The Effects of Estrogenic Endocrine Disruptors on the Osmoregulatory Functions in Euryhaline Fish

Submitted by Noura Jalal Al-Jandal, to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences in January 2011

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ABSTRACT

Osmoregulation is an essential process to maintain water and ionic balance and when euryhaline fish move between freshwater and seawater environments as part of their life cycle this presents additional osmoregulatory challenges. Migrating fish can be exposed in both environments to pollutants such as endocrine disrupting chemicals (EDCs) that include natural hormones (e.g. 17β-estradiol; E2), synthetic hormones (e.g. 17α-ethinylestradiol; EE2), and industrial chemicals (e.g. nonylphenol). The focus of this thesis was to study the effects of different categories of EDCs on the osmoregulatory functions of euryhaline fish such as three-spined sticklebacks (*Gasterosteus aculeatus*) and rainbow trout (*Oncorhynchus mykiss*). Osmoregulatory variables (such as osmolality, water and ionic content) were compared in plasma and tissues (white muscle and carcass) of rainbow trout. This validated the use of specific tissue parameters as a surrogate of plasma responses to various osmoregulatory challenges. Waterborne exposure to 17α-ethinylestradiol revealed differential sensitivity of vitellogenesis in the three-spined sticklebacks (no induction) and rainbow trout, but had a significant effect on calcium homeostasis in both species. Intraperitoneal implants of 17β-estradiol reduced $CaCO_3$ production and apparent water absorption in the intestine and increased in tissue calcium stores of seawater-acclimated trout, but fish were able to compensate and showed no overall osmoregulatory disturbance. Waterborne exposure to nonylphenol in freshwater trout was also investigated, but no effects on osmoregulation were found up to 2 ng/l. Overall, estrogens can affect osmoregulation differentially in euryhaline fish species, and sometimes at EDC levels lower than the threshold for reproductive effects (i.e. vitellogenin induction).

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CO-AUTHORS DECLARATION

I would like to declare the contribution of my co-authors in the work I have submitted for publication out of this research study.

Dr Rod Wilson contributed in Chapter 2, 3 and 5; assistance with editing the manuscripts, sampling fish and contribution in the experiment designs. Prof. Charles Tyler contributed in the experimental design in Chapter 2, and manuscript editing. Dr. Ioanna Katsiadaki contributed in Chapter 3 by hosting my visit to her laboratory in Weymouth to use her plasma vitellogenin ELISA analysis for sticklebacks, and helped in the manuscript editing for this work. Dr. Anke Lange contributed in Chapter 3 in sampling fish and comments on the manuscript. Dr. Jonathan Whittamore had a huge contribution in Chapter 5 in terminal sampling of fish, Dionex and autotitration analysis, and valuable comments on the manuscript. Dr. Eduarda Santos contributed in Chapter 5 in implantation of fish with 17β-estradiol, terminal fish sampling and manuscript editing.

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GENERAL INTRODUCTION

1. OVERVIEW

The maintenance of consistent osmotic conditions is critical for normal cell functioning in living organisms. For water-breathing animals, the strategy involved in osmotic homeostasis depends on the salinity of the external environment (McCormick and Bradshaw, 2006). A number of mechanisms are employed to regulate the intracellular and extracellular compartments (plasma, lymph, and interstitial fluid) to redress disruption of the appropriate osmotic conditions. The majority of fish live either in fresh water or in sea water, but some fish species are able to maintain their body fluids in both these environments. These fish species are termed euryhaline and some of these (particularly estuarine species) can even survive rapid fluctuations in the osmolarity in their surroundings on a daily (tidal) basis (McCormick, 2001; Marshall and Grosell, 2006). In euryhaline fish, when they migrate from freshwater to seawater environments or vice versa as a normal part of their life cycle, they may face additional exposures to different waterborne chemicals, and some of these chemicals may affect their ability to osmoregulate (Wendelaar Bonga and Lock 2008).

Osmoregulation refers to the regulation of ions and water in the intracellular and extracellular compartments in fish. In teleost fish, many of the regulatory processes are dependent on various hormones. Hormones involved include cortisol and the GH/IGF axis (GH refers to growth hormones; IGF refers to insulin-like growth factor) that play a major role in seawater acclimation and promote salt secretion (McCormick, 2001; Sangiao-Alvarellos et al., 2006; Sakamoto and McCormick, 2006). Prolactin plays a role in freshwater osmoregulation in promoting ion uptake (McCormick, 2001; Manzon, 2002).

Therefore, the interference with any of the hormone system controlling osmoregulation may be an important factor in some euryhaline or marine species and worthy of future study (Matthiessen, 2003).

The issue of endocrine disruption has received considerable research attention and became a major environmental issue, principally focusing on the effect of estrogenic compounds on fish reproduction (Körner et al., 2008), although wider effects have been studied (Matthiessen, 2003; Schlenk, 2008; Hotchkiss et al., 2008). Even though many investigations have been carried out into the effects of endocrine disruptors in fish, there are very few that have examined the effect of endocrine disruptors on osmoregulation (Bangsgaard et al., 2006), a process fundamental for fish health and survival. In Britain, endocrine disruptor effects are now of a high priority in research and the present study has focused on the potential effects of these chemicals on various aspects of osmoregulation and ion regulation in euryhaline fish.

2. ENDOCRINE DISRUPTORS

Endocrine disrupting chemicals are natural or synthetic compounds that can exert an effect on homeostasis in animals by interfering with their hormone systems. There is a growing interest in the possible threat posed by these chemicals and a significant number of *in vitro* and *in vivo* studies have been carried out to examine such effects.

In the environment, fish are exposed to multi-component mixtures of chemicals which enter water bodies via different point sources. Many of these chemicals might be toxic and pose

harmful effects on marine life. Accordingly, various studies have considered either the effect of a single chemical (Huang et al., 2010) or a mixture of chemicals (Madsen et al., 1997; Teles et al., 2004; Correia et al., 2007) to estimate the risks and the potential impacts of these chemicals and their toxicity towards wildlife. Considerable scientific evidence from such studies have indicated that some of these chemicals could mimic the action of steroid hormones and produce a similar biological response to that produced by the endogenous hormones.

Endocrine disruptors have been shown to cause serious physiological effects in the different developmental stages and various species of fish. Adverse physiological alterations reported in several studies on estrogenic chemicals include increased vitellogenin production (Arukwe et al., 2001; Teles et al., 2007; Zlabek et al., 2009), gonadal intersex (Örn et al., 2003; Tyler and Jobling, 2008), and reduced gonad growth rate and size. They have also been shown to affect fish behaviour related to reproduction such as courtship and aggression in the three-spined stickleback (*Gasterosteus aculeatus*) (Bell, 2001), male fathead minnows (*Pimephales promelas*) (Majewski et al., 2002), and male sand gobies (*Pomatoschistus minutes*) (Saaristo et al., 2010). They are also known to interfere with non-reproductive physiological and behavioural processes, such as the inhibition of the smolting of Atlantic salmon (*Salmo salar*) (Madsen et al., 1997).

The aquatic environment has been described as an ultimate sink for natural and man-made chemicals (Sumpter, 1998). Many chemicals that humans introduce into the environment have the capacity to interfere with and modulate the endocrine systems of wildlife and

mimic or antagonise the natural (endogenous) hormones. These are termed as endocrine disrupting chemicals (EDCs) (Kaminuma et al., 2000). The mechanism through which EDCs affect an organism can be via acting as a hormone receptor agonist or antagonist, through altering hormone metabolism or synthesis or excretion, or by modifying hormone receptor levels (Sonnenschein and Soto, 1998).Whether ECDs are affecting human health has not been established definitively, but there are many studies that indicate that this is the case. There is concern about their effects on reproductive ability such as the sperm counts and quality that have decreased globally (Carlsen et al., 1992; Giwercman et al., 1993, 2007; Joensen et al., 2009). For example, bisphenol A is an important environmental contaminant and is a synthetic estrogen (Ramakrishnan and Wayne, 2008). It is widely used in packaging food and drinks (Ramakrishnan and Wayne, 2008; Galloway et al., 2010) and it has been reported that it may be associated with endocrine changes in men (Galloway et al., 2010).There has been increasing attention in the last decades on the effect of EDCs in the environment. EDCs are now often classified according to their biological effect, and there are wide varieties of pollutants referred to as EDCs. There is a wide range of EDCs derived from both natural and industrial sources. They include a diverse group of agricultural chemicals, synthetic industrial and naturally occurring compounds such as estrogens pesticides, polycyclic aromatic hydrocarbons, phthalate plasticizers, alkylphenols, synthetic steroids, and natural products, e.g. phytoestrogens (Santodonato, 1997; de Alda and Barceló, 2001; Burkhardt-Holm, 2010).

Numerous man-made chemicals can mimic hormones such as organochlorine pesticides and bisphenol A. Chemicals of industrial origin are not the only ones that can act as

hormone mimics, but some natural chemicals can mimic natural estrogens or interfere with the action of endogenous hormones (Cajthaml et al., 2009). They are thought to be largely derived from those naturally excreted by women, and/or synthetic estrogen-like molecules such as 17α-ethinylestradiol (EE2) from the contraceptive pill (Tyler et al., 1998, 1999). Various industrial chemicals such as plasticizers, surfactants and pesticides, have concentrations detected in environmental samples ranging from ng/l to µg/l (Nadzialek et al., 2010).

Because of EDC's action on endogenous hormones, they can interfere with normal reproduction that is controlled by hormonal signals. The effects of EDCs on wildlife species have been extensively investigated *in vivo* (Purdom et al., 1994) and *in vitro* (Le Gac et al., 2001), where many natural fish populations are exposed to theses EDCs. These studies provided us with evidence of the negative effect(s) of EDCs such as the masculinization of female gastropod molluscs by tributylin-based antifoulant (TBT) (Matthiessen and Gibbs, 1998), and the feminization of marine and freshwater fish by estrogenic hormones and their mimics discharged in sewage and industrial effluents (Matthiessen, 2003; Jobling and Tyler, 2003).

2.1 Estrogens as endocrine disruptors

2.1.1 17α-ethinylestradiol (EE2)

Both natural and synthetic estrogens commonly enter the freshwater by sewage treatment works and the compounds responsible for estrogenic activity such as 17β-estradiol (E2) and the synthetic E2 derivate 17α-ethinylestradiol (EE2) (Desbrow et al., 1998; Williams et al.,

2003). The occurrence of EE2 in the aquatic environment is due to the insufficient removal in the wastewater treatment plant (Clouzot et al., 2008). Steroid estrogens cause a wide range of feminizing effects in fish, such as intersex and vitellogenin induction (Jobling et al., 2006). Ethinylestradiol (EE2) is a synthetic estrogen used in combination with other steroid hormones in oral contraceptives and it is an endocrine disruptor of great concern (Caldwell et al., 2008; Clouzot et al., 2010). It is considered as the primary contaminant that contributes to the estrogenic activity in surface waters in United Kingdom (Desbrow et al., 1998), and one of the most potent and significant xenoestrogens that has been detected in domestic sewage ranging from 0.2 to 7 ng/l, and up to 5 ng/l in surface waters (Desbrow et al., 1998; Belfroid et al., 1999; Larsson et al., 1999; Ternes et al., 1999).

Although EE2 detected in the environment is present in nanograms per liter, these concentrations are high enough to induce plasma vitellogenin when exposed under laboratory conditions (Lange et al., 2001; Schultz et al., 2003; Al-Jandal et al., submitted, Chapter 3). In addition, EE2 has more resistance to break down than natural estrogens (Solé et al., 2000). Fish feminization is induced from EE2 concentrations as low as 0.1 ng/l in male rainbow trout (Purdom et al., 1994). In a full life cycle of zebrafish (*Brachydanio rerio*) exposure via water at 1.67 ng/l EE2 caused vitellogenin induction (Segner et al., 2003). It has been reported that long term exposure of newly hatched roach (*Rutilus rutilus*) to 4 ngEE2/l resulted in sex reversal of males population (Lange et al., 2009).

2.1.2 17β-estradiol (E2)

17β-estradiol is a natural steroidal estrogen (Schlenk, 2008), containing an aromatic A-ring

as part of its tetracyclic molecular framework (Tapiero et al., 2002). The most important source is domestic effluents where E2 derives principally from human and veterinary medicines (Gagné et al., 2005), but also agriculture runoff (Céspedes et al., 2004). It is considered as a potent environmental contaminant (Ying et al., 2002; Dorabawila and Gupta, 2005) even at low exposure concentrations. Unfortunately, the presence of E2 received little attention until recently, although it is always present due to its secretion in mammalian urine. Estradiol levels in the environment increased over the years due to the increase in the global population in large cities and live stock-farming practices (de Alda and Barceló, 2001).

17β-estradiol plays an important role in the physiological process of fish reproduction, for example, the estrogenic constituents of the sewage effluents such as E2 can cause testis-ova in male roaches (*Rutilus rutilus*) living downstream of sewage treatment works (Jobling et al., 1998). E2-associated endocrine disruption in the reproductive system of male medaka (*Oryzias latipes*) exposed to environmentally relevant concentrations for 3 weeks induced hepatic vitellogenin concentrations and testis-ova (Kang et al., 2002). In addition, several studies showed a negative effect of E2 on fish osmoregulatory performance. A variety of salmonid fish treated with E2 showed a reduced chloride cell density and Na^+, K^+-ATPase activity in the gills (Madsen and Korgaard, 1989; Coimbra et al., 1993; Madsen et al., 1997). Furthermore, E2 treatment increases plasma vitellogenin and calcium level in teleost fish (Perrson et al., 1994; Guerreiro et al., 2002; Al-Jandal et al., submitted – Chapter 5). Vijayan et al. (2001) reported that E2 impairs the hypo-osmoregulatory capacity in the tilapia (*Oreochromis mossambicus*) due to the decrease in the branchial Na^+, K^+-ATPase

activity and decline in the metabolic activity of the liver and gills. It has been reported in previous studies that pollutants such as nonylphenol can act as endocrine-disruptors by binding to the estrogen receptor and mimic the action of 17β-estradiol (Colborn et al., 1993). Estradiol has been known to have a marked impact on calcium balance and can stimulate the calcium-binding protein production (vitellogenin) in fish, which is essential for egg production (Mosconi et al., 1998). It can induce calcium resorption from scales and bone of salmonid fish (Persson et al., 1994; Kacem et al., 2000).

2.1.3 4-nonylphenol (NP)

Nonylphenol is an organic compound and classified as xenobiotic. It is known to be a environmental estrogen (Tabata et al., 2001), and has been demonstrated to be toxic to both freshwater and marine species (McClease et al., 1981; Comber et al., 1993; Hwang et al., 2008). It is a degradation or breakdown product of nonylphenol ethoxylate (Yadetie et al., 1999) which is used in non-ionic surfactants (Yadetie et al., 1999; Harris et al., 2001). It is more persistent, toxic and estrogenic (Maguire, 1999) and bio-accumulative once in the aquatic environment (Ahel et al., 1993; Jobling et al., 1996). Nonylphenol polyethoxylates are discharged in high quantities to sewage treatment plants and directly to the environment where there is no sewage or industrial waste treatment (Maguire, 1999), then they completely degraded into NP (Ahel et al., 1994). Alkylphenol polyethoxylates are not estrogenic *per se*, however they are degraded during the sewage treatment (Sonnenschein and Soto, 1998), and the free phenols are estrogenic (Soto et al., 1991). It has been found that NP mimics 17β-estradiol in competing for the binding site of the receptor of the natural estrogen (White et al., 1994).

Nonylphenol is one of the most studied estrogen mimics, and as with other alkylphenols it is known to produce estrogenic effects (Jobling and Sumpter, 1993; White et al., 1994). Estrogenicity of NP has been reported in fish, amphibians, birds, and mammals (Hutchinson et al. 2006; Vetillard and Bailhache, 2006). It can cause the synthesis of vitellogenin (Jobling et al., 1996; Arukwe et al., 1997; Kinnberg et al., 2000) and eggshell zona radiata protein in the juvenile salmon (Arukwe et al., 1997) and inhibit testicular growth in rainbow trout (Jobling et al., 1996). Nonylphenol has been also linked to the delays in downstream migration and smolts development of Atlantic salmon (*Salmo salar*) (Madsen et al., 2004). It has been found that NP concentrations of 1 and 10 µg/l (sewage treatment plant effluent concentrations lie between this range) were not able to induce sexual differentiation in the wild rainbow trout, whereas the induction of vitellogenin and zona radiate protein were more sensitive (Ackermann et al., 2002).

2.2 Endocrine control of reproduction

The central players of the normal functioning of the endocrine system are the hypothalamus and the pituitary gland where the most fundamental physiological processes are regulated which includes reproduction. Signals from the brain control the hypothalamic secretion of gonadotropin-releasing hormones by sending signals that stimulate the pituitary gland to release gonadotropins. Gonadotropins control the production and secretion of steroid hormones from the gonads (Scholz and Klüver, 2009).

Endocrine disruptors have effects on a wide range of physiological parameters including reproduction in fish (Örn et al., 2003; Bjerregaard et al., 2006). Endocrine disrupting

chemicals can possibly perturb the hormones that regulate the reproductive function and this may lead to decrease in egg production, fertility in female fish, and reduce the gonad size or cause feminization of male fish (Arcand-Hoy and Benson, 1998). In general, the field of endocrine disruptors has mainly focused on reproduction (McCormick et al., 2005). Indeed, most of the EDCs studied to date act to interfere with the reproductive hormones, particularly estrogens or androgens (Angus et al., 2005). Steroid hormones are a large class of lipophilic molecules that act on different target sites to regulate many physiological functions (Tabb and Blumberg, 2006).

Nonylphenol is described as an environmental pollutant with endocrine disrupting characteristics and causes various dysfunctions in the male and female reproductive systems by modifying the level of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in rats (Nagao et al., 2001). In female tilapia (*Oreochromis niloticus*), nonylphenol induces changes in the gonad suggesting that it acts by mimicking sex hormones (Rivero et al., 2008). Steroid hormones such as 17β-estradiol (E2) also affect gonadal development as shown by several studies such as exposure of adult zebrafish (*Danio rerio*) to E2 resulted in secondary sexual characteristic modification in males and dose-dependent inhibition of egg production in females (Brion et al., 2004).

2.3 Vitellogenin induction as a biomarker for environmental estrogen exposure

One of the most widely used biomarkers of exposure to estrogens and their mimics in fish is the induction of vitellogenin (Tyler et al., 1996; Kime et al., 1999). Vitellogenin is a yolk precursor lipophosphoprotein in the oviparous vertebrates (Bergink and Wallace, 1974)

which is produced by the female liver stimulated by estrogen and it is normally present in very low concentrations in the plasma of immature and male organisms. Jobling and Sumpter (1993) found that the metabolites of nonylphenol polyethoxylate are estrogenic in male rainbow trout, where they considered the production of vitellogenin as an indication of estrogenic activity. White et al. (1994) investigated the ability of selected alkylphenolic compounds to stimulate vitellogenin gene expression in rainbow trout hepatocytes such as 4-octylphenol and 4-nonylphenol. Panter et al., (1998) studied the effects of 17β-estradiol and estrone on the plasma vitellogenin level and gonadosomatic index of male fathead minnows (*Pimephales promelas*). They represented the first report on the estrogenicity of steroids to male fathead minnows, and they found an increase in plasma vitellogenin when exposing to high levels of 17β-estradiol. The exposure to 1-10 ng/l of 17β-estradiol provokes feminization in male fish (Routledge et al., 1998). The presence of intersex in wild fish species indicates that endocrine disrupting chemicals are present in their environment (Gercken and Sordyl, 2002).

2.4 Non-reproductive targets of endocrine disruptors

Hormones are not only involved in regulating reproductive processes, and some research on endocrine disruptors is now focusing away from purely reproductive endpoints. An increasing number of studies have started to focus on the effect of endocrine disrupting chemicals on another physiological process such as osmoregulation. To understand the basis of these interactions, I will now describe our current knowledge of osmoregulation in fish, and the organs and mechanisms involved, before describing their endocrine control and potential interference of this by EDCs.

3. OSMOREGULATION

3.1 Osmoregulation and ion balance in fish

All living organisms either aquatic or terrestrial must maintain their water and ionic balance and the ability to regulate the osmolarity of body fluids to maintain homeostasis is fundamental to life. Marine fish maintain their hypotonic body fluids by drinking sea water, and this can expose them to dissolved contaminants via an additional route (i.e. the gut) compared to freshwater fishes. In the case of freshwater fishes, they are hyper-osmotic to their surroundings, and thus considerable amounts of water will move into their bodies down the osmotic gradient. Euryhaline fish rely on many internal mechanisms to coordinate the physiological adjustments essential for responding to alterations in the surrounding salinity.

In adult fish, hydromineral balance relies on specialized tissues and organs, namely the gills, intestine and urinary system that are under neuroendocrine control (Varsamos et al., 2005). Maintaining the osmolarity of the extracellular fluids tends to buffer the fish cells and tissues against the effects of extreme differences compared to the external freshwater or seawater. Osmotic regulation involves the intake and output of both water and ions, and the main extracellular electrolytes in all vertebrates are Na^+ and Cl^-, which is why these are the most commonly measured ions when studying osmoregulation in both freshwater and seawater fish.

3.2 Osmotic strategies

Fish either conform to environmental changes by letting their own internal conditions

follow those of the external medium, or they may regulate their own body fluids to maintain reasonable independence of the external environment. Thus fish can be classified as “osmoregulators” or “osmoconformers” depending on whether they can or cannot maintain the osmotic concentration of their extracellular fluid constant in the face of changes in external osmotic concentrations.

3.2.1 Osmoconformers:

Elasmobranchs are osmoconformers with plasma osmolality equal to or slightly higher than the surrounding marine environment (Yancey, 2005). They have evolved a strategy by reabsorbing and retaining urea and other body fluid solutes in their tissues so that plasma osmolarity remains slighter higher than that of the external seawater (Smith, 1931; Thorson, 1962; Mandrup-Poulsen, 1981). As a consequence they benefit from not having to spend metabolic resources on osmoregulation (Marshall and Grosell, 2006) and they do not need to drink seawater. However, they still face the problem of a diffusion of salts into their bodies from the high salinity concentration in the external environment. This is compensated for by salt excretion in the urine, by secretions of the rectal gland, and salt excretion via the gills (Haywood, 1973).

3.2.2 Osmoregulators:

Osmoregulators can tightly regulate their internal plasma osmolarity independent of the surrounding environment. Teleost fish are osmoregulators, and they are either hyper-osmoregulators (osmoregulate in freshwater) or hypo-osmoregulators (osmoregulate in seawater) (Marshall and Grosell, 2006). As a strategy to compensate for the water loss in

the marine environment teleost fish drink the surrounding seawater, but they are still able to achieve ionic and osmotic homeostasis by the function of the gills, gut, and urinary bladder. In freshwater teleosts the kidney plays a role in producing dilute urine to counter the diffusive water gain and the gills take up ions (Sakamoto and McCormick, 2006).

3.2.2.1 Problems for osmoregulators

Teleost fish exist in a wide range of salinity habitats thus they are faced with variety of osmotic problems. Fish challenged with an altered ambient salinity require different mechanisms to maintain homeostasis (Figure 1):

3.2.2.1.A Seawater fish and osmoregulation

The osmolality of seawater is approximately 1000 mOsm kg^{-1} and the plasma osmotic pressure of most marine teleosts is about 300-350 mOsm kg^{-1} (Shehadeh and Gordon, 1969; McDonald and Milligan, 1992). Saltwater fish are thus hypo-osmotic to the surrounding medium (Figure 1A). Therefore, they are facing the problem of diffusive salt gain and osmotic water loss to the surrounding environment and this is exacerbated by the large surface area of the gills (Genz et al., 2008). Seawater fish manage this problem using various osmoregulatory mechanisms: (1) They excrete a small volume of isotonic urine by the kidney; (2) They drink a large volume of seawater (Smith, 1930; Evans, 1993). The drinking rate varies among species and with environmental salinity and the higher the salinity the greater the rate of drinking (Røvik et al., 2001; Bartels and Potter, 2004). They process the seawater by reabsorbing monovalent ions and retaining water which takes place

in the intestine, and the excess salt load generated by these gut processes is secreted across the gills (Bartels and Potter, 2004).

3.2.2.1.B Freshwater fish and osmoregulation

In contrast with teleosts living in seawater, freshwater teleosts have body fluids that are more concentrated than the surrounding medium (Figure 1B). The osmolality of the freshwater is usually < 1 mOsm kg^{-1} and the osmotic concentration of blood in freshwater fish is 190-310 mOsm kg^{-1} (McDonald and Milligan, 1992) hence the fish are subjected to osmotic water gain and loss of ions by diffusion across gills and in the urine (Evans et al., 2005). The problem of water gain is managed by (1) low drinking rate; (2) production of a copious volume of hypotonic urine by the kidney with ion reabsorption along the excretory system; and (3) the ion losses are compensated by active uptake of monovalent and divalent ions by the gills, which is crucial for the ion homeostasis (McDonald and Wood, 1981; Bartels and Potter, 2004).

3.3 Major osmoregulatory organs

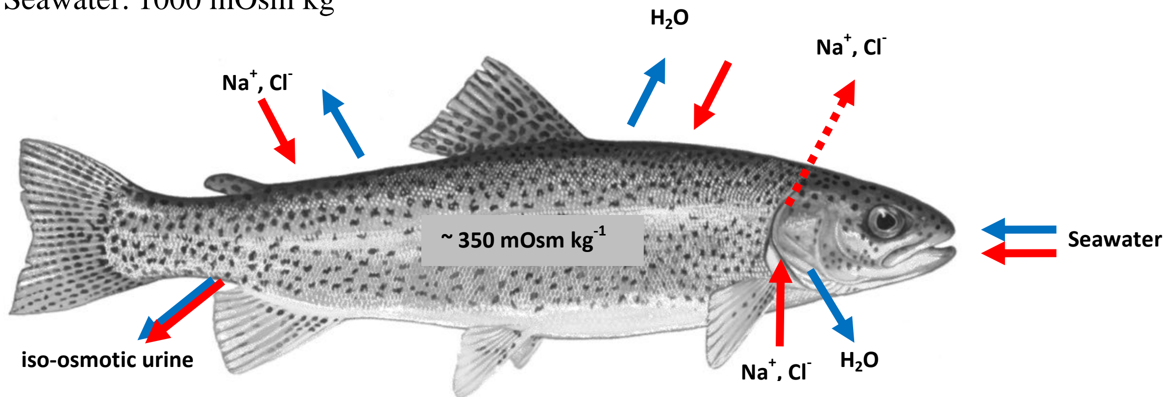
Several osmoregulatory organs play important roles in ion and water balance of fish body fluids; these include the gills, gastrointestinal tract, skin, kidney, and more specific organs, such as the rectal gland in elasmobranchs (Evans et al., 2005; Marshall and Grosell, 2006).

3.3.1 Gills

Gills are the most studied organ in fish osmoregulation and ion regulation, during fish acclimation to different salinities, the direction of ion transport is reversed, where they give

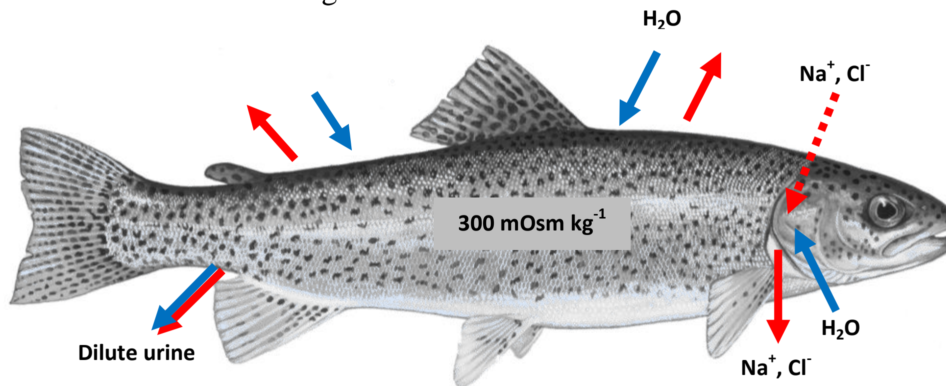
(A)

Seawater: 1000 mOsm kg^{-1}



(B)

Freshwater: < 1 mOsm kg^{-1}



Diffusion
Active
Ions
Water

Figure 1: Osmoregulation in marine and freshwater teleost (A and B, respectively). **A)** Marine teleosts face the problem of diffusive salt gain and osmotic water loss, therefore they drink large volumes of seawater, produce small amount of urine and excrete ions actively via the gills. **B)** Freshwater teleosts are subjected to osmotic water gain and diffusive ions loss. Therefore, they produce large volumes of dilute urine and take up ions actively via the gills.

an excellent model to study ion regulation and transport (Hwang and Lee, 2007). The majority of fish species use the gills as the primary site for respiratory gaseous exchange. The gills are situated on either side of the fish pharynx, and composed of two main epithelial surfaces; the lamellar and the filament epithelia (Perry, 1997). The gill epithelium of teleosts contains several different cell types; the pavement cells, which are the most abundant cell types and known to play role in the gas exchange. In freshwater teleost gills, evidence suggests that some PVCs may play an active role in ion uptake and acid-base transport by the gills (Evans et al., 2005). The mitochondria-rich cells (or MR cells) also known as chloride cells or ionocytes. In general, the mitochondria-rich cells are ovoid-shaped cells and as their name suggests, they have high densities of mitochondria in their cytoplasm (Evans et al., 2005), ovoid nuclei and high levels of the transport protein Na^+/K^+-ATPase (Laurent and Dunel, 1980). They play a role in ion transport, where in freshwater fish two subtypes of MR cells were described; α and β cell (Evans et al., 2005). The mucous cells, which produce large apical mucous secretory granules, irrespective of whether the fish is living in fresh or seawater (Wilson and Laurent, 2002).

The gills are not only the major site for gas exchange, but also for ion transport, acid-base regulation and nitrogenous waste excretion (Marshall and Grosell, 2006). To facilitate the process of gaseous exchange the gills have a high surface area with a thin epithelial layer separating the blood of the fish and aquatic environment. Various different epithelial cell types assume respiratory, osmoregulatory and excretory roles. The gill has a remarkable capacity for integrating these various functions and adjusting them to the needs of the organism. Thus, the gills are the major site for the passive loss or gain of ions and water

that occur in freshwater and seawater environments. It has become clear that the branchial epithelium is also the primary site of active transport processes and the primary site of body fluid pH regulation and nitrogenous waste secretion (Evans et al., 2005).

3.3.2 Intestine

The intestine also plays an important role in osmo- and iono-regulation, and is an important osmoregulatory organ particularly in saltwater fish, taking up water to compensate for osmotic water loss to the hyperosmotic environment (Marshall and Grosell, 2006). When saltwater is taken in during drinking in marine fish the initial desalination process starts in the oesophagus (Grosell, 2006). Seawater contains significant quantities of divalent ions, such as magnesium (Mg^{2+}) and sulphate (SO_4^{2}), but only small amounts of these are absorbed from the seawater during its passage through the intestine and become a useful biomarker for net water movement (Hickman, 1968).

Ingested seawater passes through the gastrointestinal tract where ions are differentially absorbed across the intestinal epithelium to facilitate water absorption (Taylor and Grosell, 2006). The intestine actively absorbs Na^+ and Cl^- by two apical co-transporters Na^+:Cl^- (NC) and Na^+:K^+:2Cl^- (NKCC), that facilitate water absorption. The mechanism of Na^+ and Cl^- absorption is fueled by the basolateral Na^+,K^+ ATPase (Musch et a., 1982; Grosell, 2006). The NaCl absorption can also occur via parallel antiport systems of Na^+/H^+ and Cl^-/HCO_3^- exchangers as described for the seawater-adapted tilapia (*Oreochromis mossambicu*) (Howard and Ahearn, 1988). More recently, it has been shown that a large portion of total Cl^- absorption results from the apical Cl^-/HCO_3^- exchange (Wilson et al.,

1996; Grosell et al., 2001, Grosell et al., 2005). The latter makes a significant contribution to the luminal alkalinisation in the intestine, and the high pH provides favourable conditions for Ca^{2+} and Mg^{2+} precipitation. If these divalent ions do not precipitate, they can significantly oppose water absorption (Wilson et al., 2002). The precipitation of divalent cations in the intestine of marine fish and not freshwater fish is due to the osmoregulatory strategy of fish living in hyperosmotic environment, and the need to ingest calcium-rich sea water (Figure 2).

3.3.3 Kidney and urinary bladder

Fish kidneys are long structures running dorsally in the body cavity above the gas bladder and beneath the vertebral column. Kidneys are mesonephric containing renal tubules (nephrons) and may have glomeruli in freshwater and euryhaline species. In contrast in marine fish the kidney possess greatly reduced glomeruli and some species can completely lack glomerular nephrons (Marshall and Grosell, 2006), an oddity that has resulted from the need to reduce water loss (Masini et al., 2001).

The kidney has an important function in osmoregulation in both freshwater and seawater fish, although its role is completely different under the two different environmental conditions. In freshwater, the fish face the problem of water gain and ion loss, therefore, the kidney excretes this excess water as dilute urine. While in seawater, where the fish face the problem of water loss and ion load, the primary function of the kidney is the excretion of a small volume of urine together with primarily divalent ions (Miyazaki et al., 2002; Nishimura and Fan, 2003).

