Investigation into the Effects of Pesticides on Amphibians.

A thesis submitted for the degree of Doctor of Philosophy

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Declaration

The work described in this thesis was carried out between 2004-2008 at Brunel University. This work was done independently and has not been submitted for any other degree.

Abstract

Amphibian population decline is a recognised phenomenon spanning at least the last 40 years, and it is likely that a number of factors have contributed, including environmental contamination. Amphibians are vulnerable to agrochemical uptake as they must breed in water, and often spend the aquatic phase of their lifecycle in agricultural water bodies, which may contain a complex mixture of biologically active chemicals. Endocrine disrupting compounds may cause reproductive effects in humans and wildlife, although the link between pesticides and endocrine disruption is largely unknown. Therefore, in this study, the role of pesticides in endocrine disruption, in relation to amphibian metamorphosis and reproductive development, was investigated. To achieve this objective, population data were used to select suitable field sites, water from which was tested for endocrine activity using the yeast estrogen/androgen screen, hepatocyte culture (estrogenic response), and a transgenic *Xenopus* test (thyroid disruption). Toad (*Bufo bufo*) specimens from a subsample of these sites were used to compare morphology, thyroidal, and gonadal development of caged and wild-caught tadpoles/metamorphs, to their laboratory-raised counterparts. In addition, environmentally relevant pesticides were tested for endocrine effects *in vitro*, and a short-term *in vivo* exposure was used to assess the predictive ability of the *in vitro* screens in *Xenopus*. Mortality of *Bufo bufo* was high in both laboratory-reared and caged individuals, which hindered the interpretation of results due to low *n* values. However, laboratory-reared individuals from different sites had distinct morphology and gonadal differentiation, possibly suggesting maternal transfer, a latent effect of the pond environment, and/or genetic effects. In addition, caged and wild-caught individuals were smaller, metamorphosed later, and had retarded gonadal differentiation or increased incidence of intersex, compared to their laboratory-reared counterparts. Extracts of water samples from these sites were predominantly anti-estrogenic, and/or anti-androgenic in yeast based assays, and this was also the effect observed in response to environmentally relevant pesticides tested in the same assays. Pesticides also affected ovarian steroidogenesis *in vitro*, and pentachlorophenol had a reprotoxic effect on adult female *Xenopus laevis*. Data reported in this study suggest there may be endocrine disrupting effects in native amphibians in the agricultural landscape, although further investigation is needed to confirm these findings.

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Chapter 1

Introduction & Literature Review

1.1 Overview

Over the past century, researchers from various countries have reported local amphibian declines, however, it wasn't until the first world congress of herpetology in 1989 (Cambridge U.K.), that local declines were collated and recognised as a worldwide phenomenon. Since then, amphibian population decline is a recognised trend spanning at least the last 40 years , and Houlahan *et al*. quantified this decline by analyses of datasets from North America and Europe. It is likely that a number of factors have contributed , including, habitat destruction and modification, climate change, increased UV exposure, introduction of non-indigenous species and infections (e.g. chytrid fungus, ranavirus), and adverse effects of environmental contaminants . Amphibians are vulnerable to agrochemical uptake as they must breed in water, and often spend the aquatic phase of their lifecycle in agricultural water bodies. They also have highly permeable skin, and thus are exposed to contaminants through both aquatic and terrestrial sources. Therefore, eutrophication of water bodies through fertilizer use, and use of pesticides and herbicides, could be instrumental in the decline observed .

Endocrine disruption (ED) is the process by which homeostasis of the endocrine system is altered by exogenous factors, and is thought to be contributing to decreased fertility and increased hormone sensitive cancers in humans . In men, decreased semen quality and sperm density has been reported from 1934-1996 , which has been linked to increased incidence of hypospadias and cryptochordism, and increased incidence of testicular cancer . It has been suggested that endocrine disrupting compounds may be causing the effects observed, although definitive evidence is lacking . In women, increased incidence of breast cancer has occurred in the western world, though this may be an artefact of better screening methods, rather than an actual trend . In addition, polycystic ovarian syndrome has been associated with an excess of androgen levels *in utero* , although evidence that it is a result of exogenous endocrine disruptors is scarce . However, decreased fertility, caused by disturbances to ovulation, the menstrual cycle, and spontaneous abortions, have been linked to pesticide exposure . The concept of toxicity of pesticides to wildlife was first detected when top predator birds (e.g. peregrine falcon, sparrowhawk, bald eagle, Baltic white-

tailed sea eagles) experienced severe population declines in the 1960's. This was found to be due to eggshell thinning, and was causally related to DDT, as residues were found in the eggshells of these species. Subsequent studies revealed that accumulation of DDE affected normal calcium transport and metabolism through inhibition of microsomal Ca^{2+}-ATPase in eggshell gland epithelium, which reduced shell thickness . Concern about aquatic ED was triggered by the discovery that large numbers of fish were becoming feminised in rivers in the UK. Soon after, feminisation was related to sewage effluent containing the synthetic estrogen ethinyl estradiol , and was subsequently reported to be a nationwide phenomenon . It was also shown that fish with mixed testicular and ovarian tissue, a condition called 'intersex', had reduced fertility compared to normal fish . In contrast to feminisation in fish, masculinisation of gastropod molluscs has been reported in response to tributyltin (TBT), which is used as an anti-foulant on boats. This condition is called 'imposex', and resulted in collapse of populations of Dogwhelk populations in coastal South-West England . It later became clear that the effects of TBT were widespread, and it was affecting a wide range of mollusc species. Since the use of TBT has been restricted, populations have recovered . Neither EE2 nor TBT are likely to be environmentally relevant to amphibians, as they are not generally found in effluent receiving large rivers/canals, and never in the marine environment. Instead, amphibians commonly breed in small water bodies, which are often situated in agricultural landscapes. Studies have related local amphibian decline to agrochemical usage , but endocrine endpoints were not measured in these cases. Recently, Hayes *et al*. , reported that low dose pesticide mixtures (including alachlor, atrazine, and cyfluthrin) increased corticosterone levels in *Xenopus laevis*, and inhibited metamorphosis in *Rana pipiens* (Northern Leopard frog). In addition, Reeder *et al.* reported an association between incidence of intersex with spatial and temporal declines in Cricket frog (*Acris crepitans*) populations. Atrazine levels were also found to affect hormone concentrations in *Xenopus* in their natural environment of S.Africa . Laboratory exposures to single agrochemicals have been reported to affect the endocrine system in amphibians , though negative results have also been reported .

In the UK, the period of heavy agrochemical usage was also the time when the most marked declines in anuran populations were recorded, although the causes for these declines have not been specifically linked to either agrochemicals or an endocrine

disrupting effect. Therefore, the aim of this research was to assess endocrine disruption in amphibians from UK agricultural freshwaters by field work, and to assess the endocrine disrupting potential of environmentally relevant agrochemicals in the laboratory

1.2 Population Change in the U.K.

Over the last 100 years, agricultural intensification and urban development, leading to habitat alteration and pollution, has often had deleterious effects on the native wildlife and ecosystems. Many aquatic habitats have disappeared completely due to coastal development, lowered water table, and agricultural 'improvement', particularly affecting amphibians due to their dependence on water for reproduction. Indications of declines of amphibian populations in the UK have been reported since the turn of the century , but it wasn't until the 1960's that drastic decline was documented . For example, adult frogs (*Rana temporaria*) decreased from three or four per acre in Hertfordshire in the 1930's, which was regarded to be a "fair average" for the British Isles , to an average of 0.1 adult frog (*Rana temporaria*) per acre by 1971 . Natterjack toads also decreased dramatically over this time period .

There are three species of anuran generally regarded to be native to England: 1 frog species (*Rana temporaria*), and 2 toad species (*Bufo bufo, Bufo calamita*). There are a number of populations of the Pool frog (*Rana lessonae*) and Edible frog (*Rana esculenta*) in the south east of England, resulting from frequent introductions. While it is possible that the former is native, these species, and the Natterjack toad, will not be considered further, due to their limited distribution in the UK. In addition, there are 3 urodele species in the U.K. (*Triturus helvetica, Triturus vulgaris, Triturus cristatus*), which will also not be considered further as they are not as widespread as the common frog and toad; and in the case of the latter, are heavily protected due to their rare status. Furthermore, the laboratory-based tests used in the present research were based on anuran tissue, and extrapolation to urodeles may not be accurate, due to differences in their biology and lifecycle.

1.2.1 Habitat Alteration in the U.K. in Relation to the Common Frog and Toad

Although there is little information on change of anuran population's pre-1930, it can be estimated that declines did indeed occur over this time period due to habitat loss, although to a lesser extent than in later years. The most fundamental changes in habitat were the loss of small water bodies over all England (see Table 1.1), and it was estimated that 70% of pools/marshes have been lost in the UK over the last century . There are also personal accounts of disappearance of these habitats, for example, from Yorkshire "Local farmers who remember the area in the 'twenties and earlier' speak of several small ponds and a number of minor streams…….which are no longer found" .

Historic Pond Use	**Modern Use**	**Change**
Village pond for drinking	Village pond – ornamental Reservoir – drinking	Ponds were in the community, now isolated, one reservoir supplies many people.
Farmyard pond for watering stock	Farmyard pond ornamental	No longer essential part of farm life.
Roadside pond was watering hole for passing stock	Balancing pond, road run-off, trapping pollutants	Complete change of use
Field pond/hedgerow pond was watering hole for stock	Field pond/hedgerow pond	Rarely maintained, infrequently used (water now piped to grazing stock), sites occasionally still present
Woodland pond	Woodland pond	Often completely wooded over
Ornamental lake	Ornamental lake	No change but reduced in number

		due to fewer houses with large grounds
Garden pond	Garden pond	Many now exist, important as wildlife ponds
Stew pond (fish pond for supply)	Fishing lake	Originally essential for winter food, now popular leisure activity
	Fish farms	Intensively managed
Duck pond	Duck pond	Was for supply of fowl, now ornamental
Mill pond	Mill pond	No change but little used
Stag pound to contain animals		No longer found
Icehouse pond for refrigeration		No longer found
Watercress beds	Watercress beds	Less frequent
Natural ponds where springs emerge etc.	Natural ponds where spring emerge etc.	Lowered water table has reduced numbers
	Golf course ponds	Becoming more frequent
	Garden centre ponds	Becoming more frequent

Table 1.1 – Change in pond usage over the past 100 years relating to urbanisation and agricultural intensification. Reproduced (modified) with permission from Wycherley and Anstis , and refers to Tandridge District in Surrey, but can be applied to Britain in general.

The 1930's were probably a time of resurgence of some populations due to the recession, which resulted in much agricultural land becoming derelict , whereas the 1940's heralded the acceleration of urbanisation, industrialisation, and agricultural reform, at least in part due to the demands put on the country during World War II. The changes in rural life are thought to have had an especially large impact on anurans, with intensive planting of arable crops, applications of fertilisers and pesticides, the destruction of ponds and hedgerows, and intensive forestry. In addition, traditional agricultural practices, which had formerly maintained heaths and dunes, were abandoned and natural succession occurred in the remaining areas of these habitats . At the end of this decade the first report of UK herpetofauna was published, and even though it only indicated the presence/absence of reptile and amphibian species, and not relative abundance, it is still interesting to note that at this time both the common frog and toad were present in every vice county of England . Cooke reported a slight decline in the common frog over this decade (index of change 1941-1945 = -0.04; 1946-1950 = -0.10), no information on the toad was available, but was assumed to be similar. In the 1950's, Cooke reported a more marked decline in

distribution of the common frog and toad than had been reported for the 1940's. It is probable that as well as increased habitat loss, the effects of changes made in the 1940's were only now being reflected in anuran numbers.

1.2.2 Agrochemical Usage and Decline in the Common Frog & Toad

It wasn't until the early 1960's that dramatic decreases in numbers of common frog and toads were reported and the impact of agrochemicals was implicated . For both the common frog and toad, declines on agricultural land were severe in the early 1960's and less so in late 1960's, which correlates with use of cyclodiene insecticides and DDT in the early 1960's, but a lesser effect as they were phased out of use . The pattern of decline coincides with other wildlife species, such as the Kestrel, Sparrowhawk, and Barn owl, which declined during the late 1950's and early 1960's, mainly as a result of being poisoned by agricultural chemicals . Bird declines and anuran declines were most marked in East England, which was the most intensively farmed area in England.

Area	**1951-1955**		**1956-1960**		**1961-1965**		**1966-1970**	
	Bufo	***Rana***	***Bufo***	***Rana***	***Bufo***	***Rana***	***Bufo***	***Rana***
S.W Devon/Cornwall	- 0.06	- 0.06	- 0.04	- 0.04	- 0.17	- 0.16	- 0.04	- 0.16
S Oxon/Glos	- 0.4	- 0.2	- 0.33	- 0.25	- 0.5	- 0.4	- 0.45	- 0.36
SE Sussex/Kent/ Surrey/Hants	- 0.5	- 0.21	- 0.23	- 0.19	- 0.5	- 0.4	- 0.45	- 0.4
SE Midlands London & N. home counties.	- 0.17	- 0.33	- 0.25	- 0.33	- 0.52	- 0.55	- 0.54	- 0.65
East Anglia Norfolk/ Suffolk	- 0.08	- 0.1	- 0.14	- 0.17	- 0.48	- 0.5	- 0.37	- 0.14
Midlands Warwicks	- 0.25	0	- 0.25	- 0.07	- 0.33	- 0.4	- 0.53	- 0.24
NW Midlands Ches/ Derby/Lancs	+ 0.13	0	+ 0.08	- 0.09	- 0.4	- 0.35	- 0.52	- 0.42
NE Midlands Lincs/ E & W Yorks/Notts	0	- 0.21	- 0.17	- 0.15	- 0.27	- 0.39	- 0.34	- 0.53
North Incl. N Yorks	- 0.5	- 0.25	- 0.33	- 0.29	- 0.29	- 0.23	0	- 0.35
Total	**- 0.14**	**- 0.15**	**- 0.15**	**- 0.17**	**- 0.4**	**- 0.43**	**- 0.36**	**- 0.38**

Table 1.2 – Changes in status for the common frog and toad in England. “Bufo” refers to *Bufo bufo*, the common toad, and “Rana” to *Rana pipiens*, the common frog. Index of change = (No. replies stating ‘increase’ – No. replies stating ‘decrease’)/(Total no. of replies – No. of replies stating ‘no longer found’), reproduced from Cooke .

As a result of declines in wildlife observed at the time of organochlorine pesticide use, Cooke investigated the effects of dichlorodiphenyltrichloroethane (DDT) and dieldrin on amphibians. Both pesticides caused hyperactivity, shuddering, abnormal snout development, interference with tail resorption, and mortality, although these effects were only observed at high concentrations, which would only be encountered in ponds that had been sprayed directly. In addition, the herbicide atrazine were also implicated in frog declines at this time . Collection by schoolchildren and scientists was also cited as a reason for decline up to the end of 1960 , although Cooke argued that although large numbers were taken, population decline was not correlated with areas from which they were taken.

1.2.3 Urbanisation and the Garden Pond

Population surveys during the 1970’s , and the 1980’s , revealed a more pronounced decline in the toad than the frog (Table 1.3). This was attributed to an increase in the creation of garden ponds , which are more suitable breeding habitats for frogs than toads, the latter breeding in older and larger/deeper water bodies. Indeed, during the 1980’s populations of the frog increased, whereas the toad continued to decline.

Area	**1970-1980**		**1980-1990**	
	Bufo	***Rana***	***Bufo***	***Rana***
South West	- 0.22	- 0.09	+ 0.86	- 0.07
South	- 0.09	+ 0.06	- 0.21	+ 0.37
South East	+ 0.23	+ 0.33	- 0.21	+ 0.27
West Midlands	- 0.25	- 0.06	- 0.38	+ 0.29
East Midlands	+ 0.17	+ 0.3	+ 0.06	+ 0.26
East Anglia	- 0.21	- 0.18	- 0.03	+ 0.07
North West	- 0.05	- 0.08	+ 0.4	- 0.06
North East	- 0.35	- 0.19	- 0.1	+ 0.06
Mean	**- 0.14**	**- 0.03**	**- 0.08**	**+ 0.19**

Table 1.3 – Changes of status of the Common frog and toad in England. “Bufo” refers to *Bufo bufo*, the common toad, and “Rana” to *Rana pipiens*, the common frog. Taken from Cooke & Scorgie (1983); Hilton-Brown & Oldham (1991).

Although some groups had been active in carrying toads across roads since the early 1900’s, it wasn’t until 1972 that the problem of road-associated mortality of toads was reported. The reason for toad-specific road mortality is that, unlike frogs, they display a high fidelity to their breeding site. Therefore, they may travel large distances to return to this breeding site, and consequently often need to cross roads . In 1989, concern related to road mortality was converted into action by coordination with local herpetological groups, with the setting up of the “Toads on Roads” scheme, which is run by “Froglife” (registered charity no. 1093372). However, it appears that the implementation of this scheme has not prevented toad declines, as declines are still observed in Central, South, and South Eastern areas, and especially in rural areas . The reason for this decline in toads is unknown, but one possibility is a decrease in genetic diversity of populations caused by habitat fragmentation as toads in small urban populations were found to have a lower genetic diversity than those of larger rural populations. In addition, significantly higher mortality and abnormalities were reported in tadpoles form smaller populations . They also had relatively lower genetic diversity than frogs from the same area .

Area (Rural data)	**Bufo bufo**	**Rana temporaria**
West	+ 0.05	- 0.15
Central	- 0.38	- 0.07
East/South East	- 0.3	- 0.05
North	+ 0.13	+ 0.06
Total Rural	**- 0.16**	**- 0.09**
Total Urban	**- 0.04**	**+ 0.2**

Table 1.4 – Changes in Status of Frog and Toad in England during 1990’s. Taken from Carrier and Beebee (2003).

Data from tables 1.2-1.4 were compiled and presented graphically (Figure 1.1). It is clear from this that the largest declines were observed 1960-1970, and that although frog populations appear to have recovered, toad populations have continued to decline. Although the data used for these analyses are qualitative, and were carried out by non-expert volunteers, it was similarly compiled and thus is probably unbiased.

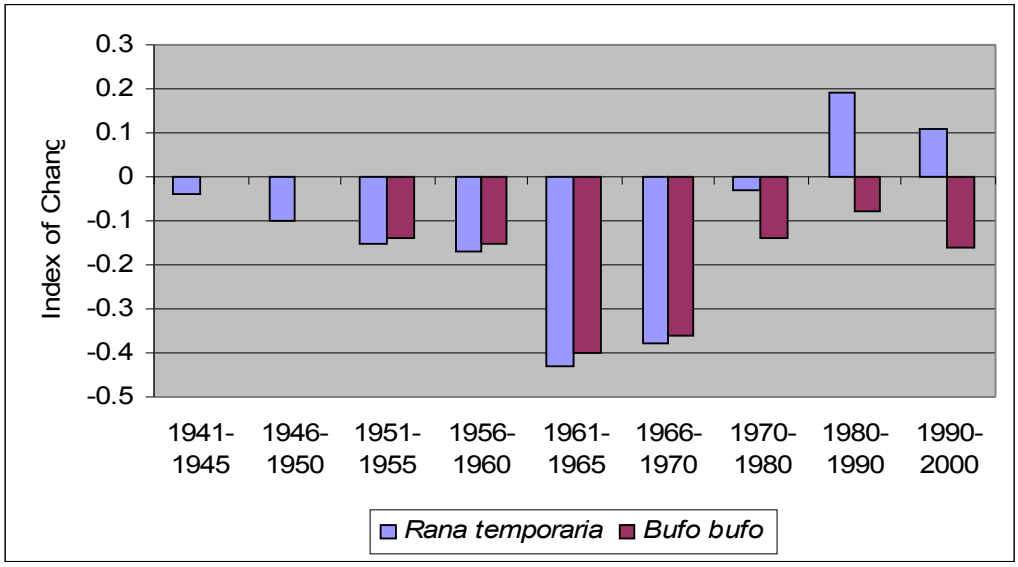


Figure 1.1 – Change in the status of the common frog, and common toad, in England in the last 60 years. (Index of change = (No. replies stating 'increase' – No. replies stating 'decrease')/(Total no. of replies – No. of replies stating 'no longer found')).

1.3 Endocrine System and Potential Targets for Endocrine Disruptors

The vertebrate endocrine system is complex and involves a range of different components to achieve homeostasis, which include periphery and target organs, and the circulatory system. Endocrine disruptors (EDs) can bind to the hormone's receptor and mimic the hormone (agonist), or bind to the receptor without eliciting a response, and thus prevent the endogenous hormone from binding (antagonist). They can also stimulate or inhibit enzymatic biotransformation, either in the target organ (steroidogenesis) or in a periphery organ (primarily the liver via hepatic biotransformation), thereby causing increased/decreased availability of the hormone. In the circulatory system, they can also act by interfering with binding to serum-binding proteins, thereby altering free hormone concentrations in the serum. Lastly, they can act upstream via the central nervous system, by interfering with the secretion of gonadotropins (luteinising hormone: LH; follicle stimulating hormone: FSH), and thyroid stimulating hormone (TSH), which in turn stimulate the production of hormones. Alterations to the endocrine system via these routes (and/or others) can lead to various effects, including altered growth and development, and sex reversal.

1.3.1 Hormone Receptors

The majority of published reports on EDs have focused on the *in vitro* capability for compounds to bind to, and initiate, hormone receptor responses. Tests have been developed to detect agents that interact directly with the hormone receptor in the absence of the endogenous ligand , or agents that inhibit binding by adding the test compound and the endogenous ligand simultaneously . The best known of these are cell proliferation assays and reporter gene expression assays. In the former, proliferation of cells that are dependant upon hormones for stimulation of growth are measured, for example, proliferation of human breast cancer cells (MCF-7) in response to estrogenic compounds . The latter is a measure of gene transcription induction following hormone receptor activation, and are based on mammalian cell lines or yeast strains. Cells are transfected with a reporter plasmid, and contain a hormone response element coupled to a reported gene, such as β-galactosidase or luciferase . The receptors that have received the most attention thus far are the estrogen receptor (ERα/β), the androgen receptor (AR), and the thyroid receptor (TRα/β) . Other receptors that have been implicated in endocrine disruption include the arylhydrocarbon receptor , the retinoic X receptor , and the pregnane X receptor , though their importance is less well understood.

In the present study three *in vitro* assays were used to detect receptor mediated (anti-) estrogens/androgens. The recombinant yeast assay was used to detect (anti-) estrogenic/androgenic activity via receptor mediated effects. The amphibian hepatocyte monolayer was used to detect estrogenicity of parent compounds and hepatic biotransformation products, via vitellogenin measurement, and the germinal vesicle breakdown assay (GVBD) was used to detect anti-androgenic activity in amphibian tissue.

1.3.2 Hepatic Biotransformation

Hepatic biotransformation often decreases the bioavailability of hormones, whereas gonadal steroidogenesis increases bioavailable hormones, resulting in dynamic

homeostatic control of hormone levels within the organism via these processes . There are several categories of reaction occurring in the liver that change the activity of steroid hormones including, hydroxylation, conjugation, and oxido-reduction, resulting in water soluble compounds that are excreted in the urine (You, 2004). In addition to metabolism of endogenous steroid hormones, the liver is also involved in the metabolism of xenobiotics. Under normal circumstances the liver acts to detoxify xenobiotics, leading to excretion of the compound, however, in some cases it metabolises a compound to a more biologically active metabolite . Induction/inhibition of biotransformation enzymes by agrochemicals may alter circulating steroid hormone levels, as has been demonstrated in field studies , and in the laboratory .

In the present study, the relative importance of parent compound and metabolite will be assessed through comparison of estrogenic activity in the yeast estrogen screen (YES), and vitellogenin (VTG) production by hepatocytes.

1.3.3 Central Nervous System and Sex Steroid Synthesis

The regulation of gonadal tissue in adult vertebrates occurs via the hypothalamo-pituitary-gonadal axis (Figure 1.2). Testosterone (T), dihydrotestosterone (DHT), and 17β-estradiol (E2) are thought to be the main controlling factors in the release of the gonadotropins LH and FSH, which in turn stimulate steroidogenic enzymes and thus stimulate biosynthesis of various steroid hormones (Figure 1.3). Steroid synthesis begins with the conversion of cholesterol to pregnenolone by cholesterol side-chain cleavage (P450scc), and subsequent conversion to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD), which together are the precursors of all other steroid hormones . In addition, the steroidogenic enzymes aromatase (P450arom) and 5α-reductase (5αR), convert androgens to estrogens (e.g. T→E2), and T to the more potent androgen DHT, respectively . In addition, two pathways of enzymatic conversion occur, the Δ5 pathway typically occurs in the adrenal cortex and stops with the production of dehydroepiandrosterone, and may be the main source of androgens needed for conversion to estrogens in females, whereas the Δ4 pathway is predominant in males and occurs within the testes . Steroid hormones influence the

hypothalamus by altering the secretion gonadotropin-releasing hormone (GnRH), which would in turn alter LH/FSH secretion, and/or act directly on the pituitary by altering its sensitivity to GnRH; and activin/inhibin are also thought to have a role in regulating FSH release . It is well established that amphibians produce GnRH , LH/FSH , and activin/inhibin , and that pituitary gonadotrophs contain LH/FSH . Neither androgenic or estrogenic receptors have been identified on the GnRH neuron, however there appears to be an abundance of receptors on other neurons, which are situated in close proximity to the GnRH neurons . The role of DHT and E2 in gonadotropin release has been shown in bullfrogs (*Rana catesbeiana*), whereby gonadectomy caused a chronic increase in levels of LH and FSH, which were remediated by implantation of DHT or E2 . DHT also acted on the pituitary directly, by increasing it's responsiveness to GnRH stimulation in gonadectomised male and female bullfrogs . Similarly, treatment of adult male and female *Xenopus laevis* with EE2 inhibited LH levels in the brain/pituitary, and treatment with methyl dihydrotestosterone (mDHT) inhibited LH production in males but not females .

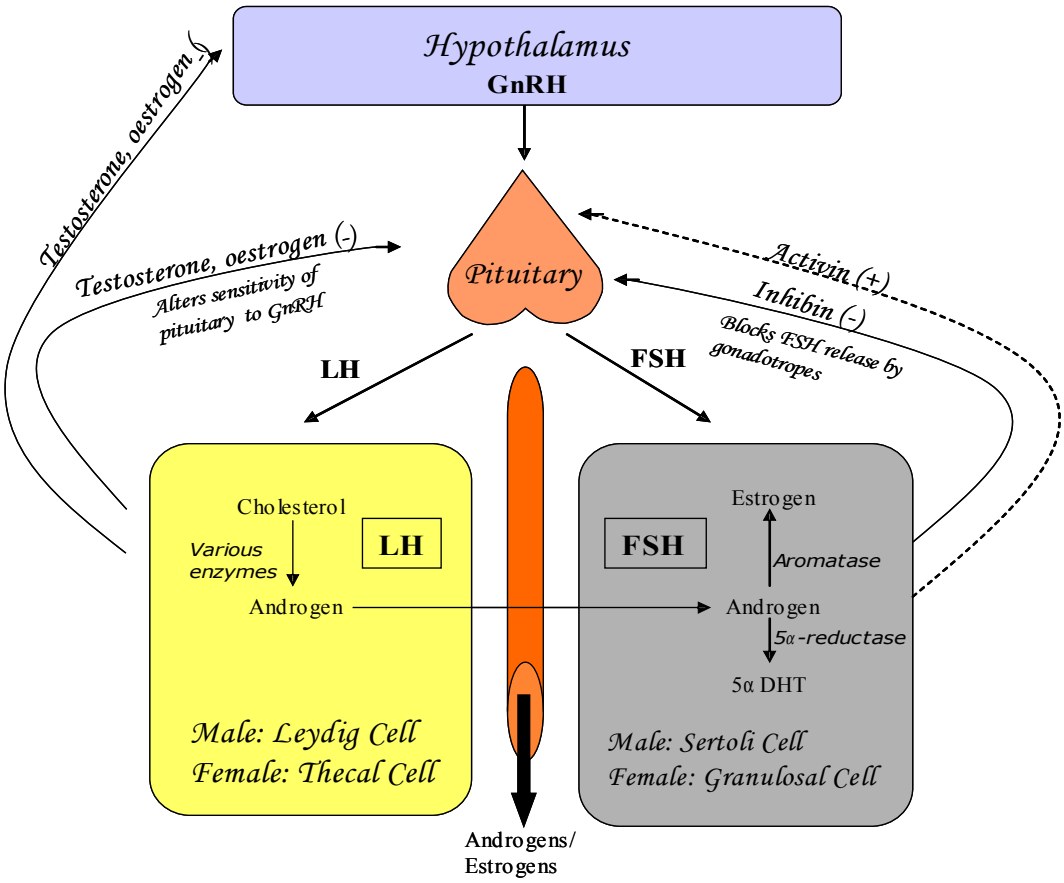


Figure 1.2. Vertebrate hypothalamo-pituitary-gonadal axis. See text for details.

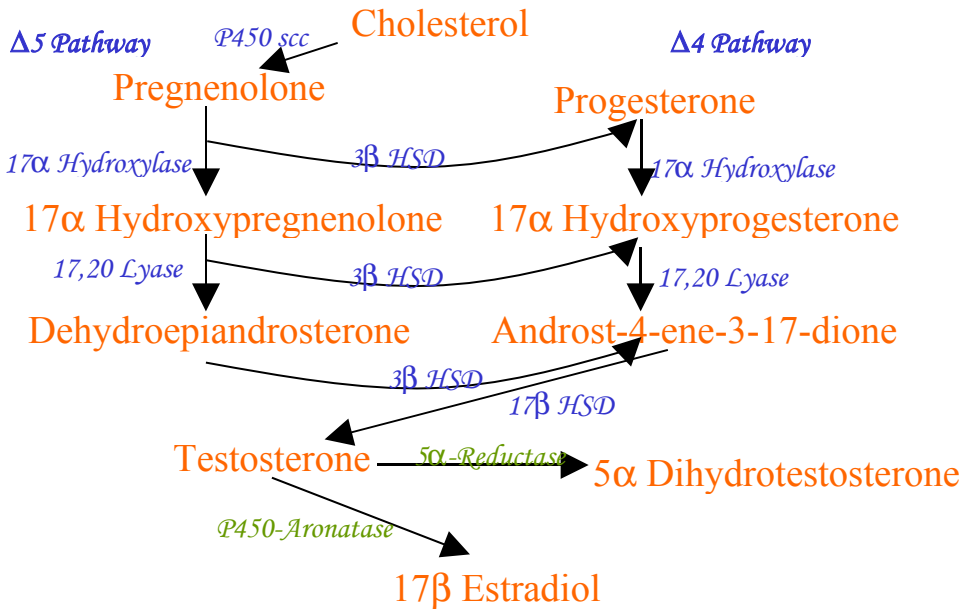


Figure 1.3. Enzymatic conversions of hormones. Note that 5α dihydrotestosterone is a more potent androgen than testosterone, it also cannot be aromatised to 17β estradiol, and that the production of androgens is necessary for the subsequent production of estrogens

In the developing amphibian, levels of LH, and FSH increase dramatically at the beginning of metamorphic climax (as does TSH, see section 1.4). There is a concurrent rise in brain GnRH content and plasma levels of FSH and LH, although this only occurs after prometamorphosis . In addition, GnRH neurons reach the median eminence at around the time when tadpoles begin to metamorphose , which indicates that the hypothalamo-pituitary-gonadal axis becomes functional at early metamorphic climax . This is sustained by the fact when 2 intraperitoneal injections of GnRH are given, LH/FSH secretion is not stimulated in prometamorphic tadpoles, but is in metamorphic climax tadpoles (Fiorentino *et al*., unpublished data, reported within Fiorentino *et al*., 1999), and plasma levels of sex steroids increase significantly from levels in premetamorphic tadpoles to levels in climax tadpoles (Di Fiore, Pinelli, D'Aniello, Rastogi, unpublished data, reported within Fiorentino *et al*., 1999). In these studies the sex of tadpoles was not reported, presumably because gonadal differentiation was not complete at the time of testing. However, it has recently been reported that during ontogeny of *Xenopus laevis*, gonadal aromatase activity was 10-fold higher in females than males at all stages where sex differentiation could be identified (Gosner stage 56-2 weeks post metamorphosis), and 5α-R was 2-fold higher in males than females from Gosner stage 60-66 . It has also been reported that DHT levels are approximately 10-fold higher in male than female bullfrogs in adults, subadults (lacked external sex specific features), and young metamorphs (recently metamorphosed), and that estradiol levels were higher in female than male subadults . The same authors reported that treatment with a GnRH agonist, increased levels of LH and FSH to a larger extent in males than females in the bullfrog (adult, subadult, and young metamorphs), and that pituitary responsiveness to a GnRH agonist occurred earlier in development in males than females (but both post-metamorphosis). In *Rana catesbeiana* it was reported that the ovary was less dependent on GnRH secretion for development than the testes , and that the pituitary of adult males was also more responsive to GnRH than adult females . This indicates that testicular development is more dependant on gonadotropin release than ovarian development. Indeed, it was reported that the tadpole (pre- and pro-metamorphic) ovarian fragments were able to synthesise and secrete estradiol independently of the pituitary, but only until metamorphosis , suggesting an independence of the ovary from gonadotropin stimulation in development. Lastly, removal of the pituitary (hypophysectomy) in larval *B. americanus*, did not affect gonadal development, but subsequent testicular

development was retarded, whereas no effect on the ovary was observed (Chang, 1955). Similarly, hypophysectomy of adult *B.arenarum* or adult *Rana esculenta* did not cause ovarian atrophy, but did provoke testicular atrophy. However, these studies operated over a short time frame (months), and eventually later stages of oogenesis and vitellogenesis are inhibited in hypophysectomised females, but no effect is observed on primary oocytes . Therefore, the hypothalamo-pituitary-gonadal axis differs in males and females during ontogeny, and these differences persist in adults.

The relative roles of LH and FSH differ in their function. In males, LH stimulates spermiation (release of spermatozoa from seminiferous tubules) and androgen production, whereas FSH stimulates spermatogenesis (development of spermatogonia to spermatozoa). In females, whereby LH stimulated ovulation, and FSH evokes ovarian growth without causing ovulation . These findings have been corroborated by more recent data, whereby FSH levels were closely correlated to vitellogenin and estradiol production in female *Rana esculenta*, and LH was closely related to androgen production in male *Rana esculenta* during the reproductive cycle in adults . During the annual reproductive cycle in *Rana esculenta*, the highest levels of gonadotropins and androgens were observed in the Spring, which coincided with reproductive activity of this species , and gonadotropins have been shown to upregulate testicular steroidogenic enzyme activity in *Xenopus laevis in vivo* and *in vitro* , which would result in the increased hormone levels observed.

In the present study, gonadal steroidogenesis was assessed by *in vitro* culture of *Xenopus* ovarian fragments. Ovulation and hormone production (P, T, E2) by tissue was measured to determine possible inhibition/stimulation of steroidogenic enzymes. Specific effects on the central nervous system were not assessed, however, the effects on the whole organism were assessed through toad sampling.

1.3.4 Sex differentiation and determination

Sex differentiation, defined as the development of testes or ovaries from undifferentiated gonad, and sex determination defined as mechanisms that direct

sex/gonadal differentiation , are fundamental endocrinological features of animals, and are highly conserved among vertebrates . Perturbation to the endocrine system via the mechanisms described above (and/or others), may ultimately result in altered fecundity of a population. In amphibians, there is considerable plasticity in phenotypic sex, whereby administration with sex steroids often results in the representative phenotype developing or mixed testicular/ovarian tissue . The sex-determining factor seems to be located in, or relayed by, the somatic tissue of the gonad, as implantation of a testis into a female causes masculinisation of the remaining gonad . However, if primordial germ cells (PGCs) are transferred to the opposite sex gonad, and the sex of the host develops normally .

In the present study, gonadal histology of *B.bufo* specimens from laboratory-reared, caged, and wild-caught individuals were used to determine effects on gonadal differentiation.

