Reproductive effects in two species of native freshwater gastropod mollusc exposed to 17β-oestradiol or an environmentally relevant mixture of oestrogenic chemicals in outdoor mesocosms

A Thesis submitted for the degree of Doctor of Philosophy

By

Alice Louise Baynes

Institute for the Environment

Brunel University

September 2009

Declaration

The work submitted in this thesis was carried out between 2004 and 2008 at Brunel University (Uxbridge, Middlesex) and Essex and Suffolk Water Treatment Works (Langford, Essex). This work was carried out independently and has not been submitted for any other degree.

Abstract

Institute for the Environment
Brunel University

Recent evidence suggests that molluscs may be sensitive to the effects of endocrine disrupting chemicals (EDCs) in a similar manner to vertebrates, such as fish. Despite this (with the exception of TBT-induced imposex in marine gastropods), molluscs have been largely overlooked in the field of endocrine disruption. Life-cycle studies were conducted in which two species of native UK freshwater gastropod molluscs (the hermaphrodite *Planorbarius corneus* and the gonochorist *Viviparus viviparus*) were exposed to either 17β-oestradiol or environmentally relevant mixtures of chemicals known to be oestrogenic to vertebrates and to be present in UK treated sewage effluents (TSE) and rivers. Adult snails were exposed for four months in outdoor mesocosms, fed by river water, over the spring and summer (breeding season) in order to examine effects on reproductive output, growth and mortality. Furthermore, offspring (F1s) were also developmentally exposed over the same period. F1 juvenile snails were then depurated in river water for nine months (over winter) after which time their growth, survival, and reproductive success were measured in further un-dosed river water mesocosm studies in the following spring/summer. Histopathology was used to determine immediate effects of chemical exposure on adult and F1 snails' reproductive health. Histopathology was also used to determine long lasting effects of chemical exposure on depurated F1s. Exposure to oestrogenic chemicals resulted in a range of effects, including modulated fecundity and growth in F0 adults, to retardation of growth, sexual development and fecundity in developmentally exposed F1s. Exposure to mixtures of oestrogenic chemicals also resulted in possible modulation of the immune system, resulting in increased parasitism and over winter mortality of exposed F1s compared to snails exposed to river water alone. Differences in sensitivity and response to exposure between the two species and the generations were also observed.

Acknowledgements

I would like to take this opportunity to acknowledge my supervisors Dr Edwin Routledge and Dr Susan Jobling for all their advice, guidance and inspiration throughout my PhD research. I would like to thank the Department for Environment, Food and Rural affairs (DEFRA) for their funding and giving me the opportunity to conduct this research. I would also like to acknowledge all the staff at the Institute for the Environment at Brunel University, and Essex and Suffolk Water Treatment works at Langford, Essex, for their day to day help during my practical research, and I would like to thank Dr Burkard Watermann from Limnomar, Germany, for his invaluable histopathological advice and expertise. I would like to say a special thank you to my fellow snail researcher Rachel Benstead for all her advice, hard work and insane amount of egg counting during the mesocosm studies and beyond. I would also like to take this opportunity to thank my friends and family for all their support, especially Sonia Chapman and Dr Jon Bolland, for all the good times and for always being their when I needed you most. Lastly I would like to thank Graham Harris. Not least for his snail wrangling skills, but, also for all his support, words of encouragement, and generally keeping me as sane as possible throughout the last push of research and the thesis writing. Thank you.

Contents

List of Figures

List of Tables

1 Introduction

1.1 Introduction

Due to a combination of stressors such as habitat loss, pollution and climate change many animals are threatened with population decline or, in extreme cases extinction. The International Union for Conservation of Nature and Natural resources (IUCN) has evaluated 25,238 (42% of described) vertebrate species and 4,116 (0.34% of described) invertebrate species. Of these evaluated species 23% of vertebrates and 51% of invertebrates were considered threatened (all critically endangered, endangered and vulnerable combined) (IUCN 2007)(IUCN 2007).

Humans discharge thousands of chemicals into the environment via air, water and soil. These chemicals come from a wide variety of sources such as energy production, agriculture, manufacturing, mining, health care, and transport. Classical toxicology generally investigates the lethality of a chemical, providing industry and policy makers with standard lethal concentrations (LC), which are used to determine acceptable concentrations of each individual chemical pollutant entering the environment. Also the general mantra of 'dilution is the solution to pollution' is used i.e. if you dilute a chemical or pollutant enough (e.g. into a river, sea or ocean) it will not have an adverse affect.

The publication of Rachel Carson's book 'Silent Spring' (Carson 1962) highlighted the possible non-lethal effects of anthropogenic pollution and linked the use of pesticides to biological effects on organisms other than their primary target. These effects can often be insidious and have far reaching population effects that remain undetected until, for example, a wild bird such as the Bald Eagle (Colborn 1991) or Asian Vulture (Green et al. 2006, Pain et al. 2008) population crashes. However, it wasn't until the 1990's that a meeting to discuss chemically induced alterations in sexual function and development (Colborn and Clement 1992) and later the publication of 'Our stolen future' (Colborn et al. 1996) brought endocrine disruption into the public eye.

There is now growing evidence of reproductive and sexual disruption caused by anthropogenic pollution in a wide range of species. Therefore, research into the effects of endocrine disrupting chemicals entering the environment is imperative so that at least some of these man-made stressors on our environment are mitigated. The occurrence of endocrine disruption in wildlife has meant scientist and regulators have had to rethink the classical toxicological ideas; direct lethality is not the only consequence of pollution, mixtures of chemicals may elicit a different response than individual chemicals alone and

adverse effects of biologically active chemicals may occur at low environmental concentrations.

1.2 The (vertebrate) Endocrine System

To understand how these chemicals disrupt biological functions some knowledge of the endocrine system is required. The endocrine system is a collection of ductless glands, which secrete hormones into the blood, which travel to target tissues where they bind to specific cellular receptors, and elicit biological responses (Figure 1.1). In addition to this, certain neurons of the nervous system can also secrete regulatory chemicals (termed neuro-hormones) into the blood, which affect specific targets. This neuro-endocrine system regulates much of the body's functions, ranging from digestion to sexual differentiation, growth and reproduction (Campbell and Reece 2005). Homeostasis is an important function of the neuro-endocrine system and hormone levels (and therefore responses) are controlled by feedback mechanisms (Figure 1.1). Although, for illustrative purposes, feedback mechanisms are often shown in isolation, they are in reality complex and cross talk between different systems enables the entire neuro-endocrine system to be integrated.

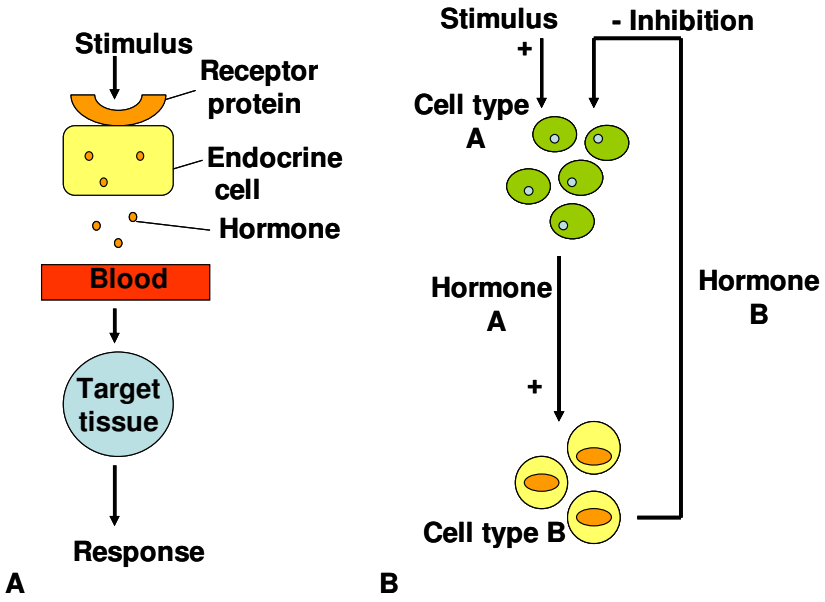

Figure 1.1 Schematic diagrams of the endocrine system.

(A) Simplified endocrine pathway: a stimulus is detected by the receptor, which in turn makes the endocrine cell produce a hormone, this travels via the blood to the target organ or tissue which produces a response (Modified from (Campbell and Reece 2005)). (B) Simplified negative feedback mechanism to maintain homeostasis: Cell type A are stimulated to produce hormone A which in turn stimulates cell type B to produce hormone B. Hormone B exerts a negative feedback regulation of hormone A (Modified from (Damstra et al. 2002)).

The vertebrate endocrine system uses three types of hormone messenger; protein peptide hormones, steroid hormones and amines derived from amino acids. Peptide hormones (the most numerous type of hormone) are usually pre-formed and then stored in secretory granules ready for release in response to the appropriate signal. Peptides require a specific secretory mechanism, which is usually triggered by an increase in intracellular calcium, or depolarisation of the cell. Upon stimulation the entire contents of the secretory granule is released (Hinson et al. 2007). Steroid hormones are made from cholesterol, and have a common core structure. Rather than storing steroid hormones, which are lipids and difficult to store, steroid secretory cells store cholesterol that can be esterified and is more easily stored. Steroid hormones are small and can diffuse across the plasma membrane down a concentration gradient and therefore do not need a specific secretory mechanism. The third group of hormones are derived from amino acids, like tyrosine, which is used to produce both thyroid hormones (thyroxine) and catecholamines (adrenaline). Like steroid secretory cells, the thyroid gland stores the precursors of thyroid hormones ready for synthesis (Hinson et al. 2007). Peptide hormones are water-soluble and can therefore be carried free in the blood, whereas steroid and thyroid hormones are lipophilic and need a carrier/binding protein to travel in the blood. These binding proteins are specific to their hormones, e.g. sex hormone binding gobulin (SHBG) or Thyroid hormone binding gobulin (THBG). Binding to these proteins increases the solubility of the hormone in the blood, increases the hormones half-life by reducing its metabolisation and enables there to be a reserve of hormone in the blood ready for use (Hinson et al. 2007).

All three types of hormone work in a similar manner in terms of signalling. Each hormone and receptor is specific to one another like a lock and key. Once a hormone binds to its receptor protein it triggers a response within the target cell. If a cell does not have the specific receptor no binding will occur and the cell will remain unresponsive (Campbell and Reece 2005). Receptors are located either embedded in the plasma membrane of the cell surface (Figure 1.2) or are intracellular within the cell nucleolus or cell cytoplasm (Figure 1.2). Steroid and thyroid hormones can pass freely across cell membranes. These hormones classically bind to intracellular receptors whereas peptides, glycoproteins and catecholamines are either too large or hydrophilic to enter the cell and so bind to cell surface receptors. When a hormone binds to a receptor on the cell surface it triggers a signal transduction pathway, which leads to a change in cytoplasmic function or a change in gene transcription in the nucleus (Figure 1.2). However, there are four main groups of cell surface receptor and they all work in slightly different ways. There are seven-transmembrane domain receptors, which are linked to G-protein activation and to

secondary messenger production or ion channel opening. Signal transmembrane domain receptors with inherent tryosin kinase activity, which function as dimers and activate kinase cascades. Signal transmembrane domain receptors without inherent kinase activity, which dimerize after binding and signal transmembrane receptors possessing guanylate cyclase activity, which act through cyclic granosine monophosphate (cGMP) generation. In each case proteins (generally enzymes) are phosphorylated, which either activates or inactivates the enzyme, and brings about a change in cellular function (Hinson et al. 2007). When a hormone binds to a nuclear receptor the general response is that of altered gene expression. Hormone-receptor complexes bind to specific regions of DNA called hormone response element (HRE), and stimulate the transcription of specific genes (Figure 1.3). Receptors located in the cytoplasm form hormone-receptor complexes, which can move into the nucleus and stimulate transcription of specific genes (Campbell and Reece 2005).

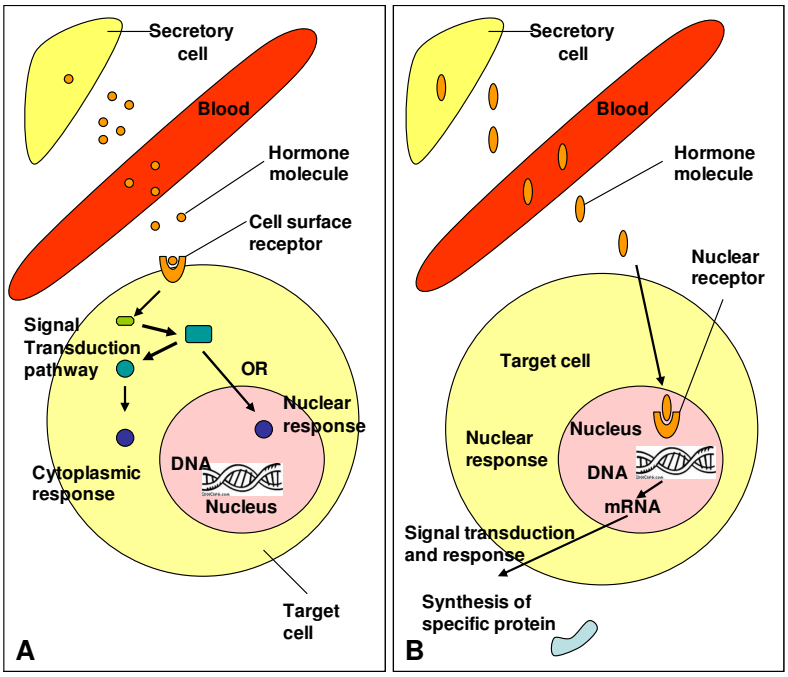

Figure 1.2 Diagrams of a cell surface receptor (A) and a nuclear receptor (B).

(A) water-soluble hormone binds to a receptor protein on the surface of a target cell, this triggers a signal transduction pathway that leads to a change in cytoplasmic function or a change in gene transcription in the nucleus. (B) a lipid-soluble hormone penetrates a target cells plasma membrane and binds to an intracellular receptor in the nucleus. The hormone-receptor complex acts as a transcription factor, typically activating gene expression (Campbell and Reece 2005).

Nuclear receptors are very similar in structure and are modular in formation with several different domains (Figure 1.3). These include (i) a variable (has a ligand-independent transactivation domain) N-terminal domain, (ii) a DNA-binding domain (DBD) which, upon activation, binds to the hormone response element (HRE), a specific sequence within

the DNA, (iii) the hinge region, (iv) a ligand-binding domain, where the hormone (ligand) binds to the receptor and at the C-terminal end (Kumar and Thompson 1999, Thompson and Kumar 2003, Kumar et al. 2004). Upon ligand binding (at the LBD) the receptors undergo structural/shape changes that allow the DBD to bind to the HRE.

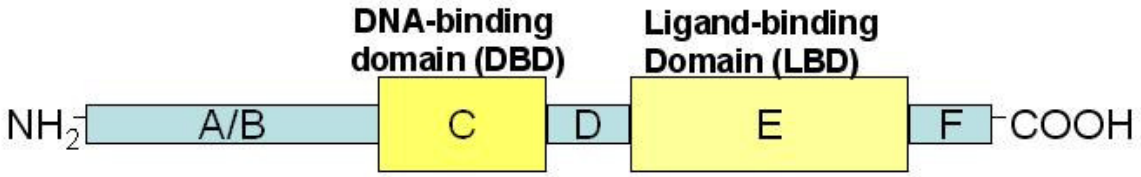

Figure 1.3 Typical structure of a nuclear receptor.

Nuclear receptors are highly structurally related and share a common structural organisation. A/B variable amino (N) -terminal domain; C well-conserved DNA-binding domain (DBD); D non-conserved hinge domain; E moderately conserved ligand-binding domain (LBD); F carboxyl (C) –terminal domain (modified from Nishikawa 2006).

As mentioned earlier, steroid hormones are all synthesised from cholesterol and have a similar basic structure. The process of steroid synthesis and steroidogenesis involves a number of proteins and enzymes. Different steroids, such as testosterone or oestradiol, can be created via different pathways. For a full schematic of human steroid pathways see Figure 1.4. However, for the purpose of illustration, one possible pathway to produce 17β-oestradiol (E2) will be described. The first step involves conversion of cholesterol (by side-chain cleavage enzyme, SCC) into Pregnenolone, which is then converted into Progesterone by 3β-Hydroxysteroid dehydrogenase. Progesterone is converted into 17α-hydroyprogesterone (by 17α-Hydroxylase), which is then converted into Androstenedione (by 17, 20-Lyase). Androstenedione is converted into Testosterone (T; by 17β-Hydroxysteroid dehydrogenase), and then to 17β-oestradiol (by Aromatase) (Hinson et al. 2007). T can also be converted to Dihydrotestoterone (DHT), a more potent androgen. The ability of enzymes to convert one hormone into another makes local paracrine (targeting of local cells) control possible.

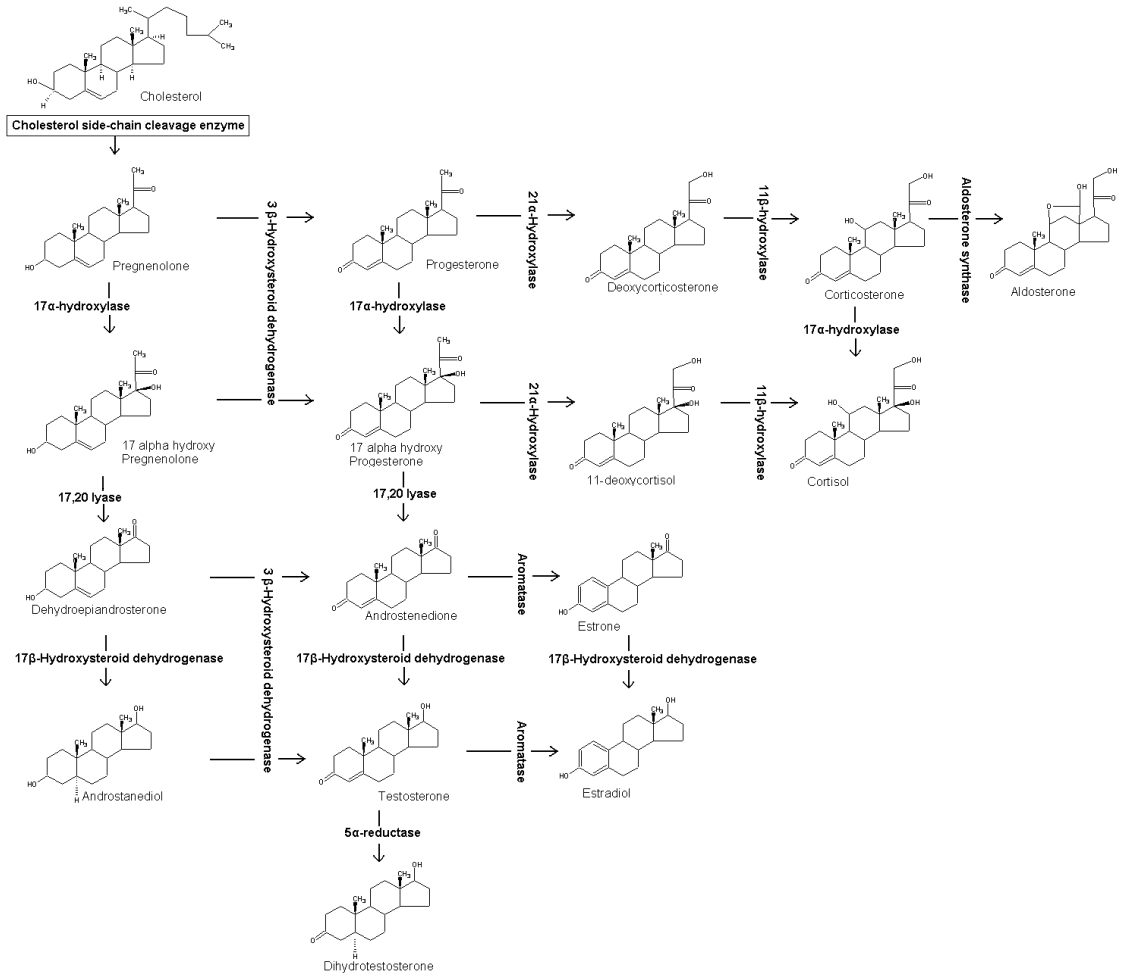

Figure 1.4 Human steroid hormone production and steroidogenic pathways.

Cholesterol is the basis for all steroid hormones. Different enzyme reactions convert one hormone to another. For example, Testosterone can be converted into 17beta-oestradiol by the enzyme aromatase, or into dihydrotestoterone (DHT), a more potent androgen, by the enzyme 5α-reductase.

The endocrine system can mediate a range of responses from quite a small number of chemical signals, because one hormone can elicit different responses when bound to different types of receptor. For example, epinephrine is a hormone involved in the 'fight or flight' response. When this hormone binds to an α-adrenergic-receptor found in intestinal blood vessels it causes the vessel to constrict. In contrast, the same hormone when bound to an β-adrenergic-receptor on a skeletal blood vessel causes the vessel to dilate. Target cells with the same receptor can also exhibit different responses if they have different signal transduction pathways and/or different effector proteins. For example, when Epinephrine binds to the β-adrenergic-receptor in liver cells, it breaks down stored glycogen and releases glucose from the cell, a totally different response to that of the blood vessel (Campbell and Reece 2005). Some endocrine glands release tropic hormones, which act directly upon another endocrine gland to release another hormone, thus acting as a cascade. For example, thyrotropic-releasing hormone (TRH) from the hypothalamus

stimulates the release of pituitary thyroid stimulating hormone (TSH), which stimulates the release of thyroxine (T4) by the thyroid gland. These cascades allow signals to be amplified and are known as an endocrine-axes (Hinson et al. 2007). They are normally set up or programmed during early development (foetal or neonatal). During this time the feedback mechanisms and sensitivity of the hypothalamus and pituitary to hormones are established.

1.3 Endocrine disruption (ED)

Endocrine disruption is a perturbation from the normal homeostasis mechanisms. One of the best-known examples of disruption to an endocrine system in humans is the disease diabetes. Blood sugar levels are normally controlled by the release of the hormone insulin by the pancreas. Perturbation of the normal endocrine function by either autoimmune destruction of the pancreatic beta cells or insulin resistance of target tissue can cause blood sugar levels to rise or fall outside normal limits which can result in effects as extreme as diabetic comma. Some types of endocrine disruption such as diabetes are long term and are caused by disease, other types can be short term and caused by an external stimulus. One example of this is the use of oral contraceptives ('the pill') by women. During the normal menstrual cycle the hypothalamus releases gonadotropin-releasing hormone (GnRH) a peptide hormone, which in turn stimulates the anterior pituitary to release follicle-stimulating hormone (FS) and luteinizing hormone (LH). FS and LH stimulate the follicle to grow and a peak in LH will trigger ovulation. The combined oral contraceptive pill contains two pharmaceutically produced hormones which mimic oestrogen and progesterone, these two hormones have a negative feedback mechanism with the hypothalamus which reduces GnRH production, which in turn stops the production of FSH and LH by the pituitary gland and as a consequence prevents ovulation occurring. An endocrine disruptor is therefore something that interferes with the endocrine systems's normal function. There is no official worldwide definition of an endocrine disruptor, although the Environmental protection agency (EPA) in the USA uses Kavlock's (Kavlock et al. 1996) description of an ED: 'An exogenous agent that interferers with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and regulation of developmental processes'. Whereas in the European Union the International Program on Chemical Safety (IPCS) definition is favoured: 'An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations' (Damstra et al. 2002).

As inferred by Kavlock's (Kavlock et al. 1996) description above, EDCs can act at multiple sites via multiple mechanisms of action, such as binding to nuclear receptors, inhibition of steroid hormone synthesis and metabolism. Primarily research has focused on those endocrine disrupting chemicals that are known to bind to nuclear hormone receptors in a similar manner to natural hormones (ligands). Once bound, these chemicals can act as agonists (eliciting the full normal response), partial agonists (eliciting only a partial response), or as antagonists (binding to the receptor but not eliciting a response). If, for example, we consider the oestrogen receptor (ER), a chemical that binds to this receptor as an agonist would be classified oestrogenic, whereas one that binds as an antagonist would be classified anti-oestrogenic, as it doesn't elicit the normal response and occupies the receptor so that other oestrogens cannot activate it (competitive binding). A chemical that binds as a partial agonist could be considered mildly oestrogenic but also as an anti-oestrogen as it can also block the receptor from being fully activated. There are several receptor groups that have been studied in relation to endocrine disruption, namely oestrogen receptors (ER), androgen receptors (AR), thyroid receptors (TR), Aryl hydrocarbon receptors (AhR) and the Retinoid receptors (RAR, RXR). Some EDCs do not bind to nuclear receptors, but instead are thought to disrupt enzymatic pathways or regulatory proteins used in producing or metabolising hormones.

1.4 Possible Endocrine Disrupting Chemicals (EDCs)

There are a growing number of chemicals that are classified as endocrine disruptors; these can come from a wide variety of sources including agriculture, industry, and domestic sewage works. This review will focus primarily on chemicals found in the aquatic environment that have oestrogenic properties; due to their relevance to my research area and also due to the wealth of literature surrounding this topic.

Steroid Oestrogens

The majority of vertebrates, some invertebrates (and indeed some plants) produce natural hormones such as oestrogen, progesterone, testosterone and thyroxin. Due to the conserved nature of hormones, and hormone receptors, the effects of chemicals often cross the taxonomic boundaries. For example, hormones excreted by humans and farm animals can impact on the endocrine system of fish. The naturally occurring oestrogens 17β-oestradiol (E2) and oestrone (E1) have been reported in the ng/l range in both treated sewage effluents (TSE) and rivers. In a survey of seven sewage treatment works (STW) effluents in the UK, Desbrow et al (Desbrow et al. 1998) found E1 to range from 1.4± 0.15 to 76.0 ±

2.9ng/l and E2 from 3.7 ± 0.6 to 48.0 ± 6.0ng/l. Johnson et al (Johnson et al. 2005) found a similar range (E1 0.2-35ng/l and E2 <0.6-13ng/l) in 17 STW effluents from across Norway, Sweden, Finland, The Netherlands, Belgium, Germany, France and Switzerland. Comparable concentrations were also found in The Netherlands (Vethaak et al. 2005), Sweden (Larsson et al. 1999), Japan (Nakada et al. 2004), Italy (Lagana et al. 2004), and in the UK (Liu et al. 2004). However, much higher concentrations have also been recorded from UK TSE, such as those reported by Rogers-Gray et al (Rogers-Gray et al. 2000), including; E1 15-220ng/l and E2 7-88ng/l. Inputs from farming (e.g. cattle) have also been measured in the UK. Matthiessen et al (Matthiessen et al. 2006) used passive samplers to measure E1 and E2 downstream of farms, and reported concentrations were in the very low ng/l range. River water concentrations of steroid estrogens have also been measured in the low ng/l range in the UK (Liu et al. 2004, Sheahan et al. 2002) and Italy (Lagana et al. 2004). Phytoestrogens have also been measured in Italian TSE at concentrations of 3-83ng/l and in river water 1-7ng/l (Lagana et al. 2004). The naturally occurring androgen testosterone (T) has been measured less frequently by researchers, but has been recorded at concentrations of 3.3-4.8ng/l in the lower Jordan River (Barel-Cohen et al. 2006) to as much as 5.5-6.22µg/l in river water samples from China (Yang et al. 2006a, Yang et al. 2006b).

Pharmaceuticals

Synthetic/pharmaceutical hormones also bind to steroid receptors, sometimes with higher affinities than their natural ligands. Often synthetic hormones have been engineered to be less prone to degradation by biological activity in order to be more effective as a medicine. However, this also means they can be more stable and persistent once excreted into the environment. The synthetic hormone that has received the most attention by researchers by far is ethinylestradiol (EE2) found in the contraceptive pill. This in part is due to its extreme potency, 11-27 times more potent than E2 in vitro (14-day juvenile rainbow trout vitellogenin assay) (Thorpe et al. 2003). Concentrations of EE2 measured in TSE are generally in the low ng/l range in the UK; <0.4-3.4ng/l (Williams et al. 2003) to 7.0 ± 3.7ng/l (Desbrow et al. 1998). Other reported concentrations include Sweden 4.5ng/l (Larsson et al. 1999), Germany 1.8ng/l (Andersen et al. 2003), The Netherlands <0.3-2.6ng/l (Vethaak et al. 2005) and Europe wide <0.8-2.8ng/l (Johnson et al. 2005). Some literature reports much higher concentrations. For instance Fernandez et al (Fernandez et al. 2007) measured EE2 from a range of domestic and industrial effluents in Canada and found concentrations generally below 5ng/l, but on one occasion as much as 178ng/l. In the Lower Jordan River, concentrations were measured at 6.1ng/l in river water, and de

Alda et al (de Alda et al. 2002) found EE2 in river sediment as high as 22.8ng/l. However, due to the complex matrices of effluents and river water these marked differences in measured concentrations of EE2 may also be related to the detection methods employed. In general analytical methods for detecting EE2 in complex matrices have been improving and the measured concentrations of EE2 have been decreasing, this makes the 178ng/l reported by Fernandez et al (Fernandez et al. 2007) seem unlikely. Other synthetic hormones such as diethystilbestrol, mestranol, norethindrone and levonorgestrel have also been measured in environmental samples in the ng/l range (Yang et al. 2006a, de Alda et al. 2002, de Alda and Barcelo 2000, de Alda et al. 2003). The synthetic androgen trenbolone acetate, used to promote beef cattle growth has also been found in receiving waters (Soto et al. 2004, Durhan et al. 2006). α-trenbolone and β-trenbolone have also been measured in cattle feed lot effluent in the USA at concentrations of up to 120ng/l and 20ng/l respectively (Wilson et al. 2008). Other pharmaceuticals such as antidepressants have been measured in river water down stream of wastewater treatment plants. Schultz et al (Schultz et al. 2008) measured a range of antidepressants in the 10 to 100 ng/l range down stream of two wastewater treatment plants in the USA. The same chemicals were also measured in river sediment (in 10's ng/l) and in fish brain homogenates (low ng/l) collected from the same sites.

Alkylphenol ethoxylates

Alkylphenol ethoxylates (APEs) have been widely used in a range of processes including pulp, paper and textile industries, pesticide formulation for agriculture and in a number of industrial and domestic cleaning products since the 1940s (Sabik et al. 2003). After use, APEs are often flushed into the sewage system and are commonly found in TSEs. Degradation of APEs in sewage works or the environment causes them to breakdown into more persistent, shorter-chain, APEs and alkylphenols (APs) (Ying et al. 2002, Ying 2006). Nonylphenol ethoxylates (NPEO) and Octylphenol ethoxylates (OPEO) are two of the most commonly used nonionic surfactants (Ying et al. 2002) and their degradation products including nonylphenol (NP) and octylphenol (OP) have been reported to be estrogenic (Jobling and Sumpter 1993, Jobling et al. 1996, Routledge and Sumpter 1996, Soto et al. 1991). Due to these effects, in the mid 1990s Alkylphenols were phased out within the European Union (EU) and NP is now characterised as a priority hazardous substance (PHS) in the Water Framework Directive (WFD) (Soares et al. 2008, Loos et al. 2008). Moreover the WFD has proposed environmental quality standards (EQS) for surface waters of 0.3µg/l NP and 0.1µg/l OP (Soares et al. 2008). A number of other countries such as Japan, Canada and the USA also monitor Alkylphenol concentrations

within their waters (Soares et al. 2008). In these countries where monitoring or prohibition have occurred, Alkylphenol concentrations in TSE have decreased from measuring hundreds of µg/l (Lye et al. 1999) Sheahan et al. 2002, Ying et al. 2002) to ng/l range (Liu et al. 2004, Sabik et al. 2003). Equally, before alkylphenol prohibition many rivers where found to be highly impacted. For example in the UK river water concentrations in the µg/l range have been recorded (Blackburn and Waldock 1995, Blackburn et al. 1999, Patrolecco et al. 2004). Whereas more recent river water concentrations across Europe have generally been reported in the ng/l range. Bolz et al (Bolz et al. 2001) reported concentrations up to 458ng/l NP and 189ng/l OP in rivers and streams in southwest Germany, Kuch and Ballschmiter (Kuch and Ballschmiter 2001) also reported low ng/l NP (6-135ng/l) river water concentrations in South Germany; attaining the WFD proposed EQS. Quednow and Puttman (Quednow and Puttmann 2008) measured NP in streams between 2003 and 2005 and found falling concentrations (in the ng/l range) over the sampling period. However, Lagana et al (Lagana et al. 2004) reported higher levels of NP between 1289-1466ng/l in the River Tiber in Italy. Short chain APs are also lipophilic (Ahel et al. 1993) and(Bolz et al. 2001) alkylphenols have also been found in high concentrations in sediment samples. Bolz et al (Bolz et al. 2001) measured concentrations up to 259µg/kg NP and 8µg/kg OP in river sediments from South West Germany, and concentrations as high as 13700µg/kg have been recorded in sediments from America (Ying et al. 2002). This means in some areas river and estuary sediment may act as source of alklyphenol pollution long after industry has stopped releasing them.

Bisphenol A

Bisphenol A (BPA) is a key raw material used in the manufacturing of polycarbonate plastic and epoxy resins (Crain et al. 2007). BPA is another oestrogen mimic, binding to the ER, although like NP and OP at a much lower affinity than the natural ligand. However, in contrast to APs BPA has not been designated as a PHS within the EU (although it is on a list of chemicals under review for possible identification as a priority substance or a PHS (Directive 2008/105/EC)). Presently there is much debate about the potency of BPA as an endocrine disruptor to both wildlife and Humans. This means there is no European EQS for BPA. However, there is evidence that BPA in the low hundreds of µg/l can induce male freshwater fish to produce a female specific protein (vitellogenin (vtg)) (Lindholst et al. 2000, Sohoni et al. 2001, Segner et al. 2003b) and can also affect testicular and germ cell development at lower concentrations (low µg/l) (Sohoni et al. 2001, Mandich et al. 2007). BPA can enter the aquatic environment via TSE and landfill leachate and, as with APs, BPA concentrations vary widely. Lagana et al (Lagana et al.

2004) recorded concentrations between 13-36ng/l in TSE in Italy. Nakada et al (Nakada et al. 2004) found BPA at concentrations of 544 ± 127ng/l in Japanese effluents. Fromme et al (Fromme et al. 2002) measured German effluents up to 702ng/l, and Lee and Peart (Lee and Peart 2000) measured BPA in a range of effluents in Canada and found concentrations between 0.010-1.08µg/l in average effluents, but as high as 149.2µg/l in some industrial ones. River water concentrations have generally been reported in the low ng/l range in Germany, Italy, UK, Japan and Switzerland (Lagana et al. 2004, Liu et al. 2004, Patrolecco et al. 2004, Bolz et al. 2001, Kuch and Ballschmiter 2001, Fromme et al. 2002, Kang and Kondo 2006, Voutsa et al. 2006) and have occasionally been reported in the high ng/l to low µg/l range (Quednow and Puttmann 2008, Heemken et al. 2001). Basheer et al (Basheer et al. 2004) measured BPA at concentrations between 0.01-2.47µg/l in coastal waters off Singapore. Sediment concentrations also varied and in Germany ranged from 0.01-0.19µg/kg (Fromme et al. 2002) to 66-343µg/kg (Heemken et al. 2001). Therefore, environmental concentrations of BPA are generally considered to be below the no effect concentration (NOEC) for endocrine disruption in fish. However, there some evidence that other groups of animals, such as molluscs, maybe more sensitive (reproductive effects at the low ng/l level) to BPA. Again, as with vertebrate exposures there have been conflicting results (Oehlmann et al. 2000, Oehlmann et al. 2006, Duft et al. 2007, Forbes et al. 2007b, Forbes et al. 2007a, Forbes et al. 2008) and further experiments maybe necessary to fully elude the threat of BPA to aquatic animals.

Organochlorines

Organochlorine pesticides (OCPs) and Polychlorinated biphenyls (PCBs) are environmentally persistent, accumulate in sediments, and bioaccumulate up tropic levels. Both OCPs and PCBs have been widely cited as potent EDs. OCPs such as DDT are used in the control of insects in agriculture and are still used to control malaria spread in some countries. The use of DDT has been phased out in many countries due to its negative impact on wildlife and possible human health issues. PCBs were used in a wide range of applications including in electrical equipment, as coolants, in PVC cables, as flame-retardants, and in paints and lubricating oils. PCB manufacturing has now been banned in light of their carcinogenic nature. OCPs and PCBs have been implicated in reproductive abnormalities and failure in a wide range of species including birds, reptiles and marine mammals (Cheek 2006, Dawson 2000). PCBs are known to interact with steroid receptors (such as ER and AhR), to interfere with thyroid function, to disrupt sex steroid enzyme activity and to affect immunity (Cheek 2006, Dawson 2000). OCPs and PCB are fairly ubiquitous in all aquatic environments. Although measured in the pg/l range, they have

been found in remote locations such as Antarctica (Tatton and Ruzicka 1967, Fuoco et al. 1995, Fuoco et al. 1994)and mountain lakes (Fernandez et al. 2005) due to their ability to be air borne. Concentrations of PCBs in industrialised countries can be high and have been measured in the hundreds or thousands of ng/l in river water and low ng/g in sediments (Xing et al. 2005, Zhang et al. 2007, Zhang et al. 2007b, Ezemonye 2006, Sprovieri et al. 2007, Castells et al. 2008). Similarly OCPs concentrations have been measured in the μg/l range in river water from China (Xing et al. 2005, Zhang et al. 2007)

Organotins

Organotins such as Tributyltin (TBT) and Triphenyltin (TPT) are used as biocides in a wide range of applications. TBT is one of the most notorious endocrine disrupting compounds found in fresh water and marine systems, where it is used as an antifouling agent in boat paint. TBT is most renowned for its masculinising effects (imposex in female dog whelks) in gastropod molluscs. Masculinisation has also been reported in other molluscs (Horiguchi et al. 2000, Horiguchi et al. 2002, Horiguchi et al. 2005) and fish (Shimasaki et al. 2003, McAllister and Kime 2003, Santos et al. 2006, Santos et al. 2006). Exposure to TBT has also been shown to disrupt ovarian function (hormone ratios, oocyte maturation) and embryo development in fish (LOEC 0.16ng/egg or 160ng/g egg) at environmentally relevant concentrations (10, 100ng/l) (Zhang et al. 2007a, Hano et al. 2007).

More recently, TBT has also been reported to induce adipogenesis in a number of vertebrates *in vivo* (Grun et al. 2006, Iguchi et al. 2007, Iguchi et al. 2008). The exact mechanisms leading to these changes are still being elucidated, but they include disruption of normal sex steroid metabolism (Bettin et al. 1996, Oberdorster and McClellan-Green 2002, LeBlanc et al. 2005, Sternberg and LeBlanc 2006, Janer et al. 2005, Janer and Porte 2007, Santos et al. 2005, McVey and Cooke 2003, Ohkimoto et al. 2005), abnormal release of neuropeptides (Oberdorster and McClellan-Green 2000, Oberdorster 2001, Oberdorster et al. 2005) and possible binding to RXR (Nishikawa 2006, Iguchi et al. 2007, Iguchi et al. 2008, Nishikawa et al. 2004, Horiguchi et al. 2007, Castro et al. 2007b, Sternberg et al. 2008a, Nakanishi 2007). A partial ban of TBT antifouling paints has led to a decline in TBT water concentrations in many areas (Antizar-Ladislao 2008). Although aquatic concentrations have been reported in the hundreds of ng/l (Fent and Hunn 1991). For example, Langston et al (Langston et al. 1987) measured TBT in seawater around Poole Harbour (UK) and reported concentrations ranging from 2-646ng Sn/l, with the highest concentrations relating to marinas with heavy boat traffic and low tidal flushing. Post partial ban, TBT is still frequently measured in the low ng/l (Fent 1996, Harino et al.

1998b, Harino et al. 1998a, Murai et al. 2005). TBT also accumulates in sewage sludge (Fent 1996) and sediments (Biselli et al. 2000, Diez et al. 2003) and is therefore still persistent in many environments. As part of the European Water framework directive TBT and its compound are now listed as priority substances (2008/105/EC) and an EQS of 2ng/l in surface waters has been set.

Triphenol tin (TPT) is also known for its masulinising effects on gastropod molluscs (Schulte-Oehlmann et al. 2000), inhibition of aromatase activity (Saitoh et al. 2001), and possible interactions with the RXR (Nakanishi 2007, Nakanishi 2008). TPT has been used in combination with TBT as a biocide in antiflouling boat paints but is also used as an agricultural and commercial fungicide. Using tissue samples of marine biota from a German specimen bank (1985-1999) TPT concentrations in North Sea mussels dropped from 98 to 7ng/g, similar patterns of decreasing TPT concentration were also found in bladder wrack, common mussels, and eelpout muscle tissues, reflecting the ceasation of using TPT as a co-toxicant in anti-fouling boat paints in the 1980s (Rudel et al. 2003). In a similar study of freshwater fish tissue sampled between 1988 and 2003 on the Elbe near Blankenese again the peak TPT concentration (253ng/g 1993) corresponded to TPT use in anti-fouling paint (Rudel et al. 2007). However, at other locations upstream on the Elbe, other rivers (e.g. Rhine) or lakes (e.g. Lake Belau) TPT concentrations correlated with its use as a fungicide (Rudel et al. 2007).

Heavy metals

Heavy metals have also been studied in relation to ED. Uranium has recently been linked with estrogenic effects in mice (Raymond-Whish et al. 2007). Cadmium (Cd) has been shown to effect cortisol steroidogensis in trout Oncorhynchus *mykiss* (Lacroix and Hontela 2006) and reduced reproductive success in fathead minnow pair breeding tests (Sellin and Kolok 2006). Cd exposure also reduced ovarian development, egg production and vitellogenin-like polypeptide proteins in the insect *Oncopeltus fasciatus* and the reduction of female germ cells and increase in apoptosis of oocytes in developmentally exposed freshwater turtles (Kitana and Callard 2008). Cd and Copper (Cu) have been linked to inhibition of ecdyson and reduced gonad somatic index (GSI) in crustaceans (Medesani et al. 2004, Rodriguez et al. 2007). Cd has also been linked to ED in humans, disrupting enzymes involved with steroidogenesis and interfering with DNA binding (Henson and Chedrese 2004).

There are a growing number of chemical which are know to have ED potential which have not been covered above. These include a number of pesticides, fungicides, antimicrobial agents, dioxins and flame-retardants. There are also a number of chemicals that have multiple modes of action. BPA for example interacts with the ER, the AR and the thyroid receptor. In addition some chemicals show evidence of ED but their exact mechanistic action is still yet to be fully eluded.

1.5 Evidence of Endocrine Disruption in the environment

Humans

There is growing concern regarding the Human health implications of exposure to endocrine disrupting chemicals. Male fertility has significantly declined in the past 50 years (Carlsen et al. 1992) and cases of prostate cancer and reproductive abnormalities in both men and women have increased (Maffini et al. 2006). A firm link between these health problems and endocrine disruption has, however, not been established. One of the most renowned cases of ED in humans is that of Diethylstilbestrol (DES); a non-steroid oestrogen given to miscarriage-susceptible pregnant women in the 1940-1970s. Later epidemiological studies have found that the pregnant women who took DES had an increased chance of developing breast cancer and had generally higher mortality rates (Colton et al. 1993, Titus-Ernstoff et al. 2006a). In addition prenatal DES exposure of daughters (whilst in the womb) also increased the chance of developing vaginal clear cell carcinoma, breast cancer, early menopause, increased reproductive abnormalities, infertility and poor pregnancy outcome (Swan 2000, Swan 2001, Hatch et al. 2006, Palmer et al. 2006, Titus-Ernstoff et al. 2006b). Prenatal exposure of sons also had reproductive repercussions, with increased risk of genital anomalies (Giusti et al. 1995). Epigenetic effects have also been reported in laboratory animals exposed to DES (Newbold 2004, Li et al. 2003, Newbold et al. 2006, Ruden et al. 2005). The pre-natal period is often seen as one of the most sensitive stages of an animal's life, as this is when much of the endocrine systems 'programming' occurs (Gore 2008)(Dickerson and Gore 2007). In addition to reproductive disruption, other ED effects have also being studied. PCB's and Dioxins are known thyroid disruptors and investigations into a link between blood or urine concentrations and reduced thyroxin have been made (Turyk et al. 2007). Thyroid function is extremely important in growth and development especially of the brain; studies of prenatal exposure to PCBs have also shown links to reduced intellectual function at only slightly higher than average human concentrations (Jacobson and Jacobson 1996).

Mammals

DDT, its metabolites (DDE and DDD) and PCBs are environmentally persistent and lipophilic which means they can magnify up a food chain and bio-accumulate in apex predators such as predatory mammals and birds. PCB and OCP exposure have been linked to the otter (*Lutra lutra*) population crash seen in the UK and the rest of Europe in the 1950s (Mason and Macdonald 2004). In a survey of dead otters from Denmark (1980-1990), otters that died of unknown causes (not road kill or drowning) had the highest levels of PCBs in their tissue (Mason and Madsen 1993). Kruuk and Conroy (Kruuk and Conroy 1996) found strong negative correlations between PCB concentration and body condition of otters in Scotland. Simpson et al (Simpson et al. 2000) found a negative correlation between PCB body burden and Vitamin A in a UK study, and suggested this as a possible reason for otter decline. Roos et al (Roos et al. 2001) in a study spanning 1968-1999 found that reduced PCB burden was linked with increasing otter populations. The majority of studies in Europe now show PCB and OCP concentrations in otter tissue and scats to be falling and that many otter populations are now recovering (Mason and Macdonald 2004, Simpson et al. 2000, Roos et al. 2001, Mason and Macdonald 1994, Elliott et al. 1999). Nevertheless, PCB concentrations in scats from populations living down stream or in coastal areas are still at levels considered to be disruptive (Lemarchand et al. 2007). In America a casual link between OCP and PCB pollution and low population numbers of the river otter (*Lontra canadensis*) and mink (*Neovison vison*) (Wren 1991) has been proposed. PCB concentrations found in dead sea otters (*Enhydra lutris nereis*) from the Californian coast were extremely high and a correlation between contamination and death by infectious diseases has been made (Nakata et al. 1998, Kannan et al. 2007). PCBs and DDT have also been measured in high concentrations in grey (*Halichoerus grypus*) and harbour seal (*Phoca vitulina*) skin/blubber samples from the UK, USA and Baltic coasts, and in each case correlations have been found between contamination and thyroid disruption (Hall and Thomas 2007, Hall and Thomas 2007, Sormo et al. 2005, Tabuchi et al. 2006, Routti et al. 2008). Similar correlations have been seen with California sea lions (*Zalophus californianus*) (Debier et al. 2005) and Polar bears (*Ursus maritimus*) with female bears most highly affected (Braathen et al. 2004).

Birds

DDE has been implicated in the disruption of normal eggshell thickness by inhibiting prostaglandin synthesis (Lundholm 1997). Since DDT has been banned in many countries the eggshell thickness of eggs in fish eating birds has increased and many populations seem to be recovering. In the Great Lakes (USA), Double-Crested Cormorants

(*Phalacrocarax auritus*) and Ring-Billed Gulls (*Larus dehawarensis*) numbers have increased (Heinz 1998). Burger et al (Burger et al. 1995) reported Common Tern (*Sterna hirundo*) and Black Skimmer (*Rynchops niger*) eggshell thickness to have increased by 50% from the 1970's to the 1990's in the New York Bight. Eggshell thickness recoveries have also been found for Grebes in Canada (Forsyth et al. 1994). However, not all fish eating birds have recovered as well. Toschik et al (Toschik et al. 2005) found that reproduction in the Osprey (*Pandion haliaetus*) appeared to still be impacted by pollution. Heinz (Heinz 1998) found the Bald Eagles (*Hailaeetus leucocephalus*) have not recovered as well as other species in the Great lakes and Burger et al (Burger et al. 1995) found the Least Tern (*S. antillarum*) shell thickness had not recovered as significantly as other terns in the New York Bight. In contrast to the birds associated with aquatic ecosystems, other predatory birds that hunt in agricultural areas have shown good signs of recovery, indicating that aquatic ecosystems may be long-term sources of contamination. Scharenberg and Looft (Scharenberg and Looft 2004) measured DDT and PCBs concentrations in Goshawk (*Accipiter gentillis*) eggs and eggshell thickness over two periods (1971-1978 and 1988-2002) in Northern Germany, and found reduction of DDT concentration over time (although PCB concentrations were not altered) and significantly lower eggshell index from eggs laid in the 1970's compare to later eggs. Indeed, eggs laid between 2001-2002 had similar index scores to those from the 18th century. The recovery in the British Sparrow Hawk (*Accipiter nisus*) has also been linked to the ban of organochlorine pesticides in UK agriculture (Newton and Wyllie 1992).

Organochlorine compounds may have further reproductive effects in birds, including possible feminisation. The normal female (egg laying) proteins vitellogenin (vtg) and zona radiata proteins (zrp) have been measured in male peregrine falcons (*Falco peregrinus*) and Jimenez et al (Jimenez et al. 2007) suggest that this may be a result of organochlorine pesticide exposure (Jimenez et al. 2007).

Fish

Examples of ED in wild fish populations are numerous, and include the masculinisation of female fish exposed to paper mill effluents; a phenomenon recorded since the early 1980's when female mosquitofish (*Gambusia affinis*) exhibiting male secondary sexual characteristics such as a gonopodium-like anal fin and male behaviour were discovered (Drysdale and Bortone 1989, Toft et al. 2004, Bortone and Cody 1999, Cody and Bortone 1997, Bortone et al. 1989, Howell et al. 1980). In other species of fish exposed to mill effluents, increased male to female sex ratios, sex steriodogenesis, reduced vtg, delayed maturation, intersex, and ovarian dysfunctions have been reported (Karels and Oikari 2000,

Karels et al. 1998, Munkittrick et al. 1998, Orn et al. 2006, Landman et al. 2008). A good example of cause and effect is the case of the Swedish female eelpouts (*Zoarces viviparus*), where a higher ratio of males was found in association with exposure to pulp mill effluents (Larsson et al. 2000). During a period of mill inactivity, sex ratios returned to normal, and then subsequently shifted towards males again when the mill was reactivated. Providing strong evidence in favour of chemicals in the effluent causing the shift in sex ratio. Masculinisation of female fish by mill effluents has been reported in a range of countries including America, Canada, New Zealand, Finland and Sweden (Parrott et al. 2006). Compounds present in Pulp/Kraft mill effluents have been shown to bind to AR in both humans and fish (Hewitt et al. 2000, Parks et al. 2001) and also bind to retinol receptors RXR and RAR (Alsop et al. 2003). However, the exact nature of the chemicals involved in the paper mill effluents are still being researched (Hewitt et al. 2008, Larsson et al. 2006, Hewitt et al. 2005).

Oestrogenic mixtures of chemicals in sewage effluents (e.g. E2, EE2, NP, OP, BPA) are believed to feminise male freshwater fish (e.g. roach and carp) in the UK, causing reduced/inhibited spermatogenesis, oocytes in the testis, feminised reproductive ducts, and the production of egg laying protein vitellogenin (vtg) (Jobling and Sumpter 1993, Jobling et al. 1995, Sumpter 1997, Jobling et al. 1998, Jobling et al. 2002, Jobling et al. 2002, Jobling and Tyler 2003, Jobling et al. 2006, Tyler and Routledge 1998, Rodgers-Gray et al. 2001, Nolan et al. 2001, van Aerle et al. 2001). Incidences of feminisation and intersex have also been reported in wild freshwater fish from France, Germany, Japan, Spain, America and Denmark (Minier et al. 2000, Gercken and Sordyl 2002, Hassanin et al. 2002, Sole et al. 2003, Kavanagh et al. 2004, Bjerregaard et al. 2006) and a growing number of reports of intersex in estuarine and marine species have also been published (Lye et al. 1997, Allen et al. 1999a, Allen et al. 1999b, Hashimoto et al. 2000, De Metrio et al. 2003, Martin-Skilton et al. 2006, Caprioli et al. 2007).

Reptiles

A number of field studies have found reproductive dysfunction in alligators (*Alligator mississippiensis*) exposed to high concentrations of organochlorines (DDT, DDE) in Florida, USA. Exposure to these compounds was associated with reduced phallus size in males (Pickford et al. 2000, Gunderson et al. 2004), and abnormal bone composition (Lind et al. 2004) and hormone profiles in females (Pickford et al. 2000, Gunderson et al. 2004, Guillette et al. 2000). Reduced aromatase activity has also been reported as a consequence of exposure (Crain and Guillette 1998). Secondary sexual characteristics have also been

affected in Snapping Turtles (*Chelydra serpentina*) found at sites with PCB and OCP pollution (de Solla et al. 2002), and Cd pollution has been implicated in increased oocyte apoptosis and reduced vtg concentration in exposed freshwater turtles (*Chrysemys picta*) (Kitana and Callard 2008, Rie et al. 2005).

Amphibians

Worldwide amphibian populations are in decline. The IUCN red list states that 29% of described amphibian species are threatened (95% of described species have been evaluated (IUCN 2007)), which has led scientists to look at endocrine disruption as a possible reason. Laboratory studies on a range of frogs have shown that certain pesticides alter tadpole metamorphosis and (possibly) sexual differentiation or sex ratio (Howe et al. 2004, Hayes et al. 2006, Hayes et al. 2002b, Hayes et al. 2002a, Hayes et al. 2003, Orton et al. 2006) and immune function (Christin et al. 2004, Brodkin et al. 2007). Exposure of adults to the pesticide methoxychlor resulted in delayed oviposition, reduced egg laying and lower plasma vtg in female *Xenopus laevis* (Pickford and Morris 2003). Embryos exposed to environmental oestrogens E2, NP and OP (10nM-10μM) had higher mortality and deformities than controls (Bevan et al., 2003), and DDE (0.01-10mg/kg body mass) exposure has been shown to affect retinol metabolism (Leiva-Presa et al. 2006, Leiva-Presa and Jenssen 2006). Developmental exposure to Atrazine (a commonly used herbicide) in the laboratory has been reported to cause retarded gonad development and testicular oocytes in male leopard frogs (*Rana pipiens*) at concentrations of 0.1 and 25ppb (0.1 and 25μg/l) (Hayes et al. 2003). Hayes et al (Hayes et al. 2003) also conducted field surveys in areas of known Atrazine use (among other herbicides and pesticides) and found similar histopathological effects. However, very little other field evidence exists for ED in amphibians. Investigations by Murphy et al (Murphy et al. 2006) with wild populations of frogs, found no correlation between Atrazine exposure (one of the most publicised pesticides with respect to amphibian ED) and hormone concentration (E2, T and 11-ketonetestosterone), aromatase activity, or gonad somatic index (GSI). However, recent field research suggests that a combination of Atrazine and phosphate exposure were the best predictors of the abundance of debilitating parasitic larval trematodes in wild northern leopard frogs (*Rana pipiens*) (Rohr et al. 2008b). Furthermore, laboratory studies showed that at environmentally relevant concentrations four pesticides (atrazine, glyphosate, carbaryl, and malathion) increased trematode parasite load in exposed frogs (Rohr et al. 2008b, Rohr et al. 2008a), suggesting that pesticides may play a role in amphibian decline via immonosupression.

Invertebrates

The vast majority of cases of ED seen in wild populations of invertebrates are related to masculinisation of female molluscs (reported in detail below). Very little field-based evidence exists for other invertebrates. Two reported cases of possible ED in invertebrates are related to crabs. Lye et al (Lye et al. 2005) sampled Green Shore Crabs (*Carcinus maenas*) from areas known to have EDCs at concentrations that affect vertebrates. Male crabs from the exposed site had increased steroid molting hormone (similar to ecdysone) reduced behavioural responses to female pheromones, reduced pleopod-length ratio and increased vtg-like proteins compared to those from a reference site. In the laboratory exposure of *C. maenas* to the estrogenic compound NP (effective concentration 1.5µg/l) reduced testis weight, increased liver weight and altered molting hormone profiles compared to controls were seen but no vtg-induction was observed (Lye et al. 2008). Ayaki et al (Ayaki et al. 2005) found what appeared to be feminised male freshwater crabs (*Geothelphusa dehaani*) in a number of rivers in Japan. 8-32% of male crabs had female openings but otherwise looked phenotypically male. No laboratory studies were carried out to confirm or refute a chemical-related cause. In laboratory exposures, disruption to timing in larval development, and storage proteins (used in vitellogenesis) have been found in barnacles (*Balanus amphitrite*, *Elminius modestus*) exposed to NP (0.01-10µg/l) or E2 (1-10µg/l) (Billinghurst et al. 2001, Billinghurst et al. 2000). Exposure of *Gammarus fossarum* (shrimp) to BPA (5, 50, 500µg/l) in an artificial stream for 103 days resulted in accelerated maturation of oocytes and a reduction in size and number of early vitellogenic oocytes (Schirling et al. 2006b). Exposure of *G. pulex* to EE2 (0.1, 1, 10, 100 µg/l) for 100 days resulted in elevated reproduction at 1 and 10 µg/l, and increased the female to male ratio to 2:1 (Watts et al. 2002). Sea urchins (*Paracentrotus lividus*) exposed to Triphenyl tin (TPT) had significantly altered androgen metabolism (225ng/l TPT), with a concentration dependant (50, 100, 225ng/l TPT) decrease in P450-aromatase activity, increased conversion of T into DHT, and possible increased 5alpha-reductase activity (Lavado et al. 2006a). Lavado et al (Lavado et al. 2006b) also exposed the crinoid *Antedon mediterranea* to several other EDCs. Exposure to methyltestosterone (MT) (1000ng/l), TPT (225ng/l), DDE (100ng/l) or fenarimol (FEN) (240 and 2400ng/l) caused a significant increase in T, whereas, exposure to cyproterone acetate (CPA) (300 and 3000ng/l) caused a significant reduction in T. MT (10ng/l) and FEN (240 and 2400ng/l) produced a significant increase in E2, whereas TPT (100 and 225ng/l) exposure significantly reduced E2 compared to controls, indicating echinoderms were likely to be affected by EDCs in the wild. Other research has focused on the effects of juvenile hormone agonists used primarily as insecticides and their possible ED effects on non-target species such as

crustaceans. Effects are possibly mediated via the ecdysteroid receptor (EcR) and can affect growth, metamorphosis, reproductive maturation and sexual differentiation ((Tuberty and McKenney 2005, Olmstead and LeBlanc 2007, LeBlanc 2007).

1.6 Effects of known vertebrate EDCs on molluscs: field and laboratory studies

The most highly cited condition of ED in molluscs is that of imposex (imposition of male sexual characteristics on a female). The condition of female gonochoristic gastropods having a penis-like structure behind their right tentacle was first observed in the UK in *Nucella lapillus* back in the 1960s (Blaber 1970). A similar condition was reported in America (Smith 1971) in another marine snail *Ilyanassa obsoleta*, at which point the term imposex was used to describe the symptoms observed. However, it wasn't until the 1980's that the connection to pollution from marinas and boats was published (Smith 1981a, Smith 1981b) and to the boat antifouling agent Tributyltin (TBT) (Smith 1981c, Smith 1981)(Smith 1981c, Smith 1981c). Further research implicated TBT in reproductive impairment (Gibbs and Bryan 1986) and population decline (Bryan et al. 1986) in some species. This research has continued and today over 150 prosobranch gastropod species are known to be affected (deFur et al. 1999). TBT does not induce full imposex characteristics (growth of penis, vas deferens) in all species of prosobranch. To illustrate, *Littorina littorea* from exposed sites, or after experimental TBT exposure (10, 100, 330, 1000ng/l TBT), did not exhibit full imposex (Matthiessen et al. 1995, Bauer et al. 1995), although reproductive abnormalities were noted in the form of reduced fecundity (100, 330, 1000ng/l TBT), hatchability (560, 1150ng/l TBT) (Matthiessen et al. 1995), and growth of the prostate as well as malformation of the oviduct (Probable threshold concentration of 15ng TBT as Sn/l) (Bauer et al. 1995). TBT and imposex have also been implicated in reduced ability to react to pheromones. Indeed, Straw and Rittschof (Straw and Rittschof 2004) reported that *I. obsoleta* (male, female and imposex) from TBT polluted sites did not react to male and female pheromones in the way non-impacted male and females did. A correlation has been found between imposex and DNA damage in *N. lapillus*, and hyperplasia of the vas deferens and penis are also correlated with increasing severity of imposex (Hagger et al. 2006).

TBT has also been implicated in disrupting other mollusc species. In the 1970's the British oyster (*Ostrea edulis*) stocks declined, and experimental evidence found that TBT could shift sex ratios in favour of more males and inhibit reproduction (Thain and Waldock 1986). In Japan, the abalone (*Haliotis madaka*) was investigated after fishery stocks fell. Histology of the gonadal tissue showed 20% of females from a polluted site had ovo-testis.

Analysis of abalone tissue showed significantly higher concentrations of butyltins and phenyltins compared to a reference site where gonad disruption was not observed (Horiguchi et al. 2000, Horiguchi et al. 2005). *Haliotis gigantea* exposed to TBT or TPT (100ng/l for 2 months) induced significant spermatogenesis to occur in females (Horiguchi et al. 2002). There is also experimental evidence that TBT can impact freshwater mollusc species. The freshwater prosobranch *Marisa cornuarietis* is masculinised at concentrations of 200ng/l TBT or 250 and 500ng/l TPT, where growth of the penis and increase of the vas deferens sequence index (VDSI) is found along with disruption to egg laying (Duft et al. 2007, Schulte-Oehlmann et al. 2000, Schulte-Oehlmann et al. 1995). Another freshwater prosobranch mollusc *Potamopyrgus antipodarum* is also affected by TBT and TPT, although not in terms of imposex induction. *P. antipodarum* females harbour embryos within a brood pouch. Upon exposure via sediment, the number of unshelled (least developed) embryo numbers in the brood pouch significantly decreased after four weeks exposure (Duft et al. 2007, Duft et al. 2003). A small number of studies have exposed fresh water pulmonate snails to TBT. Czech et al (Czech et al. 2001) exposed *Lymnaea stagnalis* to 1, 10 and 100ng/l TBT, the number of egg masses laid per snail were significantly reduced at the highest concentration, although this reduction was interpreted by the authors to be a toxic effect, rather than that of ED. In the same study, histological analysis found inflammation and degeneration in a number of tissues. Exposure of *L. stagnalis* adults and juveniles to high concentrations of TBT (230-3200ng/l) causes aberrations to the prostate gland and sperm duct, and shell growth. Embryo development and hatching success were also disrupted (Segner et al. 2003b). Experiments by Leung et al (Leung et al. 2004) on the same species found at high concentrations (1 µg/l TBT) abnormal embryonic development occurred in conjunction with significantly reduced hatchling survival and shell growth inhibition. Adult reproduction and survival was also affected at this concentration (Leung et al. 2004). In *Physa fontinalis* (another fresh water pulmonate) similar effects were observed. Abnormal embryo development occurred at 1 and 10 µg/l TBT and hatchling survival was significantly reduced at 1µg/l, as were growth and fecundity (Leung et al. 2004).

More recently, feminising effects of some chemicals on molluscs have been reported. Indeed a growing body of evidence from both laboratory and field studies suggest that many of the EDCs known to have feminising effects on reproduction in vertebrates may have similar effects on molluscs. Surveys of marine clams (*Scrobicularia plana*) in UK estuaries have revealed the widespread occurrence of intersex (oocytes present in testis) similar to that reported in fish (Chesman and Langston 2006). Exposure of undifferentiated

S. plana to a mixture of oestrogenic chemicals (E2 100µg/kg wet weight (ww), EE2 100µg/kg ww, OP 1000µg/kg ww and, NP 1000µg/kg ww) via sediment caused a significant increase in both the incidence of intersex and increased oocyte size in this species. Short-term exposure of adults (sexually differentiated) to a mixture of E2 (100µg/kg ww) or EE2 (100µg/kg ww) also resulted increased oocyte size (Langston et al. 2007). Increased oocyte size (and number) was also found in the marine mussel *Mytilus edulis* after exposure to North Sea Oil (Aarab et al. 2004). Exposure of developing *Crassostrea gigas* at 7-8 days post fertilisation to NP (1, 100µg/l), resulted in an altered sex ratio (increased females) and a 30% incidence of hermaphroditism not normally seen in this species (Nice et al. 2003). Exposure to NP at the onset of gametogenesis also affected sperm motility; 100% of control males had motile sperm but exposure to NP (1µg/l and 100µg/l) caused a significant dose dependant decrease in motile sperm (Nice 2005).

Long term (one year) exposure to sewage effluent significantly increased the ratio of females to males in *Elliptio complanata* (Blaise et al. 2003). Moreover, significant increases in alkaili-labile phosphates (ALP) were also recorded in the exposed male and (especially) female gonads compared to upstream individuals (Blaise et al. 2003). Putative vtg-like proteins measured by ALP method have been used as a biomarker in a number of bivalve studies with varying results. The ALP method measures the level of akali-labile phosphate found in an organic phase where lipoproteins, such as vitellogenins, are found (Gagne and Blaise 2000). Unlike methods such as the Enzyme-linked immunosorbent assay (ELISA) (used to detect vtg in fish) which uses specific antibodies to detect vtg, the ALP method is not specific and other lipoprotiens such as circulating cholesterol, steroid hormones, fatsoluble vitamins or triglycerides could also be measured using this indirect method. Gagne suggests to validate this method two criteria should be met, namely 1) that vitellogenic females should contain significantly more alkali-labile phosphates than males, and 2) treatment with E2 should increase the level of ALP in the target tissues (Gagne and Blaise 2000). However, even with these criteria the level of possible vtg-like proteins compared to other lipoprotiens is still undetermined. This fundamental problem may be why such opposing results have been found. Using the ALP method a number of authors have report that vtg-like proteins were elevated in female bivalves post exposure to possible estrogenic chemicals, for example in *M. edulis* exposed to a mixture of North Sea Oil and Alkylphenols (Ortiz-Zarragoitia and Cajaraville 2006) or BPA (Aarab et al. 2006). Elevated vtg-like proteins (ALP method) were also measured in female zebra mussels *Dreissena polymorpha* exposed to NP (500µg/l) (Quinn et al. 2006), whereas males

similarly exposed had significantly less vtg-like proteins. A number of other authors have reported the opposite, that male bivalves are more sensitive to exposure. Matozzo and Marin (Matozzo and Marin 2005) found male *Tapes philippinarus* to have significantly increased vtg-like proteins (ALP method) after exposure to NP (7 days to 0.1mg/l and 0.2mg/l), whereas female levels were not significantly affected. The authors also noted that control females naturally had much higher levels of vtg-like proteins than control males, which is at least in accordance with one of Gagne's criteria. In addition to the ALP method being used to measure vtg-like proteins in bivalves being questionable in its accuracy the chemical concentrations used in some of the above exposures are rather high in terms of environmental values. It is therefore possible some level of toxicity is also occurring. In a study designed to assess copper toxicity in Norwegian waters *M. edulis* were transplanted up a pollution gradient. When these mussels were analysed the females at the most polluted site had advanced gametogenesis, a higher gonad index and raised vtg-like proteins (ALP method) compared to mussels transplanted further downstream, indicating possible estrogenic effect of copper (Zorita et al. 2006). However, the mussels at the most polluted site also had significantly increased digestive gland atrophy indicating toxicity. It is therefore possible that rather than a true estrogenic affect this may instead be evidence of a survival mechanism of the mussels to spawn before it dies. Further research is therefore needed before this ALP method is truly useful in assessing ED in molluscs.

Further attempts have been made to identify biomarkers of ED in molluscs. Puinean and Rotchell (Puinean and Rotchell 2006) identified vtg mRNA and quantified gene expression in *M. edulis*. They found that males exposed (via injection, 25µg) to E2 had increased vtg gene expression compared to exposed females. Whereas, control (DMSO injected) male and female vtg gene expression did not significantly differ from one another (Puinean et al. 2006). However, in another experiment, conducted several months later, with *M. edulis* exposed to E2 via water (200ng/l) Puinean et al (Puinean et al. 2006) found no increase in either vtg or ER expression in exposed animals, although total E2 concentrations in mussel tissue had increased 15 fold, leading the authors to question the validity of using these (vtg and ER expression) biomarkers in molluscs.

In static renewal experiments, exposure of mussels (*Elliptio complanata*) to an estrogenic effluent caused serotonergic responses. Serotonin and dopamine are both implicated in sexual differentiation, gamete development, spawning and fertilisation in molluscs. Gonad serotonin concentration and cyclooxygenase (COX) activity increased at low effluent concentrations and decreased at high ones (after 45 days exposure). This was possibly

caused by a negative feedback mechanism for regulating serotonin, as high effluent concentrations also increased monoamine oxidase (MAO) activity, involved in degradation of dopamine and serotonin (Gagne and Blaise 2003, Gagne et al. 2004a). Exposure of the mussels to (concentrated) effluent via injection significantly reduced serotonin levels (in nerve ganglia and gonad), dopamine (nerve ganglia), and increased MAO in the nerve ganglia and gonad. Exposure to a low concentration of E2 (0.3µg, injected) saw an (non-significant) increase in serotonin (gonad), dopamine (nerve ganglia and gonad) and MAO (nerve ganglia and gonad), whereas, at higher concentrations (3µg, injected) both serotonin (gonad) and dopamine (nerve ganglia and gonad) levels decreased, and MAO levels increased (significant increase in gonad). Exposure to NP (3.2µg or 32µg, injected) significantly increased dopamine levels in the gonad (Gagne et al. 2004a). Whilst exposure of freshly prepared gonad slices to effluent (3% v/v, similar to 5km down stream of outfall) also stimulated oocyte release in a similar manner to serotonin in vitro(Gagne and Blaise 2003, Gagne et al. 2004a, Gagne et al. 2004b). Exposure of *P. antipodarum* to TSE known to feminise freshwater fish caused an increase of unshelled embryos in the brood pouch at low concentrations. Whereas, inhibition was seen with 100% effluent after 28 days (Jobling et al. 2004). Significant increases in unshelled embryo numbers were also produced when *P. antipodarum* was exposed to EE2, OP or BPA (Duft et al. 2007, Duft et al. 2003, Jobling et al. 2004). Exposure of *N. lapillus* to sewage effluent (known to contain BPA, OP, NP, and E2) was reported to cause a significant dose dependant increase in the percentage of mature females (Castro et al. 2007a)(Castro et al. 2007b, Castro et al. 2007a), and an increase in ER expression in the ovary. Female *N. lapillus* exposed to BPA or OP (1, 25 and 100µg/l) had significantly increased numbers of oocytes and enlarged accessory pallial glands, whereas, males of the same species had significantly reduced penis length and prostate size, and a reduction in ripe sperm stored in the vesicular seminalis, compared to control animals (Oehlmann et al. 2000). A similar stimulating effect of BPA and OP was seen in the freshwater gastropod *M. cornuarietis*, where increased oocyte number and egg laying, female malformation of the pallial oviduct and enlargement of accessory sex glands, leading to the term 'super female' to be coined (Oehlmann et al. 2000). *M. cornuarietis* were also exposed to BPA by Forbes et al (Forbes et al. 2007b, Forbes et al. 2007a) who found no reproductive effects during their exposure. However, the design and endpoints of the two experiments varied quite markedly. In the Oehlmann study (Oehlmann et al. 2000) adult snails were kept in large groups at 22^{o}C and exposed to BPA (1, 5, 25, 100µg/l nominal) for five months, a number of their offspring were also exposed (1 and 100µg/l) for a further for 12 months. In the Forbes study (Forbes et al. 2007a), adult snails were kept in breeding pairs at 25^{o}C and exposed to BPA for 12

weeks (0.1, 1, 16, 160, 640µg/l nominal). In this experiment a number of offspring were also exposed further, however only until they were 60 days post hatch. In the Forbes study (Forbes et al. 2007a) the main endpoints measured were related to egg laying, hatching and growth of offspring. In the Oehlmann study (Oehlmann et al. 2000) egg laying and hatching were also measured, however some of the most marked results were from morphological measurements of the reproductive organs; increase in female accessory sex gland volume (albumen and capsule gland), hypertrophy and rupture of glands and a number of cases where females had two vaginal openings. Comparative morphological data is absent from the Forbes study (Forbes et al. 2007a). In addition, and perhaps most importantly when comparing reproductive output, the two studies were conducted at different temperatures. As with many invertebrates temperature can play a critical role in the rate of reproductive output. Indeed in an earlier publication Forbes et al describe how under the same experimental conditions pairs of *M. cornuarietis* reproduce significantly less at 22^{o}C than at 25^{o}C (Forbes et al. 2007b) and that at the higher temperature the between-replicate variability was more than three times lower than at the higher temperature, this may suggest at the higher temperature the snails were at their upper most limit of their reproductive output, which could possibly mask other stimuli. Developmental exposure of *M. cornuarietis* to EE2 (10µg/l) or BPA (50µg/l and 100µg/l), caused significant a reduction in heart rate (10µg/l EE2 or 100µg/l BPA), and a significant increase in hatchling weight (100µg/l BPA) compared to control animals. A dose-dependant increase (EE2), or decrease (BPA) in hatching rate, was also reported (Schirling et al. 2006a). In the experiments described above neither Forbes (Forbes et al. 2007b) nor Oehlmann (Oehlmann et al. 2000) found any significant effect of BPA on hatching success in *M. cornuarietis*.

A number of experiments have also been performed with freshwater pulmonate gastropods, in particular with the pond snail *L. stagnalis*. Exposure of *L. stagnalis* and *P. corneus* eggs to an effluent that had been only mechanically treated caused 100% embryo mortality in both species. Diluting the effluent to 25% prevented mortality but reduced embryo development. Biologically treated effluent had no effect on the embryos development, as did the mechanically treated effluent after it had been left at room temperature for a year (Wagner 2000). Graded concentrations of TSE caused an increase in egg mass production and total egg mass weight per in *P. corneus*. However, results indicted that each egg mass was smaller, suggesting possible reduction in the packaging (albumin) of each egg, which could affect hatching success (Clarke et al. 2009). Exposure of adult *L. stagnalis* to EE2 (1µg/l EE2) increased the number of eggs and egg masses laid per snail, but reduced

subsequent embryo development and hatching and increased shell decalcification (Segner et al. 2003b). However, Casey et al (Casey et al. 2005) found no consistent effects on the number of egg masses laid at a concentration range of 1-10000ng/l EE2. They did, however, find an increase in egg laying malformations such as poly-nuclear eggs after 14 days exposure. Exposure of *L. stagnalis* to a combination of EE2 and cyproterone acetate (CPA; anti-androgen in vertebrates) also increased reproductive output and reduced embryos development and hatching. Egg laying was also increased after exposure to methyl-testosterone (MT) (a vertebrate AR agonist) and tamoxifen (vertebrate ER antagonist) mixture, however not to the same degree as the combination of EE2 (vertebrate ER agonist) and CPA (vertebrate ER antagonist) (Weltje et al. 2003). A study on the sea scallop, *Placopecten magellanicus*, also found E2 and P stimulated gamete release in both males and females and T stimulated gamete release in males. However, in this case, the effect of these hormones on gamete release was stopped by receptor antagonists (tamoxifen or flutamide) (Wang and Croll 2003). Czech et al (Czech et al. 2001) found no effect of MT (1, 10, 100ng/l) on egg mass laying of *L. stagnalis*. Exposure of *L. stagnalis* to Vinclozolin 250ng/l (vertebrate AR antagonist) stimulated egg mass production (Weltje et al. 2003, Lagadic et al. 2005) and reduced hatching success. However, exposure via water and food (duck weed) at a much higher concentration (250µg/l Vinclozin) reduced egg masses laying but had no effect on hatching success. In addition an increase in a testosterone-like moleclue was measured in the gonad in snails exposed via the food and water, whereas exposure via water alone had no effect on the testosterone-like moleclue's concentration (Lagadic et al. 2005). A 10-14 day exposure of *L. stagnalis* egg masses to NP (180-320µg/l) resulted in abnormal shell development and reduced hatching success (reviewed in Lagadic et al (Lagadic et al. 2007)). Lalah et al (Lalah et al. 2007) also assessed the effects of NP on embryo development and growth and found similar results at 105µg/l NP; namely a delay in growth, and an increase in embryo mortality and subsequent reduced hatching success. Again, as with some of the previous studies using NP and molluscs the concentrations tested are above or at the highest levels experienced in the aquatic environment and the results (abnormalities, reduced growth and development) may be linked to toxicity rather than ED.

A number of authors have also looked at reproductive effects of other chemicals on molluscs. Female *M. edulis* exposed to diallyl phthalate had reduced putative vtg-like phospho-proteins (ALP method) and reduced oocyte size and number, whereas males were unaffected (Aarab et al. 2006). Exposure of the pulmonate snail *P. fontinalis* to the herbicide paraquat for 7 days resulted in a reduction in egg and egg mass laying and an

increase in degenerating oocytes (Bacchetta et al. 2002). *M. cornuarietis* eggs exposed to cadmium (Cd) had increased mortality at 500µg/l and delayed development, hatching and reduced hatching weight at 250µg/l (Schirling et al. 2006a). *L. stagnalis* exposed to Cd showed reduced egg and egg mass laying at 200µg/l and above; exposed eggs had reduced and aberrant development between 25-400µg/l and no hatching occurred at 400µg/l (Gomot 1998). Coeurdassier et al (Coeurdassier et al. 2003) found *Lymnaea palustris* to be more sensitive with significantly reduced egg and egg mass laying at 80µg/l Cd, and no hatching at 40µg/l. Cd is a toxic heavy metal and is listed as one of the priority substances within the EU Water Framework Directive; the proposed EQS for inland surface waters is 0.08µg/l. The concentrations tested in the above exposures are therefore well above concentrations frequently experienced in the aquatic environment. Additionally considering that in another mollusc species (Zebra mussel) the chronic (10 week) LC50 of Cd was 130µg/l (Kraak et al. 1992) it seems that toxicity is likely to be the overriding factor (rather than ED) affecting reproductive output and hatching success in these snails.

A number of studies have found possible immunological and neuroendocrine effects of EDCs on molluscs. Canesi et al (Canesi et al. 2005, Canesi et al. 2007b, Canesi et al. 2007a, Canesi et al. 2007c) reported immune related responses from several estrogenic compounds (E2, EE2, NP, BPA) injected into *Mytilus sp*, including reduction in processes crucial to bactericidal activity (BPA), down regulation of antioxidant genes (BPA), reduced methallothioneins (BPA) and reduction in lysosomal membrane stability (BPA, E2, EE2, NP and EDC mixture). Phagocytosis could be stimulated at low (BPA 1, 5µM; EE2 1µM; NP 5µM) concentrations but inhibited at higher concentrations (100µM) of estrogenic chemicals ((Canesi et al. 2007b)). Reduction in lysosomal membrane activity in *M. edulis* exposed to E2 or P (Moore et al. 1978) and a reduction the phagocytic activity of hemocytes in *Mya arenaria* exposed to E2 (10 and 20nM, via injection) was also reported (Gauthier-Clerc et al. 2006). Champeau and Narbonne (2006) exposed *Corbicula fluminea* to either E2 (20, 200, 2000ng/l) or TBT (50, 250, 500ng Sn/l) for 15 and 30 days. They found a significant reduction in phagocytosis after 15 (2000ng/l E2) and 30 days (200ng/l, 2000ng/l E2 and 500ng Sn/l) exposure, compared to the controls. The authors reported E2 to be more effective at inhibition of phagocytosis than TBT, as only after 30 days of exposure to the highest dose of TBT caused a significant reduction. This needs to be put into context, however. The E2 concentrations found to have a significant effect (200ng/l and 2000ng/l) were far higher than those measured in the aquatic environment, whereas, the TBT concentrations tested were more environmentally relevant.

From the literature above it can be seen that there are some interesting questions arising into whether the mollusc endocrine system can be modulated by chemicals known to be ED in vertebrates. From these questions much debate has arisen amongst academics, regulators and industry. The only way this debate can be fully resolved is by conducting further research to fill the knowledge gaps that are very much present in this field. When my research was started in 2004 many of these publications (above) were not yet present in the literature. Indeed even now there is still little information regarding the possible effects of vertebrate EDC or their mixtures on UK native freshwater molluscs.

1.7 The Mollusc Endocrine System

Until recently much of the research into ED effects has focused only on vertebrates. This is because invertebrate endocrine systems are far less understood and because human health-like effects of endocrine disruption are more likely to be seen in other vertebrate systems than invertebrate systems. Both of these reasons are understandable. However, invertebrates make up around 95% of all animal species, are a fundamental part of all ecosystems and often inhabit niches where pollution is greatest (e.g. sediments). It can be argued, therefore, that they require serious attention. The IUCN has evaluated only a small percentage of all the described invertebrate species; 0.13% of insects, 1.4% of crustacean and 2.7% of molluscs. Of those evaluated 50% of insects, 83% of crustacean and 44% of mollusc species are considered under threat (IUCN 2007). The species investigated in my research thesis are in the phylum Mollusca. This is the second most species-rich group after insects (approximately 81,000 described species). Molluscs are found in a wide range of habitats and their extreme diversity is due to explosive radiation, which has occurred several times over a long evolutionary history (Wilbur 1984).

Like vertebrates and other invertebrates, the mollusc endocrine system functions to carry signals (environmental or endogenous) to a target site to elicit a response (LeBlanc et al. 1999). However, compared to vertebrates or other invertebrates, the mollusc endocrine system shows extreme diversity even within the phylum (Ketata et al. 2008). These differences can be seen though the variety of reproductive strategies employed by molluscs. For example, gastropod molluscs (slugs, snails, limpets, nudibranchs etc.), being the most diverse, can be hermaphrodites (either synchronous or sequential) or gonochorists. The majority of bivalves (mussels, scallops, clams etc.) are gonochoristic, although a number of hermaphrodites and sequential hermaphrodites also occur. Cephalopods (octopus, squid and cuttlefish etc.) are generally gonochoristic. Fertilisation

methods also vary amongst molluscs with bivalves and the majority of marine gastropods employing external fertilisation, whereas cephalopods and freshwater gastropods fertilise internally. These differences also require adaptations to external stimuli (e.g. season) and complex behavioural patterns (e.g. species specific mating).

Considering these differences makes it hard for a general mollusc (or even gastropod) endocrine system to be proposed. However, below is a review of what has been established to date:

As with vertebrates, the central nervous and endocrine system combine to regulate the body functions and to react to internal or external stimuli. The neuro-endocrine system generally consists of neurohemal organs, which contain neurosecretory cells and nerve cells, and endocrine glands such as the gonad (Ketata et al. 2008). Peptide hormone messengers are common in molluscs, and have been shown to have effects on growth and/or reproduction. Peptide hormones such as caudodorsal cell hormone (CDCH) and dorsal body hormone (DBH) have been found to be involved in the gamete release and specific behaviour of the egg laying process in the freshwater hermaphrodite (pulmonate) snail *Lymnaea stagnalis* (See Chapter 2, Figure 2.3) (Joosse 1964, Geraerts and Joosse 1975, Geraerts and Bohlken 1976, Geraerts and Algera 1976), and an egg laying hormone (ELH) has been reported in the sea hare *Aplysia californica* (Kupferma.i 1970, Kupferma.i 1972, Coggesha.Re 1970b, Coggesha.Re 1970a, Arch 1972, Arch et al. 1976a, Arch et al. 1976b). These peptides have also been found in a number of gonochoristic (prosobranch) gastropods (Ram et al. 1998). Another peptide called APGWamide has been implicated in male mating behaviour, reproduction and penis development in hermaphrodite and gonochoristic species (Smit et al. 1992, Croll and Vanminnen 1992, Fan et al. 1997, DeLange et al. 1997, de Lange and van Minnen 1998, De Boer et al. 1997, Henry et al. 2000), and has also been found in female reproductive organs in *Octopus vulgaris* (Di Cristo et al. 2005). APGWamide is synthesised in the right arterial lobe of the cerebral ganglion that is connected to the penial complex in both *Lymnaea* and *Aplysia*, and is mainly associated with male reproductive organs (Smit et al. 1992, Croll and Vanminnen 1992, Fan et al. 1997). Oberdorster et al (Oberdorster et al. 2005) suggested APGWamide to be a possible penis morphogenic factor, as males normally have higher levels than females. Females exposed to a masulinising factor (TBT induced imposex) had APGWamide levels similar to normal males. Oberdorster and McClellan-Green (Oberdorster and McClellan-Green 2000) also found that exposure to APGWamide induced masculinisation of *Ilyanassa obsoleta*. However, Santos et al (Santos et al. 2006, Santos et al. 2005) found APGWamide exposure did not cause female *Bolinus brandaris* to

be masculinised, suggesting species differences in the function of APGWamide might exist.

Some peptide hormones are clearly specific to molluscs, but others seem to be functionally, or structurally, similar between vertebrates and molluscs. Gonadotropin-releasing hormone (GnRH) is responsible for stimulating the release of lutenising hormone (LH) and follicle stimulating hormone (FSH) in vertebrates, and is a significant peptide hormone in regulating reproduction. Using antibodies, GnRH-like peptides have been detected in a wide range of molluscs including *Helisoma trivolvis* (Goldberg et al. 1993), *L.stagnalis* (Young et al. 1999), *A. californica* (Zhang et al. 2000, Zhang et al. 2008, Tsai et al. 2000, Tsai et al. 2000), *O. vulgaris* (di Cristo et al. 2002, Di Cristo et al. 2002, Iwakoshi et al. 2002, Iwakoshi-Ukena et al. 2004), bivalves (Rodet et al. 2005) and the squid (Amano et al. 2008)*Loligo bleekeri* (Amano et al. 2008). Neither the structure nor the functional role of this peptide hormone in molluscs has been fully eluded, although in general it seems to be found in the gonad and nervous tissue. Recently, Nakamura et al (Nakamura et al. 2007) reported GnRH neurons to be involved in spermatogonial proliferation in the scallop, *Patinopecten yessoensiss*. Interestingly, Tsai et al (Tsai et al. 2003) reported that *Aplysia* GnRH was unable to stimulate the release of LH from mouse pituitary. In contrast, Zhang et al (Zhang et al. 2000) found *Aplysia* bag cells were affected (reduced duration of discharge associated with egg laying stimulus) by exposure to chicken II GnRH, and Iwakoshi et al (Iwakoshi et al. 2002) found *Octopus* GnRH stimulated quail pituitary to produce LH. Hence important similarities, as well as differences, in GnRH signalling in vertebrates and invertebrates may exist.

The neuro-transmitters serotonin and dopamine have also been found to be present, and are biologically active, in molluscs. A serotonin receptor has recently been characterised in the pulmonate *Helisoma trivolvis* (Mapara et al. 2008). In a number of bivalves, serotonin has been found to reinitiate meiosis and to stimulate germinal vesical breakdown (and thus stimulating spawning) in females (Gagne et al. 2004a, Lubet and Mathieu 1990, Lippai et al. 1995, Osada et al. 1998, Martinez et al. 2000, Fong et al. 1996, Garnerot et al. 2006). Osada et al (Osada et al. 1998) found serotonin receptor formation was induced by exposure to E2 ($1x10^{-5}$ M E2 for 2 hrs at room temperature) in scallops and oysters, and Wang and Croll (Wang and Croll 2006) found E2 promoted serotonin-induced spawning in the female clam *Ruditapes philippinarum,* whereas Progesterone inhibited it. Serotonin may also play a role in gastropod reproduction, as it has been found in the CNS and ovotestis of the pulmonate snail *Biomphalaria glabrata* (Santhanagopalan and Yoshino

2000). Exposure of *B. glabrata* to serotonin has been shown to induce ovulation and increase egg laying (Manger et al. 1996, Muschamp and Fong 2001). Exposure to Methiothepin (1μM, for 30 minutes), a vertebrate serotonin receptor blocker, inhibited egg laying for a week in *B. glabrata* (Muschamp and Fong 2001). Fluoxetine a selective serotonin inhibitor (used as an anti-depressant drug) also inhibited reproduction (number of embryos) in the prosobranch *Potamopyrgus antipodarum* (Nentwig 2007). Dopamine has also been found in bivalves. In *Argopecten purpuratus,* exposure to dopamine (1-100nM) reduced oocyte release (Martinez et al. 2000)(Pani and Croll 2000) and in *Placopecten magallanicus,* spawning was found to affect dopamine levels (Pani and Croll 2000). In the gastropod *B. glabrata,* dopamine has been found in the CNS and albumen gland (Santhanagopalan and Yoshino 2000, Boyle and Yoshino 2002). Dopamine concentrations were highest in the albumen gland during packaging of eggs (Boyle and Yoshino 2002), and dopamine (10μM) was shown to significantly stimulate the secretion of proteins (un-characterised) in *in-vitro* cultures of *B. glabrata* albumen gland. Protein secretion was inhibited by co-incubation with Chlorpromazine, a dopamine antagonist (Santhanagopalan and Yoshino 2000). Dopamine also stimulates protein secretion in *H. trivolvis* albumen gland (Saleuddin et al. 2000), Mukai et al (Mukai et al. 2004) experimented with a range of dopamine receptor agonists and antagonists and suggested a dopamine D1-like receptor in the albumen gland. Therefore the role of dopamine and serotonin in molluscs appear quite different to those of mammals i.e. have (possibly) a greater direct effect on reproduction.

In addition to peptide hormones and neuro-transmitters, recent research has been focused on the possibility that vertebrate type steroid hormones are produced and/are functionally active in molluscs. Much of this work has been with three vertebrate sex hormones; 17β-estradiol (E2), testosterone (T) and progesterone (P). These hormones have been indirectly detected in many different molluscs, and endogenous levels seem to fluctuate with the reproductive cycle. In addition to this, enzymes involved in steroidogenesis (e.g. 3Beta-Hydroxysteroid dehydrogenase) and sex hormone esterifying enzymes (e.g. acyl-CoA: testoserone acyltransferase) have also been found. In *O. vulgaris,* D'Aniello et al (DAniello et al. 1996) found P, T and E2 (in the ng/g range) at higher concentrations in male tissue than females. Di Cosmo et al (Di Cosmo et al. 2001) later found that E2 and P fluctuated in the female octopus with ovary development and maturation; both authors found enzymes involved in steroidogenesis (delta 5,3beta-Hydroxysteroid dehydrogenase) within gonad tissue. Matsumoto et al (Matsumoto et al. 1997) found E2 in the ovary and testis, which increased during sexual maturation in oysters. Osada et al (Osada et al. 2004) measured E2 (HPLC) and aromatase activity (RIA) in the ovary and testis of *Patinopecten*

yessoensis and found both to increase when the ovary or testis were developing, with a peak at maturity and a drop after spawning. In the bivalve *Mya arenaria* E2 (150-400pg/g wet weight, ELISA) was measured at concentrations ten times higher than that of T in both males and females. E2 concentration was similar between the two sexes during the undifferentiated and ripe/post vitellogenic stages of the reproductive period, for the rest of the time E2 concentration was generally higher in females than males, and during the their vitellogenic/spermatocyte differentiation periods (October-November) it was significantly higher ($P<0.05$) (Gauthier-Clerc et al. 2006). There were more significant differences seen between the sexes in their T profiles. T was significantly higher in females than males before and during vitellogenesis (August-November) and during and directly after spawning (June-July) (Gauthier-Clerc et al. 2006). In the desert snails, *Sphincterochila zonata* and *S. propetarum*, a peak in T was followed by a peak in E2 during aestivation, and during active intervals a minor peak in E2 was recorded (Alon et al. 2007). In male *Ilyanassa obsoleta* (marine gastropod), T was found in its esterified form during non-reproductive periods, but an increase in free T and a decrease in esterified T occurred at the onset of mating. T was found to be lower in females, but still had a peak in free T prior to egg laying (LeBlanc et al. 2005, Gooding and LeBlanc 2004). Sternberg et al (Sternberg et al. 2008b), working with the same species, also found that T differed in the males with reproductive period, but found no change in female T concentrations, and also no relationship between E2 and reproductive phase. In the freshwater gastropod *Marisa cornuarietis,* free T was found to be similar in both sexes, whereas, total and esterified T and total, free and esterified E2, were significantly higher in males than females, sampled between March and June (low spawning season) (Janer et al. 2006).

Significant homeostatic mechanisms for sequestering steroid hormones have been demonstrated in molluscs. The ratio of esterified and 'free' hormone can alter depending on reproductive state. When the bivalve *Mytilus galloprovincialis* was exposed to 20ng/l (low), 200ng/l (medium) and 2000ng/l (high) of E2 for seven days, only at the highest concentration did free E2 elevate significantly, whereas total E2 increased in a dose dependant manner from 2-4ng/l in the control to 258ng/l in the highest exposure. The enzyme Palmitoyl-CoA (oestradiol acyltransferase) was also found to increase in a similar dose dependent manner. Histological analysis found a significant increase in gonad maturation at the highest concentration (Janer et al. 2004, Janer et al. 2005). *M. cornuarietis* were shown to cause a concentration dependent elevation of acyl-CoA (testosterone acyltransferase) after exposure to methyltestosterone (MT) (Janer et al. 2006). Gooding and LeBlanc (Gooding and LeBlanc 2004) experimentally manipulated adult *I.*

obsoleta to contain up to 300% the level of total T found in unmanipulated individuals. The concentration of esterified T found in manipulated snails increased in relation to the level of total T, whereas free T remained the same. The above results suggest both E2 and T levels are regulated in molluscs by fatty acid esterification and de-esterification processes. In mammals, sex hormones that are esterified do not directly activate their nuclear receptors. However, they are considered extremely potent, as esterified hormones are resistant to catabolism, and therefore have much extended half-lives when compared to their non-esterified counterparts (Hochberg 1998). In addition, steroid esters can act as a reservoir of biologically active hormone, which can be easily mobilised into its no-ester form and may play an important role in paracrine control (Hochberg 1998). Therefore the ability to(Hochberg 1998) produce hormone esters and then release them when needed is an important endocrine function. Molluscs may also have the ability to convert one hormone into another, in a similar manner as vertebrates. Cytochrome P450-dependent aromatisation of androgens to oestrogens has been studied in a number of molluscs (Bettin et al. 1996, Oberdorster and McClellan-Green 2002, Janer et al. 2005, Santos et al. 2002).

Another avenue for identifying whether steroid sex hormones are active in molluscs has been to identify their receptors. As mentioned earlier, vertebrate nuclear receptors bind steroid hormones to illicit altered gene expression, and it has been hypothesised that if molluscs too have these receptors they would respond similarly. The research into finding steroid receptors has been approached in a number of ways. In *O. vulgaris,* receptor binding assays (vas deferens and seminal vesicle) found receptor-like binding molecules with affinity for E2, T and P (DAniello et al. 1996). Using competition assays, Di Cosmo et al (Di Cosmo et al. 1998), found that the Progesterone receptor (PR) in *O. vulgaris* was strictly specific to P, and that it had a similar affinity for P as the vertebrate PR. A similar steroid specific binding assay also found E2 binding in the cytosol (but not the nuclear fraction) of *O. vulgaris* ovary and oviduct. They also found immunoreactivity to oestrogen receptor antibodies localised in the nuclei of follicle cells in the ovary and in oviduct cells (Di Cosmo et al. 2002). Other immunoreactivity studies have indicated the existance of possible ERs in *P. yessoensis* (Osada et al. 2003) and *M. galloprovincialis* (Canesi et al. 2004). Stefano et al (Stefano et al. 2003) found E2 to stimulate cell membrane receptors (in ganglia tissue) to release nitric oxide (NO) in *Mytilus edulis* in a similar manner to human tissue (Stefano et al. 2000). This response was inhibited by tamoxifen (a selective oestrogen receptor modulator, in invertebrates) suggesting the existence of ER cell membrane receptors in both molluscs and man.

Using molecular techniques Thornton et al (Thornton et al. 2003) were the first group to clone a non-vertebrate ER ortholog in the sea hare *A. californica*. The DNA binding domain (DBD) was very similar to that of vertebrates, but the ligand-binding domain (LBD) was found to be quite different and the ER did not to bind steroid estrogens. Therefore these receptors are often known as 'ER-like'. Further research identified molluscan ER-like orthologs in *O. vulgaris* (Keay et al. 2006), *Thais clavigera* (Kajiwara et al. 2006), *C. gigas* (Matsumoto et al. 2007), *Nucella lapillus* (Castro et al. 2007a), *M. cornuarietis* (Bannister et al. 2007) and *I. obsoleta* (Sternberg et al. 2008b). The majority of these ER-like nuclear receptors were preferentially expressed in reproductive tissues. However, none have been shown to bind to17β-estradiol. The retinoid X receptor (RXR) which has the possible natural ligand 9-*cis* retinoic acid (a form of Vitamin A) has also been found in molluscs. The RXR was first cloned by Bouton et al (Bouton et al. 2005) in the freshwater snail *Biomphalaria glabrata* and subsequently in the marine snails *T. chavigera* (Nishikawa et al. 2004) and *I. obsoleta* (Sternberg et al. 2008a). RXR gene expression was significantly higher in male *T. chavigera* than females, and was preferentially expressed in the penis and vas deferens (Horiguchi et al. 2007). Sternberg et al (Sternberg et al. 2008a) also found RXR mRNA to increase with reproductive tract recrudescence in both male and female *I. obsoleta* although at slightly different timings. Other receptors have also been investigated in molluscs. There is evidence of endogenous thyroid hormone (TH) and a peroxidase gene (AcaTPO), similar to that of vertebrates in the sea hare *A. californica* (Horiguchi et al. 2007, Heyland et al. 2006). Moreover Wu et al (Wu et al. 2007) cloned a Thyroid receptor (TR) ortholog from the limpet *Lottia gigantean* and Brown et al (Brown et al. 1995) reported on a possible Aryl hydrocarbon receptor (AhR) in the clam *Mercenaria mercenria*. The possibility that the expression of the AhR was linked to gonadal tumours found in association with polluted sites was also reported. An AhR was also successfully cloned from *M. arenaria*, however it did not bind to known ligands for vertebrate AhR (Butler et al. 2001). To date an Androgen receptor (AR) has not been cloned from a mollusc; Sternberg et al (Sternberg et al. 2008a) attempted to identify one without success from *I. obsoleta* and in our own laboratory, many attempts to clone a mollusc AR were also unsuccessful (Jobling and Routledge. pers com 2008).

1.8 Aims

The aim of this research is to determine if chemicals known to be oestrogenic to vertebrates such as fish, and to be present in UK Treated sewage effluents (TSE) and rivers, have reproductive or sexual developmental affects on two species of UK native freshwater gastropod mollusc.

2 Introduction to the Test Species and Preliminary Work

2.1 Introduction to Gastropods

Two species of fresh water gastropod molluscs were used in my research, one is a pulmonate and the other is a prosobranch. This section will start with a more detailed overview of the physiology and anatomy of these mollusc species, followed by details of experimental methods applied.

2.1.1 Pulmonates

The pulmonata are comprised of two groups; the Stylommatophora (eyes on the end of the tentacle) and the Basommatophora (eyes at the base of the tentacle). The Stylommatophora are entirely terrestrial (land slugs and snails) and the Basommatophora are mainly freshwater animals (snails) inhabiting ponds, ditches, rivers and lakes (Geraerts and Joosse 1984). There are a few marine and estuarine Basommatophorans, and even fewer terrestrial ones. Pulmonates breath air via a type of lung, which enables Basommatophoran snails to populate still waters with little dissolved oxygen content, and allows the Stylommatophora to breath air on land.

The pulmonate used in my research was a freshwater Basommatophoran, and this will be the focus of this section. The advent of molecular phylogeny has resulted in malacologists constantly updating and rearranging the classification of snails. Indeed it is doubtful that the Basommatophora are a true monophyletic group (Geraerts and Joosse 1984), so to avoid confusion I will continue to use the terminology above.

All Pulmonates are hermaphrodites, in which male and female gametes are produced within a single ovotestis before passing along a hermaphrodite duct (Figure 2.2). The gametes are then transported along separate paths (either functionally or physically), towards one or two genital openings. The junction of the hermaphrodite duct with the male (vesiculae seminalis) and female (ootheca) ducts is often referred to as the Carrefour, and at this point the albumen gland and fertilization pocket (receptaculum seminalis) also diverge (Geraerts and Joosse 1984). The female duct is largely glandular and secretes a number of nutritive and packaging materials to support and protect the developing embryos. The female duct also receives (and often stores) sperm from a copulative partner. The main function of the male duct is to transport sperm to the copulative partner. A prostate gland, sperm duct, penis and vas deferens are all generally present, although a wide range of species-specific variation in these structures occurs (Geraerts and Joosse 1984).

The ovotestis is made up of a number of sub-units called acini. Each acini produces both male and female gametes and contains their supporting cells; Sertoli and follicle cells, respectively (Figure 2.2). The epithelium of the luminal end of the acini contains both ciliated and non-ciliated cells. Male and female sex cells and their supportive cells originate from the germinal epithelial ring, a narrow band of cells extending from the luminal end of the basement membrane towards the middle region of the acini (De Jong-Brink et al. 1976). As the gametes mature they, and their supportive cells, move along the basement membrane to the bottom of the acinus (known as the vitellogenic area); this area is where oocytes and sperm mature. During their development oocytes become surrounded by the follicle cells, and in riper stages a cleft (or follicular cavity) is formed between the oocyte and the follicle cells. These stages led de Jong-Brink et al (De Jong-Brink et al. 1976) to classify five stages of oocyte maturation; stage 1 (oogonium) have no follicle cells, stage 2 follicle cells attach to the oocyte, stage 3 follicle cells surround the oocyte, stage 4 apical cleft begins to form and stage 5 a distinct apical cleft has formed. After stage 5, oocytes are either ovulated or degenerate (Figure 2.2).

During spermatogenesis the Sertoli cells maintain contact with the underlying tissue. The spermatogonia undergo mitotic division to give rise to more Spermatogonia that increase in size and then undergo meiosis I, and transform into spermatocytes. These then undergo meiosis II, which produces spermatids. Sertoli cells never divide during spermatogenesis and they are in constant contact with the developing gametes (De Jong-Brink et al. 1977). The Sertoli cells move, while carrying the developing male sex cells, to the bottom of the acini, where they lie on top of the follicle cells of vitellogenic oocytes. It is here the sperm are released (Figure 2.2) (De Jong-Brink et al. 1977).

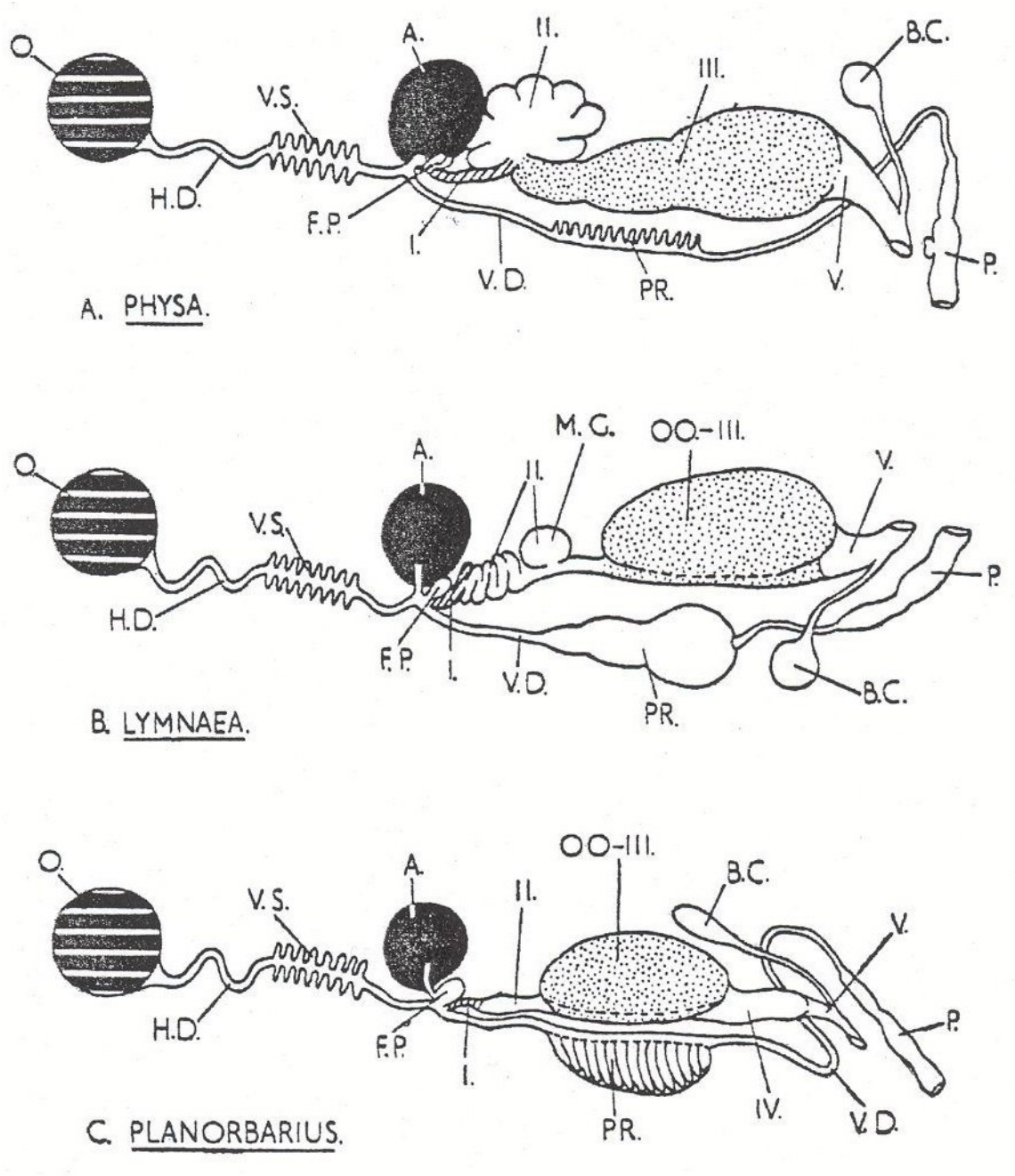

Figure 2.1 Diagram representing three families of Basommatophora hermaphrodite reproductive systems.

A Physa sp, B Lymnaea sp, C Planorbarius sp. Ovotestis, O; hermaphrodite duct, H.D.; vesiculae seminales, V.S.; fertilisation pocket F.P.; albumen gland A; ootheca OO (I, II, III, IV, V); vas Deferens V.D.; muciparous gland M.G.; Prostate P.R.; bursa copulatrix B.C.; vagina V; penis P. (Duncan, (Duncan 1975)).

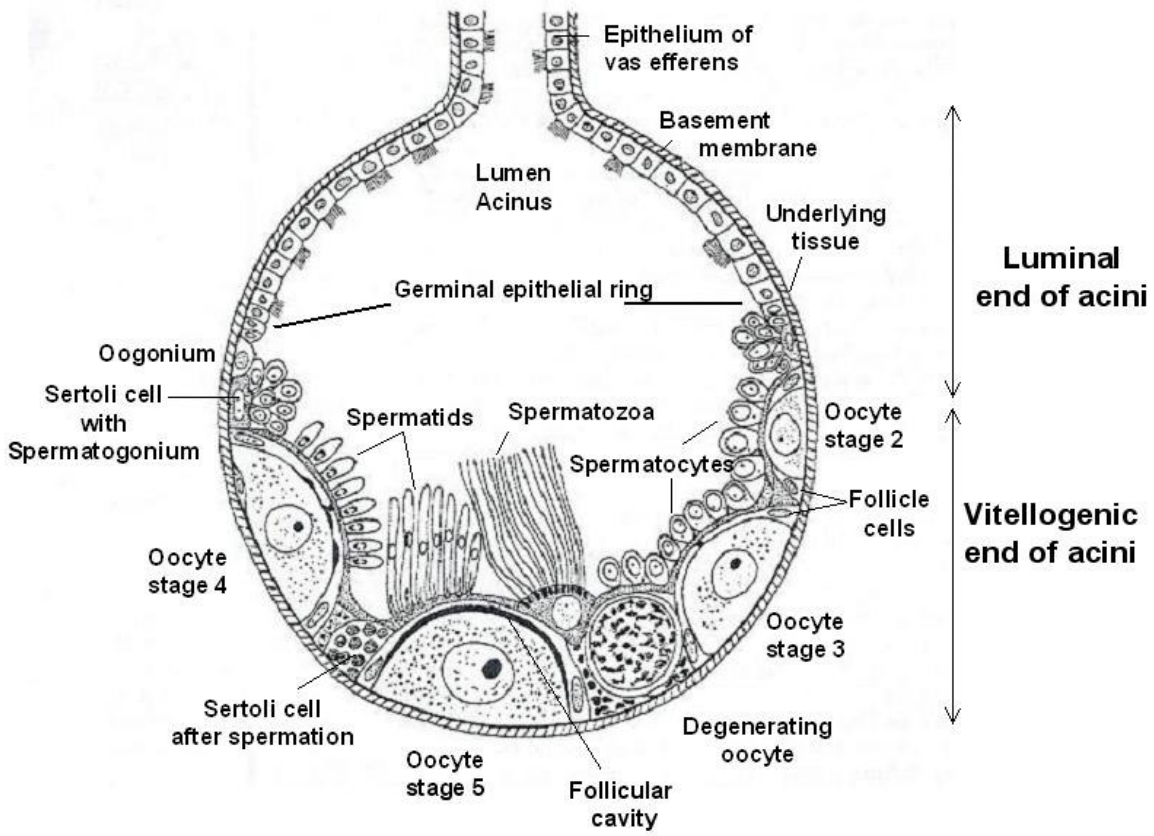

Figure 2.2 Diagram of acini from a Basommatophora ovotestis.

The ovotestis is made up of a number of acini containing both male and female gametes and their corresponding supportive cells (Sertoli and follicle cells). During early stages of gametogenesis, oogonium and spermatogonium are present at the luminal end of the acini, and as they mature migrate with their supportive cells to the vitellogenic area of the acini. Mature sperm are released into the acini lumen by their Sertoli cells. Mature (stage 5) oocytes erupt from their position in the vitellogenic area into the acini lumen, after which both sperm and oocytes are transported via the hermaphrodite duct to their separate (male or female) pathways (De Jong-Brink et al. 1976).

The neuro-endocrine control of reproduction in Basommatophora has been extensively studied in *Lymnaea stagnalis*; an egg laying Pulmonate, which inhabits freshwater lakes, pond and ditches in temperate (Northern Europe) climates. Research suggests there are six areas involved in egg laying in *L. stagnalis*: the dorsal bodies (DB), the caudodorsal cells (CDC), the lateral lobes (LL), the ovotestis, the hermaphroditic duct, and the accessory sex organs (e.g. albumen gland) (Joosse 1984). The DB, CDC and LL are all found in the cerebral ganglia of the snail's brain (Figure 2.3). The LL control much of female reproduction as they stimulate both the DB and CDC, to release dorsal body hormone (DBH) and caudodorsal hormone (CDCH). DBH stimulates the growth and differentiation of the female accessory sex organs and also stimulates vitellogenesis in the ovotestis, and CDCH stimulates a set of egg laying behaviour (see Figure 2.3) and ovulation: reviews in Ter Maat and Wayne (Ter maat 1992, Ter Maat et al. 2007, Wayne 2001). Recent evidence

suggests a role for the neuro-transmitters dopamine and serotonin in mollusc reproduction (see mollusc endocrinology Chapter 1, Section 1.7). In the albumen gland of *B. glabrata* dopamine levels were highest during packaging of eggs (Boyle and Yoshino 2002) AND dopamine stimulated protein secretion in cultured albumin gland explants in the same species (Santhanagopalan and Yoshino 2000). Serotonin exposure was also reported to increase egg laying in *B. glabrata* and blocking the serotonin receptor prevented egg laying for a week (Muschamp and Fong 2001).

Reproduction is highly seasonal in *L. stagnalis* starting in spring (May) and continuing throughout summer until early to mid September (Dogterom et al. 1985). Environmental stimuli such as temperature (Joosse and Veld 1972), photoperiod (Bohlken and Joosse 1982), dissolved oxygen (Ter maat et al. 1983) and nutrition (Scheerboom, 1978) appear to have the greatest modulating effects on reproduction and egg laying (Ter maat 1992, Ter Maat et al. 2007). Laboratory studies conducted in seven day static renewal systems revealed that snails would lay egg masses soon after tanks were replenished with clean water, and additional experiments found it was the increased dissolved oxygen that stimulated this. Clean water stimulus (CWS), via some unknown route, stimulates the CDCs, which causes the release of CDCH and the stimulation of oocyte release from the ovotestis and egg laying behaviour (Figure 2.3). This stimulatory mechanism has enabled scientists to fully eludate the timing and process of egg mass formation (Figure 2.3C). The effects of day length and temperature have also been investigated with relation to egg laying (Bohlken and Joosse 1982). It has been found that warmer temperatures and longer day length both stimulate egg laying (as would be expected in a temperate seasonal reproducer). However, day length seems to be the overriding factor. Snails kept at 8^{o}C and short day length (8 hours light: 16 hours dark) or medium day length (12L:12D) did not lay egg masses, whereas those kept at long day (16L:8D) did lay egg masses, but at a lower rate than if they were kept at warmer temperatures. Long day length also overrides the negative impact of starvation on egg laying compare to short or medium day length on starved snails (()). Dogterom et al (Dogterom et al. 1983) found that snails kept at long day length were slightly more sensitive to hormonal induction of egg laying compared to short or medium day length. However, the exact mechanism of how this happens has not been fully deduced.

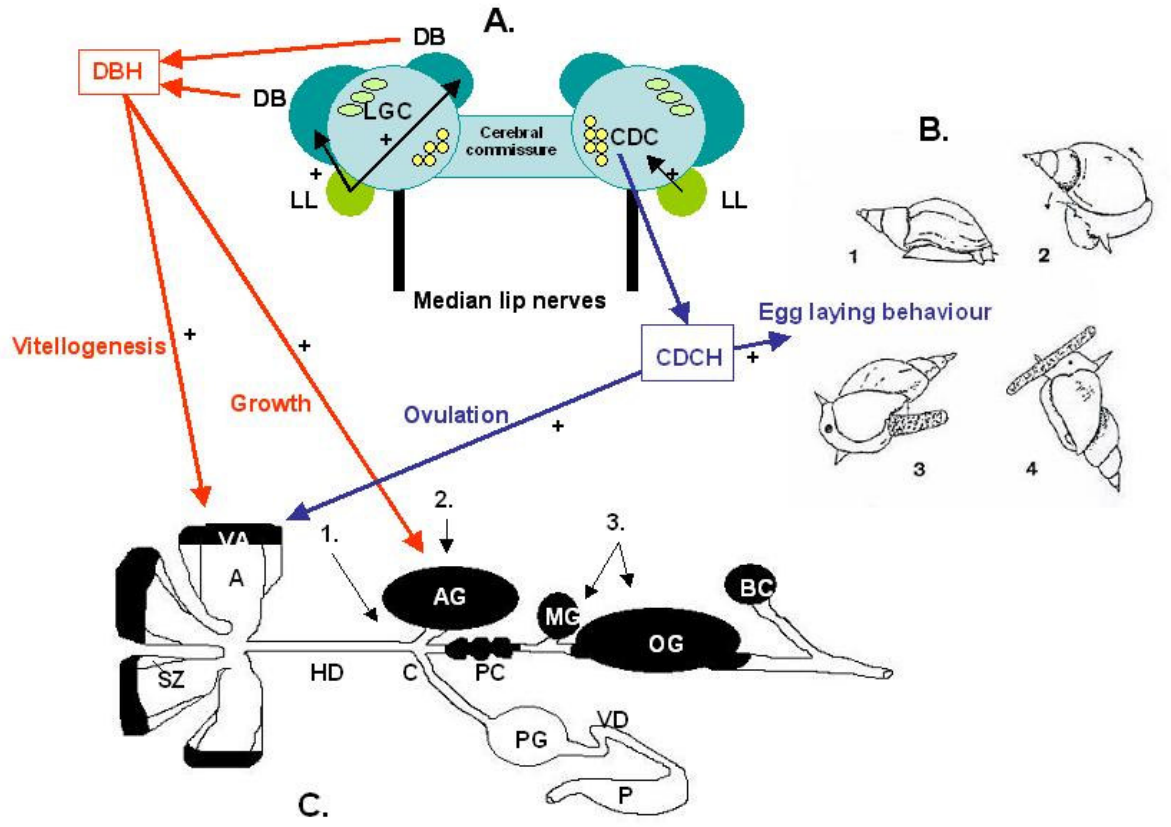

Figure 2.3 Diagrammatic representation of a transverse section through the cerebral ganglia of *Lymnaea stagnalis,* the organisation of the female endocrine system and its effects on female reproduction (egg laying).

A, Neurons in the lateral lobes stimulate the dorsal bodies and the caudodorsal cells to produce hormones. Dorsal body hormone (Red arrows) produced by the dorsal bodies stimulates vitellogenesis of oocytes in the ovotestis and the growth and differentiation of the female accessory sex organs (e.g. Albumen Gland). Caudodorsal cell hormone (blue arrows) produced by the caudodorsal cells stimulates ovulation and egg laying behaviour. + indicates stimulation. Caudodorsal cells, CDC; Caudodorsal cell hormone, CDCH; Dorsal bodies, DB; Dorsal body hormone, DBH; Light green cells, LGC; Lateral lobes, LL; (Adapted from Ter Maat, (Ter maat 1992) and Wayne (Wayne 2001). B, Phases of egg laying behaviour; 1 Resting (~50 min), 2 Turning (duration depends on size of egg mass 20-120 min), 3 Oviposition (duration depends on size of egg mass 5-15 min), 4 Inspection, usually follows oviposition (From (Ter maat 1992)). C, The various stages of egg mass formation; Oocytes mature in vitellogenic areas (VA) in the acini (A) of the ovotestis (O). 1 after ovulation the oocytes are transported via the hermaphrodite duct (HD) towards the carrefour (C), where fertilisation takes place. 2 during egg formation each egg cell is surrounded by secretion from the albumen gland (AG) and by two egg membranes secreted by the pars contorta (PC). 3 the egg mass is formed by addition of secretory products from the muciparpus (MG) and oothecal glands (OG). Bursa copulatrix, BC; penis, P; prostate gland, PG; sperm duct, SP; spermatogenic zone, SZ; vas deferens, VD; vesicuae seminales, VS (from Dogterom et al, (Dogterom et al. 1983); Joosse, (Joosse 1984)).

The male reproductive process in Pulmonates has been less studied, although a number of observations have been made. Joosse (Joosse 1964) found low temperatures to in inhibit spermatogenesis both wild and laboratory snails. Only spermatogonium and primary spermatocytes were found in snails kept under low temperature (<10^{o}C) conditions for long periods of time, suggesting spermatogenesis is temperature dependant but only at the stage of meiosis. Similar inhibition is seen in the pulmonate land snail *Helix aspersa* (Joosse 1975). As with terrestrial pulmonates, Joosse hypothesised that an androgenic brain factor may be involved in stimulating spermatogenesis (Geraerts and Joosse 1975, Boer and Joosse 1975). In contrast de Jong Brink et al (De Jong-Brink et al. 1981) suggested a steroid may be produced by the Sertoli cells, which then acts locally on spermatogenesis. Romanova et al (Romanova et al. 1996) found injecting baclofen (a Gamma-aminobutyric acid B receptor agonist) provoked a full erection in *L. stagnalis*, and more recently Muschamp and Fong (Muschamp and Fong 2001) found that blocking the serotonin receptor (Methiothepin) in *B. glabrata* induced penile erection that lasted up to 48 hours.

2.1.2 Prosobranchs

Prosobranch gastropods have quite varied reproductive biology compared to other molluscs. They exhibit two types of organisation; the basic diotocardian, in which the opening of the functional right kidney discharges gametes and fertilisation is external (i.e. sperm or eggs are generally released directly into the sea), and the more advanced monotocardians which have more glandular pallial genital ducts running parallel with the rectum, and practise internal fertilisation (Fretter 1984). Prosobranchs are the most primitive of the gastropods and are mainly gonochoristic, although in some species sex reversal does occur (Fretter 1984). In addition to the normal sexual reproduction of prosobranchs, some species exhibit parthenogenesis; one such species is *Potamopyrgus antipodarum* a native of New Zealand, which has successfully spread across Australia and Europe. In its native habitat, populations have higher numbers of males and reproduce sexually. However, in populations without males, or very low frequencies of males (as found generally in Australia and Europe) females reproduce parthenogenically. These parthenogenic groups have been found to have different numbers of chromosomes (Wallace 1992).

The second test species used in my research was a monotocardian Prosobranch, and again, the introduction will focus on this type of animal. In male monotocardians, the right kidney is not developed and instead has a long testicular duct, which also acts as a vesicula

seminalis. The testicular duct runs dorsally along the visceral mass and consists of branched tubules. At the distal end of the duct is the vas deferens, which is a short ciliated duct leading to the pallial duct and the prostate (Fretter 1984). The organisation of the male ducts differs between species, but usually sperm is produced in the tubules and passes along the sperm duct (via the vas deferens and the prostate), where secretions are added to the sperm. Sperm then passes through the duct of the penis, which is muscular, vascular and often very large. It is found on the right hand side of the snail's head, either behind or incorporated into the cephalic tentacle (Fretter 1984). Due to the complexities of internal fertilisation, female monotocardians have more elaborate reproductive systems than those of the diotocardians. Again there are variations between different species including the position and number of sperm storage/fertilisation pockets. However, generally the female system consists of an ovary at the proximal end of the reproductive tract, which runs straight along the ventral part of the visceral mass. Mature oocytes are released from the ovary along the oviduct to the fertilisation pocket (receptaculum seminalis). Fertilised embryos then travel along the oviduct to the albumen gland, where nutritional secretions are distributed by cilia, and muscle moulds them around the egg. Embryos then continue to the capsule gland, which provides the necessary protection from osmotic pressures and bacterial infection. Eggs are then either covered in another jelly secretion (in which the capsules are embedded and moulded into an egg mass), or are harboured within a brood pouch modified from the pallial duct. Both the egg mass or mature hatchling snails exit the pallial duct via the genital opening/vaginal passage (Fretter 1984).