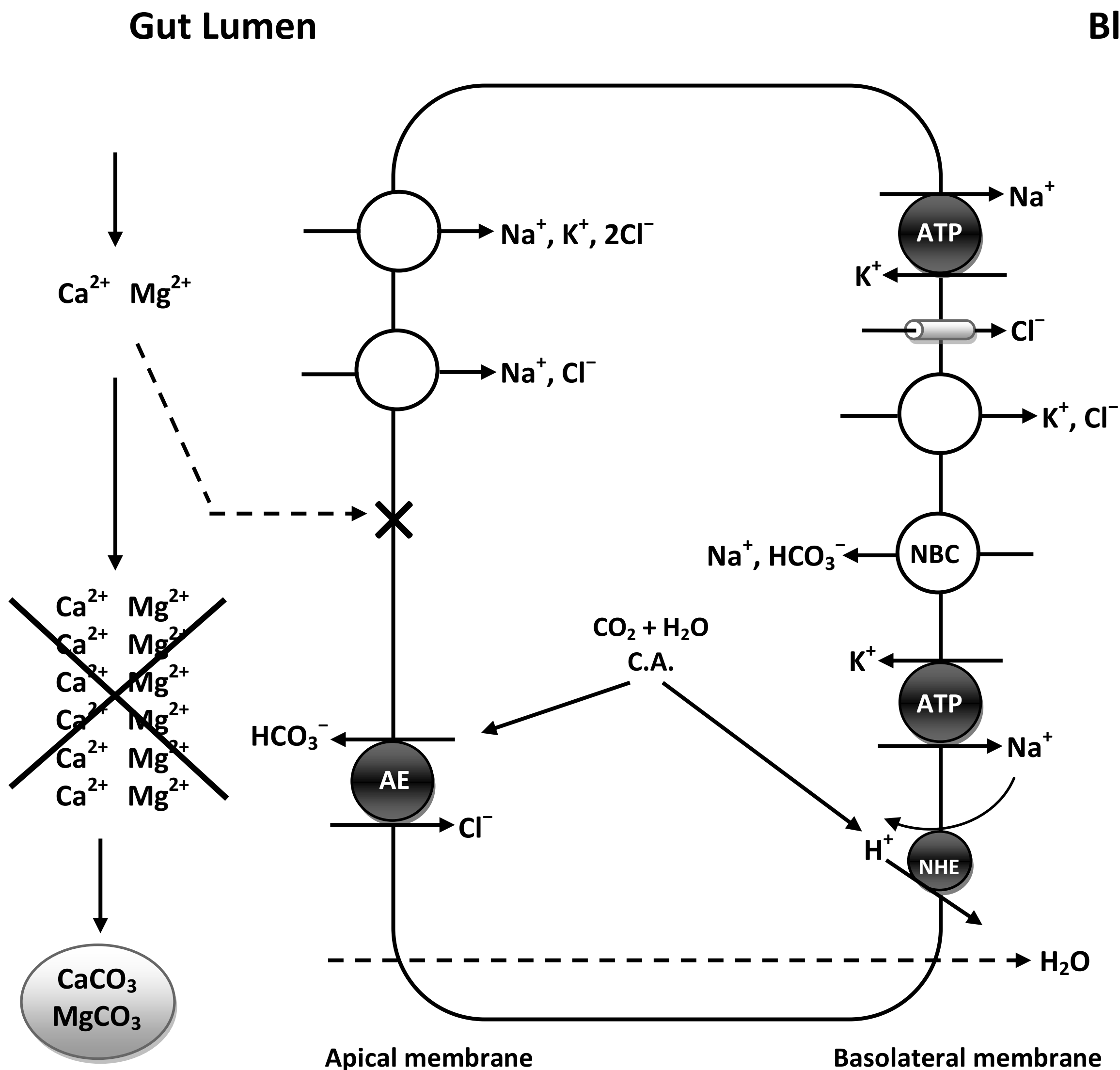


Figure 2: Schematic cellular model of the ions transport processes in the intestinal epithelium of the marine teleost intestine. Fluid absorption is driven by active NaCl absorption by two apical cotransporters (Na^+,Cl^- and Na^+,K^+,2CL^-) fuelled by basolateral Na^+-K^+-ATPase (~). Remaining Cl^- uptake occurs via Cl^-/HCO_3^- anion exchange (AE). Endogenous metabolic CO_2 provides cellular HCO_3^- catalysed by carbonic anhydrase (C.A.), and the resulting H^+ is extruded via the basolateral membrane. Basolateral import of HCO_3^- also contributes to luminal HCO_3^- via Na^+: HCO_3^- cotransporter (NBC). (Information incorporated from Grosell, 2006 and Wilson, 2011).

In most fish, the two kidneys unite and open into the urinary bladder. The urinary bladder in freshwater acts as the final location for ion uptake to generate hypotonic urine, and minimise salt loss. While in seawater fish, the urinary bladder contributes to the absorption of monovalent ions and water, which will help in concentrating the divalent ions and reducing water loss (Marshall and Gorsell, 2006).

3.3.4 Skin and opercular membrane

The contribution of the skin to fish osmoregulation is minor in comparison to the other organs described above. Mucous cells in the skin can secrete mucus that is thought be involved in osmoregulation by reducing the permeability of the skin to ions and water (Manzon, 2002). Marine teleost gobies *Gillichthys mirabilis* have mitochondria-rich cells in their skin similar to the chloride-secreting cells in the fish gills of marine fish and it has been demonstrated that these cells are responsible for anion transport across *Gillichthys* skin (Marshall and Nishioka, 1980). The skin has also been implicated as a site of Ca^{2+} uptake in the freshwater rainbow trout (*Onchoryhnchus mykiss*) (Perry and Wood, 1985). The killifish opercular epithelium has proved to be an excellent general model for Cl^- secreting epithelia, it contains typical mitochondria-rich Cl^- secreting cells. These fish have a high capacity to adapt to changes in salinity and to acclimate from low to high salinity This involves a rapid phase during which the secretion of Cl^- via chloride cells located both in the opercular epithelia and in the gill is increased rapidly (Hoffmann et al., 2002).

3.4 Euryhaline fish osmoregulation

Euryhaline fish are unusual amongst teleosts in having the ability to osmoregulate

efficiently in waters of highly variable salinity. They are able to alter their pattern of osmoregulation rapidly if sudden fluctuations in salinity occur. Euryhaline fish require additional energy for the synthesis of new salt transporting proteins as the fish moves from salt to freshwater and vice versa (Kidder et al., 2006). Gills are probably the organs that consume most of the energy during osmoregulation, kidneys play a minor role in comparison to the gills, however, several changes occur in this organ that need energy in the form of ATP. The energy requirement of the gills and kidney are maintained by oxidation of glucose and lactate, where the liver is the main site for glucose turnover, therefore the liver (non-osmoregulatory organ) metabolism is enhanced to make glucose available to fuel other metabolic and osmoregulatory processes especially in gills and kidney (Sangiao-Alvarellos et al., 2003).

The gills are a vital osmoregulatory organ in euryhaline fish. In guppy (*Lebistes reticulates*), the density of mitochondria-rich cells (MR) in the gills increases with increasing environmental salinity indicating that they are the major sites of active exchange of ions in the euryhaline fish (Erkemn and Kolankaya, 2009). However, the situation is more complex than simply the density of these cells. This is because in freshwater fish there are at least two populations of mitochondria-rich cells demonstrated in the gill epithelia which appear to have separate functions in Na^+ and Cl^- uptake (Goss et al., 2001; Perry et al., 2003). The mitochondria-rich cells in marine teleost gills are clearly very different functionally, being involved in Na^+ and Cl^- secretion rather than uptake, and the same cell type probably drives the transport of both these ions (Evans et al., 2008). Galvez et al. (2002) studied the mitochondria-rich chloride cells in the freshwater trout gill. A

novel magnetic bead separation technique was used in their lab to isolate different MR cell subtypes. Results indicated that PNA (peanut lectin agglutinin) binds only to one sub type of MR cells on the apical surface of the gill epithelium. Hence, they characterized two populations of cells; the PNA-positive (PNA^+ MR cells) and the other subtype PNA-negative (PNA^- MR cells). The intestine is also a major osmoregulatory organ in the euryhaline fish especially when they acclimated to seawater (**3.3.2**) for instance, the European eel increase their drinking rate by > 10 fold (Martinez et al., 2005).

There are two types of euryhaline teleost fish; anadromous that migrate to freshwater to spawn such as populations of Salmonidae, and catadromous fish that migrate from freshwater to spawn, such as the European eel (*Anguilla anguilla*) and flounder (*Platichthys flesus*) (Maes et al., 2007). The sheepshead minnow, *Cyprinodon variegatus*, is an excellent regulator of plasma osmolality and they are exposed to large daily fluctuations in salinity (Haney, 1999). The tilapia (*Oreochromis mossambicus*) is another species that is able to maintain the body osmolality and NaCl concentrations in a narrow range independent from the environmental salinity (Cataldi et al., 2005).

3.5 Endocrine control of osmoregulation

The ability to regulate plasma ions in the face of a changing external salinity is a necessity for fish that move between freshwater and seawater, which a few species do as part of their normal life cycle. The need to respond to rapid changes in the external environment is crucial to osmoregulatory adaptation and is brought about by the neuroendocrine system. Some hormones are involved in the development and differentiation of transport epithelia

that control the ability of fish to migrate between freshwater and seawater (McCormick, 2001). During smolting in salmonid fish, the parr-smolt transformation, where the young salmon prepare to migrate to the ocean, is effected by several hormones including cortisol and growth hormone. Other hormones, in particular androgens and estrogens may negatively influence the smolting process and impair the seawater tolerance (Madsen et al., 1997; Bangsgaard et al., 2006).

Many studies have been conducted on the hormonal control of osmoregulation in euryhaline teleosts. It has been generally accepted that prolactin (PRL) is the dominant factor in regulating hydromineral balance in freshwater and that cortisol is the dominant factor in seawater (McCormick, 2001). Hormones play a vital role in homeostatic and acclimation demands of salt and water transport. In spite of the different transport needs among vertebrates, the hormones involved are similar (McCormick and Bradshaw, 2006). For example both growth hormone (GH) (Björnsson, 1997; Sangiao-Alvarellos et al., 2006) and prolactin (Sakamoto and McCormick, 2006) play a role in osmoregulation. Seale et al. (2002) observed a significant increase in plasma growth hormone and a rapid decline in plasma prolactin levels 6 h after transfer of tilapia (*Oreochromis mossambicus*) from freshwater to 80 % seawater and a rapid increase in prolactin after transfer from seawater to freshwater. In addition, the changes in prolactin levels were inversely correlated with changes in plasma osmolality. Prolactin is generally accepted as a freshwater-adapting hormone in most euryhaline fish, and its importance varies both between and within species (Manzon, 2002). One of the earliest known functions of prolactin in teleost fish was ion uptake (Sakamoto and McCormick, 2006) and decreased water influx.

Cortisol is another steroid hormone that has been identified as a seawater-adapting hormone in a large number of fish and it is specifically involved in ion uptake under fresh water conditions (McCormick, 2001). In particular, cortisol stimulates chloride cell proliferation and differentiation in the gills of fish (Foskett et al., 1983).

The GH/IGF-1 axis plays an important role in seawater adaptation in salmonid teleosts (Sakamoto et al., 1993; Mancera and McCormick, 1998). Plasma IGF-I increases during the parr-smolt transformation and seawater-acclimation in salmonids (Lindahl et al., 1985), and IGF-I mRNA expression increased in rainbow trout (*Oncorhynchus mykiss*) gills after seawater exposure (Sakamoto and Hirano, 1991). Poppinga et al. (2007) reported that rainbow trout injected intraperitoneally with somatostatin-14 (a potent inhibitor of growth hormone) reduced seawater adaptability by inhibiting the GH–IGF-1 axis.

3.6 Endocrine disruption of osmoregulation

Endocrine disrupting compounds can interfere with the endocrine system at various levels and exert their effects by several modes of action. In addition, there is increasing evidence for the effect of endocrine disruption on osmoregulation in freshwater and seawater fish from the literature (McCormick et al., 2005; Carrera et al., 2007; Lerner et al., 2007). However, there is little or no attention given to the comparative sensitivities of different species of fish osmoregulation to endocrine disruptors.

Most of what is known about the effects of endocrine disrupting chemicals has been focused on reproduction as a major axis, although other areas of endocrine system might be

a target of endocrine disruptors. The vast majority of these studies to date have investigated the interference with reproductive endocrinology, particularly estrogens, androgens, and their critical role in fish maturation and reproduction. Estrogens such as 17β-estradiol (E2), estrone (E1) and estriol (E3) are a group of steroid hormones that can act as endocrine disruptors (Costa et al., 2010). They are predominantly female hormones, which in mammals are important for maintaining the reproductive tissues, breasts, skin and brain health. The steroids of concern for the aquatic environment due to their endocrine disruption potential are primarily estrogens and artificial estrogens used in contraceptives, which include E2, E1, E3, 17α-ethynylestradiol (EE2) and mestranol (MeEE2) (Ying et al., 2002).

Hormones are not only involved in regulating reproductive processes, and some research on endocrine disruptors is now focusing away from purely reproductive endpoints. An increasing number of studies have started to focus on the effect of endocrine disrupting chemical on another physiological process such as osmoregulation. The impact of these chemicals on osmoregulatory abilities have been addressed in several species and provide evidence of the negative interaction with the physiological and biochemical impacts (Madsen et al., 1997; Vijayan et al., 2001; Guzman et al., 2004; Carrera et al., 2007). For example, waterborne exposure to xenoestrogens in salmon (*Salmo salar*) reduces behavioural and physiological components of smoltification such as lower migration frequency (reduced migratory drive) (Bangsgaard et al., 2006). The treatment with 17β-estradiol showed an inhibitory action and impairs the hypoosmoregulatory capacity of killifish (*Fundulus heteroclitus*) (Mancera et al., 2004). Significant increase in total and

ionic Ca^{2+} have been observed in the plasma of sea bream (*Sparus aurata*) induced by peritoneal implants containing 10 µg/g E2 (Guerreiro et al., 2002). Furthermore, estrogen receptors were found on the intestine and not in the gills of the sea bream (Socorro et al., 2000) and functional estrogen receptors were identified in an intestinal epithelial cells of female rat (Thomas et al., 1993), suggesting the involvement of E2 as an estrogen on Ca^{2+} homeostasis via the intestine rather than the gills.

4. PROJECT AIM

Thousands of chemicals are reaching the environment due to human activities. It is still a challenge for scientists to study and examine the effect(s) of these chemicals on the health of aquatic organisms. Some of these chemicals can affect the endocrine system of aquatic organisms and are classified as endocrine disruptors. The concerns about these chemicals have focused for many years on the effect on reproduction, where adverse effects have been shown. Very little is known about the effects of these chemicals on other physiological processes. In the present study, the main aim and the novelty was to investigate the effects of known environmental estrogens on osmoregulation as a relatively new axis for exploration. To allow a focus on the different osmoregulatory challenges found under freshwater and seawater conditions, the present study used euryhaline species that can acclimate to both these salinities. The reproductive life cycle of many euryhaline fish species is associated with a migration between freshwater and seawater, or vice versa. This is a particularly interesting combination of physiological systems to study with respect to the influence of endocrine disrupting chemicals, e.g. if osmoregulation is also disturbed

they may not even survive the transition from one salinity extreme to the other which is essential before they can begin the main reproductive spawning events.

To accomplish this study a range of endocrine disrupting chemicals was used in this study to examine the influence(s) on the osmoregulatory ability in the euryhaline fish. The studied chemicals proved to exist in the English rivers, and they were able to exert their effect even in ng/l level, due to their persistence and high potency. The species used were rainbow trout and three-spined stickleback as model euryhaline species. However, the different body sizes of these two species creates some problems of direct comparisons, particular in relation to tissues that can be sampled and measured.

The first experiment was therefore conducted as a directly compare and validate the use of osmoregulatory parameters in both plasma and tissue digests of one species (rainbow trout) acutely exposed to different salinities to assess the usefulness of these tissues in studying ion regulation and maintenance of water balance. The second experiment used one of the widely used synthetic estrogens 17α-ethinylestradiol (EE2) to examine the relative responsiveness of rainbow trout and sticklebacks, using vitellogenin induction and the osmoregulatory ability of these species both in freshwater and subsequently seawater. Nonylphenol is an extremely relevant environmental xeno-estrogen and was included as a third experimental chapter to study the effects of three different environmentally relevant concentrations of nonylphenol on ion regulation in rainbow trout in both freshwater and following rapid seawater transfer. The last experimental chapter focused on the effect of an

endogenous estrogen (17β-estradiol), but for the first time carried out the exposure on fish already acclimated to seawater, and specifically examined the intestinal calcification process and the subsequent role this plays in ionic and osmotic homeostasis.

A comparison of osmoregulatory responses in plasma, white muscle, and carcass of rainbow trout (*Oncorhynchus mykiss*) following acute salinity challenges

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A comparison of osmoregulatory responses in plasma, white muscle and carcass of rainbow trout (*Oncorhynchus mykiss*) following acute salinity challenges

1. ABSTRACT

Euryhaline teleosts regulate their internal osmotic and ionic status across a wide range of external salinities. Studies often rely on measurements on plasma when osmoregulatory status is perturbed, whereas tissue measurements are used for small fish with limited blood volume. However, a direct comparison is lacking for plasma and tissues, so the relationships between plasma, white muscle and carcass were examined for a range of osmoregulatory variables in rainbow trout (*Oncorhynchus mykiss*) following challenge with an acute (24 hour) transfer from freshwater to a hyper-osmotic salinity of either 25 or 35. Significant increases in plasma osmolality, [Na^+], [Cl^-], [Ca^{2+}], [Mg^{2+}] were observed when salinity was increased, but not for [K^+] which was tightly and independently regulated. The water content of both tissues showed reciprocal changes to plasma osmolality. The carcass ion content only showed a significant increase at the highest ambient salinity, except for [Cl^-] which was significantly elevated at both salinities. In white muscle, ions showed significant increases, except Cl^- and Ca^{2+} which were well regulated. Measurements from both tissues can provide reliable surrogates for most of the plasma osmoregulatory variables except Cl^- and Ca^{2+} when using muscle tissue. In the case of internal regulation of K^+ both tissues provide sensitive and quantitatively similar indicators of environmental salinity disturbance, whereas plasma does not.

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2. INTRODUCTION

Fish are in a continuous and intimate contact with the surrounding medium, and most fish live in water of a substantially different osmolality and ionic content from their plasma, resulting in osmoregulatory challenges that must be compensated to allow homeostasis of osmotic and ionic variables in their internal body fluids (Kidder et al., 2006; Evans, 2010). Most teleosts are excellent osmoregulators, being able to tightly maintain a stable osmolality and ionic content of their internal milieu, and some species are also euryhaline being able to achieve this across a wide range of ambient salinity (Evans et al., 2005; Marshall and Grosell, 2006; Evans, 2010). In freshwater fish, the salt content of the blood is typically at least 100 times higher than the surrounding freshwater, whereas in marine fish it is one-third of the ambient seawater (Sakamoto and McCormick, 2006). There are essential strategies that teleost fish have evolved to balance the composition of the extracellular fluid according to the prevailing challenge presented by the external environment. Stressors such as increasing or decreasing the surrounding water salinity or exposure to an ionoregulatory toxicant can induce hydromineral disturbances in fish that therefore require regulatory mechanisms to be rapidly regulated up or down.

Freshwater fish are hyper-osmotic to the surrounding medium; therefore, they encounter the problem of ion loss and osmotic water gain (Evans et al., 2005). To compensate for this problem they produce a copious volume of extremely dilute urine (hypotonic) which involves a substantial glomerular filtration rate (Evans et al., 2005; Marshall and Grosell, 2006). To minimise ionic losses via this large urinary volume, monovalent and divalent

ions are reabsorbed by the renal epithelia (Bijvelds et al., 1996; Evans et al., 2005). However, there are still unavoidable net ionic losses via both the urine and by diffusive efflux across the gills, and these are balanced by the active uptake of monovalent and divalent ions by the gills and via the gut from dietary sources.

Seawater fish are faced with the reverse situation to freshwater fish, i.e. an osmotic loss of water and gain of inorganic ions (Evans, 2010). This water loss is balanced by continuously drinking a large volume of sea water followed by the uptake of Na^+ and Cl^- ions, and the precipitation of ingested Ca^{2+} and Mg^{2+} ion as their insoluble carbonates, by various mechanisms in the gastrointestinal tract (Grosell, 2006), that collectively play a critical role in osmoregulation by driving water absorption in the intestinal lumen (Marshall and Grosell, 2006; Whittamore et al., 2010). The extra load of Na^+ and Cl^- from the intestine is excreted via the gills and skin epithelia via a linked mechanism involving mitochondria-rich cells (Marshall and Grosell, 2006). The lack of a loop of Henle and concentrating mechanism in the kidney of teleosts prevents the production of urine which is hyperosmotic to the plasma and it appears that the main function of the kidney in marine fish is the excretion of excess divalent ions, particularly Mg^{2+} and SO_4^{2-} (Evans et al., 2005; Marshall and Grosell, 2006).

A small proportion of teleosts are euryhaline, and the physiological capacity to switch between extremes of external salinity may be the reason that teleosts are found in almost all aquatic habitats (McCormick, 2001). There are two main types of euryhaline fish, the

anadromous fish (breeding in fresh water) such as Atlantic salmon (*Salmo salar*) (Nilsen et al., 2008) and rainbow trout (*Oncorhynchus mykiss*) (Landergren, 1999), and the catadromous fish (breeding in sea water) such as the European eel (*Anguilla Anguilla*) (van Ginneken and Maes, 2005) and European flounder (*Platichthys flesus*).

Numerous studies have reported that fish challenged with an altered salinity environment, or osmoregulatory toxicants (such as acid pH and metals), experience dramatic changes in either plasma variables such as osmolality, [Na^+], [Cl^-], carcass or muscle variables such as water and ion content (Lotan, 1969; Wood et al., 1988; Wilson and Wood, 1992; Hansen et al., 1993; Nussey et al., 1995; Fielder et al., 2007). Such data are often used to infer the degree of adaptability of their osmoregulatory processes to such challenges and indicate the physiological mechanisms that are utilised in restoring a stable internal milieu in terms of ionic and water balance. However, few studies have simultaneously reported plasma and tissue osmoregulatory variables (Bath and Eddy, 1979; Beaumont et al., 2000), and so interpretation and comparison of these two different sources of data are not always straightforward.

The major aim of the present study was to compare the use of plasma and tissue osmoregulatory variables in quantifying the responses of a euryhaline fish species to various environmental salinity challenges. This is particularly relevant to studies which involve the direct comparison of fish of different sizes, as blood plasma volume is often difficult to obtain or the volume can be limiting for analysis in small sized fish. However,

the timescale and magnitude of changes in osmoregulatory variables within plasma and tissues have not previously been directly compared within the same fish.

3. MATERIALS AND METHODS

3.1 Fish

Thirty rainbow trout (*Oncorhynchus mykiss*) (body mass = 44.5 ± 1.8 g and length = 15.8 ± 0.2 cm) were obtained from Hatchlands trout farm (Devon, UK). The fish were kept in aerated, dechlorinated freshwater (Na^{+} = 390; K^{+} = 47; Ca^{2+} = 598; Mg^{2+} = 152; Cl^{-} = 400 µM; pH 7.5 and temperature 11.9 ± 0.04 °C) at the University of Exeter, and were fed daily on a commercial pelleted feed (BioMar, 3 mm, Aqualife, Denmark) at a rate of 1 % body mass per day.

3.2 Experimental design and exposure

Three aquaria were prepared for acute exposure to different salinities under static conditions, with aeration supplied to each tank. One tank was filled with the same freshwater (FW) as used for the holding condition. Two different elevated water salinities were then also prepared; i) a salinity of 25 (SW_{25}), and ii) a salinity of 35 (SW_{35}) (Table 1). The latter represents full strength oceanic seawater, and both 25 and 35 salinity treatments were made up using commercial sea salts (Tropic Marin®, Germany) added to deionised water. Conductivity (control freshwater) or salinity (seawater salinities of 25 and 35) were measured using a portable salinometer (YSI handheld probe and meter; Model 85, Yellow Springs; Ohio, USA).

Ten fish were transferred to each of the treatment tanks, and left undisturbed for 24 h before terminal sampling. Water samples were taken from the exposure chambers to measure the ambient ion concentrations by the ion chromatography (Dionex ICS-1000, Sunnyvale, CA, USA) following appropriate dilution (Table 1).

Table 1: Water chemistry in the three different salinity exposures (*na* = not applied)

Parameters	FW	SW of 25	SW of 35
Salinity	0.1	24.8	35.1
Temperature (°C)	11.9	12.0	11.8
pH	7.48	8.05	8.15
Osmolality (mOsm kg^{-1})	*na*	704	1006
Conductivity (mS)	0.20	33.9	46.8
[Na^+] (mM)	0.402	433.8	486.1
[Cl^-] (mM)	0.390	493.7	542.8
[K^+] (mM)	0.062	8.5	14.9
[Ca^{2+}] (mM)	0.591	8.7	11.7
[Mg^{2+}] (mM)	0.161	43.6	61.1

3.3 Fish sampling

Rainbow trout were anesthetized in a 100 mg/l solution of MS222 (Pharmaq Ltd, UK), prepared in water collected from the relevant exposure tank that was additionally buffered with 300 mg/l of $NaHCO_3$ (Fisher Scientific, UK) followed by vigorous aeration to restore normal dissolved CO_2 and pH levels. Blood samples (~1 ml) were collected by caudal puncture via a 23G needle into heparinised syringes (Monoparin heparin sodium, 5000 I.U./ ml; CP Pharmaceuticals, Ltd., Wrexham, UK), and were transferred to microcentrifuge tubes held on ice and containing aprotinin (10 µl; Sigma-Aldrich) to reduce enzymatic

degradation when measuring vitellogenin, and then centrifuged (13,000 rpm for 5 min at 4 °C; Heraeus by Biofuge Fresco, Kendro laboratory products, Germany) to obtain plasma. A portion of the plasma was stored at −20 °C for later analysis of vitellogenin, and the remainder was used for measuring the plasma cations (sodium, potassium, calcium and magnesium). The plasma osmolality, chloride and total protein were measured by using fresh plasma on the day of sampling.

A piece of white muscle (0.59 ± 0.03 g) was dissected from just below the dorsal fin and above the lateral line, and transferred to a pre-weighed Teflon tube. The remaining carcass without the head and the viscera (29.2 ± 1.2 g) was transferred to a pre-weighed glass vessel. The white muscle and the carcass samples were kept at 70 °C in an oven to dry for determination of their water content (%) and subsequent tissue ion analysis.

3.4 Analytical techniques

3.4.1 Analysis of plasma

Osmolality was measured on 10 µl of fresh plasma using a vapour pressure osmometer (Wescor Vapro 5520), and the chloride concentration was measured in 20 µl of fresh plasma using a chloride analyzer (Corning M925, UK). The cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) were measured by diluting the fresh plasma (× 201) for analysis by ion chromatography.

Plasma protein was measured by the colourimetric Biuret protein assay that is based on the

formation of a violet complex between the copper ions and the peptide bond linkage. The assay method used was followed by Scown et al. (2009), and then samples read on a microplate reader (Molecular Device SpectraMax 340pc) at 550 nm and the plasma protein concentrations were determined against the protein standard curve (prepared using bovine serum albumin, Sigma-Aldrich, UK).

Plasma vitellogenin was measured by the homologous vitellogenin ELISA protocols (Tyler et al., 2002). Plasma was diluted at least 1:10 prior to analysis of vitellogenin concentrations, and the detection limits of the rainbow trout vitellogenin ELISA for plasma were approximately 30 ng/ml.

3.4.2 Carcass and white muscle water and ionic content

The oven-dried white muscle and the carcass samples were weighed daily until the dry mass was constant (~ 6 days for white muscle, and ~10 days for carcass). Concentrated (69 %) nitric acid (15.6 N; AnalaR, BDH Laboratory Supplies, UK) was then added to the samples at a ratio of 5:1 (ml HNO_3 to g of dry mass) and samples were left 48 h for complete cold digestion before making the dilutions.

The white muscle acid digest was diluted 100-fold in ultra-pure water (18 MΩ; Elga-Elgastat, Maxima, UK) for [Na^+], [Ca^{2+}] and [Mg^{2+}] measurement by atomic absorption spectrophotometry (AAS; Thermo Elemental SOLAAR AAS). Another set of samples was diluted 500-fold for measuring [K^+] by AAS and [Cl^-] using a colourimetric assay (Zall et

al., 1956) where the absorbance readings were recorded on a spectrophotometer (CECIL, CE1010, 1000 Series, Cambridge, UK). All experiments were conducted with the approval of the University of Exeter Ethics Committee and under a UK Home Office license (PPL 30/2217).

3.5 Statistical analysis

All parameters were analysed for differences between treatments using one-way ANOVA by using Sigmastat 3.5 (Systat Software, Inc.). Post hoc tests used to analyse data when the normality test failed and data were considered the nonparametric Kruskal-Wallis test was performed with post hoc comparisons made using Tukey test to differ significantly when the p-value was < 0.05. Throughout this paper, the data are presented as mean ± standard error of the mean. Relationships between the plasma variables and their equivalent data for tissues (either white muscle or carcass) were analysed by linear regression, and the goodness of fit (r^2) and p value for each relationship was calculated. For all statistical analyses, $p < 0.05$ was considered significant.

4. RESULTS

4.1 Plasma, white muscle and carcass monovalent ions comparison

4.1.1 Plasma osmolality and tissue water content

All the freshwater-acclimated rainbow trout used for the salinity challenges in this study survived the 24 h exposure to the different salinities. The increased salinities caused a significant increase in the plasma osmolality of both SW_{25} and SW_{35} groups in comparison

to the FW group. In white muscle, the water content was significantly different in all three salinity treatments, with a progressive decline in the SW_{25} and SW_{35} groups in comparison to the FW group, indicating proportionally more dehydration of this tissue with increasing ambient salinity. A similar trend was observed for the carcass water content, being reduced significantly in SW_{35} group in comparison to the FW group and the SW_{25} (Figure 1A).

There were highly significant and inverse relationships between either white muscle or carcass water content and the plasma osmolality (i.e., plasma osmolality increased while tissue water contents decreased with higher environmental salinity) (Figure 1B).

4.1.2 Plasma, white muscle and carcass sodium [Na^+]

The plasma [Na^+] increased significantly in both SW_{25} and SW_{35} in comparison to FW group. The white muscle [Na^+] presented a significant increase in SW_{25} and SW_{35} groups in comparison to the FW group, and the two salinity groups are significantly different from each other. The carcass [Na^+] increased significantly in the highest salinity (SW_{35}) only in comparison to the FW and SW_{25} group (Figure 2A).

Plasma and white muscle [Na^+] showed a strongly significant relationship where they tended to increase together in response to elevated salinity. The plasma and carcass [Na^+] demonstrated a highly significant positive relationship where both variables tend to increase together (Figure 2B).

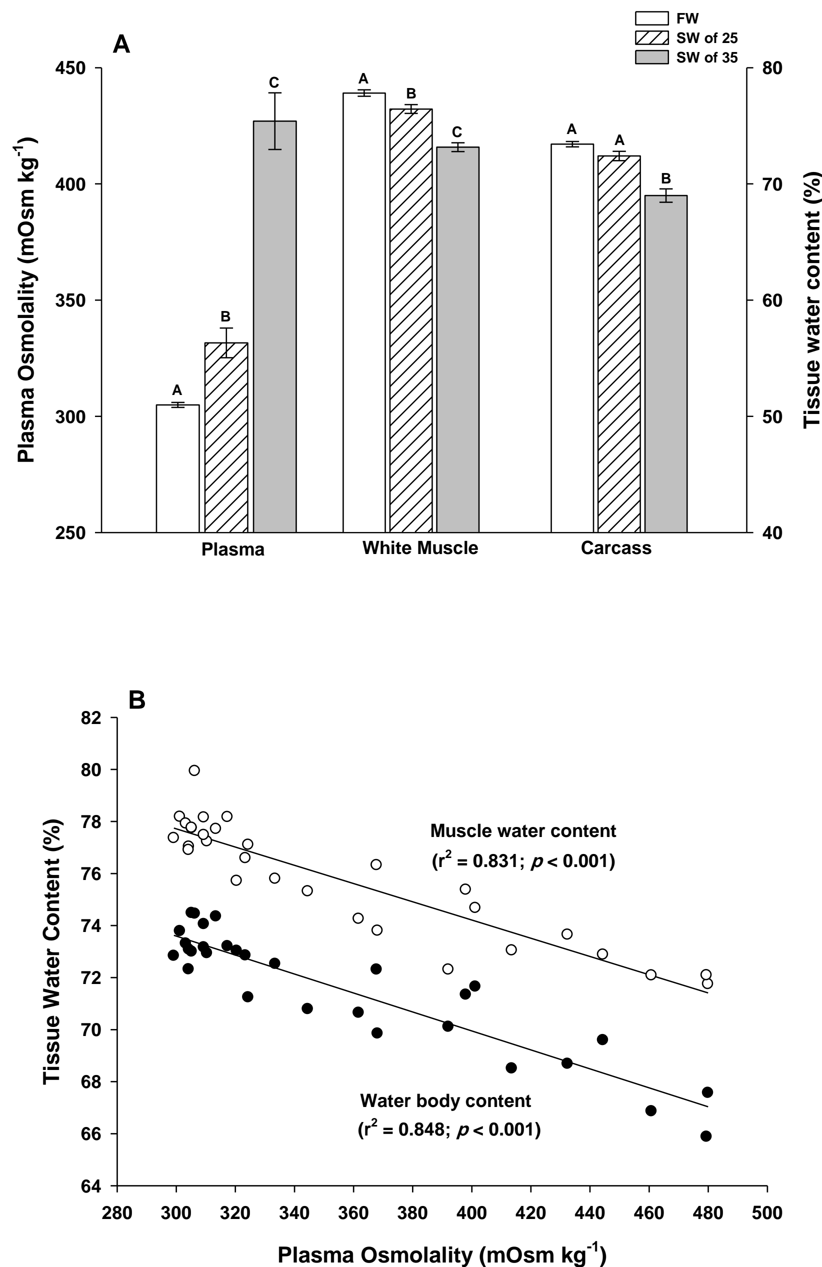

Figure 1: Plasma osmolality and various tissue water contents of freshwater-acclimated rainbow trout after 24 h exposure to different salinities (S=25 and S=35). **(A)** Mean ± SEM values for plasma osmolality, and water content of tissues across the three salinity treatments. Different letters indicate significant difference ($p < 0.05$) between the different salinity treatments. **(B)** The relationship between various tissues water contents and plasma osmolality. The linear regression equation for white muscle water content v. plasma osmolality ($y = -0.0351x + 88.239$), and for carcass water content v. plasma osmolality ($y = -0.0365x + 84.539$).