1.4 Thyroid System & Metamorphosis

Metamorphosis (a change in form or structure) is a dramatic example of postembryonic development, and is tightly controlled by hormones . In amphibians, the activity of the thyroid gland and levels of thyroid hormone (TH) change throughout metamorphosis. During premetamorphosis (when hindlimbs start to grow), TH levels are constant but low, during prometamorphosis (hindlimbs fully developed) increasing activity of the thyroid gland and increasing levels of TH are observed. The activity of the thyroid gland reaches its highest levels at the beginning of metamorphic climax (forelimbs come out of their sockets), and during the first stages of climax (tail begins to regress). In the later stages of climax, the thyroid gland activity decreases, and TH levels fall to those observed in postmetamorphic animals . Various hormones control the rate and timing of metamorphosis via inhibitory and excitatory signals acting through the hypothalamo-pituitary-thyroid axis . Corticoid and thyroid levels increase concurrently during metamorphosis of the developing tadpole , and corticosterone inhibits the rate of metamorphosis during early development and accelerates the rate during late development . The inhibitory effect may be due to negative feedback of corticosterone on the hypothalamus, thereby reducing secretion

of corticotropin-releasing factor, which in turn causes a decrease in TH production by decreasing stimulation of thyrotropin-releasing hormone . Stimulation in the later stages may occur due to the role of corticosterone in converting thyroxine (T_4) to the biologically active TH triiodothyronine (T_3) , and enhancing the binding of T_3 to its receptor . As a result of the higher levels of circulating thyroid hormones later in development, increasing corticosterone at this stage increases the rate of metamorphosis . The thyroid also seems to have a degree of autonomy during metamorphosis and a local positive feedback effect of thyroid hormone upon the thyroid hormone receptors has been reported . Studies using prolactin demonstrated the links between T_3, prolactin, and mRNA transcripts involved in autoregulation. When *Xenopus laevis* tadpoles were exposed to either T_3, or T_3 and prolactin, the prolactin inhibited the upregulation in mRNA transcripts which was observed in the T_3 only treatment. Concurrent experiments using isolated tails demonstrated that this effect on mRNA transcription could also be observed in the failure of the tail to regress in the T_3 and prolactin treatment .

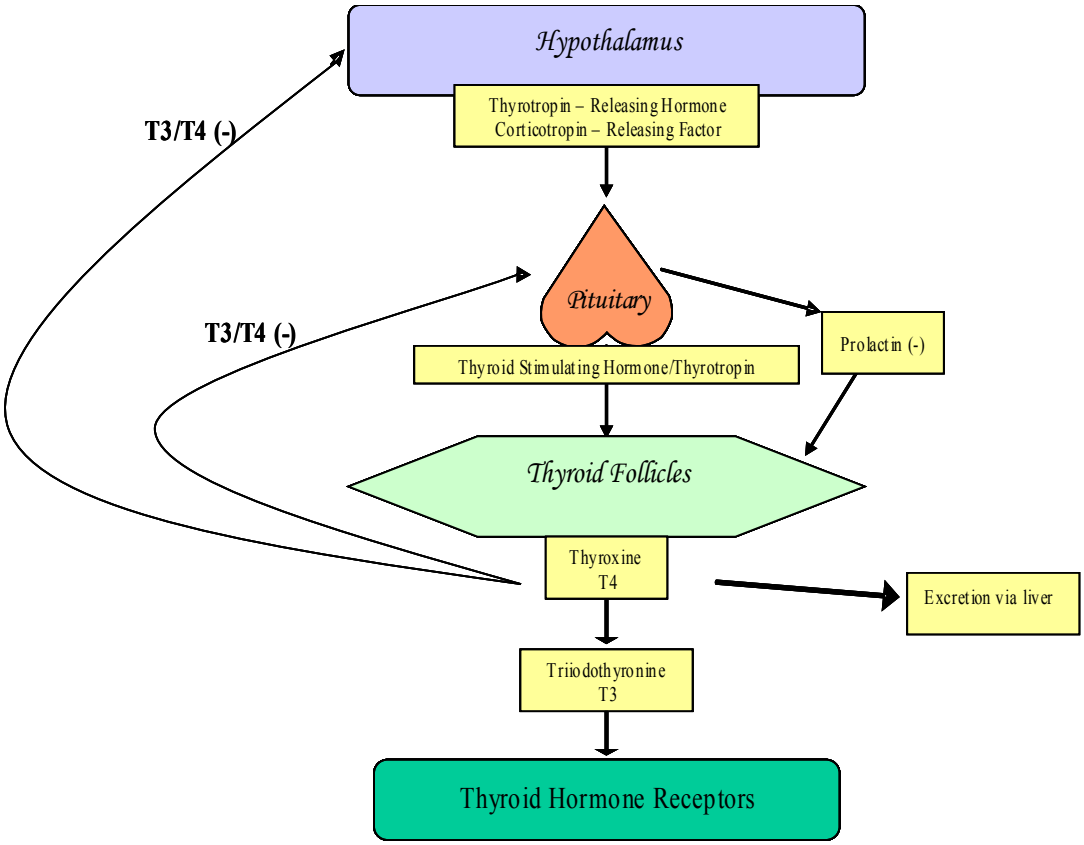


Figure 1.3. The vertebrate thyroid system (autoregulation, and influence of binding proteins not shown). See text for details.

1.4.1 Effects of External Factors on the Thyroid System

External factors, such as nutrition, competition, and temperature, can affect the rate of metamorphosis. For the developing tadpole, there is a trade-off between capitalising on an abundant food supply to attain a large size at metamorphosis, and the risk of staying in a potentially dangerous habitat. Therefore, good nutrition results in larger metamorphs, which complete metamorphosis at a younger age . Intraspecific competition and decreased food availability have been shown to increase the length of larval period , whereas a larval diet consisting of high protein caused a decrease in time to metamorphose and an increase in size at metamorphosis . Higher temperatures increase the rate of metamorphosis, as well as affecting the extent that exogenous steroids affect metamorphosis . In addition, corticosterone increases the rate of metamorphosis, and is a hormone which is related to stress, and therefore, it may be responsible for stress-induced metamorphosis, such as the acceleration of metamorphosis during pond drying . The sex steroids, estradiol and testosterone, have been shown to inhibit larval development in *Rana pipiens* , and inhibited tail resorption *in vivo* but not *in vitro* in *Xenopus laevis* ; the latter study suggesting that sex steroids do not interact with circulating thyroid hormone levels peripherally, but instead inhibit the thyroid axis more centrally . There is also some evidence that other hypothalamic substances may play a role in regulating thyrotropin, such as, somatostatin, dopamine, and glucocorticoids; which all have a inhibitory effect upon thyrotropin release .

1.4.2 Targets of the Thyroid System

The thyroid system has various possible targets for EDs, including, centrally controlled TH synthesis (hypothalamo-pituitary-thyroid axis), peripherally controlled conversion of T_4 to T_3 by deiodination enzymes, and excretion of T_3 by hepatic metabolism . The thyroid also has considerable capacity to compensate for disruption of its normal status. Thyroid follicles are capable of upregulating production of T_4, resulting in hypertrophy and hyperplasia of epithelial cells of the follicles, which can be observed histologically. Deiodination enzymes can also alter the proportion of T_3 to T_4 and thereby alter activity of circulating TH, and there is also a considerable store

of T_4 in thyroid colloid. Therefore, short term exposure to thyroid disrupting compounds may result in underestimation of the effect of these compounds . Receptor mediated effects and disruption of TH synthesis have been reported in response to a range of synthetic chemicals, including ammonium perchlorate , and various pesticides, such as DDT, amitrole, thiocarbamates , acetochlor, trifluralin, terbutryn , and organophosphates . In addition to receptor mediated effects, disruption of binding to TH-binding proteins has also been shown to be a target for some endocrine disrupting compounds, for example, polychlorintated biphenyls and DES, ioxynil and pentachlorophenol . In amphibians, thyroid sensitive endpoints, such as time taken to forelimb emergence and completion of metamorphosis, and hindlimb length, are routinely recorded. Therefore, there are more reports on the effects of pesticides on the thyroid axis than the hypothalamo-pituitary-gonadal axis.

In the present study, disruption to the thyroid axis was assessed by comparison of morphological and histological analysis of thyroid sensitive endpoints in laboratory-reared and field collected individuals.

1.5 Endocrine Disrupting Agrochemicals

The ED potential of currently used agrochemicals in the U.K. is largely unknown, and in most cases, least is known about those detected most often and at the highest concentrations. The U.S.A. has the largest world expenditure on pesticides (30% of herbicides, 24% of insecticides, 9% of fungicides, and 29% of others, Kiely *et al.*, 2004), and has a markedly different pesticide profile than the U.K. (Table 1.5). For example, of the 69 pesticides found above 0.01 µg/L in the U.K. in 2004/2005, only 7 were present in the top 25 used in the U.S.A. in 2001 (Kiely *et al.*, 2004). Therefore, pesticides that are environmentally relevant to the U.K. may not be well researched due to their relatively low global use. Herbicides and plant growth regulators were the highest use agrochemicals worldwide (37%), followed by 'others' (29%), insecticides (24%) and fungicides (9%) in 2001 . This pattern was loosely followed in the U.K., where of the 69 pesticides found above 0.01µg/L in 2004/2005, 62% were herbicides/plant growth regulators, 33% were insecticides, and 4% were fungicides (Table 1.5). In addition to the scarcity of information about environmentally relevant

pesticides, there is also generally very little information pertaining to pesticide induced ED in amphibians. Therefore, a review of the major classes of pesticides and their effects in a wide range of assays and organisms is reported here.

Table 1.5 – Levels of agrochemicals measured in U.K. freshwaters in 2004/2005 (Environment Agency Copyright 2007, duplicated as Appendix 1), and pesticide expenditure in the U.S.A. in 2001 (Kiely *et al.*, 2004).

Herbicides/Plant Growth Regulators

Mode of Action	**Type**	**Compound**	**Mean (µg/L)**	**Range (µg/L)**	**Median (µg/L)**	**Incidence (> 0.01 µg/L)**	**Median x Incidence**	**Rank UK**	**US**
Synthetic auxin	Phenoxy- acids	Mecoprop	6.593	0.028-6180	0.1	1444	144.4	1	
		MCPA	12.052	0.04-4700	0.152	615	93.48	4	
		2,4-D	40.348	0.04-18600	0.140	488	68.32	5	5
		Dichlorprop	5.112	0.04-561	0.200	120	24	9	
		2,4-DB	0.315	0.04-1.4	0.200	35	7	17	
		MCPB	0.296	0.04-1.81	0.1	65	6.5	19	
		2,4,5-T	0.077	0.02-0.352	0.059	24	1.416	44	
		4-CPA	0.165	0.04-0.995	0.075	12	0.9	49	
	Benzoic Acid	Dicamba	89.617	0.035-5090	0.1	57	5.7	21	24
		2,3,6-TBA	0.814	0.01-4.4	0.21	19	3.99	26	
	Pyridinecarboxylic acid	Fluroxypyr	0.315	0.044-4.54	0.084	26	2.184	32	
		Triclopyr	0.097	0.03-0.34	0.061	27	1.647	39	
		Clopyralid	0.093	0.04-0.37	0.05	12	0.6	53	
	Unknown	Benazolin	0.908	0.05-4.150	0.585	21	12.285	14	
Photosynthetic electron transport inhibitor	Urea	Diuron	0.652	0.016-28	0.131	785	102.835	2	
		Isoproturon	0.443	0.02-29.5	0.150	635	95.25	3	
		Chlorotoluron	0.271	0.021-5.87	0.077	217	16.709	10	
		Linuron	0.123	0.2-1.4	0.057	74	4.218	24	
		Monuron	0.346	0.04-0.94	0.196	9	1.764	36	
		Fenuron	0.129	0.07-0.213	0.124	10	1.24	46	
		Neburon	0.055	0.04-0.089	0.053	7	0.371	54	
	Triazine	Simazine	0.067	0.01-2.24	0.038	1444	54.872	6	23
		Atrazine	0.053	0.01-1.96	0.024	1464	35.136	7	2
		Trietazine	1.559	0.01-5.91	0.925	18	16.65	11	
		Terbutryn	0.134	0.01-4	0.027	148	3.996	25	

		Propazine	1.075	0.5-2.76	0.567	5	2.835	30	
	Benzothiadiazinone	Bentazone	0.121	0.04-1.75	0.07	137	9.59	15	
	Hydroxybenzonitrile	Bromoxynil	0.838	0.04-6.5	0.35	18	6.3	20	
	Pyridazinone	Chloridazon	0.573	0.04-1.73	0.32	7	2.24	31	
Uncoupler of oxidative phosphorylation	Dinitrophenol	DNOC (insecticide)	0.274	0.04-1.8	0.1	17	1.7	38	
	Unknown	Pentachlorophenol (biocide)	0.304	0.016-2.74	0.163	93	15.159	12	
Inhibits cell division (blocks microtubule function)	Carbamate	Chlorpropham	4.816	0.01-269.6	0.089	103	9.167	16	
		Carbetamide	0.264	0.05-0.744	0.297	17	5.049	23	
	Benzamide	Propyzamide	2.55	0.01-310	0.042	124	5.208	22	
	Dinitroaniline	Trifluralin	0.038	0.01-3.06	0.018	183	3.294	28	12
Inhibits cell division (blocks protein synthesis)	Chloroacetamide	Metazochlor	0.048	0.01-0.588	0.019	82	1.558	40	
Inhibts cell division (blocks sterol synthesis)	Triazole	Paclobutrazol	0.021	0.01-0.038	0.02	9	0.18	62	
Inhibiton of cell wall synthesis	Benzonitrile	Dichlobenil	0.168	0.01-10.6	0.026	122	3.172	29	
Chitin synthesis inhibitor	Benzoylurea	Diflubenzuron	0.11	0.04-0.87	0.058	21	1.218	47	
Inhibits lipid synthesis	Benzofuron	Ethofunesate	0.573	0.01-13.3	0.023	76	1.748	37	
	Thiocarbamate	Tri-allate	0.016	0.01-0.035	0.012	13	0.156	63	
Inhibits synthesis of essential amino acids	Glycine Derivative	Glyphosate	27.95	0.11-1600	0.229	149	34.121	8	1

Insecticides & Fungicides (f)

Mode of Action	**Type**	**Compound**	**Mean (µg/L)**	**Range (µg/L)**	**Median (µg/L)**	**Incidence (> 0.01 µg/L)**	**Median x Incidence**	**Rank UK**	**US**
Nerve Poison (affects sodium balance in nerves)	Organochlorine	DDT	0.025	0.01-0.094	0.02	21	0.42	53	
		TDE (DDD)	0.027	0.014-0.11	0.018	11	0.198	61	
Antagonist of the GABA receptor	Cyclodiene organochlorine	Dieldrin	5.647	0.01-153.4	0.096	69	6.624	18	

		Aldrin	0.05	0.011-0.312	0.024	15	0.36	55	
		HCH	0.019	0.01-0.055	0.017	14	0.238	60	
Cholinestersae inhibition	Organophosphate	Diazinon	0.046	0.01-0.535	0.03	451	13.53	13	
		Parathion	0.754	0.012-3	0.085	17	1.445	43	
		Mevinphos	0.33	0.01-2.53	0.03	29	0.87	50	
		Dimethoate	0.055	0.011-0.367	0.024	29	0.696	51	
		Azinphos-methyl	0.053	0.01-0.178	0.032	11	0.352	56	
		Malathion	0.03	0.01-0.109	0.019	17	0.323	57	6
		Fenitrothion	0.068	0.013-0.137	0.06	4	0.24	59	
		Propetamphos	0.019	0.01-0.034	0.016	7	0.112	64	
		Chlorfenvinphos	0.032	0.015-0.073	0.023	4	0.092	66	
		Triazophos	0.013	0.01-0.02	0.01	6	0.06	67	
	Carbamate	Pirimicarb	0.328	0.011-8.86	0.085	46	3.91	27	
Nerve poison (blocks sodium transport in nerves)	Pyrethroid	Permethrin	1166.8	0.01-26400	0.029	30	0.87	50	
		Cypermethrin	0.033	0.012-0.093	0.024	11	0.264	58	
Inhibition of digestive enzymes	Mitin	Sulcofuron	0.523	0.25-0.75	0.545	4	2.18	33	
		Flucofuron	0.293	0.18-0.52	0.255	6	1.53	41	
	Unknown	Eulan	0.107	0.02-0.7	0.07	14	0.98	48	
Uncoupler of oxidative phosphorylation	Organotin	Tributyltin	0.089	0.01-7.9	0.016	115	1.84	34	
		Dibutyltin	0.034	0.01-0.4	0.017	87	1.479	42	
Inhibition of beta-tubulin synthesis	Benzimadazole	Carbendazim (f)	0.11	0.023-0.516	0.069	20	1.38	45	
Inhibtion of ergosterol synthesis	Triazole	Flutriafol (f)	0.072	0.012-0.162	0.068	26	1.768	35	
	Morpholine	Fenpropimorph (f)	0.03	0.018-0.064	0.02	5	0.1	65	
Lipid peroxidation	Chlorphenyl/nitroaniline	Tecnazene (f)	0.013	0.01-0.21	0.011	4	0.044	68	

(f) = fungicide

1.5.1 Insecticides

1.5.1.1 Organochlorines

The organochlorine DDT was the first pesticide to be linked with adverse effects, and with endocrine disruption, in wildlife . A major route of biotransformation of DDT is dehydrochlorination to the more persistent metabolite dichlorodiphenyldichloroethylene (DDE), which has a very long half-life (10+ years in soil), and both have a high potential for bioaccumulation . DDT has been shown to be estrogenic in various test systems. VTG induction was observed in adult *Trachemys scripta* and *Xenopus laevis* injected with o,p'-DDT for 7 days , and sex reversal was observed in male *Oryzias latipes* (Japanese Medaka) eggs injected with 227 ng . Noriega & Hayes reported an estrogenic effect on coloration of *Hyperolius argus* (reed frog) when immersed in 0.1 µg/L o,p'-DDT, but not p,p'-DDT. In contrast, in *Ambystoma tigrinum*, technical grade DDT (80% p,p'-DDT & 20% o,p'-DDT) had an anti-estrogenic effect on Mullerian ducts when immersed in 10 µg/L, but DDE was estrogenic . *In situ*, DDE was associated with alligator population decline in Lake Apopka (Florida), where a major pesticide spill containing high levels of DDT was correlated with poorly organized testes and small phalli in males, and polyovular follicles and multinucleated oocytes in females . The estrogenic effects of o,p'-DDT and DDE observed *in vivo* are consistent with estrogenic activity observed *in vitro*. Both congeners of DDT were estrogenic in the E-Screen as 10 µM (Soto et al. 1994), and, when tested at lower concentrations, o,p'-DDT was a more potent estrogen than technical grade DDT or p,p'-DDT alone . DDE was also estrogenic, albeit at levels approximately 2 orders of magnitude less than o,p'-DDT . Interestingly, both DDT congeners and DDE were also anti-androgenic in various cell based test systems , and have also been shown to be anti-progestogenic . Due to their persistence in the environment and continuing use worldwide, the endocrine disrupting (EDg) activity of DDT and metabolites cause concern for wildlife and humans. However, they are of limited environmental relevance in the U.K., where use has been severely restricted since the 1960's, and were found rarely in low concentrations in 2004/05 (e.g. DDT was ranked 53, Table 1.5). Other organochlorines have also been classified as EDs , for example, methoxychlor ,

endosulfan , lindane , and toxaphene , however, similarly to DDT, they are not environmentally relevant to the U.K. Dieldrin is the most environmentally relevant organochlorine in the U.K. (ranked 18, Table 1.5), but it has been reported to have little or no ED activity , although it is highly toxic to developing amphibians at environmentally relevant concentrations (Table 1.5). Therefore, it is unlikely that organochlorine insecticides are causing ED in U.K. amphibians, but there may be a toxic effect on embryos and tadpoles.

1.5.1.2 Organophosphates and Carbamates

The use of organochlorines was largely phased out in the 1960's, and they were gradually replaced by organophosphates (OPs) and carbamates, which were more readily biodegradable. OPs are the most common type of insecticide or fungicide detected above 0.01 µg/L in the U.K. (Table 1.5). Their mode of action in the target organism is via enzyme (cholinesterase) inhibition (Tomlin, 2006). Interestingly, they possess little interaction with hormone receptors , but do affect steroidogenic enzymes. For example, dimethoate inhibited CYP450scc in Leydig tumour cell line , and diazinon inhibited cortisol secretion in adrenocortical cells . A field study investigating amphibian population decline in California associated decreasing populations with decreased acetylcholinesterase activity, and also found higher OP residues (chlorpyrifos & diazinon) in frog tissues from these individuals . Diazinon was the highest ranked insecticide or fungicide detected in the U.K. (rank 13, Table 1.5), and had a reprotoxic effect on the ovary of *Lepomis macrochirus* (Bluegill) exposed to 60 µg/L , which was accompanied by decreased estradiol levels . Other OPs have also been identified as EDs *in vivo*, for example, parathion in mice , dimethoate in rats , fish , & ewes , fenitrothion in fish , and malathion in quail . However, these compounds are rarely found in U.K. freshwaters (ranked 43-59, Table 5.1). Similarly to OPs, carbamates possess little receptor binding activity , but pirimicarb, and propamocarb induced aromatase activity in human placental microsomes , and chlorpyrifos induced various CYP450 hydroxylases in a cell transcription assay . The only carbamate found in the U.K. above 0.01 µg/L was pirimicarb (ranked 27, Table 1.5), however, the maximum concentration it was detected at was 8.9 µg/L, compared to 11900 µg/L (50 µM) used by Andersen *et al*. (2002), and therefore, carbamates are unlikely to be effecting amphibians in the UK.

1.5.1.3 Pyrethroids

The insecticidal properties of pyrethrum, a product made from Chrysanthemum flowers has long been known, and natural pyrethrins served as a model for the development of synthetic pyrethroids. Natural pyrethrins degredate very quickly in sunlight (hours), however, synthetic pyrethroids are more stable and can last for months or even years in soil (Tomlin, 2006). The often adsorb strongly to soil particles, but during storm events are washed into nearby water bodies, thereby inducing a spike in contamination level . They are widely used insecticides, and examples include permethrin, cypermethrin, fenvalerate and deltamethrin, although only permethrin and cypermethrin were detected in the U.K. in 2004/05 (ranked 50 & 58, respectively, Table 1.5). They have an agonistic effect on the estrogen receptor in the μM range, which has been shown in various cell based test systems , and metabolites of deltamethrin and cypermethrin are also anti-androgenic over a similar range . Permethrin and cypermethrin have also been shown to induce transcription of CYP450 hydroxylases , which catalyze the hydroxylation of testosterone, and therefore could result in lowered levels of circulating testosterone. Indeed, cypermethrin has been associated with decreased anogenital distance and relative prostate weight in male rat pups after perinatal exposure , which are indicative endpoints of anti-androgenic activity , and could result from decreased circulating testosterone levels. Cypermethrin also decreased hatching rate, and increased time to metamorphosis in *Rana arvalis* tadpoles exposed to 1 & 10 μg/L , however, levels found in the environment were below this level (maximum measured concentration: 0.0002 μM, 0.093 μg/L). Permethrin, on the other hand, was found at much higher levels (maximum measured concentration: 67.5 μM, 26400 μg/L), which are similar to effective concentrations *in vitro*, indicating a possible effect on amphibians at point source areas.

1.5.2 Herbicides

1.5.2.1 Triazines

The triazines are widely used herbicides worldwide, atrazine was the second most commonly used pesticide in the U.S.A. in 2001 , and simazine and atrazine were among the highest ranked agrochemicals in the UK (6 & 7 respectively, Table 1.5). Research has indicated that atrazine is an endocrine disruptor in rats , fish , and amphibians . In amphibians, the most commonly reported effect was feminisation/demasculinisation upon exposure to 0.1-25 µg/L, either through decreased testosterone levels , or histological abnormalities . Concentrations at the lower end of this range are comparable to levels found in the U.K. environment (maximum measured concentration 1.96 µg/L, Table 1.5), and thus are a cause for concern. Most studies have reported that triazines or their metabolites do not interact with hormone receptors , but have been reported to affect steroidogenic enzymes *in vitro* . Atrazine upregulated aromatase in human adrenocortical cells , but this effect could not be verified *in vivo* . Atrazine and the metabolite deethylatrazine, inhibited 5α-R and 17βHSD activity in male rat pituitary cell suspension, and in male rats *in vivo* . In addition, atrazine inhibited adenocorticotropic hormone (ACTH) stimulated cortisol secretion in steroidogenic cells of the rainbow trout and *Rana catesbeiana,* but not *Xenopus laevis* ; indicating a species specific response. Concentrations eliciting a response *in vitro* were in the low µM range (1 µM = 215 µg/L), which is higher than levels found in the environment, however, due to effects observed in aquatic organisms *in vivo* this compound remains a cause for concern, as does the less tested triazine, simazine.

1.5.2.2 Phenoxy-Carboxylic Acids

The phenoxy herbicides 2-methyl-4-chlorophenoxyacetic acid (MCPA), dichlorophenoxyacetic acid (2,4-D), and mecoprop are among the highest ranked agrochemicals in the U.K. (1, 4, & 5 respectively, Table 1.5), and 2,4-D was also the 5th most commonly used pesticide in the U.S. in 2001 , however, their effects on humans and wildlife are not well defined. MCPA caused testicular degeneration in the seminiferous tubules of rats fed 112 mg/kg/day , and increased hepatic enzyme activity in female newts exposed to 800 mg/L , however, this is much higher than levels found in the environment (maximum detected concentration: 4.7 mg/L). Mecoprop was not estrogenic or anti-estrogenic *in vitro* , but had a slightly toxic effect on murine embryos exposed to 0.5 µg/L . 2,4-D decreased circulating thyroxine

levels in ewes exposed to 30 mg/kg/week, but, estradiol, cortisol and LH levels were unaffected . *In vitro*, *Xenopus* oocyte maturation was irreversibly inhibited by exposure to 10 mM 2,4-D , however, this corresponds to 2.2 g/L, which is much higher than levels found in the environment (maximum detected concentration: 18.6 mg/L). However, the LC50 of 2,4-D to *Bufo melanosticus* larvae was 8.05 mg/L , suggesting that toxicity could occur at point source locations.

1.5.2.3 Ureas

The ureas are also highly ranked herbicides in the UK, for example, diuron, chlorotoluron, linuron and isoproturon were ranked 2nd, 3rd, 10th, & 24th, respectively (Table 1.5). However, similarly to the phenoxy herbicides, little is known of their ED effects, especially in the case of chlorotoluron, where no reports were available. Diuron had no effect on fish testicular or ovarian steroidogenesis *in vitro* at 0.1 or 1 mM . In addition, no effect was observed on prostate 5α-R activity up to 100 μM , or placental aromatase activity up to 50 μM . It also did not interact with the estrogen receptor , but was weakly anti-androgenic . Considering it's weak/absent endocrine effects *in vitro*, perhaps it is not surprising that diuron had minimal effects on reproductive parameters in rats exposed *in vivo* . At similar concentrations, linuron also did not interact with the estrogen receptor , and didn't inhibit placental aromatase acitivity . However, it had approximately 3 times more potent anti-androgenic activity than diuron , and inhibited prostate 5α-R activity by 50% at 86 μM . *In vivo*, several studies have shown anti-androgenic activity of linuron on reproductive parameters in rats, and the mechanism of action was hypothesised to be at least partly via antagonism of the androgen receptor . Anti-androgenic activity has also been demonstrated in an aquatic exposure study, whereby inhibition of spiggin production in female Stickleback exposed to 150 μg/L was reported (Katsiadaki et al. 2006). However, in the latter study, no effect was observed at 15 μg/L, which is still an order of magnitude higher than the maximum concentration detected in the U.K. in 2004/2005 (1.4 μg/L), indicating it may not pose a risk to amphibians. Isoproturon is the least well studied of the phenoxy herbicides, and to the author's knowledge, just

one publication has tested for endocrine effects *in vitro*, and no estrogenic or anti-estrogenic activity was observed . However, it has been shown to cause mortality and developmental deformities in *Bombina bombina* (fire-bellied toad) tadpoles exposed to 0.1-100 µg/L , which is within the range of environmental levels (0.02-29.5 µg/L, Table 1.5).

1.5.2.4 Other Herbicides

Other herbicides that do not fall into the above categories but are environmentally relevant include, dicamba, 2,3,6-trichlorobenzoic acid (2,3,6-TBA), benazolin, bentazone, bromoxynil, pentachlorophenol (PCP), trifluralin, and glyphosate (see Table 1.5 for details). Of these, no information pertaining to the EDg potential of dicamba, 2,3,6-TBA, benazolin, bentazone, or bromoxynil could be obtained. PCP is used as an insecticide, fungicide, and herbicide and thus has a wide range of applications, agriculturally, and industrially, but its primary use is to protect timber from wood-boring insects and fungal rots (Tomlin, 2006). PCP use has been restricted in Europe since 1991 , but it was still detected in surface waters in the U.K.in 2004/05, and was ranked 12th (Table 1.5). It has previously been reported to have anti-estrogenic activity *in vitro* , although estrogenic and anti-androgenic activity were not observed , and it had no effect on aromatase activity . In a series of publications, Beard and Rawlings have demonstrated various reproductive effects in mammals, for example, decreased whelping rate in mink , increased severity of oviductal intraepithelial cysts in ewes , and seminiferous tubule atrophy in rams . Interestingly, serum hormone and gonadotropin levels were unaffected in these studies, suggesting a direct toxic effect on reproductive tissues. Indeed, *in vitro* PCP irreversibly inhibited ovulation of Zebrafish oocytes at > 0.6 µM , and caused toxicity in rat sertoli cells at 10 nM . Furthermore, PCP reduced the number of eggs laid, and their subsequent hatching rates, and induced formation of testis-ova in Japanese medaka exposed to 50-200 µg/L . The maximum measured concentration in 2004/05 was 2.74 µg/L (0.01 µM), which is lower than effective concentrations reported above, however it has the potential to bioaccumulate ; and therefore, may pose a risk to amphibians.

Trifluralin (dinitroaniline herbicide, ranked 28th in UK and 12th in US) decreased thyroxine, increased estradiol, and decreased LH concentrations in ewes fed 35 mg/kg/week for 5 weeks . However, it did not interact with the estrogen or androgen

receptor in a gene transcription assay , or the estrogen receptor in a proliferation assay , and had no effect on thyroid responsive endpoints in mice . Glyphosate is a glycine derivative and was ranked 8th in the U.K. and 1st in the U.S.A. (Table 1.5). The herbicide formulation Roundup®, which contains glyphosate as the active ingredient, caused formation of testis-ova and increased time to metamorphosis in larval *Rana pipiens*, whereas glyphosate alone did not have an effect . Roundup ® also had a more potent excitatory effect on aromatase activity in human placental cells than glyphosate alone . These effects were probably due to the adjuvents present in Roundup ®, as they are designed to facilitate entry into the cell . In support of this theory, the most effective formulations of glyphosate based herbicides in *R.pipiens* were those containing the surfactant polyethoxylated tallowamine (POEA), which also elicited a response alone . Roundup ® also decreased E2 levels, and reproductive success of Jundía (*Rhamda quelen*) exposed to 3.6 mg/L for 40 days . This suggests that the 'active' ingredient of a pesticide may not always be the compound most likely to affect wildlife, but little is known about the relative effects of pesticide adjuvents . The mechanism of action of glyphosate/Roundup ®, may be via inhibition of steroidogenic acute regulatory protein (StAR) expression, which transports cholesterol to the inner mitochondrial membrane for conversion to steroid hormones . The maximum measured concentration of glyphosate in the UK was 1.6 mg/L (Table 1.5), but glyphosate had no effect on amphibians when tested alone . Roundup ® is the most common formulation of glyphosate (Tomlin, 2006), however, the aquatic levels of POEA are unknown, and therefore, the risk of glyphosate based pesticides to amphibians in the UK is also unknown.

1.5.3 Fungicides

Fungicides constitute the smallest group of agrochemicals found in U.K. freshwaters, and only four (carbendazim, flutriafol, fenpropimorph, and tecnazene) were found above 0.01 µg/L in 2004/05 (Table 1.5). Carbendazim is a metabolite of the benzimidazole fungicide benomyl, and this conversion occurs rapidly in water (~ 2 hours) and animal tissues (Tomlin, 2006). It is reported to cause infertility, testicular atrophy and abnormal spermiogenesis, and occlusion of the effernet ductules in rats . In addition, it caused histological alterations in thyroid and adrenals, of rat pups

exposed in utero , and adults , but no effects on other thyroid or pituitary hormones were observed . The toxic mode of action in the target organism is by inhibition of mitosis via inhibition of beta-tubulin synthesis, which is similar to the physiological effects observed in rats where decreasing presence of microtubules in the Sertoli cells of the testis was reported . It is debatable as to whether this constitutes endocrine disruption per se, or general toxicity, as the endocrine system is not affected, although it causes major disruption of an endocrine organ. No information could be obtained on the other fungicides detected, although flutriafol is an azole fungicide, and this class of fungicides has been shown to inhibit aromatase in vitro . Other fungicides are also EDs, for example, vinclozolin is a potent anti-androgen in vivo and in vitro , which is thought to be due to it's metabolites , and mancozeb inhibited spermatogenesis in mice , and rats . In addition, mancozeb affected the thyroid system and increased the weight of the thyroid gland in hemicastrated rats , and inhibited the iodide pump essential for the production of T4 . It also inhibited cortisol secretion in Xenopus adrenocortical cells in vitro . However, these fungicides were not present in the UK (Table 1.5).

1.5.4 Nitrate

There is some evidence that nitrate could have an endocrine disrupting effect on steroidogenesis in rats, and possibly in alligators. Panesar reported that nitrate inhibited gonadotropin-stimulated steroidogenesis in mouse leydig tumour cells, possibly via conversion to nitric oxide and consequent inhibition of intracellular cyclic adenosine monophosphate or inhibition of cytochrome P-450 enzymes. Panesar & Chan also demonstrated that nitrite and, to a lesser degree nitrate, inhibited androgen steroidogenesis, resulting in decreased testosterone production in mouse leydig tumour cells (LOEC's: 5 mM & 40 mM). The same authors also exposed rats to nitrate and nitrite to 50 mg/L in drinking water (as sodium nitrite or sodium nitrate), which caused a reduction in circulating corticosterone and testosterone levels; and concluded that the possible mechanism of action was via inhibition of cytochrome P-450 enzymes. There is also circumstantial evidence that nitrate causes a decrease in testosterone concentration in alligators, as a strong negative correlation was found between these two variables, where pesticide contamination was minimal . Where

nitrate concentrations in lake water exceeded 10 mg/L, testosterone levels in juvenile alligators fell by 50%, and they were also reported to have smaller penises (Louis Guillette, pers. comm.). Further to this investigation, Guillette & Edwards reported that increased nitrate concentrations (up to 5 mg/L) were correlated with decreased sperm counts and increased teste weight in mosquitofish collected from nitrate-contaminated springs.

1.5.5 Mixtures

Mixtures are more environmentally relevant than single chemicals, but are not useful in determining the mechanism of action of individual chemicals, as it is not possible to attribute an effect to a specific compound. In addition, making a 'typical' mixture that is representative of the agricultural environment is difficult, as compounds and concentrations in the environment will differ greatly depending on meteorological, geological, and geographical factors, as well as the crop type. This is different from riverine environments, where sewage effluent and industrial effluent discharge a fairly well known composition of compounds in the environment, and in the case of sewage treatment works, this discharge is also fairly constant. Typical estrogenic compounds from these pollution sources include EE2, bisphenol-A, and alkylphenols, which have been well studied both singly , and to a lesser extent in mixtures . These estrogenic compounds are generally accepted to act in an additive manner, whereby exposure to a mixture containing a concentration of each compound that did not have an effect, causes an effect when combined . Sumpter *et al.* successfully modeled the effects of mixtures of estrogens on fish in a riverine environment, however, effects of mixtures of agrochemicals are more complex to predict. This is partly because, unlike synthetic estrogens, which function by stimulating the estrogen receptor, agrochemicals have diverse modes of action (Table 1.5), and partly because less is known about their individual ED effects.

1.5.5.1 Laboratory

In amphibians, mixture studies have mainly focused on thyroid and growth endpoints, such as time to, and size at, metamorphosis. Hayes *et al*. reported that mixtures of

agrochemicals had a more pronounced effect on amphibian time and size at metamorphosis than any compound alone, indicating an additive effect. Sullivan & Spence , and Allran & Karasov analysed the mixture effect of atrazine and nitrate on growth parameters and metamorphosis *Xenopus laevis*, but no effect was observed. However, Orton *et al.* reported a mixture effect of altered gonadal development and sex ratios in *Rana pipiens* exposed to the same compounds at similar concentrations. In addition, Gray *et al.* reported that vinclozolin and procymidone acted additively in the Hershberger assay, and Birkoj *et al*. reported that a combination of deltamethrin, methiocarb, prochloraz, simazine, and tribenuron-methyl were anti-androgenic in rats, where no effects were observed with the single compounds at the same doses; suggesting an additive effect.