The ovary and oogenesis were studied in the deep-sea prosobranch *Bathynerita naticoidea*, in which the ovary was described as an acinous organ containing oocytes in all stages of oogenesis. Follicle cells were found to be closely associated with the developing oocytes (Eckelbarger and Young 1997). Oogenesis has also been studied in the marine prosobranch *Ilyanassa obsoleta* (Taylor and Anderson 1969). Early pre-vitellogenic stage groups of oocytes were joined together by maculae adhaerentes (spot desmosome) which were not found in more developed stages. Prior to vitellogenesis the oocytes were seen to enlarge greatly. Once vitellogenesis commenced the area where oocytes were previously attached turns into a cleft, which increases in size throughout vitellogenesis. Post-vitellogenic oocytes were characterised by a large eccentrically placed nucleus and a distinct nuclear envelope. Follicle cells were found to incompletely cover the oocyte, and during oocyte maturation spread over the oocyte so that pre-vitellogenic oocytes had follicle cells two to three cells thick whereas vitellogenic oocytes had follicle cells one or two cells thick. It was also found that follicle cells were in much closer association with pre-vitellogenic

oocytes than those of oocytes having completed vitellogenesis. Follicle cells were implicated in oocyte nutrition and protein secretion due to their structural similarities to those of vertebrates (Taylor and Anderson 1969).

Walker and Macgregor (Walker and MacGregor 1968) investigated spermatogenesis in *Nucella lapillus* a marine prosobranch. They found spermatogenesis to be very similar to that seen in vertebrates in that spermatogonia undergo mitotic division to give rise to more spermatogonia, which then divide into spermatocytes, which undergo meiosis producing spermatids, which in turn become spermatozoa. The gonad was made up of a number of branched tubules that contained spermatogonia, spermatocytes, spermatids and mature sperm. The spermatogonia were found in groups around the periphery, and the mature sperm clustered in the centre of the tubule with the tails towards the lumen. Groups of spermatocytes and spermatids were found in clusters scattered throughout the tubules, it was also found that cells within a group passes through spermatogenesis at the same time (Walker and MacGregor 1968). Some prosobranch gastropods, in addition to producing fertile eusperm, also produce infertile parasperm (Buckland-Nicks 1998). Parasperm is associated with internal fertilisation and sperm competition within the female. A number of possible types of parasperm have been identified including those that transport eusperm to an optimal pre-fertilisation site, those that reduce the female's drive to re-mate; either via chemical interactions or via 'nuptial gifts', some form a plug which prevents subsequent mating success by another mate, and others create an hostile environment for incoming rival sperm (Buckland-Nicks 1998).

Unfortunately, detailed studies of the endocrine system of Prosobranch gastropods have not been carried out. Therefore, their endocrine control is somewhat more of a mystery. This is partially due to few prosobranch species lending themselves to laboratory culture in the way many pulmonates do. Moreover, their nerve cells, which may provide neuro-hormones are relatively small, and therefore more difficult to work with (Fretter 1984). Reproduction in several marine species is tied to lunar and tidal changes, and many species exhibit reproduction in relation to temperature and nutritional status (Fretter 1984). Some information is available as highlighted in the mollusc endocrinology section above. Perhaps counter intuitively, most of the literature on vertebrate-type steroids and their receptors in gastropods are associated with Prosobranchs, especially marine Prosobranchs. ER-like receptors have been found in *Thais clavigera* (Kajiwara et al. 2006), *Nucella lapillus* (Castro et al. 2007a), *M. cornuarietis* (Bannister et al. 2007) and *I. obsoleta* (Sternberg et al. 2008b). This is likely due to where researchers have focused their efforts,

rather than a lack of receptors in pulmonates. Indeed, the first gastropod ER-like was found in the hermaphrodite Opisthobranch (sea hare) *Aplysia californica*, and other receptors (such as the RXR) have been found in both pulmonate and prosobranch species.

2.2 Introduction to the test species

2.2.1 Test species 1 – *Planorbarius corneus* (Pulmonate)

Below is an introduction to the first of the two freshwater snail species used in this research.

2.2.1.1 Ecology, habitat and distribution

Planorbarius corneus or the Greater Ramshorn snail is one of Britain's largest species of fresh water Pulmonate snail growing to a size of 25-35mm in diameter. As with all Pulmonate gastropods *P. corneus* has a 'lung' or pulmonary cavity, which is connected with the outside via the pneumostome and gives it the ability to respire atmospheric air (Fretter 1975). The mantle cavity is filled with air, allowing the snail to descend to the bottom of ponds or stagnant pools to feed on submerged aquatic plants or decaying matter. Intermittently the snail will surface to refill its air supply. In addition to this it also has a slip of mantle which is highly vascularised which, protruded into the water, may act as an accessory gill (Fretter 1975). *P. corneus* is found in ponds and slow moving bodies of water, such as canals and drainage ditches, throughout most of central and eastern England. It is less numerous in northern, south-western England, Wales and Ireland (Kerney 1999) see Figure 2.4.

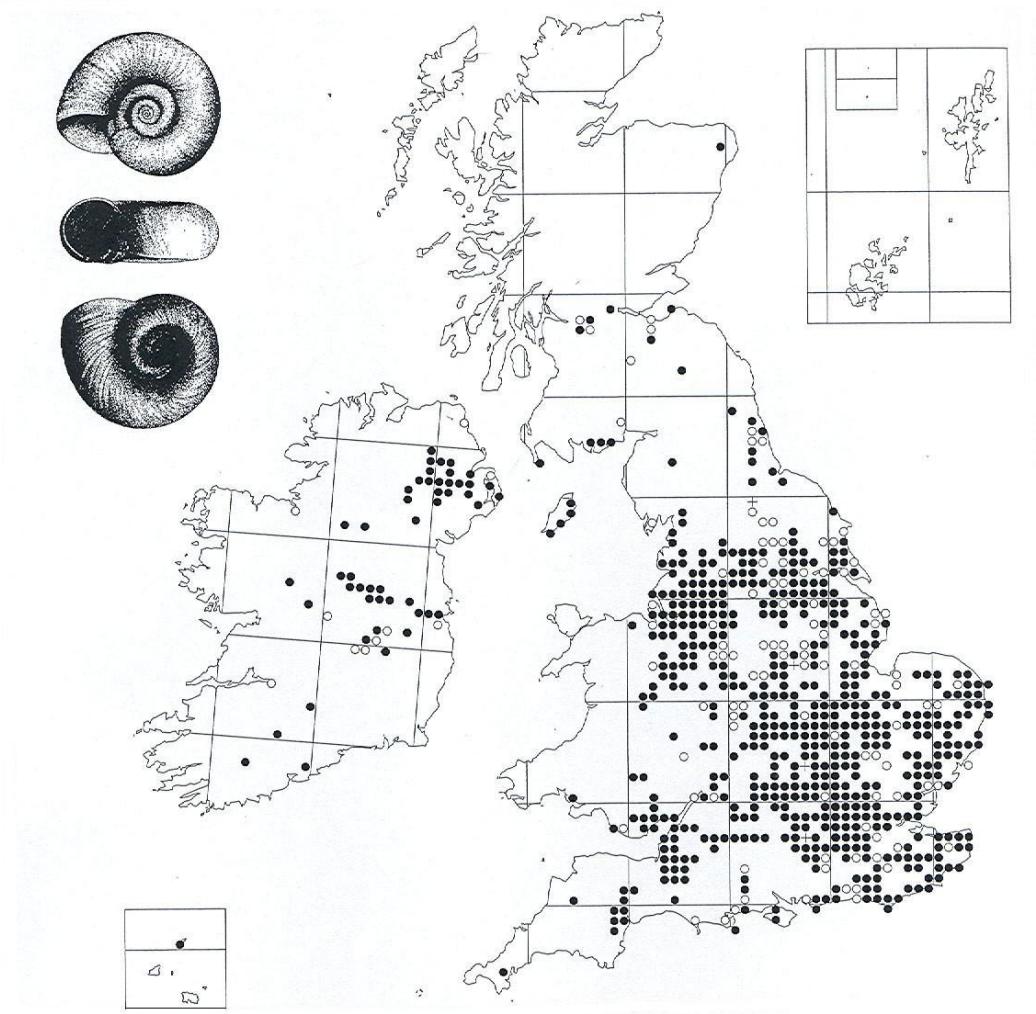

Figure 2.4 Map of the UK distribution of the Great ram's-horn snail, *Planorbarius corneus*.

Filled circles are records made in or after 1965, open circles are records made prior to 1965, crosses indicate fossil occurrence (lateglacial to postglacial) (from Kerney, (Kerney 1999)).

2.2.1.2 Life cycle and reproduction

The life history and reproduction of *Planorbarius corneus* have not been widely studied in the field. A brief study (June-September 1962) conducted by Berrie (Berrie 1963) on a population in a pond near Reading concluded *P. corneus* had an annual life cycle in the wild. From Berrie's observations, the species had a restricted period of reproduction concluding by mid June, the adults died before August, to be replaced by their progeny the following year. A more comprehensive study by Costil and Daguzan (Costil and Daguzan 1995b) conducted fortnightly over 17 months, found a much more complex life strategy. Cohorts were designated to snails of similar size to determine when each group of snails were laid, empty shells (dead snails) were also analysed, along with the presence of egg masses. *P. corneus* were seen to reproduce from the beginning of May until November. It was found that *P. corneus* life-cycle had some adaptive plasticity depending on

environmental conditions; an annual life cycle with two generations was observed in 1987, whereas only one spring generation seemed to be present in 1986 and 1988 (Costil and Daguzan 1995b). It was concluded that high summer mortality of larger snails was due to reproductive effort, whereas smaller snails die from probable predation. It was also recognised that the extreme climatic conditions (cold and hot) as well as lack of food and mineral resources had particularly unfavourable effects on population densities of *P. corneus* (Costil and Daguzan 1995b). As with many pulmonate gastropods, *P. corneus* is easily cultured in the laboratory (Fretter 1975); they have no larval stage and young hatchling snails feed on the same herbivorous diet as the adults. Under laboratory conditions *P. corneus* has been found to live to over 4 years old at 15^{o}C (Costil and Daguzan 1995a) and once sexually active to breed more or less continuously until old age. *P. corneus* generally lays flat almost concentric circular egg masses only one egg deep. The eggs are slightly orange in pigmentation and are encased in a gelatinous mass. This mass sticks the eggs to rocks or foliage in the wild or any hard surface such as glass or pipe work in the laboratory (personal observation). As mentioned above, the embryonic development of *P. corneus* as with other freshwater Pulmonates is direct and takes place within eggs. The eggs comprise of a zygote surrounded by perivitelline fluid and a membrane, embedded in jelly and enclosed in a common egg mass (Costil 1997). The development of embryos located in the same egg mass is not synchronous. The time between the first egg hatching and the last can be as much as 12 days at low temperatures and less than one day at 15-25^{o}C (Costil 1997).

2.2.1.3 Reproductive Morphology and Physiology

The Hermaphrodite system

Like other Basommatophora gastropods, *P. corneus* is a simultaneous hermaphrodite and therefore its reproductive system is physiologically complex. Both male and female gametes are produced in a single ovotestis, which travel along the oviduct before separating into the male or female ducts Figure 2.1C. At the junction (Carrefour) of the male, female and hermaphrodite ducts the fertilisation pocket and the albumen gland are also found, in this region it has been particularly difficult to ascertain details of morphological and functional roles (Fretter 1975). Duncan (Duncan 1960) studied both the morphology and histology of the reproductive system of *P. corneus* and found an intimate association between the male and female systems compared to that of other Basommatophora such as Lymnaea, Physa or Ancylus. The vas deferens is embedded in the surface of the oothecal (or capsule gland) and the oviduct passes right through the

prostate (Duncan 1960). This makes it difficult to dissect out each individual part without damaging the tissue (personal observation).

Male gametes (produced in the ovotestis) pass along the hermaphrodite duct to the vas deferens, via the prostate gland before reaching the muscular penis, which enables internal fertilisation to occur. The female gametes (produced by the ovotestis) pass first into the fertilization pocket and then to the albumen gland, which adds a nutritive secretion to the fertilized egg. The egg then passes along the oviduct on to an area of oothecal (or capsule) gland, which gives the egg its protective wrapping, after which it passes through another part of the oviduct out to the vagina. The oviduct of Basommatophora have been found to be more complex than in other Pulmonates (e.g. terrestrial). This is probably associated with the production of more elaborate egg capsules appropriate to fresh water (Fretter 1975) and extended nutrition for direct development within the egg.

The ovotestis

The ovotestis of *P. corneus* is similar to that of other Pulmonates. The ovotestis is comprised of a number of acini, each containing both male and female gametes at various stages of development along with their supportive Sertoli and follicle cells (Figure 2.2). See Section 2.1.1, for detailed description and diagrams of Pulmonate ovotestis. Timing and growth of male and female gametes in the pulmonate are thought to be controlled by a range of hormones, which are stimulated by both biotic and a-biotic factors (see Section 2.1.1 and Figure 2.3 for details).

2.2.2 Test species 2 – *Viviparus viviparus* (Prosobranch)

Below is an introduction to the second of the two freshwater snail species used in this research.

2.2.2.1 Ecology, habitat and distribution

Viviparus viviparus or the 'Common River Snail' is a gonochoristic prosobranch gastropod and, like *P. corneus*, is one of the UK's largest freshwater gastropods; adults reach a size of 25-35 mm shell length (Kerney 1999). *V. viviparus* is generally found in large deep water bodies which are well oxygenated, and contain high levels of suspended organic matter such as major lowland rivers or canals, and is a characteristic species of English canal systems (Kerney 1999). Figure 2.6 shows the UK distribution of *V. viviparus*. Eleutheriadis and Lazaridou-Dimitriadou (Eleutheriadis and Lazaridoudimitriadou 1995), found that dissolved oxygen was the most significant water chemistry parameter associated with density of *V. contectus* populations in several water bodies in N. Greece. Growth

rings have been used with some success to age *V. viviparus*, although, events such as cold periods or lack of food are just as likely to produce the typical winter slowing of growth seen with growth rings, and may lead to miscalculation in age. Maximum age has been calculated using growth rings as 6 years old but with extremes as much as 11 years old (Fretter and Graham 1962). *V. viviparus* is both a grazer of algae and a filter feeder, although adults seem primarily to filter feed (personal observation). Hockelmann and Pusch (Hockelmann and Pusch 2000) found seston (food particle) removal rate increased with snail size in *V. viviparus*. Cook (Cook 1949) reported that *V. viviparus* filter feed in several positions; it was observed that by moving along the muddy bottom and pushing their foot into the mud they could stir up silt and organic matter in front of them, also they could feed on passing matter by half burying themselves in the mud with the aperture of the shell upper most and the foot protruding slightly (Figure 2.5). In both cases snails inhale water from the left hand side of the mantle, water passes over the gill and food accumulates in the 'food-collecting' gutter in the mantle cavity, at which point a mucus string is formed; the snail can then roll this into a ball and periodically turn its head to eat the accumulation (Cook 1949).

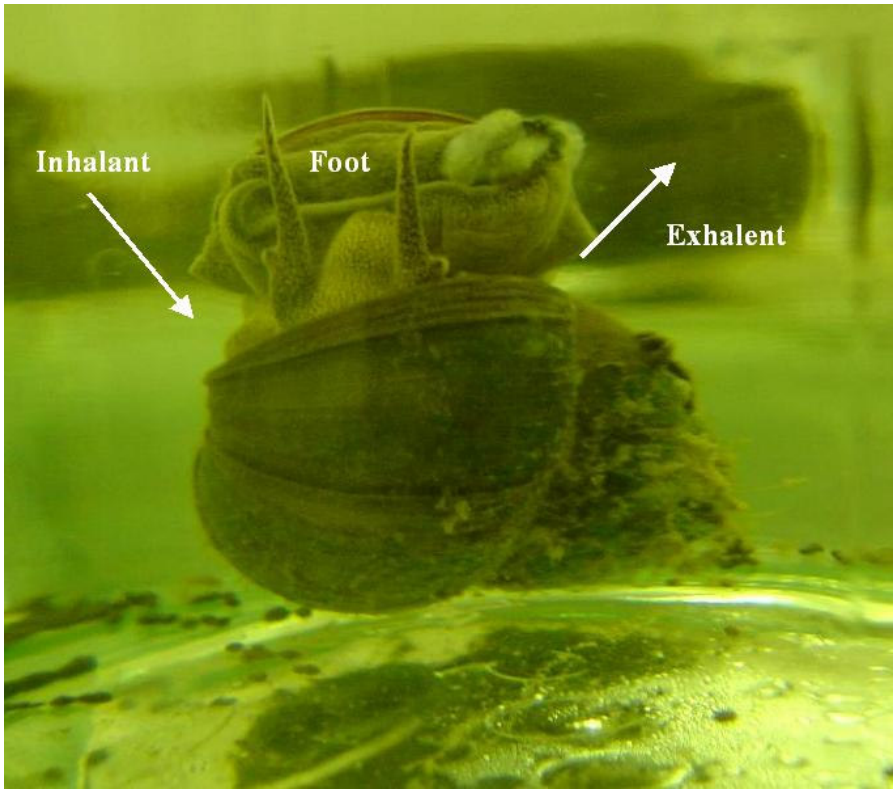

Figure 2.5 Photograph of an adult female *V. viviparus* in classic feeding position.

The snail is sitting on its back with its foot upper most. The white arrows indicate flow of water; in through the mantle cavity, over gills, and then exhaled out.

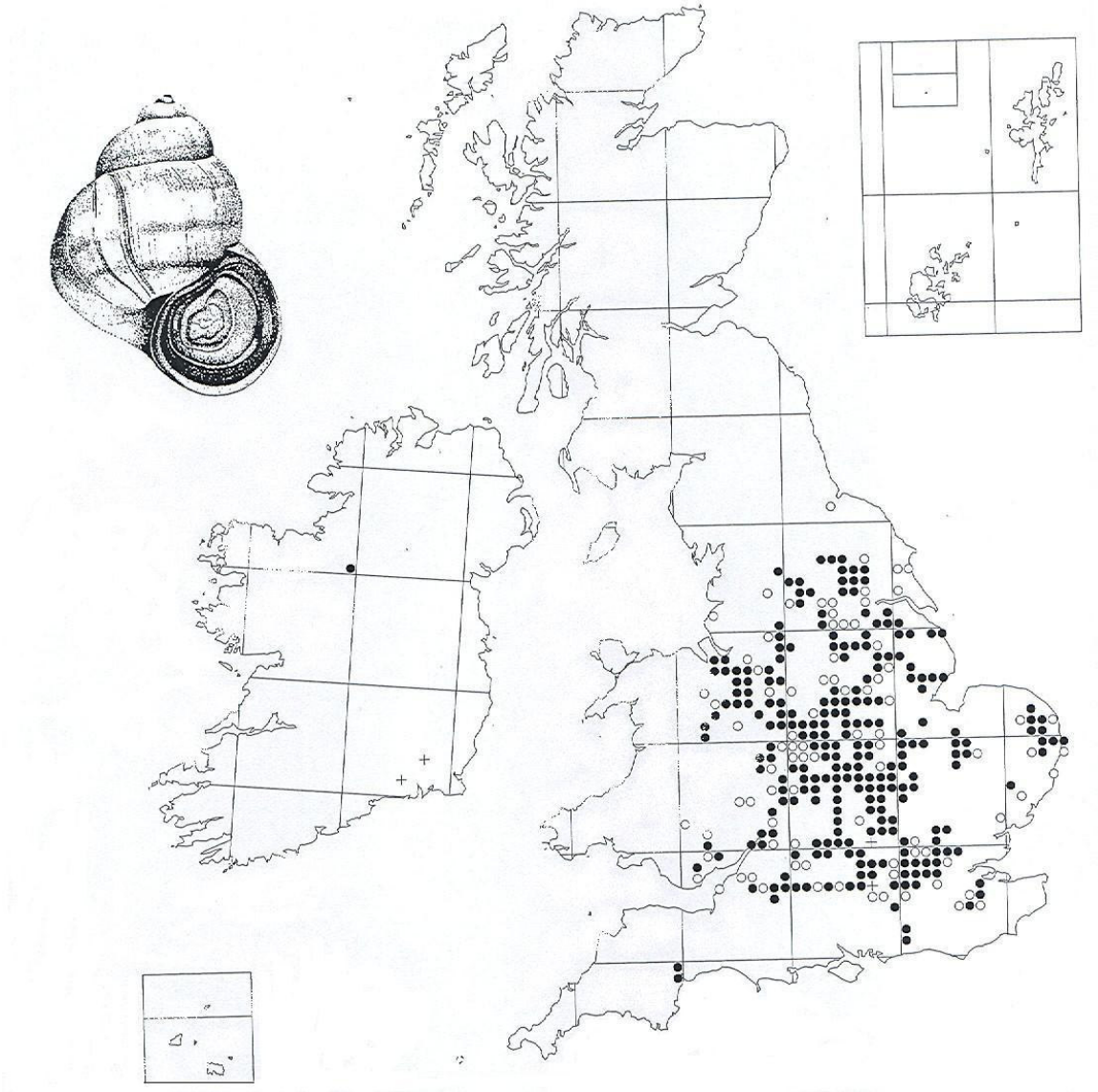

Figure 2.6 Map of the UK distribution of the Common river snail, *V.viviparus*.

Filled circles are records made in or after 1965, open circles are records made prior to 1965, crosses indicate fossil occurrence (lateglacial to postglacial) (Kerney 1999).

2.2.2.2 Life cycle and reproduction

Sex in *Viviparus* sp. has been found to be determined genetically; ZW = female, ZZ = male (Barsiene et al. 2000). Fertilisation is internal, both male and female are highly promiscuous and mate with a number of partners within each breeding season. The life cycle and reproduction of several members of *Viviparus* have been studied (including *V. viviparus*), they show similar trends; *Viviparus* sp. are relatively long lived (section 2.2.2.1 above) compared to other freshwater gastropods, and are iteroparous. In the Worcester-Birmingham canal, Young (Young 1975) described a mainly biannual life cycle, with female *V. viviparus* reaching sexual maturity above 12 mm shell length. Snails spent the spring and summer months on the canal walls and then migrated to the muddy bottom in winter. Adults reached a maximum shell length of 36 mm. Males and females are easily

distinguished by the appearance of the right cephalic tentacle; in females both tentacles are slender and pointed (Figure 2.7) whereas in males the right hand one is larger and thicker and contains the penis and penis pouch (Figure 2.7 and Figure 2.8). This sexual morphology was first described in *Viviparus interextus* by Blinney in 1865 (van Cleave and Lederer 1932). Sex differences in size and lifespan (males smaller and shorter lived than females) have also been reported in *Viviparus* sp including; *V. contectoides* (van Cleave and Lederer 1932), *V. malleatus* (Stanczykowksa et al. 1971) and *V. georgianus* (Browne 1978). There are conflicting reports for the species *V. ater*, where females were reported to be larger than males in Lake Alserio, Northern Italy (De Bernardi et al. 1976), but no difference was observed in Lakes Maggiore (Italy) and Zurich (Switzerland) (Ribi and Gebhardt 1986). Several authors report skewed sex ratios (generally biasing females) in *V. contectoides* (van Cleave and Lederer 1932), *V. georgianus* (Browne 1978), *V. contectus* (Jezewski 2004) and *V. viviparus* (Jezewski 2004, Jakubik 2006, Jakubik 2007, Jakubik 2003). These differences are attributed to a number of reasons including time of year the sampling was conducted, shorter lifespan of males or preference to differing environmental conditions (e.g. better for hatchling release).

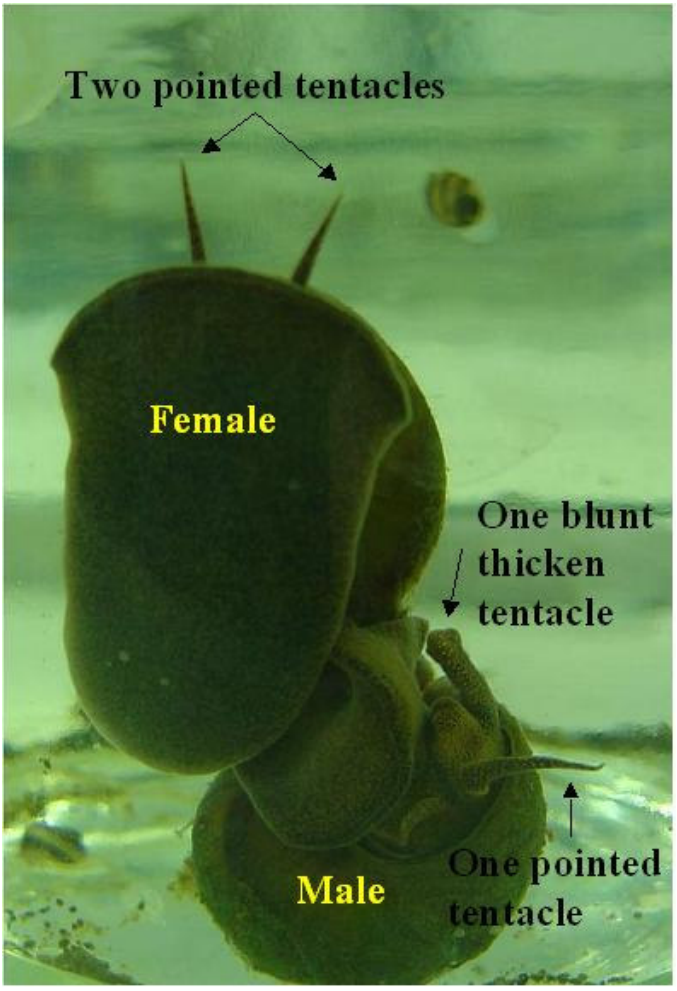

Figure 2.7 Photograph of adult male and female *V. viviparus* in a jam jar of water.

The female is distinguishable as both her tentacles are slender and pointed, whereas, the male's right tentacle is thicker and enlarged.

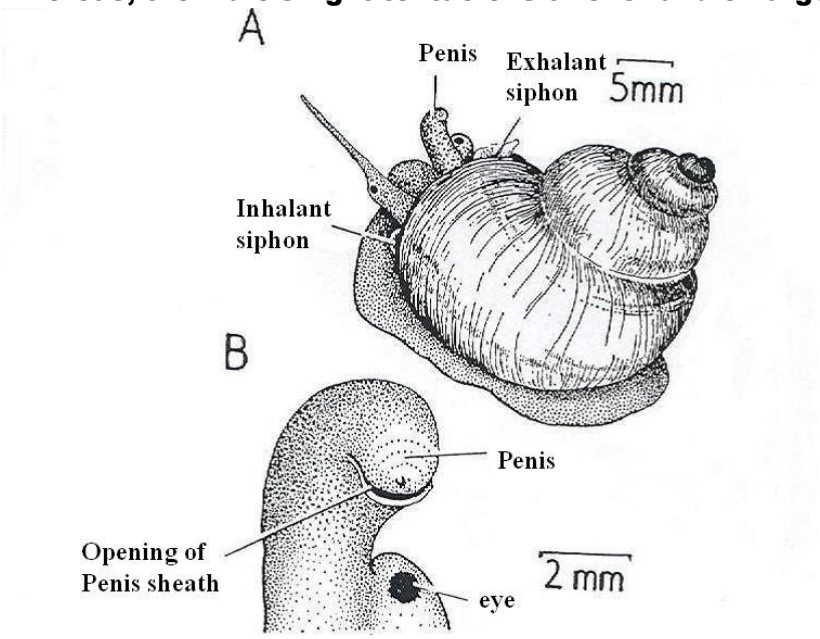

Figure 2.8 Diagram of an adult male *V. viviparus*:

(A) Whole animal. (B) details of penis, from Fretter and Graham, (Fretter and Graham 1962).

Females

During mate finding, females are passive in comparison to males (Ribi and Katoh 1998). Females can store sperm for some time, allowing continuous fertilisation of eggs, and can continue to release hatchlings when isolated for up to two years in *V. ater* (Trub and Ribi 1997). However, this may not be the preferred method of reproduction, as in the same species Oppliger et al (Oppliger et al. 2003) found that females which had multiple mating partners had more offspring than those with just one. In natural populations mating is frequent, on average 60 times each between April and November in one population of *V. ater* (Staub and Ribi 1995). Females harbour developing embryos within their brood pouch (Figure 2.9) for a number of months (depending on species and season) and give birth to well developed young; consequently fecundity is low compared to egg laying gastropods such as *P. corneus*. Embryos (Figure 2.10) harboured by females are not all the same age/maturity and are released individually when they reach maturity and when conditions are favourable (Fretter and Graham 1962). In the Worcester-Birmingham canal Young (Young 1975) found newly hatched *V. viviparus* throughout the spring and summer. A certain amount of plasticity in reproduction and life span has been observed depending on biotic and abiotic factors. Two populations of *Viviparus ater* studied in Swiss (Lake Zurich) and Italian (Lake Maggiore) lakes showed differing strategies with respect to reproduction (Ribi and Gebhardt 1986, Gebhardt and Ribi 1987), in Lake Zurich where conditions were favourable, females grew faster and larger than those from Lake Maggiore (less favourable - water temperature, algae growth, dissolved calcium and increased parasitism). Females from Lake Zurich reproduced at a constant or slightly increased rate throughout their life. Females from Lake Maggiore, however, decreased reproduction with age. In caged studies it was found that comparably sized Lake Maggiore females produced more offspring than Lake Zurich females, however Lake Zurich hatchlings were much larger and (it was presumed) required longer gestation (Ribi and Gebhardt 1986). This plasticity is favourable to population survival as females, which may suffer higher mortality or cost of survival, need to produce more offspring faster than females living under favourable conditions (Gebhardt and Ribi 1987). It was also found that in Lake Zurich two peaks in offspring release occurred (May and August) compared to one (July) in Lake Maggiore which, again, highlights the unfavourable conditions and higher cost and investment required per female to reproduce in Lake Maggiore (Ribi and Gebhardt 1986). Inter-population variation (demographic and biometric) were observed in Lake Alserio (Italy) between two populations of *V. ater* with different habitat conditions (De Bernardi et al. 1976), differences in male lifespan and female fecundity were reported by Browne

(Browne 1978) in *V. georgianus* inhabiting different lakes around New York (USA). In this study it was found that, in all four sites, female fecundity reached a maximum at two years old, and at three years old embryo numbers were either reduced (two sites) or totally absent (two sites), however, embryo size was not compared. As stated above the number of embryos harboured in the brood pouch and overall female fecundity can vary greatly with age, size and habitat conditions. In *V. viviparus* collected from the Zegrzynski Reservoir (Central Poland) female fecundity increased with shell size; no embryos were found in females under 8.1 mm shell length, embryos per female ranged from 0.9-6.7 in females 8.1-12 mm shell length, 1.1-9.6 in females 12.1-25 mm shell length and 2.0-9.1 in the largest size group 25.1-35.0 mm shell length (Jakubik 2007). In the same study area, the average the number of embryos harboured by female *V. viviparus* in outflow zones of rivers sites (high flow, increased organics) are double those in the reservoir (Jakubik 2006).

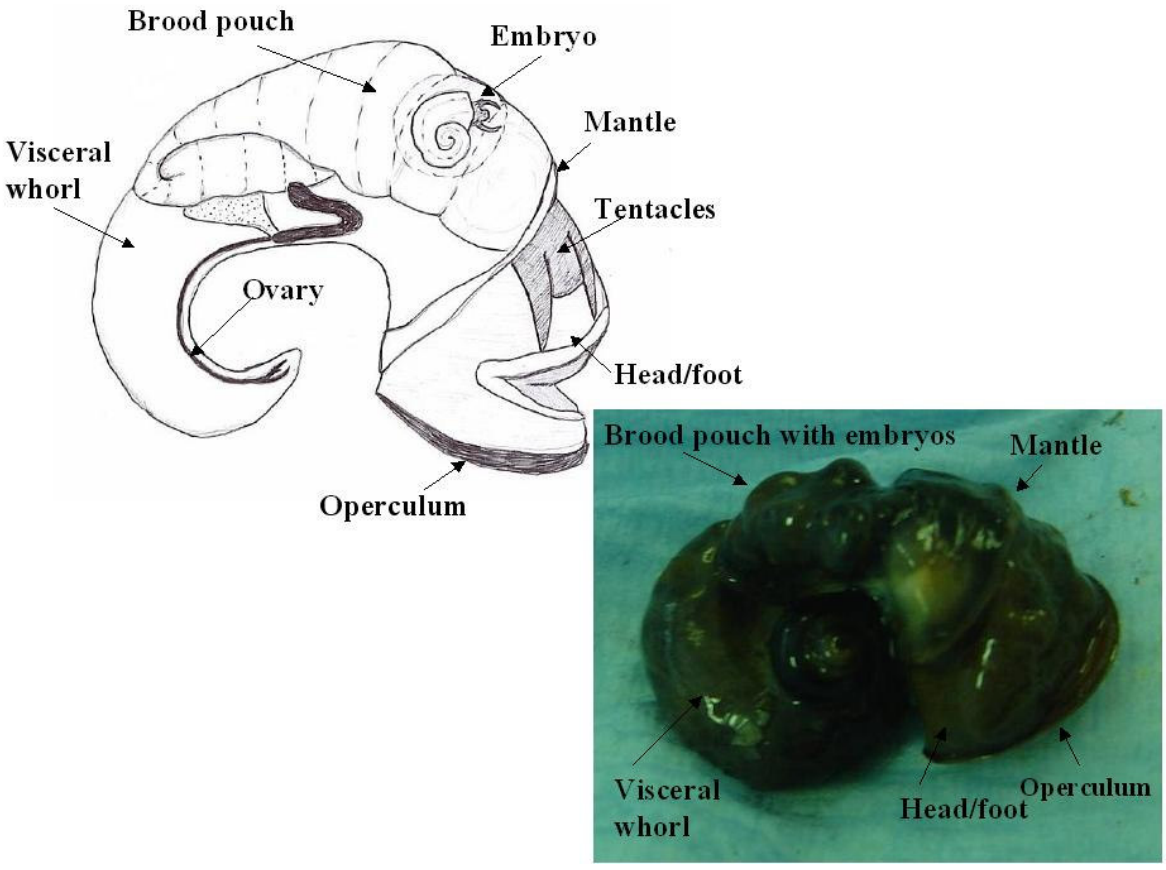

Figure 2.9 Diagram and photograph of a female *V. viviparus* with the shell removed, observed from the right hand side.

The head/foot, operculum, mantle, visceral whorl and brood pouch – with embryos inside (seen as lumps in the brood pouch in the photo) are highlighted on both the diagram and photograph. The diagram also indicates the position of the ovary within the visceral whorl. Diagram adapted from Bottke, (Bottke 1972).

Figure 2.10 Photograph of *V.viviparus* shelled embryos in a weigh boat having been dissected out of an adult females brood pouch.

The embryos range in size, with the largest almost ready to be released by the female. The distinctive strips on the shell are already visible.

Males

In many of the *Viviparus sp.* studied, males are significantly smaller than females. This variation has been ascribed by a number of authors to the different life and reproductive strategies developed between males and females. Males actively seek out mates and have been reported to move almost twice as far as females (*V. ater*; (Ribi and Gebhardt 1986)). Copulation is frequent, occurring every 3-4 days during the mating season (Staub and Ribi 1995), and it has been suggested most the time and energy invested in this, rather than feeding, may result in reduced growth rate and longevity (Browne 1978). Male *Viviparus* produce two types of sperm; normal fertile eupyrene sperm and a second non-fertile oligopyrene sperm (Oppliger et al. 1998). The exact function of this oligopyrene sperm is not known but it could provide extra nutrition to females (Oppliger et al. 1998), aid transport of eupyrene sperm (oligopyrene sperm is extremely motile), or deceive females about the quantity of eupyrene sperm provided by the male (Oppliger et al. 2003). In experimental and natural conditions it has been found that oligopyrene sperm quantity increases with increasing male competition (more males than females within a group), and that increased oligopyrene sperm length increases fertilisation success in *V. ater* (Oppliger et al. 2003). It was also found that sperm concentration (oligopyrene plus eupyrene) was positively correlated with male shell length (Oppliger et al. 2003). The possibility of size assortative mating in *V. ater* was investigated by Staub and Ribi (Staub and Ribi 1995) in Lake Zurich. As males increase sperm concentration with increasing size and females

increase either the size or number of offspring with increasing size it could be expected that both male and female would prefer larger partners. It was found that a constant 40-45% of mating pairs were of the same size class over the mating period examined (April-October). Mating pairs with larger females were most frequent in spring and early summer, whereas mating pairs with larger males were more common in late summer and autumn (Staub and Ribi 1995). It has been suggested the switch from larger males in the spring to larger females in the autumn may be in-part due to females migrating to deeper water in autumn or it may be due to changing partner preference (Staub and Ribi 1995). Under experimental conditions it was found that female *V. ater* and *V. contectus* were quite passive during mating and frequently allowed males of a different species to copulate with them (Ribi and Katoh 1998). Overall a positive correlation was found between the sizes of mating pairs in *V. ater*, although a wide range of partner size combinations occurred, indicating any size assortative mating was not due to incompatibility between partners (Staub and Ribi 1995).

Embryos

Adults mate throughout the summer months and embryos (Figure 2.10) are generally first observed in the posterior end of the brood pouch in mid to late summer. Embryos develop within nutritive egg sacs harboured inside the female's brood pouch. Although no further direct nutrition is provided by the mother, the brood pouch does provide a protective atmosphere (Fretter and Graham 1962). Release of hatchlings varies depending on geographic range and environmental conditions, and under prime conditions two broods can be released in one year; one in early spring (harboured over previous autumn/winter), the other late summer (harboured over spring/summer). *V. viviparus* from the Zegrzynski Reservoir, Poland (Jakubik 2006) and Worcester-Birmingham canal, UK (Young 1975) had their first release in early spring (March to April), followed by another in late summer (August to September). Whereas, *V. ater* in Lake Zurich (Ribi and Gebhardt 1986) start slightly later, with their first release in May and their second in August. The literature also reports many single releases, which start from March to April for *V. contectoides* in the Illinoise area, USA (van Cleave and Lederer 1932), April to May for *V. contectoides* in the New York area, USA (van Cleave and Lederer 1932), May for *V. ater* in Lake Zurich, Switzerland (Ribi and Gebhardt 1986), May to June for *V. ater* in Lake Alserio, Northern Italy (De Bernardi et al. 1976), and as late as May to July for *V. ater* in Lake Baikal and River Angara, Southern Siberia (Ropstorf and Sitnikova 2006) and July for *V. ater* Lake Maggiore, Italy (Ribi and Gebhardt 1986). Hatchling *Viviparus sp.* although large and well developed, are still susceptible to predation by fish, such as Roach (*Rutilus rutilus*), Bream

(*Barbus barbus*), Rudd (*Scardinius erythrophtalmus*) and Tench (*Tinca tinca*) (Keller and Ribi 1993) and by birds such as Ducks (*Anas platyrhyncos*) (De Bernardi et al. 1976). However, it was calculated in Lake Zurich that around 10 percent of young survived their first summer (Keller and Ribi 1993).

2.2.2.3 Reproductive Morphology and Physiology

Male

Male *V. viviparus* are conspicuous due to their large club like right tentacle, which holds the penis and penis pouch. The male reproductive system is made up of a large gonad comprised of branched tubules held within the visceral mass of the body. Each tubule of the adult snail contains all stages of spermatogenesis and supportive Sertoli cells. Younger spermatogenic stages (spermatogonium, spermatocytes and spermatids) are found in clumps attached to the tubule walls (Figure 2.16A and B). Mature spermatozoa are found in the lumen area and vesicula seminalis (duct and sperm storage area). The sperm duct runs almost the length of the ventral area of the body and has a thick layer of muscle along its length (Fretter and Graham 1962). The vas deferens then leads to the Prostate, which extends to the base of the penis (Fretter and Graham 1962). As stated above, the penis is held within the right cephalic tentacle, 'the vas deferens runs through the right tentacle to a finger-like process which normally lies folded back in a pouch on the right side and opens at its tip' (Fretter and Graham 1962) (Figure 2.11).

Female

In *V. viviparus* the pallial oviduct is elaborate as a consequence of internal fertilisation. Seminal fluid is received from the male by the bursa copulatrix and sperm is stored in the receptaculum seminis. The pallial duct is further modified to form the brood pouch, in which the developing embryos are harboured (Fretter and Graham 1962). The albumen and shell glands also develop from the pallial oviduct. Sperm is transmitted to the receptaculum via sperm channels found on the ventral wall of the brood pouch (Fretter and Graham 1962). The ovary of female *V. viviparus* is small in comparison to many egg laying gastropods. It is tube like and runs the length of the ventral area of the visceral mass (Figure 2.9). Developing oocytes are seen close to the ovary wall (Figure 2.17) and are found in association with supportive follicle cells. Once fertilised embryos are coated in nutritive secretions from the albumen gland and then covered with a protective shell. Encased embryos are then held within the brood pouch (on a stalk) with the most developed closest to the vagina, there is no evidence that embryos obtain nourishment

directly from the female other than the albumen and yolk provided within the capsule (Fretter and Graham 1962).

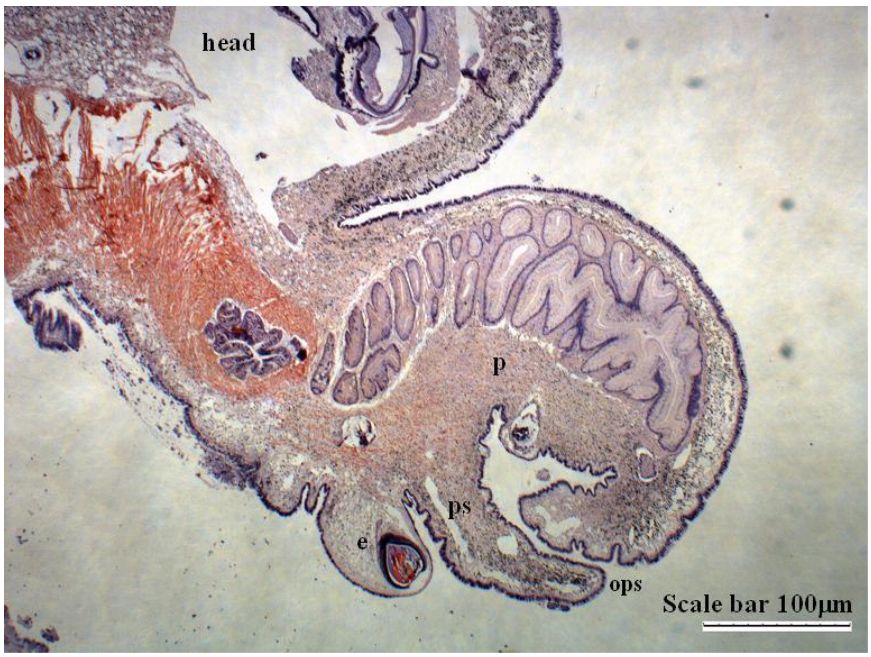

Figure 2.11 Photomicrograph of an adult male *V.viviparus* right tentacle with penis complex at x20 magnification.

5µm section stained with H & E. eye – e; opening of penis sheath – ops; penis – p; penis sheath – ps;

2.3 Preliminary work and optimisation

Before commencing the main mesocosm experiments, a number of smaller experiments and procedures were conducted with both the species of snail. These were mainly to optimise histopathological techniques or to provide information that would feed into the main experiment design, such as normal sexual development and time to sexual maturity.

2.3.1 *Planorbarius corneus*

A number of preliminary studies were conducted with *P. corneus* within the laboratory environment. These were primarily to 1) optimise dissection, tissue preparation, fixation and histopathological techniques that would be required during the main mesocosm studies, 2) determine the time period over which sexual development occurs, which led to the bases for the exposure period in the mesocosm studies, 3) assess 'normal' (not chemically exposed) gonad structure histopathologically.

2.3.1.1 *Planorbarius corneus* acquisition

All adults for these studies were obtained from Blades Biological (Cowden, Edenbridge, Kent, TN8 7DX. England.), and were collected from a lowland ditch near Worthing, Sussex, U.K. (exact location not disclosed), which has no STWs inputs.

2.3.1.2 General laboratory culture conditions

Water

Before entering the header tank in the snail room, the water was filtered using two filters to de-chlorinate it. The temperature of water entering the snail room was largely determined by external (outdoor) temperatures. The header tank water could be heated to17-18^{o}C (when needed) using a number of small, submerged domestic aquarium water heaters.

Tanks

Two sizes of tank were used throughout. Large 80 litre glass holding tanks were used to house adults and larger juveniles when not being used in experiments. Each 80-litre tank had a plastic down pipe from a header tank and could be used with constant flow, under static renewal, or with an internal fluval filter pump. Smaller 9 litre glass tanks were used for adult breeding groups (to provide egg masses for development work) and to grow-on larger juveniles. These were generally used under static renewal conditions but could be used with constant flow if necessary. 500 ml glass jam jars were also frequently used to house egg masses and hatchlings under static renewal conditions, and to transport and narcotise snails.

Temperature and photoperiod

The snail room temperature was maintained at 17-18^{o}C by the use of an internal air-conditioning unit. The photoperiod for all work conducted in the snail room was 12 hours light: 12 hours dark (12: 12) and this was controlled externally by a digital timer switch.

2.3.1.3 General laboratory husbandry

During non-experimental periods adult and maturing juvenile snails were housed in the large 80 l tanks at a density of less than 1 adult snail per litre. Snails were fed on fish flake food and either organic carrot, lettuce or cucumber twice weekly and any uneaten flake food or rotting vegetation was siphoned out prior to feeding. Dead snails were also removed during this cleaning and feeding time.

Collection of egg masses

Once egg masses had been laid they were easily observed stuck to the glass or pipe-work in the aquaria. Egg masses laid on flat surfaces were removed using a sharp safety razor blade pressed against the edge of the egg mass and run underneath it; care was taken not to damage the eggs inside the mass. If the egg mass was laid on pipe-work or corner a thumbnail could often be used in the same manner. Once removed egg masses were placed in 500ml jam jars containing tank water and labelled with the parent tank reference and date until further use. Jam jars were kept at the same temperature and light regime as parents.

Egg mass hatching and hatchling husbandry

Egg masses were housed in 500ml glass jam jars with a 12:12 light regime. Every 48 hours 50% of the water was renewed with clean de-chlorinated snail room water and any dead snails or waste food was siphoned out with a pipette. Hatchlings were fed on finely crushed fish flakes after every water change. At 4 and 6 weeks post hatch the hatchlings were moved to a clean jam jar and given 100% new water, any dead snails were removed. At two months old hatchlings were moved to either 80 l or 9 l tanks as required. Cleaning and feeding continued every 48 hours.

2.3.1.4 Preparing *P. corneus* for dissection or histological fixation

Before any snail dissection or fixation could occur, snails had to be narcotised in 5% w/v Magnesium Chloride (MgCl) (≤99%, Sigma-Aldrich) solution for 30 minutes, or until the snail had stopped movement, which ever was the longer. MgCl solution was made in de-chlorinated tap water (or if prepared in the field, river water). Once the snails were fully narcotised they were blotted dry and any large patches of algae, mucus or detritus where removed. Shell diameter (Figure 2.12) and shell aperture (Figure 2.12) were measured using digital callipers. Total weight was measured using an electronic balance. Once weighed and measured, the snail shell was removed using a large G-Clamp (adults) or a smaller one for juveniles. The snail was placed flattest side down onto the bottom part of the G-clamp. The top clamp was then slowly screwed down until the shell cracked. The shell could then be peeled off, working from the head end backwards. The muscular attachment between the shell and the body tissue was pinched out with forceps. Care was taken not to damage the soft tissue, especially as the gonad was harboured in the finer whorls in the middle of the shell. When handling the de-shelled snail with forceps, the mantel was used to hold the tissue, to avoid damaging any reproductive organs. Once the snail was removed from the shell it was washed in a petri dish containing Phosphate

buffered solution (PBS) made using PBS tablets (Oxiod) dissolved in distilled water, following the manufactures guidelines. This was to remove any additional shards of shell, and to see if any parasitic digenea cercaria emerged (which were visible by eye). After the shell was removed, the soft body weight could be measured on the electric balance. Generally, after shell removal, snails were fixed whole for histopathology, in order to minimise the risk of damaging the fragile tissue.

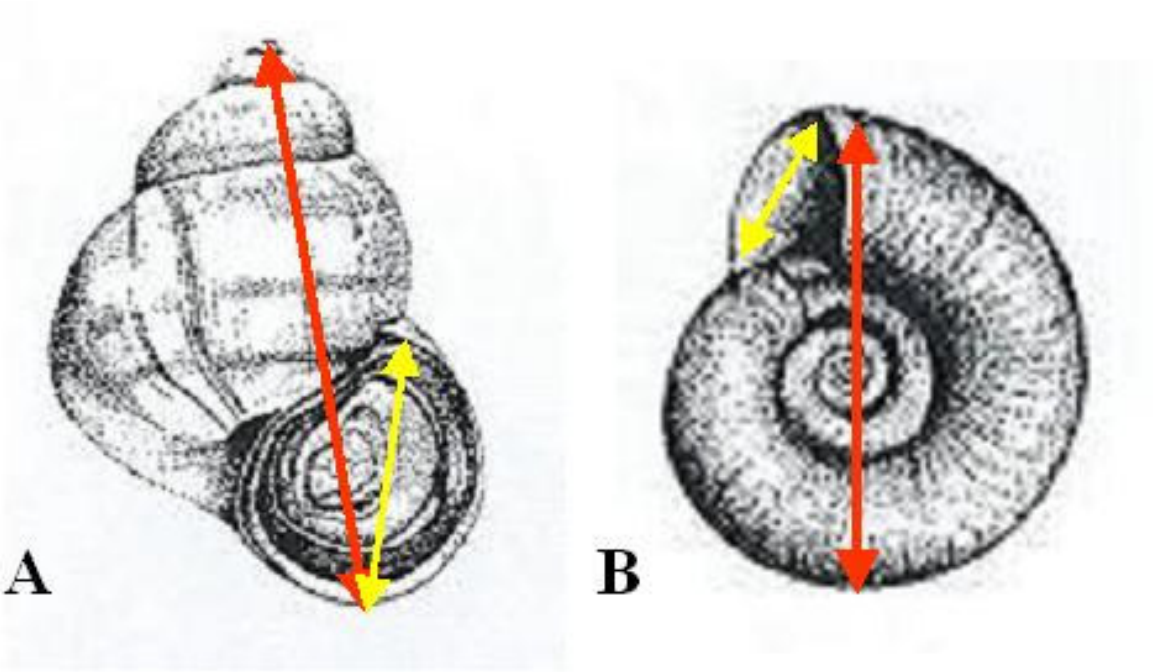

Figure 2.12 Diagrams to illustrate the points used to measure shell length/diameter (Red arrows) and aperture (Yellow arrows).

A, *Viviparus viviparus* Shell length and aperture B, *Planorbarius corneus* shell diameter and aperture. Pictures not to scale. Measurements were taken using digital callipers to two decimal places.

2.3.2 *Viviparus viviparus*

As with *P. corneus*, there was very little literature relating to the histopathology of *V. viviparus* and therefore some preliminary work was required to provide this information. However, unlike *P. corneus V. viviparus* are not easily cultured under laboratory conditions (additional experiments conducted by myself but not presented here) and therefore wild specimens had to be used instead.

2.3.2.1 *Viviparus viviparus* acquisition

Unlike *P. corneus* a biological supplier could not be found for *V. viviparus,* therefore all adult and juvenile *V. viviparus* were collected from the Grand Union Canal near Uxbridge, Middlesex. They were collected from approximately 500m of canal bank, 250m either side of the Ordnance Survey (OS) Reference TQ 052 814 (Figure 2.13). Snails were collected

from the east bank of the canal at a maximum depth of approximately 700 mm (arms length). They were transported from the canal site in food grade plastic buckets containing canal water at a maximum density of 4 snails per litre.

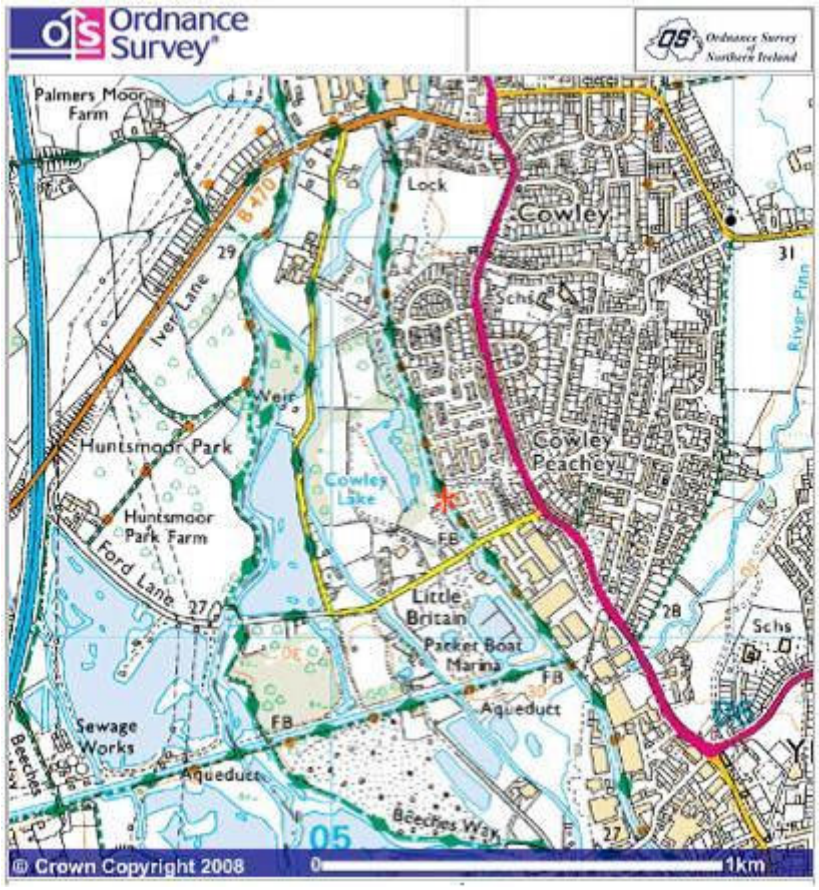

Figure 2.13 Ordnance Survey (OS) map of the Grand Union Canal *Viviparus viviparus* collection area.

Red asterisk (*) indicates OS reference TQ 052 814, snails were collected from around 500 meters of canal bank approximately 250 m either side of this point.

2.3.2.2 General protocol for preparing *V.viviparus* for dissection or histological fixation

Before any snail dissection or fixation could occur, snails had to be narcotised in 5% w/v MgCl solution for 30 minutes, or until the snail had stopped movement and the operculum gaped slightly, which ever was longer. MgCl solutions were made in de-chlorinated tap water (or if prepared in the field, river water). Once snails were fully narcotised they were blotted dry and any large patches of algae, mucus or detritus where removed. Shell length and shell aperture (operculum height) (Figure 2.12) were then measured using digital callipers. The total weight was measured using an electronic balance. The snail shell was then removed using a large G-Clamp (adults) or a smaller one for the juveniles. The snail was positioned with its operculum flat on the bottom of the G-Clamp, the top half of the

clamp was then slowly screwed down until the shell cracked along the first (largest) or second whorl of the shell. Forceps were then used to push the operculum (and attached snail) through the shell opening towards the shell. In this way the first whorl of the shell was completely removed from the snail. Each snail was attached to its shell by a piece of muscular tissue on its mid-ventral area. This was detached by pinching it with the tips of the forceps. Once the snail was detached from this point, the body could be more easily removed from the shell. This was accomplished by either teasing it out with the forceps (by holding onto the operculum and gently twisting), or by holding the shell uppermost (with the head/foot hanging below) and gently shaking the body loose in a circular motion. The weight of the body pulled the rest of the tissue free of the shell. Once the snail was removed from its shell it was rinsed in PBS and the soft tissue was weighed on the digital balance (to three decimal places). When handling the de-shelled snail with forceps, the operculum or mantel were always used as holding points, to avoid damaging any reproductive organs. As previously described for *P. corneus V. viviparus* were generally fixed whole for histological investigation to prevent damage to the delicate tissues.

2.3.2.3 Protocol for *V. viviparus* embryo removal

Embryos were dissected out of the female snail's brood-pouch into a petri dish containing PBS. Fine dissection scissors were used to make an incision laterally along the brood-pouch, forceps were used to sweep the embryos out, the incision was followed until no further embryos could be seen and removed. Care was taken to minimise disturbance to other organs. Once all the embryos were removed from the female the number of shelled (most mature) and un-shelled (least mature) embryos were counted. Shell growth on embryos was quite obvious and the distinctive strips were already apparent on many embryos. Once the embryos were removed the female body was re-weighed.

2.3.3 Preliminary experiments – Histopathology

There is very little information in the literature on the histopathology of either *P. corneus* or *V.viviparus*. Histopathological endpoints were used in the two mesocosm experiments; therefore it was important to gain some baseline information on this species before measuring any possible effects on histopathology due to chemical exposure. Therefore, laboratory rearered *P. corneus* or wild caught *V. viviparus* were used to determine 'normal' histopathology of the developing and sexually active gonad.

2.3.3.1 Preliminary Experiment 1: Ontogeny of sexual development in laboratory reared *P. corneus* from day of hatch to sexual maturity

Experiment 1 – Introduction

No information or histological description could be found in the literature of how the gonad of *P. corneus* develops. How the gonad develops and how long this takes was important information with regard to how long the mesocosm experiments should last and interpreting the resulting histopathology of developmentally exposed F1 snails. Therefore, the following work was conducted prior to designing the two chemically dosed experiments (Chapters 4 and 5).

Experiment 1 – Materials and Methods

Adult snail husbandry

When used to produce eggs for the developmental study each breeding group comprised of 6 Adult snails (>20mm Diameter). These were placed in 9 l glass aquaria. Ambient room temperature was kept at 17^{o}C with a 12:12 light regime. A 50% water change was conducted every 48 hours. Fresh water was piped from the header tank. Any uneaten fish flakes were removed using a siphon, after which snail were fed with fresh food. Egg masses were collected frequently, every 24 to 48 hours.

Husbandry of hatchlings and young snails

For two weeks the six breeding group tanks were inspected daily for egg masses and any found would be removed from the 9 l parent tanks. Egg masses laid on the same day were pooled and placed in 500 ml jam jars filled with tank water at a maximum density of 10 egg masses per jar, 50% of the water was changed in each jam jar every 48 hours. Jam jars were inspected daily for hatching activity.

Hatchling snails were very delicate and direct handling generally caused damage and subsequent death, however the following method proved very successful. When snails hatched they crawled off the egg mass in search of food and came to sit on the glass of the jam jar. This meant that egg masses with un-hatched eggs could be carefully removed and placed in a new jam jar, and any newly hatched snails not yet left the surface of the egg mass could easily be washed off it using a small jet of water from a pipette. The jam jar with newly hatched snails could then be relabelled with the date of hatch and hatchlings could be fed with a small slice of organic carrot. This process was repeated until all snails had hatched. The only other method found to be successful in handling young hatchlings was to use a small 1ml plastic pipette with the nozzle cut diagonally (to produce a larger

bore), small hatchlings could then be carefully sucked into the pipette and transferred to wherever they needed to be moved to without damage e.g. another jam jar or to narcotise or to fixative solutions.

Young hatchlings were kept in jam jars until they were two months post hatch, 50% of the water was changed every 48 hours, and any dead snails or rotted food were removed using a pipette. At 4 and 6 weeks post hatch the hatchlings were moved to a clean jam jar and given 100% new water. Hatchlings were fed after every water change on small slices of organic carrot and finely ground fish flakes. Small pieces of cuttlefish bone were placed in each jam jar to provide calcium for shell growth.

At 2 months post hatch, young snails were moved into the 9 l glass aquaria. The same 48 hour water changing and feeding regime was continued, any rotten food was removed using a siphon during water changing, and cuttlefish bone was again placed in the tanks. Young snails aged 2-3 months were kept a density of roughly 30-40 snails per 9 l aquaria (minimum of 225ml of water per snail), then at 3-4 months between 10-15 snails per 9 l aquaria (minimum of 600ml of water per snail). Due to the occasional natural mortality and continual sacrificing for histology density was not constant. During the whole experiment room temperature was kept at 17^{o}C with a light regime of 12 hours on and 12 hours off.

Sampling regime

Hatchlings and young snails were sacrificed on the first day of hatch and then at 3, 5, 7, 9, 11, 13, 15, 19, 21, 23, 27, 30, 34, 42, 49, 56, 63, 70, 77, 84, 91, 98, 105 and 112 days post hatch (dph). After all samples were taken surplus young snails were moved to a larger 80 l holding tank until further use.

Hatchling sampling protocol

Up to 1-month post hatch a pipette was used to remove around 10-20 hatchlings from their 500ml glass jam jar and placed in 5% MgCl solution for a maximum of 5 minutes to narcotise them. Over 1 month post hatch either a pipette or a spatula was used (where appropriate) to remove 6-10 young snails from their jam jar or tank and placed in 5% MgCl solution for a maximum of 20 minutes to be narcotised. After being narcotised hatchlings and young snails were fixed whole (with shell) in Kahel's fixative (Appendix III for recipe). Histologically fixed snails were kept in screw top glass vials at room temperature until further processing (approximately 3 weeks).

Histology processing

All cassettes were labelled with the species code and age. Fixed hatchlings under 1 month old were tiny and hard to see so before being placed into histology cassettes they were first dyed with eosin for easier recognition when embedding and sectioning. Using a pipette hatchlings were removed from the Kahel's fixative and placed in a watch glass with a few drops of eosin for 30-60 seconds. The tiny hatchlings were also prone to loss through the holes in the histology cassette so; after wetting in (70% IMS) a single piece of cassette sized Wattmanns (RA Lamb) filter paper was fitted to the bottom of the cassette. Once prepared the fixed and eosin stained hatchlings could be pipetted onto the middle of the lined cassette, and another piece of wetted filter paper was placed on top of the hatchling snails making a sandwich and preventing sample loss. The cassette lid could then be fitted and the cassette transferred to a large beaker of 70% IMS until needed. Fixed snails over 1 month old were removed from the Kahel's fixative either using a pipette or spatula as appropriate. Any large bits of shell were removed using a fine dissecting needle and forceps, although most of the shell had become jelly-like at this stage (approximately 3 weeks of fixation) and most samples were minimally handled to reduce tissue damage. Once past the filter paper sandwich stage snails were processed at a maximum of 3 per cassette. Hatchling and young snails were then processed and embedded following the protocol as given in Chapter 3, Sections 3.1.2 and 3.1.3.

Sectioning, staining and cover-slipping

Wax blocks were kept on ice prior to sectioning on a rotary microtome (Leica RM2255). Wax embedded hatchlings were serially section at 5um every 25um for young hatchlings from day of hatch to 77dph, and then every 50um for hatchlings of 84dph and older. The methods for staining and cover slipping in section 2.3.5 were followed.

Slide reading

Slides were read on an Olympus (BX51) light microscope at low (x20), medium (x100) and high (x400) magnifications. Photomicrographs were tacked a digital camera (Q Imaging Micropublisher 5.0RTV) linked to a personal computer. Q Capture Pro 5.1 software was used to capture and view images from the digital camera; see Section 3.1.7 for details.

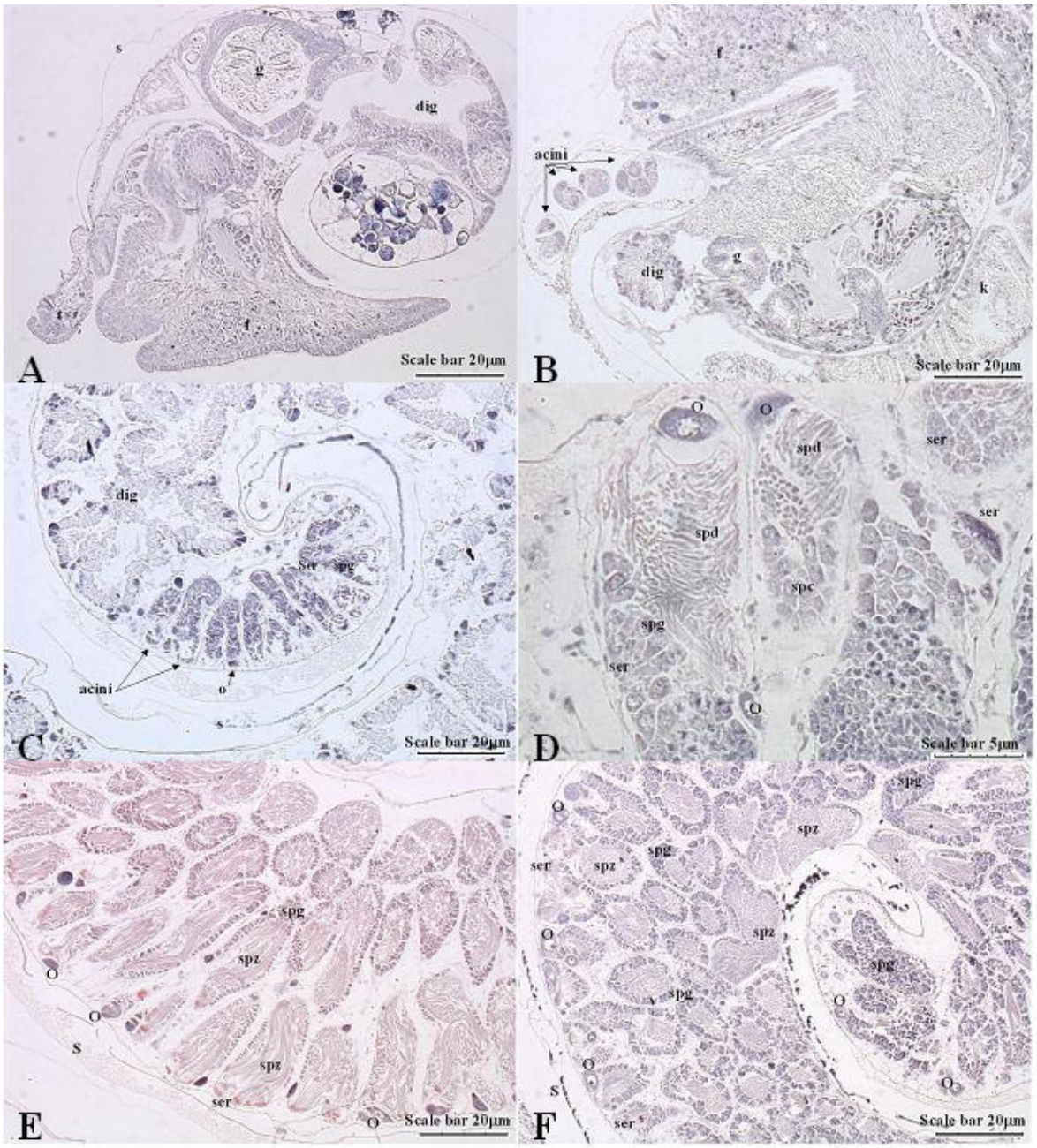

Figure 2.14 Photomicrograph of *P. corneus* hatchlings showing the gonad at different stages of development.

(A) Day of hatch with no gonad visible (x100); (B) 23 Days post hatch small acini first become apparent (x100); (C) 63 days post hatch a number of acini with Sertoli cells, spermatogonium, spermatocytes and early stage oocytes (1 and 2) (x100); (D) 77 days post hatch with a number of acini with spermatogenesis up to spermatids stage and early oogenesis (x400); (E) 84 days post hatch with a large part of the visceral mass with fully spermatogenesis (x100); (F) 91 days post hatch acini have full spermatogenesis and oogenesis to stage 3 (x100). 5µm sections stained with H & E. digestive tissue – dig; foot – f; gut – g; kidney – k; oocyte – O; shell – S; Sertoli cells – ser; spermatocytes – spc; spermatids – spd; spermatogonium – spg; spermatozoa – spz; tentacle – t.

Experiment 1 - Results

No gonad was observed in snails from day of hatch to 19dph (Figure 2.14). Formation of the gonad containing early stages of acini was observed in hatchlings ranging in age from 21 to 56dph (Figure 2.14). Recognisable adult looking gonad structure and large numbers of sertoli cells were first observed in the gonad of 49dph snails and by 63dph sertoli cells with attached spermatogonium and spermatocytes were seen along with stage 1 and 2 oocytes (De Jong-Brink et al. 1976) (Figure 2.14). At 70 days post hatch, all hatchlings had a gonad containing an increasing number of acini with early spermatogenesis and oogenesis. Full spermatogenesis with spermatozoa in the lumen was seen in a few individuals of 77dph and all hatchlings analysed by 84dph (Figure 2.14). By 105dph stage 4 oocytes (De Jong-Brink et al. 1976) were observed along with supportive follicle cells and the beginnings of a follicular gap. Stage 5 and degenerating oocytes were not observed in any hatchlings up to 112dph at which point sampling finished.

Experiment 1 - Discussion

As previously stated, the reason why this piece of work was conducted was that no literature could be found on *P. corneus* gonad development and this information was required before the mesocosm studies could commence. Although there is no literature on *P. corneus* for comparison, there is a comprehensive study of gonad and reproductive tract development in *Lymnaea stagnalis* by Fraser (Fraser 1946) from 5 days post lay (embryos) to 16 weeks post hatch. In *P. corneus* hatchlings, the gonad consisting of early acini with germinal cells was first observed from 21 dph, whereas, in *L. stagnalis* this was first observed at around 1 week post hatch (Fraser 1946). At 14 days post hatch Fraser noted that the gonad contained more lobes and spermatogonium, which is earlier than found in *P. corneus*. Sertoli cells were first observed in the gonad of 49dph snails and spermatogonium at 63dph in *P. corneus*. This is where the largest differences in gonad development start to occur as Fraser does not report spermatocyte appearance until approx 91 dph, whereas in *P. corneus* spermatocytes are seen from 63dph. In addition to this, Fraser does not report oocytes in the acini until approx 112dph (shell length 10 mm) at which time full spermatogenesis was occurring. Indeed all *P. corneus* hatchlings observed from 70dph contained early stages of oogenesis. Full spermatogenesis was first seen at 77dph and was ubiquitous by 84dph. Later stage oocytes were seen in *P. corneus* hatchlings along with supportive follicle cells by 105dph. It is surprising that Fraser reports full spermatogenesis and early oocytes appearing as late as 112 days (16 weeks) post hatch as other authors report egg laying in this species at 9 (Bohlken and Joosse 1982) or 10 (Janse et al. 1989) weeks post hatch. Fraser reports the water temperature in the hatchling aquaria as 22-25^{o}C

with a mean of 23°C. Therefore temperature does not account for the late development reported by Fraser, as Bohlken and Joosse raised their snails at 20°C. Spermatogenesis occurs earlier than oogenesis in many Basommatophora. In *L. stagnalis* a 2-3 week period of mating as a male occurs before egg laying (Van Duivenboden 1983). This has not been observed in *P. corneus*, and as oocyte and sperm development is much closer in timing this phenomenon may not be present in this species.

Overall, these results indicate that *P. corneus* has a fully developed gonad with both mature sperm and eggs after 16 weeks (or 4 months). It was therefore decided that the mesocosm study should be conducted for at least this time period so that mature F1 offspring could be assessed for their reproductive development.

2.3.3.2 Preliminary experiment 2: Description of gonad histopathology of *P. corneus*; reared and housed under laboratory conditions until sexually active

Experiment 2 – Introduction

There was very little information in the literature regarding the structure and histopathology of the *P. corneus* gonad while sexually active. Histopathology of *P. corneus* gonad structure and activity were important endpoints in the two mesocosm experiment. It was therefore necessary to conduct this experiment to provide this key information.

Experiment 2 – Materials and Methods

Six young laboratory raised snails (see above), which had been observed laying eggs, were removed from their tank by hand and placed in a 500ml jam jar containing tank water. They were then narcotised in 5% MgCl solution for 30 minutes or until they withdrew into their shells and stopped roaming about, whichever was longest. Narcotised snails were then weighed and measured according to the method stated in Section 2.3.1.4. Snails were removed from their shells and fixed in Bouin's fixative following the method in Chapter 3, Section 3.1.1. The methods for cassetting (Chapter 3, Section 3.1.1) and processing (Section 3.1.2) and embedding (Section 3.1.3) were followed. Chilled blocks were sectioned on a rotary microtome at 5µm, at least 10 sections were taken from each block. Sections were then dried for 24 hours, stained and cover-slipped as given in the method Section 3.1.5. Each slide was read on a light microscope at low (x20), medium (x100) and high (x400) magnifications. Photomicrographs were taken using digital camera and QCapture Pro 5.1 software as before.

Experiment 2 - Results

The sexually mature gonad is made up of a number of acini. Each acini contains both spermatogenic and oogenic cells of all stages of maturity. The acini wall has more than 70% of the area covered with successive stages of spermatogenesis. Only the very luminal end is free of either spermatogenic or oogenic cells. Thick clumps of spermatogonium are found attached to sertoli cells, which in turn are attached to the acini walls mainly towards the luminal end of the acini. These spread, mature and proliferate through spermatocytes to spermatids as they move towards the vitellogenic end (still in association with their sertoli cells), once mature they are released as spermatozoa into the acini lumen. Very few immature spermatogenic cells are present in the lumen of the acini or the central lumen, which combines at the end of each acini. Oocytes are also attached to the acini walls, stage 1 oocytes (De Jong-Brink et al. 1976) or oogonium are found attached to the acini walls anywhere from the luminal end to the vitellogenic end of the acini and are motile beneath or adjacent to a layer of sertoli cells. Larger oocytes of stages 2 and 3 (De Jong-Brink et al. 1976) with attached follicle cells are generally found moving towards the vitellogenic end of the acini, stage 4 and 5 oocytes are found at the vitellogenic end of the acini where they become much enlarged beneath a layer of sertoli cells. Once oocytes reach stages 4 and 5 follicle cells are visible surrounding the whole oocyte and a cleft or gap can be seen forming at the top of the oocyte (De Jong-Brink et al. 1976). It seems that when the oocyte ovulates this cleft ruptures and the oocyte is released into the lumen (personal observation), released oocytes appears to be motile and heads towards the central lumen. Degenerating oocytes, which have not ovulated, can also be observed at the vitellogenic end of the acini as well as degenerating or reabsorbing follicle and sertoli cells. Figure 3.2A and Figure 3.3 are annotated photomicrographs of the gonad from a sexually active *P. corneus*, highlighting gonad organisation.

Experiment 2 - Discussion

The gonad of *P. corneus* is similar in structure to that described in other Basommatophora including *Lymnaea stagnalis* (Joosse and Reitz 1969) and *Biomphalaria glabrata* (De Jong-Brink et al. 1976). Developing spermatogenic cells can be seen with their supportive sertoli cells, maturing as they move towards the acini vitellogenic area. Oocytes at all stages of development can also be easily observed as well as their follicle cells. As this species has not been frequently used as a toxicological test species, this ‘normal’ (non chemically exposed) gonad histopathology of *P. corneus* provides vital comparative material for post chemical exposure (i.e. mesocosm study) specimens.

2.3.3.3 Preliminary Experiment 3: Reproductive development of *V. viviparus* embryos harboured by a female from the Grand Union Canal

Experiment 3 – Introduction

As found with *P. corneus,* there is very little literature regarding *V. viviparus* developmental biology. However, unlike *P. corneus*, *V. viviparus* could not be successfully cultured in the laboratory, therefore to investigate the histopathology of gonad development of *V. viviparus,* wild adult specimens harbouring embryos were brought into the laboratory for examination.

Experiment 3 – Materials and Methods

To assess the level of sexual identity and development in embryos harboured within the brood pouch, one adult female *V. viviparus* from the Grand Union Canal was sacrificed and had the embryos dissected from the brood pouch (see Section 2.3.2.3). Ten shelled embryos were removed. Each embryo had the shell length and width measure using electronic callipers in millimetres and was then weighted in grams on an electronic balance before the shell was carefully removed with a dissecting needle and forceps. De-shelled *V. viviparus* embryos were then fixed in Bouin's fixative for an hour before being transferred to 70% IMS. Embryos were stored in 70% IMS in 5 ml glass screw top vials at room temperature prior to histological processing (See Chapter 3, Section 3.1.2) and embedding (See Chapter 3, Section 3.1.3). *V. viviparus* embryos were serially sectioned at 5µm every 50µm and then stained with H & E (See Chapter 3, Section 3.1.5).

Experiment 3 - Results

Embryo shell length ranged from 5.45 mm to 7.54 mm, shell aperture was 3.91-5.75 mm and total weight ranged from 0.04 g to 0.12 g. *V.viviparus* shelled embryos were well developed and the ducts which would form into the sperm duct or oviduct/ brood pouch were visible in all ten of the embryos analysed. Five of the embryos had a reproductive duct that was observed within the right tentacle (Figure 2.15C), suggesting they were male. Three had ducts that did not enter the right tentacle but opened on to the mantles (Figure 2.15A) edge, suggesting these were female. In two embryos a reproductive duct was observed but only in its ventral position, which, at this age, was similar in both males and females.

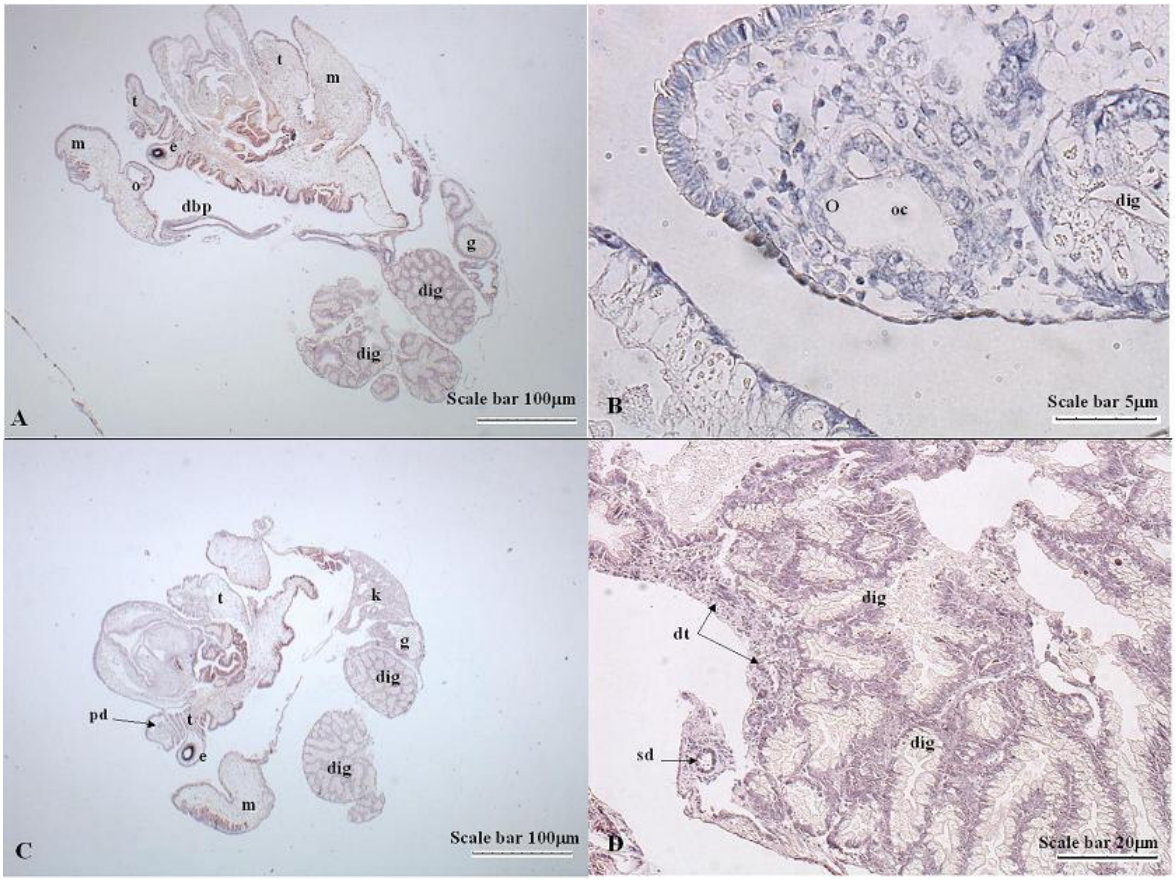

Figure 2.15 Photomicrographs of male and female *V. viviparus* embryos.

5µm section stained with H & E. (A) a female embryo at x20 magnification. (B) an ovary of a female embryo at x400 magnification. (C) a male embryo at x20 magnification, and (D) a sperm duct and developing gonad tubules of a male embryo at x100 magnification. Developing brood pouch –dbp; developing (gonad) tubules – dt; Digestive tissue – dig; eye – e; gut – g; kidney – k; mantle – m; ovary – O; opening – o; ovarian cavity – oc; sperm duct – sd; tentacle – t; penis duct – pd;

Experiment 3 - Discussion

A light and electron microscope study by Griffond (Griffond 1978) found that the female gonad of *V. viviparus* could be observed in a differentiated state in individuals of 4-5 mm shell length, whereas in males this did not occur until around 9-10 mm. A large amount of variation in maturation rate was also observed in both male and female embryos in both this (my) and Griffond's study. Griffond (Griffond 1978) describes the germ cells of male and female as being indistinguishable from each other. However, Griffond reports that the morphology of the gonad can be used to separate the sexes; the female gonad has a larger lumen or ovarian cavity (Figure 2.15B) when compared to the developing male gonad (Figure 2.15D), and male germ cells proliferate far more and thus do not mature until later than seen in females. From these observations a structure considered to be a rudimentary ovary (few germ cells, large lumen) was observed in one of the embryos sampled (Figure

2.15B). Therefore it was possible to determine the sex of even very young (pre-hatching) snails.

2.3.3.4 Preliminary Experiment 4 - Histopathology of sexually mature male and female *V. viviparus* from the Grand Union Canal

Experiment 4A - Introduction

A preliminary investigation into the histopathology of adult *V. viviparus* gonad was conducted prior to the start of the mesocosm study. This was conducted so that a reference of 'normal' gonad tissue could be produced to compare with later chemically exposed individuals.

Experiment 4B – Introduction

In addition to the initial experiment, a further assessment of the overall health, parasite load, sex ratio and sex related differences of the adult *V. viviparus* population to be used in the 2006 mesocosm experiments (Chapters 4 and 5) was conducted (using snails collected from the Grand Union Canal at the same time as those intended for use in the mesocosms).

Experiment 4 – Materials and Methods

For Experiment 4A six *V. viviparus* of various sizes were collected from the Grand Union Canal. For Experiment 4B 30 adult snails were randomly picked from the large group of snails collected for the mesocosm studies.

All adult snails were narcotised and prepared for fixation following the protocol given in Section 2.3.2.2. The methods for cassetting (Chapter 3, Section 3.1.1) and processing (see Chapter 3, Section 3.1.2) and Embedding (see Chapter 3, Section 3.1.3) were followed. Chilled blocks were sectioned on a rotary microtome at 5µm, at least 10 sections were taken from each block. Sections were then dried for 24 hours, stained and cover-slipped as given in the method in Chapter 3, Section 3.1.5. Each slide was read on a light microscope at low (x20), medium (x100) and high (x400) magnifications. Photomicrographs were taken using digital camera and QCapture Pro 5.1 software as before. In the male *V. viviparus* a scoring system (modified from Johnsen (Johnsen 1970)) was used to identify the level of spermatogenesis observed in each individual, this score could then be used for statistical analysis. In the females a simpler method, counting oocytes, was used.

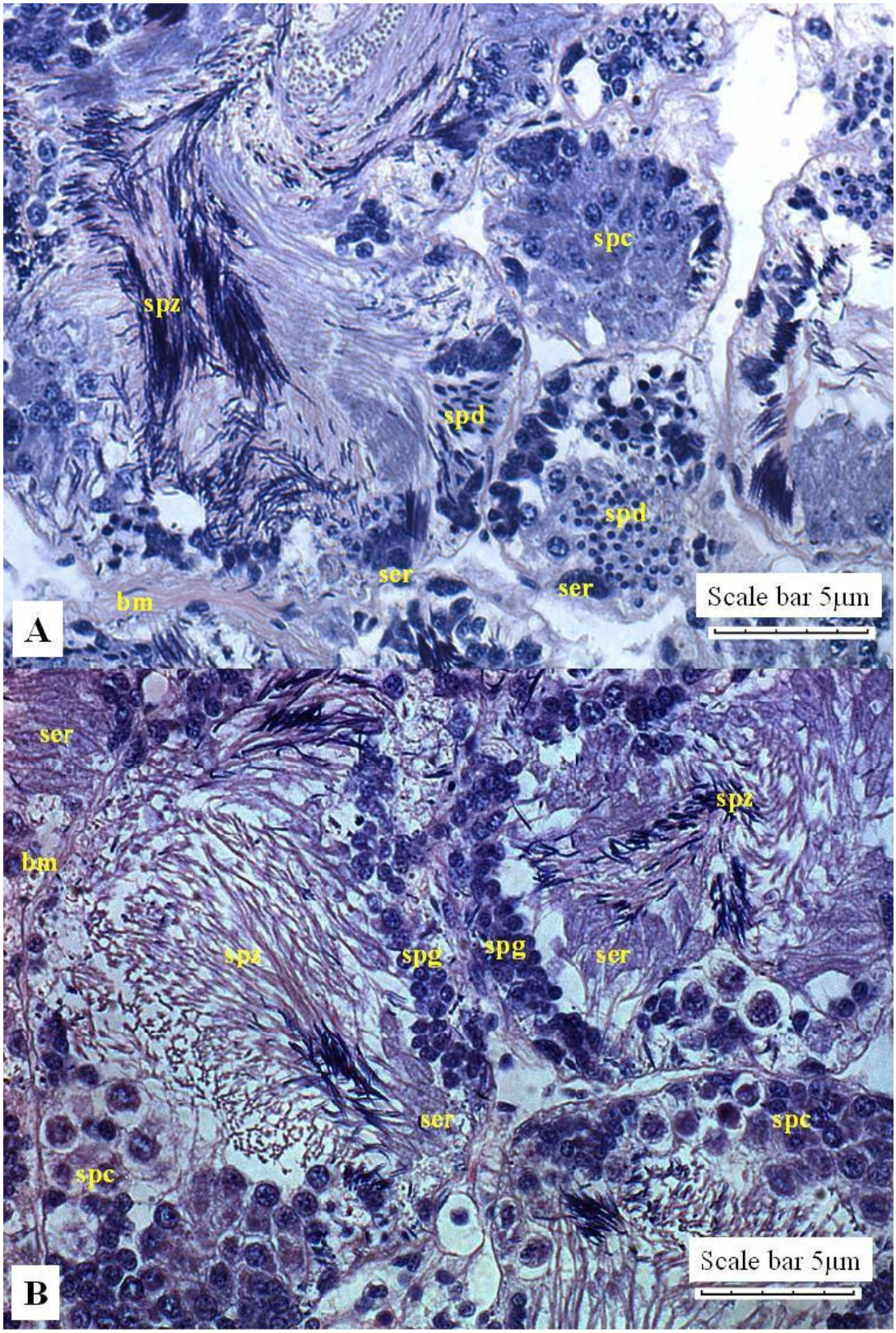

Figure 2.16 Photomicrographs of *V. viviparus* male gonads showing tubules with full spermatogenesis (x400).

5µm thickness, stained with H & E. (A) Adult (shell length 24.62mm). (B) Mature juvenile (shell length 13.91mm). Spermatozoa – spz; Spermatids – spd; Spermatocyte – spc; Spermatogonium – spg; Sertoli cell – ser; basement membrane –bm. N.B sertoli cells can take different forms depending on stage of spermatogenesis.

Male – Spermatogenesis was scored from 1 to 10. Table 2.1 defines the scoring protocol. To determine spermatogenic cell type slides were analysed at x400 magnification. A mean score from the slides analysed was produced for each individual for comparison and statistical analysis. Figure 2.16A and Figure 2.16B are photomicrographs of tubules with full spermatogenesis. Spermatogenic cells, supportive cells (sertoli cells) and general structures are highlighted.

Female – For each section of gonad analysed the total number of oocytes were counted under x400 magnification. The mean number of oocytes per section was used for statistical analysis. Figure 2.17 is a photomicrograph of a female *V. viviparus* gonad, the ovary with developing oocytes are clearly visible.

Table 2.1 Spermatogenesis Scoring protocol for *V. viviparus*.

Score	Description
1.	No germ cells present
2.	Germ cells only
3.	Germ cells and sertoli cells visible
4.	Spermatogonium present
5.	Spermatocytes present
6.	Spermatids present
7.	Spermatozoa present in <30% of tubules
8.	Spermatozoa present in 30-50% of tubules
9.	Spermatozoa present in 50-70% of tubules
10.	Spermatozoa present in >70% of tubules

Score 1 indicates no gonad development, Score 10 indicates full spermatogenesis, an average (mean) score was calculated for each individual from a number of section of gonad. This scoring system was adapted from Johnsen (Johnsen 1970).

Histological analysis of wild-caught *V. viviparus* revealed some parasitism. As wild-caught snails would be used in the mesocosm experiments, it was necessary to devise a scoring method.

Parasite score – For male and female *V. viviparus* the same parasite scoring method was used. Two types of parasite infection were found in *V. viviparus*. Digenean parasites were found in an encysted (non-reproductive) form in the head-foot and muscular tissue. Digenean parasites were also found in their asexual reproductive form (sporocysts) in the digestive tissue. Table 2.2 below defines the scoring protocol for the encysted parasites. A mean score from the slides analysed was produced for each individual. Figure 2.18A and Figure 2.18B are two photomicrographs of infected *V. viviparus* tissue showing both types of infection. For the asexual reproductive form of infection in the digestive tissue the following recording method was used;- < 10% of digestive/ reproductive tissue infected, 10-30% of digestive/ reproductive tissue infected, 30-50% of digestive/ reproductive tissue

infected, 50-70% of digestive/ reproductive tissue infected, and > 70% of digestive/ reproductive tissue infected. The tissue was viewed at x20 magnification and a 10x10 graticule was used to estimate the level of parasite infection.

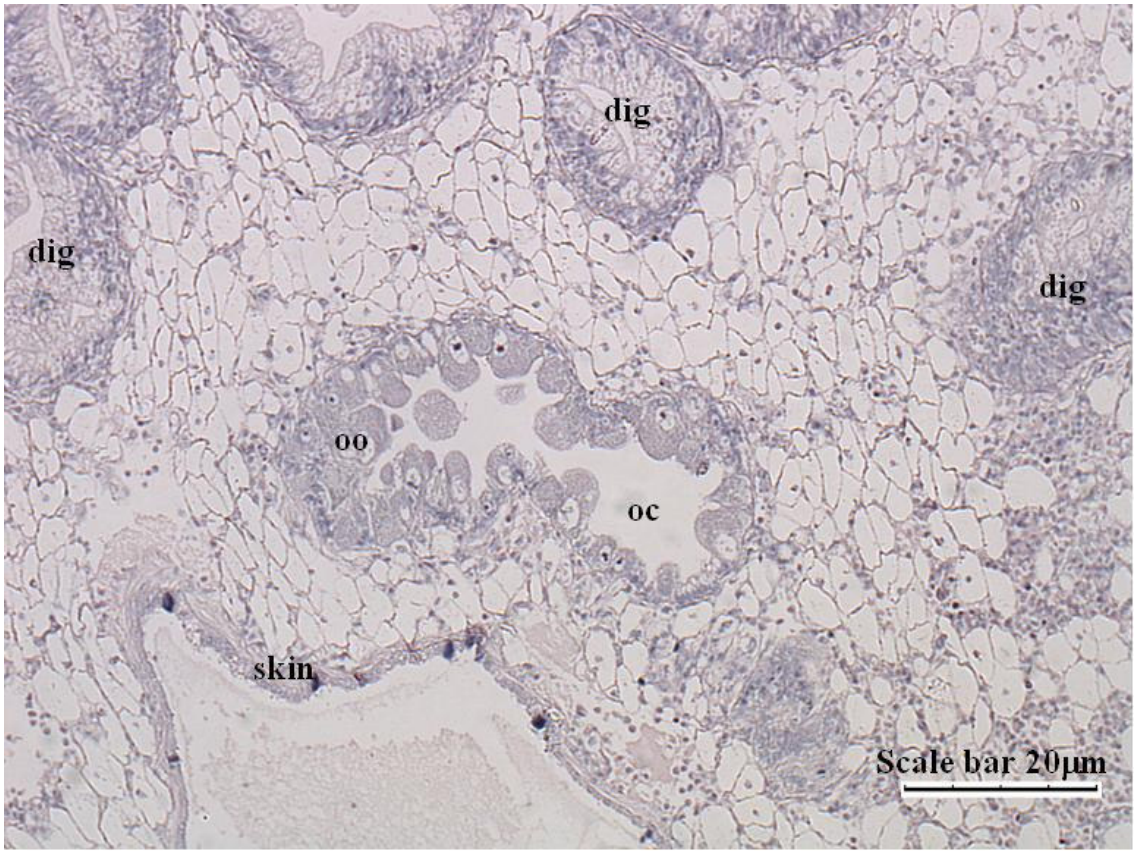

Figure 2.17 Photomicrograph of *V. viviparus* adult female gonad showing the ovary with developing oocytes (x100).

5µm thickness, stained with H & E. Oocytes – oo; Ovarian cavity – oc; Digestive tissue – dig;

Table 2.2 Parasite scoring protocol for encysted digeneans in *V.viviparus*, two types of parasite infection were found in *V.viviparus*.

Score	Description
1.	No parasites observed
2.	Encysted parasites only, < 5 cysts
3.	Encysted parasites only, 5-15 cysts
4.	Encysted parasites only, 15-30 cysts
5.	Encysted parasites only, > 30 cysts

The numbers of encysted parasite were counted to provide a score. A mean score from all the slides analysed was produced for each individual.

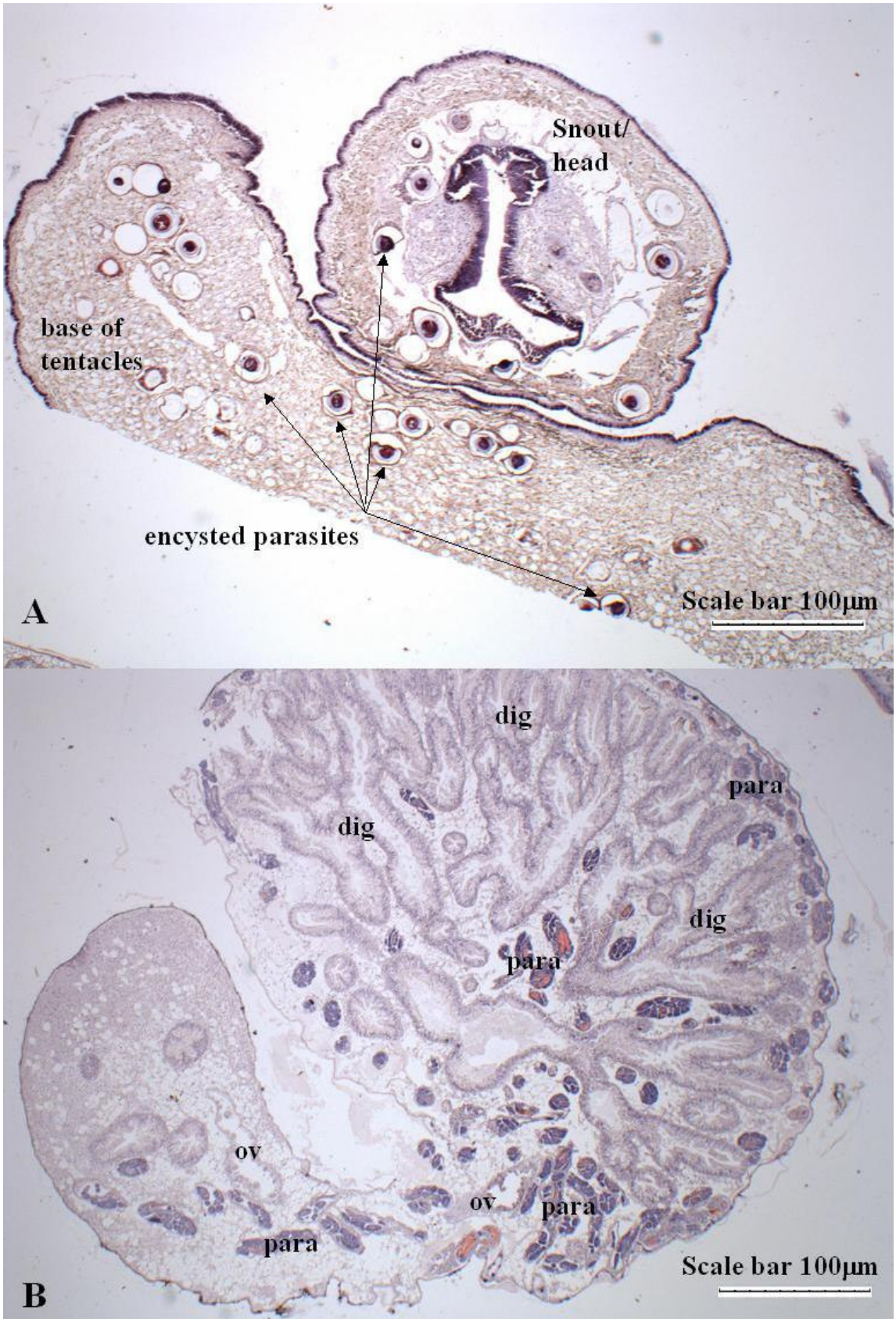

Figure 2.18 Photomicrographs of parasitically infested *V. viviparus*.

Histological sections (5µm thickness) stained with H & E. A, photomicrograph of an adult female's head in longitudinal section (x20), snout and tentacles are visible, as are encysted parasites. B, photomicrographs of an adult female V.viviparus tail, areas of parasite infection are visible as dark purple or pink staining. Digestive tissue – dig; ovary – ov; parasites – para;

Experiment 4B - Results
Male

Of the 30 'health check' *V. viviparus,* 12 were presumed male from examining external morphology of the right tentacle (however, one was misidentified and was actually a small female). The shell height ranged from 17.34 to 29.71 mm (mean 22.88 mm), shell aperture 13.15-20.76 mm (mean 16.75 mm) (Figure 2.19). Total weight ranged from 1.97 to 6.02 g (mean 3.69 g) and soft body weight (shell removed) was 0.75-2.47 g (mean 1.39 g) (Figure 2.19). There was a strong significant positive correlation observed between shell length and total body weight (Spearman's rank correlation P<0.001) and between shell length and soft body weight (P<0.001). Five males were analysed histologically; full spermatogenesis was observed in all males as was the penis complex within the right tentacle (Figure 2.11).

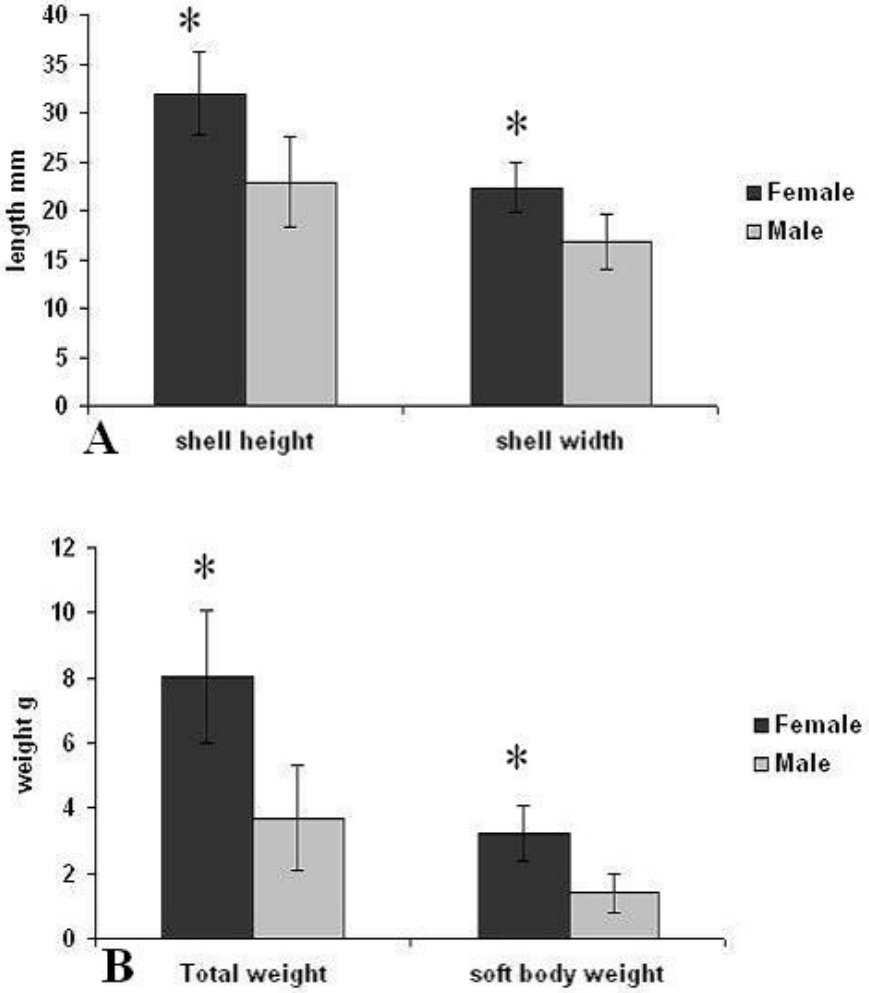

Figure 2.19 Adult *V. viviparus* mean shell size and weight from the 'health check group'.

(A) Mean shell height and width of health check females (n=18) and males (n=12). (B) Mean total weight and soft body weight (shell removed) of health check females and males. Error bars show standard deviation, star (*) indicates significant difference (P<0.001) between males and females.

Female

18 of the 30 'health check' *V. viviparus* were presumed female from examining external morphology (right tentacle). Only one female (smallest 17.2 mm) was initially misidentified as a male (making a total of 19 females). Shell height ranged from 17.2 mm to 37.34 mm with a mean of 31.95 mm, shell aperture ranged from 14.06 to 27.18 mm with a mean of 22.32 mm (Figure 2.19). Total weight ranged from 1.80 to 11.34 g (mean 8.05 g), soft body weight (shell removed) was 0.70-4.35 g (mean 3.24 g) (Figure 2.19). There was a strong significant positive correlation observed between shell length and total body weight (Spearman's rank correlation $P<0.001$) and between shell length and soft body weight ($P<0.001$). Two of the 19 females did not contain shelled embryos when dissected. The total number of embryos per female was not recorded. 16 of the 19 female snails were analysed histologically, the mean number of oocytes per section of ovary analysed ranged from 6.5 to 32 oocytes. There was a positive correlation observed between mean oocyte number and size (as measured by shell length), however R^2 was low ($R^2 = 0.1145$), Spearman's rank correlation found an almost significant value of $P= 0.06$. Figure 2.20 is a photomicrograph of a brood pouch containing embryos at 100 times magnification.

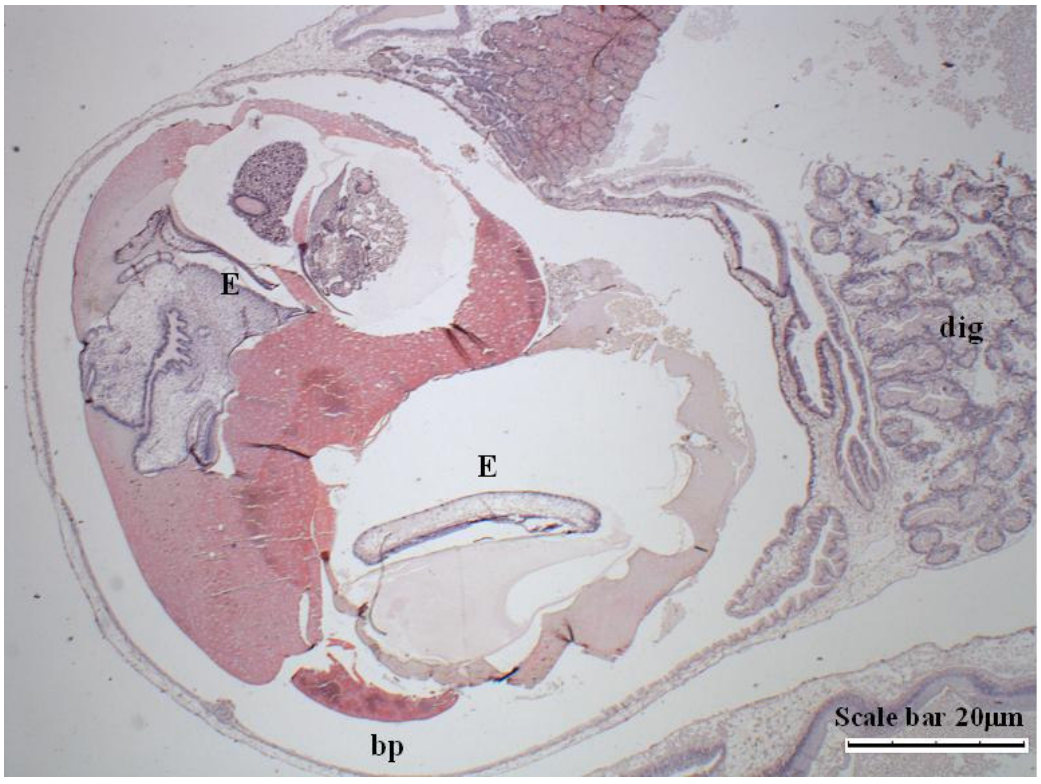

Figure 2.20 Photomicrograph of an adult female *V. viviparus* brood pouch harbouring embryos at x100 magnification.

5µm section stained with H & E. digestive tissue – dig; embryo – E; brood pouch – bp;

Comparison of male and female size

Female *V. viviparus* collected from the Grand Union Canal were significantly larger than males; t-test of shell height P<0.001, shell aperture P<0.001, total weight P<0.001 and soft body weight P<0.001.

Parasite load and abundance

As these snails were obtained from wild populations there was a level of parasitism present. Sixteen females and five male adult *V. viviparus* snails were histologically analysed, of these 100% of the females and 66.7% of the males were found to have some level of parasite infection. Two types of infection were observed. The first and most common were Digenean (Trematode) metacercariae encysted within the head, food and muscle tissue (Figure 2.18A and Figure 2.21). The second, which was only observed in 25% of females, was a sporocyst (trematode) infection of the digestive tissue (Figure 2.18B). Some sporocyst infections were extremely heavy, with approximately 30-50% of the digestive tissue replaced by asexually reproducing larval trematodes. A slight positive correlation was observed between size (as measured by shell length) and mean parasite score in *V. viviparus*. However, correlations were low R^2 = 0.2536 (male and female combined), R^2 = 0.0008 (male alone) and R^2 = 0.0154 (female alone) and none were statistically significant (P>0.05).

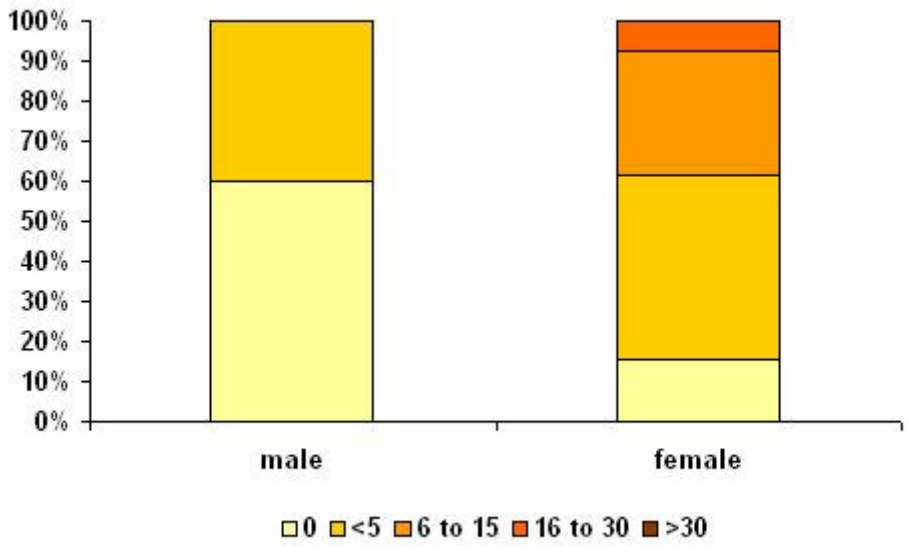

Figure 2.21 Percentage of snails with each specific parasite score (encysted parasites) in adult male and female *V. viviparus* from the 'health check group'.

Colour code indicates severity of infection – pale yellow no parasites to brown more than 30 encysted parasites per section of snail analysed.

Effect of parasite loads on adult *V. viviparus* spermatogenesis

Total spermatogenesis was observed in all male *V. viviparus* analysed, however, the male with the lowest mean spermatogenesis score of 9.9 out of 10 also had the highest average parasite score of 1.444.

Effect of parasite loads on adult *V. viviparus* oocyte number

All females had some level of parasite infection so it was impossible to directly compare parasitised and non-parasitised snail's oocyte number. Mean encysted parasite score in females ranged from 1.125 to 4, with an overall mean parasite score of 2.26. A slight negative (but non-significant) trend was observed between mean parasite score and mean oocyte numbers. Females with digestive tissue infection by Digenean sporocyst had mean oocytes numbers ranging from 7.63 to 24.13, with an overall mean of 13.29 oocytes per section. Whereas, females with just encysted Digenean infections had mean oocytes numbers ranging from 6.5 to 32.0, with an overall mean of 21.52 oocytes per section.

Experiment 4 - Discussion

Adult size

Maximum female shell length (37.34 mm) was generally similar to the value of 38 mm reported in the literature for Worcester-Birmingham Canal, UK (Young 1975) and 35 mm Zegrzynski Reservoir, Poland (Jakubik 2006). The maximum male shell length reported here (29.71 mm) is slightly larger than by other authors including Jakubik (Jakubik 2006) of 20 mm. Female adult *V. viviparus* were significantly larger ($P<0.001$ t-test) than males. This difference in size has been documented in *V. viviparus,* and for other *Viviparus sp.,* by several authors (see Section 2.2.2.1).

Parasite infection

The frequency of parasite infection reported here (100% in females and 66 % in males) is higher than found in the available literature. To illustrate, in Lake Glubokoe (Russia) around 48% of *V.viviparus* (male and female) were infected (Nikitina 1986) and a maximum of 32% infection (male and female) was recorded in the River Narew (Poland) (Jezewski 2004). However, only 21 snails were sampled here and with the majority being large and sexually active, so this may not be an adequate representation of the population within the Grand Union Canal. Another reason for the discrepancy between levels of infection may be due to the sampling methods employed. In the survey conducted by Nikitina (Nikitina 1986), only mature cercariae (free swimming) were counted after they had naturally emerged from the snail host, no dissection or histopathology was conducted

so metacercariae (encysted) would not have been detected. Jezewski (Jezewski 2004) did find metacercariae of several species with up to 21.33% infection in *V.viviparus* from the River Narew.

Effect of parasite load on spermatogenesis

With such a small sample size (n = 5) it was not possible to see any trend in spermatogenesis and parasite load (full spermatogenesis was observed in all 5 snails). Many factors may influence the level of impact that parasites have on spermatogenesis, including time of infection (before or after gonad development), level of infection, species of parasite, and life stage (cercariae/ metacercariae) of the parasite. Further sampling of the population would be necessary to better elude effects of parasitism on male reproduction.

Effect of parasite load on oocyte number and embryos

Again with such small sample size (n = 19) it is difficult to determine a trend between parasite load and fecundity in the female *V. viviparus* sampled. All females carried some level of parasite infection, so comparisons of parasitised versus non-parasitised snails were not possible. Although a negative correlation between parasite load and oocyte number was observed, it was not statistically significant. In addition to this, females with sporocyst infections had a lower average oocyte number than those with just encysted parasites (13.29 ± 9.38 and 21.52 ± 7.38, respectively), indicating that sporocyst infection may provide a larger stress to the animal than encysted parasites. No literature could be found on the relationship between parasite infection and oocyte number in *Viviparus sp*. There are reports that a parasite infection can affect the number of embryos harboured by 2-4 times in *V. viviparus* and *V. fasciatus* (Samochwalenko and Stanczykowska 1972). Unfortunately the actual number of embryos in the brood pouch of each female snail was not recorded here (just the presence or absence). No literature could be found on the relationship between female size and oocyte number in *Viviparus sp.* Jakubik (Jakubik 2007) reports a positive relationship of increased female size on the number of embryos harboured in the brood pouch. Two of the 19 female snails from this collection did not harbour embryos in the brood pouch; one was the smallest female (17.2 mm shell diameter) sampled, and had a parasite score of 2.6 and mean oocyte number of 14. A female snail of this size would be expected to have mature embryos. Indeed, Jakubik (Jakubik 2007) found females above 8.1 mm shell length harboured embryos. However, owing to the time of year they were sampled (late May), fully formed embryos may have already been released. The other was a larger female 31.6 mm shell diameter and had a mean parasite score of 3 and an average of 19 oocytes per section. Later histological

analysis revealed a number of small unshelled embryos at the posterior end of the brood pouch, and therefore she may have also recently released all of her shelled embryos.

2.4 Discussion

The information gained, from both the literature and the preliminary experiments, was vital for devising the mesocosm experiments (Chapters 4 and 5). It was important to understand some basic information on ecology and habitat, so that the experiments could be designed appropriately for the specific species used, such as providing the right type of food or conditions. It was also important to know the different reproductive strategies employed by the different test species, before designing the dosed experiments. Due to the 'gap' in the literature regarding developmental biology and histopathology (of the reproductive organs) of my test species, it was essential to provide this information myself. In doing this, I have obtained some important baseline information, which I can compare with the chemically exposed snails from my mesocosm studies.

3 Materials and Methods

3.1 General Histological Methods

3.1.1 Fixation

Unless otherwise stated all tissues were fixed for histological analysis in at least three times their volume of Bouin's solution (Sigma-Aldrich), for a maximum of 24 hours. Samples were then rinsed twice in 70% Industrial Methylated Spirits (IMS, Charles Tenant) (70% IMS: 30 % distilled water) and then stored in 70% IMS at room temperature.

Post fixation tissue preparation

Prior to processing, snail tissue was placed in biopsy cassettes. Unless otherwise stated the whole snail was sliced transversely (Figure 3.1A) into 3-4 mm sections or rounds using a sharp disposable microtome blade (Shandon MB35 premier). The tissue was then placed in descending size order, from top to bottom, in the biopsy cassette. In the case of *V. viviparus* the operculum was also removed and discarded. The snout and tentacles were also removed so that longitudinal sections could be taken from them. The majority of the foot and the radula (where possible) were not taken for histological analysis. These were instead retained in the fixative bottle. Each specimen was designated its own biopsy cassette (Histosette II) and labelled with the individual snail reference number.

3.1.2 Tissue Processing

Tissue processing removed any water and/or remaining fixative from the tissue by submerging the tissue into a series of increasing alcohol concentrations. The alcohol was then removed by a clearing agent (Histoclear II, RA Lamb) and the tissue was immersed into molten paraffin wax (Lamb wax W1). Finally a vacuum was used to impregnate wax deep into the tissue, replacing the initial liquids. All tissues were processed in a Shandon Citadel 1000 automatic carousel tissue processor (Thermo-Shanndon). Table 3.1 gives the standard immersion times for each solution used. At the end of processing the tissue remained in the final bath of hot wax until it was transferred for embedding (approximately 30-60 minutes).

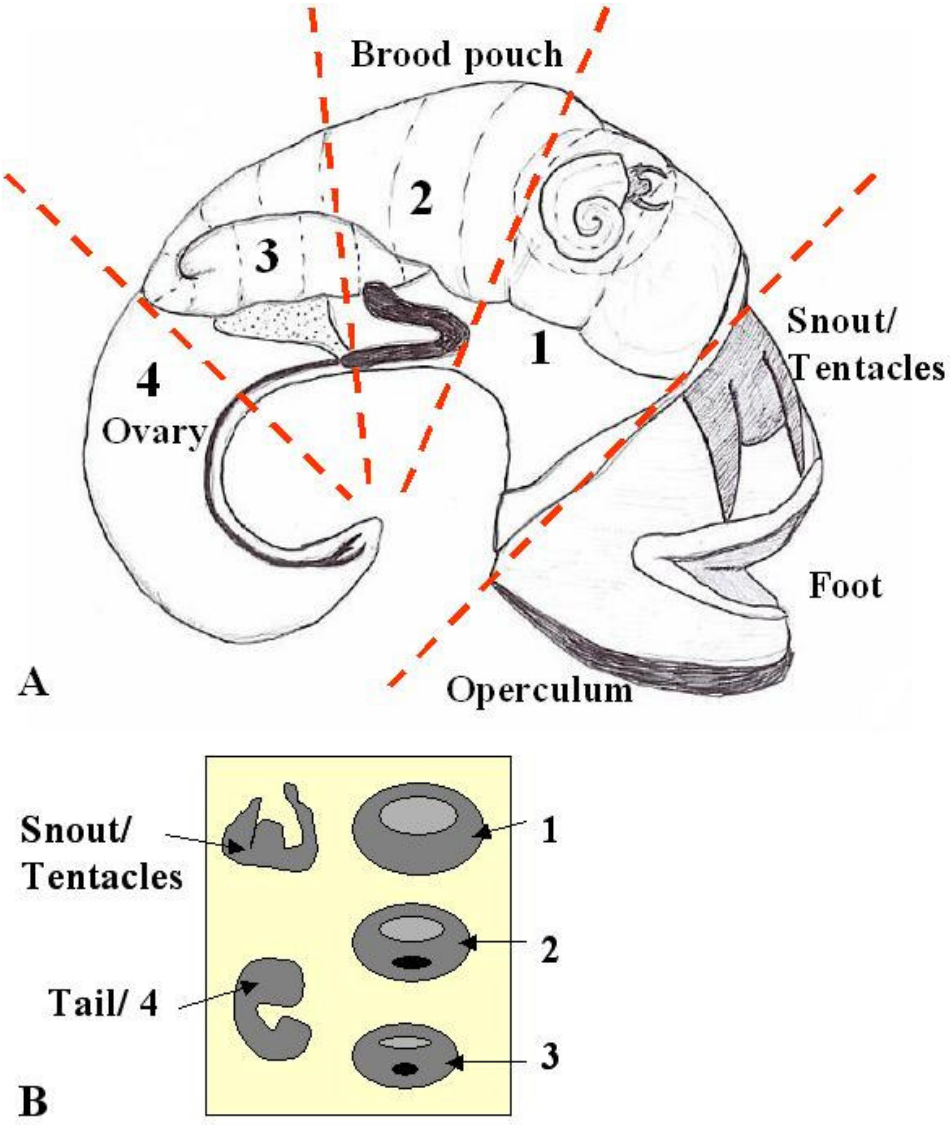

Figure 3.1 Diagrams to illustrate the preparation of whole preserved snail (for illustration female *V. viviparus* used) for cassetting, processing and embedding.

A, Each snail was sliced transversely into 3-4 mm section (rounds) using a sharp disposable microtome blade starting from just behind the tentacles; Red dotted lines indicate cut. The same method was used for both V. viviparus and P. corneus, the head-foot, operculum (*V. viviparus* only) and radular area were not used for histological analysis. In the case of *V. viviparus* the snout and tentacles were also removed so that longitudinal sections could be made. B, Diagram to illustrate arrangement of snail tissue in base mould (cream coloured area) and subsequent wax block from which histological sections were taken. Transverse slices of tissue (grey) were placed into the base mould in descending order (i.e. 1-4 from Figure 3.1A), the frontal end always faced towards the bottom of the mould. With V. viviparus snails the snout and tentacles were placed so that longitudinal sections would be achieved (tentacles were not taken for *P. corneus*).

Table 3.1 Standard immersion time for each solution used for processing tissue in the Shandon Citadel 1000 automatic carousel tissue processor.

Step	Solution	Immersion time (hrs)
1	70% IMS	3
2	90% IMS	2.5
3	95% IMS	1.5
4	100% IMS	1.5
5	100% IMS	1.5
6	100% IMS	1.5
7	100% IMS	1.5
8	Histoclear II	1.5
9	Histoclear II	1.5
10	Histoclear II	1.5
11	Hot paraffin wax	1.25
12	Hot paraffin wax (Vacuum)	1.25
		Total 20 hours

The tissue was submerged in increasing concentrations of alcohol - Industrial Methylated Sprits (IMS), to remove any water or fixative from the tissue. Histoclear II, a clearing agent, was used to remove the alcohol in preparation for wax impregnation of the tissue. Finally the tissue was submerged in molten wax, a vacuum (step 12) was used to pull the wax through the tissue so that it was fully impregnated.