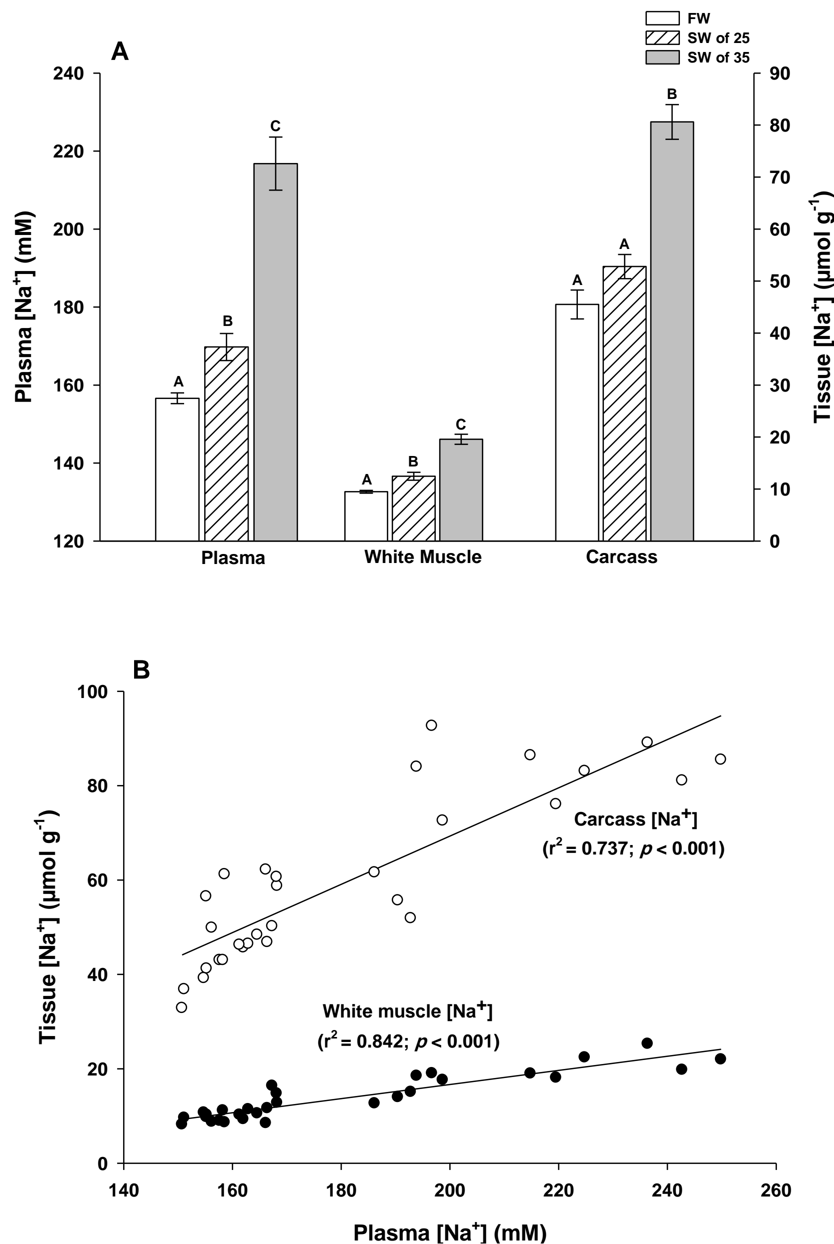

Figure 2: Plasma [Na^+] and various tissue [Na^+]of freshwater-acclimated rainbow trout after 24 h exposure to different salinities (S=25 and S=35). **(A)** Mean ± SEM values for plasma Na^+, and tissues Na^+ across the three salinity treatments. Different letters indicate significant difference ($p < 0.05$) between the different salinity treatments. **(B)** The relationship between various tissues Na^+ and plasma Na^+. The linear regression equation for white muscle Na^+ v. plasma Na^+ ($y = 0.15x - 13.314$), and for carcass Na^+ v. plasma Na^+ ($y = 0.512x - 32.985$).

4.1.3 Plasma, white muscle and carcass chloride [Cl^-]

Plasma [Cl^-] increased significantly with increasing ambient salinity with all three treatments being significantly different from each other. In contrast, no change was detected in the white muscle [Cl^-] in any of the groups. The carcass [Cl^-] followed the trend of plasma Cl^- in terms of increasing with raising the ambient salinity and all the groups were significantly different (Figure 3A).

The plasma and white muscle [Cl^-] showed a positive and significant relationship, but noticeably weaker than the relationship for Na^+. Plasma and carcass [Cl^-] showed a highly significant relationship (Figure 3B).

4.1.4 Plasma, white muscle and carcass potassium [K^+]

Plasma [K^+] did not differ between the three salinities. The white muscle K^+ showed a significant increase in the SW_{35} group in comparison to the FW group only. The carcass K^+ increased significantly with elevating the surrounding salinity in the SW_{35} in comparison to both the FW and SW_{25} groups (Figure 4A). Accordingly, there was no significant relationship between either the white muscle or carcass K^+ content and the plasma [K^+] (Figure 4B).

4.2 Plasma, white muscle and carcass divalent ions comparison

4.2.1 Plasma, white muscle and carcass [Ca^{2+}]

The plasma [Ca^{2+}] increased significantly with elevated ambient salinity in both the SW_{25}

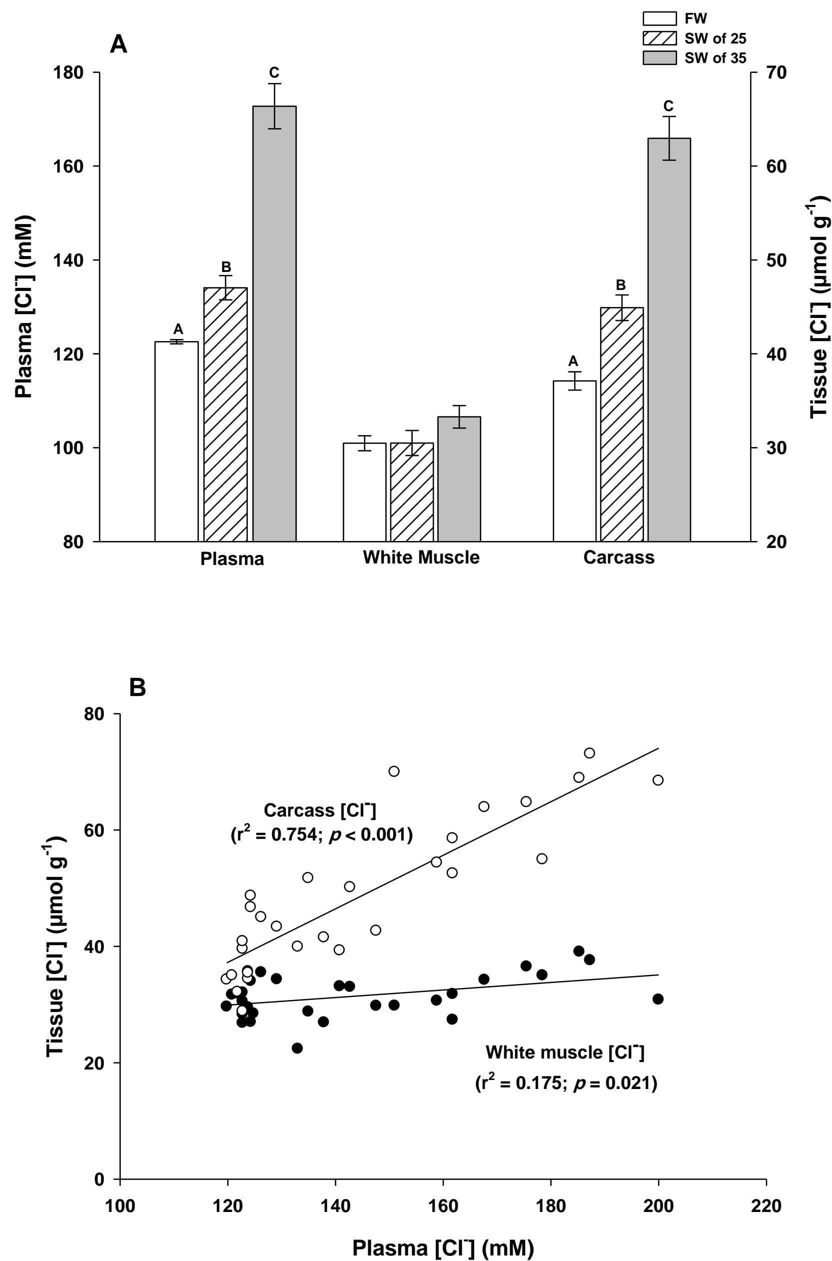

Figure 3: Plasma [Cl^-] and various tissue [Cl^-] of freshwater-acclimated rainbow trout after 24 h exposure to different salinities (S=25 and S=35). **(A)** Mean ± SEM values for plasma Cl^-, and tissues Cl^- across the three salinity treatments. Different letters indicate significant difference ($p < 0.05$) between the different salinity treatments. **(B)** The relationship between tissue Cl^- and plasma Cl^-. The linear regression equation for white muscle Cl^- v. plasma Cl^- ($y = 0.065x + 22.126$), and for carcass Cl^- v. plasma Cl^- ($y = 0.460x - 17.965$).

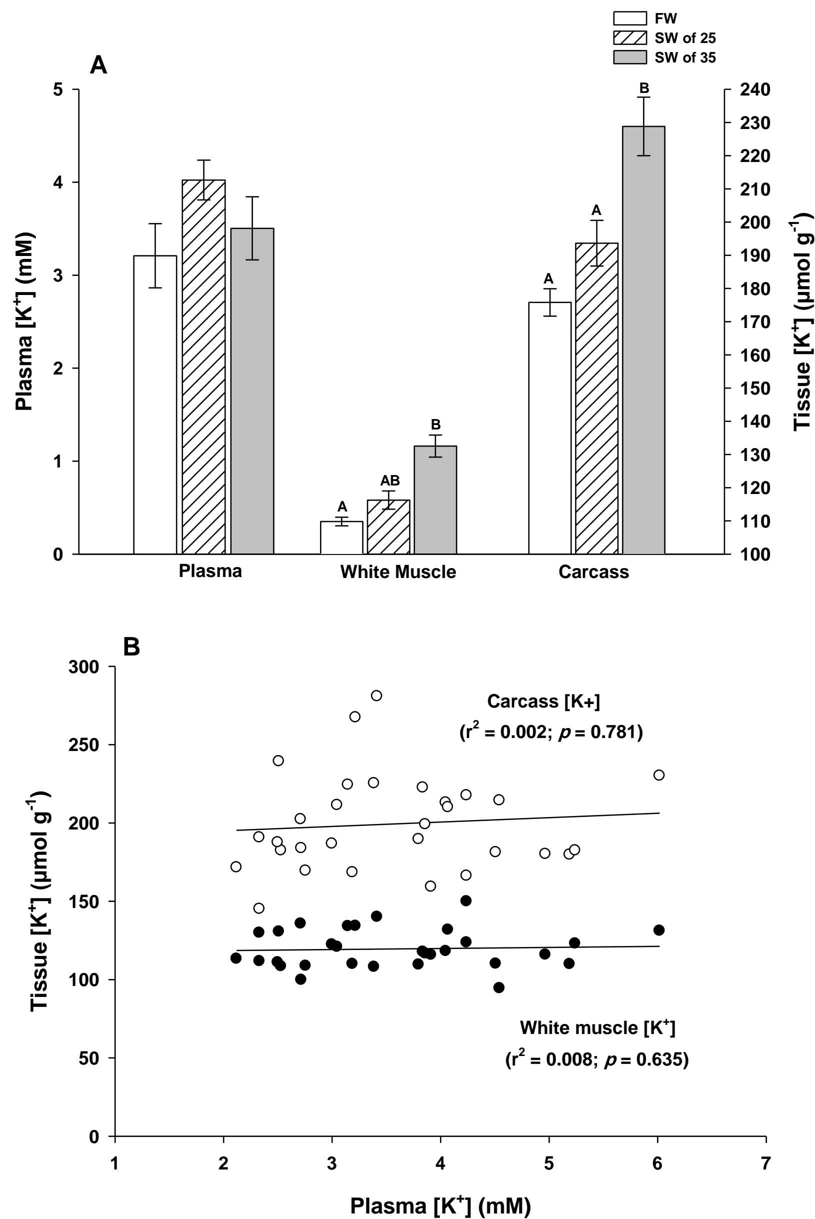

Figure 4: Plasma [K^+] and various tissue [K^+] of freshwater-acclimated rainbow trout after 24 h exposure to different salinities (S=25 and S=35). **(A)** Mean ± SEM values for plasma K^+, and tissues K^+ across the three salinity treatments. Different letters indicate significant difference ($p < 0.05$) between the different salinity treatments. **(B)** The relationship between various tissues K^+ and plasma K^+. The linear regression equation for white muscle K^+ v. plasma K^+ ($y = 0.670x + 117.147$), and for carcass K^+ v. plasma K^+ ($y = 2.789x + 189.427$).

and SW_{35} groups in comparison to the FW group. White muscle [Ca^{2+}] was extremely tightly regulated and showed no significant difference between the FW group and either the SW_{25} or SW_{35} groups, although the two high salinity groups were significantly different to each other. The carcass [Ca^{2+}] increased significantly in the highest salinity group SW_{35} in comparison to the FW and SW_{25} groups (Figure 5A).

There was a no significant relationship between the plasma and white muscle [Ca^{2+}], while the carcass Ca^{2+} presented a positive relationship with the plasma [Ca^{2+}] (Figure 5B).

4.2.2 Plasma, white muscle, and carcass magnesium [Mg^{2+}]

The plasma [Mg^{2+}] increased significantly in the SW_{25} and SW_{35} in comparison to FW group. Both tissues (white muscle and carcass) followed the same trend in terms of increasing significantly in the high salinity groups (Figure 6A). There were strong positive relationships between the plasma [Mg^{2+}] and both the white muscle and carcass Mg^{2+} contents (Figure 6B).

4.3 Plasma protein and vitellogenin

There was no significant effect (p = 0.063) of salinity on plasma protein in any of the groups (FW = 3.49 ± 0.1; SW $_{25}$ = 3.52 ± 0.1; SW_{35}= 3.87 ± 0.2 g/dl). No significant differences (p = 0.312) in plasma vitellogenin were observed after exposing the fish to different salinities (FW = 2.34 ± 1.1; SW $_{25}$ = 1.37 ± 0.3; SW_{35}= 2.82 ± 1.0 µg/ml).

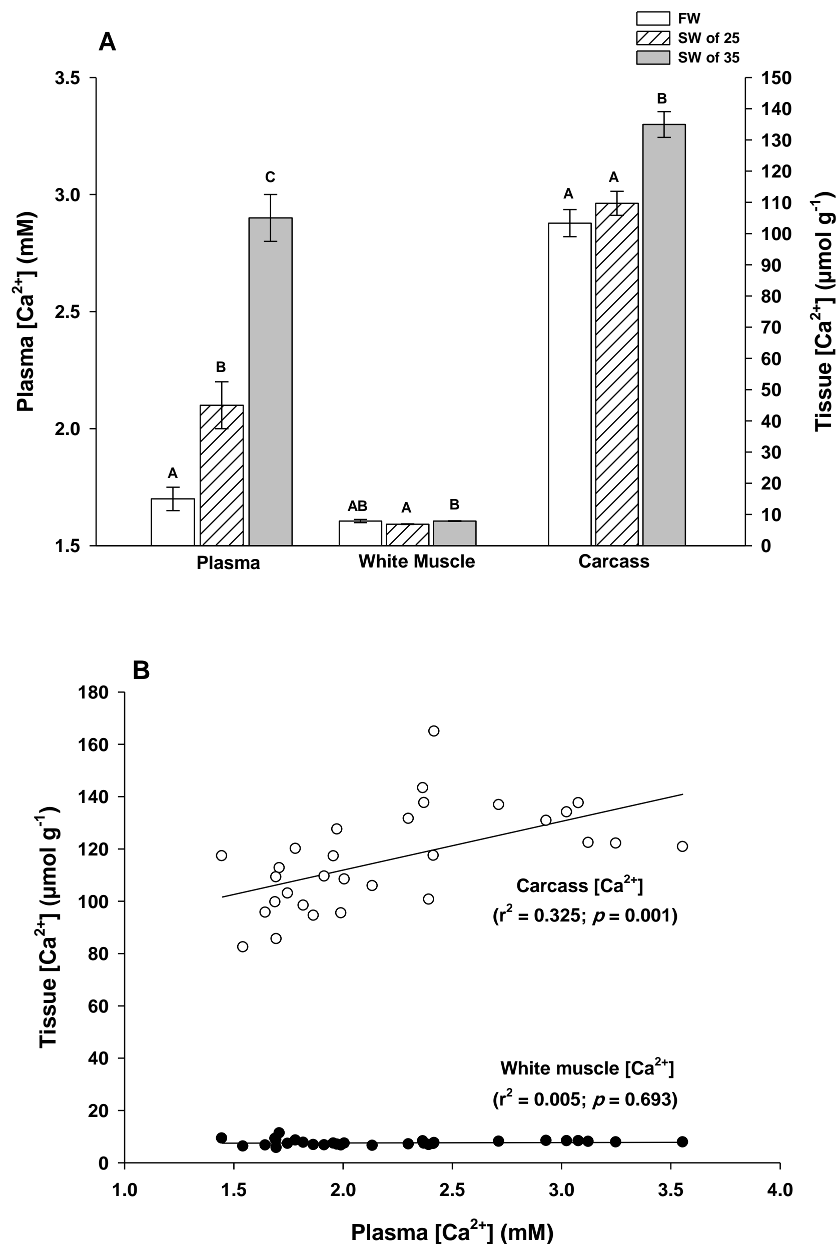

Figure 5: Plasma [Ca^{2+}] and various tissue [Ca^{2+}] of freshwater-acclimated rainbow trout after 24 h exposure to different salinities (S=25 and S=35). **(A)** Mean ± SEM values for plasma Ca^{2+}, and tissues Ca^{2+} across the three salinity treatments. Different letters indicate significant difference ($p < 0.05$) between the different salinity treatments. **(B)** The relationship between various tissues Ca^{2+} and plasma Ca^{2+}. The linear regression equation for white muscle Ca^{2+} v. plasma Ca^{2+} ($y = 0.145x + 7.263$), and for carcass Ca^{2+} v. plasma Ca^{2+} ($y = 18.650x + 74.594$).

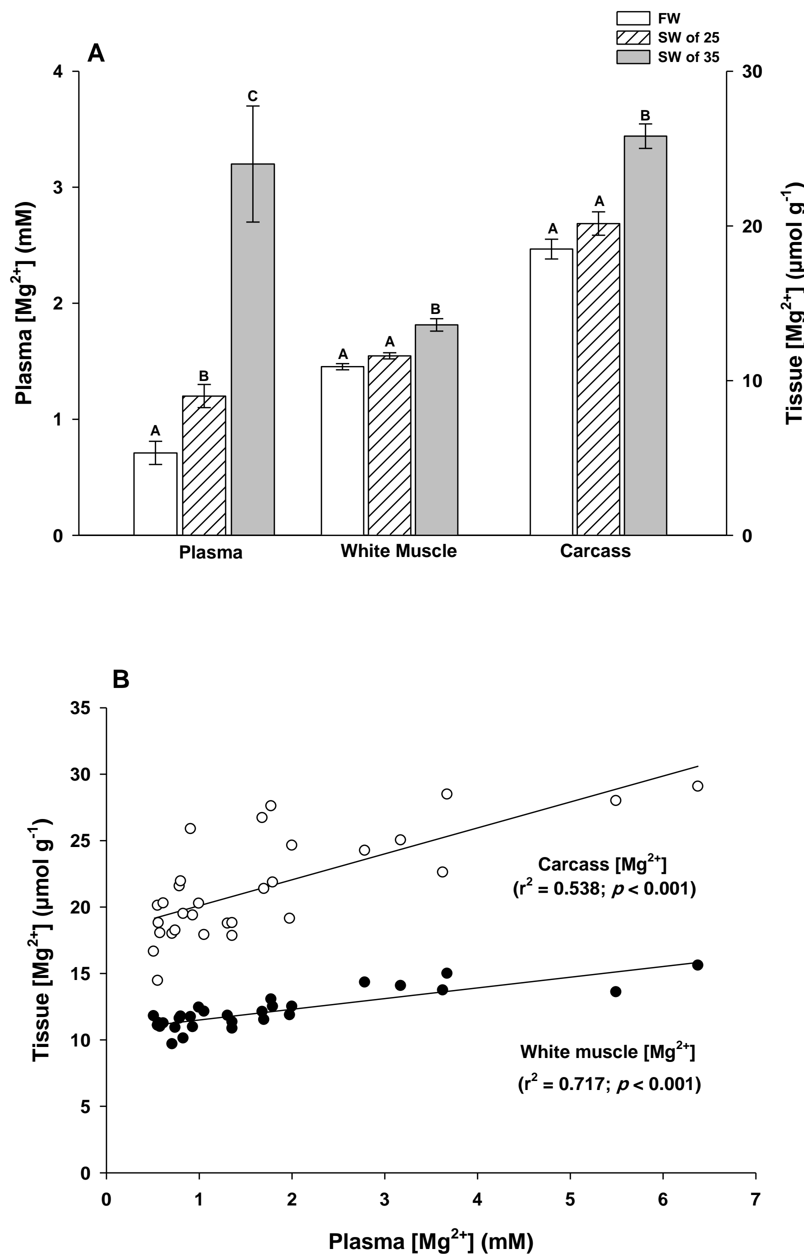

Figure 6: Plasma [Mg^{2+}] and various tissue [Mg^{2+}] of freshwater-acclimated rainbow trout after 24 h exposure to different salinities (S=25 and S=35). **(A)** Mean ± SEM values for plasma Mg^{2+}, and tissues Mg^{2+} across the three salinity treatments. Different letters indicate significant difference ($p < 0.05$) between the different salinity treatments. **(B)** The relationship between various tissues Mg^{2+} and plasma Mg^{2+}. The linear regression equation for white muscle Mg^{2+} v. plasma Mg^{2+} ($y = 0.804x + 10.699$), and for carcass Mg^{2+} v. plasma Mg^{2+} ($y = 1.951x + 18.149$).

5. DISCUSSION

5.1 Plasma osmolality comparison with white muscle and carcass water content

The present study established the relationships between the plasma, white muscle and carcass for a range of osmoregulatory variables. Several significant relationships were detected between the plasma and the two tissue compartments which indicate that in many cases the tissue data can be a suitable surrogate of the plasma osmoregulatory status in response to environmental challenge when no plasma samples are available. However, for some of these variables, certain compartments do not appear to be suitable for indicating disturbances to internal ion or osmotic regulation. In general, as expected, there were significant changes in the majority of the plasma, white muscle and carcass ions with increasing the ambient salinity, and the observed changes in these internal compartments were generally in parallel (or reciprocal in the case of water content).

After transfer to different salinities, an initial rapid phase of internal changes occurs in fish. The pronounced increase in the plasma osmolality was evidence of hyperosmotic stress due to the osmotic loss of water and the diffusive influx of ions during the acute exposure to elevated salinities. There was ~ 9 and 40 % increase in the plasma osmolality in the salinity of 25 and 35, respectively, in comparison to trout in freshwater. The elevated plasma osmolalities appear to be accounted for by the parallel increases in the inorganic salts (Leray et al., 1981) as the sum of all the measured plasma ions underwent very similar increases. Several studies have reported plasma osmolality to increase after 24 h transfer from freshwater to seawater in different euryhaline species such as coho salmon

(*Oncorhynchus kisutch*) (Young et al., 1995) and Atlantic salmon (*Salmo salar*) (Rydevik et al., 1990).

As predicted, the tissue water content decreased after 24 h exposure to increased ambient salinity due to the osmotic loss of water from the fish. The degree of initial dehydration during such acute challenges has been previously shown to depend on the extent of the salinity change (Hwang et al., 1989) and this was true in the present study. Regression analysis showed that the decreases in water content of both white muscle and carcass during a dehydration stress quantitatively reflect the simultaneous increases in plasma osmolality, and thus water content from either compartment can be successfully used as a surrogate for, or to predict the plasma osmolality status.

Lotan (1973) indicated that the euryhaline teleost (*Aphanius dispar*) increased the water content in muscle after transfer to freshwater from 300 % seawater because of the cells swelling where no volume regulation occurred during the first 24 h. The swelling of the muscle cells was due to uptake of water from the extracellular fluids of the fish body. Hence, in our study the opposite happened, the significant decrease in the white muscle water content detected can be explained by the shrinkage of muscle cells (Lundgreen et al., 2008). Interestingly, it has been observed that the muscle tissue showed a higher water content than the carcass in our study. The same finding was reported by Bath and Eddy (1979) where they examined the exposure of rainbow trout to sudden changes in salinity. The most obvious explanation is that the carcass measurement includes a large fraction

which will be the skeletal material, and bone has a very low water content compared to other tissue (Cameron, 1985) such as muscle.

5.2 Plasma, white muscle and carcass monovalent ions

Seawater contains considerably higher levels of all the monovalent ions, Na^+, Cl^- and K^+, than the plasma of teleost fish. The acute transfer from freshwater to seawater therefore rapidly reverses the external-to-internal diffusion gradients for all these ions resulting in greater uptake across the gills as well as via ingested seawater in the gut (Bath and Eddy, 1979). The increase in ambient salinity caused a general increase in plasma ions in comparison to the freshwater fish except for the plasma K^+ which was tightly regulated in all treatments. The plasma K^+ results presumably indicates that rainbow trout may have an adequate and rapidly induced mechanism for maintaining the plasma K^+ balance, and the rate of flux between both the external medium and the extracellular fluids, and between the intracellular and extracellular fluids to regulate a very stable plasma concentration of K^+. In contrast to the plasma K^+, both the white muscle and carcass K^+ contents increased significantly with ambient salinity. Accordingly, there was no significant relationship between the K^+ content of either tissue and the plasma [K^+]. Although there is clearly an extra load of K^+ from the seawater entering the fish plasma (presumably via the gills and from ingested seawater in the gut), we speculate that this extra load of K^+ is very rapidly transferred from the plasma to the other body tissues. Therefore, the second conclusion from this study is that the tissues can provide very good indicators of disturbance to internal

K^+ regulation caused by external salinity challenge, whereas the plasma does not indicate this because it is so rapidly regulated.

Elevations in both [Na^+] and [Cl^-] were detected in the plasma of SW_{25} and SW_{35} fish in comparison to the FW group as expected (see above). Parallel increases were observed in both white muscle and carcass for Na^+. However, white muscle would appear to be a slightly better indicator of plasma Na^+, as the mean value for the carcass Na^+ content of fish transferred to a salinity of 25 was not statistically distinguishable from the freshwater group. This was similar to the study of Prodocimo et al. (2007) on a freshwater rainbow trout that showed proportional increases in plasma Na^+ at several elevated salinities, but carcass Na^+ was only elevated in full strength seawater.

By contrast, to sodium, increases in tissue Cl^- that paralleled those in plasma were only observed in the carcass. Although there was a statistically significant relationship between white muscle [Cl^-] and plasma [Cl^-] across all three treatments, the ability to predict one from the other was extremely low (r^2 = 0.175), and there were no significant differences between the mean values of the three groups. Thus, muscle [Cl^-] has no value as a surrogate for plasma [Cl^-] during such an osmoregulatory challenge, whereas carcass [Cl^-] provides a viable alternative. Therefore, the third conclusion is that white muscle [Na^+] is the best indicator of plasma [Na^+], whereas carcass Cl^- is the only option as a surrogate for plasma [Cl^-].

5.3 Plasma, white muscle and carcass divalent ions

Both plasma Ca^{2+} and Mg^{2+} results showed increases that were proportional to the ambient salinity. Fuentes et al, (1997) also reported that the plasma Ca^{2+} and Mg^{2+} increased in rainbow trout in response to elevated external salinity, which was attributed to either increased uptake of these ions via the gut (paralleling increased drinking rate following acute salinity transfer in rainbow trout; Perrott et al., 1992) or accelerated brachial influxes. A large increase in Ca^{2+} uptake in rainbow trout after increasing the salinity (Prodocimo et al., 2007) was explained by increased activity of branchial basolateral Na^{+}/Ca^{2+} exchanger (Verbost et al., 1994).

There was no significant relationship between white muscle and plasma Ca^{2+}, due to impressively tight regulation of the total Ca^{2+} content of the white muscle concentration with changing the ambient salinity except between the two salinity groups across the range of salinities used. White muscle Ca^{2+} can clearly not be used as an indicator of salinity disturbances to internal Ca^{2+} regulation. By contrast, a significant relationship was obtained between the carcass Ca^{2+} data and the plasma [Ca^{2+}], although the r^2 value was low (0.325), which indicates a limited ability to predict plasma levels if only carcass measurements are made. In addition, only the mean value of the SW_{35} group was significantly different from the freshwater group. This no doubt reflects the vast amount of background Ca^{2+} already sequestered within the carcass (primarily in the bones) which forms a rather slowly exchangeable pool (Cameron, 1985) with new Ca^{2+} entering the body from the external sea water over the previous 24 hour salinity challenge. Thus, although the carcass offers a

potentially useful surrogate for disturbances to Ca^{2+} regulation, it is not quite as sensitive as plasma measurements.

Both the white muscle and carcass Mg^{2+} data showed a strong and significant relationship with the plasma [Mg^{2+}]. However, for both tissues only the mean value of the SW_{35} group was significantly different from the freshwater group, these tissue measurements were less sensitive than plasma at detecting environmental disturbance to Mg^{2+} balance. This is probably related to the fact that while magnesium is found as only a minor contributor (relative to calcium) to the solid component of bone and scales, Mg^{2+} is present either ionized, complexed or protein bound in the cells of all tissues at rather high levels, being the second most abundant intracellular cation after K^{+}. Thus, the intracellular compartment serves as a very large reservoir of carcass Mg^{2+} relative to the small percentage present in the extracellular fluid (Bijvelds et al., 1998). Therefore, acute increases in Mg^{2+} uptake via the gills or gut will produce more noticeable changes in plasma than in any tissue compartment.

In conclusion, measurements of Ca^{2+} and Mg^{2+} in the carcass can provide a fairly useful surrogate for the plasma levels of these divalent cations. The same is true for white muscle Mg^{2+}, whereas this tissue is cannot be used as an indicator of plasma Ca^{2+}, due to the exceptionally rapid regulation of intracellular levels of this ion during acute environmental disturbance.

5.4 Plasma protein and vitellogenin

Measuring the total plasma protein is a broad indicator of health, stress and well being (Riche, 2007). More specific to osmotic disturbances, plasma protein has been used to indicate rapid (hours/days) changes in plasma fluid volume because protein movements into and out of the vascular system are much slower than fluid shifts (McDonald et al., 1980). Therefore, fish undergoing dehydration due to an acute seawater challenge would be expected to have elevated plasma [protein]. Although there was a trend for increasing plasma [protein] with ambient salinity in the present study, this was not quite significant ($p = 0.063$). Generally, stress is also known to reduce plasma vitellogenin levels in fish (Carragher et al., 1989), and fish in this study being stressed by change in salinity, but no significant change of the plasma vitellogenin induction observed in this study. In another words, that shows the acute salinity challenge alone does not affect plasma vitellogenin, and any other effect on plasma vitellogenin observed in this PhD study in the next coming chapters must be due to the prior exposure to endocrine disruptor.

6. CONCLUSIONS

1. The water content of the white muscle and the carcass can successfully reflect reciprocal changes in the plasma osmolality in response to acute challenge with increased salinity. This validates the study of osmotic regulation using either of these tissues or plasma, for example when comparing data based on different sizes within the same species, and potentially species where sampling plasma is problematic.

2. Sodium content from white muscle provides the best reflection of acute disturbances to plasma Na^+ regulation, whereas only Cl^- data from the carcass and not the white muscle can be used as an indicator of plasma Cl^- changes.

3. Plasma K^+ is exceptionally well regulated in response to high salinity challenge, and the K^+ levels from either white muscle tissue or the carcass provide the only useful indication of disturbances to internal plasma regulation.

4. Calcium content from the carcass but not the white muscle provides a reasonably sensitive surrogate for plasma Ca^{2+}. Plasma Mg^{2+} regulation can also be predicted from either of these tissues.