1.5.5.2 Field

Field studies in agricultural areas have also attempted to describe the endocrine disrupting effects of agrochemicals, though in the absence of chemical analysis, it is not known if agrochemicals indeed caused reported effects. In a series of papers Guillette and co-workers reported that alligators from a contaminated site had abnormally small phalli, skewed sex hormone ratios (estrogen:testosterone), and males had reduced testosterone concentrations . The authors related these parameters to a pesticide spill containing the organochlorines dicofol and DDT (both congeners) in the contaminated lake, although agricultural land also bordered the lake. Further research indicated that these effects may have been caused by altered hepatic biotransformation enzyme activity, which was observed in alligators from the contaminated lake compared to the reference site. Particularly, activity was sexually dimorphic in the reference site, but this dimorphism was lost in individuals from the contaminated sites . However, although hepatic enzyme activity was reduced in the contaminated lakes, this was not correlated with plasma sex steroid levels , suggesting it may not be a good measure of perturbation to hormone levels. In a distinct water catchment, the impact of agricultural activity was assessed to determine if the effects observed previously were site-specific. Similar effects were observed, whereby female alligators from the most contaminated site had reduced plasma testosterone and estradiol, and males from this site had smaller phallus size , indicating the effects may be related to agrochemicals. There is very little comparative research using

amphibians, however, Fort *et al.* reported that exposure of *Xenopus laevis* to pond water and sediment extracts from reference and polluted ponds caused a delay in metamorphosis.

1.6 Conclusion

It is clear that there has been a decline in populations of the common frog and toad in the U.K. over the past hundred years, and that in some parts of the country this decline is continuing, especially in the case of toads. Habitat modification is the most obvious, and indeed the most important, cause of population decline up to the present, however, agrochemicals may also play a significant role. The extent to which decline is related to agrochemical usage is unknown, and this is exacerbated by the lack of knowledge of ED effects of currently used pesticides in the U.K. Most of the reported data on the ED effects of pesticides are from mammalian cell lines, and are focused on the ability of a single compound, which is often not relevant to the U.K. environment, to stimulate hormone receptors. Considering that many pesticides function by inhibiting active processes, such as electron transport or enzyme activity, these routes of ED are underrepresented in the literature. For example, most organochlorines act by antagonism of the GABA receptor, and many have receptor mediated effects in test systems , whereas OPs and carbamates act by inhibiting acetylcholinesterase, and are less active at the receptor but inhibit enzymes . In addition, at present there are no standardised tests using an amphibian model for testing endocrine disruption, and therefore there is a need for rapid and cost-effective screening tools to detect and characterise EDs in relation to amphibians. Standardised *in vitro* and *in vivo* tests are generally of mammalian origin and have limited applicability to amphibians. One exception to this is the *Xenopus* Metamorphosis Assay (XEMA), which is currently undergoing standardisation and validation . The U.K. is in a unique situation concerning long-term qualitative population data, however, since 1990 UK herpetological research has all but disappeared (excluding the work by Beebee). Therefore, the aim of this study was to assess whether there is evidence for alterations in thyroid function and reproductive parameters in native amphibian populations, specifically in relation to agrochemicals. The objectives were:

1. To use *in vitro* tests and population data for selection of sites with varying levels of agrochemical input, and to compare development of caged and wild-caught tadpoles/metamorphs from these sites to their laboratory-reared counterparts. Measured endpoints included morphometric parameters, thyroid and gonad histology.
2. To develop and validate amphibian bioassays to better estimate risk of compounds to amphibians. The bioassays that were developed (with varying success) were: Hepatocyte culture and VTG induction to test for (anti-) estrogens; germinal vesicle breakdown assay (GVBD) for testing (anti-) androgens; use of a transgenic tadpole to test for (anti-) thyroids; oocyte ovulation to test for inhibition/stimulation of steroidogenesis. These bioassays were used to detect ED activity of water extracts from selected sites, and environmentally relevant pesticides.

Chapter 2

Endocrine Disruption in Native Amphibians

2.1 Overview

Feminisation of fish caused by sewage effluent in rivers , and imposex caused by TBT in molluscs , are well characterised examples of ED in the environment. Amphibians typically inhabit agricultural environments, and although pesticides are often shown to have EDg activity in laboratory exposures (see Chapter 1, section 1.5), evidence of ED in environmentally exposed individuals is less well defined. Pesticides have been associated with tadpole abnormalities in caged studies , and declining amphibian populations have been associated with decreased cholinesterase activity and increasing concentrations of organophosphates . In addition, Fellers *et al*. reported higher concentrations of DDE, γ-chlordane, and *trans*-nonachlor in whole frog tissues from a declining population than a stable population, and associated this to agrochemical spray drift. Similarly, Davidson & Knapp reported that spray drifts of pesticides were instrumental in decline of *Rana muscosa*, and had a more significant contribution than the presence of fish in ponds. Although these studies demonstrate a probable link between amphibian population declines and pesticides, they do not specifically indicate evidence of ED. Reeder *et al*. addressed this question by histological analysis of gonads of Cricket frogs (*Acris crepitans*) in Illinois (USA). Firstly (1998), a significant correlation between levels of polychlorinated biphenyls (PCBs) and polychlorinated dibenzofurans with incidence of intersex was reported,

and this correlation also approached significance with concentrations of atrazine ($p = 0.07$). Secondly (2005), using historical specimens over the 1830-2001, incidence of intersex was shown to increase during the period of industrial growth (1930-1945), was highest during the period of industrialisation and use of organochlorines (1946-1959), and decreased when sales of DDT were restricted (1960-1979). In addition, the incidence was highest in urban industrialised areas, intermediate in agricultural areas, and lowest in non-agricultural/industrial areas, indicating that industrial contaminants are stronger inducers of intersexuality than agrochemicals; but that both probably contributed to the effects observed. In reptiles, Guillette *et al.* reported small phalli, skewed sex hormone ratios, and reduced testosterone concentrations in males collected from a lake contaminated with a pesticide spill (organochlorines), compared to those collected from a reference site (see Chapter 1, section 1.5.5.2 for details). In addition to *in situ* experimental designs, another approach to assessing ED in the environment is to use water extracts in *in vitro* or short-term *in vivo* screens. Although environmental samples from agricultural sites have been analysed for pesticide residues , and for toxicity to amphibians , there is a lack of published research on the endocrine effects of agricultural extracts. To the authors knowledge, endocrine endpoints have been assessed only once , whereby pond extracts inhibited the thyroid axis (observed as tail resorption inhibition), although the proximity of these ponds to agricultural land was not reported.

The field work component of this project was designed to investigate the evidence for ED in native amphibians in the U.K. agricultural environment. Water sampling was carried out over two breeding seasons (July 2004 and April-June 2005), and animal specimens were collected in the third breeding season (April-August 2006). During the first breeding season, due to limited time available (the present project began in March 2004), a water sample from only one location was taken and processed by solid-phase extraction. Training in two amphibian ED bioassays (hepatocyte monolayer and transgenic assay), was completed using this sample as the test substrate. In the second breeding season, water samples were collected by passive accumulation devices (PADs), but due to unforeseen problems with the amphibian bioassays, were instead tested for ED activity using the yeast estrogen/androgen screens (Routledge *et al*. 1996, Sohoni and Sumpter, 1998). In the third breeding season, toads were sampled from selected sites across England and Wales, and

compared morphologically and histologically to their laboratory-reared counterparts. Water samples were also collected from these sites with PADs, however, these samples were not available for testing.

2.2 Breeding Season One

2.2.1 Introduction

The selected site was situated in a drainage ditch in the fens in East Anglia, due to the high agricultural intensity in this area and the presence of toad populations (Arnold Cooke, pers. comm.). A site situated in fenland was also chosen because of its topography, as the water bodies consist of a network of irrigation ditches (where amphibians breed), and thus broadly similar water pesticide profiles could be expected across this region. Water was extracted by solid-phase extraction with C_{18} and OASIS cartridges. The former extracts more lipophilic compounds, whereas the latter extract hydrophilic compounds and intermediate compounds, although there is also overlap between the cartridges . Extraction of water samples by solid-phase extraction is a validated technique and has been used previously, both in laboratory and in field samples . In the present study, both extracted samples (C_{18} and OASIS) were tested in two amphibian bioassays: the hepatocyte monolayer for estrogenic activity, and the transgenic tadpole assay for thyroidal activity (in association with Towa-Kagaku, Japan).

2.2.1.1 Hepatocyte Monolayer

The hepatocyte monolayer assay is based on the production of vitellogenin (VTG) in response to stimulation of hepatic ERs by estrogenic compounds. VTG is a

phospholipoglycoprotein egg-yolk precursor protein and is secreted by the liver of mature females in oviparous vertebrates. Following secretion, it enters the bloodstream and is incorporated into oocytes as yolk, and thus causes growth and development of the ovarian follicle; a process called vitellogenesis. Synthesis of VTG occurs naturally in females in response to circulating estrogens, but this does not occur naturally in males, due to the low circulating levels of endogenous estrogens in the bloodstream. However, males can be induced to produce VTG in response to exogenous estrogen stimulation, and therefore it is a useful biomarker of estrogenic exposure in males . In addition, the *in vitro* response can be indicative of the *in vivo* effect (Jones *et al.*, 2000), and due to the large range in potential VTG concentrations produced by the liver, VTG induction is a useful bioassay for samples with unknown estrogenicity (Sumpter and Jobling, 1995). Hepatocyte culture has been found to be relatively insensitive to estradiol compared with human cell lines, and yeast based assays (Jones *et al.*, 2000). However, liver cells have the advantage of possessing a relatively complete set of biotransformation activities , so the effects of hepatic metabolites are concurrently tested *in vitro*, which may result in a more accurate prediction of *in vivo* effects (Smeets *et al.*, 1999). VTG has often been used to assess the response of fish to sewage treatment works effluent , and has also been indicated in estrogenic pollution in the marine environment *in vivo* . Fish hepatocyte culture has been widely used for testing contaminants including pesticides, for example, DDT , dieldrin, and aldrin induced VTG production in Rainbow trout (Okoumassoun *et al.*, 2002a) and Channel Catfish (Monteverdi and Di Guilio, 1998) hepatocytes, whereas methoxychlor did not . In addition, the dicarboximide fungicide procymidone was estrogenic using rainbow trout hepatocytes . Species-specific differences in the VTG response to estrogenic chemicals seem to be marginal in fish (Sumpter and Jobling, 1995), however, differences in sensitivity between species have been reported. For example, Smeets *et al.* reported induction of VTG by methoxychlor using Carp hepatocytes, whereas no effect was observed in Channel Catfish hepatocytes (Schlenk *et al.*, 1997), and Monteverdi and Di Giulio (1999) reported different magnitude of effects of synthetic estrogens in Channel Catfish hepatocytes compared to Rainbow trout. There are no studies comparing the VTG response to estrogens in fish and amphibians, and therefore it is unknown if extrapolation between fish and amphibians is accurate, however, it is likely that the general effect would be the same (Sumpter and Jobling, 1995). VTG, as a biomarker of estrogen exposure, has been used to test

synthetic estrogens in *Xenopus laevis in vitro*, using isolated hepatocytes , and *in vivo* following intraperitoneal injection of adults . Few studies have used isolated amphibian hepatocytes for testing agricultural compounds, but Rankouhi *et al.* reported no estrogenic activity of methoxychlor using *Rana temporaria* hepatocytes, and Lutz and Kloas (1999) reported weak estrogenic activity of DDT using *Xenopus laevis* hepatocytes. In addition, aquatic exposure to toxaphene and dieldrin stimulated VTG production in adult *Xenopus* alone but not in combination , and injection with DDT in adult *Xenopus* and *Trachemys scripta* also stimulated VTG . To the authors knowledge, VTG stimulation has only been used once as a biomarker of estrogenicity *in vivo* in relation to agricultural pollution, whereby Okoumassoun *et al.*, reported that VTG was significantly correlated with organochlorine pesticide levels in fish (Tilapia). In addition, Hurter *et al.* used liver slices from *Xenopus* to test environmental samples from sewage effluent, lake water, and dam water. All were found to be estrogenic, but it was not reported what types of pollutants the lake and dam water were likely to contain.

2.2.1.2 Transgenic Tadpole Assay

In contrast to estrogenic effects of compounds, which are thought to be primarily receptor-mediated, the thyroid axis contains multiple possible sites of action, and relatively little is known about the main targets for endocrine disruptors (see Chapter 1, section 1.4.2). Therefore, in order to test the thyroid system effectively, an *in vivo* experimental design was chosen in preference to *in vitro* tests. At present, the '*Xenopus* Metamorphosis Assay' (XEMA) is being validated by the Organisation for Economic Co-operation and development (OECD), to be used as a short-term screen to test for thyroid disrupting chemicals . In XEMA, *Xenopus* tadpoles are exposed to a water control, a positive control, a negative control, or the test compound, for a period of between 7 and 28 days. At the end of the exposure period, thyroid sensitive morphological endpoints, such as developmental stage and hindlimb length (HLL), are measured for comparison with the controls (Opitz *et al.*, 2005). The transgenic tadpole assay used here follows a similar experimental design as XEMA. However, the test organism carries a transgene containing the TRβ gene promoter sequence linked to a green fluorescent protein gene, which results in a visible response when the TRβ is stimulated/inhibited by endogenous or exogenous thyroid active substances

. It is thought that TRβ plays the prime role in inducing the changes taking place at the climax stage of metamorphosis, because the expression level of the gene correlates well with the rise in the active form of thyroid hormone (T_3) in blood during metamorphosis . In addition, *Xenopus* TRβ gene was shown to contain the thyroid responsive element (TRE) in its transcriptional regulatory region and has previously been shown to increase expression during premetamorphosis and metamorphic climax after treatment with T_3 (Oofusa *et al.*, 2001). It was anticipated that by using the transgenic tadpole in preference to normal *Xenopus* tadpole, it would be possible to decrease the exposure time needed and to increase sensitivity of the assay, as gene transcription changes may be detected before morphological changes occur . TH stimulates a series of morphological changes that occur during metamorphosis of the anuran tadpole to the adult frog . Exposure studies using anurans as the test organism often measure thyroid sensitive morphometric endpoints, such as time to metamorphic climax or forelimb emergence, and hindlimb length, as standard experimental protocol. Coady *et al.* and Carr *et al.* reported weak inhibition of metamorphosis in response to environmentally relevant exposure to atrazine in *Xenopus*, although Orton *et al.* and Allran and Karasov reported no such effect on *Rana pipiens* larvae. Howe *et al.* reported that glyphosate based pesticides, such as Roundup ®, increased the time to metamorphosis in *Rana pipiens* tadpoles at environmentally relevant concentrations, and Fordham *et al.* reported that malathion had the same effect, but only at concentrations above 1000 µg/L, which are highly unlikely to be found in the environment. Howard *et al.* reported that carbaryl, chlorpyrifos, and imidacloprid increased the time to metamorphosis at 0.1 x LC50 in *Pseudacris triseriata, Bufo americanus* and *Rana sphenocephala*, although the environmental relevance of this study is also questionable due to the high concentrations used (imidacloprid: 18450-46800; carbaryl: 5158-6317; chlorpyrifos: 112-132 µg/L). In addition, Fort *et al.* reported that methoxychlor delayed hindlimb differentiation, inhibited rate of tail resorption, and caused follicular hyperplasia in the thyroid glands of *Xenopus* tadpoles exposed to 0.1 mg/L. In addition, in a mesocosm mixture exposure (methyl mercury, atrazine, monosodium methanearsonate, & chlorpyrifos), atrazine and chlorpyrifos concentrations (concentration range: atrazine – 0 to 230 µg/L; chlorpyrifos – 0 to 4.65 µg/L) were correlated with delayed metamorphosis in *Hyla chrysoscelis* . In contrast, Cheek *et al.* reported decreased time to forelimb emergence in *Xenopus* tadpoles when exposed to acetochlor and T_3, although acetochlor had no effect alone.

To the author's knowledge, no field studies with amphibians related to agrochemical exposure have been reported. However, in alligators, histological alterations in the thyroid gland have been reported from an agriculturally contaminated site compared to a reference site . The thyroid gland from individuals collected from the contaminated site had increased epithelial cell area and decreased colloid, which are both indicators of suppressed thyroid gland activity. In addition, ammonium perchlorate (a by-product of explosives) has been shown to retard metamorphosis and cause histological abnormalities in laboratory-exposed amphibians , and this effect was corroborated by effects observed in the field (Theodorakis *et al.*, 2006).

2.2.2 Methods

2.2.2.1 Water Sampling

The selected site in breeding season 1 was Ibberson's Pump station (IP) in East Anglia (x,y coordinates = 535900, 288000). Pesticides detected in this area in 2004/2005 were 2,4-D (max: 60 ng/L, percentage of times detected: 14%), atrazine (45 ng/L, 86%), bentazone (200 ng/L, 14%), chloropropham (2140 ng/L, 100%), dieldrin (3 ng/L, 4%), diuron (1008 ng/L, 100%), mecoprop (120 ng/L, 43%), simazine (235 ng/L, 100%), and tributyltin (2 ng/L, 6%) (see Appendix 2, pp 288-297). Water samples were collected from IP in 12 x 2.5 litre amber glass bottles (total = 30 litres) on 27th July 2004, and 0.5 % methanol (MeOH: 12.5 ml per bottle) was added to each bottle to minimise biodegradation of the sample. At the time of water collection physical parameters at the test site were also noted and water quality parameters were measured (temperature (temp), pH, dissolved oxygen (DO), conductivity), at the time of water collection. Bottles were stored overnight at 4^{0}C at Brunel University. On the following day, samples were filtered with glass microfibre filters (GF/F circles 150 mm, Whatman, UK), which have a pore size of 0.7 µm, to remove debris prior to solid-phase extraction. The filtered samples were then passed through either C_{18} (Waters, UK), or OASIS (Waters, UK) cartridges (1500 ml sample/cartridge (x10) for C_{18}, and 750 ml/cartridge (x20) for OASIS), for a total of 15 L water sample per cartridge type. Cartridges were primed prior to addition of the

sample by passing 5ml of distilled water, followed by methanol, and again by distilled water, using a flow rate of 5-10 ml/minute. Pre-filter discs and cartridges were stored at -80C until shipment to Towa-Kagaku Company Ltd. (Hiroshima, Japan) on dry ice (4 day shipment) in August 2004. The glass microfilters were eluted in methanol, by ultrasonic wave (yamato 2510 BRANSON) for 5 min. Cartridges were eluted with methanol (C_{18} = 30ml/cartridge, OASIS = 5ml/cartridge) using a flow rate of ~ 5ml/minute. Extracts from filters and cartridges were combined for each cartridge type (separate samples for C_{18} and OASIS extracts), and placed in round bottomed flasks for evaporation (yamato rotary evaporator RE 440), for approximately 2 hours. Samples were further evaporated with nitrogen gas, until they had a volume of 1.5 ml, which resulted in x10000 concentration of the original water sample (15 L). Methanol (MeOH) stocks were stored at 4^0C until use in bioassays.

2.2.2.2 Hepatocyte Monolayer

2.2.2.2.1 Culture Media

Culture media used for hepatocyte culture was: 50% Leibovitz L-15 medium (Sigma), containing: 1 μg/ml insulin (dissolved in 10 mM HCl solution, Sigma), 10 nM dexamethasone (Sigma), 0.05% glucose (Sigma) and antibiotics (50 units/ml of penicillin and 50 μg/ml streptomycin). Collagenase (Wako Pure Chemicals Co., Japan) solutions (0.1 % and 0.06 %) were prepared in perfusion media: 0.55% NaCl, 0.014% KCl, 1 mM pyruvate, 0.1% glucose, 0.02% NaHCO 3 , 0.5% BSA, 10 mM HEPES (pH 7.4). The 0.06% solution was used for *in situ* liver perfusion, and the 0.1 % solution was used for incubation of the perfused liver. All media were sterilized by filtration prior to use (DURAPORE Membrane Filter, Millipore: 0.22 μm).

2.2.2.2.2 Hepatocyte Culture

To avoid contamination of cell culture by micro-organisms, frogs were cleaned by dipping them in 10-20 mg/L KMn_2O_4 for 1-2 hr, and were anaesthetised by injection with 0.5-1 ml of 20 mg/ml MS-222. Hepatocytes were isolated from adult male *Xenopus laevis* liver according to a one-step perfusion method. The body cavity was opened and a needle connected to silicon tubing and a peristaltic pump (MASTER

FLEX 6-600 RPM, Cole-Parmer Instrument Co.), was inserted through the heart into the hepatic vein. 100-200 ml collagenase solution was perfused through the liver over a time span of 5-10 minutes. The partially digested liver was then removed from the body cavity, minced with scissors, and incubated in collagenase solution (10-20 ml) at 25°C for 15-30 min, whilst gently shaking. Following incubation, the tissue was suspended by glass pipette (large pore size: > 3 mm), and sieved through a sterilized nylon mesh (120 μm pore size) to remove structural tissue. The cell suspension was centrifuged at 200 rpm (20g) for 2 min, the supernatant was removed by pipette, and the cell pellet was re-suspended in media. This step was repeated 3/4 times to ensure complete removal of collagenase solution. In some cases the cell suspension contained a dark layer of pigment cells, which were removed by gently expelling media on to the upper phase of the cells, and then removing the supernatant. The cell density was counted and adjusted to a cell concentration of 1 x 10^5 cells/ml with culture media. On average, 3 x 10^7 - 5 x 10^7 hepatocytes were prepared from one mature male *Xenopus laevis*. The cells were inoculated into 96-well tissue culture plates at a density of 3-4 x 10^4 cells/well (0.3 ml of the cell suspension for each well), and incubated at 22^0C. On the following day the culture medium was replaced with fresh culture medium, 0.2 ml was removed from each well and replaced with 0.2 ml of fresh medium, and this was repeated twice. The cells are cultured for 2/3 days to allow growth of cells on the substratum of the plates, and to thereby form a monolayer (Figure 2.1). In some cases the cells did not extend on the substratum after 3 days, and were therefore discarded. *In situ* liver perfusion with collagenase results in hepatocytes that are structurally intact, and they retain many of their normal *in vivo* characteristics, such as the ability to consume or produce glucose, respond to a variety of hormones, and synthesise lipids, RNA and protein .

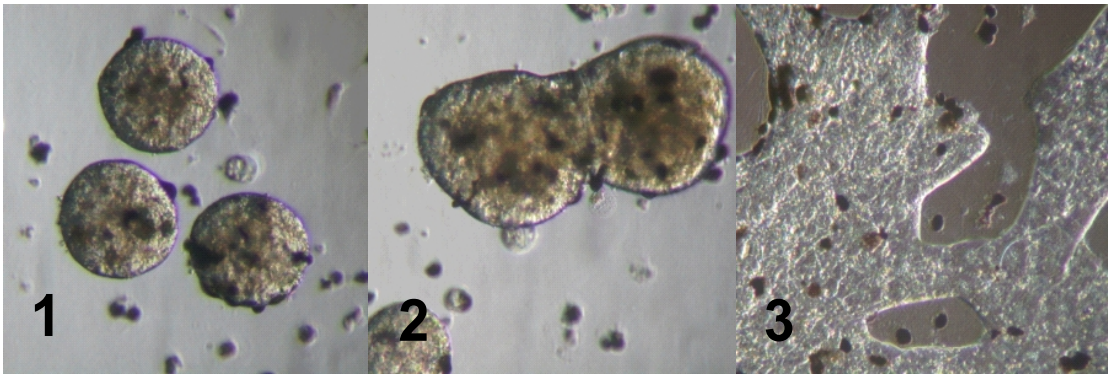


Figure 2.1. Isolated *Xenopus* hepatocytes on day 1 (1), day 3 (2), and day 6 (3) of culture, showing recovery of cells (1,2), and formation of a monolayer (3)**.** x200 magnification (courtesy of N.Mitsui, Towa-Kagaku, Japan).

2.2.2.2.3 Exposure to Test Compounds

The cell culture media were replaced with fresh media (0.2 ml removed, 0.2 ml added, 0.2 ml removed) to remove metabolic by-products of cell respiration and replenish nutrients, prior to the start of exposure. 50 µl of fresh medium was then added, resulting in a total of 150 µl in the wells. Hepatocytes were exposed to media only, a solvent control, estradiol (positive control), or a water extract, by addition of 150 µl of test media (a 2-fold dilution). Final concentrations of E2 in the positive control wells were: 1.11, 0.37, 0.11, and 0.037 nM, and each well also contained 0.01% methanol (MeOH). Final concentrations of extracts were: 0.25, 0.5, 1, 2.5, 5, and 10-fold of the original water sample. Due to serial dilution of the sample extracts in media, MeOH concentrations in these wells ranged from 0.1 – 0.0025, and therefore, 0.1, 0.05, and 0.025 % MeOH were used as additional solvent controls. The cells were exposed to these concentrations twice over the 8 day incubation period. The first exposure was at 2/3 days after inoculation (depending on cell recovery), and the second exposure occurred 3 days after the first exposure. The test media were removed 3 days after the second exposure for analysis of vitellogenin and albumin concentrations by ELISA. Each sample and controls were tested in triplicate wells, and this experimental design was also repeated a total of 3 times.

2.2.2.2.4 ELISA Analysis

After 6 days of exposure, 0.2 ml of the culture media was removed and added to 96-well microtiter plates for dilution. Test media from the 1.1 and 0.3 nM estradiol exposure medias were diluted 10-fold, and 0.1 and 0.03 nM estradiol exposure media was diluted 2-fold, with sample diluent solution from the ELISA kit (phosphate buffered saline, PBS). Concentration of VTG in culture media was measured using a sandwich ELISA for VTG (kindly donated by Japan Envirochemicals), and according

to the manual of the VTG-ELISA kit. Briefly, samples were added to plates that had been pre-coated with VTG antibody, followed by addition of the second antibody, and a colometric reagent . Albumin (ALB) levels were also measured using a sandwich ELISA, and were used to verify cell viability. The remaining diluted media samples were further diluted by 5-fold or 25-fold to achieve a total of a 50-fold dilution for use in the ALB ELISA (previously diluted either 10-fold or 2-fold for VTG analysis). For the ALB ELISA, 96-well microtitre plates (MaxisorpII, Nunc) were coated with 50 µl of 5 µg/ml solution (in PBS: Phosphate Buffered Saline) per well of albumin antibody, covered, and incubated at 4^0C overnight. Following incubation, wells were washed 3 times with 0.1% Tween 20-containing PBS (tPBS), and excess liquid was removed by tapping onto paper towel. This washing step was repeated prior to addition of each component, and plates were incubated for 1 hour at room temperature after addition of each component. Addition to reagents to wells were as follows: 300 µl of blocking reagent (0.5% Blockace in tPBS); 50 µl of standard albumin antigen or sample; 50 µl of the second antibody (HRP-labelled); 100 µl of the chromogen (TMBZ) in TMBZ buffer (Japan Envirochemicals Ltd., Japan); 100 µl of 1M H_2SO_4 solution. Absorbance readings were determined with a spectrophotometer (Muliskan JX, Thermo labsystems) at 450 and 600 nm.

2.2.2.3 Transgenic Assay

2.2.2.3.1 Tadpoles

Spawning of transgenic *Xenopus laevis* was induced by injection with human chorionic gonadotropin (HCG). Eggs and tadpoles were maintained at 22 ± 1 ^{0}C, pH 7.4 ± 0.3, and dissolved oxygen (DO) 7.3 ± 0.5, and a 12:12 hour light:dark cycle, prior to and during exposure. During the pre-exposure period, a complete water change was performed every 48 hours, and tadpoles were fed Sera Micron *ad libitum* (Sera GmbH, Heinsberg, Germany). These tadpoles were of variable fluorescence and on the day before exposure tadpoles were separated into groups according to their fluorescence and stage, using a fluorescence dissecting microscope (MZ FLIII, Leica). Categories for fluorescence were --, -+, or ++, and only ++ tadpoles (see Figure 2.2) of Gosner stage 51/52 were selected for the exposure experiments. Stage 52 was chosen as the starting stage as low but increasing levels of endogenous thyroid

hormone are present at this stage , and therefore it was anticipated that this would allow identification of both antagonistic (suppression of fluorescence relative to controls) and agonistic effects (increase in fluorescence relative to controls).

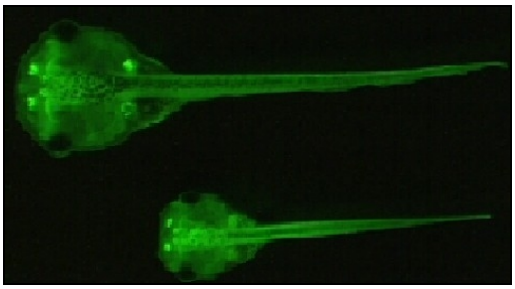


Figure 2.2 Transgenic *Xenopus* tadpoles.

2.2.2.3.2 Experimental Design

The day after tadpole selection, 80 tadpoles were placed in individual glass containers, and total length, hindlimb length, and fluorescence were measured before being randomly assigned to tanks. No significant differences in these parameters were observed between tanks at this stage (ANOVA $p > 0.05$). Larvae were reared in aged tap water only, or containing: 0.01% methanol (methanol control), thyroxine (T_4, 2 µg/L), propylthiouracil (PTU, 20 mg/L), or C_{18}/OASIS extracts (0.25, 0.5, or 1-fold of original water concentration), for 10 days. Two replicate tanks containing 4 L were used per treatment, and three larvae were placed in each tank. Water quality parameters (temperature, pH, and DO) were measured daily prior to feeding. Total length, HLL, stage, and fluorescence were measured prior to exposure, and on days 5, and 10. On days 5 and 10, tadpoles were removed carefully with a net and placed in individual glass containers. Total length was measured with callipers, and the other parameters were measured by taking photographs (Leica DC 200 camera), both with and without the fluorescence filter on the microscope (MZ FLII, Leica). Photographs were analysed with Image-Pro plus (version 4). Weight was also measured after euthanisation on day 10, to observe if the tadpoles had normal growth over the exposure period. During the first 5 days of exposure, 100 mg/tadpole Sera Micron (Sera GmbH, Heinsberg, Germany) was added to the tanks daily, and after day 5 this was increased to 150 mg/tadpole. A 100% water change was performed on day 5 of exposure after the tadpoles had been removed for measurements, and tadpoles were then returned to their respective tanks.

2.2.2.3.3. Calculations

The dissolved oxygen was measured twice with a 1 minute interval and the average of these two measurements was reported. The fluorescence density was measured and this number was divided by the total amount of pixels in the photograph to obtain the 'fluorescence/pixel', this was to normalise data as photographs were mistakenly taken at different pixel densities.

2.2.2.3.4 Statistics

Data were normally distributed therefore, differences between conditions were tested using ANOVA, and differences between pairs were tested using the student's *t*-test.

2.2.3 Results

2.2.3.1 Hepatocyte Monolayer

The addition of E2 induced VTG production in a dose dependant manner up to 0.37 nM, and each well contained the same amount of viable cells, as measured by ALB production (Figure 2.3, A). In addition, MeOH had no effect on VTG production, and good replication between plates was also observed (Figure 2.3, B).

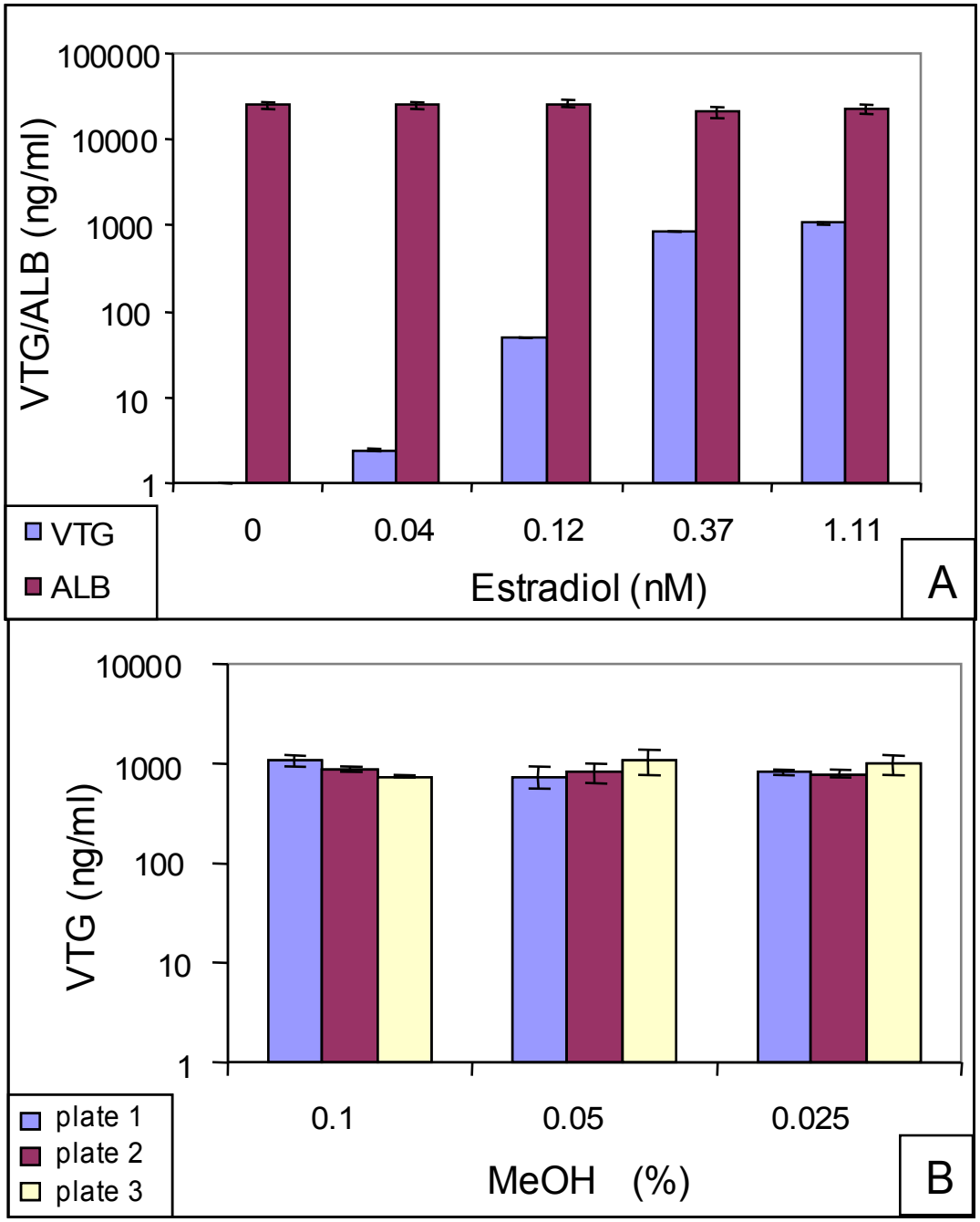


Figure 2.3. Effects of E2 (Panel A) and MeOH (Panel B) on vitellogenin (VTG) and albumin (ALB) production by cultured *Xenopus* hepatocytes. Values are means ± SE based on triplicate wells.

Exposure of cultured hepatocytes to both C_{18} and OASIS extracts significantly increased VTG production in a dose dependant manner at 2.5-fold, 5-fold, and 10-fold concentrations of the original water sample (ANOVA $P < 0.01$). The OASIS extract also induced higher VTG production than the C_{18} extract, but only at 10-fold

concentration of the extract (*t*-test $P = 0.02$). In addition, there was the same number of viable cells in each well, as measured by albumin production (Figure 2.4).

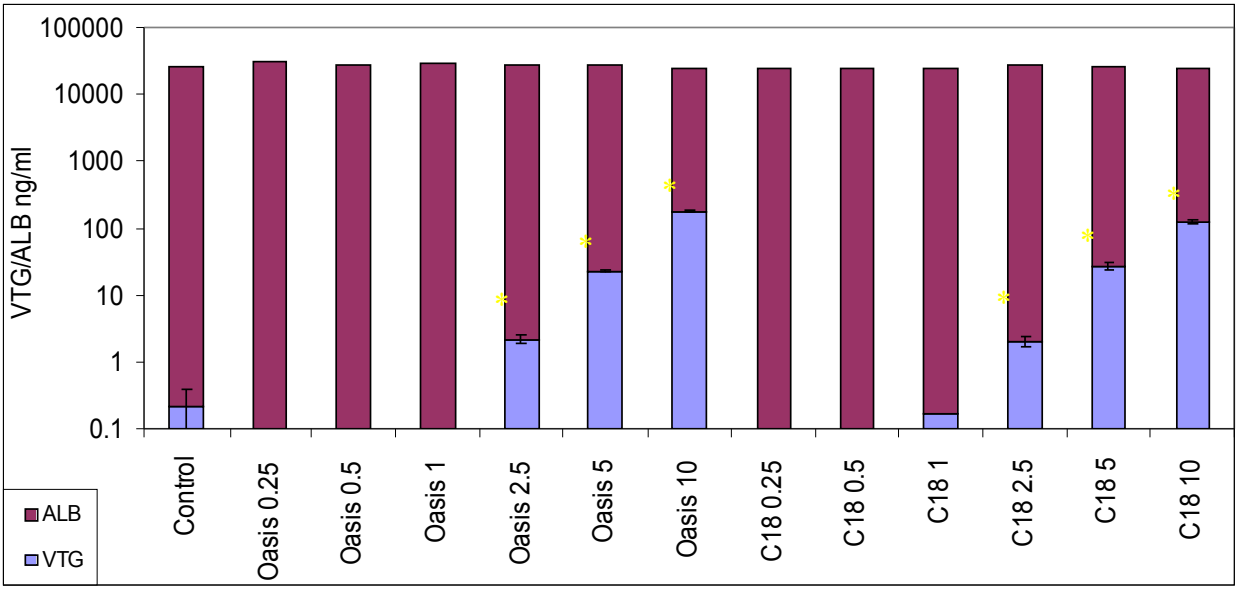


Figure 2.4. The effect of water sample extracts (Oasis or C18) obtained from IP, on vitellogenin (VTG) and albumin (ALB) production of cultured *Xenopus* hepatocytes. Cells were exposed to 0.25, 0.5, 1, 2.5, 5, and 10-fold of the original water sample, or media only (control). Values are means ± SE based on triplicate wells, and over 3 separate plates. * denotes significant difference from control.