3.1.3 Embedding

Biopsy cassettes containing processed tissue were placed into the hopper of a wax dispensing unit (65^{o}C, RA Lamb) of molten paraffin wax (Lamb wax W1, melting point 57-58^{o}C). Reusable stainless steel base moulds (various sizes, RA Lamb) were also heated in the hopper. Each cassette was removed individually using a long pair of forceps and placed on a mini hotplate (80^{o}C, RA Lamb). A base mould of the correct size (for the volume of tissue) was also removed from the heated wax, and placed on the hot plate (it was important to keep everything hot to avoid wax solidifying). The cassette lid was removed and discarded (care was taken not to discard any tissue attached). The base mould was filled with molten wax from the dispenser, and carefully re-placed on the hotplate (to maintain molten wax). Using electric heated forceps (65 ^{o}C, RA Lamb) the processed tissue was carefully removed from the biopsy cassette and placed into the heated mould. The tissue was organised in descending size order (top to bottom) (Figure 3.1B) and the front most part (of the snail) was always placed facing the bottom of the mould. Once all the tissue was in place the bottom half of the cassette was placed onto of the mould and more molten wax was poured into the base mould/cassette. The base mould (containing the arranged tissue) and cassette combination were then carefully placed onto a cold plate (-8 ^{o}C, RA Lamb), to set the tissue-wax-cassette together. The wax was allowed to cool and set for approximately 15 minutes. The solid wax block (containing tissue) with attached cassette was then removed from the base mould. Blocks were then trimmed of excess wax using a blunt scalpel and stored at room temperature.

3.1.4 Sectioning

All the tissue was sectioned using a rotary microtome (Leica RM2235) and disposable microtome blades (Shanndon MB35 premier 35°/ 80 mm), at a thickness of 5µm. Each section was floated on a distilled water bath (42°C, Paraffin section mounting bath, Barnstead/Electrothermal) until flattened. Each tissue section was floated onto a Histobond glass slide (RA Lamb). Sections were ordered so that the first section removed was closest to the frosted end. The next section removed was positioned below it, and so on. Between two and eight sections of tissue were placed on each slide depending on the size of the tissue. Each slide was labelled with the individual sample reference and slide number on the opaque (frosted) end (with pencil). Slides were left to dry on warming racks (45°C, Photox dishwarmer) overnight (minimum 12 hours) prior to staining.

There were a number of different sectioning protocols used for the snails from the mesocsom exposures. F1 snails (sampled in September 2006) had their entire body (minus head/foot) serially sectioned with tissue sections taken every 100µm. This provided a maximum of 21 slides (each with 6-8 sections of tissue per slide) per snail. Due to the time consuming laborious nature of serially sectioning, staining, and then analysing the whole snail, this was not performed for the adult snails or the breeding study F1s. For adult *P. corneus* four sections of gonad were taken per snail every 100µm. For adult *V. viviparus* ten sections of gonad and tentacles were taken per snail every 100µm. For the F1 breeding study snails (both *V. viviparus* and *P. corneus*) five sections were taken of the gonad (and, tentacles for *V. viviparus*) per snail every 100µm. In each case (other than serially sectioned F1s) a number of sections were taken prior to the ones kept for analysis; to make sure the centre of the gonad had been reached (i.e. not peripheral tissue or just wax).

3.1.5 Staining and mounting/ coverslipping

Slides were stained with Harris's Haematoxylin (RA Lamb) and 1% Aqueous Eosin (RA Lamb) manually in batches of 25 slides per slide rack. The standard immersion times for each solution are given in Table 3.2 below.

After staining, the slides were left to dry in a fume cupboard for two to five minutes or until the majority of the histoclear II (RA Lamb) had evaporated. Small droplets of histomount (RA Lamb) were placed onto each section of tissue and a glass coverslip (22 x 50 mm, No. 1 thickness, RA Lamb) was mounted onto the slide. Care was taken to remove any air bubbles from around the tissue and excess histomount was removed with lens tissue

before the slides were placed on their cardboard drying racks. The slides were dried in a fume cupboard for at least 24 hours.

Table 3.2 Table of standard immersion times for each solution used to manually stain tissue with Harris' Haematoxylin (blue/purple colour) and Eosine (pink colour).

Step	Solution	Purpose	Time (mins)
1	Histoclear II	Dissolved wax	15
2	100% IMS	Step-wise hydration	2
3	90% IMS	Step-wise hydration	2
4	70% IMS	Step-wise hydration	2
5	Tap water	Full hydration	2
6	Harris's Haematoxylin	Colour binds to nucleus	10-15
7	Tap water	Removed excess stain	10-15
8	Acid-IMS (70% IMS plus 1% Hydrochloric acid)	Dechlorination	20-30 seconds
9	Tap water	Rinse	20-30 seconds
10	Saturated solution of $LiCO_3$ in distilled water	Salt, raises ph	20-30 seconds
11	Tap water	Rinse	20-30 seconds
12	Eosin 1% aqueous	Counter stain for cytoplasm	30-60 seconds
13	Tap water	Removed excess stain	5
14	70% IMS	Step-wise dehydration	2
15	90% IMS	Step-wise dehydration	2
16	100% IMS	Step-wise dehydration	5
17	Histoclear II	Remove IMS, binding agent	5

Time given in minutes unless other wise stated. Tissue was step-wise hydrated to allow stain to penetrate cells (Haemotoxylin and Eosin function in aqueous solution) and then step-wise dehydrated to prevent oxidisation after staining. The purpose of each solution used is noted.

3.1.6 Slide blinding

All the slides made from the snails sampled from the mesocsom studies were blinded prior to analysis, to prevent bias. To do this the opaque end of the slide, on which the reference number was written, was covered in masking tape (both sides of the slide). As the masking tape was a little opaque at times a small white sticker was stuck to the masking tape so that the reference could no longer be seen (masking tape was easier to remove after analysis than stickers alone). The slide number was re-written on the sticker so that the sectioning order could be maintained. Each group of slides belonging to one snail were kept together

using elastic bands. When all the slides were blinded, and bound with an elastic band, they were placed in a large cardboard box. Snails from both the oestrogenic mixtures and E2 mesocosm exposures were mixed together in the same box. However, the two species were not mixed together. The different groups of snails i.e. adults, September sampled F1s (sampling period 1) and F1 breeding study snails (sampling period 2) were also put in separate boxes due to the different analysis methods (see section on histopathological analysis: Section 3.2). When the time came to analyse the tissue, a group of slides representing one snail, was picked out of the box and given a new (blinded) reference number. The slides were then analysed using the appropriate protocol. Once all the slides were analysed the masking tape (and stickers) was removed and the blinded reference number was linked with the original reference number.

3.1.7 Light Microscopy and Photography

All slides were viewed under an Olympus (BX51) light microscope. Slides were viewed using x2, x10 and x40 objectives (total magnification x20, x100 and x 400 respectively). Photomicrographs were taking using a digital camera (Q Imaging Micropublisher 5.0RTV) linked to a personal computer. Q Capture Pro 5.1 software was used to capture and view images from the digital camera.

3.2 Histopathological analysis

As little previous research had been conducted to look at disruption to the gonad in these two species, it was necessary to devise and optimise a number of scoring methods to provide data for analysis. In the case of *V. viviparus* two fairly simple methods for determining stage of spermatogenesis or number of oocytes present is described in Chapter 2, Section 2.3.3.4. However in the case of the more complex ovotestis of the *P. corneus* a more comprehensive method was required to evaluate disruption. To start with two or three snails from either RW exposed or non-exposed (laboratory bred) were processed, serially sectioned whole, and stained using the methods described in Sections 3.1.2 to 3.1.5. All the sections of these snails were analysed to see how far the gonad extended, and to see if the structure of the gonad or maturity of gametes changed depending on location along the gonad. After this range finding work, a number of individuals from the exposed groups were also analysed (prior to blinding) to assess the types of disruption that might occur. A couple of pilot studies with a small number of F1s and adults were used to test several scoring methods. Once the scoring methods had been optimised, and the gonad range finding was conducted, the following methods were used to analyse the sections of gonad.

3.2.1 General gonad scoring methods used for *P. corneus*

Snails sampled at different points of the mesocosm studies (adults, F1s and F1 breeding study snails) were sectioned using different protocols. This meant that different methods were also used when choosing which sections of gonad to analyse. For the F1 *P. corneus* sampled in September (sampling period one) five sections of gonad were scored using the scoring methods (below). To find which of the sections to score the following method was followed: All sections of F1 snail were analysed for the presence of gonad tissue. The number of sections per slide that contained were recorded as were the total number of gonad sections per snail. The section closest to the middle of the gonad was then located, as were the sections a third of the way into the gonad (i.e. a third from the start, and a third from the end). These three sections plus two from either side of the midsection (i.e. one before middle, and one after) were used for scoring analysis. For the adult and F1 breeding study snails (i.e. those not wholly serially sectioned) all sections of gonad taken (four and five, respectively) were scored using the *P. corneus* scoring methods (below).

Gonad structure in the *P. corneus* gonads from exposed and non-exposed mesocosms differed in several ways, seen in both the gametes and supporting cells. Consequently, one scoring method that adequately described all of these differences could not be devised. Instead a number of scoring systems were employed relating to a process, such as oogenesis, or area of the acini, such as the vitellogenic area. The different anomalies in gonad structure are described below:

Percentage of different oogenesis stages present – oogenesis stages were defined as stage 1, stage 2, stage 3, stage 4, stage 5, and degenerating (De Jong-Brink et al. 1976). Stage 1 (oogonium) was the youngest (least developed), and stage 5 was ready for ovulation, with a well developed follicle cavity ((fc), see Figure 3.3 and Figure 3.4). The number of each stage present was recorded for each section of gonad analysed, the total number of oocytes was then calculated (by adding all the stages). The percentage of each stage present was then calculated i.e. percentage of stage 1 oocytes = (total number of oocytes ÷ number of stage 1 oocytes) x 100. For each snail the average (mean) percentage was used for comparison and statistical analysis.

Vitellogenic area activity of acini – This was measured by first counting the total number of acini per section of gonad. Then counting the number of acini that had active vitellogenic areas (i.e. which had maturing oocytes (stages 4 or 5), sperm and/or degenerating oocytes, follicle or sertoli cells). Acini with no cells attached to this area were

counted as not active, see Figure 3.2 for examples of active (A) and non-active (B) vitellogenic areas. The percentage of active vitellogenic areas was then calculated i.e. percentage of acini with active vitellogenic areas = (total number of acini/number of acini with active vitellogenic areas) x 100. This was then simplified into a score, with, 1 = <10% of acini with active vitellogenic areas, 2 = 10-30% of acini with active vitellogenic areas, 3 = 30-50% of acini with active vitellogenic areas, 4 = 50-70% of acini with active vitellogenic areas, and 5 = >70% of acini with active vitellogenic areas. For each snail the average (mean) score was used for comparisons and statistical analysis.

Acini wall cover – To estimate the percentage acini wall cover (in developing oocytes, sperm and supportive cells), for each section analysed, a 10 x 10 graticule was placed into the microscope eyepiece, and the tissue was viewed at x400 magnification. A score of 5 was given if more than 70% of the acini walls were covered in developing germ and/or supportive cells, a score of 4 was given if 50-70% were covered, 3 if 30-50% were covered, 2 if 10-30% were covered and 1 if less than 10% of the acini walls were covered in cells. An average percentage cover for each section was calculated (the percentage cover per field of view were added together ÷ the number of fields of view taken) so that each section was given a score. Then an average (total section score ÷ number of sections scored) score was used for comparisons and statistical analysis.

Level of acini with immature spermatogenic cells in lumen - This was measured by first counting the total number of acini per section of gonad. Then, the numbers of acini with immature spermatogenic cells (spermatogonium, spermatocytes and spermatids) or sertoli cells sloughed into the lumen were counted. The percentage of acini with immature spermatogenic cells sloughed into the lumen were then calculated i.e. percentage of acini with immature spermatogenic cells sloughed into the lumen = (total number of acini/number of acini with sloughed immature spermatogenic cells) x 100. This was then simplified into a score, with, 1 = <10% of acini with sloughed immature spermatogenic cells, 2 = 10-30% of acini with sloughed immature spermatogenic cells, 3 = 30-50% of acini with sloughed immature spermatogenic cells, 4 = 50-70% of acini with sloughed immature spermatogenic cells, and 5 = >70% of acini with sloughed immature spermatogenic cells. For each snail the average (mean) score was used for comparison or statistical analysis. See Figure 3.4 and Figure 3.5 for examples of immature spermatogenic cells sloughed into the lumen.

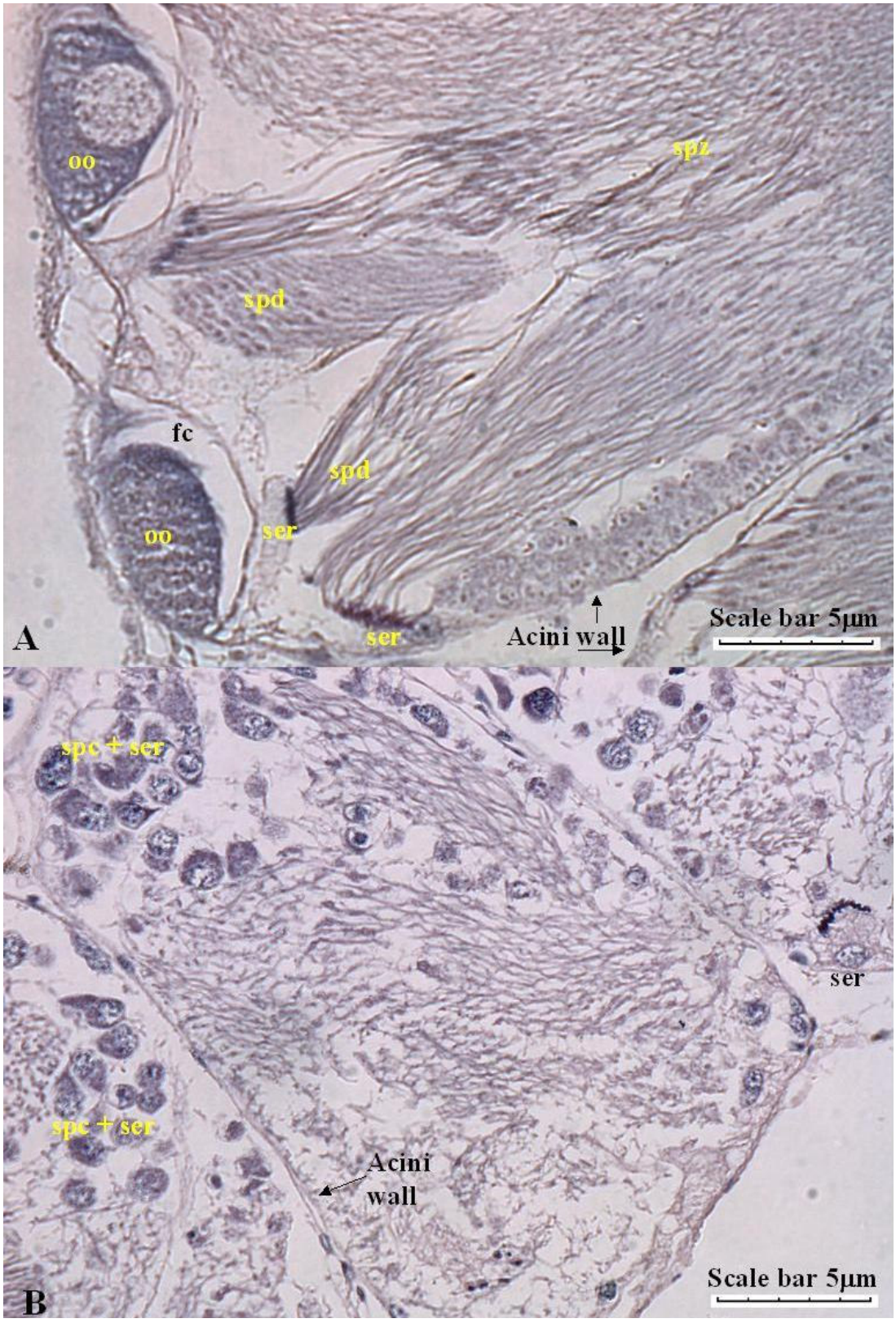

Figure 3.2 Photomicrographs of *P. corneus* gonad acini at 400 x magnification.

(A) Active vitellogenic area with maturing oocytes and spermatids with supporting sertoli cells, the acini walls are covered in spermatogenic cells, the acini lumen is filled with spermatozoa. (B) non-active vitellogenic area, no maturing oocytes present, many spermatocytes and sertoli cells sloughed into the acini lumen, acini walls bare of spermatogenic cells. Histological sections (5µm) stained with H&E. Spermatozoa – spz; Spermatids – spd; Spermatocyte – spc; Spermatogonium – spg; Sertoli cell – ser; Oocyte – oo; follicle cavity – fc;

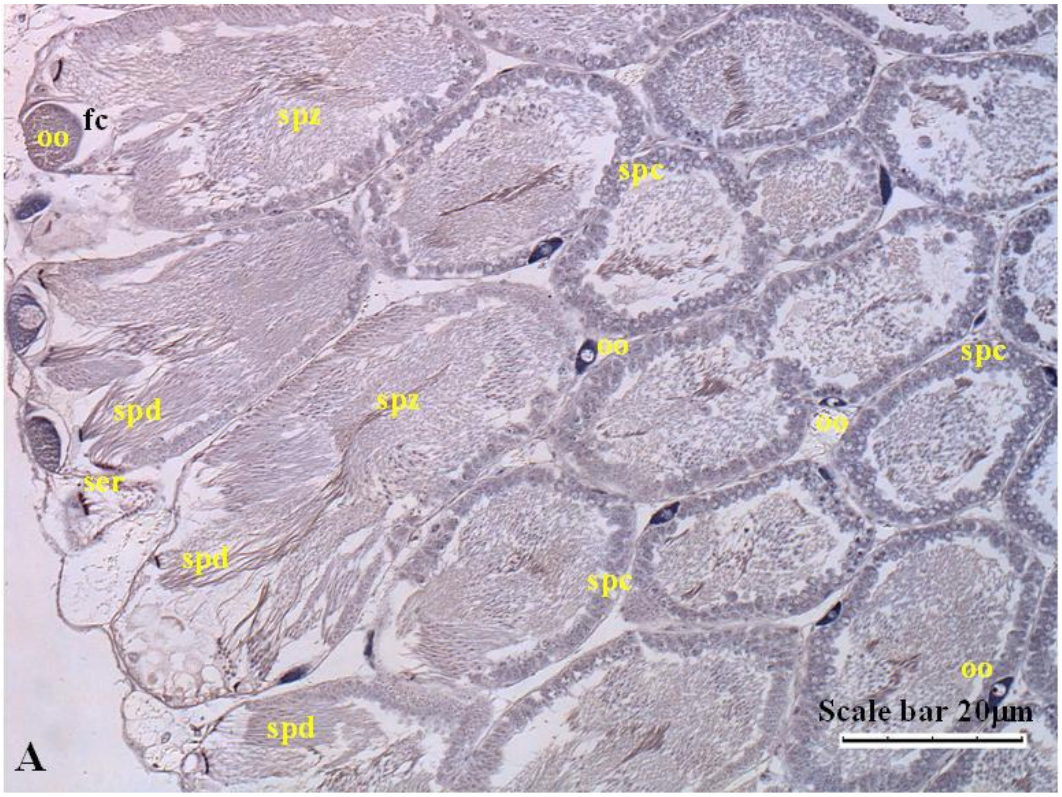

Figure 3.3 Photomicrograph of *P. corneus* healthy gonad with a number of well defined acini at 100 times magnification.

Acini walls are covered in spermatogenic and oogenic cells, majority of vitellogenic areas are active. Acini lumen filled with spermatozoa. Full range of spermatogenesis, oogenesis and supporting cells. Histological sections (5μm) stained with H&E. Digestive tissue – dig; Spermatozoa – spz; Spermatids – spd; Spermatocyte – spc; Spermatogonium – spg; Sertoli cell – ser; Oocyte – oo; follicle cavity – fc;

Binary variables (scored as Yes or No) included:

For the each of the below the whole section was analysed.

Sertoli cells attached to acini walls – yes/no (if no, there were no Sertoli cells attached to the acini walls per section analysed)

Acini wall disruption – yes there was disruption (broken/ displaced walls) or no (intact walls).

Mature spermatogenic cells in the lumen only – Recorded as either yes there were only mature spermatogenic cells (spermatozoa) in the acini lumen or no there were other (immature or sertoli cells) spermatogenic cells in the acini lumen.

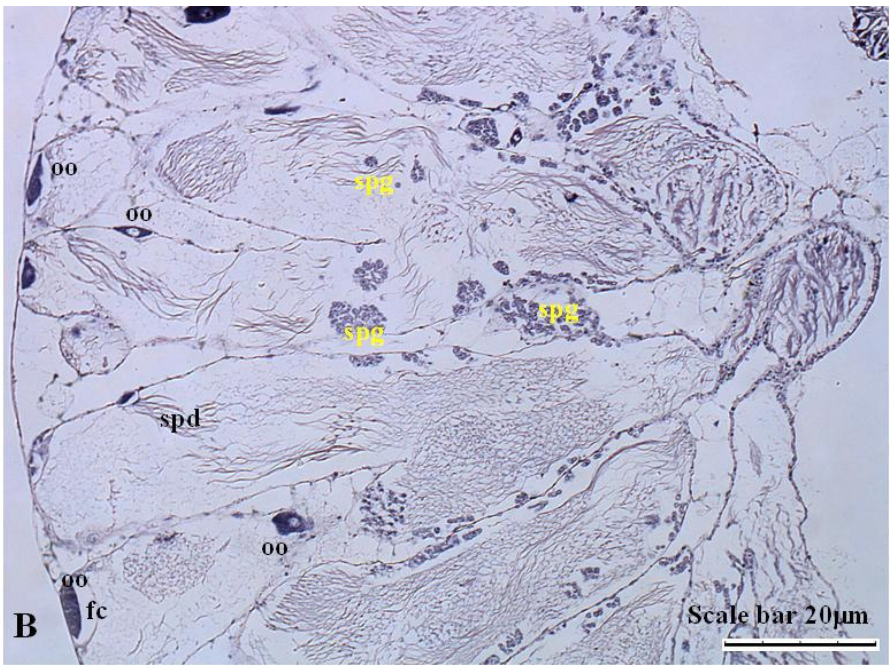

Figure 3.4 Photomicrograph of *P. corneus* partially disrupted gonad with with a number of definable acini at 100 times magnification.

Acini walls only covered in patches of spermatogonium and a couple of oocytes. Some vitellogenic areas not active, whilst other only have oocytes and not spermatogenic cells. Some spermatogonium and oocytes have sloughed into the acini lumen. Histological sections (5µm) stained with H&E. Digestive tissue – dig; Spermatozoa – spz; Spermatids – spd; Spermatocyte – spc; Spermatogonium – spg; Sertoli cell – ser; Oocyte – oo; follicle cavity – fc;

Immature spermatogenic cells sloughing into lumen (Figure 3.4 and Figure 3.5) – For this each of the types of immature spermatogenic cells were recorded separately (i.e. spermatogonium, spermatocytes and spermatids). These were recorded, as either yes there were immature spermatogenic cells (of each type separately) sloughed into the lumen or no there were not.

Sertoli cells sloughing into lumen (Figure 3.4 and Figure 3.5) – yes/no

Once all the sections had been scored, the number per sections scoring yes (e.g. analysed 2 out of 5 sections analysed scored yes) was used for comparison of different snails.

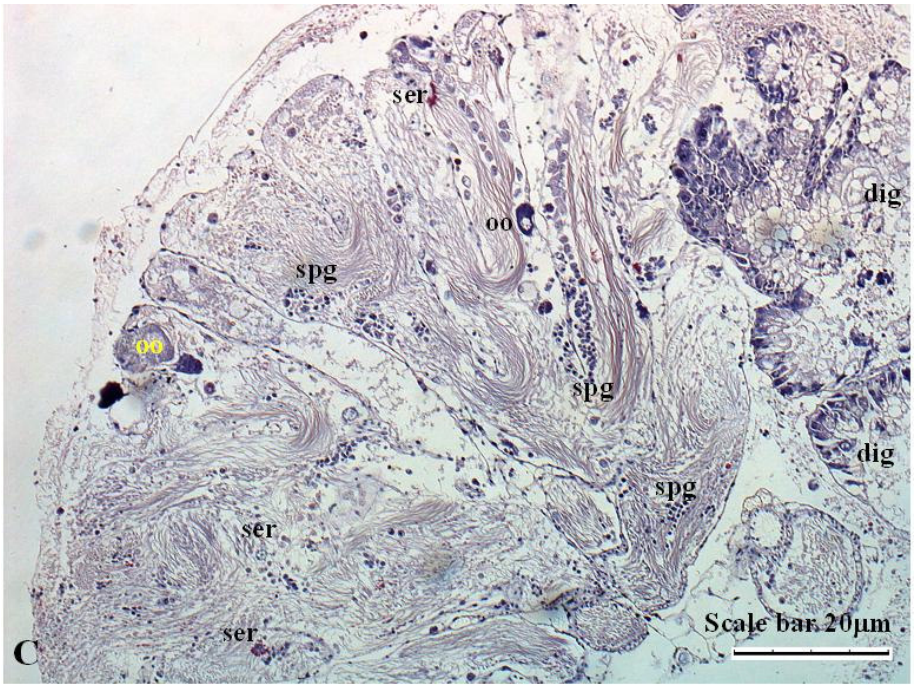

Figure 3.5 Photomicrograph of *P. corneus* heavily disrupted gonad showing acini at 100 times magnification.

Acini walls are disrupted and ill defined. Acini walls are only covered in a few spermatogonium. The majority of vitellogenic areas are not active. Spermatogonium and sertoli cells sloughed into the acini lumen. Histological sections (5µm) stained with H&E. Digestive tissue – dig; Spermatozoa – spz; Spermatids – spd; Spermatocyte – spc; Spermatogonium – spg; Sertoli cell – ser; Oocyte – oo; follicle cavity – fc;

Parasite score – Each snail was also assessed for parasite infection. The type of parasite infection seen in *P. corneus* was different than those seen in *V. viviparus* (see Chapter 2, Section 2.3.3.4), therefore a different scoring system was used. When an infestation was seen, a 10 x 10 graticule was used to estimate the percentage of tissue infected. A score of 1 indicated no observed parasites in the tissue sections analysed (same section as for gonad analysis). A score of 2 indicated less than 50% of the snail's reproductive or digestive tissue was infected with parasites. A score of 3 indicated more than 50% of the snail's reproductive or digestive tissue was infected with parasites. For each snail the average (mean) score was used for comparison and statistical analysis. Figure 3.6 illustrates what a Digenean parasite infection in *P. corneus* looks like under the light microscope at 100 times magnification, in a prepared histological section.

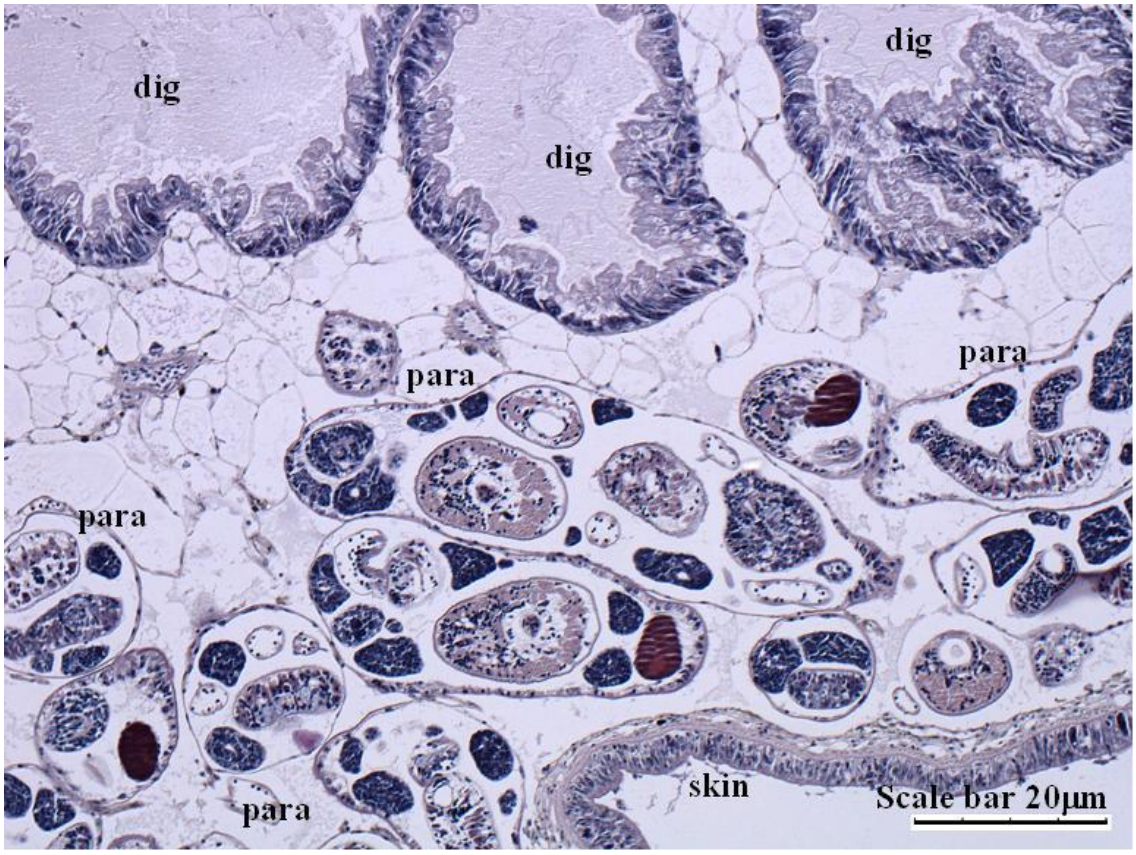

Figure 3.6 Photomicrograph of *P. corneus* digestive tissue (100x) with parasitic Digenean infection.

Each sac contains a number of developing larval digenea; in some cases the larvae's ventral or oral sucker can be observed. Histological sections (5µm) stained with H&E. Digestive tissue – dig; parasite infection – para;.

3.2.2 General scoring methods used for *V. viviparus*

Snails sampled at different points in the mesocosm studies (adults, F1s and F1 breeding study snails) were sectioned using different protocols. This meant that different methods were also used when choosing which sections of gonad to analyse. For the F1 *V.viviparus* sampled in September (sampling period one) five sections of gonad were scored using the scoring methods (below). To find which of the sections to score the following method was followed. All sections of F1 snail were analysed for the presence of gonad tissue. The number of sections containing gonad per slide were recorded, as were the total number of gonad sections per snail. The section closest to the middle of the gonad was then located, as were the sections a third of the way into the gonad (i.e. a third from the start, and a third from the end). These three sections plus two from either side of the midsection (i.e. one before middle, and one after) were used for scoring analysis (Chapter 2, Section 2.3.3.4). For the adult and F1 breeding study snails (i.e. those not wholly serially sectioned) all sections of gonad taken (ten and five, respectively) were scored using the *V. viviparus* scoring methods (Chapter 2, Section 2.3.3.4).

Sex determination – The sex of an individual was determined by the presence of sex cells (spermatogenesis – male, or oogenesis - female), of secondary sexual organs (i.e. penis/sperm duct within right tentacle – male, or no duct in right tentacle plus brood pouch - female), and by sex glands (prostate – male, or capsule and albumen gland – female).

Parasite score – The same sections of tissue analysed for spermatogenesis or number of oocytes were also assessed for their parasite score (Chapter 2, Section 2.3.3.4).

3.3 Water sample preparation for Yeast Estrogen Screen (YES)

Extraction and concentration of oestrogenic chemicals from water samples

Extraction and concentration of oestrogens or oestrogenic chemicals from water samples was achieved using a Supelco 'visiprep' vacuum manifold (Figure 3.7C) and reverse-phase C18 cartridges (Sep-Pak C18, Waters Associates, inc). Each C18 cartridge was positioned into the port of the extraction manifold with the silicone liner inserted into the stainless steel needle beneath, and plastic sample well above (Figure 3.7A). The vacuum unit was then attached to the manifold and switched on. The C18 cartridge was primed by adding to the sample well 5ml of double distilled water, and then 5ml of methanol (these were sucked thought the cartridge by the vacuum) (Figure 3.7B). After priming it was important that the C18 cartridge did not dry out so another 5ml of double distilled water was added while the well bung was applied. This bung had and attached plastic straw, which was suspended into the water sample at the other end, weighed down by a stainless steel weight (Figure 3.7C). The vacuum then pulled the water sampled through the plastic straw, into the sample well, and through the C18 cartridge (where the oestrogens were bound). The wastewater dripped through the disposable liner and into the base of the manifold, where it was removed via a rubber tube to a collecting vessel for disposal (Figure 3.7C). Once all of the water sample had passed through the C18 cartridge, the bung and attached straw were removed. The C18 cartridge was left to dry with the vacuum on for at least 30 minutes to remove any water. The manifold lid with attached cartridges was then removed so that double solvent rinsed (twice with methanol, once with ethanol) glass collecting vials could be placed below each cartridge. The manifold lid was then replaced so that the C18 cartridge/ disposable liners were aligned with the collecting vials. The C18 cartridges were eluted with 5ml of methanol under a vacuum at approximately 2ml/min (Figure 3.7D). Then the methanol-elutant was dried (evaporated) under nitrogen gas using the supelco 'visidry' attachment for the extraction manifold. The sample was then re-suspended in 1 ml of ethanol (unless other volume stated). The concentration factor (calculated as the volume

of water extracted/the volume of ethanol the sample was re-suspended in) was recorded. Vials containing samples in ethanol were stored with tight fitting lids at 4ºC to prevent evaporation.

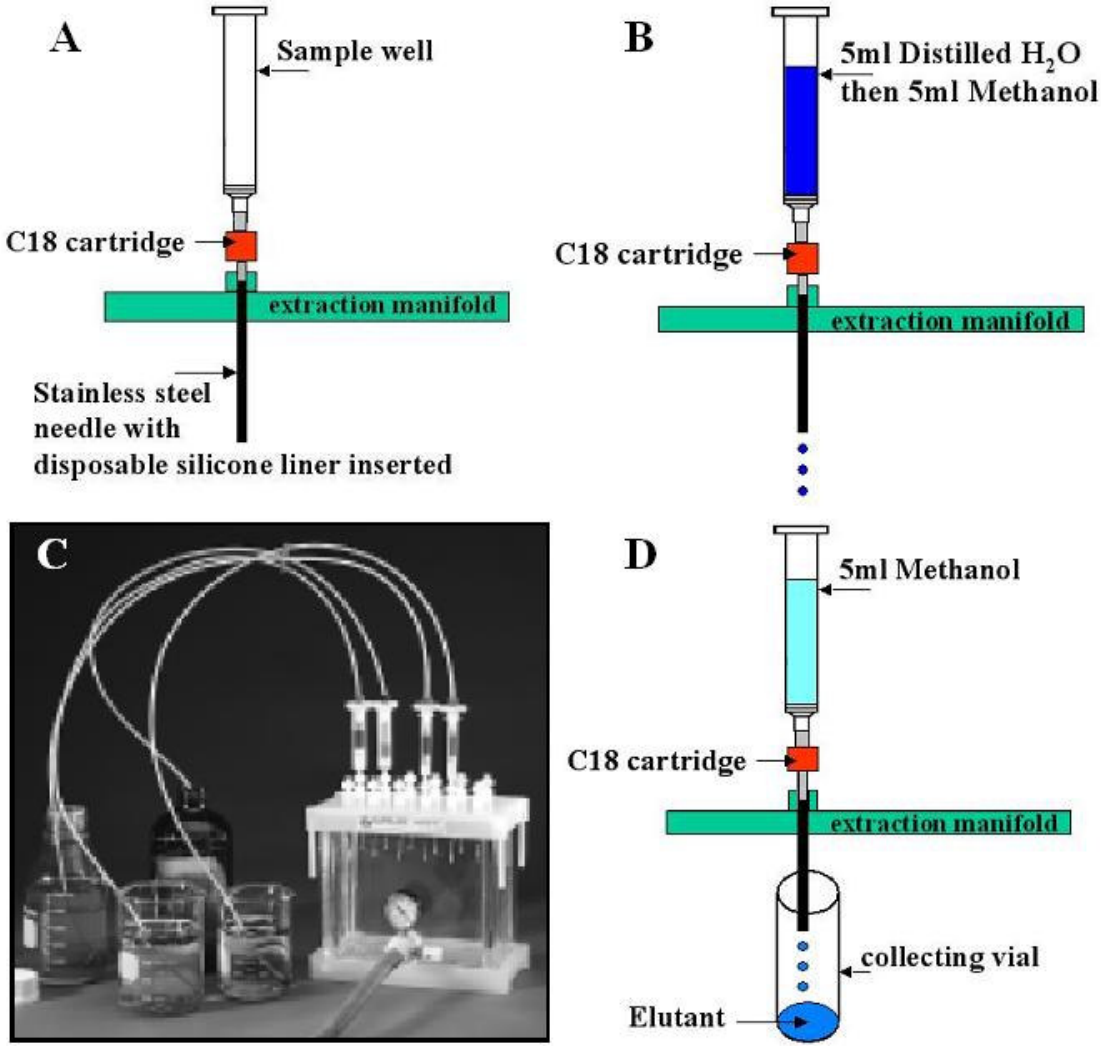

Figure 3.7 Diagrams illustrating extraction manifold at various stages of sample extraction.

(A) The C18 cartridge and sample well were positioned into the manifold port above the stainless steel needle with inserted disposable liner. (B) The C18 cartridge was primed with 5ml of double distilled water and then 5ml of methanol under vacuum (~2ml/min). (C) The bungs were placed on the sample well with their attached straws, which were suspended in the water sample vessels (left hand side of photo). The pressure provided by the vacuum pulled the sample water up the straw, into the sample well, and down through the C18 cartridge. Wastewater dripped out of the disposable liners into the base of the manifold, and exited via a tube (centre of the manifold in photo). (D) Once dry, the sample was eluted from the C18 cartridge with 5ml of methanol. The elutant was collected in a double solvent washed glass vial. Photograph from Supelco advertising literature (www.sigmaaldrich.com, 2008).

3.4 Yeast Estrogen Screen (YES) Assay

The yeast assay was carried out in a type II laminar flow cabinet. The protocols and recipes for the component parts of the growth medium can be found in the Appendix I.

3.4.1 The assay

Day 0 - Preparation of yeast growth medium for assay. The growth medium was prepared by adding 5 ml glucose solution, 1.25 ml L-aspartic acid solution, 0.5 ml vitamin solution, 0.4 ml L-threonine solution, and 125 µl copper (II) sulfate solution to 45 ml of sterile minimal medium. This mixture was then transferred to a sterile conical flask. To this 125µl of 10X concentrated yeast stock was added from a cryogenic vial. The inoculated growth medium was then incubated at 28˚C for approximately 24 hour on an orbital shaker.

Day 1 - Preparation of yeast assay plates and assay medium. A 96-well microtitre plate was used to serially dilute each assay sample (prepared from water extraction, Section 3.3) in absolute ethanol. 100µl of absolute ethanol were pipetted into each row of wells, accept for the first well. 100µl of sample was pipetted into the first and second wells. 100µl of the 50:50 mixture of sample and ethanol was removed from the second well and transferred to the third well. This was mixed three times by pulling the liquid up and down the pipette. Once the sample was well mixed, 100µl was taken from this well and transferred into the fourth well, and so on until all 12 wells had been completed (the final well contained 200µl). The 17β-estradiol standard curve dilution was prepared in the same manner. The 17β-estradiol stock for the standard curve was prepared in absolute ethanol at $2x10^{-7}$ M (54.48 µg/L) concentration (Appendix I). From each of the dilution wells in the dilution plate a 10µl aliquot was transferred to a new 96-well microtitre assay plate, starting from the most dilute (most concentrated on the left, least on the right). Each sample dilution was replicated so that two identical rows were produced. Between each double row of sample/standard curve one row was left completely empty (blank). In addition to the samples, blank and standard curve rows two rows of 10µl absolute ethanol (same as used for dilution preparation) were included. This ensured any actively via contamination of the solvent was known and accounted for.

Once all assay plates were complete they were left to evaporate to dryness in the type II laminar flow cabinet. While the assay plates dried the assay medium was prepared. For every 2 1/2 96-well assay plates, 50ml of assay medium was required. The assay medium was prepared in a similar manner to that of the growth medium with a couple of differences; firstly, 0.5ml of Chlorophenol red-β-D-galactopyranoside (CPRG) was added

to the growth medium. Secondly, instead of inoculating with 125µl of concentrated yeast stock from the cryogenic vial, this medium was inoculated with $4x10^7$ yeast cells from the 24-h culture prepared on Day 0, per 50ml of growth medium. This was calculated by taking two 100µl aliquots of the 24-hour culture, each of these were placed into a well of a 96-well microtiter plate. The absorbance of the duplicate 24-hour culture were read at 640nm using a spectrophotometer plate reader, and the average value was used to extrapolate the number of cells per ml using the graph shown in Figure 3.8. The volume of 24-hour culture needed to give 4×10^7 cells per 50ml of growth medium was calculated using the following equation.

Vol of 24-hour culture = 4×10^7 / No. cells calculated from graph

Once the volume of 24-hour culture to be added was found, it was added to the new assay medium, and mixed well. By this time all of the ethanol had evaporated from the 96-well assay plates. 200µl of the yeast inoculated assay medium was added to each well of the assay plate using a multichannel pipette. Each plate was then sealed round its edges using autoclave tape, to prevent evaporation of medium whilst incubated (which may affect the concentration of CPRG and therefore the background or measured response at 540nm). Each plate was then shaken vigorously for 2 minutes on a titre plate shaker before being incubated at 32˚C in a naturally ventilated heating cabinet.

Day 2 – Assay plate maintenance. On day two the assay plates were removed from the incubator and shaken vigorously for 2 minutes on a plate shaker, to mix and disperse the multiplying yeast cells. After which, the plates were returned to the incubator at 32˚C.

Day 3 – Reading assay plates. After three days of incubation the assay plates were again vigorously shaken for 2 minutes on a plate shaker. The plates were then left for approximately 1 hour to re-settle. The assay plates were then read on a spectrophotometer plate reader. The plates were read at an absorbance of 540nm (for colour) and 620nm (for turbidity). The plates were then left at room temperature and read again the following day (after shaking and resting) if a longer period of incubation was needed.

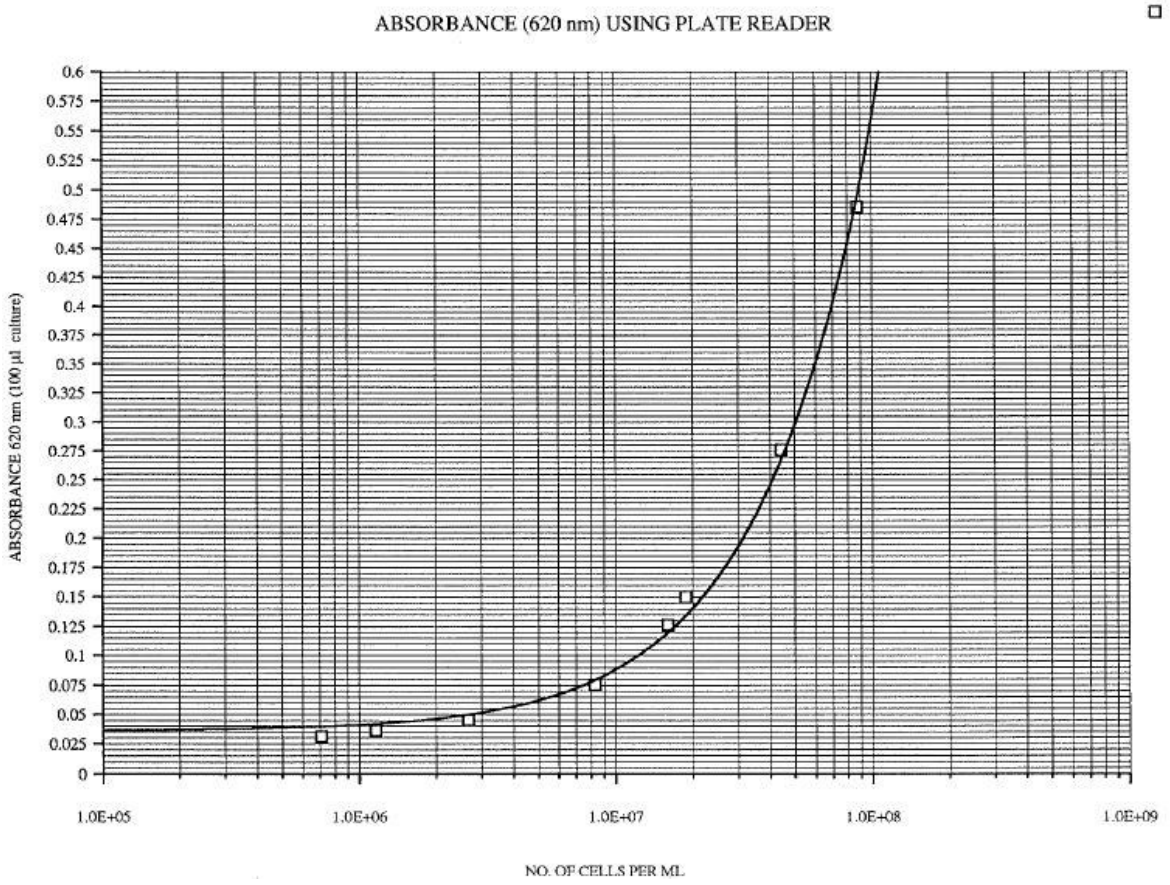

Figure 3.8 Graph used to calculate volume (ml) of 24-hour yeast culture to be added to 50ml of assay medium (see equation above).

On the Y-axis is the absorbance at 620nm for 100µl of 24-hour yeast culture. On the X-axis is the number of cells per ml.

3.4.2 Yeast assay calculations

Firstly, the colour of the assay medium (from the 540nm reading) was corrected for turbidity (from the 620nm reading). The following equation was used to obtain corrected values.

Corrected value = Sample absorbance (540nm) – [sample absorbance (620nm) – blank absorbance (620nm)]

Calculating oestrogenic equivalents

The oestrogenic equivalents were calculated by first plotting the corrected absorbance values (see correction calculation above) at 540nm against the relative concentration of each sample, the E2 standard curve was also plotted (as was the ethanol blank, to check for contamination). When plotted the E2 standard and sample dilutions produced sigmoidal curves. Both replicates (rows) were plotted on the same graph. If the two sets of points were not similar the assay was re-run. Points on the curves that were maximal (flattened area at top of curve), or due to toxicity to the yeast (sudden drop in absorbance) were

discarded. Only the linear part of the graph was used for calculations. The mid point of this area was obtained, and the two corrected absorbance values either side of the mid point were taken. The well numbers of these two points were noted. These corrected absorbance values at 540nm were then extrapolated from the standard curve. Then each value was multiplied to find the concentration in the first well (i.e. before serial dilution). These values were then multiplied by the molecular weight of 17β-estradiol (272.4), to provide a concentration in weight per volume, rather than a Molar concentration. These values were then multiplied by 20 to account for the volume of assay medium, and then divided by the original concentration factor (Volume of water extracted/volume of ethanol eluted, Section 3.3) to give the concentration of the initial water sample in g/l. A mean of the two values was used as the final oestrogenic equivalent concentration of the original sample.

3.5 Mesocosm Experiments

3.5.1 Experiment overview

The two exposures, E2 (Chapter 4) and oestrogenic mixtures (Chapter 5), ran in parallel. The exposure period ran for 16 weeks from early May until late August 2006. Seven mesocosm tanks were used in total (Figure 3.9). Each fed with water from the River Chelmer. At the start of the experiment each tank held groups of caged adult snails of both species. Mortality and reproductive output of adult snails were recorded fortnightly over the 16 weeks of the exposure. Offspring (F1) produced by the adults were re-released into the parental tanks (see individual species sections for details). After chemical (E2 or mixture) exposure was terminated, all adult snails were sacrificed, biometric data was recorded and adult snails were histologically fixed for histopathological analysis. After the adult snails were removed, all F1 snails from each tank were also removed from the tanks. The number of F1s from each tank and their size-class were recorded. A number of F1s were sacrificed, had their biometric data recorded, and were also fixed for histopathological analysis. The remaining F1s were re-released back into their original tanks; which were filled with river water only. These F1s were left in the tanks to over-winter and depurate. The following spring the tanks were again scoured for surviving F1s and their number recorded. A number of these depurated F1s were placed into breeding groups in a similar manner to their parents. A further un-exposed breeding study was conducted over 14 weeks for the *P. corneus* and 18 weeks for the *V. viviparus*. F1 reproductive output and mortality were recorded fortnightly. At the end of these F1 breeding studies, the snails were sacrificed, their biometric data were recorded, and the snails were fixed for histopathological analysis. In total the experiments ran for one year

and four months and over a thousand snails were analysed. Figure 3.10 shows a schematic of the experiment timeline and at which point sampling occurred.

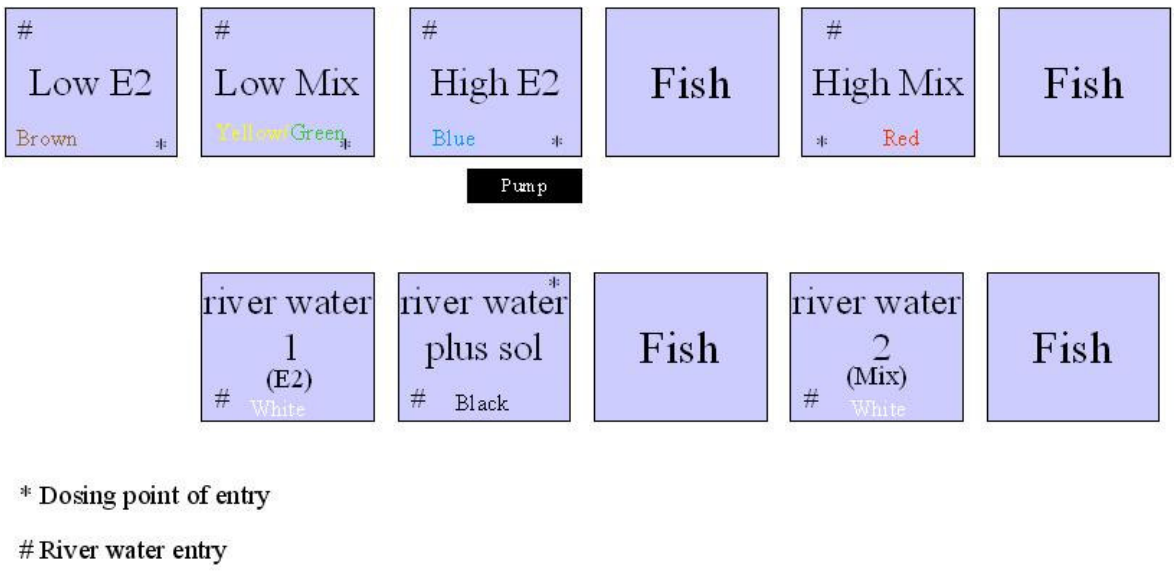

Figure 3.9 Diagram of the layout of mesocosm tanks at Langford water treatment works.

The diagram shows the position of the tanks used for each experiment E2 (low E2, high E2 and river water 1) and oestrogenic mixtures (low mix, high mix, river water 2 and river water plus solvent). # Indicates the point of entry for the river water for each tank. * Indicates the dosing point of entry, dosing was via peristaltic pump located next to the high E2 tank. The colours labelled on the tanks indicate the colour coding used for dosing reservoirs/pump tubing/sample bottles etc. The tanks labelled 'Fish' were not part of these experiments, and were used by other investigators.

As previously stated, both the E2 and the oestrogenic mixtures experiments were run in parallel. This situation was an unfortunate necessity due to lack of time, i.e. each experiment took 18 months to run and had to start in the spring. However, ideally each experiment would have been conducted separately. This would have allowed for replicate treatment tanks to be used (as more tanks would have been available), which would have reduced the possible risk of interpreting 'tank effects' as 'treatment effects'. The experimental set up used had 'replicate' groups of adults with in one treatment tank, to assess reproductive output. These groups of adult snail could be considered 'pseudo-replicates' and conducting statistical analysis on the egg laying data produced by these groups could prove contentious. Running the two experiments separately would have also allowed possible refinements to experimental design.

3.5.2 The Mesocosm Tanks

The mesocosm tanks were situated on site at Langford Water Treatment Works (WTW), Essex. Langford WTW is part of Essex and Suffolk Water and is in close proximity to both the River Blackwater and the River Chelmer (Figure 3.11). The mesocosms are a set of tanks 1m^3 in size (Figure 3.12). These could be fed by River water from either the River Chelmer or the River Blackwater, by effluent from the water treatment plant or a mixture of river water and effluent. In these experiments only River Chelmer water was used. For the E2 exposure three tanks were used: one tank held just river water (reference tank, RW), one tank held river water plus the high E2 (HE2) concentration, and another tank held river water plus the low E2 (LE2) concentration. For the Mixture exposure four tanks were used: one tank held just river water (reference tank, RW), one held river water plus Ethanol (solvent tank, RW+S), one held river water plus the high concentration of the oestrogenic mixture (HM), and the other held river water plus the low concentration of the oestrogenic mixture (LM).

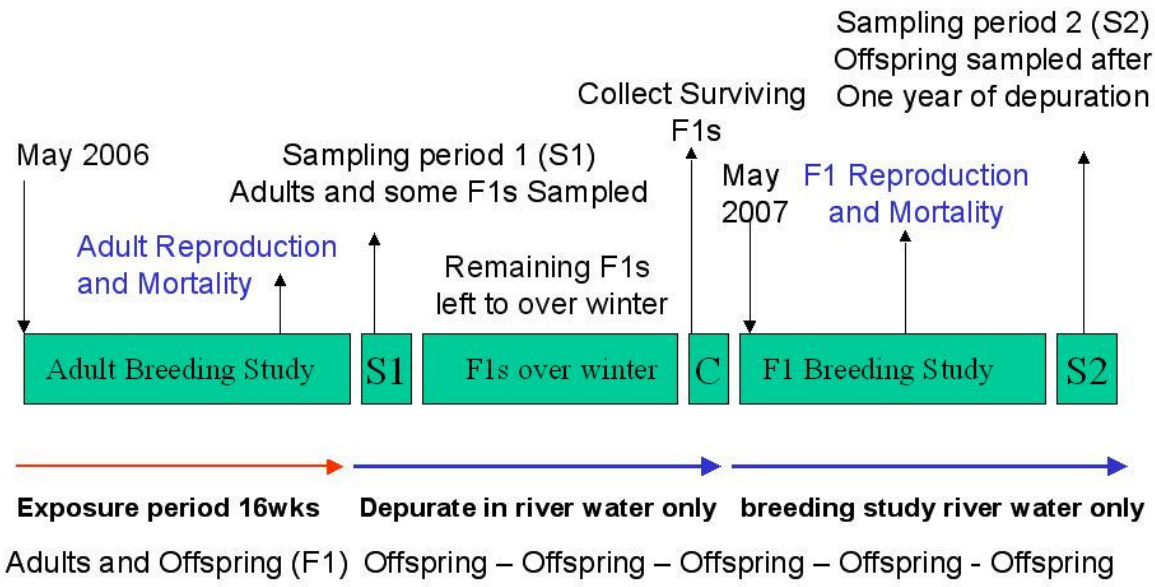

Figure 3.10 Diagram representing the timeline of the mesocosm experiments, indicating the different experiment aspects and their sampling points.

The initial Exposure period ran for 16 weeks, during this time adults and their offspring (F1) were exposed. Adult reproduction and mortality were recorded fortnightly. At the end of the exposure period the first sampling occurred (S1), all adults and a number of F1s were sampled to collect morphometric and histopathological data on growth and reproductive development/fitness. After this time, the remaining F1s were left to depurate over winter in river water. In spring 2007 the surviving over wintered F1s were collected and placed in breeding groups for the F1 breeding study. Reproductive output and mortality were recorded fortnightly. At the end of the breeding study F1 snails were sampled (S2), again, to collect morphometric and histopathological data. The whole life-cycle experiment ran from May 2006 until August 2007.

Each tank contained a number of snail cages. Adult snails were housed in cylindrical plastic cages (approximately 80cm high and 50cm diameter) made from sturdy gardening mesh (mesh holes approximately 4 mm^2) and sewn together using nylon fishing wire (Figure 3.13). These cages were suspended from wooden broom handles above the tank via stainless steel 2-3 mm diameter wire (Figure 3.12) Glass inserts within the cages were multifunctional, they provided weight and structure to the cages but also provided additional roaming and grazing areas for the adult snails (algae and biofilm grow on the glass surface), as well as an egg-laying surface for the *P. corneus*. The cages and their glass inserts had been successfully used by Clarke et al (Clarke et al. 2009) in an earlier mesocosm experiment. Some running repairs were required to prevent snail escape and replace any perished fishing line; therefore cages were inspected fortnightly over the exposure period. The *V. viviparus* cages in the E2 exposure and F1 breeding study also had an additional 'sock' of finer netting (approximately $1mm^2$) placed over them, in an attempt to monitor if small neonates were escaping from the $4mm^2$ holes in the primary cages. Between sampling periods chicken wire was placed over the top of all the tanks to prevent predation of snails by birds.

Figure 3.11 Ordinance Survey Map of Langford village, Essex.

The Essex and Suffolk water treatment works (labelled Wks) and their reservoirs are shown at the centre of the map, this is the area the mesocosm tanks are housed, allowing access to river water from either the River Chelmer (labelled, running west to south-east), or The River Blackwater (not labelled, running north-west to south-east).

Mesocosm tank water was extracted from the river via a pump; this water was then coarsely filtered by gravel to remove large objects of flotsam or detritus. From here the water was pumped directly to the mesocosm tanks. There was the possibility to have the water pass through an additional activated carbon filtrated to remove any smaller particles and biologically active materials e.g. Estradiol. This method had been previously used with an experiment exposing the same two gastropod species to mixtures of effluent and river water (Clarke 2006). However, it was found that the particulate matter, minerals and algae removed were an important food source for the snails, so pre-filtration with activated charcoal was not used in this experiment. Once coarsely filtered, the river water ran into the tanks through a series of aquarium (non-toxic) grade plastic pipes. Pipes, pipe joints and 'weld on wet and dry' solvent cement were all purchased from Tropical Marine Centre (TMC), pipe diameters ranged from 40-80mm (1.5-3.0 inches). Water exited the tanks via an overflow pipe; water from dosed tanks was then gravity filtered through activated carbon to remove any EDCs before being returned to the river. Water from the un-dosed tanks went directly back to the river down stream of the abstraction point.

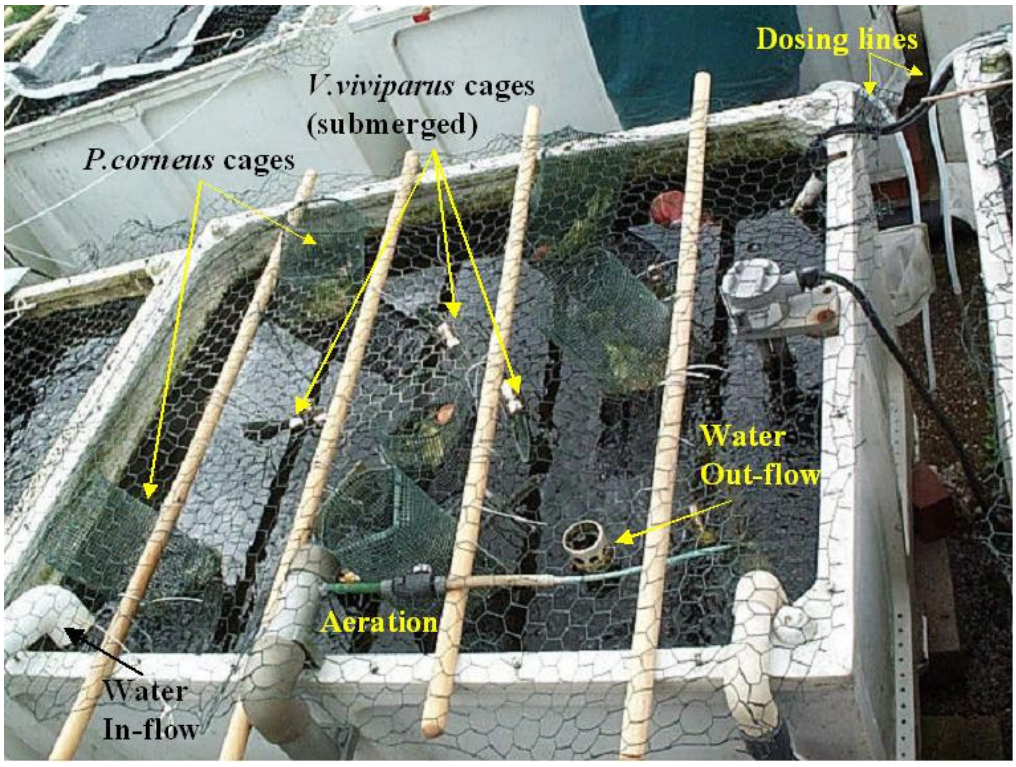

Figure 3.12 Photo showing an example of a mesocosm tank from the oestrogenic mixtures exposure.

Adult snail cages are suspended by wooden broom handles; The *P. corneus* cages were positioned so that the air breathing snails can reach the surface, but not escape, The *V. viviparus* cages were submerged, providing the best habitat possible for this deeper water (gilled) species. River water flows into the tank (bottom left), and out through the down pipe (bottom middle). Chicken wire prevented predation of snails by birds.

The post-dosing carbon filter was made from a large 45-gallon steel drum (880mm High and 580mm diameter) fitted with a fine mesh above the base on which the activated carbon was held. Activated carbon was supplied by Essex and Suffolk Water and was rod shaped (approx 3x1mm) or granular forms (approx 1-1.5mm diameter). Water was gravity fed from the dosed mesocosm tanks via a series of plastic 80mm (approx) diameter pipes into the top of the steel drum; its lid was tightly fixed down by means of a clamp. The water then percolated through the activated carbon and exited via another pipe in to a slues, which fed into the river down stream of the inflow pump. Effluent from this activated carbon filter was monitored for the EDCs dosed in the experiment to confirm their removal.

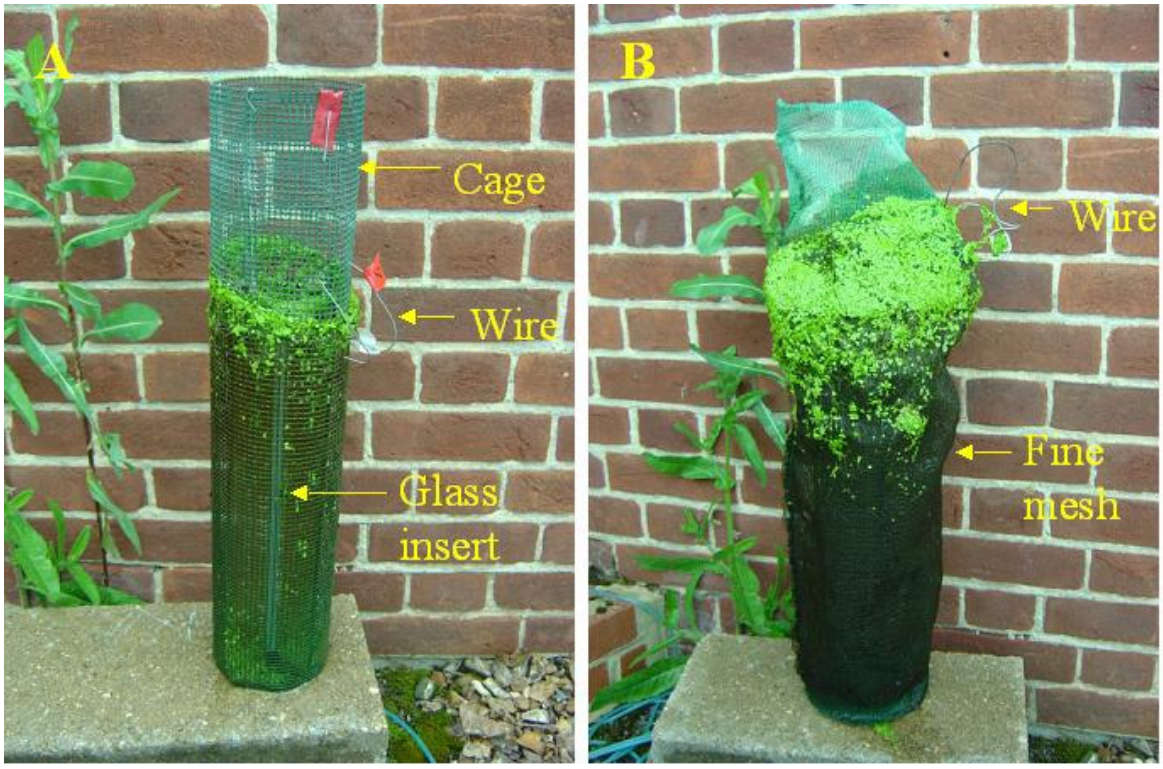

Figure 3.13 Photos of snail cages.

(A) *P. corneus* or *V. viviparus* cylindrical cage made from sturdy plastic garden mesh (holes are approximately 4 mm2) sewn together with nylon fishing wire. The cage was suspended in the tank via wire (shown in both A and B). The glass insert is visible in this photograph; the glass insert provided an egg laying/ additional feeding surface and weighted the cage down so it did not float. (B) *V. viviparus* cylindrical cage with additional fine mesh cover to prevent neonate escape, used in the E2 exposure only. Glass insert also present but not visible in this photograph.

3.5.3 Dosing

Dosing was accomplished via the use of a Watson Marlow multi-channel peristaltic pump (Sci-Q 200 series) and fine Watson Marlow 1.02 mm bore (white toggle each end, Watson Marlow) surgical grade silicone tubing. The silicone tubing ran from the mixing reservoir (weighted by large stainless steel nut) via the peristaltic pump into the required tank at a nominal rate of 1.5ml/min, while the river water flowed into the tank at a rate of 3 l/min. Each stock bottle, dosing reservoir, and pump line was colour coded with electrical tape to prevent cross contamination. The mixing reservoirs were made from large 10 litre black plastic buckets with lids. The mixing reservoirs were rinsed with tap water and refilled ever 3-4 days with a mixture of tap water and dosing stock solution or tap water and Ethanol (solvent tank) at a concentration of 1 ml stock solution (or Ethanol) to 1 l tap water.

Stock solution preparation

All stock solutions were prepared in the laboratory at Brunel University; fresh stock solutions were made every four weeks. Stock bottles (1 litre) were washed and given double solvent rinse (methanol and ethanol) prior to new stock storage. Approximately 500ml of each stock solution was prepared. Ethanol (Hayman Ltd) was used as the carrier solvent in all stock solutions. Each chemical was weighed on an electronic balance (to four decimal places) before being added to approximately 100ml ethanol w/v to make individual 'super' concentrated stocks. These 'super stocks' were then combined; 62.5ml (8 chemicals/500ml) of each were added to produce the mixture stock solution (or as a single chemical for the E2 only stocks, Chapter 4). Personal Protective Equipment (PPE) was worn while preparing and handling stock solutions. Stock solutions were stored in a domestic type refrigerator at 4^{o}C in 1 litre amber glass bottles. When transported they were stored in insulated (polystyrene) boxes with icepacks until transferred to another domestic type refrigerator on site at Langford WTW. Stocks were diluted 1000 times when added to the mixing reservoirs at Langford (1ml of stock to 1 litre water), and then diluted a further 2000 times when dosed to the mesocosm tank (1.5ml/min reservoir stock to 3000ml/min river water). Table 3.3 and Table 3.4 (below) give the nominal weight of each oestrogenic chemical added to the stock, the concentration of the stocks and the final concentration in the mesocosm tanks for the mixtures and E2 experiments, respectively.

Table 3.3 Nominal weight of each oestrogenic chemical to be added to Ethanol to produce 'super stocks', nominal concentration of mixtures stocks, and nominal concentration in mesocosm tanks for the mixtures experiment

	Low oestrogenic mixture			High oestrogenic mixture		
Oestrogenic chemical	Weight of chemical to be added in mg	Concentration in mixture stock bottle mg/l	Final concentration in tank ng/l	Weight of chemical to be added in mg	Concentration in mixture stock bottle mg/l	Final concentration in tank ng/l
E1	20	40	20	100	200	100
E2	3	6	3	10	20	10
EE2	0.5	1	0.5	1.5	3	1.5
NP	1500	3000	1500	6000	12000	6000
NP1EO	1000	2000	1000	5000	10000	5000
NP2EO	2000	4000	2000	7000	14000	7000
OP	150	300	150	600	1200	600
BPA	50	100	50	100	200	100

Table 3.4 Nominal weight of E2 added to 500ml of Ethanol to produce stocks, nominal concentration of E2 stocks, and nominal concentration in mesocosm tanks for the E2 experiment

	Low 17β-Estradiol (E2)			High 17β-Estradiol (E2)		
Chemical	Weight of chemical to be added in mg	Concentration in stock bottle mg/l	Final concentration in tank ng/l	Weight of chemical to be added in mg	Concentration in stock bottle mg/l	Final concentration in tank ng/l
E2	10	20	10	100	200	100

Dosing maintenance

Tap water was used in the dosing reservoir in an effort to reduce the level of microbes or algae in the mixing reservoir that could metabolise the solvent and/or chemicals dosed and also produce clogging masses, which could affect the dosing rate. In an outdoor environment however, it is impossible to keep the mixing reservoirs sterile, and some level of microbial growth was seen in all mixing reservoirs, especially during hot weather. When this was observed the microbial scum was rinsed out of the dosing reservoirs with tap water. Pump rates from the dosing reservoirs and river water pumps were measured and adjusted to as close to nominal (1.5 ml/min and 3 l/min accordingly) every 3-4 days. The surgical tubing was inspected every 3-4 days and any blockages present were either dislodged or the tubing was replaced. The overflow pipes were also unblocked of weed if, and when, necessary.

The post dosing carbon filter was regularly checked and maintained. Regular agitation and replacement of activated carbon was necessary to facilitate the volume of water removal

from the tanks required. As the river water from the mesocosm experiment was not pre-carbon filtered before reaching the tanks, large quantities of organic matter were present and this often clogged the filter and slowed the progress of water. In addition the shape of the activated carbon used differed during the exposure period, rod shaped carbon was used for the first four weeks, the supply of which ran out, and was then replaced with round or granular carbon. The granular carbon clogged more frequently and thus required more frequent changing. The River water pump and coarse filter were regularly checked and maintained by Mr David Walker of Essex and Suffolk Water.

3.5.4 Adult snail allocation, reproduction and mortality sampling regimes

3.5.4.1 *Viviparus viviparus* allocation to breeding groups: Mixture experiment

Eleven adult snails were randomly designated to each of the six replicate cages per tank. For each snail the length and aperture (Chapter 2, Section 2.3.2.2) were measured in millimetres using electronic callipers, to two decimal places. The weight was measured in grams, to three decimal places, using an electronic balance. Each group of snails was then placed into their cage labelled A-F (Figure 3.14) along with the glass insert and suspended in the required tank so that the cage was fully submerged. Cages were closed at the top end by means of folding the netting over and securing it with a large stainless steel bulldog clip; this provided a seal to stop the adult snails and offspring escaping while underwater. *V. viviparus* snails were not given additional food during the exposure as it was considered that enough natural food would be available from the river water.

3.5.4.2 *V. viviparus* allocation to breeding groups: E2 experiment

As for the Mixtures experiment (above), except only three replicate cages per tank were used, labelled A-C. In addition a finer mesh cage cover was used (Figure 3.13) for these reproductive groups, to prevent small neonates from escaping.

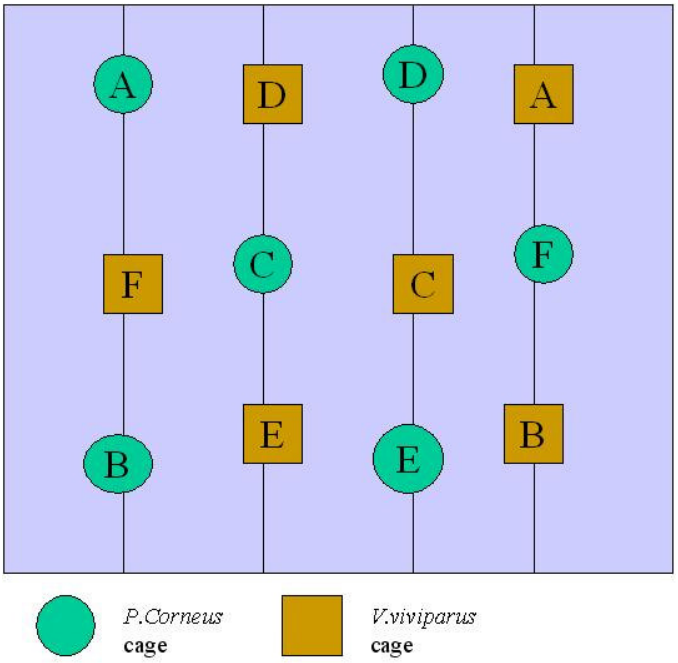

Figure 3.14 Diagram of an oestrogenic mixture exposed mesocosm tank showing the arrangement of snail cages.

Green circles represent *P. corneus* cages (labelled A-F), brown squares represent *V. viviparus* cages (labelled A-F), and lines represent wooden broom handles from which snail cages were suspended.

3.5.4.3 *V. viviparus* adult reproduction and mortality sampling regime: Mixture and E2 experiments

Each cage was carefully emptied fortnightly. The number of offspring were counted and then released into the main area of the corresponding tank. The number and shell length/aperture of any dead adult snails were also recorded (the soft parts of dead snails degrade rapidly and dead snails are easy to identify by their odour and appearance). Living adult snails were then replaced into their cage and re-suspended in their tank as detailed above.

3.5.4.4 *Planorbarius corneus* allocation to breeding groups: Mixture experiment

Six adult snails were randomly designated to each of the six replicate cages per tank. For each snail the length and aperture (Chapter 2, Section 2.3.1.4) were measure in millimetres using electronic callipers, to two decimal places. The weight was measured in grams, to three decimal places, using an electronic balance. Each group of snails was then placed into their cage labelled A-F (Figure 3.14) along with their glass insert and suspended in the required tank so that the top of the cage was above the water line. Around 10cm of cage protruded above the glass insert and waterline, to prevent the snails escaping but allowing them to the surface to breathe. *P. corneus* were fed twice a week on approximately 30 g

organic carrot and approximately 30 g flake fish food per cage. Any rotting carrot left from previous feedings was removed fortnightly.

3.5.4.5 *Planorbarius corneus* allocation to breeding groups: E2 experiment

The allocation was as for mixtures experiment (above) except three replicate cages per tank were used (Labelled A-C) each with nine adult snails. Feeding quantity was doubled.

3.5.4.6 *P. corneus* adult reproduction and mortality sampling regime: Mixture and E2 experiments

Cages were carefully emptied fortnightly and the glass inserts removed. The number of masses and eggs in each mass was counted by eye and recorded. The egg mass covered glass insert was then suspended in the appropriate tank (Mixture experiment only), so that the eggs could hatch and the offspring would be released into the tank. The number and shell length/aperture of any dead adult snails were recorded (the soft parts of dead snails degrade rapidly and dead snails are easy to identify by their odour and appearance). Living adult snails were replaced into their cage, given a new glass insert, re-suspended in their tank, and fed as detailed above.

3.5.5 Water sampling for chemical analysis

Five sets of water samples were taken for chemical analysis during the sixteen-week exposure period. Water samples from each tank of the chemically exposed tanks, the solvent tank and the carbon filter outflow were taken monthly from the start of the exposure (weeks 0, 4, 8, 12 and 16). Water samples from the two river tanks were taken on alternate months. Water samples were also taken from the river water inflow, which fed the tanks at weeks eight, twelve and sixteen. Samples were analysed for E1, E2, EE2, NP, OP and BPA from the mixtures tanks, solvent tank, river water tanks and carbon filter effluent. Only E1, E2 and EE2 were measured from the high and low E2 exposure tank samples and river water influent samples. Additional water samples were also taken at week 16 from the two RW tanks (oestrogenic mixture and E2 study) and the RW+S tank to measure the concentrations of 19 metals and minerals (Barium, Boron, Calcium, Iron, Lithium, Magnesium, Manganese, Potassium, Sodium, Strontium, Sulphate, Tributyltin, Aluminium, Cadmium, Chromium, Copper, Lead, Nickel and Zinc).

3.5.5.1 Water chemistry protocol

Water samples were couriered to the Environment Agency's National Laboratory Service (NLS) in Nottingham (UK) for analysis. See Appendix II for protocol.

3.5.6 Water sampling for YES analysis

Six sets of water samples were taken for yeast screen analysis during the sixteen-week exposure period. Water samples were taken at weeks 2, 6, 8, 10, 12 and 16 from the two E2 dosed tanks (HE2 and LE2), the two mixture dosed tanks (HM and LM) and the solvent tank (RW+S). Water samples were also taken at weeks 8, 10, 12 and 16 from the river water only tanks (alternate sampling), the river water influent and the carbon filter effluent. The water samples taken at weeks 8, 12 and 16 were simultaneously sampled with the water for chemical analysis.

3.5.6.1 Protocol for water samples for YES analysis

Each water sample was approximately 1000 ml. Samples were stored in 1000 ml, solvent rinsed, amber glass bottles. These samples were stored in polystyrene cool boxes with freezer packs while transported from Langford WTW to Brunel University. Once at the university they were kept in a refrigerator at 4^{o}C overnight. The method for water sample extraction onto C18 cartridges was followed (Section 3.3). However, due to the suspended solids the total 1000 ml of sample was often not extracted, a maximum extraction time of 6 hours was used, and the volume extracted in this time was recorded. Other than these exceptions the general method for preparing samples (eluting, drying down and re-suspending) for the yeast screen were followed (Section 3.3). Samples in ethanol were stored at 4^{o}C. The methods stated in Sections 3.4 and 3.4.2 were followed for preparing yeast mediums, running the yeast screen assay, and interpreting the results.

3.5.7 End of exposure (sampling period one)

The dosing pumps were turned off on the 25th of August, four days prior to the sampling of adult snails. River water continued to flow into all tanks during this time. All adult snails were sacrificed over a two-day period in late August 2006.

3.5.7.1 Adult *Viviparus viviparus* and *Planorbarius corneus* sampling methods

Each cage was removed from its tank and the cage letter recorded. The glass insert and snails were then carefully removed. Any dead snails had their length and aperture measured using the digital callipers and recorded. All other snails were narcotised in 5% MgCl solution for 30 minutes prior to being further handled. Once narcotised snails were given a reference number and had the following measurements recorded: shell length, shell aperture and total weight (shell on). Snails were then carefully removed from their shells using the methods in Chapter 2, Sections 2.3.2.2 and 2.3.1.4, and their soft tissue weighed.

Once the shell had been removed any abnormalities or obvious parasite infections were noted. *V. viviparus* snails could then be sexed. Female snails had their brood pouch dissected out (Chapter 2, Section 2.3.2.3) and the number of embryos were counted and recorded as either shelled (oldest) or unshelled (youngest). The body was then weighed again (minus the embryos) before being fixed in Bouin's fixative. Male snails also had the total penis length recorded before being fixed in Bouin's fixative. For *P. corneus* once the shell had been removed the body was then weighed again before being fixed in Bouin's fixative. After 24 hours the Bouin's fixative was removed and replaced with 70% IMS (*V. viviparus* and *P. corneus*). This IMS was again replaced with new 70% IMS four days later. The tissue was stored in IMS at room temperature.

3.5.7.2 F1 snail sampling method

All F1 snails were sampled over the month of September 2006. For each mesocosm tank the same method was followed. The fish guard was placed into the tank around the down pipe and the down pipe was unscrewed and the tank drained. Once the tank was drained it was scoured by hand and all the snails were removed from the tank, mud and filamentous algae (this took several hours per tank). Snails were then separated into their different species, and again into rough size classes i.e. above or below 10 mm in shell length (*V. viviparus*) or shell diameter (*P. corneus*). These were then counted and recorded. Then 50 *V. viviparus* and 40 *P. corneus* from the above 10 mm size class were placed in large food grade plastic buckets containing river water, ready to be transported back to Brunel University. Then the mesocsom tanks were re-filled with river water and the remainder of the F1 snails were replaced back into the tanks to over-winter. This was a laborious process when conducted solo and a maximum of two tanks could be sampled in one day. F1 snails were then transported back to Brunel University snail room and held in their buckets with constant aeration at 17^{o}C 12:12 light:dark regime until they were sacrificed and fixed for later histological analysis. The method for weighing, measuring, narcotising and shell removal was followed for F1 as for the adult *P. corneus* and *V. viviparus* (Chapter 2, Sections 2.3.1.4 and 2.3.2.2), the only deviations being that F1 *V. viviparus* did not have their penis length taken or brood-pouches dissected. Sampling and fixing the F1s took a further 3-4 days after each tank drain down. Therefore, it took the whole of September 2006 to sample the seven tanks, and there were several weeks between some tank sampling dates.

3.5.8 Over-wintering of F1 snails

At the beginning of October 2006 the river water pump was switched off and the remaining F1s were left to over winter in static river water. By this time the water in the mesocosm tank had been renewed many times, however the sediment and some of the plant matter were left in the tanks to provide food and shelter to the over-wintering F1s. Due to a combination of high mortality in the river water solvent mesocosm tank and competition for mesocosm tanks by other scientists, none of the F1 individuals from this group were over-wintered. Also due to this pressure for mesocosm tank space the two river water tanks F1s were combined for over-wintering and subsequent F1 reproductive studies.

3.5.8.1 Measuring over-winter success of F1 snails

The following March the tanks were re-visited and the river water pump turned back on. Each tank was again drained down and snails were removed from the mud and weed counted, and placed into size classes of <10mm, 10-15mm, 15-20mm and above 20mm shell length (*V. viviparus*) or shell diameter (*P. corneus*). The numbers in each size class of F1 surviving the winter were recorded.

3.5.9 Developmentally exposed and then depurated F1 reproductive output study

For the purpose of the F1 reproductive study the E2, Mixture and River Water developmentally exposed groups were treated to the same protocol.

3.5.9.1 Protocol for *V. viviparus*

At the end of March 2007, the *V. viviparus* F1 breeding study groups were organised. Over wintered F1s from the two E2 exposures (HE2 and LE2), the two mixture exposures (HM and LM) and the pooled river water exposure (RW) were kept in separate 2 litre buckets of river water during allocation. Snails were divided in to size classes of above or below 10 mm shell length prior to allocation, only snails above 10 mm shell length were used in the F1 breeding study (where possible). Each treatment group was allocated three replicate breeding cages, each containing two males and nine females. *V. viviparus* snails were observed while crawling about to designate their sex; those with an enlarged right tentacle were presumed to be male. All *V. viviparus* breeding cages (E2, Mix, and RW developmentally exposed; 15 in total) were placed in one river water only mesocosm tank, at a flow rate of approximately 3 l/min. Reproductive output and mortality were measured fortnightly using the same protocol as used for the adult snails (see method above). The F1 *V. viviparus* breeding study continued for 19 weeks over the spring and summer of 2007.

3.5.9.2 Protocol for *P. corneus*

At the end of April 2007, the *P. corneus* F1 breeding study groups were organised. Over wintered F1s from the two E2 exposures (HE2 and LE2), the two mixture exposures (HM and LM) and the pooled river water exposure (RW) were kept in separate 2 litre buckets of river water during allocation. Snails were divided in to size classes of above or below 10 mm shell diameter prior to allocation. Only snails above 10 mm shell length were used in the F1 breeding study (where possible). Breeding groups of six snails were allocated to each cage. Unfortunately due to high over-winter mortality only one breeding group could be allocated to the HM developmentally exposed *P. corneus*. All *P. corneus* breeding cages (E2, Mix, and RW developmentally exposed; 13 in total) were placed in one river water only mesocosm tank, at a flow rate of approximately 3 l/min. Reproductive output and mortality were measured fortnightly using the same protocol as used for the adult snails (see method above). The F1 *P. corneus* breeding study continued for 14 weeks over the spring and summer of 2007.

3.5.10 End of F1 breeding study (sampling period two)

Surviving *P. corneus* from the F1 breeding study were sampled on the 1st of August 2007. Surviving *V. viviparus* from the F1 breeding study were sampled the following week over two days (8th and 9th of August). Snails (*V. viviparus* and *P. corneus*) were sampled using the same protocol as used in the adult sampling method (sampling period one, above). The only diversion from the original protocol was that male penis length was not measured in F1 *V. viviparus*.

3.6 Statistical methods

All statistical analysis was conducted using the statistics package SPSS V15.0.1.1 for windows. Statistical significance was accepted at $P < 0.05$ for all comparisons. Data sets that did not have acceptable homogeneity of variance, or a normal distribution were analysed using non-parametric tests. For single comparisons a t-test was used, and if the data were non-parametric a Mann-Whitney test was used. For multiple comparisons, a One way ANOVA was used, followed by post hoc analysis of Least Significant Difference (LSD). If the data were non-parametric, a Kruskal-Wallis test was used followed by post hoc analysis using the Mann-Whitney test. Pearson's correlation or Spearmann's rank order correlations were used to find significance for correlated data. Analysis using co-variants were also conducted using ANCOVA; bonferrni was used for post hoc analysis.

With hindsight other more enlightening statistical methods could have been used on the data produced during this research. Principle component analysis would have been very useful to determine which endpoints measured were most significant. This would have allowed a sharper focus to be drawn on the more important aspects of the histopathology results.

4 Effects of exposure to 17β-oestradiol on reproduction in two species of UK native fresh water gastropod mollusc

4.1 Introduction

In the past much of the research on the effects of EDC on molluscs has focused on the masculinising agent TBT. When this project began, several research papers by a single German laboratory indicated that molluscs may also be sensitive to vertebrate feminising agents such as BPA, NP and OP(Oehlmann et al. 2000, Duft et al. 2003), and treated sewage effluents or EE2 (Jobling et al. 2004) and also be sensitive to the anti-androgens Cyproterone acetate and vinclozolin (Tillmann et al. 2001)..In a later publication, by the same German laboratory, *M. cornuarietis* were seen to be more sensitive to BPA's effects at lower temperatures, or rather, that at higher temperatures reproduction was already highly stimulated and therefore possibly masked the impact of chemical stimulation (Oehlmann et al. 2006). The low concentrations at which some of the effects were observed in these publications raised concern about UK gastropod molluscs, which have a seasonal reproductive cycle, to whether or not reproduction and sexual development may also be affected by exposure to oestrogenic chemicals. In addition, previous research in our own laboratory has indicated that a treated sewage effluent (Clarke et al. 2009) caused dose-dependent effects on the seasonal cycle of reproduction in a native gastropod. Therefore, the work presented in this chapter was conceived to see whether E2 alone would also elicit similar reproductive effects in two species of gastropod, under similar experimental conditions.

The developmental stages of many animals are extremely sensitive to ED. Exposure during the programming of the endocrine system can have long term and far reaching effect on growth, reproduction, and sexual development (Gore 2008) . These are important factors to consider when assessing the implications of ED at the population or ecosystem level. The focus of this experiment, therefore, was to assess the effects of developmental exposure of 17β-oestradiol (E2) on sexual development and subsequent reproductive fitness in the two chosen test species of gastropod mollusc. The effects of exposure to E2 on adult reproduction and mortality were also measured, the two test periods together constituting a whole life cycle.

Due to the difficulties encountered rearing and maintaining both species of gastropod in the laboratory, and because of the knowledge that external cues such as photoperiod and temperature are important parts of their seasonal reproductive cycle, it was decided to conduct the exposure in outdoor mesocosms, under natural photoperiodic and climatic conditions. The two test species, *P. corneus* and *V. viviparus,* provide contrasting

reproductive strategies and life histories broadly representative of many UK mollusc species.

Role of steroid oestrogens in reproduction in the test species

The steroid oestrogen 17β-oestradiol (E2) naturally occurs in vertebrates where it is important in sexual development in both males and females. However, inadvertent exposure to E2 can alter normal sexual development and function (in vertebrates). Therefore, E2 is often used as a positive control (or benchmark) to assess oestrogenic effects in vertebrate test species. At the time my research was initiated there was tantalising evidence indicating molluscs have varying levels of sex steroids (E2 and T) at different times of their breeding cycles, and that they can metabolise and store hormones in a similar manner to vertebrates (Di Cosmo et al. 2001, Gooding and LeBlanc 2004, Janer et al. 2004, Janer et al. 2005) and an ER-like nuclear receptor had recently been found in a gastropod mollusc (Thornton et al. 2003). With this initial evidence that vertebrate type sex steroids and EDCs may be biologically active in molluscs and important in regulating reproduction, it can be hypothesised that 17β-oestradiol would affect molluscs, perhaps in a similar manner to that of vertebrates. Furthermore, inappropriate concentrations or timing of application may have deleterious affects on reproduction, which could have knock on effect at the population level. It is prudent therefore to investigate the possible ED effects of the vertebrate oestrogen, 17β-oestradiol (E2), on molluscs.

4.1.1 Previous research of 17β-oestradiol on the test species

In a previous experiment conducted by Rachel Benstead during the summer of 2004, *Planorbarius corneus* and *Viviparus viviparus* adults were exposed to high concentrations of 17β-oestradiol (E2, 20ng/l or 200ng/l nominal plus stripped river water control) over 12 weeks. Actual measured concentrations of E2 were much higher than nominal at 50ng/l and 350ng/l. Reproductive output measured in eggs laid per snail in the *P. corneus* was significantly ($P<0.05$) inhibited at both concentrations of E2 compared to the stripped river water alone. Mortality was also significantly increased in the higher concentration of E2 (Benstead 2005). In *V. viviparus*, reproductive output (offspring released per snail) was significantly increased ($P<0.05$) in the highest concentration of E2. In a preliminary study a small number of developmentally exposed *V. viviparus* F1s were collected for some investigatory histopathology. Upon analysis, I found that a number of the E2 exposed F1 *V. viviparus* individuals contained both male and female characteristics (e.g. brood pouch and oocytes and vas deferens and spermatogenesis) and were therefore intersex. The percentage of intersex individuals was 14% in the 350ng/l E2 tank (n=29) and 5% in the

50ng/l E2 tank (n=20). Unfortunately, the production of F1 was only incidental to the experimental design and only two F1 were collected from the stripped river water (reference) tank and both of these were normal females. This initial experiment provided some tantalising results; adult reproduction was affected in both species and normal sexual development was possibly affected by early exposure in *V. viviparus*. However the very high concentrations of E2 also caused high mortalities and the small number of F1s sampled in the un-dosed mesocosm made it difficult to assess the true affect of E2 on development.

4.1.2 Aims

The aim of this experiment was to assess the effects of E2 exposure on adult reproduction and sexual development in two species of gastropod mollusc, *V. viviparus* and *P. corneus*, this time working with larger numbers of snails and at lower concentrations of E2 than in the previous experiment.

4.2 Method

Two species of freshwater gastropod (*V. viviparus* and *P. corneus*) were exposed to 17β-oestradiol in a freshwater mesocosm experiment. The mesocosm tanks were housed at Essex and Suffolk Water's Langford water treatment works site. In the dosed experiment, three 685 litre ($1m^3$) purpose built tanks were fed with river water, which was abstracted from the River Chelmer via a submerged pump. One tank contained river water only (Tank 1: River water (RW)). Two further tanks were dosed with either 'low' or 'high' concentrations of 17β-oestradiol (Tank 2: River water plus 10ng/l 17β-oestradiol (nominal) (LE2); Tank 3: River water plus 100ng/l 17β-oestradiol (nominal) (HE2)). See Chapter 3 Section 3.5, which states the full materials and methods and the design of the set up.

Adult gastropods and their subsequent offspring were exposed over 16 weeks from the beginning of May until the end of August 2006. This study ran in collaboration with Rachel Benstead (Environment Agency/Brunel University), who collected data on the effects of 17β-oestradiol exposure on the adults reproductive output/fecundity. At the start of the experiment each tank was furnished with six mesh cages of snails, three for each species, each containing either nine adult *P. corneus* or eleven adult *V. viviparus*. Reproductive output of the adult snails was measured fortnightly, either by counting the number of eggs laid (*P. corneus*) or neonates released (*V. viviparus*). Offspring (F1s) produced by the exposed adults were also developmentally exposed in the same mesocosm tank in which their parents were housed. Adult snail mortality was also recorded

fortnightly. Tank water samples were taken on weeks 0, 4, 8, 12 and 16 for chemical analysis (E1, E2 and EE2). Water samples were also taken on weeks 2, 6, 8, 10, 12 and 16 from the two E2 dosed tanks and on weeks 8, 10, 12 and 16 from the river water tank for Yeast Estrogen Screen (YES) analysis. The water samples taken at weeks 8, 12 and 16 were simultaneously sampled for the YES and water chemical analysis. When dosing culminated (end of August 2006), all the adults and a number of the F1s were sacrificed and fixed for histopathological analysis of reproductive health and development (see Chapter 3 Section 3.5.7). The remaining F1s from each of the treatments (river water only (RW); low 17β-oestradiol (LE2); high 17β-oestradiol (HE2)) were counted and then depurated in river water in their exposure tanks over autumn-winter 2006 (see Section 3.5.8). The surviving depurated F1s were then counted and groups of snails were assessed for their reproductive output in two further un-dosed mesocosm (*P. corneus* over 14 weeks, *V. viviparus* over 18 weeks) experiments, conducted spring-summer 2007 (see Section 3.5.9 for full materials and methods). At the end of the F1 breeding study (August 2007) these snails were sacrificed and fixed for histopathological analysis of reproductive health and development (see Section 3.5.10).

4.3 Results

4.3.1 Physical tank parameters

Temperature

Water temperature fluctuated over the 16 weeks of exposure with a low of 14^{o}C measured in May and a peak of 26^{o}C at the end of July (Figure 4.1).

Flow Rate

Over the entire experiment water flow into the tanks was kept to approximately 3l/min (RW 3.24 ± 0.59, LE2 3.11 ± 0.54 and HE2 2.84 ± 0.42). Flow rates did vary day to day, and the flow meters needed frequent adjustment to keep flow rates equal. However, overall there were no significant differences in flow rate between the three tanks (P = 0.127, ANOVA).

Dosing Pump

The dosing pump rate was kept at approximately 1.5ml/min (LE2 1.41 ± 0.26 and HE2 1.39 ± 0.25). Blockages occurred occasionally (especially in hot weather) and tubing needed replacement periodically. No significant difference was, however, found between the high E2 and low E2 dose pump rates (P = 0.422, Mann-Whitney).

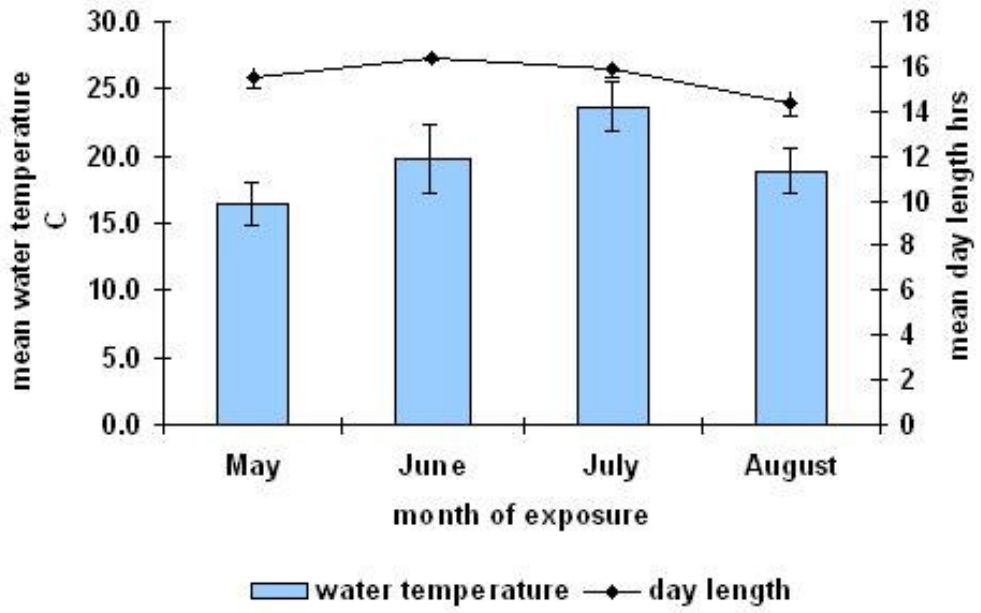

Figure 4.1 Mean measured tank water temperature (°C) and mean day length (hours) for each month over the 16 week exposure period using adult (F0) snails.

Day length data obtained from UK Met office website. Error bars indicate standard deviation.

4.3.2 Biological (YES) and chemical analysis of tank water during adult and F1 exposure

4.3.2.1 Chemical analysis results

The measured concentrations (chemical analysis) of steroids varied between the tanks and the sampling points (see Table 4.1).

E1

E1 concentration was lowest in the river water inlet (<1-1.38ng/l) (which fed all the tanks) and river water tank (<1-2.03ng/l). In the LE2 tank concentrations ranged from <1-15ng/l and in the HE2 tank they ranged from <1-30.6ng/l.

E2

E2 concentration in the RW tank was 10.1 and 5.71ng/l on the first two sampling occasions (week 0 and 4, respectively) after which it dropped to <0.3ng/l (detection limit). Unfortunately the river water inlet, which fed the tanks, was not sampled at weeks 0 and 4 so it is unknown whether the unexpectedly high concentrations of E2 were also found at the river water inlet. In the LE2 tank, the E2 concentration ranged from 1.59-28.6ng/l; the highest E2 measured concentration (232ng/l) was in the HE2 tank at week four of the exposure.

EE2

The highest concentration of EE2 was measured in the river water inlet (3.73ng/l, week 16), which fed all the tanks. At the same sampling time, the RW tank also had a high EE2 concentration (2.34ng/l), however, in the LE2 and HE2 tanks the EE2 concentration was much lower (0.583ng/L and 0.634ng/l).

Table 4.1 Measured concentration of steroid oestrogens from the three exposure tanks plus the river water pump inlet measured over the 16 week exposure.

E1					
Week	**0**	**4**	**8**	**12**	**16**
Low E2 tank	2.05	<1	15	1.3	1.46
High E2 tank	5.15	30.6	5.7	5.2	<1
River water tank	2.03	<1	1.05	<1	<1
River water inlet	ns	ns	<1	<1	1.38
E2					
Week	**0**	**4**	**8**	**12**	**16**
Low E2 tank	8.76	4.82	28.6	1.59	2.84
High E2 tank	22.7	232.0	41.7	6.38	1.25
River water tank	10.1	5.71	<0.3	<0.3	<0.3
River water inlet	ns	ns	<0.3	<0.3	0.428
EE2					
Week	**0**	**4**	**8**	**12**	**16**
Low E2 tank	ns	0.638	0.659	1.09	0.634
High E2 tank	ns	0.658	0.452	1.46	0.583
River water tank	ns	0.375	0.37	1.37	2.34
River water inlet	ns	ns	0.375	0.997	3.73

E1 and E2 were measured in the tank water on weeks 0, 4, 8, 12 and 16. EE2 was measured in the tank water on weeks 4, 8, 12 and 16. E1, E2 and EE2 were measured in the river inlet water on weeks 8, 12 and 16. All concentrations measured in nano-grams per litre (ng/l). Not sampled; ns. Water chemistry provided by Environment Agency's National Laboratory Service, Nottingham.

Kruskal-Wallis analysis of the measured chemistry results found no significant difference in E1 (P=0.098) or EE2 (P=0.951) concentrations between each of the three mesocsom tanks and river water inlet. However, a significant difference was found for the E2 (P=0.039). Post hoc analysis (Mann-Whitney) found the HE2 tank to have a significantly higher concentration of E2 compared to the RW tank and the river inlet (P=0.045 and 0.024, respectively). The LE2 tank also had a significantly higher concentration of E2 compare to the river inlet (P=0.024). However, no significant difference in E2 concentration was found between the HE2 and LE2 tanks (P=0.347), the LE2 tank and the RW tank (P=0.245) or between the RW tank and the river inlet (P=0.608). Figure 4.2 (below) gives the mean concentrations of each steroid over the 16-week exposure.

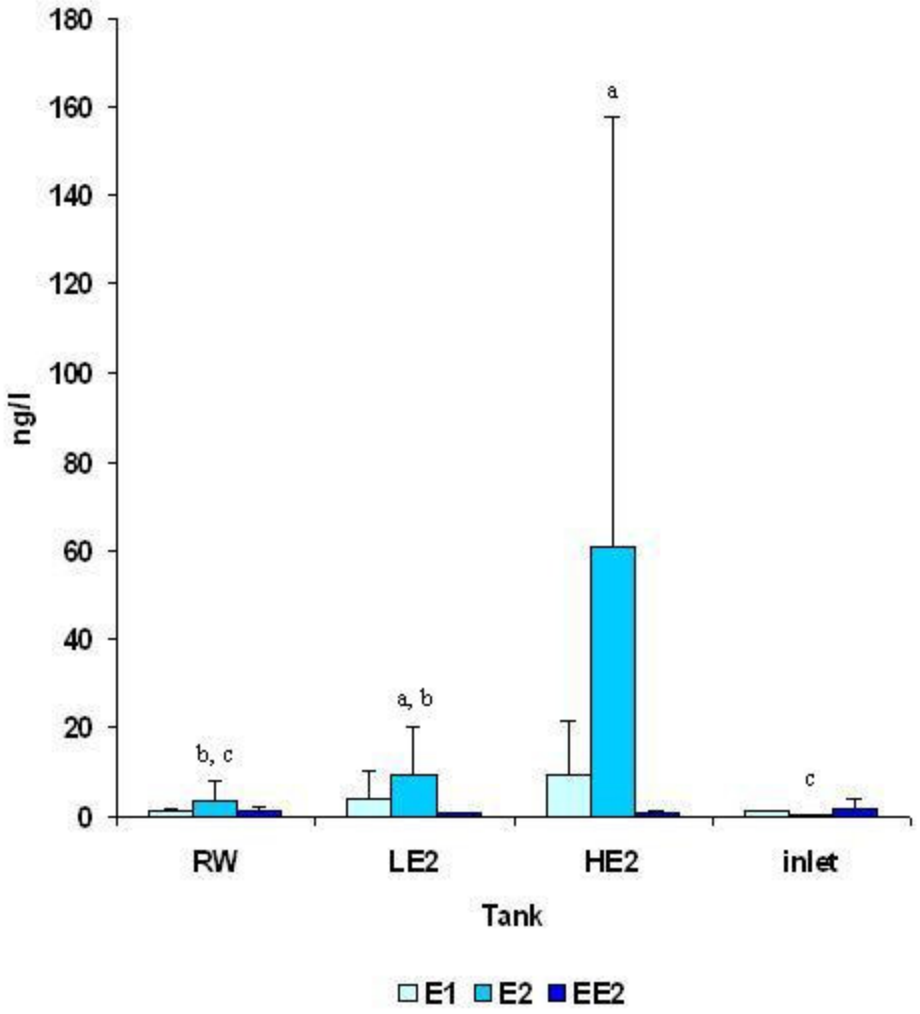

Figure 4.2 Mean measured concentration of steroid estrogens from the three exposure tanks (RW, LE2 and HE2) plus the river water pump inlet (inlet).

E1 and E2 were measured in the tank water on weeks 0, 4, 8, 12 and 16. EE2 was measured in the tank water on weeks 4, 8, 12 and 16. E1, E2 and EE2 were measured in the river inlet water on weeks 8, 12 and 16. All concentrations measured in nano-grams per litre (ng/L). Error bar shows standard deviation. Letters (a, b, c, d) denotes statistical similarity of E2 measured concentrations. Water chemistry provided by Environment Agency's National Laboratory Service, Nottingham.

4.3.2.2 Predicted 17β-oestradiol concentration of dosed tank water samples

The chemical analysis revealed much lower concentrations of E2 than were expected from the nominal dosing; therefore attempts were made to investigate why this might have happened. Firstly the possibility that concentrations were lower due to possible increased dilution was investigated

.

Using the measured dosing pump rate, and the river water flow rate into the two exposure tanks, actual dilution factors from each of the dosing reservoirs were calculated. The E2 concentrations were then predicted for each of the water sampling points (chemical and

YES). The predicted concentrations (Table 4.2) calculated were on average about 20% above the nominal concentration.

Table 4.2 Predicted E2 concentrations (ng/l) for each of the two dosed mesocsom tanks, low concentration of 17β-oestradiol (LE2) and high concentration of 17β-oestradiol (HE2)

Week	LE2 tank water	HE2 tank water
0	17.82	139.16
2	18.71	178.65
4	6.91	173.82
6	5.19	87.64
8	9.55	94.54
10	14.21	83.20
12	15.36	92.24
16	16.64	102.27
Mean	13.05 ± 5.16	118.94 ± 39.33

The predictions were made using the measured dosing pump rate and water flow rate to produce the actual dilution factor into the tanks for each sampling point. Mean ± standard deviation.

4.3.2.3 Predicted oestrogen equivalents (EEQ) of the mesocosm tank water using the measured water chemistry results.

Predicted EEQs were made to compare the measured concentration of steroids from the chemical analysis with the measured EEQ from the Yeast Estrogen Screen (YES) assay. To predict the oestrogen equivalent (EEQ) of the mesocosm tank water as measured in the yeast oestrogen screen (YES), 1ng/l E2 or EE2 were assumed to equal 1ng/l EEQ (Segner et al. 2003a, Van den Belt et al. 2004) and 1ng/l E1 was equal to 0.5ng/l EEQ (Van den Belt et al. 2004). Table 4.3 gives the mean measured concentrations of E1, E2 and EE2 plus the resultant predicted EEQs.

4.3.2.4 Oestrogen equivalents of the mesocosm tank water, as measured in Yeast Oestrogen Screen (YES) assay

Table 4.4 shows the YES assay results (EEQ), these all were lower than the nominal concentrations for the two exposed tanks (HE2~100ng/l; LE2~10ng/l).

Table 4.3 Mean measured concentration of steroid estrogens and mean predicted YES EEQ from these measured concentrations for the three exposure tanks plus the river water pump inlet.

	E1	E2	EE2	Predicted EEQ
Low E2 Tank	4.16 ± 6.07	9.32 ± 11.11	0.76 ± 0.22	12.01 ± 14.06
High E2 Tank	9.53 ± 11.93	60.81 ± 97.00 *	0.79 ± 0.46	66.20 ± 102.90
River Water Tank	1.22 ± 0.46	3.34 ± 4.45	1.11 ± 0.94	4.84 ± 4.05
River Pump inlet	1.13 ± 0.22	0.34 ± 0.07	1.70 ± 1.78	2.61 ± 1.97

All concentrations measured in nano-grams per litre (ng/l), mean ± standard deviation. Water chemistry provided by Environment Agency's National Laboratory Service, Nottingham. YES EEQ calculated by assuming 1ng/l E2 or EE2 were equal to 1ng/l EEQ (Segner et al. 2003a, Van den Belt et al. 2004) and that 1ng/l E1 was equal to 0.5ng/l EEQ (Van den Belt et al. 2004). Asterisk indicates significant difference from River water tank.

Table 4.4 Measured EEQs for the three tanks (River water only, RW; Low concentration of 17β-estradiol, LE2; High concentration of 17β-estradiol, HE2) and the river water inlet (that fed all tanks) from the yeast oestrogen screen (YES).

	Week 2	Week 6	Week 8	Week 10	Week 12	Week 16	Mean
RW	ns	ns	0	1.67	0	1.75	0.86 ± 0.99
LE2	1.74	1.11	5.98	0.88	0	1.15	1.81 ± 2.12
HE2	6.78	0	14.72	3.66	1.71	6.14	5.5 ± 5.20
River inlet	ns	ns	2.01	0	0	0.95	0.74 ± 0.96

Water samples taken at weeks 8, 12 and 16 (highlighted in grey) were split between water chemistry analysis and yeast screen analysis, and are therefore directly comparable. No water sample was taken from the river inlet or RW tank on weeks 2 and 4. Mean ± standard deviation. All measured in ng/l. Not sampled; ns.

Analysis of the YES assay results by One way ANOVA found no significant difference between the EEQs from each of the mesocsom tank samples and the river inlet sample (P= 0.087).

4.3.2.5 Comparison of the predicted E2 concentration and the measured EEQ

The actual EEQ measured by YES (Table 4.4) in each tank was lower than predicted E2 concentration from the dilution factor (Table 4.2). Indeed, statistical analysis found that the predicted E2 concentrations from the dilution factors were significantly higher than the measured EEQ in the YES assay ($P<0.001$, T-test).

4.3.2.6 Comparison of predicted EEQs from chemical analysis and actual measured EEQ from YES assay

The mean EEQ predictions were higher than those actually measured in the YES assay. However, only water samples taken at weeks 8, 12 and 16 are directly comparable (chemistry and YES samples) as they were sampled at exactly the same time as each other. In general, the predicted EEQs (for weeks 8, 12 and 16) were higher than those actually measured in the YES assay, with the exceptions of week 8 from the river inlet sample (2.01ng/l EEQ) and week 16 from the HE2 tank (6.14ngl EEQ). Analysis by one way ANOVA found no significant differences ($P<0.05$) between the predicted EEQ (from the chemical analysis) and the actual EEQ measured in the YES assay. A significant positive correlation (Pearson's correlation, 0.849, $P<0.001$) was also found between the predicted EEQ from the chemicals analysis and the actual EEQ measured in the YES, weeks 8, 12 and 16, when all the treatments (i.e. RW, LE2, HE2 and inlet) were pooled (due to small sample size).

4.3.2.7 Oestrogen equivalent measured by YES in the dosing reservoir water

In an attempt to identify where or when the E2 had been lost from the test system, water samples were taken from the dosing reservoirs (prior to entering in mesocosm tank). The first sample was taken shortly after the dosing stock was mixed into the reservoir and a second sample was taken after 72hrs. These samples were then extracted and run in the YES assay.

The mean EEQ values were reduced by around 30-50% 72 hours after dosing (two samples taken in July). For example, the mean initial (30 minutes after mixing stock with water) value from the HE2 mixing reservoir was 180.3 ± 77.8 µg/l EEQ, and after 72 hours it was 49.0 ± 17.6 µg/l EEQ. The initial mean value in the LE2 mixing reservoir was 24.4 ± 7.8 µg/l EEQ, and after 72 hours it was 12.9 ± 4.0 µg/l EEQ. The expected initial EEQs would have been 200µg/l (HE2) and 20µg/l (LE2) given that the stock concentration was diluted 1000 time (1ml/l) in the dosing reservoir.

4.3.3 Effects of 17β-oestradiol exposure on adult *P. corneus*

4.3.3.1 Reproduction

Rachel Benstead collected the adult reproductive output and survival data, 'The adult *P. corneus* showed a complex response to 17β-oestradiol in terms of the numbers of eggs produced, with induction occurring at low concentrations (LE2) and inhibition at higher concentrations (HE2)' (Benstead 2007) see Figure 4.3, below.

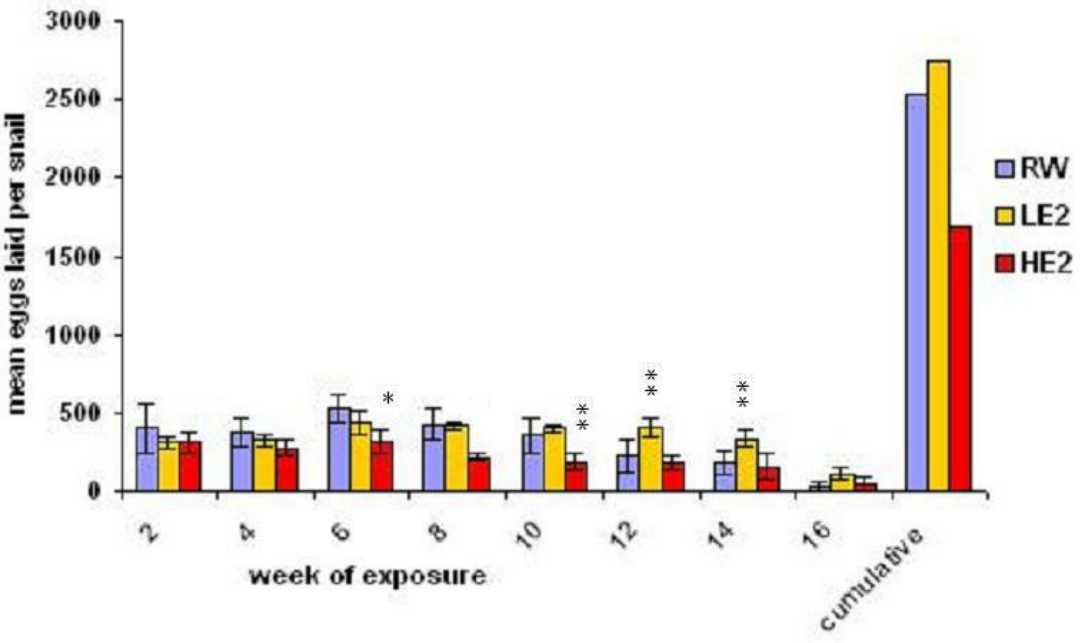

Figure 4.3 Mean number of eggs laid per adult *P. corneus* over the 16 week E2 experiment

Snails exposured to river water (RW), low concentration of 17β-oestradiol (LE2) or high concentration of 17β-oestradiol (HE2). Error bars show standard deviation. One star (*) indicates significant difference from RW tank (P<0.05), double star indicates significant difference from all other treatment

The general trend seen with the RW tank snails was that initially egg laying was fairly constant, with a peak in egg laying around midsummer (week 6), after which egg laying steadily declined through mid to late summer. The LE2 exposed snails laid eggs at a fairly constant rate over the spring and summer, only declining in late summer (after week 14). The HE2 exposed snails consistently laid fewer eggs per snail compare to the RW and the LE2 exposed snails (Figure 4.3). Statistical analysis found significant differences in the number of eggs laid per snail from the different treatment groups for weeks 6 (P=0.044, ANOVA), 10 (P=0.029, ANOVA), 12 (P=0.024, ANOVA) and 14 (P=0.039, ANOVA). No significant differences were found at weeks 2 (P=0.514, ANOVA), 4 (P=0.275, ANOVA) or week 8 (P=0.061, K-W), at week 16 the effects were marginally significant at the 95% level (P=0.055, ANOVA). Analysis of the cumulative number of eggs laid per snail (Kruskal-Wallis) found no significant difference between the three treatment groups (P=0.066). Post hoc analysis found at week 6 the RW exposed snails had laid significantly more eggs per snail than HE2 exposed snails (P=0.016, LSD). At week 10 HE2 expose snails laid significantly less eggs per snails than both RW and LE2 exposed snails (P= 0.033 and 0.013, respectively). Whereas at week 12 the RW and HE2 exposed snails laid significantly less eggs per snail than the LE2 snails (P= 0.026 and 0.011, respectively).

This trend continued at week 14; the LE2 snails laid significantly more eggs per snail than the RW or HE2 snails (P= 0.038 and 0.019, respectively) (see Figure 4.3).

4.3.3.2 Survival

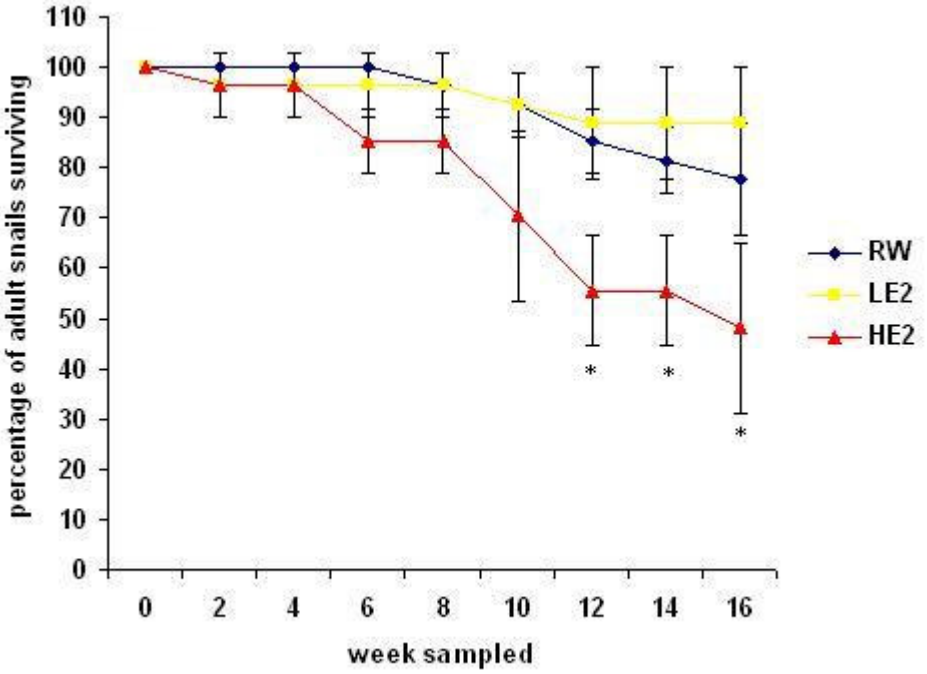

Figure 4.4 The percentage of *P. corneus* adults surviving at each sampling period during the E2 experiment

Snails exposed to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2) over 16 weeks. Error bars show standard deviation. Star (*) indicates significant difference from other treatments.

Of the 27 (9 per cage) adult *P. corneus* placed each mesocosm tank at the start of the exposure, 21 (77.8%) survived until the end of the exposure from the RW treatment, 24 (88.9%) survived from the LE2 treatment and 13 (48.1%) survived from the HE2 treatment. The HE2 exposed snails survival dropped steeply between weeks 4 and 6 and then again after week 8 compare to that of snails from the RW or LE2 exposures (Figure 4.4).

Statistical analysis (K-W) found no significant differences in survival between the treatments at weeks 2, 4 and 6 (P=0.565, 0.565, 0.056, respectively) or at weeks 8 and 10 (P=0.125 and 0.079, ANOVA). Analysis by one way ANOVA did find significant differences between the three treatment groups survival at weeks 12, 14 and 16 (P=0.011, 0.014 and 0.024, respectively).

After week 16 of the exposure the dosing pumps administering the E2 to the tanks were switched off and the tanks were left to depurate in running river water over the weekend. After the weekend the adult snails were removed from the tanks for the final time. During this short depuration a further 6 adult snails from the HE2 exposure tank and 1 snail from the LE2 exposure tank had died; leaving a total of 21 snails from the RW tank, 23 from the LE2 tank and 7 from the HE2 tank. At this point the remaining snails were sampled; their size and weight were measured and the soft tissue was fixed for histology (see Section 3.5.7), the data from these specimens are presented below.

4.3.3.3 Size and Weight

Morphometric data was collected to see whether E2 exposure had impacted the growth of the snails. Mean shell diameter, total weight and soft body weight were similar over all treatments (Table 4.5). No significant differences were found between the treatment groups in surviving adult shell diameter (P=0.816), total weight (P=0.811), or soft body weight (P=0.801). Spearman's rank order was used to correlate shell diameter, total weight and soft body weight. Significant positive correlations were found between shell diameter and total weight (RW; 0.805 P= 0.017, LE2; 0.741 P= 0.008, HE2; 0.893 P= 0.007) and shell diameter and soft body weight (RW; 0.876 P= 0.007, LE2; 0.794 P<0.001, HE2; 0.893 P= 0.007) for adults from all treatments. However, significant positive correlations between total weight and soft body weight were only found in RW (0.881 P= 0.002) and LE2 (0.759 P= 0.003) exposed adults. HE2 exposed adults had a positive, but not significant, correlation (0.643 and P=0.119).

Table 4.5 Shell diameter, total weight and soft body weight of surviving adult F0 *P. corneus* from the E2 experiment

	River Water exposed	Low E2 exposed	High E2 exposed
Shell diameter mm	26.5 ±1.6	26.9 ± 2.4	26.2 ± 2.1
Total weight g	2.9 ± 0.6	2.8 ± 0.8	2.7 ± 0.6
Soft body weight g	1.3 ± 0.3	1.3 ± 0.5	1.2 ± 0.3

Snails sampled after four months of exposure to river water (RW), low concentration of 17β-oestradiol (LE2) or high concentration of 17β-oestradiol (HE2). Mean ± standard deviation.

4.3.4 Effects of 17β-oestradiol exposure on adult *P. corneus* gonad histopathology

As stated in Section 4.3.3.2, a total of 51 adult snails (RW; 21, LE2; 23, HE2; 7) were sampled and fixed for histopathological analysis from this experiment. However, due to time constraints a maximum of 10 specimens were sectioned, stained and analysed from each treatment (RW, n = 10; LE2, n = 10; HE2, n = 7). Four sections of gonad were analysed per adult snail. As the main focus of this research was to be on the possible

effects of developmental exposure to E2, rather than on adult exposure, this low number of samples analysed was decided upon as a compromise between available time and quantity of data. However, it is my intention to complete the analysis for the remaining specimens when time/funding is available.