Differential osmoregulatory responses to 17α-ethinylestradiol between three-spined sticklebacks (*Gasterosteus aculeatus*) and rainbow trout (*Oncorhynchus mykiss*)

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1. ABSTRACT

The osmoregulatory effects of waterborne exposure to 17α-ethinylestradiol (EE2) were investigated in three-spined stickleback (*Gasterosteus aculeatus*) and rainbow trout (*Onchorhynchus mykiss*). Mixed sex groups of both species were exposed in freshwater to nominal EE2 concentrations of 5 and 10 ng/l for 7 days and then held in clean freshwater for 21 days before being subjected to a 24 h seawater challenge. After 7 days of exposure to EE2, plasma vitellogenin was not affected in sticklebacks, but was significantly elevated by 3- and 4-orders of magnitude (at 5 and 10 ng/l of EE2, respectively) in both male and female rainbow trout and remained elevated throughout the subsequent 21 days in clean water. In rainbow trout, ion concentrations and osmolality were measured in plasma, whereas in sticklebacks ion and water content were measured in carcass due to their smaller size. Exposure to 5 ng/l EE2 in freshwater increased plasma osmolality in rainbow trout (in both sexes), but had no effect on plasma Na^+ or Cl^-. Subsequent seawater challenge increased plasma osmolality by approximately 10% in control rainbow trout but there were no additional influence of the prior EE2 treatments. Interestingly, male trout previously exposed to 5 ng/l EE2 were better able to hypo-regulate plasma Na^+ compared to controls during seawater challenge. In sticklebacks, regulation of carcass water content was unaffected by the exposure to EE2. However, in contrast to rainbow trout plasma Na^+, male sticklebacks exposed to EE2 (10 ng/l) were less able to hypo-regulate carcass Na^+ content in response to subsequent seawater challenge. For the divalent ions, plasma [Ca^{2+}] and

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[Mg^{2+}] were significantly elevated in both sexes of rainbow trout after 7 days EE2 exposure in freshwater, and after their subsequent seawater challenge plasma [Ca^{2+}] was also elevated above controls in females. In sticklebacks carcass Ca^{2+} content (but not Mg^{2+}) changed in opposite directions in males (increased) and in females (decreased) in response to EE2 with the latter only being a delayed effect, 3 weeks after the freshwater exposure. These data indicate a greater estrogenic sensitivity to EE2 in freshwater rainbow trout compared with sticklebacks under identical conditions. They also show that exposure to EE2 can affect the regulation of divalent ions even in the absence of vitellogenin induction (for Ca^{2+} in sticklebacks), and can also have delayed and less predictable effects on the regulation of monovalent ions (Na^+) when fish are subsequently presented with an osmoregulatory challenge such as elevated salinity.

2. INTRODUCTION

There is growing evidence that many chemicals discharged into the environment can affect endocrine functions in fish, and alter the normal processes of development and reproduction (Tyler and Routledge, 1998; Weber et al., 2003). The pharmaceutical estrogen, 17α-ethinylestradiol (EE2), used in oral contraceptives and in post-menopausal treatments, enters surface waters via effluent discharges from wastewater treatment works (WWTW) and has been implicated as one of the primary contaminants contributing to the feminisation of fish in freshwaters (Jobling et al., 2006). Reported median concentrations of EE2 in WWTW effluents range from 1-17 ng/l and from below the detection limit to 15 ng/l in surface waters (Zha et al., 2007). Despite the low environmental concentrations,

EE2 is exquisitely potent and laboratory studies have demonstrated the induction of vitellogenin (the precursor of yolk in females) in male fish of many species (Kime and Nash, 1999) at waterborne concentrations as low as 0.1 ng/l (Purdom et al., 1994). Vitellogenin is therefore used in fish as a sensitive biomarker of exposure to estrogens and their mimics (Eidem et al., 2006). Altered dynamics of vitellogenin induction in females can impact their reproductive capabilities (Ankley et al., 2001; Thorpe et al., 2007) and very high levels of vitellogenin induction can cause adverse health effects such as kidney failure (Hutchinson et al., 2006; Liney et al., 2006). 17α-ethinylestradiol has also been shown to induce disruptions in the development of the gonadal duct, cause intersex and even lead to complete sex reversal in roach (*Rutilus rutilus*) at concentrations of only 4 ng/l (Lange et al., 2008; Lange et al., 2009). Long term exposure to EE2 at concentrations between 4 and 6 ng/l has been shown to cause reproductive failure in both laboratory maintained fish populations (zebrafish; Nash et al., 2004) and wild fish populations (fathead minnows; Kidd et al., 2007).

Although endocrine disruption has received considerable attention, most of this research has been directed at the effects on the reproductive axis, and to a lesser extent on development (as reviewed in Tyler et al., 1998; Vos et al., 2000). Environmental estrogens can affect other physiological processes in aquatic organisms. For example, exposure to nonylphenol has been shown to alter olfaction in fish (Moore and Waring, 1996), EE2 and fenitrothion (an anti-androgenic pesticide) have been shown to disrupt behaviours associated with aggression and courtship in the three spined stickleback (*Gasterosteus*

aculeatus) (Bell, 2001; Sebire et al., 2009, respectively), and EE2 can alter growth in tilapia (*Oreochromis nilotica*) (Shved et al., 2007).

Osmoregulation is a key physiological process in fish and its effective control is especially crucial for euryhaline species that move between fresh and brackish/salt waters as part of their natural behaviour. Although osmotic and ionic regulation have the potential to be impacted by endocrine disruptors comparatively few studies have investigated how exposure to such chemicals influences osmoregulation compared to reproductive endpoints. Vijayan et al. (2001) and Lerner et al. (2007) found osmoregulatory effects of 17β-estradiol (E2) in tilapia and in Atlantic salmon (*Salmo salar)*, respectively, but the responses were not identical. Freshwater tilapia showed elevated plasma [Na^+] after injection with 17β-estradiol, but also exhibited a reduced ability to regulate plasma osmolality after seawater challenge (Vijayan et al., 2001). In Atlantic salmon, waterborne exposure to 17β-estradiol (2 µg/l) and nonylphenol (10 and 100 µg/l) caused reduced plasma [Na^+] in freshwater, but elevated [Cl^-] in seawater. On the other hand, Moore et al. (2003) found no effects on osmoregulation in Atlantic salmon smolts in freshwater exposed to 5-20 µg/l nonylphenol. Some EDCs (such as EE2 and NP) can bioconcentrate many thousand fold (Larsson et al., 1999; Gibson et al., 2005) and thus for migrating fish, delayed effects on osmoregulation after an EDC exposure is entirely possible. The effect of EDCs were further investigated to study the influence on ion ATPase activities in different fish species. Sexually immature sea bream (*Sparus auratus*) injected intraperitoneally with coconut oil and 200 µgNP/g body mass reduced kidney Na^+, K^+ ATPase activity (Carrera et al., 2007). Prolonged

treatment with 2 μg g^{-1} E2 in the euryhaline killifish, *Fundulus heteroclitus* decreased gill K^+-*p*NPPase activity only following transfer from SW to 1 ppt SW (Mancera et al., 2004).

The aim of the present study was to assess how a short term exposure (7 days) to EE2 (at environmentally relevant concentrations) in fresh water affected the subsequent osmoregulatory capabilities in freshwater and following the challenge of an acute transfer to seawater in two species, rainbow trout (*Oncorhynchus mykiss*) and three-spined stickleback (*Gasterosteus aculeatus*), that both have strong euryhalinity (Perry et al., 2006; Tudorache et al., 2007). The response and relative sensitivity of the fish to EE2 exposure was quantified by measurements of vitellogenin induction and effects on osmoregulatory capability assessed via measurements of ion levels and osmolality of plasma (in rainbow trout), or ion and water content of the carcass (in sticklebacks). A separate study has assessed the comparability of these compartments for assessing osmotic and ionic regulatory parameters within the same species (Al-Jandal and Wilson, submitted, Chapter 2), allowing us to make direct comparisons between the effects on trout and sticklebacks in the present study.

3. MATERIALS AND METHODS

3.1 Fish

One hundred immature rainbow trout (*Oncorhynchus mykiss*) and 300 three-spined sticklebacks (*Gasterosteus aculeatus*) of mixed sex for both species, were obtained from Houghton Springs fish farm (Dorset, UK). The sticklebacks and the rainbow trout were

kept in separate aerated, holding tanks (155 litre capacity, receiving dechlorinated freshwater; Na^+ = 390; K^+ = 47; Ca^{2+} = 598; Mg^{2+} = 152; Cl^- = 400 µM; titratable alkalinity (to pH 4.0) = 0.85 mM; pH 7.5; temperature 10.4 ± 1.5 °C) at the University of Exeter and were fed daily at rates of 1 % of total body mass for sticklebacks and 0.5 % of total body mass for rainbow trout. Sticklebacks were fed on a mixture of brine shrimp, blood worms and white mosquito larvae (Tropical Marine Centre, Bristol, UK), administered twice daily. Rainbow trout were fed daily on a commercial pelleted feed (BioMar, 5 mm, Aqualife, Denmark). Previous studies have shown that commercial fish food may contain estrogenic substances that can elevate vitellogenin levels in the plasma (Pelissero et al., 1991; Pelissero and Sumpter, 1992; Matsumoto et al., 2004), but the food used in the present study has previously been shown to be free of significant estrogen content by Thorpe et al. (2000). They reported that a feeding ration of 1 % of body weight/day did not result in any changes in vitellogenin concentrations in the plasma of juvenile male or female rainbow trout as used in the present study. Food was withheld 2 days prior to and during the 7 days EE2 exposure period. To allow identification of individual trout, 3 months before the EE2 exposure fish were anaesthetised in MS222 (Pharmaq Ltd, UK; 100 mg/l buffered with 300 mg/l of $NaHCO_3$; Fisher Scientific, UK) and implanted with PIT tags (Avid plc UK, size 8 mm) subdermally in the left upper flank taking care to avoid the lateral line and red muscle. Assessments were made of the ion levels in a sub group of rainbow trout (plasma) and sticklebacks (carcass) 10 days prior to the EE2 exposure.

For trout, body mass was recorded (109.2 ± 2.1 g) and blood samples (300 µl) were

collected by caudal puncture with a 20-gauge needle into chilled heparinised syringes (Monoparin heparin sodium, 5000 I.U./ ml, CP Pharmaceuticals, Ltd., Wrexham, UK) and the blood was transferred immediately into microcentrifuge tubes (held on ice) containing aprotinin (~1 µl per 100 µl blood; Sigma-Aldrich) to reduce enzymatic degradation of vitellogenin. Blood samples were centrifuged (13,000 rpm for 5 min at 4 °C; Heraeus by Biofuge fresco, Kendro laboratory products, Germany) to obtain plasma. Half of the fresh plasma was stored on ice for analysis of osmolality and chloride and to make a dilution for cation analysis, and the remainder was frozen (−80 °C freezer) for later analysis of vitellogenin. For sticklebacks, a sub-group of the population was anaesthetised and killed by cerebral blow. Body mass was recorded (0.57 ± 0.07 g) and the fish blood samples were collected by caudal severance into heparinised haematocrit tubes from the open ends of the caudal artery/vein complex.

3.2 Experimental design and exposure

A flow-through system comprising of six aquaria (155 l each) was used for exposing both fish species simultaneously to EE2. Freshwater was supplied from a header tank by gravity to three mixing bottles (1200 ml/min each), which were dosed with EE2 to provide nominal water concentrations of EE2 of either zero (i.e. control), 5 ng/l or 10 ng/l. The output from each mixing bottle was split into two, each one delivering 600 ml/min of the required concentration to one of two replicate aquarium tanks per treatment. The mixing bottles received EE2 from a dosing stock bottle via a peristaltic pump (Watson Marlow 205U, UK; using mediprene tubing - Yellow/Yellow, Elkay Laboratory Products, UK, Ltd).

The dosing stock solution of EE2 (5 µg/l) was prepared in a 10 l darkened bottle (avoiding exposure to light). To generate the dosing stock, a concentrated stock solution of EE2 was prepared in acetone (20 mg/l) from which 2.5 ml were added to the dosing bottle. The acetone was allowed to evaporate before adding 2 l of deionised water and stirring the solution for 2 h. Eight litres of deionised water were then added to produce 10 l of the dosing stock. This approach was adopted to avoid the use of solvent in the exposure tanks. The dosing of the fish tank exposure system was initiated 10 days before adding the fish to ensure equilibration of EE2 between the water phase and all dosing and exposure tank surfaces. On the first days of the exposure, 12 rainbow trout and 24 sticklebacks were placed in each tank. The sticklebacks were held in mesh cages (Boyu Industries Co., Ltd., China) within the tank to avoid conflict with the rainbow trout. A small test had been previously carried out by adding the two species together in the same tank in the above manner after 2 days starvation. Video analysis showed no aggressive or predatory behaviour from the trout and no stress related behaviour was observed in the sticklebacks within their mesh cages (e.g. tight shoaling or avoidance of the cage edges).

Fish were not fed during the 7 days exposure in fresh water. For ethical/welfare reasons a few fish (rainbow trout) were removed from two of the tanks and terminated; 4 fish from one of the control tanks and 2 fish were terminated from one of the highest concentration (10 ng/l) tanks. At the end of the 7 days exposure, the rainbow trout were anaesthetised (as above), blood sampled by caudal puncture and then returned to the same exposure tank. Twelve of the 24 sticklebacks were also removed from each of the exposure tanks and

sacrificed (as above) for collection of blood and tissue samples (gonads). The remaining 12 sticklebacks in each tank and the blood-sampled rainbow trout were then kept in EE2-free freshwater for 21 days (i.e. a 3 weeks depuration period) to give the rainbow trout enough time to recover from the blood sampling and both species to recover from EE2 exposure. Following this 21 days depuration period, fish in all tanks were exposed to seawater (salinity = 25 ‰) for 24 hours in a semi-static system, and then a final sampling undertaken (see below). The desired salinity was achieved in each tank by adding pre-determined volumes of a hypersaline solution made up using commercial sea salts (Tropic Marin®, Germany) giving the following conditions: Osmolality = 714.7 ± 5.4 mOsm kg^{-1}; [Na^+] = 248.3 ± 11.6 mM; [Cl^-] = 369.8 ± 3.1 mM; [K^+] = 7.9 ± 0.04 mM; [Ca^{2+}] = 7.0 ±0.2 mM; [Mg^{2+}] = 42.0 ± 2.1 mM. Unfortunately, there was a technical error in two of the exposure tanks (one of the control replicate tanks and one of the 10 ng/l replicate tanks) during sampling of the rainbow trout after the SW challenge test. As a result, although all fish survived, data from these two tanks are not available, and individual fish numbers are therefore, reduced in these two treatments in seawater.

3.3 Sampling

3.3.1 Fish sampling

Sticklebacks were placed in anaesthetic (as above) before being terminated by a schedule 1 method of the UK Home Office regulations (brain destruction), weighed and blood sampled. The blood was collected in heparinised micro haematocrit capillary tubes (~50 µl capacity; Bilbate limited, UK), centrifuged using a micro haematocrit centrifuge (Gelman

Hawksley Ltd) and the plasma collected (~3 µl) and stored at −80ºC until analysis for vitellogenin by ELISA (Katsiadaki et al., 2002; Hahlbeck et al., 2004). The rainbow trout were blood sampled as detailed above and the blood was centrifuged and the plasma separated into two tubes and one aliquot kept at −80ºC until analysis for vitellogenin, and the other aliquot kept on ice and used directly to measure the osmolality and ions (see below). Gonads and kidneys were dissected from all fish for histology and sex confirmation.

All experiments were conducted with the approval of the University of Exeter Ethics Committee and under a UK Home Office license (PPL 30/2217).

3.4 Analytical techniques

3.4.1 Determination of EE2 concentration in exposure water

Water samples from the exposure tanks were stored at −20ºC until analysis to confirm the EE2 concentrations by gas chromatography/mass spectrometry (GCMS) by the Environment Agency's National Laboratory Service in Nottingham, UK (Kelly, 2000). Briefly, 50 ml of 100 % methanol (Fisher Scientific, UK) and 10 ml of glacial acetic acid were added to 1 litre of the water sample followed by extraction on solid-phase cartridges (SPE; C18, Sep-Pak and Waters, Waters Corporation, Milford, Massachusettes, USA). Cartridges were pre-conditioned with 5 ml of 100 % methanol and followed by 5 % methanol and in HPLC water (Fisher Scientific, UK). Samples extracted onto the cartridges were dried with nitrogen gas for 10-15 min, wrapped in aluminium foil and kept in −20 ºC

until analysis. The GC-MS technique used had a detection limit of 0.1 ng EE2/l (Kelly, 2000). The osmolality and [Cl^-] of the seawater tank exposure were measured as described for plasma ions (see below).

3.4.2 Vitellogenin analysis (ELISA protocol)

Plasma vitellogenin was measured for both rainbow trout and sticklebacks by homologous vitellogenin ELISA protocols (Katsiadaki et al., 2002 and Tyler et al., 2002). Rainbow trout plasma was diluted at least 1:10 prior to analysis of vitellogenin concentrations. Detection limits of the rainbow trout and sticklebacks vitellogenin ELISA for plasma were approximately 30 ng/ml and 20 ng/ml, respectively.

3.4.3 Plasma and carcass ion analysis

Stickleback carcasses (i.e. without the head and gut) were dried in an oven (70 °C) overnight, and weighed daily until the dry mass was constant. The carcasses were digested in 6 ml of concentrated 69 % nitric acid (15.6 N) (HNO_3) (AnalaR, BDH Laboratory Supplies, UK) overnight. Following complete digestion, 100 µl of the acid digest solution were diluted by addition of 10 ml of ultra-pure water (18 MΩ; Elga-Elgastat, Maxima, UK). For rainbow trout plasma, the osmolality was measured on 10 µl of fresh plasma using a vapour pressure osmometer (Wescor Vapro 5520, USA). Chloride concentration was measured in 20 µl of fresh trout plasma using a chloride analyzer (Corning M925, UK), whereas in sticklebacks [Cl^-] was measured in carcass digests using a colourimetric chloride assay (CECIL, CE1010, 1000 Series, Cambridge, UK; Zall et al., 1956). Cation

concentrations ([Na^+], [Ca^{2+}] and [Mg^{2+}]) were measured in rainbow trout plasma and stickleback carcass digests using atomic absorption spectrophotometry (PYE Unicam SP9, Philips, model no. SP9800; in flame emission mode for [Na^+], and atomic absorption mode for [Ca^{2+}] and [Mg^{2+}]). For calcium and magnesium measurements lanthanum chloride ($LaCl_3$) was added (0.1 % w/v final concentration) to all standards and samples to avoid sulphate and phosphate interference (Pybus et al., 1970). For the rainbow trout plasma, two dilutions were prepared; 100-fold to measure [Ca^{2+}] and [Mg^{2+}], and 1000-fold for measuring [Na^+].

3.5 Histology

To assess the status of sexual development, the dissected organs were fixed in Bouin's solution (Raymond A. Lamb, Eastbourne, UK) for 4 h and subsequently washed twice in 70 % industrial methylated spirit (IMS). The samples were then dehydrated in a graded series of IMS up to 100 %, cleared in xylene and then embedded in paraffin wax (Sigma-Aldrich, Poole, UK) using a Shandon tissue processor (Citadel 2000, Thermo Electron Corporation, Runcorn, UK). The blocks were sectioned (5 µm), sections floated on a water bath and then collected onto glass slides. Sections were stained using Harris's haematoxylin and eosin (ThermoShandon) using an automated stainer (Shandon Varistain XY automated stainer, Shandon Life Sciences Ltd., UK), treated with Histomount (National Diagnostic) and left overnight to dry at room temperature before examination by light microscopy (Zeiss Axioskop 40 microscope, Carl Zeiss, Oberkochen, Germany). Digital images were taken using an Olympus DP70 charge-coupled device camera (Olympus Optical) coupled to

analySIS 3.2 software (Soft Imaging System, Munster, Germany).

3.6 Statistical analysis

Vitellogenin and the ion concentration data were analysed using one-way ANOVA followed by unpaired t-test analysis to compare the treatment groups using Sigmastat 3.5 (Systat Software, Inc.). Kruskal Wallis (non parametric ANOVA) was used to analyse data when the normality test failed ([Na^+] in male rainbow trout after EE2 exposure, [Cl^-] in males sticklebacks after EE2 exposure; [Mg^{2+}] and [Na^+] in males sticklebacks after seawater challenge, vitellogenin in males sticklebacks after EE2 and after seawater challenge, and vitellogenin in females after EE2 exposure. Data were considered to differ significantly when the p value was < 0.05. Vitellogenin data were not normally distributed and were log transformed to achieve variance homogeneity. Throughout this paper, the data are presented as mean ± standard error of the mean. Data after freshwater EE2 exposure were tested separately from the data after SW challenge.

4. RESULTS

4.1 Determination of tank water concentrations of EE2

The mean concentrations of EE2 measured within the two exposure treatments were both greater than 80 % of the nominal concentrations (Table 1).

4.2 Plasma vitellogenin

After 7 days exposure to EE2, plasma vitellogenin concentrations in male trout increased

Table 1: Measured EE2 concentrations in the exposure tanks during the 7 days exposure in freshwater, determined by GCMS technique (Kelly, 2000; see Materials and Methods for full details). Values are means ± SEM where n = 6.

Treatment	Mean [EE2] (ng/l)	Range (ng/l)	% of nominal for mean value
Control	< 0.1*	0.0-0.0	n/a
5 ng/l EE2	4.34±0.07	4.13-4.49	86.7
10 ng/l EE2	8.12±0.21	7.53-8.64	81.2

**mean value is below the detection limit of 0.1 ng/l*

significantly from 0.5 µg/ml in the controls to 265 µg/ml and 4410 µg/ml in the 5 and 10 ng EE2/l exposure groups, respectively. Plasma vitellogenin concentrations in female trout followed a similar pattern, and increased from 0.1 µg/ml in controls to 386 µg/ml and 1654 µg/ml in the low and high EE2 exposure groups, respectively. After 3 weeks in clean freshwater and a further 24 h in clean sea water, the vitellogenin concentrations were still elevated above controls for both sexes in the two EE2 exposure groups, with little sign of clearance (Figure 1A).

In the sticklebacks, plasma vitellogenin concentrations were measured at 0.2 µg/ml in control males, whereas in females they were almost 4-fold higher at 713 µg/ml; some of the control females (n = 6) were approaching sexual maturity. After 7 days exposure to EE2 in freshwater, there was no significant difference between any of the treatment groups for either sex and this was also the case following 3 weeks in clean freshwater and the 24 h seawater challenge (i.e. there was no delayed effect of the EE2 on vitellogenin induction; Figure 1B).

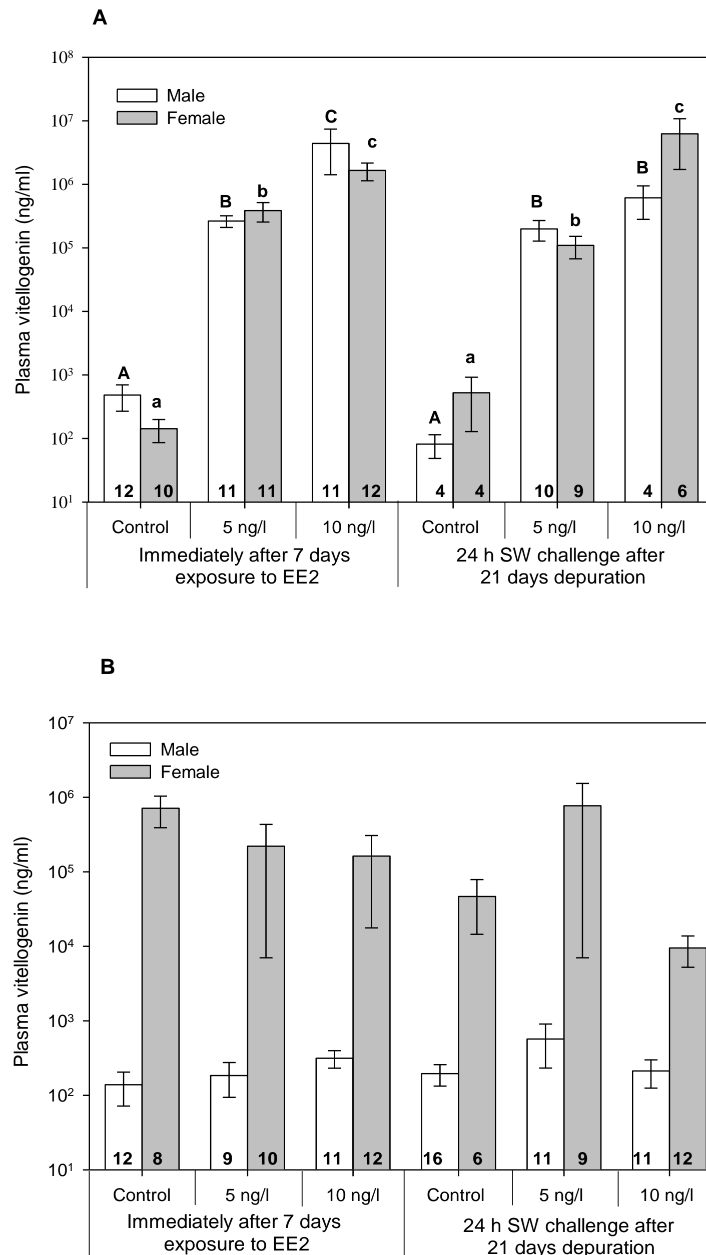

Figure 1: Plasma vitellogenin concentration in male and female rainbow trout **(A)** and three-spined sticklebacks **(B)** immediately after a 7 days exposure to EE2 in freshwater and after a 24 h exposure to sea water following a 21 days depuration period in freshwater after the initial EE2 exposure. Each column represents the mean ± SEM. Asymmetric error bars are due to logarithmic scale of vitellogenin data. Different letters above bars (capital letters for males and small letters for females) indicate significant differences compared to the control within a given time point ($P < 0.05$, Tukey's multiple comparison). Numbers in the bars indicate *n* of replicates of samples analysed.

4.3 Osmoregulatory variables

Prior to the EE2 exposures plasma or carcass ion levels in rainbow trout and sticklebacks were within the normal range established in previous studies (Flik et al., 1997; Handy et al., 2002; Tkatcheva et al., 2007). In rainbow trout plasma, osmolality and ion levels were Osm = 304.9 ± 0.8 mOsm kg^{-1}, [Cl^-] = 131.7 ± 0.4 mM, [Na^+] = 147.9 ± 1.7 mM, [K^+] = 2.8 ± 0.1 mM, [Ca^{2+}] = 2.3 ± 0.1 mM, [Mg^{2+}] = 0.5 ± 0.0 mM. In sticklebacks the carcass water content was 72.0 ± 1.4 %, and ion contents were [Cl^-] = 39.5 ± 6.2 µmol g^{-1}, [Na^+] = 57.0 ± 3.7 µmol g^{-1}, [K^+] = 75.1 ± 2.0 µmol g^{-1}, [Ca^{2+}] = 485 ± 20 µmol g^{-1}, [Mg^{2+}] = 28.4 ± 2.4 µmol g^{-1}.

After 7 days exposure to 5 ng EE2/l in freshwater, the plasma osmolality in both male and female rainbow trout was significantly elevated in comparison with the respective controls. Following exposure to seawater, after the 21 days recovery period, the plasma osmolality showed no differences between treatments in both sexes (Figure 2A), but was higher in all of these groups compared with their respective values after 7 days exposure to EE2 in freshwater. In the sticklebacks, after 7 days of EE2 exposure, the carcass water content was similar in controls and both EE2 treatment groups (73-75 %), and did not differ significantly from the pre-exposure values. After exposure to seawater (following the 21 days depuration period) the body water contents (71-72 %) were slightly depressed compared with the freshwater values, but there were no statistically significant differences between treatment groups (Figure 2B).

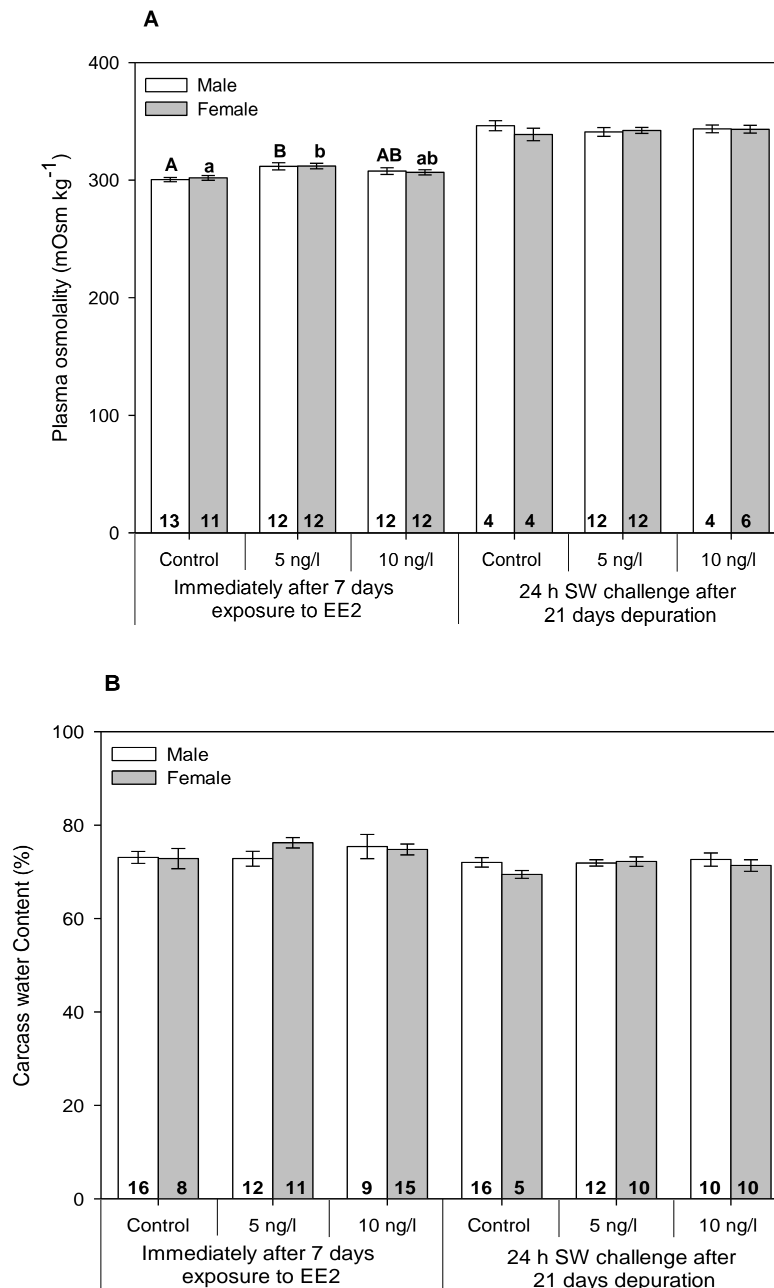

Figure 2: **(A)** Plasma osmolality of the rainbow trout (mixed sex) and **(B)** the carcass water content in sticklebacks immediately after a 7 days EE2 exposure and after a 24 h exposure to sea water following a 21 days depuration period in freshwater after the initial EE2 exposure. Numbers in the bars indicate *n* of replicates.

In rainbow trout, plasma [Na^+] in both sexes showed no significant changes after EE2 exposure in fresh water, but after the 24 h seawater challenge, males in the 5 ng EE2/l group had a significantly lower [Na^+] compared with controls. The females showed a similar, but non-significant, response pattern as in the males for plasma [Na^+] (Figure 3A). In the sticklebacks, carcass [Na^+] did not differ between the treatment groups after exposure to EE2 in fresh water. However, a significant difference was observed in sticklebacks after seawater challenge with values being elevated above controls in the high EE2 group males, and in the low EE2 group females (Figure 3B).

There was no effect of the EE2 treatments on plasma [Cl^-] of trout for either sex, either in freshwater or after the 24 h seawater challenge (Figure 4A), although plasma [Cl^-] was raised by ~15 mM in all groups following seawater challenge relative to their freshwater values. There was no effect of EE2 treatment on carcass [Cl^-] of sticklebacks for either sex after 7 days exposure in freshwater. The data for carcass [Cl^-] in sticklebacks after the depuration period (seawater challenge) were not included because of samples loss due of a technical error (Figure 4B).

17α-ethinylestradiol exposure caused concentration-dependent increases in plasma [Ca^{2+}] in both male and female rainbow trout in freshwater. Following the seawater challenge (after the 21 days depuration period) there were no significant differences between the treatment groups for [Ca^{2+}] in males, but the [Ca^{2+}] increased significantly compared to

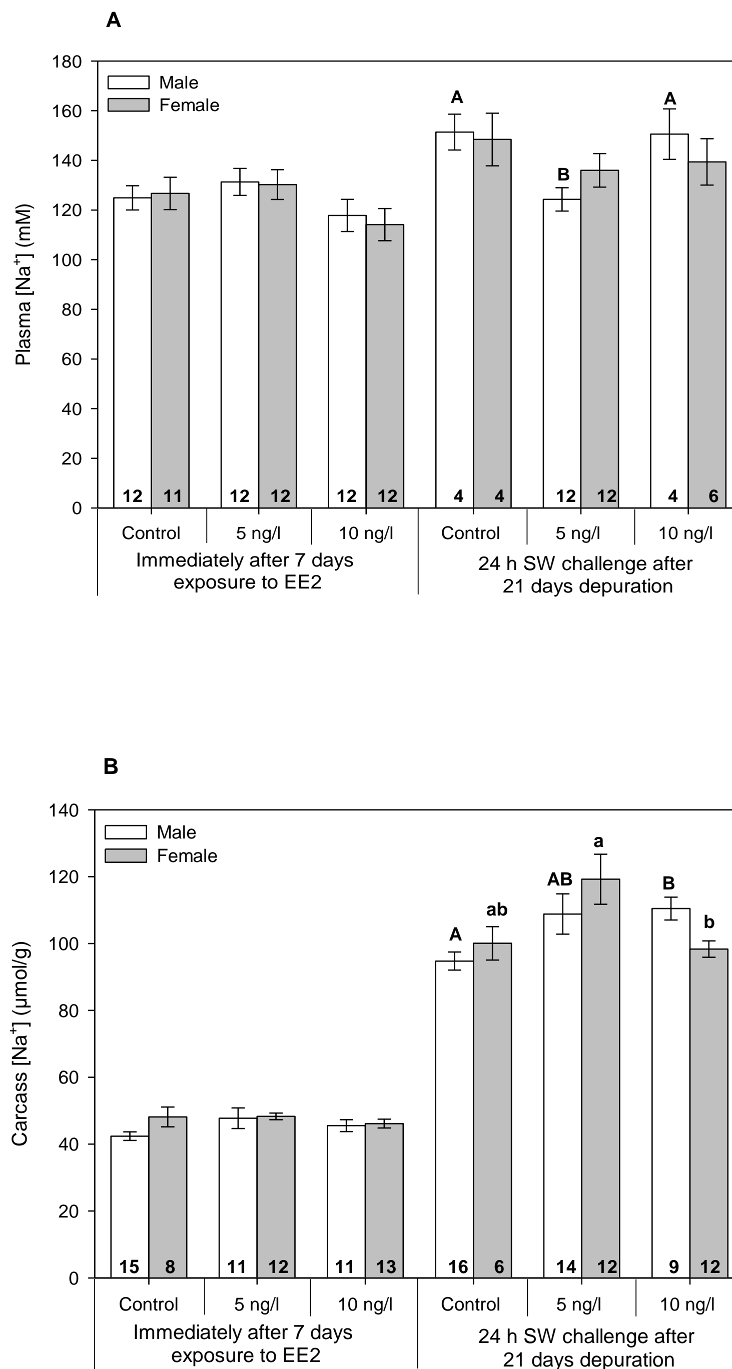

Figure 3: **(A)** [Na^+] in the plasma of rainbow trout and **(B)** [Na^+] in carcass digest in sticklebacks immediately after a 7 days EE2 exposure and after a 24 h exposure to sea water following a 21 days depuration period in freshwater after the initial EE2 exposure. Numbers in the bars indicate *n* of replicates.