2.2.3.2 Transgenic Tadpole Assay

A total of 10 % mortality was observed over all tanks, and within treatments it was highest in control and PTU treatments (22 %), followed by MeOH, 0.5-fold Oasis, 0.25-fold C_{18}, and T_4 (11 %), mortality was not observed in other conditions. Significant differences between conditions was not observed in total body length over the experimental period (days 0, 5, & 20), or weight at day 10 (average weight over all treatments 198.9 ± 6.9, ANOVA $p > 0.05$). T_4 treatment significantly increased the stage of tadpoles at day 10 compared to all other treatments (*t*-test $p < 0.01$, Figure 2.5). HLL and fluorescence were increased in the T_4 treated tadpoles at day 5, compared to all treatments (*t*-test $p < 0.05$), except the OASIS 0.5-fold (*t*-test $p > 0.22$). At day 10 the HLL and fluorescence were increased compared to all treatments (*t*-test $p < 0.026$, Figure 2.6). HLL and fluorescence response was similar across

treatments, and no significant difference between these parameters was observed at day 5 or 10 (*t*-test $p > 0.05$).

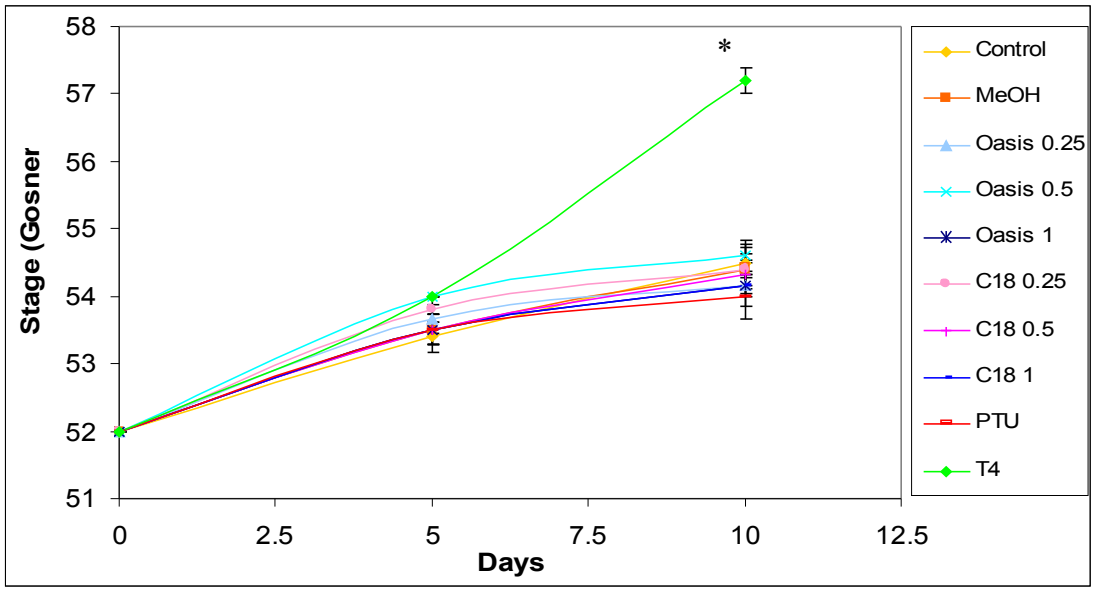


Figure 2.5 – Stage measured over the 10 day exposure period. Tadpoles were exposed to aged tap water (control) MeOH (0.01 %), or 0.25, 0.5, or 1-fold concentration of either Oasis or C18 water extracts (in MeOH). * denotes significant difference of T_4 treated tadpoles compared to all other treatments. Values are mean ± SE, n = 6, 5 or 4 (depending on mortality).

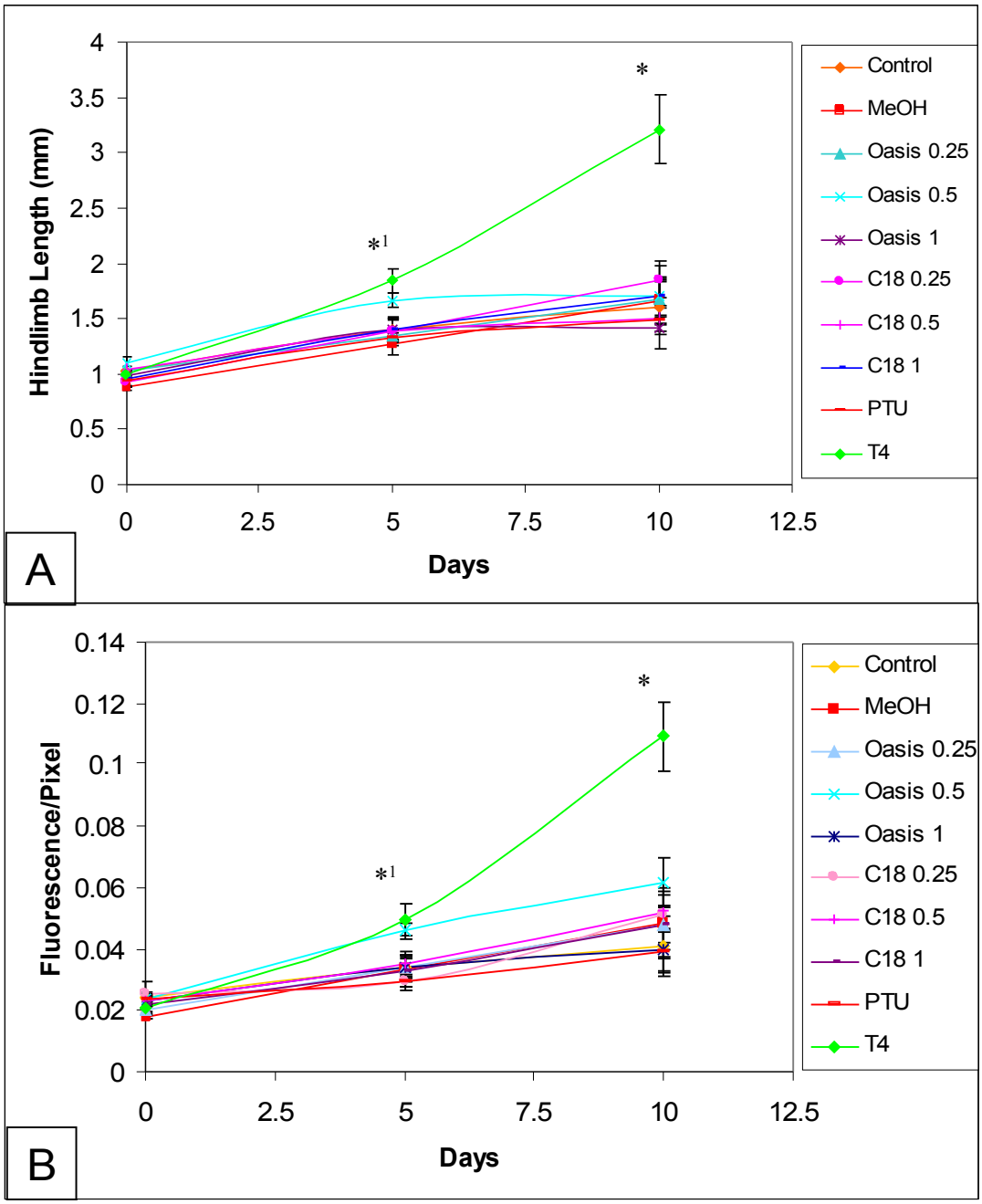


Figure 2.6 – Hindlimb length (Panel A) and fluorescence (Panel B) measured over the 10 day exposure period. Tadpoles were exposed to aged tap water only (control), MeOH (0.01 %), or 0.25, 0.5, or 1-fold concentration of either Oasis or C18 water extracts (in MEOH). * denotes significant difference of T_4 tadpoles compared to all other treatments. *[1] denotes significant difference of T_4 tadpoles compared to all treatments except Oasis 0.5-fold. Values are mean ± SE, n = 6, 5 or 4 (depending on mortality).

2.2.4 Discussion

2.2.4.1 Hepatocyte Monolayer

Estrogenic activity was observed in response to the extracts at ≥ 2.5-fold concentration of the original water sample. During the extraction process it is probable that the nature of the water sample changed to some degree, potentially including the breakdown of estrogenic compounds . Therefore, activity observed at the lower concentrations (2.5 and 5-fold) indicate that amphibians at IP may be exposed to estrogenic compounds. Although various organochlorines , and the fungicide procymidone , have been shown to induce VTG production, these compounds are not prevalent in the UK (see Appendix 1). However, pentachlorophenol (PCP), which is found more often in the U.K., has been shown to upregulate vitellogenin production in fish hepatocytes *in vitro* and *in vivo* . However, it was not detected in this area (Appendix 2 pp 288-297). Atrazine (max: 28 ng/L), simazine (max: 235 ng/L), mecoprop (max: 120 ng/L), dieldrin (max: 3 ng/L), diuron (max: 1008 ng/L) were detected in this area, however, these pesticides have been reported not to interact with the estrogen receptor . To the author's knowledge, the receptor mediated effects of other pesticides found in this area (2,4-D (max: 60 ng/L), chloropropham (max: 2140 ng/L), tributyltin (max: 2 ng/L)) are not known. The sample extract contained a mixture of compounds, resulting in estrogenic activity, however, the identities of these compounds are unknown. Reports on the interaction of pesticide mixtures at the estrogen receptor have focused on pesticides that are not present in the UK environment . However, the most commonly reported interaction of pesticides was of an additive effect, and pesticide mixtures have also been reported to act additively in amphibian growth and development . Therefore, although the nature of the mixture is unknown, and pesticides detected in this area are not reported to interact with the estrogen receptor (or have not been tested), additive effects may have contributed to estrogenic activity observed. In addition to pesticides, the samples may have contained natural and/or synthetic estrogens from sewage effluent or manure fertiliser. The nearest sewage treatment plant to IP was located approximately 20 km away, and due to the topography of the fens (connected irrigation ditches), may have contributed to the estrogenic activity observed in the sample.

Despite lack of chemical analysis of the extract, the hepatocyte monolayer assay has proved useful for detecting estrogenic activity of aquatic agricultural samples. There is generally a good correlation between *in vitro* and *in vivo* measures of VTG induction in response to a wide range of contaminants, and therefore *in vitro* VTG production is considered to be a reliable endpoint to predict effects *in vivo* (Jones *et al.*, 2000). However, it is not known how increased VTG levels could affect the health of individuals or populations. In females, the possible effects range from healthier offspring, due to increased resources in the early stages of life, to less healthy adults, due to rapid use of energy stores . In males, it is not known if VTG induction is associated with feminisation or lowered fecundity, although elevated VTG levels in the plasma have been associated with intersexuality in wild roach , and intersex fish have been reported to have reduced fertility .

2.2.4.2 Transgenic Tadpole Assay

None of the treatments affected mortality or total length, indicating there were not treatment-specific effects on tadpole growth. T_4 stimulated all of the thyroid sensitive endpoints measured, but neither PTU nor the water extracts had any effect. The weight and developmental stage of tadpoles after 10 days was decreased compared to normal *Xenopus* development (O. Tooi pers. comm., Opitz *et al*., 2005). After 10 days, it could be expected that tadpoles in the control water would have reached stage 56/57, and have a weight of approximately 500 mg, however, in this experiment, they only reached stage 54, and had an average weight of 198 mg. It is unknown why the tadpoles had retarded growth and development. Previous experiments in the same laboratory reported a significant inhibition of development when exposed to the same concentration of PTU for 7 and 14 days (O. Tooi pers. comm.). Perhaps the lack of effect of PTU was due to adverse experimental conditions, such as food shortage or poor water quality, which affected the overall growth and development of the tadpoles. Indeed, there was a total of 10% mortality across all groups in the 10 day exposure period, which is relatively high for *Xenopus laevis* . The lack of effect of PTU may have been caused by this slow development as at stage 52 very low endogenous thyroid hormone is detected in the developing tadpole, and it isn't until stage 54/55 that thyroid hormone levels begin to increase substantially (Opitz *et al*.,

2005). Therefore, perhaps the circulating levels of endogenous T_4 were too low for PTU (or the water samples) to have an observable antagonistic effect over the exposure period.

It was anticipated that fluorescence would be a more sensitive endpoint for detection of perturbation to the thyroid system, than HLL, but this was not found to be the case. Fluorescence was measured by fluorescence density of the selected area, and therefore, confounding factors such as shadow or melanocytes may have altered the results. This problem was exacerbated by the method of selecting the area for fluorescence measurement using a computer mouse, leading to possible erroneous selection of areas of shadow at the edge of the hindlimb. Therefore, it is possible that a dark area of shadow or melanocyte could considerably affect the fluorescence density measured. Modifications to the method used for measuring fluorescence may sufficiently reduce confounding effects of shadows and melanocytes. For example, the tadpole will fluoresce for some time after euthanisation, and therefore it would be possible to measure fluorescence in homogenised tissue at the end of exposure. However, as optimisation of this assay was beyond the scope of the present study, and there was no indication that fluorescence would prove to be more sensitive than HLL, this assay was not used again.

2.3 Breeding Season Two

2.3.1 Introduction

The aim of the field work undertaken during breeding season two was to identify agricultural water bodies in England and Wales with toad (*Bufo bufo*) populations and endocrine disrupting activity. Sites were selected based on estimated agrochemical input, and presence of toad populations. It was important that sites had reported toad populations, as a subsample of sites were selected for toad sampling in breeding season three. Water extracts were obtained from selected sites by passive accumulation devices (PADs), rather than the grab sampling method used in breeding season one, resulting in time-integrated extracts (see section 2.3.1.1). These extracts

were then tested for (anti-) estrogenic and (anti-) androgenic activity using the yeast estrogen/androgen screens.

2.3.1.1 Passive Accumulation Devices (PADs)

Measuring pesticide levels in the aquatic environment by time-integrated sampling methods overcame the risk of missing episodic contamination due to storm events and pesticide run-off . Sediment, biota, and PADs have been used to measure pesticide levels, and there are problems associated with each method. Using sediment samples, it is impossible to assess the influence of factors such as sediment bioturbation/resuspension events, sediment sorbent quality, and degradation rates . Using biota will give the most accurate prediction of what the animal is being exposed to *in situ*, as the influence of depuration and uptake rates, and uptake through diet, are all incorporated . However, mortality may occur and uptake and depuration rates may be influenced by the condition of the animal. Most importantly for the present study, PAD deployment produces an extracted sample that can be used for testing in laboratory bioassays, as well as for chemical analysis. Grab-samples can also be used to obtain extracted samples, however, there are practical considerations of collecting and extracting large volumes of water for use in bioassays. In addition, grab-sampling does not distinguish between bioavailable and non-bioavailable fractions of the sample (Kot *et al.*, 2000), whereas PADs, comprised of a porous membrane filled with sequestration media, are designed to mimic the parts of animals that are involved in uptake and bioconcentration (e.g. gills and lipid).

The semipermeable membrane device (SPMD) is one of the most widely used PADs and consists of a thin film of high purity triolein (1,2,3-tri-[cis-9-octacenoyl]glycerol), sealed inside a layflat, thin-walled tube of nonporous, low density polyethylene. The diameters of the transient cavities in the tubing are about 10 Å, preventing uptake of contaminant molecules associated with dissolved organic matter or particulates , and thereby allowing only bioavailable compounds to be sequestered . Huckins *et al*. developed the SPMD, and found similar uptake rates of purified Grass Carp (*Ctenopharyngodon idella*) lipid and the synthetic lipid triolein, although triolein sequestered higher concentrations. The mass of an analyte sequestered by the SPMD is affected by: The concentration of compound in water, although this does not affect

the uptake rate; temperature of the water (higher temperature results in faster uptake rates); duration of exposure; biofouling of the membrane; and most importantly, the log Kow of the compound (Kot *et al*., 2000). SPMDs sequester non-ionic hydrophobic compounds with a log Kow > 3. A linear relationship between uptake rate and log Kow was reported for selected classes of compounds, such as organochlorines and PCBs, with a log Kow between 3-6 . A decrease in sequestration rates was observed for compounds with log Kow values > 6, which is similar to the pattern observed in bioconcentration rates in fish . Indeed, studies comparing uptake rate of PCBs and PAHs by SPMDs and biota reported similar sequestration profiles across a range of compounds, although SPMDs generally sequestered higher concentrations than biota . Other classes of pesticides that have been shown to be sequestered by SPMDs include hydrophobic pyrethroids , hydrophobic organophosphates , and others . Uptake rates of compounds with log Kow's of < 3, which includes many pesticides, have rarely been tested, and thus are not well defined. Wang *et al.* reported a similar pattern of sequestration of relatively hydrophilic compounds to more hydrophobic compounds, whereby SPMDs sequestered nitroaromatics with log Kow's of 1.4-2.4 to a larger extent than goldfish, and the pattern of uptake was similar. In addition, atrazine was detected in field deployed SPMDs and has a log Kow of 2.61 (Wang *et al.*, 1999a). However, Sabaliunas and Sodergren (1997) reported that two hydrophilic herbicides, propachlor (log Kow=2.3) and alachlor (log Kow=2.1), were not sequestered in a laboratory study, suggesting that log Kow values may not be accurate in estimating sequestration of more hydrophilic compounds. Lastly, with the exception of dieldrin, trifluralin (Sabaliunas and Sodergren, 1997), and atrazine , few compounds that are environmentally relevant to the UK have been tested in laboratory exposures or measured in extracts of field deployed devices, and therefore it is unknown to what extent pesticides here will be taken up by the SPMDs.

Due to concern over pesticide bioaccumulation, many modern pesticides have log Kow's < 3, and therefore may not be sequestered by SPMDs. In order to extract the more hydrophilic compounds from the selected sites, the polar organic chemical integrative sampler (POCIS), which sequesters compounds with a log Kow < 4, was also deployed. The POCIS is comprised of a sorbent mixture (80:20 (weight:weight) Isolute ENV + :Ambersorb 1500 dispersed on S-X3 BioBeads) sandwiched between

two polyethersulfone membranes . They were developed much more recently than SPMDs, and thus there are less data available on compounds that they sequester. However, laboratory exposures of POCIS have shown them to sequester a number of environmentally relevant pesticides, including diazinon, diuron, isoproturon, atrazine , and atrazine metabolites (Petty *et al.*, 2004). Furthermore, in a field deployment in Denmark, 29 out of 46 polar pesticides were detected with POCIS, including previously unreported pesticides that are environmentally relevant to the UK, such as bentazone, 2,4-D, MCPA, and fenpropimorph . In a field deployment in the Thames estuary, diuron and isoproturon were identified in the extracts, and using laboratory calibration data, environmental levels were estimated. Grab samples were also taken at the time of POCIS deployment, for comparison to estimated environmental levels. Levels measured in the grab samples were between 1.2 to 4.5-fold different from those estimated using the POCIS, with one sample below the limit of detection in POCIS but present at 40 ng/L in grab sample (Alvarez *et al.*, 2004), indicating that further calibration studies are needed for accurate estimation of environmental levels.

The rationale for using PADs was to obtain an extract of pond water that was integrative over a developmentally relevant time period in the endocrine system of amphibians. The period of water sampling coincided with metamorphosis from early tadpole to climax stages of metamorphosis, thus encompassing the period when gonadal differentiation and thyroid axis stimulation occur.

2.3.1.2 Yeast Screen

Receptor mediated ED occurs through mimicking (agonism) or blocking (antagonism) endogenous hormone receptor binding. To date, the majority of *in vitro* tests for endocrine disruption have focused on hormone receptors, with estrogen receptor agonism and androgen receptor antagonism being the most common . The recombinant yeast screen consists of yeast cells transfected with the human estrogen (yeast estrogen screen: YES) or androgen receptor (yeast androgen screen: YAS), and a colour change is observed if (anti-) estrogenic/androgenic compounds are present. It is a sensitive, reproducible, rapid, and ethical technique, and a high throughput of compounds is possible . However, yeast have limited capacity for metabolism, and

therefore can only provide preliminary data for various compounds that may be active *in vivo* . Pesticides have been shown to possess (anti-) estrogenic and anti-androgenic activity in the yeast screen, although androgenic activity has not been observed . For example, o,p'- DDT and p,p'-DDT have estrogenic and anti-androgenic activity , and diuron was weakly estrogenic (Noguerol *et al.*, 2006). In addition, fenarimol (fungicide) and dicofol (insecticide) were estrogenic, although other pesticides tested, such as, PCP, chlorpyriphos, diuron and linuron were reported to have no effect on the estrogen or androgen receptor . In contrast, Jung *et al.* (2004) reported that PCP was anti-estrogenic, though no effect was observed with other pesticides, such as, aldicarb, carbaryl, and trifluralin. Several pyrethroid insecticides, including permethrin, fenvalerate and cypermethrin, and their metabolites have been shown to possess (anti-) estrogenic and anti-androgenic activity , and the metabolites of pyrethroids appear to be more potent EDs than the parent compounds (Tyler *et al*., 2000). As mentioned, the composition of the extracts I collected was unknown, and only the pyrethroids, PCP, and diuron are present in the UK, and have been shown to be active in the yeast screen.

Various studies have used yeast screens to assess estrogenic activity of environmental samples in relation to sewage treatment works , however, to the author's knowledge the YES has only been used once in relation to pesticide contamination. Petty *et al.* (2004) used POCIS samplers in a constructed wetland, which received urban wastewater and inflow from the Missouri river. Atrazine and its metabolites, propoxur (a carbamate insecticide), and nonylphenol were analytically identified in the extract, which was also found to be estrogenic in the YES. Estrogenicity may have been due to the nonylphenol in the extract, as the "alkylphenol" effect of creeping across plates was observed.

2.3.1.3 Species Selection

As mentioned previously (Chapter 1, section 1.2) populations of the Common Toad, *Bufo bufo,* have been decreasing in the south of England at least since the 1940's. There are various possible reasons for this decline, although road associated mortality is probably the most important . Toad-specific mortality on roads occurs because, unlike frogs, toads display high fidelity to one breeding site and may travel large

distances and cross roads to reach their breeding site . It was calculated that 24-40 cars per hour killed approximately 50% of migrating *B.bufo* . More recently, Hels and Buchwald modelled the probability of survival of an individual toad crossing a road, and concluded that its chances of survival depended on the number of vehicles passing per unit time and the width of the tyres. Since both of these parameters are likely to have increased over the years, it is likely that road-associated mortality has also increased. In the UK, local groups had been active in carrying toads across roads since the early 1900's, but it wasn't until the 1989 that coordination of local herpetological groups occurred, with the setting up of the "Toads on Roads" scheme (run by Froglife, registered charity: 1088255). A by-product of this scheme is historical recording of the number of toads carried across roads, as well as any dead toads observed on the road and, therefore, trends in populations at individual sites over time can be analysed. If a toad population is stable, then the numbers killed by traffic should increase over time, as the volume of traffic increased, however, if traffic levels become too high then toad losses may become unsustainable and the population will decline .

Despite the toads on roads scheme there is evidence to suggest that the common toad is still decreasing in Central, South, and South Eastern areas, and especially in rural areas . However, the findings reported in that study should be treated with caution, as they were based on questionnaires sent to local groups, whereby participants were requested to indicate population status (increase, decline, stable, extinct, uncertain) for sites that had been monitored for at least 5 years. If indeed this recent decline is substantiated, it may be due to a decrease in genetic diversity of populations caused by habitat fragmentation , as toads in small urban populations were found to have a lower genetic diversity than those of larger rural populations, and tadpoles from the former displayed significantly higher mortality and abnormalities . They also had relatively lower genetic diversity than common frogs in the same area .

In addition to the possible decline in this species in England, toads were also chosen in preference to frogs for a number of other reasons. Firstly, the toads on roads data indicate, at the very least, where a toad population exists, and at best the trend of the population over time, assisting in site selection. Secondly, due to their high fidelity to breeding sites, it can be expected that any effects seen are ongoing at that site, and

may be related to the toad count data. Thirdly, frogs breed primarily in garden ponds (see Chapter 1), which would not typically give an accurate assessment of effects caused by chemicals in the agricultural environment.

2.3.2 Methods

2.3.2.1 Site Selection

Toads on roads data were combined with pesticide use intensity data (POPPIE database, Environment Agency, U.K.). The POPPIE database is an Environment Agency resource consisting of estimated surface level concentrations of pesticides, which have been analytically verified, on a catchment level basis. Using these two datasets, 10 sites were chosen across England and Wales with varying levels of pesticides and with known toad populations. These sites were located in 4 clusters (Figure 2.7). The first cluster was located in E. Anglia, and contained a low, a medium, and a high risk site. The low risk site, Woodwalton fen (abbreviation = WW, grid reference = TL225837), was an enclosed pond located within the fenlands. It was the only site without a toad population but was chosen as a reference site for the medium and high risk sites in E.Anglia. The medium risk site, 20 foot river (20', TL322972) and was located in a drainage ditch in the fens, which forms part of a connected network of canals. The high risk site, Sporle (SP, TF854102), was an isolated pond that was surrounded by arable agriculture. The second cluster was located in the North of England and also contained a low, a medium, and a high risk. The low risk site, Little Hayfield (LH, SK031879), was located in Derbyshire at the bottom of a small valley, where no agricultural activity was visible. The medium risk site, Repton Shrubs (RS, SK310230), was located in Derbyshire, in a pond bordering pastural agriculture. The high risk site, Oxton Bogs (OB, SK615512), was located in Nottinghamshire, and was a pond surrounded by arable agriculture. The third cluster was located in the midlands and contained a medium and a high risk site. The medium risk site, Church Lench pool (CL, SP025505), was located in Herefordshire. The pool was used for fishing, and orchards were observed in close proximity to the pool. The high risk site, Layes Pool (LP, SO802658), was located in Worcesteshire, and bordered arable agriculture on one side and pasture on the other. The final cluster was

based on toad populations rather than agrochemical levels. It was comprised of two low risk sites, Yatton (YT, ST425752) and Pant-y-Llyn (PYL, SN606166), the former had a decreasing population and the latter an increasing population of toads. YT was situated on the edge of North Somerset County Nature Reserve (which is a SSSI: Site of Special Scientific Interest), and no visible agriculture could be observed. The pond at PYL is a turlough, and thus has no groundwater inflow or outflow, and was situated at the edge of a SSSI (Carmel Woods). Please note, site selection work was undertaken primarily by Dr. Daniel Pickford (Brunel University, U.K.), with limited input from the author.

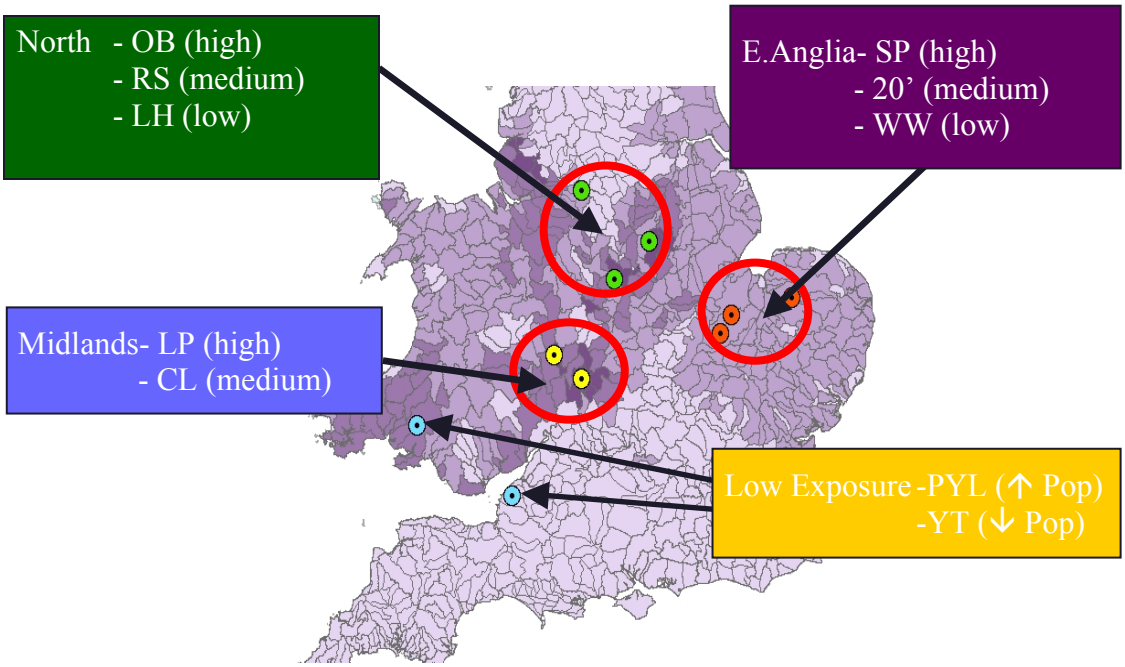


Figure 2.7. Map showing selected sites in England and Wales. Higher total agrochemical use intensity is indicated by darker background colour. See text for details.

2.3.2.2 Water Sampling

Water samples were obtained by deployment of SPMD and POCIS samplers at each site for 29 days during April-May 2005. This is a typical exposure time for these devices, and saturation of samplers was not expected over this time period , and was

designed to coincide with tadpole thyroidal and gonadal development. Three membranes of each type were deployed in steel cages and submerged approximately 0.5 metres below the water surface. The water depth was measured, and a galvanised steel chain was cut to the appropriate length. The chain was attached to a buoy at one end, threaded through loops in the steel cage containing the PADs, and tied round a concrete building block at the other end (sampling PADs). Membranes and deployment devices were obtained from Exposmeter (Tavelsjo, Sweden). They were placed in the pond, where they were suspended in the water column, and thus it was presumed that normal fluctuations in water level over the sampling period would not affect submersion. As a procedural control for each site ('field blank'), one membrane of each type was exposed to air for the duration of deployment, and again at retrieval, of the sampling PADs. Field blanks were stored at -20^{0}C in the intervening period (29 days). Both sampling PADs and field blanks were stored at -20^{0}C after arrival at Brunel University, and were transported to and from the sampling sites on ice. Water quality parameters (temperature, pH, DO, specific conductivity, and salinity) were measured (YSI 556 handheld multiparameter system, YSI Hydrodata) at 3 points within 2 metres of the deployed PADs, at deployment and retrieval of PADs. In addition, 3 water samples taken from the same sites were measured for nitrite, nitrate, phosphate, iodine (Hanna C200 36 parameter colorimeter, Hanna Instruments), and water hardness (General Hardness test kit, Nutrafin).

2.3.2.3 Sample Extraction

Elution of chemicals bound to the SPMD's followed the official guidelines , and the POCIS followed instructions outlined previously . All glassware used in the extraction process were solvent rinsed (MeOH x 2 followed by ethanol (EtOH) x1) and dried in a fume hood prior to use. All solvents used in the extraction process were HPLC grade. Each SPMD was rinsed in 200 ml hexane in a glass beaker for 20/30 seconds to remove surface adherents. The SPMD was inspected for holes in the membrane, and any observed were isolated by heat sealing. They were then placed in a stainless steel tray and washed with running tap water and surface debris was removed by scrubbing with a toothbrush. Following this, the SPMD was submerged in 1-M HCl for 30 seconds, rinsed with tap water, submerged in acetone and isopropanol for 30

seconds each, and was then dried on solvent rinsed aluminium foil. Each SPMD was dialysed in 250 ml glass bottles by incubation with hexane (200 mls, 18 hours, 18^0C), followed by further incubation with fresh hexane (200mls, 4 hours, 18^0C). The hexane from these two extractions were combined, and evaporated with nitrogen gas. Each POCIS membrane was cut open with a solvent rinsed stainless steel blade and the sorbent was collected on solvent rinsed aluminium foil. Sorbent was mixed from 3 membranes retrieved from each cage (one cage at each site). The sorbent was placed in a glass gravity flow chromatography column fitted with a glass wool plug and stopcock. Sorbent was dialysed by incubation with eluent (1:1:8 ratio of methanol:toluene:dichloromethane, 25 mls, 2 minutes) in the glass column. The stopcock was then opened, and a further 50 mls of eluent was passed through the sorbent. Extracts were collected in 100 ml glass bottles, and dried with nitrogen gas. EtOH (10 mls) was added to each glass bottle and mixed vigorously (x2), followed by decanting into a glass vial (28 ml volume), and again, evaporation of EtOH. Glass vials containing dried SPMD and POCIS extracts, were re-suspended in 2.04 mls of EtOH per membrane.

2.3.2.3.1 Calculation of Uptake Rate

In this study, it was not possible to accurately estimate ambient levels at sampling sites, as in the absence of chemical analysis it was not known what the samples contained, and therefore, uptake rates were impossible to determine. In addition, uptake rates of the majority of pesticides that are environmentally relevant to the U.K. aquatic environment are not known, making extrapolations using the literature difficult. Therefore, an estimate based on uptake rates of organochlorine pesticides was used to determine a concentration factor of the extract. Although organochlorines are generally not present in the UK at concentrations previously used for calibration studies of SPMDs , ambient concentration of compounds does not affect the uptake rate by PADs, which was expected to be linear for the duration of the exposure period (Huckins *et al.*, 2002b; Alvarez *et al*., 2004). Nevertheless, it should be noted that the concentration factor is a relative value of the quantity of compound extracted per Litre of water, per day of deployment, used within this study, and not an estimation of environmental levels. Calculation of the concentration factor comprised of several stages:

1. Data reported in Huckins *et al.*, was used to correlate uptake rate and log Kow of organochlorines at 13.7^{0}C, which was the average temperature over all sites over the sampling period (Figure 2.8).

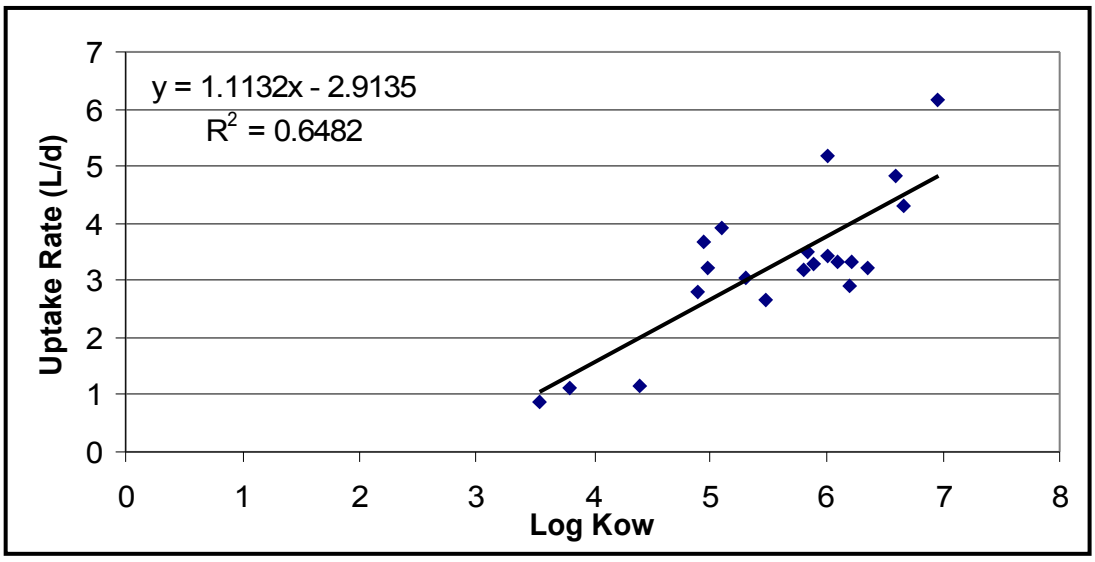


Figure 2.8. Uptake rate (Litres/Day) of a range of organochlorines with varying log Kow's at 13.7^{0}C by a SPMD membrane.