Due to the complex nature of the *P. corneus* gonad, analysis was split between different areas or cell types within the gonad (Chapter 2, Section 3.2.1 for details).

All adult *P. corneus* analysed (from all treatment groups) had sertoli cells attached to the acini walls. A dose-dependant decrease in normal vitellogenic area activity (maturing oocytes with follicle cells and/or sperm with sertoli cells) was found (Table 4.6). Analysis of the mean scores by One way ANOVA, however, found no significant difference between the three treatment groups (P = 0.060).

All adult *P. corneus* analysed (from all treatments) had intact acini walls. Adult snails from the RW exposure had the highest amount of cell (developing oocytes, sperm and supporting cells) coverage along the acini walls. Adult snails from the LE2 treatment had the least (Table 4.7) cell cover. Analysis of the mean scores using One way ANOVA found a significant difference between the groups (P = 0.005); post hoc analysis of LSD found a significant difference between the RW and LE2 snails (P = 0.001) but not between the RW and HE2 (P = 0.111) or between the two E2 treatments (P = 0.115).

Table 4.6 Percentage of adult *P. corneus* snails with varying level of active vitellogenic areas from the E2 experiment

Percent of acini with active vitellogenic area	Score	Percentage of adult snails from the River Water exposure (RW)	Percentage of adult snails from the Low E2 exposure (LE2)	Percentage of adult snails from the High E2 exposure (HE2)
<10%	1	0	0	14.5
10-30%	2	0	20	0
30-50%	3	10	30	14.5
50-70%	4	50	30	71
>70%	5	40	20	0

***P.corneus* sacrificed after four months exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 10; LE2, n = 10; HE2, n = 7). Four sections of gonad were analysed per adult snail.**

Table 4.7 Percentage of adult *P. corneus* snails with varying level of acini wall cell cover from the E2 experiment.

Percent of acini wall covered by germ cells and/or supportive cells	Score	Percentage of adult snails from the River Water exposure (RW)	Percentage of adult snails from the Low E2 exposure (LE2)	Percentage of adult snails from the High E2 exposure (HE2)
<10%	1	0	10	0
10-30%	2	0	20	29
30-50%	3	40	50	14
50-70%	4	10	20	43
>70%	5	50	0	14

***P.corneus* sacrificed after four months exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 10; LE2, n = 10; HE2, n = 7). Four sections of gonad were analysed per adult snail.**

Sloughing of spermatogenic cells into the acini lumen

Overall snails from the HE2 exposure had the highest percentage of affected snails for all four cell types (Sertoli cell, spermatogonium, spermatocyte and spermatids) sloughing in to the lumen (Table 4.8).

Sertoli cells sloughing into lumen

Adult snails from the RW treatment had the least number of sections of gonad with Sertoli cells sloughing into the acini lumen, whereas, snails from the HE2 treatment had the highest level (Table 4.8). Analysis of the mean number of sections affected by Kruskal-Wallis Test found a significant difference between groups (P = 0.025). Post hoc analysis using Mann-Whitney Test found a significant difference between the RW and HE2 treated snails (P= 0.010), but not between the RW and LE2 (P= 0.133) or between the E2 treatments (P= 0.105).

Spermatogonium sloughing into lumen

Spermatogonium cell sloughing into the lumen was most frequent in HE2 exposed snails and least frequent in RW exposed snails (Table 4.8). However, Kruskal-Wallis Test found no significant difference between the three treatments (P= 0.095).

Spermatocyte sloughing into the lumen

Snails from the HE2 treatment had the highest frequency of sections of gonad with spermatocytes sloughing into the acini lumen, snails from the LE2 and RW treatment had similar levels of spermatocyte sloughing (Table 4.8). One way ANOVA of the mean

number of sections affected found no significant difference between the three groups (P = 0.094).

Spermatids sloughing into the lumen

Spermatids were frequently found sloughed into the lumen in all treatments; however, snails from the HE2 exposure were more affected by spermatids sloughing into the lumen than those from the LE2 or RW (Table 4.8). Analysis of the mean number of sections affected by One way ANOVA found no significant difference between the three treatment groups (P= 0.226).

Mature spermatogenic cells in the lumen

A dose-dependant decrease in the number of sections with just mature spermatozoa in the acini lumen was found (Table 4.9). None of the sections analysed from any of the HE2 exposed snails had only mature spermatogenic cells in the lumen. However, analysis of the mean number of sections with only spermatozoa in the lumen by Kruskal-Wallis Test found no significant difference between the three treatments (P= 0.197).

Occurrence of acini with immature spermatogenic cells in lumen

The percentage of acini (per section of gonad) affected by immature spermatogenic cells sloughing into the lumen increased in a dose dependent manner (Table 4.10). One way ANOVA of the mean scores found no significant difference between the three groups (P= 0.124).

Oogenesis stages present

The percentage of different stages of oocytes present in the gonad was similar across all treatments. The HE2 exposed snails had a slightly higher percentage of younger stages of oocytes and slightly less mature stages compared to RW or low E2-exposed snails (Table 4.11). One way ANOVA of the mean percentages found no significant difference between treatments for the stage 1, 2, 4 and the degenerating oocytes (P= 0.662, 0.806, 0.433 and 0.593), and Kruskal-Wallis Test found no significant difference between the treatments of stage 3 and 5 oocytes (P= 0.768 and 0.347).

Table 4.8 Percentage of adult *P. corneus* snails with varying level of immature spermatogenic cells sloughing into the acini lumen from the E2 experiment

Number of sections affected	Percentage of adults affected by Sertoli cell sloughed into lumen			Percentage of adults affected by Spermatogonium sloughed into lumen			Percentage of adults affected by Spermatocyte sloughed into lumen			Percentage of adults affected by Spermatid sloughed into lumen		
	RW	LE2	HE2	RW	LE2	HE2	RW	LE2	HE2	RW	LE2	HE2
None	70	40	14	20	10	0	40	50	0	30	30	14.3
1 out of 4	20	30	29	10	10	0	10	20	29	20	30	0
2 out of 4	10	20	57	10	0	0	30	0	0	0	30	28.6
3 out of 4	0	10	0	10	20	0	10	0	14	30	0	28.6
4 out of 4	0	0	0	50	60	100	10	30	57	20	10	28.6
	a	a, b	b (0.01)	A	a	a	a	a	a	a	a	a

P.corneus **sacrificed after four months exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 10; LE2, n = 10; HE2, n = 7). Four sections of gonad were analysed per adult snail. Letters (a, b) denote statistical similarity between groups and the number in brackets = P value.**

Table 4.9 Percentage of adult *P. corneus* snails with varying numbers of sections of gonad with only mature spermatozoa in the acini lumen from the E2 experiment.

No. of sections with only mature spermatozoa in the acini lumen	Percentage of adult snails from the River Water exposure (RW)	Percentage of adult snails from the Low E2 exposure (LE2)	Percentage of adult snails from the High E2 exposure (HE2)
None	60	70	100
1 out of 4	20	10	0
2 out of 4	10	0	0
3 out of 4	0	10	0
4 out of 4	10	10	0

P.corneus **sacrificed after four months exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 10; LE2, n = 10; HE2, n = 7). Four sections of gonad were analysed per adult snail.**

Parasitism

No parasites were observed in any of the adult snails from the RW, LE2 or HE2 mesocosms.

Table 4.10 Percentage of adult *P. corneus* snails affected by varying percentage of acini with immature spermatogenic cells sloughing into the lumen from the E2 experiment

Percentage of acini affected by immature spermatogenic cells sloughing into the lumen	Score	Percentage of adult snails from the River Water exposure (RW)	Percentage of adult snails from the Low E2 exposure (LE2)	Percentage of adult snails from the High E2 exposure (HE2)
<10%	1	50	30	28.6
10-30%	2	30	50	14.2
30-50%	3	20	0	0
50-70%	4	0	0	28.6
>70%	5	0	20	28.6

***P.corneus* sacrificed after four months exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 10; LE2, n = 10; HE2 n = 7). Four sections of gonad were analysed per adult snail.**

Table 4.11 The mean percentage of different stages of oogenesis in adult *P. corneus* from the E2 experiment

Stage of oocyte maturation	Mean percent of oogenesis stage after River Water exposure	Mean percent of oogenesis stage after Low E2 exposure	Mean percent of oogenesis stage after High E2 exposure
Stage 1	33.5 ± 10.0	31.7 ± 5.7	35.7 ± 8.6
Stage 2	21.7 ± 4.6	20.9 ± 3.2	20.6 ± 1.6
Stage 3	20.8 ± 5.5	21.1 ± 2.3	23.0 ± 7.3
Stage 4	11.0 ± 4.7	12.4 ± 5.5	9.0 ± 5.4
Stage 5	0.8 ± 1.2	0.3 ± 0.4	0.9 ± 0.8
Degenerating	12.1 ± 5.3	13.5 ± 6.5	10.8 ± 3.6

***P.corneus* sacrificed after four months exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 10; LE2, n = 10; HE2, n = 7). Four sections of gonad were analysed per adult snail. Stage 1 (oogonium) is the youngest stage of oogenesis, stage 5 is ready to be ovulated, oogenesis stages based on description by de Jong-Brink et al, (De Jong-Brink et al. 1976).**

4.3.5 Effects of 17β-oestradiol on developmentally exposed F1 *P. corneus*

4.3.5.1 Survival and growth at end of exposure

At the end of exposure, the total number of F1 *P. corneus* found in the mesocosm tanks increased dose-dependently; RW (164), LE2 (274), and HE2 (483). The percentage of snails above 10 mm shell diameter (normal minimum sexually active size) was highest in the RW tank (73%), whereas in the HE2 and LE2 tanks, 48 and 46% of the snails were above 10 mm shell diameter, respectively. Table 4.13 gives the number of F1s above and below 10mm shell diameter.

Table 4.12 Total number and number per size class of *P. corneus* F1s from each mesocosm tank after dosing ended (September 2006).

Shell Diameter	River Water (RW)	Low dose E2 (LE2)	High dose E2 (HE2)
<10 mm	45 (27%)	149 (54%)	250 (52%)
>10 mm	119 (73%)	125 (46%)	233 (48%)
Total	164	274	483

Percentage of each size range given in brackets

Size and weight at the end of exposure

Approximately 40 F1s of above 10 mm shell diameter (presumed sexually mature) were removed from each treatment for further analysis in September 2006 (after the end of exposure). Of these, specimens the F1s from the high E2 dose tank were significantly smaller (shell diameter and aperture) and lighter (total weight and soft body weight) than those from the low E2 and River water treatments ($P<0.001$), see Figure 4.5. There was a significant ($P<0.001$) positive correlation between shell diameter and total weight, and shell diameter and soft body weight in all treatments.

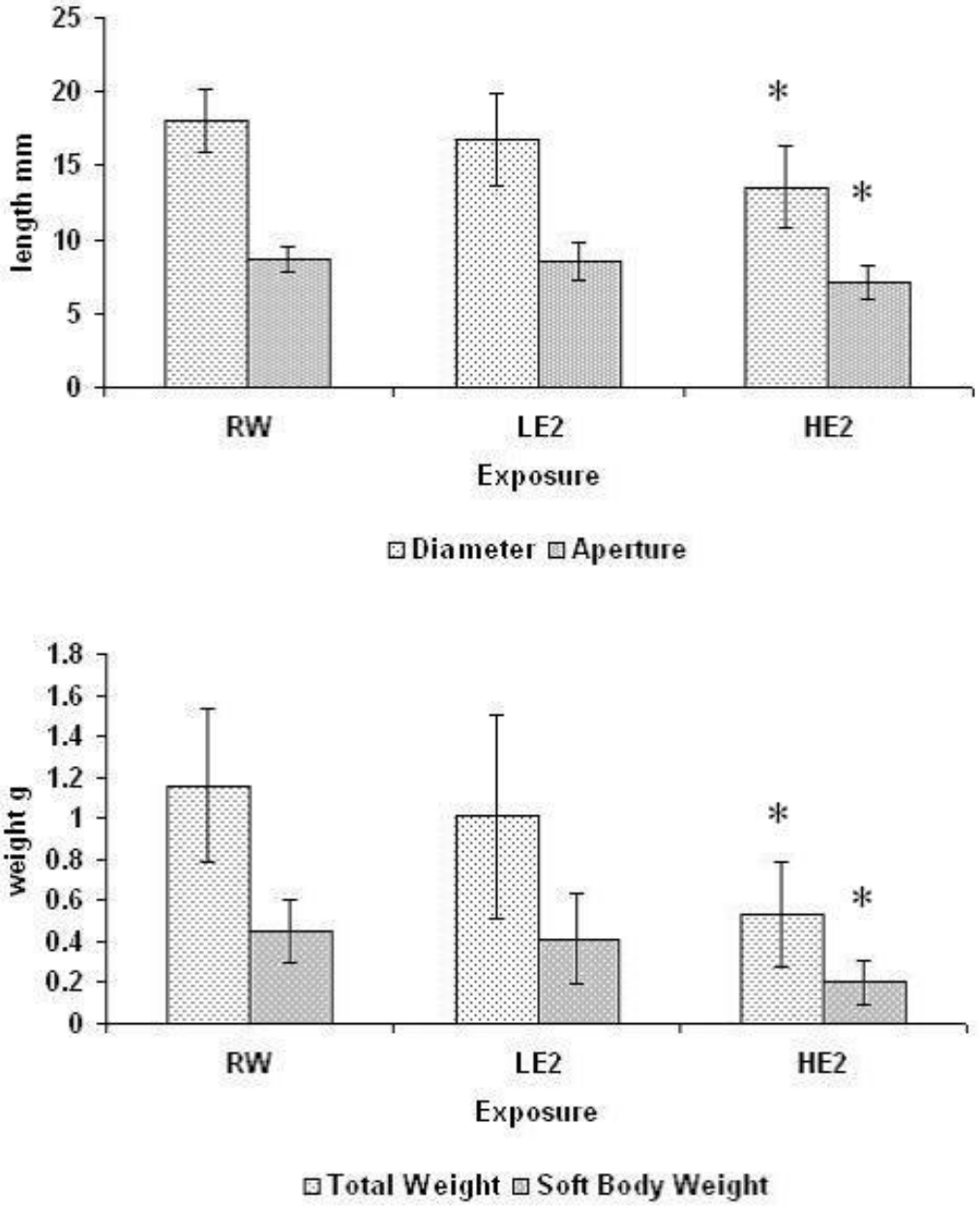

Figure 4.5 Mean shell length and aperture (A) and mean total weight and soft body weight (B) of the F1 *P. corneus* sampled from the three treatment tanks in September 2006.

Error bars indicate standard deviation around the mean. Star (*) indicates significant difference (P<0.05) from other treatments.

4.3.6 Histopathology of F1 *P. corneus* immediately after exposure

As stated above, in September 2006 (after dosing finished) approximately 40 F1s from each treatment (river water; RW, low dose of 17β-oestradiol; LE2 and high dose 17β-oestradiol; HE2) were taken from the tanks and fixed for histopathological analysis. However, due to time constraints only around half (~20 from each treatment) of these specimens were sectioned, stained and analysed; the data from these specimens is presented in the following sections. It is my intention to complete the analysis for the remaining specimens when time/funding is available.

Due to the complex nature of the *P. corneus* gonad, analysis was split between different areas or cell types within the gonad. The majority of snails sampled from all treatments had all stages of oogenesis and spermatogenesis present. However, exposure to 17β-oestradiol did increase disruption to the gonad structure as described below.

4.3.6.1 Effects of E2 exposure on the F1 hermaphrodite gonad

One snail from the HE2 treatment had no sertoli cells attached to the acini walls in four out of five sections of gonad analysed. All other snails (from all three treatments) had sertoli cells attached to the acini walls in five out of five sections. A dose-dependant decrease in normal vitellogenic area activity (maturing oocytes with follicle cells and/or sperm with Sertoli cells) was found (Table 4.13 and Figure 4.6). When the mean scores were analysed (Mann-Whitney Test) significant differences were found between the RW and E2 exposed snails (LE2 $P = 0.008$ and HE2 $P < 0.001$) and between E2 exposed snails ($P = 0.002$).

Table 4.13 Percentage of F1 *P. corneus* snails with each of the different levels of acini vitellogenic area activity from the E2 experiment

Percent of acini with active vitellogenic area	Score	Percentage of F1 snails from the River Water exposure (RW)	Percentage of F1 snails from the Low E2 exposure (LE2)	Percentage of F1 snails from the High E2 exposure (HE2)
<10%	1	0	0	16
10-30%	2	0	0	11
30-50%	3	0	20	47
50-70%	4	21	35	21
>70%	5	79	45	5
		a	b	C

***P. corneus* sacrificed after developmental exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on a sub-sample of F1s from each treatment (RW, n = 18; LE2, n = 20; HE2, n = 21). Five sections of gonad were analysed per snail. Letters (a, b, c) denote statistical similarity between groups.**

The HE2 exposed snails had more frequent disruption to the acini wall than LE2 or RW exposed snails. The LE2 exposed snails had the least disruption (Table 4.14). Kruskal-Wallis test of the number of sections affected found a significant difference between treatments (P= 0.009). Post hoc analysis using Mann-Whitney Test found a significant difference between the LE2 and HE2 treatments (P= 0.003) but not between HE2 and RW (P= 0.088) or between LE2 and RW treatments (P= 0.166).

Table 4.14 Percentage of F1 *P. corneus* snails with varying level of acini wall disruption from the E2 experiment

Number of sections affected by acini wall disruption	Percentage of F1 snails from the River Water exposure (RW)	Percentage of F1 snails from the Low E2 exposure (LE2)	Percentage of F1 snails from the High E2 exposure (HE2)
None	73	90	47
1 out of 5	11	5	19
2 out of 5	0	5	0
3 out of 5	3	0	10
4 out of 5	11	0	14
5 out of 5	0	0	10
	a, b	a	B

***P. corneus* sacrificed after developmental exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on a sub-sample of F1s from each treatment (RW, n = 18; LE2, n = 20; HE2, n = 21). Five sections of gonad were analysed per snail. Letters (a, b) denote statistical similarity between groups.**

Snails from the HE2 treatment had the least amount of cell (developing oocytes, sperm and supporting cells) cover of the acini walls, and those from the RW had less than those from the LE2 treatment (Table 4.15). When the scores were analysed using One way ANOVA a significant difference between groups was found (P<0.001) and a post hoc test of LSD found that the LE2 snails had a significantly higher score than the HE2 or RW snails (P <0.001 and <0.001) but no significant difference was found between the HE2 and RW exposed snails (P = 0.227).

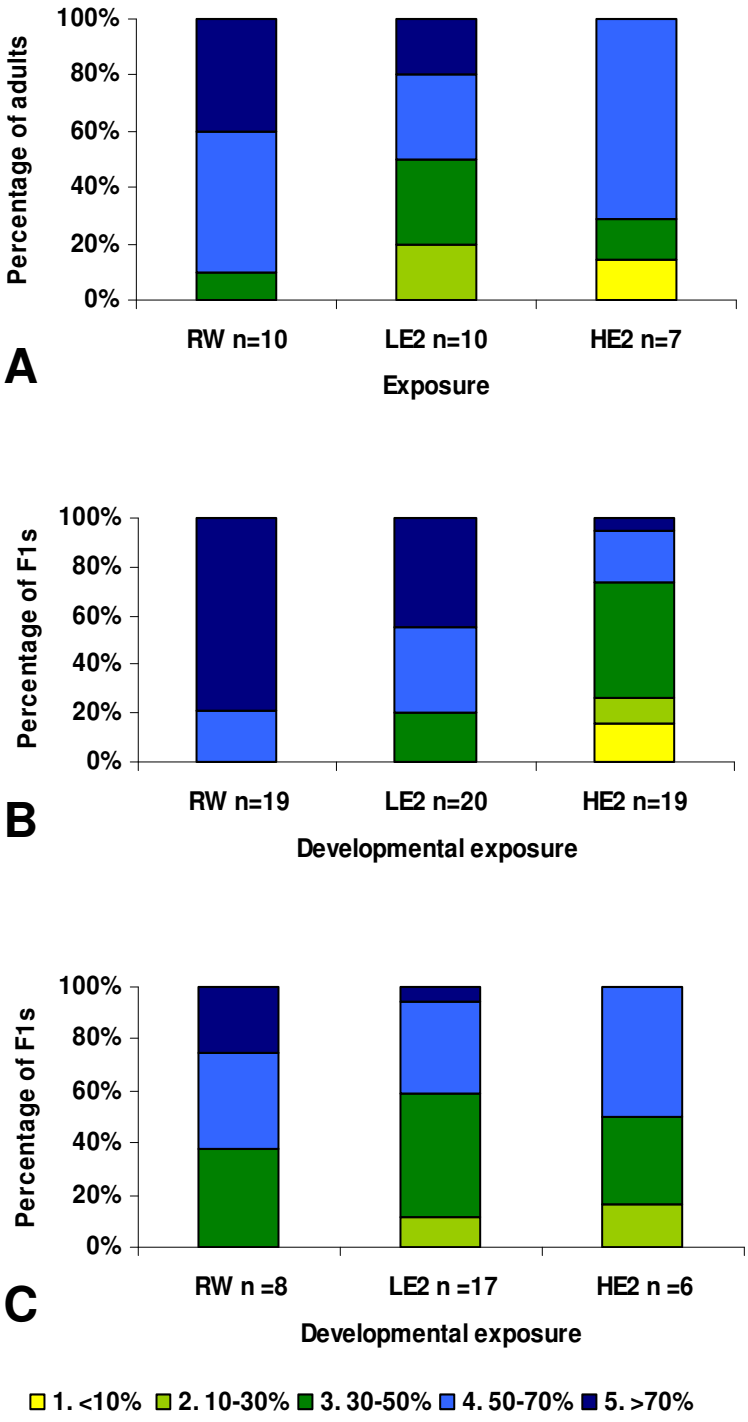

Figure 4.6 Vitellogenic area activity in *P. corneus* from the E2 experiment

Snails exposed to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2) as adults during reproductive period (A) or developmentally (B and C). Adult snails (A) sampled directly after exposure (end August 2006), F1s (B) sampled directly after developmental exposure (September 2006) or after one year of depuration in river water (early August 2007). Percentage of snails with varying level of vitellogenic area activity (Sperm/oocyte maturation and supportive cells); Score 1 (yellow) lowest level of activity <10% of acini contain active vitellogenic area, Score 5 (dark blue) highest level of activity >70% of acini contain active vitellogenic area. n= number of snails analysed.

Table 4.15 Percentage of F1 *P. corneus* snails with varying level of acini wall cell cover from the E2 experiment

Percent of acini wall covered by germ cells and/or supportive cells	Score	Percentage of F1 snails from the River Water exposure (RW)	Percentage of F1 snails from the Low E2 exposure (LE2)	Percentage of F1 snails from the High E2 exposure (HE2)
<10%	1	0	0	20
10-30%	2	47	5	35
30-50%	3	41	21	35
50-70%	4	12	53	10
>70%	5	0	21	0
		a	B	a

***P. corneus* sacrificed after developmental exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on a sub-sample of F1s from each treatment RW n = 18, LE2 n = 20 and HE2 n = 21, five sections of gonad were analysed per snail. Letters (a, b) denote statistical similarity between groups.**

Immature spermatogenic and supportive cells sloughing into the lumen

Sertoli cells sloughing into the lumen

The number of sections analysed with Sertoli cells sloughed into the lumen increased in the snails from the E2 treatments compare to those from RW (Table 4.16). No significant difference (P= 0.258) between treatments was found using Kruskal-Wallis Test.

Spermatogonium sloughing into the lumen

Spermatogonium sloughing was very common in snails from all three mesocosm tanks, and a dose-dependant increase in the mean percentage of snails with the highest level (five out of five sections affected) was observed (Table 4.16). One way ANOVA, however found no significant difference between treatments (P= 0.307).

Spermatocytes sloughing into the lumen

Spermatocyte sloughing into the lumen was less frequent than that of the spermatogonia, and the number of sections affected by spermatocyte sloughing was similar between the HE2 and the RW. In comparison, the LE2 treated snails had a higher frequency of affected sections (Table 4.16). One way ANOVA found no significant difference between treatments (P= 0.119).

Spermatids sloughing into the lumen

There was a dose-dependant increase in spermatids sloughing into the lumen (Table 4.16). A significant difference was found between treatments when analysed using One way

ANOVA (P= 0.009). Post hoc analysis using LSD found the RW exposed snails to have significantly less spermatids sloughing than the HE2 exposed snails (P= 0.002), no significant difference was found between the RW and the LE2 (P= 0.082) snails or between the two E2 dosed snails (P= 0.153).

Table 4.16 Percentage of F1 *P. corneus* snails with varying levels of immature spermatogenic cells sloughed into the acini lumen from the E2 experiment

Number of sections affected	Percentage of F1s with Sertoli cells sloughing into lumen			Percentage of F1s with Spermatogonium sloughing into lumen			Percentage of F1s with Spermatocyte sloughing into lumen			Percentage of F1s with Spermatid sloughing into lumen		
	RW	LE2	HE2	RW	LE2	HE2	RW	LE2	HE2	RW	LE2	HE2
None	78.9	55	66.7	5.3	10	4.8	63.2	40	71.4	42.1	20	14.3
1 out of 5	15.8	25	9.5	5.3	0	0	21.1	25	14.35	21.1	25	14.3
2 out of 5	5.3	10	0	10.5	0	0	10.5	25	9.5	21.1	15	14.3
3 out of 5	0	5	14.3	10.5	15	0	5.3	0	4.8	10.5	10	23.8
4 out of 5	0	5	4.8	15.8	15	23.8	0	5	0	5.3	30	4.8
5 out of 5	0	0	4.8	52.6	60	71.4	0	5	0	0	0	28.6
	a	A	a	a	a	a	a	a	a	a	a, b	b

***P. corneus* sacrificed after developmental exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on a sub-sample of F1s from each treatment (RW, n = 18; LE2, n = 20; HE2, n = 21). Five sections of gonad were analysed per snail. Letters (a, b) denote statistical similarity between groups.**

Mature spermatogenic cells in the lumen

Spermatozoa only

Less than 50% of the snails from all of the treatments had only mature spermatogenic cells in the lumen. Snails from the HE2 dose had the highest percentage of sections affected by early spermatogenic stages sloughing into the acini lumen per snail (Table 4.17). Kruskal-Wallis test found a significant difference between treatments (P= 0.016). Post hoc analysis using the Mann-Whitney test found HE2 to have significantly less sections of gonad with

only mature spermatozoa in the lumen compared to LE2 (P= 0.017) or RW (P= 0.005) exposed snails. However, there was no significant difference between the RW and LE2 (P=0.608) exposed snails.

Table 4.17 Percentage of F1 *P.corneus* snails with varying number of sections of gonad with only mature spermatozoa in the acini lumen from the E2 experiment.

No. of sections with just mature spermatozoa in the lumen	Percentage of F1 snails from the River Water exposure (RW)	Percentage of F1 snails from the Low E2 exposure (LE2)	Percentage of F1 snails from the High E2 exposure (HE2)
None	52.6	60	90.5
1 out of 5	15.8	15	9.5
2 out of 5	10.5	15	0
3 out of 5	10.5	0	0
4 out of 5	5.3	0	0
5 out of 5	5.3	10	0
	a	a	b

***P. corneus* sacrificed after developmental exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2)Histological analysis was performed on a sub-sample of F1s from each treatment (RW, n = 18; LE2, n = 20; HE2, n = 21). Five sections of gonad were analysed per snail. Letters (a, b) denote statistical similarity between groups.**

Occurrence of acini with immature spermatogenic cells in lumen

The number of acini affected by immature spermatogenic cells sloughing into the lumen increased dose dependently (Table 4.18). When these scores were analysed using Kruskal-Wallis test a significant difference between groups was found (P= 0.003). Post hoc analysis using the Mann-Whitney Test found no significant difference between the two E2 exposed groups (P = 0.232). The RW exposed snails had significantly less than those from the LE2 treatment (P = 0.011) and HE2 treatment (P = 0.002).

Oogenesis stages present

A dose dependant increase in the ratio of young to old oocytes was found in *P. corneus* developmentally dosed to E2. E2 exposed snails had a higher percentage of young oocytes compared to those from the RW exposure (Table 4.19). Kruskal-Wallis Test found significant differences between treatments in stage 1 (P <0.001) oocytes. Post hoc analysis using the Mann-Whitney Test found snails from the RW treatment had significantly less stage 1 oocytes compared to E2-exposed snails (LE2 P = 0.003, HE2 P <0.001), but no significant difference was found between the different E2 exposures (P = 0.109). One way ANOVA found significant differences between groups in stage 2 (P =0.001), 4 (P = 0.032) and degenerating oocytes (P = 0.011). Post hoc analysis using LSD found a similar trend as

in stage 2 oocytes where RW had significantly less than LE2 (P = 0.001) and HE2 (P = 0.002), but E2 treatments were not significantly different from one another (P = 0.760). Post hoc analysis (LSD) of stage 4 oocytes found RW snails had significantly more stage 4 oocytes than HE2 exposed snails (P = 0.010) but no significant difference between LE2 and RW (P = 0.088), or LE2 and HE2 (P = 0.357). Post hoc analysis of the degenerating oocytes found RW to have significantly more than LE2 (P = 0.027) or HE2 (P = 0.004) but not between the two E2 treatments (P= 0.455).

Table 4.18 Proportion of F1 *P. corneus* snails found with varying percentage of acini with immature spermatogenic cells sloughing into the lumen from the E2 experiment

Percentage of acini affected by immature spermatogenic cells sloughing into the lumen	Score	Percentage of F1 snails from the River Water exposure (RW)	Percentage of F1 snails from the Low E2 exposure (LE2)	Percentage of F1 snails from the High E2 exposure (HE2)
<10%	1	100	60	42.9
10-30%	2	0	35	38.0
30-50%	3	0	5	9.5
50-70%	4	0	0	4.8
>70%	5	0	0	4.8
		a	b	b

***P.corneus* sacrificed after developmental exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on a sub-sample of F1s from each treatment (RW, n = 18; LE2, n = 20; HE2, n = 21). Five sections of gonad were analysed per snail. Letters (a, b) denote statistical similarity between groups.**

Parasitism

No parasites were observed in any of the sampled F1 *P. corneus* snails from the RW, LE2 or HE2 mesocosms.

Table 4.19 Mean percentage of different stages of oogenesis in F1 *P. corneus* from the E2 experiment.

Stage of oocyte maturation	Mean percent of oogenesis stage after River Water exposure	Mean percent of oogenesis stage after Low E2 exposure	Mean percent of oogenesis stage after High E2 exposure
Stage 1	8.3 ± 3.8 a	13.2 ± 4.1 b	18.3 ± 10.5 b
Stage 2	13.7 ± 5.1 a	20.4 ± 7.5 b	19.9 ± 5.0 b
Stage 3	22.3 ± 4.3 a	26.4 ± 7.6 a	23.4 ± 5.9 a
Stage 4	27.7 ± 7.5 a	23.4 ± 7.1 ab	21.2 ± 8.3 b
Stage 5	16.8 ± 9.5 a	10.2 ± 8.0 a	12.5 ± 9.6 a
Degenerating	11.2 ± 7.6 a	6.3 ± 5.3 b	4.7 ± 7.3 b

***P. corneus* sacrificed after developmental exposure to, River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on a sub-sample of F1s from each treatment (RW, n = 18; LE2, n = 20; HE2, n = 21). Five sections of gonad were analysed per snail. Stage 1 (oogonium) are the youngest stage of oogenesis, stage 5 is ready to be ovulated, oogenesis stages based on description by de Jong-Brink et al, (De Jong-Brink et al. 1976). Letters (a, b) denote statistical similarity between groups.**

4.3.7 Effects of early developmental exposure to 17β-oestradiol and then depuration on F1 *P. corneus*

4.3.7.1 Survival and growth during depuration in river water

The percentage survival over-winter was higher in the two E2-exposed groups compared to RW. 19% of hatchlings survived in the RW, 28% from the LE2 and 25% from the HE2 (Table 4.20).

Table 4.20 F1 *P. corneus* were left to depurate in river water over winter following developmental exposure.

F1 P.corneus	River Water (RW)	Low E2 (LE2)	High E2 (HE2)
Left to over-winter September 2006	322	240	442
Surviving March 2007	60	68	109
Percent survival	19%	28%	25%

Snails exposed to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Number left in each treatment tank, number surviving until March 2007 and percentage over winter survival.

However, between March and April 2007 a large percentage (49%) of the remaining HE2 developmentally exposed snails died (compared to 29% of the LE2 and 20% of the RW developmentally exposed F1 snails).

Developmentally exposed F1 *P. corneus* snails were measured in April 2007 (after eight months of depuration); the HE2 developmentally exposed F1 snails were on average smaller than the LE2 or the RW F1s. In contrast, the LE2 F1s were large and had the highest percentage of snails in the largest size class (>20 mm shell diameter) (Table 4.21).

Table 4.21 Total number and percentage (in brackets) of different shell size classes of all surviving F1 *P. corneus* after developmental exposure

Percentage Shell Size class	River Water developmentally exposed (n=48)	Low E2 developmentally exposed (n=28)	High E2 developmentally exposed n=(56)
<10 mm shell diameter	5 (10%)	3 (6%)	12 (21%)
10-15 mm shell diameter	15 (31%)	10 (21%)	28 (50%)
15-20 mm shell diameter	25 (52%)	27 (56%)	15 (27%)
> 20 mm shell diameter	3 (6%)	8 (17%)	1 (2%)

Snails exposed to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2) followed by eight months depuration in river water over autumn/winter 2006. F1 snails were measured April 2007. Total number of snails = n.

4.3.7.2 Reproductive output

After winter depuration, a total of 18 F1 developmentally exposed snails (>10mm in shell diameter) from each treatment were placed into three reproductive groups (six snails per group) housed in mesh cages in the same manner as the adults in the dosed study. These cages were all housed in one mesocosm tank that was fed with river water (see Section 3.5.9 for details).

Egg laying was delayed by up to six weeks in both the LE2 and the HE2 exposed snails compared to those from the RW exposure, despite the larger size of the LE2 exposed snails. The RW developmentally exposed F1s started laying egg masses by week 2 (first sampling point) whereas only one egg mass containing 10 eggs was laid by week 5 by the HE2 developmentally exposed F1s. Moreover, snails from this group (HE2) laid no further eggs until week 8, when the LE2 developmentally exposed snails also started to lay eggs (Figure 4.7). The mean numbers of eggs laid per snail were compared at each sampling point. Statistical analysis found significant differences between the three groups at weeks 2 (P= 0.021, K-W) and 5 (P= 0.035, K-W), but not at weeks 6 (P= 1.000, K-W), 8 (P= 0.067, ANOVA), 10 (P= 0.162, ANOVA), 12 (P= 0.619, K-W) or 14 (P= 0.281, K-W). The RW developmentally exposed snails laid significantly more eggs per snail at weeks 2 and 5 compare to the LE2 exposed snails (wk 2 P= 0.034 and wk 5 P= 0.037) and the HE2

exposed snails (wk 2 P= 0.034 and wk 5 P= 0.046). The mean cumulative number of eggs laid per snail was also reduced in both LE2 and HE2 developmentally exposed F1 compared to RW; cumulatively over the 14 weeks of the F1 breeding study RW developmentally exposed snails laid 112 eggs per snail compared to 6 by the LE2 and 45 by the HE2 developmentally exposed F1s. However, One way ANOVA found no significant difference in the cumulative number of eggs laid per snail (P= 0.197).

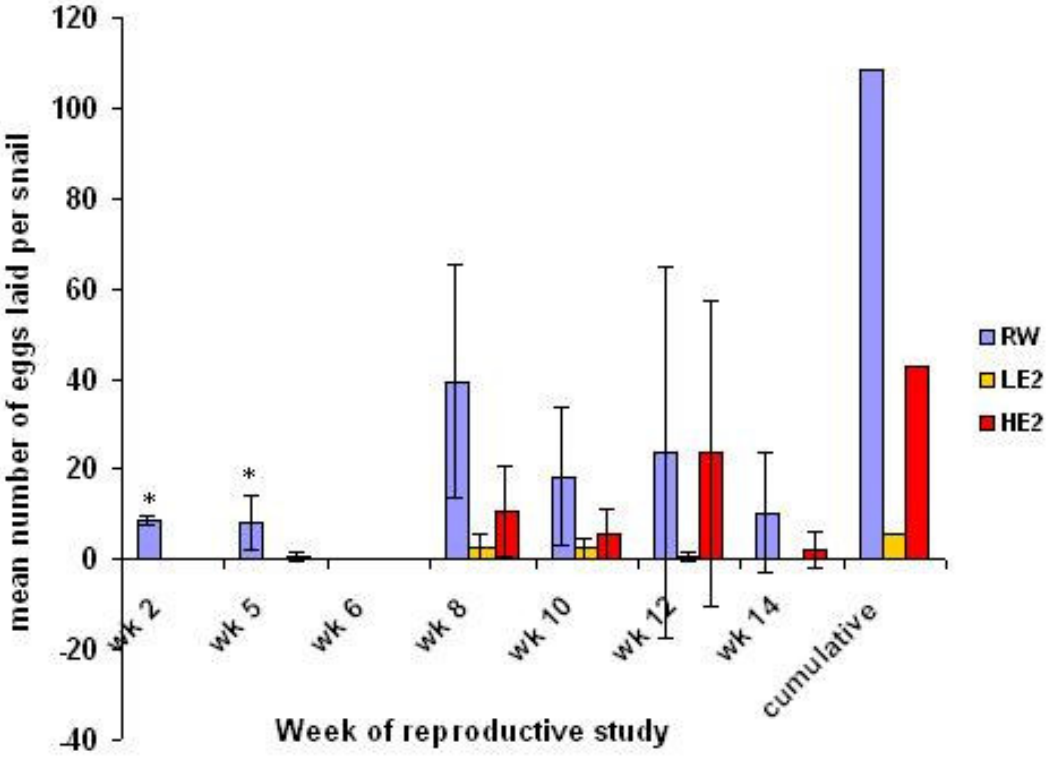

Figure 4.7 Mean number of eggs per individual *P. corneus* from F1 E2 experiment

Snails developmentally exposed to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2) and then depurated in river water over autumn/winter 2006. Mean number of eggs laid per snail over each time period during spring/summer 2007 and cumulatively over the entire 14 week study. Error bars give standard deviation, star (*) indicates significant difference from other treatments P<0.05.

The number of egg masses laid per individual snail followed a similar trend to the number of eggs laid per snail, in that the RW developmentally exposed snails laid the most egg masses and the LE2 developmentally exposed snails laid the least. Statistical analysis found significant differences between the three treatments for the mean number of egg masses laid per snail at weeks 2 (P=0.001, ANOVA) and 8 (P=0.030, ANOVA) but not at weeks 5, 8, 10, 12 or 14 (no egg masses were laid at week 6). At weeks 2 and 8 the RW

developmentally exposed snails laid significantly more egg masses per snail compared to the LE2 developmentally exposed (P=0.001, 0.015) and the HE2 developmentally exposed (P=0.001, 0.029) snails (Figure 4.8).

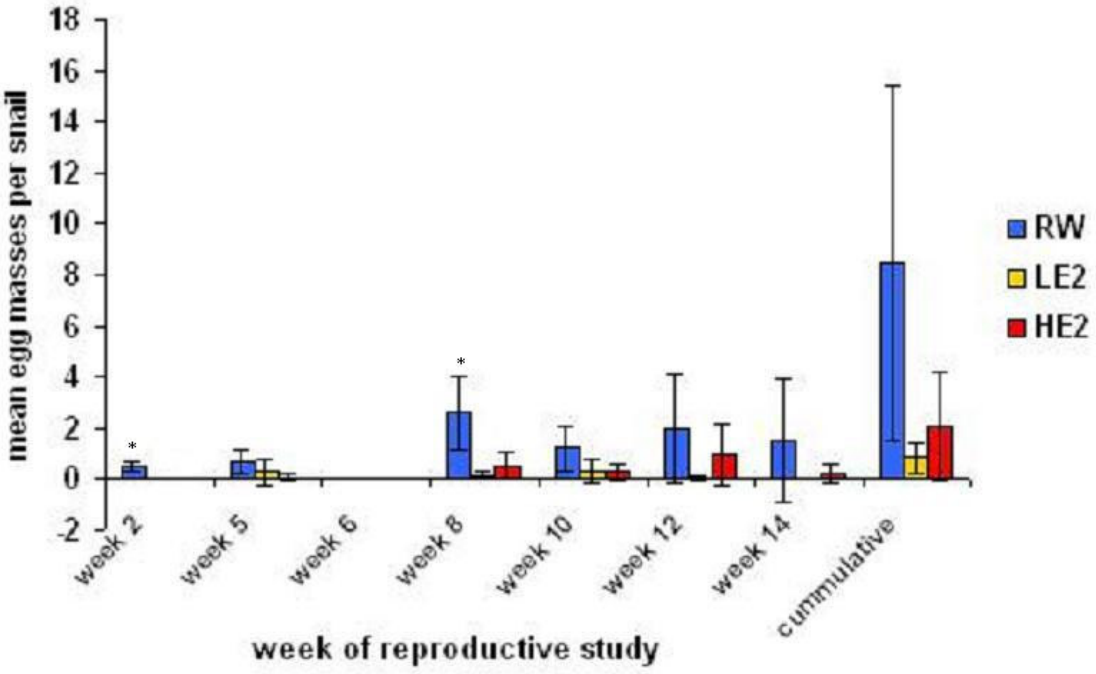

Figure 4.8 The mean number of egg masses laid per individual *P. corneus* from F1 E2 experiment.

Snails developmentally exposed to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2) and then depurated in river water over autumn/winter 2006. Mean number of egg masses laid per snail over each time period during spring/summer 2007 and cumulatively over the entire 14 week study. Error bars give standard deviation. Star (*) indicates significant difference from other treatments P<0.05.

The mean number of eggs per egg mass for the RW developmentally exposed snails peaked at week 14 (24.8 ± 10.4 eggs per mass), the LE2 exposed snails laid their largest egg masses at week 8 (15.3 ± 3.8 eggs per mass) and the HE2 exposed snails their largest masses at week 12 (22.9 ± 8.7 eggs per mass). On average, the LE2 developmentally exposed snails laid the smallest egg masses and the HE2 developmentally exposed snails laid the largest egg masses (Table 4.22).

Table 4.22 Mean number of eggs per egg mass laid by *P. corneus* F1s developmentally exposed and then depurated in river water over autumn/winter 2006.

	Week 2	Week 5	Week 6	Week 8	Week 10	Week 12	Week 14
RW	17.1 ± 6.5	12 ± 3.3	-	15.4 ± 4.8	15.6 ± 6.2	16.5 ± 5.6	24.8 ± 10.4
LE2	-	-	-	15.3 ± 3.8	6.5 ± 6.3	11.0 ± 0	-
HE2	-	10 ± 0	-	20.1 ± 5.1	21.0 ± 8.2	22.9 ± 8.7	11.7 ± 4.0

Snails developmentally exposed to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Mean number of eggs per mass laid over each time period during spring/summer 2007. Dash (-) indicates no egg-masses laid.

4.3.7.3 Survival and growth

Survival over the 14 week breeding study was lowest in the HE2 developmentally exposed snails (33%), and highest in the LE2 snails (94%) compared to 44% of RW F1s. Significant differences between LE2 and RW/HE2 were found from week 2 onwards (Figure 4.9). Over the whole breeding study (14 weeks), only one snail from the LE2 group died compared to 10 from the RW group and 12 from the HE2 group.

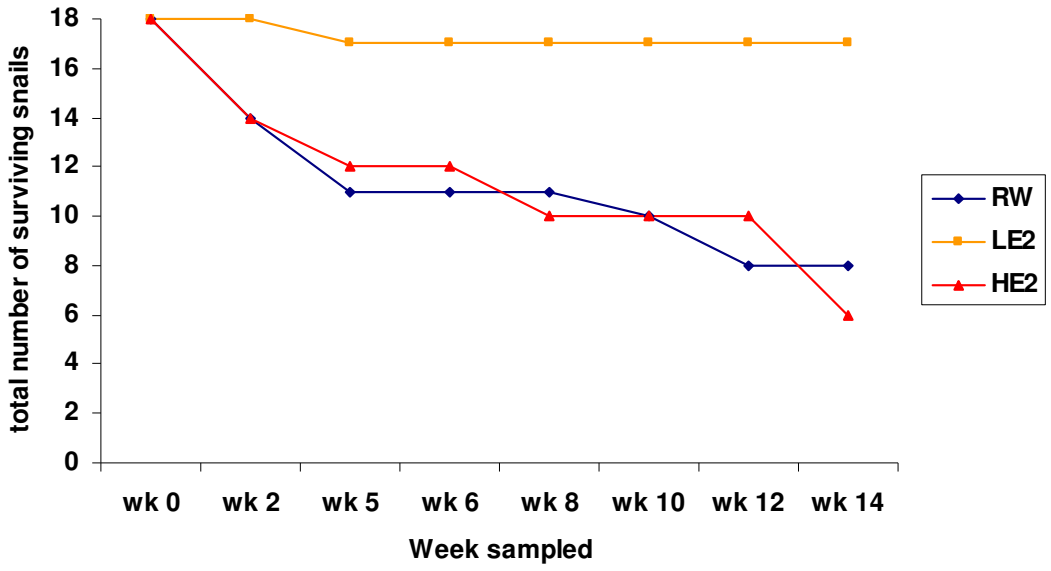

Figure 4.9 Survival of depurated snails over 14 weeks of reproductive study conducted Spring/Summer 2007.

***P.corneus* F1s developmentally exposed to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2) and then depurated in river water over autumn/winter 2006.**

No significant differences were found in the F1 snail shell diameter or weight between treatment groups at the start (diameter P=0.566 and total weight P=0.265) or finish

(diameter P=0.475, total weight P=0.216 and soft body weight P=0.302) of the F1 breeding study (Table 4.23). There were also no significant differences between the mean shell diameter (P= 0.903) or weight (P=0.644) of snails from each reproductive group (three groups per treatment). The mean shell diameter and total weight did increased in all treatments over the 14 week breeding study; shell diameter increased by 10.8% in the RW F1s, 10.9% in the LE2 F1s and 11.7% in the HE2 F1s, and total weight increased by 25.9% in RW F1s, 25.3% in LE2 F1s and 32.7% in HE2 F1s. Table 4.23, below, gives the mean shell diameter and total weight at the start and end of the F1 breeding study.

Table 4.23 Shell diameter and total weight of *P. corneus* at the start and end of the un-dosed F1 breeding study

	River Water (RW)	Low E2 (LE2)	High E2 (HE2)
Start of study shell diameter (mm)	14.6 ± 1.9	15.1 ± 1.5	14.9 ± 1.3
End of study shell diameter (mm)	16.3 ± 1.6	17.0 ± 1.1	16.8 ± 0.6
Start of study total weight (g)	0.7 ± 0.3	0.8 ± 0.2	0.7 ± 0.2
End of study total weight (g)	0.9 ± 0.2	1.1 ± 0.2	1.0 ± 0.1

F1 *P. corneus* developmentally exposed to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2) and then depurated in river water for eight months.

To assess growth Spearman's rank order was used to correlate shell diameter and total weight at the start of the F1 breeding study, and shell diameter and total weight and soft body weight at the end. At the start of the breeding study, significant positive correlations were found between shell diameter and total weight (RW; 0.595 P<0.001, LE2; 0.936 P<0.001, HE2; 0.898 P<0.001). At the end of the F1 breeding study significant positive correlations were also found for shell diameter and total weight (RW; 0.952 P<0.001, LE2; 0.781 P<0.001, HE2; 0.829 P= 0.042). However, significant positive correlations for shell diameter and soft body weight and total weight and soft body weight were only found in the RW and LE2 developmentally exposed snails (RW diameter/soft body weight; 0.810 P=0.015, RW total weight/soft body weight; 0.905 P=0.002 and LE2 diameter/soft body weight; 0.806 P<0.001, LE2 total weight/soft body weight; 0.634 P= 0.006). The HE2 developmentally exposed and depurated F1s did not have significant correlations for shell diameter/soft body weight (0.429 P=0.397) or for soft body weight/total weight (0.086 P=0.872).

4.3.8 Effect of developmental exposure to 17βestradiol and then depuration in river water on histopathology of the gonad

All of the snails sampled at the end of the F1 breeding study were subject to histopathological analysis (RW, n = 8; LE2, n = 17; HE2, n = 6). Five sections of gonad were analysed per snail.

All snails sampled (from all three treatments) had Sertoli cells attached to the acini walls in all of the sections of gonad analysed. A dose-dependant decrease in normal vitellogenic area activity (maturing oocytes with follicle cells and/or sperm with sertoli cells) was found in snails developmentally exposed to E2 and then depurated in river water for approximately one year (Table 4.24 and Figure 4.6). However, analysis of the average scores by One way ANOVA found no significant differences between the three treatment groups (P= 0.106).

Table 4.24 Percentage of F1 *P. corneus* snails with each of the different levels of vitellogenic area activity from the depurated E2 expriment

Percent of acini with active vitellogenic area	Score	Percentage of depurated F1 snails from the River Water exposure (RW)	Percentage of depurated F1 snails from the Low E2 exposure (LE2)	Percentage of depurated F1 snails from the High E2 exposure (HE2)
<10%	1	0	0	0
10-30%	2	0	6	17
30-50%	3	13	29	33
50-70%	4	49	53	50
>70%	5	38	12	0

***P. corneus* sacrificed after developmental exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2) and then depuration for approximately one year in river water. Histological analysis was performed on all surviving snails from the F1 breeding study (RW n = 8, LE2 n = 17, HE2 n = 6). Five sections of gonad were analysed per snail.**

The majority of F1 snails (from all treatments) had intact acini walls in all sections of gonad analysed. One snail from the RW exposure had three out of five sections with intact acini walls and one snail from the LE2 exposure had two out of five sections with intact acini walls; the rest of the snails from the RW and the LE2 exposures had five out of five sections with intact acini walls. The remaining snails from the HE2 treatment had five out of five sections with intact acini walls. Analysis by One way ANOVA found no significant difference between the three treatments (P= 0.772). The HE2 F1 snails developmentally exposed and then depurated had the least amount of cell (developing oocytes, sperm and supporting cells) coverage around of the acini walls, whilst the LE2 F1 snails had the most

(Table 4.25). Analysis by One way ANOVA of the mean scores found no significant difference between the three treatments (P= 0.258).

Table 4.25 Percentage of depurated F1 *P. corneus* snails with varying level of acini wall cell cover from the depurated E2 experiment

Percent of acini wall covered by germ cells and/or supportive cells	Score	Percentage of depurated F1 snails from the River Water exposure (RW)	Percentage of depurated F1 snails from the Low E2 exposure (LE2)	Percentage of depurated F1 snails from the High E2 exposure (HE2)
<10%	1	0	0	0
10-30%	2	0	0	0
30-50%	3	13	6	33
50-70%	4	25	24	17
>70%	5	62	70	50

***P. corneus* sacrificed after developmental exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on all surviving snails from the F1 breeding study (RW, n = 8; LE2, n = 17; HE2, n = 6). Five sections of gonad were analysed per snail.**

Immature spermatogenic and supportive cells sloughed into lumen

Sertoli cells sloughing into lumen

The number of sections analysed with Sertoli cells sloughed into the lumen was highest in the snails from the RW developmental exposure and lowest in the HE2 developmental exposure (Table 4.26). However, analysis of the mean number of sections affected by sloughed Sertoli cells in the lumen by One way ANOVA found no significant difference between the groups (P= 0.313)

Spermatogonium sloughing into lumen

Spermatogonial sloughing into the acini lumen was most frequent in snails developmentally exposed to LE2 and least frequent in snails developmentally exposed to HE2 (Table 4.26). However, analysis of the mean number of sections affected by sloughed spermatogonium cells in the lumen by Kruskal-Wallis test found no significant difference between the groups (P= 0.817).

Spermatocyte sloughing into the lumen

The number of sections affected by spermatocytes sloughing into the acini lumen was highest in HE2 developmentally exposed snails and lowest in RW snails (Table 4.26). However, analysis of the mean number of sections affected by sloughed spermatocyte cells in the lumen by One way ANOVA found no significant difference between the groups (P= 0.861).

Spermatids sloughing into the lumen

The number of sections analysed with spermatids sloughed into the lumen was highest in the snails from the RW developmental exposure and lowest in the HE2 developmental exposure (Table 4.26). However, analysis of the mean number of sections affected by sloughed spermatids in the lumen by One way ANOVA found no significant difference between the groups (P= 0.910).

Table 4.26 Percentage of depurated F1 *P. corneus* snails with varying level of immature spermatogenic cells sloughed into the acini lumen from the depurated E2 experiment

Number of sections affected	Percentage of depurated F1s affected by Sertoli cell sloughed into lumen			Percentage of depurated F1s affected by Spermatogonium sloughed into lumen			Percentage of depurated F1s affected by Spermatocyte sloughed into lumen			Percentage of depurated F1s affected by Spermatid sloughed into lumen		
	RW	LE2	HE2	RW	LE2	HE2	RW	LE2	HE2	RW	LE2	HE2
None	37.5	47	66.6	25	0	50	37.5	35.3	33.3	25	17.6	16.7
1 out of 5	12.5	29	16.6	0	11.8	0	12.5	29.4	0	0	0	16.7
2 out of 5	12.5	12	0	12.5	11.8	0	0	5.9	33.3	12.5	17.6	0
3 out of 5	0	0	16.6	0	5.9	50	12.5	0	0	12.5	17.6	33.3
4 out of 5	25	0	0	25	47.1	0	37.5	17.6	16.7	12.5	17.6	16.7
5 out of 5	12.5	12	0	37.5	23.5	0	0	11.8	16.7	37.5	29.4	16.7
Stats	a	A	a	a	a	a	a	a	a	a	a	a

***P.corneus* sacrificed after developmental exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on all surviving snails from the F1 breeding study (RW, n = 8; LE2, n = 17; HE2, n = 6). Five sections of gonad were analysed per snail. Letter (a) indicates statistical similarity.**

Mature spermatogenic cells in the lumen

Spermatozoa only

The percentage of snails with only mature spermatozoa free in the acini lumen was highest in the snails from the RW developmental exposure and decreased in a dose-dependant

manner (Table 4.27). However, analysis of the mean number of sections affected by One way ANOVA found no significant difference between the three groups (P= 0.983).

Table 4.27 Percentage of depurated F1 *P. corneus* snails with varying numbers of sections of gonad with only mature spermatozoa in the acini lumen from the depurated E2 experiment

No. of sections with just mature spermatozoa in the lumen	Percentage of F1 snails from the River Water exposure (RW)	Percentage of F1 snails from the Low E2 exposure (LE2)	Percentage of F1 snails from the High E2 exposure (HE2)
None	50	41.2	50
1 out of 5	25	35.3	16.6
2 out of 5	0	11.7	33.3
3 out of 5	0	11.7	0
4 out of 5	0	0	0
5 out of 5	25	0	0
	a	a	a

***P. corneus* sacrificed after developmental exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on all surviving snails from the F1 breeding study (RW, n = 8; LE2, n = 17; HE2, n = 6). Five sections of gonad were analysed per snail. Letter (a) indicated statistical similarity.**

Table 4.28 Percentage of depurated F1 *P. corneus* snails affected by varying percentage of acini with immature spermatogenic cells sloughing into the lumen from the depurated E2 experiment

Percentage of acini affected by immature spermatogenic cells sloughing into the lumen	Score	Percentage of F1 snails from the River Water exposure (RW)	Percentage of F1 snails from the Low E2 exposure (LE2)	Percentage of F1 snails from the High E2 exposure (HE2)
<10%	1	50	58.8	33.3
10-30%	2	12.5	23.5	66.7
30-50%	3	37.5	11.7	0
50-70%	4	0	5.8	0
>70%	5	0	0	0
		a	a	a

***P. corneus* sacrificed after developmental exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on all surviving snails from the F1 breeding study (RW, n = 8; LE2, n = 17; HE2, n = 6). Five sections of gonad were analysed per snail. Letter (a) indicates statistical similarity.**

Occurrence of acini with immature spermatogenic cells in lumen

Depurated snails from the LE2 exposure had the highest percentage of snails with both the least (<10% of acini) and most (>70% of acini) level of immature spermatogenic cells

sloughed into the lumen (Table 4.28). No apparent trend could be observed and analysis by One way ANOVA found no significant difference between the three groups (P= 0.755).

Oogenesis stages present

The percentages of oocytes at each stage of oogenesis were overall quite similar; 4% on average of oocytes were at Stage 5 (mature) from all three groups (Table 4.29). Larger differences were observed in Stage 2 and degenerating oocytes between the RW and the HE2 exposed snails. One way ANOVA found no significant differences between the groups for any oocyte stage. P values were large for Stage 1 (P= 0.367), Stage 3 (P= 0.940), Stage 4 (P= 0.438), Stage 5 (P= 0.918), and degenerating oocytes (P= 0.198). Whereas P values were lower for Stage 2 (P= 0.060) oocytes.

Table 4.29 Mean percentage of different stages of oogenesis in depurated F1 *P. corneus* from the E2 experiment

Stage of oocyte maturation	Mean percent of oogenesis stage after River Water exposure	Mean percent of oogenesis stage after Low E2 exposure	Mean percent of oogenesis stage after High E2 exposure
Stage 1	19.9 ± 5.5	25.0 ± 8.2	22.4 ± 11.3
Stage 2	21.8 ± 4.7	18.4 ± 4.3	16.4 ± 3.0
Stage 3	20.0 ± 4.2	20.5 ± 8.3	19.4 ± 3.7
Stage 4	19.9 ± 6.5	16.2 ± 7.2	16.6 ± 7.7
Stage 5	3.6 ± 3.3	4.2 ± 4.2	3.7 ± 3.0
Degenerating	14.8 ± 8.3	15.8 ± 8.5	22.1 ± 5.6

***P. corneus* sacrificed after developmental exposure to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Histological analysis was performed on all surviving snails from the F1 breeding study (RW, n = 8; LE2, n = 17; HE2, n = 6). Five sections of gonad were analysed per snail. Stage 1 (oogonium) are the youngest stage of oogenesis, and Stage 5 are ready to be ovulated (oogenesis stages based on description by de Jong-Brink et al., (De Jong-Brink et al. 1976).**

Parasitism

No parasites were observed in any of the sampled F1 snails from the RW, LE2 or HE2 mesocosms

4.3.9 Effects of 17β-oestradiol exposure on adult *V. viviparus* reproduction and survival

As with the *P. corneus* data, the adult *V. viviparus* reproduction and survival data were collected by Rachel Benstead.

4.3.9.1 Number of neonates released per snail over reproductive study

On average, the cumulative number of neonates released per snail was highest in the HE2 exposed snails; the RW exposed snails released an average of 16.6 neonates each, LE2 released 16.2 and HE2 released 19.2. The numbers of neonates released per snail were similar across the three exposures until week 12 (RW = 15.4; LE2 = 15.3; HE2 = 15.9), after which snails from the HE2 exposure released a larger number of neonates. Analysis by One way ANOVA found significant differences between treatments at weeks 14 (P=0.017) and 16 (P=0.013) (and an almost significant p value of 0.055 at week 8). At weeks 14 and 16, significant differences were found between the number of neonates released by the HE2 and the LE2 exposed snails (P= 0.009 and 0.006, weeks 14 and 16 respectively) and between the HE2 and the RW (P=0.014 and 0.014, weeks 14 and 16 respectively) snails, (Figure 4.10).

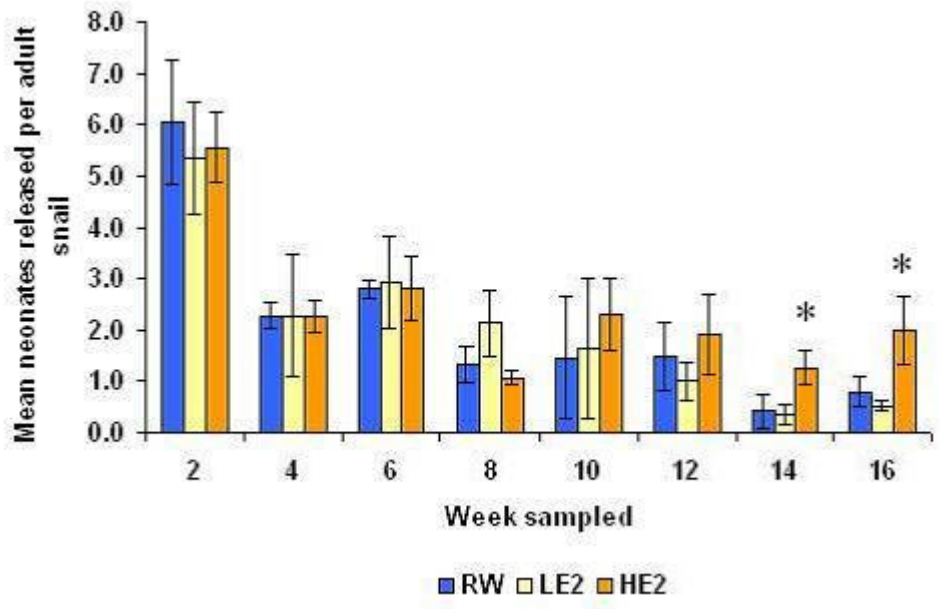

Figure 4.10 Mean number of neonates released per adult snails in E2 experiment over the 16 week exposure period.

Adult *V. viviparus* exposed to River Water (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). Error bars show standard deviation. Star (*) indicates significant difference from other treatments (P<0.05 LSD). Adult *V. viviparus* reproduction data collected by Rachel Benstead.

4.3.9.2 Number of embryos harboured per female at the end of E2 exposure study

The mean number of embryos harboured by adult females at the end of the exposure was highest from the LE2 group (33 embryos per female) and lowest from the HE2-exposed group (27 embryos per female); the RW snails had 31 embryos per female. Analysis by one way ANOVA found no significant difference between the three exposure groups (P=

0.237). However, when the number of embryos were split between shelled (most mature) and unshelled (least mature), there were significant differences found between the groups for the number of unshelled embryos per female (P<0.001), but no significant differences for the shelled embryos (P= 0.065; see Table 4.30). Post hoc analysis (LSD) showed significant differences between the number of unshelled embryos harboured by females exposed to LE2 compared to RW (P<0.001) and HE2 (P= 0.003).

Table 4.30 Mean number of shelled (most mature) and unshelled (least mature) embryos harboured per surviving female after 16 week of exposure in the E2 experiment.

Embryos	River Water (RW)	Low E2 (LE2)	High E2 (HE2)
Shelled	22.8 ± 10.5 a	16.7 ± 7.4 a	17.5 ± 7.7 a
Un-Shelled (developing)	8.4 ± 5.1 a	16.4 ± 7.8 b	9.2 ± 6.5 a

V. viviparus **adult females exposed to River Water (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2). The number of females analysed for each groups were RW n = 21, LE2 n = 22, HE2 n = 13, mean ± Standard deviation. Letter (a, b) indicates statistical similarity.**

4.3.9.3 Survival of adult *V. viviparus* over E2 exposure study

Adult survival was highest from the LE2 exposure and lowest from the HE2 exposure. On average 72.7% of the snails from the RW, 84.8% from the LE2 and 44.1% from the HE2 exposure survived until week 16 of the study. The level of adult mortality from the HE2 groups increased steeply towards the end of the exposure; between week 14 and week 16 survival dropped by 31% (Figure 4.11). Analysis by Kruskal-Wallis found no significant difference in percentage survival during weeks 2 (P=0.102) and 4 (0.195), and one way ANOVA found no significant difference between the three groups at weeks 6 (P= 0.132) and 8 (P= 0.248). However significant differences were found at week 10 (P= 0.048 K-W) and at week 12 (P=0.028 ANOVA). Marginally significant differences at the 95% level were found at weeks 14 (P= 0.055 ANOVA) and 16 (P= 0.054 K-W). Post hoc analysis found a significant difference in survival between the RW and the LE2 exposed snails at week 10 (P= 0.034 M-W). However, no significant differences were found between the RW and the HE2 exposed snails (P= 0.121 M-W) or between the LE2 and the HE2 snails (P= 0.105 M-W) at this time. At week 12 significant differences were found between the RW and the LE2 (P= 0.021 LSD) exposed snails, and the LE2 and the HE2 snails (P= 0.016 LSD), but not between the RW and the HE2 snails (P= 0.820 LSD).

After week 16 of the exposure the dosing pumps administering the E2 to the tanks were switched off and the tanks were left to depurate in running river water over the weekend. After the weekend the adult snails were removed from the tanks for the final time. During this short depuration time one adult *V. viviparus* snail from each exposure tank died; leaving a total of 23 snails from the RW tank, 27 snails from the LE2 and 14 snails from the HE2 tank. At this point the remaining snails were sampled; their size and weight were measured and the soft tissue was fixed for histology (see Section 3.5.7), the data from these specimens are presented below.

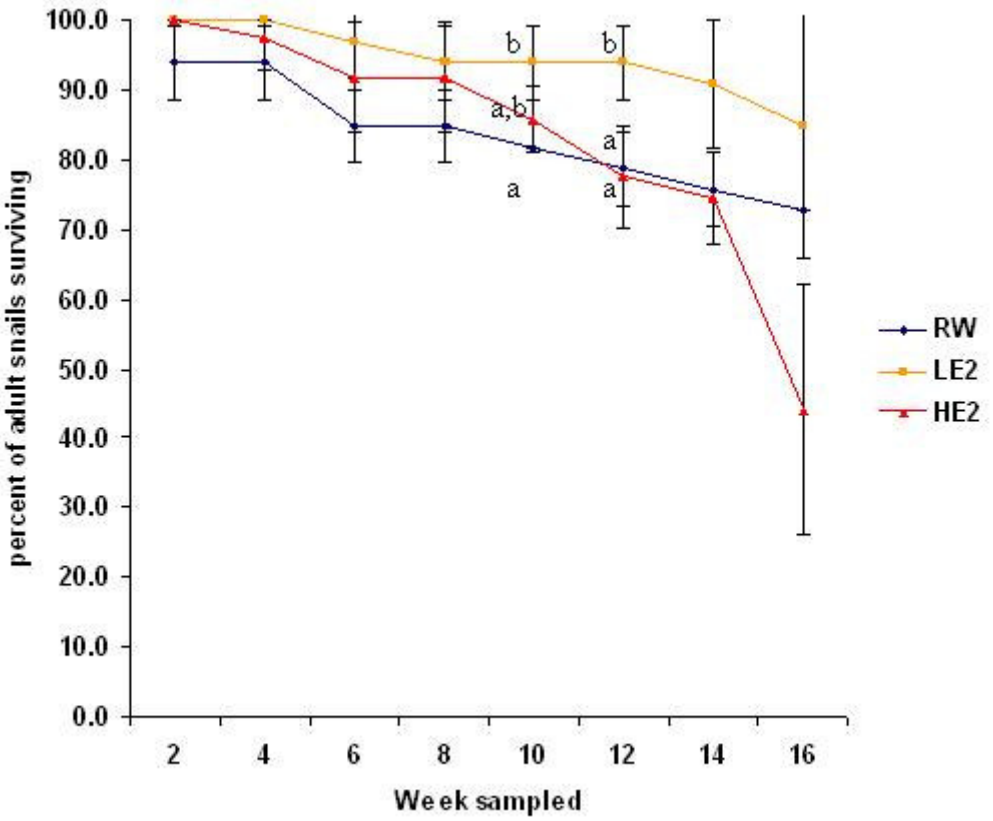

Figure 4.11 percentage of adult *V. viviparus* surviving over 16 week E2 exposure

Snails exposed to River water only (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2) as adults during reproductive period. Error bars show standard deviation. Letters (a, b) indicated statistical similarity.

4.3.9.4 Size and growth of adult *V. viviaprus*

F0 Females size and weight at the end of the exposure period

Mean values for shell length, soft body weight and soft body weight with embryos removed were similar across all treatments (Table 4.31). The total weight at the end of the

exposure period was lower in snails from the HE2 treatment (Mean total weight RW 8.3 ± 2.1g, LE2 8.2 ± 1.4g, HE2 5.3 ± 3.0g). No significant differences were found between the treatment groups for soft body weight (P= 0.224, ANOVA) or soft body weight embryos removed (P= 0.679, K-W). However, significant differences were found for shell length (P= 0.041, K-W) and total weight (P= 0.010, K-W). Post hoc analysis found no significant differences between shell length or total weight between the LE2 and the RW-exposed snails (length P= 0.768, weight P= 0.781, M-W). However, significant differences were observed between the LE2 and the HE2 groups for both shell length and total weight (length P= 0.007, total weight P= 0.004, M-W), and for total weight between the RW and the HE2 exposed snails (P= 0.017, M-W) (but not for length P= 0.091 M-W). Significant positive correlations (Spearman's rank order) were found for shell length and total weight, and shell length and soft body weight for the RW (shell length/total weight 0.948 P<0.001, shell length/soft body 0.453 P= 0.039), the LE2 (shell length/total weight 0.931 P<0.001 and soft body weight 0.817 P<0.001) and the HE2 (shell length/total weight 0.588 P= 0.035, shell length/soft body weight 0.665 P= 0.013) exposed females. Significant positive correlations were also found between shell length and soft body weight after embryos had been removed for the RW (0.918 P<0.001) and the LE2 (0.773 P<0.001), but not for the HE2-exposed females (0.489 P= 0.090). To assess whether the size of the adult female was related to the number of embryos harboured, Spearman's rank order correlations were performed. A significant negative correlation was found between shell length and the number of shelled embryos a female harboured in RW exposed snails (-0.489 P= 0.024), whereas a significant positive correlation was found in LE2 exposed snails (0.451 P= 0.035), no significant correlation was found in HE2 snails. These correlations can be a useful tool when trying to assess whether females are recruiting a large number of small embryos, as apposed to a small number of larger embryos, and is discussed further in Section 4.4.3, later. No significant correlation was found between shell length and the number of unshelled embryos harboured by females from any treatment group.

F0 Male size and weight at the end of the exposure period

The mean shell length, total weight and soft body weight of males at the end of the exposure decreased in a dose-dependant manner (Table 4.32). However, due to the small sample size (RW, n=2; LE2, n=5; HE2, n=1) statistical analysis was not conducted.

Table 4.31 Mean shell length, total weight and soft body weight of surviving adult *V. viviparus* females after four months of exposure in the E2 experiment

	River Water exposed	Low E2 exposed	High E2 exposed
Shell length mm	33.0 ± 3.2 a, b	32.5 ± 3.2 a	32.5 ± 2.9 b
Total weight g	8.3 ± 2.1 a	8.2 ± 1.4 a	5.3 ± 3.0 b
Soft body weight g	3.1 ± 0.9 a	3.3 ± 0.7 a	3.1 ± 0.6 a
Soft body weight embryos removed g	1.8 ± 0.5 a	1.8 ± 0.2 a	1.9 ± 0.3 a

Snails exposed to river water (RW), low concentration of 17β-oestradiol (LE2) or high concentration of 17β-oestradiol (HE2). RW n= 21, LE2 n=22, HE2 n= 13. Mean ± standard deviation, letter (a, b) indicate statistical similarity.

Table 4.32 Mean shell length, total weight and soft body weight of surviving adult *V. viviparus* males after four months of exposure in the E2 experiment

	River Water exposed	Low E2 exposed	High E2 exposed
Shell length mm	31.4 ± 3.8	25.1 ± 2.7	21.01
Total weight g	7.0 ± 2.4	4.1 ± 1.2	2.66
Soft body weight g	2.7 ± 0.8	1.5 ± 0.3	1.00

Snails exposed to river water, low concentration of 17β-oestradiol, or high concentration of 17β-oestradiol. RW n= 2, LE2 n=5, HE2 n=1. Mean ± standard deviation.

4.3.10 Effects of 17β-oestradiol exposure on F0 adult *V. viviparus* gonad histopathology

As stated in section 4.3.9.3, a total of 64 adult snails (RW; 23(♀=21, ♂=2), LE2; 27(♀=22, ♂=5), HE2; 14(♀=13, ♂=1)) were sampled and fixed for histopathological analysis from this experiment. All the male specimens (RW, n= 2; LE2, n= 5; HE2, n= 1) were sectioned stained and analysed. However, due to time constraints a maximum of 11 female specimens were sectioned, stained and analysed from each treatment (RW, n= 11; LE2, n= 11; HE2, n=10). Ten sections of gonad were analysed per adult snail. As the main focus of this research was to be on the possible effects of developmental exposure to E2, rather than on adult exposure, this low number of samples analysed was decided upon as a compromise between available time and quantity of data. However, it is my intention to complete the analysis for the remaining specimens when time/funding is available.

Analysis of F0 Female *V. viviparus*

The mean number of oocytes per section of gonad analysed for adult females increased in a dose-dependant manner (Table 4.33). The RW exposed females had a mean of 7.5 oocytes per section analysed whereas the LE2 exposed females had 9 oocytes per section and the

HE2 females had 10.6 oocytes per section. However, no statistically significant difference was found between the three treatments (P= 0.576, ANOVA).

Table 4.33 Mean oocytes per section of gonad analysed From F0 *V. viviparus* females from the E2 experiment

	River Water (RW)	Low 17β-oestradiol (LE2)	High 17β-oestradiol (HE2)
Mean oocytes per section analysed	7.5 ± 5.8 a	9.0 ± 4.5 a	10.6 ± 9.2 a

Snails exposed to; River Water only (RW), river water plus Low 17β-oestradiol (LE2) or river water plus High 17β-oestradiol (HE2). Ten sections analysed per adult female. RW n= 11, LE2 n= 11, HE2 n=10 mean ± standard deviation. Letter (a) indicates statistical similarity.

Correlations between female size or weight and reproductive potential

To assess whether the size of female affects the mean number of oocytes per section counted, a number of correlations were conducted. Over the three treatments, significant positive correlations between the mean oocyte number and shell length (0.477 P<0.001), total weight (0.517 P<0.001) and soft body weight (0.528 P<0.001) were found, suggesting larger females would on average have a higher mean number of oocytes per section of tissue analysed. Therefore, analysis of covariance (ANCOVA) was used to see if the mean number of oocytes were significantly different between the groups when co-analysed with; shell length, total weight, or soft body weight. Once co-analysed for size or weight, significant differences were found between the treatment groups mean oocyte number (P= 0.004, 0.007, 0.008, respectively). Post hoc analysis of this co-variant data (Bonferroni) showed HE2 and LE2 exposed F0 females to have significantly more oocytes per section than RW exposed snails when co-analysed for; shell length (HE2 P=0.008, LE2 P=0.017), total weight (HE2 P=0.025, LE2 P= 0.015), or soft body weight (HE2 P=0.034, LE2 P=0.015). Whereas, no significant differences were found between the HE2 and LE2 exposed snails.

Analysis of F0 male *V. viviparus*

The mean spermatogenic score (see Section 3.2.2) was marginally lower in the E2 exposed snails compared to the RW exposed snails (Table 4.34). The RW males scored an average of 10, the LE2 scored 9.8, and the HE2 scored 9.9 out of a maximum score of 10. However, due to the small sample size (RW, n=2; LE2, n=5; HE2, n=1) statistical analysis was not conducted.

Table 4.34 Mean spermatogenesis score per section of gonad analysed in F0 *V. viviparus* males from the E2 experiment

	River Water (RW)	Low 17β-oestradiol (LE2)	High 17β-oestradiol (HE2)
Mean spermatogenesis score per section analysed	10.0 ± 0.0	9.78 ± 0.2	9.9 ± 0.0

Snails exposed to River Water only (RW), river water plus Low 17β-oestradiol (LE2) or river water plus High 17β-oestradiol (HE2). Ten sections analysed per adult male, Section 3.2.2 gives details of scoring method. RW n= 2, LE2 n= 5, HE2 n= 1, mean ± Standard deviation.

Parasitism

As with the adult *V. viviparu*s from the 'health check' group (Chapter 2, Section 2.3.3.4), the majority of adult *V. viviparus* had some level of parasitism, either just encysted parasites (metacercaria) or infections in the digestive tissue (sporocyst stage). The experimental set up (use of wild caught snails and non-carbon-filtered river water) means that parasitism of snails has become a feature of this research (and this thesis). The possible effect (or interaction) these parasites may have on their snail hosts will be touched on briefly in this chapter (in terms of possible reproductive effects), but will be more fully explored in Chapters 5 and 6.

Parasitism of F0 Females

Only three adult female *V. viviparus* analysed had no parasitic infection (RW = 1; HE2 = 2). Encysted parasites (metacercaria) found in the muscular tissue of the head and the foot were most frequent, but infections of the digestive tissue (sporocyst stage) were also found (Table 4.35).

Table 4.35 Percentage of adult female F0 *V. viviparus* snails with different types of parasite infections from the E2 experiment

	River Water (RW)	Low 17β-oestradiol (LE2)	High 17β-oestradiol (HE2)
Encysted parasites	72.7%	100%	70%
Infection of the digestive tissue	18.2%	0	10%
No infection	9.1%	0	20%

Snails sacrificed after four months of exposure to River Water only (RW), river water plus Low 17β-oestradiol (LE2) or river water plus High 17β-oestradiol (HE2). RW, n = 11; LE2, n = 11; HE2, n = 10. Ten sections were analysed per snail. Encysted parasites (metacercaria) found in the muscular tissue of the head and the foot, infections of the digestive tissue (sporocyst stage).

The intensity of infection (number or area infected) for both encysted parasites and digestive tissue parasites were also recorded. The intensity of infection by encysted parasites in adult female *V. viviparus* was highest in the RW exposure and lowest in the HE2 exposure (Figure 4.12). The digestive tissue infection in the one HE2 female covered more than 70% of the tissue whereas the infections seen in the RW females were less severe with between 30-50% of the tissue affected.

Parasitism of F0 Males

Only two adult male *V. viviparus* had no parasitic infection (both LE2), none of the males had an infection of the digestive tissue. The majority had encysted parasites however (Table 4.36). The intensity of infection by encysted parasites in adult male *V. viviparus* was higher in the RW exposed snails whilst snails from the LE2 had a lower infection level (there was only one male form the HE2 exposure), see Figure 4.12.

Table 4.36 Percentage of adult male F0 *V. viviparus* snails with different types of parasite infections from the E2 experiment

	River Water (RW)	Low 17β-oestradiol (LE2)	High 17β-oestradiol (HE2)
Encysted parasites	100%	60%	100%
Infection of the digestive tissue	0	0	0
No infection	0	40%	0

Snails sacrificed after four months of exposure to River Water only (RW), river water plus Low 17β-oestradiol (LE2) or river water plus High 17β-oestradiol (HE2). RW, n = 2; LE2, n = 5; HE2, n = 1. Ten sections were analysed per snail. Encysted parasites (metacercaria) found in the muscular tissue of the head and the foot, infections of the digestive tissue (sporocyst stage).

Correlations between parasite infection and reproductive potential

Many parasites can affect their hosts ability to reproduce successfully, either via some means of chemical castration or via possible reduced nutrition (See Chapter 5, Section 5.5.4.1 for more details), therefore Spearman's rank order correlations were performed on the RW-exposed adults (to avoid complications of chemical exposure) to see if parasite infection could be correlated with reproductive potential. No significant correlations were found between encysted parasite score and oocyte number (-0.091, P=0.790), un-shelled embryo number (-0.290, P= 0.386), or shelled embryo number (-0.191, P=0.574). The numbers of males from the RW exposure were too small to perform statistical correlations; both snails had the highest spermatogenic score of 10, which means full spermatogenesis was occurring in more than 70% of tubules. No digestive tissue infections were observed

in these males. The number of females with digestive tissue infections was too small to correlate. However, the mean numbers of embryos harboured by females (RW) with digestive tissue infections were much lower than those with just encysted parasites (Table 4.37), indicating that this type of infection may affect reproductive output.

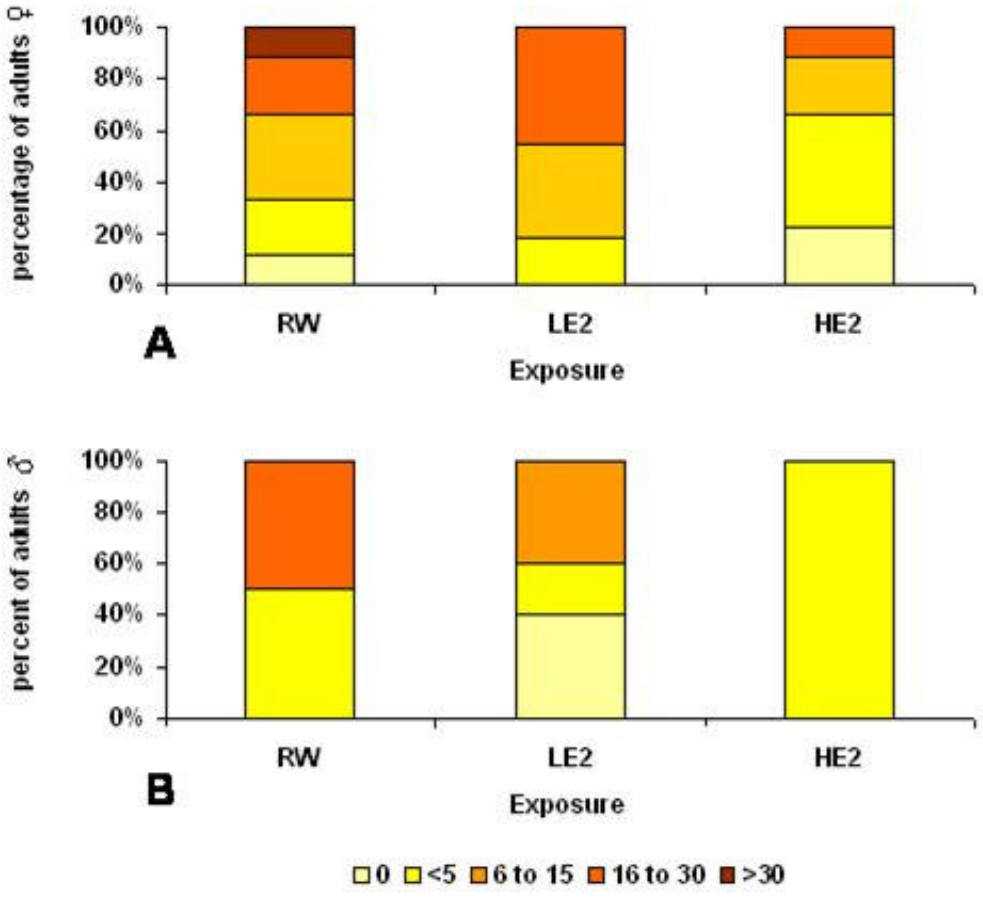

Figure 4.12 Intensity of encysted parasite infections in adult *V. viviparus* after four months E2 exposure.

Snails exposed to river water only (RW), river water plus low 17β-oestradiol (LE2), or river water plus high 17β-oestradiol (HE2). (A) Percentage of F0 females with varying intensities of parasite infection. (B) Percentage of F0 males with varying intensities of parasite infection. RW n= 21♀, 2♂; LE2 n= 22♀, 5♂; HE2 n= 13♀, 1♂. 10 sections per adult snail were analysed. Pale yellow indicates no parasites found, yellow indicates less than 5 parasites per section, light orange indicates 6-15 parasites per section, dark orange indicates 16-30 parasites per section, brown indicates more than 30 parasites per section.

Table 4.37 Mean number of embryos harboured by F0 female *V. viviparus* exposed to RW only split by parasite infection.

	Non-infected females (RW)	Infected females with encysted parasites only (RW)	Infected females with digestive tissue parasites (RW)
Mean embryos harboured per female	25.0 ± 0	35.1 ± 12.1	15.5 ± 13.4

Females had either; no infection, encysted parasites only or an infection of the digestive tissue, mean ± standard deviation.

4.3.11 Effects of 17β-oestradiol exposure on F1 *V. viviparus* growth, survival and reproductive development

4.3.11.1 Number and size of juveniles collected at the end of the exposure

The total number of neonates released into the tanks by the adult *V. viviparus* was; 500 in the RW tank, 522 in the LE2 tank and 601 in the HE2 tank. The number of juveniles (F1s) held in the mesocosm tanks at the end of the 16 weeks also increased with E2 exposure. The RW tank had 119 neonates, LE2 tank had 175 and HE2 tank had 160. Therefore the percentage of neonates surviving until the end of dosing period was 23.8% in RW, 33.5% in LE2 and 26.6% in the HE2 tank. The percentage of snails above 10 mm shell length (normal minimum sexually active size) was, however, highest in the RW tank at 82%, whilst the LE2 tank and the HE2 tanks had 74% and 63%, respectively. Table 4.38 below lists the number of F1s above and below 10mm shell diameter.

Table 4.38 Total number (and number per size class) of *V. viviparus* F1s from each mesocosm tank after dosing ended (September 2006).

Shell Length	River Water (RW)	Low dose E2 (LE2)	High dose E2 (HE2)
<10 mm	22 (18%)	46 (26%)	60 (37%)
>10 mm	97 (82%)	129 (74%)	100 (63%)
Total	119	175	160

In September 2006 (after dosing finished), approximately 50 F1s from each treatment (river water; RW, low dose of 17β-oestradiol; LE2 and high dose 17β-oestradiol; HE2) were taken from the tanks and fixed for histopathological analysis. Due to time constraints it was decided that 20 specimens of each sex from each treatment would be sectioned, stained and analysed. The F1s were sexed once back at the laboratory using their secondary sexual characteristics i.e. enlarged right tentacle equals a male. However, once these specimens were analysed histopathologically it became apparent that in some cases the initial observed sex (from morphology) was incorrect, therefore the actual numbers per sex were; RW, 20 males and 20 females; LE2, 16 females and 25 males; and HE2, 21

females and 20 males. Due to this misidentification of sex from morphology, only the data from those specimens of known sex (i.e. histopathologically analysed) are shown below.

4.3.11.2 Size and weigh of F1 *V. viviparus* end the end of exposure

F1 Female *V. viviparus* size and weight

The shell length, shell aperture, total weight and soft body weight of F1 females were analysed (Figure 4.13). One way ANOVA found no significant difference between treatments for shell length (P= 0.405), shell aperture (P= 0.689) or total weight (P= 0.162). However, a significant difference was found between treatments for the soft body weight (P= 0.043). Post hoc analysis (LSD) found the F1 female *V. viviparus* from the HE2 exposure to be significantly lighter than the RW exposed snails (P= 0.017), but not the LE2 snails (P= 0.077), and the RW and LE2 were not significantly different (P= 0.580) from each other either.

F1 Male *V. viviparus* size and weight

The shell length, shell aperture, total weight and soft body weight of F1 males were analysed (Figure 4.14). Kruskal-Wallis Test found significant differences between developmental exposures for shell length (P= 0.027), shell aperture (P= 0.004), total weight (P= 0.002) and soft body weight (P= 0.002). Post hoc analysis using the Mann-Whitney test found the HE2 exposed F1 male's shell length, shell aperture, total weight and soft body weight to be significantly smaller/lighter than the RW (P=0.007, 0.002, <0.001, <0.001 respectively) or the LE2 (P=0.046, 0.008, 0.006, 0.005, respectively) exposed snails. No significant differences were found between LE2 and RW exposed snails for shell length, shell aperture, total weight or soft body weight (P= 0.802, 0.973, 0.891, 0.604, respectively).

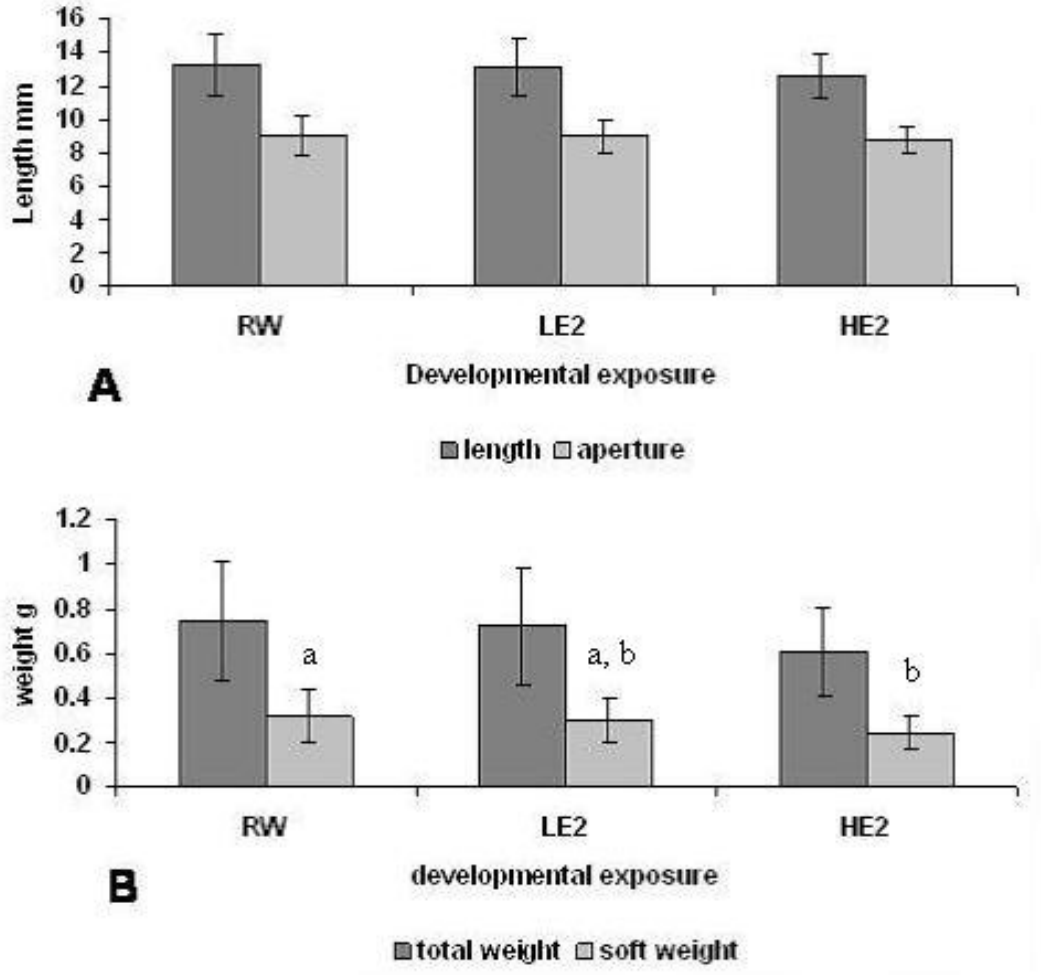

Figure 4.13 The size and weight of F1 *V. viviparus* female from E2 experiment.

Snails developmentally exposed to River Water only (RW), river water plus Low 17β-oestradiol (LE2) or river water plus High 17β-oestradiol (HE2). (A) Mean shell length and shell aperture. (B) Mean total weight and soft body weight. RW n= 20, LE2 n= 16 and HE2 n= 21. Error bars show standard deviation. Letter (a, b) indicate statistical similarity.

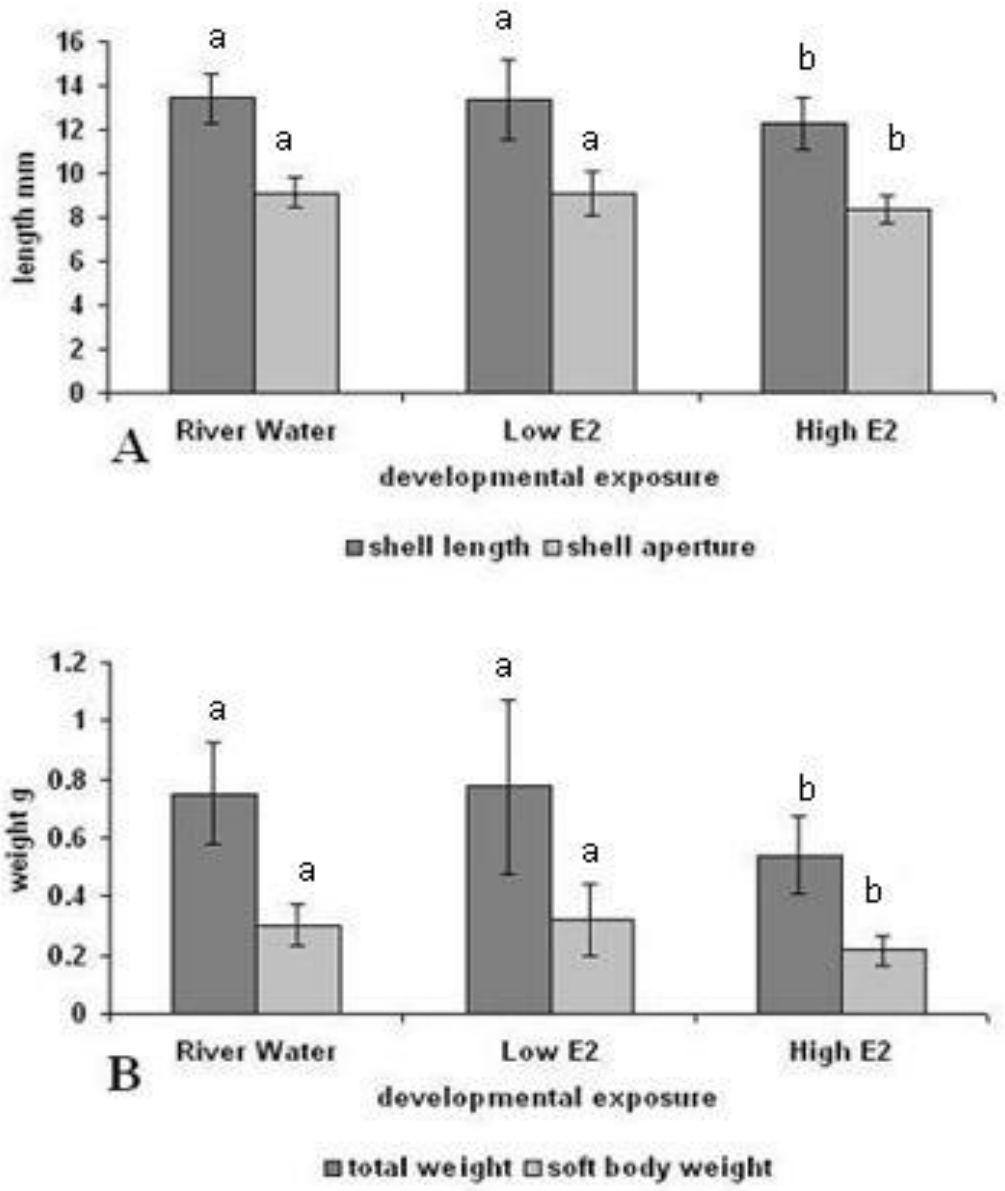

Figure 4.14 Size and weight of F1 *V. viviparus* males from E2 experiment.

Snails developmentally exposed to River Water only (RW), river water plus Low 17β-oestradiol (LE2) or river water plus High 17β-oestradiol (HE2). (A) Mean shell length and shell aperture. (B) Mean total weight and soft body weight. RW n= 20, LE2 n= 25 and HE2 n= 20. Error bars show standard deviation. Letter (a, b) indicate statistical similarity.

4.3.12 Histopathology of F1 *V. viviparus* gonad immediately after exposure to 17β-oestradiol

As previously stated a sub-sample (approximately 20 of each sex) of the total number (~50 per treatment) of F1s collected after developmental exposure were sectioned, stained and analysed histologically.

F1 female mean oocyte number

The mean number of oocytes per section analysed decreased in a dose-dependant manner (Figure 4.15). The RW developmentally exposed females had an average of 5.7 oocytes per section, the LE2 exposed females had 2.8 and the HE2 exposed females had an average of 2 oocytes per section analysed. Kruskal-Wallis test showed a significant difference between the mean oocyte numbers in each treatment (P= 0.002), whilst post hoc analysis (M-W) found no significant difference between the high and low E2 treatments (P= 0.247), significant differences were found between the HE2 and the RW exposed snails (P= 0.001) and the LE2 and the RW exposed snails (P= 0.008).

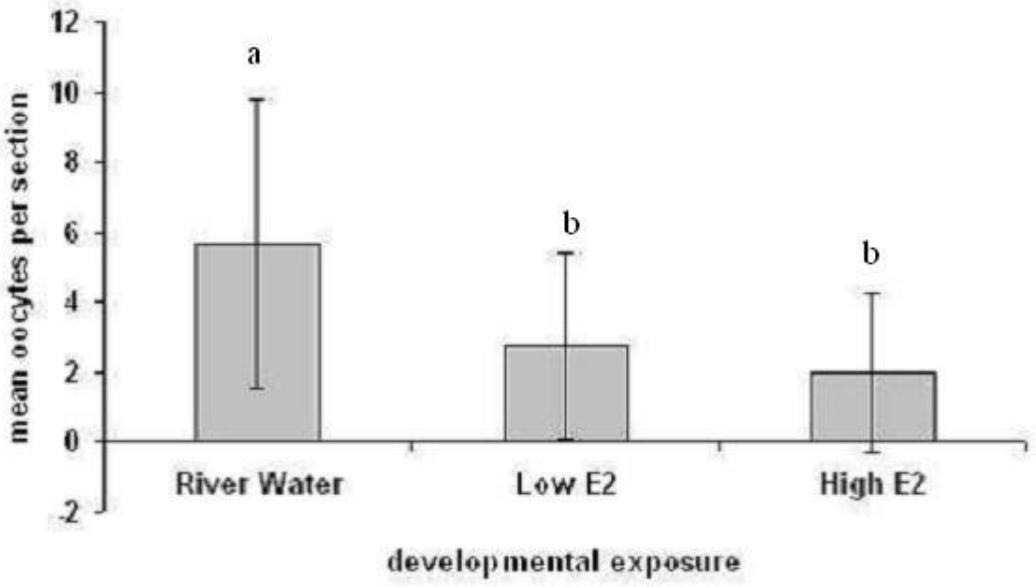

Figure 4.15 The mean number of oocytes recorded per section analysed of F1 *V. viviparus* females from E2 experiment.

Snails developmentally exposed to either, River Water only (RW), river water plus Low 17β-oestradiol (LE2) or river water plus High 17β-oestradiol (HE2). Sampled in September 2006, RW n= 20, LE2 n= 16 and HE2 n= 21. Five sections analysed per female. Error bars show standard deviation. Letter (a, b) indicate statistical similarity.

Correlation between F1 female size and sexual development

To assess the impact of body size or weight on the number of oocytes counted per section, Spearman's rank order correlations were conducted. Correlations found female F1 length to be significantly positively correlated to total weight (0.974, P< 0.001), soft body weight (0.949, P<0.001) and mean oocyte number (0.553, P<0.001). A significant positive correlation was also found between soft body weight and mean oocyte number (0.599, P<0.001), indicating that on average larger snails had a larger number of oocytes per section of tissue analysed.

To analyse whether the significant reduction in oocyte number with 17β-oestradiol exposure was actually due to the lower soft body weight found in E2 snails, mean oocyte number was co-analysed with soft body weight. A significant difference in oocyte number between the three treatments was still found (P= 0.008, ANCOVA). A significant difference was also found when mean oocyte number was analysed with shell length (P=0.004) or total weight (P=0.007) as a covariant. Post hoc analysis (Bonferroni) found RW exposed snails to have significantly high mean oocyte number compare to LE2 (soft body weight; P=0.015, shell length; P=0.017, total weight; P=0.015) or HE2 (soft body weight; P=0.034, shell length; P=0.008, total weight; P=0.025) exposed snails. However, no significant differences were found between the LE2 and HE2 exposed snails.

F1 male spermatogenesis score

The mean spermatogenesis score of F1 *V. viviparus* decreased in a dose-dependant manner (Figure 4.16). The RW exposed males had a mean score of 9 (full spermatogenesis in 50-70% of tubules), the LE2 had a score of 8 (full spermatogenesis in 30-50% of tubules) and the HE2 exposed snails had a mean score of 6 (spermatids present). Kruskal-Wallis test found a significant difference between the treatments (P<0.001) and post-hoc analysis using the Mann-Whitney test found the HE2 snails to have a significantly lower score than the LE2 (P= 0.003) or the RW (P<0.001) exposed snails. The LE2 exposed snails also had significantly lower spermatogenesis scores compared to RW exposed snails (P= 0.016).

Correlations between F1 male size and sexual development

To assess if F1 male spermatogenesis score was linked to body size or weight, Spearman's rank order correlations were used. Correlations found F1 male length to be significantly positively correlated to total weight (0.944, P<0.001), soft body weight (0.914, P<0.001) and to spermatogenic score (0.704, P<0.001). Significant positive correlations were also found between total weight and spermatogenic score (0.781, P<0.001) and between soft body weight and spermatogenic score (0.718, P<0.001), indicating that on average larger F1 males would have a higher spermatogenic score.

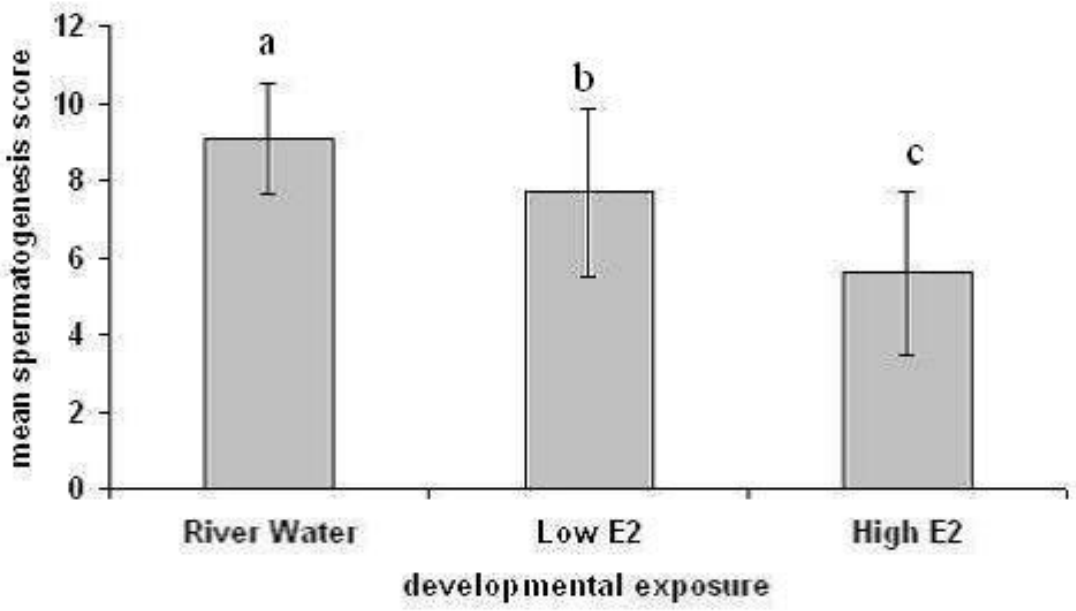

Figure 4.16 The mean spermatogenesis score of F1 *V.viviparus* males from E2 experiment.

Snails developmentally exposed to either, River Water only (RW), river water plus Low 17β-oestradiol (LE2) or river water plus High 17β-oestradiol (HE2). Sampled in September 2006, RW, n= 20; LE2, n= 25; HE2, n= 20. Score 1 - no germ cells present, Score 10 - full spermatogenesis in more than 70% of tubules. Five sections of gonad were analysed per male. Error bars show standard deviation. Letters (a, b, c) indicate statistical similarity.

To analyse whether the significant reduction in spermatogenic score with increasing dose of 17β-oestradiol was due to the smaller size of E2 exposed snails, spermatogenesis score was co-analysed with shell length, total weight or soft body weight. In each case a significant difference in spermatogenic score was still found between the three treatment groups ($P<0.001$ for each, ANCOVA). Post hoc analysis (Bonferroni) of the co-variant data showed the RW exposed males to have significantly higher spermatogenesis score compare to HE2 (shell length; $P<0.001$, total weight; $P<0.001$, soft body weight; $P<0.001$) or LE2 (shell length; $P=0.016$, total weight; $P=0.009$, soft weight; $P=0.006$) exposed snails. However, no significant differences were found between the LE2 and HE2 exposures ($P=0.057$, 0.335, 0.553, respectively).

Parasitism

No parasites were found in any of the F1 males or females examined from the three exposures.

4.3.13 Effects of 17β-oestradiol exposure and then depuration in river water on F1 *V. viviparus* reproduction, survival, and growth

4.3.13.1 F1 *V. viviparus* Survival

***V. viviparus* F1 over-winter survival**

The percentage survival over-winter was higher in the LE2-exposed group compare to the RW and the HE2 exposures. 53% of hatchlings survived in the RW, 94% from the LE2 and 55% from the HE2 (Table 4.39).

***V. viviparus* F1 survival during the breeding study**

Over the F1 breeding study, survival was high in all groups of snails and was highest in the LE2 developmental exposure group (LE2, 97%; HE2, 88%; RW, 85%).

Table 4.39 Number of F1 *V. viviparus* left to depurate over winter after developmental exposure, and number surviving after depuration.

F1 *V.viviparus*	River Water	Low E2	High E2
Left to over-winter September 2006	520	125	108
Surviving March 2007	273	117	59
Percent survival	53%	94%	55%

Number left in each treatment tank in September 2006, number surviving until March 2007 and percentage over winter survival. The RW tank was a pooling of RW exposed F1 from the E2 and Mixtures studies, hence the higher over-winter number when compare to initial September count.

4.3.13.2 Effects of 17β-oestradiol exposure and then depuration in river water on F1 *V. viviparus* reproductive output

None of the F1 females from the three developmental exposures released any neonates during the 18-week breeding study. At the end of the study, the brood pouches of female snails were dissected to count the number of embryos harboured. The percentage of F1 females harbouring embryos was highest from the LE2 developmental exposure; RW 12.5%, LE2 36% and HE2 5%. The mean number of shelled and unshelled embryos was also highest in the LE2 developmentally exposed females (Table 4.40). Statistical analysis (Kruskal-Wallis test) found significant differences in the total number of embryos (P= 0.009) as well as the number of shelled (P= 0.011) and unshelled (P= 0.013) embryos harboured by F1 females. Post hoc analysis (Mann-Whitney test) found the LE2 snails to have significant more embryos compared to the HE2 snails (total embryos P= 0.008, shelled P= 0.018, unshelled P= 0.009). The LE2 snails also had significantly more embryos than the RW exposed snails (total embryos P= 0.037, shelled P= 0.037). No significant

difference was found between HE2 and RW snails (total embryos P= 0.369, shelled P= 0.361, unshelled P= 0.369), or between the RW and the LE2 in unshelled embryo number (P= 0.052).

Table 4.40 The mean number of embryos (shelled, unshelled or total) harboured in the brood pouch per F1 female *V. viviparus* at the end of the 18 week F1 breeding study.

	Shelled embryos	Un-Shelled embryos	Total embryos
River Water developmental exposed	0.04 ± 0.2 a	0.33 ± 1.1 a	0.38 ± 1.3 a
Low E2 developmental exposed	0.56 ± 1.2 b	0.80 ± 1.2 a	1.36 ± 2.1 b
High E2 developmental exposed	0.00 ± 0.0 a	0.05 ± 0.2 b	0.05 ± 0.2 a

***V. viviparus* had been developmentally exposed to river water (RW), river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2) and then depurated in river water for eight months before being allocated into breeding groups. Mean ± standard deviation. Letter (a, b) indicates statistical similarity.**

4.3.13.3 Effects of 17β-oestradiol exposure and then depuration in river water on F1 *V. viviparus* size, weight and growth

At the beginning of the F1 breeding study, mean shell length (P= 0.929 ANOVA), aperture (P= 0.987, ANOVA) and total weights (P= 0.986 ANOVA) were similar between developmental dose groups. However, by the end of the 18-week breeding study, snails from the LE2 developmental exposure had grown larger and heavier than both the RW and the HE2 exposed snails. Statistically significant differences were found between the treatments shell length and aperture (P<0.001, ANOVA), total weight (P=0.001, ANOVA) and soft body weight (P<0.001, K-W). Post hoc analysis found the LE2 developmentally exposed snails to be significantly larger/heavier in shell length, shell aperture, total weight and soft body weight than both the RW (P<0.001) and the HE2 (P<0.001, 0.001 0.009, 0.032, respectively) exposed snails. No significant differences were found between the RW and HE2 exposed snails. On average the LE2 snails had increased shell length by 24.7% compare to RW 16.6% and HE2 18.7%. Total weight had increased by 44.5% from the LE2 developmentally exposed snails compared to 30.6% from RW and 35.8% from the HE2 groups (Table 4.41).

Mean female size at end of the F1 breeding study

At the end of the breeding study the F1 females from the LE2 developmental exposure had on average larger shell length, shell aperture, total weight and soft body weight, than those from the HE2 and RW. Using One way ANOVA significant differences were found between the three groups for shell length (P= 0.001), shell aperture (P= 0.004) and total weight (P= 0.021). Post hoc analysis (LSD) found the LE2 developmentally exposed snails to be significantly larger than both the HE2 and the RW exposed snails in shell length (RW P= 0.001, HE2 P= 0.004) and shell aperture (RW P= 0.005, HE2 P= 0.003), no significant differences were found between the RW and the HE2 exposed snails (shell length P=0.669, shell aperture P= 0.777). Post hoc analysis of the total weight found a significant difference between LE2 and RW (P= 0.007) but not between the LE2 and HE2 (P= 0.061) or between HE2 and RW (P=0.467) exposed snails. Analysis using Kruskal-Wallis found a significant difference between groups in soft body weight (P= 0.002), and post hoc analysis found a significant difference between the LE2 and RW developmentally exposed snails (P<0.001), but not between LE2 and HE2 (P= 0.068) or between HE2 and RW (P= 0.220) snails.

Mean male size at end of the F1 breeding study

At the end of the breeding study the F1 males from the LE2 developmental exposure had, on average, larger shell length, shell aperture, total weight and soft body weight, than those from the HE2 and the RW (Table 4.43). Analysis by One way ANOVA found significant differences between the groups in shell length (P= 0.029) and total weight (P= 0.036), and almost significant differences between shell aperture (P= 0.051) and soft body weight (P= 0.058). Post hoc analysis of shell length found significant differences between the LE2 and both the RW and HE2 (P= 0.023, 0.021 respectively) snails, but no significant difference was found between the RW and the HE2 (P= 0.694) snails. Analysis of total weight followed the same pattern, significant differences were found between the LE2 and the RW (P=0.021) and the HE2 (P=0.036) snails, but no significant difference was found between the HE2 and the RW (P= 0.496) snails.

Table 4.41 The mean size, weight, and growth of *V. viviparus* F1 snails developmentally exposed in the E2 experiment and then depurated in river water

Mean	River Water (RW) developmentally exposed and then depurated	Low E2 (LE2) developmentally exposed and then depurated	High E2 (HE2) developmentally exposed and then depurated
Shell length (mm) Start	12.91 ± 1.14 a	12.89 ± 1.53 a	12.78 ± 1.68 a
Shell length (mm) End	15.48 ± 1.30 a	17.11 ± 1.28 b	15.72 ± 1.51 a
Shell length percentage increase	16.6%	24.7%	18.7%
Shell aperture (mm) Start	8.89 ± 0.71 a	8.86 ± 0.99 a	8.85 ± 0.91 a
Shell aperture (mm) End	10.45 ± 0.71 a	11.21 ± 0.64 b	10.50 ± 0.84 a
Shell aperture percentage increase	15%	20.9%	15.7%
Total weight (g) Start	0.73 ± 0.17 a	0.73 ± 0.27 a	0.72 ± 0.26 a
Total weight (g) End	1.05 ± 0.24 a	1.32 ± 0.28 b	1.12 ± 0.32 a
Total weight percentage increased	30.6%	44.5%	35.8%

Snails exposed to river water (RW), low concentration of 17β-oestradiol (LE2) or high concentration of 17β-oestradiol (HE2). Mean shell length, shell aperture and total weight of snails at time of allocated to breeding study groups (March 2007) and then at the termination of the 18-week study. Mean increase in size given a percentage. Males and females analysed together. Mean ± standard deviation. Letter (a, b) indicates statistical similarity.

Table 4.42 The mean size and weight of female F1s at end of the 18 week un-dosed breeding study.

	River Water (RW) developmentally exposed and then depurated	Low E2 (LE2) developmentally exposed and then depurated	High E2 (HE2) developmentally exposed and then depurated
Shell length mm	15.5 ± 1.3 a	16.9 ± 1.2 b	15.7 ± 1.8 a
Shell aperture mm	10.5 ± 0.7 a	11.2 ± 0.6 b	10.4 ± 1.0 a
Total weight g	1.0 ± 0.2 a	1.3 ± 0.3 b	1.1 ± 0.3 ab
Soft body weight g	0.5 ± 0.1 a	0.6 ± 0.1 b	0.6 ± 0.2 ab

Size and weight of *V. viviparus* female snails developmentally exposed to river water (RW), low concentration of 17β-oestradiol (LE2) or high concentration of 17β-oestradiol (HE2) and then depurated in river water. Mean ± standard deviation. Letters (a, b) indicate statistical similarity.

Table 4.43 The mean size and weight of male F1s at end of the 18 week un-dosed breeding study.

	River Water (RW) developmentally exposed and then depurated	Low E2 (LE2) developmentally exposed and then depurated	High E2 (HE2) developmentally exposed and then depurated
Shell length mm	15.5 ± 1.4 a	17.7 ±1.5 b	15.9 ± 1.3 a
Shell aperture mm	10.1 ± 0.8 a	11.4 ± 0.8 a	10.6 ± 0.7 a
Total weight g	1.0 ± 0.3 a	1.4 ± 0.3 b	1.2 ± 0.3 a
Soft body weight g	0.5 ± 0.1 a	0.7 ± 0.2 a	0.6 ± 0.2 a

Size and weight of *V. viviparus* F1 male snails developmentally exposed to river water (RW), low concentration of 17β-oestradiol (LE2) or high concentration of 17β-oestradiol (HE2) and then depurated in river water. Mean ± standard deviation. Letters (a, b) indicate statistical similarity.

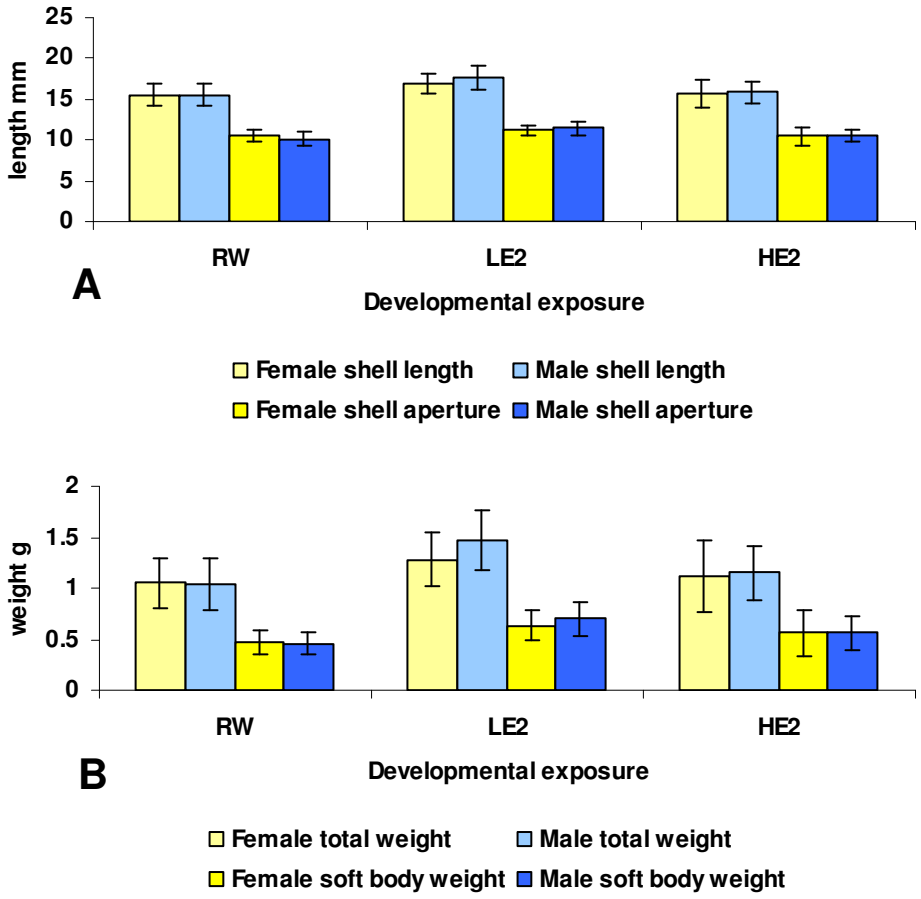

Figure 4.17 Size and weight of males and females from the F1 breeding study.

F1 *V. viviparus* snails developmentally exposed to river water (RW), low concentration of 17β-oestradiol (LE2) or high concentration of 17β-oestradiol (HE2) and then depurated in river water. Error bars show standard deviation.

Comparison of male and female size at end of the F1 breeding study

It was observed that male F1 *V. viviparus* snails from the LE2 developmental exposure were larger (shell length, aperture, total weight and soft body weight) than F1 females (similarly exposed) at the end of the F1 breeding study, although not significantly so.

Males and Females from the RW and HE2 exposures were closer in size to one another (Figure 4.17 above)

4.3.14 Gonad histopathology of F1 *V.viviparus* developmentally exposed to 17β-oestradiol and then depurated in river water for a year

All surviving F1s were sampled at the culmination of the F1 breeding study (after approximately one year of depuration). All F1s were analysed histologically. From the RW exposure, 4 males and 24 females were analysed, from the LE2 exposure 25 females and 7 males were analysed and from the HE2 exposure 20 females and 8 males were analysed. Five sections of gonad were analysed per snail.

Post F1 breeding study female oocyte number

The mean oocyte number was highest in snails developmentally exposed to RW only. The HE2 and LE2-exposed snails had similar mean numbers of oocytes (Table 4.44 below). Analysis using the Kruskal-Wallis Test found a significant difference between treatments (P= 0.041). Post hoc analysis using the Mann-Whitney test found a significant difference between the RW and the LE2 (P= 0.018) developmentally exposed snails, but not between the RW and the HE2 (P= 0.247) snails, or between the LE2 and the HE2 (P= 0.117) snails.

Table 4.44 The mean oocyte numbers of *V. viviparus* F1 female snails from the depurated E2 experiment

	River Water (RW)	Low E2 (LE2)	High E2 (HE2)
Mean oocyte number	4.0 ± 4.06 a	2.0 ± 2.70 b	1.7 ± 1.68 a, b

Snails developmentally exposed to river water (RW), low concentration of 17β-oestradiol (LE2) or high concentration of 17β-oestradiol (HE2) and then depurated in river water for approximately one year. Mean ± standard deviation. RW, n= 24; LE2, n= 25; HE2, n= 20. Letters (a, b) indicate statistical similarity

Correlations between F1 female size and reproductive potential

Correlations were used to assess the relationship between body size and mean oocyte number. A positive correlation between F1 females shell length and total weight was found for all three treatments (P<0.001 Spearman's rank order), and between shell length and soft body weight (RW P<0.001, LE2 P<0.001 and HE2 P=0.002). A significant positive correlation was also found between the total weight and mean oocyte number for F1s from the RW exposure (0.481 P=0.020), but not for those from the LE2 (0.200 P=0.339) or HE2 (-0.011 P=0.965). Positive correlations were also seen between shell length and mean oocyte number (0.369 P= 0.084) and soft body weight and mean oocyte number (0.343

P=0.109) for RW F1s. However, these correlations were not statistically significant and became less significant in the E2 exposed F1s.

Throughout this study it has been found that there is a relationship between female *V. viviparus* size and mean number of oocytes; larger/heavier females tend to have more oocytes per section of tissue. Therefore the mean number of oocytes was analysed with shell length, total weight or soft body weight as co-variants. Analysis by ANCOVA found a significant difference between the treatments (P= 0.002, 0.004, 0.008, respectively ANCOVA). Post hoc analysis (Bonferroni) of this co-variant data was also conducted. Significant differences were found between the RW and the LE2 developmentally exposed females co-analysed for shell length (P=0.002), total weight (P=0.004) and soft body weight (P=0.010), and between RW and HE2 exposed females for total weight (P=0.049) and soft body weight (P=0.046), but not for shell length (P=0.052). However, no significant differences were found between the E2 treatments.

Post F1 breeding study male spermatogenesis score

The mean spermatogenesis score was highest from *V. viviparus* developmentally exposed to LE2 and then depurated for a year (Table 4.45). However, the difference between developmental exposure groups was small and analysis by Kruskal-Wallis showed no significant difference (P= 0.371).

Table 4.45 The mean spermatogenesis score of *V. viviparus* F1 male snails from the depurated E2 experiment

	River Water (RW)	Low E2 (LE2)	High E2 (HE2)
Mean spermatogenesis score	9.95 ± 0.1	10.0 ± 0	9.88 ± 0.2

Snails developmentally exposed to river water (RW), low concentration of 17β-oestradiol (LE2) or high concentration of 17β-oestradiol (HE2) and then depurated in river water for approximately one year. 1= least developed, 10 = most developed. Mean ± standard deviation RW, n= 4; LE2, n= 7; HE2, n= 8.