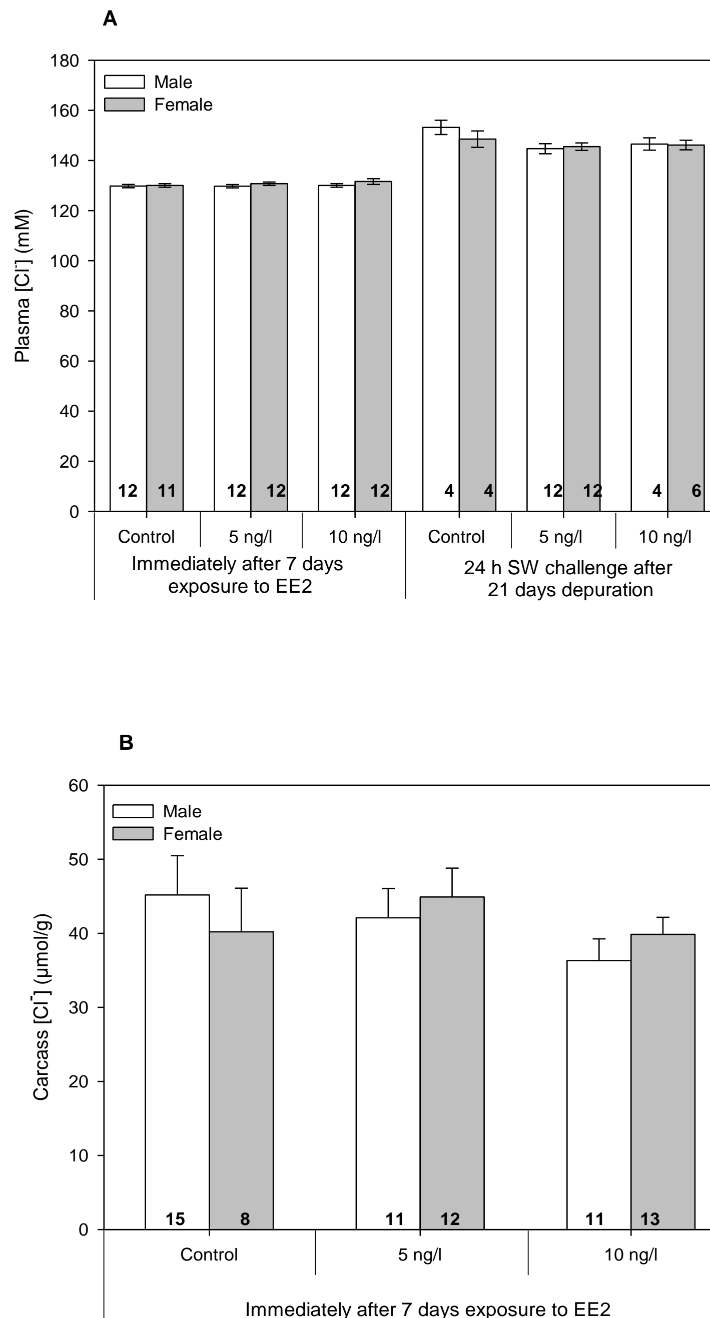

Figure 4: **(A)** Plasma [Cl$^-$] of rainbow trout immediately after a 7 days exposure to EE2 and after a subsequent 24 h exposure to sea water following a 21 days depuration period in freshwater after the initial EE2 exposure, and **(B)** and sticklebacks carcass [Cl$^-$] after 7 days exposure to EE2 ([Cl$^-$] data for the seawater challenge is missing due to a technical problem). Numbers in the bars indicate *n* of replicates.

controls in both treatments in female trout (Figure 5A). In sticklebacks, carcass [Ca^{2+}] was significantly higher in males in the high EE2 exposure group in fresh water compared with controls, but no differences were observed for the females. Following the 24 h seawater challenge, carcass [Ca^{2+}] showed no significant differences between males, but decreased significantly in females in the treatment groups in comparison to the control (Figure 5B).

In rainbow trout, EE2 exposure induced a small significant increase in plasma [Mg^{2+}] in both males and females. After the seawater challenge, there was an increase in plasma [Mg^{2+}] generally in both sexes compared to their freshwater values, but there were no significant differences between treatments (Figure 6A). In sticklebacks, there were no significant differences in the carcass [Mg^{2+}] between the EE2 treatment groups, either after the 7 days freshwater exposure or following the seawater challenge (Figure 6B), although all values were ~25 % higher in seawater compared to their freshwater values.

4.4 Histological findings

In control rainbow trout ovaries consisted of primary oocytes at the perinuclear stage, while the testes contained predominantly spermatogonia (i.e. both sexes were immature). Exposure to EE2 at the adopted concentrations and short duration (7 days) revealed no obvious changes in the gonads in trout. In sticklebacks, the gonads were more mature and in females, ovaries contained oogonia, primary and secondary oocytes and vitellogenic oocyte, and the testes contained spermatogonia A & B, spermatocytes, spermatids and spermatozoa. There were no signs of overt sexual disuption (i.e. effects on germs cells) for

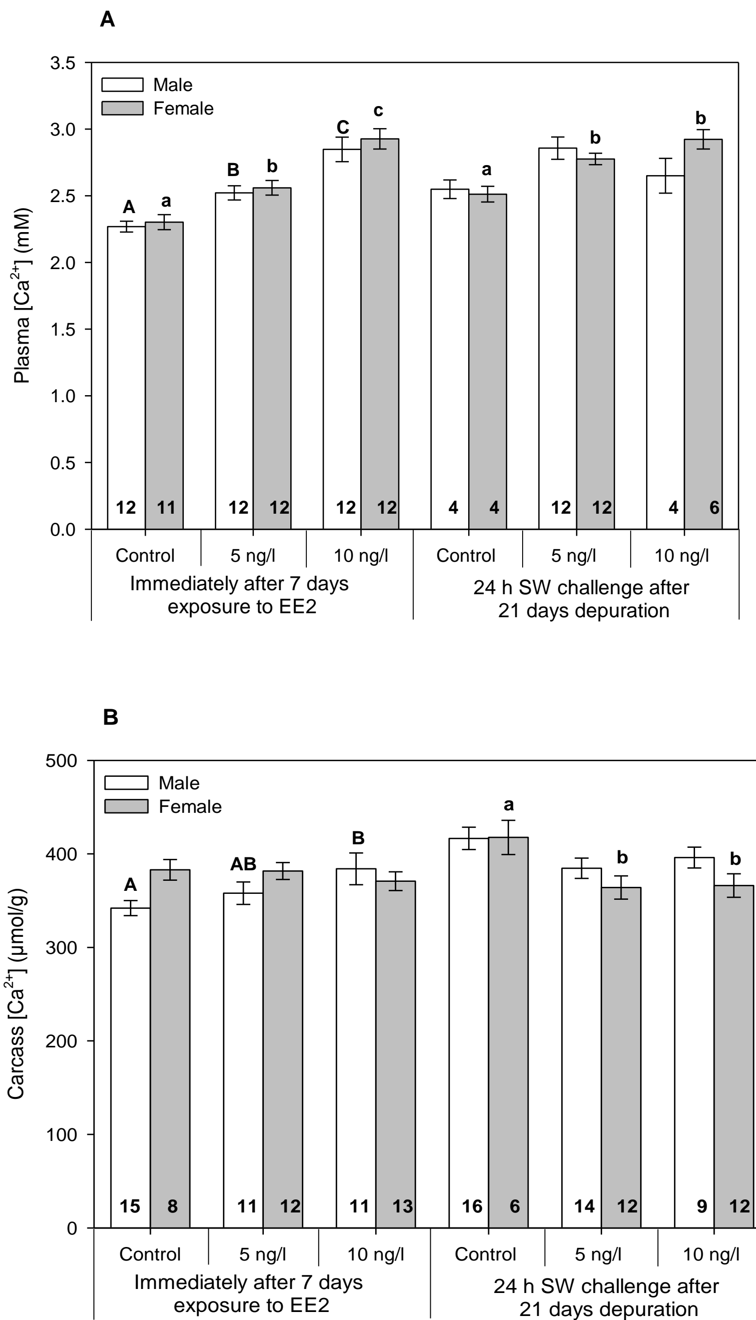

Figure 5: **(A)** [Ca^{2+}] in plasma of rainbow trout and **(B)** [Ca^{2+}] in carcass digest of sticklebacks immediately after a 7 days EE2 exposure and after a 24 h exposure to sea water following a 21 days depuration period in freshwater after the initial EE2 exposure. Numbers in the bars indicate *n* of replicates.

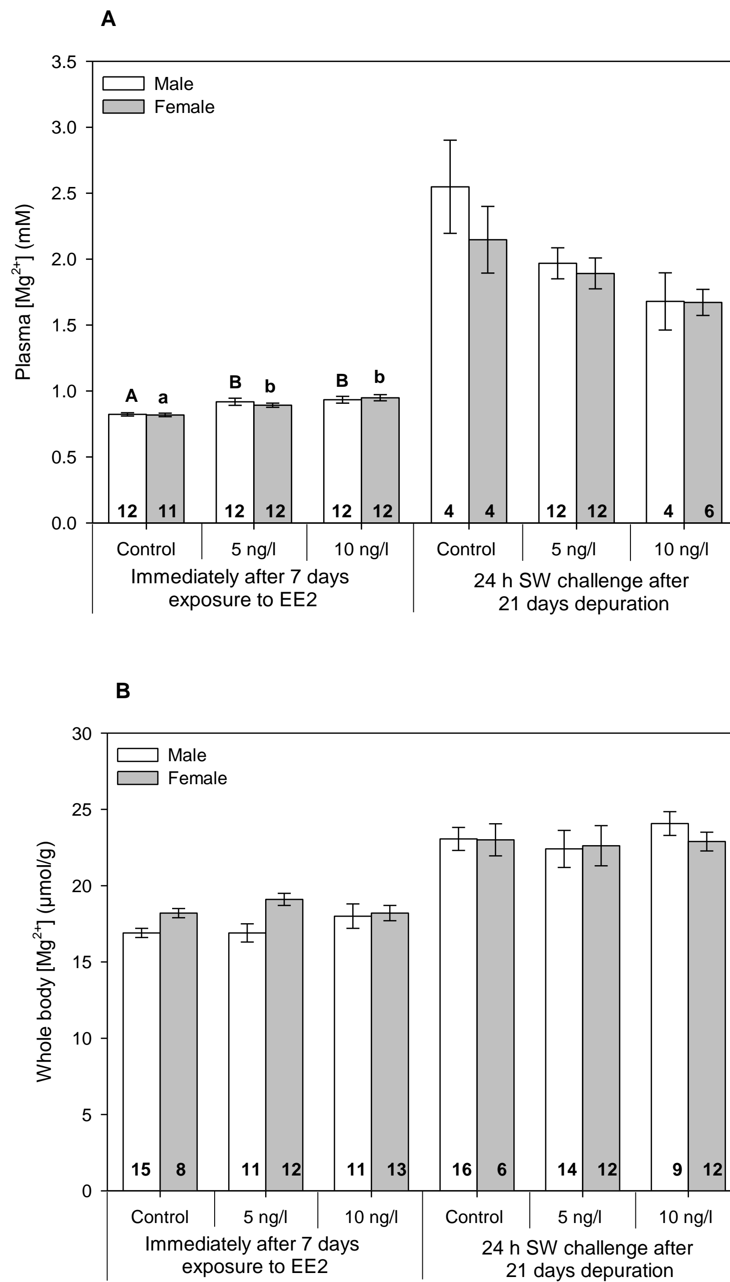

Figure 6: **(A)** [Mg^{2+}] in plasma of rainbow trout and **(B)** [Mg^{2+}] in carcass digest of sticklebacks immediately after a 7 days EE2 exposure and after a 24 h exposure to sea water following a 21 days depuration period in freshwater after the initial EE2 exposure. Numbers in the bars indicate *n* of replicates.

the EE2 exposure, as expected for the short exposure duration employed. Histological analysis of kidney samples similarly showed no signs of overt structural disruptions by the EE2 exposure.

5. DISCUSSION

5.1 Overview

There is a concern globally about the effect of endocrine disruptors on fish and their potential to affect a wide range of physiological functions (Goodhead and Tyler 2008). In the present study, the immediate and latent effects of exposure to one of the most well known endocrine disruptors, 17α-ethinylestradiol (EE2), were studied with respect to the osmoregulatory capabilities of two euryhaline fish species. The EE2 freshwater exposure regime adopted was short (7 days) followed by a 21 days period for recovery and depuration, before a 24 h seawater exposure to investigate their subsequent capability in response an osmoregulatory challenge (Madsen et al., 1997; Staurnes et al., 2001; McCormick et al., 2005).

5.2 Comparative estrogenic responses in rainbow trout and stickleback

Rainbow trout were more sensitive to the waterborne estrogenic treatment compared with the three-spined stickleback exposed simultaneously in the same tanks, as measured by vitellogenin induction responses. This agrees with previous separate studies on these two species where vitellogenin induction has been reported in rainbow trout exposed to concentrations of EE2 as low as 0.1 ng/l (Purdom et al., 1994), whereas for sticklebacks,

threshold concentrations are somewhat higher; e.g. 53.7 ng/l, in males (Andersson et al., 2007) or between 10 and 20 ng/l in males (Katsiadaki and co-workers, personal communication). Previous studies have further shown rainbow trout to be more sensitive to other environmental estrogens when compared with other fish species too, including roach (17β-estradiol; Routledge et al., 1998) and zebrafish (4-*tert*-octyphenol; Van den Belt et al., 2003). In the latter study, the zebrafish and trout were held at different temperatures (25-29 °C and 12-17 °C, respectively), and therefore, temperature could have influenced the differential responses seen in this case. However, Körner et al. (2008) showed that increased temperature actually elevated the vitellogenin mRNA expression in juvenile brown trout after exposure to EE2, and therefore the different sensitivities of rainbow trout and zebrafish do not appear to be explained simply by temperature.

Exposing two species within the same tanks separated by a screen ensures identical exposure conditions when comparing two species and so removes any doubt about comparability of the precise exposure concentrations. However, this dual exposure regime could potentially have induced complicating factors in one or both species. However, as mentioned in the Methods section a prior test showed no aggressive or stress-related behaviour and the osmoregulatory variables measured in fish from the exposure tanks were within the normal range found for these species held in their mono-species stock tanks. This would indicate minimal influence of the dual exposure conditions on their respective responses to the EE2 exposure.

In the present study, there were no obvious sex-related differences in the induction of vitellogenin and the persistence of vitellogenin in the circulation in rainbow trout after EE2 exposure was similar to that reported in the Atlantic salmon after exposure to both 4-tert-octylphenol and 17β-estradiol (Bangsgaard et al., 2006) supporting the contention that a short term exposure to EE2 can have long lasting effects.

To our knowledge, there are no previous studies that have directly compared rainbow trout and sticklebacks exposed under the same exposure conditions, and no studies have used similar short term exposures (7 days) to examine longer lasting and delayed effects. However, two previous studies have exposed juvenile rainbow trout for a longer period (2 weeks) to a range of EE2 levels (1-100 ng/l in Verslycke et al., 2002) where vitellogenesis was induced after two weeks exposure to 4.5 ng/l EE2 as a component of treated sewage effluent in Larsson et al. (1999).

5.3. Effect of EE2 on osmotic regulation

5.3.1 Osmolality and water body content

The effect of EE2 on osmotic regulation in freshwater also differed between the two study species, as did their response to the later seawater challenge. Ion concentrations and osmolality were measured in rainbow trout plasma samples whereas carcass water and ion contents were used in sticklebacks making direct comparison of these variables between species more complicated. Nevertheless, the relative effects of EE2 on osmotic regulation are still apparent. For Na^+ and Cl^-, we have assumed that changes in plasma concentrations

will approximate changes in carcass levels of these ions, therefore allowing qualitative comparison between the trout and stickleback. This assumption is partially based on a number of studies examining the ionoregulatory effects of exposure to acidification and to toxic metals where comparable responses in the blood and in carcass ions have been shown (e.g. Wood et al., 1988; Wilson and Wood, 1992). In addition we have recently completed a comparative study on the relationship between the carcass and plasma measurements within the same rainbow trout after 24 hour exposures to different salinities to induce a range of osmoregulatory challenges (Al-Jandal and Wilson, Chapter 2). This methodological study showed a clear and strong relationship for carcass versus plasma comparisons for all the osmoregulatory variables presented in the present study (i.e., water content/osmolality, Na^+, Cl^-, Ca^{2+} and Mg^{2+}). This justifies the comparison of trends for these particular osmoregulatory parameters in carcass of sticklebacks and plasma of trout within the current study. However, it is worth noting that the goodness of fit between plasma and carcass data for Ca^{2+} and Mg^{2+} was not as high as for the monovalent ions. This is almost certainly because the quantity of these divalent ions in plasma is small compared to the rest of the body. The vast majority of calcium is contained within the skeletal compartment (Cameron, 1985), and Mg^{2+} which is much more abundant in the intracellular compartment of all cells (Morgan and Potts, 1995; Bijvelds et al., 1998). So, small changes in the fluxes of these divalent cations (e.g. across the gills or gut) are more likely to induce measureable changes against the low background in the plasma, than against the high background in the carcass. Clearly these factors need to be considered when comparing data from trout and sticklebacks in the present study.

The slight changes in plasma osmolality on exposure to EE2 were common to both sexes in rainbow trout. There was about a 10 % increase in plasma osmolality in all treatment groups in the 24 h seawater challenge (after 21 days recovery) relative to the values in freshwater. This response was as expected for an acute seawater challenge for salmonids (Hwang et al., 1989; Marshall et al., 1999; Al-Jandal et al., submitted, Chapter 2), i.e. as a result of passive uptake of ions and loss of water due to the sudden reversal of osmotic and ion gradients. However, there were no differences between the treatments groups with respect to plasma osmolality of seawater-challenged rainbow trout, i.e. no delayed effect of EE2 on seawater transfer tolerance was seen.

In the sticklebacks, the carcass water content was not affected by the EE2 treatment in freshwater or after the seawater challenge (following 21 days depuration in freshwater). Clearly the sticklebacks were able to maintain water balance effectively in both freshwater and seawater environments, despite an exposure to EE2. Rainbow trout showed a rapid water loss during the first 24 hours of acclimation to the salinity increase. This might suggest that EE2 at the exposure concentrations adopted did not affect the balance between passive water movements and the urine production (in freshwater) or drinking rate (in seawater) in sticklebacks. It was also notable that carcass water content was regulated extremely well in response to the acute seawater challenge in sticklebacks, with no significant difference between treatments. In a separate study where we directly compared tissue water content with plasma osmolality within the same fish in response to acute salinity challenge, we found that carcass water content was validated as a surrogate of

plasma osmolality (Al-Jandal and Wilson, submitted, Chapter 2). Thus sticklebacks appear to have a more rapid induction of osmoregulatory homeostasis than trout following such an acute salinity challenge, indicating a higher degree of day-to-day euryhalinity.

5.3.2 Effect on monovalent ions

Disruption of ion regulation usually leads to a decrease in plasma or carcass ions when in freshwater, and an increase when in seawater, i.e. the normal regulation of these internal ion levels is impaired and they follow the prevaling gradient between the internal and external environment. In freshwater rainbow trout the plasma [Na^+] and [Cl^-] were not affected after the 7 days exposure to EE2, indicating that EE2 either had no effect on the relevant ionoregulatory processes, or that the active uptake and passive loss processes responded equally in the hypo-osmotic freshwater environment (Wood, 1992). Rather than impairing the tolerance to a high salinity challenge, prior exposure to EE2 appeared to "improve" some aspects of ion regulation following an acute seawater challenge, at least in terms of the plasma [Na^+] the male fish previously exposed to 5 ng EE2/l in freshwater. At this stage we do not know which aspect of seawater osmoregulation might be responsible for this enhanced hypo-regulation of Na^+ (e.g. gill versus gut, or active versus passive transport processes). However, this overall response differs from a study by Vijayan et al. (2001) who found that intraperitoneal injection of a different estrogenic compound, 17β-estradiol, into freshwater-acclimated Mozambique tilapia (*Oreochromis mossambicus*) reduced their ability to hypo-regulate plasma [Na^+] after transfer to 50 % seawater relative to sham-injected controls, which was explained by reduction in gill Na^+K^+ ATPase

activities by 17β-estradiol. In another study, aqueous exposure of freshwater juvenile Atlantic salmon to 17β-estradiol and environmentally relevant concentrations of nonylphenol disrupted their freshwater ion regulatory ability by decreasing plasma sodium levels (Lerner et al., 2007), again differing from a lack of EE2 effect in freshwater in the present study.

In the sticklebacks, EE2 exposure did not affect carcass Na^+ regulation in freshwater, however, following the seawater challenge, male fish subsequently had increased [Na^+] in comparison with controls. This is similar to the effect noted in tilapia in 50 % seawater after injection with 17β-estradiol (Vijayan et al, 2001) which was explained by inhibition of gill excretion of Na^+ via the Na^+/K^+-ATPase. However, we cannot rule out other effects of EE2, such as on the passive permeability of the gill or skin, Na^+ handling by the gut, which could also potentially explain higher plasma Na^+ levels.

The obvious concern would be that estrogenic compounds in the environment may have a negative influence on the movement of fish in the wild between different salinities (e.g. Madsen et al., 1997). It would appear that for sodium regulation following EE2 exposure at least, sticklebacks appear to follow the more obvious trend of impaired regulation in seawater, whereas trout were either unaffected (at 10 ng/l) or have improved hypo-regulation of sodium (at 5 ng/l) when acutely transferred to seawater. However, we should bare in mind that sodium is just one of the important ions that must be internally regulated and sampling was limited to just one timepoint, 24 h after seawater transfer. Nevertheless,

at the very least such differential species responses to EE2 for sodium regulation suggests that prediction of estrogenic effects on osmotic regulation may not be as predictable as reproductive effects.

5.3.3 Effect on divalent ions

In rainbow trout, the ion most affected after exposure to EE2 in freshwater was [Ca^{2+}]. This is perhaps not surprising as it is well established that increased plasma [Ca^{2+}] is known to parallel elevated vitellogenin concentrations in fish, and indeed plasma [Ca^{2+}] has been used as an indirect measure of vitellogenin (Gillespie and de Peyster, 2004; Lv et al., 2006). In both sexes of rainbow trout, there were [EE2]-dependent increases in the plasma [Ca^{2+}] after 7 days of exposure in freshwater. A number of studies have shown an increase in [Ca^{2+}] due to estrogenic exposure. Persson et al. (1994) found that freshwater juvenile rainbow trout injected with 17β-estradiol experienced increased calcium uptake at the gill as well as enhanced vitellogenesis and concluded that these were linked processes. An increase in both plasma calcium and vitellogenin after 17β-estradiol treatment was also observed by Carragher and Sumpter (1991). The increased calcium level in fish plasma is not necessarily derived from external sources only, but could also be from the considerable stores of calcium within scales and bones (Rotllant et al., 2005), and studies have shown that plasma calcium can be supplied from the scales under specific physiological challenges, such as treatment with 17β-estradiol (Armour et al., 1997; Persson et al., 1997).

Following 3 weeks depuration, and then a 24 h seawater challenge, the difference in [Ca^{2+}]

between controls and EE2-exposed trout persisted in females but not in the males. It is likely that with the removal of the estrogenic stimulus (EE2), although vitellogenin was still present in the circulation, there would have been a reduced synthesis of new vitellogenin, and potentially also a reduced drive to take up or mobilise further Ca^{2+}. However, as the vitellogenin concentrations had not declined by this time, and were not different between the two sexes, we cannot offer any clear explanation for this difference in [Ca^{2+}] between male and female trout. Nevertheless, the potential for a disconnection between plasma [Ca^{2+}] and vitellogenin levels further supports the principle that estrogenic control of Ca^{2+} regulation can be independent of vitellogenin levels. Thus plasma Ca^{2+} cannot be reliably used as indirect measure of vitellogenin in contrast to previous suggestions (Gillespie and de Peyster, 2004; Lv et al., 2006).

In sticklebacks, the body levels of Ca^{2+} increased after EE2 exposure (in freshwater) in males only, and there were no significant treatment effects after seawater challenge. This finding for elevated Ca^{2+} in males was not associated with a parallel increase in plasma vitellogenin (which did not occur in sticklebacks, in contrast with in trout). This further supports the above proposal that Ca^{2+} regulation was affected independently of any influence of EE2 on vitellogenesis (i.e. different to the conclusion of Persson et al., 1994).

Interestingly, there was a significant decrease in the carcass [Ca^{2+}] in the female sticklebacks 3 weeks after the end of the EE2 exposure. We can speculate that this indicates a delayed effect of EE2 on the redistribution of Ca^{2+} between somatic and gonadal tissues.

The reason why this was only observed in the stickleback females could be related to the greater maturity of their ovaries compared to trout in this study (see section 3.4 “Histological Findings” in the Results section). Perhaps EE2 directly stimulates Ca^{2+} uptake into ovaries, independently of vitellogenin induction, but only in ovaries that are already primed by the maturation process. This Ca^{2+} uptake from the plasma would ultimately draw from stores in somatic tissues that would manifest as a reduction in carcass Ca^{2+} levels.

Prolactin is known to function as a hypercalcemic hormone in freshwater teleost fish (Flik et al., 1986). In euryhaline fish such as the three-spined sticklebacks, prolactin cells become highly active in freshwater (Wendelaar Bonga and Greven, 1978) as it is usually a calcium-poor external medium and prolactin levels increase as a response to maintain the normal internal calcium levels (Flik et al., 1989). Estrogenic exposure could affect the synthesis of prolactin, for example Poh et al. (1997) reported that the prolactin content in tilapia was elevated by treatment with 17β-estradiol. Pickford et al. (1970) also reported that prolactin may elevate the plasma Na^+ in seawater conditions (as we found here for carcass [Na^+] in the male sticklebacks exposed to EE2), which was attributed to a decrease in gill Na^+/K^+-ATPase activity. Interestingly, prolactin appears to have sex-specific roles in many vertebrates, including paternal care in male sticklebacks (Schradin and Anzenberger, 1999).

It is therefore interesting to speculate that EE2 somehow interferes with the role of prolactin in male sticklebacks, and their subsequent Ca^{2+} regulation in freshwater and Na^+

regulation in seawater, but not in female sticklebacks. However, we have no current measurements of prolactin to support this hypothesis.

Plasma Mg^{2+} was increased after EE2 exposure in both male and female rainbow trout in freshwater. Similar to calcium, plasma Mg^{2+} levels have been used as an indirect indicator of the level of vitellogenin (Arukwe and Goksøyr, 2003; Lv et al., 2006). However, in rainbow trout, although plasma Mg^{2+} was elevated following seawater challenge relative to levels in freshwater, the same trend between EE2 treatments and controls was not evident after the seawater challenge, even though vitellogenin was still greatly elevated. Freshwater fish depend primarily on dietary sources for magnesium, but have secondary mechanisms for Mg^{2+} uptake via the gills in freshwater (Bijvelds et al., 1998). Given that the rainbow trout in this study were not fed during the exposure it seems likely that the higher level of Mg^{2+} in the EE2 treatments under freshwater conditions was due to enhanced gill uptake. Alternatively, the magnesium pool of the bones and scales may be used as a reserve to maintain normal magnesium levels in soft tissues when magnesium intake is low (Cowey et al., 1977; Reigh et al., 1991; Bijvelds et al., 1996). In carp (*Cyprinus carpio*) with low magnesium status, it has been shown that magnesium is mobilised from the bones and is replaced by Na^{+} (Vandervelden et al., 1992). Therefore, we cannot rule out a potential effect of EE2 on Mg^{2+} release from internal stores instead of (or in addition to) an effect on gill uptake of Mg^{2+}.

6. CONCLUSION

This study has demonstrated that rainbow trout had a greater sensitivity to the estrogenic effects of EE2 exposure, with no vitellogenin induction at all in the sticklebacks, even at the highest concentration used (10 ng/l). In contrast, EE2 had significant effects on osmoregulatory function in both these euryhaline species, even in the absence of vitellogenin induction (in sticklebacks), demonstrating a greater sensitivity of osmoregulation to estrogenic stimulation compared with the commonly used reproductive endpoint of vitellogenin induction. Furthermore, there were some intriguing differences between the species with respect to specific osmoregulatory responses. Such differences included an improved hypo-regulation of plasma sodium during seawater challenge in male rainbow trout (in the 5 ng/l exposure group), in contrast with an impaired carcass sodium regulation during seawater challenge in male sticklebacks. A further interesting finding was the disconnection between internal Ca^{2+} levels and plasma vitellogenin (in freshwater male sticklebacks immediatley after 7 days EE2 exposure, and in the trout following 3 weeks depuration and seawater challenge), which suggests differences in the control (or sensitivity) of these two processes by estrogenic chemicals. Overall, osmoregulatory impacts of estrogenic exposure have been demonstrated in two euryhaline species, but such effects may be less predictable than the better studied reproductive impacts.

Effects of a sublethal waterborne exposure to 4-nonylphenol in freshwater and seawater on ion regulation capabilities in rainbow trout (*Oncorhynchus mykiss*)

Effects of a sublethal waterborne exposure to 4-nonylphenol in freshwater and seawater on ion regulation capabilities in rainbow trout (*Oncorhynchus mykiss*)

1. ABSTRACT

The osmoregulatory effects of waterborne exposure to nonylphenol (NP) were examined in rainbow trout (*Onchorhynchus mykiss*). Immature female rainbow trout were exposed to three environmentally relevant concentrations (0.5, 2 and 8 µg NP/l) for 7 days in freshwater, directly followed by acute seawater challenge test (24 h). The measured concentrations of nonylphenol in the exposure tanks were only one third of the nominal concentrations. After 7 days exposure to nonylphenol, there was no vitellogenin induction observed in any of the treatments, although significantly higher levels of plasma vitellogenin were detected in the plasma samples of the fish exposed to the seawater test. in comparison to the freshwater, and that could be due to the difference in the ELISAs assays sensitivities. The effect on the osmoregulatory variables could not be established due to technical error (aeration stopped) in one of the sampling days lead to significant differences between the replicate tanks of each treatments. As an alternative, the effect of low oxygen level occurred was investigated to study the potential effects on the ions levels. It was very clear that the tanks affected by the low oxygen presented a significant lower effect of plasma osmolality, Na^+ and Cl^-, K^+ in the fish exposed 7 days to nonylphenol in freshwater. The increase in ventilation as a normal physiological response to the low oxygen caused increase in the gill functional surface area, and promotes oxygen uptake and increases the ions efflux rate.

2. INTRODUCTION

In the last 20 years, much research has been focused on a group of chemicals present in the aquatic environment that can be either natural or man-made (synthetic), which have the ability to interfere with the organism's normal endocrine function (Arcand-Hoy and Benson, 1998; Tyler et al., 1998; Moore et al., 2003). These so-called endocrine disrupting chemicals (EDCs) can disrupt the hormonal system in vertebrates by mimicking natural hormones, inhibiting their production by affecting synthesis pathways, or alteration of their excretion dynamics (Soares et al., 2008). Hormones affected have included those associated with growth, development (e.g. thyroid hormones) and sexual development and function (including androgens and estrogens). Most of the studies on EDCs have focused on chemicals that affect estrogenic response pathways, and many of these EDCs elicit an estrogenic effect by binding to and activating the estrogen receptor(s) (Jobling et al., 2003).

Endocrine disrupting chemicals include a broad class of chemicals such as the main naturally occurring estrogens in all vertebrates, for example estradiol-17β (E2), estrone (E1) and estriol (E3) (Tyler et al., 1998), and the synthetic ones include 17α-ethinylestradiol (EE2) (Van den Belt et al., 2003), and the estrogen mimics nonylphenol (NP) (Matthiessen et al., 2006). Phytoestrogens are plant compounds that exist widely in numerous plants such as soybeans. Phytoestrogens such as genistein and equol can act either as estrogen agonists or antagonists (Herman et al., 1995).