2. Pesticide concentrations in catchments that contained a sampling site were collated (using POPPIE database), and the average concentration of each pesticide from all sites between April-June was calculated
3. The organochlorine equation (Figure 2.8) was applied to pesticides with an average of > 1 ng/L and a log Kow > 3 (a total of 80 pesticides were found above >1 ng/L, and of these 27 compounds had a log Kow > 3).
4. The average uptake rate of these pesticides was calculated (1.4 L/d), was multiplied by 29 (days spent in the water) and by 3 (3 membranes per sample were deployed), resulting in an equivalent of 122.4 Litres over the sampling period for each site.
5. A x 20000 stock was prepared by adding 2.04 mls ethanol per membrane (6.12 mls for sampling PADs, 2.04 mls for the field blanks). Therefore, the composition of compounds that were in 122.4 Litres of water, were reduced to a volume of 6.12 mls.

The calculated uptake rate was similar to a previous study using SPMDs, where the composition of the water was unknown .

2.3.2.4 Yeast Screen

The recombinant yeast estrogen screen (YES), including preparation of the growth medium, has been described previously . Briefly, stimulation of the transfected human estrogen receptor causes the release of β-galactosidase, which metabolises a chromogenic substrate (chlorophenol red-β-D-galactopyranoside), and thereby causes a colour change in the media, which is measured by absorbance (540 nm, Spectramax 340pc, Molecular Devices, CA, USA). The growth of yeast cells was also measured (at 620 nm), and the 620 nm value of the EtOH only wells ('blank') was subtracted from the 620 nm value of the exposed well, and this value was subtracted from the absorbance value at 540 nm of the corresponding well. The yeast androgen screen follows the same principle, but yeast were transfected with the human androgen receptor instead of the human estrogen receptor . The anti-screens function by inhibition of receptor binding, via co-incubation with the agonist (E2 or T), and the test compound. Standards for each screen were as follows: E2 for the estrogen screen (10 to 0.0049 nM); 4-Hydroxytamoxifen (OH-T) for the anti-estrogen screen (25 to 0.01 μM); T for the androgen screen (50 to 0.02 nM); and flutamide (FLUT) for the anti-androgen screen (50 to 0.02 μM). They were run in duplicate in each assay, and ethanol and media only controls were also run in each assay. Standards were serially diluted 12 times, in two-fold steps, and 10 μL of each concentration were then added to a 96-well microtiter plate and allowed to evaporate to dryness. After evaporation, 200 μL of medium containing CPRG and yeast (8 x 10^5 cells/ml) were added to each well. For the anti-estrogen/androgen screens, 0.25 nM E2, or 2.5 nM T, was added to the media prior to addition to the wells. Initially, sampling and field blank extracts were tested at x2000 concentration (x10 dilution of the concentrated extract), in duplicate in each screen. To achieve this concentration, 20 μL of extract was added per well, evaporated to dryness, and subsequently diluted x 10 by the addition of the yeast containing growth media (200 μL). Subsequently, samples were serially diluted in two-fold steps over 12 wells (x2000 - x0.97 concentration), and the field blanks were again tested at x2000. Therefore, the top concentration (x2000) of sampling and

field blank extracts were tested twice, and the dose response curve of the sampling extracts was tested once.

2.3.2.4.1 Calculations

Hormone equivalents were calculated for each screen using the following equation :

$$\text{EE/OHE/TE/FE} = \frac{EC_{50}\ (\text{Standard})}{EC_{50}\ (\text{Sample})}$$

EE = E2 equivalents, OHE = OH-T equivalents, TE = T equivalents, FE = FLUT equivalents (FEs). Equivalents were calculated where the absorbance value that represents the EC_{50} of the standard was reached by the samples. The field blank extract absorbance values were converted to percentage values to allow comparison between assays.

2.3.3 Results

PADs were lost from RS in the Northern cluster, they were found lying on the bank when the site was visited for PAD retrieval. In addition, the PADs at LH were half submerged in the bottom sediment of the pond upon retrieval. Biofouling of the membranes can lower the exchange rate between SPMDs and ambient water , but was minimal at all sites.

2.3.3.1 Site Characteristics

Water quality varied between sites, but values were within those expected for normal amphibian development (Table 2.1). Temperature, and pH were similar between sites, whereas conductivity, salinity, and hardness differed markedly. These values were correlated, and were highest at WW & 20', which also had the lowest altitude (1 & -1 metres). DO levels also differed between sites. Phosphate was detected at more sites, and in higher concentrations, than nitrate. Iodine was detected at varying levels at all sites except WW. Toad tadpoles were not observed at WW, or LP, but where

observed, development was similar across sites, except at OB, where it was more advanced.

	WW	20'	SP	LH	OB	YT	PYL	CL	LP
Temp (^{0}C)	13.9 (0.9)	15.6 (0.2)	12.3 (0.4)	13.5 (1.6)	14.6 (1.45)	13.3 (2.1)	11.8 (1.5)	14.4 (1.2)	15.5 (0.05)
Cond.(µS/cm)	1322 (62)	2866 (55)	491 (41.6)	152 (17)	1113 (128)	462 (73.2)	187 (14)	500 (34)	494 (89)
Salinity (ppt)	0.81 (0.1)	1.67 (0.2)	0.3 (0.03)	0.1 (0.01)	0.7 (0.06)	0.29 (0.03)	0.12 (0)	0.31 (0.02)	0.30 (0.06)
Oxygen(mg/L)	6.1 (1.5)	14.5 (1.9)	12 (4)	10.2 (0.7)	8.6 (1.47)	13.2 (1.68)	9.62 (0.75)	10.8 (1.28)	11.21 (1.9)
pH	6.82 (0.1)	8.02 (0.2)	7.6 (0.7)	6.2 (0.1)	7.46 (0.39)	7.91 (0.2)	7.09 (0.17)	6.91 (0.14)	7.92 (.011)
Hard. (mg/L)	807 (93)	773 (53.5)	250 (30.1)	93 (13.4)	397 (3.34)	233 (0^1)	86.67 (0^1)	253 (0^1)	240 (20.1)
Nitrate (mg/L)	0	0	0	0	1.67 (0.97)	0	0.05 (0.05)	0	0
Nitrite (mg/L)	0	0	0	0	0.04 (0)	0	0	0	0
Phos. (mg/L)	0.5 (0.06)	2.47 (0.7)	1.31 (0.9)	0.48 (0.16)	0.64 (0.15)	1.57 (0^1)	0.4 (0.28)	0.31 (0^1)	0.522 (0.2)
Iodine (mg/L)	0	0.08 (0.1)	0.1 (0.1)	0.03 (0)	0.03 (0)	0.05 (0.05)	0.02 (0.02)	0.03 (0.03)	0.8 (0.37)
Altitude (m)	1	-1	58	206	71	5	145	96	45
T. Abundance	None	Few	Many	Few	Some	Few	Few	Many	None
Stage (G)*		28	28	28-29	30-33	28	28	29	

Table 2.1. Water quality at the sampling sites. Values are means (SE) values measured at PAD deployment and retrieval, *n* = 2. Temp = temperature, Cond = specific conductivity, Hard = hardness, Phos = phosphate, T = Toad. G = Gosner. * = stage observed at time of PAD retrieval. (0^1) indicates only one value is available for this parameter.

2.3.3.2 Hormonal Activity

None of the samples from the SPMD extracts were estrogenic (data not shown), whereas most of the POCIS samples displayed some estrogenic activity. The most strongly estrogenic extracts were WW and 20'. OB, SP, PYL, YT, CL & LH were weakly estrogenic, though no estrogenic activity was observed at LP (Figure 2.9). All SPMD extracts except for OB and WW were anti-estrogenic (Figure 2.10), and at the highest concentration tested YT and LP were more strongly anti-estrogenic than the standard. Substantially less anti-estrogenic activity was observed in response to the POCIS extracts, where only LP was anti-estrogenic, although WW was estrogenic in this screen (Figure 2.11). None of the samples tested were androgenic (data not shown). Anti-androgenic activity was observed in response to SPMD extracts (Figure 2.12), and WW, YT, & LP were the strongest anti-androgens. Similarly to anti-estrogenic activity, anti-androgenic activity was less pronounced in response to the POCIS extracts, but was also highest at WW & LP (Figure 2.13).

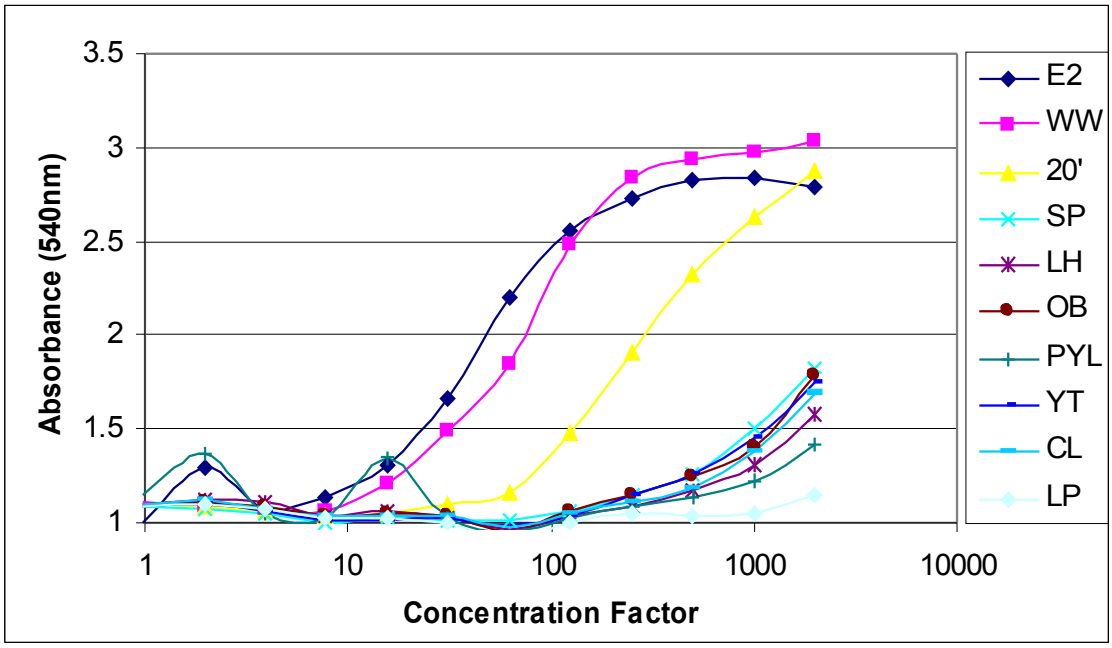


Figure 2.9. Estrogenic activity of POCIS extracts from each site in the YES. Extracts were serially diluted in 2-fold steps, over the concentration range of x2000-x0.97 of the nominal water concentration. 17β-estradiol (E2) was also serially diluted in 2-fold steps, over the concentration range of 10-0.0049 nM. Plates were incubated at 28^0C for 7 days. Values are calculated absorbance values (see section 2.3.2.6), and each data point represents a single value. WW = woodlwalton fen, 20' = twenty foot river, SP = sporle, LH = little hayfield, OB = oxton bogs, PYL – Pant-y-Llyn, YT = yatton, CL = church lench, LP = layes pool.

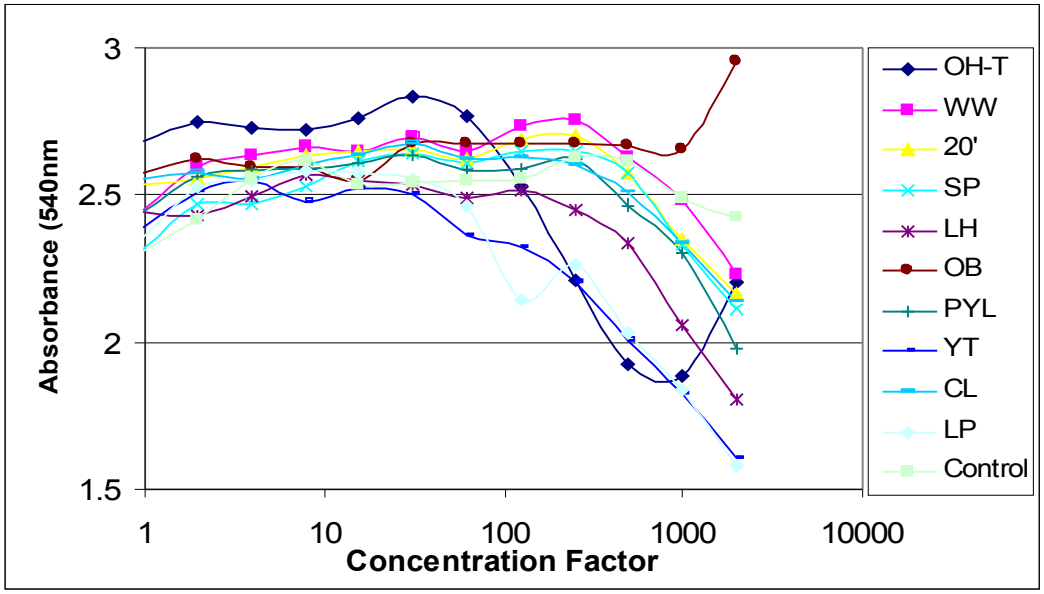


Figure 2.10. Anti-estrogenic activity of SPMD extracts from each site. Extracts were serially diluted in 2-fold steps, over the concentration range of x2000-x0.97 of the nominal water concentration. Hydroxytamoxifen (OH-T) was also serially diluted in 2-fold steps, over the concentration range of 25-0.01 µM. Plates were incubated at 28^{0}C for 5 days. Values are calculated absorbance values (see section 2.3.2.6), and each data point represents a single value. Control = ethanol only exposed wells, for other abbreviations see Figure 2.9.

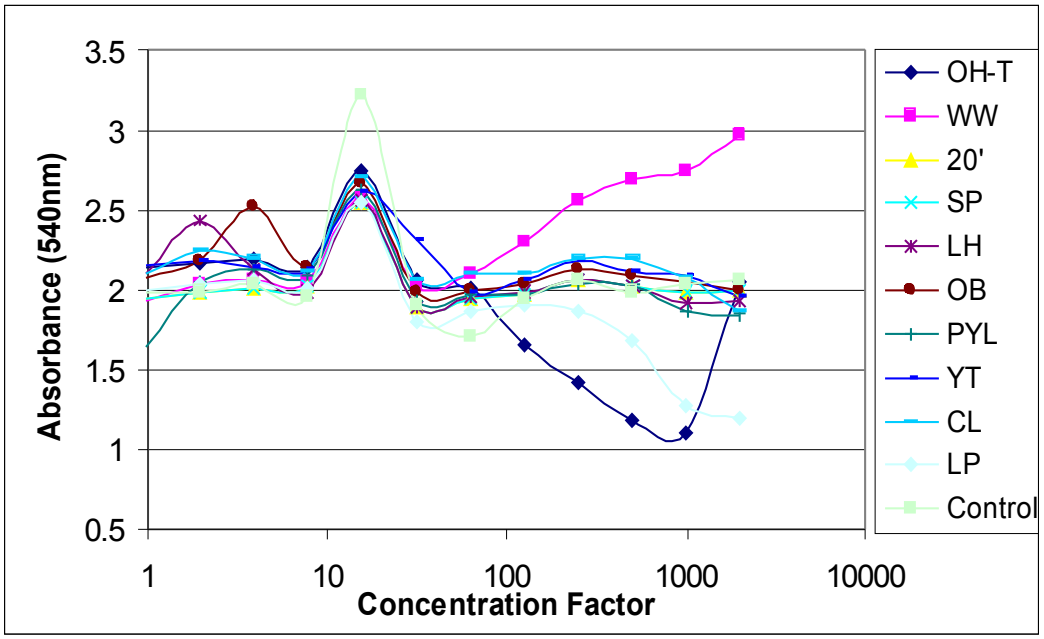


Figure 2.11. Anti-estrogenic activity of POCIS extracts from each site. Each data point represents a single value. For experimental details see Figure 2.10, for other abbreviations see Figure 2.9.

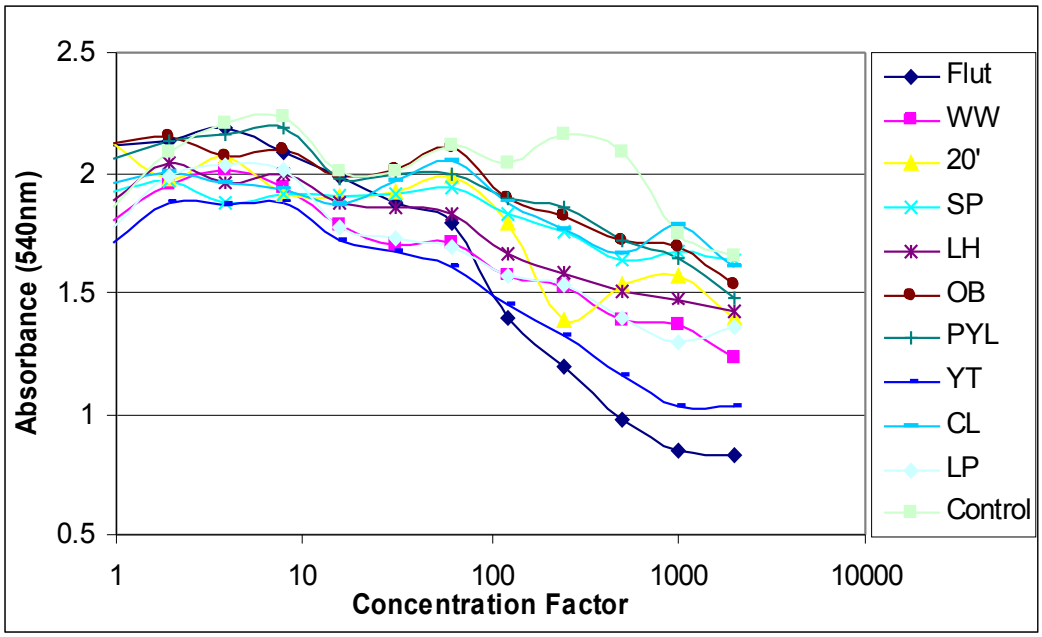


Figure 2.12. Anti-androgenic activity displayed by SPMD extracts from each site. Each data point represents a single value. Extracts were serially diluted in 2-fold steps, over the concentration range of x2000-x0.97 of the nominal water concentration. Flutamide (Flut) was also serially diluted in 2-fold steps, over the concentration range of 50-0.02 µM. Plates were incubated at 28^{0}C for 5 days. Values are calculated absorbance values (see section 2.3.2.6), and each data point represents a single value. Control = ethanol only exposed wells, for other abbreviations see Figure 2.9.

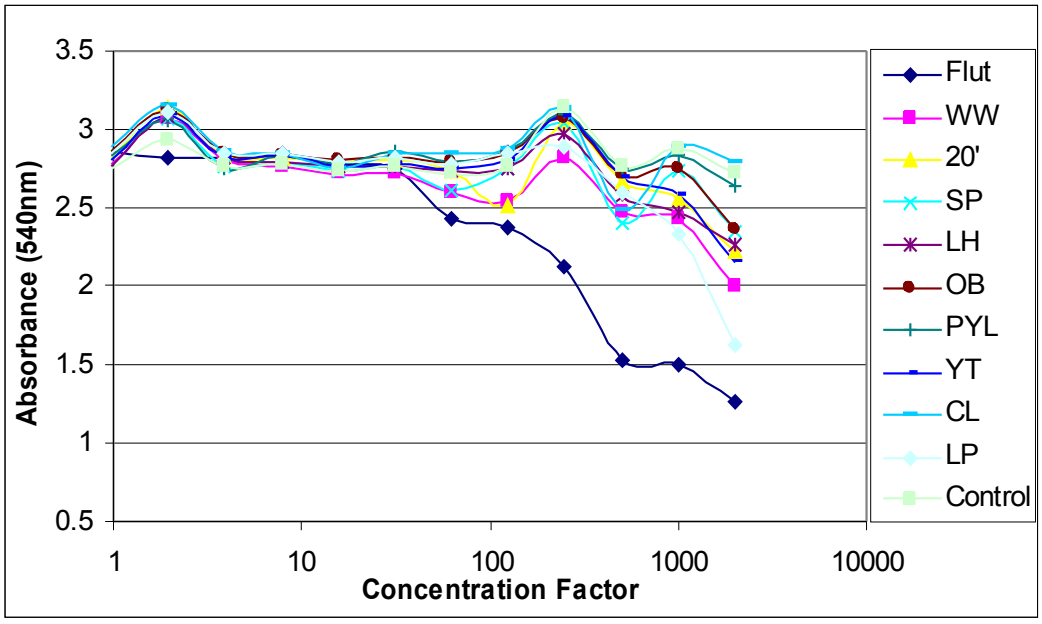


Figure 2.13. Anti-androgenic activity of POCIS extracts from each site. Each data point represents a single value. For experimental details see Figure 2.12, for other abbreviations see Figure 2.9.

Endocrine equivalents from SPMD and POCIS extracts were calculated, and combined to assess the overall estrogenic, anti-estrogenic, and anti-androgenic activity from each site, and to compare with the original risk estimated from the POPPIE database (Table 2.2). The POPPIE database did not predict endocrine response in the yeast screen in all cases except at LP, where high risk was correlated with a high anti-estrogenic/androgenic activity. Where a particular type of endocrine activity was observed, it originated primarily from one type of PAD across all sites, thus, estrogenic activity was observed in the POCIS extracts, and anti-estrogenic/androgenic activity in the SPMD extracts. Lastly, each extract was active in at least one screen.

SITE	YES E2 eq. (nM)		Total YES	A-YES OHT eq. (nM)		Total A-YES	A-YAS Flut eq. (nM)		Total A-YAS	RISK
	S	P		S	P		S	P		
WW	0	0.71	**0.71**	0	0	**0**	0.25	0.15	**0.4**	**L**
20'	0	0.17	**0.17**	0.15	0	**0.15**	0.06	0.01	**0.07**	**M**
SP	0	0.01	**0.01**	0.18	0	**0.18**	0	0.01	**0.01**	**H**
LH	0	0.01	**0.01**	0.4	0	**0.4**	0	0.01	**0.01**	**L**
OB	0	0.01	**0.01**	0	0	**0**	0	0.01	**0.01**	**H**
PYL	0	0.01	**0.01**	0.2	0	**0.2**	0	0	**0**	**L**
YT	0	0.01	**0.01**	1	0	**1**	0.69	0.01	**0.7**	**L**
CL	0	0.01	**0.01**	0.17	0	**0.17**	0	0	**0**	**M**
LP	0	0	**0**	0.86	0.3	**0.89**	0.25	0.2	**0.45**	**H**
Total	**0**	**0.94**	**0.94**	**2.96**	**0.3**	**2.99**	**1.25**	**0.4**	**1.47**	

Table 2.2. Endocrine equivalent values for each site and in different membranes. S = SPMD, P = POCIS, L = Low, M = Medium, H = High, eq. = equivalent. The nominal value of 0.01 was given to extracts that had weak endocrine activity (no part of the curve reached the EC50 value).

2.3.3.3 Field Blanks

Endocrine activity was also observed in field blank extracts, and is presented as percentages of the maximal response (YES: no response = 0 %, maximal stimulatory response = 100 %; anti-YES/YAS: no response = 100 %, maximal inhibitory response

= 0 %). There appeared to be a background level of activity from both types of PADs, which was more pronounced in the POCIS, compared to the SPMD, extracts. Background estrogenicity of POCIS field blanks were ~20 %, and SPMD field blanks were < 5 % (Figure 2.14, A). Background anti-estrogenic effects of POCIS and SPMD extracts was ~80 %, and background anti-androgenic effects for POCIS was ~70 %, and SPMD ~ 80 % (Figure 2.14, B). In addition to background activity, POCIS field blank extracts from YT were highly estrogenic in the YES (103.9 %, Figure 2.14, A), and the anti-YES (158.8 %, Figure 2.14, B). Three SPMD field blanks were estrogenic (WW = 22.1 %; LH = 36.7 %; YT = 37.4 %, Figure 2.14, A), although no estrogenic activity was observed in the sampling SPMDs from these sites. In addition, WW and 20' were anti-androgenic (87.3 % and 69.2 %, respectively, Figure 2.14, B).

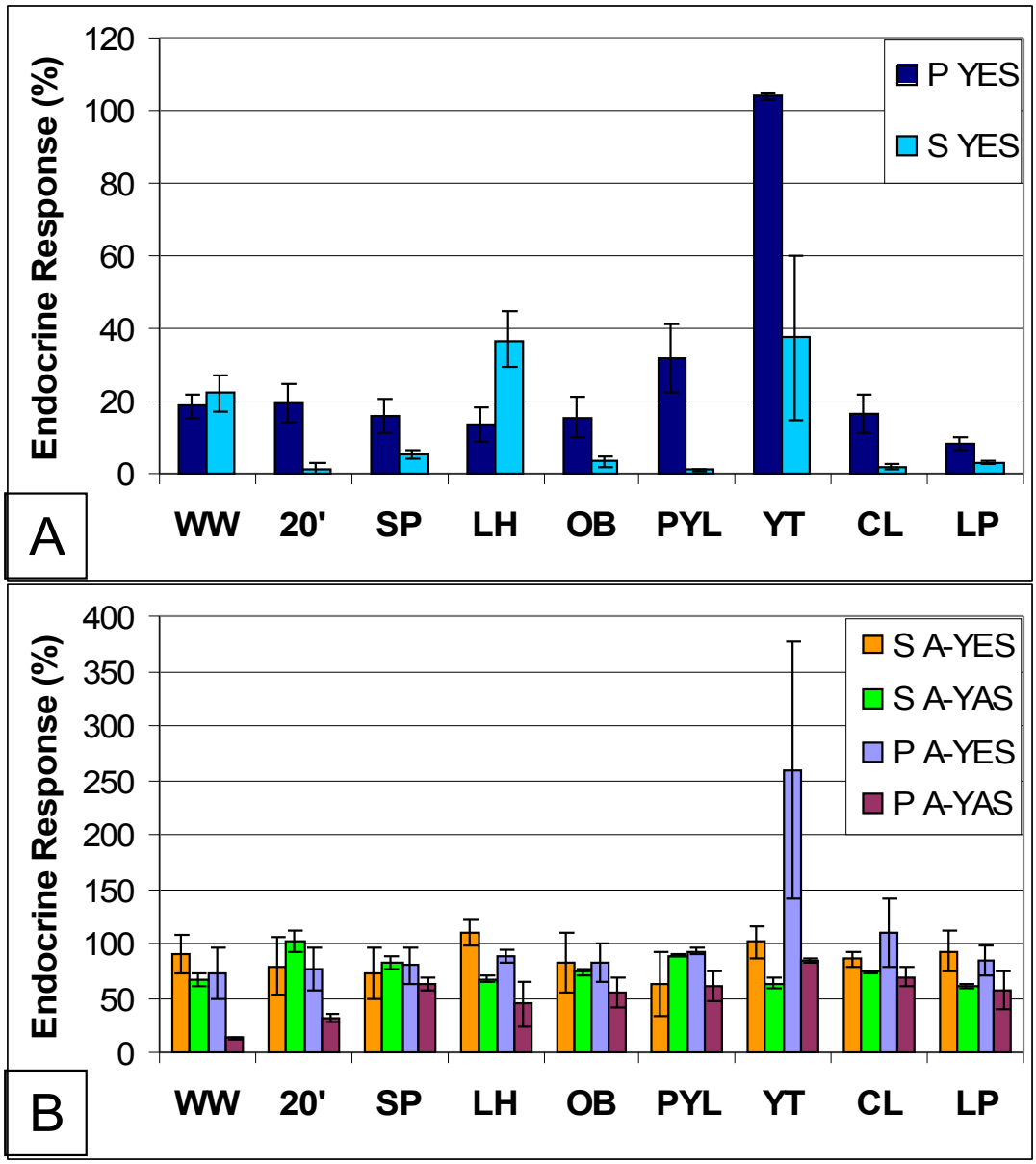


Figure 2.14. Endocrine activity of field blanks (% of maximal stimulatory/inhibitory response). For the purpose of this representation, the no effect level for the yeast estrogen screen is 0 % (Panel A), and for the anti-estrogen/androgen screens is 100 % (Panel B). Values are mean ± se of duplicate wells, run over two assays. P = POCIS extract, S = SPMD extract, YES = yeast estrogen screen, A-YES = yeast anti-estrogen screen, A-YAS = yeast anti-androgen screen.

2.3.4 Discussion

2.3.4.1 Hormonal Activity

All sites were active in at least one screen, and samples were generally more potent in the anti-YES/YAS than the YES (no activity observed in the YAS). The highest estrogenic activity was observed at WW (POCIS), the highest anti-estrogenic activity was observed at YT (SPMD) followed by LP (POCIS & SPMD), and the highest anti-androgenic activity at YT (SPMD), followed by WW & LP (POCIS & SPMD). The amount of activity at sites was not related to estimated risk, which may have been due to a number of factors. Firstly, the POPPIE database reports pesticide concentrations on a catchment level basis, and extrapolation from such a large area to estimate risk in a small, sometimes isolated, water body was probably inaccurate. Secondly, the most recent dataset that was available was from 1998, and pesticide profiles are likely to have changed considerably since then. Lastly, in the absence of analytical chemistry, it is unknown if pesticide concentrations in the samples was correlated with estimated risk, however, even if this correlation exists, it would not necessarily correspond to endocrinological effects observed. Due to these reasons, this dataset was not used as a guide of agrochemical pollution in further experiments (see Appendix 1 for UK pesticide concentrations in 2004/2005).

Estrogenic activity was observed in the POCIS extracts and not the SPMD extracts, and only at WW and 20'. WW was designed to be the low risk site for the E.Anglia cluster, however, it was highly estrogenic. The site at WW is comprised of 20 artificial ponds, and it was discovered that some of ponds had been used for a mesocosm exposure study . They were dosed with 1 mg/L of either diquat or dichlobenil, and although it is not known which ponds were exposed, neither of these compounds are persistent in the environment , so it is highly unlikely they would still be present in the ponds. It was also discovered that Roundup ® (active ingredient Glyphosate) had been used between 2001-2003 to remove scrub from around the ponds (A.Cooke, pers comm), however, again, glyphosate is not a persistent pesticide (Tomlin, 2006). Interestingly, the activity profile of sampling extracts was similar at WW and 20', which may indicate that the PADs were similarly exposed. There may

have been groundwater contamination if the water table was elevated, as WW is situated only 1 metre above sea level, and WW and 20' had very similar water quality profiles, which were distinct from other sites. This included highly elevated conductivity and salinity values, suggesting there may have been seawater input. The pond at WW may also have been contaminated by pesticides via spray drift , as SPMD field blanks were estrogenic, and POCIS field blanks were anti-androgenic. Spraying was observed on adjacent land during PAD deployment, and it is known that SPMDs can sample air-borne contaminants, such as PCBs , DDE , and pyrethroid insecticides , however, uptake of air borne contaminants by POCIS has not been reported. Unlike the other sites that were isolated ponds, 20' was situated in an irrigation ditch forming a network of canals, and thus resembled a riverine environment. Synthetic and natural estrogens are known to be sampled by the POCIS membrane , and weak estrogenic activity connected to sewage outflow has been identified in this area . Therefore, PADs may have been contaminated with natural and synthetic estrogens, causing the estrogenic effects observed at WW and 20'.

Anti-estrogenic/androgenic activity was more widespread than estrogenic activity, and the highest activity was observed at YT and LP. Pesticides detected in the area surrounding both these sites were atrazine (max: 0.16 µg/L), MCPA (max: 0.22 µg/L), mecoprop (max: 1.03 µg/L). Other pesticides detected near to YT (appendix 2, pp 260-280) were fluroxypyr (max: 0.06 µg/L), and simazine (max: 0.06 µg/L), and near to LP (appendix 2, pp 296-318) included, 2,4-D (max: 0.24 µg/L), isoproturon (max: 1.9 µg/L), and PCP (max: 2.7 µg/L). Apart from anti-estrogenic effect of PCP , the receptor mediated affects of these compounds have either not been tested or no activity was observed (see Chapter 1, section 1.5). Therefore, the source of the anti-estrogenic/androgenic activity observed at various sites is difficult to identify (but see Chapter 4), but due to the widespread and potent anti-activity, it is important that these effects are tested more thoroughly in relation to pesticide exposure.

The endocrine activity observed in the field blanks was unexpected. Petty *et al.* reported no uptake of pesticides or pharmaceuticals in field blanks of POCIS, and no effect was observed in the YES. In contrast, SPMD field blanks were reported to be toxic to developing amphibians to the same extent as sampling membranes (Bridges *et al.*, 2004). The authors' did not discuss the possible causes for toxicity observed,

however, despite the distinct endpoints compared to the present study, residual effects of field blanks are also indicated. As mentioned, SPMDs can sample air-borne contaminants, and alkyl hydrocarbons and phthalates have been identified in field blanks in two separate deployments . Some phthalates are estrogenic , and this effect has been observed in the YES (Harris *et al.*, 1997). They are ubiquitous industrial pollutants and have been measured atmospherically in areas as diverse as the North Sea , the Arctic , and the urban environment , therefore, they may be contributing to estrogenic effects of field blanks. In other studies using field deployed SPMDs, field blanks were not reported to have been used , or possible effects were not reported , and therefore, comparisons cannot be made.

As described, it was not possible to estimate environmental levels of pesticides, therefore, it is not known if activities observed are environmentally relevant. However, in a similar experimental design with SPMDs, Bridges *et al*. estimated an uptake rate of 1 litre/day/SPMD and used this value to estimate risk to native amphibians. The authors' rationale was that uptake of hydrophobic compounds varied from 0.01-2 L/day/SPMD, and uptake in invertebrates and fishes ranged from 0.03-8 L/day (see Bridges *et al*., 2004 and references therein), and thus the estimate of 1 L/d/SPMD was considered 'conservative'. In the present study, an uptake rate of 1.4 L/day/PAD was estimated, which would result in a relatively more dilute sample if reconstituted to 'ambient' levels, than that used be Bridges *et al.* (2004). The authors' reported tadpole deformities in response to extracts tested at estimated ambient levels, and their concentration was 12% more concentrated than LOEC reported here (x20 concentration of extract). Uptake rate by POCIS may have been somewhat slower, as Matthiessen *et al*. reported an uptake rate of 0.129 L/day/POCIS of E2, and Alvarez *et al.* (2004) reported uptake rates of 0.045 L/day/POCIS for diuron, and 0.086 L/day/POCIS for isoproturon. Therefore, it is likely that POCIS extracts were substantially more dilute than SPMD extracts. Indeed SPMD extracts were more active in the anti-YES/YAS, although only the POCIS extract was estrogenic. Therefore, despite the unknown nature of the extracts, and the uncertainty of ambient levels, there is evidence that amphibians may be at risk of EDs at these sites.

2.3.4.2 Site Selection

In E.Anglia, the low risk site (WW) was highly estrogenic, moderately anti-androgenic, and weakly anti-estrogenic, which was surprising as it was a pond located inside a nature reserve. It was meant as a reference site, but was endocrinologically active, and did not have a toad population. Therefore, it was decided that 20' would be used in breeding season three as it had high activity and an amphibian population. The sites in the Northern cluster were problematic. Firstly, one of the samplers was taken out of the water and left on the bank (RS), so this sample was lost. Secondly, the high risk site had the lowest activity of all the sites tested (OB). Thirdly, although moderately high activity was identified at the low risk site (LH) the PADs were partly submerged in sediment when retrieved, therefore it is not known if endocrine effects observed were derived from uptake from the water or the sediment. Furthermore, due to practicalities of repeated sampling in breeding season three, sites in the North of England would only have been used if results from this stage were encouraging. Therefore, it was decided that sites in the north of England would not be used in breeding season three. In the midlands cluster, the high risk site (LP) was highly anti-estrogenic and anti-androgenic, and the medium risk site was moderately anti-estrogenic (CL). LP and CL are thought to have distinct agrochemical input compared to sites from E.Anglia, as the former is bordered by pasture and arable agriculture, whereas the latter is situated near orchards. The pattern of endocrine effects was also distinct, as no estrogenic activity was observed, but potent anti-estrogenic and anti-androgenic effects were observed. In addition, these sites were situated at higher altitudes, had lower conductivity/salinity values, and no activity in the field blanks was observed (above background levels). Therefore, due to the distinct nature of these sites, it was decided that one would be selected for use in breeding season three. No tadpoles were observed at LP, however, the activity was high at this site, whereas low activity was observed at CL, but tadpoles were also observed. Water visibility was poor at LP during PAD deployment/ retrieval, and D.Pickford and the author were informed by the landowners that toadspawn and toads were often observed. Therefore it was decided to use LP in breeding season three.