Correlations between F1 male size and reproductive potential

Significant positive correlations (Spearman's rank order) were found for shell length and total weight for LE2 (0.821 P= 0.023) and HE2 (0.905 P= 0.002) developmentally exposed males. A significant positive correlation was also found for soft body weight and shell length in the LE2 exposed males (0.821 P= 0.023), but not for those from the HE2 (0.690 P= 0.058). No significant correlations were found for the RW exposed males, possibly due to the small sample size (n=4). No significant correlations were found between mean

spermatogenesis score and shell length, total weight or soft body weight for any of the exposure groups, although this could be expected given that the highest scores (9-10, full spermatogenesis) was achieved by most individuals.

Parasitism

None of the F1 *V. viviparus* analysed from the F1 breeding study were parasitised.

4.4 Discussion

4.4.1 Physical parameters, water chemistry and YES assay

The water temperature within the mesocosm tanks varied with weather and season, which was to be expected using this type of experimental set up. The fluctuating temperature and day length are important seasonal cues, which can stimulate or inhibit reproduction. They were important in the design of the experiment, as recent evidence suggests that gastropods may be especially sensitive to vertebrate EDCs during times of increasing or reducing reproduction (Oehlmann et al. 2006, Clarke et al. In Press, Benstead 2006). The flow rate of the water in to the mesocosm tanks fluctuated, as did the pump rates. These fluctuations were not statistically significant between the tanks. However, they may play an important role when considering the water chemistry (below), as any deviation from the desired dilution factor could have an impact of the overall tank concentrations of 17β-oestradiol.

Chemical analysis of the mesocosm tank water found the average concentration of E1 and E2 to be highest in the HE2 exposure tank, followed by the LE2 tank. The lowest mean concentration of E1 and E2 was measured in the river water inlet, which fed all tanks. The RW tank had slightly higher mean concentrations (E1 and E2) than the inlet. Surprisingly, the highest mean concentration of EE2 was measured in the river water inlet, followed by the RW tank, with lower mean concentrations measured in the two dosed mesocosm tanks. In addition to the presence of EE2 (which will be discussed further below) in the RW tank, high E2 concentrations (10.1 and 5.71ng/l) were measured in the RW tank during the first two sampling occasions (weeks 0 and 4 respectively). The river water inlet was not sampled at these points, nor were water samples taken for the YES assay. This makes it difficult to confirm, or refute, the chemical analysis. It is always possible that contamination of the RW tank occurred. However, every attempt was made to prevent this. The RW tank was situated on a separate row of tanks to that of the dosed tanks and dosing reservoir stocks were prepared and housed at ground level (below tanks) to prevent any possible spills. When the tanks were sampled for adult reproductive output, the RW tank would be sampled first, and then the tanks would be sampled in order from low to high

concentrations. In addition, dosing stocks were always prepared at the end of the day, after other sampling had occurred. Whilst preparing the stocks, gloves were worn and then immediately discarded and all stock bottles, dosing reservoirs, pump tubing etc were colour coded to prevent cross contamination. As gastropods are known to produce E2 (Janer and Porte 2007), it is possible that a certain amount was released by the gastropods themselves, however the concentrations measured for the rest of the exposure period (weeks 8, 12 and 16) were below the limit of detection (LOD, 0.3ng/l) for E2.

Statistical analysis of the water chemistry (over the entire exposure) found no significant difference between each of the exposure tanks or the river water inlet, for any of the steroid oestrogens (E1, E2, EE2) measured. This is almost entirely due to the wide variation of measured concentrations within each tank rather than between them. Measured concentrations of E2 ranged from 1.25ng/l to 232ng/l in the HE2 tank. Therefore, the smaller range of (1.59ng/l to 28.6ng/l E2) measured in the LE2 tank resides within the range of concentrations measured in the HE2 tank, and no significant difference can be found. E1 concentrations also varied, with the maximum variation found in the HE2 tank. The maximum variation between measured concentrations of EE2 was found in the river water inlet.

Analysis of the mesocosm tank water using the YES assay found the highest average EEQ was from the HE2 tank (5.5 ± 5.20 ng/l EEQ) samples, followed by the LE2 tank (1.81 ± 2.12ng/l EEQ). The RW tank and the river inlet had lowest EEQ (0.86 ± 0.99ng/l EEQ and 0.74 ± 0.96ng/l EEQ, respectively). All the EEQ from the two dosed tanks were well below the nominal concentrations strived for (HE2 ~ 100ng/l, LE2 ~ 10ng/l). The HE2 tank water EEQ ranged from 1.71% to 14.72% of nominal and the LE2 tank water EEQ ranged from 8.8% to 59.8% of nominal. Statistic analysis found the predicted (from the dilution factor) EEQ were significantly higher than the measured EEQ in both tanks.

These differences between predicted/nominal and actual EEQ however, were not unexpected. The use of river water (complete with algae, bacteria, other biota and suspended sediments) was always going to be a confounding factor in terms of the stability of E2 in the water column. Firstly there were large volumes of suspended sediments accompanying the river water in each tank. At the culmination of the 16-week exposure period, approximately 50-70mm depth of sediment had been deposited on to the bottom of each tank. E2 has been reported to absorb to both bed and suspended sediments (Holthaus et al. 2002), with 80-90% of sorption occurring within 24 hours. Bacteria living in river

water have been reported to degrade E2 into E1, reducing its oestrogenic potency in both the YES assay and vertebrate models (Van den Belt et al. 2004). Jurgens et al (Jurgens et al. 2002) report E2 to have a half-life (t½) of 0.2-9 days (d) in a range of river waters from the UK, incubated at 20°C (E1 was degraded at a similar rate). Similarly Matsuoka et al (Matsuoka et al. 2005) reported E2 to be completely degraded within 5d in summer sampled Japanese river water. Both authors reported no significant reduction in E2 if the river water samples had been autoclaved prior to spiking. E2 degradation has also been reported in anaerobic river sediments with t½ of 0.11-0.66d UK (Jurgens et al. 2002) and 0.24-1.5d New Zealand (Sarmah and Northcott 2008). Due to the wide spread nature of these bacteria, it is not impossible that sorption and biodegradation of oestrogens occurred within the mesocosm tanks, as it does in rivers. Furthermore, it is possible that by constantly providing E2 as a nutrient source, E2-consuming bacteria were encouraged to proliferate in the two dosed tanks. Moreover, with tank water temperatures reaching 26°C, rates of removal may well have been high. Another possible route of E2 removal from the water column may be via uptake by algae and biota. During the exposure study, an abundance of biological material was found in the mesocosm tank, ranging from filamentous algae, freshwater sponges and Bryozoa, to aquatic invertebrates such as small crustacean and leeches. A small number of fish fry were also in evident in all the tanks, in addition to the gastropods in the test system. Takahashi et al (Takahashi et al. 2003) reported periphytons can bioaccumulate E2 64-1200 times that of the exposure concentration and that the benthos tested could bioaccumulate E2 around 100-106 times. Indeed, sorption of E2 to suspended organic matter and uptake in algae may well have provided another route of exposure (via consumption) to both gastropod species tested in this system.

When the EEQs were predicted (by the dilution factor), degradation of E2 was not accounted for. Some degradation of E2 to E1 is apparent from the water chemistry. E1 was not added to the tank and therefore must have come from the river water feeding the tanks, and/or from the degradation of E2 within the tank, and/or from animals within the tank. The same source of river water fed each of the tanks, so is unlikely to be the cause of differences in E1 seen between the tanks. The same can be said for the animals within the tanks. It is therefore likely that the E1 was, for the largest part, from the degradation of E2. This is especially evident when looking at the water chemistry reported for the HE2 tank at week 4, where an extremely high (232ng/l) concentration of E2 is reported along side a high concentration of E1 (30.6ng/l). However this does not follow for every sampling period.

In contrast to E1, EE2 cannot arise from the degradation of E2, and was not added to the tanks, and so must have come from the river water. It is possible that the river water contained higher than normal amount of EE2; the summer of 2006 was especially warm and dry. Therefore the normal dilution of TSE upstream of the river water inlet may have been much lower than average. However the markedly different concentration measured between the river inlet/RW tank and the two dosed tanks is still an issue. It has generally been reported that EE2 does not biodegrade as quickly as E2 in river water. Matsuoka et al (Matsuoka et al. 2005) report EE2 t½ as 14d and Jurgens et al (Jurgens et al. 2002) similarly report EE2 t½ at 17d compared to 1.2d for E2 under the same conditions. However, recent research reports that EE2 can be degraded by bacteria to E1, if co-incubated with enough E2. Pauwel et al (Pauwels et al. 2008) cultured six species of E2 degrading bacteria (sampled from a compost heap). After 21 days of incubating EE2 (1.0 ± 0.1mg/l at 28°C) with the different bacteria, no significant degradation was observed (if it was the only source of carbon). However, co-incubating with 1-5mg/l of E2 gave t½ of 41 ± 14 h to 14 ± 3 h. Increasing the ratio of E2:EE2 increased degradation, so that at a ratio of 5:1, 95% of EE2 was removed in 5 days (Pauwels et al. 2008). The concentrations tested by Pauwel et al (Pauwels et al. 2008) are orders of magnitude higher than those encountered in my experimental work. There is evidence (for E2 degradation) however, that concentration is not important in determining degradation rates. Jurgens et al (Jurgens et al. 2002) tested a wide range of E2 concentrations (20ng/l to 500µg/l) in river water and found similar rates across the range. It could therefore be possible that the high E2 levels dosed to the HE2 and LE2 tanks facilitated the degradation of EE2.

Although the predicted EEQs (from the chemical analysis) were generally higher than the actual measure EEQs, they were not found to be significantly different from those of the actual EEQ concentrations.

Water samples (for YES analysis) were taken from the two dosing reservoirs to assess how stable the E2 was in the dosing water. Samples from both the LE2 and HE2 reservoirs were taken 30 minutes after the stocks were mixed with the tap water and then again after 72 hours. The nominal concentrations of the dosing reservoirs were 200µg/l (HE2) and 20µg/l (LE2). The HE2 reservoir sample (taken after 30 minutes of mixing) had around 90% of the nominal EEQ (180 ± 77.8µg/l EEQ). However, after the same time in the LE2 reservoir, the measured EEQ was around 122% of the nominal concentration (24.4 ± 7.8µg/l EEQ). After 72 hours in the HE2 reservoir around 25% of nominal E2 concentration was measured (49.0 ± 17.6µg/l EEQ), which was around 27% of the 30

minute EEQ. Whereas, in the LE2 reservoir 65% of the nominal concentration was measured (12.9 ± 4.0µg/l EEQ), around 53% of the 30 minute EEQ. It is possible that both sorption and bacterial action affected the dosing reservoirs. In the LE2 reservoir, it is possible that over the dosing period E2 had sorbed onto the inside of the plastic container. The process of sorption is somewhat fluid and it is therefore possible that by adding fresh dosing stock (of a low E2 concentration) to the reservoir some of the E2 (previously stuck to the plastic) was released into the reservoir water in an attempt to form a new equilibrium. It is of course also possible that the high EEQ measured in the reservoir was due to incomplete mixing of the stock solution. Although the water used to make the dosing reservoir stock was tap water, and not river water, it is still possible that bacteria were present in the dosing reservoirs. Indeed, a scummy film (assumed to be bacterial) was observed in all the dosing reservoirs by the end of the 16-week exposure. Attempts were made to rinse this out with tap water, but the reservoirs were never sterilised in any manner. Without chemical analysis of the reservoir stock samples, it is difficult to determine whether bacterial action was to blame for the reduced EEQs from the 30-minute sample to the 72-hour sample. However, it is highly probable that a certain amount of degradation occurred.

Overall the variability in both the water chemistry and YES assay results make it difficult to give any definitive amounts of E2 in each of the mesocosm tanks. However, both indicate that the HE2 tank had on average higher concentrations of E2 than the LE2 or RW tanks. It is possible that both the chemical analysis and the YES assay result are not as accurate as would be desired. The river waters complex matrix of suspended organic mater may have hampered the chemical analysis. Moreover, it is possible that the volume of water extracted for the YES assay was insufficient to properly detect the E2 in the sample. There was a high level of suspended organics in the water samples taken from the mesocosm tanks. This prevented large volumes of water being easily extracted using the method stated in Chapter 3. In hindsight, other methods could have been tried; such as using multiple C18 cartridges per water sample (and then eluting all of them into a pooled sample), or to filter the water sample prior to extraction may have been more effective. It would certainly be prudent to assess some of these methods if the experiment was to be repeated. The fact that water samples were only taken in tandem (for YES and analytical chemistry) for the last three sampling periods is somewhat frustrating. Again this would be rectified if the experiments were repeated.

Many of the problems encountered with the water chemistry and YES assay are almost certainly related to the mesocosm set up its self. These systems are fundamentally different from the more easily controlled world of the indoor laboratory. The suspended organic material was actively encouraged in this system to provide a food source for the test species. It is part of the 'almost real world' aspect of using mesocosms. There are always costs and benefits in different types of experimental set ups. The benefit of using these large outdoor mesocosms is that one gets a better idea of how the animals behave under different seasonal conditions, which would be difficult to mimic within the laboratory. There are also 'man hours' to cost into any experiment, and many more would certainly have been needed if a similar experiment were to be run within a laboratory. For example, the offspring of the adult snails were more or less left to look after them-selves over winter, as the mesocosm environment provided all the food and shelter required. This allowed time for the histopathology to be conducted. However, the disadvantages of using the mesocosms are most evident in terms of the reliability of introducing chemicals into the water.

4.4.2 Effect of 17β-oestradiol exposure on survival and growth

Throughout the experiment, the main trend observed with respect to survival was that snails (of both species) had the highest survival rate in the LE2 tank, with the lowest generally being from the HE2 tank. For both species adult percentage survival at the end of the 16-week exposure period were remarkably similar with 72.7% of *V. viviparus* adults and 77.8% of *P. corneus* adults surviving in the RW tank, 84.8% and 88.9% from the LE2 tanks, and 44.1% and 48.1% from the HE2 tank respectively. It is somewhat surprising that in both species survival was so similar, as *V. viviparus* are known to be longer lived than *P. corneus* and could therefore be expected to be more robust.

In contrast to the adults, at the end of the exposure period, the total number of surviving neonates (*V. viviparus*) was highest in the LE2 tank (175), followed by the HE2 tank (160), and the RW tank (119). The total number of neonates released by adult *V. viviparus* was highest in the HE2 (601), followed by LE2 (552) and RW (507). Therefore early percentage survival of neonates was higher in the LE2 tank (33.5%) compared to HE2 (26.6%) or RW (23.5%). A similar trend of higher survival in the LE2 tank was also found for the over-wintering F1s. 94% of *V. viviparus* F1s survived the winter from the LE2 tank compare to 53% from the RW tank and 55% from the HE2 tank.

The survival pattern of the *P. corneus* F1s was somewhat different to *V. viviparus*: 28% from the LE2 tank survived the winter compare to 25% from the HE2 tank and 19% from the RW tank. However, during early spring, *P. corneus* from the HE2 tank suffered almost twice as many mortalities as those from the LE2 and RW tanks. The lower F1 survival of the *P. corneus* compared to the *V. viviparus* is likely to be due to differences in life strategies. As discussed in Chapters 2, *V. viviparus* has low fecundity compare to that of *P. corneus.* Instead of producing a large number of young, it invests its energy into producing a small number of larger, well-developed offspring. These offspring are therefore far better equipped to survive the winter. The trend of LE2 survival continued through the un-dosed F1 breeding study. 97% of the LE2 developmentally exposed *V. viviparus* F1s survived to the end of the F1 breeding study compare to 88% from the HE2 groups and 85% from the RW groups. For *P. corneus* 94% of the LE2 developmentally exposed snails survived compared to 44% from the RW and 33% from the HE2 exposures

At the end of the 16-week exposure, adult female *V. viviparus* from the HE2 exposure were significantly lighter than those from the LE2 and RW exposures. *V.viviparus* RW exposed males were almost twice as heavy (once the shell was removed) than males from the LE2 or HE2 exposures. Whereas, there were no significant differences in size or weight of the adult *P. corneus* post-exposure. However, the normal highly significant positive correlation found between total weight (shell on) and soft body weight (shell removed) was lost in the HE2 exposed snails. Immediately after developmental exposure, F1 *V. viviparus* also showed optimal growth in the RW only treatment with 85% of the population above 10mm in shell length compare to 75% from the LE2 exposure and 63% from the HE2 exposure. It is possible, however, that the smaller percentage of 10mm+ offspring in the HE2 tank also relates to the significantly higher reproductive output exhibited by the *V. viviparus* adults towards the end of the exposure period (weeks 14-16) in this tank. F1 *P. corneus* showed a similar size/growth trend to the *V. viviparus*, with 73% of RW exposed snails measuring above 10mm in shell diameter, compared to 46% of HE2 and LE2 snails. When the sub-samples were taken of the F1s for histopathological analysis, it was found that F1 female *V. viviparus* from the HE2 exposure had significantly lower soft tissue weight compare to those from the RW, even though overall shell sizes were similar. F1 male *V. viviparus* were affected to a greater extent, with HE2 exposed males significantly smaller (shell length and aperture, total and soft body weight) than those in the RW or LE2 exposures. *P. corneus* developmentally exposed to the HE2 treatment were also significantly smaller (shell length and aperture, total and soft body weight) than those from the LE2 and RW exposures. During the un-dosed F1 breeding study the LE2 exposed and

then depurated *V. viviparus* grew larger and heavier than the RW or HE2 exposed snails. The size and weight of depurated F1 *P. corneus* from the different developmental exposures were not significantly different from each other at the beginning or the end of the un-dosed F1 breeding study. However, in a striking similarity to their parents, the HE2 developmentally exposed F1s had also lost their significant positive correlation between total weight (with shell) and soft body weight (without shell). In addition, they had also lost their normal significant positive correlation between shell diameter and soft body weight. It seems therefore that although exposure during adult hood or during early developmental stages to high concentrations of E2 can have toxic effects (increased mortality, retarded growth), that exposure during these times to low concentrations may prove beneficial in terms of survival. In the case of *V. viviparus*, F1s it also seems that developmental exposure to LE2 increased growth rates later, when the snails were depurated.

4.4.3 Effect of 17β -oestradiol exposures on reproduction and sexual development

On average (over the whole 16 week exposure), HE2 *V. viviparus* snails released the most offspring per snail, with RW and LE2 releasing slightly lower numbers (Figure 4.10). From week 10 onwards, HE2 exposed snails released a higher number of neonates per adult *V. viviparus*, however significant differences between treatments were not observed until week 14 of the exposure study. This delayed trend could be expected: female *V. viviparus* harbour their offspring over winter (pre-exposure) for release in the spring, and therefore any impact of exposure on reproduction would not be seen until summer. The higher reproductive output seen in the HE2 exposed snails may, in part, be related to the higher mortality rate experienced in the HE2 tank (Figure 4.9). Females may have prematurely released their offspring due to increasing stress or threat of mortality (Ribi and Gebhardt 1986). Unfortunately the size of offspring released was not recorded: these data would have been beneficial in understanding whether the HE2 had somehow stimulated the growth of the offspring (thus facilitating earlier release) or if, on the other hand, they were prematurely released (aborted young). When the female's brood-pouches were dissected at the end of the exposure, those from the HE2 tank had on average the lowest number of embryos per female (Table 4.30), indicating premature release was the likely explanation (rather than them having more embryos to release). Females from the LE2 exposure had the highest number of embryos. Interestingly, females from the LE2 treatment harboured significantly more un-shelled (youngest) embryos when compared to the RW or HE2 females. These un-shelled embryos would likely represent eggs produced by the female during the exposure period, whereas the shelled embryos were perhaps the un-shelled

embryos present at the start of the exposure. The number of unshelled embryos produced by the prosobranch mollusc *Potamopyrgus antipodarum* has been used in recent research to evaluate the oestrogenic or androgenic effects of a number of EDCs and TSE (Duft et al. 2007, Duft et al. 2003, Jobling et al. 2004, Jobling et al. 2003). Jobling et al (Jobling et al. 2004) reported that exposure to EE2 induced a similar reproductive response in *P. antipodarium* as found in the fathead minnow (*Pimephales promelas*), a commonly used fish test species, with un-shelled embryo production (or egg laying) stimulated at low concentrations and inhibited at higher concentrations. This 'inverted U-shaped' response may also be evident in my research, although the limited number of concentrations tested is a hindrance to fully understanding the complete response. A number of different mechanisms may be at play here. When the adult female *V. viviparus* were analysed histologically, a trend was seen of increased oocyte number inline with increasing E2 dose, which could be evidence of E2 mediated stimulation on oogenesis. Accelerated oogenesis has been reported in grayling (*Thymallus thymallus*) exposed to concentrations of E2 as low as 1ng/l (Lahnsteiner et al. 2006). However, as discussed above, in the HE2 exposure tank there was also a higher level of mortality and altered/retarded growth when compared to the RW and the LE2 exposures: evidence of toxicity. The energy required to produce eggs with a large enough food reserve for many months of 'gestation' must be significant. Therefore, any evidence of E2 stimulation on embryo production may have been concealed by toxic stress to the females at higher E2 concentrations. It is also possible that toxicity and stress may also have affected the male's ability to mate. Histopathological assessment of the adult male *V. viviparus* gonad, however, indicated full spermatogenesis was occurring in all treatment groups. It is however possible that E2 exposure negatively affected sperm quality parameters (e.g. motility) not measured in this research. There are reports of reduced sperm quality after E2 exposure in vertebrate species. Gill-Sharma et al (Gill-Sharma et al. 2001) reported significantly reduced sperm motility in adult rats exposed to E2 (<10µg/kg/day). In a fresh water fish (Grayling) sperm density, volume and motility were negatively affected by pre-spawning exposure to ≥ 1ng/l E2 (Lahnsteiner et al. 2006). In a species such as *V. viviparus*, which practices internal fertilisation, sperm quality may be vital to fertilisation success.

Table 4.46 Comparison of effects in the E2 experiment on *V. viviparus* adults, F1s directly after developmentally exposed, and F1s after developmental exposure and then depuration in river water for one year

	Adult exposed for 16 weeks		F1s developmentally exposed		F1s developmentally exposed and then depurated	
	LE2	HE2	LE2	HE2	LE2	HE2
Neonates released per snail	—	↑	N/A	N/A	—	—
Total embryos per female	—	—	N/A	N/A	↑	—
Shelled embryos per female	⇣	⇣	N/A	N/A	↑	—
Un-shelled embryos per female	↑	—	N/A	N/A	⇡	—
Mean oocytes	↑	↑	↓	↓	↓	↓
Spermatogenic score	—	—	↓	↓	—	—
Size and growth	—	⇣	—	↓	↑	—
Survival	↑	⇣	E ⇡ O ⇡	E ⇡ O —	⇡	—
Parasitic infection	⇣	⇣	—	—	—	—

Snails expoed to; river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2) in comparison to snails exposed to river water only (RW). Key to symbols: dashed horizontal lines no difference in effect seen when compared to RW; dashed vertical lines show a trend but not a statistically significant one when compared to RW; solid vertical line shows a significant difference (P< 0.05) when compare to RW; arrow head indicates an increase or decrease compared to RW; N/A, Not applicable; E, survival during exposure; O, survival over winter.

When the number of shelled (oldest) embryos harboured were correlated with their mother's shell length, a significant negative correlation was found in the RW exposed snails, whereas a significant positive correlation was found in the LE2 exposed snails (no significant correlation was found in HE2 snails). It could be assumed that larger females should harbour more shelled offspring (causing a positive correlation). Research by Jakubik (Jakubik 2006, Jakubik 2007) on *V. viviparus* from the Zegrzynski Reservoir (Central Poland) found female fecundity (total embryo number) increased with shell size,

and that fecundity could double in areas with good water flow and food supplies. However, in contrast Ribi and Gebhart (Ribi and Gebhardt 1986) found that in a population of *V. ater,* which had little threat of early mortality, large females might alternatively produce fewer larger offspring (causing a negative correlation). This perhaps indicates that although the LE2 females may be harbouring a larger number of offspring they may be smaller in comparison to those from the RW exposure. However, the size of embryos was, unfortunately, not measured. No significant correlation was found between female shell length and the number unshelled embryos harboured in any treatment group.

Reproductive output in the adult *P. corneus* was induced at the LE2 concentration but was inhibited at the HE2 concentration. The stimulation of egg production (LE2) was especially evident during the normal end of summer decline (after the summer solstice peak). Snails from the LE2 exposure laid significantly more eggs at weeks 12, 14 and 16 than their RW counterparts, Figure 4.3 illustrates this, Figure 4.18A shows the mean number of eggs laid per snail in the eight weeks leading up to the summer solstice: when normally egg laying is at its peak, and Figure 4.18B shows the eight week period after the solstice: when normal egg laying is in decline. A similar response was also observed in the same species exposed to graded concentrations of TSE (Clarke et al. 2009). Inappropriate stimulation of reproduction has also been observed in fish. EE2 (5-6ng/l) exposed Fathead minnows continued to express VTG mRNA and protein after the normal end of the breeding season (Kidd et al. 2007). Egg laying by HE2 snails was reduced from week 6 of the exposure, which was shortly followed by a sharp drop in percentage survival. Again it seems that with this species at the higher concentration, any stimulating effects that might occur are masked by toxicity and stress.

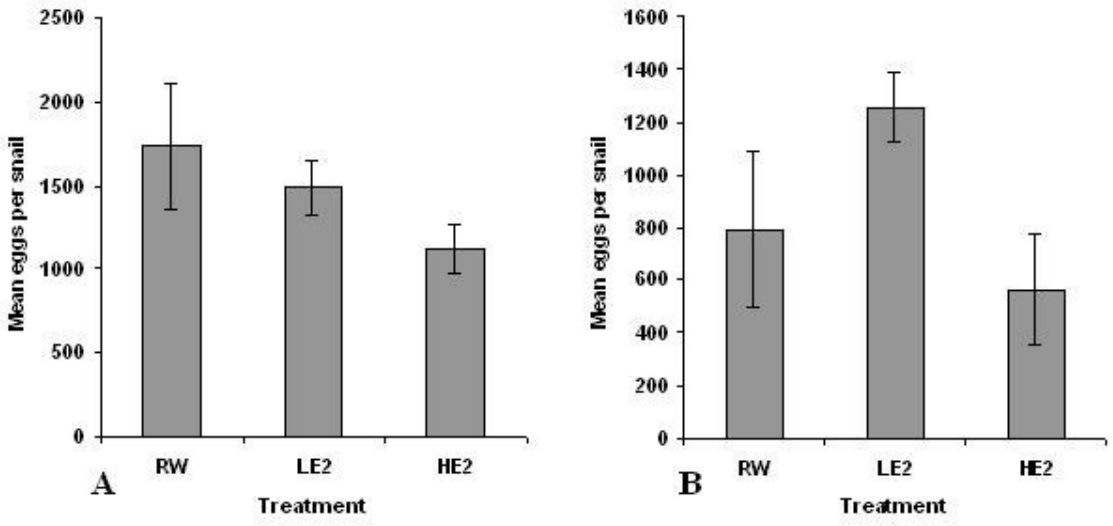

Figure 4.18 Mean eggs laid per snail from the E2 experiment.

Snails exposed to river water only (RW), river water plus a low concentration of 17β-oestrodiol (LE2) and river water plus a high concentration of 17β-oestrodiol (HE2) split between the first eight weeks of the exposure period (A) and the last eight weeks of the exposure period (B).

Histopathological analysis of the adult gonad of *P. corneus* found some interesting effects of exposure relating to the gametes supporting cells. The Sertoli cells, spermatogenic supportive cells, seemed to be especially sensitive. Significant increases in the numbers of Sertoli cells and spermatogonium sloughed into the acini lumen (where they were presumed no longer functional) were found in the adult HE2 exposed snails. The HE2 exposed snails also suffered increased spermatocyte and spermatid sloughing into the lumen compared to that of RW and LE2 snails, albeit a non-significant one. Negative affects of E2 exposure on Sertoli cells have been reported in a number of vertebrates. Rasmussen et al (Rasmussen et al. 2005) reported E2 exposure (0.5µg/l E2, continuous flow through) to disrupt Sertoli cell structure and function in the eelpout (*Zoarces viviparus*), resulting in impairment of spermatogenesis and the lobular structure of the testis. Proliferation and degenerative changes of Sertoli cells were observed in adult fathead minnows (*P. promelas*) exposed to E2 (0.0625-1000nM) for 14 days (Miles-Richardson et al. 1999a). Exposure of adult male rats to E2 (0.1mg/kg) for 10 days significantly reduced their sperm count and the number of litters sired (Aleem et al. 2006). At concentrations >10µg/kg/day E2, Sertoli cell function was disrupted in adult rats (Gill-Sharma et al. 2001): this E2 induced disruption to Sertoli cells was prevented by co-exposure to an anti-oestrogen, suggesting the effect was ER mediated (Gill-Sharma et al. 2001). The developmentally exposed F1 *P. corneus* also showed spermatogenic disruption: the E2 developmentally exposed snails had a significantly higher percentage of acini

affected by Sertoli cell and immature spermatogenic cells sloughing into the lumen compared to the RW exposed snails. In addition to Sertoli cell disruption, a significant dose-dependent reduction in the percentage of active vitellogenic areas (maturing oocytes and sperm) was found (a similar but non-significant trend was also observed in the adult snails, Table 4.47). This suggests the follicle cells (oocyte supportive cells) may also be affected. There are several ways the follicle cells may have been affected to result in reduction of mature oocytes in the vitellogenic area. Firstly the ability of the follicle cells to transport the developing oocytes to the vitellogenic area may have been inhibited, and thus promoting empty vitellogenic areas. Secondly, the follicle cells may have been disrupted in their ability to support oocyte maturation (which normally occurs in the vitellogenic area). When the different stages of oocyte maturation were assessed, the E2 developmentally exposed snails were found to have a significantly higher percentage of young oogenesis stages, and a lower percentage of mature stages when compare to the RW snails (a similar but non-significant trend was observed in the adult snails, Table 4.47). In vertebrates, there has been less research focused on the effects of E2 on female germs cells than on male germ cells. However, it has been reported that in sexually mature fathead minnows exposed to E2 (0.0625-1000nM), increased numbers of primary stage oocytes were recorded, with reduced numbers of later stages of development (Miles-Richardson et al. 1999a). Therefore, the reduced activity observed in the vitellogenic area could be due to the combined effects of Sertoli cell disruption (and sloughing) and a reduction in oocyte maturation. There has been little research on the possible toxicological effects on gonad histopathology of molluscs (especially hermaphrodites). Recent research has, however, also indicated Sertoli cell dysfunction (immature spermatogenic cells found in lumen) in *P. corneus* exposed either as adults or developmentally to TSE (Clarke et al. 2009). However, due to the staggered sampling of the F1 *P. corneus*, it is also important to consider the possibility of seasonal changes in gonad function. The HE2 exposed F1s were sampled 9 days earlier than the LE2 and RW F1s. Therefore changes in water temperature and day length may also have affected the snails. From my own additional laboratory work on *P. corneus* (Appendix V), I found that reduced temperature (below 15^{o}C) initially caused sertoli and immature spermatogenic cells to slough into the lumen. During the F1 sampling period (14-23 September), midday water temperatures were measured at 16-18^{o}C. The level at which the spermatogenic and sertoli cell sloughing occurs under different natural conditions is unfortunately undocumented.

Impacts on spermatogenesis and oogenesis were also found in the E2 developmentally exposed F1 *V. viviparus*. The F1 females from both E2 exposures had significantly less

oocytes per section (of gonad analysed) than the RW exposed snails. Male spermatogenesis score (sexual development) was significantly affected in a dose dependant manner. The HE2 exposed males had on average only spermatid present in the gonad compared to full spermatogenesis in 50-70% of tubules in RW exposed males. Although Sertoli cells were not seen to detach from the tubule basement membrane (as seen in *P. corneus*) this (retarded spermatogenesis) still might be evidence of Sertoli cell dysfunction. Developmental exposure to steroid oestrogens has been reported to disrupt normal spermatogenesis in vertebrates. For example, day old rats exposed to E2 (1µg) resulted in altered gonocyte migration, reduced cell proliferation and increased apoptosis (Vigueras-Villasenor et al. 2006). Similar results were reported (reduced germ and Sertoli cell volume (per testis) in adults, increased apoptosis) when Atanassova et al (Atanassova et al. 1999) exposed developing rats to 10µg/kg EE2.

After a year of depuration in river water, female oocyte number still suffered a dose-dependant decrease. Whereas, in depurated males, spermatogenesis had fully recovered. It seems therefore that although the effects on spermatogenesis were transient in developing *V. viviparus,* the effects on oocyte number were permanent. This may be especially important in such a long-lived species, as a short exposure at the developmental stage could have implications for recruitment for several years. For both the F1s and the adults of this species, it would have been very informative to continue the experiment for some time, to fully appreciate the long-term effects of exposure. In vertebrates, Kidd et al (Kidd et al. 2007) report that chronic exposure to the synthetic oestrogen EE2 (5-6ng/l) lead to near extinction of Fathead minnows (*P. promelas*) from one of Canada's experimental lakes. Early signs of effects on these fish included retarded spermatogenesis in males and altered oogenesis in females (Kidd et al. 2007), similar findings as observed in *V. viviparus* exposed to E2 in my research.

Table 4.47 Summary of histolopathological effects seen in *P. corneus* adults directly after exposure, F1s developmentally exposed and F1s developmentally exposed and then depurated for one year from the E2 experiment

	Adult exposed for 16 weeks		F1s developmentally exposed		F1s developmentally exposed and then depurated	
	LE2	HE2	LE2	HE2	LE2	HE2
Sertoli cells attached to acini walls	–	–	–	↑	–	–
Percentage of acini with active vitellogenic area	↓	↓	↓	↓	↓	↓
Area of acini walls covered by sex cells	↓	↓	↑	↓	↑	↓
Acini wall integrity	–	–	↑	↓	–	↑
Sertoli cells sloughed into acini lumen	↑	↑	↑	↑	–	↓
Spermatogonium sloughed into acini lumen	↑	↑	↑	↑	–	↓
Spermatocytes sloughed into acini lumen	–	↑	↑	–	–	–
Spermatids sloughed into acini lumen	–	↑	↑	↑	–	–
Only spermatozoa in acini lumen	–	↓	–	↑	↓	↓
Percentage of acini affected by immature spermatogenic cells sloughed into lumen	–	↑	↑	↑	–	–

Snails exposed to; river water plus a low concentration of 17β-oestradiol (LE2) or river water plus a high concentration of 17β-oestradiol (HE2) in comparison to snails exposed to river water only (RW). Key to symbols: dashed horizontal lines no difference in effect seen when compared to RW; dashed vertical lines show a trend but not a statistically significant one when compared to RW; solid vertical line shows a significant difference (P< 0.05) when compare to RW; arrow head indicates an increase or decrease compared to RW.

Given the histopathological evidence (reduced oocyte number and lower spermatogenic score) of the *V. viviparus* directly after developmental exposure to E2, the F1 breeding study results were somewhat of a surprise: the LE2 developmentally exposed and then depurated females harboured significantly more embryos (total and shelled) than the RW (or HE2) exposed snails. A large proportion of the females from all treatment groups did

not harbour embryos. However, the lack of embryos in the majority of RW females was somewhat unexpected. Jakubik (Jakubik 2007) reports that female *V. viviparus* from the Zegrzynski Reservoir (Central Poland) had between 0.9-6.7 embryos per female at a shell length of 8.1-12mm and 1.1-9.6 embryos per female at a shell length of 12.1-25mm. At the end of the F1 breeding study the females were around 15-18mm in shell length. The lack of embryos harboured may therefore be a result of the experimental set up. From the RW developmentally exposure, 12.5% of *V. viviparus* female's harboured embryos compared to 36% from LE2 exposed and only 5% from HE2 exposed. The lower embryo number and percentage of females harbouring embryos in the RW tank may have been density related. The initial over-wintering density of *V. viviparus* offspring in the RW tank was over four times that of the LE2 or HE2 tanks (the densities in these tanks were similar). Combined with the number of *P. corneus* housed in the same tanks to over winter, the densities of juvenile snails were 0.8 l/snail in the RW tank compare to 1.8 l/snail in the LE2 tank and 1.2 l/snail in the HE2 tank. *V. viviparus* are known to aggregate in extremely high densities under good conditions. Jakubik (Jakubik 2003) reported densities over 800 individuals/m^2 in the Zegrzynski Reservoir. However, Browne and Richardson (Browne 1978) report these aggregations are not observed when food sources are limited. The percentage of F1 *V. viviparus* surviving the winter period from the RW tank was much lower than seen in the LE2 tank. During the over-wintering period, the river water pump had to be removed from the river and switched off (leaving all the tanks static), reducing the food and possibly dissolved oxygen (although the aeration pump was still active). The increased over-wintering density may therefore have caused increased competition for food and resources, hampering reproduction or the female's ability to produce the nutrition needed for eggs, and therefore possibly reducing the number of embryos, the effects of density will be discussed further in Chapter 6. The reason the density was so much higher in the RW tank was due to the pooling of two RW tanks (due to a lack of availability of tanks). However, the significantly lower number of embryos harboured by females from the HE2 exposure compared to those from the LE2 tank is less likely to be an affect of density. The reduced oocyte number combined with lower spermatogenic score seen in HE2 *V. viviparus* F1s directly post exposure is likely to be key. Due to the long gestation period of these animals, mating must occur in their first autumn for females to release offspring the following spring-summer. In this experiment, males from the HE2 exposure (on average) were not producing any mature sperm in September, which would have prevented many of the snails from mating successfully.

Effects of E2 developmental exposure on depurated reproduction were also seen in F1 *P. corneus*. Egg laying was delayed in the both the HE2 and LE2 developmentally exposed snails compared to those from the RW only. Indeed, LE2 developmentally exposed and then depurated snails did not start laying egg masses until eight weeks into the experiment (compared to from the beginning in RW snails). LE2 reproductive period was less than half that of the RW, only lasting for six weeks. During the short time they did lay eggs LE2 snails also laid at a much lower rate. Over the 14 weeks of the study the mean number of eggs laid per snail was only 6 for LE2 exposed snails compare to 112 from the RW. The HE2 snails were affected in a similar manner but to a lesser extent than those from the LE2 exposure. Snails from the two E2 treatments were not stunted and LE2 snails survived the F1 breeding study better than those from the RW exposure. Therefore toxicity does not seem to be a factor affecting the reproductive output of depurated snails. In *P. corneus,* as are other Basommatophora, egg laying is regulated by both biotic and a-biotic factors; the most prominent of these being day length, water temperature and nutritional state (Joosse 1984, Bohlken and Joosse 1982, Costil and Daguzan 1995a, Costil 1997, Scheerboom 1978). In experiments with another UK native pulmonate *L. stagnalis*, day length was found to be the overriding external factor in controlling reproductive output (Bohlken and Joosse 1982, Bohlken et al. 1986). *P. corneus* normally start laying egg in early spring and continue until late summer (Clarke et al. 2009, Costil and Daguzan 1995b). However, in the case of the E2 developmentally exposed groups they did not start laying eggs until around week 8 of the study. The summer solstice (longest day) fell between weeks 8 and 10, when normally maximum reproductive output is achieved.

As reported in Chapter 2, pulmonate gastropods have several types of neuro-secretory cells found in the cerebral ganglia, which control growth and reproduction. Growth is controlled by products produced by the light green cells (LGC), whereas egg laying is controlled by the lateral lobes (LL). The LL stimulates the caudodorsal cells (CDC) to produce caudodorsal cell hormone (CDCH), which controls ovulation and oviposition. The LL also stimulated the dorsal bodies (DB) to produce dorsal body hormone (DBH), which stimulates the growth and differentiation of the female accessory sex organs and stimulates vitellogenesis in the ovotestis (Chapter 2, Section 2.1.1 and Figure 2.3). However, the LL also suppresses the action of the LGC. So there is a balance between growth and reproduction. Day length has an important function with regard to egg laying, however the exact mechanism is unknown (Joosse 1984, Bohlken and Joosse 1982, Bohlken et al. 1984). My results are suggestive that developmental exposure may have disrupted the normal response to increased day length that induces egg laying. In vertebrates, disruption

to the hypothalamic-pituitary-gonadal (HPG) axis during its development or 're-programming' by inappropriate exposure to steroid oestrogens (or other EDCs) has been reported to cause permanent alterations in adult reproductive behavior or function (Gore 2008, Dickerson and Gore 2007, Ottinger et al. 2008, Ottinger and Abdelnabi 1997). It is therefore possible that similar disruption to the developing neuro-endocrine system of gastropods could occur. The neuro-endocrine control of the prosobranchs has not been investigated to the same level as pulmonates. However, the increased growth rate but reduced oocyte number observed in developmentally exposed *V. viviparus* may also be due to disruption of the growth-reproduction balance.

When the gonads of the developmentally exposed *P. corneus* F1s were analysed after approximately one year of depuration, a dose-dependant decrease in active vitellogenic area was still in evidence. However, sloughing of Sertoli and immature spermatogenic cells was not significantly different between the three groups. This, combined with the data obtained from the *V. viviparus*, suggest that the effects of E2 on Sertoli cells and spermatogenesis are activational, rather than organisational. It is not known, however, how long some of these effects persist, as histological samples were not taken at the beginning of the F1 breeding study.

5 Effects of exposure to environmentally relevant estrogenic mixtures on reproduction of two UK native fresh water gastropod molluscs

5.1 Introduction

The feminising effects of some treated sewage effluents to vertebrates, such as fish, have been well documented (Jobling et al. 2002, Rodgers-Gray et al. 2001, Jobling et al. 2003, Harries et al. 1996, Liney et al. 2005, Vajda et al. 2008). Domestic and industrial effluents, and therefore river water, contain thousands of chemicals. However, research has been conducted in the past to identify the oestrogenic components of these effluents, using methods such as toxicity identification and evaluation (TIE). Using these techniques, a number of chemicals have been implicated in the feminisation of fish, including the natural steroid oestrogen 17β-oestradiol (E2), the synthetic steroid oestrogen 17α-ethynylestradiol (EE2), the plastics component bisphenol A (BPA), and the alkylphenol ethoxylates; nonlyphenol (NP) and octylphenol (OP) (Desbrow et al. 1998, Jobling and Sumpter 1993, Routledge and Sumpter 1996, Tyler and Routledge 1998, White et al. 1994, Routledge and Sumpter 1997, Routledge et al. 1998). In 2004, when this research project was initiated, there were a small number of publications indicating that molluscs could also be sensitive to domestic and industrial effluents (Blaise et al. 2003, Gagne et al. 2004b, Jobling et al. 2004). It is therefore suggested that these same chemicals identified from effluents to be oestrogenic to fish, may also be responsible for the feminising effects seen in molluscs. Indeed, as previously stated in Chapter 3, a small number of studies have assessed reproductive effects of single chemical exposures (BPA, EE2, NP, OP) to gastropod molluscs (Segner et al. 2003b, Oehlmann et al. 2000, Oehlmann et al. 2006, Duft et al. 2007, Duft et al. 2003, Jobling et al. 2004). However, at the time this research was instigated, no published studies had investigated the reproductive and/or developmental effects of environmentally relevant mixtures of these EDCs on gastropod molluscs.

5.1.1 Previous research of oestrogenic mixtures on the test species

To my knowledge no previous research on the effects of oestrogenic mixtures has been conducted with the gastropod species *V. viviparus* or *P. corneus*. However, research has been conducted on these two species with graded concentrations of Treated Sewage Effluent (TSE) (Clarke et al. 2009). This produced evidence of disruption of the reproductive cycle of the adult gastropods and to the reproductive development of their developmentally-exposed offspring. However, as previously mentioned, TSE contains thousands of chemicals and compounds, which can affect animals in many different ways (nutrition, toxic, androgenic, anti-androgenic, oestrogenic, anti-oestrogenic, etc). This can make identifying oestrogenic effects troublesome. Therefore, the purpose of this experiment was to test a mixture of environmentally relevant oestrogenic chemicals, which are known to be endocrine disruptors to vertebrates such as fish, on native gastropod

molluscs. This experiment was devised to provide a more 'real world' exposure than that presented in Chapter 4 (high concentrations of a single chemical), but without the added complexities and confounding factors that a whole effluent study may present. As in the E2 experiment (Chapter 4), the effects of exposure on both the sexually mature adult animal, and the developing offspring were assessed. The same two species of native UK freshwater gastropods were chosen, as was the use of the mesocsom set up. The reasoning behind choosing these species and the mesocsom set up were the same as for the E2 study (Chapter 4). This replication of species and experimental design also provided the opportunity for comparisons to be made between the effects of a single oestrogenic chemical and an oestrogenic mixture.

5.2 Aims

The aim of this experiment was to assess the effects of exposure to environmentally relevant oestrogenic mixtures on adult reproduction and sexual development in two species of native gastropod mollusc, *V. viviparus* and *P. corneus*.

5.3 Method

Two species of freshwater gastropod (*V. viviparus* and *P. corneus*) were exposed to environmentally relevant estrogenic mixtures in a freshwater mesocosm experiment. The mesocosm tanks were housed at Essex and Suffolk water's Langford water treatment works site. In the dosed experiment four 685 litre ($1m^3$) purpose built tanks were fed with river water, which was abstracted from the River Chelmer via a submerged pump. Two tanks were dosed with either 'high' (nominal doses typical of treated sewage effluent) or 'low' (nominal doses typical of river water) concentrations of a mixture of estrogenic chemicals namely the steroids Estrone (E1; high, 100ng/l; low, 20ng/l), 17β-oestradiol (E2; high, 10ng/l; low, 3ng/l) and 17α-Ethinlyestradiol (EE2; high, 1.5ng/l; low, 0.5ng/l) and the synthetic industrial chemicals Nonylphenol (NP; high, 6µg/l; low, 1.5µg/l) and its ethoxylates NP1EO (high, 5µg/l; low, 1µg/l) and NP2EO (high, 7µg/l; low, 2µg/l), Octylphenol (OP; high, 600ng/l; low, 150ng/l) and Bisphenol A (BPA; high, 100ng/l; low, 50ng/l). A third tank contained river water and the carrier solvent ethanol (RW+S) and a fourth tank contained river water only (RW). See Chapter 3 Section 3.5, which states the full materials and methods.

At the start of the experiment, each tank was furnished with twelve mesh cages of snails, six for each species, each containing either six adult *P. corneus* or eleven adult *V. viviparus*. Adult gastropods and their subsequent offspring were exposed over 16 weeks

from the beginning of May until the end of August 2006. The reproductive output of the adult snails was measured fortnightly either by counting the number of eggs laid (*P. corneus*) or neonates released (*V. viviparus*). The offspring (F1s) produced by the exposed adults were also developmentally exposed in the same mesocosm tank in which their parents were housed. Adult snail mortality was recorded fortnightly. Tank water samples were taken on weeks 0, 4, 8, 12 and 16 for chemical analysis (E1, E2, EE2, NP, OP, BPA). Water samples were also taken on weeks 2, 6, 8, 10, 12 and 16 from the two dosed tanks and on weeks 8, 10, 12 and 16 from the river water tank for Yeast Estrogen Screen (YES) analysis. The water samples taken at weeks 8, 12 and 16 were simultaneously sampled for the YES and water chemical analysis. When dosing culminated (end of August 2006) all the adults and a number of the F1s were sacrificed and fixed for histopathological analysis of reproductive health and sexual development (see Section 3.5.7). The remaining F1s from three of the treatments (river water only (RW); low concentration of the oestrogenic mixture (LM); high concentration of the oestrogenic mixture (HM)) were counted and then depurated in river water over autumn-winter 2006 (see Section 3.5.8). The surviving depurated F1s were then recounted and groups of snails were assessed for their reproductive output in two further un-dosed mesocosm (*P. corneus* over 14 weeks, *V. viviparus* over 18 weeks) experiments, conducted spring-summer 2007 (see Section 3.5.9 for full materials and methods). At the end of the F1 breeding study (August 2007), these snails were sacrificed and fixed for histopathological analysis of reproductive health and development (see Chapter 3, Section 3.5.10).

5.4 Results

5.4.1 Physical tank parameters

Temperature

Water temperature fluctuated over the 16 weeks of exposure with a low of 14^{o}C measured in May and a peaked of 26^{o}C at the end of July (Figure 4.1).

Flow Rate

Over the entire experiment water flow into the tanks was kept to approximately 3l/min (RW 3.3 ± 0.6, RW+S 3.0 ± 0.6, LM 3.2 ± 0.6, HM 3.2 ± 0.7), flow rates did vary day to day and the flow meters needed frequent adjustment to keep flow rates equal. However, statistical analysis (ANOVA) found no significant difference between the four treatment tanks (P= 0.252).

Dosing Pump

The dosing pump rate was kept at approximately 1.5ml/min (RW+S 1.6 ± 0.1, LM 1.5 ± 0.2, HM 1.4 ± 0.2). Blockages, however, occurred occasionally (especially in hot weather) and tubing needed to be replaced periodically. Statistical analysis (Kruskal-Wallis) found a significant difference between the dosing pump rates ($P<0.001$). Post hoc analysis (Mann-Whitney) found the RW+S to have significantly higher dosing rate compared to that of the HM pump rate ($P<0.001$). However, no significant difference was found between RW+S and LM ($P= 0.097$) rates, or between LM and HM ($P= 0.075$) rates.

Concentration of solvent (Ethanol) in tanks

The river water flow rate and dosing pump rate dictated the concentration of solvent (Ethanol) in the RW+S, the LM and the HM tanks. Due to a combination of higher dosing pump rate and lower water flow in the RW+S tank the average the concentration of ethanol in the RW+S tank was higher than in the LM or the HM tanks. Figure 5.1 illustrates average concentration of ethanol in the three tanks over the 16 week dosing period.

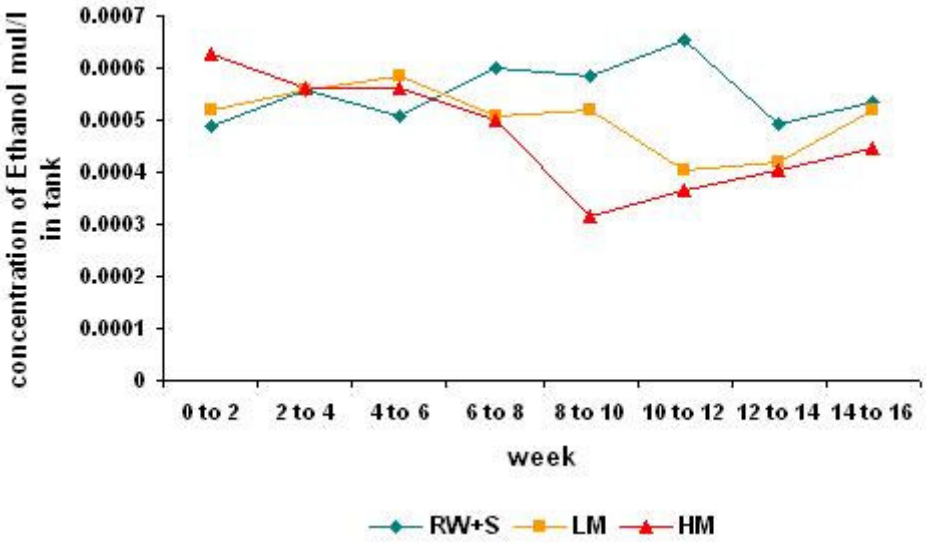

Figure 5.1 Concentration of carrier solvent (Ethanol) in the mesocsom tanks during the adult and F1 exposure in the oestrogenic mixture experiment.

River water plus solvent (RW+S), low oestrogenic mixture (LM) and high oestrogenic mixture (HM). The concentration of solvent was calculated from the measured dosing pump rate and water flow rate into each tank. Concentration of solvent measured in µl/l.

5.4.2 Chemical and Biological (YES) analysis of mesocosm water samples

Measured concentrations of steroid and non-steroid estrogens from the mesocosm tank water during adult and F1 exposure

5.4.2.1 Water chemistry results

E1

The maximum E1 concentration was measured at 155ng/l at week twelve from the HM tank. Mean E1 concentrations over the entire study were lowest in the river pump inlet at 1.13 ± 0.22ng/l. The RW and RW+S tanks had similar mean E1 concentrations of 1.22 ± 0.46ng/l and 1.37 ± 0.63ng/l. The LM tank had a mean concentration of 30.96 ± 48.04ng/l and the HM tank had the highest E1 mean concentration of 60.54 ± 61.01ng/l (Table 5.1).

E2

The highest E2 measured concentration was at week 0 from the LM tank; 27.4ng/l. The mean measured concentrations of E2 ranged from 0.34 ± 0.09ng/l in the river pump inlet to 10.87 ± 7.84ng/l in the HM tank and 11.28 ± 11.46ng/l in the LM tank (Table 5.1).

EE2

The maximum measured concentration of EE2 (5.53ng/l) was found in the HM tank at week 12. Mean measured concentrations of EE2 ranged from 1.11 ± 0.94ng/l in the RW tank and 1.18 ± 0.91ng/l in the RW+S tank to 3.26 ± 2.39ng/l in the HM tank (Table 5.1). The mean measured concentrations of both the steroid and non-steroid oestrogens are given in Table 5.3.

NP

The NP limit of detection varied between sampling dates, the LOD was 0.1µg/l at weeks 0 and 16, and 0.5µg/l at weeks 4, 8 and 12. NP was not measure above the LOD for either of the RW or RW+S tanks for any of the sampling times. The highest measured concentration was 3.82µg/l at week 12 from the HM tank. Mean concentrations of NP ranged from 0.34 ± 0.22µg/l for both the RW and RW+S tanks to 1.47 ± 1.67µg/l in the HM tank (Table 5.2).

OP

The OP limit of detection varied between sampling dates. During weeks 0 and 16 LOD was 0.2µg/L and at weeks 4, 8 and 12 it was 1ug/l. None of the water samples taken from any of the tanks had measured concentrations of OP above the limit of detection (Table 5.2)

Table 5.1 Measured concentration (ng/l) of steroid oestrogens from the four exposure tanks plus the river water pump inlet measured over the 16 week exposure.

E1					
Week	**0**	**4**	**8**	**12**	**16**
Low mixture tank	2.92	21.1	116	5.73	9.05
High mixture tank	11.2	51.7	79.8	155	5.02
River water tank	2.03	<1	1.05	<1	<1
River water plus solvent tank	2.46	<1	<1	<1	<1
River water inlet	ns	ns	<1	<1	1.38
E2					
Week	**0**	**4**	**8**	**12**	**16**
Low mixture tank	27.4	3.96	22.9	0.836	1.32
High mixture tank	5.94	11.6	10.5	24.8	1.49
River water tank	10.1	5.71	<0.3	<0.3	<0.3
River water plus solvent tank	7.01	0.624	<0.3	<0.3	<0.3
River water inlet	ns	ns	<0.3	<0.3	<0.3
EE2					
Week	**0**	**4**	**8**	**12**	**16**
Low mixture tank	ns	4.4	4.11	1.21	1.76
High mixture tank	ns	5.11	1.18	5.53	1.21
River water tank	ns	0.375	0.37	1.37	2.34
River water plus solvent tank	ns	0.237	0.974	1.06	2.43
River water inlet	ns	ns	0.375	0.997	3.73

E1 and E2 were measured in the tank water on weeks 0, 4, 8, 12 and 16. EE2 was measured in the tank water on weeks 4, 8, 12 and 16. E1, E2 and EE2 were measured in the river inlet water on weeks 8, 12 and 16. All concentrations measured in nano-grams per litre (ng/l). Not sampled; ns. Water chemistry provided by Environment Agency's National Laboratory Service, Nottingham.

BPA

The BPA limit of detection varied throughout the exposure period and between water samples. The minimum limit of detection achieved for BPA was 0.04µg/l and the maximum was 1µg/L. The minimum detection limit (<0.04µg/l) was recorded for all tank water samples (RW, RW+S, LM and HM) taken at week 16. The minimum (<0.04µg/l) level was also recorded for RW and RW+S samples taken at week four, and for RW+S and LM water samples taken at week 0. The highest concentration of BPA actually measured

was 0.506µg/l from the HM tank at week 8. Mean measured concentrations of BPA ranged from 0.08 ± 0.08µg/l from the RW+S tank to 0.33 ± 0.32µg/l from the HM tank (Table 5.2).

Table 5.2 Measured concentration (µg/l) of non-steroid oestrogens (Nonylphenol, Octylphenol and Bisphenol A) from the four exposure tanks measured over the 16 week exposure.

NP					
Week	**0**	**4**	**8**	**12**	**16**
Low mixture tank	0.128	0.822	<0.5	<0.5	0.147
High mixture tank	0.118	2.65	0.6	3.82	0.170
River water tank	<1	<0.5	<0.5	<0.5	<0.1
River water plus solvent tank	<1	<0.5	<0.5	<0.5	<0.1
River water inlet	ns	ns	ns	ns	ns
OP					
Week	**0**	**4**	**8**	**12**	**16**
Low mixture tank	<0.2	<1	<1	<1	<0.2
High mixture tank	<0.2	<1	<1	<1	<0.2
River water tank	<0.2	<1	<1	<1	<0.2
River water plus solvent tank	<0.2	<1	<1	<1	<0.2
River water inlet	ns	ns	ns	ns	ns
BPA					
Week	**0**	**4**	**8**	**12**	**16**
Low mixture tank	<0.04	0.046	<0.2	<0.1	<0.04
High mixture tank	<0.1	0.180	0.506	<0.8	<0.04
River water tank	<0.16	<0.04	<0.2	0.139	<0.04
River water plus solvent tank	<0.04	<0.04	0.223	0.076	<0.04
River water inlet	ns	ns	ns	ns	ns

All concentrations measured in micro-grams per litre (µg/l). Not sampled; ns. Water chemistry provided by Environment Agency's National Laboratory Service, Nottingham

APEs

In addition to the above results nonylphenol ethoxylates (NP1EO and NP2EO) and octylphenol ethoxylates (OP1EO and OP2EO) were also measured in tank water (RW, RW+S, LM and HM) at weeks 0, 4, 8, 12 and 16. However, none were measured above the

limit of detection (LOD). The LOD for all four ethoxylates were <1µg/l at weeks 0 and 16, and <5µg/l at weeks 4, 8 and 12 with the exception of the HM tank at week 4 which had a LOD of <10µg/l for NP1EO and NP2EO. The nominal concentrations of NP1EO and NP2EO dosed to the low and high mixture tanks were 1µg/l and 5µg/l of NP1EO and 2µg/l and 7µg/l NP2EO, respectively.

Table 5.3 Mean measured and nominal concentration of steroid and non-steroid oestrogens from the oestrogenic mixtures experiment.

Tank	E1 (ng/l)	E2 (ng/l)	EE2 (ng/l)	NP (µg/l)	BPA (µg/l)	OP (µg/l)
RW	0.92 ± 0.7 a	3.25 ± 4.4 a,c	1.11 ± 0.9 a	0.17 ± 0.1 a	0.07 ± 0.05 a	0.34 ± 0.2 a
RW+S	1.07 ± 0.9 a	1.65 ± 2.7 a	1.18 ± 0.9 a	0.17 ± 0.1 a	0.07 ± 0.1 a	0.34 ± 0.2 a
LM nominal	**20**	**3**	**0.5**	**1.5**	**0.05**	**0.15**
LM actual	30.96 ± 48.0 b	11.28 ± 11.5 b,c	2.87 ± 1.6 a	0.32 ± 0.3 a	0.12 ± 0.2 a	0.34 ± 0.2 a
HM nominal	**100**	**10**	**1.5**	**6.0**	**0.1**	**0.6**
HM actual	60.54 ± 61.0 b	10.87 ± 7.8 b	3.26 ± 2.4 a	1.47 ± 1.7 a	0.23 ± 0.2 a	0.34 ± 0.2 a
Inlet	0.79 ± 0.5	0.24 ± 0.1	1.70 ± 1.8	not measured	not measured	not measured

River Water only tank (RW), River Water plus Solvent tank (RW+S), Low estrogenic Mixture tank (LM), High estrogenic Mixture tank (HM) and river water pump inlet (river water fed all tanks) over 16 week dosing period. Mean steroid oestrogen (Estrone E1, 17β-oestradiol E2, Ethinylestradiol EE2) concentrations measured in nanograms per litre (ng/l). Mean non-steroid oestrogens (Nonylphenol NP, Bisphenol A BPA, Octylphenol OP) concentrations meansured in micro-grams per litre (µg/l). Half LOD was used to calculate the mean. Mean ± standard deviation. Letters (a, b, c) indicate statistical similarity. Water chemistry provided by Environment Agency's National Laboratory Service, Nottingham.

Due to the differences seen between the nominal concentrations dosed into the tanks and the actual measure concentrations of each chemical from the water chemistry, a number of predictions were made. These were an attempt to understand where these differences arose. Comparisons were made between the actual concentrations and the predicted concentrations from the dilution factor or between the predicted EEQ from the actual chemical analysis and EEQ from YES assay.

5.4.2.2 Predicted concentrations of steroid and non-steroid oestrogens for the two mixture exposure tanks

Using the measured dosing pump rate and the river water flow rate into the two exposure tanks, actual dilution factor from each of the dosing reservoirs were calculated (Table 5.4 and Table 5.5).

Table 5.4 Predicted concentrations of steroid and non-steroid oestrogens (ng/l) in the low oestrogenic mixtures tank (LM).

Week	E1 (20)	E2 (3)	EE2 (0.5)	NP (1500)	BPA (50)	OP (150)
0	17.6	2.6	0.4	1318.7	44.0	131.9
2	18.2	2.7	0.5	1368.1	45.6	136.8
4	29.3	4.4	0.7	2200.0	73.3	220.0
6	22.0	3.3	0.6	1650.0	55.0	165.0
8	14.1	2.1	0.4	1054.9	35.2	105.5
10	20.0	3.0	0.5	1500.0	50.0	150.0
12	17.3	2.6	0.4	1297.3	43.2	129.7
16	22.3	3.3	0.6	16.71.4	55.7	167.1

The predictions were made using the predicted chemical concentrations in the mixing reservoir (from the amount added as stock) divided by the actual dilution factor into the tanks for each sampling point (Chemical and YES analysis). Numbers in brackets are nominal concentrations ng/l.

Table 5.5 Predicted concentrations of steroid and non-steroid oestrogens (ng/l) in the high oestrogenic mixtures tank (HM).

Week	E1 (100)	E2 (10)	EE2 (1.5)	NP (6000)	BPA (100)	OP (600)
0	133.3	26.7	2.0	8000.0	133.3	800.0
2	140.0	28.0	2.1	8400.0	140.0	840.0
4	130.0	26.0	2.0	7800.0	130.0	780.0
6	98.3	19.7	1.5	5897.6	98.3	589.8
8	63.7	12.8	1.0	3824.2	63.7	382.4
10	62.0	12.4	0.9	3720.0	62.0	372.0
12	74.6	14.9	1.1	4478.1	74.6	447.8
16	86.7	17.3	1.3	5200.0	86.7	520.0

The predictions were made using the predicted chemical concentrations in the mixing reservoir (from the amount added as stock) divided by the actual dilution factor into the tanks for each water sampling point (Chemical and YES analysis). Numbers in brackets are nominal concentrations ng/l.

5.4.2.3 Comparison of the predicted concentrations of each chemical dosed to the mesocosms and the actual measured concentrations from the chemical analysis

Chemical analysis was taken on weeks 0, 4, 8, 12 and 16. The average measured concentrations of EE2 and BPA were higher than the predicted ones in both the LM and the HM tanks. The LOD of OP was higher than the predicted concentration of OP for both tanks. The average measured concentrations of NP were lower than the predicted concentration in both the LM and HM tanks. Average measured concentrations of E1 and

E2 were higher than predicted in the LM tank, whereas, they were below the predicted concentrations in the HM tank. Statistical analysis found no significant difference between the predicted concentration and the measured concentration for E1 (P=0.630, t-test), E2 (P=0.753, M-W), OP (P=0.596, M-W) and BPA (P=0.341, M-W) from the LM tank. Significant differences were found between the measured and predicted concentrations for EE2 (P=0.013, M-W), and NP (P=0.001, t-test) from the LM tank. For the HM tank no significant difference was found between the predicted and the measured concentrations of E1 (P=0.265, T-test), E2 (P=0.112, t-test), EE2 (P=0.413, M-W), OP (P=0.096, t-test) and BPA (P=0.904, t-test). Only the measured concentration of NP from the HM tank was found to be significantly lower than the predicted concentration (P=0.005, t-test).

5.4.2.4 Oestrogen equivalents (EEQ) of the mesocosm tank water, as measured in Yeast Oestrogen Screen (YES)

Table 5.6 and Figure 5.2 show the measured YES EEQs for the mesocosm tanks and river inlet for each sampling period, and the mean for the entire 16 weeks. Statistical analysis found the EEQs from the YES assay to significantly differ between the tanks (P=0.007, K-W). The EEQ were found to be significantly higher in the LM or HM tanks compared to the RW tank (P=0.032). Both the LM and HM tanks also had significantly higher EEQs when compared to the RW+S tank (P=0.009), and the HM tank had a significantly higher EEQ than the river inlet (P=0.032). No significant differences were found between the RW and the RW+S tanks (P=0.337), the RW tank and the river inlet (P=1.000), the RW+S tank and the river inlet (P=0.471), the LM tank and the river inlet (P=0.054) or between the two mixtures tanks (P=0.522).

5.4.2.5 Predicted oestrogen equivalents (EEQ) of the two dosed mesocsom tanks using the predicted concentrations of each chemical from the dilution factor of the stocks

The predicted chemical concentrations in Tables 6.3.2 and 6.3.3 were used to predict the EEQs in the YES assay (Table 5.7). To predict the oestrogen equivalent (EEQ) of the mesocosm tank water as measured in the yeast oestrogen screen 1ng/l E2 or EE2 were assumed to equal 1ng/l EEQ (Segner et al. 2003a, Van den Belt et al. 2004). Whereas, E1 was assumed to be about half as potent as E2 in the yeast screen and therefore 1ng/l E1 was equal to 0.5ng/l EEQ (Van den Belt et al. 2004). The NP was assumed to be 4000 times less potent than E2 and the BPA 10000 times less potent (Beresford et al. 2000). The OP was assumed to be 2000 times less potent than E2 (Segner et al. 2003a).

Table 5.6 Measured EEQ ng/l for each mesocosm tank from the oestrogenic mixture experiment from the yeast oestrogen screen (YES) assay

	Week 2	Week 6	Week 8	Week 10	Week 12	Week 16	mean
RW	-	-	0	1.67	0	1.75	0.86 ± 0.99 a
RW+S	1.06	0	0	0	0	0.89	0.33 ± 0.51 a
LM	3.25	1.96	80.57	5.84	0.85	3.83	16.05 ± 31.66 b,c
HM	9.25	3.28	11.77	3.31	27.27	0.87	9.29 ± 9.72 b
Inlet	-	-	2.01	0	0	0.95	0.74 ± 0.96 a,c

River water only, RW; river water plus solvent, RW+S; low oestrogenic mixture, LM; high oestrogenic mixture, HM, and the river water inlet (which fed all tanks). Mean EEQ ± standard deviation. Letters (a,b,c) indicate statistical similarity. No water samples were taken for analysis from the RW tank or river inlet at weeks 2 and 6. Water samples taken at weeks 8, 12 and 16 were taken simultaneously for both YES assay and Chemical analysis (highlighted).

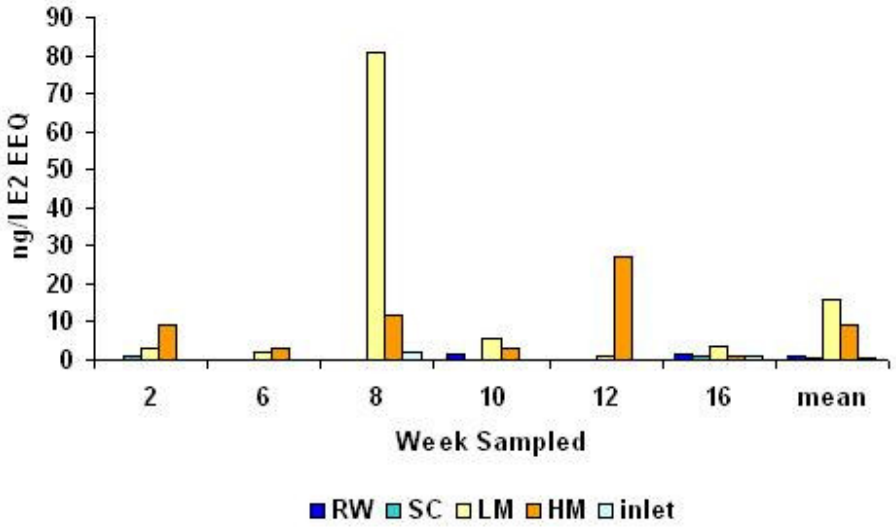

Figure 5.2 EEQs as measured in the yeast oestrogen screen (YES) assay for each sampling period for each of the mesocosm tanks in the oestrogenic mixtures experiment.

River water only, RW; river water plus solvent, RW+S; low oestrogenic mixture, LM; high oestrogenic mixture, HM and the river inlet (which fed all tanks). No water samples were taken from the RW tank and river inlet during weeks 2 and 6.

Table 5.7 Predicted EEQs (ng/l) for the two dosed mesocosm tanks from the oestrogenic mixtures experiment

	Week 2	Week 6	Week 8	Week 10	Week 12	Week 16	Predicted mean
LM	12.7	15.4	9.8	14.0	12.1	15.5	13.2 ± 2.2
HM	102.6	72.1	46.7	45.5	54.7	63.5	64.2 ± 21.4

Low oestrogenic mixture, LM; high oestrogenic mixture, HM. Predictions based on the predicted chemical concentrations in Tables 6.3.2 and 6.3.3. Mean ± standard deviation.

5.4.2.6 Comparison of the predicted EEQ from the chemical predictions and the measured EEQ from the YES assay

The average measured EEQ from the LM tank was slightly higher than the predicted EEQ from the predicted chemical concentrations. However, the average measured EEQ from the HM tank was much lower than the predicted EEQ. Statistical analysis (M-W) found the LM measured EEQ not to be significantly different from the predicted EEQ (P=0.055). Whereas, the predicted EEQs from the HM tank was significantly higher than the measured EEQ (P=0.002).

5.4.2.7 Predicted oestrogen equivalents (EEQ) of the mesocosm tank water using the measured water chemistry results

Table 5.8 gives the predicted EEQ for the mesocosm tank water samples from the measured water chemistry. To predict the oestrogen equivalent (EEQ) of the mesocosm tank water as measured in the yeast oestrogen screen the NP was assumed to be 4000 times less potent than E2 and the BPA 10000 times less potent (Beresford et al. 2000). The OP was assumed to be 2000 times less potent than E2 (Segner et al. 2003a). For measurements (OP, NP and BPA) below LOD the LOD was used in the calculations.

5.4.2.8 Comparison of the predicted EEQ from the chemical analysis and the measured EEQ from the YES assay

Only water samples taken at weeks 8, 12 and 16 were compared, as these were the only ones taken simultaneously for both chemical and YES analysis. The predicted and measured EEQs were compared for all the mesocsom tanks (RW, RW+S, LM, HM) and the river water inlet. On average, the predicted EEQs from the chemical analysis were higher than those actually measured in the YES assay, with the exception of the sample taken at week 8 from the river inlet (2.01ng/l EEQ).

5.4.2.9 Oestrogen equivalent (EEQ) measured by YES in the dosing reservoir water

To assess whether the reduced concentration of dosed chemicals or EEQ (from the nominal) occurred before entering the tank (i.e. in the mixing reservoirs), water samples were taken from each reservoir (RW+S, LM and HM) shortly after a new stock had been prepared and then again after 72hrs (i.e. just before the stock would normally be renewed). These water samples were then analysed in the YES assay. The mean EEQ values were reduced by around 30-60% in the mixtures dosing reservoirs after 72hrs (two samples taken in July). For example the mean initial (30 minutes after mixing stock with water) value from the HM mixing reservoir was 192 ± 169 µg/l EEQ and after 72 hours it was 83 ± 33 µg/l EEQ. The initial mean value in the LM mixing reservoir was 114 ± 112 µg/l EEQ and after 72 hours it was 82 ± 35 µg/l EEQ. The expected initial EEQs would have been around 27.9µg/l in the LM reservoir and 146.6µg/l in the HM reservoir. Samples were also taken and analysed from the river water plus solvent (RW+S) mixing reservoir, no oestrogenic activity was detected in any of these samples.

Table 5.8 Predicted EEQ in ng/l for the mesocsom tanks, in the oestrogenic mixtures experiment, from the measured chemical analysis.

	Week 0	Week 4	Week 8	Week 12	Week 16	Predicted Mean
RW	11.3	7.2	1.8	2.8	3.3	5.3 ± 3.9
RW+S	8.4	2.0	2.4	2.5	3.5	3.8 ± 2.6
LM	29.0	19.6	85.7	5.6	7.7	29.5 ± 32.8
HM	11.7	43.7	52.3	109.4	5.5	44.5 ± 41.5
Inlet	-.	-	1.2	1.8	4.85	2.6 ± 1.97

River water, RW; river water plus solvent, RW+S; low oestrogenic mixture, LM; high oestrogenic mixture, HM, and the river water inlet (that fed all tanks) Water samples taken at weeks 8, 12 and 16 (highlighted) were taken simultaneously for water chemistry and YES analysis, and therefore can be compared directly.

5.4.3 Effects of exposure to estrogenic mixtures on adult *P. corneus* reproduction and survival

5.4.3.1 Survival

Adult snail survival was lower in RW exposed snails compared to RW+S, LM and HM groups until week 10 (Figure 5.3), after which snails from the RW+S exposure suffered high mortalities. The RW, LM and HM groups each had 25 out of a total of 36 snails surviving at the end of the 16 week exposure. Only 5 out of 36 from the RW+S tank survived (Figure 5.3). Significant differences in the number of surviving snails were found

at week 4 (P= 0.015, K-W), 12 (P= 0.46, K-W), 14 (P< 0.001, ANOVA) and 16 (P<0.001, ANOVA). At week 4 the RW exposed snails were found to have significantly lower survival than RW+S (P= 0.019, M-W) and HM (P= 0.019, M-W) but no significant difference was found between RW and LM (P= 0.093). At week 12, the RW+S snails survival was significantly lower than those from the LM tank (P= 0.017), but no significant differences (P> 0.05) in survival was found between the other treatment groups. At both weeks 14 and 16 RW+S had significantly lower survival than RW, LM or HM (P< 0.001, LSD) exposed snails (Figure 5.3).

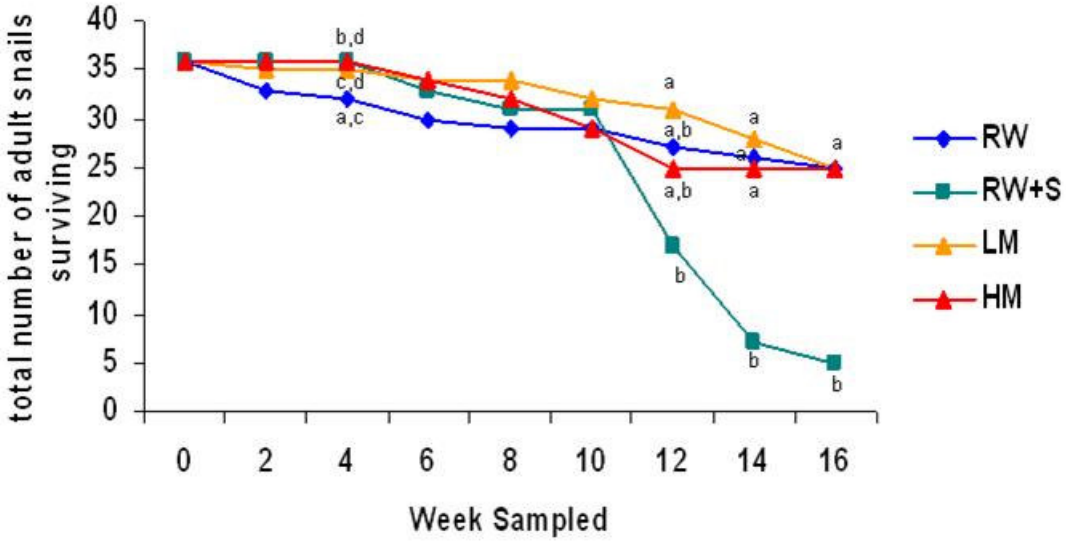

Figure 5.3 Total number of surviving adult *P. corneus* counted at each fortnightly sampling point from each treatment in the oestrogenic mixture experiment.

Snails exposed to River Water only (RW), River Water plus Solvent (RW+S), Low oestrogenic Mixture (LM) or High oestrogenic Mixture (HM). A Total of 36 adult snails were allocated to each treatment group. Letters (a, b, c, d) indicate statistical similarity.

After week 16 of the exposure the dosing pumps administering the oestrogenic mixture to the tanks were switched off and the tanks were left to depurate in running river water over the weekend. After the weekend the adult snails were removed from the tanks for the final time. During this short depuration a further 3 adult snails died. Two snails from the RW exposure tank and 1 snail from the LM exposure tank; leaving a total of 23 snails from the RW tank, 5 from the RW+S tank, 24 from the LM tank and 25 from the HM tank. At this point the remaining snails were sampled; their size and weight were measured and the soft

tissue was fixed for histology (Section 3.5.7), the data from these specimens are presented in section 5.4.3.3.

5.4.3.2 Reproduction

Mean eggs laid per adult snail

The mean numbers of eggs laid per snail per fortnight were similar at each sampling point between the four exposure groups. Egg laying was well underway when the sampling period started in May. Peak egg laying occurred at week four (1st June 2006) for the RW+S exposed snails (355.1 eggs/snail/fortnight) and week six (15th June) for the snails in the three other exposure groups. At week 6 the LM exposed snails laid the highest number of eggs per snail (344.7), the RW exposed snails laid a maximum of 336.2 eggs per snail and the HM exposed snails laid 296.7 eggs per snail. After week 6 in all treatment groups egg laying declined, however LM snail continued to lay more eggs than the other exposure groups at week 16 (23rd August) (Figure 5.4). Statistical analysis found no significant differences between the mean numbers of eggs laid per snail per fortnight between the four treatment groups.

Mean egg masses laid per snail

The mean number of egg masses (EMs) laid per snail per fortnight was at its peak for the LM and HM exposed snails at week 2 (the first sampling point). The LM snails laid a mean of 8.9 EMs per snail and the HM exposed snails laid 7.9 EMs per snail over the two week period. Snails exposed to RW+S had their peak in egg mass laying at week 4 (8.9 EMs per snail). Snails from the RW exposure peaked later at week 6. The LM and HM exposed snails also had a second lower peak at week 6. Whereas, RW+S exposed snails had their second peak at week 10, just prior to the high mortality recorded in this group. After week 6 the LM, HM and RW exposed snails steadily decreased the number of egg masses laid per snail. However, the LM exposed snails laid more egg masses than HM or RW exposed snails (Figure 5.5). Over the entire sixteen weeks of exposure LM exposed snails laid the most EM per snail (Table 5.9). No statistically significant differences were found between the four treatments groups mean egg masses laid per snail per fortnight.

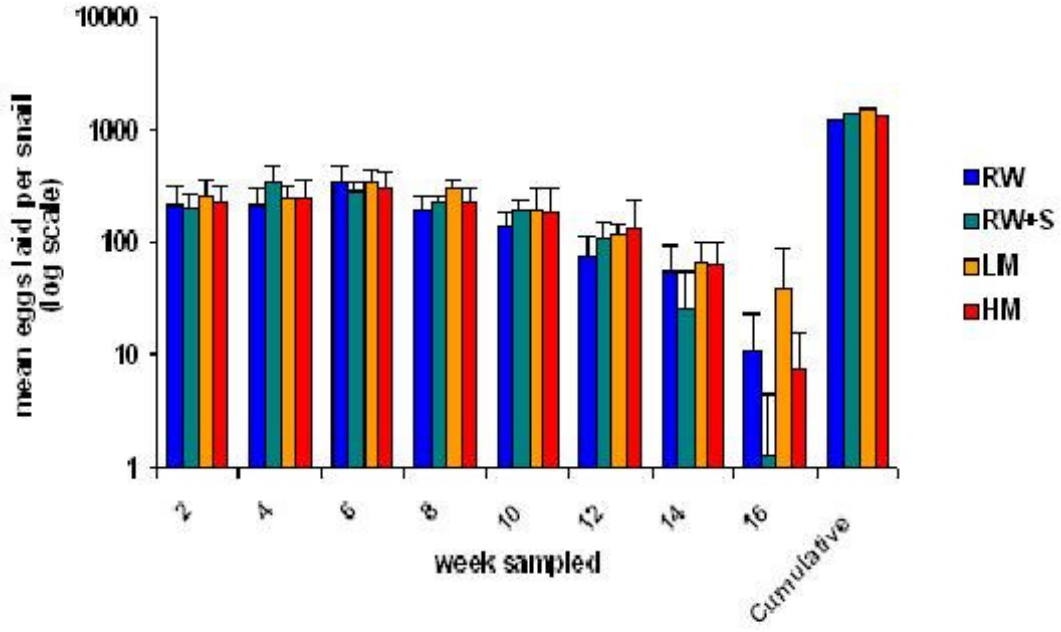

Figure 5.4 Adult *P. corneus* reproductive output during oestrogenic mixtures experiment.

Snails exposured to River water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). Mean number of eggs laid per adult snail per fortnight and cumulatively, error bar indicate standard deviation.

Table 5.9 Total number of egg masses laid per snail over sixteen weeks of exposure by adult *P. corneus* in the oestrogenic mixtures experiment

	River Water (RW)	River Water plus Solvent (RW+S)	Low oestrogenic Mixture (LM)	High oestrogenic Mixture (HM)
Total egg masses (EMs) laid per snail	36.0 ± 2.99	38.9 ± 2.96	43.2 ± 2.67	36.4 ± 2.61

Snails exposed to; River water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). Mean ± Standard deviation.

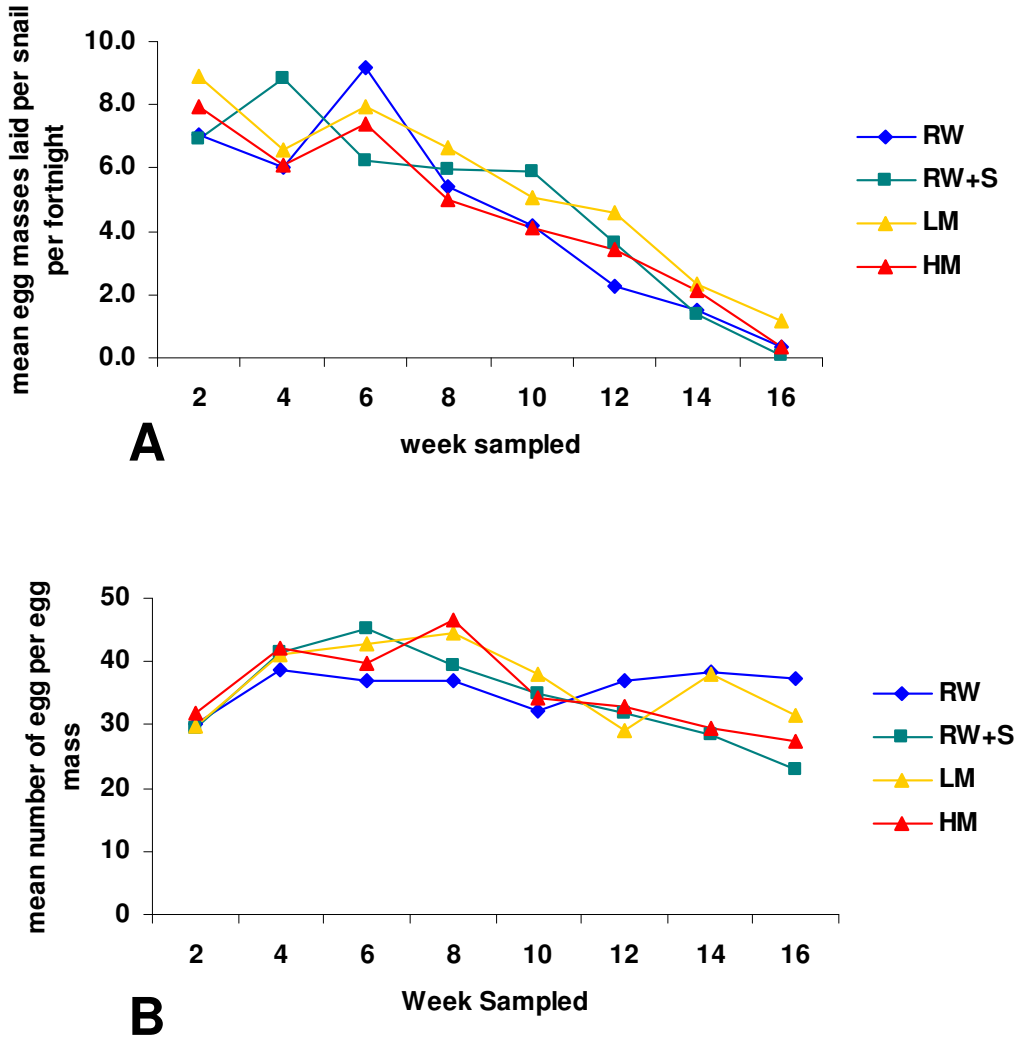

Figure 5.5 Number of egg masses and eggs per egg mass laid by *P. corneus* adults during oestrogenic mixtures experiment.

Snails exposed to either River water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). (A) mean number of egg masses laid per adult snail per fortnight. (B) mean number of eggs in each egg mass.

Mean eggs per egg mass

At the first sampling point the average numbers of eggs per egg mass were similar across the four treatments group with between 30-32 eggs per mass (Figure 5.5). The RW+S exposed snails had a peak of 45.2 eggs per egg mass at week 6, after which a steep decline occurred (down to 23 eggs per mass at week 16). The LM and HM exposed snails both peaked at week 8 with the LM exposed snail having 44.4 eggs per mass and the HM exposed snails having 46.6 eggs per mass. After week 8 the HM exposed snail's eggs per mass dropped more steeply than those from the LM exposure. The eggs per mass in the RW exposed snails increased slightly at week 4 (to 38.5 egg per mass) and then remained at between 37 and 38.3 eggs per mass for the rest of the exposure period, apart from a

slight drop at week 10 (32.3 eggs per mass). Over the whole exposure period the LM exposed snails laid the largest egg masses (i.e. with the most eggs per mass) with a mean of 36.8 eggs per mass. The RW+S snails laid the smallest with 34.2 eggs per mass. The HM and RW exposed snails laid similarly sized masses (35.6 and 35.9 eggs per mass, respectively). A significant difference was found for the number of eggs per egg mass laid at week 8 (P= 0.002, ANOVA). Post hoc analysis found the RW and RW+S exposed snails to have significantly less eggs per mass compared to the LM (P= 0.006, 0.048, LSD) and the HM (P= 0.001, 0.006, LSD) exposed snails; there were no significant differences between the RW and RW+S (P= 0.332, LSD) or between the LM and HM exposed snails egg mass sizes (P= 0.357, LSD).

5.4.3.3 Size and weight

At the start of the experiment the mean shell size (diameter and aperture) were similar across all treatment groups. The mean shell diameter ranged from 21.9 to 22.5 mm, and the mean shell aperture ranged from 9.5 to 9.9 mm. The mean total weight was also similar between groups ranging from 1.8 to 2.0 grams. One way ANOVA found no significant differences between the four treatment groups for shell diameter (P= 0.633), shell aperture (P= 0.505) or total weight (P= 0.575).

After sixteen weeks of exposure mean shell diameters were smallest in the RW exposure (24.3mm) and largest in RW+S exposed snails (25.9mm). The mean total body weight (shell on) was also lowest in the RW exposed snails (24.0g) and largest in RW+S (25.9g) (however, only 5 snails from the RW+S exposure survived to the end of exposure). One way ANOVA found no significant differences between the four treatment groups after 16 weeks of exposure. However, a low P value (P= 0.065) was found for mean shell diameter.

Growth over the 16 weeks of exposure was highest from the RW+S exposed snails. However, an extremely high mortality was also observed in this group (see above). The next highest growth rate was found in snails exposed to the HM. These snails grew on average 14.8% in shell diameter and 30.7% in total weight (Table 5.10 below) compare to 9.1% increase in shell diameter and 21.4% increase in total weight seen in the RW exposed snails.

Table 5.10 Size weight and growth of adult *P. corneus* before and after exposure in the oestrogenic mixtures experiment

	River Water (RW)	River Water plus Solvent (RW+S)	Low oestrogenic Mixture (LM)	High oestrogenic Mixture (HM)
START Shell diameter mm	22.1 ± 2.2	21.9 ± 2.1	22.5 ± 2.7	22.0 ± 2.1
FINISH Shell diameter mm	24.3 ± 2.1	25.9 ± 2.2	25.4 ±1.6	25.8 ± 2.3
Growth %	9.1%	15.7%	11.2%	14.8%
START Total weight g	1.9 ± 0.6	1.9 ± 0.5	2.0 ± 0.7	1.8 ± 0.5
FINISH Total weight g	2.4 ± 0.6	2.8 ± 0.9	2.5 ± 0.6	2.6 ± 0.7
Growth %	21.4%	33.7%	20.2%	30.7%

Snails expoed to River water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). The mean shell length and total weight given plus mean percentage growth over the 16 weeks. Mean ± standard deviation

5.4.4 Effects of oestrogenic mixture exposure on adult *P. corneus* gonad histopathology

As stated in section 5.4.3.1, a total of 77 adult snails (RW; 23, RW+S; 5, LM; 24, HM; 25) were sampled and fixed for histopathological analysis from this experiment. However, due to time constraints a maximum of 20 specimens were sectioned, stained and analysed from each treatment (RW; 20, RW+S; 5, LM; 18, HM; 18). Four sections of gonad were analysed per adult snail. As the main focus of this research was on the possible effects of developmental exposure to the oestrogenic mixture, rather than on adult exposure, this low number of samples analysed was decided upon as a compromise between available time and quantity of data.

Due to the complex nature of the *P. corneus* gonad, analysis was split between different areas or cell types within the gonad.

All snails from the RW and RW+S had four out of four sections with sertoli cells attached to the acini walls. One snail from the LM had one out of four sections with no sertoli cells attached to the acini walls. One snail from the HM exposure had all four sections of gonad with no sertoli cells attached; however, this was the parasitised individual and could to be

due to the infection. A dose-dependant decrease in normal vitellogenic area activity (maturing oocytes with follicle cells and/or sperm with sertoli cells) was found see Table 5.11 and Figure 5.10. However, this reduction was not statistically significant P= 0.539 (K-W).

Adult snails from the RW+S exposure had the highest amount of cell cover (developing oocytes, sperm and supporting cells) of the acini walls. Adult snails from the HM treatment had the least (

Table **5.12**). A significant difference was found between the four exposure groups for acini wall cover P= 0.010 (ANOVA). Post hoc analysis found HM exposed snails to have significantly less acini wall covered than RW (P= 0.003 LSD) or the RW+S (P= 0.011) exposed snails. No significant difference was found between the other treatment groups.

Table 5.11 Percentage of Adult *P. corneus* snails with varying levels of active vitellogenic areas from the oestrogenic mixtures experiment

Percent of acini with active vitellogenic area	Score	Percentage of adult snails from the River Water exposure (RW)	Percentage of adult snails from the River pus Solvent Water exposure (RW+S)	Percentage of adult snails from the Low oestrogenic mixture exposure (LM)	Percentage of adult snails from the High oestrogenic Mixture exposure (HM)
<10%	1	0	0	7%	0
10-30%	2	5 %	0	0	0
30-50%	3	0	0	0	18%
50-70%	4	45%	40%	50%	53%
>70%	5	50%	60%	43%	29%
		a	a	a	a

Snails sacrificed after four months exposure to River water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). Histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 20; RW+S, n = 5; LM, n = 18; HM n = 18, (one parasitised individual was omitted)). Four sections of gonad were analysed per adult snail. Letter (a) indicates statistical similarity.

Only one snail, the heavily parasitised individual from the HM exposure, had any acini wall disruption, which was probably due to the infestation. All the other adult *P. corneus* analysed (from all treatments) had intact acini walls.

Table 5.12 Percentage of Adult *P. corneus* snails with varying level of acini wall cell cover from the oestrogenic mixtures experiment

Percent of acini wall covered by germ cells and/or supportive cells	Score	Percentage of adult snails from the River Water exposure (RW)	Percentage of adult snails from the River pus Solvent Water exposure (RW+S)	Percentage of adult snails from the Low oestrogenic mixture exposure (LM)	Percentage of adult snails from the High oestrogenic Mixture exposure (HM)
<10%	1	0	0	0	6%
10-30%	2	5%	0	29%	12%
30-50%	3	20%	0	0	35%
50-70%	4	15%	20%	29%	35%
>70%	5	60%	80%	42%	12%
		a	a	a,b	B

Snails sacrificed after four months exposure to either River water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). Histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 20; RW+S, n = 5; LM, n = 18; HM, n = 18 (one parasitised individual was omitted)). Four sections of gonad were analysed per adult snail. Letters (a, b) indicate statistical similarity.

Sertoli cells sloughing into lumen

The adult snails from the RW+S treatment had the least number of sections of gonad with Sertoli cells sloughing into the acini lumen, whilst snails from the HM treatment had the highest level (Table 5.13). A significant difference was found between the four treatment groups P= 0.015 (K-W). Post hoc analysis found the RW exposed snails to have significantly less Sertoli cell sloughing compare to the LM (P= 0.050 M-W) and HM (P= 0.004) exposed snails but not the RW+S (P= 0.617) exposed snails. However, no significant differences were found between the RW+S exposed snails and the LM (P= 0.194) or HM (P= 0.079) exposed snails or between LM and HM (P= 0.335) exposed snails.

Spermatogonium sloughing into lumen

Spermatogonium cell sloughing into the acini lumen was found most frequently in the LM exposed snails and least frequent in RW+S exposed snails (Table 5.13). However, no significant difference was found between the four treatment groups (P= 0.477, K-W).

Spermatocytes sloughing into the lumen

Spermatocyte sloughing into the lumen was found most frequently in the HM exposed snails and least frequent in RW+S exposed snails (Table 5.13). No significant difference was found between the four treatment groups P= 0.063 (K-W).

Table 5.13 The percentage of adult *P. corneus* snails with varying level of immature spermatogenic cells sloughing into the acini lumen from the oestrogenic mixtures experiment

Number of sections affected	Percentage of Adults with Sertoli cells sloughing into lumen				Percentage of Adults with Spermatogonium sloughing into lumen				Percentage of Adults with Spermatocyte sloughing into lumen				Percentage of Adults with Spermatid sloughing into lumen			
	RW	RW+S	LM	HM	RW	RW+S	LM	HM	RW	RW+S	LM	HM	RW	RW+S	LM	HM
None	95	100	71.4	52.9	30	20	7.1	23.5	85	80	50	41.2	35	40	21	29.4
1 out of 4	5	0	7.1	17.6	10	20	0	11.8	0	20	0	11.8	20	20	0	17.6
2 out of 4	0	0	14.3	5.9	5	40	21.4	11.8	0	0	0	11.8	5	20	36	5.9
3 out of 4	0	0	7.1	17.6	10	0	21.4	5.9	0	0	21	5.9	10	20	7.1	29.4
4 out of 4	0	0	0	5.9	45	20	50	47.1	15	0	29	29.4	30	0	36	17.6
	A	ab	b	b	a	a	a	a	a	a	A	a	a	a	A	a

Snails sacrificed after four months exposure to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). Histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 20; RW+S, n = 5; LM, n = 18; HM, n = 18 (one parasitised individual omitted)). Four sections of gonad were analysed per adult snail. Letters (a, b) indicate statistical similarity.

Spermatids sloughing into the lumen

Spermatid sloughing into the lumen was found most frequently in the LM exposed snails and least frequently in RW+S exposed snails (Table 5.13). No significant difference was found between the four treatments (P= 0.531, ANOVA) for spermatids sloughing into the lumen.

Mature spermatogenic cells in the lumen

Spermatozoa only

The adult snails exposed to the RW+S had the highest frequency of sections of gonad with just mature spermatogenic cells in the acini lumen, snails exposed to the LM had the lowest (Table 5.14). No significant difference was found between the four treatment groups (P= 0.120 K-W).

Table 5.14 The percentage of adult *P. corneus* snails with varying numbers of sections of gonad with only mature spermatozoa in the acini lumen from the oestrogenic mixtures experiment

No. of sections with only mature spermatozoa in the acini lumen	Percentage of adult snails from the River Water exposure (RW)	Percentage of adult snails from the River pus Solvent Water exposure (RW+S)	Percentage of adult snails from the Low oestrogenic mixture exposure (LM)	Percentage of adult snails from the High oestrogenic Mixture exposure (HM)
0 out of 4	45%	20%	64%	64.7%
1 out of 4	10%	0	29%	5.9%
2 out of 4	5%	40%	0	0
3 out of 4	15%	20%	0	23.5%
4 out of 4	25%	20%	7%	5.9%
	A	a	a	a

Snails sacrificed after four months exposure to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). Histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 20; RW+S, n = 5; LM, n = 18; HM, n = 18 (one parasitised individual omitted)). Four sections of gonad were analysed per adult snail. Letters (a) indicate statistical similarity.

Occurrence of acini with immature spermatogenic cells in lumen

The percentage of acini (per section of gonad) affected by immature spermatogenic cells sloughing into the lumen was lowest in snails exposed to the RW+S and highest in those exposed to the LM (Table 5.15). No significant difference was found between the four treatment groups P= 0.581 (ANOVA).

Oogenesis stages present

The percentage of different stages of oocytes present in the gonad was similar across all treatments (Table 5.16). The mean percentage of early oocyte stages (one and two) was highest in LM exposed snails. The mean percentage of mature oocytes (stage five) was highest in RW+S exposed snails. One way ANOVA found significant differences between the groups for stage 2 (P= 0.005) and 3 (P= 0.015) oocytes. No significant difference was found for stage 1 (P= 0.140), 4 (P= 0.168), 5 (P=0.487) oocytes or degenerating oocytes (P= 0.057). Post hoc analysis found snails from the HM exposure to have a significantly lower percentage of stage 2 oocytes compare to those snails exposed to the RW (P= 0.024 LSD) or the LM (P<0.001). No significant difference was found in the percentage of stage 2 oocytes between the other treatment groups. The LM exposed snails were found to have a significantly lower percentage of stage 3 oocytes than the RW (P= 0.001 LSD) or the HM (P= 0.026) exposed snails. No significant difference in the percentage of stage 3 oocytes was found between the other treatment groups.

Table 5.15 The percentage of adult *P. corneus* snails affected by varying percentage of acini with immature spermatogenic cells sloughing into the lumen from the oestrogenic mixtures experiment

Percentage of acini affected by immature spermatogenic cells sloughing into the lumen	Score	Percentage of adult snails from the River Water exposure (RW)	Percentage of adult snails from the River pus Solvent Water exposure (RW+S)	Percentage of adult snails from the Low oestrogenic mixture exposure (LM)	Percentage of adult snails from the High oestrogenic Mixture exposure (HM)
<10%	**1**	55%	80%	36%	41.2%
10-30%	**2**	20%	0	43%	17.6%
30-50%	**3**	15%	20%	0	23.5%
50-70%	**4**	10%	0	0	5.9%
>70%	**5**	0	0	21%	11.8%
		a	a	a	a

Snails sacrificed after four months exposure to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). Histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 20; RW+S, n = 5; LM, n = 18; HM, n = 18 (one parasitised individual was omitted)). Four sections of gonad were analysed per adult snail. Letter (a) indicates statistical similarity.

Table 5.16 The mean percentage of different stages of oogenesis in adult *P. corneus* from the oestrogenic mixture experiment

Stage of oocyte maturation	Mean percent of oogenesis stage after River Water exposure	Mean percent of oogenesis stage after River Water plus Solvent exposure	Mean percent of oogenesis stage after Low oestrogenic Mixture exposure	Mean percent of oogenesis stage after High oestrogenic Mixture exposure
Stage 1	22.4 ± 9.1 a	17.2 ± 3.5 a	26.7 ± 6.5 a	22.6 ± 8.7 a
Stage 2	24.9 ± 4.3 a	24.1 ± 3.8 ab	27.3 ± 3.6 a	21.8 ± 4.2 b
Stage 3	25.8 ± 5.0 a	23.5 ± 2.6 ab	20.4 ± 4.2 b	24.2 ± 4.9 a
Stage 4	12.4 ± 5.1 a	17.7 ± 3.4 a	12.3 ± 4.2 a	13.8 ± 5.7 a
Stage 5	1.5 ± 1.4 a	2.4 ± 3.2 a	1.5 ± 1.6 a	1.1 ± 1.4 a
Degenerating	13.0 ± 5.2 a	15.2 ± 3.1 a	11.9 ± 4.5 a	16.5 ± 5.3 a

Snails sacrificed after four months exposure to either River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). Histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 20; RW+S, n = 5; LM, n = 18; HM, n = 18 (one parasitised individual was omitted)). Four sections of gonad were analysed per adult snail. Stage 1 (oogonium) is the youngest stage of oogenesis, stage 5 is ready to be ovulated. Oogenesis stages based on description by de Jong-Brink et al (De Jong-Brink et al. 1976). Letters (a, b) indicate statistical similarity.

Parasitism

Histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 20; RW+S, n = 5; LM, n = 14; HM, n = 18). Of all these snails, only one showed parasitic infection. The one case of infection was extremely heavy and had eliminated the whole gonad. The infected snail came from the HM exposure.

5.4.5 Effects of oestrogenic mixtures on developmentally exposed F1 *P. corneus*

5.4.5.1 Survival and growth at end of exposure

At the end of the exposure the total numbers of F1 *P. corneus* found in the mesocosm tanks were highest in the oestrogenic mixture exposed tanks. A total of 2129 F1s were collected from the LM tank. 461 F1 were collected from the HM tank, 239 from the RW tank and 213 from the RW+S tank (Table 5.17). However, the vast majority of F1s collected from the LM tank were very small (young hatchlings). The percentages of F1 snails above 10 mm shell diameter (normal minimum sexually active size) were highest from the RW (44%) and the RW+S tanks (39%). The percentages of F1s above 10 mm from the oestrogenic mixture tanks were low (HM, 5% and LM, 6%).

Table 5.17 The total number and number per size class of *P. corneus* F1s from each mesocosm tank after dosing ended (September 2006).

Shell Diameter	River Water (RW) exposed	River Water plus Solvent (RW+S) exposed	Low oestrogenic Mixture (LM) exposed	High oestrogenic Mixture (HM) exposed
<10 mm	134 (56%)	129 (60%)	2000 (94%)	440 (95%)
>10 mm	105 (44%)	84 (40%)	129 (6%)	21 (5%)
Total	239	213	2129	461

Percentage of each size range given in brackets

5.4.5.2 Size and weight

Approximately 40 (only 20 from HM) F1s of above 10 mm shell diameter (presumed sexually mature) were removed from each treatment for further analysis in September 2006 (after the end of exposure). A sub-sample of 20 F1s from each treatment were analysed histologically. The F1s developmentally exposed to the HM were significantly smaller (shell diameter; mean 12.4 ± 2.14 mm) and lighter (total weight; mean 0.39 ± 0.2 g and soft body weight; mean 0.17 ± 0.08 g) than those from the LM (diameter; mean 14.0 ± 1.5 mm (P= 0.005), total weight; mean 0.6 ± 0.19 g (P= 0.002), soft body weight; 0.25 ± 0.08 g (P= 0.004)), the RW+S (diameter; 14.2 ± 2.0 mm (P= 0.002), total weight; 0.60 ± 0.23 g (P= 0.001), soft body weight; 0.24 ± 0.08 g (P= 0.004)) or the RW (diameter; 14.9 ± 1.4

mm (P< 0.001), total weight; 0.68 ± 0.17 g (P< 0.001), soft body weight; 0.26 ± 0.08 g (P< 0.001)) exposed snails (Figure 5.6).

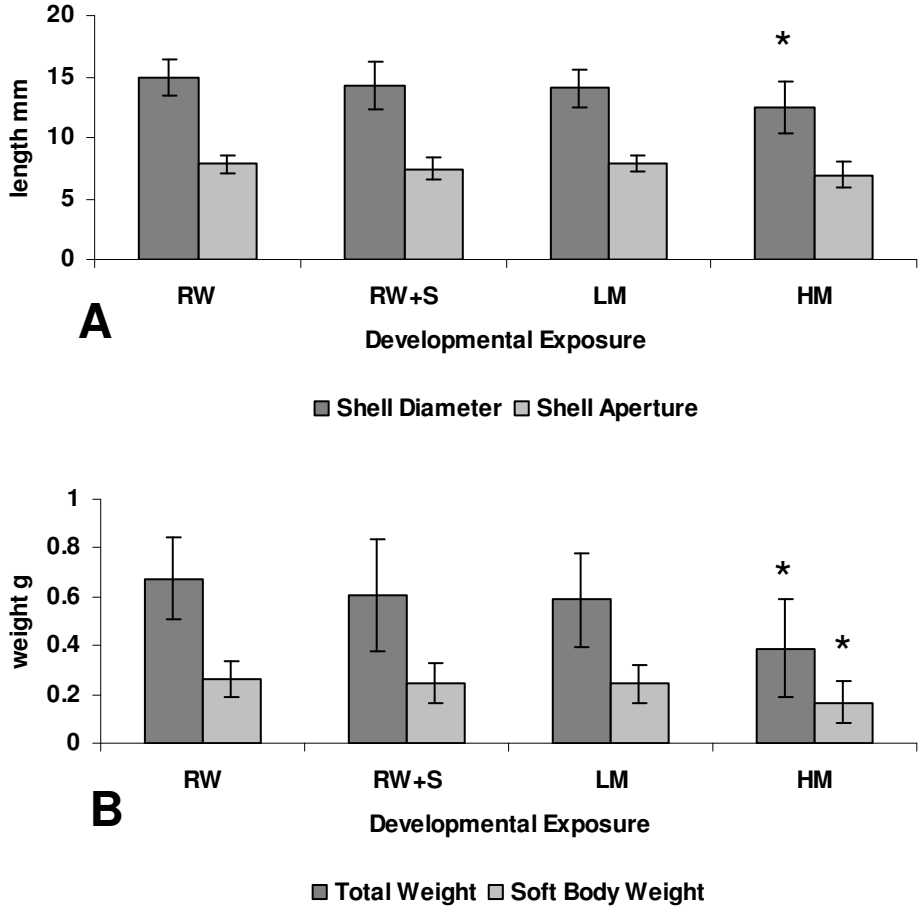

Figure 5.6 Size and weight of the F1 *P. corneus* sampled from the four treatment tanks in September 2006.

Mean shell length and aperture (A) and mean total weight and soft body weight (B). Error bars indicate standard deviation around the mean. Star (*) indicates significant difference (P<0.05) from other treatments (ANOVA).

5.4.6 Histopathology of *P.* corneus F1 immediately after exposure to oestrogenic mixtures

As stated above, in September 2006 (after dosing finished) approximately 40 F1s from each treatment (river water; RW, river water plus solvent; RW+S, low dose oestrogenic mixture; LM and high dose oestrogenic mixture; HM) were taken from the tanks and fixed for histopathological analysis. However, due to time constraints only around half (RW n=20, RW+S n=21, LM n=21, HM n=20) of these specimens were sectioned, stained and analysed; the data from these specimens is presented in the following sections.

Due to the complex nature of the *P. corneus* gonad analysis was split between different areas or cell types within the gonad. The majority of snails sampled from all treatments had all stages of oogenesis and spermatogenesis present.

5.4.6.1 Effects on the hermaphrodite gonad

One snail from the HM and the RW treatments had no Sertoli cells attached to the acini walls in one out of five sections of the gonad analysed. One snail from the RW+S exposure also had no Sertoli cells attached to the acini walls in one out of five sections of gonad analysed, another RW+S exposed snail had no Sertoli cells attached to the acini wall in two out of five sections analysed. All other snails (from all four treatments) had Sertoli cells attached to the acini walls in five out of five sections. Analysis using Kruskal-Wallis Test found no significant difference between the four exposure groups (P= 0.546).

A dose dependant decrease in normal vitellogenic area activity (maturing oocytes with follicle cells and/or sperm with Sertoli cells) was found (Table 5.18 and Figure 5.10). Analysis using One way ANOVA found a significant difference between the four exposure groups (P= 0.015). Post hoc analysis (LSD) found the HM exposed F1 have significantly less active vitellogenic areas compared to those from the LM (P= 0.020), the RW+S (P=0.008) and the RW (P= 0.004) exposures. No significant differences in vitellogenic area activity were found between the other treatment groups.

Table 5.18 The percentage of F1 *P. corneus* snails with varying level of acini vitellogenic area activity from the oestrogenic mixtures experiment

Percent of acini with active vitellogenic area	Score	Percentage of F1 snails from the River Water exposure (RW)	Percentage of F1 snails from the River pus Solvent Water exposure (RW+S)	Percentage of F1 snails from the Low oestrogenic mixture exposure (LM)	Percentage of F1 snails from the High oestrogenic Mixture exposure (HM)
<10%	1	10%	0	0	6%
10-30%	2	10%	5%	5%	24%
30-50%	3	0	30%	37%	29%
50-70%	4	45%	50%	47%	41%
>70%	5	35%	15%	11%	0
		a	a	a	b

Snails sacrificed after developmental exposure to River Water only (RW, n=20), River Water plus Solvent (RW+S, n=21), river water plus Low oestrogenic Mixture (LM, n=21) or river water plus High oestrogenic Mixture (HM, n=20). Five sections of gonad were analysed per F1 snail. Letters (a, b) indicate statistical similarity.

F1 *P. corneus* from the LM developmental exposure had the least acini wall disruption (Table 5.19). Those from the RW+S had the most. Analysis using Kruskal-Wallis Test found no significant difference between the four different exposure groups (P= 0.114).

Table 5.19 The percentage of F1 *P. corneus* snails with varying level of acini wall disruption from the oestrogenic mixtures experiment

Number of sections affected by acini wall disruption	Percentage of F1 snails from the River Water exposure (RW)	Percentage of F1 snails from the River pus Solvent Water exposure (RW+S)	Percentage of F1 snails from the Low oestrogenic mixture exposure (LM)	Percentage of F1 snails from the High oestrogenic Mixture exposure (HM)
0 out of 5	55%	35%	68%	65%
1 out of 5	0	10%	16%	5%
2 out of 5	0	15%	0	10%
3 out of 5	20%	15%	5%	10%
4 out of 5	10%	15%	0	10%
5 out of 5	15%	10%	11%	0
	a	a	a	a

Snails sacrificed after developmental exposure to River Water only (RW, n=20), River Water plus Solvent (RW+S, n=21), river water plus Low oestrogenic Mixture (LM, n=21) or river water plus High oestrogenic Mixture (HM, n=20). Five sections of gonad were analysed per F1 snail. Letter (a) indicates statistical similarity.

The amount of cell (developing oocytes, sperm and supporting cells) cover of the acini walls was highest in F1 *P. corneus* from the LM exposure and lowest in RW+S exposed snails (Table 5.20). Analysis using Kruskal-Wallis test found a significant difference between groups ($P<0.001$). Post hoc analysis (M-W) found no significant differences between the HM and the LM exposed snails ($P= 0.246$) or between RW and RW+S exposed snails ($P= 0.355$). However, the RW and RW+S exposed snails had significantly less acini wall cover when compared to the HM ($P= 0.037$ and 0.004 respectively) or the LM ($P< 0.001$ and <0.001, respectively) exposed snails.

Table 5.20 The percentage of F1 *P. corneus* snails with varying level of acini wall cell cover from the oestrogenic mixtures experiment

Percent of acini wall covered by germ cells and/or supportive cells	Score	Percentage of F1 snails from the River Water exposure (RW)	Percentage of F1 snails from the River pus Solvent Water exposure (RW+S)	Percentage of F1 snails from the Low oestrogenic mixture exposure (LM)	Percentage of F1 snails from the High oestrogenic Mixture exposure (HM)
<10%	1	10%	25%	0	5%
10-30%	2	45%	55%	5%	30%
30-50%	3	30%	20%	30%	10%
50-70%	4	5%	0	35%	25%
>70%	5	10%	0	30%	30%
		a	a	b	b

Snails sacrificed after developmental exposure to River Water only (RW, n=20), River Water plus Solvent (RW+S, n=21), river water plus Low oestrogenic Mixture (LM, n=21) or river water plus High oestrogenic Mixture (HM, n=20). Five sections of gonad were analysed per F1 snail. Letters (a, b) indicate statistical similarity.

5.4.6.2 Immature spermatogenic and supportive cells sloughing into the lumen

Sertoli cells sloughing into the lumen

F1s from the RW+S had the least number of sections of gonad with Sertoli cells sloughing into the acini lumen, whilst the LM exposed snails had the most (Table 5.21). No significant difference was found between the four exposure groups (P= 0.664, ANOVA).

Spermatogonium sloughing into the lumen

F1s from the LM exposure had the least number of sections of gonad with spermatogonium sloughing into the acini lumen, whilst the HM exposed snails had the most (Table 5.21). No significant difference was found between the four exposure groups (P= 0.308, ANOVA).

Spermatocyte sloughing into the lumen

F1s from the RW+S exposure had the least number of sections of gonad with spermatocytes sloughing into the acini lumen, whilst the RW exposed snails had the most (Table 5.21). No significant difference was found between the four exposure groups (P= 0.157, K-W).

Spermatids sloughing into the lumen

F1s from the RW exposure had the least number of sections of gonad with spermatids sloughing into the acini lumen, the HM exposed snails had the most (Table 5.21). One way ANOVA found a significant difference between exposure groups (P= 0.002). Post hoc analysis found the RW and RW+S exposed snails to have significantly less spermatids sloughing in to the lumen compared to the LM (P=0.004 and 0.013, respectively, LSD) and HM (P= 0.002 and 0.008, respectively) exposed snails. No significant difference was found between the RW and the RW+S (P= 0.662) exposed snails or between the LM and the HM (P= 0.861) exposed snails.

Mature spermatogenic cells in the lumen

Spermatozoa only

F1s from the RW had the highest number of sections of gonad with only spermatozoa free in the acini lumen, whilst HM exposed snails had the least (Table 5.22). However no significant difference was found between the exposure groups (P= 0.279, K-W).

Occurrence of acini with immature spermatogenic cells in lumen

The percentage of acini (per section of gonad) affected by immature spermatogenic cells sloughing into the lumen was highest in snails exposed to RW and lowest from those exposed to RW+S (Table 5.23). No significant difference was found between the four exposure groups (P= 0.801, ANOVA).

Table 5.21 The percentage of F1 *P. corneus* snails with varying level of immature or supportive spermatogenic cells sloughing into the acini lumen from the oestrogenic mixtures experiment

Number of sections affected	Percentage of F1s with Sertoli cell sloughing into lumen				Percentage of F1s with Spermatogonium sloughing into lumen				Percentage of F1s with Spermatocyte sloughing into lumen				Percentage of F1s with Spermatids sloughing into lumen			
	RW	RW+S	LM	HM	RW	RW+S	LM	HM	RW	RW+S	LM	HM	RW	RW+S	LM	HM
None	65	60	50	55	10	5	0	0	45	65	50	40	55	35	10	5
1 out of 5	0	15	10	10	5	0	10	0	20	30	5	10	0	15	10	10
2 out of 5	10	20	15	10	0	0	10	0	5	0	15	10	10	15	10	15
3 out of 5	15	0	10	15	10	5	5	15	0	0	5	25	10	15	15	20
4 out of 5	10	0	5	5	10	20	30	15	5	0	10	0	15	5	20	10
5 out of 5	0	5	10	5	65	70	45	70	25	5	15	15	10	15	35	40
	A	A	a	a	a	a	a	a	a	a	a	a	a	a	b	b

Snails sacrificed after developmental exposure to River Water only (RW, n=20), River Water plus Solvent (RW+S, n=21), river water plus Low oestrogenic Mixture (LM, n=21) or river water plus High oestrogenic Mixture (HM, n=20). Five sections of gonad were analysed per F1 snail. Letters (a, b) indicate statistical similarity.

Table 5.22 The percentage of F1 *P. corneus* snails with varying numbers of sections of gonad with only mature spermatozoa in the acini lumen from the oestrogenic mixtures experiment

No. of sections with only mature spermatozoa in the acini lumen	Percentage of F1 snails from the River Water exposure (RW)	Percentage of F1 snails from the River pus Solvent Water exposure (RW+S)	Percentage of F1 snails from the Low oestrogenic mixture exposure (LM)	Percentage of F1 snails from the High oestrogenic Mixture exposure (HM)
0 out of 5	70	70	55	85
1 out of 5	5	25	35	10
2 out of 5	10	0	5	5
3 out of 5	0	0	0	0
4 out of 5	5	0	5	0
5 out of 5	10	5	0	0
	a	a	a	a

Snails sacrificed after developmental exposure to River Water only (RW, n=20), River Water plus Solvent (RW+S, n=21), river water plus Low oestrogenic Mixture (LM, n=21) or river water plus High oestrogenic Mixture (HM, n=20). Five sections of gonad were analysed per F1 snail. Letter (a) indicates statistical similarity.

Oogenesis stages present

The mean percentage of oogenesis stages present was most similar between RW and RW+S exposed F1s (Table 5.24). LM developmentally exposed snails had the highest

percentage of young oocytes (stage 1 and 2) and also had the highest percentage of mature oocytes (stage 5). Statistical analysis (ANOVA) found significant differences between the percentage of stage 1 (P= 0.003) and 2 (P= 0.043) oocytes between the four treatments but not stage 3 (P= 0.237), stage 4 (P= 0.144) or stage 5 oocytes (P= 0.680). Post hoc analysis (LSD) found RW and RW+S exposed snails to have a significantly lower percentage of stage 1 oocytes compare to snails from the LM (P= 0.019, 0.021) and the HM (P= 0.003, 0.003). No significant difference was found in the percentage of stage 1 oocytes between the RW+S and the RW (P=0.974) exposed snails or between the LM and the HM (P=0.469) exposed snails. Post hoc analysis found LM developmentally exposed snails to have a significantly higher percentage of stage 2 oocytes compare to HM (P= 0.005) exposed snails. No statistical differences were found for the percentage of stage 2 oocytes between the other treatment groups.

Table 5.23 Proportion of F1 *P. corneus* snails found with varying percentage of acini with immature spermatogenic cells sloughing into the lumen from the oestrogenic mixtures experiment

Percentage of acini affected by immature spermatogenic cells sloughing into the lumen	Score	Percentage of F1 snails from the River Water exposure (RW)	Percentage of F1 snails from the River pus Solvent Water exposure (RW+S)	Percentage of F1 snails from the Low oestrogenic mixture exposure (LM)	Percentage of F1 snails from the High oestrogenic Mixture exposure (HM)
<10%	1	50	65	60	50
10-30%	2	25	15	15	15
30-50%	3	0	10	10	25
50-70%	4	5	5	10	5
>70%	5	20	5	5	5
		a	a	a	a

Snails sacrificed after developmental exposure to River Water only (RW, n=20), River Water plus Solvent (RW+S, n=21), river water plus Low oestrogenic Mixture (LM, n=21) or river water plus High oestrogenic Mixture (HM, n=20). Five sections of gonad were analysed per F1 snail. Letter (a) indicates statistical similarity.

Parasitism

Histological analysis was performed on a sub-sample of 20 F1s from each treatment. Five sections of gonad were analysed per F1 snail. None of the F1 *P. corneus* analysed had a parasitic infection.

Table 5.24 The mean percentage of different stages of oogenesis in F1 *P. corneus* from the oestrogenic mixtures experiment

Stage of oocyte maturation	Mean percent of oogenesis stage after River Water exposure	Mean percent of oogenesis stage after River Water plus Solvent exposure	Mean percent of oogenesis stage after Low oestrogenic Mixture exposure	Mean percent of oogenesis stage after High oestrogenic Mixture exposure
Stage 1	10.7 ± 7.9 a	10.7 ± 9.8 a	16.5 ± 7.7 b	18.3 ± 5.2 b
Stage 2	15.8 ± 4.9 ab	14.9 ± 7.0 ab	18.0 ± 5.0 a	13.0 ± 4.6 b
Stage 3	24.3 ± 6.5 a	20.3 ± 6.6 a	22.2 ± 5.2 a	12.6 ± 6.5 a
Stage 4	23.9 ± 11.4 a	23.7 ± 13.4 a	22.2 ± 7.2 a	29.5 ± 9.3 a
Stage 5	10.1 ± 6.4 a	10.7 ± 9.8 a	12.8 ± 6.9 a	11.6 ± 6.3 a
Degenerating	15.3 ± 12.1 a	19.7 ± 12.6 a	8.3 ± 7.4 a	5.9 ± 7.9 a

Snails sacrificed after developmental exposure to either River Water only (RW, n=20), River Water plus Solvent (RW+S, n=21), river water plus Low oestrogenic Mixture (LM, n=21) or river water plus High oestrogenic Mixture (HM, n=20). Five sections of gonad were analysed per F1 snail. Stage 1 (oogonium) is the youngest stage of oogenesis, stage 5 is ready to be ovulated. Oogenesis stages based on description by de Jong-Brink et al (De Jong-Brink et al. 1976). Letters (a, b) indicate statistical similarity.