Synthetic and natural steroid estrogens are of particular concern in affecting wildlife health. Due to their human origin, steroid estrogens are regularly measured in the domestic sewage

effluents throughout United Kingdom, Japan, Australia, and the United States (Desbrow et al., 1998; Johnson et al., 2000; Niven et al., 2001; Komori et al., 2004; Johnson et al., 2007; Ying et al., 2009). Although the measured concentrations are usually low, in the ng/l range, in lab experiments they have been shown to induce estrogenic activities in various animals including vitellogenin induction and intersex in some fish species (Örn et al., 2003; Van den Belt et al., 2003; Balch et al., 2004; Hahlbeck et al., 2004; Balch and Metcalfe, 2006; Lange et al., 2009). It is worth noting that some xenoestrogens, such as 4-nonlyphenol, have been detected at higher concentrations up to μg/l (Johnson et al., 2005).

The aquatic environment is the ultimate sink for most chemicals whether natural or man-made. They may be disposed of via drains to sewage treatment works or in landfills sites, industrial wastes, agriculture and food/drug processing. Either the chemicals or their degradation products will eventually enter the aquatic environment (Sumpter, 1998; Barse et al., 2007). There is strong evidence that steroid estrogens discharged by the human population via sewage works are a major cause of endocrine disruption in fish in the UK (Jobling et al., 1998).

In fish, the major routes of uptake of EDCs are the gills, the gut via food, and the skin especially in small fish where there is a large surface area to volume ratio (Lien and McKim, 1993). These chemicals reach the blood stream directly from gill uptake, reaching the target organs without passing through the liver first, unlike the situation in mammals (Sohoni et al., 2001).

Possible reproductive disruption in wild fish was initially found through the observation of the induction of vitellogenin in male fish, which has subsequently proven to be a useful biomarker of exposure to chemicals with estrogenic activity (Örn et al., 2003; Vethaak et al., 2005), and has been applied widely to screen and test for estrogens and for estrogenic activity of effluent discharges (Knudsen et al., 1997; Rodgers-Gray et al., 2000; Liney et al., 2006).

Nonylphenol is one of the most studied estrogen mimics that appears to effect development in several organisms (Hemmer et al., 2002; Yadetie and Male, 2002). Nonylphenol belongs to the alkylphenols and is one of the degradation products of alkylphenol polyethoxylates. It is a xenobiotic used in the production of nonylphenol ethoxylates surfactants. Due to the wide use of nonylphenol ethoxylates they can reach sewage treatment works in considerable amounts where they incompletely degraded to nonylphenol (Soares et al., 2008). The major source of nonylphenol into the aquatic environment is through the discharge of effluents from sewage treatment works (Ahel et al., 1996; Langford et al., 2005).

The chemical and physical properties of nonylphenol influence its degradation. It is a hydrophobic compound with low solubility in water (Soares et al., 2008), and can become associated with organic matter (adsorbed) in sediments (John et al., 2000). Furthermore, it is a semi-volatile organic compound capable of water/air exchange (Soares et al., 2008). Sunlight can decrease the concentration of nonylphenol in the surface layers of natural waters by photolysis (Neamtu and Frimmel, 2006), whereas in sediment the half-life of

nonylphenol is 60 years (Shang et al., 1999), and can be biodegraded by microorganisms (Chang et al., 2004). In UK rivers, water samples collected during 1994 and 1995 showed nonylphenol concentrations ranging from <0.2 to 30 μg/l depending on the proximity to industrial discharges (Blackburn et al., 1999).

Nonylphenol is a chemical of a significant environmental concern due to its estrogenic effects and is hazardous to aquatic life (Lech et al., 1996; Nimrod and Benson, 1996; Schwaiger et al., 2002; Kurihara et al., 2007; Hwang et al., 2008). The evidence of nonylphenol estrogenicity is mainly based on the induction of vitellogenin in male or immature female fish (Sumpter and Jobling, 1995; Christiansen et al., 1998; Huang et al., 2008). The mechanism by which nonylphenol exerts its estrogenic and endocrine disrupting effect *in vivo* could be through a direct-acting mechanism, for instance binding to estrogen receptor (Yadetie et al., 1999), or via indirect mechanisms like increased plasma 17β-estradiol (E2) in fathead minnows (*Pimephales promelas*) (Giesy et al., 2000). One of the major effects of nonylphenol was to increase the plasma concentration of the endogenous estrogen (E2). Vitellogenin induction has been suggested to be a sensitive E2-specific biomarker and nonylphenol was found to mimic the natural hormone 17β-estradiol by competing with the natural steroid estrogen for binding site of the receptor (Giesy et al., 2000).

Nonylphenol has also been identified as an anti-androgen by using the yeast detection system for the anti-androgenic and androgenic effects of chemicals. The results suggested nonylphenol affects multiple steps of the activation and function of androgen receptors (Lee

et al., 2003). Several studies reported the adverse effects of nonylphenol on the development of male reproductive tract during perinatal exposure of male rats (Lee, 1998; de Jager et al., 1999).

Most attention regarding the biological activity of nonylphenol has been focused on its effects on reproductive processes. Less attention has been paid to the actions of nonylphenol on other physiological processes such as osmoregulation. The majority of previous studies have concentrated on the impact of endocrine disruptors on reproduction by using vitellogenin induction as a biomarker for exposure to an estrogen. The primary objective of the present study was to examine the effect of nonylphenol on the osmoregulatory capabilities of rainbow trout. The novelty of this study is that it will assess the effects of 3 different environmentally relevant concentrations (0.5, 2 and 8 µg/l) of nonylphenol on ion regulation in fish under the same exposure conditions. Nonylphenol was chosen because it is an extremely relevant environmental xeno-estrogen and widely dispersed in water, sediment and many biota (Ahel et al., 1993, 1994). Rainbow trout was used for this study as it is widely used in the study of endocrine disruption, due to its well described endocrinology and osmoregulatory physiology, the availability of body sizes that facilitate sufficient volumes of blood for such studies (Christiansen et al., 2000), and its a ability to acclimate to seawater as a good euryhaline species that lends itself to studies of the osmoregulatory impacts of toxicants.

3. MATERIALS AND METHODS

3.1 Fish

Immature triploid female rainbow trout (*Oncorhynchus mykiss*) (300 fish) were obtained from Hatchlands trout farm (Devon, UK). The rainbow trout were kept in aerated, freshwater holding tanks (dechlorinated freshwater; [Na^{+}] = 432.5 ± 18.7; [K^{+}] = 63.9 ± 3.0; [Ca^{2+}] = 165.2 ± 2.1; [Mg^{2+}] = 154.6 ± 4.6; Cl^{-} = 400 µmol l^{-1}; pH 7.5; temperature 10.4 ± 1.5 °C) at Exeter University, and were fed daily on a commercial feed (pellets) (BioMar, 5 mm, Aqualife, Denmark) at rates of 1 % body mass. Food was withheld two days prior to the nonylphenol exposure.

From the stock fish, 24 trout were anesthetized in MS222 (Pharmaq Ltd, UK), (100 mg/l buffered with 300 mg/l of $NaHCO_3$; Fisher Scientific, UK) for the collection of a blood sample for analysis of ion levels and vitellogenin prior to the exposure study. Blood samples (100 µl) were collected by caudal puncture using 26G needles into heparinised (Monoparin heparin sodium (mucous), 5000 I.U./ ml, CP Pharmaceuticals, Ltd., Wrexham, UK) syringes held on ice and transferred into microcentrifuge tubes containing aprotinin (10 µl) (Sigma-Aldrich) to reduce enzymatic degradation of vitellogenin, and then centrifuged (13,000 rpm for 5 min at 4 ºC; Heraeus by Biofuge fresco, Kendro laboratory products, Germany) to obtain plasma. A portion of the plasma was stored on ice for analysis of osmolality and chloride, and cations analysis. The remainder was frozen (−80 °C freezer) for later analysis of vitellogenin. Body mass and total length was also recorded (35.6 ± 3.0 g; 15.7 ± 4.5 cm respectively).

3.2 Experimental design and exposure

A flow through system comprising of 10 aquaria (99 l each) was used for exposing rainbow trout to nonylphenol. Freshwater was supplied from a header tank by gravity to five mixing bottles (1200 ml/min each), which were dosed with nonylphenol to provide water concentrations of 0.5, 2, and 8 µg NP/l. Dilution water and solvent controls (ethanol at 0.0001 %) were included (ethanol was used as a solvent vehicle for the nonylphenol exposures). The output from each mixing bottle was split into two, each one delivering 600 ml/min of the required concentration to one of two replicate aquarium tanks. The mixing bottles received nonylphenol from the stock bottles via a peristaltic pump (Watson Marlow 205U, UK; using mediprene tubing - white/white, Elkay Laboratory Products, UK, Ltd) (Figure 1).

The nonylphenol stock solution (800 mg in 100 ml ethanol) was prepared in a dark bottle and kept at 4°C. From this stock, the dosing bottles were prepared fresh every 2 days. The system required replenishing with 2 L of each stock daily, therefore, 4 L of each bottle was prepared fresh to deliver the required concentration in the exposure tanks. All the dosing bottles contained 0.1 % ethanol, and the nonylphenol concentrations were 0.5, 2 and 8 mg/l). The dosing of the system was initiated 4 days before the fish exposure to ensure equilibration of nonylphenol between the water phase and all dosing and exposure surfaces. After initial dosing for 4 days, 24 rainbow trout were placed into 5 tanks only (one replicate tank for each treatment). After a further two days, the second set of replicate tanks received 24 fish for each tank and treated similar to the first set of tanks. The rationale for a staggered experimental design was because it was only possible to analyse 60 fish/day,

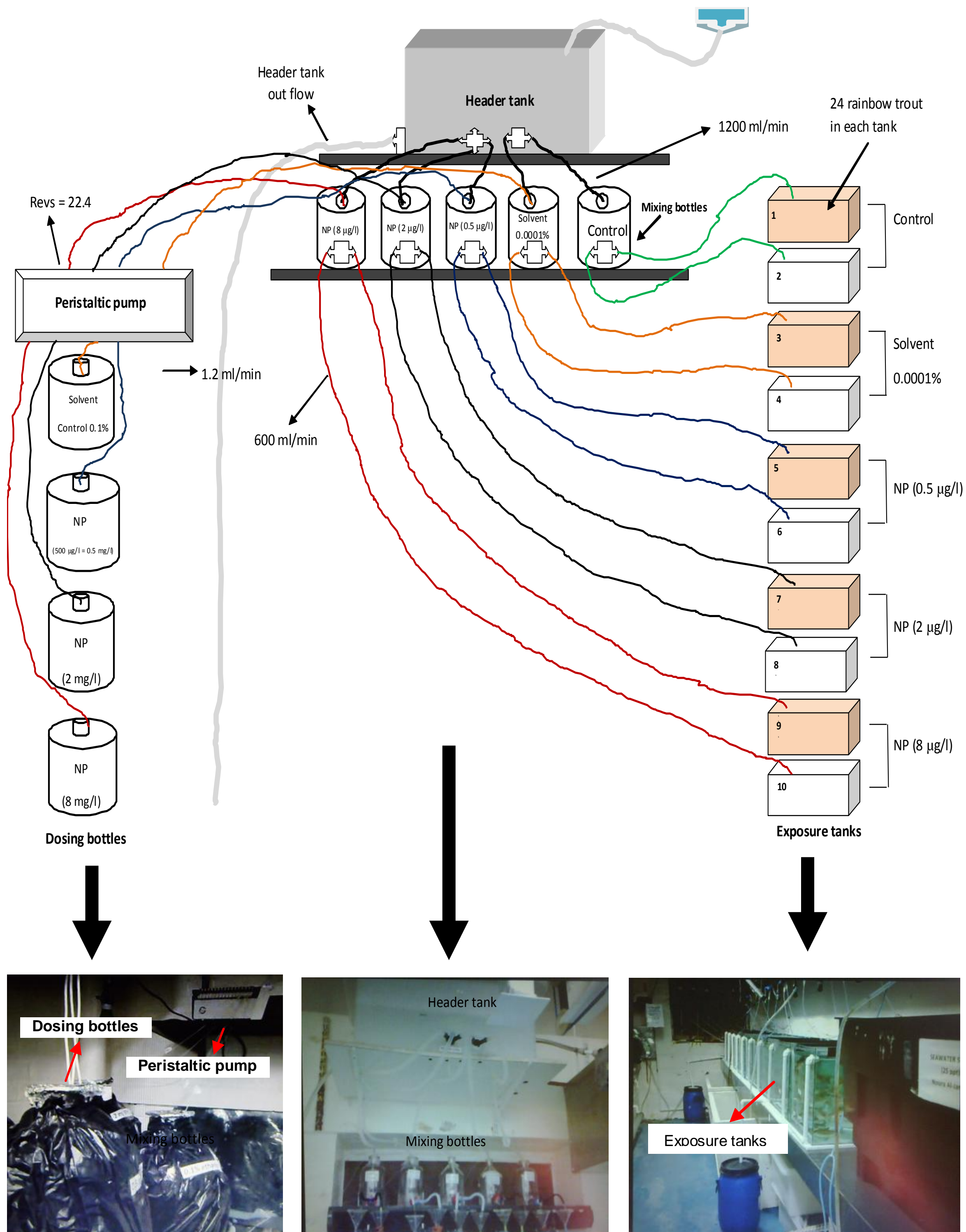

Figure 1: Showing the exposure plan in schematic drawing (above), and pictures taken from the real exposure in the laboratory.

where the terminal sampling was designed to be completed in 4 days. The exposure to nonylphenol was carried out in freshwater for 7 days, and then 12 fish only from the 24 fish in each tank were sampled from each exposure, including controls. The remaining 12 fish in each nonylphenol exposure regime were then subjected to a seawater challenge. On day 7 of the exposure rainbow trout (n = 60; 12 from each tank) were anaesthetized (as above) and blood sampled by caudal puncture.

The remaining 12 trout in each tank were then exposed to seawater after flushing the tanks at a fast flow rate (3.8 l/min) for 2 h, to remove all the nonylphenol. The seawater challenge test (salinity = 25 ‰), was undertaken to study the effect of a 7 day exposure to nonylphenol on the subsequent ability of the fish to osmoregulate in freshwater and after seawater challenge. The seawater used for this challenge was made up using commercial sea salts (Tropic Marin®, Germany) [Osmolality = 700 ± 3 mOsm kg^{-1}; [Na^+] = 273.6 ± 10.8 mM; [Cl^-] = 342.7 ± 2.4 mM; [K^+] = 7.5 ± 0.3 mM; [Ca^{2+}] = 6.4 ±0.3 mM; [Mg^{2+}] = 28.6 ± 1.1 mM].

3.3 Fish sampling

Rainbow trout were sampled after the seawater challenge as detailed above. Blood collected was centrifuged and the plasma separated into two tubes, one of them kept at −80ºC until analysis for vitellogenin, and the other sample was kept on ice, and used directly to measure osmolality and [Cl^-].

3.4 Analytical techniques

3.4.1 Determination of chemical concentrations in exposure water

Water samples from the exposure tanks were taken on day 0 and 7 of the exposure to nonylphenol and kept in glass bottles collected previously from the Environment Agency's National Laboratory Service, Starcross, UK. Water samples were kept in dark in box with ice and taken to the analytical laboratory. The osmolality and [Cl^-] of the tank exposure water were measured by vapour pressure osmometer and chloride analyzer in the Exeter laboratory.

3.4.2 Plasma ions analysis

Rainbow trout plasma vitellogenin was measured by homologous vitellogenin ELISA, (Tyler et al., 2002). Detection limits of the rainbow trout was 40-80 ng/ml. Plasma osmolality was measured using a Wescor Vapro 5520 vapour pressure osmometer with 10 µl of the fresh plasma. [Cl^-] was measured in trout using a chloride meter (Corning M925, Chloride analyzer, UK) with 20 µl of fresh plasma. Sodium, Ca^{2+}, K^+ and Mg^{2+} were measured by using atomic absorption spectrophotometery (AAS; PYE Unicam SP9, Philips; with Na^+ and K^+ in FES mode, and Ca^{2+} and Mg^{2+} in AAS mode). For calcium and magnesium standards and samples, lanthanum chloride ($LaCl_3$) was added to reduce sulphate and phosphate interference (Pybus et al., 1970), two dilutions were prepared, ×100 to measure [Ca^{2+}], [Mg^{2+}] and [K^+], whereas ×1000 were prepared for measuring [Na^+]. All experiments were conducted with the approval of the University of Exeter Ethics Committee and under a UK Home Office license (PPL 30/2217).

3.5 Statistical analysis

All parameters were analysed for differences between treatments using one-way ANOVA by using Sigmastat 3.5 (Systat Software, Inc.). Post hoc tests used to analyse data when the normality test failed and significant differences between groups were determined using the non-parametric Kruskal-Wallis One Way ANOVA on ranks followed by the Dunn's Method to determine differences between groups. Differences between groups were considered to differ significantly when the p-value was < 0.05. Throughout this paper, the data are presented as mean ± standard error of the mean.

4. RESULTS

4.1 Determination of tank water concentrations of nonylphenol

The measured nonylphenol concentrations in the exposure tanks are shown in (Table 1). The mean concentration of nonylphenol within the three exposure treatments were between 26 % and 36 % of the nominal concentrations.

4.2 Plasma vitellogenin

After 7 days exposure to nonylphenol in freshwater, no induction of vitellogenin was detected in the plasma of the exposed fish. The concentrations measured in plasma of fish from all the exposure tanks were < 200 ng/ml. However, after seawater challenge test, the vitellogenin concentrations measured in fish from all the exposure tanks were > 300 ng/ml (Figure 2). No vitellogenin induction was observed after exposure to the highest level of nonylphenol (2 µg/l measured, nominal = 8 µg/l).

Table 1: Measured nonylphenol concentrations in the exposure tanks during the 7 day exposure in freshwater. Values are means ± SEM.

Treatment	Mean of [NP] (µg/l)	Range (µg/l)	% of nominal for mean value
Control	< 0.125	0.125 -0.125	n/a
Solvent control	< 0.125	0.125 -0.125	n/a
0.5 µg NP/l	0.181± 0.03	0.125 - 0.251	36.2
2 µg NP/l	0.611 ± 0.05	0.529 -0.730	30.6
8 µg NP/l	2.093 ± 0.19	1.64 – 2.54	26.2

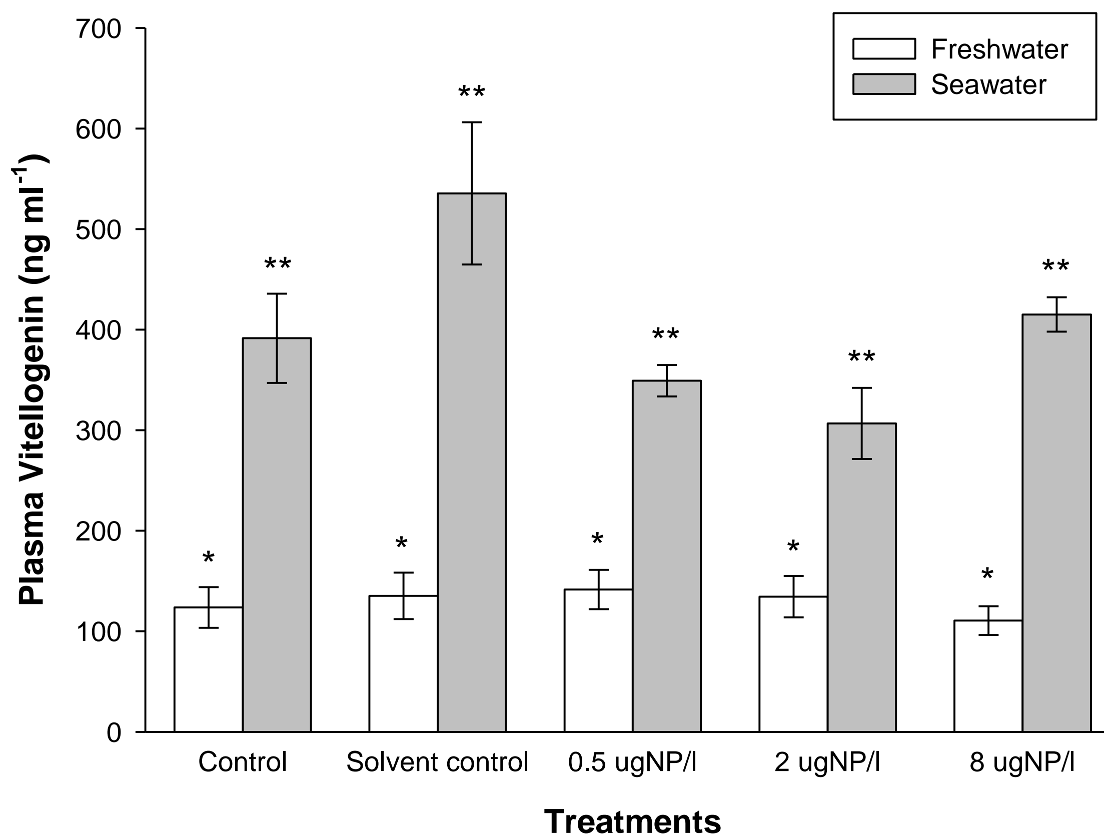

Figure 2: Plasma vitellogenin concentration in rainbow trout after 7 days exposure to nonylphenol and after a 24 h seawater challenge test. Each column represents the mean ± SEM

4.3 Osmoregulatory variables

Due to an unfortunate error on the sampling day for one set of replicate tanks, where the aeration stopped accidently. Therefore, fish sampling was delayed for 2 h to allow the affected fish to recover from oxygen depletion stress. After sampling the fish, all the parameters relevant to fish osmoregulation were measured. The data from the exposure in freshwater showed significant differences in the mean values between the replicate tanks within each treatment after applying ANOVA, especially in osmolality (almost 10-30 mOsm kg^{-1} difference), chloride, sodium and potassium. The mean values were significantly lower in the tanks where the aeration was interrupted (Table 2A).

Table 2A: Showing plasma osmolality and ions between the replicate tanks after 7 days exposure to nonylphenol in freshwater. Number of fish in each tank (*n* = 12). Data missing for control T1 due to technical error. The values are the means ± SE of the replicate tanks. Asterisks indicate significant difference between replicate tanks, and grey shaded rows are tanks affected by lack of aeration.

Treatments	Tanks codes	Osmolality (mOsm kg^{-1})	[Cl^-] mM	[Na^+] mM	[K^+] mM	[Ca^{2+}] mM	[Mg^{2+}] mM
Control	T1	299.9 ± 3.0	122.4 ± 1.1	none	none	none	none
	T2	278.8 ± 6.5*	113.7 ± 3.0*	128.8 ± 3.5	1.8 ± 0.2	2.0 ± 0.1	0.6 ± 0.1
Solvent Control	T3	295.2 ± 2.6	122.0 ± 0.9	147.0 ± 1.7	3.7 ± 0.3	2.0 ± 0.1	0.9 ± 0.2
	T4	282.3 ± 5.8	116.1 ± 1.8*	134.0 ± 2.7*	1.9 ± 0.1*	2.1 ± 0.1	0.8 ± 0.1
0.5 µgNP/l	T5	303.6 ± 2.6	125.6 ± 0.9	143.9 ± 1.0	3.1 ± 0.1	1.9 ± 0.04	0.7 ± 0.03
	T6	280.6 ± 4.2*	115.9 ± 1.5*	134.2 ± 1.8*	2.5 ± 0.1*	2.1 ± 0.1*	0.8 ± 0.03
2 µgNP/l	T7	300.0 ± 3.1	126.6 ± 1.2	145.9 ± 1.7	3.3 ± 0.1	2.0 ± 0.1	0.9 ± 0.1
	T8	272.0 ± 6.1*	113.9 ± 1.6*	132.9 ± 2.3*	3.5 ± 0.3*	2.2 ± 0.2	1.1 ± 0.2
8 µgNP/l	T9	295.8 ± 3.6	121.8 ± 1.1	144.8 ± 1.3	3.2 ± 0.2	2.1 ± 0.1	0.8 ± 0.1
	T10	278.6 ± 6.2*	113.1 ± 2.6*	133.7 ± 3.6*	3.3 ± 0.2*	2.4 ± 0.3	1.2 ± 0.2

After seawater challenge, the ANOVA for the intact replicate tanks were applied to investigate if there are any differences between the replicate tanks after the error occurred during the freshwater exposure. There were no significant differences between the replicate tanks detected for all the treatments, except for the osmolality and [Mg^{2+}] in the highest exposure tanks (8 µg NP/l), the [Cl^-] showed significant difference between the replicate tanks of 2 and 8 µg NP/l exposure treatments (Table 2B).

Table 2B: Showing plasma osmolality and ions between the replicate tanks after acute seawater challenge. The values are the means ± SE of the replicate tanks. Asterisks indicate significant difference between replicate tanks, and grey shaded rows are tanks affected by lack of aeration.

Treatments	Tanks	Osmolality (mOsm kg^{-1})	[Cl^-] mM	[Na^+] mM	[K^+] mM	[Ca^{2+}] mM	[Mg^{2+}] mM
Control	T1	365.7 ± 5.1	156.6 ± 2.9	190.9 ± 2.9	3.5 ± 0.2	3.1 ± 0.1	2.2 ± 0.2
	T2	375.6 ± 4.7	160.5 ± 2.3	191.6 ± 2.8	4.1 ± 0.2	2.1 ± 0.1	1.7 ± 0.2
Solvent Control	T3	355.6 ± 6.6	151.1 ± 2.7	184.3 ± 3.1	4.3 ± 0.1	3.1 ± 0.1	2.0 ± 0.1
	T4	354.0 ± 8.4	151.5 ± 3.6	176.0 ± 4.2	4.1 ± 0.2	1.8 ± 0.1	1.6 ± 0.2
0.5 µgNP/l	T5	367.0 ± 8.1	154.7 ± 4.1	189.5 ± 6.8	4.0 ± 0.3	3.0 ± 0.3	2.5 ± 0.6
	T6	359.3 ± 8.8	157.1 ± 4.2	181.5 ± 6.3	3.1 ± 0.2	2.0 ± 0.1	1.4 ± 0.1
2 µgNP/l	T7	363.5 ± 9.3	150.0 ± 4.0	184.6 ± 4.0	3.5 ± 0.2	3.1 ± 0.1	2.0 ± 0.2
	T8	385.8 ± 8.0	162.5 ± 2.7*	178.6 ± 6.4	3.8 ± 0.4	1.6 ± 0.2	1.4 ± 0.2
8 µgNP/l	T9	358.2 ± 4.7	148.1 ± 2.3	190.5 ± 3.4	4.1 ± 0.3	3.1 ± 0.1	2.2 ± 0.1
	T10	384.5 ± 8.3*	160.1 ± 2.5*	180.5 ± 3.4	3.7 ± 0.2	2.0 ± 0.1	1.5 ± 0.2*

5. DISCUSSION

The influence of nonylphenol on osmoregulatory system has been previously studied in salmonids. Most of the exposure regimes used by the previous studies were applied by using nonylphenol injected into the fish (Madsen et al., 2004; McCormick et al., 2005; Carrera et al., 2007). Some studies used the aqueous exposure route but with higher levels

than those used in the present study (Arsenault et al., 2004; Lerner et al., 2007) which aimed to examine the effect of environmentally relevant concentrations of nonylphenol in freshwater and the ability of this xeno-estrogen to impair the salinity tolerance immediately after direct exposure to an endocrine disruptor.

5.1 The concentration of nonylphenol in the exposure tanks

The measured concentrations of nonylphenol in the exposure tanks after 7 days, were approximately one third of the nominal concentrations in all the exposure concentrations. Similar studies have reported actual concentrations which were approximately 1/3 to 1/5 of the nominal concentrations (Nimrod and Benson, 1998; Nichols et al., 2001; Schoenfuss et al., 2008). The reduced concentrations could be due to several reasons such as degradation of nonylphenol in the exposure tanks. Alternatively the lower measured concentrations may have been related to an incomplete solubilisation of nonylphenol in the stock solution (aqueous solubility limit of NP = 5.24 ± 0.11 mg NP/l at 14 °C, Ahel and Giger, 1993) although unfortunately we did not measure the stock solution concentration. Other potential factors include volatilization of nonylphenol, adsorption to tubing or the glass tanks. Adsorption to organic matter present in the tanks can also occur (Villeneuve et al., 2002), and fish themselves may absorb some nonylphenol (Pickford et al., 2003). However, in our study we have used a flow through system which makes these factors unlikely to happen. Furthermore, the pump's flow rate was not checked for accuracy during and after the study to ensure the delivery of the proper concentration required for the study. Since the loss was around 70 % in all the doses the pump's flow rate may also be a factor.

Other studies that have exposed fish to nonylphenol have found widely differing recoveries of nonylphenol in the water. Pickford et al., (2003) exposed male fathead minnows (*Pimephales promelas*) to nonylphenol through waterborne exposure for 2 weeks at nominal concentrations of 1, 10 or 50 µg/l, and the measured concentrations in the dosed tanks were between 72-129 % of nominals. One possible reason why the measured concentrations of nonylphenol were more closely aligned with nominals in that study was that their exposure tanks were dosed for 7 days with nonylphenol prior to addition of the fish, whereas in our study we pre-dosed the system for only 4 days, that longer period of exposure might have helped. This suggestion is further supported by another study, where dosing of nonylphenol (nominal concentrations 1, 10 and 100 µg/l) were initiated 3 weeks prior to adding fish, and the measured concentrations were between 70 % and 85 % of nominals (Harris et al., 2001). However, although lower levels of nonylphenol were obtained in the exposure tanks than anticipated, they are still close to the relevant environmental concentrations which should not be considered as a total loss.

5.2 Plasma vitellogenin

The exposure to nonylphenol for 7 days revealed no vitellogenin induction in the plasma of rainbow trout. The results showed no vitellogenin induction which could be due to the low exposure level and short exposure period. Also, it turned out that the nominal exposure levels did not reach to the exposure tanks. Several studies have exposed fish to nonylphenol at concentrations between 1 and 50 µg NP/l and have reported either a non-significant effect on plasma vitellogenin concentrations and/or great variability among fish in the same treatment (Pickford et al., 2003; Van den Belt et al., 2003). Similar to our result in terms of

vitellogenin induction, Atlantic salmon (*Salmo salar* L.) smolts were exposed in freshwater to 5, 10, 15, and 20 µg NP/l for a 30-day period where no vitellogenin induction detected (Moore et al., 2003). There was also no detectable vitellogenin in juvenile Atlantic salmon treated with 10 µg NP/l for 21 days in freshwater (Lerner et al., 2007).

It is not known why there was an elevated level of vitellogenin in all fish after the seawater challenge in the present study. One possible reason is a consequence of a stress response after being subjected to the accidentally reduced oxygen level, but this is speculation only. Indeed, generally, stress is known to reduce plasma vitellogenin levels in fish (Carragher et al., 1989), rather than increase them, so this seems an unlikely explanation. However, the increase observed after seawater challenge was actually rather small relative to the orders of magnitude changes commonly observed in response to significant estrogenic stimulation. It is therefore wise to interpret these data with caution, and it is quite likely that the small difference may reflect different performance of the ELISA assays for samples analysed on these different experimental days.

On the other hand, several studies have documented that hypoxia (low oxygen) acts as an endocrine disruptor of fish reproduction by affecting hormones secreted by the hypothalamus-pituitary-gonadal-liver system (Wu et al., 2003; Landry et al., 2007; Thomas et al., 2007). But, there is no direct evidence that hypoxia can affect the vitellogenin level. Male fathead minnows (*Pimephales promelas*) were exposed to a mixture of estrogenic chemicals under hypoxic conditions (< 2 mg of O_2/l) and the results revealed no effect of hypoxia on vitellogenin response (Brian et al., 2009).

One possible reason might explain the difference in the plasma vitellogenin levels in seawater from the freshwater is the difference sensitivity between different ELISAs carried out. As the plasma samples used to measure the vitellogenin were divided for different ELISAs in different days.

5.3 Osmoregulatory variables

The reduction in oxygen that occurred due to the accidental loss of aeration in the exposure tanks would have added an additional stress to the exposed fish besides the exposure to nonylphenol. The gills carry out multiple tasks such as gas exchange and ion transport (Evans et al., 2005). In general, freshwater fish face the problem of gaining water and losing ions, and the major ions in plasma, Na^+ and Cl^-, have particularly high rates of loss via branchial diffusion, even under normoxic conditions (Wood, 1992). Data showed a significant decrease in these ions in the tanks affected by oxygen depletion in comparison to their unaffected replicate tanks within each treatment (Table 2A). That can be explained by the extra stress, as fish would normally increase their ventilation as a normal physiological response to lower oxygen levels (Evans et al., 2005). Higher ventilation helps to assist in the extraction of enough oxygen from the water to satisfy the normal oxygen demand. However, a consequence of elevated ventilation could also be increased diffusive ion loss across the gills (Gonzalez and McDonald, 1992, 1994) and may result in decreasing the level in the plasma detected in the affected tanks. An increase in the gill functional surface area, as during hyper-ventilation, promotes oxygen uptake and increases the Na^+ efflux rate, whereas reduction in surface areas cause the opposite. This relationship between oxygen uptake and diffusive ion fluxes is called the osmo-respiratory compromise.

A study on freshwater adapted rainbow trout experimentally showed a relationship between oxygen consumption and Na^+ loss, where Na^+ loss increased whenever oxygen consumption increased (Gonzalez and McDonald, 1992). Another reason could be the down regulation of the uptake channels and/or Na^+-K^+-ATPase activity to make energetic savings during hypoxia (Wood et al., 2007). In freshwater fish hyperventilation (due to low oxygen) is expected to cause reduced plasma ions (especially Na^+ and Cl^-), whereas hyperventilation in seawater fish will cause the opposite (increased plasma ions, especially Na^+, Cl^-, and Mg^{2+} that have the biggest gradients across the gills). The results support the above ideas on low oxygen supply and decrease in the plasma osmolality, Na^+ and Cl^- level in freshwater fish accordingly.