In the cluster based on toad populations, the highest overall activity of all sites was observed at the site with reported declining populations (YT), and moderate to low activity at the site with increasing populations (PYL). YT borders a SSSI, and was thought to be a relatively unpolluted site. Compared to other sites, high phosphate

levels were measured at YT, possibly indicating contamination with fertiliser. A livestock farm was observed bordering one side of the pond, and animal waste was present on the path between the farm and the pond. Relatively low activity was observed at PYL, and a healthy toad population had been recorded. In addition, low phosphate and nitrate levels were recorded. Therefore, PYL served as the 'reference' site for comparison of 'polluted' sites, and YT was also used in breeding season three.

Therefore, in breeding season three, 4 sites were analysed further: 20' river in the fens (arable), LP in the midlands (arable and pasture), YT in Somerset (unknown pollutants), and PYL in Camarthenshire ('reference site').

2.4 Breeding Season Three

2.4.1 Introduction

The aim of breeding season three was to assess the impact of selected sites on survivorship, morphology, sex determination/differentiation, and the thyroid axis in the common toad (*Bufo bufo*). Caged and wild caught individuals from each site were analysed morphometrically, and the gonad and thyroid were analysed histologically. Toadspawn from each site was also collected and reared in aged tap water ("laboratory-reared") for comparison to caged and wild-caught individuals. Laboratory-reared individuals from each site also eliminated confounding variables caused by regional differences in gonadal development within the same species, which is a well known phenomenon on amphibians . Although water extracts were obtained with PADs, these samples were not available for analysis in the present project.

2.4.1.1 Gonadal Development in Bufo

A description of sex differentiation in amphibians has been described previously (see Chapter 1, sections 1.3.3 & 1.3.4), therefore comparative features of Bufonidae species will be described here. Spermatogenesis and oogenesis of Bufonidae species follow the same pattern as that of other vertebrates , but retarded gonadal

development in comparison to other amphibians has been reported . Distinction of sexes by histological analysis has been reported to occur approximately at forelimb emergence in various species, including, *B.japonicus formosus* , *B.bufo* , and *B.lentiginosus* . Sex races in species of Bufonidae have often been reported as semi-differentiated, whereby, all individuals first develop as females, and sex is permanently differentiated by completion of metamorphosis . Since gonadal development of only post-metamorphic individuals will be analysed in the present study, the question of sex races may not be relevant. However, one study reported that differentiation of the permanent sex was not complete by the fifteenth day after metamorphosis . Therefore, a predominance of females at early stages of gonadal differentiation may be an artefact of differentiation rates, rather than a result of external factors. In a series of papers King demonstrated that there was no effect of temperature, nutrition, egg ripeness, or source of spermatozoan, on sex ratios in *B.lentiginosus*. In a total of over 10000 animals analysed, deviation of sex ratio was rarely more than 10% of the expected 50:50 male:female ratio. In other laboratory studies, the sex ratio of *B.bufo* did not deviate by more than 10 % , or *B.americanus* .

2.4.1.1.1. Effects of Exogenous compounds on Sexual Differentiation in Bufonids

Various compounds, including gonadotropins and steroid hormones, have been shown to influence gonadal differentiation, and species-specific differences have also been reported. Larval treatment with 0.1 mg/L E2 induced feminisation of gonads in *B.bufo* and *B.americaus* , but no effect was observed in *B.vulgaris* exposed to 1 mg/L or *B.boreas* exposed to 0.3 mg/L . In addition, retardation of differentiation was observed in *B.americus* tadpoles exposed to 0.5 or 1 mg/L E2 (Chang, 1955). Testosterone was less effective than E2, and had no effect on gonadal differentiation in *B.boreas* exposed to 0.4 mg/L , or *B.bufo* exposed to 1 mg/L . In addition, methyltestosterone (MT) had no effect on *B.americanus* exposed to 0.02, 0.05, or 0.1 mg/L, but retardation of development was observed in tadpoles exposed to 0.5 mg/L (Chang, 1955). Responses to estrogens and androgens may have been due to a local effect on the gonad in low doses, and inhibition of gonadotropin release in higher doses, leading to retardation of development. Indeed, DHT had a potent inhibitory effect on gonadal differentiation in *B.bufo* (Petrini and Zaccanti, 1998), and is thought to have a stronger negative feedback effect in the hypothalamo-pituitary-gonadal

system than testosterone (see Chapter 1, section 1.3.3). FSH may upregulate 5α-R activity, resulting in higher DHT levels, as has been observed in rats , as larval treatment with FSH caused masculinisation of gonads of *B.vulgaris* , and the same effect was observed in injection of metamorphosed, but undifferentiated, *B.arenarum* (1 month post-metamorphosis) with FSH . It has been reported that ovarian differentiation in Bufonids is less dependant on gonadotropins than testicular differentiation, and that the pituitary of adult male toads is more responsive to GnRH than adult females (see Chapter 1, section 1.3.3), therefore, feminisation could be explained by a lack of gonadotropins. However, injection of undifferentiated *B.arenarum* (1 month post-metamorphosis) with LH caused feminisation. Perhaps LH had a negative feedback effect on the hypothalamus, resulting in decreased FSH production, which may be required for masculinisation of genetic males. Interestingly, treatment of *B.bufo* with a 5α-reductase inhibitor (17βC: 4-androstene-3-one-17β-carboxylic acid), enhanced gonadal differentiation of both sexes (Petrini and Zaccanti, 1998), and exposure to an aromatase inhibitor (4-OHA: 4-hydroxy-4-androstene-3,17-dione) caused masculinisation in *B.bufo* (Pertini and Zaccanti, 1998). There are not enough background data to accurately interpret these results, however, it seems likely that both gonadotropins, through the hypothalamo-pituitary-gonadal axis, and hormones, through a local effect on the gonad, influence sexual differentiation in toads.

2.4.1.1.2. Bidder's Organ

Bufonidae species are unique among amphibians in that they possess a Bidder's organ (BO), which lies anteriorly to the gonad and is present in juveniles of both sexes. It normally persists in adult males, but becomes continuous with the ovary in females after approximately 1 year , and thus often disappears completely . It has been detected in all Bufonidae species studied thus far, including *B.canorus, B.viridis, B.fowleri, B.americanus, B.vulgaris, B.lentiginosus, B.quercicus* , and *B.bufo* . BO develops much more rapidly than the gonad, and attains a large size before it is possible to ascertain sex of the individual . The BO is a rudimentary ovary, and its development consists of two oogenic growth phases, and therefore, a bimodal distribution of oocyte size can be observed in early life (< 60 days post metamorphosis). The first phase begins at Gosner stage 34 , and the second at

completion of metamorphosis (Zaccanti *et al.*, 1971, Falconi *et al*., 2007). In females, a clear distinction between BO and the ovary can be observed, and in males a 'mesogonad' with the appearance of an immature ovary located between BO and the teste was sometimes observed (Zaccanti *et al*., 1971). It has been reported that removal of the ovary may induce development of BO into a functional ovary, but this has not been well studied (reported in Witschi, 1933). In contrast, many studies have reported the development of a functional ovary upon orchidectomy. This 'ovary' can produce fertilizable eggs, although, orchidectomised toads only develop rudimentary oviducts, and therefore cannot release the eggs . BO oocytes from orchidectomised toads and ovaries are functionally similar, both in protein expression , and steroidogenic enzyme activity (3β-HSD, 17β-HSD) . Bufonids are not true hermaphrodites, as they do not possess functional ovarian and testicular tissue simultaneously, however, it has been proposed that BO is evidence of hermaphroditic ancestry of Bufonids, and perhaps all amphibians (Witschi, 1933). Interestingly, if male *Xenopus* larvae are treated with estrogens for a short time near the critical sex-determining period, the cephalic portion of the gonad develops into an ovarian-like structure, and the posterior develops into a teste , suggesting the potential for a cephalic ovarian structure in other species.

Development and maintenance of gonadal function in amphibians is at least partly controlled by gonadotropins (see Chapter 1, section 1.3.3), and BO degenerated after removal of the pituitary in adult *B.bufo* , but increased in weight after pituitary implantation in adult *B.arenarum* ; suggesting gonadotropins also have a role in BO maintenance. Indeed, gonadotropin treatment (PMSG: pregnant mare's serum gonadotropin; & HCG: human chorionic gonadotropin) enhanced the stimulating effect of orchidectomy on BO development in *B.woodhousii* (Pancak-Roessler and Norris, 1991), and gonadectomy in *Rana esculenta* has been reported to increase gonadotropin levels . Therefore, the development of BO into an ovary following orchidectomy may be due to the lack of negative feedback on the pituitary, leading to an increase in gonadotropin levels (Pancak-Roessler and Norris, 1991). Interestingly, BO size was not affected by gonadotropins in animals with intact testes, despite increased gonadotropin levels (Pancak-Roessler and Norris, 1991), and similarly, bidderian levels of 3β-HSD and 17β-HSD were stimulated by gonadotropins in orchidectomised toads, but this response was not observed in intact toads (Pancak-

Roessler and Norris, 1991). Increased incidence of atretic follicles was also observed in intact males treated with gonadotropins (Pancak-Roessler and Norris, 1991), and many degenerated bidderian oocytes were found in *B.japonicus formosus* at the same time of year as enhanced gonadotropin levels . Thus, the presence of the teste maintains the development of BO in an undifferentiated state, even in the presence of increased gonadotropin levels, and may induce oocyte atresia (Pancak-Roessler and Norris, 1991). The nature of this control is unknown, however, androgens may play a role as amphibian teste size increases in the breeding season, and this increase is positively correlated with testosterone levels , and an inverse relationship between BO and teste size has been reported . In support of this hypothesis, reproductively active male *B.woodhousii* are reported to have smaller BOs, compared to non-reproductively active individuals , and testosterone treatment caused atrophy of BO in adult *B.melanostictus* , and inhibition of BO growth in developing *B.bufo* . In addition, treatment with testosterone propionate or MT depressed growth of BO, including a reduction in oocyte number and size, in larval *B.vulgaris* . Both testosterone and DHT have been reported to decrease the size of the BO, and inhibit its development in larval *B.bufo* , and treatment with FSH, which had previously been reported to cause masculinisation, reduced the size of BO and of bidderian oocytes in *B.vulgaris* . The androgen responsible for inhibiting BO development *in vivo* may be DHT, as treatment with a 5α-R inhibitor (17βC: 2 mg/L), accelerated BO differentiation, and increased BO size, in larval *B.bufo* . Although, in a later study, the same laboratory reported the opposite effect on BO in response to 17βC, under similar experimental conditions (Petrini & Zaccanti, 1998), therefore, to elucidate the role of androgens in bidderian differentiation and growth requires further investigation. The reported effects of estrogens on BO are generally similar to that of androgens; that is, inhibition of development in larvae and degeneration in adults is normally observed, at least in response to high concentrations. In a larval study using *B.vulgaris formosus*, exposure to 0.5-1 mg/L estradiol or estrone caused retardation of the development of BO (Takahashi, 1956), as was observed in larval *B.bufo* exposed to 0.1 mg/L estradiol (Petrini and Zaccanti, 1998). In adults, orchidectomised or intact *B.bufo* were treated with diethylstilbestrol dipropionate (DES) implants for 10 months. In intact toads, treatment with high concentrations (1000-100 µg/week) caused degeneration of the diplotenic oocytes in BO, whereas low doses (50-1 µg/week), enhanced development of BO. In orchidectomised toads, larger BOs were

observed, and enhanced BO development was observed in toads receiving 1 µg/week DES . Similarly, Basu & Mondal treated intact *B.melanstictus* adult males with an estradiol implant, resulting in dosage of approximately 1 mg/week, and reported degeneration of bidderian oocytes. Therefore, in adults, an inhibitory effect of estrogens on BO is reported at high concentrations, whereas a stimulatory effect is reported at lower concentrations. Similarly to effects observed on the gonad, the reported effect on BO may be due to negative feedback by the exogenous compounds, leading to reduced gonadotropin release at high concentrations, but a local stimulatory effect at lower concentrations.

2.4.1.2 Thyroidal Development

The morphology , and function of the thyroid system in Bufonidae does not appear to be distinct from other amphibians. Therefore, for a general description of the amphibian thyroid system see Chapter 1 (section 1.4).

2.4.2 Methods

2.4.2.1 Experimental Design

In March/April 2006 toadspawn was collected from the 4 sites selected from breeding season two (Yatton: YT; Pant-y-Llyn: PYL; twenty-foot river: 20'; Layes pool: LP), and reared in aged tap water in the laboratory and in cages deployed at each site. Spawn was collected from between 2-4 different strings, although it is not known whether these originated from different females. 9 groups of 100 eggs were counted and placed in separate 50 ml centrifuge tubes with site water. The developmental stage of embryos at this time was between gastrulation and late cleavage . At 18^0C, embryos of *Rana pipiens* reach the late cleavage stage at 21 hours post-fertilisation (Rugh, 1951), therefore, it is likely that the eggs collected had been laid in the previous few days. Four cages were deployed at each site, and 100 eggs were placed in each cage. The remaining eggs (5 x 100) were transported to the laboratory in a cooled ice box. Cage design was similar to that used by Cooke . Square plastic plant pots (Wyevale Garden centre, 35x35x25), were leached in tap water for 48 hours. The

middle panels on each side of the pot were removed, leaving a large rectangular gap on each side. The pots were then lined with fine plastic mesh (~ 1 mm diameter) to allow free flow of water and nutrients into the cage, but prevent tadpoles from escaping. Bubble-wrap was tied around the rim of the pot to assist flotation of the cage, so that tadpoles could reach the water surface (Figure 2.15). Upon deployment of the cages, ~2 metres of nylon wire was tied round each bottom corner of the cage, and the other end of this wire was tied around a breezeblock.

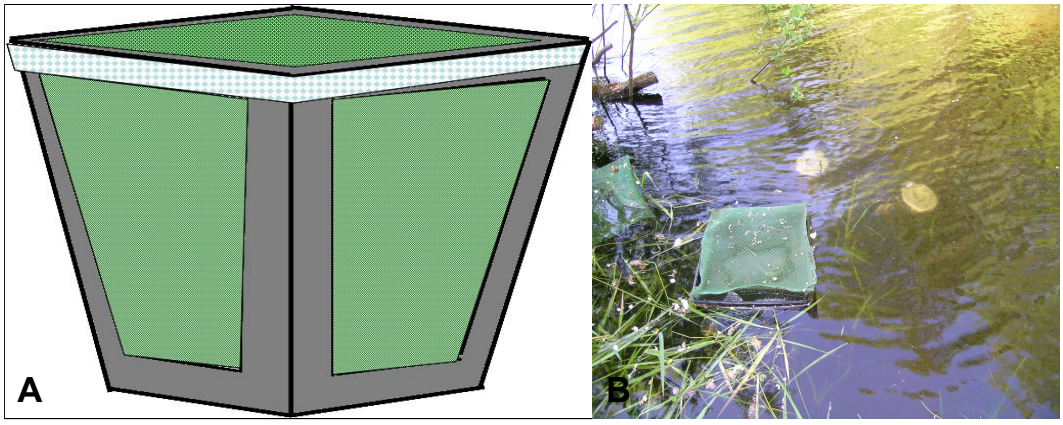


Figure 2.15. Diagram of the cage used for tadpole sampling at selected sites (Panel A), and a photograph of the cage at PYL (Panel B).

Laboratory-reared individuals were kept in 20 Litre glass aquaria containing 12 Litre aged tap water, in a shaded outside environment. 5 replicate tanks were used per site, each containing 100 eggs collected previously. During embryo development prior to hatching (~3 weeks), water quality (temperature, pH, DO), and ammonia (NH_3^+) levels were tested every 7 days. Ammonia values were converted to unionised ammonia (NH_3), using temperature, and pH values. Conductivity (µS/cm) was also measured once at the beginning of exposure. Water quality was good, and therefore, no water change was carried out during this period. At hatching (~ 3 weeks post collection), the number of hatched individuals and abnormal larvae were counted. Any abnormal embryos were removed and fixed in 10% neutral buffered formalin (NBF). The laboratory-reared individuals were culled to maintain good water quality, resulting in 40 larvae per tank. At this time, tanks were emptied and cleaned, before returning larvae to their respective tanks. Following hatching, water change (75%) was conducted every 48 hours, and water quality was measured prior to each change. There is a critical period in metamorphosis when mass mortality occurs by drowning,

if metamorphosing individuals cannot reach air (F.Orton, unpublished observation). Therefore, when tail regression began to be observed (~ 6 weeks post hatch), most of the water was drained from tanks, and they were tilted to allow emergence of metamorphs. Sphagnum moss was also added to the tanks, to facilitate emergence. To avoid loss by drowning of caged individuals, they were brought to the laboratory when forelimb emergence (FLE) occurred at each site, and were kept under the same regime as laboratory-reared individuals, except site water was used instead of aged tap water. The young metamorphs were transferred to vivariums on completion of metamorphosis. Vivariums were made from plastic rat boxes (21x33x19 cm), covered with muslin cloth, which was held in place with elastic. The bottom of the vivarium was originally covered with compost, followed by a layer of sphagnum moss. However, when the transferred metamorphs were checked the next day, almost 100% mortality had occurred. Therefore, the vivariums were cleaned, and the bottom was covered with damp sphagnum moss only. A shallow ceramic dish filled with water was also placed in the corner of each vivarium. Animals were initially fed wingless Drosophila (Blades Biological, UK), and when they were larger, micro-crickets (Blades Biological, UK), every 48 hours. The vivariums were also checked for moisture at feeding, and water was added to sphagnum moss and ceramic dishes where necessary.

Time of spawn-deposition by amphibians is controlled mainly by temperature. Due to distinct environments at each site, detection of spawn, and therefore cage deployment, was carried out at different times at each site (YT – 29.03.06; PYL – 2.04.06; 20' – 3.04.06; LP – 5.04.06). Development of embryos and therefore time to hatching, also varied between sites, therefore, sampling was carried out on different dates from each site according to hatching date (see Figure 2.16). Animals were sampled at 5 time points following hatching (5, 7, 9, 12, and 15 weeks post hatch), based on date when all embryos had hatched (hatch date: YT – 21.04.07, PYL & 20' – 24.04.07; LP – 25.04.07). These sampling points will be described as time points (TP) 1, 2, 3, 4, and 5, from this point onwards. At each time point, wild caught individuals were sampled, when found, from each site. At larval stages, tadpoles were caught with a net, and after metamorphosis, the area surrounding the pond was searched for metamorphs. TP 1 & 2 occurred prior to metamorphosis, and therefore samples were of larval individuals taken from their cages at the various sites. Between TP 2-3,

metamorphosis occurred in most of the individuals, and the remaining caged individuals were brought to the laboratory by sampling TP 3. Mortality was measured at 3 times during exposure: Firstly, initial hatching success, secondly, during larval period (up to TP 2), and finally, during metamorphic period (up to end of exposure). In addition, the incidence of forelimb emergence (FLE) was noted at TP 2 (see Figure 2.16). In the field environment, water quality parameters (temperature, pH, DO, and conductivity) were measured (YSI 556 handheld multiparameter system, YSI Hydrodata UK) at the time of egg retrieval, hatching, and TP 1, 2, and 3 (5 times).

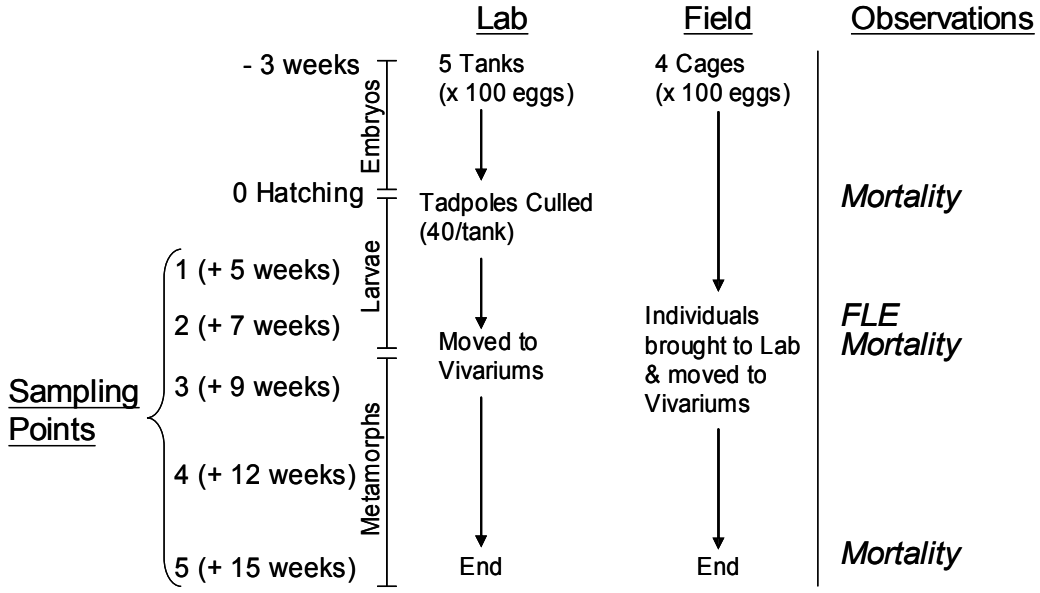


Figure 2.16. Experimental design of laboratory (Lab) and field site (Cage) observations and sampling points during embryonic, larval, and post-metamorphic development of *Bufo bufo*. See text for details.

2.4.2.2 Animals

At each sampling TP, laboratory-reared, caged, and wild caught specimens were anaesthetised by immersion in MS-222, and sacrificed by pithing. Laboratory-reared individuals were dabbed on tissue to remove excess water, and weighed (wgt). The total length (TL), snout-vent length (SVL), and the hindlimb length (HLL), was measured with digital callipers, and the developmental stage was noted . Samples were then fixed in neutral buffered formalin (NBF: Sigma, U.K.). Caged and wild specimens were fixed in NBF prior to morphological measurements, due to practicalities of taking measurements in the field environment. Therefore, correction

factors for weight, TL, SVL, and HLL, were calculated using measurements from before and after NBF treatment of laboratory-reared specimens (see Appendix 3). HLE, developmental stage, and FLE are thyroid sensitive morphological endpoints. Due to size differences between groups, hindlimb length was expressed as a proportion of snout-vent length (HLL/SVL). A condition factor was also calculated by dividing the weight of the individual by SVL (wgt/SVL). Specimens were dissected by opening the body cavity, the intestines were removed. The gonads were removed for histological analysis by dissection of the middle portion of the animal by cutting just below the forelimbs and above the hindlimbs. The head was also cut away from the body, and this tissue contained the thyroid. Tissue was placed in Bouins' solution for decalcification (20 hours), followed by 70% industrial methylated spirits (IMS: 24 hours). They were placed in a tissue processor (Shandon Citadel 2000, Thermo Fisher Scientific, UK), and gradually dehydrated in IMS (70%, 90%, 100%), followed by immersion in Histoclear (RA Lamb, U.K.), and were embedded with paraffin wax. The heads were embedded in a vacuum oven (model 3606-1CE, Labline Instruments Incorporated, USA) to ensure entry of wax into cartilagenous tissue. Gonads were sectioned longitudinally at 7μM, and each section was mounted on a glass slide. The thyroids were sectioned transversally at 5 μM, and two sections were kept every 30 μM, as recommended by OECD guidelines (unpublished). Specimens from time point 1 were not analysed as they were too undeveloped for thyroidal or gonadal analysis. Morphological parameters and thyroid histology was analysed in specimens taken from TP 2, 3, 4, & 5, and gonad histology in specimens from TP 3, 4, & 5.

2.4.2.3 Histological Analysis

Analysis of all histological sections was undertaken blind, whereby identifying labels on slides were covered and coded prior to analysis.

2.4.2.3.1 Thyroidal Analysis

Animals were split into developmental stages prior to analysis, using TK tadpole development table , to allow comparison between sites. The groups were: premetamorphic (pre: TK stage 5-11), prometamorphic (pro: TK stage 12-19), climax (clim: TK stage 20-24), and metamorphs (TK stage 25). In most cases, pre, pro, and

clim individuals were found at TP 2, and metamorphs at later TPs. Since developmental stage was the same for all metamorphs, they were sub-divided according to TP (3, 4, or 5) to show changes of the thyroid organ over time.

2.4.2.3.1 Sex Characterisation

The sex of the individual was characterised as female, male, intersex, or undifferentiated. Ovaries were characterised by reduction of the medulla, leading to formation on an ovarian cavity, and the presence of oogonial cell nests or oocytes. Testes were characterised by a well developed medulla, and organisation of spermatogonia into early seminiferous tubules . Intersex individuals contained a minimum of one testicular oocyte per teste examined. Undifferentiated individuals could not be characterised as female or male due to absence of features described above.

2.4.2.3.2 Cytometric Analysis

Cytometric analysis of BO and gonads was undertaken over several stages. Firstly, the number of sections containing the right BO, gonad, or thyroid was counted. Secondly, the section containing the largest area of tissue was selected by observing the middle section, and the preceding/proceeding 3 sections. Photographs were then taken for further analysis of each tissue type (Olympus BXSI microscope, Micropublisher 5.0 RTV camera, QCapturePro 5.1 software). One photograph was taken at x100 magnification, which was used for measuring the area of the organ. The size of each organ was calculated by multiplication of the area at the largest point by the number of tissue containing sections. BOs, testes, and thyroids were ovoid in shape, and ovaries lobular, and thus the size of the organs were a relative measure for use within this study, not absolute values. Using this photograph of BO or ovary, the number of diplotenic oocytes with a visible nucleus was counted, and total number was calculated by multiplication of counted oocytes by the number of tissue containing sections. A further 3 photographs were taken at x 400 or x 1000 magnification of BO

and ovary, which were used for measuring the size of second growth phase (SGP) and first growth phase (FGP) diplotenic oocytes. To ensure standard oocyte measurements between photographs, only oocytes with a visible nucleus were measured (the nucleus was generally found in only 1 section). The larger oocytes, which are also more centrally located, pertain to the first oogenic wave (FGP oocytes), and the smaller and more peripherally located ones pertain to the second oogenic wave (SGP oocytes) . Every section containing testicular tissue was checked for the presence of testicular oocytes (TOs), and where found, the number was counted. The incidence of testicular oocytes were calculated as number of individuals containing testicular oocytes divided by total number of females identified at that time point (normal females + intersex). Severity of testicular oocytes was calculated as the number of testicular oocytes per intersex individual. The thyroids were analysed using OECD guidelines (unpublished). A scoring system (0-5, 0 indicating no perturbation from the normal state, and 5 indicating extreme change) was used to assess epithelial cell hypertrophy and hyperplasia, and colloid quality and depletion. The epithelial cell hypertrophy and hyperplasia values were added together to give a measure of activity of the gland, and the colloid quality and depletion values were added together to give a measure of colloid perturbation of the gland. The Bidder's somatic index (BSI), testicular somatic index (TSI), ovarian somatic index (OSI), and thyroid somatic index (ThSI) were calculated by dividing the volume of the organ by the weight of the individual. It is possible that by calculating the relative size of the organ by histological sections instead of weight could introduce an artefact into the data. However, a normalising factor was necessary due to the large differences in size from laboratory-reared, caged, and wild-caught individuals, and organs were too small to dissect from the body cavity.

2.4.2.4 Statistical Analysis

Data were analysed for normality, and where normally distributed, were analysed for differences between groups using ANOVA. Where not normally distributed, pairs were analysed with Wilcoxon's test and groups with Kruskal Wallis's test. If significant differences were observed at this point, Dunnett's test was used on both parametric and non-parametric data to determine where these differences lay. Relationships between multiple variables were analysed using ANCOVA.

2.4.3 Results

2.4.3.1 Water Quality

There was no difference in water quality between tanks containing laboratory-reared individuals over the exposure period (ANOVA $p > 0.05$). Values were within the range expected for normal tadpole development (Table 2.3). There was no difference in temperature, and pH, between laboratory conditions and field sites (ANOVA $p > 0.05$). However, DO was significantly higher at YT and 20' (Dunnett's $p = 0.013$ & 0.008) compared to laboratory conditions. Conductivity also varied, and was significantly higher at 20' (Dunnett's $p < 0.0001$), and lower at YT and PYL (Dunnett's $p = 0.018$ & < 0.0001), compared to laboratory conditions.

	Laboratory	YT	PYL	20'	LP
T (^{0}C)	15.6 ± 0.02	15.2 ± 1.32	13.9 ± 1.6	15.9 ± 1.6	14.4 ± 1.5
pH	8.03 ± 0.00	8.13 ± 0.14	8 ± 0.1	8 ± 0.2	8.4 ± 0.6
DO (mg/L)	7.6 ± 0.05	11.1 ± 1.3*	8.9 ± 0.5	10.9 ± 0.6*	10.1 ± 1.7
C (µS/cm)	817.4 ± 4.2	500.5 ± 16.3*	212.3 ± 12.5*	2485.2 ± 228*	654 ± 33.6
NH_3 (µg/L)	29.7 ± 0.72	N/A	N/A	N/A	N/A

Table 2.3. Water quality in laboratory tanks and from field sites over the larval period, values are mean ± SE. For laboratory measurements $n = 29$, for field measurements $n = 5$. * denotes significant differences in water quality from field sites compared to the laboratory conditions

2.4.3.2 Mortality

Mortality was high in both laboratory-reared and caged individuals, but overall survival was lower in the caged individuals (YT = 2%; PYL = 3.5%; 20' = 8%; LP = 0%), than the laboratory individuals (YT = 11.5%; PYL = 14.5%; 20' = 23%; LP =

23.5%), and was below 30% in all cases (Figure 2.17). In laboratory-reared individuals, hatching success was fairly high, and larvae also had good survival, however, high mortality was observed between larval and metamorphic stages. In the caged individuals, an opposite trend was observed, whereby there was low hatching success (except PYL, Figure 2.17, B), but mortality was less pronounced during larval and metamorphic periods. In addition, there were significantly more abnormal larvae observed in laboratory-reared individuals from LP compared to other sites (Dunnett's $p = 0.02$, data not shown).

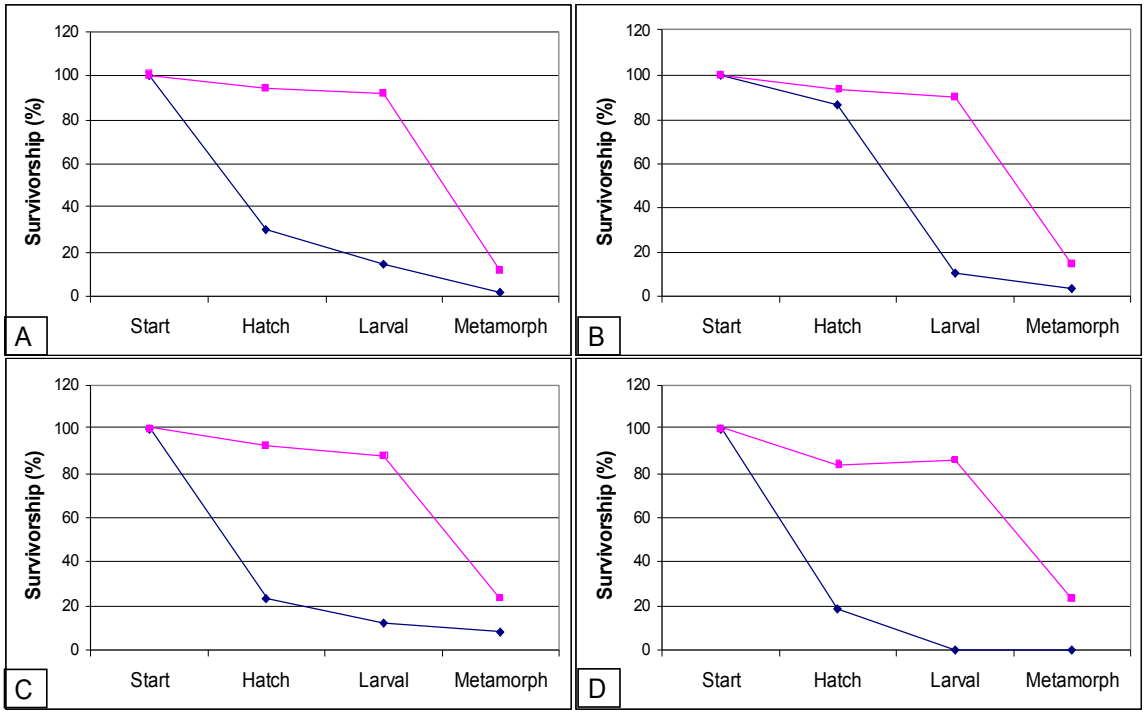


Figure 2.17. Survivorship of laboratory (pink line) and caged (blue line) individuals from YT (Panel A), PYL (Panel B), 20' (Panel C), and LP (Panel D). Values are means from either 5 replicate tanks (laboratory) or 4 replicate cages (caged).

2.4.3.3 Morphometrics

Individuals from LP were not analysed morphologically due to 100 % mortality in caged individuals at an early stage of the experiment, and absence of wild-caught individuals. Morphometric comparisons between laboratory-reared individuals showed significant differences in growth and development parameters between YT, PYL, and 20' (Table 2.4). Generally, individuals from YT were bigger (wgt, SVL,

wgt/SVL) than those from PYL or 20'. This difference persisted over the experimental period for individuals from 20', but individuals from PYL were only significantly smaller than YT at TP 3. Laboratory-reared individuals from PYL developed more slowly (stage, HLL, HLL/SVL) than those from YT or 20', and this difference also persisted over the experimental period. Percentage of FLE of laboratory-reared individuals (at TP 2) was also significantly lower in individuals from PYL compared to YT and 20'. Significant differences between laboratory-reared, caged, and wild caught individuals within each site were also observed (Table 2.4). Caged and wild caught individuals were smaller (wgt, SVL, wgt/SVL) and developed more slowly (stage, HLL, HLL/SVL) than their respective laboratory-reared counterparts at each site. These differences persisted over the experimental period at PYL and YT (but only until TP4 at 20'), but became less pronounced over time. In addition, percentage of FLE was significantly lower in caged individuals from PYL and 20' compared to their laboratory-reared counterparts, but this effect was not observed at YT. By time point 5, differences between laboratory-reared, caged, and wild caught individuals were less pronounced at all sites, and only weight (YT), SVL (YT, PYL), and HLL/SVL (20'), were significantly different. At YT and 20', few significant differences in growth or development between caged and wild caught individuals was observed, however, at PYL wild caught individuals were larger and more developed than caged individuals (TP3 – only TP where both caged and wild caught individuals were present).