5.4.7 Effects of developmental exposure to estrogenic mixtures and then depuration in river water on F1 *P. corneus*

5.4.7.1 Survival and growth during depuration in river water

The percentage of F1 over-winter survival was highest in *P. corneus* from the RW developmental exposure (19%) compared to the much lower percentage survival of those from the LM (6%) or HM (2%), (Table 5.25).

Table 5.25 Survival of F1 *P. corneus* left to depurate over winter after oestrogenic mixtures developmental exposure

F1 *P. corneus*	River Water	Low Mixture	High Mixture
Left to over-winter September 2006	322	2090	440
Surviving March 2007	60	123	8
Percent survival	19%	6%	2%

Snails exposed to River water only (RW), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). Number of F1s left in each treatment tank to over winter, number of F1s surviving until March 2007 and percentage over winter survival.

Between March and April 2007 the percentage survival of the RW (80%) and the LM (85.4%) developmentally exposed snails were similar. However, the HM developmentally exposed snails continued to have a lower percentage survival (62.5%).

Developmentally exposed F1 *P. corneus* snails were measured in April 2007 (after eight months of depuration). The exposed snails were on average smaller than those from the RW exposure. None of the HM or the LM developmentally exposed snails reached a shell diameter above 20 mm (Table 5.26).

Table 5.26 The percentage of different shell size classes of F1 *P. corneus* after developmental exposure to River water (RW), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM) followed by eight months depuration in river water during autumn/winter 2006.

Percentage Shell Size class	River Water developmentally exposed	Low Mixture developmentally exposed	High Mixture developmentally exposed
<10 mm shell diameter	10%	38%	40%
10-15 mm shell diameter	31%	60%	60%
15-20 mm shell diameter	52%	2%	0
> 20 mm shell diameter	6%	0	0

F1 snails measured April 2007.

5.4.7.2 Reproductive output

Reproductive output was severely impacted in the HM developmentally exposed *P. corneus* compared to those exposed to RW or LM (Table 5.27). Over the entire 14 weeks of the un-dosed F1 breeding study, the HM exposed snails laid the least number of eggs and egg masses (EMs) (4 EMs containing a total of 17 eggs). The LM exposed snails laid the most (141 EMs containing 2329 eggs). However due to differences in snail numbers and mortality (see survival below), reproductive output per snail is a better comparison of reproductive output.

Table 5.27 Total number of eggs and egg masses laid by F1 *P. corneus* over 14 week un-dosed F1 breeding study

	River Water (RW)	Low oestrogenic Mixture (LM)	High oestrogenic Mixture (HM)
Total egg masses laid	79	141	4
Total eggs laid	1255	2329	17

Snails developmentally exposed to River Water (RW), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM) and then depuration over winter in river water.

Egg laying in the RW and the LM developmentally exposed snails was underway when the sampling period started in May. Peak egg laying occurred at week 8 (19th June 2007) for the RW and the LM exposed snails. However, LM developmentally exposed snails laid

almost twice as many eggs per snail at this point (RW 39.4 eggs/snail, LM 68.7 eggs/snail). The HM developmentally exposed snails only laid eggs between weeks 8 and 10, laying a mean of 4.3 eggs per snail. After week 8 all the RW and the LM developmentally exposed snails egg laying declined. The mean number of eggs laid per snail was similar for the LM and the RW groups from week 10 until the end of the breeding study (Figure 5.7). Statistical analysis (t-test) found RW developmentally exposed snails had laid significantly more eggs per snail at week 2 (P= 0.001) but not at weeks 5 (P=0.655), 6 (P=0.205), 10 (P=0.995), 12 (P=0.507, M-W) or 14 (P=0.876). The cumulative number of eggs laid per snail was not found to be significantly different between the RW and the LM exposed snails (P=0.755). The HM egg laying could not be analysed as it only had one replicate group.

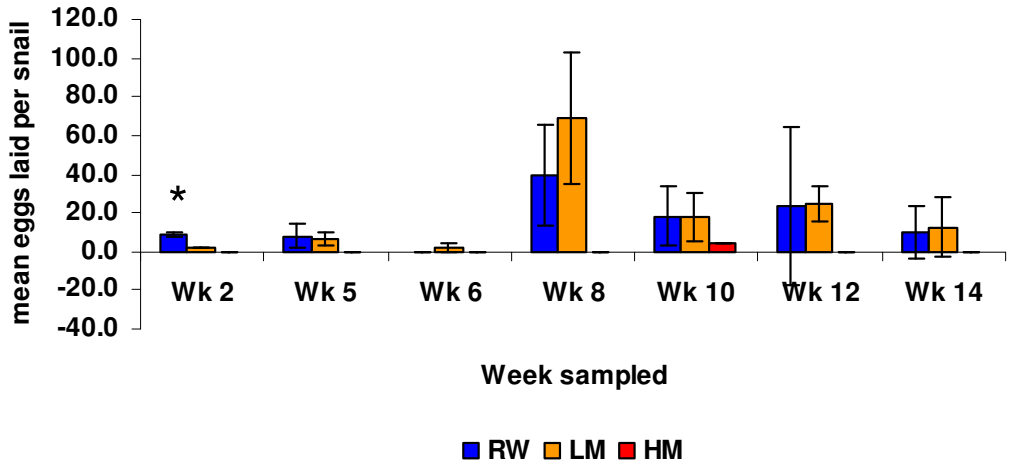

Figure 5.7 Mean egg laid per snail during the F1 breeding study by *P. corneus* from the oestrogenic mixtures experiment.

F1s previously developmentally exposed to River Water (RW), Low oestrogenic Mixture (LM) or High oestrogenic Mixture (HM) and then depurated over autumn/winter in river water. Error bars show standard deviation. Star (*) indicates significant difference from LM.

Overall, the mean numbers of egg masses laid per snail were similar between the RW and the LM developmentally-exposed groups. However, at week 8 (during the peak in egg mass laying) the LM developmentally exposed snails laid a larger amount (3.9 ± 1.1) of egg masses per snail compared to the RW (2.6 ± 1.4) exposed snails. The HM developmentally exposed snails laid a maximum of 1 egg mass per snail at week 10 (Figure 5.8A). After week 8, the number of egg masses laid per snail declined in both the RW and the LM developmentally-exposed snails. No significant (t-test) differences were found between the mean number of egg masses laid by snails developmentally-exposed to

RW or LM at weeks 2 (P= 0.060), 5 (P=0.725), 6 (P=0.132), 8 (P=0.286), 10 (P=0.960), 12 (P= 0.983) or 14 (P=0.695) or between the cumulative numbers of egg masses laid per snail (P= 0.729). The HM developmentally exposed snails could not be analysed, due to it only having one replicate group.

At the first sampling point (week 2) the average numbers of eggs per egg mass were 18.0 ± 4.1 for the RW exposed snails, 12.0 ± 2.0 for the LM, and 0 (no egg masses laid) for the HM snails. The highest mean number of eggs per egg mass were recorded at week 14 for both RW (23.8 ± 2.6) and LM (19.6 ± 8.0) snails. The HM developmentally-exposed snails only laid egg masses at week 10 (4.3 eggs per egg mass). The LM and the RW developmentally exposed snails laid similarly sized egg masses at weeks 5, 8, 10 and 12 (neither RW nor HM snails laid egg masses at week 6, Figure 5.8B). However, over the 14-week breeding study the RW developmentally exposed snails (16.2 eggs per egg mass) laid on average larger egg masses than those from the LM (14.8 eggs per egg mass) exposure. However, statistical analysis (t-Test) found no significant difference between the number of eggs per egg mass from the LM and RW developmentally-exposed snails for weeks 2 (P= 0.084), 5 (P= 0.783), 8 (P= 0.350), 10 (0.694), 12 (P=0.977) or 14 (P= 0.539). No significant difference was found between the mean number of eggs per egg mass laid over the entire breeding study (P= 0.513) by the RW and the LM developmentally-exposed snails. The HM group could not be compared as it only had one replicate, and only laid egg masses at one sampling period.

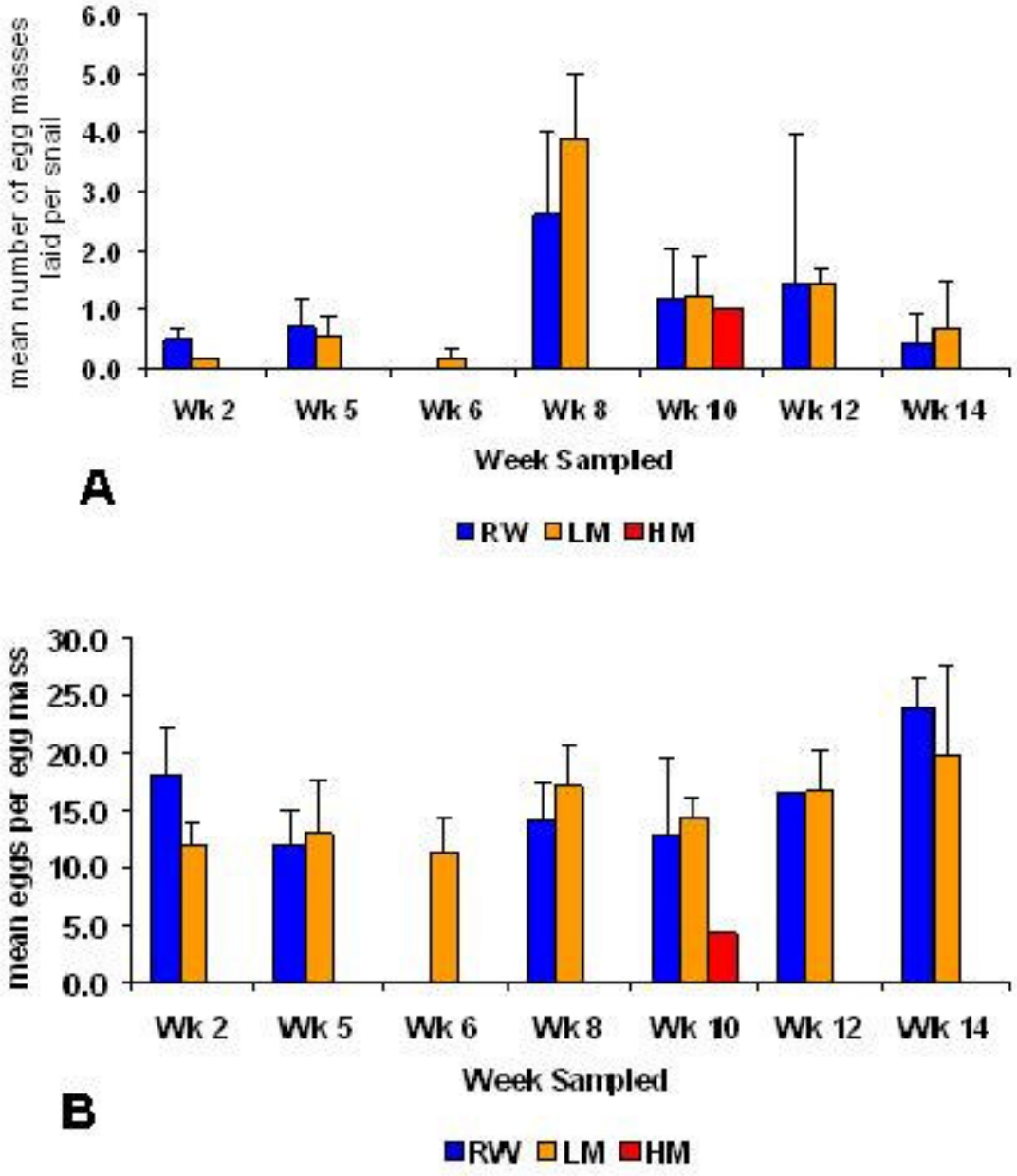

Figure 5.8 A, Mean number of egg masses laid per snail and B, mean number of eggs per egg mass laid by F1 *P. corneus* during the F1 breeding study.

Snails developmentally exposed to River Water only (RW), Low oestrogenic Mixture (LM) or High oestrogenic Mixture (HM) and then depurated over autumn/winter in river water only. Error bar gives standard deviation.

5.4.7.3 Survival and growth

Due to the high level of over-winter mortality suffered by the HM developmentally exposed snails, only one replicate breeding group of four snails was used. The RW and LM exposure groups had three replicates; each containing six snails. Over the 14 weeks of breeding study, the RW exposed snails suffered the highest percentage mortality (44% surviving). The HM developmentally exposed snails had 50% (2 snails) by the end of the study and the LM developmentally exposed snails had 88.9% surviving (Figure 5.9).

Statistical analysis (Mann-Whitney) of the percentage of surviving snails was found to be significantly higher in the LM developmentally-exposed snails compared to the RW exposed snails at weeks 2, 5, 6 and 8 (P= 0.034). The LM developmentally-exposed snails also had a significantly higher percentage survival compared to the RW snails at weeks 10 (P= 0.013), 12 (P= 0.047) and 14 (P= 0.047, t-test).

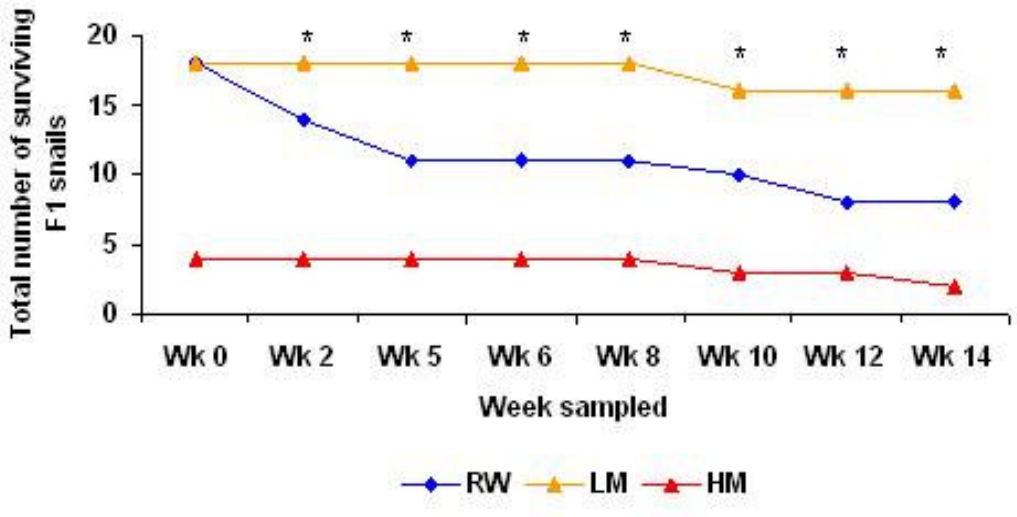

Figure 5.9 Survival of depurated snails over 14 week un-dosed F1 reproductive study conducted Spring/Summer 2007.

***P. corneus* F1s developmentally exposed to River Water only (RW), Low oestrogenic Mixture (LM) or High oestrogenic Mixture (HM) and then depurated over autumn/winter in river water only. A Total of 18 (LM and RW) or 4 (HM) snails were allocated to each group at week 0. Star (*) indicates significant difference from RW. Statistical analysis of HM not conducted due to low numbers.**

At the start of the F1 breeding study, the F1s from the RW developmental exposure were larger and heavier then those from the LM or HM exposures (Table 5.28). The RW snails had a mean shell diameter of 14.6 ± 1.9 mm, compared to 13.9 ± 0.6 mm (LM snails) and 11.8 ± 1.6 mm (HM snails). Significant differences were found between the groups for shell length (P= 0.018 K-W), shell aperture (P= 0.015) but not for total weight (P= 0.059). Post-hoc analysis found RW snails to be significantly larger than HM exposed snails for shell diameter (P= 0.027) and shell aperture (P= 0.014). The LM developmentally-exposed snails were also significantly larger than the HM snails in shell diameter (P= 0.006) and shell aperture (P= 0.017). No significant difference was found between the RW and the LM developmentally exposed snail's shell diameter (P= 0.200) or shell aperture (P= 0.137). At the end of the 14-week reproductive study the RW developmentally exposed snails were still larger than the LM or the HM snails. However, statistical analysis

(ANOVA) found no significant differences between the developmental exposure groups in shell diameter (P= 0.725), shell aperture (P= 0.162), total weight (P= 0.667) or soft body weight (P= 0.654). The percentage growth over the breeding study was highest in the HM group at 25.5% for shell diameter and 56.6% for total weight. The percentage growth was 12.3% for shell length in the LM snails and 10.8% for the RW snails. The percentage increase in total weight was 27.5% for the LM snails and 25.9% for the RW snails.

Table 5.28 Size and weight of F1 *P.corneus* before and after un-dosed F1 breeding study.

	River Water (RW)	Low oestrogenic Mixture (LM)	High oestrogenic Mixture (HM)
Start of study Shell diameter mm	14.6 ± 1.9 a	13.9 ± 0.9 a	11.8 ± 1.6 b
End of study Shell d mm	16.3 ± 1.6 a	15.8 ± 1.3 a	15.8 ± 2.1 a
Start of study Total weight g	0.7 ± 0.3 a	0.6 ± 0.1 a	0.35 ± 0.2 b
End of study Total weight g	0.9 ± 0.23 a	0.8 ± 0.2 a	0.8 ± 0.3 a

Snails developmentally exposed to river water (RW), low oestrogenic mixture (LM) or high oestrogenic mixture (HM) and then depurated in river water for eight months. Shell diameter and total weight at the start and end of the 14 week un-dosed F1 breeding study. Mean ± standard deviation, letters (a, b) indicate statistical similarity.

Spearman's rank order was used to correlate the shell diameter and total weight at the start of the F1 breeding study, and the shell diameter, total weight and soft body weight at the end. Significant positive correlations were found for both the RW and the LM developmentally-exposed snails between shell diameter and weight at the start of the breeding study (P<0.001). After the breeding study, significant positive correlations were also found for the RW and LM snails shell diameter and weight (P<0.001, <0.001, respectively), shell diameter and soft body weight (P= 0.015, <0.001, respectively), and total weight and soft body weight (P= 0.002, <0.001, respectively). Correlations could not be performed on snails from the HM due to low numbers.

5.4.8 Effect of developmental exposure to oestrogenic mixtures and then depuration in river water on histopathology of the gonad

Histological analysis was performed on all surviving snails from the F1 breeding study (RW, n = 8; LM, n = 16; HM, n = 2). Five sections of gonad were analysed per snail.

All the snails samples (from all three treatments) had Sertoli cells attached to the acini walls in all of the sections of gonad analysed.

A dose dependant decrease in normal vitellogenic area activity (maturing oocytes with follicle cells and/or sperm with Sertoli cells) was found in snails developmentally exposed and then depurated in river water for approximately one year (Table 5.29 and Figure 5.10). However, no significant difference was found between the three groups (P= 0.133, ANOVA).

Table 5.29 The percentage of F1 *P. corneus* snails with varying level of acini vitellogenic area activity from the oestrogenic mixtures experiment

Percent of acini with active vitellogenic area	Score	Percentage of depurated F1 snails from the River Water exposure (RW)	Percentage of depurated F1 snails from the Low Mixture exposure (LM)	Percentage of depurated F1 snails from the High Mixture exposure (HM)
<10%	1	0	0	0
10-30%	2	0	12%	50%
30-50%	3	13%	47%	50%
50-70%	4	49%	35%	0
>70%	5	38%	6%	0

Snails sacrificed after developmental exposure to River water only (RW), river water plus low oestrogenic mixture (LM) or river water plus high oestrogenic mixture (HM) and then depuration for approximately one year in river water. Histological analysis was performed on all snails from the F1 breeding study (RW, n = 8; LM, n = 16; HM, n = 2). Five sections of gonad were analysed per snail.

The majority of F1 snails (from all treatments) had intact acini walls in all the sections of gonad analysed. One snail from the RW exposure had three out of five sections with intact acini walls. Three of the LM snails showed disruption to the acini wall, one had four out of five sections intact, one had three out of five intact and one had no sections intact. One of the two HM snails had disruption in two out of five sections analysed. No significant difference was found between the exposure groups (P= 0.708, ANOVA). The HM developmentally exposed and then depurated snails had the highest amount of cell (developing oocytes, sperm and supporting cells) cover of the acini walls. However, only two snails were sampled due to the high mortality suffered in this group. The RW exposed snails had a higher amount of cell cover than the LM exposed snails (Table 5.30). No significant difference was found between the exposure groups (P= 0.142, Kruskal-Wallis).

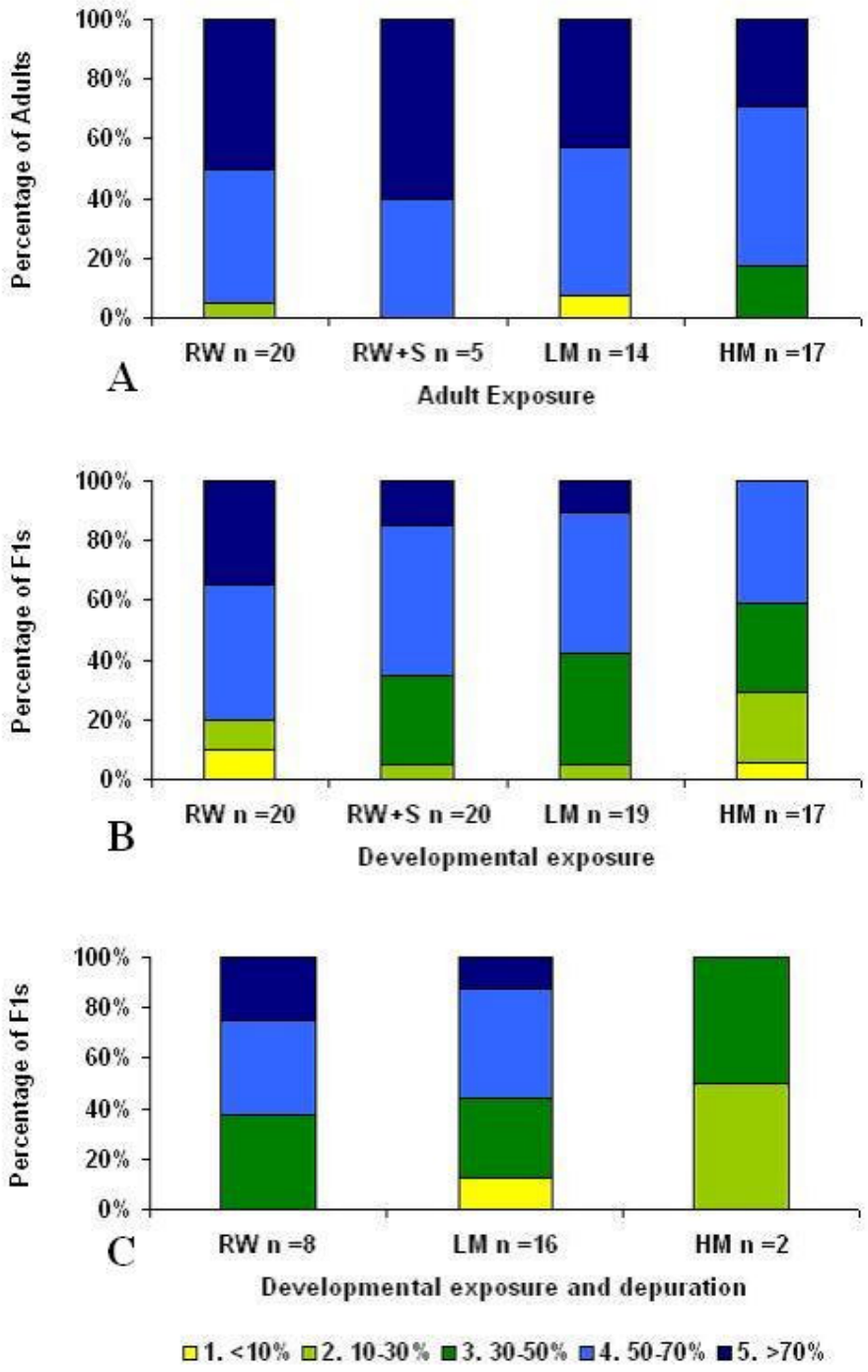

Figure 5.10 Percentage of snails with varying level of vitellogenic area activity (Sperm/oocyte maturation and supportive cells).

P. corneus **either exposed to either River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM) as adults during reproductive period (Histogram A) or developmentally (Histograms B and C). (A) Adult snails sampled directly after exposure (end August 2006). (B) F1s sampled directly after developmental exposure (September 2006). (C) F1s sampled after one year of depuration in river water (early August 2007). Score 1 (yellow) lowest level of activity <10% of acini contain active vitellogenic area, Score 5 (dark blue) highest level of activity >70% of acini contain active vitellogenic area, n= number of snails analysed.**

Table 5.30 The percentage of depurated F1 *P. corneus* snails with varying level of acini wall cell cover from the oestrogenic mixtures experiment

Percent of acini wall covered by germ cells and/or supportive cells	Score	Percentage of depurated F1 snails from the River Water exposure (RW)	Percentage of depurated F1 snails from the Low Mixture exposure (LM)	Percentage of depurated F1 snails from the High Mixture exposure (HM)
<10%	1	0	0	0
10-30%	2	0	12.5	0
30-50%	3	13	31.25	0
50-70%	4	25	12.5	0
>70%	5	62	43.75	100

Snails sacrificed after developmental exposure to River water only (RW), river water plus low oestrogenic mixture (LM) or river water plus high oestrogenic mixture (HM) and then depuration for approximately one year in river water. Histological analysis was performed on all snails from the F1 breeding study (RW n = 8; LM, n = 16; HM, n = 2). Five sections of gonad were analysed per snail.

Immature spermatogenic and supportive cells sloughed into lumen

Sertoli cells sloughing into lumen

The number of sections analysed with Sertoli cells sloughed into the lumen were similar between the LM and the RW exposed snails (Table 5.31). The HM had the lowest percentage of sections with Sertoli cell sloughing. No significant difference was found between the exposure groups (P= 0.988, ANOVA).

Spermatogonium sloughing into lumen

Spermatogonium sloughing into the acini lumen was most frequent in snails developmentally exposed to HM (Table 5.31). No significant difference was found between the exposure groups (P= 0.416, ANOVA).

Spermatocytes sloughing into the lumen

Spermatocytes sloughing into the lumen was most frequent in snails developmentally exposed to the HM, and lowest in those exposed to RW only (Table 5.31). Statistical analysis found no significant difference was found between the exposure groups (P= 0.134 K-W).

Spermatids sloughing into the lumen

Spermatids sloughing into the lumen were most frequent in snails developmentally exposed to the RW, the LM snails showed a similar trend (Table 5.31). No significant difference was found between the exposure groups (P= 0.997, ANOVA)

Mature spermatogenic cells in the lumen

Spermatozoa only

The percentage of snails with only mature spermatozoa free in the acini lumen was highest in the snails from the RW developmental exposure and decreased in a dose dependant

manner (Table 5.32). No significant difference was found between the exposure groups (P= 0.589, ANOVA).

Table 5.31 The percentage of depurated F1 *P. corneus* snails with varying level of immature spermatogenic cells sloughed into the acini lumen from the oestrogenic mixtures experiment

Number of sections affected	Percentage of depurated F1s with Sertoli cell sloughing into lumen			Percentage of depurated F1s with Spermatogonium sloughing into lumen			Percentage of depurated F1s with Spermatocyte sloughing into lumen			Percentage of depurated F1s with Spermatid sloughing into lumen		
	RW	LM	HM	RW	LM	HM	RW	LM	HM	RW	LM	HM
None	37.5	37.5	0	25	12.5	0	37.5	37.5	0	25	12.5	0
1 out of 5	12.5	18.75	50	0	0	0	12.5	6.25	0	0	25	0
2 out of 5	12.5	6.25	0	12.5	18.75	0	0	18.75	0	12.5	0	50
3 out of 5	0	6.25	50	0	18.75	0	12.5	6.25	0	12.5	12.5	0
4 out of 5	25	18.75	0	25	18.75	0	37.5	6.25	0	12.5	18.75	50
5 out of 5	12.5	12.5	0	37.5	31.25	100	0	25	100	37.5	31.25	0
	A	A	a	a	a	a	a	a	a	A	a	a

Snails sacrificed after developmental exposure to River water only (RW), river water plus low oestrogenic mixture (LM) or river water plus high oestrogenic mixture (HM) and then depuration for approximately one year in river water. Histological analysis was performed on all snails from the F1 breeding study (RW, n = 8; LM, n = 16; HM, n = 2). Five sections of gonad were analysed per snail. Letters (a) indicate statistical similarity.

Table 5.32 The percentage of depurated F1 *P. corneus* snails with varying numbers of sections of gonad with only mature spermatozoa in the acini lumen from the oestrogenic mixtures experiment

Number of sections with just mature spermatozoa in the lumen	Percentage of F1 snails from the River Water exposure (RW)	Percentage of depurated F1 snails from the Low Mixture exposure (LM)	Percentage of depurated F1 snails from the High Mixture exposure (HM)
0 out of 5	50	56.25	100
1 out of 5	25	12.5	0
2 out of 5	0	6.25	0
3 out of 5	0	12.5	0
4 out of 5	0	6.25	0
5 out of 5	25	6.25	0
	a	a	A

Snails sacrificed after developmental exposure to River water only (RW), river water plus low oestrogenic mixture (LM) or river water plus high oestrogenic mixture (HM) and then depuration for approximately one year in river water. Histological analysis was performed on all snails from the F1 breeding study (RW n = 8; LM, n = 16; HM. n = 2). Five sections of gonad were analysed per snail. Letter (a) indicates statistical similarity.

Occurrence of acini with immature spermatogenic cells in lumen

The depurated snails from the LM exposure had the highest percentage of snails with the most (>70% of acini) level of immature spermatogenic cells sloughed into the lumen. However, no trend was apparent between treatments (Table 5.33). No significant difference was found between the exposure groups (P= 0.944, ANOVA).

Table 5.33 The percentage of depurated F1 *P. corneus* snails affected by varying percentage of acini with immature spermatogenic cells sloughing into the lumen from the oestrogenic mixtures experiment

Percentage of acini affected by immature spermatogenic cells sloughing into the lumen	Score	Percentage of F1 snails from the River Water exposure (RW)	Percentage of depurated F1 snails from the Low Mixture exposure (LM)	Percentage of depurated F1 snails from the High Mixture exposure (HM)
<10%	1	50	50	0
10-30%	2	12.5	25	100
30-50%	3	37.5	18.75	0
50-70%	4	0	0	0
>70%	5	0	6.25	0
		a	a	A

Snails sacrificed after developmental exposure to River water only (RW), river water plus low oestrogenic mixture (LM) or river water plus high oestrogenic mixture (HM) and then depuration for approximately one year in river water. Histological analysis was performed on all snails from the F1 breeding study (RW, n = 8; LM, n = 16; HM, n = 2). Five sections of gonad were analysed per snail. Letter (a) indicates statistical similarity.

Table 5.34 The mean percentage of different stages of oogenesis in depurated F1 *P. corneus* from the oestrogenic mixtures experiment

Stage of oocyte maturation	Mean percent of oogenesis stage after River Water exposure	Mean percent of oogenesis stage after LM exposure	Mean percent of oogenesis stage after HM exposure
Stage 1	19.9 ± 5.5 a	22.7 ± 7.5 a	34.2 ± 11.2 a
Stage 2	21.8 ± 4.7 a	20.4 ± 3.5 a	16.8 ± 0.1 a
Stage 3	20.0 ± 4.2 a	19.6 ± 6.5 a	16.0 ± 2.2 a
Stage 4	19.9 ± 6.5 a	15.8 ± 5.3 a	24.9 ± 5.4 a
Stage 5	3.6 ± 3.3 a	1.9 ± 2.4 a	3.1 ± 1.9 a
Degenerating	14.8 ± 8.3 a	19.6 ± 10.1 a	5.1 ± 6.0 a

Snails sacrificed after developmental exposure to River water only (RW), river water plus low oestrogenic mixture (LM) or river water plus high oestrogenic mixture (HM) and then depuration for approximately one year in river water. Histological analysis was performed on all snails from the F1 breeding study (RW, n = 8; LM, n = 16; HM, n = 2). Five sections of gonad were analysed per snail. Stage 1 (oogonium) are the youngest stage of oogenesis, stage 5 is ready to be ovulated, oogenesis stages based on description by de Jong-Brink et al (De Jong-Brink et al. 1976). Letters (a) indicate statistical similarity.

Oogenesis stages present

The HM developmentally exposed snails had the highest percentage of young oocytes (Stage 1 and 2). The RW exposed snails had the highest percentage of mature (stage 5) oocytes (Table 5.34, above). No significant differences were found between the exposure groups for stage 1 (P= 0.059, ANOVA), stage 2 (P=0.264), stage 3 (P=0.666), stage 4 (0.061), stage 5 (P= 0.333) or degenerating oocytes (P= 0.111).

Parasitism

No parasites were found in any of the F1 snails analysed (from any of the exposure groups).

5.4.9 Effects of estrogenic mixtures exposure on adult *V. viviparus* reproduction and survival

5.4.9.1 Number of neonates released per snail over reproductive study

On average, the cumulative number of neonates released per snail was highest in the RW exposed snails; RW exposed snails released 14.7 neonates each, RW+S 13.2 neonates each, LM released 12.4 and HM 13.6. However, no significant difference was found between the four treatment groups (P= 0.451, ANOVA). Neonate release was at its peak at week 2 (first sampling period), after which the number of neonates released declined rapidly (Figure 5.12). The mean number of neonates released per snail per fortnight was similar over all exposures until week 8. Statistical analysis (ANOVA) found significant differences between the groups at weeks 8 (P= 0.002) and 10 (P= 0.042). Post hoc analysis (LSD) found at week 8 the RW exposed snails released a significantly greater number of neonates compared to the RW+S (P= 0.002), LM (P.0.001) and the HM (P= 0.012) exposed snails. No significant differences were seen between the numbers of neonates released by the other treatment groups. At week 10, post hoc analysis found the LM exposed snails released significantly less neonates compared to the RW (P= 0.009) and RW+S (P=0.048) exposed snails. At the same time point, the HM exposed snails released an almost significantly lower number of neonates compare to the RW (P=0.057) snails. No significant differences were found between the numbers of neonates released by the other treatment groups.

Number of embryos harboured per female at the end of exposure

The mean numbers of embryos harboured by adult females at the end of the exposure were highest from the RW exposure (30.5 embryos per female) and lowest from the HM

exposure (27.5 embryos per female). However no significant difference was found between the treatment groups (P=0.630, ANOVA). Embryos were designated into two groups shelled (most mature) and un-shelled (least mature). The RW exposed females harboured the highest number of shelled embryos but the lowest number of un-shelled embryos. Conversely, the LM exposed females harboured the least number of shelled embryos and the highest number of un-shelled embryos (Table 5.35 below). No significant difference was found between the numbers of shelled (P= 0.128, ANOVA) or unshelled (P=0.416, Kruskal-Wallis) embryos harboured per female at the end of exposure.

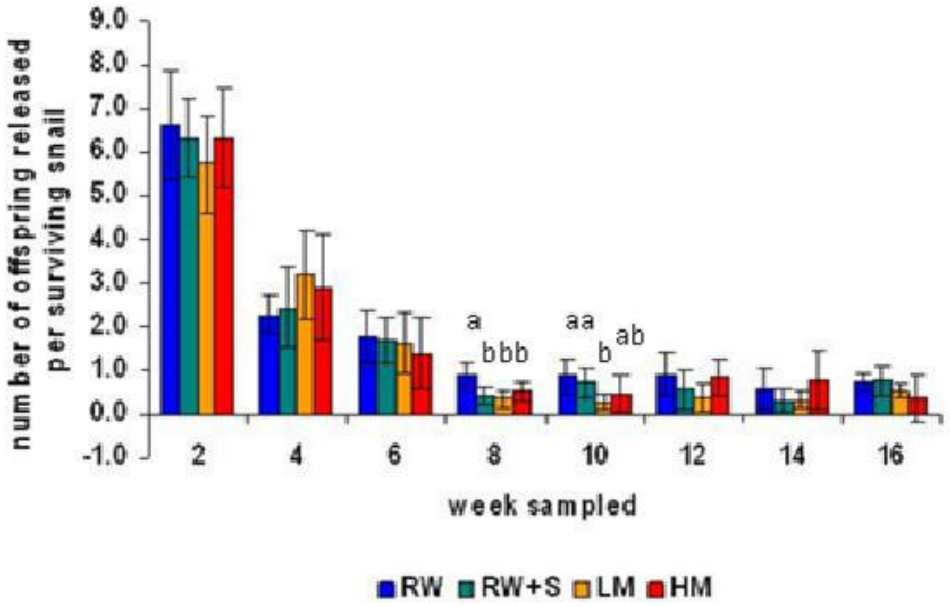

Figure 5.11 Mean number of neonates released per adult snails over 16 week exposure period by adult *V. viviparus* in the oestrogenic mixtures experiment.

Snails exposed to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). Error bars show standard deviation. Letter (a,b) indicates statistical similarity.

Table 5.35 Mean number of shelled (most mature) and unshelled (least mature) embryos harboured per female *V. viviparus* after 16 week of exposure in the oestrogenic mixtures experiment.

Embryos	River Water (RW)	River Water plus Solvent (RW+S)	Low oestrogenic Mixture (LM)	High oestrogenic Mixture (HM)
Shelled	26.5 ± 11.1	23 ± 11.5	18.6 ± 9.7	23.25 ± 9.6
Un-Shelled	4.0 ± 3.5	5.7 ± 5.1	8.3 ± 6.8	4.25 ± 4.3
Total	30.5 ± 12.2	28.7 ± 12.5	26.9 ± 10.7	27.5 ± 9.8

***V. viviparus* snails exposed to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). The number of females analysed for each groups were RW n = 33, RW+S n = 27, LM n = 36, HM n = 28. Mean ± standard deviation.**

5.4.9.2 Survival

Survival of adults during the dosed reproductive study was highest from the LM exposure and lowest from the RW+S exposure (Figure 5.12). On average, 60.6% of the snails from the RW, 54.5% from the RW+S, 65.2% from the LM, and 56.6% from the HM exposure survived until week 16 of the study. Up until week 10, the RW+S snails were surviving at the same level as the LM exposed snails. However, after week 10, the RW+S snail survival dropped steeply. No significant differences were found between the four treatment groups for the number of adults surviving at each of the fortnightly sampling periods.

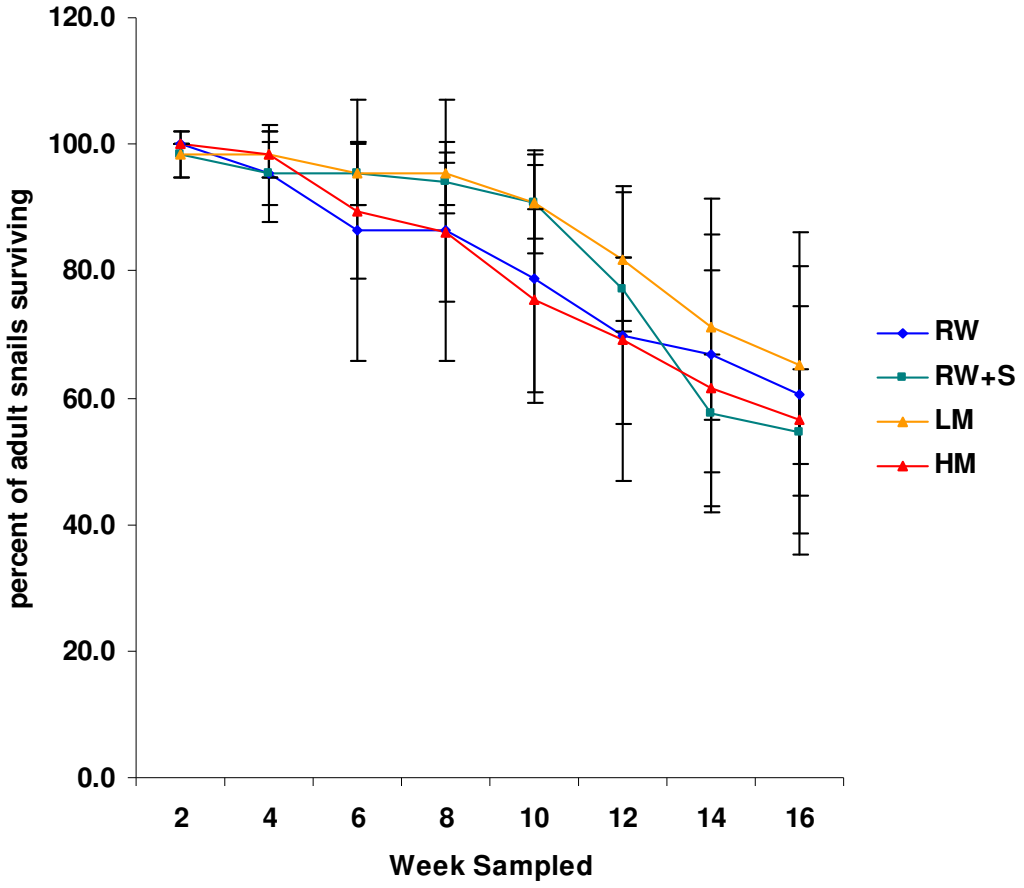

Figure 5.12 *V. viviparus* adults survival during dosed reproductive study.

Snails exposed to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM) as adults during reproductive period. Percentage of adults (males and females) surviving over exposure period from each exposure. Error bars indicate standard deviation.

After week 16 of the exposure, the dosing pumps administering the oestrogenic mixture to the tanks were switched off and the tanks were left to depurate in running river water over the weekend. After the weekend the adult snails were removed from the tanks for the final time. During this short depuration time several (RW; n=3, RW+S; n=3, LM; n=2, HM; n=5) adult *V. viviparus* snails from each exposure tank died. This left a total of 37 snails

from the RW tank, 33 snails from the RW+S tank, 41 snails from the LM and 30 snails from the HM tank. At this point the remaining snails were sampled; their size and weight were measured and the soft tissue was fixed for histology (see Section 3.5.7), the data from these specimens are presented below.

5.4.9.3 Size and weight pre exposure

The mean values for shell length, shell aperture and total weight pre exposure were similar across the exposure groups. On average snails from the RW+S group were slightly larger in both shell size and weight (Table 5.36). No significant differences were found between pre exposure groups in shell length (P=0.793, ANOVA), shell aperture (P=0.776, ANOVA), or total weight (P=0.448, ANOVA).

Table 5.36 Size and weight of *V. viviparus* adults pre exposure from the oestrogenic mixtures experiment

	River Water (RW)	River Water plus Solvent (RW+S)	Low oestrogenic Mixture (LM)	High oestrogenic Mixture (HM)
Shell length mm	32.7 ± 3.8	33.0 ± 4.1	32.4 ± 3.1	32.4 ± 3.8
Shell aperture mm	26.6 ± 2.6	26.9 ± 2.9	26.5 ± 2.6	26.4 ± 2.6
Total weight g	8.8 ± 2.3	8.9 ± 2.5	8.3 ± 2.0	8.6 ± 2.3

Snails exposed to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). Mean shell length, shell aperture and total weight. Mean ± standard deviation.

5.4.9.4 Adult *V. viviparus* Size, Weight and Growth post exposure

Female *V. viviparus* size and weight post exposure

The mean values for shell length, shell aperture, total weight, soft body weight and soft body weight embryos removed were similar over all treatments (Table 5.37). Females from the RW exposure were on average the largest and heaviest, whilst those from the HM exposure were smallest and lightest. However, statistical analysis (ANOVA) found no significant differences between the exposure groups for shell length (P=0.607), shell aperture (P=0.721), total weight (P=0.566), soft body weight (P=0.574) or soft body weight after embryos were removed (P=0.521).

Male *V. viviparus* size and weight post exposure

The male *V. viviparus* from the LM exposure were on average larger and heavier than those from other exposure groups, males from the HM were smallest (Table 5.38). No

significant differences were found between the four exposure groups in shell length (P=0.372), shell aperture (P= 0.377), total weight (P= 0.414), or soft body weight (P= 0.281).

Table 5.37 Shell length, shell aperture, total weight, soft body weight and soft body weight embryos removed of adult *V. viviparus* females from the oestrogenic mixtures experiment

	River Water (RW)	River Water plus Solvent (RW+S)	Low oestrogenic Mixture (LM)	High oestrogenic Mixture (HM)
Shell length mm	33.3 ± 2.2	32.8 ± 4.2	32.4 ± 2.7	32.1 ± 3.0
Shell aperture mm	17.6 ± 1.2	17.4 ± 1.7	17.3 ± 1.3	17.0 ± 1.3
Total weight g	8.5 ± 1.4	8.3 ± 2.2	8.0 ± 1.6	7.8 ± 1.6
Soft body weight g	3.2 ± 0.6	3.2 ± 0.9	3.0 ± 0.7	3.0 ± 0.6
Soft body weight embryos removed g	1.9 ± 0.4	2.0 ± 0.6	1.9 ± 0.5	1.8 ± 0.3

Snails sampled after four months of exposure. Snails exposed to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). The number of females analysed for each groups were RW n = 33, RW+S n = 27, LM n = 36, HM n = 28. Mean ± standard deviation.

Table 5.38 Size and weight of *V. viviparus* males after four months of exposure in the oestrogenic mixtures experiment.

	River Water (RW)	River Water plus Solvent (RW+S)	Low oestrogenic Mixture (LM)	High oestrogenic Mixture (HM)
Shell length mm	26.3 ± 4.1	28.5 ± 3.7	29.6 ± 1.5	25.6 ± 4.0
Shell aperture mm	14.9 ± 1.2	15.5 ± 1.6	16.2 ± 0.7	14.8 ± 1.0
Total weight g	4.9 ± 1.7	5.6 ± 1.6	6.3 ± 0.6	4.5 ± 1.6
Soft body weight g	1.6 ± 0.5	1.9 ± 0.6	2.2 ± 0.5	1.4 ± 0.7

Snails expoed to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). Shell length, shell aperture, total weight, soft body weight of surviving adult males. The number of males analysed for each group were RW n=4, RW+S n=6, LM n=5, HM n=2. Mean ± standard deviation.

5.4.10 Effects of exposure to estrogenic mixtures on adult gonad histopathology

As stated in section 5.4.3.1 a total of 141 adult snails (RW; 37(♀=33, ♂=4), RW+S; 33(♀=27, ♂=6), LM; 41(♀=36, ♂=5) HM; 30(♀=28, ♂=2)) were sampled and fixed for histopathological analysis from this experiment. All the male specimens (RW, n= 4; RW+S, n= 6; LM, n=5 HM, n= 2) were sectioned stained and analysed. However, due to time constraints a maximum of 23 female specimens were sectioned, stained and analysed from each treatment (RW, n= 23; RW+S, n=22; LM, n= 21; HM, n=21). Ten sections of gonad were analysed per adult snail. As the main focus of this research was on the possible effects of developmental exposure to oestrogenic, rather than on adult exposure, this low number of samples analysed was decided upon as a compromise between available time and quantity of data.

F0 Female *V. viviparus*

The female *V. viviparus* from the RW exposure had the highest mean number of oocytes per section of gonad analysed (11.0 oocytes per section). The females from the LM exposure had around half as many oocytes per section (4.2 oocytes per section) (Figure 5.13). A significant difference was found between the four exposure groups (P= 0.001, ANOVA). Post hoc analysis (LSD) found RW females to have significantly more oocytes per section compare to the LM (P< 0.001), HM (P= 0.001) and RW+S (P= 0.049) exposed snails.

Correlations between F0 female size/weight and reproductive potential (mean oocytes)

Over the four treatments significant positive correlations between the mean oocyte number and shell length (0.459 P<0.001), total weight (0.348 P=0.007) and soft body weight (0.520 P<0.001) where found, suggesting larger females would on average have higher mean number of oocytes per section of tissue analysed. Analysis of covariance (ANCOVA) was used to see if the mean number of oocytes was significantly different between the groups when co-analysed for shell length, total weight or soft body weight. Once co-analysed no significant differences were found between the treatment groups mean oocyte number (shell length; P=0.763, total weight; 0.755, soft body weight; 0.673). Indicating that although no significant differences were found between the sizes of the snails from the different treatment groups, the smaller snail size and weight seen in the mixture exposed snails did significantly affect the mean oocyte number, leading to significant differences to be seen between the groups.

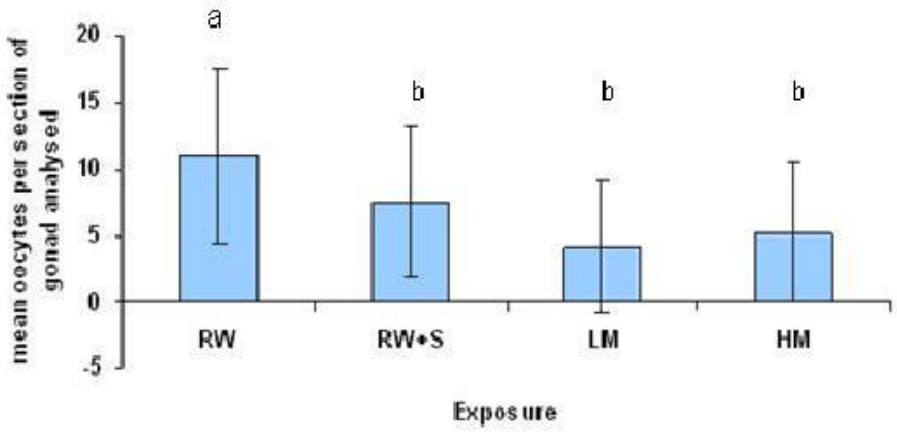

Figure 5.13 Mean number of oocytes per section of gonad analysed for female *V. viviparus* after four months of exposure in the oestrogenic mixtures experiment.

Snails exposed to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). A sub sample of females from each group were sectioned and analysed histologically (RW, n = 23; RW+S, n = 22; LM, n = 21; HM, n = 21). Ten section of gonad were analysed per snail. Mean number ± standard deviation. Letters (a,b) indicate statistical similarity.

F0 Male *V. viviparus*

The mean spermatogenesis score was lowest in males from the RW+S exposure. Mean scores from the other three exposures were similar (Table 5.39). Although the number of snails was low in some treatments, statistical analysis was conducted. No significant difference was found between the four exposure groups (P= 0.396 K-W).

Table 5.39 Mean spermatogenesis score of male *V. viviparus* after four months of exposure in the oestrogenic mixtures experiment

	River Water (RW)	River Water plus Solvent (RW+S)	Low oestrogenic Mixture (LM)	High oestrogenic Mixture (HM)
Mean spermatogenesis score	10.0 ± 0.1	7.9 ± 3.6	9.9 ± 0.2	10.0 ± 0.0

Snails exposed to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). All males were analysed histologically (RW, n= 4; RW+S, n= 6; LM, n= 5; HM, n =2). Ten sections of gonad were analysed per snail. Mean score ± standard deviation.

Parasitism

As with the 'health check group' (Section 2.3.3.4), the majority of adult *V. viviparus* had some level of parasitism.

F0 Females

Only four of the female snails analysed had no parasite infection (2 RW and 2 RW+S). Encysted parasites (metacercaria) found in the muscular tissue of the head and the foot were most frequent, but infections of the digestive tissue (sporocyst stage) were also found (Table 5.40).

Table 5.40 Percentage of adult female *V. viviparus* snails with different types of parasite infections from the oestrogenic mixtures experiment

	River Water (RW)	River Water plus Solvent (RW+S)	Low oestrogenic Mixture (LM)	High oestrogenic Mixture (HM)
Encysted parasites	82.6%	77.3%	90.5%	85.7%
Infection of the digestive tissue	8.7%	13.6%	9.5%	14.3%
No infection	8.7%	9.1%	0.0	0.0

Snails sacrificed after four months of exposure to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). A sub sample of females from each group were sectioned and analysed histologically (RW, n = 23; RW+S, n = 22; LM, n = 21; HM, n = 21). Ten section of tissue were analysed per snail. Encysted parasites (metacercaria) found in the muscular tissue of the head and the foot, infections of the digestive tissue (sporocyst stage).

The intensity of infection (number or area infected) for both encysted parasites and digestive tissue parasites were also recorded. The intensity of infection by encysted parasites in adult female *V. viviparus* was highest in the HM exposure and lowest in the RW only exposure (Figure 5.14). Of the females with digestive tissue parasites all three from the RW+S had the highest level of infection (>70% of digestive tissue infected). One female from the RW, LM and HM also had the highest level of infection (>70% of digestive tissue infected), with the remaining infected snails having between 30-50% of the digestive tissue infected.

Males

Only one male was found to have no parasite infection and this was from the RW exposure. The majority of males had encysted parasites (metacercaria) found in the muscular tissue of the head and the foot, one had an infection of the digestive tissue (Table 5.41). The intensity of infection by encysted parasites in adult male *V. viviparus* was highest in the LM exposure and lowest in the RW only exposure (Figure 5.14). Only one

male (RW+S) had digestive tissue parasites. This individual was highly infested with more than 70% of the digestive tissue infected.

Table 5.41 Percentage of adult male *V. viviparus* snails with different types of parasite infections from the oestrogenic mixtures experiment

	River Water (RW)	River Water plus Solvent (RW+S)	Low oestrogenic Mixture (LM)	High oestrogenic Mixture (HM)
Encysted parasites	75%	83.3%	100%	100%
Infection of the digestive tissue	0	16.7%	0	0
No infection	25%	0	0	0

Snails sacrificed after four months of exposure to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). All adult males were analysed histologically (RW, n= 4; RW+S, n= 6; LM, n= 5; HM n =2). Ten sections of tissue were analysed per snail. Encysted parasites (metacercaria) found in the muscular tissue of the head and the foot, infections of the digestive tissue (sporocyst stage).

Correlations between parasite infection and reproductive potential

Some parasites can affect their host's ability to reproduce, either via some method of chemical castration or via misappropriation of energy that should have been spent on reproduction. The possible effects of parasitic infection will be discussed later in this Chapter and in Chapter 6. In an attempt to assess whether the parasites found in this study had an effect on their hosts ability to reproduce, a number of correlations were performed.

Spearman's rank order correlations were performed on RW exposed adults to see if parasite infection had an effect on reproductive potential (mean oocyte numbers or the number of embryos harboured). No significant correlations were found between the mean oocyte number and the mean encysted parasite score (correlation coefficient 0.056, P= 0.824), the mean shelled embryos harboured and the mean encysted parasite score (correlation coefficient 0.139, P=0.548), or between the mean un-shelled embryos harboured and the mean encysted parasite score (correlation coefficient 0.062 P= 0.790). The number of females with digestive tissue infections was too small to correlate. However the mean number of embryos harboured by females (RW and RW+S combined) with digestive tissue infections were much lower than those with just encysted parasites (Table 5.42).

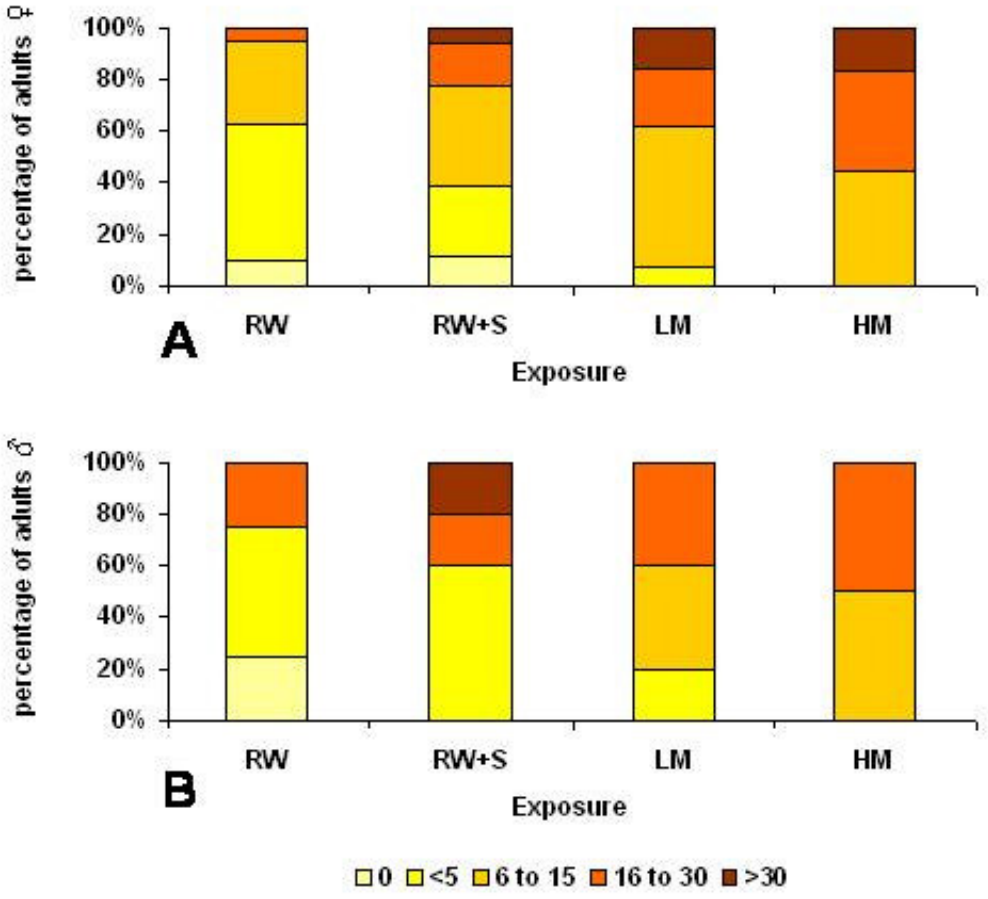

Figure 5.14 Intensity of encysted parasite infections in adult *V. viviparus* after four months exposure in the oestrogenic mixtures experiment.

Snails exposed to river water only (RW), river water plus solvent (RW+S), river water plus low oestrogenic mixture (LM), or river water plus high oestrogenic mixture (HM). (A) The percentage of females with varying intensities of parasite infection. (B) The percentage of males with varying intensities of parasite infection. RW n= 33♀, 4♂; RW+S n= 27♀, 6♂; LM n= 36♀, 5♂; HM n= 28♀, 2♂. 10 sections of tissue per adult snail were analysed. Pale yellow indicates no parasites found, yellow indicates less than 5 parasites per section, light orange indicates 5-15 parasites per section, dark orange indicates 15-30 parasites per section, brown indicates more than 30 parasites per section.

The number of males from the RW exposure was too small to correlate mean spermatogenesis score with mean encysted parasite score. Of the male *V. viviparus* sampled, one had a digestive tissue parasite infection, which had taken over the majority (>70%) of the digestive tissue; in this male, spermatogenesis was absent.

Table 5.42 The mean number of embryos harboured by female *V. viviparus* exposed to RW or RW+S split by parasite infection. Females had no infection, encysted parasites only, or an infection of the digestive tissue.

	Non-infected females (RW n=2 and RW+S n=2)	Infected females with encysted parasites only (RW n=19 and RW+S n=17)	Infected females with digestive tissue parasites (RW n=2 and RW+S n=3)
Mean embryos harboured per female	29.75 ± 8.4	33.2 ± 9.7	6.2 ± 3.9

Mean ± standard deviation.

5.4.11 Effects of oestrogenic mixture exposure on F1 *V. viviparus* growth, survival and reproductive development

5.4.11.1 Number and size of juveniles collected at the end of exposure

The number of juveniles (F1s) collected from the mesocosm tanks at the end of the 16-week dosing period was highest in the RW tank (553) and lowest in the RW+S tank (100) (Table 5.43). The total number of neonates released into each tank by the adult snails was; 901 in the RW tank, 829 in the RW+S tank, 798 in the LM tank, and 810 in the HM tank. Therefore, the percentage survival of neonates during the exposure period was 61.4% in the RW tank, 12.1% in the RW+S tank, 31.3% in the LM tank and 17.3% in the HM tank. The percentage of snails above 10 mm shell length (normal minimum sexually active size) was highest in the RW exposure tank (80%). The RW+S tank had 49% of F1s above 10mm shell length, the LM tank had 60% of F1s above 10mm and the HM tank had 64% of F1s above 10mm shell length (Table 5.43).

Table 5.43 The number of *V. viviparus* F1s collected at the end of adult reproductive study.

	River Water (RW)	River Water plus Solvent (RW+S)	Low oestrogenic Mixture (LM)	High oestrogenic Mixture (HM)
<10 mm shell length	110 (19.9%)	51(51%)	100 (40%)	50 (36%)
>10 mm shell length	443 (80.1%)	49 (49%)	150 (60%)	90 (64%)
Total F1s	553	100	250	140

Adults and offspring exposed to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). The number of F1s above and below 10mm in shell length (normal minimum sexually active size), Percentage in brackets.

5.4.11.2 Size and weight of juvenile *V. viviparus*

In September 2006 (after dosing finished) approximately 50 F1s from the river water plus solvent (RW+S), low dose oestrogenic mixture (LM) and high dose oestrogenic mixture (HM) and 100 F1s from the river water (RW) were taken from the tanks and fixed for histopathological analysis. Due to time constraints it was decided that 20 specimens of each sex from each treatment would be sectioned, stained and analysed. The F1s were sexed once back at the laboratory using their secondary sexual characteristics i.e. enlarged right tentacle for males. However, once these specimens were analysed histopathologically it became apparent that in some cases the initial observed sex (from morphology) was incorrect, therefore the actual numbers were; 21 males and 15 females from the RW exposure, 26 males and 13 females from the RW+S, 21 males and 20 females from the LM and 24 males and 18 females from the HM exposure.

F1 Female *V. viviparus*

The shell length, shell aperture, total weight and soft body weight were analysed (Figure 5.15). On average the RW developmentally exposed females were largest and those from the HM exposure were smallest. Analysis of shell length and shell aperture by One way ANOVA found significant differences between exposure groups (P= 0.004 and 0.003 respectively). Post hoc analysis (LSD) found females from the RW to have significantly larger shell length and aperture compared to females from the HM (P= 0.001 and 0.001) or RW+S (P= 0.005 and 0.002) exposure. Females from the HM also had significantly smaller shell length than females from the LM (P= 0.030) exposure. However, no significant differences were found between the shell lengths of RW+S and HM (P= 0.789), or between LM and RW (P= 0.174) and no significant differences were found between the shell apertures of the snails from the other treatment groups. The total weight of the females was analysed using the Kruskal-Wallis test. A significant difference was found between the four treatment groups (P= 0.012). Post hoc analysis found that the total weight of F1 females from the RW exposure was significantly more than the HM (P= 0.001) and RW+S (P= 0.023) exposed females, but not compare to LM (P= 0.069) exposed snails. A significant difference was also found between the four exposure groups for soft body weight (shell removed) (P= 0.039 ANOVA). Post hoc analysis (LSD) found that only RW and HM (P= 0.009) were significantly different from one another (RW heavier than HM).

F1 Males *V. viviparus*

The shell length, shell aperture, total weight and soft body weight were analysed. F1 males from the RW exposure were on average largest and HM exposed males were smallest

(Figure 5.16). Analysis of shell length and aperture by One way ANOVA found significant differences between exposure groups (P=0.003, 0.010 respectively). Post hoc analysis (LSD) found the HM developmentally exposed snails to be significantly smaller than the RW (P= 0.001, 0.004), the LM (P=0.002, 0.013) for shell length and aperture, and the RW+S for shell length only (P= 0.048). The RW snails also had significantly larger shell aperture than the RW+S (P= 0.032) exposed snails. Significant differences were also found between exposure groups for total and soft body weights (P= 0.006 and 0.017 respectively, ANOVA). Post hoc analysis (LSD) found HM developmentally exposed snails to be significantly lighter (total and soft body weight) compared to the RW (P= 0.001, 0.010) exposed snails, the RW+S (P= 0.012, 0.019) exposed snails, and the LM (P= 0.017, 0.006) exposed snails.

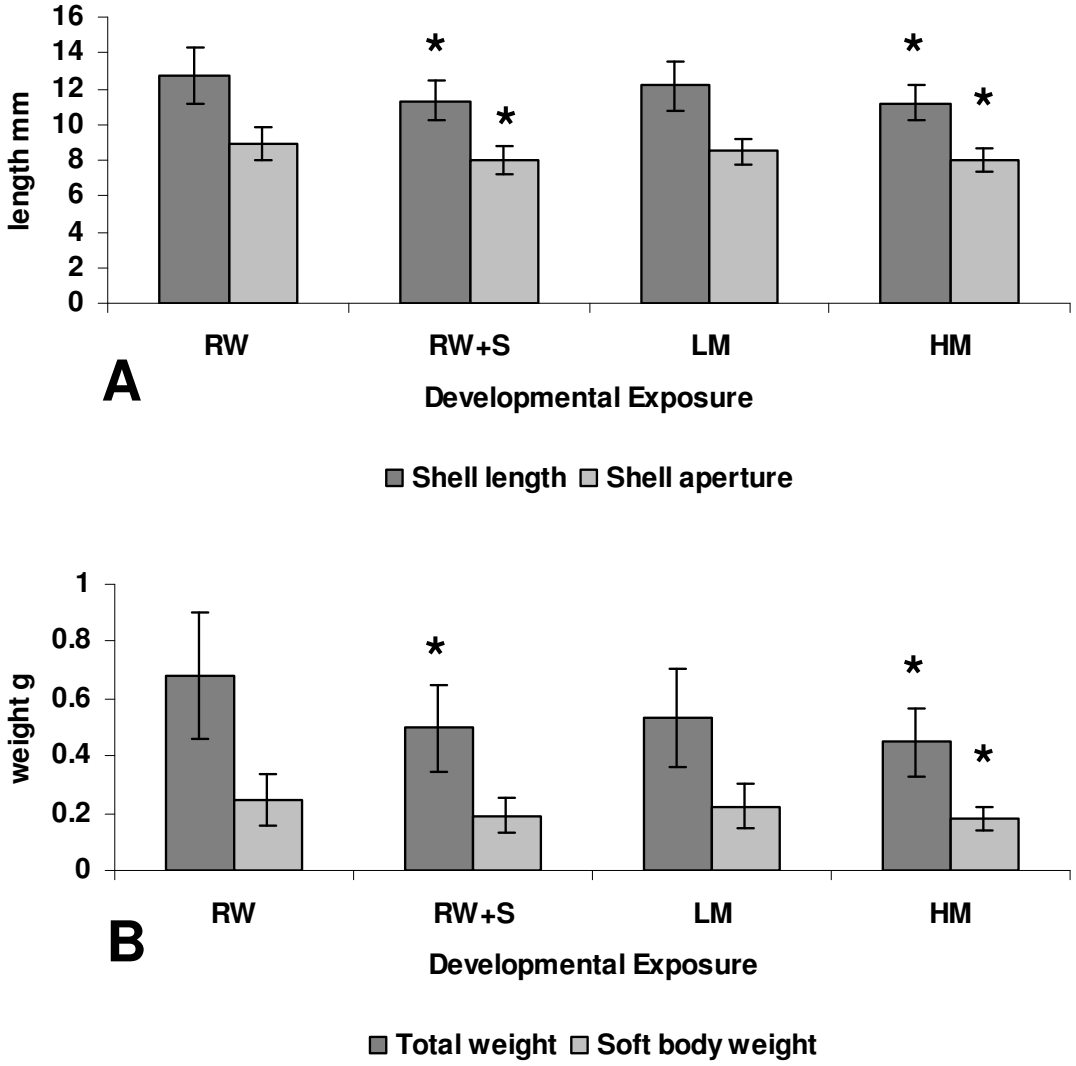

Figure 5.15 Size and weight of F1 *V. viviparus* females sampled September 2006 after the oestrogenic mixtures experiment.

Snails developmentally exposed to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). RW n = 15, RW+S n = 13, LM n = 20, HM n = 18. (A) Mean shell length and shell aperture. (B) Mean total weight and soft body weight. Error bars show standard deviation, Stars (*) indicate significant difference (P<0.05) from RW.

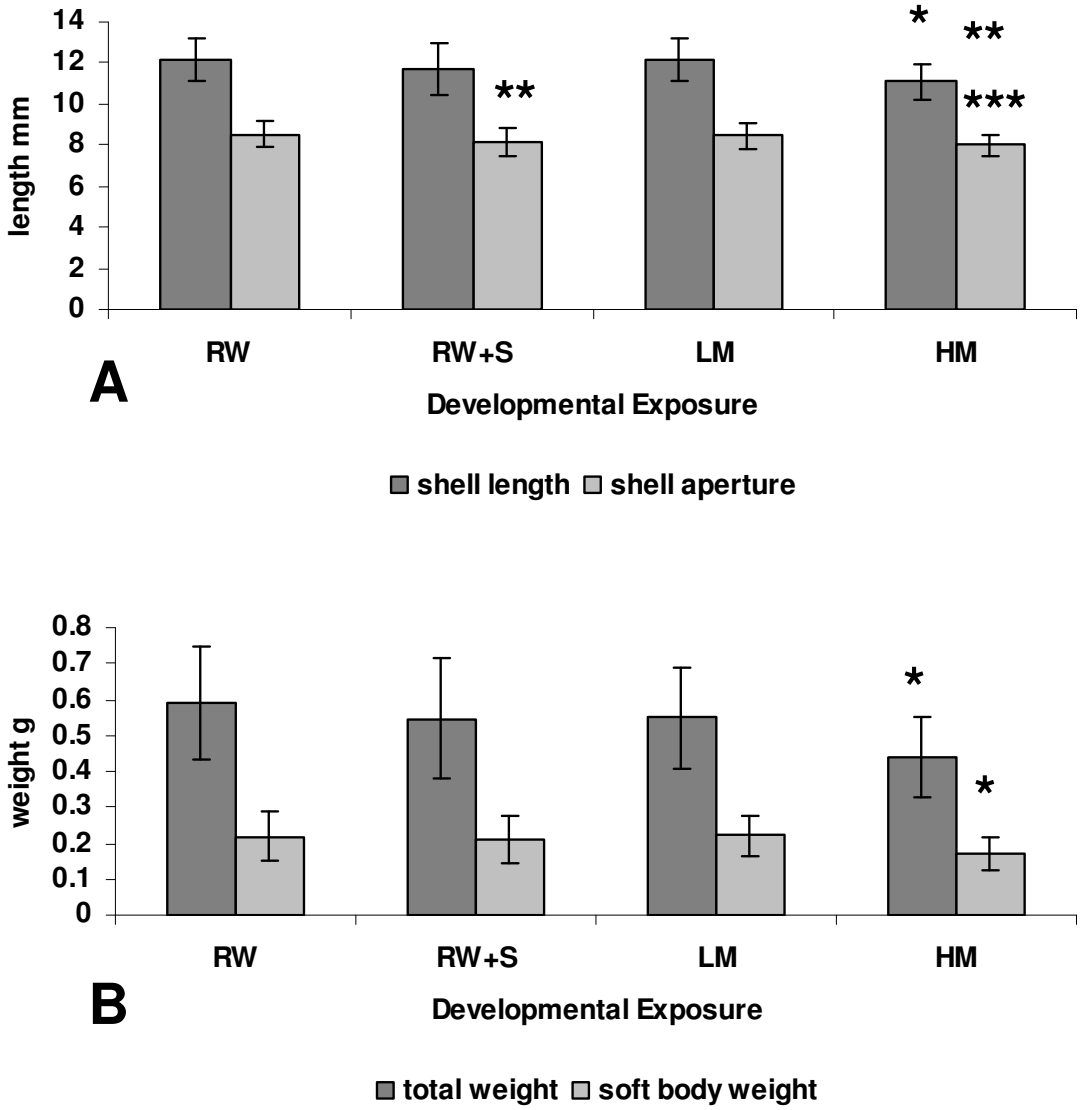

Figure 5.16 Size and weight of F1 *V. viviparus* males sampled September 2006 after the oestrogenic mixtures experiment.

Snails developmentally exposed to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). RW n = 21, RW+S n = 26, LM n = 21, HM n = 24. (A) Mean shell length and shell aperture. (B) Mean total weight and soft body weight. Error bars show standard deviation. Star * indicate significant difference (P<0.05) from all other exposure groups, double star ** indicates significant difference from RW exposure group, triple star * indicates significant difference from LM exposure group.**

5.4.12 Effects of oestrogenic mixture exposure on the histopathology of *V. viviparus* F1 gonad immediately after developmental exposure

Approximately 40 F1s (from each exposure group) from the September 2006 collection period were analysed histologically (RW, 21 males and 15 females; RW+S, 26 males and 13 females; LM, 21 males and 20 females; HM, 24 males and 18 females).

Female oocyte number immediately after developmental exposure to oestrogenic mixtures

The mean number of oocytes per section of gonad analysed was highest in F1s developmentally exposed to the RW and lowest in those exposed to the HM. The RW developmentally exposed females had an average of 3.8 ± 4.5 oocytes per section, those exposed to RW+S had 2.6 ± 2.3 oocytes per section, LM had 3.1 ± 1.8 and HM had 1.6 ± 1.6 oocytes per section (Figure 5.17). Analysis using Kruskal-Wallis test found a significant difference between the exposure groups (P= 0.023). Post hoc analysis (Mann-Whitney) found the HM exposed females to have significantly less oocytes per section compared to the RW (P= 0.045) and the LM (P= 0.002) exposed snails. However, no significant differences were found for the mean oocytes number between the other treatment groups.

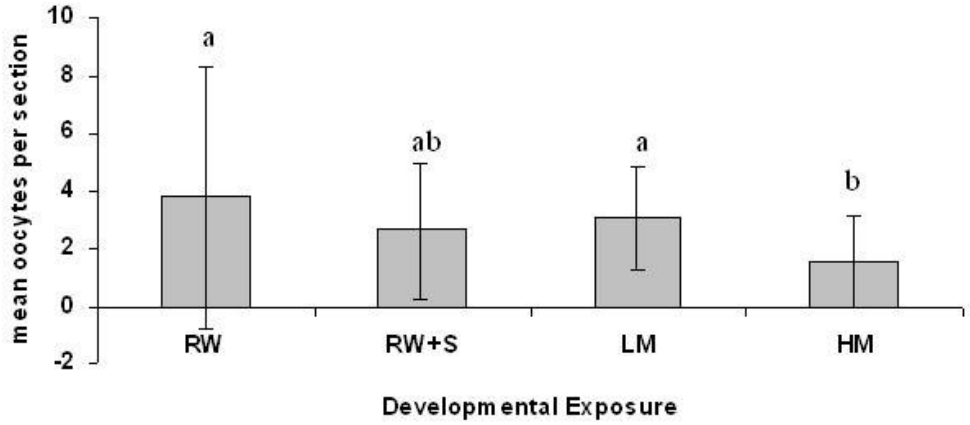

Figure 5.17 Mean oocytes per section of gonad analysed of F1 *V. viviparus* females sampled September 2006 after oestrogenic mixture experiment.

Snails developmental exposed to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM), RW n = 15, RW+S n = 13, LM n = 20, HM n = 18. Five sections analysed per female. Error bars indicate standard deviation. Letters (a, b) indicate statistical similarity.

Correlations between F1 female size and sexual development

Significant positive correlations (Pearson's) were found for the RW, RW+S, LM and HM developmentally exposed snail's between shell length and total weight (RW; 0.980 P<0.001, RW+S; 0.953 P<0.001, LM; 0.973 P<0.001, HM; 0.913, P<0.001) and shell length and soft body weight (RW; 0.915 P<0.001, RW+S; 0.950, P<0.001, LM; 0.913 P<0.001, HM; 0.713 P=0.001). Significant positive correlations were also found between shell length and mean oocyte number (0.577, P=0.031), total weight and mean oocyte

number (0.636, P=0.015), and soft body weight and mean oocyte number (0.778, P=0.001) for the RW exposed snails, indicating that on average larger snails would have higher mean number of oocytes per section of tissue analysed. However, no significant correlations were found for the other three treatments between shell length and mean oocyte number (RW+S; 0.564 P=0.090, LM; 0.144 P=0.569, HM; 0.054, P=0.842), total weight and mean oocyte number (RW+S; 0.615 P=0.059, LM; 0.117 P=0.645, HM; -0.085 P=0.756) or between soft body weight and mean oocyte number (RW+S; 0.595 P=0.70, LM; 0.007 P=0.977, HM; -0.036 P=0.894).

When the mean oocytes number was analysed with shell length, total weight or soft body weight as co-variance (ANCOVA) the significant differences (in oocyte number) were no longer evident (P= 0.763, 0.755, 0.673 respectively), indicating that perhaps the lower oocyte number in HM exposed snails maybe linked to their significantly smaller size.

Male spermatogenesis score immediately after developmental exposure to oestrogenic mixtures

Mean spermatogenesis scores were highest in snails from the RW+S exposure and lowest in snails exposed to the HM (Figure 5.18). Analysis by Kruskal-Wallis found a significant difference between the four exposure groups (P= 0.005). Post hoc analysis (Mann-Whitney) found HM males to have a significantly lower spermatogenesis score compare to the RW (P=0.011), RW+S (P= 0.002) or LM (P= 0.007) exposed snails. No significant difference was found between the RW and the RW+S (P= 0.324) exposed snails, the RW and the LM (P= 1.00) exposed snails, or between the RW+S and the LM (P= 0.372) exposed snails.

Correlations between F1 male size and sexual development

Significant positive correlations (Pearson's) were found for the RW, RW+S, LM and HM developmentally exposed snail's between their shell length and total weight (RW; 0.955 P<0.001, RW+S; 0.974 P<0.001, LM; 0.966 P<0.001, HM; 0.941 P<0.001), between shell length and soft body weight (RW; 0.918 P<0.001, RW+S; 0.949 P<0.001, LM; 0.930 P<0.001, HM; 0.886 P<0.001), shell length and mean spermatogenesis score (RW; 0.830 P<0.001, RW+S; 0.771 P<0.001, LM; 0.742 P<0.001, HM; 0.604, P=0.002), total weight and spermatogenesis score (RW; 0.800 P<0.001, RW+S; 0.749 P<0.001, LM; 0.796 P<0.001, HM; 0.626, P=0.001) and soft body weight and mean spermatogenesis score (RW; 0.792 P<0.001, RW+S; 0.819 P<0.001, LM; 0.795 P<0.001, HM; 0.551, P=0.006). These indicate that larger juvenile males would have higher spermatogenesis scores.

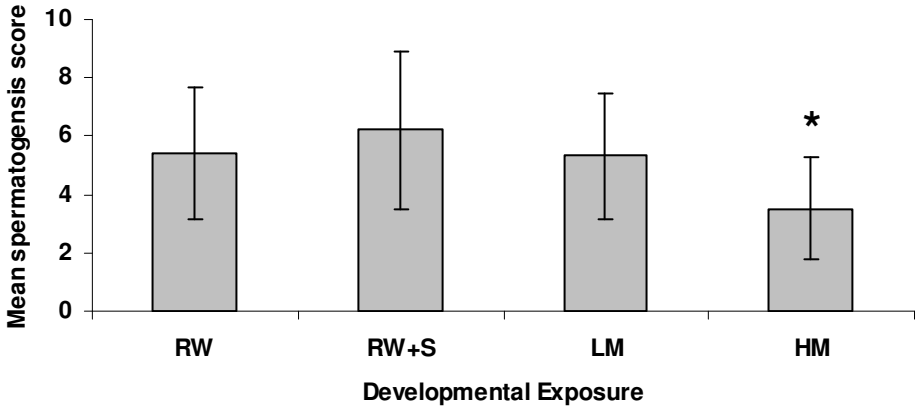

Figure 5.18 Mean spermatogenesis score of F1 *V. viviparus* males sampled September 2006 after oestrogenic mixture experiment.

Snails developmentally exposed to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM). RW n = 21, RW+S n = 26, LM n = 21, HM n = 24. Score 1 - no germ cells present, Score 10 - full spermatogenesis in more than 70% of tubules. Five sections of gonad analysed per male. Error bars give standard deviation. Star (*) indicates significant difference (P<0.05) from all other exposure groups.

To analyse whether the significant reduction in spermatogenic score with high dose of oestrogenic mixture was due to the smaller size of mixture exposed snails, spermatogenesis score was co-analysed with shell length, total weight or soft body weight. In each case a significant difference was still found between the three treatments (P<0.001, 0.011, 0.007 respectively, ANCOVA). Post hoc analysis (Bonferroni) of the co-variant data showed the RW+S exposed males to have significantly higher spermatogenesis score compare to HM (shell length; P=0.002, total weight; P=0.021, soft body weight; P=0.010) and LM (shell length; P=0.003, soft weight; P=0.046) as well as the RW (shell length; P=0.008) exposed snails. No significant differences were found between the RW+S and LM when co-analysed for total weight (P=0.183), or between the RW+S and RW when co-analysed for total weight (P=0.060) or soft body weight (P=0.246), or between the LM and HM exposures for any co-variant (P=1.000).

Parasitism

Encysted parasites (metacercaria) were found in the muscular tissue of the head and the foot in the majority of F1s from the mixture exposures in both male and female snails (Table 5.44), but not in those exposed to RW or RW+S. No parasitic infections of the digestive tissue were seen in any of the F1s analysed.

Table 5.44 Percentage of snails from each exposure group with encysted parasite infections.

	River Water (RW)	River Water plus Solvent (RW+S)	Low oestrogenic Mixture (LM)	High oestrogenic Mixture (HM)
F1 males	0	0	100%	95.7%
F1 females	0	10%	94.7%	100%

F1 *V. viviparus* snails sampled September 2006 after developmental exposure to River Water only (RW), River Water plus Solvent (RW+S), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM).

The severity of parasite infection (increasing number of parasites per section analysed) was also measured using a parasite score. The severity of parasite infection increased with dose in both male and female snails (

Figure 5.19). Statistical analysis (Kruskal-Wallis) found the mean parasite score to be significantly different between exposures for *V. viviparus* F1s (male $P<0.001$, female $P<0.001$). Post hoc analysis found that the RW and the RW+S exposed snails to have a significantly lower parasite scores than the LM (male $P<0.001$, female $P<0.001$) or the HM (male $P<0.001$, female $P<0.001$) developmentally exposed snails. No significant differences in parasite score were found between the HM and the LM developmentally exposed *V. viviparus* (male $P=0.398$, female $P=0.061$), or between the RW and the RW+S developmentally exposed snails (male $P=1.000$, female $P=0.683$).

Correlation between reproductive potential and parasite infection

No significant correlations (Spearman's) were found between the mean parasite score and the mean number of oocytes per section for the HM (0.328, $P=0.215$) or the LM (0.002, $P=0.993$) developmentally exposed females. RW and RW+S snails did not have sufficient parasite infections to be correlated.

A significant positive correlation (Spearman's) was found between the mean parasite score and the mean spermatogenic score (0.449, $P=0.032$) of the HM developmentally exposed males. However, a slight (not significant) negative correlation (Spearman's) was found between the mean spermatogenesis score and the mean parasite score (-0.081, $P=0.735$) of LM developmentally exposed males. A positive (but not significant) correlation was also found between shell length and mean parasite score in the LM (0.325, $P=0.175$) and the HM (0.383, $P=0.075$) developmentally exposed snails.

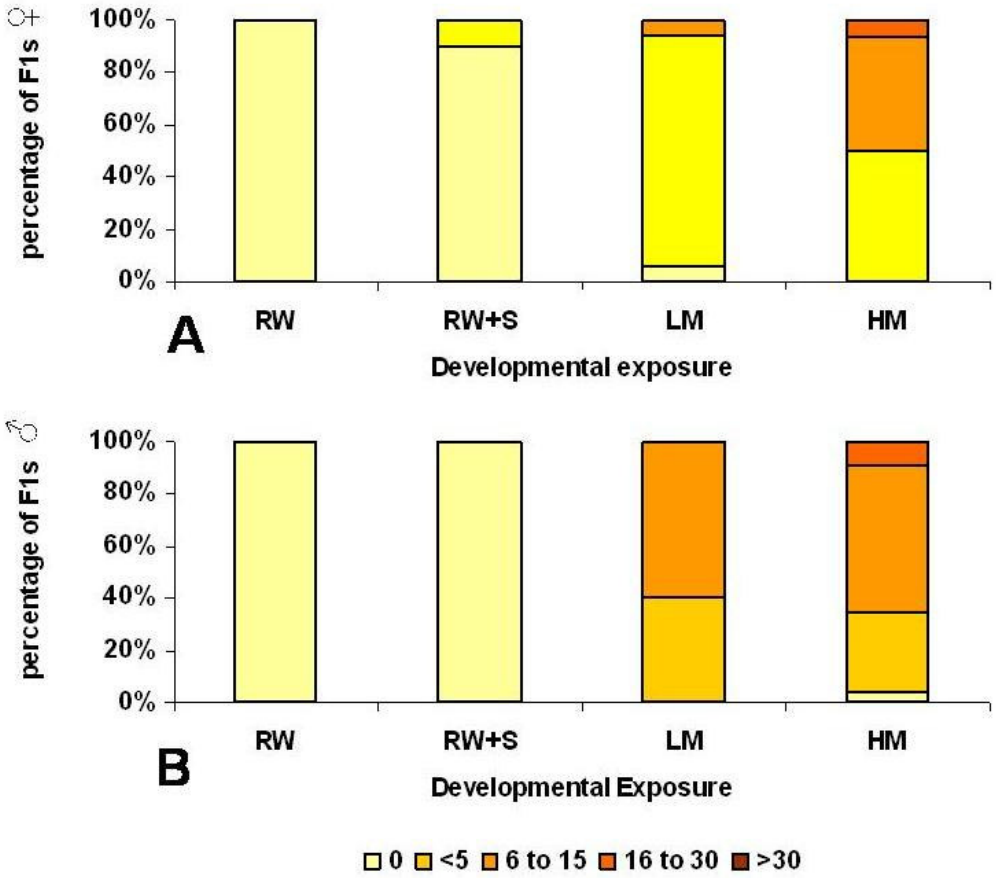

Figure 5.19 Encysted parasite infections in F1 *V. viviparus* after oestrogenic mixtures experiment.

Snails developmentally exposed to river water only (RW), river water plus solvent (RW+S), river water plus low oestrogenic mixture (LM), or river water plus high oestrogenic mixture (HM). (A) the percentage of females *V. viviaprus* with varying intensities of parasite infection. (B) the percentage of males *V. viviparus* with varying intensities of parasite infection. 5 sections per snail were analysed. Pale yellow indicates no parasites found, yellow indicates less than 5 parasites per section, light orange indicates 5-15 parasites per section, dark orange indicates 15-30 parasites per section, brown indicates more than 30 parasites per section.

5.4.13 Effects of early developmental exposure to oestrogenic mixtures, and then depuration in river water on F1 *V. viviparus*

5.4.13.1 Survival and growth during depuration in river water

The percentage survival over-winter was higher in the HM exposed group compared to RW and LM. 53% of hatchlings survived in the RW, 33% from the LM and 75% from the HM (Table 5.45).

Table 5.45 Number of F1 *V. viviparus* left to depurate over winter after developmental exposure.

F1 *V. viviparus*	River Water	Low oestrogenic mixture (LM)	High oestrogenic mixture (HM)
Left to over-winter September 2006	520	200	84
Surviving March 2007	273	66	63
Percent survival	53%	33%	75%

Number left in each treatment tank in September 2006, number surviving until March 2007 and percentage over winter survival.

5.4.13.2 Reproductive output during F1 breeding study (spring-summer 2007)

None of the F1 females from the three developmental exposures released any neonates during the 18 week breeding study (Table 5.46). At the end of the study the brood pouch of female snails were dissected to count the number of embryos harboured. None of the mixture developmentally exposed females (LM, n=17; HM n=15) harboured any embryos (shelled or unshelled) compared to 12% of F1 females from the RW exposure (RW, n=25).

Table 5.46 The mean number of embryos (shelled or unshelled) harboured in the brood pouch per F1 female *V. viviparus* at the end of the 18 week F1 breeding study.

	Shelled embryos	Un-Shelled embryos
River Water developmental exposed (n=25)	0.04	0.33
Low Mix developmental exposed (n=17)	0	0
High Mix developmental exposed (n=15)	0	0

***V. viviparus* had been developmentally exposed to River Water only (RW), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM) and then depurated in river water for eight months before being allocated into breeding groups.**

5.4.13.3 Survival and growth during F1 breeding study

Over the F1 breeding study, percent survival was highest from the HM developmental exposure group and lowest from the LM exposure group (RW, 84.8%; LM, 78.8%; HM, 96.7%). At the beginning of the F1 breeding study, developmentally exposed snails were smaller than those from the RW exposure. Mean shell length ($P<0.001$, ANOVA), shell aperture ($P<0.001$, ANOVA) and total weight ($P<0.001$, ANOVA) were significantly different between the three developmental exposure groups. Post hoc analysis (LSD) found the RW exposed snails to be significantly larger than the LM (shell length $P<0.001$, shell aperture $P<0.001$ and total weight $P<0.001$) and the HM (shell length $P<0.001$, shell

aperture P<0.001 and total weight P<0.001) exposed snails. The LM exposed snails were also significantly larger than the HM (shell length P= 0.026, shell aperture P=0.022 and total weight P=0.020) exposed snails.

At the end of the F1 breeding study, females from the LM developmental exposure had on average larger shell length, shell aperture, total weight and soft body weight than the other two exposure groups (Table 5.47). However, statistical analysis (ANOVA) found no significant differences between the exposure groups (shell length P= 0.428, shell aperture P= 0.770, total weight P= 0.715, soft body weight P= 0.222).

Table 5.47 Size and weight of *V. viviparus* F1 female after un-dosed breeding study.

	River Water (RW) developmentally exposed and then depurated	Low oestrogenic Mixture (LM) developmentally exposed and then depurated	High oestrogenic Mixture (HM) developmentally exposed and then depurated
Shell length mm	15.5 ± 1.3	16.0 ± 1.1	15.7 ± 1.1
Shell aperture mm	10.5 ± 0.7	10.6 ± 0.6	10.5 ± 0.7
Total weight g	1.0 ± 0.2	1.1 ± 0.3	1.0 ± 0.2
Soft body weight g	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1

Snails developmentally exposed to River Water only (RW), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM) and then depurated in river water. Mean length or weight ± standard deviation. (RW, n = 25; LM, n = 17; HM, n = 15)

At the end of the F1 breeding study the males from the RW developmental exposure were on average larger than those from the LM or HM exposures (Table 5.48). However, statistical analysis (ANOVA) found no significant differences between the exposure groups (shell length P= 0.330, shell aperture P= 0.617, total weight P= 0.866, soft body weight P= 0.249).

Comparison of male and female size at end of the F1 breeding study

Females from the mixture exposures were on average larger than similarly exposed males. Whereas, males and females from the RW exposure were of similar size (Table 5.47 and Table 5.48) to each other. A t-tests found no significant differences between the size of the RW males and females shell length (P= 0.964), shell aperture (P= 0.356), total weight (P= 0.913) or soft body weight (P= 0.866). Similarly no significant differences were found between the HM male and female shell length (P= 0.106), shell aperture (P= 0.092), total weight (P= 0.213) or soft body weight (P= 0.351). When the LM males and females were compared, the females were found to be significant larger in shell length (P= 0.019, Mann-

Whitney), shell aperture (P= 0.033, M-W) and soft body weight (P= 0.050, t-test). No significant difference was found between total weight (P= 0.330, t-test) of the RW males and females.

Table 5.48 Size and weight of *V. viviparus* F1 males after un-dosed breeding study.

	River Water (RW) developmentally exposed and then depurated	Low oestrogenic Mixture (LM) developmentally exposed and then depurated	High oestrogenic Mixture (HM) developmentally exposed and then depurated
Shell length mm	15.5 ± 1.4	14.3 ± 1.5	15.1 ± 1.3
Shell aperture mm	10.1 ± 0.8	9.8 ± 0.9	10.1 ± 0.7
Total weight g	1.0 ± 0.3	1.0 ± 0.4	1.0 ± 0.2
Soft body weight g	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1

Snails developmentally exposed to River Water only (RW), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM) and then depurated in river water. Mean length or weight ± standard deviation. (RW, n = 4; LM, n = 7; HM, n = 15)

5.4.14 Effect of developmental exposure to oestrogenic mixtures and then depuration in river water on histopathology of the gonad

All the F1s were sampled at the culmination of the F1 breeding study (after approximately one year of depuration). All the F1s were sectioned stained and analysed histologically (RW, 24 ♀ and 4 ♂; LM, 18 ♀ and 7 ♂; HM 17 ♀ and 15 ♂). Five sections of gonad were analysed per snail.

Post F1 breeding study female oocyte number

The mean oocyte number was highest in snails developmentally exposed to the RW, and reduced in a dose dependant manner (Table 5.49). Statistical analysis (Kruskal-Wallis) found a significant difference between the three exposure groups (P= 0.001). Post hoc analysis (Mann-Whitney) found RW snails to have significantly more oocytes compared to the LM (P= 0.009), and the HM (P<0.001) developmentally exposed snails. No significant difference was found between the LM and the HM (P= 0.349) exposed snails.

Correlations between female F1 size and reproductive potential

Spearman's rank order was used to correlate F1 female *V. viviparus* size and weight to reproductive potential (mean oocytes number). The RW, LM and HM exposed females had significant positive correlations between shell length and total weight (RW; 0.927 P<0.001, LM; 0.800 P<0.001, HM; 0.900 P<0.001), and shell length and soft body weight (RW;

0.866 P<0.001, LM; 0.749 P= 0.001, HM; 0.786 P=0.001). The RW exposed females also had a significant positive correlation between mean oocytes and total weight (0.481, P= 0.020). Whereas, no significant correlations were found for the LM and HM snails (LM; 0.027 P=0.920, HM; -0.113, P=0.689).

Table 5.49 Mean oocyte numbers per section of gonad analysed of *V. viviparus* F1 female snails from the depurated oestrogenic mixtures experiment

	River Water (RW)	Low oestrogenic Mixture (LM)	High oestrogenic Mixture (HM)
Mean oocyte number	4.0 ± 4.1	1.4 ± 2.4	0.8 ± 1.5
	A	b	b

Snails developmentally exposed to River Water only (RW), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM) and then depurated in river water for approximately one year. five sections analysed per snail. Mean ± standard deviation (RW, n = 25; LM, n = 17; HM, n = 15). Letter (a, b) indicates statistical similarity.

Throughout this study it has been found that there is a relationship between *V. viviparus* snail size and mean number of oocytes; larger/heavier females tend to have more oocytes per section of tissue. Therefore the mean number of oocytes was analysed with shell length, total weight or soft body weight as co-variants. Analysis by ANCOVA found a significant difference between the treatments (P<0.001, 0.001, 0.001, respectively). Post hoc analysis (Bonferroni) of this co-variant data was also conducted. Significant differences were found between the RW and both the HM and LM when co-analysed for shell length (P=0.001 and 0.005, respectively), total weight (P=0.003 and 0.008, respectively) and for soft body weight (P=0.001 and 0.007, respectively). No significant differences were found between HM and LM snails (P=1.000).

Post F1 breeding study male spermatogenesis score

Mean spermatogenesis score was lowest in LM developmentally exposed snails and highest in RW exposed snails (Table 5.50). However statistical analysis (Kruskal-Wallis) found no significant difference between the three groups (P= 0.572).

Correlations between male F1 size and reproductive potential

No significant correlations were found for the RW developmentally exposed males (n=4). However, the shell length and total weight were positively correlated (0.800, P=0.200) and shell length and soft body weight were positively correlated (0.800, P=0.200). Shell length, total weight and soft body weight were also positively correlated to mean spermatogenesis

score were positively correlated (0.775, P=0.225).). Significant positive correlations were found for the LM (n=6) and HM (n=15) exposed males between shell length and soft body weight (LM; 0.886 P=0.019, HM; 0.966 P<0.001). For the HM developmentally exposed males significant positive correlations were also found between shell length and soft body weight (0.882, P<0.001), shell length and mean spermatogenesis score (0.549, P=0.034), total weight and mean spermatogenesis score (0.636, P=0.011) and between soft body weight and mean spermatogenesis score (0.706, P=0.003). Whereas for the LM exposed males positive, but not significant, correlations were found between the shell length and total weight (0.657, P=0.156), shell length and mean spermatogenesis score (0.577, P=0.231), total weight and mean spermatogenesis score (0.698, P=0.123) and between soft body weight and mean spermatogenesis score (0.395, P=0.439).

Table 5.50 Mean spermatogenesis score per section of gonad analysed of *V. viviparus* F1 male snails from the depurated oestrogenic mixtures experiment

	River Water (RW)	Low oestrogenic Mixture (LM)	High oestrogenic Mixture (HM)
Mean spermatogenesis score	9.95 ± 0.1	8.94 ± 1.83	9.61 ± 1.01

Snails developmentally exposed to River Water only (RW), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM) and then depurated in river water for approximately one year. Spermatogenesis score; 1= no germ cells, to 10 = full spermatogenesis in >70% of tubules. Five sections analysed per snail. Mean ± standard deviation (RW, n = 4; LM, n = 7; HM, n = 15).

As positive correlations between size/weight and mean spermatogenic scores were present in the juvenile male *V. viviparus* snails, ANCOVA analysis was performed to see if significant differences in mean spermatogenesis scores could be observed when co-analysed for shell length, total weight or soft body weight. No significant differences were found between the treatments (shell length; P=0.827, total weight; P=0.316, soft body weight; P=0.530).

Parasitism

The majority of F1s from the developmental exposures had encysted parasite infections. However, one of the F1s from the RW exposure had parasites. The severity of parasite infection increased with dose (Figure 5.20). Mean parasite scores were analysed statistically (Kruskal-Wallis) for both male and female snails. Significant differences between treatments were found for males (P= 0.001) and females (P<0.001). Post hoc analysis (Mann-Whitney) found RW exposed snails to have a significantly lower parasite

score compared to LM (male P=0.039, female P<0.001), and HM (male P=0.002, female P<0.001) developmentally exposed snails. The LM exposed snails were also found to have a statistically lower parasite score compare to the HM (male P=0.014, female P= 0.041) exposed snails.

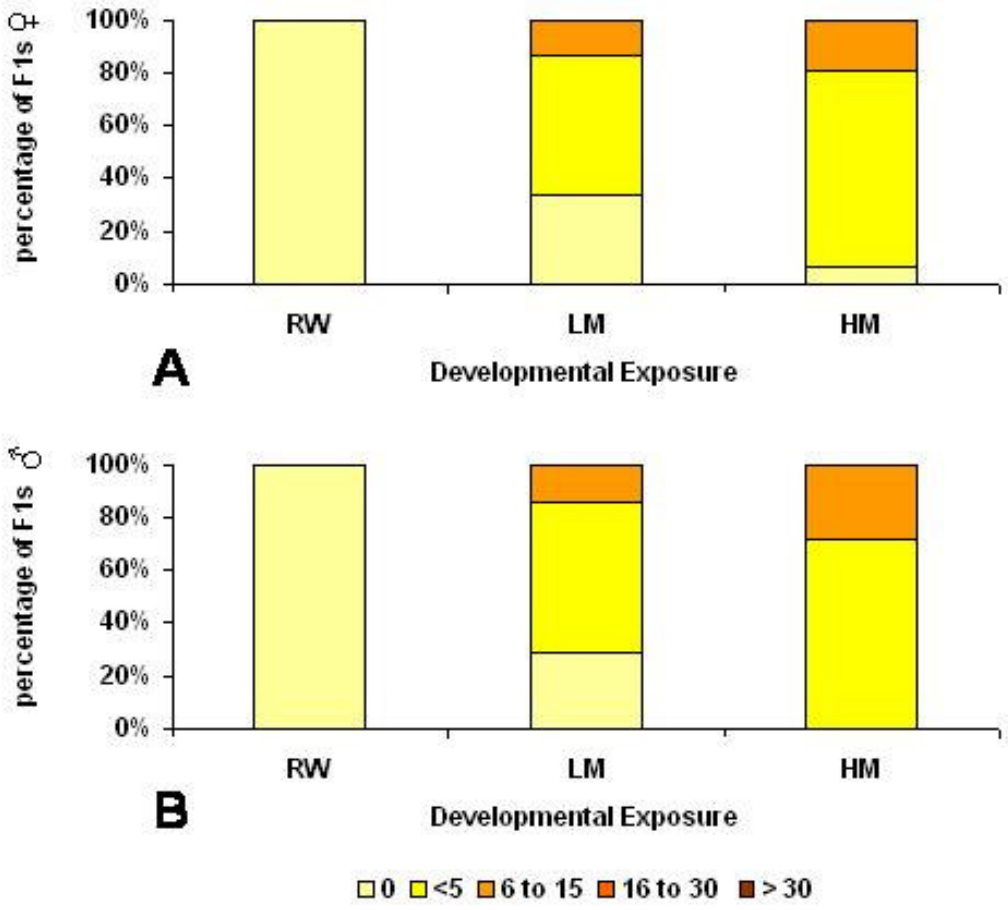

Figure 5.20 Parasite load of F1 *V. viviparus* after oestrogenic mixtures experiment.

Snails developmentally exposed to River Water only (RW), river water plus Low oestrogenic Mixture (LM) or river water plus High oestrogenic Mixture (HM) and then depuration in river water for a year. (A) percentage of F1 females with varying intensity of encysted parasite infection. (B) percentage of F1 males with varying intensity of encysted parasite infection. Intensity of infection scored from 0 encysted parasites per section analysed (pale yellow) to a maximum of >30 encysted parasites per section (brown). Five sections of tissue analysed per F1 snail.

Slight (but not significant) negative correlations were found between the mean parasite scores and the mean spermatogenesis scores (LM; -0.308, P=0.553, HM; -0.126, P=0.654).

Correlations between parasite score and reproductive potential

There were no significant correlations (Spearman's) found between the parasite score and the spermatogenesis score for either the LM (-0.308, P=0.553) or the HM (-0.126, P= 0.654) developmentally exposed and then depurated males. No significant correlations were found between mean parasite score and mean oocyte number for the LM or HM developmentally exposed females. A very slight positive (but not significant) correlation was found between the mean parasite score and the mean oocyte number in the LM developmentally exposed females. Whereas, a slight (but not significant) negative correlation was found between the mean parasite score and mean oocyte number of the HM developmentally exposed females.

5.5 Discussion

5.5.1 Physical parameters, water chemistry and YES assay

As with the E2 exposure (Chapter 4), the water temperature within the mesocosm tanks varied with weather and season, and was expected within this type of experimental set up. The fluctuating temperature and day length are important seasonal cues, which can stimulate or inhibit reproduction in gastropods (Chapter 1, Section Figure 2.2). These cues were important in the design of the experiment as recent evidence suggests that gastropods may be especially sensitive to vertebrate EDCs during times of increasing or reducing reproduction (Oehlmann et al. 2006, Benstead 2007, Clarke et al. In Press). The flow rate of the water in to the mesocsom tanks fluctuated, as did the pump rates. The dosing pump rate was significantly faster into the RW+S tank than into the HM tank and the RW+S also had a slightly (but not significantly) lower flow rate of river water into the tank compared to the other tanks. As a consequence of the combination of the higher dosing pump rate and lower flow rate, the RW+S tank had a higher concentration of carrier solvent (ethanol) compared to the LM or the HM tanks (Figure 6.3.1). However, the maximum concentration of Ethanol in the RW+S tank (0.000654µl/l) fell well below the OECD guideline of a maximum of 100µl/l (Hutchinson et al. 2006). The water flow and dosing pump rates were not significantly different between the two dosed exposure tanks.

Chemical analysis of the mesocosm tank water found the average concentration of E1, EE2, NP and BPA to be highest in the HM tank (OP was below the LOD in all tanks). The average E2 concentration was highest in the LM tank. The lowest average concentrations of E1 and EE2 were measured in the RW tank and the lowest average concentrations of E2 and BPA were measured in the RW+S tank. The average NP concentration was the same in both the RW and RW+S tanks. As was found in the E2 mesocsom study (Chapter 4), a wide variation in measured concentrations of each chemical was experienced. The

measured concentrations of each chemical were generally much lower than the nominal concentrations, with the exception of EE2. When the measured concentrations of each chemical were compared to the predicted concentration (from the dilution factor) they too were lower. However, only OP and NP were significantly lower than the predicted concentrations. The measured concentration of EE2 in the LM tank was the only chemical dosed to be significantly higher than the predicted concentration.

The generally lower than expected concentrations of the alkylphenols and BPA in both the dosed mesocosm tanks is likely to be related to the experimental set up; namely the use of river water (complete with algae, bacteria, other biota and suspended sediments). As has been discussed for steroid oestrogens (Chapter 4, Section 4.4.1), both alkylphenols and BPA are subject to sorption to sediments, up-take by biota and bacterial degradation within the mesocosm tanks. There were large volumes of suspended sediments accompanying the river water in each tank; at the culmination of the 16-week exposure period approximately 50-70mm depth of sediment had been deposited on to the bottom of each tank. BPA has the lowest affinity for absorption to sediments, compared to OP and NP, as it only weakly binds to the sediment (Ying et al. 2003). However, this could still be a possible route by which the BPA dosed into the mesocosm could be removed from the water column. On the other hand the BPA may have been degraded. There are many published reports that BPA is metabolised by bacteria found in river water. Kang and Kondo (Kang and Kondo 2002b, Kang and Kondo 2002a) and Ike et al (Ike et al. 2000) report that BPA-degrading bacteria are common in river water. The case for these BPA degrading bacteria being widely present was questioned, however, when Ying et al (Ying et al. 2003) reported that in their ground water samples BPA was not degraded after 70 days. They suggest that specific BPA degrading bacteria may proliferate in polluted areas, which may well be true as there are many reports of BPA being degraded in sewage sludge. Kang and Kondo (Kang and Kondo 2002b) report that BPA degradation increased with both bacterial count and water temperature and that no degradation was observed in autoclaved samples. Ike et al (Ike et al. 2000) report that although many bacteria degrade BPA, the majority of them cannot fully mineralise the BPA, and persistent metabolites are formed. Suzuki et al (Suzuki et al. 2004) also report BPA metabolites in river water. Some of these metabolites were also found to be possible xeno-oestrogens, as they bound to the human αER (Suzuki et al. 2003). This obviously leaves us with the possibility that although the BPA could have been partially removed from the mesocosm tanks, the oestrogenic metabolites may still be present. BPA may have also accumulated in animal tissue within the tank. Kang and Kondo (Kang and Kondo 2006) report concentrations of BPA in freshwater snail tissue

(11.0 ± 3.4ng/g), over eleven times that of the river water they inhabited. Takahashi et al (Takahashi et al. 2003) report that BPA could be bioaccumulated by periphytons (18-650 times) and benthos (8-170). Unfortunately none of the animals within the mesocosm were analysed for uptake or bioaccumulation of any of the chemicals dosed. APEs are well known to biodegrade into metabolites under aerobic conditions (Ying et al. 2002, Ying 2006, Ahel et al. 1993, Langston et al. 2005), and increased temperature increases the degradation rate (Ying et al. 2002). The alkylphenols NP and OP have a relatively high affinity to organic rich sediments compared to water, due to their structure and slightly hydrophobic nature (Ying et al. 2002, Ying 2006, Langston et al. 2005). Ying et al (Ying et al. 2003) reported that NP had a sorbtion coefficient of 195 and that OP had a sorbtion coefficient of 90.9, and that they both strongly bound to the aquifer sediments tested. Johnson et al (Johnson et al. 1998) have reported that the distribution coefficient (K-d) of OP was between 6-700 l/kg in a range of river bed sediments from the UK (Aire, Calder and Thames), and that suspended sediments may adsorb 5-35 times the amount of OP compare to bed sediments. As would be expected, increased organic matter and finer particle size also increased the level of sorbtion (Johnson et al. 1998). NP had a K-d of 1902 l/kg in River Avon water and sediment samples (Langston 2008). From the above information it would be expected that a large proportion of both the alkylphenols, but perhaps especially the NP, would have sorped onto the suspended and settled organic matter, which was present in all the mesocsom tanks. In addition to the sorbtion of the OP and NP, as with the BPA, biota may have also played a part in their removal from the water column. Takahashi et al (Takahashi et al. 2003) report that periphytons can bioaccumulate NP by 160-650 times, and that benthos can bioaccumulate NP around 63-990 times. Ahel et al (Ahel et al. 1993) report extremely high bioaccumulation factors of NP, up to 10000 times, in the filamentous blanket weed *Cladophora glomerata*. It is therefore interesting to note that filamentous blanket weed was present in all the mesocsom tanks and grew vigorously throughout the experiment. Microalgae (*Isochrysis galba*) have also been reported to bioconcentrate NP by 6940 times; the uptake was extremely fast with 77% of the NP added to the water accumulated intracellularly after 1 hour (Correa-Reyes et al. 2007). The sorption of NP and OP to suspended organic matter and uptake in algae may well have provided an additional route of exposure (via consumption) to both gastropod species tested in this system. Both NP and OP have been reported to accumulate in molluscs. Indeed, concentrations of 2740ng/g NP and 820ng/g OP have been reported in oysters and 870ng/g NP and 400ng/g OP in marine snails from an industrial area of Taiwan (Chin-Yuan et al. 2005). The higher accumulation reported in the oyster may be due to their filter feeding nature. Lietti et al (Lietti et al. 2006) have reported bioaccumulation

factors between 1098-1918, in 7 day laboratory exposures of the clam *Tapes philippinarum*.

Water samples (for YES analysis) were taken from the two dosing reservoirs to assess how stable the oestrogenic chemicals were between renewal periods. Samples from both the LM and the HM reservoirs were taken 30 minutes after the stocks were mixed with the tap water, and then again after 72 hours. The predicted EEQ concentrations of the dosing reservoirs were 27.9µg/l in the LM reservoir and 146.6µg/l in the HM reservoir. The HM reservoir sample taken after only 30 minutes had past, had around 131% of the predicted EEQ and the LM reservoir had around 408% of the predicted EEQ. After 72 hours in the HM reservoir the EEQ had reduced to around 57% of the predicted EEQ, whereas in the LM reservoir the measured EEQ had reduced slightly to around 294% of the predicted EEQ. It is possible that both sorption and bacterial action affected the dosing reservoirs. As discussed in Chapter 4 (Section 4.4.1), it is possible that over the dosing period a large amount of chemical sorped onto the plastic surface of the dosing reservoir. When the dosing reservoir was refilled with water and the mixtures stock, a new state of equilibrium may have released some of the sorped chemicals back into the water. When compared to the sediment K-d for OP (6-700l/kg) and NP (1902l/kg), E2 has a relatively low K-d of 4-14l/kg (Holthaus et al. 2002, Johnson et al. 1998, Langston 2008). This may also account for some of the unexplained high concentrations of the steroid oestrogens measured in the dosed mesocsom tanks. In the HM dosing reservoir, bacterial action may have also played a role in the reduced EEQ measured over the renewal period. Although the water used to make the dosing reservoir stock was tap water, and not river water, it is still possible that bacteria were present in the dosing reservoirs. As observed in the E2 dosing reservoirs (Chapter 4) a film (assumed to be bacterial) had been observed in all the dosing reservoirs (including the solvent) by the end of the 16-week exposure. It is therefore possible that a certain amount of degradation of oestrogenic chemicals occurred before they reached the mesocosm tanks.

As with the E2 mesocosm study (Chapter 4), there were un-expectantly high concentrations of E2 in the un-dosed tanks in the first two water samples. The river inlet, which fed all the tanks, was not sampled at this time. The source of this E2 is therefore unknown. As discussed in Chapter 4 (Section 4.4.1), all attempts to prevent contamination were in place. The high concentrations of EE2 measured towards the end of the experiment in the RW, RW+S and river inlet are also of concern due to the highly oestrogenic nature of EE2. Concentrations of EE2 were also higher than expected in the two dosed mesocosm

tanks earlier in the experiment. Whereas, when the highest concentrations of EE2 were measured in the un-dosed tanks, lower concentrations were measured in the dosed tanks. A similar confounding pattern was observed in the E2 experiment (Chapter 4) and therefore the same reasoning may apply. Over the course of the dosing period a large colony of oestrogen metabolising bacteria may have grown in the dosed mesocsom tanks. Although EE2 had been reported to be relatively stable in the environment, recent research indicates if EE2 is co-incubated with enough E2 it can be metabolised by bacteria (Pauwels et al. 2008). In Pauwel et al (Pauwels et al. 2008), however, the EE2 was not metabolised until all the E2 had been converted to E1. Therefore, at the beginning of the study there may have been less competition for carbon resources (due to lower bacterial counts, algae and sediment) and therefore E2 and E1 were preferentially metabolised. Whereas, by the end of the study the bacteria may have been forced to metabolise the EE2 possibly leading to a lower concentration in the mixture tanks compared to the river inlet and un-dosed tanks.

There are a number of other confounding chemical analysis measurements including BPA measured in the RW and the RW+S tanks. Chemical analysis of BPA, NP and OP was not performed in the river water inlet; therefore the source of BPA contamination is unknown. The BPA may have been present in the river water, or it is possible for it to have leached out of the plastics used in the tank or pipes used in the mesocsom design. The hot weather during the exposure may have facilitated its mobilisation from the plastic into the water. However, the chemical analysis itself is somewhat frustrating. The different levels of detection (LOD) reported for BPA, NP and OP vary quite considerably, sometimes within the same set of samples. For example the LOD for BPA ranged from 0.04-1.00µg/l. The LOD of BPA was therefore well above the nominal dosed concentration (50 and 100ng/l) on some occasions. This similarly affected the OP chemistry, whereby none of the water samples had measured concentrations of OP above the LOD. This suggests that the clean up process used for the water samples was not entirely adequate (or repeatable) for the environmentally relevant concentrations used in this experiment. The measured EEQs from the YES assay were significantly correlated with the predicted EEQs from the chemical analysis. However, the YES assay only gives the overall oestrogenicity as detected in the human ER. NP, OP and BPA are thousands of times less potent within the yeast screen compared to the natural ligand E2. Therefore, the difference between 40 and 1000ng of BPA would easily be masked by a couple of ng of E2. If this experiment were to be repeated, it would be important to include the chemists in discussions when designing the experiment, so as to confirm that the concentrations dosed were practical to measure.

5.5.2 Effect of oestrogenic mixture exposure on survival and growth

Unlike the E2 exposure (Chapter 4), there was no general trend in survival across both species and generations. However, survival was lowest for both species in the RW+S tank, where only 14% of the *P. corneus* adults survived until the end of the 16 weeks study, compared to 69% in all other treatments. The *V. viviparus* adult survival was also lowest in the RW+S tank. For both species the adult percentage survival in the RW+S tank was on a par with the other treatments until week ten. After week ten, the percentage survival dropped steeply. The exact reason for this steep increase in mortality after week ten is unknown. The F1 survival over the dosing period was affected in a similar manner. The initial percentage survival was calculated from the total number of neonates or eggs released into each tank compared to the number of F1s collected in September, after the dosing had finished. The F1 *V. viviparus* had the highest initial percentage survival in the RW tank (61.4%) and the lowest in the RW+S tank (12.0%). The F1 *P. corneus* had their highest initial percentage survival in the LM tank (4.0%) and the lowest in the RW+S tank (0.5%). Growth of the F1 *V. viviparus* was also negatively affected in the RW+S tank during exposure, much more so than in the dosed tanks. However, adult growth rates were not similarly affected. After 16 weeks of exposure, adult *V. viviparus* were on average smallest from the HM treatment but not significantly so. Similarly, exposed *P. corneus* were found to be smallest in the RW tank compare to all the other treatments, the RW exposed snails had significantly smaller shell diameters compare to the HM exposed snails. *P. corneus* are known to eat bio-film (personal observation). It is possible that the solvent (ethanol) used in the RW+S, LM and HM tanks increased the available bio-film in these tanks. Therefore, it provided an additional food source to these snails. In the same species exposed to TSE (over spring and summer), a similar increase in adult size was observed by Clarke et al (Clarke et al. 2009). Adult mussels (*Elliptio complanata*) exposed to municipal effluents in a caged study also showed increased growth downstream compared to the upstream site (Blaise et al. 2003). However, the F1 *P. corneus* collected from the mixtures tanks were smaller than those from the RW or RW+S tank. When a sub-sample of F1s were measured, the HM developmentally exposed *P. corneus* were found to be significantly smaller (shell diameter, total weight and soft body weight) than F1s from all the other exposure groups. Similarly, F1 *V. viviparus* exposed to the HM were found to be significantly smaller than the RW exposed snails. Developmental exposures to steroid oestrogens or oestrogenic chemicals such as alkylphenols or BPA have been reported to reduce juvenile growth in a number of vertebrate species. Developmental exposure of two species of frog (*Rana sylvatica* or *Rana pipiens* tadpoles) to steroid oestrogens (E2 and EE2) or OP (0.25-10μM) reduced tadpole body size (Hogan et al. 2006). In the Japanese

medaka (*Oryzias latipes*), OP (50µg/l) or BPA (200µg/l) exposure from post fertilisation also significantly reduced growth (Ramakrishnan and Wayne 2008, Knorr and Braunbeck 2002). Both BPA (0.2-20ppb) and NP (0.2-2.0ppb) have also been reported to significantly reduce growth in young viviparous swordtail fish (*Xiphophorus helleri*), and mixtures of the two chemicals were reported to inhibit growth at a higher level than single chemical exposure alone (Kwak et al. 2001).

In an attempt to identify the cause of the increased mortality of both species in the RW+S tank, additional water chemistry samples were taken from the RW+S and RW tanks at week 16. 19 metals, compounds and essential elements were tested for (Appendix IV). However, the only difference observed between the tanks was the lower concentration of calcium measured in the RW tank (6.10mg/l) compared to the RW+S tank (94.1mg/l), or the RW tank used in the E2 study (96.8mg/l). The lower level of calcium in the RW tank could have been a factor in the reduced shell size of some of the adult snails in this tank, or may have been due to the high number of growing F1 *V. viviparus*. There is a possibility that the carrier solvent (ethanol) had an impact on the snail survival. Although the solvent was not measured in the tanks, a prediction of the amount entering the tanks can be made from the dosing pump rate and the volume of river water entering the tank. The average dosing pump rate in the RW+S was higher than the HM or the LM pump rates, and the water flow rate into the tank was on average lower. Therefore, the predicted concentration of ethanol in the RW+S tank was elevated compared to those from the LM or the HM tanks. This increase was especially evident between weeks ten and twelve, which coincided with the beginning of the adult snail survival crash in the RW+S tank. Hutchinson et al (Hutchinson et al. 2006) reviewed the use of carrier solvents in eco-toxicology testing. Acute (48 hour) toxicity of ethanol was reported to be in the thousands of mg/l for a range of species, including the invertebrate *Daphnia magna* and the fish *Cyprinus carpio* (Hutchinson et al. 2006). Hutchinson et al (Hutchinson et al. 2006) concluded that to avoid any adverse 'solvent effects' (including altered reproduction and enzymatic processes) solvent concentrations should be kept below 20µl/l. The maximum predicted concentration of ethanol in the RW+S tank was 0.000654µl/l compare to a maximum of 0.000583µl/l in the mixtures tank. Therefore, it seems unlikely that this was the cause of the high mortality. The reason for the high mortality observed in the RW+S tank still remains a mystery. However, it is my personal opinion that cross contamination may have occurred from the fish tank (from a different experiment) adjacent to it (Figure 3.9). During the summer of 2006 this particular fish tank was being treated for white spot (a common fish disease) with Malachite Green, which is known to be toxic to many

invertebrates, including molluscs. For this reason (and due to pressure on tank space) it was decided not to continue the F1s from the RW+S tank for over winter survival and F1 breeding study. Incidentally, the RW+S tank was also the only mesocsom tank, from both the E2 and the oestrogenic mixtures exposures, not to harbour any juvenile fish. These fish (dace, perch and gudgen) presumably entered the tanks as fry via the water pump, and were discovered (and removed) when the tanks were drained down to count the F1 snails in September 2006.

Over-winter survival of F1 *P. corneus* was reduced in a dose-dependant manner. Only 2% of the F1s counted in September 2006 survived from the HM tank, 6% survived from the LM tank and 19% survived from the RW tank. In the LM tank, at least, this high mortality is likely to be due to very small size/ young stage of development the *P. corneus* F1 was at in September. The vast majority of the 2000 F1s below 10mm in shell diameter in this tank were fairly new hatchlings. The high number of hatchlings in the LM exposure tank was partly due to the extended egg laying (and therefore hatching) period, exhibited by the parent snails. The small hatchlings obviously did not have the capacity to over-winter successfully. This may have also been an important factor in the HM exposed *P. corneus* over-winter success. None of the HM exposed snail replaced into the tank to depurate in September 2006 were above 10mm in shell diameter (as these had been sampled for histopathology). The LM and the HM developmentally exposed *P. corneus* were also found to be smaller compared to the RW developmentally exposed snails after depuration. However, during the F1 breeding study, the LM developmentally exposed snails had twice the percentage survival rate compared to the RW exposed snails. Due to the high over winter mortality only four HM developmentally exposed snails could be put in the F1 breeding study; of these, 2 survived until sampling occurred at the end of the 14-week study. Over the same time period the LM and HM snails grew well, however, they were still smaller than the RW snails after a year of depuration.