Due to the loss of aeration at a key stage of the experiment, the data obtained were not complete in terms of a complete set from replicate tanks for each treatment. To study the potential effects of nonylphenol on the osmoregulatory variables was the major aim of this study and this was clearly hampered by this problem. Therefore, the investigation of the potential physiological effects of low oxygen level on the osmoregulatory variables has been the focus in the latter part of the discussion.

The influence of 17β-estradiol on intestinal calcium carbonate precipitation and osmoregulation in seawater-acclimated rainbow trout (*Oncorhynchus mykiss*)

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Noura J. Al-Jandal, Jonathan M. Whittamore, Eduarda M. Santos, and Rod W. Wilson

The influence of 17β-estradiol on intestinal calcium carbonate precipitation and osmoregulation in seawater-acclimated rainbow trout (*Oncorhynchus mykiss*)

1. ABSTRACT

The intestine of marine teleosts produces carbonate precipitates from ingested calcium as part of their osmoregulatory strategy in sea water. The potential for estrogens to control the production of intestinal calcium carbonate and so influence osmoregulation was investigated in seawater-acclimated rainbow trout following intraperitoneal implantation of 17β-estradiol (E2) at two doses (0.1 and 10 μg E2 g^{-1}). Levels of plasma vitellogenin provided an indicator of estrogenic effect, increasing significantly by 3 and 4 orders of magnitude at the low and high dose, respectively. Plasma osmolality and muscle water content were unaffected, whereas E2-treated fish maintained lower plasma [Na^+] and [Cl^-]. Plasma [Ca^{2+}] and [Mg^{2+}] and muscle [Ca^{2+}] increased with vitellogenin induction, whilst the intestinal excretion of calcium carbonate was reduced. This suggests that elevated levels of circulating E2 may enhance Ca^{2+} uptake *via* the intestine by reducing $CaCO_3$ formation that normally limits intestinal availability of Ca^{2+}. Increasing E2 also caused a significant decline in intestinal fluid [SO_4^{2-}], suggesting a reduction of water absorption by the intestine. Together with elevated [Na^+] and [Cl^-] and reduced [HCO_3^-] in intestinal fluid, we speculate that E2 may influence water absorption *via* a number of potential intestinal processes; 1) reduced NaCl cotransport, 2) reduced Cl^- uptake *via* Cl^-/HCO_3^- exchange, and 3) reduced precipitation of Ca^{2+} and Mg^{2+} carbonates. Despite these effects on intestinal ion and water transport, overall osmoregulatory status was not compromised in E2 treated fish, suggesting the possibility of compensation by other organs.

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2. INTRODUCTION

2.1 Osmoregulation by marine teleost fish

Marine teleost fish are osmoregulators, maintaining their body fluids hypo-osmotic (~300 mOsm kg^{-1}) to seawater (~1000 mOsm kg^{-1}) and therefore face the problem of osmotic water loss and the passive gain of ions (Evans et al., 2005; Marshall and Grosell, 2006). To overcome this potential dehydration they continuously drink the surrounding seawater at rates of 1-5 ml kg^{-1} h^{-1} (Smith, 1930; Evans, 1993; Fuentes et al, 1996; Marshall and Grosell, 2006) and excrete the excess salt load across the gills (Na^+ and Cl^-), and in the rectal fluid and urine (Ca^{2+}, Mg^{2+} and SO_4^{2-}) (Marshall and Grosell, 2006). The gastrointestinal tract therefore has a key role in processing ingested seawater, and absorbing water (Shehadeh and Gordon, 1969; Marshall and Grosell, 2006).

2.2 Role and mechanism of the intestine in osmoregulation

The principal and most intensively studied driving force for water absorption in the intestine of seawater teleosts is the simultaneous absorption of Na^+ and Cl^- (Smith, 1930; Skadhauge, 1974; Usher et al., 1991; Marshall and Grosell, 2006). This absorptive process is ultimately fuelled by the basolateral Na^+,K^+ ATPase (NKA) which generates an electrochemical Na^+ gradient sufficient to drive the apical uptake of Na^+ and Cl^- via two cotransporters; Na^+:Cl^- (NC) and Na^+:K^+:2Cl^- (NKCC) (Musch et al., 1982; Grosell, 2006; 2010). A second direct driving force for fluid absorption in the marine teleost intestine involves the apical anion exchanger (AE) Cl^-/HCO_3^- which may contribute as much as 70 % of the total Cl^- and water absorption by this epithelium under non-fed conditions (Grosell et al., 2005; Grosell, 2006; 2010). The secreted bicarbonate has two major sources; the first

being the hydration of the endogenous metabolic CO_2 that is mediated by cytosolic carbonic anhydrase (Walsh et al., 1991; Wilson et al., 1996; Wilson and Grosell, 2003; Grosell et al., 2005; 2007; Grosell and Genz, 2006). The H^+ ions liberated from this CO_2 hydration are mostly transported across the basolateral membrane, either via Na^+/H^+ exchange (NHE) or a V-type H^+-ATPase, resulting in acid absorption proportional to HCO_3^- secretion at the apical membrane (Grosell and Taylor, 2007; Grosell et al., 2009; Whittamore et al., 2010). The second source of cellular bicarbonate is direct basolateral entrance via a Na^+:HCO_3^- cotransporter (NBC1) allowing for the basolateral uptake of both Na^+ and HCO_3^- driven by the basolateral Na^+,K^+ ATPase (Grosell and Genz, 2006; Grosell et al., 2009; Grosell, 2010). A third indirect driving force for water absorption is the precipitation of divalent cations (Ca^{2+} and Mg^{2+} from ingested seawater). The presence of elevated concentrations of HCO_3^- (30-110 mM) and high luminal pH (up to 9) in the intestinal fluid provide favourable conditions for the precipitation of calcium and magnesium carbonates. In the absence of carbonate precipitation, the poorly absorbed Ca^{2+} and Mg^{2+} would rise to very high levels (67 and 353 mM, respectively) which would clearly retard osmotic water absorption from the intestine (Wilson et al., 2002). Removal of most of the ingested Ca^{2+} and a smaller proportion of the ingested Mg^{2+} by alkaline precipitation conveniently avoids an excessive accumulation of these ions, reducing the luminal osmolality and thereby maximising the potential for water absorption by the intestine (Wilson et al., 2002; Marshall and Grosell, 2006; Whittamore et al., 2010).

The ingestion of seawater presents the intestine with a large potential source of Ca^{2+} for absorption and since systemic Ca^{2+} is very tightly regulated within the intracellular and

extracellular fluids the intestine has an important role in Ca^{2+} homeostasis. Since the gills do not participate in the excretion of divalent ions (Flik and Verbost, 1993) any Ca^{2+} absorbed by the intestine, and not sequestered by any of the internal pools (such as bones, otoliths, scales, gonads etc.), will be removed by the kidneys. However, given the infrequent and small amounts of urine produced by marine teleosts (Marshall and Grosell, 2006), the kidneys are very limited in their ability to excrete excess divalent ions such as Ca^{2+}. It is therefore not surprising that the renal excretion rates of Ca^{2+} by marine teleosts are estimated as being low (Hickman, 1968; Björnsson and Nilsson, 1985). Despite this, relatively few studies have considered the role of the intestine in Ca^{2+} homeostasis in marine teleosts. Under resting inter-digestive conditions, many studies show that little Ca^{2+} is actually absorbed across the intestine into the blood. *In vivo* the vast majority (> 80 %) of Ca^{2+} entering the intestine can be accounted for as excreted in the rectal fluid, with ~40-60 % in the form of solid $CaCO_3$ (Wilson and Grosell, 2003) thus the entry of Ca^{2+} into the body seems to be considerably limited. However, other studies report a net absorption of Ca^{2+} across the intestine (Sundell and Björnsson, 1988; Flik et al., 1990; Flik and Verbost, 1993), and in particular, the intestine can contribute in times of extra demand for Ca^{2+} such as gonadal maturation. Given that marine teleosts precipitate $CaCO_3$ as part of their osmoregulatory strategy, which also helps to limit the Ca^{2+} availability for absorption from the ingested water to maintain Ca^{2+} homeostasis (Wilson et al., 2002; Wilson and Grosell, 2003), this raises the question of whether there is any coordination or integration of these processes during periods of elevated Ca^{2+} demand.

2.3 The role of estrogens in fish osmoregulation

Sex steroid hormones include androgens and estrogens and these hormones are key coordinators of the reproductive development and function in males and females respectively. The function of estrogens is well conserved in vertebrates and includes the regulation of oocyte growth within the gonads and secondary sex characteristics and behaviours (Nagahama et a., 1995; Tyler and Sumpter, 1996). In addition, estrogens play a role in multiple processes in the body, including in the regulation of metabolism (Chen et al., 2009), the regulation of growth (Yu et al., 1979; Simm et al., 2008), bone development (Warner and Jenkins, 2007) and immune function (Wenger and Segner, 2008).

Estradiol has also been shown to play a role in the regulation of ionic and osmotic homeostasis, and the physiological processes involved in salt and water transport in fish (Mancera et al., 2004; Carrera et al., 2007). The effects reported include inhibition of key osmoregulatory variables (Mancera et al., 2004; McCormick et al., 2005; Lerner et al., 2007). For example, significant increases in total and ionic Ca^{2+} have been observed in the plasma of sea bream (*Sparus aurata*) following intraperitoneal implants containing 10 µg E2/ g body mass (Guerreiro et al., 2002; Guzman et al., 2004). Further indirect evidence supporting the involvement of estradiol in osmoregulation is provided by the expression of estrogen receptors in the intestine in a number of fish species including the sea bream (Socorro et al., 2000) and fathead minnows (*Pimephales promelas*) (Filby and Tyler, 2005). In the rainbow trout, four estrogen receptors have been described and expression of all four genes was demonstrated in the digestive tract (Nagler et al., 2007). Estrogen hormones such as E2 exert their effect principally (but not exclusively) by binding and activating the

nuclear estrogen receptors (Edwards, 2005). It has been suggested that estrogens and their receptors expressed in the gills in salmonids (Rogers et al., 2000) may be involved in osmoregulation as E2 and a variety of xenoestrogens antagonize metabolic, morphological, and physiological changes that take place during smoltification (Miwa and Inui, 1986; Madsen et al., 2004).

More specifically, a role of estradiol in calcium balance is supported by the additional need for Ca^{2+} during sexual maturation in females. During gonadal maturation, the females require abundant Ca^{2+} to provide oocytes with a ready store for subsequent skeletal development of their offspring. The enhanced demand of Ca^{2+} at such times can be supplied from various sources, under the influence of estrogens. In salmonids, estradiol can induce Ca^{2+} resorption from the scales and bones (Mugiya and Watabe, 1977; Carragher and Sumpter, 1991; Persson et al., 1994) and from the ambient water (Persson et al., 1994). Due to the continual ingestion of Ca^{2+}-rich seawater the intestine of marine fish has potential access to a readily available pool of Ca^{2+} which can be used to supply developing oocytes during sexual maturation. Here we hypothesise that the intestine could feasibly respond to estrogenic stimulus by enhancing Ca^{2+} uptake. The putative enhancement of Ca^{2+} uptake via the intestine may coincide with a reduction in the elimination of Ca^{2+} as $CaCO_3$, leading to increased Ca^{2+} transport by the intestinal epithelium.

2.4 The aim of the study

A number of previous studies have examined the effect of estradiol on hypo-osmoregulation in teleosts (Madsen and Korsgaard, 1991; Vijayan et al., 2001; Guzman et

al., 2004; Mancera et al., 2004). However, only one study has investigated the potential for calciotropic hormones (parathyroid hormone-related protein and stanniocalcin) to regulate bicarbonate and calcium transport by the marine teleost intestine *in vitro* (Fuentes et al., 2010). To our knowledge, this is the first study examining the potential estrogenic control of intestinal $CaCO_3$ formation and excretion and the subsequent consequences for ionic and osmotic homeostasis in a seawater-adapted teleost, the rainbow trout, *in vivo*.

3. MATERIAL AND METHODS

3.1 Experimental animals and acclimation to seawater

Immature female rainbow trout (*Oncorhynchus mykiss* Walbaum) (n = 44) (194.1 ± 3.5 g and 24.8 ± 0.2 cm, respectively) were obtained from Houghton Springs fish farm (Devon, UK) and kept in aerated, dechlorinated freshwater (pH 7.5 and temperature 11.2 ± 0.3 °C) at the University of Exeter. Fish were left unfed for two days before being transferred to a salinity of 10 ppt (approximately iso-osmotic) for 48 h, otherwise they were maintained on commercial pellet feed (BioMar, 5 mm, Aqualife, Denmark). Salinity was then increased to 20 ppt for a further 24 h and then to full strength seawater (salinity of 33 ppt; Na^+ = 420; K^+ = 10; Ca^{2+} = 10; Mg^{2+} = 47; Cl^- = 455; SO_4^{2-} = 21 mM), where the fish were left to acclimate for 10 days before starting the experiments. All fish experiments described in this study were conducted according to UK Home Office guidelines.

3.2 Implantation of 17β-estradiol (E2)

After acclimation to seawater, fish were anaesthetised in a 50 mg/l solution of MS222 (Pharmaq Ltd, UK) buffered with 150 mg/l $NaHCO_3$, followed by prolonged aeration to

restore normal CO_2 and pH levels. Estradiol was dissolved in coconut butter (Sigma-Aldrich, UK) to achieve the concentrations required for the experiment (Control = coconut butter only (vehicle); Low dose = 0.1 mg E2/ml; High dose = 10 mg E2/ml). Fish were randomly allocated to one of the three treatments (10 fish per treatment) before intraperitoneal (i.p.) injection at a dose of 1 µl per gram of fish, to achieve the required doses of 0.1 mg and 10 mg of E2 per kg of fish body mass. After injection, the needle was withdrawn and ice was applied to the injection site to help solidify the implant and avoid losses of the injected coconut butter. Fish were then transferred to individual chambers (18 litres capacity) containing aerated seawater.

3.3 *In vivo* experimental procedures and sampling

A semi-static system was used, whereby fish were maintained in their individual chambers for two weeks before terminal sampling. Intestinal precipitates of calcium carbonate excreted into the chamber were collected twice daily (10.00 and 17.00) from each fish, and 80 % of the water in each chamber was renewed every 48 h.

Two weeks after E2 implantation fish were administered an overdose of anaesthetic (300 mg/l buffered MS222), followed by blood sampling (~1 ml) via caudal puncture. The blood sample was divided into two aliquots, one added to 10 µl of aprotinin (Sigma-Aldrich) to reduce enzymatic degradation of vitellogenin, and the second without aprotinin for ion measurements. Blood samples were then centrifuged (13,000 rpm for 5 min at 4 ºC; Heraeus by Biofuge fresco, Kendro laboratory products, Germany) and the plasma was separated from the blood cells. The fresh plasma was used to measure osmolality and

chloride, and the remainder was subsequently diluted for ion analysis. The abdominal cavity was then opened by a ventral midline incision and the entire intestine ligated to collect fluid for analysis. A piece of white muscle was removed (2.09 g ± 0.06) from immediately below the dorsal fin for ion and water content measurement. The peritoneal cavity was then examined to confirm the integrity of the injected implant of coconut oil.

3.4 Analytical techniques for plasma and muscle variables

Osmolality was measured on 10 µl samples using a vapour pressure osmometer (Wescor Vapro 5520, USA). Cations (Na^+, Ca^{2+} and Mg^{2+}) and anions (Cl^- and SO_4^{2-}) were measured by ion chromatography (Dionex ICS-1000, Sunnyvale, CA, USA) on samples of plasma, intestinal fluids and seawater, following appropriate dilution. Total CO_2 of intestinal fluids was measured using a carbon dioxide analyser (Mettler Toledo model 965D, Columbus, OH, USA). The pH of intestinal fluid was determined using an Accumet combined microelectrode (Fisher Scientific, Loughborough, UK) connected to Hanna HI 8314 pH meter.

White muscle samples were transferred to a pre-weighed Teflon tube and then to an oven (70 °C) where they were dried to a constant mass. The dried samples were digested with 5 volumes of nitric acid (15.6 N; AnalaR, BDH Laboratory Supplies, UK). Following complete digestion, two sets of dilutions were prepared (100× and 500×) and 10 % (w/v) lanthanum chloride (Fisher Scientific, UK) was added to achieve 0.1 % (w/v) for measurement of divalent ions by atomic absorption spectrophotometry (Thermo Elemental SOLAAR AAS, UK). Muscle chloride was measured on 100-fold diluted digests using a

colourimetric assay (Zall et al., 1956) and the absorbance read on a microplate reader (Molecular Devices, Spectra MAX 340pc, USA) at 480 nm. Plasma vitellogenin was measured as an indicator of the exposure to 17β-estradiol using a homologous ELISA, according to previously described protocols (Tyler et al., 2002). Plasma was diluted at least 1:10 prior to analysis of vitellogenin concentrations, resulting in a detection limit for this study of 30 ng/ml.

3.5 Determination of carbonate content of intestinal precipitates

The collected $CaCO_3$ precipitates were rinsed with deionised water before being oven dried (45 °C) overnight. The next day, the dry weight was taken before adding 1 ml of 5 % (w/v) sodium hypochlorite (Fisher Scientific, UK) and left for 4 h to digest the organic mucus component. Samples were then rinsed 3 times with deionised water, centrifuged before being oven-dried for a further 24 h before and the final dry mass taken. The cleaned, dried inorganic samples of the precipitates were then sonicated (Vibra-Cell, Sonics and Materials Inc., Newtown, CT, USA) in 20 ml of ultrapure water (Maxima Ultrapurewater, ELGA, UK) for double end-point titration to determine the bicarbonate equivalent content.

The bicarbonate equivalents (HCO_3^- + $2CO_3^{2-}$) content of these precipitates was determined using the double titration method of Hills (1974), as described by Wilson et al. (2002) using an autotitrator (TIM845 titration manager and SAC80 automated sample changer, Radiometer, France). Samples were gassed with N_2 to remove all HCO_3^- and CO_3^{2-} as gaseous CO_2 during acidification and to ensure a stable pH measurement when returning to the starting pH (Wilson et al., 2002). Subsequently, samples were re-acidified to ensure

complete dissolution of Ca^{2+} and Mg^{2+} that had been liberated during titration of the precipitates. These acidified samples were then diluted for analysis of Ca^{2+} and Mg^{2+} content by ion chromatography.

3.6 Calculations and data analysis

Hickman (1968) and Genz et al. (2008) have previously used SO_4^{2-} concentrations in rectal fluid as a surrogate of intestinal fluid absorption based on the assumption that intestinal epithelium is impermeable $MgSO_4$ therefore considering it as a potential endogenous marker for water absorption. In the present study seawater and intestinal [SO_4^{2-}] for each fish were measured and used to obtain an estimate of fractional water absorption (%) by the intestine by using the formula:

$$\text{Fractional Water Absorption} = 100 - (\text{Seawater } [SO_4^{2-}]/\text{Intestinal } [SO_4^{2-}] \times 100)$$

The concentration of bicarbonate equivalents ($HCO_3^- + 2CO_3^{2-}$) in the intestinal fluid were calculated according to Henderson-Hasselbach equation using measurements of total CO_2 and pH:

$$[HCO_3^-] = [TCO_2]/(1+10^{(pH-9.46)})$$

The total CO_2 (mM) content [TCO_2] is the sum of [molecular CO_2] + [HCO_3^-] + [CO_3^{2-}]. Therefore, the carbonate (CO_3^{2-}) fraction was calculated by the following:

$$[CO_3^{2-}] = [TCO_2] - [HCO_3^-]$$

Hence, Total HCO_3^- equivalent = $[HCO_3^-] + 2[CO_3^{2-}]$

Vitellogenin data were log transformed prior to analysis. Significant differences between treatments were determined using One Way Analysis of Variance (ANOVA) followed by the Student-Newman-Keuls Method to determine significant differences between individual groups, when data was normally distributed and had approximate equal variance. For data that failed to meet these assumptions, significant differences between treatment groups were determined using the non-parametric Kruskal-Wallis One Way ANOVA on ranks followed by the Dunn's Method to determine differences between individual groups. Differences between treatment groups were considered to be significant when $P < 0.05$, and highly significant when $P < 0.001$. As an additional indication of the effect of estrogenic stimulation on physiological variables, regression analyses were performed comparing these against plasma vitellogenin concentrations (log transformed) of individual fish. Correlation analysis was carried out by linear regression, and the Pearson correlation coefficient (R) was calculated. For all statistical analyses, $p < 0.05$ was considered significant. All statistical analysis were conducted using Sigmastat 3.5 (Systat Software, Inc.).

4. RESULTS

4.1 Plasma vitellogenin and osmoregulatory variables

Seawater-acclimated rainbow trout implanted with E2 for two weeks, showed highly significant inductions of plasma vitellogenin (Figure 1) indicating strong and dose-dependent estrogenic response. The E2 implants did not affect plasma osmolality at either

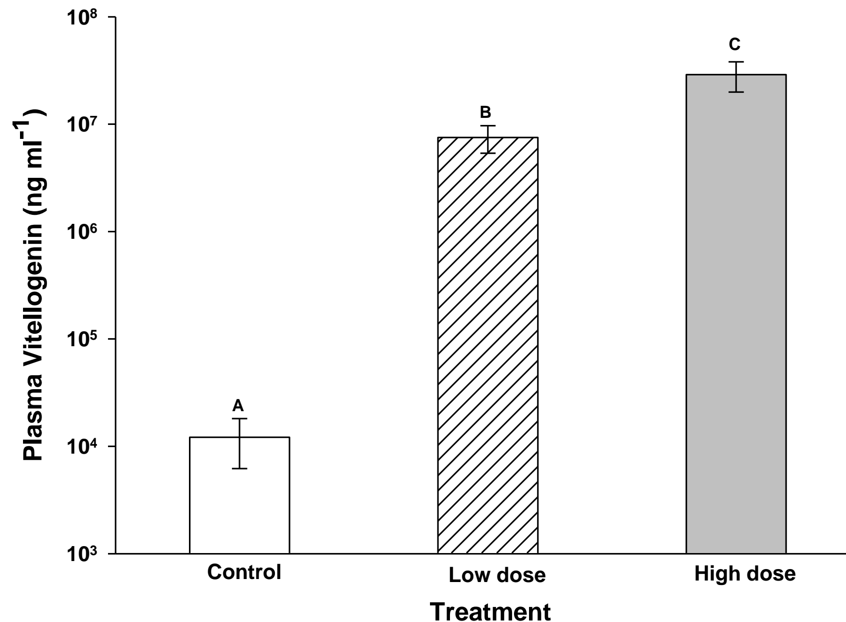

Figure 1: Plasma vitellogenin concentration in seawater-acclimated rainbow trout after 2 weeks implant with E2 (0.1 and 10 µg E2 g^{-1}). Each column represents the mean ± SEM. Asymmetric error bars are due to logarithmic scale of vitellogenin data. Different letters above bars indicate significant differences between treatment groups within a given time point (*P* < 0.001, Student-Newman-Keuls method).

dose. However, increasing levels of circulating vitellogenin were associated with significant decreases in both plasma [Na^+] and [Cl^-] (Figure 2). Estrogen exposure, as expected, caused a dose-dependent and highly significant increase in plasma [Ca^{2+}], and plasma [Mg^{2+}] also increased significantly, but in the high dose group only (Figure 3). No significant differences were detected in the plasma [SO_4^{2-}] (Table 1).

Concurrent with observations of plasma osmolality, E2 treatment did not affect muscle water content of the seawater-acclimated rainbow trout in any of the treatment groups (control group = 78.2 ± 0.3 %; low dose group = 78.2 ± 0.2 %; high dose group = 77.5 ±

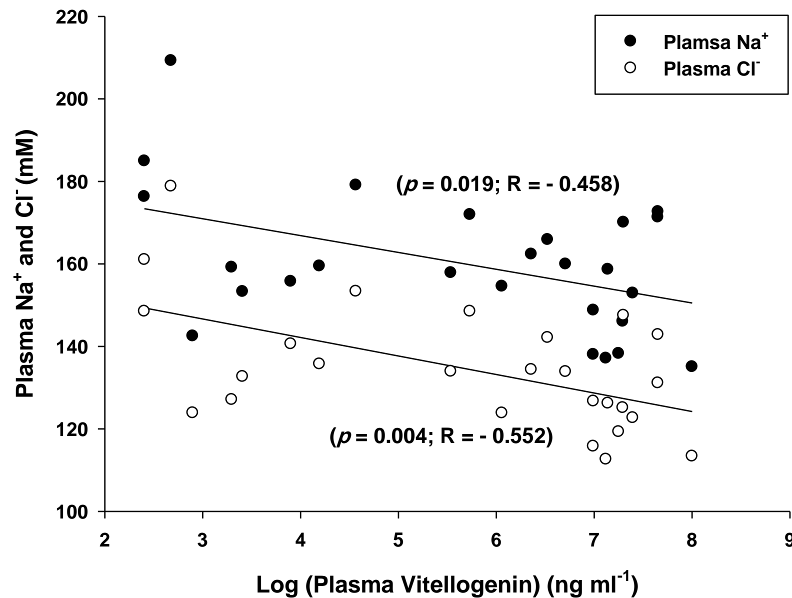

Figure 2: Relationship between the plasma vitellogenin and plasma Na^+ and Cl^-. Correlation analysis was carried out by linear regression, and the Pearson correlation coefficient (R) was calculated.

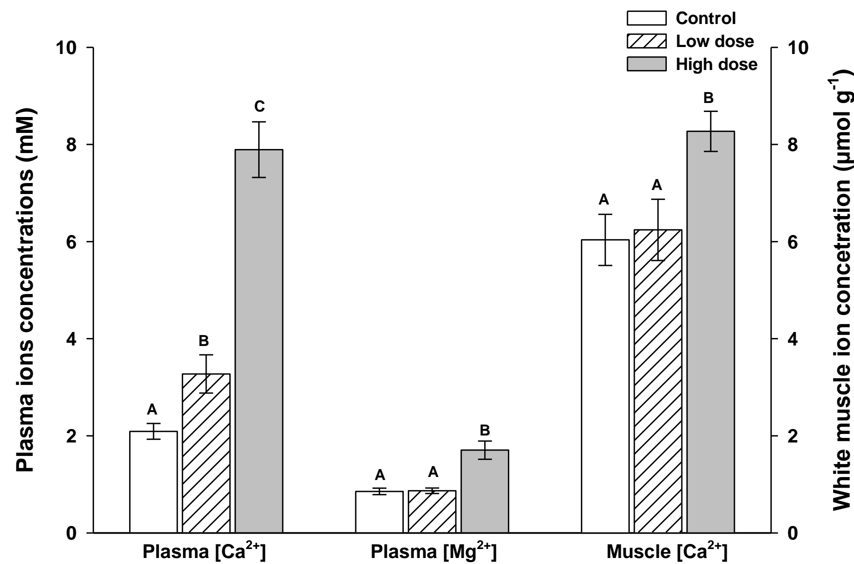

Figure 3: Plasma $[Ca^{2+}]$, $[Mg^{2+}]$ and white muscle $[Ca^{2+}]$ in seawater-acclimated rainbow trout treated with E2 (0.1 and 10 µg E2 g^{-1}) for two weeks. Different letters above bars indicate significant differences between treatment groups. Each column represents the mean ± SEM.

Table 1: Plasma osmoregulatory variables and muscle water content in seawater-acclimated rainbow trout implanted with either control or one of two doses of 17β-estradiol (0.1 and 10 μg E2 g^{-1}) for two weeks. Different letters after each number indicate significant differences between treatment groups.

	Muscle water content (%)	**Osmolality** (mOsm kg^{-1})	**[Na^+]** mM	**[Cl^-]** mM	**[SO_4^{2-}]** mM
Control	78.2 ± 0.3	322.8 ± 8.0	168.8 ± 6.8^a	144.6 ± 5.9^a	1.16 ± 0.2
Low Dose	78.2 ± 0.2	315.9 ± 5.1	157.8 ± 4.0	132.7 ± 4.2	0.69 ± 0.1
High Dose	77.5 ± 0.3	326.2 ± 8.3	153.2 ± 4.9^b	126.5 ± 3.5^b	0.91 ± 0.2

0.3 %). Calcium concentrations in white muscle increased significantly by almost 40 % in the high dose group in comparison to the control group (Figure 3).

4.2 Intestinal fluid chemistry and fractional water absorption

The 17β-estradiol had no significant effect on intestinal fluid osmolality. Intestinal [Na^+] was significantly elevated by 33 % in the high dose group in comparison to controls. No significant effect was detected in [Cl^-] although mean values followed the same upward trend as the intestinal [Na^+] with increasing E2 dose (Table 2). Under control conditions the intestinal fluid pH was distinctly alkaline (pH = 8.41 ± 0.06), with high concentrations of HCO_3^- equivalents (95.2 ± 5.8 meq l^{-1}). The pH and [HCO_3^-] decreased significantly in the high dose group by 0.2 pH units and 22 meq l^{-1}, respectively, in comparison to the control (Figure 4). Magnesium and SO_4^{2-} concentrations were significantly lower in the high dose group in relation to the control group by 23 and 27 %, respectively, with no differences observed in relation to Ca^{2+} (Table 2). The estimated fractional water absorption based on [SO_4^{2-}] was significantly reduced by 17 % at the highest E2 dose group (Figure 5).

Table 2: Intestinal fluid osmolality and ions concentrations in the seawater-acclimated rainbow trout implanted with either control or one of two doses of 17β-estradiol (0.1 and 10 µg E2 g^{-1}) for two weeks. Different letters after each number indicate significant between treatment groups.

	Osmolality ($mOsm\ kg^{-1}$)	**[Na^+] mM**	**[Cl^-] mM**	**[Ca^{2+}] mM**	**[Mg^{2+}] mM**	**[SO_4^{2-}] mM**
Control	332.3 ± 11.3	43.4 ± 3.2[a]	74.9 ± 6.0	6.7 ± 0.7	152 ± 8.3[a]	68.8 ± 3.8[a]
Low Dose	313.3 ± 7.0	49.5 ± 3.6	81.5 ± 5.3	7.7 ± 0.5	139.0 ± 13.6	59.6 ± 4.9
High Dose	344.3 ± 17.0	64.5 ± 5.3[b]	87.2 ± 6.1	6.9 ± 0.8	116.6 ± 9.8[b]	50.3 ± 4.7[b]

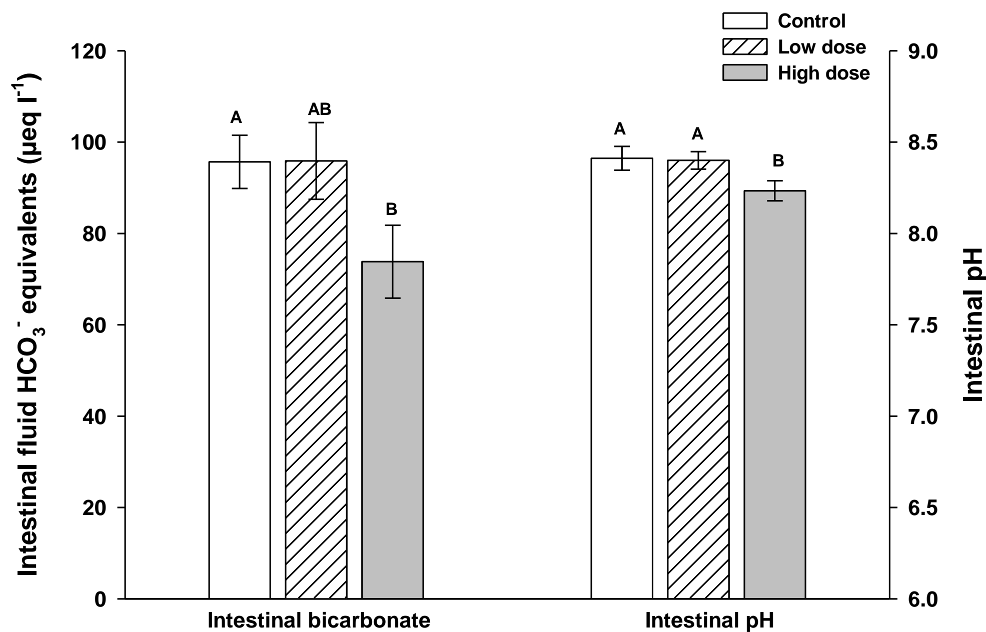

Figure 4: Intestinal fluid HCO_3^- equivalent and pH in the seawater-acclimated rainbow trout after 2 weeks implant with E2 (0.1 and 10 µg E2 g^{-1}). Different letters above bars indicate significant differences between treatment groups. Each column represents the mean ± SEM.