	Yatton				Pant-y-Llyn				20’			
	a/b	x/y/z	x/y/z	x/y/z	a/b	x/y/z	x/y/z	x/y/z	a/b	x/y/z	x/y/z	x/y/z
Time Point 2	**Lab**		**Cage**	**Wild**	**Lab**		**Cage**	**Wild**	**Lab**		**Cage**	**Wild**
N	25		13	5	25		10	None	25		16	5
Stage (TK)	$19^{ab}{}_{x}$		18_{xy}	16_{y}	$18^{a}{}_{x}$		7.5_{y}	----------	$20^{b}{}_{x}$		16_{y}	14_{z}
Wgt (mg)	$436\ (23)^{a}{}_{x}$		$189\ (10)_{y}$	$258\ (15)_{y}$	$394\ (16)^{ab}{}_{x}$		$113\ (15)_{y}$	----------	$351\ (7)^{b}{}_{x}$		$317\ (7)_{y}$	$371\ (3)_{x}$
SVL (mm)	$13.9\ (0.2)^{a}{}_{x}$		$10.7\ (0.1)_{y}$	$11.7\ (0.4)_{z}$	$13.6\ (0.4)^{a}{}_{x}$		$8\ (0.4)_{y}$	----------	$12.7\ (0.1)^{b}{}_{x}$		$11.5\ (0)_{y}$	$12.2\ (0.3)_{z}$
HLL (mm)	$12.4\ (0.3)^{a}{}_{x}$		$8.1\ (0.7)_{y}$	$8.3\ (0.7)_{y}$	$11.4\ (0.3)^{b}{}_{x}$		$1.3\ (0.1)_{y}$	----------	$11.8\ (0.3)^{ab}{}_{x}$		$6.2\ (0.2)_{y}$	$4.8\ (0.8)_{z}$
FLE (%)	44.4^{a}		24.4	----------	$26.5^{b}{}_{x}$		0_{y}	----------	$62.7^{c}{}_{x}$		0_{y}	----------
Wgt/SVL	$31\ (1.2)^{a}{}_{x}$		$17.7\ (0.9)_{y}$	$22\ (0.7)_{y}$	$29.2\ (1.1)^{ab}{}_{x}$		$13.8\ (1.1)_{y}$	----------	$27.7\ (0.5)^{b}$		27.6 (0.6)	30.4 (1.9)
HLL/SVL	$0.9\ (0.03)^{ab}{}_{x}$		$0.76\ (0.06)_{y}$	$0.7\ (0.04)_{y}$	$0.85\ (0.03)^{a}{}_{x}$		$0.15\ (0.02)_{y}$	----------	$0.93\ (0.02)^{b}{}_{x}$		$0.54\ (0.02)_{y}$	$0.39\ (0.06)_{z}$
Time Point 3	**Lab**		**Cage**	**Wild**	**Lab**		**Cage**	**Wild**	**Lab**		**Cage**	**Wild**
N	13		4	9	14		10	10	21		13	5
Stage (TK)	25_{x}		25_{x}	24.5_{y}	25_{x}		18.5_{y}	21_{z}	25_{x}		25_{x}	24.5_{y}
Wgt (mg)	$271\ (15)^{a}{}_{x}$		$79\ (7)_{y}$	$71\ (4)_{y}$	$219\ (9)^{b}{}_{x}$		$139\ (13)_{y}$	$226\ (11)_{x}$	$212\ (8)^{b}{}_{x}$		$133\ (5)_{y}$	$172\ (14)_{xy}$
SVL (mm)	$14.4\ (0.3)^{a}{}_{x}$		$8.3\ (0.2)_{y}$	$7.9\ (0.1)_{y}$	$13.6\ (0.2)^{b}{}_{x}$		$11.1\ (0.2)_{y}$	$13.8\ (0.3)_{x}$	$13.3\ (0.2)^{c}$		13.3 (0.1)	13.1 (0.5)
HLL (mm)	$16.4\ (0.5)^{a}{}_{x}$		$10.4\ (0.3)_{y}$	$9.8\ (0.2)_{y}$	$14.6\ (0.3)^{b}{}_{x}$		$6.5\ (1.1)_{y}$	$13.4\ (0.5)_{x}$	$15.1\ (0.2)^{b}{}_{x}$		$13.9\ (0.2)_{y}$	$13.7\ (0.6)_{y}$
Wgt/SVL	$18.6\ (0.8)^{a}{}_{x}$		$8.5\ (0.6)_{y}$	$7.8\ (0.4)_{y}$	$16\ (0.5)^{b}{}_{x}$		$12.4\ (1.1)_{y}$	$16.3\ (0.6)_{x}$	$15.8\ (0.4)^{b}{}_{x}$		$10\ (0.3)_{y}$	$13\ (0.7)_{z}$
HLL/SVL	$1.13\ (0.02)^{a}{}_{x}$		$0.99\ (0.01)_{y}$	$0.96\ (0.02)_{y}$	$1.07\ (0.02)^{b}{}_{x}$		$0.6\ (0.11)_{y}$	$0.97\ (0.02)_{x}$	$1.14\ (0.01)^{a}{}_{x}$		$1.05\ (0.01)_{y}$	$1.04\ (0.02)_{y}$
Time Point 4	**Lab**		**Cage**	**Wild**	**Lab**		**Cage**	**Wild**	**Lab**		**Cage**	**Wild**
N	5		2	5	10		4	None	15		10	1
Stage (TK)	25		25	25	25^{-x}		19^{-y}	----------	25		25	25
Wgt (mg)	$364\ (54)^{a\text{-}x}$		$109\ (6)^{-y}$	$107\ (29)^{-y}$	$301\ (18)^{ab\text{-}x}$		$62\ (16)^{-y}$	----------	$277\ (16)^{b}$		234 (18)	268 (0)
SVL (mm)	$15.3\ (0.9)^{-x}$		$10.3\ (0.3)^{-y}$	$10.6\ (0.8)^{-y}$	$14.7\ (0.4)^{-x}$		$8.3\ (0.2)^{-y}$	----------	$14.0\ (0.2)^{-x}$		$12.7\ (0.3)^{-y}$	15.8 (0)
HLL (mm)	$18.2\ (0.5)^{a\text{-}x}$		$13.5\ (0)^{-y}$	$10.7(0.9)^{-y}$	$17.3\ (0.5)^{ab\text{-}x}$		$7.3\ (3.1)^{-y}$	----------	$16.8\ (0.3)^{b}$		16.9 (0.4)	16.8 (0)
Wgt/SVL	$23.3\ (2.6)^{-x}$		$11.8\ (0.2)^{-y}$	$10.7\ (1.9)^{-y}$	$20.4\ (0.7)^{-x}$		$7.5\ (1.8)^{-y}$	----------	19.8 (1.1)		18.2 (1.0)	13 (0.7)
HLL/SVL	1.2 (0.05)		1.21 (0.03)	1.18 (0.03)	1.18 (0.02)		0.89 (0.4)	----------	$1.21\ (0.02)^{-x}$		$1.29\ (0.02)^{-y}$	1.06 (0)
Time Point 5	**Lab**		**Cage**	**Wild**	**Lab**		**Cage**	**Wild**	**Lab**		**Cage**	**Wild**

N	5	2	None	5	None	5	10	9	None
Stage (TK)	25	25	----------	25	----------	25	25	25	----------
Wgt (mg)	575 (55)$^{a}_{x}$	294 (7)$_{y}$	----------	624 (93)^{a}	----------	523 (69)	386 (36)^{b}	337 (39)	----------
SVL (mm)	17.6 (0.8)$^{a}_{x}$	13.9 (0.8)$_{y}$	----------	18.5 (0.8)$^{a}_{x}$	----------	15.9 (0.7)$_{y}$	15.7 (0.4)^{b}	14.7 (0.5)	----------
HLL (mm)	20.6 (0.9)	14.7 (0)	----------	19.1 (0.2)	----------	17.5 (0.8)	18.6 (0.4)	19.1 (0.6)	----------
Wgt/SVL	32 (1.9)	23.7 (1.9)	----------	33.2 (3.5)	----------	32.5 (2.9)	24.2 (1.5)	22.4 (1.8)	----------
HLL/SVL	1.15 (0.02)^{a}	1.15 (0.07)	----------	1.03 (0.02)$^{b}_{x}$	----------	1.1 (0.02)$_{y}$	1.19 (0.02)$^{a}_{x}$	1.2 (0.01)$_{y}$	----------

Table 2.4. Morphometric parameters measured in laboratory-reared, caged, and wild individuals from each site and time point. Values are means (± se), except for stage & FLE, which are median and percentage values respectively. Letters denote significant differences found within each time point for the various endpoints measured. a/b = differences between laboratory-reared individuals from each site at each time point. x/y/z = differences between laboratory-reared, caged, and wild individuals within each site at each time point. Absence of a letter following a value indicates no significant differences were observed

2.4.3.4 Thyroidal Analysis

The thyroids are paired glands located in the neck of the amphibian and consist of numerous follicles, surrounded by epithelial cells, and filled with colloid (Figure 2.18, A-J). A clear distinction can be observed between the thyroids of prometamorphic and climax tadpoles taken from time point 2 (Figure 2.18, A-D), and those taken from metamorphs (Figure 2.18, E-J). The thyroids of tadpoles are larger, and composed of more follicles of different sizes (Figure 2.18, A & C), compared to those from metamorphs (Figure 2.18, E-J). The epithelial cells are elongated in tadpoles (hypertrophy), indicating stimulation by thyroid stimulating hormone (Figure 2.18, B, D), whereas they are cuboidal in metamorphs, indicating reduced stimulation (Figure 2.18, F, H, J). In addition, in some specimens there was more than one layer of epithelial cells for each follicle (hyperplasia), also indicating increased glandular activity (Figure 2.18, A-J). The colloid in these photos is of good quality and no depletion can be observed, as would be expected in un-impacted individuals. However, colloid degeneration was observed in some individuals (Figure 2.19, A, B, C, D), which included bubbles near to the epithelial cells in minimal cases (Figure 2.19, A), with those bubbles becoming larger in moderate cases (Figure 2.19, B), leading to increased colloid depletion in severe cases (Figure 2.19, C), and and complete loss of the colloid from some follicles in very severe cases (Figure 2.19, D). These changes to the colloid were not necessarily linked to changes in epithelial cells (Figure 2.19, A & D), although stimulation of epithelial cells in conjunction with colloid depletion was also observed (Figure 2.19, B & C). Similarly, increased activity of the thyroid was not always associated with changes in the colloid (Figure 2.19, E), although in some cases both occurred simultaneously (Figure 2.19, F). In some individuals there also appeared to by a high proportion of epithelial cells for each follicle (Figure 2.19, G & H). Occasionally, eosinophilic cells were observed in the thyroid (Figure 2.19, F), which may be connective tissue , although it is unknown why it was found in a few specimens.

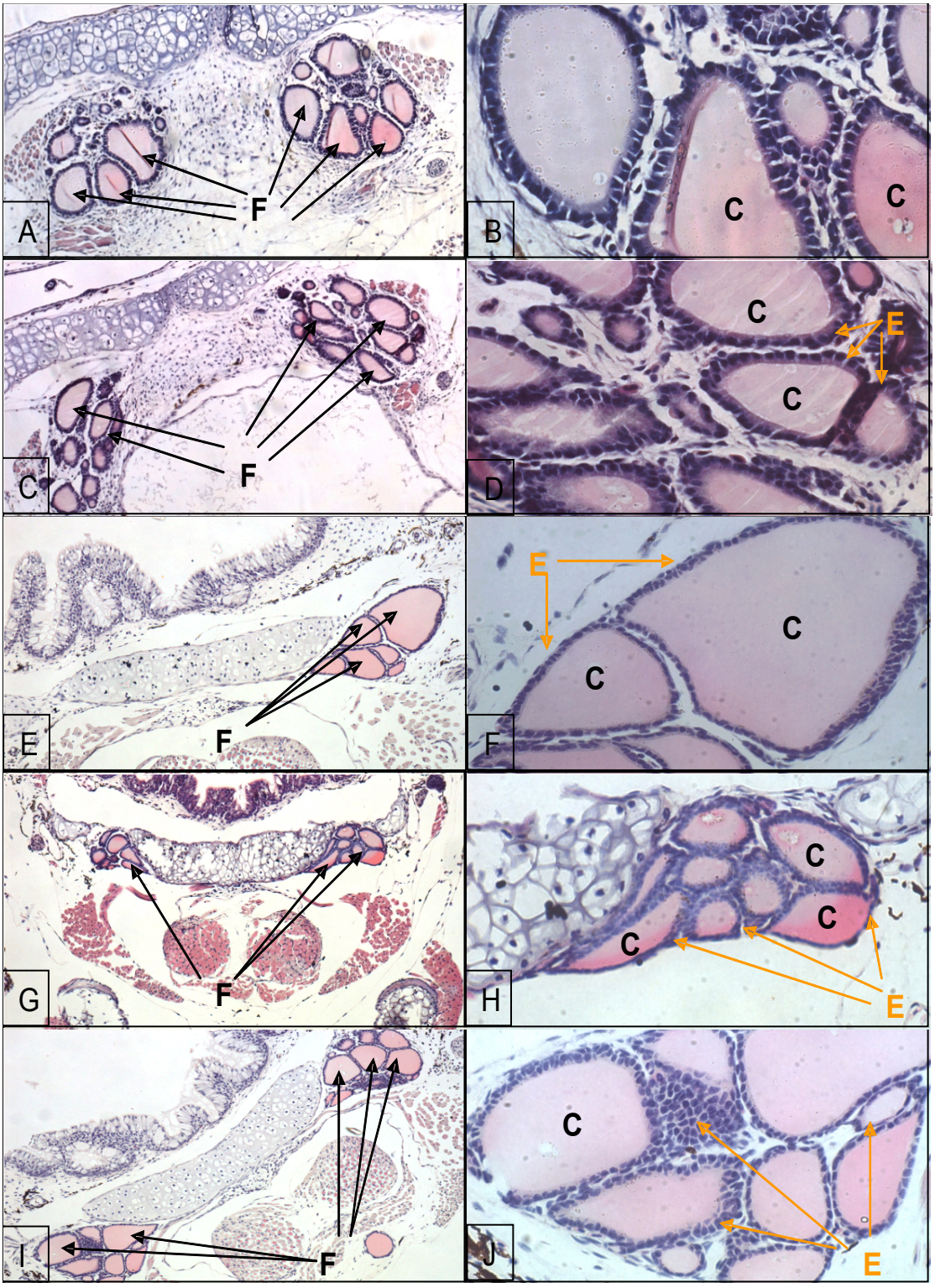


Figure 2.18. Histological sections showing thyroidal development in Bufo tadpoles and metamorphs, photos taken at x 100 (Panels A, C, E, G, I) and x 400 (Panels B, D, F, H, I) magnification. Panels A & B represent the thyroid of tadpoles in the prometamorphic stage of development, and panels C & D tadpoles in the climax stage of development. Panels E & F represent metamorphs from time point 3, G & H from time point 4, and I & J from time point 5. F = follicle, E = epithelial cell, C = colloid.

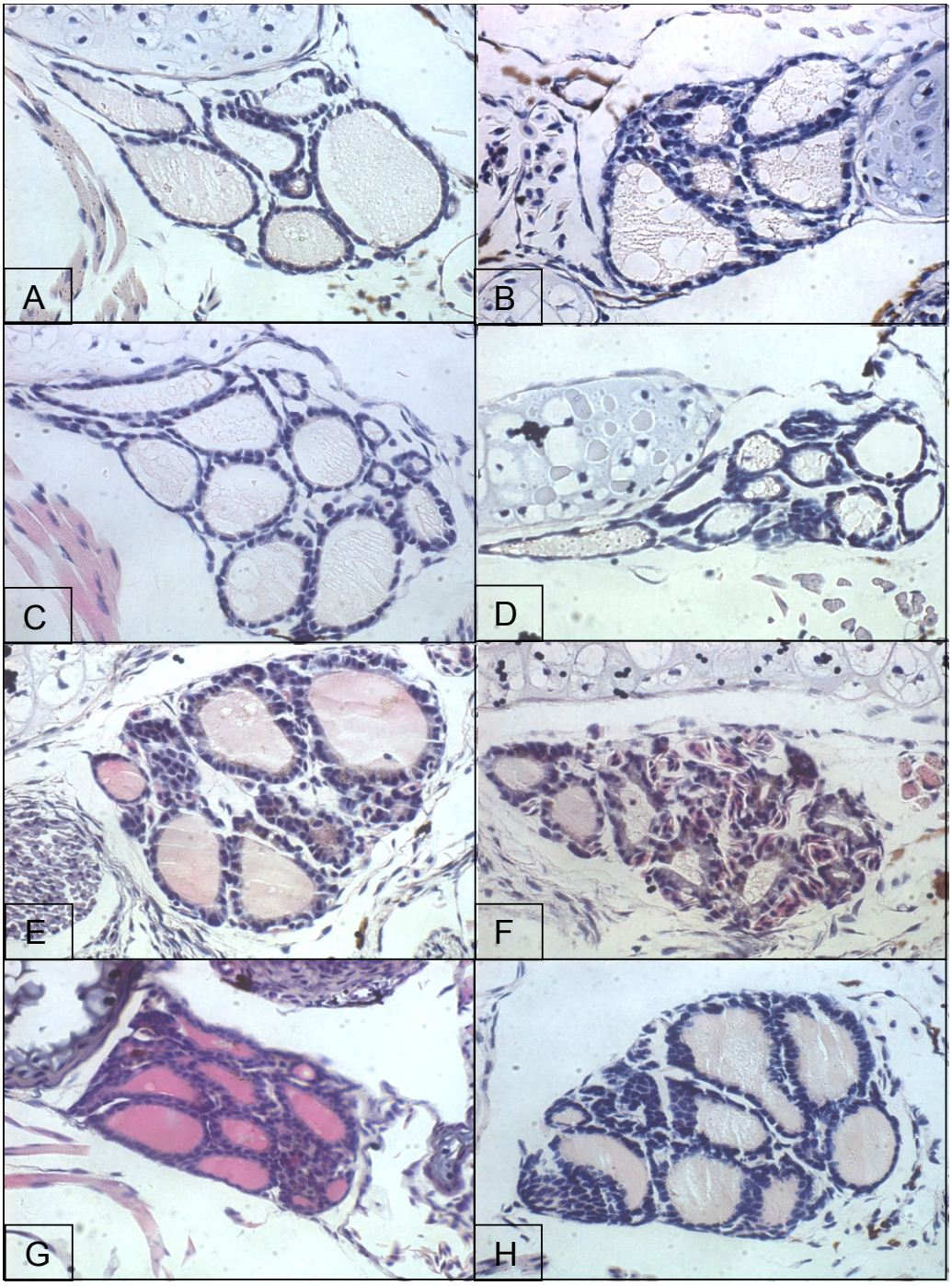


Figure 2.19. Examples of thyroidal abnormalities in Bufo tadpoles and metamorphs. All photos taken at x 400 magnification. Panels A-D show colloid degeneration ranging from minimal (Panel A) to very severe (Panel D). Panels E & F show hypertrophy, and G & H show hyperplasia. Panel F also shows presence of unknown eosinophilic cells in the thyroid. Panel A = caged specimen from PYL (TP 3); Panel B = wild caught specimen from YT (TP 4); Panel C = caged specimen from 20' (TP 5); Panel D = laboratory-reared specimen from YT (TP 4); Panel E = wild caught specimen from PYL (TP 3); Panel F = laboratory-reared specimen from 20' (TP 3); Panel G = wild caught specimen from PYL (TP 5); Panel H = laboratory-reared specimen from 20' (TP 3).

The ThSI increased up to time point 3, and then decreased at TPs 4 & 5, and was similar between sites in laboratory-reared individuals (Figure 2.20, A). At all sites, the ThSI was smaller in caged and wild caught individuals compared to laboratory-reared individuals, in at least one time point. At YT (Figure 2.20, B), the ThSI of prometamorphic tadpoles were significantly smaller in caged (Dunnett's $p = 0.011$) and wild caught (Dunnett's $p = 0.017$) individuals. The ThSI of wild caught individuals at TP 4 was significantly greater than laboratory-reared individuals (Dunnett's $p = 0.04$). A similar trend was observed for the caged individuals at this time point, but it could not be verified statistically as the thyroid of only one individual was analysed (one was lost during processing, Figure 2.20, B). At PYL (Figure 2.20, C), ThSI of caged prometamorphic individuals was smaller than laboratory-reared individuals (Dunnett's $p < 0.0001$). Other comparisons were difficult due to the slow development at this site, which resulted in all cage and wild caught individuals to be at climax stage at TP 3. At 20' (Figure 2.20, D), the ThSI of caged and wild-caught individuals was always smaller than laboratory-reared individuals (Dunnett's $p < 0.001$), except at TP 5. At all sites, prometamorphic and climax groups from caged and wild caught individuals were represented by tadpoles at an earlier stage of developmental stage, than groups comprised of laboratory-reared individuals (data not shown). Therefore, to compare caged and wild caught individuals to their representative laboratory-reared counterparts, stage was plotted against ThSI for premetamorphic, prometamorphic, and climax tadpoles, and tadpoles/metamorphs from TP 3 (Figure 2.21). ThSI was not significantly affected by treatment (laboratory-reared, caged, or wild caught) at YT (Figure 2.21, A, ANCOVA $p = 0.33$) or PYL (Figure 2.21, B, ANCOVA $p = 0.43$), but was significantly affected at 20' (Figure 2.21, C, ANCOVA $p < 0.0001$). In addition, ThSI was significantly affected by developmental stage at YT (Figure 2.21, A, ANCOVA $p < 0.0001$) and 20' (Figure 2.21, C, $p < 0.0001$), but not at PYL (Figure 2.21, B, ANCOVA $p = 0.27$).

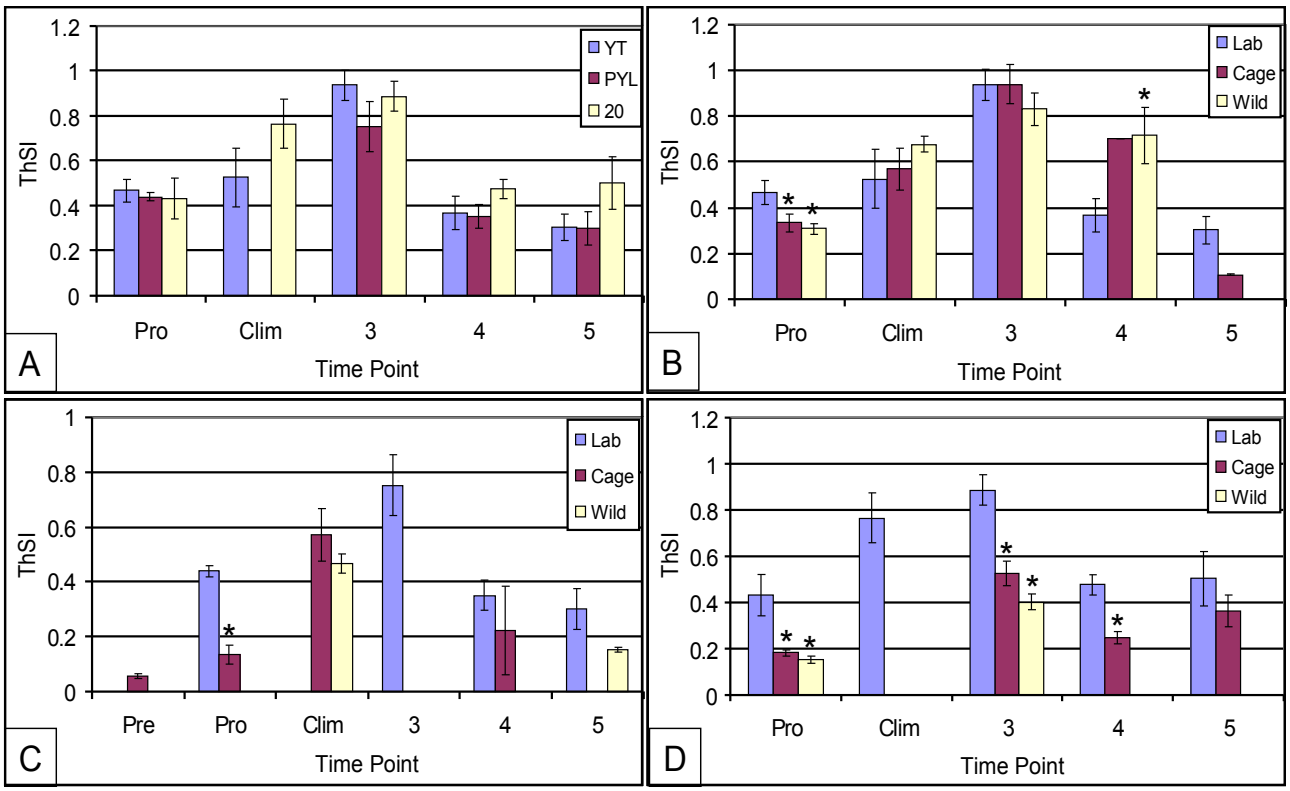


Figure 2.20. ThSI of laboratory-reared, caged and wild-caught individuals of premetamorphic (pre), prometamorphic (pro), and climax (clim) stage tadpoles, and metamorphs sampled at TP 3, 4, and 5. Panel A = laboratory-reared comparison, Panel B = individuals from YT, Panel C = individuals from PYL, Panel D = individuals from 20'. * denotes significant differences of caged or wild-caught individuals compared to their laboratory-reared counterparts. Values are mean ± SE, for n values see Table 2.4. Missing bars indicate an absence of sampled individuals, except PYL (Panel C), where ThSI of clim tadpoles were obtained at TP3, so no values for TP3 were available.

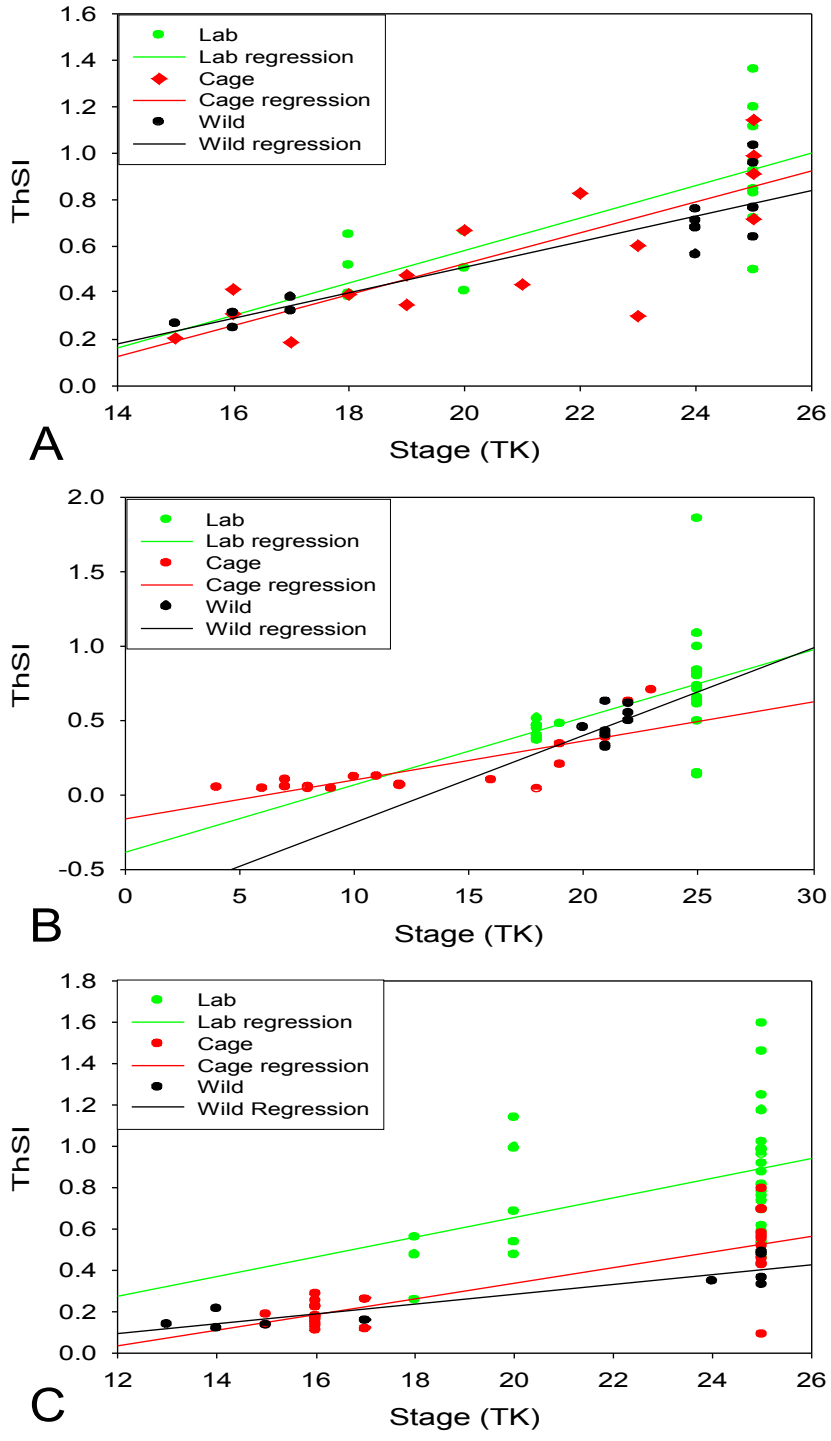


Figure 2.21. ThSI of laboratory-reared, caged and wild-caught individuals plotted against developmental stage (TK = Taylor-Kollros). Premetamorphic, prometamorphic, and climax tadpoles, and tadpoles/metamorphs from TP 3 were plotted. Each point represents one individual. Panel A = YT, Panel B = PYL, Panel C = 20'. Lines are linear regressions; YT: $r^2 = 0.68$, $p < 0.0001$ (Lab: $r^2 = 0.58$, $p = 0.0002$; Cage: $r^2 = 0.62$, $p = 0.0002$; Wild: $r^2 = 0.82$, $p < 0.0001$); PYL: $r^2 = 0.52$, $p < 0.0001$ (Lab: $r^2 = 0.14$, $p = 0.07$; Cage: $r^2 = 0.63$, $p < 0.0001$; Wild: $r^2 = 0.17$, $p = 0.24$); 20': $r^2 = 0.67$, $p < 0.0001$ (Lab: $r^2 = 0.16$, $p = 0.03$; Cage: $r^2 = 0.67$, $p < 0.0001$; Wild: $r^2 = 0.82$, $p = 0.0003$)

Activity of the thyroid gland was similar between sites of laboratory-reared individuals from YT, PYL and 20', and the general trend was a decrease in activity over time (Figure 2.22, A). Activity was decreased in caged and wild caught individuals from YT in prometamorphic tadpoles (Figure 2.22, B, Wilcoxon p = 0.0059), no effects were observed at PYL (Figure 2.22, C), and activity was decreased in caged individuals from 20' at time point 3 compared to laboratory-reared individuals (Figure 2.22, D, Wilcoxon p = 0.0023).

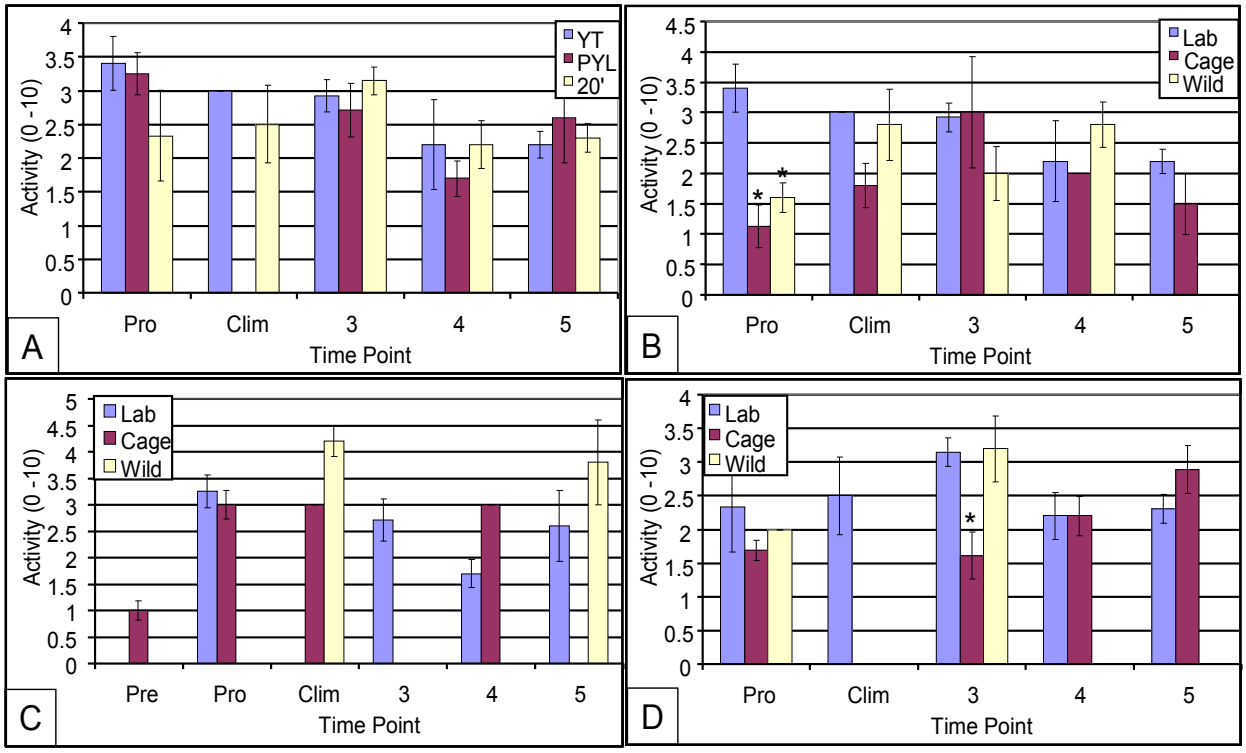


Figure 2.22. The activity of the thyroid gland in laboratory-reared, caged, and wild-caught individuals in premetamorphic (pre), prometamorphic (pro), and climax (clim) stage tadpoles, and metamorphs sampled at TP 3, 4, and 5. Panel A = laboratory-reared comparison, Panel B = individuals from YT, Panel C = individuals from PYL, Panel D = individuals from 20'. * denotes significant differences between caged or wild-caught individuals and their laboratory-reared counterparts. Values are mean ± SE, for n values see Table 2.4.

The quality of the colloid was similar between laboratory-reared individuals from each site (Figure 2.23, A). The colloid quality was worse (high value) in prometamorphic tadpoles from YT (Figure 2.23, B) and 20' (Figure 2.23, D) in caged and wild-caught individuals, though this difference was only significant at 20' (Wilcoxon p = 0.002). No difference was observed in individuals from PYL (Figure 2.23, C), or at later TP in any sites.

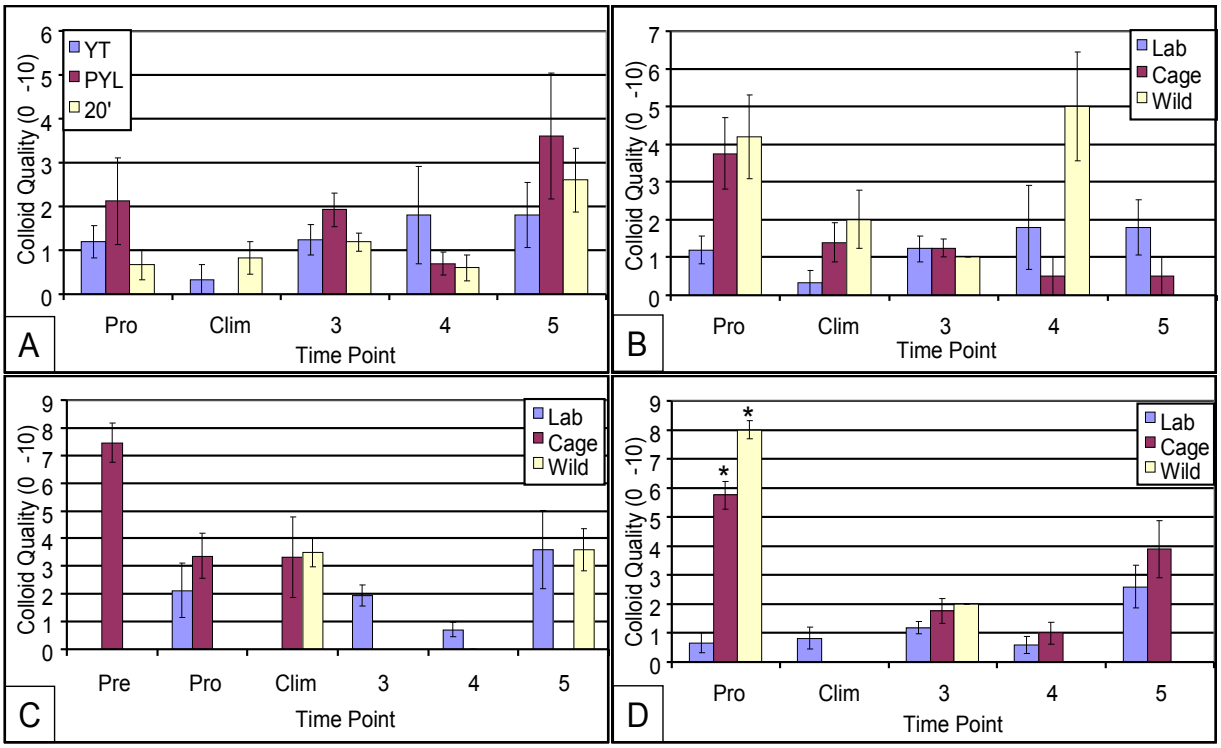


Figure 2.23. The colloid quality of the thyroid gland in laboratory-reared, caged and wild-caught individuals during premetamorphic (pre), prometamorphic (pro), and climax (clim) stage tadpoles, and metamorphs sampled at TP 3, 4, and 5 (0 = no disturbance to colloid, 10 = severely impacted colloid). Panel A = laboratory-reared comparison, Panel B = individuals from YT, Panel C = individuals from PYL, Panel D = individuals from 20'. * denotes significant differences of caged and wild-caught individuals compared to their laboratory-reared counterparts. Values are mean ± SE, for n values see Table 2.4.

2.4.3.5 Bidderian and Gonadal Development

Morphological differences in the shape of BO and bidderian oocytes, was observed between individuals (Figure 2.24, A-F). BOs contained both FGP and SGP oocytes (Figure 2.24 C,D), and had a layer of epithelial cells surrounding each oocyte (Figure 2.24, A-F). Multi-nucleate oocytes were often observed (Figure 2.24 A,B), and atretic oocytes were also observed occasionally (Figure 2.24, D).