No dose-dependent trend was observed for over winter survival of F1 *V. viviparus*. From the RW tank 53% (273 snails) survived until the spring compare to 33% (66 snails) from the LM tank and 75% (63 snails) from the HM tank. The high percentage survival of the HM exposed *V. viviparus* is in total contrast to the *P. corneus* survival. The overall density of snails in the HM tank was quite low (1.3 l/snail) at the beginning of winter compare to the RW (0.8 l/snail) and LM (0.3l /snail) tanks. Therefore competition for resources (e.g. dissolved oxygen and food) would have been less. Also the death of so many *P. corneus* must have impacted the biological oxygen demand (and therefore dissolved oxygen) of the

static river water in this tank. Although the tanks were aerated, this may have had a negative effect on the *V. viviparus* ability to respire (via the gill). It is unfortunate that the river water pump had to be removed over the winter period (leaving the tanks static), as it is likely a flow through tank would have supported many more *V. viviparus* F1s. However, Essex and Suffolk water could not maintain the pump over the winter.

At the beginning of the F1 breeding study (end of over-wintering) F1 *V. viviparus* size and weight decreased in a dose dependant manner. The oestrogenic mixture developmentally exposed snails were significantly smaller than those from the RW exposure, and the HM exposed snails were also significantly smaller than the LM exposed snails. However, by the end of the F1 breeding study these differences in size had disappeared, and no significant differences between developmental exposures were found. During the F1 breeding study the percentage survival of F1 *V. viviparus* was again highest in the HM developmentally exposed snails (96.7%) and lowest in the LM developmentally exposed snails (78.8%). This similar pattern of survival suggests that perhaps density was not the only issue at play with respect to over winter survival (the possible effects of density will be discussed further in Chapter 6).

5.5.3 Effect of oestrogenic mixture exposures on reproduction and sexual development

Over the entire 16 weeks of exposure, the *V. viviparus* snails from the RW tank released the most neonates (14.7) per snails, with RW+S, LM and HM exposed snails releasing slightly lower numbers (13.2, 12.4 and 13.6, respectively). There was no significant difference between reproductive outputs of adult *V. viviparus* from different treatments during the first six weeks of the exposure study. As previously stated in Chapter 4 this was to be expected as female *V. viviparus* harbour their offspring over winter (pre-exposure) for release in the spring. At week 8 the RW exposed snails released a significantly higher number of neonates per snails compared to the RW+S, the LM and the HM exposed snails, and at week 10 the LM exposed snails continued to release significantly less neonates compared to the RW exposed snails. At the end of the 16-week dosed study the adult female *V. viviparus* had their brood pouches dissected and the number of embryos within them recorded. As with the total neonates released over the breeding study, the RW exposed females harboured the most embryos (30.5) and the LM females harboured the least (26.9). However, no significant difference was seen between the four exposure groups. When the embryos were split between shelled (most mature) and unshelled (youngest) embryos, the RW exposed females were found to have higher numbers of

shelled embryos compare to the LM exposed females. Whereas, the LM exposed females harboured on average twice as many unshelled embryos compared to the RW exposed females. As mentioned in Chapter 4, the number of unshelled embryos produced by the prosobranch mollusc *Potamopyrgus antipodarum* has been used in recent research to evaluate the oestrogenic effects of an oestrogenic mixture (namely TSE). In *P. antipodarum* the unshelled embryo number was stimulated by low concentrations of TSE but inhibited at higher concentrations (Jobling et al. 2004). Similar inverted U-shaped dose responses were seen when the same species was exposed to EE2, BPA, OP or NP (Duft et al. 2003, Jobling et al. 2004). Similarity can be drawn between the two species responses. However, several differences are also evident. The concentrations of EE2, BPA, OP and NP generally used in the *P. antipodarum* exposures were much higher than used in my experiment but, the duration of the experiments were shorter, Duft et al (Duft et al. 2003) report the LOEC (for BPA) to reduce over time from 30µg/kg at weeks 2 and 4, to 1µg/kg at week 8. The combination of chemicals in my experiment may also have an additive effect. Mixtures of chemicals at individually low effect or no effect concentrations have been found to elicit oestrogenic effects in fish (Brian et al. 2007). There is also the issue of the possible inverted U-shaped dose response; with the LM exposed females harbouring more unshelled embryos than HM exposed females. Whether the lower number of unshelled embryos observed in the HM exposed females was due to increased toxicity or to the possibly less stimulation due to lower E2 concentration compared to the LM tank is impossible to know. Further experiments with more reliable chemistry and more doses are needed to fully elude the dose response.

Histological analysis of the females after 16 weeks exposure found the RW exposed snails to have significantly more oocytes than the exposed females (LM and HM). This perhaps indicates a certain level of disruption to oogenesis rather than stimulation. However, this significant difference was no longer in evidence when co-analysed for size (ANCOVA), indicating that size differences between the groups had caused an artefact in the results. Survival was not significantly effected by exposure; although both the male and female *V. viviparus* from the HM were smaller than their RW counterparts. The increase in the number of unshelled embryos in the LM exposed females may be evidence of reduced growth rate of the embryos rather than reproductive stimulation. Reduced growth was observed in both the *P. corneus* and *V. viviparus* offspring in the oestrogenic mixtures tanks. As discussed above (Section 5.5.2), BPA, OP and NP exposure in developing fish has resulted in retarded growth (Ramakrishnan and Wayne 2008, Knorr and Braunbeck 2002, Kwak et al. 2001). The mixture of oestrogenic chemicals may have also affected

male reproductive ability. Although full spermatogenesis was observed in the histopathology of the exposed males, effects on sperm motility were not assessed. As discussed in Chapter 4 (Section 4.4.3), exposure to steroid estrogens can negatively affect sperm motility in rats (Gill-Sharma et al. 2001) and fish (Lahnsteiner et al. 2006). There are reports of OP, BPA and NP also negatively affecting sperm motility in vertebrates. Exposure of adult rats to OP (150 and 450mg/kg) significantly reduced sperm motility (Bian et al. 2006). OP (100µl) also inhibited seminal fluid production in the eelpout (*Zoarces viviparus*) (Rasmussen and Korsgaard 2004). Exposure of adult brown trout (*Salmo trutta*) to BPA (1.75 and 2.40µg/l) significantly reduced sperm quality (density, motility and velocity) (Lahnsteiner et al. 2005), and NP exposure negatively affected sperm motility in the Japanese madaka (*Oryzias latipes*) both in vivo (20 and 100µg/l) and in vitro (100µmol/l) (Kawana et al. 2003, Hara et al. 2007). NP exposure has also been reported to negatively affect sperm motility in molluscs. Nice (Nice 2005) exposed the pacific oyster (*Crassostrea gigas*) to NP (1 and 100µg/l) during gametogenesis (72 hours). The percentage of oysters with motile sperm reduced in a dose dependant manner with 100% of the control and solvent control oysters having motile sperm compare to 30% from the 1µg/l and 10% from the 100µg/l NP exposures (Nice 2005). This suggests that mollusc (or at least bivalve mollusc) male reproduction may be extremely sensitive to NP pollution.

The reproductive output of adult *P. corneus* was not significantly different between the different exposures. However, differences in the patterns of reproduction were observed. Snails from the RW+S exposure had their reproductive peak (eggs laid per snail) earlier (week 4) the other three groups (week 6). The maximum number of eggs per egg mass was attained at week 6 for the same group and then dropped off steeply. These results may have been an early indicator of toxicity to the snails. It has been documented in several species (especially invertebrates) that reproduction will increase in the face of adversity to counter the negative effect of mortality.

The RW, LM and the HM exposed *P. corneus* had their reproductive peak (maximum eggs per snail) at week 6 when the RW exposure group had their peak in laying egg masses. The RW exposed snail's mean number of eggs per mass increased between week 2 and 4 and then levelled out for the rest of the study. Whereas, both the LM and HM exposed snail's egg masses continued to increase in size until week 8. After week 6, all the treatment groups reduced their egg and egg mass laying per snail. However, the LM exposed snails reduced at a slower rate; by week 16 the LM exposed snails were laying on average more than twice as many eggs per snails than the RW exposed snails. Over the whole experiment

the snails from the LM exposure laid the both the highest number of egg masses and the largest egg masses (i.e. higher number of eggs per mass). Some similar results were reported when *P. corneus* was exposed to TSE (Clarke et al. 2009). The number of egg masses laid by snails exposed to 50% or 100% TSE significantly increased compared to the control. However, although Clarke et al (Clarke et al. 2009) found the cumulative number of egg masses laid per snail increased in TSE exposed snails, they also found that later in the summer the weight of each egg mass was reduced, but the number of eggs per mass was not. The likelihood is that this may be a result of reduced nutritive packaging/albumen in each egg. Reduced hatching success or lower nourishment of hatchlings may then follow. The stunting of the HM and the LM exposed hatchlings in my experiments may also be evidence of reduced developmental nutrition.

Mice exposed to BPA are reported to have up-regulated dopamine D1 receptor function (Suzuki et al. 2003). This is of interest in molluscs as dopamine has a stimulatory effect with regard to protein secretion of the albumen gland in fresh water pulmonate gastropods (Santhanagopalan and Yoshino 2000), and this activity has been demonstrated to be mediated via a D1-like receptor (Mukai et al. 2004). Therefore similar disruption to receptor function in molluscs could impact the volume of albumen deposited around each egg. Interestingly, E2 (0.85µg/l) exposure has been reported to inhibit yolk deposition in adult guppies (*Poecilia reticulata*) (Kinnberg et al. 2003).

As in the E2 study (Chapter 4), histological analysis of the adult gonad of *P. corneus* found some effects of exposure relating to the gamete supporting cells. Again the Sertoli cells, spermatogenic supportive cells, seemed to be especially sensitive. Significant increases of Sertoli cells sloughing were found in both the LM and HM exposed snails compared to the RW exposed snails. Overall immature spermatogenic cells were most frequently found sloughed into the acini lumen in the mixture-exposed snails. The HM exposed snails also exhibited a significant reduction in the amount of developing sex cells (male and female) covering the acini walls compare to the RW or the RW+S exposed snails. As discussed in Chapter 4 (Section 4.4.3) E2 has been reported to have negative affects on Sertoli cell function in vertebrates (Gill-Sharma et al. 2001, Rasmussen et al. 2005, Miles-Richardson et al. 1999a, Aleem et al. 2006). BPA, NP and OP have also been shown to affect Sertoli cell structure and function; Reduced Sertoli cell viability was found in vitro cultured rat cells exposed to BPA (150-200µM) or OP (30-60µM) (Qian et al. 2006, Iida et al. 2003), Eelpout (*Zoarces viviparus*) exposed to OP (50 and 100µg/l) for three weeks resulted in altered Sertoli cell structure (Rasmussen et al. 2005), and Fathead minnows exposed to NP (1.1 and 3.4µg/l) experienced changes in the number and size of Sertoli cells at

concentration that did not affect external sexual characteristics (e.g. fat pad) (Miles-Richardson et al. 1999b).

In a similar trend to the E2 exposed *P. corneus* (Chapter 4), oogenesis and vitellogenic area activity was also affected in *P. corneus* exposed to oestrogenic mixtures. The LM exposed snails had the highest percentage of early stage oocytes (Stage 1 and 2), and a dose dependant (but not significant) reduction in active vitellogenic area (maturing oocytes and sperm) was also observed in the oestrogenic mixture exposed snails. Similarly, in an hermaphroditic vertebrate, the fish *Rivulus marmoratus*, exposure to NP (150 and 300µg/l) inhibited testicular growth, oogenesis and the normal structure of the hermaphrodite gonad (Tanaka and Grizzle 2002).

Similar effects were found in the developmentally exposed *P. corneus*. The vitellogenic area active was also reduced in a dose-dependant fashion, and HM developmentally exposed F1s had significantly less active vitellogenic areas compared to all other treatment. The RW and RW+S F1 snails had significantly less spermatid sloughing compare to the mixture exposed snails. However, Sertoli cell, spermatogonium and spermatocyte sloughing did not generally follow the same trend. Interpretation of the spermatogenic cell sloughing is complicated by the long period over which the F1s were sampled. All the mesocosm tanks (both E2 and mixtures experiments, 7 tanks in total) were sampled in September 2006. However, the HM tank was sampled 8 days before the LM tank, and the RW and RW+S tanks were sampled a further 21 days later. This was due to the long period of time it took to drain down each tank, sample and histologically fix the F1s. Therefore changes in water temperature and day length may also have affected the snails. From my own the laboratory work on *P. corneus* (Appendix V) I have found that reduced temperature (below 15^{o}C) initially caused Sertoli and immature spermatogenic cells to slough into the lumen, although reduced temperatures did not affect vitellogenic area activity. During the F1 sampling period (1-30th September) midday water temperatures were measured at 16-18^{o}C. However, night-time temperatures were not measured and it is assumed that towards the end of September water temperatures did drop below 15^{o}C. This may have caused the increased Sertoli cell and immature spermatogenic cell sloughing observed in some of the RW and RW+S exposed snails. The effect of day length on spermatogenic cells has not been documented; this would have also varied through the sampling period. With hindsight it would have been better to sub-sample a few snails from each tank at each time point. However, the majority of the F1s (especially the *V. viviparus*) inhabited the bottom of the tank, in amongst the mud and detritus, and this

was why the drain-down method was favoured. Despite this, the percentage of different stages of oogenesis present in the F1 *P. corneus* was affected in a similar manner to the exposed adults. The RW and the RW+S developmentally exposed snails had a significantly lower percentage of stage 1 (youngest) oocytes compare to the LM and the HM exposed snails. Interestingly, maternal exposure to BPA has been reported to affect oogenesis in female mouse offspring (Susiarjo et al. 2007).

Impacts on spermatogenesis and oogenesis were also found in developmentally exposed *V. viviparus*. The HM and RW+S developmentally exposed females had significantly less oocytes (per section of tissue) compare to the RW and the LM exposed snails. However, mean oocyte number was significantly correlated to the size and weight of the RW exposed snails, and the female F1s from the different treatments were of greatly varying size. Therefore, the mean oocyte number was analysed with snail size or weight as a co-factor. After this analysis no significant differences in mean oocyte number were found between the treatments. The RW F1 females had significant positive correlations between oocyte number and size (shell length, weight). However, in the RW+S, the LM and the HM developmentally exposed snails correlations between oocyte number and snail size were not significant, suggesting there was still possible perturbation to the 'normal' relationship between growth and sexual maturation. In contrast, and somewhat unexpectedly mean spermatogenesis score was highest in the RW+S exposed male *V. viviparus*. The males from the HM exposure had a significantly lower spermatogenesis score compared to all other treatments. Therefore sexual development in both male and female *V. viviparus* was significantly disrupted by exposure to the HM, although in the RW+S, the disruption (of unknown aetiology) was predominantly seen in females.

Following depuration, the F1 *V. viviparus* were placed in breeding groups. Over the 18 weeks of the un-dosed breeding study none of the F1 females released any offspring. It is unknown at what age female *V. viviparus* first release offspring. The F1 females were roughly a third of the size of the adult females collected for the first breeding study. It is therefore not totally unexpected that offspring were not released during the F1 breeding study. At the end of the F1 breeding study (August) the depurated females had their brood-pouches dissected and the embryos removed. None of the mixture-exposed females harboured any embryos compared to 12.5% of females from the RW exposure. This overall lack of embryos in the broodpouch was unexpected. Jakubik (Jakubik 2007) reports that female *V. viviparus* from the Zegrzynski Reservoir (Central Poland) contained between 0.9-6.7 embryos per female at a shell length of 8.1-12mm and 1.1-9.6 embryos

per female at a shell length of 12.1-25mm. At the end of the F1 breeding study the female *V. viviparus* were around 15-16mm in shell length and would therefore be expected to harbour embryos. The low number of females harbouring embryos from the RW treatment may have resulted from the high density of F1s over-wintered in this tank (the possible effects of density will be discussed in detail in Chapter 6). The density *V. viviparus* F1s was between 2.6 and 6.1 times that of the LM and the HM tanks, respectively. As discussed in Chapter 4 this high density was due to a combination of pooling two RW tank and the lower reproductive output of the adult *V. viviparus* from the oestrogenic mixture tanks. On the other hand this does not account for the complete lack of embryos harboured by the mixture-exposed females. The long gestation period of these animals may be key in the reason for this. The *V. viviparus* needed to mate in the previous autumn for females to harbour embryos the following spring. The reduced oocyte number combined with lower spermatogenic score seen in the LM and HM developmentally exposed snails in September 2006 may have affected their ability to mate. In the *V. viviparus* F1 males, spermatogenesis had recovered after a year of depuration, but female oocyte number were still significantly reduced in both the LM and the HM exposed snails compared to the RW exposed snails. This suggests oogenesis may be negatively affected by developmental exposure to oestrogenic chemicals even after a long period of depuration. Interestingly, maternal exposure of the sea urchin *Strongylocentrotus purpuratus* to E2 or OP resulted in embryos being less sensitive than normal to E2 (Roepke et al. 2006). This reduced sensitivity to hormonal cues may result in differences in growth and development (over an animal's entire life), although the function of E2 in sea urchins is not fully eluded at this time. With a long-lived, slow growing species such as *V. viviparus* this could have far reaching effects. Developmental exposure to certain EDCs may not only affect reproductive potential directly. Indeed, recent research with laboratory rodents and BPA show effects on behaviour and neurology. Developmental exposure to BPA has been reported to alter sexually dimorphic brain morphology (Patisaul and Polston 2008) and to alter behaviour in terms of exploration, emotional responses, play and reproduction in mice and rats (Patisaul and Polston 2008, Dessi-Fulgheri et al. 2002, Gioiosa et al. 2007). Behavioural alterations have also been reported in adult rats (0.040mg/kg/day BPA) with regard to maternal behaviour (Della Seta et al. 2005). Interestingly BPA has also been reported to alter ER-α proliferation and distribution in the hypothalamus of male and female rats (Ceccarelli et al. 2007). Maternal exposure of the sea urchin (*S. purpuratus*) to OP or E2 has also been reported to increase mRNA of an orphan steroid receptor (SpSHR2) in the eggs (Roepke et al. 2006). The similar effects

seen in such wide taxonomic groups point to further routes by which EDCs may impact on the neuro-endocrine and reproductive systems.

In contrast to *V. viviparus*, the depurated *P. corneus* showed similar patterns of reproduction. The RW exposed snails laid significantly more eggs per snail at the start of the breeding study (week 2), but thereafter no significant differences were observed. The LM developmentally exposed snails laid more eggs per snail over the entire breeding study compared to the RW exposed snails, mirroring their exposed parents. However, the HM developmentally exposed snails were severely impacted in terms of reproduction. The high over winter mortality observed in this group resulted in only four snails being allocated to the breeding group. For the RW and the LM developmentally exposed snails peak egg laying occurred during midsummer (week 8). The HM developmentally exposed snails only laid eggs between weeks 8 and 10 and laid a maximum of 4.3 eggs per snail. This indicates that the long day length stimulated all the groups of snails to lay eggs during this time, even those negatively affected by chemical exposure. Joosse (Joosse 1984) reported similar finding in *L. stagnalis* subjected to reduced temperatures and starvation, highlighting the fact that the drive to reproduce in these snails overrides all others.

Similarities were found between the developmentally exposed snails sampled immediately after exposure (September 2006) and after a year of depuration (August 2007). The vitellogenic area activity again decreased in a dose dependant manner. The HM developmentally exposed and then depurated snails had an almost significantly (P=0.056) lower percentage of active vitellogenic areas compare to the RW exposed snails. Immature spermatogenic cell sloughing was still impacted by oestrogenic mixture exposure although only significantly so for spermatocytes (HM). Oogenesis was still affected after a year of depuration, with the HM developmentally exposed snails having a significantly higher percentage of young oocytes (stage 1) compared to RW exposed snails. These results indicate that many of the effects seen in both the exposed adult and offspring snails are still apparent long after the exposure has ceased.

5.5.4 Freshwater gastropods and their parasites

In addition to growth and reproductive endpoints, the level of parasitic infection was also evaluated in the snails in this test system and became an additional theme in this research. Gastropods (both pulmonate and prosobranch) are known to be susceptible to parasitism, especially to larval trematode (Digenea) infection. Gastropods and digeneans have a long evolutionary history and have been associated for at least 200 million years (Blair et al.

2001). Digenea have complex life histories and normally require two or more hosts to complete their lifecycle. Molluscs, especially gastropods and bivalves, frequently provide the first, and sometimes second, intermediate host. Whereas, vertebrates such as fish, birds and mammals are normally the definitive host.

Here follows some basic information about Digenean parasites life history. Digenea only reproduce sexually in their definitive host (vertebrate), the eggs produced in this host are generally excreted in the faeces (Figure 5.21). The faeces are either ingested by the first intermediate host, or hatch in the water into ciliated miracidia, which locate their host and burrow into their tissue. Once in their first intermediate host (e.g. bivalves or gastropods) they become mother sporocysts that replicate asexually producing large numbers of daughter sporocysts (or rediae). These again reproduce asexually to become free-living cercaria which burst out of the first intermediate hosts tissue. In species that require only one intermediate host they either, (i) do not become free living, but instead wait as a cyst (or sporocyst) to be ingested by the definitive host or, (ii) locate their definitive host as cercaria. Some parasites that do not require a second intermediate host will encyst on plants, again waiting to be ingested. Where a second intermediate host is needed, the cercaria locates this host (e.g. mollusc, frog, fish) and burrow into the tissue and become a metacercarial cyst. Here they lie dormant encysted in the tissue until their final/definitive host ingests them. A change in pH normally initiates the maturation of the parasite to its adult stage and the whole cycle begins again (Figure 5.21). Differences exist between parasite species; some metacercariae do not lie dormant and instead have a period of growth and development. This process requires nutrition from the host, and the ability to evade the host's immune system. Consequently these types of metacercarial cysts are more complex in structure and function. Some metacercaria do not need to encyst at all as they inhabit an area of the body less prone to immune reaction (Fried and Graczyk 1997). For example, the genus *Diplostomum* accumulates in the eye (lens and aqueous humor) of freshwater fish. This has the additional benefit of reducing the host's eyesight, and increases the chance of predation and the completion of the parasite's life cycle.

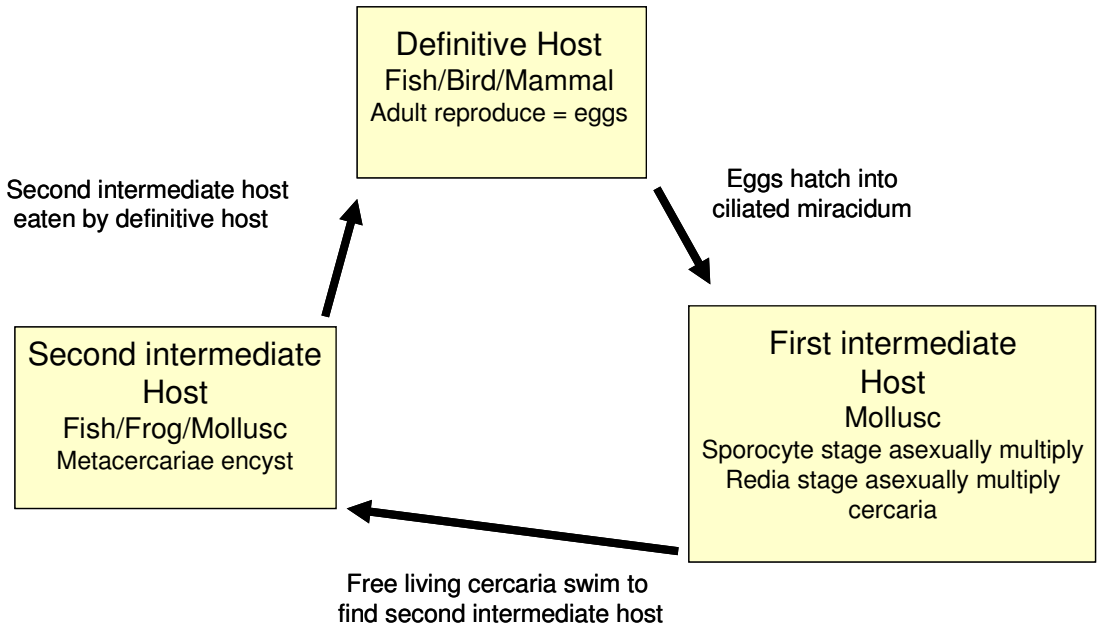

Figure 5.21 Diagram representing an example of a Digenean (Trematode) lifecycle with two intermediate hosts.

Frequently the effects of parasitism on the first intermediate host are the most destructive (Langand and Morand 1998). Replicating larvae can infest large areas of reproductive or digestive tissue. Parasitic infestation of the first intermediate host often reduces fecundity. This process is host and parasite specific, and can occur because, (1) the parasite gains nutrition from the host, and therefore is an energy drain, (2) the parasite infects the reproductive tissue and physically castrates the host, (3) the parasite chemically castrates the host; preventing reproduction. In addition to reducing fecundity, damaging the digestive tissue reduces the lifespan of the host and consequently the length of time the parasite can use the host as a resource. Castration of the host either physically or chemically is therefore more advantageous to the parasite than removing the digestive tissue (Hall et al. 2007, Taskinen et al. 1997). Curtis et al (Curtis 2007) found infected *L. obsoleta* grow much slower than un-infected individuals but that they lived for possibly twice as long.

5.5.4.1 Parasite and their effect on their hosts fecundity

When observing histological preparations of infected mollusc tissue it is easy to see the physical impact that the developing trematode larvae have on the host reproductive tissue (Probst and Kube 1999). The chemical castration obviously needs more detailed investigations. The host-parasite relationship has been well studied in the pulmonate gastropods and the digenea from the Schistosomatidae, as humans are a possible definitive host. Work with *Lymnaea stagnalis* infected with *Trichobilharzia ocellata* has shown that

parasitic effects on reproduction were due mainly to interference of female endocrine control(De Jong-Brink et al. 1988, De Jong-Brink 1992, De Jong-Brink et al. 1992), and studies with *B. glabrata* infected with *Schistosoma mansoni* have found that the male reproductive side was still able to function six weeks after female reproduction was inhibited (Cooper et al. 1996). A neuro-peptide, Schistosomin, released by the infected snail itself (from its CNS) has been identified as the substance involved in female castration (Hordijk et al. 1991b, Schallig et al. 1991b). Schistosomin treatment inhibits the activity of the albumen gland (Joosse et al. 1988) and reduces the activity of the CDC, and thereby inhibits egg laying (Hordijk et al. 1991b, Hordijk et al. 1991b, Schallig et al. 1991a, Hordijk et al. 1991c, Hordijk et al. 1991a, Hordijk et al. 1992). Similarly Schistosomin neuro-peptides have also been found to occur in other pulmonate species (*Biomphalaria glabrata* and *B. pfeifferi*) in response to infection by parasites belonging to the Schistosomatidae (*Schistosoma mansoni*), whereas infection by another digenea (Diplostomatidae) did not elicit Schistosomin release in *L. stagnalis* (De Jong-Brink et al. 1991). Schallig et al (Schallig et al. 1991a) found the release of Schistosomin co-occurred with the appearance of cercaria, and de Jong Brink et al (De Jong-Brink et al. 1995) have suggested Schistosomin to be a cytokine-like factor involved in the immune and stress response to invading parasites. Hordijk et al (Hordijk et al. 1992) also found that Schistosomin increased the excitability of the Light Green Cells (LGC), which are involved in regulating growth. Therefore Schistosomin may be a natural regulator of snail reproduction and growth. In addition to this, Crewsoyen and Yoshino (Crewsoyen and Yoshino 1995) found sporocysts of *S. mansoni* produced/released polypeptides in in-vitro cultures and Manger et al (Manger et al. 1996) found that infection of *B. glabrata* by the same parasite reduced dopamine and serotonin in the CNS and serotonin in the plasma. If infected snails were exposed to serotonin (via tank water) they were stimulated to lay eggs again.

A consequence of castration in many species of gastropod is gigantism, food resources normally allocated to reproduction (and possibly locomotion) are thought to enable this increased growth, although this again depends on specific host and parasite and also the timing of infection (before or after sexual maturation) (Mouritsen and Jensen 1994, Gorbushin 1997, Keas and Esch 1997, Gerard and Theron 1997, Sorensen and Minchella 1998, Munoz-Antoli et al. 2007, Negovetich and Esch 2008, Taskinen and Saarinen 1999). Infection by castrating parasites has also been linked to fecundity compensation (Hall et al. 2007), whereby a newly infected snail will increase reproduction prior to castration. Blair and Webster (Blair and Webster 2002, Blair and Webster 2007) found that *B. glabrata*

exposed to *S. mansoni* but without successful infection also increased reproductive output. This may be supported by Kirst's (Krist 2001) findings that wild populations exposed to high parasite loads reproduce more than those with low parasite load. However, fecundity compensation has not been found in all species. *P. antipodarum* exposed to *Microphallus sp* did not undergo castration or reduced growth (Krist and Lively 1998), however this may be related to *P. antipodarum* ability to be parthenogenic. Rupp (Rupp 1996) reported altered mating behaviour in *B. glabrata* infected with *S. mansoni*, whereby infected snails preferred to mate with uninfected snails. The author suggests this is evidence for the 'good genes hypothesis', however it was also noted that infected snails mated as males more frequently (owing to female castration) and therefore it may be that uninfected snails would mate more readily as females.

As with other prey-predator or host-parasite interactions, some animals have evolved methods to mitigate or stop infection. These interactions are often referred to as an 'arms race' as every time a host finds a way of defending itself the parasite evolved to compensate. Several strategies exist and each has its benefits and costs. One method is to be resistant to the infection; this requires increased investment into immune function. Langand et al (Langand and Morand 1998) investigated the cost of resistance in *Biomphalaria glabrata* resistant to *Echinostoma caprioni* and found resistant individuals took longer to mature than non-resistant counterparts. Resistant *B. glabrata* were also found to be less tolerant to Cd exposure (Salice and Roesijadi 2002). Borges et al (Borges et al. 1998) found some *B. glabrata* completely resistant to *S. mansoni* infection whereas others were partially tolerant. Miller et al (Miller et al. 2006) state the host tolerance reduces the individual's risk of mortality, but increases the prevalence of parasites in the community. Langand and Movand (Langand and Morand 1998) demonstrated with two strains of *B. glabrata* one susceptible to *E. caproni* the other not, that there was high heritability in both strains, indicating both had positive attributes when it came to selection.

Measurements of growth, mortality and reproduction are frequently made to assess toxicological or endocrine disruption effects in test species. It is therefore important to investigate and understand the effects parasitism can have of these end points and to know whether your test animal/population has an infection.

5.5.5 Other effects of exposure to oestrogenic mixtures

In light of the above information, it was an important finding that a large number of the *V. viviparus* (adult and offspring) from the oestrogenic mixtures experiment (and a number of

the adults from the E2 exposure) were parasitised. There have been a number of studies determining the parasitic fauna of *V. viviparus* in different wild habitats. In a survey of snails from Lake Glubokoe (Russia), 48% of *V. viviparus* were found to be highly parasitised by six different species of larval trematode, and some snails were infected with two species at once (Nikitina 1986). The trematode populations were also studied in *V. viviparus* from River Wieprz, River Narew and the Zegrzynski Reservoir (Poland), and infection frequency was found to vary from 7.3 percent in the reservoir, to 21.2 percent in the River Wieprz and 32 percent in the River Narew (Jezewski 2004). Jezewski (Jezewski 2004) also noted that female snails were more frequently parasitised than males (50% compared to 15.32%) in the River Narew. Although the species of trematode reported in these two surveys were different, in both cases the family Echinostomatidae were one of the most dominate. In my experiments it was impossible to determine (it was attempted) the species present from histological preparation and in any case the effects of individual trematode species on *V. viviparus* growth, development and fecundity could not be found in the literature. However, Samochwalenko and Stanczykowska (Samochwalenko and Stanczykowska 1972) found over 50% infestation of *V. viviparus* and *V. fasciatus*, infections that reduced embryo numbers by 2-4 times of that in un-parasitised females. Zhokhov (Zhokhov 1993) found that infections of *V. viviparus* by *Echinoparyphium sp* increased with age. This could mean that older females, who would normally contribute a large proportion of reproductive output, may have reduced fertility.

In my mesocosm exposures it was found that the oestrogenic mixtures exposed *V. viviparus* had higher levels of encysted (metacerial digenea) parasite infection. This trend was observed in both the adults and F1s. The exposed F1s had significantly higher infection intensities (parasite score) than RW or RW+S exposed snails. The depurated F1s showed a similar trend with the addition that the HM developmentally exposed snails had significantly higher parasite scores compared to the LM exposed snails, indicating a dose dependant increase in infection rate. There was also a change in the sex related infection rate in the oestrogenic mixture exposed snails between the F0 and F1 generations of snails. Jezewski (Jezewski 2004) reported that female *V. viviparus* had increased parasite infection compare to males and a similar trend was evident in the pre and post-exposure adult *V. viviparus*. However, in the developmentally exposed F1s this trend was reversed, and the F1 males had a higher intensity of infection than the F1 females.

When taken together, the results of this study pose two questions, (1) did exposure to oestrogenic mixtures affect the *V. viviparus* immune system and thus their increase

susceptibility to parasite infection and (2) were the negative affects observed on reproduction caused by exposure to oestrogenic mixtures, increased parasite infection, or possibly a combination of the two?

There is evidence of oestrogen and oestrogenic chemicals having an impact on mammalian immune function. In humans this is most evident by the high proportion of women who suffer autoimmune problems (Ahmed 2000). Rodent studies have demonstrated that environmental oestrogens, such as OP, can promote splenic lymphocyte cell death (NairMenon et al. 1996), and that BPA can modulate lymphocyte proliferation with lower concentrations stimulating and higher concentrations inhibiting proliferation (Jontell et al. 1995). BPA has also been reported to alter macrophage function in rodents (Segura et al. 1999) and NP has been reported to affect (decrease phagocytic activity) the immune system of rainbow trout (Hebert et al. 2009). There is also growing evidence that environmentally relevant mixtures of chemicals such as TSE and agricultural runoff have immunomodulatory affects. It is possible that immunotoxicity and endocrine disruption could be related effects. Field studies of the freshwater bream (*Abramis brama*) found parasite infections (*L. intestinalis*) were elevated at a site of heavy pollution (Hecker and Karbe 2005). Caged studies of another freshwater fish species (Roach) reported increased digenean infections down stream of Kraft mill effluent (Jeney et al. 2002). Roach exposed to TSE were found to display immunotoxic and genotoxic effects at low concentrations not found to have classic ED effects (male vtg stimulation, intersex) (Liney et al. 2006) . Recently, Filby et al (Filby et al. 2007b, Filby et al. 2007a) also exposed fathead minnows to TSE and also reported genotoxicity and modulated immune function, including reduced lymphocytes indicative of immunosuppression, whereas, exposure of fathead minnows to EE2 (in the same test system) had no effect on the immune system (Filby et al. 2007b, Filby et al. 2007a). In the European eel (*Anguilla anguilla*) experimentally exposed to PCBs, normal antibodies to the nematode *Anguillicola crassus* were not produced, and infection rates were higher (Sures and Knopf 2004). A number of studies have also reported lowered immune function and/or increased parasite infection in amphibians exposed to mixtures of pesticides (Hayes et al. 2006, Christin et al. 2003) and agricultural runoff (Kiesecker 2002). Kiesecker (Kiesecker 2002) reported increased digenea metacercariae (*Ribeiroia sp.*) and reduced eosinophils circulating in the blood of wood frogs (*Rana sylvatica*) with increasing exposure to agricultural runoff. The parasitically infected frogs were also smaller than similarly exposed (but not infected) frogs. Leopard frogs (*Rana pipiens*) exposed to a mixture of pesticides were found to be immuno-suppressed (Hayes et al. 2006). Both control and exposed frogs were tested positive for

flavobacteria, but only frogs exposed to the mixture of pesticides showed signs of pathology (Hayes et al. 2006). Additionally, Hayes et al (Hayes et al. 2006) reported that clawed frogs (*X. laevis*) exposed to mixtures of pesticides had elevated corticosterone, an indicator of stress. Stress is a highly conserved reaction across taxa (Ottaviani and Franceschi 1996) and stress is known to reduce immune response. Some parasites are thought to use this response to facilitate their infection (Sures 2006). Indeed, a number of studies have found exposure to stress hormones alone also increases parasite infection. Harris et al (Harris et al. 2000) reported hydrocorisone acetate implants in a number of salmonids (Salmon, Trout, Charr), significantly increased infection rate and monogenean parasite (*Gyrodactylus salaris*) frequency compared to sham operated fish. Similarly, exposure to corticosterone glucocoticosteroid (an amphibian stress hormone) as tadpoles significantly increased *Alaria sp.* (mesocerceriae) infection and reduced circulating eosinohilic granulocytes in the grey tree frog (*Hyla versicolor*) (Belden and Kiesecker 2005). These results suggest that perhaps not only do certain chemicals have the ability to modulate the immune system, but that the mixture itself may have a multiple stressing effects that disrupt immune function. Therefore, low concentrations of a mixture of chemicals may elicit responses that would not be evident in a single chemical exposure.

There has been very little research published into the effects of digenean metacercarial infections of gastropods, or their affects on gastropod reproduction, whereas, digenean sporocyst infections in gastropods have been more widely investigated. Their negative or modulatory impacts on reproductive output in pulmonates has been of special interest to a number of researchers, and is discussed above. A number of species of digenean sporocyst have the ability to chemically castrate or inhibit reproduction, the best-known example of which is the *L. stagnalis-T. ocellata* interactions (de Jong-Brink et al. 2001). In my research, sporocyst infections (of the digestive tissue) were observed to reduce the number of embryos harboured by adult female *V. viviparus* (Chapters 2, 4 and 5). Whether this was due to reduced nutrition of the snail or some kind of chemical disruption is, however, unknown. Unfortunately the effects of metacercarial cysts seem to be less straightforward. As discussed earlier, the encysted parasites may be considered dormant and only waiting to be ingested by their next host. However, it is still important to asses their possible impacts to reproduction, as so little literature is available. When the RW and RW+S exposed adult females were assessed, slight (but not significant) positive correlations were found between parasite score and mean oocyte number, shelled embryos harboured, and unshelled embryos harboured. The RW and RW+S females with metacercarial infections were found to harbour slightly more embryos that those without infection. This however may be

related to the size or age of the snail rather than a stimulatory effect of infection. A slight (but not significant) correlation was found between the shell length and mean parasite score of pre-exposed/health check (Chapter 2, Section 2.3.3.4) *V. viviparus*, and Zhokhov (Zhokhov 1993) also reported digenean infection increased with age in *V. viviparus*. Similar results were found when the parasite score was correlated with oocyte number or shell length in the developmentally exposed (LM and HM) F1s. Although in the developmentally exposed and then depurated snails only the LM exposed snails continued to show the slight positive correlation. The HM developmentally exposed snails showed a slight (but not significant) negative correlation. The HM exposed snails had a significantly higher infection intensity compare to the LM exposed snails. Therefore whether this negative correlation is related to the higher number of parasites or to the developmental exposure is unknown. It is important to note that none of the RW developmentally exposed *V. viviparus* had parasites to make comparisons with. Also due to the lack of embryos harboured by the F1 developmentally exposed snails, the effect of metacercarial infection on embryos harboured could not be assessed. Further research including a large survey of wild *V. viviparus* with various intensities of infection would be needed to fully elude possible effects of this type of digenean infection on female reproduction.

Male reproduction can also be negatively affected by trematode infection. Spermatogenesis was inhibited in vitro cultures of *Ilyanassa obsolete* gonad co-incubated with *Zoogonus lasius* extracts (Pearson and Cheng 1985). Only one of the male *V. viviparus* had a sporocyst infection of the digestive tissue in my experiment. In this individual spermatogenesis was completely absent. The small number of adult males sampled makes correlating the parasite score (metacercarial infection) with spermatogenic score difficult. However, full spermatogenesis was evident in all the other adult males analysed. As with the female F1s, only the oestrogenic mixture exposed males had metacercarial infections. In the HM developmentally exposed snails the mean spermatogenesis score and parasite score were significantly positively correlated. However, the LM developmentally exposed males did not exhibit this correlation and indeed showed a slight (but not significant) negative correlation. This slight negative correlation was also found in both the LM and HM developmentally exposed and then depurated F1s. It is important to note that full spermatogenesis was found in all the depurated male F1s (RW, LM, HM) at this time, and therefore it can be assumed the metacercarial infection did not affect spermatogenesis to a great degree in either the adults or developing offspring. Further controlled experiments where snails are exposed to either the oestrogenic mixture alone or in combination with parasite cercaria (similar to those performed by Kiesecker, (Kiesecker 2002)) would be

extremely useful in determining (a) whether increased parasite infection was definitely related to oestrogenic mixture exposure and (b) if these parasites had any additional affects on reproduction and development.

In contrast to the findings that *V. viviparus* exposed to the oestrogenic mixtures had increased levels of parasitism, no trend was observed in the infection of *P. corneus*. The literature suggests that *P. corneus* can be heavily and widely parasitised by larval trematodes (Digenea) in the wild. A survey of molluscs conducted in two small ponds and one swamp in the Czech Republic between 1998 and 2000 found 78.2% of the *P. corneus* sampled to be infected with larval trematodes (Faltynkova 2005) and in a larger survey of 12 species of planorbid snails (7628 snails) in Central Europe between 1998 and 2006 found *P. corneus* to be the most widely and heavily infected of the 12 species (Faltynkova et al. 2008). These differences in pattern of infection may relate to definitive hosts present up-stream in the river water, which fed the mesocosm tanks. Gibson and Bray (Gibson and Bray 1994) conducted a survey from a host-parasite database of 119 digenea families. They found that generally, digenea that had a fish as its vertebrate host favoured prosobranchs and bivalves as their first intermediate host. Those that had frogs, birds, or mammals as their vertebrate host favoured pulmonates as their intermediate host. Therefore perhaps the river contained a larger population of fish, which would supply digenean parasites, which would favour a prosobranch rather than a pulmonate host. It is also possible that the *P. corneus,* as a shorted lived species, which were parasitically infected had already died by the time sampling occurred.

6 Discussion

6.1 Major finding of this research

The main aims of these mesocosm experiments were to assess the possible effects of E2 or oestrogenic mixture exposure to reproduction and sexual development in two species of gastropod mollusc. In this experiment, E2 exposure was seen to modulate adult reproduction in both species, reducing the normal late-summer reproductive decline in P. corneus, and increasing the number of neonates released and new embryos harboured by *V. viviparus*. Using histopathological analysis, effects on oogenesis and spermatogenesis were observed in both species, with the F1s being especially sensitive to E2 and oestrogenic mixture exposure. An additional effect of exposure to the oestrogenic mixtures was increased parasitic infection, indicating possible effects to the snail's immune system. The mechanisms by which these effects took place are as yet unkown. Molluscs are known to have estrogen-like receptors, however, it is not known if steroids (or steroid mimics) can modulate the endocrine system of gastropods via these receptors, in the same way they have been observed to do in vertebrates.

6.2 Dosing

For the E2 study the results showed that the actual concentrations of the dosed oestrogen in the treatment tanks were highly variable throughout the study, such that statistically significant differences between the RW and the dosed tanks, but not between the low and high dosed tanks, could be found. Similarly, in the oestrogenic mixtures experiment, the measured concentrations of each chemical dosed were generally lower than the nominal concentrations, with the exception EE2. The variability in the actual concentrations of dosed chemicals relative to the nominal in both experiments is likely to be related to experimental set up, namely the use of river water (complete with algae, bacteria and suspended sediments). Steroid oestrogens, alkylphenols and BPA are all subject to sorbtion to sediment, bacterial degradation and up-take by biota within the mesocosm tanks. In both experiments there was also microbial growth in the dosing reserviours, which may have led to degradation of chemicals even before they entered the mesocosm tanks. In both the E2 and the oestrogenic mixtures experiments, there were unexpectedly high concentrations of E2 in the un-dosed tanks, and EE2 in both the un-dosed tanks and the river water inlet. Notwithstanding this, in both experiments, the biological results generally suggest the high dose tanks did recive a higher dose than the low dose tanks.

6.3 Comparison of effects between single oestrogenic chemical exposure and oestrogenic mixture exposure on two species of freshwater gastropods

6.3.1 Test species 1 - *Planorbarius corneus*

There are a number of similarities between the effects seen in the E2 and the oestrogenic mixtures exposures. Both the LE2 and the LM exposed adults laid the highest number of eggs over the breeding study. Both these groups also continued to lay eggs at a higher rate than the other exposure groups during the normal autumn decline, suggesting inappropriate stimulation of reproduction. The E2 exposure seemed to cause more significant effects of exposure than the oestrogenic mixtures study. However, the trends were broadly similar to each other and to the effects reported by Clarke et al (Clarke et al. 2009), when the same species was exposed to TSE. This suggests that environmentally relevant mixtures of oestrogenic chemicals may have a modulating effect on *P. corneus* reproduction. Table 6.1 illustrates the trends in the histological endpoint measured in the adult snails from the two exposures. Some striking similarities are present. Both the oestrogenic mixtures and the E2 exposure had significant impacts on Sertoli cells and the immature spermatogenic cells they support. The sloughing of immature spermatogenic cells has also been reported by Clarke et al (Clarke et al. 2009), in the same species exposed to TSE. Oestrogenic exposure (E2 or mixtures) also affected oogenesis, with higher percentages of young stages of oocytes in exposed snails. However, whether this is a result of stimulation or perhaps some kind of follicle cell dysfunction would require further research. The vitellogenic area of the acini, where both oocytes and sperm mature, was also negatively affected by exposure to oestrogenic mixtures or E2 alone. The developmentally exposed F1s showed similar trends in their histopathology (Table 6.2). Oestrogenic exposure (LE2, HE2 and HM) significantly reduced the percentage of active vitellogenic areas. Immature spermatogenic cell and Sertoli cells sloughing generally increased after developmental exposure to either E2 or the oestrogenic mixture. Similarly, the percentage of young oocytes was significantly higher in E2 and oestrogenic mixture exposed snails compared to RW exposed snails.

In addition to the similarities in histopathology of the snails between the two different exposures there were also a number of differences. For instance the HE2 exposure seemed to be toxic to the adult *P. corneus*, resulting in lower percentage survival, and a reduced correlations between size and weight at the end of the exposure. In contrast, the HM exposure did not cause an increase in percentage mortality and indeed at the end of the exposure F0 *P. corneus* from the HM exposure had significantly larger shells than those from the RW exposure. Differences could also be observed between the phenotypes of the animals from the adult exposure and developmental exposure. *P. corneus* adult exposure to

E2 or oestrogenic mixtures resulted in larger numbers of offspring in the tanks at the end of the exposure. However, in both cases, the oestrogenic developmentally-exposed offspring were smaller (length and weight) than their RW counterparts, significantly so in both the HE2 and the HM. However, once depurated, again there were marked differences between the two exposures. The oestrogenic-mixture exposed snails suffered extremely high over-winter mortality and grew poorly compared to RW developmentally exposed snails (Table 5.28). Whereas, the E2 exposed snails had higher over-winter survival compared to the RW developmentally exposed snails, and the LE2 developmentally exposed snails grew larger than those from the RW exposure (Table 4.23). It was therefore surprising that these large and presumably healthy E2 exposed (and then depurated) snails exhibited both delayed and reduced reproduction compared to the RW exposed snails (Figure 4.8). As discussed in Chapter 4, these results could indicate a disruption to the normal balance of growth and reproduction wich is controlled by the light green cells (LGC) and the lateral lobes (LL). This would be an interesting area for further research if the results could be replicated. Perhaps laboratory exposures could determine if the day length threshold for egg laying can be modulated by developmental exposure to E2. This type of experiment may also enlighten some of the unknown aspects of pulmonate neuro-endocrinology relating to the stimulation of the LL. Disruption to the normal pattern of reproduction could be disastrous for a temperate species, which obviously relies heavily on external cues to time reproduction with food availability and clement conditions for growth. Also, for a species that is known to be highly fecund reducing the period over which reproduction occurs could have large population-level effects.

As discussed above, adult snails exposed to the oestrogenic mixtures survived and grew well. This cannot be said for the developmentally exposed F1 *P. corneus*. Indeed the overriding effect of the HM developmental exposure was toxicity, resulting in markedly reduced growth and survival. This inevitably also affected reproductive output. This highlights how important full lifecycle studies can be in determining the effects chemicals (or mixtures) may have at a population level. With a species that lives for only a few years, a whole population may be wiped out very quickly. Unlike marine gastropods, freshwater snails do not have a planktonic larval stage; therefore re-colonisation could take a long period of time. The loss of an important detritivore and prey item may also alter the wider ecology of the environment. The LM developmentally-exposed *P. corneus* were also negatively impacted by exposure. Again reduced over winter survival and growth were observed (Table 5.25 and Table 5.26). However, during the F1 breeding study, these snails showed good survival, growth and reproductive output compared to the RW exposed snails

(Figure 5.7, Figure 5.8, Figure 5.9 and Table 5.28). This indicates that at the lower 'polluted river' (LM) concentration individual snail reproductive output may not be as severally affected. Although, the reduced over-winter survival of these F1s would still be of consequence when assessing population health.

Table 6.1 Comparison of trends in the histological endpoints measured in adult (F0) *P. corneus* from the 17β-oestradiol and oestrogenic mixtures experiments

Endpoint	17β-oestradiol	Oestrogenic mixtures
Vitellogenic area activity	Dose dependant ↓	Dose dependant ↓
Acini wall covered in germ cells	LE2 significantly less than RW	HM significantly less than RW
Sloughing of Sertoli cells	Dose dependant ↑, HE2 significantly more than RW	Dose dependant ↑, LM and HM significantly more than RW
Sloughing of spermatogonium	Dose dependant ↑	Dose dependant ↑
Sloughing of spermatocytes	HE2 ↑ than RW or LE2	Dose dependant ↑
Sloughing of spermatids	Dose dependant ↑	LM ↑ than RW+S
Mature spermatozoa only	Dose dependant ↓	Dose dependant ↓
Percentage of acini affected by immature spermatogenic cell sloughing	Dose dependant ↑	LM ↑ than RW+S
Oogenesis	E2 exposure ↑ in percentage of young stages	LM ↑ in percentage of young stages

The histopathology of the depurated F1 *P. corneus* showed less marked effects when compared to the recently exposed snails in both the E2 and the oestrogenic mixture exposed snails (Table 6.3). However, many of the same trends were still apparent (reduced vitellogenic area activity, altered oogenesis, sloughing of spermatogenic cells) after a year of depuration. In a species that only lives for perhaps 2-3 years, these effects may hamper reproduction for a large proportion of the animal's lifetime.

Table 6.2 Comparison of trends in the histological endpoints measured in F1 *P. corneus* from the 17β-oestradiol and oestrogenic mixtures experiments

Endpoint	17β-oestradiol	Oestrogenic mixtures
Vitellogenic area activity	Dose dependant ↓, LE2 and HE2 significantly lower than RW	Dose dependant ↓, HM significantly lower than RW
Acini wall covered in germ cells	LE2 significantly more than RW or HE2	LM and HM significantly more than RW
Sloughing of Sertoli cells	Dose dependant ↑	LM ↑
Sloughing of spermatogonium	Dose dependant ↑	No trend observed
Sloughing of spermatocytes	LE2 ↑	RW ↑
Sloughing of spermatids	Dose dependant ↑, HE2 significantly more than RW	Dose dependant ↑, LM and HM significantly more than RW
Mature spermatozoa only	HE2 significantly less than RW or LE2	HM ↓
Percentage of acini affected by immature spermatogenic cell sloughing	RW significantly less than LE2 and HE2	RW ↑
Oogenesis	E2 exposure ↑ in percentage of young stages, LE2 and HE2 significantly more young oocytes	LM ↑ in percentage of young stages, LM and HM significantly more young oocytes

Table 6.3 Comparison of trends in the histological endpoints measured in exposed and then depurated F1 *P. corneus* from the 17β-oestradiol and oestrogenic mixtures experiments

Endpoint	17β-oestradiol	Oestrogenic mixtures
Vitellogenic area activity	Dose dependant ↓	Dose dependant ↓
Acini wall covered in germ cells	HE2 lowest, LE2 highest	No trend observed
Sloughing of Sertoli cells	RW highest	HM ↑
Sloughing of spermatogonium	HE2 lowest, LE2 highest	HM ↑
Sloughing of spermatocytes	Dose dependant ↑	Dose dependant ↑
Sloughing of spermatids	Dose dependant ↓	No trend observed
Mature spermatozoa only	Dose dependant ↓	Dose dependant ↓
Percentage of acini affected by immature spermatogenic cell sloughing	No trend observed	Dose dependant ↑
Oogenesis	No trend observed	HM higher percentage of stage 1 and 4 oocytes

6.3.2 Test species 2 - *Viviparus viviparus*

The adult *V. viviparus* experienced quite different effects of exposure to either E2 or oestrogenic mixtures. The HE2 exposed snails released a significantly higher number of neonates during the last month of exposure compared to the LE2 and the RW exposed snails (Figure 4.10). However, this was accompanied by high mortality (Figure 4.11), and was presumably due to premature neonate release as a result of toxicity. Both the male and female HE2-exposed adult *V. viviparus* were smaller, and the females harboured less embryos, at the end of the 16 week exposure, again suggesting toxicity at this

concentration. In contrast, the LE2 females harboured significantly more unshelled embryos, indicating possible reproductive stimulation (Table 4.30). There were no significant effects of oestrogenic mixture exposure on adult size or weight (Table 5.37 and Table 5.38). In this experiment the RW exposed snails released the highest number of neonates. This indicates that the oestrogenic mixture exposure may have inhibited reproduction in this species. The histopathology of the female gonad can shed some light on these differing reproductive outputs. In the E2 study, the mean number of oocytes increased in a dose-dependant manner, again suggesting possible (E2) stimulatory effects of female reproduction. However, in the mixtures study, the oestrogenic mixture exposed adult females had less oocytes compared to the RW exposed snails. This suggests the oestrogenic mixtures affected the female's reproduction in a different manner, perhaps through suppression of oogenesis or via some toxic mechanism. Adult male spermatogenesis scores were not significantly affected by either E2 or oestrogenic mixture exposure.

Developmental exposure to the oestrogenic mixtures or E2 suppressed spermatogenesis in a dose-dependant manner. The LE2, the HE2 and the HM developmentally exposed males had significantly lower spermatogenesis scores compared to RW exposed F1s (indicating that different life stages differ in their susceptibility). Mean oocyte number was also reduced in a similar manner in the E2 F1 exposed snails, both directly after exposure, and after a years depuration. However, in the mixtures experiment, the F1 RW+S exposed snails also had reduced oocyte numbers compare to the RW snails (as did the LM and HM snails) sampled in September 2006. However, these differences in mean oocyte number were found to be related to the smaller female body size exhibited by the RW+S, LM and HM snails (after analysis with ANCOVA). However, after depuration in river water it was found that the LM and HM females did have significantly less oocytes compared to the RW snails, even after accounting for differences in size. F1 size was affected in both experiments; snails were significantly smaller from both the HE2 and the HM at the end of the dosed exposure. During the F1 breeding study both the LM and the LE2 developmentally exposed snails grew more than the RW exposed snails (Table 4.31, Table 4.32, Table 5.47 and Table 5.48). The LE2 developmentally-exposed and depurated females harboured significantly more embryos than the RW or the HE2 developmentally-exposed snails, this maybe related to over-wintering density and is discussed further in Section 6.4 (below). The mixture-exposed snails did not harbour any embryos. At the end of the breeding study, the mean number of oocytes was reduced in a dose dependant manner, for both E2 and oestrogenic mixture exposures. These results show that in an

exposure during early development may have long-term effects. There were a number of other quirks observed in the developmentally exposed *V. viviparus* worth noting. (1) The LE2 developmentally exposed males grew larger than the LE2 females (which according to the literature is unusual), (2) the misidentification of F1 males from morphology alone increased in a dose-dependent manner, for example, some males appeared to have a female-like pointed right tentacle, this was especially apparent in the HM exposed males. These observations suggest that exposure to oestrogenic chemicals may also alter sex specific morphology. Therefore, if the experiments were to be repeated, the morphology of the exposed snails may be another useful endpoint.

These experiments indicate along with a growing body of other publications, that molluscs may be sensitive to vertebrate EDCs. Indeed, molluscs may be extremely sensitive to certain EDC action. NP, OP and BPA have all been reported to affect mollusc reproduction at environmentally relevant concentrations (Oehlmann et al. 2000, Oehlmann et al. 2006, Duft et al. 2007, Duft et al. 2003, Nice 2005, Jobling et al. 2004).

6.4 Possible alternative explanation - Density

At the start of the exposure, the total number of snails per tank was 60 (See Section 3.5.4 for details) in the E2 exposure (RW, LE2 and HE2) and 102 in the oestrogenic mixtures exposure (RW, RW+S, LM, HM), resulting in a density of 0.09 snails per litre (sn/l) and 0.15 sn/l, respectively. However, after the initial set-up, the density of snails in each exposure tank was constantly changing with snail recruitment (neonates released or hatched) and/or mortality. As can be seen from Table 6.4, the density of snails in each tank at the end of the 16-week exposure varied quite widely. Therefore, the question has to be asked; could the reproductive and developmental effects measured in these experiments be a product of different densities, rather than of differing chemical exposure.

The effect of population density on sexual development is not well documented in the literature. However, the effects of density on growth and adult reproductive success or fecundity have been frequently studied. In general, the theory is that high population density leads to limited resources, such as food, which reduces growth, which in turn reduces fecundity. This type of density-mediated effect is especially evident in animals that require a high body condition to sustain energy intensive reproduction, such as mammalian pregnancy. A good example of this is demonstrated by Stewart et al (Stewart et al. 2005), who experimentally altered two populations of North American Elk (high or low density), and then monitored reproductive success. As might be expected, the higher density

population had significantly lower pregnancy rates (adjusted for age) compared to the low density group.

Table 6.4 The number of snails collected from each tank at the end of the 16-week exposure study and the resulting density of snails in each tank.

	PC			VV			Density snail/litre	
	All F1s	F1s >10mm shell diameter	Adults	All F1s	F1s >10mm shell length	Adults	All snails	Adults + F1s >10mm shell diameter/length
RW (mix)	239	105	25	553	443	37	1.25	0.89
RW+S	213	84	5	100	49	33	0.51	0.25
LM	2129	129	25	250	150	41	3.56	0.50
HM	461	21	25	140	90	30	0.96	0.24
RW (E2)	164	119	21	119	97	24	0.48	0.38
LE2	274	125	24	175	129	28	0.73	0.45
HE2	483	233	13	160	100	15	0.98	0.53

Each tank had a 685 litre capacity; initial density in the E2 experimental tanks (RW, LE2, HE2) were 0.09 sn/l, and in the Mixtures experimental tanks (RW, RW+S, LM, HM) 0.15 sn/l. The density of 'all snails' is given to represent the total density of snails at the end of the exposure study, the density of 'adult + F1s over 10mm shell diameter/length' is given in an attempt to show the F1s which would have been resident in the tank over a longer period.

To fully understand the role (if any) of density on growth, development and reproduction in each of the exposure tanks, elegant modelling techniques would need to be employed. As these data have not been collected, a more simplistic approach must be used. To achieve this, adult and F1 snails held at different initial densities in the E2 reference (RW^{E2}) or Mixtures reference (RW^{mix}) tanks were compared. The RW^{E2}tank held 3 replicate cages of each species (total 60 adult snails), whereas, the RW^{mix} had six replicate cages (total 102 adult snails). There were 11 Viviparus in each replicate cage in both experiments, but there were 9 Planorbids (RW^{E2}) and 6 Planorbids (RW^{mix}). Given the larger total number of adult snails in the Mixtures RW tank (around 48% more than in the E2 RW tank), it was not surprising that at the end of the 16 week exposure the density of F1 snails in the Mixture RW tank was more than double that seen in the E2 RW tank (Table 6.4, above).

There is very little information in the literature about the effect of density or nutrition on *V. viviparus*. However, in one study by Brown (Brown and Richardson 1992)(Brown and Richardson 1992, Brown and Richardson 1992) growth and reproduction were compare in two species of viviparid prosobranch snail (*V. subpurpureus* and *Campeloma decisum*) in

two waters in south Louisiana USA (Brown and Richardson 1992). The first site had higher levels of coarse and fine organic mater (food) but also had lower water temperature, lower dissolved oxygen, lower pH and lower water hardness when compared to the second site. However, the density of snails at the first site was quadruple that of the second, indicating that when large quantities of food are present these species could adapt to multiple unfavourable conditions (Brown and Richardson 1992). To assess the effect of density on reproductive output of *V. viviparus* in my experiments, the numbers of neonates released by the F0 females in the two RW tanks were compared. On average over the 16-week study, the snails from the RW^{E2} tank (lower density) released more neonates per adult than the Mixture RW tank (higher density) (16.6 and 14.7, respectively), and at week 6 this was significantly greater (P=0.024, M-W). At the end of the 16-week breeding study, there were no significant differences in the total numbers of embryos harboured in the brood pouch by the F0 females. However, there were differences when the embryos were split between shelled (mature) and unshelled (immature) embryos. The number of unshelled embryos harboured by the females from the RW^{E2} (lower density) tank (8.4 ± 5.1) was significantly (P=0.001, t-test) higher than the RW^{mix} (higher density) tank (4.0 ± 3.5). The occurrence of earlier life-stage embryos in the brood pouch of the RW^{E2} snails was probably due to the fact that they had already released more of their shelled embryos. It is possible that snails held at the higher density (RW^{mix}) had less food and therefore less energy for reproduction, resulting in a longer gestation period (lower reproductive output) and a higher likelihood of finding shelled embryos in the brood pouch at the end of the experiment. Therefore density (possibly via differences in nutrition) may have affected reproductive output in the F0 *V. viviparus*. This information is extremely important when considering the results of the E2 F1 breeding study (Table 4.40). In this study, remaining F1 snails from the RW^{E2} and RW^{mix} were combined and overwintered together. All snails (reference and treatment) were placed into breeding groups the following spring. The F1 snails developmentally exposed to RW harboured significantly less embryos in the brood pouch compared to the LE2 females at the end of the breeding study. This result was contrary to the histopathological evidence collected from the developmentally exposed F1s prior to overwintering, where a combination of a higher spermatogenic score and oocyte number was observed in the RW exposed F1s compared to the LE2 F1s. However, as RW developmentally exposed F1s were overwintered at much higher densities than E2 exposed snails (Table 6.5) it is possible that density may have directly (nutrition) or indirectly affected reproduction in this group, thereby masking any effect of E2 exposure on embryo recruitment. Therefore in this case, density may affect the interpretation of the data obtained from the *V. viviparus* F1 breeding study.

In contrast to the differences found in F0 *V. viviparus* unshelled embryo numbers, the shell length, shell aperture, total weight, soft body weight and soft body weight with embryos removed were all almost identical between the two reference tanks. Therefore, differences in density did not appear to affect adult growth. Moreover, and importantly (for my results), there were no significant differences between the mean oocyte numbers for the two (density) groups of F0 females, suggesting that oogenesis was not affected by density, or that this endpoint is not a reliable measure of density-effects on reproduction (based on observed differences in neonate production mentioned earlier). As previously mentioned, *Viviparus* snails are known to naturally congregate in high densities. Therefore, they might be well adapted for such conditions and may not be expected to show gross changes to gonad development.

Table 6.5 Total number of *P. corneus* and *V. viviparus* F1s and overall density of snails at the start and end of the over-wintering (depuration) period

	Start of over-wintering period			End of over-wintering period		
	P. corneus	***V. viviparus***	**Total density sn/l**	***P. corneus***	***V. viviparus***	**Total density sn/l**
River Water tank (RW)	332	520	1.23	60	273	0.48
Low 17-βoestradiol tank (LE2)	240	125	0.53	68	117	0.27
High 17-βoestradiol tank (HE2)	442	108	0.80	109	59	0.25
Low oestrogenic mixture tank (LM)	2096	200	3.36	123	66	0.28
High oestrogenic mixture tank (HM)	440	84	0.77	8	63	0.10

Snails per litre, Sn/l.

The same analysis was conducted for the F1 *V. viviparus* snails immediately after the end of the 16-week F0 dosed study. The RW^{mix} (higher density) F1 females were slightly smaller than their RW^{E2} (lower density) counterparts, however there were no statistically significant differences between the two groups for shell length, shell aperture, total weight, or soft body weight. Also, similarly to the F0 females there were no significant differences in mean oocyte number between the high and low-density groups. This is important information given the significantly lower number of oocytes observed in the F1s developmentally exposed to E2 (both immediately after exposure and after depuration in

river water) and oestrogenic mixtures (after depuration). This information suggests that in the case of oocyte number, chemical exposure had a greater effect than density.

In contrast to the F1 females, significant differences were found between the F1 *V. viviparus* males from the two density groups: F1 males from the RW^{mix} (higher density) tank were significantly smaller and lighter, and had a significantly lower spermatogenesis score compared to those from the RW^{E2} (lower density) tank. This indicates that (1) sex-specific density effects may occur, that (2) the developing males may be more sensitive to increased density than developing females, and (3) that higher density negatively affected growth and sexual development in males. Therefor sex-specific differences may occur in developing *V. viviparus* with regard to density.

There is very little information in the literature relating to the effect of density in prosobranch snails. However, a number of laboratory studies have been conducted relating to effects on oxygen consumption in *Viviparus contectoides* under different conditions, including increased density and reduced nutrition. The oxygen consumption of male and female *V. contectodies* were measured at a range of densities (2, 4, 6 or 8 snails) with either single sex or mix sex (1:1 ratio) groups. No effect on oxygen consumption was observed in any group tested when compared to snails housed individually (Studier and Pace 1978). Therefore density its self does not seem to impact the snails (oxygen consumption) directly. However, in another experiment, oxygen consumption of male and female *V. contectodies* were measured after snails were starved for 3, 9 and 12 days. After corrections for weight were made, it was observed that male and female snails reacted differently to starvation (Studier and Pace 1978). Oxygen consumption rose significantly in male snails when compared to pre-starvation rates, whereas, in females snails oxygen consumption slowly dropped over time and was significantly lower than pre-starvation rates by 9 days of starvation. The authors felt that differences in energy reserves may account for the differences in oxygen consumption in males and females and that males having fewer energy reserves, may have increased food-finding activity. Although in my study the F1s would have been young and less likely to have food reserved, it may be that differences in behaviour exhibited by males and females account for the differing growth reactions to density. Whatever the causes, the fact that males were smaller at the higher density makes interpreting the F1 *V. viviparus* spermatogenesis data in the exposure studies complicated. This is especially true in the E2 experiment, where the (end of exposure) density increased in a dose-dependent manner and spermatogenesis score decreased dose-dependently. This suggests the possibility that density is the only factor

influencing gonad development in the male *V. viviparus* in this experiment. However, the same explanation does not fit with the results from the mixtures experiment. Although the highest spermatogenic score was observed in the lowest density tank (RW+S), the next lowest density tank had the lowest spermatogenic score (HM). This suggests that the chemicals may have had a greater influence on spermatogenesis than density in this case. Also, if you assume there is no effect of chemical exposure on spermatogenesis, and plot (or correlate) the snail density against the mean spermatogenic scores, from all seven of the treatment tanks, there is no significant trend (Figure 6.1). Therefore, in the mixtures experiment at least, there is little evidence to suggest that density was the only factor affecting spermatogenic development. Nevertheless, density does affect growth (and spermatogenic score) and differences in density might explain the discrepancy between the spermatogenic scores of the F1 males from the RW (lower spermatogenic score and higher density) and the RW+S (higher spermatogenic score and lower density) tanks in the oestrogenic mixtures experiment. These results highlight the need to control density in these types of experiment (discussed further in Section 6.5).

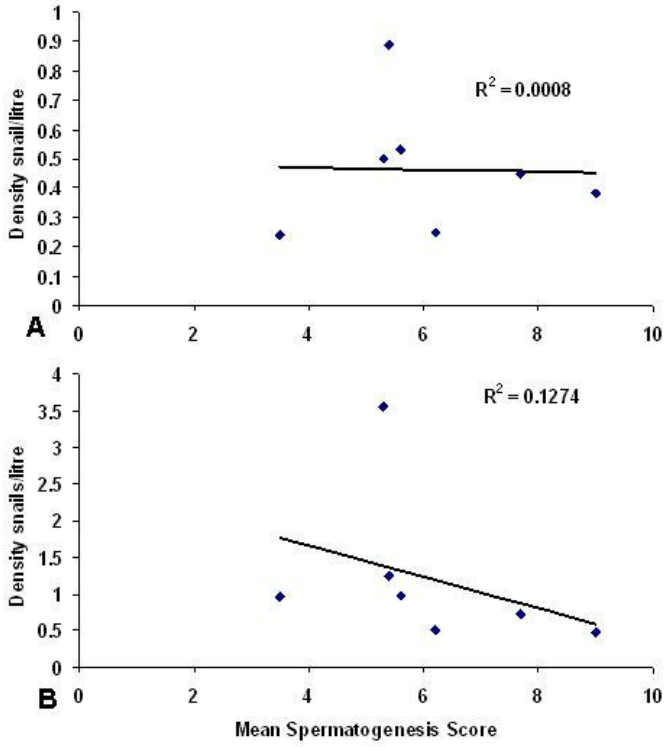

Figure 6.1 Mean spermatogenesis score in F1 V. viviparus plotted against the density of snails in each tank at the end of the 16-week exposure study.

A, density calculated using all adults plus F1s over 10mm in shell diameter/length. B, density calculated using all snails (F1s of all sizes and adults). See Table 6.5 **for details. Pearson's correlation were not significant for either A -0.028 P= 0.953 or B -0.357 P= 0.432.**

No literature could be found on the effect of density on *P. corneus* growth, development and fecundity. However, a number of studies on other Basommatophora have been conducted and summarised by Jordaens et al (Jordaens et al. 2007). Members of the families Planorbidae (*Biomphalaria glabrata*, *Bulinus abyssinicus*, *Bulinus forkalii, Bulinus truncates,* and *Helisoma duryi*) Physidae (*Physa gyrin,* and *Physa heterostropha pomilia*) and Lymnaeidae (*Lymnaea stagnalis*) were all found to be negatively affected by crowding in terms of fecundity and growth rate/body size (Jordaens et al. 2007). To assess the effect of density on *P. corneus* from my study, the reproductive outputs of the snails from the two RW tanks were compared. The *P. corneus* snails from the RW^{E2} (low density) tank laid more eggs per snail than those from the RW^{mix} tank (high density). Indeed, although the pattern of reproduction was the same, the RW^{E2}snails laid significantly more eggs per snail at weeks 4, 8, 10, 12, 14 and cumulatively over the 16 weeks of exposure, again suggesting density plays a very important role in fecundity in this species. It can be assumed that the increased density in the RW^{mix} tank lead to increased competition for resources, such as food or minerals. No literature could be found on the effect of nutrition on *P. corneus* fecundity. There is, however, literature on the more widely studied species *Lymnaea stagnalis* and it is assumed that *P. corneus* would be affected in a similar manner. Scheerboom (Scheerboom 1978) found that the level of food quality, food consumption and food assimilation were positively related to egg production in *L. stagnalis*. Further work by Joosse (Joosse 1984), also on *L. stagnalis,* found reproductively active snails removed from large breeding tanks with high density and food competition and placed in isolation laid up to four times as many eggs per snail. The idea that the higher density (RW^{mix}) tank had limited resources is also supported by the size of the F0 snails at the end of the 16-week exposure; the lower density (RW^{E2}) *P. corneus* were significantly larger in shell diameter and total weight (P= 0.004 and 0.029 t-test, respectively) than the higher density (RW^{mix}) snails. Similar analysis was also conducted for the F1 *P. corneus,* and a similar trend was observed in the F1 snails as seen with the F0 generation; the lower density (RW^{E2}) snails were significantly larger and heavier than those from the higher density tank (RW^{mix}).

The histopathological endpoints of the F0 *P. corneus* (cell coverage of the acini walls, vitellogenic area activity, sloughing of Sertoli cells or immature spermatogenic cells, percentage of acini with only mature sperm in the lumen, percentage of oogenesis stages) were also compared between the high and low-density RW tanks (t-test). No significant differences were found between the RW^{E2} (low density) snails and the RW^{mix} (high density) snails. This indicates the differing densities found in the two RW tanks did not

impact these endpoints. The histopathological endpoints were also compared in the F1 generation; only the percentage of Stage 5 oocytes was significantly different between the two density groups (RW^{mix}having on average less than RW^{E2}). Additionally, for the RW^{mix}F1s all the histopathological endpoints measured were correlated (Pearson's) with shell length to assess whether there were any significant relationships between the endpoints and size; no significant correlations were found. This indicates that the histopathology seen (such as Sertoli cell sloughing) in the F0 and F1 snails are not size dependent and unlikely to be related to density or growth.

Although having different densities in each tank has made interpreting some of the data more complex, having all the offspring grow and develop in the parental tanks was part of the initial experimental plan. Unlike many marine mollusc species, freshwater gastropods do not have a planktonic larval stage, which would facilitate dispersal. Therefore, in the wild it is likely that increased reproductive activity in adults would lead to increased density of juveniles in close proximity. Indeed, the density of snails in each tank itself may also be a product of exposure. Certainly in the E2 experiment, the number of *V. viviparus* neonates released into the tank increased dose-dependently, and although the E2 exposed juveniles at the end of the 16-week exposure were smaller there was also an increase in their percentage survival, which again reinforced the increase in density.

6.5 Limitations

As with all experiments, there are a number of limitations of this work, which should be recognised. Firstly, and perhaps most importantly, these experiments have not been repeated. Due to the long time period required (over 16 months) to conduct these life-cycle experiments, there was no possibility for repetition or refinement during my PhD research. My colleague, Rachel Benstead, has conducted a similar E2-dosed mesocosm study in 2004 (Benstead 2005). The main purpose of that exposure was to investigate F0 reproduction in *P. corneus* and *V. viviparus* (egg laying/neonate release); no histopathological endpoints were measured and the F1 generation was not fully investigated. However, during these experiments, similar trends in F0 reproduction were found, namely E2-exposure altered the pattern of reproduction in *P.corneus* (extending the egg laying season) and *V. viviparus* (increased reproductive output towards the end of the experiment). Similarities were also seen in mortality, in that the high E2 concentrations increase mortality (Benstead 2005). Repetition of the developmental exposures would be most enlightening and the other limitations given below would need to be considered to refine the experimental set-up.

The use of river water and the effect this had on the chemical analysis of the water samples was somewhat limiting when trying to interpret the biological results. It is important to highlight there were no true control tanks, in that the diluent river water already contained variable amounts of E2 and most likely other chemicals. As previously mentioned in the exposure chapters (Chapter 4 and 5), the use of river water was fundamental to the mesocosm design, to recreate (as far as possible) the natural environment, with all the interactions that go with it and to provide natural food for the *V. viviparus*. However, it also provided some complicating factors such as varying limits of detection for chemical analysis, un-dosed chemicals in the reference tanks, sediment, bacteria and algae; all of which may have affected the level of dosed chemicals, and parasites. The use of river water did provide a more 'real world' aspect to the experiment and in this sense the experiment was very successful. It is unlikely that in a river receiving effluent, the chemicals within it would remain in the water column at a fixed concentration. Degradation by bacteria, sorbtion to sediments, and uptake by biota are naturally occurring process. The use of river water also gave some very interesting (and unanticipated) results, such as the parasite infection rate of *V. viviparus*, which would not have occurred if a 'clean' water source had been used. However, I am aware it also limits the impact of some of the results, as it makes it difficult to prove without doubt that the effects seen are caused by chemical exposure. Therefore, it would have been advantageous if chemical analysis of snail tissue, filamentous algae and sediments had been conducted. Additionally the level of detection of all the chemicals dosed should have been fully discussed with the chemist analysing the samples, so that LOD were not above or close to the nominal concentrations dosed.

Wild caught snails were used in these experiments, and to a certain extent this too was a limitation. Unlike a laboratory animal the history (genetic background, age, previous chemical exposure, nutrition, parasite status) of my snails was unknown. Therefore, large amounts of variation in the reaction to stressors, such as chemical exposure, could be expected, and did occur. This was an unfortunate consequence of using relatively 'new to toxicological testing' group of animals (such as gastropod molluscs), rather than using more traditional laboratory breed species such as a rodent or a fish. If the experiment were to be repeated, or if freshwater gastropods were to be used in further investigations with EDC (as I feel they should be), it would be important to have sustainable laboratory cultures. The use of laboratory-cultured individuals would certainly remove a proportion of the individual variability experienced thus far.

The differing density of snails encountered in these experiments is another limitation when interpreting the results. As discussed above (Section 6.4), the different density of snails may have been a product of the chemical exposure, however, it makes a direct cause and effect relationship difficult to deduce. In a laboratory experiment, density would have been more easily controlled, and the effects seen in the animals would only be due to the chemical exposure. However, the mesocosm set up did not lend its self to constant readjustment of density. As stated above (Section 6.4), density did affect some endpoints (adult reproduction, growth, F1 spermatogenesis). However, it did not affect all endpoints measured, especially those relating histopathology such as the mean oocyte number in *V. viviparus* and sloughing of immature spermatogenic cells/Sertoli cells or vitellogenic area activity in *P. corneus*. Therefore, on balance, effects of chemical exposure can still be deduced.

The significant increase in parasite infection observed in the oestrogenic mixture exposed *V. viviparus* F1 was an additional, and possibly important, impact of exposure. However, it was also a confounding factor when trying to assess reproductive and growth endpoints, which were the initial focus of the study. As discussed in Chapter 5 Section 5.5.4 and 5.5.5, some parasite are know to modulate their hosts fecundity and growth. Therefore if the experiment were to be repeated, additional measures would need to be taken to stop parasite infection. There are several ways this could be achieved. (1) By preventing the parasite entering the mesocosm tanks e.g. UV radiation or finer filtration. (2) By protecting the snails from infection by perhaps using a finer mesh surrounding the snail cages. This method may have the added benefit that some snails could be protected and others not. Perhaps this would enable comparisons to be made between infected and non-infected snails reproduction and development.

Another possible limitation to the work is the use of a single exposure tank per treatment. In an ideal world, a larger number of tanks would have been used, however, in reality this was not possible as the footprint of such an experiment would be enormous and in practical to run without a large team of people. Within each tank the adult snails were split into ‘replicate’ breeding groups, which given the nature of the mesocosm tank would have experienced slightly different physical, chemical and biological conditions, allowing each group to be considered independent. However, all the offspring were held as one big group. This made statistical analysis of some of the data impossible.

6.6 Further Work

As with much research, there are many more questions raised by the results of this study than perhaps answers. Further research relating to gastropods and endocrine disrupting chemicals is surely necessary to better elude the problems faced by gastropods (and molluscs in general) in the polluted environment. To aid this, further research on basic gastropod neuro-endocrinology would also be extremely important. This may provide evidence of the mechanisms behind some of the effects observed in my work. For example detail information on the function and activity of receptors (ER-like, RXR and suggested D1) or neuro-endocrine cells (LL, LGC, DB, CDC) in relation to sexual development, growth and reproduction would be advantageous. Additionally, vast numbers of toxicological tests conducted are with single chemicals at environmentally irrelevant concentrations. Therefore, research into the effects of EDC mixtures and effluents on reproduction and immune system function (in any taxa) would be vitally important in understanding the 'real world' effects of our anthropogenic pollutants.

I, myself, would be especially interested in the relationship between chemical exposure and immune function. Recent research suggests there maybe a link between metabolic and immune related diseases, such as diabetes, and exposure to chemicals initially regarded as reproductive endocrine disruptors (Newbold et al. 2008, Lang et al. 2008, Nadal et al. 2009). Therefore finding animal models, which can be used to further investigate these effects would be extremely important.

6.7 Conclusions

Historically there has been very little research into oestrogenic ED effects on molluscs. This has generally been related to a lack in the basic understanding of molluscan neuro-endocrinology. However, my (and other recently published) research indicates that some molluscs are sensitive to EDCs known to be in the aquatic environment and to affect vertebrate reproduction. The effects reported here, such as stimulated reproductive output, inappropriate reproductive timing, Sertoli cell dysfunction, inhibited spermatogenesis, modulated oogenesis, and stunted juvenile growth have all also been reported in vertebrates exposed to environmental oestrogens. Whether these effects are mediated through traditional nuclear receptors or via some non-genomic mechanism is still up for debate. However, rather than shying away from research into the effects our anthropogenic waste may be having on this large and diverse taxonomic group, we should be investing more resources into understanding both the normal endocrinology and its possible disruption by pollutants in molluscs.

References

Aarab N., S. Lemaire-Gony, E. Unruh, P. D. Hansen, B. K. Larsen, O. K. Andersen, and J. R. Narbonne. 2006. Preliminary study of responses in mussel (Mytilus edilus) exposed to bisphenol A, diallyl phthalate and tetrabromodiphenyl ether. Aquatic Toxicology **78**:S86-S92.

Aarab N., C. Minier, S. Lemaire, E. Unruh, P. D. Hansen, B. K. Larsen, O. K. Andersen, and J. F. Narbonne. 2004. Biochemical and histological responses in mussel (*Mytilus edulis*) exposed to north sea oil and to a mixture of north sea oil and alkylphenols. Marine Environmental Research **58**:437-441.

Ahel M., J. McEvoy, and W. Giger. 1993. Bioaccumulation of the lipophilic metabolites of nonionic surfactants in freshwater organisms. Environmental Pollution **79**:243-248.

Ahmed S. R. 2000. The immune system as a potential target for environmental estrogens (endocrine disrupters): a new emerging field. Toxicology **150**:191-206.

Aleem M., V. Padwal, J. Choudhari, N. Balasinor, P. Parte, and M. K. Gill-Sharma. 2006. Estradiol affects androgen-binding protein expression and fertilizing ability of spermatozoa in adult male rats. Molecular and cellular endocrinology **253**:1-13.

Allen Y., P. Matthiessen, A. P. Scott, S. Haworth, S. Feist, and J. E. Thain. 1999a. The extent of oestrogenic contamination in the UK estuarine and marine environments - further surveys of flounder. Science of the Total Environment **233**:5-20.

Allen Y., A. P. Scott, P. Matthiessen, S. Haworth, J. E. Thain, and S. Feist. 1999b. Survey of estrogenic activity in United Kingdom estuarine and coastal waters and its effects on gonadal development of the flounder Platichthys flesus. Environmental Toxicology and Chemistry **18**:1791-1800.

Alon G., L. S. Shore, and Y. Steinberger. 2007. Correlation between levels of sex hormones (progesterone, testosterone, and estrogen) and ecophysiological-behavior stages in two species of desert snails (Sphincterochila zonata and Sphincterochila prophetarum) in the Northern Negev Desert. General and comparative endocrinology **151**:122-127.

Alsop D., M. Hewitt, M. Kohli, S. Brown, and G. Van der Kraak. 2003. Constituents within pulp mill effluent deplete retinoid stores in white sucker and bind to rainbow trout retinoic acid receptors and retinoid X receptors. Environmental Toxicology and Chemistry **22**:2969-2976.

Amano M., Y. Oka, Y. Nagai, N. Amiya, and K. Yamamori. 2008. Immunohistochemical localization of a GnRH-like peptide in the brain of the cephalopod spear-squid, Loligo bleekeri. General and comparative endocrinology **156**:277-284.

Andersen H., H. Siegrist, B. Halling-Sorensen, and T. A. Ternes. 2003. Fate of estrogens in a municipal sewage treatment plant. Environmental science & technology **37**:4021-4026.

Antizar-Ladislao B. 2008. Environmental levels, toxicity and human exposure to tributyltin (TBT)-contaminated marine environment. A review. Environment international **34**:292-308.

Arch S. 1972. Biosynthesis of Egg-Laying Hormone (Elh) in Bag Cell Neurons of Aplysia-Californica. Journal of General Physiology **60**:102-&.

Arch S., P. Earley, and T. Smock. 1976a. Biochemical Isolation and Physiological Identification of Egg-Laying Hormone in Aplysia-Californica. Journal of General Physiology **68**:197-210.

Arch S., T. Smock, and P. Earley. 1976b. Precursor and Product Processing in Bag Cell Neurons of Aplysia-Californica. Journal of General Physiology **68**:211-225.

Atanassova N., C. McKinnell, M. Walker, K. J. Turner, J. S. Fisher, M. Morley, M. R. Millar, N. P. Groome, and R. M. Sharpe. 1999. Permanent effects of neonatal estrogen exposure in rats on reproductive hormone levels, sertoli cell number, and the efficiency of spermatogenesis in adulthood. Endocrinology **140**:5364-5373.

Ayaki T., Y. Kawauchino, C. Nishimura, H. Ishibashi, and K. Arizono. 2005. Sexual disruption in the freshwater crab (Geothelphusa dehaani). Integrative and Comparative Biology **45**:39-42.

Bacchetta R., P. Mantecca, and G. Vailati. 2002. oocyte degeneration and altered ovipositiory activity induced by paraquat in the freshwater snail *Physa fontinalis* (Gastropoda: Pulmonata). Journal of Molluscan studies **68**:181-186.

Bannister R., N. Beresford, D. May, E. J. Routledge, S. Jobling, and M. Rand-Weaver. 2007. Novel estrogen receptor-related transcripts in Marisa cornuarietis; a freshwater snail with reported sensitivity to estrogenic chemicals. Environmental science & technology **41**:2643-2650.

Barel-Cohen K., L. S. Shore, M. Shemesh, A. Wenzel, J. Mueller, and N. Kronfeld-Schor. 2006. Monitoring of natural and synthetic hormones in a polluted river. Journal of environmental management **78**:16-23.

Barsiene J., G. Ribi, and D. Barsyte. 2000. Comparative karyological analysis of five species of Viviparus (Gastropoda : Prosobranchia). Journal of Molluscan Studies **66**:259-271.

Basheer C., H. K. Lee, and K. S. Tan. 2004. Endocrine disrupting alkylphenols and bisphenol-A in coastal waters and supermarket seafood from Singapore. Marine Pollution Bulletin **48**:1145-1167.

Bauer B., P. Fioroni, I. Ide, S. Liebe, J. Oehlmann, E. Stroben, and B. Watermann. 1995. Tbt Effects on the Female Genital System of Littorina-Littorea - a Possible Indicator of Tributyltin Pollution. Hydrobiologia **309**:15-27.

Belden L. K., J. M. Kiesecker. 2005. Glucocorticosteroid hormone treatment of larval treefrogs increases infection by Alaria sp trematode cercariae. Journal of Parasitology **91**:686-688.

Benstead R. 2007. Personal comunication

Benstead R. 2006. Personal comunication

Benstead R. 2005. Personal comunication

Beresford N., E. J. Routledge, C. A. Harris, and J. P. Sumpter. 2000. Issues arising when interpreting results from an in vitro assay for estrogenic activity. Toxicology and applied pharmacology **162**:22-33.

Berrie A. D. 1966. Growth and seasonal changes in the reproductive organs of *Lymnaea stagnalis* (L). Proc. malac. Soc. Lond. **37**:83-92.

Berrie A. D. 1963. Life-cycle of *Planorbarius corneus* L. Nature **198**:805-806.

Bettin C., J. Oehlmann, and E. Stroben. 1996. TBT-induced imposex in marine neogastropods is mediated by an increasing androgen level. Helgolander Meeresuntersuchungen **50**:299-317.

Bian Q., J. Qian, L. C. Xu, J. F. Chen, L. Song, and X. R. Wang. 2006. The toxic effects of 4-tert-octylphenol on the reproductive system of male rats. Food and Chemical Toxicology **44**:1355-1361.

Billinghurst Z., A. S. Clare, and M. H. Depledge. 2001. Effects of 4-n-nonylphenol and 17 beta-oestradiol on early development of the barnacle Elminius modestus. Journal of experimental marine biology and ecology **257**:255-268.

Billinghurst Z., A. S. Clare, K. Matsumura, and M. H. Depledge. 2000. Induction of cypris major protein in barnacle larvae by exposure to 4-n-nonylphenol and 17 beta-oestradiol. Aquatic Toxicology **47**:203-212.

Biselli S., K. Bester, H. Huhnerfuss, and K. Fent. 2000. Concentrations of the antifouling compound Irgarol 1051 and of organotins in water and sediments of German North and Baltic Sea marinas. Marine pollution bulletin **40**:233-243.

Bjerregaard L. B., B. Korsgaard, and P. Bjerregaard. 2006. Intersex in wild roach (Rutilus rutilus) from Danish sewage effluent-receiving streams. Ecotoxicology and environmental safety **64**:321-328.

Blaber S. J. M. 1970. The occurrence of a penis-like outgrowth behind the right tentacle in spent females of *Nucella lapillus* (L.). Proc. malac. Soc. Lond **39**:231-233.

Blackburn M. A., M. J. Waldock. 1995. Concentrations of alkylphenols in river and estuaries in England and Wales. Water research **29**:1623-1629.

Blackburn M. A., S. J. Kirby, and M. J. Waldock. 1999. Concentrations of Alkyphenol Polyethoxylates entering UK estuaries. Marine Pollution Bulletin **38**:109-118.

Blair D., G. M. Davis, and B. Wu. 2001. Evolutionary relationships between trematodes and snails emphasizing schistosomes and paragonimids. Parasitology **123**:S229-S243.

Blair L., J. P. Webster. 2007. Dose-dependent schistosome-induced mortality and morbidity risk elevates host reproductive effort. Journal of Evolutionary Biology **20**:54-61.

Blair L., J. P. Webster. 2002. Host reproductive fitness: the influence of increasing parasite pressure in a Biomphalaria glabrata. Invertebrate Reproduction & Development **41**:243-249.

Blaise C., F. Gagne, M. Salazar, S. Salazar, S. Trottier, and P. -. Hansen. 2003. Experimentally induced feminisation of freshwater mussels after long-term exposure to a municipal effluent. Fresenius Environmental Bulletin **12**:865-870.

Boer H. H., J. Joosse. 1975. Endocrinology. Pages 245-307 *In* V. Fretter and J. Peake, editors. Pulmonates. Functional Anatomy and Physiology, Academic Press Inc, London.

Bohlken S., J. Joosse. 1982. The Effect of Photoperiod on Female Reproductive Activity and Growth of the Fresh-Water Pulmonate Snail Lymnaea-Stagnalis Kept Under Laboratory Breeding Conditions. International Journal of Invertebrate Reproduction **4**:213-222.

Bohlken S., J. Joosse, R. Vanelk, and W. P. M. Geraerts. 1986. Interaction of Photoperiod and Nutritive State in Female Reproduction of Lymnaea-Stagnalis. International Journal of Invertebrate Reproduction and Development **10**:151-157.

Bohlken S., M. Vanderfluit, J. Joosse, and W. P. M. Geraerts. 1984. The Lateral Lobes are Not Involved in the Transmission of Photoperiodic Stimuli which Influence Egg-Production in the Hermaphrodite Pulmonate Snail Lymnaea-Stagnalis. Proceedings of the Koninklijke Nederlandse Akademie Van Wetenschappen Series C-Biological and Medical Sciences **87**:25-31.

Bolz U., H. Hagenmaier, and W. Korner. 2001. Phenolic xenoestrogens in surface water, sediments, and sewage sludge from Baden-Wurttemberg, sout-west Germany. Environmental Pollution **115**:291-301.

Borges C. M. C., C. P. de Souza, and Z. A. Andrade. 1998. Histopathologic features associated with susceptibility and resistance of Biomphalaria snails to infection with Schistosoma mansoni. Memorias do Instituto Oswaldo Cruz **93**:117-121.

Bortone S. A., R. P. Cody. 1999. Morphological masculinization in poeciliid females from a paper mill effluent receiving tributary of the St. Johns River, Florida, USA. Bulletin of environmental contamination and toxicology **63**:150-156.

Bortone S. A., W. P. Davis, and C. M. Bundrick. 1989. Morphological and Behavioral Characters in Mosquitofish as Potential Bioindication of Exposure to Kraft Mill Effluent. Bulletin of environmental contamination and toxicology **43**:370-377.

Bottke W. 1972. Zur morphologie des ovars von *Viviparus contectus* (Millet 1813), (Gastropoda, Prosobranchia) I die follikelzellen. Z Zellforsch mikrosk anat **133**:103-118.

Bouton D., H. Escriva, R. L. de Mendonca, C. Glineur, B. Bertin, C. Noel, M. Robinson-Rechavi, A. de Groot, J. Cornette, V. Laudet, and R. J. Pierce. 2005. A conserved retinoid X receptor (RXR) from the mollusk Biomphalaria glabrata transactivates transcription in the presence of retinoids. Journal of Molecular Endocrinology **34**:567-582.

Boyle J. P., T. P. Yoshino. 2002. Monoamines in the albumen gland, plasma, and central nervous system of the snail Biomphalaria glabrata during egg-laying. Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology **132**:411-422.

Braathen M., A. E. Derocher, O. Wiig, E. G. Sormo, E. Lie, J. U. Skaare, and B. M. Jenssen. 2004. Relationships between PCBs and thyroid hormones and retinol in female and male polar bears. Environmental health perspectives **112**:826-833.

Brian J. V., C. A. Harris, M. Scholze, A. Kortenkamp, P. Booy, M. Lamoree, G. Pojana, N. Jonkers, A. Marcomini, and J. P. Sumpter. 2007. Evidence of estrogenic mixture effects on the reproductive performance of fish. Environmental science & technology **41**:337-344.

Brodkin M. A., H. Madhoun, M. Rameswaran, and I. Vatnick. 2007. Atrazine is an immune disruptor in adult northern leopard frogs (Rana pipiens). Environmental Toxicology and Chemistry **26**:80-84.

Brown D. J., R. J. Vanbeneden, and G. C. Clark. 1995. Identification of 2 Binding-Proteins for Halogenated Aromatic-Hydrocarbons in the Hard-Shell Clam, Mercenaria-Mercenaria. Archives of Biochemistry and Biophysics **319**:217-224.

Brown K. M., T. D. Richardson. 1992. Phenotypic Plasticity in the Life Histories and Production of 2 Warm-Temperate Viviparid Prosobranchs. Veliger **35**:1-11.

Browne R. A. 1978. Growth, Mortality, Fecundity, Biomass and Productivity of 4 Lake Populations of Prosobranch Snail, Viviparus-Georgianus. Ecology **59**:742-750.

Bryan G. W., P. E. Gibbs, L. G. Hummerstone, and G. R. Burt. 1986. The Decline of the Gastropod Nucella-Lapillus Around Southwest England - Evidence for the Effect of Tributyltin from Antifouling Paints. Journal of the Marine Biological Association of the United Kingdom **66**:611-640.

Buckland-Nicks J. 1998. Prosobranch Parasperm: Sterile Germ Cells that Promote Paternity? Micron **29**:267-280.

Burger J., K. Viscido, and M. Gochfeld. 1995. Eggshell Thickness in Marine Birds in the New-York Bight - 1970s to 1990s. Archives of Environmental Contamination and Toxicology **29**:187-191.

Butler R. A., M. L. Kelley, W. H. Powell, M. E. Hahn, and R. J. Van Beneden. 2001. An aryl hydrocarbon receptor (AHR) homologue from the soft-shell clam, Mya arenaria: evidence that invertebrate AHR homologues lack 2,3,7,8-tetrachlorodibenzo-p-dioxin and beta-naphthoflavone binding. Gene **278**:223-234.

Campbell N., J. Reece. 2005. Biology. Pearson Education, .

Canesi L., M. Betti, L. C. Lorusso, C. Ciacci, and G. Gallo. 2005. 'In vivo' effects of Bisphenol A in *Mytilus* hemocytes: modulation of kinase-mediated signalling pathways. Aquatic toxicology **71**:73-84.

Canesi L., C. Borghi, C. Ciacci, R. Fabbri, L. Vergani, and G. Gallo. 2007a. Bisphenol-A alters gene expression and functional parameters in molluscan hepatopancreas. Molecular and cellular endocrinology **276**:36-44.

Canesi L., L. C. Lorusso, C. Ciacci, M. Betti, M. Rocchi, G. Pojana, and A. Marcomini. 2007b. Immunomodulation of *Mytilus* hemocytes by individual estrogenic chemicals and environmentally relavent mixtures of estrogens: In vitro and in vivo studies. Aquatic Toxicology **81**:36-44.

Canesi L., C. Borghi, R. Fabbri, C. Ciacci, L. C. Lorusso, G. Gallo, and L. Vergani. 2007c. Effects of 17 beta-estradiol on mussel digestive gland. General and comparative endocrinology **153**:40-46.

Canesi L., C. Ciacci, M. Betti, L. C. Lorusso, B. Marchi, S. Burattini, E. Falcieri, and G. Gallo. 2004. Rapid effects of 17 beta-estradiol on cell signaling and function of Mytilus hemocytes. General and comparative endocrinology **136**:58-71.

Caprioli R., C. Costa, E. Cataldi, C. Boglione, and S. Cataudella. 2007. First record of female germ cells in the testis of a wild Mediterranean northern bluefin tuna Thunnus thynnus L. 1758. Journal of fish biology **71**:620-622.

Carlsen E., A. Giwercman, N. Keiding, and N. E. Skakkebaek. 1992. Evidence for Decreasing Quality of Semen during Past 50 Years. British medical journal **305**:609-613.

Carson R. 1962. Silent Spring. Houghton Mifflin, .

AnonymousA comparison of freshwater pulmonate and prosobranch mollucsc responces to steroidal oestrogens. ; 22-26 May 2005; ; 2005. 105 p.

Castells P., J. Parera, F. J. Santos, and M. T. Galceran. 2008. Occurrence of polychlorinated naphthalenes, polychlorinated biphenyls and short-chain chlorinated paraffins in marine sediments from Barcelona (Spain). Chemosphere **70**:1552-1562.

Castro C., C. Melo, R. Guillot, I. Mendes, S. Queiros, D. Lima, M. A. Reis-Henriques, and M. M. Santos. 2007a. The estrogen receptor of the gastropod *Nucella lapillus*: Modulation following exposure to an estrogenic effluent? Aquatic Toxicology **84**:465-468.

Castro L. F. C., D. Lima, A. Machado, C. Melo, Y. Hiromori, J. Nishikawa, T. Nakanishi, M. A. Reis-Henriques, and M. M. Santos. 2007b. Imposex induction is mediated through the Retinoid X Receptor signalling pathway in the neogastropod Nucella lapillus. Aquatic Toxicology **85**:57-66.

Ceccarelli I., D. Della Seta, P. Fiorenzani, F. Farabollini, and A. M. Aloisi. 2007. Estrogenic chemicals at puberty change ER alpha in the hypothalamus of male and female rats. Neurotoxicology and teratology **29**:108-115.

Cheek A. O. 2006. Subtle sabotage: endocrine disruption in wild populations. Revista de biologia tropical **54**:1-19.

Chesman B. S., W. J. Langston. 2006. Intersex in the clam *Scrobicularia plana:* a sign of endocrine disruption in estuaries. Biology letters **2**:420-422.

Chin-Yuan C., L. Li-Lian, and D. Wang-Hsien. 2005. Determination of xenoestrogens Alkylphenols in oyster and snail tissues by extractive steam distillation and gas chromatography-mass spectrometry. Journal of the chines chemical society **52**:1257-1262.

Christin M. S., L. Menard, A. D. Gendron, S. Ruby, D. Cyr, D. J. Marcogliese, L. Rollins-Smith, and M. Fournier. 2004. Effects of agricultural pesticides on the immune system of Xenopus laevis and Rana pipiens. Aquatic Toxicology **67**:33-43.

Christin M. S., A. D. Gendron, P. Brousseau, L. Menard, D. J. Marcogliese, D. Cyr, S. Ruby, and M. Fournier. 2003. Effects of agricultural pesticides on the immune system of Rana pipiens and on its resistance to parasitic infection. Environmental Toxicology and Chemistry **22**:1127-1133.

Clarke N. 2006. Personal comunication

Clarke N., E. J. Routledge, A. Garner, D. Casey, R. Benstead, D. Walker, B. Watermann, K. Gnass, A. Thomsen, and S. Jobling. 2009. Exposure to Treated Sewage Effluent Disrupts Reproduction and Development in the Seasonally Breeding Ramshorn Snail (Subclass: Pulmonata, Planorbarius corneus). Environmental science & technology **43**:2092-2098.

Cody R. P., S. A. Bortone. 1997. Masculinization of mosquitofish as an indicator of exposure to kraft mill effluent. Bulletin of environmental contamination and toxicology **58**:429-436.

Coeurdassier M., A. De Vaufleury, and P. M. Badot. 2003. Bioconcentration of cadmium and toxic effects on life-history traits of pond snails (Lymnaea palustris and Lymnaea stagnalis) in laboratory bioassays. Archives of Environmental Contamination and Toxicology **45**:102-109.

Coggesha.Re. 1970a. A Cytologic Analysis of Ovotestis in Aplysia. Anatomical Record **166**:291-&.

Coggesha.Re. 1970b. Cytologic Analysis of Bag Cell Control of Egg Laying in Aplysia. Journal of Morphology **132**:461-&.

Colborn T. 1991. Epidemiology of Great-Lakes Bald Eagles. Journal of toxicology and environmental health **33**:395-453.

Colborn T., C. Clement. 1992. *Chemically-Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection*. Princeton Scientific Publishing Co, New Jersey, USA.

Colborn T., J. Peterson Myers, and D. Dumanoski. 1996. Our Stolen Future: Are We Threatening Our Own Fertility, Intelligence, and Survival?-A Scientific Detective Story. E P Dutton, .

Colton T., E. R. Greenberg, K. Noller, L. Resseguie, C. Vanbennekom, T. Heeren, and Y. Q. Zhang. 1993. Breast-Cancer in Mothers Prescribed Diethylstilbestrol in Pregnancy - further Follow-Up. Jama-Journal of the American Medical Association **269**:2096-2100.

Cook P. M. 1949. A Ciliary feeding mechanism in *Viviparus viviparus* (L.). J. Moll. Stud. **27**:265-271.

Cooper L. A., S. E. Larson, and F. A. Lewis. 1996. Male reproductive success of Schistosoma mansoni-infected Biomphalaria glabrata snails. Journal of Parasitology **82**:428-431.

Correa-Reyes G., M. T. Viana, F. J. Marquez-Rocha, A. F. Licea, E. Ponce, and R. Vazquez-Duhalt. 2007. Nonylphenol algal bioaccumulation and its effect through the trophic chain. Chemosphere **68**:662-670.

Costil K. 1997. Effect of temperature on embryonic development of two freshwater pulmonates, *Planorbarius corneus* (L.) and *Planorbis planorbis* (L.). Journal of Molluscan studies **63**:293-296.

Costil K., J. Daguzan. 1995a. Effect of Temperature on reproduction in *Planobarius corneus* (L.) and *Planorbis planorbis* (L.) throughout the life span. Malacologia **36**:79-89.

Costil K., J. Daguzan. 1995b. Comparative life cycle and growth of two freshwater gastropod species, *Planorbarius corneus* (L.) and *Planorbis planorbis.*. Malacologia **37**:53-68.

Crain D. A., L. J. Guillette. 1998. Reptiles as models of contaminant-induced endocrine disruption. Animal Reproduction Science **53**:77-86.

Crain D. A., M. Eriksen, T. Iguchi, S. Jobling, H. Laufer, G. A. LeBlanc, and L. J. Guillette. 2007. An ecological assessment of bisphenol-A: Evidence from comparative biology. Reproductive Toxicology **24**:225-239.

Crewsoyen A. E., T. P. Yoshino. 1995. Schistosoma-Mansoni - Characterization of Excretory-Secretory Polypeptides Synthesized In-Vitro by Daughter Sporocysts. Experimental parasitology **80**:27-35.

Croll R. P., J. Vanminnen. 1992. Distribution of the Peptide Ala-Pro-Gly-Trp-Nh2 (Apgwamide) in the Nervous-System and Periphery of the Snail Lymnaea-Stagnalis as Revealed by Immunocytochemistry and Insitu Hybridization. Journal of Comparative Neurology **324**:567-574.

Curtis L. A. 2007. Larval trematode infections and spatial distributions of snails. Invertebrate Biology **126**:235-246.

Czech P., K. Weber, and D. R. Dietrich. 2001. Effects of endocrine modulating substances on reproduction in the hermaphrodite snail *Lymnaea stagnalis* L. Aquatic toxicology **53**:103-114.

Damstra T, Barlow S, Bergman A, Kavlock R, Van Der Kraak G. Global assessment of the state-of-the-science of endocrine disruptors. World Health Organisation; 2002. Report nr WHO/PCS/EDC/02.2.

DAniello A., A. Di Cosmo, C. Di Cristo, L. Assisi, V. Botte, and M. M. DiFiore. 1996. Occurrence of sex steroid hormones and their binding proteins in Octopus vulgaris lam. Biochemical and biophysical research communications **227**:782-788.

Dawson A. 2000. Mechanisms of endocrine disruption with particular reference to occurrence in avian wildlife: A review. Ecotoxicology **9**:59-69.

de Alda M. J. L., D. Barcelo. 2000. Determination of steroid sex hormones and related synthetic compounds considered as endocrine disrupters in water by liquid chromatography-diode array detection-mass spectrometry. Journal of Chromatography a **892**:391-406.

de Alda M. J. L., S. Diaz-Cruz, M. Petrovic, and D. Barcelo. 2003. Liquid chromatography-(tandem) mass spectrometry of selected emerging pollutants (steroid sex hormones, drugs and alkylphenolic surfactants) in the aquatic environment. Journal of Chromatography a **1000**:503-526.

de Alda M. J. L., A. Gil, E. Paz, and D. Barcelo. 2002. Occurrence and analysis of estrogens and progestogens in river sediments by liquid chromatography-electrospray-mass spectrometry. Analyst **127**:1299-1304.

De Bernardi R., O. Ravera, and B. Oregioni. 1976. Demographic structure and biometric characteristics of *Viviparus ater* Cristofori and Jan (Gasteropoda: Prosobranchia) from Lake Alserio (Northern Italy). J. Moll. Stud. **42**:310-318.

De Boer P. A. C. M., A. Ter Maat, A. W. Pieneman, R. P. Croll, M. Kurokawa, and R. F. Jansen. 1997. Functional role of peptidergic anterior lobe neurons in male sexual behavior of the snail Lymnaea stagnalis. Journal of neurophysiology **78**:2823-2833.

De Jong-Brink M. 1992. Neuroendocrine Mechanisms Underlying the Effects of Schistosome Parasites on their Intermediate Snail Host. Invertebrate Reproduction & Development **22**:127-138.

de Jong-Brink M., M. J. M. Bergamin-Sassen, and M. S. Soto. 2001. Multiple strategies of schistosomes to meet their requirements in the intermediate snail host. Parasitology **123**:S129-S141.

De Jong-Brink M., M. Elsaadany, and M. S. Soto. 1991. The Occurrence of Schistosomin, an Antagonist of Female Gonadotropic-Hormones, is a General Phenomenon in Hemolymph of Schistosome-Infected Fresh-Water Snails. Parasitology **103**:371-378.

De Jong-Brink M., M. M. Elsaadany, and H. H. Boer. 1988. Trichobilharzia-Ocellata - Interference with Endocrine Control of Female Reproduction of Lymnaea-Stagnalis. Experimental parasitology **65**:91-100.

De Jong-Brink M., L. P. C. Schot, H. J. N. Schoenmakers, and M. J. M. Bergamin-Sassen. 1981. A biochemical and quantative electron microscope study on steriodogenesis in ovotestis and digestive gland of the pulmonate snail *Lymnaea stagnalis*. General and comparative endocrinology **45**:30-38.

De Jong-Brink M., H. H. Boer, T. G. Hommes, and A. Kodde. 1977. Spermatogenesis and the role of Sertoli Cells in the Freshwater Snail *Biomphalaria glabrata*. Cell and Tissue Research **181**:37-58.

De Jong-Brink M., A. de Wit, G. Kraal, and H. H. Boer. 1976. A light and electron microscope study on oogenesis in the freshwater Pulmonate snail *Biomphalaria glabrata.*. Cell and Tissue Research **171**:195-219.

De Jong-Brink M., R. M. Hoek, A. B. Smith, M. J. M. Bergamin-sassen, and W. Lageweg. 1995. Schistosoma Parasites Evoke Stress Responses in their Snail Host by a Cytokine-Like Factor Interfering with Neuroendocrine Mechanisms. Netherlands Journal of Zoology **45**:113-116.

De Jong-Brink M., P. L. Hordijk, D. P. E. J. Vergeest, H. D. F. H. Schallig, K. S. Kits , and A. Ter maat. 1992. The Antigonadotropic Neuropeptide Schistosomin Interferes with Peripheral and Central Neuroendocrine Mechanisms Involved in the Regulation of

Reproduction and Growth in the Schistosome-Infected Snail Lymnaea-Stagnalis. Progress in brain research **92**:385-396.

de Lange R. P. J., J. van Minnen. 1998. Localization of the neuropeptide APGWamide in gastropod molluscs by in situ hybridization and immunocytochemistry. General and comparative endocrinology **109**:166-174.

De Metrio G., A. Corriero, S. Desantis, D. Zubani, F. Cirillo, M. Deflorio, C. R. Bridges, J. Eicker, J. M. de la Serna, P. Megalofonou, and D. E. Kime. 2003. Evidence of a high percentage of intersex in the Mediterranean swordfish (Xiphias gladius L.). Marine pollution bulletin **46**:358-361.

de Solla S. R., C. A. Bishop, and R. J. Brooks. 2002. Sexually dimorphic morphology of hatchling snapping turtles (Chelydra serpentina) from contaminated and reference sites in the Great Lakes and St Lawrence River basin, North America. Environmental Toxicology and Chemistry **21**:922-929.

Debier C., G. M. Ylitalo, M. Weise, F. Gulland, D. P. Costa, B. J. Le Boeuf, T. de Tillesse, and Y. Larondelle. 2005. PCBs and DDT in the serum of juvenile California sea lions: associations with vitamins A and E and thyroid hormones. Environmental Pollution **134**:323-332.

deFur P. L., M. Crane, and L. J. Tattersfield. 1999. Conclusions and recommendations. Endocrine Disruption in Invertebrates: Endocrinology, Testing, and Assessment :271-279.

DeLange R. P. J., F. A. VanGolen, and J. VanMinnen. 1997. Diversity in cell specific co-expression of four neuropeptide genes involved in control of male copulation behaviour in Lymnaea stagnalis. Neuroscience **78**:289-299.

Della Seta D., I. Minder, F. Dessi-Fulgheri, and F. Farabollini. 2005. Bisphenol-A exposure during pregnancy and lactation affects maternal behavior in rats. Brain research bulletin **65**:255-260.

Desbrow C., E. J. Routledge, G. C. Brighty, J. P. Sumpter, and M. Waldock. 1998. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. Environmental science & technology **32**:1549-1558.

Dessi-Fulgheri F., S. Porrini, and F. Farabollini. 2002. Effects of perinatal exposure to bisphenol A on play behavior of female and male juvenile rats. Environmental health perspectives **110**:403-407.

Di Cosmo A., C. Di Cristo, and M. Paolucci. 2002. A estradiol-17 beta receptor in the reproductive system of the female of Octopus vulgaris: Characterization and immunolocalization. Molecular reproduction and development **61**:367-375.

Di Cosmo A., C. Di Cristo, and M. Paolucci. 2001. Sex steroid hormone fluctuations and morphological changes of the reproductive system of the female of Octopus vulgaris throughout the annual cycle. Journal of Experimental Zoology **289**:33-47.

Di Cosmo A., M. Paolucci, C. Di Cristo, V. Botte, and G. Ciarcia. 1998. Progesterone receptor in the reproductive system of the female of Octopus vulgaris: Characterization and immunolocalization. Molecular reproduction and development **50**:451-460.

Di Cristo C., J. Van Minnen, and A. Di Cosmo. 2005. The presence of APGWamide in Octopus vulgaris: a possible role in the reproductive behavior. Peptides **26**:53-62.

di Cristo C., M. Paolucci, and A. Di Cosmo. 2002. GnRH and FMRFamide: two neuro-bioactive molecules involved in Octopus vulgaris reproduction. Journal of Physiology-London **543**:2P-3P.

Di Cristo C., M. Paolucci, J. Iglesias, J. Sanchez, and A. Di Cosmo. 2002. Presence of two neuropeptides in the fusiform ganglion and reproductive ducts of Octopus vulgaris: FMRFamide and gonadotropin-releasing hormone (GnRH). Journal of Experimental Zoology **292**:267-276.

Dickerson S. M., A. C. Gore. 2007. Estrogenic environmental endocrine-disrupting chemical effects on reproductive neuroendocrine function and dysfunction across the life cycle. Reviews in Endocrine & Metabolic Disorders **8**:143-159.

Diez S., J. Jimenez, and J. M. Bayona. 2003. Recent status and historical trends of the organotin contamination in sediments from the Western Mediterranean Sea. Ciencias Marinas **29**:547-560.

Dogterom G. E., R. Thijssen, and H. Vanloenhout. 1985. Environmental and Hormonal-Control of the Seasonal Egg-Laying Period in Field Specimens of Lymnaea-Stagnalis. General and comparative endocrinology **57**:37-42.

Dogterom G. E., S. Bohlken, and J. Joosse. 1983. Effect of the Photoperiod on the Time Schedule of Egg Mass-Production in Lymnaea-Stagnalis, as Induced by Ovulation Hormone Injections. General and comparative endocrinology **49**:255-260.

Drysdale D. T., S. A. Bortone. 1989. Laboratory Induction of Intersexuality in the Mosquitofish, Gambusia-Affinis, using Paper-Mill Effluent. Bulletin of environmental contamination and toxicology **43**:611-617.

Duft M., U. Schulte-Oehlmann, L. Weltje, M. Tillmann, and J. Oehlmann. 2003. Stimulated embryo production as a parameter of estrogenic exposure via sediments in the freshwater mudsnail *Potamopyrgu antipodarum*. Aquatic Toxicology **64**:437-449.

Duft M., C. Schmitt, J. Bachmann, C. Brandelik, U. Schulte-Oehlmann, and J. Oehlmann. 2007. Prosobranch snails as test organisms for the assessment of endocrine active chemicals - an overview and a guideline proposal for a reproduction test with the freshwater mudsnail *Potamopyrgus antipodarum*. Ecotoxicology **16**:169-182.

Duncan C. J. 1975. Reproduction. Pages 309-365 *In* V. Fretter and j. Peake, editors. Pulmonates. Functional Anatomy and physiology. Academic press, London.

Duncan C. J. 1960. The Genital System of the Freshwater Basommatophora. Proceedings of the zoological society london **135**:339-356.

Durhan E. J., C. S. Lambright, E. A. Makynen, J. Lazorchak, P. C. Hartig, V. S. Wilson, L. E. Gray, and G. T. Ankley. 2006. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. Environmental health perspectives **114**:65-68.

Eckelbarger K. J., C. M. Young. 1997. Ultrastructure of the ovary and oogenesis in the methane-seep mollusc Bathynerita naticoidea (Gastropoda: Neritidae) from the Louisiana slope. Invertebrate Biology **116**:299-312.

Eleutheriadis N., M. Lazaridoudimitriadou. 1995. Density and Growth of Fresh-Water Prosobranch Snails (Bithynia-Graeca and Viviparus-Contectus) in Relation to Water Chemistry in Serres, Northern Greece. Journal of Molluscan Studies **61**:347-352.

Elliott J. E., C. J. Henny, M. L. Harris, L. K. Wilson, and R. J. Norstrom. 1999. Chlorinated hydrocarbons in livers of american mink (Mustela vison) and river otter (Lutra canadensis) from the Columbia and Fraser River basins, 1990-1992. Environmental monitoring and assessment **57**:229-252.

Ezemonye L. I. 2006. Concentrations and distribution of polychlorinated biphenyls (PCBs) in aquatic environment of niger delta of Nigeria (water and sediments). Discovery and Innovation **18**:160-169.

Faltynkova A. 2005. Larval trematodes (Digenea) in molluscs from small water bodies near Ceske Budejovice, Czech Republic. Acta Parasitologica **50**:49-55.

Faltynkova A., V. Nasincova, and L. Kablaskova. 2008. Larval trematodes (Digenea) of planorbid snails (Gastropoda : Pulmonata) in Central Europe: a survey of species and key to their identification. Systematic parasitology **69**:155-178.

Fan X. M., R. P. Croll, B. Wu, L. Fang, Q. Shen, S. D. Painter, and G. T. Nagle. 1997. Molecular cloning of a cDNA encoding the neuropeptides APGWamide and cerebral peptide 1: Localization of APGWamide-like immunoreactivity in the central nervous system and male reproductive organs of Aplysia. Journal of Comparative Neurology **387**:53-62.

Fent K. 1996. Organotin compounds in municipal wastewater and sewage sludge: Contamination, fate in treatment process and ecotoxicological consequences. Science of the Total Environment **185**:151-159.

Fent K., J. Hunn. 1991. Phenyltins in Water, Sediment, and Biota of Fresh-Water Marinas. Environmental science & technology **25**:956-963.

Fernandez M. P., M. G. Ikonomou, and I. Buchanan. 2007. An assessment of estrogenic organic contaminants in Canadian wastewaters. Science of the Total Environment **373**:250-269.

Fernandez P., G. Carrera, and J. O. Grimalt. 2005. Persistent organic pollutants in remote freshwater ecosystems. Aquatic Sciences **67**:263-273.

Filby A. L., E. M. Santos, K. L. Thorpe, G. Maack, and C. R. Tyler. 2007a. Gene expression profiling for understanding chemical causation of biological effects for complex mixtures: A case study on Estrogens. Environmental science & technology **41**:8187-8194.

Filby A. L., T. Neuparth, K. L. Thorpe, R. Owen, T. S. Galloway, and C. R. Tyler. 2007b. Health impacts of estrogens in the environment, considering complex mixture effects. Environmental health perspectives **115**:1704-1710.

Fong P. P., R. Deguchi, and K. Kyozuka. 1996. Serotonergic ligands induce spawning but not oocyte maturation in the bivalve Mactra chinensis from central Japan. Biological Bulletin **191**:27-32.

Forbes V. E., J. Aufderheide, R. Warbritton, N. van der Hoeven, and N. Caspers. 2007a. Does bisphenol A induce superfeminization in *Marisa cornuarietis*? Part II: Toxicity test results and requirements for statistical power analyses. Ecotoxicology and Environmental Safety **66**:319-325.

Forbes V. E., H. Selck, A. Palmqvist, J. Aufderheide, R. Warbritton, N. Pounds, R. Thompson, N. van der Hoeven, and N. Caspers. 2007b. Dose bisphenol a induce superfeminization in *Marisa cornuarietis*? Part I: Intra- and Inter-laboratory variability in test endpoints. Ecotoxicology and Environmental Safety **66**:309-318.

Forbes V. E., R. Warbritton, J. Aufderheide, N. Van Der Hoeven, and N. Caspers. 2008. Effects of Bisphenol a on Fecundity, Egg Hatchability, and Juvenile Growth of Marisa Cornuarietis. Environmental Toxicology and Chemistry **27**:2332-2340.

Forsyth D. J., P. A. Martin, K. D. Desmet, and M. E. Riske. 1994. Organochlorine Contaminants and Eggshell Thinning in Grebes from Prairie Canada. Environmental Pollution **85**:51-58.

Fraser L. A. 1946. The embryology og the reproductive tract of *Lymnaeas stagnalis appressa* say. Transactions of American microscopical society **65**:279-298.

Fretter V. 1975. Introduction. Pages xi-xxviii *In* V. Fretter and j. Peake, editors. Pulmonates, Functional Anatomy and Physiology, Academic Press, London.

Fretter V. 1984. Prosobranchs. Pages 1--45 *In* K. M. Wilbur, A. S. Tompa, N. H. Verdonk, and Van den Biggelaar, J. A. M., editors. The Mollusca - Reproduction, Academic Press, Inc, London.

Fretter V., A. Graham. 1962. British Prosobranch Mollusca, Their functional anatomy and ecology. Adlard and sons ltd, Dorking.

Fried B., T. K. Graczyk. 1997. Advances in Trematode Biology. CRC Press, New York.

Fromme H., T. Kuchler, T. Otto, K. Pilz, J. Muller, and A. Wenzel. 2002. Occurrence of phthalates and bisphenol A and F in the environment. Water research **36**:1429-1438.

Fuoco R., M. P. Colombini, and C. Abete. 1994. Determination of Polychlorobiphenyls in Environmental-Samples from Antarctica. International journal of environmental analytical chemistry **55**:15-25.

Fuoco R., M. P. Colombini, C. Abete, and S. Carignani. 1995. Polychlorobiphenyls in sediment, soil and sea water samples from Antarctica. International journal of environmental analytical chemistry **61**:309-318.

Gagne F., C. Blaise. 2003. Effects of municipal effluents on serotonin and dopamine levels in the freshwater mussel Elliptio complanata. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **136**:117-125.

Gagne F., C. Blaise. 2000. Organic alkali-labile phosphates in biological materials: A generic assay to detect vitellogenin in biological tissues. Environmental toxicology **15**:243-247.

Gagne F., M. Fournier, and C. Blaise. 2004a. Serotonergic effects of municipal effluents: Induced spawning activity in freshwater mussels. Fresenius Environmental Bulletin **13**:1099-1103.

Gagne F., C. Blaise, and J. Hellou. 2004b. Endocrine disruption and health effects of caged mussels, Elliptio complanata, placed downstream from a primary-treated municipal effluent plume for 1 year. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **138**:33-44.