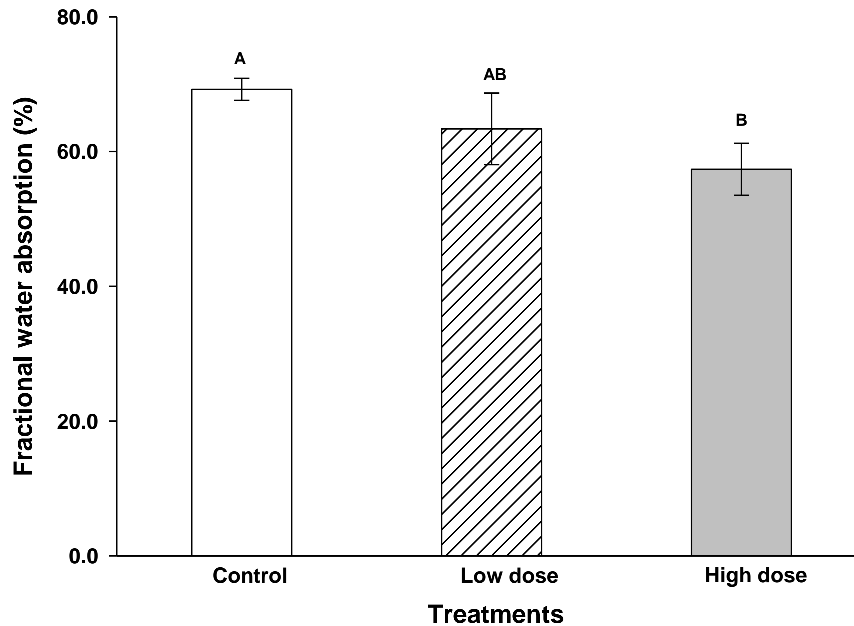

Figure 5: Fractional water absorption in the intestine of the seawater-acclimated rainbow trout after two weeks implant with E2 (0.1 and 10 µg E2 g^{-1}). Different letters above bars indicate significant differences between treatment groups. Each column represents the mean ± SEM.

4.3 Intestinal excretion of calcium carbonate precipitates

The $CaCO_3$ excretion rate was significantly reduced following exposure to E2 (Figure 6A), with a 50 % reduction observed in the mean value of the high dose group in comparison to the control group during the second week after implantation. A similar relationship was observed in the first week (declining $CaCO_3$ excretion with increasing plasma vitellogenin) although this was not significant (Figure 6B). During the second week, the rate of excretion of Ca^{2+} within the precipitates was also significantly reduced by 40 % in the high dose group compared to the control (10.5 ± 2.0 versus 17.6 ± 2.1 µmol kg^{-1} h^{-1}, respectively). The rate of Mg^{2+} excretion within the precipitates showed a similar significant decrease, and this was apparent in both the low and high dose groups (2.0 ± 0.3 and 2.2 ± 0.2

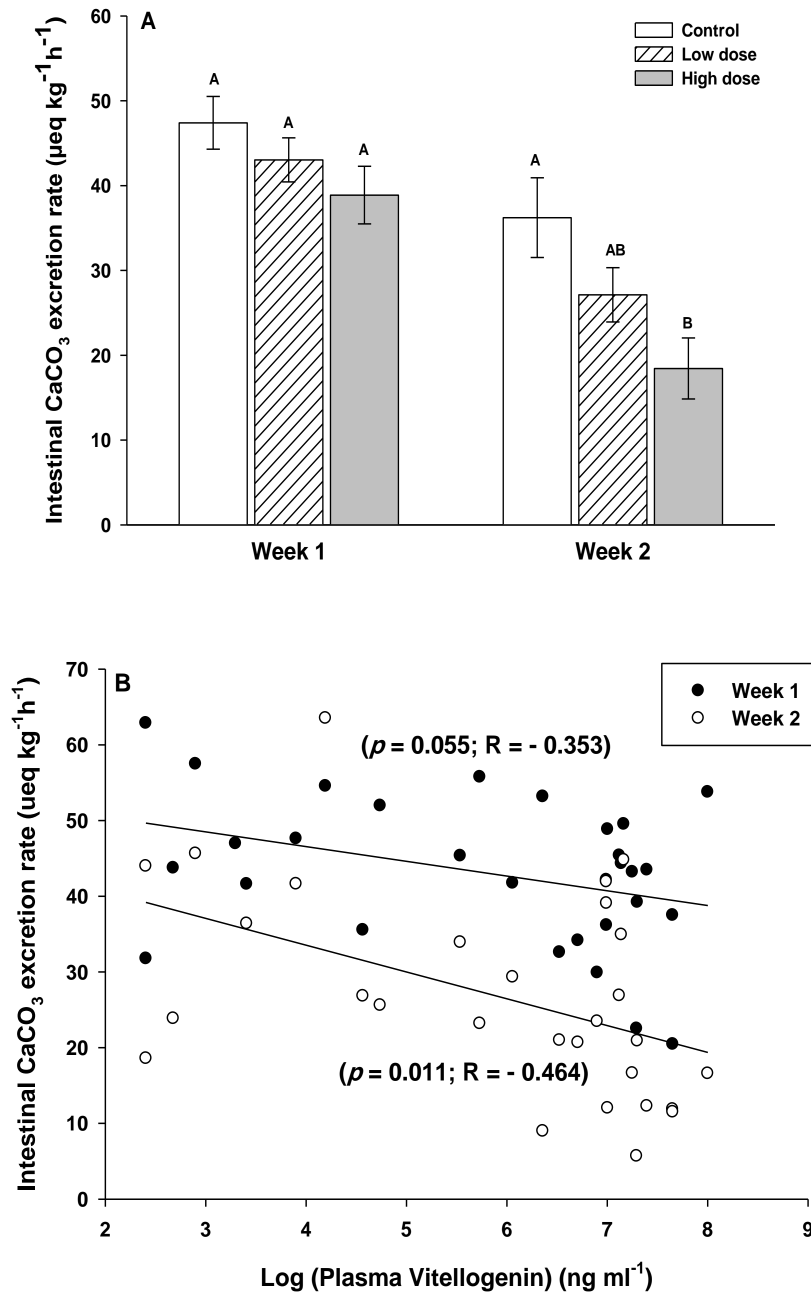

Figure 6: **(A)** Precipitate carbonate excretion rate in the seawater-acclimated rainbow trout after 2 weeks implant with E2 (0.1 and 10 µg E2 g^{-1}). Different letters above bars indicate significant differences between treatment groups. Each column represents the mean ± SEM. **(B)** Relationship between the precipitate carbonate excretion rate and the plasma vitellogenin. Correlation analysis was carried out by linear regression, and the Pearson correlation coefficient (R) was calculated.

μmol $kg^{-1}h^{-1}$, respectively) in comparison to the control (3.0 ± 0.3 μmol $kg^{-1}h^{-1}$) during the first week, but only significantly reduced in the high dose group in comparison to the control (1.1 ± 0.2 and 2.5 ± 0.6 μmol $kg^{-1}h^{-1}$, respectively) during the second week.

5. DISCUSSION

The major aim of this study was to investigate the possible effect(s) of 17β-estradiol (E2) on the handling of calcium by the intestine, in particular $CaCO_3$ formation, and the implications this may have in terms of osmoregulation. To achieve this we experimentally elevated the circulating estrogen levels using intraperitoneal implants of 17β-estradiol. Most of the measured parameters within the present data set were compatible with our hypothesis in terms of the reduction in $CaCO_3$ formation and excretion, and the subsequent potential for enhanced absorption of ingested divalent ions (particularly Ca^{2+}).

5.1 Plasma vitellogenin as a marker of elevation of circulating E2

Vitellogenin is a complex precursor protein for yolk production in oviparous vertebrates produced by the liver. It has been widely used as a biomarker for exposure to estrogens based on circulating levels in the plasma of immature female or male fish (Sumpter and Jobling, 1995). Plasma vitellogenin was therefore measured in the present study as an indicator of the biological response to the E2 implantation treatments and their estrogenic stimulation. Elevated levels of plasma vitellogenin were clearly observed in a dose-dependent manner for the low and high E2 treatments. The concentrations of vitellogenin in the control group corresponded to those expected in female rainbow trout during early gonadal development (Bon et al., 1997).

5.2 The role of E2 in promoting absorption of divalent cations via the intestine

Treatment with E2 was associated with enhanced uptake of Ca^{2+} into the body, indicated by very large elevations in calcium concentration in both the plasma and muscle. The most obvious routes for enhanced uptake of Ca^{2+} from the marine environment are the gills and the intestine. Although we have no direct evidence of E2 directly enhancing Ca^{2+} uptake from the intestine the potential scope for enhanced uptake is clear given that less Ca^{2+} was excreted by the intestine in the form of precipitated carbonates in fish treated with E2. Under resting conditions, an important function of this intestinal precipitation process is thought to be limitation of intestinal absorption of Ca^{2+} from ingested seawater as 40-60 % of ingested Ca^{2+} is excreted in the form of solid $CaCO_3$ (Wilson and Grosell, 2003). Seawater fish live in a hypercalcemic environment, and any Ca^{2+} absorbed which is in excess of growth requirements must be excreted, which has both an energetic cost and in the case of renal excretion it enhances the risk of kidney stones. The latter is due to the high concentrations of urinary SO_4^{2-} in marine fish and the insolubility of calcium sulphate (Wilson and Grosell, 2003) in addition to the very low urine flow rates in marine teleosts (Hickman Jr. and Trump, 1969; McDonald et al., 1982; Fletcher, 1990). The reduced intestinal carbonate precipitation in response to E2 would be a logical component of an estrogenic stimulation of Ca^{2+} absorption processes in the intestine. Indeed, the significant decrease in excretion of both Ca^{2+} and Mg^{2+} as carbonate precipitates supports the suggested effect of E2 on the balance of these two divalent cations in the intestine of marine fish. In addition, the role of the gills in the Ca^{2+} uptake cannot be neglected. Sea bream (*Sparus aurata*) fulfil the extra Ca^{2+} demand by utilising the environmental Ca^{2+} to protect the internal calcified structures (Guerreiro et al., 2002). However, although the latter

study concluded that the intestinal uptake of Ca^{2+} was not affected by E2, they only considered measurements based on intestinal fluid, and did not take into account any changes in Ca^{2+} present in carbonate precipitates.

Plasma divalent cations, [Ca^{2+}] and [Mg^{2+}], were both increased in a dose-dependent manner after treatment with E2, perhaps not surprisingly given that these two ions are known to increase in parallel with the elevated plasma vitellogenin in fish (Guerreiro et al., 2002). Indeed, an increase in plasma Ca^{2+} and Mg^{2+} following estrogenic stimulation have previously been used as indirect indicators of effective vitellogenin induction (Gillespie and de Peyster, 2004). Highly elevated [Ca^{2+}] in white muscle of the high dose group suggests that extra Ca^{2+} was accumulating in more than one major compartment (i.e. plasma as well as muscle). A key question for the present study is the source of the extra calcium that builds up in plasma and white muscle in response to E2, and in particular whether some of this could be due to enhanced uptake by the intestine.

Calcium uptake in the intestinal tract follows a similar cellular route to those in the gills, and occurs via active transport mechanisms (Flik et al., 1990). The cytoplasm of enterocytes has very low free Ca^{2+} levels and is electrically negative relative to the intestine lumen, which subsequently generates an electrochemical gradient to drive Ca^{2+} across the apical brush border membrane into the enterocytes. Enterocytes from the Atlantic cod (*Gadus morhua*) demonstrated a presence of voltage-gated L-type Ca^{2+} channels mainly located on the apical side of the cell which suggests the involvement of these channels in the entry of Ca^{2+} into the enterocytes (Larsson et al., 1998). The subsequent basolateral

extrusion of Ca^{2+} to the extracellular fluid occurs via transporters which include Ca^{2+}-ATPase (Flik et al., 1996). Arjmandi et al. (1994) showed that E2 administration in rats promotes intestinal absorption of Ca^{2+} *in vivo*. The latter supports the idea that there is a route whereby Ca^{2+} can be absorbed from the intestine, which could potentially fulfil the increased need of Ca^{2+} in female fish, to support the vitellogenic growth of oocytes during gonadal maturation.

The present study draws attention to the potential for estrogens to influence the calcification process by the intestine of marine fish. In an elegant study by Fuentes et al. (2010) it has been reported that endocrine factors such as the parathyroid hormone-related protein (PTHrP) and stanniocalcin 1 have an antagonistic action in the control of Ca^{2+} and HCO_3^- movement in the intestine of seawater fish such as sea bream (*Sparus auratus*). PTHrP increased Ca^{2+} uptake and reduced HCO_3^- secretion, whereas stanniocalcin 1 had the opposite effect, reversing Ca^{2+} net flux from absorptive to secretory, and promoting intestinal HCO_3^- secretion. In their *in vitro* study Fuentes et al. (2010) measured the HCO_3^- and Ca^{2+} transport rates but did not measure the $CaCO_3$ precipitation process itself. The present study is therefore the first to investigate any hormonal regulation of the production and excretion of $CaCO_3$ precipitates by the intestine of marine fish *in vivo*, and the first to show the potential for estrogenic control of this process. Having shown this using exogenous (implants) of E2, this raises the question of whether natural elevation of endogenous estrogens (as occurs during sexual maturation of female fish) will have a similar effect. This may be important to know for two reasons. Firstly, whether estrogens in sexually maturing females act directly on the intestine to control carbonate precipitation,

the supply of Ca^{2+} for gonadal uptake, and ultimately affect osmoregulation. Secondly, it may help in refining estimates of the global contribution of marine fish to the inorganic carbon cycle via their gut excretion of $CaCO_3$ precipitates (Wilson et al., 2009). Currently such spatial models of carbonate production by fish populations do not differentiate between immature and sexually mature fish, apart from considering body size (Jennings and Wilson, 2009; Wilson et al., 2009).

5.3 Effect of E2 on calcium homeostasis

The implantation with E2 revealed a significant increase in plasma Ca^{2+} levels. It is interesting to note that intraperitoneal injection of E2 in freshwater acclimated fish, rather than seawater acclimated fish such as in the present study, had no effect on Ca^{2+} absorption across the intestine of rainbow trout given 10 μg E2/g body mass (Mugiya and Ichi, 1981). However, freshwater fish are constantly faced with an osmotic water influx and therefore have extremely low drinking rates (Flik and Verbost, 1993). In freshwater fish that are not feeding, the gut is therefore much less important than the gills in terms of uptake of exogenous Ca^{2+} (Perry and Wood, 1985). E2 treatment of freshwater rainbow trout *Oncorhynchus mykiss* increases the uptake of Ca^{2+} from the water (presumably via the gills) along with mobilization from scales (Carragher and Sumpter, 1991; Persson et al., 1994, 1995, 1998) during the induction of vitellogenin.

5.4 Effect of E2 on the plasma and the white muscle osmoregulatory variables

Several previous studies have presented a negative effect of E2 on the osmoregulatory performance and ion balance in freshwater, seawater and euryhaline fish following acute

transfer between different salinities (Madsen and Korsgaard, 1991; Coimbra et al., 1993; Vijayan et al., 2001). Our study, however, showed no overall effect on the ability to maintain plasma osmolality or muscle water content, despite evidence in support of an inhibition of the ion transport processes in the intestine that are associated with water absorption. In a previous study, the whole body water content of three-spined sticklebacks (*Gasterosteus aculeatus*) and the plasma osmolality of rainbow trout, were not affected during a 24 hour seawater challenge after exposure to ethinylestradiol in freshwater, and both species were able to hypo-osmoregulate effectively (Al-Jandal et al., submitted, Chapter 3). The reason for the differences between our data and that published in the literature are not entirely clear but may be a consequence of the dose of E2 chosen or physiological differences in responsiveness between the fish used in each study, or differences between acute salinity challenges (previous studies) and fish maintained in a constant salinity for the present study.

Plasma [Na^+] and [Cl^-] showed a significant decrease at the high E2 dose, which suggests that these fish were not compromised in their ability to excrete excess Na^+ and Cl^- from their plasma (performed by the gills) and no overall osmoregulatory stress was detected (plasma or muscle). Indeed, fish undergoing treatment with E2 appeared better able to hypo-regulate Na^+ and Cl^- (relative to their ambient sea water) compared to the control fish. In a previous study we have found that male freshwater trout exposed to waterborne EE2 at 5 ng/l were also better able to hypo-regulate plasma Na^+ compared to controls during a subsequent seawater challenge (Al-Jandal et al., submitted, Chapter 3). The results suggest that the fish were able to compensate and hypo-osmoregulate despite the changes taking

place in the intestine. The potential mechanisms by which estrogenic stimulation may improve hypo-regulation of these plasma ions remains to be investigated.

5.5 Effect of E2 on ion and water handling processes in the intestine

The intestine of seawater fish plays an essential role in compensating for whole body osmotic water loss. We hypothesised that the treatment with E2 may induce a negative effect on bicarbonate secretion and calcium carbonate precipitation by the intestine and in turn the rate of intestinal water absorption. The present data supports this hypothesis. Although the actual transport rates of Na^+, Cl^-, and HCO_3^- by the intestine were not measured, the concentration of these ions within the intestinal fluid observed across treatments were affected by E2 in a manner consistent with changes in transport. Similarly, the measured excretion of precipitated calcium carbonates was also substantially reduced. Furthermore, the intestinal concentration of sulphate (used as a surrogate marker for fluid absorption) indicated that fractional absorption of water by the intestine was also reduced by E2. A potential caveat to this statement is that E2 could have increased the intestinal absorption of SO_4^{2-} and the apparent change in fluid absorption is in fact an artefact of lowered intestinal [SO_4^{2-}] rather than fluid movements. However, assuming that sulphate transport was not affected, and close to negligible as previously found (Hickman, 1968; Genz et al., 2008), a reduction in the intestinal water absorption can occur for the two reasons described below.

(1) Reduction in solute-linked water absorption driven by apical NaCl cotransport and Cl^-/HCO_3^- exchange in the intestine:

There was a general pattern of increased intestinal Na^+ and Cl^- concentrations and decreased plasma Na^+ and Cl^- concentration in response to E2. Together with the reduction in HCO_3^- levels in the intestinal fluid, these data suggest a reduction in Na^+ and Cl^- absorbed by the intestine via both NaCl cotransport and via Cl^-/HCO_3^- exchange. Both these transport systems drive an important fraction of the net water absorption in the intestine of marine fish (Grosell, 2006; Grosell et al., 2009; Whittamore et al., 2010), so any inhibition of these processes would be expected to directly interfere with water absorption. Usually, the intestinal absorption of the Na^+ and Cl^- from the ingested seawater is followed by water absorption, leaving behind the poorly absorbed Mg^{2+} and SO_4^{2-} in elevated concentrations in the intestinal fluid (Smith, 1930; Grosell et al., 2001).

E2 has been found to reduce the Na^+/K^+ ATPase activity in the gills of salmonids (Madsen et al., 1997). Although the same basolateral transporter is the underlying driving force for apical NaCl co-transport in the intestine, we can only speculate at this stage as to whether E2 has a direct influence on any of the individual transporters involved in NaCl uptake or Cl^-/HCO_3^- exchange within the intestine of marine fish, which could include any of the various apical and basolateral transport components involved (Grosell et al., 2007; Whittamore et al. 2010). However, it is worth pointing out that using an *in vitro* preparation of the toadfish intestine, Tresguerres et al. (2010) found no effect of luminally applied E2 on the short circuit current under symmetrical conditions. This suggests that E2 may not necessarily influence NaCl cotransport.

Estrogen was found to act through estrogen receptors to modulate fluid reabsorption in the adult mouse efferent ductules of the reproductive tract by regulating the expression of ion transporters involved in Na^{+} and Cl^{-} movement (Lee et al., 2001). Furthermore, it has been found that E2 can inhibit Cl^{-} transport processes in the isolated distal colon of female rats (Condliffe et al., 2001), showing the potential E2-driven mechanisms for regulating ion transport processes in the intestine of other vertebrates.

(2) Reduced water absorption due to decreased precipitation of Ca^{2+} and Mg^{2+} carbonates:

Another potential cause for reduced water absorption in the present study is the significantly decreased production of carbonate precipitates within the intestine. The divalent cations Ca^{2+} and Mg^{2+} present in the ingested seawater are poorly absorbed by the intestine and would become extremely concentrated in the intestine because of water absorption driven by the previous described mechanisms (Wilson et al., 2002). The prevention of excessive accumulation of these cations by precipitation as their insoluble carbonates normally aids in both Ca^{2+} homeostasis (preventing excessive intestinal absorption) and in promoting further water absorption by reducing intestine fluid osmolality (Wilson et al., 2002; Wilson and Grosell, 2003). The reduction in $CaCO_3$ excretion associated with reduced intestinal fluid HCO_3^{-} levels in response to E2 would presumably mean that less water absorption is driven by this alkaline precipitation mechanism. However, although $CaCO_3$ is predicted to permit additional fluid absorption by the intestine, there is currently no direct experimental evidence that this process of $CaCO_3$ formation drives intestinal fluid absorption (Wilson et al., 2002; Whittamore et al., 2010).

6. CONCLUSION

Studying Ca^{2+} balance has mostly focused on freshwater and euryhaline fish rather than marine fish especially with regard to the intestine as an osmoregulatory organ. Our novel finding is that E2 induces a major Ca^{2+} accumulation in plasma and muscle which is in parallel with plasma vitellogenin increases, and reduces calcium carbonate production by the intestine in seawater-acclimated rainbow trout. The findings reported here have implications for understanding of at least three areas of marine teleost biology. Firstly, the physiology of intestinal $CaCO_3$ production and associated ion transport mechanisms in relation to natural reproductive maturation in female teleosts. Secondly, the potential for exogenous xenoestrogens to influence these processes. Thirdly, they might help to refine the model estimates of $CaCO_3$ production by marine teleost fish and subsequently their contribution to the global inorganic carbon cycle (Wilson et al., 2009).

GENERAL DISCUSSION

Endocrine disrupting chemicals are a pervasive problem, and have become a major issue in the scientific community and the public due to their widespread occurrence and deleterious effects in animals. The effect of endocrine disruptors in wildlife is not yet fully understood and it is strongly believed that these chemicals are far more widespread than what is currently confirmed. Further research is required in this field besides the effects on reproduction, as many other physiological systems are regulated by hormonal systems, such as osmoregulation. For this PhD study I have focused my attention on the effect of several endocrine disrupting chemicals on the osmo- and iono-regulatory functions of euryhaline teleost fish. The results have indicated significant and largely negative effects on these vital processes and have highlighted some interesting avenues for future investigation.

When studying osmoregulatory variables, using plasma is the most common strategy to achieve fundamental information, whereas some studies have examined other tissues (e.g. muscle or carcass) as a source of osmoregulatory parameters, particularly when a source of plasma is limited for any reason (e.g. small animal size, or all the plasma available is required for other parameter measurements). However, the latter approach using tissues has never been rigorously validated as a suitable surrogate for all the potential osmoregulatory variables that can be measured in plasma. In the present study, such a validation was attempted, and has highlighted to fish physiologist the most robust variables that can be directly compared between plasma and tissue compartments (Chapter 2; Al-Jandal and Wilson, 2011). The results obtained presented a reciprocal changes between the plasma osmolality and the body water content, and parallel changes for most (but not all) of the ions that are of routine interest in such studies. This information has been directly useful in

a comparative study of two euryhaline species of different body size in Chapter 3 of the present PhD study (Al-Jandal et al., submitted), and should be of value to other studies that involve similar comparisons. This study provided the first useful methodical tool and clear description of how the plasma and tissue analyses correlate. This information will be of particular interest to osmoregulatory physiologists that work in species in which plasma sampling is troublesome.

One of the endocrine disrupting chemicals of great concern examined was the synthetic estrogen 17α-ethinylestradiol (EE2). It is used commonly in the contraceptive pill, and known for its high potency, occurrence, and persistent nature (Santos et al., 2007). Measured concentrations in UK freshwaters can reach 3.4 ng/l (Williams et al., 2003). This study presented the ability to expose two different species (different body size) in the same exposure conditions to avoid any practical differences that might a ffect the direct comparison. The results showed different sensitivity or response to endocrine disruptors differs between species. For example, rainbow trout (*Onchorhynchus mykiss*) showed a higher sensitivity when exposed to EE2 in comparison to the three-spined sticklebacks (*Gasterosteus aculeatus*), at least in terms of plasma vitellogenin induction (see Chapter 3; Al-Jandal et al., submitted).

The study of EE2 on the osmoregulatory capability of the three-spined stickleback (*Gasterosteus aculeatus*) and rainbow trout (*Onchorhynchus mykiss*) revealed a particularly intriguing effect of this estrogen on calcium homeostasis, as internal Ca^{2+} levels were upregulated in both species despite their very different responses in terms of vitellogenin

induction. Estrogenic stimulation of vitellogenin production and Ca^{2+} accumulation normally go hand-in-hand, so to observe a separation of these processes in the sticklebacks (no vitellogenin but elevated carcass Ca^{2+}) suggests differential sensitivity of the liver vitellogenin production and the uptake of environmental Ca^{2+} via the osmoregulatory organs.

Calcium balance in freshwater, seawater and euryhaline fish is controlled by a flexible combination of branchial uptake, absorption in the gut and renal excretion which are considered as essential processes for Ca^{2+} homeostasis. It would be interesting to further investigate Ca^{2+} dynamics after the exposure to EE2 to dissect out which of these organs and transport systems are primarily involved in the changes in internal Ca^{2+} levels in response to this endocrine disruption. The precise molecular mechanism by which EE2 may modulate Ca^{2+} regulation (in the absence of vitellogenesis) will be of great interest. An attempt was made to investigate this further in the present PhD study, but the number of male three-spined sticklebacks required was unfortunately too large to carry out the planned experiments. Instead, a further study (Chapter 5, Al-Jandal et al., submitted) was carried out to examine the effect of another estrogen (17β-estradiol; E2) on Ca^{2+} and water balance with a particular focus on the calcification process in the gut which is known to limit excessive Ca^{2+} uptake and promote water absorption under normal circumstances in seawater fish. A number of previous studies have examined the effect of endocrine disrupting chemicals on Ca^{2+} balance in freshwater teleosts, whereas the effect of estrogens on Ca^{2+} balance in marine fish and specifically the role of the gut was first examined in the present PhD study. The data obtained makes an important contribution to the role of

estrogenic compounds on the regulation of Ca^{2+} transport by the gut of marine fish especially when considering female fish with high estrogen levels during the natural maturing process. The present findings will also have potentially important repercussions for understanding how fish within pollution-related studies when considering environmental endocrine disruptors, although this is likely to be limited in geographical location to areas close to coastlines with large human populations.

The estrogenic control of gut $CaCO_3$ production in fish also has intriguing implications for an environmental issue of global significance, given the recently discovered contribution of gut calcium carbonate excretion to the total input of precipitated carbonate minerals into the marine inorganic carbon cycle (Wilson et al., 2009). After the industrial revolution there was an increase in the production of CO_2, this atmospheric build up of CO_2 dissolves in the ocean which makes the ocean more acidic that in turn changes the seawater carbonate chemistry which is expected to affect the production of the marine calcifying microalgae. The high concentration of Ca^{2+} in the ocean reacts with the bicarbonate producing insoluble calcium carbonate in the process of calcification (Figure 1). The vast majority of oceanic calcification is known to be from planktonic organisms (Feely et al., 2004) and is conventionally attributed to coccolithophores and foraminifera in particular. In the past, scientists have believed that the ocean's supply of $CaCO_3$ minerals (e.g. calcite and aragonite), which dissolve to make seawater more alkaline, came primarily from the external skeletons of microscopic marine plankton. However, Wilson et al. (2009) revealed that fish contribute a significant fraction of the ocean calcium carbonate production as a by-product of their osmoregulatory need to drink a large volume of seawater, and in turn this

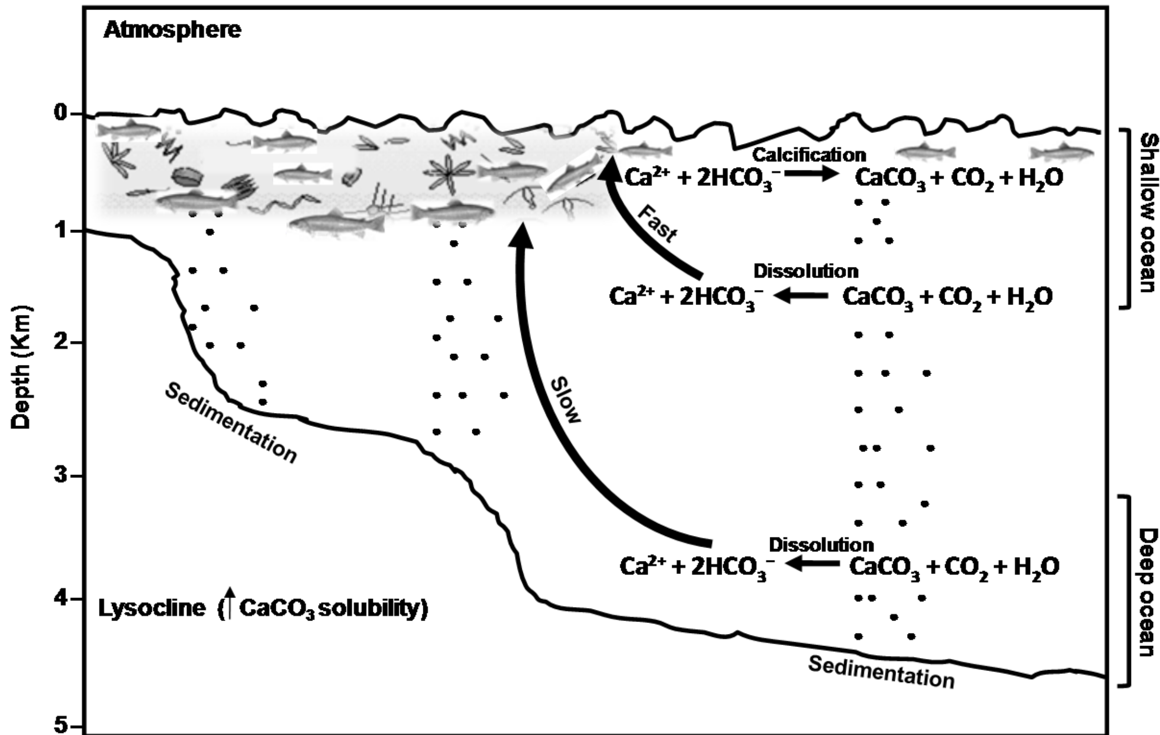

Figure 1: Schematic diagram showing the contribution of marine fish to the marine inorganic carbon cycle. Marine teleosts produce carbonate precipitates in the upper layer (≈ 500 m). Any carbonates that dissolve in shallow waters (e.g. <1 km deep) could make a major contribution to restoring the pH and alkalinity in the surface waters as vertical circulation within this region is rapid. If fish carbonates sink to the much deeper ocean before dissolving then the restoration of alkalinity (bicarbonate) and pH in the surface ocean will be much slower as vertical mixing across this depth will take 500-1000 years (Millero, 2007). Furthermore, dissolution may not occur at all if fish carbonates are buried within deep sediments, in which case they will not help to restore the pH balance of the surface ocean.

non-skeletal, piscine source of $CaCO_3$ may have important influences the pH balance in the ocean.

As mentioned earlier the gut plays a major role in osmoregulation in seawater fish and part of this involves the production of calcium carbonate ($CaCO_3$). The responses observed to an experimentally applied estrogen raise the possibility that estrogens (both natural endogenous levels and uptake of exogenous contaminants) may influence this process in

the gut, and therefore might affect the percentage of $CaCO_3$ contribution in the ocean by marine fish as modelled by Wilson et al. (2009). The results presented in Chapter 5 reveal potentially important insights into this crucial process in the fish gut. Although the results obtained from this study showed a significant effect on $CaCO_3$ production, the effect on the global contribution of fish to the marine inorganic carbon cycle in the real environment might not be big when considering the following:

1. Fish body size, where the large sexually mature fish are small contributors to the carbonate production in comparison to the small juvenile fish (Wilson et al., 2009).
2. Maturation cycle is seasonal rather than continuous for individual adult fish, therefore although the effect of estrogens on fish gut production of $CaCO_3$ is important during sexual maturation, the overall effect of estrogens on the percentage of contribution of fish is globally is not likely to be large on this scale when considering whole populations.
3. When considering the synthetic estrogens, the presence of these in the marine environment is low due to the high dilution, therefore, the effect of endocrine disruptors is likely to be localised to areas next to the coast, and hence the contribution to the global cycle in the open ocean is not likely to be high.
4. Fish gender plays a role in the matter of natural estrogens, where in this PhD the effect of an endogenous estrogen was examined in female fish only, whereas in the ocean (real environment) there are male and female fish. But when considering the synthetic estrogens from environmental pollution and their effect on fish, in that case the male fish might be included, where the vitellogenin synthesis will occur

due to the exposure to the endocrine disruptors and therefore might affect the $CaCO_3$ production in male fish too.

It would be very interesting to investigate the effect of more endocrine disrupting chemicals on intestinal carbonate precipitation and osmoregulation. In particular, studying the effect of nonylphenol effect on the intestinal carbonate precipitation of other euryhaline species (e.g. flounder) would be of value, given the environmental relevance of this chemical, and the rapid movements of this species between freshwater and seawater extremes during tidal salinity changes within their estuarine environment. Nonylphenol has been shown to mimic the effect of estradiol and it is a ubiquitous contaminant in water, sediment, and fauna (Lye et al., 1999; Gibson et al., 2005; Jin et al., 2008). The chance for the presence of nonylphenol in the sewage effluent in the estuaries where the flounder might exist will be a good rationale for investigation of the effect of nonylphenol on the $CaCO_3$ production in flounder.

The effect of nonylphenol on euryhaline fish was investigated in this study. Unfortunately, the aim of the study was hampered by a technical error and the data could not be investigated further. Although there were not high quality data to include and provide a lot of useful information to add to the thesis, it is important to include this study in the thesis for the scientist in the field to learn from our mistakes.

In general, these studies make an important contribution to the field of natural endocrine control processes as well as endocrine disruption showing that these chemicals have effects

on euryhaline osmoregulation. The results indicated either an improvement of hypo-osmoregulation after exposure to endocrine disruptor in seawater (Chapter 3 and Chapter 5), or negative effect on osmoregulation as a process itself. This study provided two aspects of research, which were physiological (Chapter 2 and Chapter 5), and ecotoxicological (Chapter 3 and Chapter 4) to cover as much range of endocrine disruptors and different exposure scenarios (waterborne, endogenous implant).

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