Garnerot F., J. Pellerin, C. Blaise, and M. Mathieu. 2006. Immunohistochemical localization of serotonin (5-hydroxytryptamine) in the gonad and digestive gland of Mya arenaria (Mollusca : Bivalvia). General and comparative endocrinology **149**:278-284.

Gauthier-Clerc S., J. Pellerin, M. Fournier, and J. Amiard. 2006. Immunological and biochemical responses in *Mya arenaria* (Mollusca Bivalvia) exposed *in vivo* to estradiol-17B. Comparative Biochemistry and Physiology, part C **144**:228-234.

Gebhardt M., G. Ribi. 1987. Reproductive Effort and Growth in the Prosobranch Snail, Viviparus-Ater. Oecologia **74**:209-214.

Geraerts W. P. M., J. Joosse. 1984. Freshwater snails (Basommatophora). Pages 142-207 *In* K. M. Wilbur, A. S. Tompa, N. H. Verdonk, and Van den Biggelaar, J. A. M., editors. The mollusca, Reproduction, Academic Press. Inc, London.

Geraerts W. P. M., L. H. Algera. 1976. The stimulatory effects of the dorsal body hormone on cell differentiation in the female accessory sex organ of the hermaphrodite freshwater snail *Lymnaea stagnalis*. Gen.Comp.Endocrinol. **29**:109-118.

Geraerts W. P. M., S. Bohlken. 1976. The control of ovulation in the hermaphrodite freshwater snail *Lymnaea stagnalis* by the neurohormone of the caudo-dorsal cells. Gen.Comp.Endocrinol. **28**:350-357.

Geraerts W. P. M., J. Joosse. 1975. Control of Vitellogenesis and of Growth of Female Accessory Sex Organs by Dorsal Body Hormone (Dbh) in Hermaphroditic Freshwater Snail Lymnaea-Stagnalis. General and comparative endocrinology **27**:450-464.

Gerard C., A. Theron. 1997. Age. Oecologia **112**:447-452.

Gercken J., H. Sordyl. 2002. Intersex in feral marine and freshwater fish from northeastern Germany. Marine environmental research **54**:651-655.

Gibbs P. E., G. W. Bryan. 1986. Reproductive failure in populations of the dog-whelk, Nucella lapillus , caused by imposex induced by tributyltin from antifouling paints. Journal of the Marine Biological Association of the United Kingdom **66**:767-777.

Gibson D. I., R. A. Bray. 1994. The Evolutionary Expansion and Host-Parasite Relationships of the Digenea. International journal for parasitology **24**:1213-1226.

Gill-Sharma M. K., S. D'Souza, V. Padwal, N. Balasinor, M. Aleem, P. Parte, and H. S. Juneja. 2001. Antifertility effects of estradiol in adult male rats. Journal of endocrinological investigation **24**:598-607.

Gioiosa L., E. Fissore, G. Ghirardelli, S. Parmigiani, and P. Palanza. 2007. Developmental exposure to low-dose estrogenic endocrine disruptors alters sex differences in exploration and emotional responses in mice. Hormones and behavior **52**:307-316.

Giusti R. M., K. Iwamoto, and E. E. Hatch. 1995. Diethylstilbestrol Revisited - a Review of the Long-Term Health-Effects. Annals of Internal Medicine **122**:778-788.

Goldberg J. I., R. Garofalo, C. J. Price, and J. P. Chang. 1993. Presence and Biological-Activity of a Gnrh-Like Factor in the Nervous-System of Helisoma-Trivolvis. Journal of Comparative Neurology **336**:571-582.

Gomot A. 1998. Toxic effects of cadmium on reproduction, development, and hatching in the freshwater snail Lymnaea stagnalis for water quality monitoring. Ecotoxicology and environmental safety **41**:288-297.

Gooding M. P., G. A. LeBlanc. 2004. Seasonal variation in the regulation of testosterone levels in the eastern mud snail (Ilyanassa obsoleta). Invertebrate Biology **123**:237-243.

Gorbushin A. M. 1997. Field evidence of trematode-induced gigantism in Hydrobia spp. (Gastropoda: Prosobranchia). Journal of the Marine Biological Association of the United Kingdom **77**:785-800.

Gore A. C. 2008. Developmental programming and endocrine disruptor effects on reproductive neuroendocrine systems. Frontiers in neuroendocrinology **29**:358-374.

Green R. E., M. A. Taggart, D. Das, D. J. Pain, C. S. Kumar, A. A. Cunningham, and R. Cuthbert. 2006. Collapse of Asian vulture populations: risk of mortality from residues of the veterinary drug diclofenac in carcasses of treated cattle. Journal of Applied Ecology **43**:949-956.

Griffond B. 1978. Sexualisation de la gonade de *Viviparus viviparus* L. (mollusque gasteropode prosobranche a sexes separes). Rous's Archives of Developmental Biology **184**:213-231.

Grun F., H. Watanabe, Z. Zamanian, L. Maeda, K. Arima, R. Cubacha, D. M. Gardiner, J. Kanno, T. Iguchi, and B. Blumberg. 2006. Endocrine-disrupting organotin compounds are potent inducers of adipogenesis in vertebrates. Molecular Endocrinology **20**:2141-2155.

Guillette L. J., D. A. Crain, M. P. Gunderson, S. A. E. Kools, M. R. Milnes, E. F. Orlando, A. A. Rooney, and A. R. Woodward. 2000. Alligators and endocrine disrupting contaminants: A current perspective. American Zoologist **40**:438-452.

Gunderson M. P., D. S. Bermudez, T. A. Bryan, S. Degala, T. M. Edwards, S. A. E. Kools, M. R. Milnes, A. R. Woodward, and L. J. Guillette. 2004. Variation in sex steroids and phallus size in juvenile American alligators (Alligator mississippiensis) collected from 3 sites within the Kissimmee-Everglades drainage in Florida (USA). Chemosphere **56**:335-345.

Hagger J., A., M. H. Depledge, J. Oehlmann, S. Jobling, and T. Galloway S. 2006. Is there a causal association between Genotoxicity and the Imposex effect? Environmental health perspectives **114**:20-26.

Hall A. J., G. O. Thomas. 2007. Polychlorinated biphenyls, DDT, polybrominated diphenyl ethers, and organic pesticides in United Kingdom harbor seals (Phoca vitulina) - Mixed exposures and thyroid homeostasis. Environmental Toxicology and Chemistry **26**:851-861.

Hall S. R., C. Becker, and C. E. Caceres. 2007. Parasitic castration: a perspective from a model of dynamic energy budgets. Integrative and Comparative Biology **47**:295-309.

Hano T., Y. Oshima, S. G. Kim, H. Satone, Y. Oba, T. Kitano, S. Inoue, Y. Shimasaki, and T. Honjo. 2007. Tributyltin causes abnormal development in embryos of medaka, Oryzias latipes. Chemosphere **69**:927-933.

Hara Y., C. A. Struessmann, and S. Hashimoto. 2007. Assessment of short-term exposure to nonylphenol in Japanese medaka using sperm velocity and frequency of motile sperm. Archives of Environmental Contamination and Toxicology **53**:406-410.

Harino H., M. Fukushima, S. Kawai, and K. Megumi. 1998a. Measurement of butyltin contamination of water and sediment in Osaka Bay, Japan. Applied Organometallic Chemistry **12**:819-825.

Harino H., M. Fukushima, Y. Yamamoto, S. Kawai, and N. Miyazaki. 1998b. Contamination of butyltin and phenyltin compounds in the marine environment of Otsuchi Bay, Japan. Environmental Pollution **101**:209-214.

Harries J. E., D. A. Sheahan, S. Jobling, P. Matthiessen, P. Neall, E. J. Routledge, R. Rycroft, J. P. Sumpter, and T. Tylor. 1996. A survey of estrogenic activity in United Kingdom inland waters. Environmental Toxicology and Chemistry **15**:1993-2002.

Harris P. D., A. Soleng, and T. A. Bakke. 2000. Increased susceptibility of salmonids to the monogenean Gyrodactylus salaris following administration of hydrocortisone acetate. Parasitology **120**:57-64.

Hashimoto S., H. Bessho, A. Hara, M. Nakamura, T. Iguchi, and K. Fujita. 2000. Elevated serum vitellogenin levels and gonadal abnormalities in wild male flounder (Pleuronectes yokohamae) from Tokyo Bay, Japan. Marine environmental research **49**:37-53.

Hassanin A., S. Kuwahara, Nurhidayat, Y. Tsukamoto, K. Ogawa, K. Hiramatsu, and F. Sasaki. 2002. Gonadosomatic index and testis morphology of common carp (Cyprinus carpio) in rivers contaminated with estrogenic chemicals. Journal of Veterinary Medical Science **64**:921-926.

Hatch E. E., R. Troisi, L. A. Wise, M. Hyer, J. R. Palmer, L. Titus-Ernstoff, W. Strohsnitter, R. Kaufman, E. Adam, K. L. Noller, A. L. Herbst, S. Robboy, P. Hartge, and R. N. Hoover. 2006. Age at natural menopause in women exposed to diethylstilbestrol in utero. American Journal of Epidemiology **164**:682-688.

Hayes T., K. Haston, M. Tsui, A. Hoang, C. Haeffele, and A. Vonk. 2003. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (Rana pipiens): Laboratory and field evidence. Environmental health perspectives **111**:568-575.

Hayes T., K. Haston, M. Tsui, A. Hoang, C. Haeffele, and A. Vonk. 2002a. Herbicides: Feminization of male frogs in the wild. Nature **419**:895-896.

Hayes T. B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. A. Stuart, and A. Vonk. 2002b. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Sciences of the United States of America **99**:5476-5480.

Hayes T. B., P. Case, S. Chui, D. Chung, C. Haeffele, K. Haston, M. Lee, V. P. Mai, Y. Marjuoa, J. Parker, and M. Tsui. 2006. Pesticide mixtures, endocrine disruption, and amphibian declines: Are we underestimating the impact? Environmental health perspectives **114**:40-50.

Hebert N., F. Gagne, D. Cyr, J. Pellerin, C. Blaise, and M. Fournier. 2009. EFFECTS OF 4-NONYLPHENOL ON THE IMMUNE SYSTEM OF RAINBOW TROUT (Oncorhynchus mykiss). Fresenius Environmental Bulletin **18**:757-761.

Hecker M., L. Karbe. 2005. Parasitism in fish - an endocrine modulator of ecological relevance? Aquatic Toxicology **72**:195-207.

Heemken O. P., H. Reincke, B. Stachel, and N. Theobald. 2001. The occurrence of xenoestrogens in the Elbe river and the North Sea. Chemosphere **45**:245-259.

Heinz G. H. 1998. Contaminant effects on Great Lakes fish-eating birds: a population perspective. Principles and Processes for Evaluating Endocrine Disruption in Sildlife :141-154.

Henry J., C. Zatylny, and P. Favrel. 2000. HPLC and electrospray ionization mass spectrometry as tools for the identification of APGWamide-related peptides in gastropod and bivalve mollusks: comparative activities on Mytilus muscles. Brain research **862**:162-170.

Henson M. C., P. J. Chedrese. 2004. Endocrine disruption by cadmium, a common environmental toxicant with paradoxical effects on reproduction. Experimental biology and medicine **229**:383-392.

Hewitt L. M., J. L. Parrott, K. L. Wells, M. K. Calp, S. Biddiscombe, M. E. McMaster, K. R. Munkittrick, and G. J. Van der Kraak. 2000. Characteristics of ligands for the Ah receptor and sex steroid receptors in hepatic tissues of fish exposed to bleached kraft mill effluent. Environmental science & technology **34**:4327-4334.

Hewitt L. M., T. G. Kovacs, M. G. Dube, D. L. MacLatchy, P. H. Martel, M. E. McMaster, M. G. Paice, J. L. Parrott, M. R. Van den Heuvel, and G. J. Van der Kraak. 2008. Altered reproduction in fish exposed to pulp and paper mill effluents: Roles of individual compounds and mill operating conditions. Environmental Toxicology and Chemistry **27**:682-697.

Hewitt L. M., M. G. Dube, S. C. Ribey, J. M. Culp, R. Lowell, K. Hedley, B. Kilgour, C. Portt, D. L. MacLatchy, and K. R. Munkittrick. 2005. Investigation of cause in pulp and paper environmental effects monitoring. Water Quality Research Journal of Canada **40**:261-274.

Heyland A., D. A. Price, M. Bodnarova-Buganova, and L. L. Moroz. 2006. Thyroid hormone metabolism and peroxidase function in two non-chordate animals. Journal of Experimental Zoology Part B-Molecular and Developmental Evolution **306B**:551-566.

Hinson J., P. Raven, and C. Shern. 2007. The Endocrine System: Systems of the Body Series. Elsevier Health Sciences, .

Hochberg R. B. 1998. Biological esterification of steroids. Endocrine reviews **19**:331-348.

Hockelmann C., M. Pusch. 2000. The respiration and filter-feeding rates of the snail Viviparus viviparus (Gastropoda) under simulated stream conditions. Archiv Fur Hydrobiologie **149**:553-568.

Hogan N. S., D. R. S. Lean, and V. L. Trudeau. 2006. Exposures to estradiol, ethinylestradiol and octylphenol affect survival and growth of Rana pipiens and Rana sylvatica tadpoles. Journal of Toxicology and Environmental Health-Part A-Current Issues **69**:1555-1569.

Holthaus K. I. E., A. C. Johnson, M. D. Jurgens, R. J. Williams, J. J. L. Smith, and J. E. Carter. 2002. The potential for estradiol and ethinylestradiol to sorb to suspended and bed sediments in some English rivers. Environmental Toxicology and Chemistry **21**:2526-2535.

Hordijk P. L., M. De Jong-Brink, A. Ter maat, A. W. Pieneman, J. C. Lodder, and K. S. Kits. 1992. The Neuropeptide Schistosomin and Hemolymph from Parasitized Snails Induce Similar Changes in Excitability in Neuroendocrine Cells Controlling Reproduction and Growth in a Fresh-Water Snail. Neuroscience letters **136**:193-197.

Hordijk P. L., R. H. M. Ebberink, M. De Jong-Brink, and J. Joosse. 1991a. Isolation of Schistosomin, a Neuropeptide which Antagonizes Gonadotropic-Hormones in a Fresh-Water Snail. European Journal of Biochemistry **195**:131-136.

Hordijk P. L., H. Vanloenhout, R. H. M. Ebberink, M. De Jong-Brink, and J. Joosse. 1991b. Neuropeptide Schistosomin Inhibits Hormonally-Induced Ovulation in the Fresh-Water Snail Lymnaea-Stagnalis. Journal of Experimental Zoology **259**:268-271.

Hordijk P. L., H. D. F. H. Schallig, R. H. M. Ebberink, M. De Jong-Brink, and J. Joosse. 1991c. Primary Structure and Origin of Schistosomin, an Anti-Gonadotropic Neuropeptide of the Pond Snail Lymnaea-Stagnalis. Biochemical Journal **279**:837-842.

Horiguchi T., M. Kojima, T. Matsuo, H. Shiraishi, M. Morita, and Y. Adachi. 2002. Tributyltin and triphenyltin induce spermatogenesis in ovary of female abalone, *Haliotis gigantea*. Marine environmental research **54**:679-684.

Horiguchi T., N. Takiguchi, h. S. Cho, M. Kojima, M. Kaya, H. Shiraishi, M. Morita, H. Hirose, and M. Shimizu. 2000. Ovo-testis and disturbed reproductive cycle in the giant abalone, *Haliotis madaka*: possible linkage with organotin comtamination in a site of population decline. Marine Environmental research **50**:223-229.

Horiguchi T., M. Kojima, N. Takiguchi, M. Kaya, H. Shiraishi, and M. Morita. 2005. Continuing observation of disturbed reporductive cycle and ovarian spermatogenesis in the giant abalone, *Haliotis madaka* from an organotin-contaminated site of japan. Marine pollution bulletin **51**:817-822.

Horiguchi T., T. Nishikawa, Y. Ohta, H. Shiraishi, and M. Morita. 2007. Retinoid X receptor gene expression and protein content in tissues of the rock shell Thais clavigera. Aquatic Toxicology **84**:379-388.

Howe C. M., M. Berrill, B. D. Pauli, C. C. Helbing, K. Werry, and N. Veldhoen. 2004. Toxicity of glyphosate-based pesticides to four North American frog species. Environmental Toxicology and Chemistry **23**:1928-1938.

Howell W. M., D. A. Black, and S. A. Bortone. 1980. Abnormal Expression of Secondary Sex Characters in a Population of Mosquitofish, Gambusia-Affinis-Holbrooki - Evidence for Environmentally-Induced Masculinization. Copeia :676-681.

Hutchinson T. H., N. Shillabeer, M. J. Winter, and D. B. Pickford. 2006. Acute and chronic effects of carrier solvents in aquatic organisms: a critical review. Aquatic toxicology **76**:69-92.

Iguchi T., H. Watanabe, Y. Ohta, and B. Blumberg. 2008. Developmental effects: oestrogen-induced vaginal changes and organotin-induced adipogenesis. International journal of andrology **31**:263-268.

Iguchi T., Y. Katsu, T. Horiguchi, H. Watanabe, B. Blumberg, and Y. Ohta. 2007. Endocrine disrupting organotin compounds are potent inducers of imposex in gastropods and adipogenesis in vertebrates. Molecular & Cellular Toxicology **3**:1-10.

Iida H., K. Maehara, M. Doiguchi, T. Mori, and F. Yamada. 2003. Bisphenol A-induced apoptosis of cultured rat Sertoli cells. Reproductive Toxicology **17**:457-464.

Ike M., C. S. Jin, and M. Fujita. 2000. Biodegradation of bisphenol A in the aquatic environment. Water Science and Technology **42**:31-38.

IUCN. 2007. international union for the conservation of nature and natural resources - red list. **2008**:.

Iwakoshi E., K. Takuwa-Kuroda, Y. Fujisawa, M. Hisada, K. Ukena, K. Tsutsui, and H. Minakata. 2002. Isolation and characterization of a GnRH-like peptide from Octopus vulgaris. Biochemical and biophysical research communications **291**:1187-1193.

Iwakoshi-Ukena E., K. Urena, K. Takuwa-Kuroda, A. Kanda, K. Tsutsui, and H. Minakata. 2004. Expression and distribution of octopus gonadotropin-releasing hormone in the central nervous system and peripheral organs of the octopus (Octopus vulgaris) by in situ hybridization and immunohistochemistry. Journal of Comparative Neurology **477**:310-323.

Jacobson J. L., S. W. Jacobson. 1996. Intellectual impairment in children exposed to polychlorinated biphenyls in utero. New England Journal of Medicine **335**:783-789.

Jakubik B. 2007. Egg number-female body weight relationship in freshwater snail (Viviparus viviparus L.) population in a reservoir. Polish Journal of Ecology **55**:325-336.

Jakubik B. 2006. Reproductive pattern of Viviparus viviparus (Linnaeus 1758) (Gastropoda, Viviparidae) from littoral aggregations in a through-flow reservoir (Central Poland). Polish Journal of Ecology **54**:39-55.

Jakubik B. 2003. Year-to-year stability of aggregations of Viviparus viviparus (Linnaeus 1758) in littoral zone of lowland, rheophilic reservoir (Central Poland). Polish Journal of Ecology **51**:53-66.

Janer G., G. A. LeBlanc, and C. Porte. 2005. A comparative study on androgen metabolism in three invertebrate species. General and comparative endocrinology **143**:211-221.

Janer G., R. Lavado, R. Thibaut, and C. Porte. 2004. Effects of 17B-estradiol exposure in the mussel *Mytilus galloprovincialis.*. Marine environmental research **58**:443-446.

Janer G., C. Porte. 2007. Sex steroids and potential mechanisms of non-genomic endocrine disruption in invertebrates. Ecotoxicology **16**:145-160.

Janer G., R. Lavado, W. Thibaut, and C. Porte. 2005. Effects of 17 beta-estradiol exposure in the mussel Mytilus galloprovincialis: A possible regulating role for steroid acyltransferases. Aquatic Toxicology **75**:32-42.

Janer G., A. Lyssimachou, J. Bachmann, J. Oehlmann, U. Schulte-Oehlmann, and C. Porte. 2006. Sexual dimorphism in esterified steroid levels in the gastropod Marisa cornuarietis: The effect of xenoandrogenic compounds. Steroids **71**:435-444.

Janse C., W. C. Wildering, and C. M. Popelier. 1989. Age-Related-Changes in Female Reproductive Activity and Growth in the Mollusk Lymnaea-Stagnalis. Journals of Gerontology **44**:B148-B155.

Jeney Z., E. T. Valtonen, G. Jeney, and E. M. Jokinen. 2002. Effect of pulp and paper mill effluent (BKME) on physiological parameters of roach (Rutilus rutilus) infected by the digenean Rhipidocotyle fennica. Folia parasitologica **49**:103-108.

Jezewski W. 2004. Occurrence of Digenea (Trematoda) in two Viviparus species from lakes, rivers and a dam reservoir. Helminthologia **41**:147-150.

Jimenez B., G. Mori, M. A. Concejero, R. Merino, S. Casini, and M. C. Fossi. 2007. Vitellogenin and zona radiata proteins as biomarkers of endocrine disruption in peregrine falcon (Falco peregrinus). Chemosphere **67**:S375-S378.

Jobling S., D. Sheahan, A. Osborne, P. Matthiessen, and J. P. Sumpter. 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. Environmental toxicology and chemistry **15**:194-202.

Jobling S., C. R. Tyler. 2003. Endocrine disruption in wild freshwater fish. Pure and Applied Chemistry **75**:2219-2234.

Jobling S., J. P. Sumpter. 1993. Detergent Components in Sewage Effluent are Weakly Estrogenic to Fish - an In-Vitro Study using Rainbow-Trout (Oncorhynchus-Mykiss) Hepatocytes. Aquatic Toxicology **27**:361-372.

Jobling S., M. Nolan, C. R. Tyler, G. C. Brighty, and J. P. Sumpter. 1998. Widespread sexual disruption in wild fish. Environmental science & technology **32**:2498-2506.

Jobling S., T. Reynolds, R. White, M. G. Parker, and J. P. Sumpter. 1995. A Variety of Environmentally Persistent Chemicals, Including some Phthalate Plasticizers, are Weakly Estrogenic. Environmental health perspectives **103**:582-587.

Jobling S., N. Beresford, M. Nolan, T. Rodgers-Gray, G. C. Brighty, J. P. Sumpter, and C. R. Tyler. 2002. Altered sexual maturation and gamete production in wild roach

(Rutilus rutilus) living in rivers that receive treated sewage effluents. Biology of reproduction **66**:272-281.

Jobling S., D. Casey, T. Rodgers-Gray, J. Oehlmann, U. Schulte-Oehlmann, S. Pawlowski, T. Baunbeck, A. P. Turner, and C. R. Tyler. 2004. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent (vol 65, pg 205, 2003). Aquatic Toxicology **66**:205-+.

Jobling S., D. Casey, T. Rodgers-Gray, J. Oehlmann, U. Schulte-Oehlmann, S. Pawlowski, T. Baunbeck, A. P. Turner, and C. R. Tyler. 2003. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. Aquatic Toxicology **65**:205-220.

Jobling S., R. Williams, A. Johnson, A. Taylor, M. Gross-Sorokin, M. Nolan, C. R. Tyler, R. van Aerle, E. Santos, and G. C. Brighty. 2006. Predicted exposures to steroid estrogens in UK rivers correlate with widespread sexual disruption in wild fish populations. Environmental health perspectives **114**:32-39.

Jobling S., S. Coey, J. G. Whitmore, D. E. Kime, K. J. W. Van Look, B. G. McAllister, N. Beresford, A. C. Henshaw, G. C. Brighty, C. R. Tyler, and J. P. Sumpter. 2002. Wild intersex roach (Rutilus rutilus) have reduced fertility. Biology of reproduction **67**:515-524.

Johnsen S. G. 1970. Testicular Biopsy Score Count a Method for Registration of Spermatogenesis in Human Testes Normal Values and Results in 335 Hypo Gonadal Males. Hormones (Basel) **1**:2-25.

Johnson A. C., H. -. Aerni, A. Gerritsen, M. Gibert, W. Giger, K. Hylland, M. Jurgens, T. Nakari, A. Pickering, M. J. -. Suter, A. Svenson, and F. E. Wettstein. 2005. Comparing steroid estrogen, and nonylphenol content across a range of European sewage plants with different treatment and management practices. Water Research **39**:47-58.

Johnson A. C., C. White, T. J. Besien, and M. D. Jurgens. 1998. The sorption potential of octylphenol, a xenobiotic oestrogen, to suspended and bed-sediments collected from industrial and rural reaches of three English rivers. Science of the Total Environment **210**:271-282.

Jontell M., C. T. Hanks, J. Bratel, and G. Bergenholtz. 1995. Effects of Unpolymerized Resin Components on the Function of Accessory Cells Derived from the Rat Incisor Pulp. Journal of dental research **74**:1162-1167.

R. Reinboth, editor. Structural and Endocrinological Aspects of Hermaphroditism in Pulmonate Snails, with Particular Reference to *Lymnaea stagnalis* (L.). Intersexuality in the Animal Kingdom; 1975. 158 p.

Joosse J. 1964. Dorsal bodies and dorsal neurosecretory cells of the cerebral ganglia of *Lymnaea stagnalis* L. Arch. Neerl. Zool. **15**:1-103.

Joosse J., C. J. Veld. 1972. Endocrinology of reproduction in the hermaphrodite gastropod *Lymnaea stagnalis*. Gen.Comp.Endocrinol. **18**:599-600.

Joosse J., D. Reitz. 1969. Functional anatomical aspects of the ovotestis of *Lymnaea stagnalis*. Malacologia **9**:101-109.

Joosse J. 1984. Photoperiodicity, Rhythmicity and Endocrinology of Reproduction in the Snail Lymnaea-Stagnalis. Ciba Foundation Symposia **104**:204-217.

Joosse J., R. Vanelk, S. Mosselman, H. Wortelboer, and J. C. E. Vandiepen. 1988. Schistosomin - a Pronase-Sensitive Agent in the Hemolymph of Trichobilharzia-Ocellata-Infected Lymnaea-Stagnalis Inhibits the Activity of Albumin Glands Invitro. Parasitology research **74**:228-234.

Jordaens K., L. Dillen, and T. Backeljau. 2007. Effects of mating, breeding system and parasites on reproduction in hermaphrodites: pulmonate gastropods (Mollusca). Animal Biology **57**:137-195.

Jurgens M. D., K. I. E. Holthaus, A. C. Johnson, J. J. L. Smith, M. Hetheridge, and R. J. Williams. 2002. The potential for estradiol and ethinylestradiol degradation in English rivers. Environmental Toxicology and Chemistry **21**:480-488.

Kajiwara M., S. Kuraku, T. Kurokawa, K. Kato, S. Toda, H. Hirose, S. Takahashi, Y. Shibata, T. Iguchi, T. Matsumoto, T. Miyata, T. Miura, and Y. Takahashi. 2006. Tissue preferential expression of estrogen receptor gene in the marine snail, *Thais clavigera*. General and comparative endocrinology **148**:315-326.

Kang J., F. Kondo. 2006. Bisphenol A in the surface water and Freshwater Snail collected from Rivers around a secure landfill. Environmental contamination and technology **76**:113-118.

Kang J. H., F. Kondo. 2002a. Bisphenol a degradation by bacteria isolated from river water. Archives of Environmental Contamination and Toxicology **43**:265-269.

Kang J. H., F. Kondo. 2002b. Effects of bacterial counts and temperature on the biodegradation of bisphenol A in river water. Chemosphere **49**:493-498.

Kannan K., E. Perrotta, N. J. Thomas, and K. M. Aldous. 2007. A comparative analysis of polybrominated diphenyl ethers and polychlorinated biphenyls in southern sea otters that died of infectious diseases and noninfectious causes. Archives of Environmental Contamination and Toxicology **53**:293-302.

Karels A., A. Oikari. 2000. Effects of pulp and paper mill effluents on the reproductive and physiological status of perch (Perca fluviatilis L.) and roach (Rutilus rutilus L.) during the spawning period. Annales Zoologici Fennici **37**:65-77.

Karels A. E., M. Soimasuo, J. Lappivaara, H. Leppanen, T. Aaltonen, P. Mellanen, and A. O. J. Oikari. 1998. Effects of ECF-bleached kraft mill effluent on reproductive steroids and liver MFO activity in populations of perch and roach. Ecotoxicology **7**:123-132.

Kavanagh R. J., G. C. Balch, Y. Kiparissis, A. J. Niimi, J. Sherry, C. Tinson, and C. D. Metcalfe. 2004. Endocrine disruption and altered gonadal development in white perch (Morone americana) from the lower Great Lakes region. Environmental health perspectives **112**:898-902.

Kavlock R. J., G. P. Daston, C. DeRosa, P. FennerCrisp, L. E. Gray, S. Kaattari, G. Lucier, M. Luster, M. J. Mac, C. Maczka, R. Miller, J. Moore, R. Rolland, G. Scott, D. M. Sheehan, T. Sinks, and H. A. Tilson. 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the US EPA-sponsored workshop. Environmental health perspectives **104**:715-740.

Kawana R., C. A. Strussmann, and S. Hashimoto. 2003. Effect of p-Nonylphenol on sperm motility in Japanese medaka (Oryzias latipes). Fish Physiology and Biochemistry **28**:213-214.

Keas B. E., G. W. Esch. 1997. The effect of diet and reproductive maturity on the growth and reproduction of Helisoma anceps (Pulmonata) infected by Halipegus occidualis (Trematoda). Journal of Parasitology **83**:96-104.

Keay J., J. T. Bridgham, and J. W. Thornton. 2006. The Octopus vulgaris estrogen receptor is a constitutive transcriptional activator: Evolutionary and functional implications. Endocrinology **147**:3861-3869.

Keller G., G. Ribi. 1993. Fish Predation and Offspring Survival in the Prosobranch Snail Viviparus-Ater. Oecologia **93**:493-500.

Kerney M. 1999. Atlas of the land and freshwater Molluscs of Britain and Ireland. Harley Books, Colchester, England.

Ketata I., X. Denier, A. Hamza-Chaffai, and C. Minier. 2008. Endocrine-related reproductive effects in molluscs. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **147**:261-270.

Kidd K. A., P. J. Blanchfield, K. H. Mills, V. P. Palace, R. E. Evans, J. M. Lazorchak, and R. W. Flick. 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proceedings of the National Academy of Sciences of the United States of America **104**:8897-8901.

Kiesecker J. M. 2002. Synergism between trematode infection and pesticide exposure: A link to amphibian limb deformities in nature? Proceedings of the National Academy of Sciences of the United States of America **99**:9900-9904.

Kinnberg K., B. Korsgaard, and P. Bjerregaard. 2003. Effects of octylphenol and 17 beta-estradiol on the gonads of guppies (Poecilia reticulata) exposed as adults via the water or as embryos via the mother. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **134**:45-55.

Kitana N., I. P. Callard. 2008. Effect of cadmium on gonadal development in freshwater turtle (Trachemys scripta, Chrysemys picta) embryos. Journal of Environmental Science and Health Part A-Toxic/hazardous Substances & Environmental Engineering **43**:262-271.

Knorr S., T. Braunbeck. 2002. Decline in reproductive success, sex reversal, and developmental alterations in Japanese medaka (Oryzias latipes) after continuous exposure to octylphenol. Ecotoxicology and environmental safety **51**:187-196.

Kraak M. H. S., D. Lavy, W. H. M. Peeters, and C. Davids. 1992. Chronic Ecotoxicity of Copper and Cadmium to the Zebra Mussel Dreissena-Polymorpha. Archives of Environmental Contamination and Toxicology **23**:363-369.

Krist A. C. 2001. Variation in fecundity among populations of snails is predicted by prevalence of castrating parasites. Evolutionary Ecology Research **3**:191-197.

Krist A. C., C. M. Lively. 1998. Experimental exposure of juvenile snails (Potamopyrgus antipodarum) to infection by trematode larvae (Microphallus sp.): infectivity, fecundity compensation and growth. Oecologia **116**:575-582.

Kruuk H., J. W. H. Conroy. 1996. Concentrations of some organochlorines in otters (Lutra lutra L) in Scotland: Implications for populations. Environmental Pollution **92**:165-171.

Kuch H. M., K. Ballschmiter. 2001. Determination of endocrine-disrupting phenolic compounds and estrogens in surface drinking water by HRGC-(NCI)-MS in the picogram per liter range. Environmental science and technology **35**:3201-3206.

Kumar R., E. B. Thompson. 1999. The structure of the nuclear hormone receptors. Steroids **64**:310-319.

Kumar R., B. H. Johnson, and E. B. Thompson. 2004. Overview of the structural basis for transcription regulation by nuclear hormone receptors. Essays in Biochemistry: Nuclear Receptor Superfamily **40**:27-39.

Kupferma.i. 1972. Studies on Neurosecretory Control of Egg-Laying in Aplysia. American Zoologist **12**:513-&.

Kupferma.i. 1970. Stimulation of Egg Laying by Extracts of Neuroendocrine Cells (Bag Cells) of Abdominal Ganglion of Aplysia. Journal of neurophysiology **33**:877-&.

Kwak H. I., M. O. Bae, M. H. Lee, Y. S. Lee, B. J. Lee, K. S. Kang, C. H. Chae, H. J. Sung, J. S. Shin, J. H. Kim, W. C. Mar, Y. Y. Sheen, and M. H. Cho. 2001. Effects of nonylphenol, bisphenol A, and their mixture on the viviparous swordtail fish (Xiphophorus helleri). Environmental Toxicology and Chemistry **20**:787-795.

Lacroix A., A. Hontela. 2006. Role of calcium channels in cadmium-induced disruption of cortisol synthesis in rainbow trout (Oncorhynchus mykiss). Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **144**:141-147.

Lagadic L., M. Coutellec, and T. Caquet. 2007. Endocrine disruption in aquatic pulmonate molluscs: few evidences, many challenges. Ecotoxicology **16**:45-59.

AnonymousInfluence of the complexity of aquatic test system on the fate of vinclozolin and its reprotoxic effects in *Lymnaea stagnalis*. ; 22-26 May 2005; ; 2005. 105 p.

Lagana A., A. Bacaloni, I. De Leva, A. Faberi, G. Fago, and A. Marino. 2004. Analytical methodologies for determining the occurance of endocrine disrupting chemcials in sewage treatment plants and natural waters. Analytica chimica acta **501**:79-88.

Lahnsteiner F., B. Berger, A. Kletzl, and T. Weismann. 2006. Effect of 17 beta-estradiol on gamete quality and maturation in two salmonid species. Aquatic Toxicology **79**:124-131.

Lahnsteiner F., B. Berger, M. Kletzl, and T. Weismann. 2005. Effect of bisphenol A on maturation and quality of semen and eggs in the brown trout, Salmo trutta f. fario. Aquatic Toxicology **75**:213-224.

Lalah J. O., G. F. Severin, K. -. Schramm, D. Lenoir, A. Behechti, K. Guenther, and A. Kettrup. 2007. Effects of a Branche *p*-Nonlyphenol Isomer (4(3',6'-dimethyl-3'-hepyl0-

phenol0 on embryogenesis in *Lymnaea stagnalis* L. Archives of Environmental contamination and toxicology **52**:104-112.

Landman M. J., S. Taylor, and M. R. van den Heuvel. 2008. Carotenoid depletion and ovarian dysfunction in common bully exposed to a New Zealand pulp mill effluent. Aquatic Toxicology **87**:303-309.

Lang I. A., T. S. Galloway, A. Scarlett, W. E. Henley, M. H. Depledge, R. B. Wallace, and D. Melzer. 2008. Association of urinary bisphenol A concentration with medical disorders and laboratory abnormalities in adults. Jama-Journal of the American Medical Association **300**:1303-1310.

Langand J., S. Morand. 1998. Heritable non-susceptibility in an allopatric host-parasite system: Biomphalaria glabrata (Mollusca) Echinostoma caproni (Platyhelminth digenea). Journal of Parasitology **84**:739-742.

Langston W. J. 2008. Personal comunication

Langston W. J., G. R. Burt, and B. S. Chesman. 2007. Feminisation of male clams *Scrobicularia plana* from estuaries in Southwest UK and its induction by endocrine-disrupting chemicals. Marine ecological progress series **333**:173-184.

Langston W. J., G. R. Burt, B. S. Chesman, and C. H. Vane. 2005. Partitining, bioavailability and effects of oestrogens and xeno-oestrogens in the aquatic environment. J. Mar. Biol. Ass. U.K. **85**:1-31.

Langston W. J., G. R. Burt, and M. J. Zhou. 1987. Tin and Organotin in Water, Sediments, and Benthic Organisms of Poole Harbor. Marine pollution bulletin **18**:634-639.

Larsson D. G. J., M. Adolfsson-Erici, J. Parkkonen, M. Pettersson, A. H. Berg, P. -. Olsson, and L. Forlin. 1999. Ethinlyoesradiol - an undesired fish contraceptive? Aquatic Toxicology **45**:91-97.

Larsson D. G. J., M. adolfsson-Erici, and P. Thomas. 2006. Characterization of putative ligands for a fish gonadal androgen receptor in a pulp mill effluent. Environmental Toxicology and Chemistry **25**:419-427.

Larsson D. G. J., H. Hallman, and L. Forlin. 2000. More male fish embryos near a pulp mill. Environmental Toxicology and Chemistry **19**:2911-2917.

Lavado R., M. Sugni, M. D. C. Carrievali, and C. Porte. 2006a. Triphenyltin alters androgen metabolism in the sea urchin Paracentrotus lividus. Aquatic Toxicology **79**:247-256.

Lavado R., A. Barbaglio, M. D. C. Carnevali, and C. Porte. 2006b. Steroid levels in crinoid echinoderms are altered by exposure to model endocrine disruptors. Steroids **71**:489-497.

LeBlanc G., A., M. Goodin P., and M. Sternberg. 2005. Testosterone-Fatty Acid Esterification: A unique target for endocrine toxicity of Tributyltin to Gastropods. Intergrated Comparative Biology **45**:81-87.

LeBlanc G. A. 2007. Crustacean endocrine toxicology: a review. Ecotoxicology **16**:61-81.

LeBlanc G. A., P. M. Campbell, P. den Besten, R. P. Brown, E. S. Chang, J. R. Coats, P. L. deFur, T. Dhadialla, J. Edwards, L. M. Riddiford, M. G. Simpson, T. W. Snell, M. Thorndyke, and F. Matsumura. 1999. The endocrinology of invertebrates. Endocrine Disruption in Invertebrates: Endocrinology, Testing, and Assessment :23-106.

Lee H. B., T. E. Peart. 2000. Bisphenol A contamination in Canadian municipal and industrial wastewater and sludge samples. Water Quality Research Journal of Canada **35**:283-298.

Leiva-Presa A., B. M. Jenssen. 2006. Effects of p,p '-DDE on retinoid homeostasis and sex hormones of adult male European common frogs (Rana temporaria). Journal of Toxicology and Environmental Health-Part A-Current Issues **69**:2051-2062.

Leiva-Presa A., A. S. Mortensen, A. Arukwe, and B. M. Jenssen. 2006. Altered hepatic retinol and CYP26 levels in adult European common frogs (Rana temporaria) exposed to p,p '-DDE. Marine environmental research **62**:S10-S15.

Lemarchand C., C. Amblard, Y. Souchon, and P. Berny. 2007. Organochlorine compounds (pesticides and PCBs) in scats of the european otter (Lutra lutra) from an actual expanding population in central france. Water Air and Soil Pollution **186**:55-62.

Leung K. M. Y., N. J. Morley, E. P. M. Grist, D. Morritt, and M. Crane. 2004. Chronic toxicity of tributyltin on developmet and reproduction of hermaphroditic snail *Physa fontinalis*: Influence of population density. Marine environmental research **58**:157-162.

Li S. F., R. Hansman, R. Newbold, B. Davis, J. A. McLachlan, and J. C. Barrett. 2003. Neonatal diethylstilbestrol exposure induces persistent elevation of c-fos expression and hypomethylation in its exon-4 in mouse uterus. Molecular carcinogenesis **38**:78-84.

Lietti E., M. G. Marin, V. Matozzo, S. Polesello, and S. Valsecchi. 2006. Bioaccumulation of the estrogenic compound 4-nonylphenol in the clam Tapes philippinarum. Marine environmental research **62**:S231-S232.

Lind P. M., M. R. Milnes, R. Lundberg, D. S. Bermudez, J. Orberg, and L. J. Guillette. 2004. Abnormal bone composition in female juvenile American alligators from a pesticide-polluted lake (Lake Apopka, Florida). Environmental health perspectives **112**:359-362.

Lindholst C., K. L. Pedersen, and S. N. Pedersen. 2000. Estrogenic response of bisphenol A in rainbow trout (Oncorhynchus mykiss). Aquatic Toxicology **48**:87-94.

Liney K. E., S. Jobling, J. A. Shears, P. Simpson, and C. R. Tyler. 2005. Assessing the sensitivity of different life stages for sexual disruption in roach (Rutilus rutilus) exposed to effluents from wastewater treatment works. Environmental health perspectives **113**:1299-1307.

Liney K. E., J. A. Hagger, C. R. Tyler, M. H. Depledge, T. S. Galloway, and S. Jobling. 2006. Health effects in fish of long-term exposure to effluents from wastewater treatment works. Environmental health perspectives **114**:81-89.

Lippai M., I. Gobet, M. Tomkowiak, Y. Durocher, C. Leclerc, M. Moreau, and P. Guerrier. 1995. Thimerosal Triggers Meiosis Reinitiation in Oocytes of the Japanese Clam

Ruditapes-Philippinarum by Eliciting an Intracellular Ca2+ Surge. International Journal of Developmental Biology **39**:401-407.

Liu R., J. L. Zhou, and A. Wilding. 2004. Simultaneous determination of endocrine disrupting phenolic compounds and steroids in water by solid-phase extraction-gas chromatography-mass spectrometry. Journal of Chromatography A **10022**:179-189.

Loos R., J. Wollgast, J. Castro-Jimenez, G. Mariani, T. Huber, G. Locoro, G. Hanke, G. Umlauf, G. Bidoglio, P. Hohenblum, W. Moche, S. Weiss, H. Schmid, F. Leiendecker, T. Ternes, A. Navarro Ortega, A. Hildebrandt, D. Barcelo, P. Lepom, I. Dimitrova, O. Nitcheva, S. Polesello, S. Valsecchi, S. Boutrup, O. Sortkjaer, R. de Boer, and J. Staeb. 2008. Laboratory intercomparison study for the analysis of nonylphenol and octylphenol in river water. Trac-Trends in Analytical Chemistry **27**:89-95.

Lubet P., M. Mathieu. 1990. Endocrine Regulation in Bivalve Mollusks. Annee Biologique **29**:235-252.

Lundholm C. E. 1997. DDE-induced eggshell thinning in birds: Effects of p,p'-DDE on the calcium and prostaglandin metabolism of the eggshell gland. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **118**:113-128.

Lye C. M., C. L. J. Frid, M. E. Gill, D. W. Cooper, and D. M. Jones. 1999. Estrogenic alkylphenols in fresh tissue, sedimeets, and water from the UK Tyne and Tees estuaries. Environmental science and technology **33**:1009-1014.

Lye C. M., M. G. Bentley, and T. Galloway. 2008. Effects of 4-nonylphenol on the endocrine system of the shore crab, Carcinus maenas. Environmental toxicology **23**:309-318.

Lye C. M., M. G. Bentley, A. S. Clare, and E. M. Sefton. 2005. Endocrine disruption in the shore crab Carcinus maenas - a biomarker for benthic marine invertebrates? Marine Ecology-Progress Series **288**:221-232.

Lye C. M., C. L. J. Frid, M. E. Gill, and D. McCormick. 1997. Abnormalities in the reproductive health of flounder Platichthys flesus exposed to effluent from a sewage treatment works. Marine pollution bulletin **34**:34-41.

Maffini M. V., B. S. Rubin, C. Sonnenschein, and A. M. Soto. 2006. Endocrine disruptors and reproductive health: The case of bisphenol-A. Molecular and cellular endocrinology **254**:179-186.

Mandich A., Bottero S., Benfenati E., Cavasco A., Erratico C., Maggioni S., Massari A., Pedemonte F., Vigano. L. 2007. In vivo exposure of carp to graded concentrations of bisphenol A. General and Comparative Endocrinology; 23rd Biennial Conference of European Comparative Endocrinologists. **153** (1-3) 15-24 .

Manger P., J. Y. Li, B. M. Christensen, and T. P. Yoshino. 1996. Biogenic monoamines in the freshwater snail, Biomphalaria glabrata: Influence of infection by the human blood fluke, Schistosoma mansoni. Comparative Biochemistry and Physiology A-Physiology **114**:227-234.

Mapara S., S. Parries, C. Quarrington, K. C. Ahn, W. J. Gallin, and J. I. Goldberg. 2008. Identification, molecular structure and expression of two cloned serotonin receptors from the pond snail, Helisoma trivolvis. Journal of Experimental Biology **211**:900-910.

Martinez G., A. Z. Olivares, and L. Mettifogo. 2000. In vitro effects of monoamines and prostaglandins on meiosis reinitiation and oocyte release in Argopecten purpuratus Lamarck. Invertebrate Reproduction & Development **38**:61-69.

Martin-Skilton R., R. Lavado, R. Thibaut, C. Minier, and C. Porte. 2006. Evidence of endocrine alteration in the red mullet, Mullus barbatus from the NW Mediterranean. Environmental Pollution **141**:60-68.

Mason C. F., S. M. Macdonald. 2004. Growth in otter (Lutra lutra) populations in the UK as shown by long-term monitoring. Ambio **33**:148-152.

Mason C. F., S. M. Macdonald. 1994. Pcbs and Organochlorine Pesticide-Residues in Otters (Lutra-Lutra) and in Otter-Spraints from Southwest England and their Likely Impact on Populations. Science of the Total Environment **144**:305-312.

Mason C. F., A. B. Madsen. 1993. Organochlorine Pesticide-Residues and Pcbs in Danish Otters (Lutra-Lutra). Science of the Total Environment **133**:73-81.

Matozzo V., M. G. Marin. 2005. Can 4-nonylphenol induce vitellogenin-like proteins in the clam *Tapes philippinarum*? Environmental research **97**:43-49.

Matsumoto T., A. M. Nakamura, K. Mori, I. Akiyama, H. Hirose, and Y. Takahashi. 2007. Oyster estrogen receptor: cDNA cloning and immunolocalization. General and comparative endocrinology **151**:195-201.

Matsumoto T., M. Osada, Y. Osawa, and K. Mori. 1997. Gonadal estrogen profile and immunohistochemical localization of steroidogenic enzymes in the oyster and scallop during sexual maturation. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology **118**:811-817.

Matsuoka S., M. Kikuchi, S. Kimura, Y. Kurokawa, and S. Kawai. 2005. Determination of estrogenic substances in the water of Muko River using in vitro assays, and the degradation of natural estrogens by aquatic bacteria. Journal of Health Science **51**:178-184.

Matthiessen P., D. Arnold, A. C. Johnson, T. J. Pepper, T. G. Pottinger, and K. G. T. Pulman. 2006. Contamination of headwater streams in the United Kingdom by oestrogenic hormones from livestock farms. Science of the Total Environment **367**:616-630.

Matthiessen P., R. Waldock, J. E. Thain, M. E. Waite, and S. Scropehowe. 1995. Changes in Periwinkle (Littorina-Littorea) Populations Following the Ban on Tbt-Based Antifoulings on Small Boats in the United-Kingdom. Ecotoxicology and environmental safety **30**:180-194.

McAllister B. G., D. E. Kime. 2003. Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (Danio rerio). Aquatic Toxicology **65**:309-316.

McVey M. J., G. M. Cooke. 2003. Inhibition of rat testis microsomal 3 beta-hydroxysteroid dehydrogenase activity by tributyltin. Journal of Steroid Biochemistry and Molecular Biology **86**:99-105.

Medesani D. A., L. Susana, L. Greco, and E. M. Rodriguez. 2004. Interference of cadmium and copper with the endocrine control of ovarian growth, in the estuarine crab Chasmagnathus granulata. Aquatic Toxicology **69**:165-174.

Miles-Richardson S. R., V. J. Kramer, S. D. Fitzgerald, J. A. Render, B. Yamini, S. J. Barbee, and J. P. Giesy. 1999a. Effects of waterborne exposure of 17 beta-estradiol on secondary sex characteristics and gonads of fathead minnows (Pimephales promelas). Aquatic Toxicology **47**:129-145.

Miles-Richardson S. R., S. L. Pierens, K. M. Nichols, V. J. Kramer, E. M. Snyder, S. A. Snyder, J. A. Render, S. D. Fitzgerald, and J. P. Giesy. 1999b. Effects of waterborne exposure to 4-nonylphenol and nonylphenol ethoxylate on secondary sex characteristics and gonads of fathead minnows (Pimephales promelas). Environmental research **80**:S122-S137.

Miller M. R., A. White, and M. Boots. 2006. The evolution of parasites in response to tolerance in their hosts: The good, the bad, and apparent commensalism. Evolution **60**:945-956.

Minier C., G. Caltot, F. Leboulanger, and E. M. Hill. 2000. An investigation of the incidence of intersex fish in Seine-Maritime and Sussex regions. Analusis **28**:801-806.

Moore M., N., D. Lowe M., and P. Fieth E. M. 1978. Responses of Lysosomes in the digestive cells of the common mussel, *Mytilus edulis*, to sex steriods and cortisol. cell and tissue research **188**:1-9.

Mouritsen K. N., K. T. Jensen. 1994. The Enigma of Gigantism - Effect of Larval Trematodes on Growth, Fecundity, Egestion and Locomotion in Hydrobia-Ulvae (Pennant) (Gastropoda, Prosobranchia). Journal of experimental marine biology and ecology **181**:53-66.

Mukai S. T., L. Kiehn, and A. S. M. Saleuddin. 2004. Dopamine stimulates snail albumen gland glycoprotein secretion through the activation of a D1-like receptor. Journal of Experimental Biology **207**:2507-2518.

Munkittrick K. R., M. E. McMaster, L. H. McCarthy, M. R. Servos, and G. J. Van Der Kraak. 1998. An overview of recent studies on the potential of pulp-mill effluents to alter reproductive parameters in fish. Journal of Toxicology and Environmental Health-Part B-Critical Reviews **1**:347-371.

Munoz-Antoli C., A. Marin, R. Toledo, and J. G. Esteban. 2007. Effect of Echinostoma friedi (Trematoda : Echinostomatidae) experimental infection on longevity, growth and fecundity of juvenile Radix peregra (Gastropoda : Lymnaeidae) and Biomphalaria glabrata (Gastropoda : Planorbidae) snails. Parasitology research **101**:1663-1670.

Murai R., S. Takahashi, S. Tanabe, and I. Takeuchi. 2005. Status of butyltin pollution along the coasts of western Japan in 2001, 11 years after partial restrictions on the usage of tributyltin. Marine pollution bulletin **51**:940-949.

Murphy M. B., M. Hecker, K. K. Coady, A. R. Tompsett, E. B. Higley, P. D. Jones, L. H. Du Preez, K. R. Solomon, J. A. Carr, E. E. Smith, R. J. Kendall, G. Van Der Kraak, and J. P. Giesy. 2006. Plasma steroid hormone concentrations, aromatase activities and GSI in ranid frogs collected from agricultural and non-agricultural sites in Michigan (USA). Aquatic Toxicology **77**:153-166.

Muschamp J. W., P. P. Fong. 2001. Effects of the serotonin receptor ligand methiothepin on reproductive behavior of the freshwater snail Biomphalaria glabrata: Reduction of egg laying and induction of penile erection. Journal of Experimental Zoology **289**:202-207.

Nadal A., Alonso-Magdalena P., Soriano S., Quesada I., Ropero A. B. 2009. The pancreatic beta-cell as a target of estrogens and xenoestrogens: Implications for blood glucose homeostasis and diabetes. Molecular and Cellular Endocrinology; 16th European Congress on Obesity. MAY 25. 63-68.

NairMenon J. U., G. T. Campbell, and C. A. Blake. 1996. Toxic effects of octylphenol on cultured rat and murine splenocytes. Toxicology and applied pharmacology **139**:437-444.

Nakada N., H. Nyunoya, M. Nakamura, A. Hara, T. Iguchi, and H. Takada. 2004. Identification of estrogenic compounds in wastewater effluent. Environmental toxicology and chemistry **23**:2807-2815.

Nakamura S., M. Osada, and A. Kijima. 2007. Involvement of GnRH neuron in the spermatogonial proliferation of the scallop, Patinopecten yessoensiss. Molecular reproduction and development **74**:108-115.

Nakanishi T. 2008. Endocrine disruption induced by organotin compounds; organotins function as a powerful agonist for nuclear receptors rather than an aromatase inhibitor. Journal of Toxicological Sciences **33**:269-276.

Nakanishi T. 2007. Potential toxicity of organotin compounds via nuclear receptor signaling in mammals. Journal of Health Science **53**:1-9.

Nakata H., K. Kannan, L. Jing, N. Thomas, S. Tanabe, and J. P. Giesy. 1998. Accumulation pattern of organochlorine pesticides and polychlorinated biphenyls in southern sea otters (Enhydra lutris nereis) found stranded along coastal California, USA. Environmental Pollution **103**:45-53.

Negovetich N. J., G. W. Esch. 2008. Life history cost of trematode infection in Helisoma anceps using mark-recapture in Charlie's Pond. Journal of Parasitology **94**:314-325.

Nentwig G. 2007. Effects of pharmaceuticals on aquatic invertebrates. Part II: The antidepressant drug fluoxetine. Archives of Environmental Contamination and Toxicology **52**:163-170.

Newbold R. R. 2004. Lessons learned from perinatal exposure to diethylstilbestrol. Toxicology and applied pharmacology **199**:142-150.

Newbold R. R., E. Padilla-Banks, and W. N. Jefferson. 2006. Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. Endocrinology **147**:S11-S17.

Newbold R. R., Padilla-Banks E., Jefferson W. N., Heindel J. J. 2008 Effects of endocrine disruptors on obesity. International Journal of Andrology; 4th Copenhagen Workshop on Endocrine Disrupters; MAY 28-31, 2007; APR; 2008. 201-208.

Newton I., I. Wyllie. 1992. Recovery of a Sparrowhawk Population in Relation to Declining Pesticide Contamination. Journal of Applied Ecology **29**:476-484.

Nice H. E. 2005. Sperm motility in the pacific oyster (*Crassostrea gigas*) is affected by nonylphenol. Marine Pollution Bulettin **50**:1668-1674.

Nice H. E., D. Morritt, M. Crane, and M. Thorndyke. 2003. Long-term and transgenerational effects of nonylphenol exposure at a key stage in the development of Crassostrea gigas. Possible endocrine disruption? Marine Ecology-Progress Series **256**:293-300.

Nikitina E. N. 1986. Trematode Larvae in Snails of Lake Glubokoe. Hydrobiologia **141**:139-141.

Nishikawa J. 2006. Imposex in marine gastropods may be caused by binding of organotins to retinoid X receptor. Marine Biology **149**:117-124.

Nishikawa J., S. Mamiya, T. Kanayama, T. Nishikawa, F. Shiraishi, and T. Horiguchi. 2004. Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. Environmental science & technology **38**:6271-6276.

Nolan M., S. Jobling, G. C. Brighty, J. P. Sumpter, and C. R. Tyler. 2001. A histological description of intersexuality in the roach. Journal of fish biology **58**:160-176.

Oberdorster E. 2001. Peptide Hormones Versus Steroid Hormones: Case studies from Snail and Turtle Populations. Annals of the New York academy of science **948**:75-79.

Oberdorster E., P. McClellan-Green. 2002. Mechanisms of imposex induction in the mud snail, Ilyanassa obsoleta: TBT as a neurotoxin and aromatase inhibitor. Marine environmental research **54**:715-718.

Oberdorster E., P. McClellan-Green. 2000. The neuropeptide APGWamide induces imposer in the mud snail, Ilyanassa obsoleta. Peptides **21**:1323-1330.

Oberdorster E., J. Romano, and P. McClellan-Green. 2005. The neuropeptide APGWamide as a penis morphogenic factor (PMF) in gastropod mollusks. Integrative and Comparative Biology **45**:28-32.

Oehlmann J., U. Schulte-Oehlmann, M. Tillmann, and B. Markert. 2000. Effects of Endocrine Disruptors on Prosobranch Snails (Mollusca: Gastropoda) in the Labortaory. Part I: Bisphenol A and Octylphenol as Xeno-Estrogens. Ecotoxicology **9**:383-397.

Oehlmann J., U. Schulte-Oehimann, J. Bachmann, M. Oetken, I. Lutz, W. Kloas, and T. A. Ternes. 2006. Bisphenol A induces superfeminization in the ramshorn snail Marisa cornuarietis (Gastropoda : Prosobranchia) at environmentally relevant concentrations. Environmental health perspectives **114**:127-133.

Ohkimoto K., Y. Sakakibara, M. Suiko, H. Yoshikawa, M. C. Liu, and H. Tamura. 2005. Biocides, tributyltin and triphenyltin, as possible inhibitors of the human sulfotransferase involved in the estrogen homeostasis. Pesticide biochemistry and physiology **81**:32-38.

Olmstead A. W., G. A. LeBlanc. 2007. The environmental-endocrine basis of gynandromorphism (intersex) in a crustacean. International Journal of Biological Sciences **3**:77-84.

Oppliger A., D. J. Hosken, and G. Ribi. 1998. Snail sperm production characteristics vary with sperm competition risk. Proceedings of the Royal Society of London Series B-Biological Sciences **265**:1527-1534.

Oppliger A., Y. Naciri-Graven, G. Ribi, and D. J. Hosken. 2003. Sperm length influences fertilization success during sperm competition in the snail Viviparus ater. Molecular ecology **12**:485-492.

Orn S., A. Svenson, T. Viktor, H. Holbech, and L. Norrgren. 2006. Male-biased sex ratios and vitellogenin induction in zebrafish exposed to effluent water from a Swedish pulp mill. Archives of Environmental Contamination and Toxicology **51**:445-451.

Ortiz-Zarragoitia M., M. P. Cajaraville. 2006. Biomarkers of Exposure and Reproduction-Related Effects in Mussels Exposed to Endocrine Disruptors. Environmental Contamination and Toxicology **50**:361-369.

Orton F., J. A. Carr, and R. D. Handy. 2006. Effects of nitrate and atrazine on larval development and sexual differentiation in the northern leopard frog Rana pipiens. Environmental Toxicology and Chemistry **25**:65-71.

Osada M., H. Tawarayama, and K. Mori. 2004. Estrogen synthesis in relation to gonadal development of Japanese scallop, *Patinopecten yessoensis*: gonadal profile and immunolocalization of P450 aromatase and estrogen. Comparative Biochemistry and Physiology, Part B **139**:123-128.

Osada M., T. Takamura, H. Sato, and K. Mori. 2003. Vitellogenin synthesis in the ovary of scallop, Patinopecten yessoensis: Control by estradiol-17 beta and the central nervous system. Journal of Experimental Zoology Part A-Comparative Experimental Biology **299A**:172-179.

Osada M., A. Nakata, T. Matsumoto, and K. Mori. 1998. Pharmacological characterization of serotonin receptor in the oocyte membrane of bivalve molluscs and its formation during oogenesis. Journal of Experimental Zoology **281**:124-131.

Ottaviani E., C. Franceschi. 1996. The neuroimmunology of stress from invertebrates to man. Progress in neurobiology **48**:421-440.

Ottinger M. A., M. A. Abdelnabi. 1997. Neuroendocrine systems and avian sexual differentiation. American Zoologist **37**:514-523.

Ottinger M. A., E. Lauoie, N. Thompson, A. Barton, K. Whitehouse, M. Barton, M. Abdelnabi, M. Quinn Jr., G. Panzica, and C. Viglietti-Panzica. 2008. Neuroendocrine and behavioral effects of embryonic exposure to endocrine disrupting chemicals in birds. Brain Research Reviews **57**:376-385.

Pain D. J., C. G. R. Bowden, A. A. Cunningham, R. Cuthbert, D. Das, M. Gilbert, R. D. Jakati, Y. Jhala, A. A. Khan, V. Naidoo, J. L. Oaks, J. Parry-Jones, V. Prakash, A. Rahman, S. P. Ranade, H. S. Baral, K. R. Senacha, S. Saravanan, N. Shah, G. Swan, D. Swarup, M. A. Taggart, R. T. Watson, M. Z. Virani, K. Wolter, and R. E. Green. 2008. The race to prevent the extinction of South Asian vultures. Bird Conservation International **18**:S30-S48.

Palmer J. R., L. A. Wise, E. E. Hatch, R. Troisi, L. Titus-Ernstoff, W. Strohsnitter, R. Kaufman, A. L. Herbst, K. L. Noller, M. Hyer, and R. N. Hoover. 2006. Prenatal

diethylstilbestrol exposure and risk of breast cancer. Cancer Epidemiology Biomarkers & Prevention **15**:1509-1514.

Pani A. K., R. P. Croll. 2000. Catechol concentrations in the hemolymph of the scallop, Placopecten magellanicus. General and comparative endocrinology **118**:48-56.

Parks L. G., C. S. Lambright, E. F. Orlando, L. J. Guillette, G. T. Ankley, and L. E. Gray. 2001. Masculinization of female mosquitofish in kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. Toxicological Sciences **62**:257-267.

Parrott J. L., M. E. McMaster, and L. M. Hewitt. 2006. A decade of research on the environmental impacts of pulp and paper mill effluents in Canada: Development and application of fish bioassays. Journal of Toxicology and Environmental Health-Part B-Critical Reviews **9**:297-317.

Patisaul H. B., E. K. Polston. 2008. Influence of endocrine active compounds on the developing rodent brain. Brain Research Reviews **57**:352-362.

Patrolecco L., S. Capri, S. De Angelis, S. Polesello, and S. Valsecchi. 2004. Determination of endocrine disrupting chemicals in environmental solid matrices by extraction with a non-ionic surfactant (Tween 80). Journal of Chromatography a **1022**:1-7.

Pauwels B., K. Wille, H. Noppe, H. De Brabander, T. V. de Wiele, W. Verstraete, and N. Boon. 2008. 17 Alpha-Ethinylestradiol Cometabolism by Bacteria Degrading Estrone, 17 Beta-Estradiol and Estriol. Biodegradation **19**:683-693.

Pearson E. J., T. C. Cheng. 1985. Studies on Parasitic Castration - Occurrence of a Gametogenesis-Inhibiting Factor in Extract of Zoogonus-Lasius (Trematoda). Journal of invertebrate pathology **46**:239-246.

Pickford D. B., I. D. Morris. 2003. Inhibition of gonadotropin-induced oviposition and ovarian steroidogenesis in the African clawed frog (Xenopus laevis) by the pesticide methoxychlor. Aquatic Toxicology **62**:179-194.

Pickford D. B., L. J. Guillette, D. A. Crain, A. A. Rooney, and A. R. Woodward. 2000. Plasma dihydrotestosterone concentrations and phallus size in juvenile American alligators (A. mississippiensis) from contaminated and reference populations. Journal of Herpetology **34**:233-239.

Probst S., J. Kube. 1999. Histopathological effects of larval trematode infections in mudsnails and their impact on host growth: what causes gigantism in Hydrobia ventrosa (Gastropoda : Prosobranchia)? Journal of experimental marine biology and ecology **238**:49-68.

Puinean A. M., J. M. Rotchell. 2006. *Vitellogenin* gene experssion as a biomarker of endocrine disruption in the vertebrate, *mytilus edulis*. Marine Environmental Research **62**:S211-S214.

Puinean A. M., P. Labadie, E. M. Hill, M. Osada, M. Kisida, R. Nakao, A. Novillo, I. P. Callard, and J. M. Rotchell. 2006. Laboratory exposure to 17B-estradiol fails to induce vitellogenin and estrogen receptor gene expression in the marine invertebrate *Mytilus edulis*. Aquatic Toxicology **79**:376-383.

Qian J., Q. Bian, L. B. Cui, J. F. Chen, L. Song, and X. R. Wang. 2006. Octylphenol induces apoptosis in cultured rat Sertoli cells. Toxicology letters **166**:178-186.

Quednow K., W. Puttmann. 2008. Endocrine disruptors in freshwater streams of Hesse, Germany: Changes in concentration levels in the time span from 2003 to 2005. Environmental Pollution **152**:476-483.

Quinn B., F. Gagne, C. Blaise, M. Costello, J. G. Wilson, and C. Mothersill. 2006. Evaluation of the lethal and sub-lethal toxicity and potential endocrine disrupting effects of nonylphenol on the zebra mussel (*Dreissena polymorpha*). Comparative Biochemistry and Physiology, part C **142**:118-127.

Ram J. L., C. S. Gallardo, M. L. Ram, and R. P. Croll. 1998. Reproduction-associated immunoreactive peptides in the nervous systems of prosobranch gastropods. Biological Bulletin **195**:308-318.

Ramakrishnan S., N. L. Wayne. 2008. Impact of bisphenol-A on early embryonic development and reproductive maturation. Reproductive Toxicology **25**:177-183.

Rasmussen T. H., B. Korsgaard. 2004. Estrogenic octylphenol affects seminal fluid production and its biochemical composition of eelpout (Zoarces viviparus). Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **139**:1-10.

Rasmussen T. H., S. J. Teh, P. Bjerregaard, and B. Korsgaard. 2005. Anti-estrogen prevents xenoestrogen-induced testicular pathology of eelpout (Zoarces viviparus). Aquatic Toxicology **72**:177-194.

Raymond-Whish S., L. P. Mayer, T. O'Neal, A. Martinez, M. A. Sellers, P. J. Christian, S. L. Marion, C. Begay, C. R. Propper, P. B. Hoyer, and C. A. Dyer. 2007. Drinking water with uranium below the US EPA water standard causes estrogen receptor-dependent responses in female mice. Environmental health perspectives **115**:1711-1716.

Ribi G., M. Gebhardt. 1986. Age specific fecundity and size of offspring in the prosobranch snail, *Viviparus ater*. Oecologia **71**:18-24.

Ribi G., M. Katoh. 1998. Weak preference for conspecific mates in the hybridizing snails Viviparus ater and V-contectus (Mollusca Prosobranchia). Ethology Ecology & Evolution **10**:383-392.

Rie M. T., N. Kitana, K. A. Lendas, S. J. Won, and I. P. Callard. 2005. Reproductive endocrine disruption in a sentinel species (Chrysemys picta) on Cape Cod, Massachusetts. Archives of Environmental Contamination and Toxicology **48**:217-224.

Rodet F., C. Lelong, M. P. Dubos, K. Costil, and P. Favrel. 2005. Molecular cloning of a molluscan gonadotropin-releasing hormone receptor orthologue specifically expressed in the gonad. Biochimica Et Biophysica Acta-Gene Structure and Expression **1730**:187-195.

Rodgers-Gray T. P., S. Jobling, C. Kelly, S. Morris, G. C. Brighty, M. J. Waldock, J. P. Sumpter, and C. R. Tyler. 2001. Exposure of juvenile roach (Rutilus rutilus) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. Environmental science & technology **35**:462-470.

Rodriguez E. M., D. A. Medesani, and M. Fingerman. 2007. Endocrine disruption in crustaceans due to pollutants: A review. Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology **146**:661-671.

Roepke T. A., E. S. Chang, and G. N. Cherr. 2006. Maternal exposure to estradiol and endocrine disrupting compounds alters the sensitivity of sea urchin embryos and the expression of an orphan steroid receptor. Journal of Experimental Zoology Part A-Comparative Experimental Biology **305A**:830-841.

Rogers-Gray T. P., S. Jobling, S. Morris, C. Kelly, S. Kirby, A. Janbakhsh, J. E. Harries, M. J. Waldock, J. P. Supter, and C. R. Tyler. 2000. long-term temporal changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. Environmental science and technology **34**:1521-1528.

Rohr J. R., T. R. Raffel, S. K. Sessions, and P. J. Hudson. 2008a. Understanding the net effects of pesticides on amphibian trematode infections. Ecological Applications **18**:1743-1753.

Rohr J. R., A. M. Schotthoefer, T. R. Raffel, H. J. Carrick, N. Halstead, J. T. Hoverman, C. M. Johnson, L. B. Johnson, C. Lieske, M. D. Piwoni, P. K. Schoff, and V. R. Beasley. 2008b. Agrochemicals increase trematode infections in a declining amphibian species. Nature **455**:1235-U50.

Romanova E. V., S. S. Rubakhin, and K. S. Rozsa. 1996. Behavioral changes induced by GABA-receptor agonists in Lymnaea stagnalis L. General pharmacology **27**:1067-1071.

Roos A., E. Greyerz, M. Olsson, and F. Sandegren. 2001. The otter (Lutra lutra) in Sweden - population trends in relation to Sigma DDT and total PCB concentrations during 1968-99. Environmental Pollution **111**:457-469.

Ropstorf P., T. Sitnikova. 2006. On the reproduction and growth of some endemic gastropods of Lake Baikal. Hydrobiologia **568**:115-127.

Routledge E. J., J. P. Sumpter. 1997. Structural features of alkylphenolic chemicals associated with estrogenic activity. Journal of Biological Chemistry **272**:3280-3288.

Routledge E. J., J. P. Sumpter. 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environmental Toxicology and Chemistry **15**:241-248.

Routledge E. J., D. Sheahan, C. Desbrow, G. C. Brighty, M. Waldock, and J. P. Sumpter. 1998. Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. Environmental science & technology **32**:1559-1565.

Routti H., M. Nyman, B. M. Jenssen, C. Backman, J. Koistinen, and G. W. Gabrielsen. 2008. Bone-related effects of contaminants in seals may be associated with vitamin D and thyroid hormones. Environmental Toxicology and Chemistry **27**:873-880.

Rudel H., P. Lepper, and J. Steinhanses. 2003. Retrospective monitoring of organotin compounds in marine biota from 1985 to 1999: Results from the German Environmental Specimen Bank. Environmental science & technology **37**:1731-1738.

Rudel H., J. Muller, J. Steinhanses, and C. Schroter-Kermani. 2007. Retrospective monitoring of organotin compounds in freshwater fish from 1988 to 2003: Results from the German environmental specimen bank. Chemosphere **66**:1884-1894.

Ruden D. M., L. Xiao, M. D. Garfinkel, and X. Y. Lu. 2005. Hsp90 and environmental impacts on epigenetic states: a model for the trans-generational effects of diethylstibesterol on uterine development and cancer. Human molecular genetics **14**:R149-R155.

Rupp J. C. 1996. Parasite-altered behaviour: Impact of infection and starvation on mating in Biomphalaria glabrata. Parasitology **113**:357-365.

Sabik H., F. Gagne, C. Blaise, D. J. Marcogliese, and R. Jeannot. 2003. Occurrence of alkylphenol polyetoxylates in the St. Lawrence River and their bioconcentration by mussels (*Elliptio complanata*). Chemosphere **51**:349-356.

Saitoh M., T. Yanase, H. Morinaga, M. Tanabe, Y. M. Mu, Y. Nishi, M. Nomura, T. Okabe, K. Goto, R. Takayanagi, and H. Nawata. 2001. Tributyltin or triphenyltin inhibits aromatase activity in the human granulosa-like tumor cell line KGN. Biochemical and biophysical research communications **289**:198-204.

Saleuddin A. S. M., S. T. Mukai, K. Almeida, and G. Hatiras. 2000. Membrane transduction pathway in the neuronal control of protein secretion by the albumen gland in Helisoma (mollusca). Acta Biologica Hungarica **51**:243-253.

Salice C. J., G. Roesijadi. 2002. Resistance to cadmium and parasite infection are inversely related in two strains of a freshwater gastropod. Environmental Toxicology and Chemistry **21**:1398-1403.

Samochwalenko T., A. Stanczykowska. 1972. Fertiliy differentiation of two species of Viviparidae (*Viviparus fasciatus* Mull. and *Viviparus viviparus* L.) in some environments. Ecology **20**:479-492.

Santhanagopalan V., T. P. Yoshino. 2000. Monoamines and their metabolites in the freshwater snail Biomphalaria glabrata. Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology **125**:469-478.

Santos M. M., L. Filipe, C. Castro, M. N. Vieira, J. Micael, R. Morabito, P. Massanisso, and M. A. Reis-Henriques. 2005. New insights into the mechanism of imposex induction in the dogwhelk *Nucella lapillus*. Comparative Biochemistry and Physiology, part C **141**:101-109.

Santos M. M., M. A. Reis-Henriques, M. N. Vieira, and M. Sole. 2006. Triphenyltin and tributyltin, single and in combination, promote imposex in the gastropod Bolinus brandaris. Ecotoxicology and environmental safety **64**:155-162.

Santos M. M., C. C. ten Hallers-Tjabbes, N. Vieira, J. P. Boon, and C. Porte. 2002. Cytochrome P450 differences in normal and imposex-affected female whelk Buccinum undatum from the open North Sea. Marine environmental research **54**:661-665.

Santos M. M., M. Micael, A. P. Carvalho, R. Morabito, P. Booy, P. Massanisso, M. Lamoree, and M. A. Reis-Henriques. 2006. Estrogens counteract the masculinizing effect of tributyltin in zebrafish. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **142**:151-155.

Sarmah A. K., G. L. Northcott. 2008. Laboratory degradation studies of four endocrine disruptors in two environmental media. Environmental Toxicology and Chemistry **27**:819-827.

Schallig H. D. F. H., M. J. M. Sassen, P. L. Hordijk, and M. De Jong-Brink. 1991a. Trichobilharzia-Ocellata - Influence of Infection on the Fecundity of its Intermediate Snail Host Lymnaea-Stagnalis and Cercarial Induction of the Release of Schistosomin, a Snail Neuropeptide Antagonizing Female Gonadotropic-Hormones. Parasitology **102**:85-91.

Schallig H. D. F. H., P. L. Hordijk, P. W. Oosthoek, and M. De Jong-Brink. 1991b. Schistosomin, a Peptide Present in the Hemolymph of Lymnaea-Stagnalis Infected with Trichobilharzia-Ocellata, is Produced Only in the Snails Central-Nervous-System. Parasitology research **77**:152-156.

Scharenberg W., V. Looft. 2004. Reduction of organochlorine residues in Goshawk eggs (Accipiter gentilis) from northern Germany (1971-2002) and increasing eggshell index. Ambio **33**:495-498.

Scheerboom J. E. M. 1978. Influence of Food Quantity and Food Quality on Assimilation, Body Growth and Egg-Production in Pond Snail Lymnaea-Stagnalis (L) with Particular Reference to Haemolymph-Glucose Concentration. Proceedings of the Koninklijke Nederlandse Akademie Van Wetenschappen Series C-Biological and Medical Sciences **81**:184-197.

Schirling M., A. Bohlen, R. Triebskorn, and H. -. Kohler. 2006a. An invertebrate embryo test with the apple snail *Marisa cornuarietis* to assess effects of potential developmental and endocrine disruptors. Chemosphere **64**:1730-1738.

Schirling M., D. Jungmann, V. Ladewig, K. U. Ludwichowski, R. Nagel, H. R. Kohler, and R. Triebskorn. 2006b. Bisphenol A in artificial indoor streams: II. Stress response and gonad histology in Gammarus fossarum (Amphipoda). Ecotoxicology **15**:143-156.

Schulte-Oehlmann U., M. Tillmann, B. Markert, and J. Oehlmann. 2000. Effects of Endocrine Disruptors on Prosobranch Snails (Mollusca: Gastropoda) in the Laboratory. Part II: Triphenyltin as a Xeno-Androgen. Ecotoxicology **9**:399-412.

Schulte-Oehlmann U., C. Bettin, P. Fioroni, J. Oehlmann, and E. Stroben. 1995. Marisa cornuarietis (Gastropoda, Prosobranchia): a potential TBT bioindicator for freshwater environments. Ecotoxicology **4**:372-384.

Schultz M. M., Furlong E. T., Kolpin D. W., Werner S. L., Schoenfuss H. L., Barber L. B., Blazer V. S., Norris D. O. and Vajda A. M. Occurence and distribution of antidepressant pharmaceuticals in two U.S. stream, sediments and fish brain tissues exposed to wastewater effluent. ; 3-7 August 2008; .

Segner H., J. M. Navas, C. Schafers, and A. Wenzel. 2003a. Potencies of estrogenic compounds in in vitro screening assays and in life cycle tests with zebrafish in vivo. Ecotoxicology and environmental safety **54**:315-322.

Segner H., K. Caroll, M. Fenske, C. R. Janssen, G. Maack, D. Pascoe, C. Schafers, G. F. Vandenbergh, M. Watts, and A. Wenzel. 2003b. Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. Ecotoxicology and environmental safety **54**:302-314.

Segura J. J., A. Jimenez-Rubio, N. Olea, J. M. Guerrero, and J. R. Calvo. 1999. In vitro effect of the resin component bisphenol A on substrate adherence capacity of macrophages. Journal of endodontics **25**:341-344.

Sellin M. K., A. S. Kolok. 2006. Cd exposures in fathead minnows: Effects on adult spawning success and reproductive physiology. Archives of Environmental Contamination and Toxicology **51**:594-599.

Sheahan D. A., G. C. Brighty, M. Daniel, J. Kirby Sonia, M. R. Hurst, J. Kennedy, S. Morris, E. J. Routledge, J. P. Sumpter, and M. J. Waldock. 2002. Estrogenic activity measured in sewage treatment works treating industrial inputs containing high concentrations of alkylphenolic compounds - a case study. Environmental toxicology and chemistry **21**:507-514.

Shimasaki Y., T. Kitano, Y. Oshima, S. Inoue, N. Imada, and T. Honjo. 2003. Tributyltin causes masculinization in fish. Environmental Toxicology and Chemistry **22**:141-144.

Simpson V. R., M. S. Bain, R. Brown, B. F. Brown, and R. F. Lacey. 2000. A long-term study of vitamin A and polychlorinated hydrocarbon levels in otters (Lutra lutra) in south west England. Environmental Pollution **110**:267-275.

Smit A. B., C. R. Jimenez, R. W. Dirks, R. P. Croll, and W. P. M. Geraerts. 1992. Characterization of a Cdna Clone Encoding Multiple Copies of the Neuropeptide Apgwamide in the Mollusk Lymnaea-Stagnalis. Journal of Neuroscience **12**:1709-1715.

Smith B. S. 1981a. Male characteristics in the female*Nassarius obsoletus*: variations related to locality, season and year. Veliger **23**:212-216.

Smith B. S. 1981b. Reproductive anomalies in stenoglossan snails related to pollution from marinas. J. appl. Toxicol **1**:15-21.

Smith B. S. 1981c. Male characteristics on female mud snails caused by antifouling bottom paints. J Appl Toxicol :22.

Smith B. S. 1971. Sexuality in the American mud snail,*Nassarius obsoletus* Say. Proc. malac. Soc. Lond. **39**:377.

Smith B. S. 1981. Tributyltin compounds induce male characteristics on female mud snails Nassarius obsoletus = Ilyanassa obsoleta. J Appl Toxicol **1**:141-4.

Soares A., B. Guieysse, B. Jefferson, E. Cartmell, and J. N. Lester. 2008. Nonylphenol in the environment: A critical review on occurrence, fate, toxicity and treatment in wastewaters. Environment international **34**:1033-1049.

Sohoni P., C. R. Tyler, K. Hurd, J. Caunter, M. Hetheridge, T. Williams, C. Woods, M. Evans, R. Toy, M. Gargas, and J. P. Sumpter. 2001. Reproductive effects of long-term exposure to bisphenol a in the fathead minnow (Pimephales promelas). Environmental science & technology **35**:2917-2925.

Sole M., D. Raldua, F. Piferrer, D. Barcelo, and C. Porte. 2003. Feminization of wild carp, Cyprinus carpio, in a polluted environment: plasma steroid hormones, gonadal morphology and xenobiotic metabolizing system. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **136**:145-156.

Sorensen R. E., D. J. Minchella. 1998. Parasite influences on host life history: Echinostoma revolutum parasitism of Lymnaea elodes snails. Oecologia **115**:188-195.

Sormo E. G., I. Jussi, M. Jussi, M. Braathen, J. U. Skaare, and B. M. Jenssen. 2005. Thyroid hormone status in gray seal (halichoerus grypus) pups from the Baltic Sea and the Atlantic Ocean in relation to organochlorine pollutants. Environmental Toxicology and Chemistry **24**:610-616.

Soto A. M., H. Justicia, J. W. Wray, and C. Sonnenschein. 1991. Para-Nonyl-Phenol - an Estrogenic Xenobiotic Released from Modified Polystyrene. Environmental health perspectives **92**:167-173.

Soto A. M., J. M. Calabro, N. V. Prechtl, A. Y. Yau, E. F. Orlando, A. Daxenberger, A. S. Kolok, L. J. Guillette, B. le Bizec, I. G. Lange, and C. Sonnenschein. 2004. Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in eastern Nebraska, USA. Environmental health perspectives **112**:346-352.

Sprovieri M., M. L. Feo, L. Prevedello, D. S. Manta, S. Sammartino, S. Tamburrino, and E. Marsella. 2007. Heavy metals, polycyclic aromatic hydrocarbons and polychlorinated biphenyls in surface sediments of the Naples harbour (southern Italy). Chemosphere **67**:998-1009.

Stanczykowksa A., E. Magnin, and A. Dumouchel. 1971. Studies of three *Viviparus malleatus* populations of the Montreal region. 1. Growth, fecundity, biomass, and annual production. Canadian Journal of Zoology **49**:1431-1441.

Staub R., G. Ribi. 1995. Size Assortative Mating in a Natural-Population of Viviparus-Ater (Gastropoda, Prosobranchia) in Lake Zurich, Switzerland. Journal of Molluscan Studies **61**:237-247.

Stefano G. B., W. Zhu, K. Mantione, D. Jones, E. Salamon, J. J. Cho, and P. Cadet. 2003. 17-beta estradiol down regulates ganglionic microglial cells via nitric oxide release: Presence of an estrogen receptor beta transcript. Neuroendocrinology letters **24**:130-136.

Stefano G. B., P. Cadet, C. Breton, Y. Goumon, V. Prevot, J. P. Dessaint, J. C. Beauvillain, A. S. Roumier, I. Welters, and M. Salzet. 2000. Estradiol-stimulated nitric oxide release in human granulocytes is dependent on intracellular calcium transients: evidence of a cell surface estrogen receptor. Blood **95**:3951-3958.

Sternberg R. M., G. A. LeBlanc. 2006. Kinetic characterization of the inhibition of acyl coenzyme A: Steroid acyltransferases by tributyltin in the eastern mud snail (Ilyanassa obsoleta). Aquatic Toxicology **78**:233-242.

Sternberg R. M., A. K. Hotchkiss, and G. A. Leblanc. 2008a. Synchronized expression of retinoid X receptor mRNA with reproductive tract recrudescence in an imposex-susceptible mollusc. Environmental science & technology **42**:1345-1351.

Sternberg R. M., A. K. Hotchkiss, and G. A. LeBlanc. 2008b. The contribution of steroidal androgens and estrogens to reproductive maturation of the eastern mud snail Ilyanassa obsoleta. General and comparative endocrinology **156**:15-26.

Stewart K. M., R. T. Bowyer, B. L. Dick, B. K. Johnson, and J. G. Kie. 2005. Density-dependent effects on physical condition and reproduction in North American elk: an experimental test. Oecologia **143**:85-93.

Straw J., D. Rittschof. 2004. Responses of mud snails from low and high imposex sites to sex pheromones. Marine pollution bulletin **48**:1048-1054.

Studier E. H., G. L. Pace. 1978. Oxygen-Consumption in Prosobranch Snail Viviparus-Contectoides (Mollusca-Gastropoda) .4. Effects of Dissolved-Oxygen Level, Starvation, Density, Symbiotic Algae, Substrate Composition and Osmotic-Pressure. Comparative Biochemistry and Physiology A-Physiology **59**:199-203.

Sumpter J. P. 1997. Environmental control of fish reproduction: a different perspective. Fish Physiology and Biochemistry **17**:25-31.

Sures B. 2006. How parasitism and pollution affect the physiological homeostasis of aquatic hosts. Journal of helminthology **80**:151-157.

Sures B., K. Knopf. 2004. Individual and combined effects of cadmium and 3,3 ', 4,4 ', 5-pentachlorobiphenyl (PCB 126) on the humoral immune response in European eel (Anguilla anguilla) experimentally infected with larvae of Anguillicola crassus (Nematoda). Parasitology **128**:445-454.

Susiarjo M., T. J. Hassold, E. Freeman, and P. A. Hunt. 2007. Bisphenol a exposure in utero disrupts early oogenesis in the mouse. Plos Genetics **3**:e5.

Suzuki T., Y. Nakagawa, I. Takano, K. Yaguchi, and K. Yasuda. 2004. Environmental fate of bisphenol A and its biological metabolites in river water and their xeno-estrogenic activity. Environmental science & technology **38**:2389-2396.

Suzuki T., K. Mizuo, H. Nakazawa, Y. Funae, S. Fushiki, S. Fukushima, T. Shirai, and M. Narita. 2003. Prenatal and neonatal exposure to bisphenol-A enhances the central dopamine D1 receptor-mediated action in mice: Enhancement of the methamphetamine-induced abuse state. Neuroscience **117**:639-644.

Swan S. H. 2001. Intrauterine exposure to diethylstilbestrol: Long-term effects in humans. Apmis **109**:S210-S221.

Swan S. H. 2000. Intrauterine exposure to diethylstilbestrol: Long-term effects in humans. Apmis **108**:793-804.

Tabuchi M., A. Veldhoen, N. Dangerfield, S. Jeffries, C. C. Helbing, and P. S. Ross. 2006. PCB-related alteration of thyroid hormones and thyroid hormone receptor gene expression in free-ranging Harbor seals (Phoca vitulina). Environmental health perspectives **114**:1024-1031.

Takahashi A., T. Higashitani, Y. Yakou, M. Saitou, H. Tamamoto, and H. Tanaka. 2003. Evaluating bioaccumulation of suspected endocrine disruptors into periphytons and benthos in the Tama River. Water Science and Technology **47**:71-76.

Tanaka J. N., J. M. Grizzle. 2002. Effects of nonylphenol on the gonadal differentiation of the hermaphroditic fish, Rivulus marmoratus. Aquatic Toxicology **57**:117-125.

Taskinen J., M. Saarinen. 1999. Increased parasite abundance associated with reproductive maturity of the clam Anodonta piscinalis. Journal of Parasitology **85**:588-591.

Taskinen J., T. Makela, and E. T. Valtonen. 1997. Exploitation of Anodonta piscinalis (Bivalvia) by trematodes: Parasite tactics and host longevity. Annales Zoologici Fennici **34**:37-46.

Tatton J. O., J. H. A. Ruzicka. 1967. Organochlorine Pesticides in Antarctica. Nature **215**:346-348.

Taylor G. T., E. Anderson. 1969. Cytochemical and fine structural analysis of oogenesis in the gastropod, *Ilyanassa obsoleta*. Journal of Morphology **129**:211-248.

Ter maat A. 1992. Egg laying in the hermaphrodite pond snail Lymnaea stagnalis. Prog. Brain. Res **92**:345-360.

Ter maat A., J. C. Lodder, and M. Wilbrink. 1983. Induction of egg-laying in the pond snail *Lymnaea stagnalis* by environmental stimulation of the release of ovulation hormone from caudo-dorsal cells. Int. J. Invertebr. Reprod **6**:239-347.

Ter Maat A., C. Zonneveld, J. A. G. M. de Visser, R. F. Jansen, K. Montagne-Wajer, and J. M. Koene. 2007. Food intake, growth, and reproduction as affected by day length and food availability in the pond snail Lymnaea stagnalis. American Malacological Bulletin **23**:113-120.

Thain J. E., M. J. Waldock. 1986. THE IMPACT OF TRIBUTYLTIN (TBT) ANTIFOULING PAINTS ON MOLLUSCAN FISHERIES. WATER SCIENCE AND TECHNOLOGY **18**:193-202.

Thompson E. B., R. Kumar. 2003. DNA binding of nuclear hormone receptors influences their structure and function. Biochemical and biophysical research communications **306**:1-4.

Thornton J. W., E. Need, and D. Crews. 2003. Resurrecting the Ancestral Steroid Receptor: Ancient Origin of Estrogen Signaling. Science **5640**:1714-1717.

Thorpe K. L., R. I. Cummings, T. H. Hutchinson, M. Scholze, G. C. Brighty, J. P. Sumpter, and C. R. Tyler. 2003. Relative potencies and combination effects of steroidal estrogens in fish. Environmental science & technology **37**:1142-1149.

Tillmann M., U. Schulte-Oehlmann, M. Duft, B. Markert, and J. Oehlmann. 2001. Effects of endocrine disruptors on prosobranch snails (Mollusca : Gastropoda) in the laboratory. Part III: Cyproterone acetate and vinclozolin as antiandrogens. Ecotoxicology **10**:373-388.

Titus-Ernstoff L., R. Troisi, E. E. Hatch, J. R. Palmer, L. A. Wise, W. Ricker, M. Hyer, R. Kaufman, K. Noller, W. Strohsnitter, A. L. Herbst, P. Hartge, and R. N. Hoover. 2006a. Mortality in women given diethylstilbestrol during pregnancy. British journal of cancer **95**:107-111.

Titus-Ernstoff L., R. Troisi, E. E. Hatch, L. A. Wisei, J. Palmer, M. Hyer, R. Kaufman, E. Adam, W. Strohsnitter, K. Noller, A. L. Herbst, J. Gibson-Chambers, P. Hartge, and R. N. Hoover. 2006b. Menstrual and reproductive characteristics of women whose

mothers were exposed in utero to diethylstilbestrol (DES). International journal of epidemiology **35**:862-868.

Toft G., E. Baatrup, and L. J. Guillette. 2004. Altered social behavior and sexual characteristics in mosquitofish (Gambusia holbrooki) living downstream of a paper mill. Aquatic Toxicology **70**:213-222.

Toschik P. C., B. A. Rattner, P. C. McGowan, M. C. Christman, D. B. Carter, R. C. Hale, C. W. Matson, and M. A. Ottinger. 2005. Effects of contaminant exposure on reproductive success of ospreys (Pandion haliaetus) nesting in Delaware River and bay, USA. Environmental Toxicology and Chemistry **24**:617-628.

Trub H., G. Ribi. 1997. High fecundity of hybrids between the sympatric snail species Viviparus ater and V-contectus (Gastropoda: Prosobranchia). Heredity **79**:418-423.

Tsai P. S., T. A. Maldonado, and J. B. Lunden. 2003. Localization of gonadotropin-releasing hormone in the central nervous system and a peripheral chemosensory organ of Aplysia californica. General and comparative endocrinology **130**:20-28.

Tsai P. S., T. A. Maldonado, T. R. Pak, and L. Zhang. 2000. Characterization of a Gonadotropin-Releasing Hormone System in an opisthobranch mollusk, Aplysia californica. American Zoologist **40**:1238-1239.

Tuberty S. R., C. L. McKenney. 2005. Ecdysteroid responses of estuarine crustaceans exposed through complete larval development to juvenile hormone agonist insecticides. Integrative and Comparative Biology **45**:106-117.

Turyk M. E., H. A. Anderson, and V. W. Persky. 2007. Relationships of thyroid hormones with polychlorinated biphenyls, dioxins, furans, and DDE in adults. Environmental health perspectives **115**:1197-1203.

Tyler C. R., E. J. Routledge. 1998. Oestrogenic effects in fish in English rivers with evidence of their causation. Pure and Applied Chemistry **70**:1795-1804.

Vajda A. M., L. B. Barber, J. L. Gray, E. M. Lopez, J. D. Woodling, and D. O. Norris. 2008. Reproductive disruption in fish downstream from an Estrogenic wastewater effluent. Environmental science & technology **42**:3407-3414.

van Aerle R., M. Nolan, S. Jobling, L. B. Christiansen, J. P. Sumpter, and C. R. Tyler. 2001. Sexual disruption in a second species of wild cyprinid fish (the Gudgeon, gobio gobio) in United Kingdom freshwaters. Environmental Toxicology and Chemistry **20**:2841-2847.

van Cleave H. J., L. G. Lederer. 1932. Studies on the life cycle of the snail, *Viviparus contectoides*. Journal of Morphology **53**:499-522.

Van den Belt K., P. Berckmans, C. Vangenechten, R. Verheyen, and H. Witters. 2004. Comparative study on the in vitro in vivo estrogenic potencies of 17 beta-estradiol, estrone, 17 alpha-ethynylestradiol and nonylphenol. Aquatic Toxicology **66**:183-195.

Van Duivenboden Y. A. 1983. Transfer of semen accelerates the onset of egg-laying in female copulants of the hermaphrodite freshwater snail *Lymnaea stagnalis*. Int. J. Invertebr. Reprod **6**:249-257.

Vethaak D. A., J. Lahr, S. M. Schrap, A. C. Belfroid, G. B. J. Rijs, A. Gerritsen, J. De Boer, A. S. Bulder, G. C. M. Grinwis, R. V. Kuiper, J. Legler, T. A. J. Murk, W. Peijnenburg, H. J. M. Verhaar, and P. de Voogt. 2005. Integrated assessment of estrogenic contamination and biological effects in the aquatic envrionment of the Netherlands. Chemosphere **59**:511-524.

Vigueras-Villasenor R. M., N. A. Moreno-Mendoza, G. Reyes-Torres, D. Molina-Ortiz, M. C. Leon, and J. C. Rojas-Castaneda. 2006. The effect of estrogen on testicular gonocyte maturation. Reproductive Toxicology **22**:513-520.

Voutsa D., P. Hartmann, C. Schaffner, and W. Giger. 2006. Benzotriazoles, alkylphenols and bisphenol a in municipal wastewaters and in the Glatt River, Switzerland. Environmental Science and Pollution Research **13**:333-341.

Wagner A. 2000. Influence of sewage from different water treatment plants in the Cracow Region (Poland) on early development stages of water snails. Lakes and Reservoirs: Research and management **5**:83-88.

Walker M., H. C. MacGregor. 1968. Spermatogenesis and the structure of the mature sperm in *Nucella Lapillua* (L). Journal of cell science **3**:95-104.

Wallace C. 1992. Parthenogenesis, Sex and Chromosomes in Potamopyrgus. Journal of Molluscan Studies **58**:93-107.

Wang C. D., R. P. Croll. 2003. Effects of sex steroids on in vitro gamete release in the sea scallop, *Placopecten magellanicus*. Invertebrate reproduction and development **44**:89-100.

Wang C. D., R. P. Croll. 2006. Effects of sex steroids on spawning in the sea scallop, Placopecten magellanicus. Aquaculture **256**:423-432.

Watts M. M., D. Pascoe, and K. Carroll. 2002. Population responses of the freshwater amphipod Gammarus pulex (L.) to an environmental estrogen, 17 alpha-ethinylestradiol. Environmental Toxicology and Chemistry **21**:445-450.

Wayne N. L. 2001. Regulation of seasonal reproduction in mollusks. Journal of Biological Rhythms **16**:391-402.

Weltje. L., Scholz, C., van Doornmalen, J., Markert, C., Endocrine disruption in the hermaphroditic pondsnail *Lymnaea stagnalis*. ; 27 April-1 May 2003; SETAC ; 2003. 189 p.

White R., S. Jobling, S. A. Hoare, J. P. Sumpter, and M. G. Parker. 1994. Environmentally Persistent Alkylphenolic Compounds are Estrogenic. Endocrinology **135**:175-182.

Wilbur K. 1984. The Mollusca. **7**. Academic Press.

Williams R. J., A. C. Johnson, J. J. L. Smith, and R. Kanda. 2003. Steroid estrogens profiles along river stretches arising from sewage treatment works discharges. Environmental science & technology **37**:1744-1750.

Wilson, V. S., M. C. Cardon, J. Thornton, J. J. Korte, J. E. WELCH, L. E. GRAY, JR., and P. C. Hartig. In vitro detection of androgenic and estrogenic activity in complex environmental effluent samples: lessons learned. ; SETAC Sydney 3-7th August 2008; .

Wren C. D. 1991. Cause-Effect Linkages between Chemicals and Populations of Mink (Mustela-Vison) and Otter (Lutra-Canadensis) in the Great-Lakes Basin. Journal of toxicology and environmental health **33**:549-585.

Wu W., E. G. Niles, and P. T. Loverde. 2007. Thyroid hormone receptor orthologues from invertebrate species with emphasis on Schistosoma mansoni. Bmc Evolutionary Biology **7**:.

Xing Y., Y. Lu, R. W. Dawson, Y. Shi, H. Zhang, T. Wang, W. Liu, and H. Ren. 2005. A spatial temporal assessment of pollution from PCBs in China. Chemosphere **60**:731-739.

Yang L. H., T. G. Luan, and C. Y. Lan. 2006a. Solid-phase microextraction with on-fiber silylation for simultaneous determinations of endocrine disrupting chemicals and steroid hormones by gas chromatography-mass spectrometry. Journal of Chromatography a **1104**:23-32.

Yang L. H., C. Y. Lan, H. T. Liu, J. Dong, and T. G. Luan. 2006b. Full automation of solid-phase microextraction. Analytical and Bioanalytical Chemistry **386**:391-397.

Ying G. 2006. Fate, behavior and effects of surfactant and their degradation products in the environment. Environment international **32**:417-431.

Ying G., B. Williams, and R. Kookana. 2002. Environmental fate of alkylphenols and alkylphenol ethoxylates - a review. Environment international **28**:215-226.

Ying G. G., R. S. Kookana, and P. Dillon. 2003. Sorption and degradation of selected five endocrine disrupting chemicals in aquifer material. Water research **37**:3785-3791.

Young K. G., J. P. Chang, and J. I. Goldburg. 1999. Gonadotrophin-releasing hormone neuronal system of the freshwater snail Helisoma trivolvis and Lymnaea stagnalis: possible involvement in reproduction. Journal of comparative neurology **404**:427-437.

Young M. R. 1975. The life cycles of six species of freshwater molluscs in the Worcester-Birmingham canal. Proc. malac. Soc. Lond **41**:533-548.

Zhang L., N. L. Wayne, N. M. Sherwood, H. R. Postigo, and P. Tsai. 2000. Biological and immunological characterization of Multiple GnRH in an Opisthobranch Mollusk *Aplysia californica*. General and comparative endocrinology **188**:77-89.

Zhang J. L., Z. H. Zuo, Y. X. Chen, Y. Zhao, S. Hu, and C. G. Wang. 2007a. Effect of tributyltin on the development of ovary in female cuvier (Sebastiscus marmoratus). Aquatic Toxicology **83**:174-179.

Zhang J. Y., L. M. Qiu, J. He, Y. Liao, and Y. M. Luo. 2007b. Occurrence and congeners specific of polychlorinated biphenyls in agricultural soils from Southern Jiangsu, China. Journal of Environmental Sciences-China **19**:338-342.

Zhang L. H., J. A. Tello, W. M. Zhang, and P. S. Tsai. 2008. Molecular cloning, expression pattern, and immunocytochemical localization of a gonadotropin-releasing hormone-like molecule in the gastropod mollusk, Aplysia californica. General and comparative endocrinology **156**:201-209.

Zhang P., J. M. Song, Z. G. Liu, G. X. Zheng, N. X. Zhang, and Z. P. He. 2007. PCBs and its coupling with eco-environments in Southern Yellow Sea surface sediments. Marine pollution bulletin **54**:1105-1115.

Zhokhov A. E. 1993. Age Structure and Seasonal Dynamics of Invasion of a Pond Snail, Viviparus-Viviparus, Population with Parthenitas of Trematodes. Zoologichesky Zhurnal **72**:17-25.

Zorita I., M. Ortiz-Zarragoitia, M. Soto, and M. P. Cajaraville. 2006. Biomarkers in mussels from a copper site gradient (Visnes, Norway): An integrated biochemical, histochemical and histological study. Aquatic Toxicology **78**:S109-S116.

Appendix I

YEAST SCREEN PROTOCOLS

PREPARATION AND STORAGE OF MINIMAL MEDIUM AND MEDIUM COMPONENTS

Minimal medium and medium components prepared in glassware contaminated with an oestrogenic chemical will lead to elevated background expression. Glassware, spatulas, stirring bars, etc., must be scrupulously cleaned, and should not have had prior contact with steroids.
Rinse glassware, spatulas, stirring bars twice with absolute ethanol, and leave to dry. Alternatively, wash twice with methanol, and once with ethanol.

Minimal Medium (pH 7.1)
Add 13.61 g KH_2PO_4, 1.98 g $(NH_4)_2SO_4$, 4.2 g KOH pellets, 0.2 g $MgSO_4$, 1 ml $Fe_2(S0_4)_3$ solution (40 mg/50 ml H_20), 50 mg L-leucine, 50 mg L-histidine, 50 mg adenine, 20 mg L-arginine-HCl, 20 mg L-methionine, 30 mg L-tyrosine, 30 mg L-isoleucine, 30 mg L-lysine-HCl, 25 mg L-phenylalanine, 100 mg L-glutamic acid, 150 mg L-valine, and 375 mg L-serine to 1 L double-distilled water. Place on heated stirrer to dissolve.
Dispense 45 ml aliquots into glass bottles.
Sterilise at 121˚C for 10 min, and store at room temperature.

D-(+)-Glucose
Prepare a 20% w/v solution.
Sterilise in 20 ml aliquots at 121˚C for 10 min.
Store at room temperature.

L-Aspartic Acid
Make a stock solution of 4 mg/ml.
Sterilise in 20 ml aliquots at 121˚C for 10 min.
Store at room temperature.

Vitamin Solution
Add 8 mg thiamine, 8 mg pyridoxine, 8 mg pantothenic acid, 40 mg inositol, and 20 ml biotin solution (2 mg/100 ml H_2O) to 180 ml double-distilled water.
Sterilise by filtering through a 0.2-µm pore size disposable filter, in a laminar air flow cabinet. Filter into sterile glass bottles in 10 ml aliquots.
Store at 4˚C.

L-Threonine
Prepare a solution of 24 mg/ml.
Sterilise in 10 ml aliquots at 121˚C for 10 min.
Store at 4˚C.

Copper (II) Sulfate
Prepare a 20 mM solution.
Sterilise by filtering through a 0.2-µm pore size filter, in a laminar flow cabinet. Filter into sterile glass bottles in 5 ml aliquots.
Store at room temperature.

Chlorophenol red-ß-D-galactopyranoside (CPRG)
Make a 10 mg/ml stock solution. Sterilise by filtering through a 0.2-µm pore size filter into sterile glass bottles, in a laminar flow cabinet.
Store at 4˚C.

PREPARATION AND STORAGE OF YEAST STOCKS

All yeast work carried out in a type II laminar flow cabinet.

SHORT TERM STORAGE (-20˚C) - 10X concentrated yeast stock culture

Day 1
Prepare growth medium by adding 5 ml glucose solution, 1.25 ml L-aspartic acid solution, 0.5 ml vitamin solution, 0.4 ml L-threonine solution, and 125 µl copper (II) sulfate solution to 45 ml minimal medium. Transfer to a sterile conical flask (final volume of approximately 50 ml). Add 125 µl of 10X concentrated yeast stock from cryogenic vial stored at -20˚C. Incubate at 28˚C for approximately 24 hour on an orbital shaker, or until turbid. Yeast from a cryovial stored at -80˚C can also be used, but may take longer to produce a turbid culture.

Day 2
Add growth medium to two conical flasks (each with a final volume of approximately 50 ml). Add 1 ml yeast from 24-h culture to each flask.
Incubate at 28˚C for approximately 24 hour on an orbital shaker, or until turbid.

Day 3
Transfer each 24-h culture to a sterile 50-ml centrifuge tube.
Centrifuge the cultures at 4˚C for 10 min at 2,000 g.
Decant the supernatant, and resuspend each culture in 5 ml of minimal medium with 15% glycerol (add 8 ml sterile glycerol to 45 ml minimal medium).
Transfer 0.5 ml aliquots of the 10X concentrated stock culture to 1.2-ml sterile cryovials.
Store at -20˚C for a maximum of 4 months.

LONG-TERM STORAGE (-70˚C/-80˚C)

Preparation of minimal medium agar slopes (1% agar)

Add 1 g bacteriological grade agar to 90 ml minimal medium.

Autoclave, and allow to cool to 50˚C before adding the growth medium components;
- 10 ml glucose,
- 2.5 ml L-aspartic acid,
- 1 ml vitamin solution,
- 0.8 ml L-threonine solution, and
- 250 µl copper (II) sulfate.

Swirl, and quickly pour into sterile universals (approx. 10 ml per universal). Leave the universals at an angle of approximately 45˚, so that the agar sets forming a slope.

Preparation of yeast

Using a sterile loop, take some yeast from a cryovial (stored at either -20˚C or -80˚C), and spread this over the surface of the slope.
Incubate the slopes at 32˚C for 3 days.

Add 1 ml sterile glycerol (100 %) to each universal, and resuspend the cells using a sterile loop.
Aliquot the glycerol yeast suspension to cryovials (0.5 ml per cryovial), and store at -70˚C or -80˚C.

PREPARATION AND STORAGE OF CHEMICALS

Glassware must be scrupulously cleaned since contamination may give rise to false positives.
Rinse all glass bottles twice with absolute ethanol (or twice with methanol, and once with ethanol), and leave to dry.

Weigh chemicals directly into bottles.

Prepare the 17ß-estradiol stock for the standard curve in absolute ethanol, at $2x10^{-7}$ M (54.48 µg/L).

Stock solutions of test chemicals are prepared in absolute ethanol at $2x10^{-2}$ M or 2 g/L, although this concentration may vary, depending on potency, toxicity etc.

If the chemical is insoluble in ethanol, try another solvent, such as minimal medium. However, certain solvents will melt plastic plates.

Appendix II

CHEMISTRY PROTOCOL

Chemistry Protocol obtained from the Environment Agency's National Laboratory Service (NLS), Nottingham, UK.

For APEs
Aqueous sample is solvent extracted using 50/50 v/v diethyl ether/iso-hexane. Extract is concentrated prior to GC-MS with EI detection (operated in SIM mode). The calibration standards were a technical mix of isomers for NP, pure p-tert OP, and similarly a mix of isomers for NP-ethoxylates and single isomerfor OP-ethylate.

BPA
Spiked with deuterated BPA before solvent extracted with DCM. Extract is then dried before derivatisation using trifluoroacetic anhydride. Same GC-MS system as for APEs.

Oestrogens
Spiked with deuterated ostrogen analogues prior to extraction with a styrene divinyl benzene polymer SPE cartridge. Steriods are then deabsorbed with DCM. Extract is concentrated and cleaned-up using gel permeation chromatography fractionation. Extract then undergoes a solvent exchange to 95/5v/v isohexane/propan-2-ol and is cleaned up using normal pahse chromatography in an amino LC column. It is then evaporated to incipient dryness and disolved in methanol. Analysed using HPLC with negative ion atmospheric photo-ionisation interface and MS time-of-flight detection.

Appendix III

Kahel's Fluid recipe

Kahel's Fluid was prepared in a fume cupboard and stored in acid resistant glass bottle. NBF, ethanol and glacial acetic acid were added to the distilled water.

Six parts Neutrally Buffered Formalin (NBF)
Fifteen parts 95% Ethanol
One part Glacial Acetic Acid
Thirty parts distilled water

Appendix IV

Trace metals, minerals and compounds measured from the two river water mesocsom tanks (E2 study and oestrogenic mixtures study) and the river water plus solvent tank (oestrogenic mixtures study) at week 16 of the dosed mesocsom studies (August 2006). Chemical analysis provided by Environment Agency's National Laboratory Service (NLS), Nottingham, UK. (< Limit of detection).

	E2 Study River water tank	**Mixtures Study River water tank**	**Mixtures Study River water plus solvent tank**
Barium µg/l	26.9	26.8	26.6
Boron µg/l	110	<100	<100
Calcium mg/l	96.8	6.10	94.1
Iron µg/l	<30.0	<30.0	<30.0
Lithium µg/l	9.92	10.3	9.88
Magnesium mg/l	9.38	9.20	9.13
Manganese µg/l	<10.0	<10.0	<10.0
Potassium mg/l	11.0	10.6	10.7
Sodium mg/l	62.6	59.8	60.2
Strontium µg/l	419	409	405
Sulphate SO_4 mg/l	102	98.4	98.4
TBT µg/l	<0.002	<0.002	<0.002
Aluminium µg/l	<10.0	<10.0	<10.0
Cadmium µg/l	<0.10	<0.10	<0.10
Chromium µg/l	0.506	<0.500	<0.500
Copper µg/l	1.89	20.6	1.88
Lead µg/l	<0.40	<0.40	<0.40
Nickel µg/l	<5.0	<5.0	<5.0
Zinc µg/l	5.16	8.67	5.32

Appendix V

Histological description of *P. corneus* gonad during reduced temperatures under laboratory conditions

The affect of reduced temperature on *P. corneus* gonad was assessed over 8 weeks. Approximately 40 laboratory raised sexually mature snails (~10-20mm diameter) were removed from their 80 l holding tank (17-18^{o}C) and moved to a new 80l flow through tank (15^{o}C). Due to the time of year (October) it was possible to use the dropping external ground water temperature to slowly reduce the temperature in the tank by around 1^{o}C per week until it reached 11^{o}C where it stabilised. The reduced temperature tank was inspected ever three to four days for the first four weeks for egg masses. At this point snails were also fed, any waste flake food from previous feedings was removed using a siphon, and uneaten carrot was left unless mouldy. Water temperatures were recorded twice weekly before cleaning and feeding.

Effect of reduced temperature on egg laying

No egg masses were laid after snails were transferred to the reduced temperature tank.

Effect of temperature reduction on the histopathology of the acini walls

No affect was seen on wall integrity over the 8 weeks of temperature reduction. A small amount of disruption was observed in the gonad at week 8 but this was due to artefact during histological sectioning and may have been in part due to over fixation of the tissue. At week 0 before temperature reduction the acini walls were thickly covered in spermatogenic, oogenic or supportive cells, with less than a third of the walls bare of cells (mainly at the luminal end). From week 1 to week 3 cell cover was slightly reduced, becoming less thick and in places gaps between clumps of spermatogenic cells could be observed. By week 4 bare patches could also be observed towards the vitellogenic end of the acini walls, this patchiness was still observed at week 8.

Effect of temperature reduction on the histopathology of the vitellogenic end of the acini

All of the gonad sections observed from snails sampled at weeks 0, 1, 3, 4 and 8 had a score of 5 which equals >70% of the acini have full vitellogenic ends i.e. those which contained oocytes, maturing spermatogenic cells or degenerating supporting cells (sertoli or follicle). During week 2, vitellogenic area activity was reduced slightly. Snails scored an

average of 4.6, which equalled two out of five sections (per snail) of gonad scored 4 (50-70% of acini have active vitellogenic areas) with the remainder scoring 5.

Effect of temperature reduction on the histopathology of spermatogenic and oogenic cells

Figure Aii, is photomicrographs of the gonad of laboratory reared *P. corneus* at week 0 (before temperature reduction) and weeks 1, 2 and 4 (after temperature reduction). On average only 10% of the sections of gonad observed had any immature spermatids and sertoli cells sloughed into the lumen prior to water temperature reduction. Sloughing of immature spermatogenic cells (spermatogonium, spermatocytes and spermatids) and sertoli cells increased markedly after one week of temperature reduction, around 70% of the sections observed had sloughed sertoli cells free in the lumen and 90% of the sections observed had spermatogonium, spermatocytes and spermatids free in the lumen. None of the sections observed from week 1 had only spermatozoa in the lumen (as generally observed in sexually active gonads). Between 80-100% of the sections observed from week 2 snail gonads had sloughed immature and/or sertoli cells, 80% had spermatogonium and spermatocytes, 90% had sertoli cells and 100% had spermatids sloughed into the lumen of the acini. 3 to 4 Weeks after temperature reduction started the level of sloughing had reduced slightly and in 30-40% of the sections observed spermatozoa were the only cells seen in the lumen. 20-50% of the sections observed had sertoli and spermatids free in the lumen, 30-40% had spermatocytes, and 60% had sloughed spermatogonium. By week 8 the percentage of sections with only spermatozoa in the lumen had increased to 60% and the number with sloughed sertoli cells, spermatogonium or spermatids had been reduced to 20%. No spermatocytes were observed sloughed into the lumen at this stage. No trend was observed in the percentage of different stages of oogenesis after 1, 2, 3, 4 or 8 weeks of temperature reduction (Figure Ai).

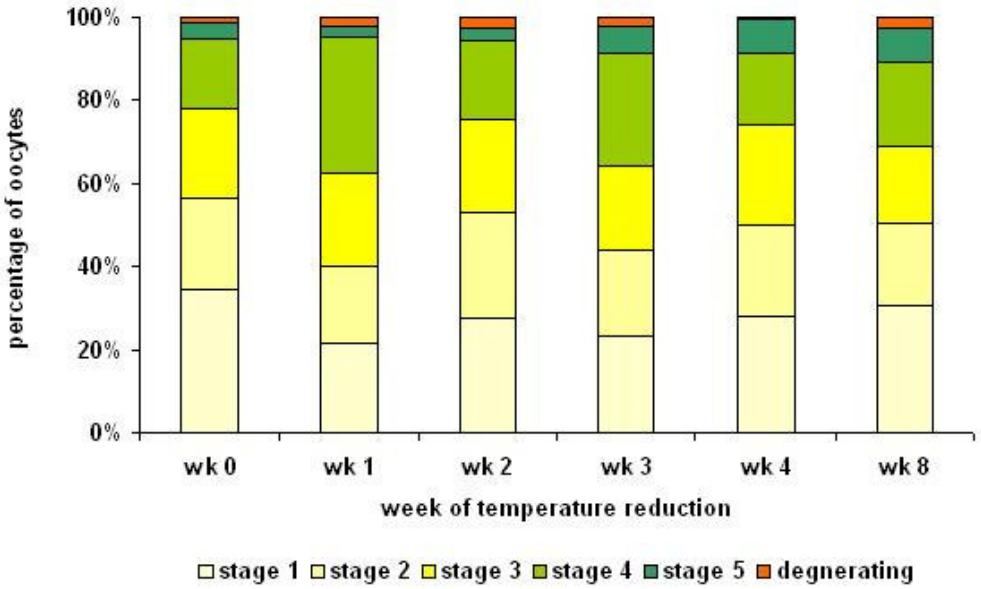

Figure Ai, Percentage of oocytes at different developmental stages during the temperature reduction experiment.
Week 0, before temperature reduction. Five sections of gonad were analysed per adult snail. Stage 1 (oogonium) is the youngest stage of oogenesis, stage 5 is ready to be ovulated, oogenesis stages based on description by de Jong-Brink et al, (De Jong-Brink et al. 1976).

Discussion and comparison to the literature

In agreement with other *P. corneus* housed below 15°C (Costil and Daguzan 1995a) no egg masses were laid by snails after they had been moved to the temperature reduced tank. However, it is possible to get *L. stagnalis* to breed at 8°C if housed under a LD light regime (16:8 L:D) (Dogterom et al. 1983). Very little information on the effect of temperature reduction (or autumnal season) on the Basommatophoran gonad was found. Berrie (Berrie 1966) sampled *L. stagnalis* at different times of year from a pond in Lanarkshire, and the internal composition of histological sections of gonad was measured. Gonad composition was split into three classes; developing spermatozoa, mature spermatozoa or empty. In December (1957) 25.6% of the gonad was occupied by developing spermatozoa, 2.4% by mature spermatozoa and 72% was empty in snails with a shell length between 11.25 and 22.25 mm. At the same time larger snails (31.5-32 mm shell length) had 13.4% of the gonad occupied by maturing spermatozoa and 86.6% empty. In May (1958) snails of 15.5-30 mm shell diameter had 41.7% developing spermatozoa, 23% mature spermatozoa and 35.3% empty, and snails with shell length of 26-34.5 mm had 31.6% of the gonad filled with developing spermatozoa, 19.3% mature spermatozoa and 49.1% empty (Berrie 1966). A general trend of decreased mature and developing spermatozoa and increased emptiness during winter can be observed from the data. Oocytes were also counted and less were observed in winter (1.8 and 78.5 in 1957 and 1958, respectively) than spring (48.4 and 155.5). One other study, also on *L. stagnalis*

cooled a laboratory bred snail from 20°C to 8°C for 70 days (Boer and Joosse 1975). A photomicrograph indicates spermatogenesis has halted (only spermatogonium and spermatocytes are visible), and the lumen is empty except for many mature oocytes, indicating either oogenic maturation still occurs at this temperature (Boer and Joosse 1975) or that mature oocytes are stored in the lumen for some time. All stages of spermatogenesis were still observed in *P. corneus* gonads after temperature reduction of up to 8 weeks; however the percentage of spermatogenic cells covering the acini walls did reduce especially after 4 and 8 weeks of temperature reduction. Large quantities of immature sperm were seen sloughing into the lumen within the first few weeks of temperature reduction. The level of sloughing was greatly reduced by weeks 3 and 4 and had almost halted by week 8. By week 8 of temperature reduction the area of lumen filled by spermatozoa had been reduced, suggesting that longer periods of reduced temperature would lead to the empty gonad/lumen seen by Berrie (Berrie 1966) and Boer and Joosse (Boer and Joosse 1975). No accumulation (or percentage increase) of mature oocytes was observed in temperature reduced *P. corneus* as found by Boer and Joosse (Boer and Joosse 1975) in *L. stagnalis*.

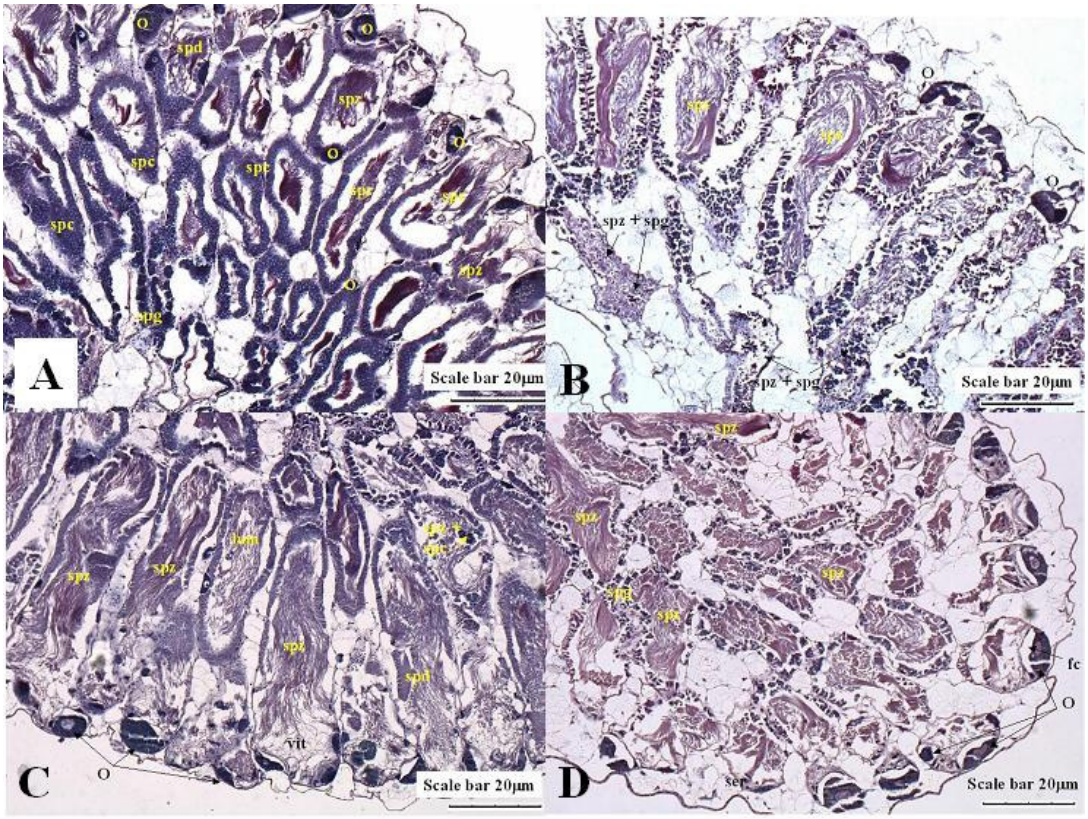

Figure Aii Photomicrograph of *P. corneus* temperature reduced gonad.
(A) week 0 before temperature reduction, spermatogenic cells cover the acini wall thickly, spermatogenic and oogenic cells fill the vitellogenic area, spermatozoa fill the lumen; (B) 1 week after temperature reduction, some immature spermatogenic cells slough into the lumen, vitellogenic area quite full of oocyte and maturing spermatogenic cells; (C) 2 weeks after temperature reduction, immature spermatogenic cells still seen sloughing into lumen, vitellogenic area has become patch with spermatogenic cells, but many oocytes still evident; (D) 3 weeks after temperature reduction, thin covering of spermatogenic cells on acini walls, lumen still filled with spermatozoa, vitellogenic area dominated by mature oocytes. 5µm sections stained with H & E. Spermatogonium - spg; Spermatocytes - spc; Spermitids - spd; Spermatozoa - spz; Sertoli cells - ser; Follicle cavity - fc. Oocytes - O; Vitellogenic area - vit; Lumen - lum.