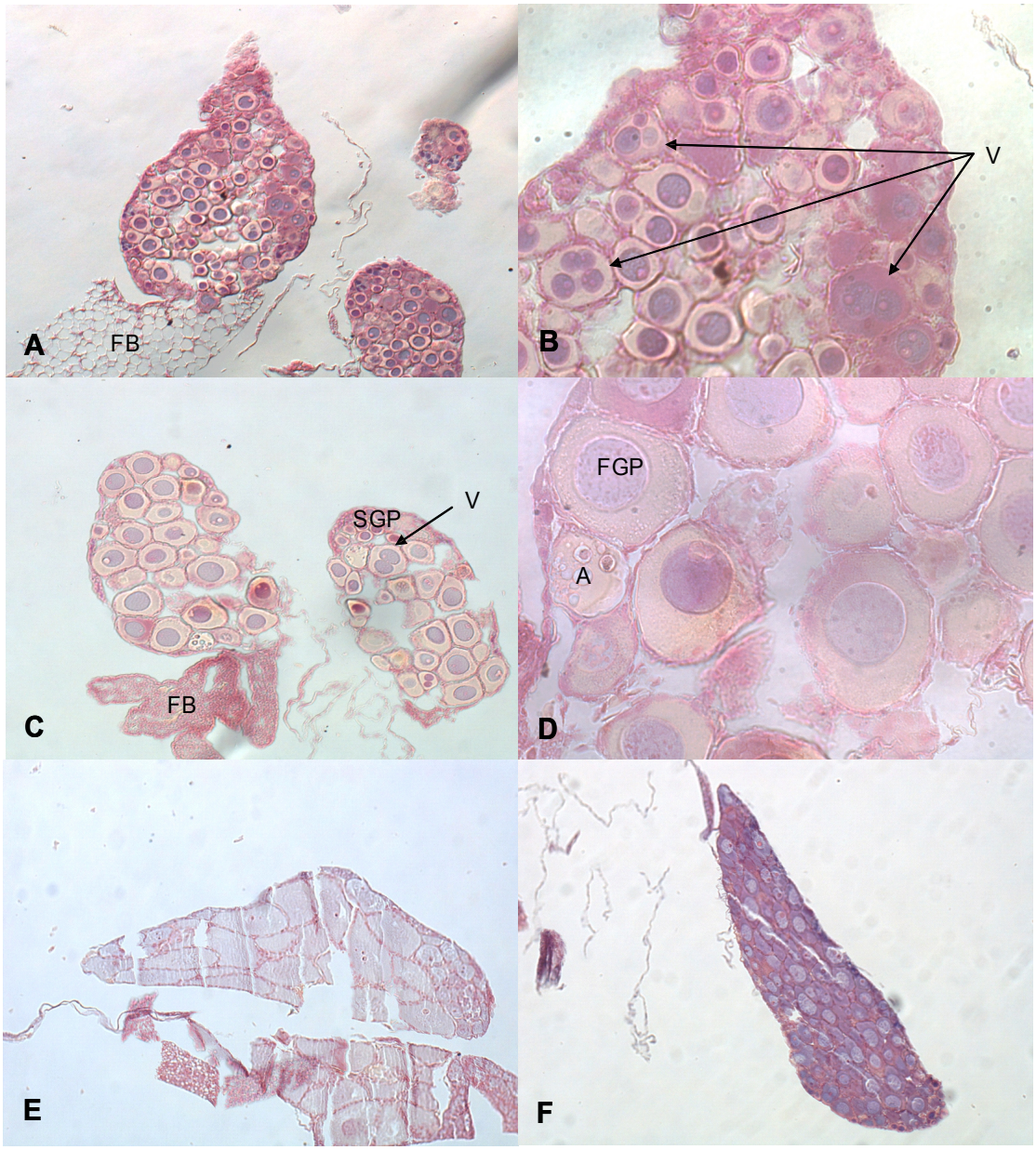


Figure 2.24. Features of BO observed in laboratory-reared individuals. Panel's A, C, E & F = x 100 magnification; Panels B & D = x 400 magnification. FB = fat body,

FGP = first growth phase oocyte, SGP = second growth phase oocyte, V = oocyte containing >1 nuclei, A = atretic oocyte.

Four sexes were characterised during histological analysis, undifferentiatied (Figure 2.25), female (Figure 2.26, 2.27), male (Figure 2.28) and intersex (Figure 2.28). Undifferentiated gonads were either, long and thin, but lacking in an ovarian cavity (Figure 2.25, A & B), or shorter but lacking testicular features (Figure 2.25 C & D). Early ovarian development is characterised by reduction of the medulla, resulting in formation of an ovarian cavity (Figure 2.26, A-F). In some individuals, diplotenic oocytes were observed before the ovarian cavity could be well characterised (Figure 2.26, A,B). In other individuals, a prominent ovarian cavity was observed, but germ cells were in oogonial cell nests (Figure 2.26, C-F). Oogonia gradually increase in size during development towards becoming oocytes (Figure 2.26, D,F). As the ovary develops, the ovarian cavity becomes more pronounced, and oocytes become larger (Figure 2.27, A-F). In addition, FGP and SGP oocytes were observed in the older metamorphs (Figure 2.27, C-F). Testicular development was more retarded than ovarian development and development did not proceed past the spermatogonial stage (Figure 2.28, A-D), even in individuals 6 weeks post metamorphosis (Figure 2.28, C,D). However, they were identified by medullary development, and the presence of smaller densely packed germ cells. The shape of the teste changed over time, and became shorter and rounder in older metamorphs (Figure 2.28, A-D), TOs were observed in some individuals (intersex), and several were often observed in one teste (Figure 2.28, E,F).

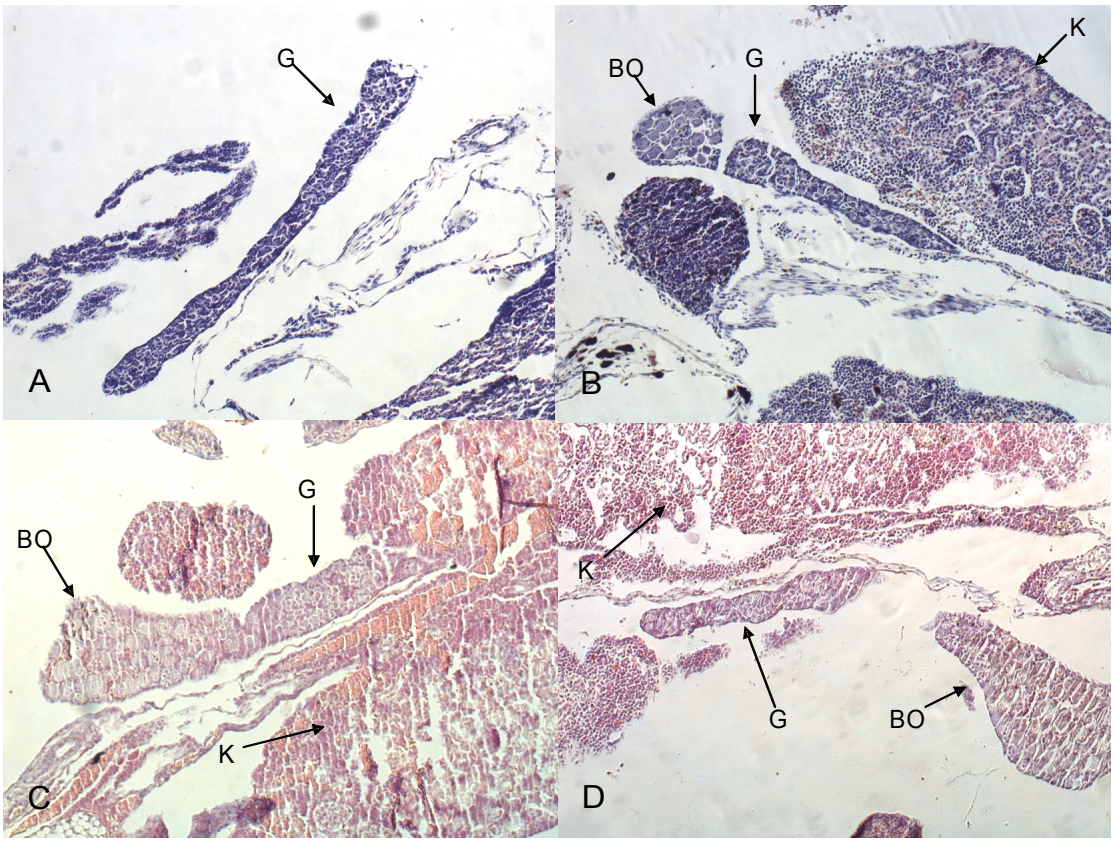


Figure 2.25. Undifferentiated gonads from young metamorphs, all taken at x 100 magnification. Some were long and thin, but lacking an ovarian cavity (Panel A,B) and others were shorter, but could not clearly be characterised as testes (Panel C,D). G = Gonad, BO = Bidder's organ, K = Kidney.

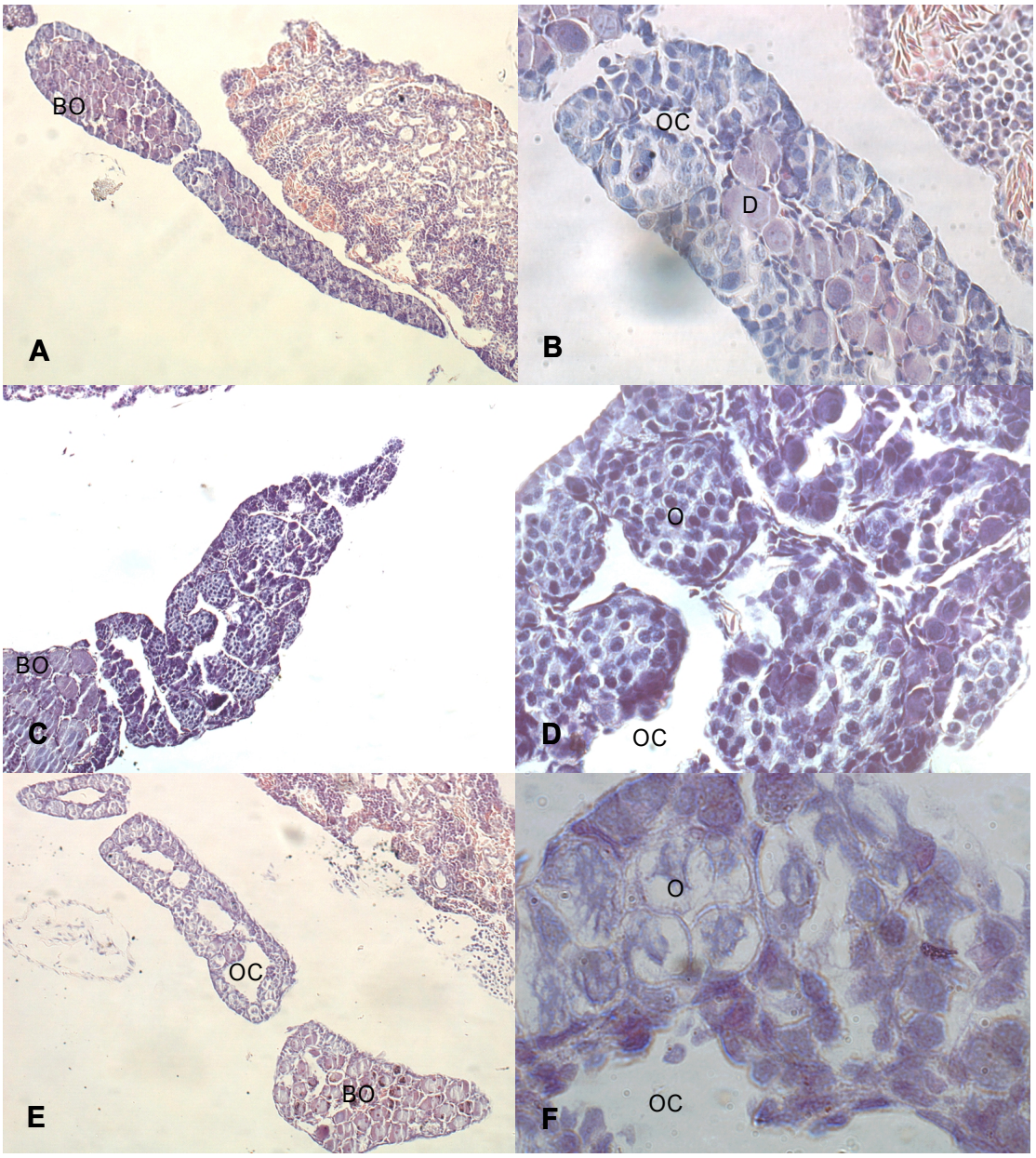


Figure 2.26. Early ovarian development in young metamorphs (all taken from TP 3). Panels A, C, & E = x 100 magnification; Panels B, D, & F = x 400 magnification. BO = Bidder's organ, OC = ovarian cavity, D = diplotenic oocyte, O = oogonial nest. See text for details.

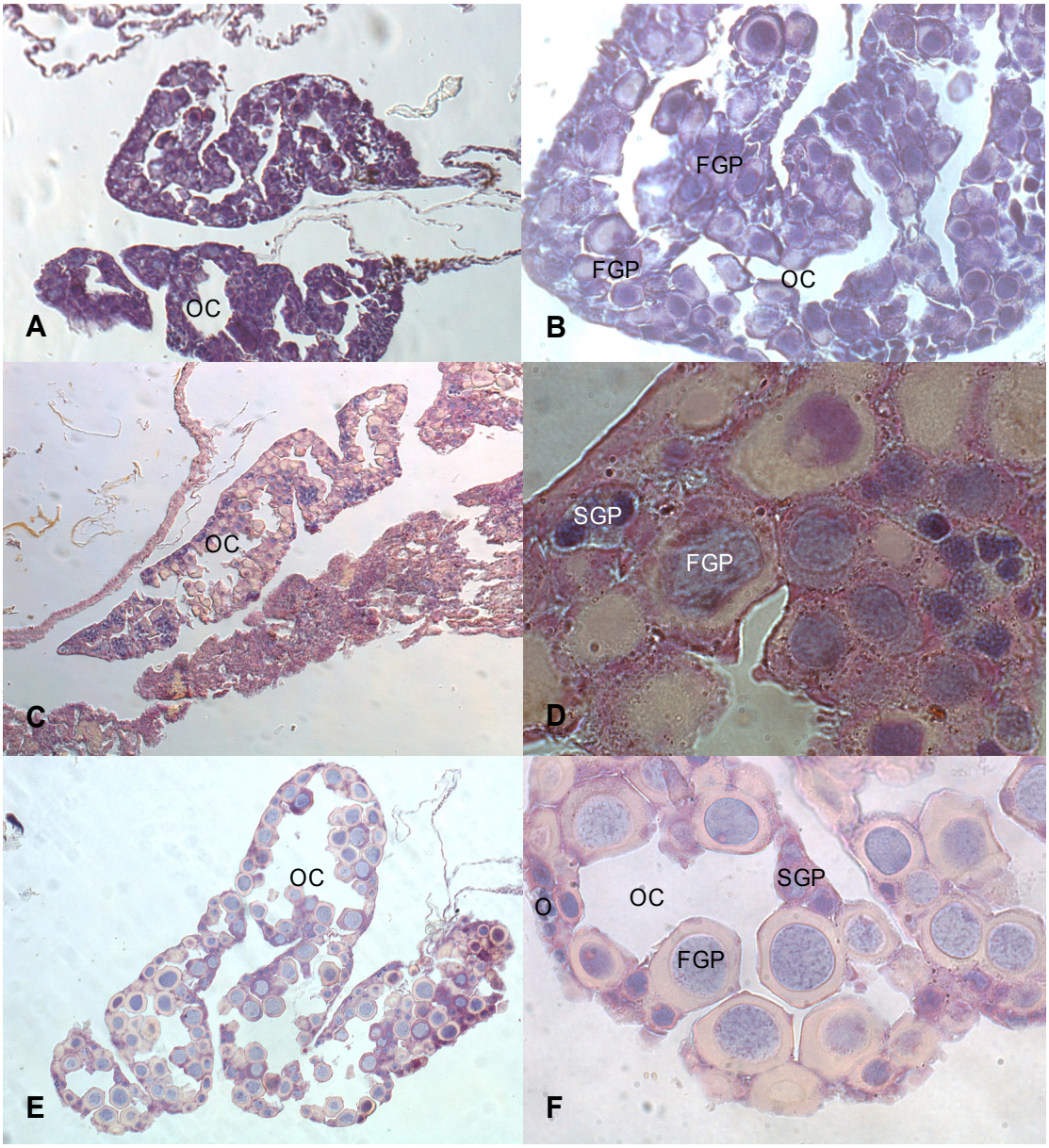


Figure 2.27. Ovaries from older metamorphs, showing ovarian development (time points (TP) 3, 4 & 5). Panel A & B = ovary from TP 3 taken at x 100 & x 400 magnification, respectively; Panel C & D = ovary from TP 4 taken at x 100 and x 400 magnification, respectively; Panel E & F = ovary from TP 5 taken at x 100 and x 400 magnification, respectively. OC = ovarian cavity, FGP = first growth phase oocyte, SGP = second growth phase oocyte, O = oogonia.

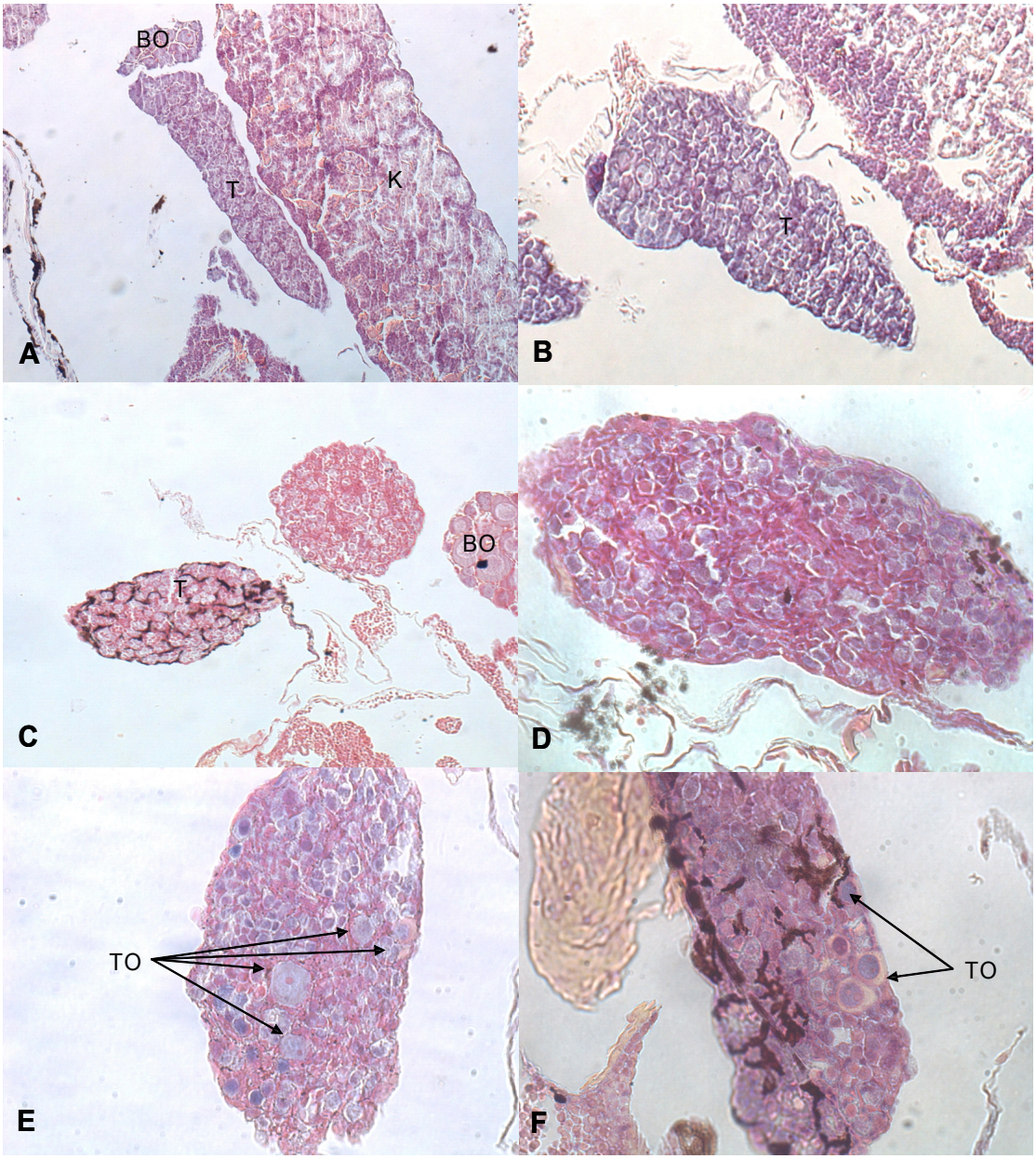


Figure 2.28. Testes from metamorphs showing testicular development and testicular oocytes from different time points (TP). Panel A = teste from TP 3 (x 100); Panel B = teste from TP 4 (x 100); Panel C = teste from TP 5 (x 100); Panel D = teste from TP 5 (x 400); Panel E = teste showing TO's (x 400); Panel F = teste showing TO's (x 400). BO = Bidder's organ, T = teste, TO = testicular oocyte.

Perturbations from development in laboratory-reared individuals (Figure 2.26-2.28), were observed in caged and wild individuals from each site. At YT, retardation of gonadal development was observed (Figure 2.29), at PYL unusual gonads were observed in wild-caught individuals (desribed below, Figure 2.30), and at 20' a high incidence of intersex individuals were identified (Figure 2.31). Caged and wild-caught individuals from YT were undifferentiated at TP4 (Figure 2.29, A & B, respectively), which was never observed at other sites. In addition, ovarian development was retarded (Figure 2.29, C-G). At TP3, oogonial cell nests could be observed in laboratory-reared individuals (Figure 2.29, C), whereas oogonia were less developed in caged individuals, and the only distinguishing feature was an ovarian cavity (Figure 2.29, D). At TP4, an ovary containing diplotenic oocytes was observed in laboratory-reared individuals (Figure 2.29, E), whereas only a few small oocytes and oogonial cell nests could be observed in caged individuals (Figure 2.29, F). At TP5, ovaries from both laboratory-reared and caged individuals were well developed, but ovaries and diplotenic oocytes were larger in laboratory-reared individuals (Figure 2.29, H), compared with the caged individual (Figure 2.29, G). Males could not be compared at YT because they were never observed in caged individuals. At PYL, ovarian and testicular development in laboratory-reared individuals followed the normal pattern (Figure 2.30, A-F). All caged individuals were undifferentiated at TP3, and those from TP4 were lost, therefore their development could not be compared. However, wild-caught individuals were retrieved at TP5, and gonads were unusual (Figure 2.30, G & H). The ovary contained small oocytes but no ovarian cavity (Figure 2.28, G), and the teste was also underdeveloped compared to laboratory-reared individuals from the same time point, and had a dense area of cells in the medulla (Figure 2.30, H). At 20', ovarian and testicular development was similar in laboratory-reared (Figure 2.31, A & C) and caged individuals (Figure 2.31, B & D), at TP3 (Figure 2.31, A & B) and TP4 (Figure 2.31, C & D). However, a high incidence of testicular oocytes was observed in both laboratory-reared and caged individuals (Figure 2.31, E-H). There seemed to be more TOs in laboratory-reared (Figure 2.31, E & G) than caged (Figure 2.31, F &H) individuals. No ovaries were observed at TP5 in caged individuals for comparison.

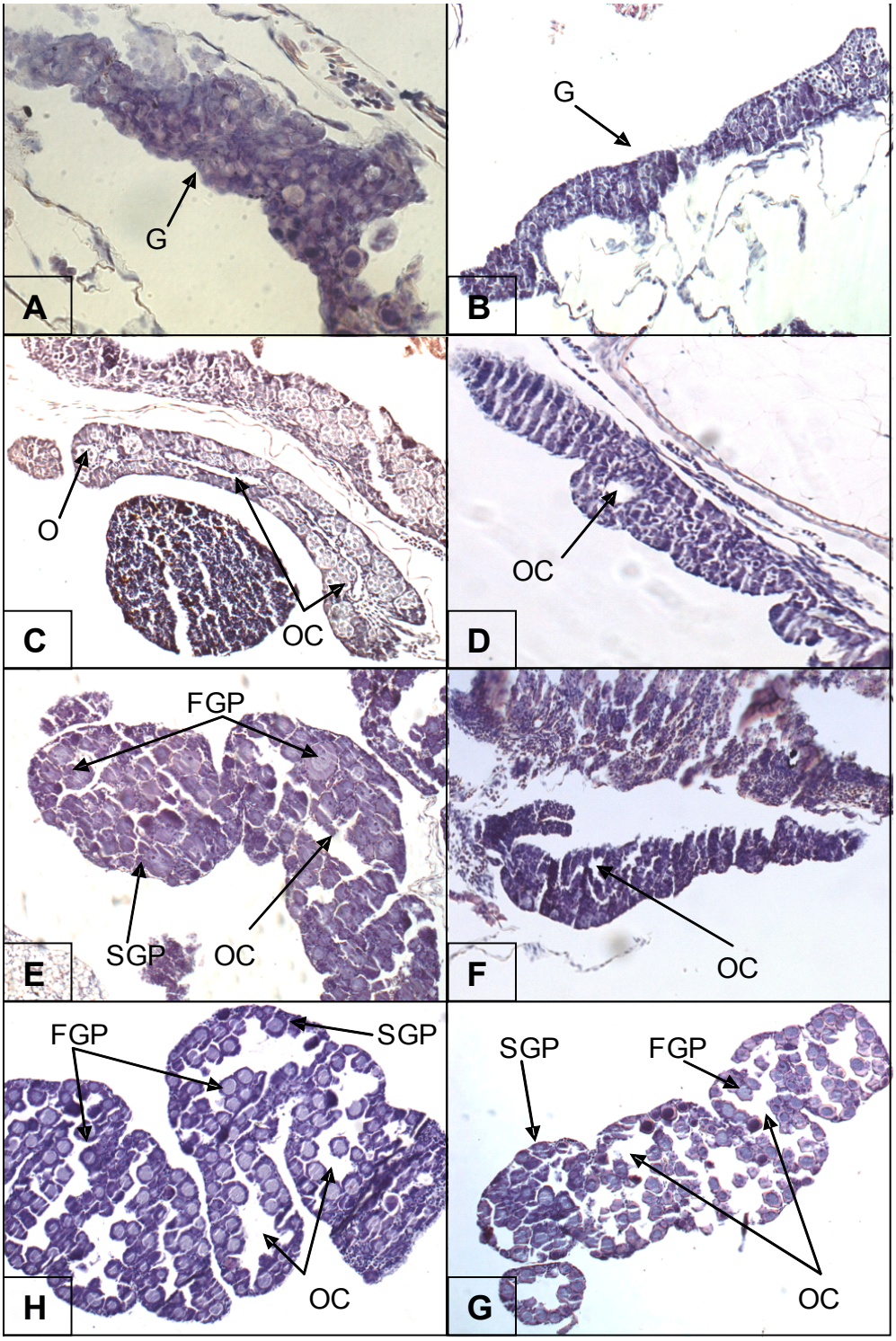


Figure 2.29. Gonadal development at YT at different time points (TP). Panel A = undifferentiated gonad from caged individual (x 400, TP4); Panel B = undifferentiated gonad from wild-caught individual (x 100 TP4); Panel C = immature ovary from laboratory-reared individual (x 100 TP3); Panel D = caged individuals (x 100 TP3); Panel E = ovary from laboratory-reared individual (x 100 TP4); Panel F = ovary from caged individual (x 100 TP4); Panel H = ovary from laboratory-reared individual (x 100 TP5); Panel G = ovary from caged individual (x 100 TP5). For list of abbreviations see Figure 2.27.

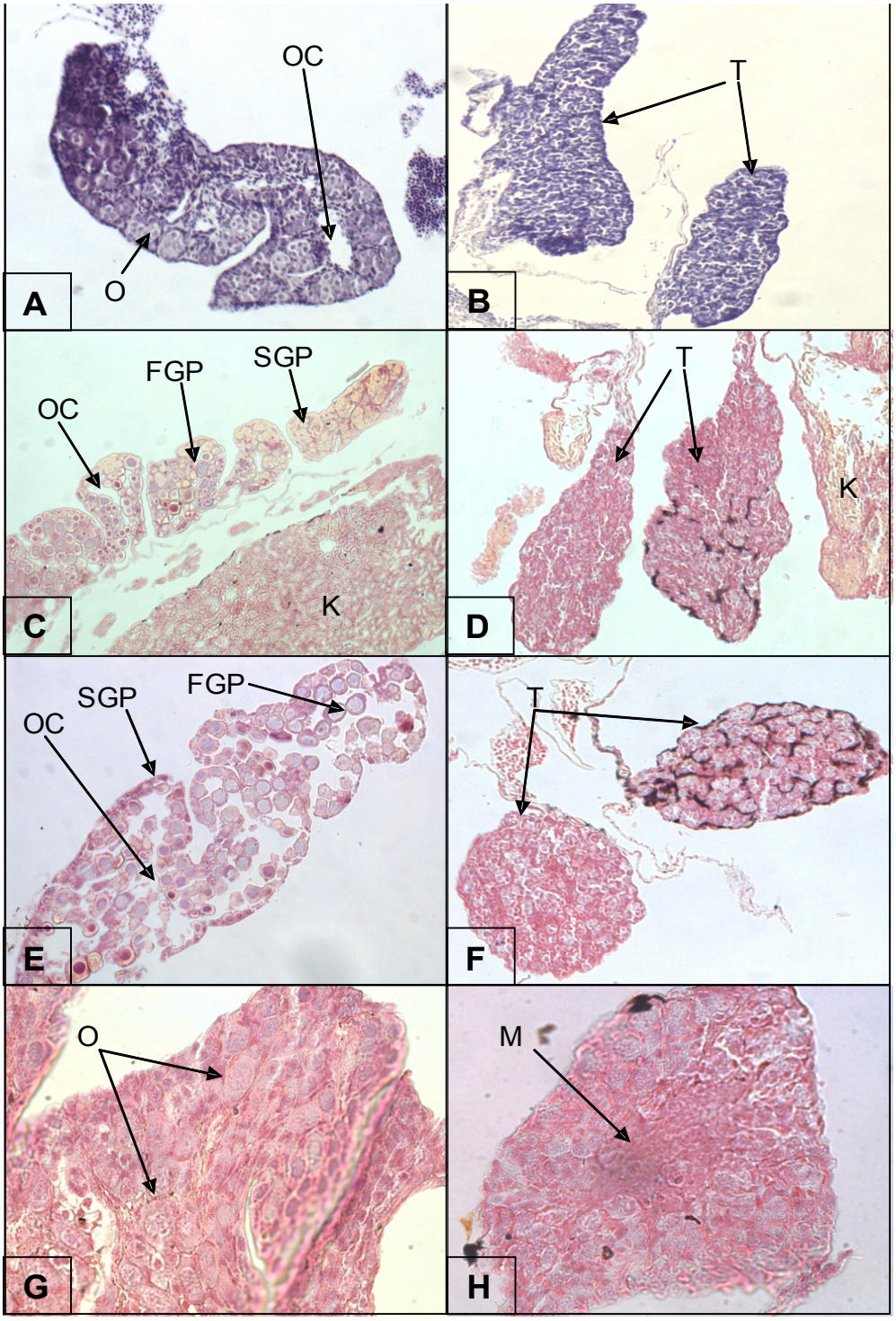
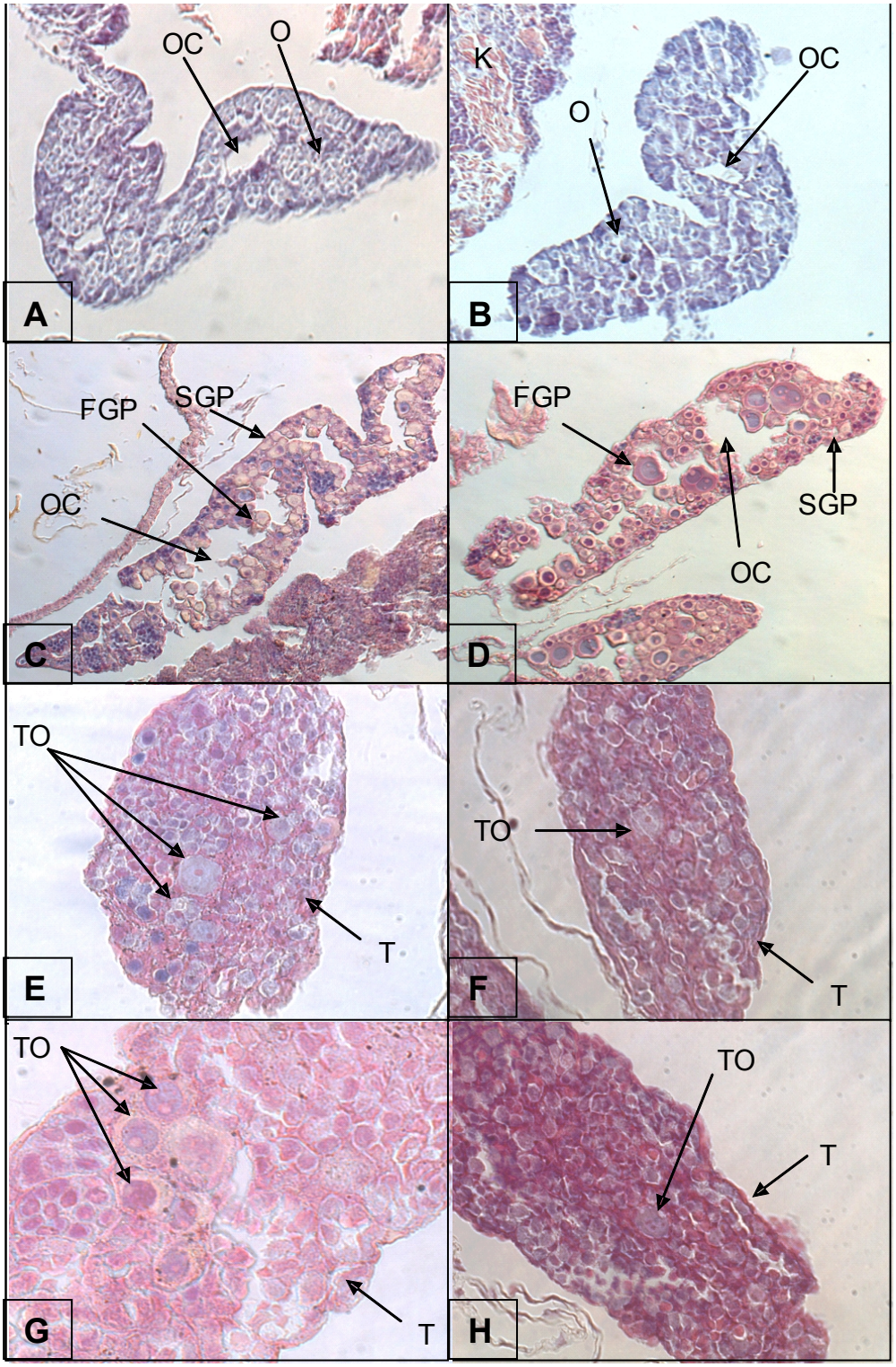


Figure 2.30. Gonadal development at PYL at different time points (TP). Panel A = immature ovary from laboratory-reared individual (TP3); Panel B = teste from laboratory-reared individual (TP3); Panel C = ovary from laboratory-reared individual (TP4); Panel D = teste from laboratory-reared individual (TP4); Panel E = ovary from laboratory-reared individual (TP5); Panel F = teste from laboratory-reared individual (TP5); Panel G = ovary from wild-caught individual (TP5); Panel H = teste from wild-caught individual (TP5). T = teste, M = medullary, for list of other abbreviations see Figure 2.27.



Figre 2.31. Gonadal development at 20' from different time points (TP). Panel A & B = immature ovary from laboratory-reared and caged individual (x 100, TP3), respectively; Panel C & D = ovary from laboratory-reared and caged individual (x 100, TP4), respectively; Panel E & F = intersex gonads from laboratory-reared and caged individuals (x 400, TP4), respectively; Panel G & H = intersex gonads from laboratory-reared and caged individuals (x 400, TP5), respectively. TO = testicular oocyte, for other abbreviations see Figure 2.27.

2.4.3.6 Sex Ratios

Using the histological sex characterisations described, *n* values of different sexes at each time point were determined for YT, PYL, and 20' (Table 2.5). In laboratory-reared individuals, sex ratios from pooled time points were significantly different between sites (Chi^2 p = 0.01), more undifferentiated individuals were observed at YT, and more intersex at 20' (Table 2.5). Comparison of sex ratios at YT and PYL from laboratory-reared, caged, and wild caught caught individuals were not analysed statistically due to the low *n* values at these sites. However, it can be observed that more undifferentiated individuals were observed in caged and wild caught individuals from YT and PYL, compared to their laboratory-reared counterparts (Table 2.5). Sex ratios changed over time in laboratory-reared individuals (pooled from different sites and split into time points, Chi^2 p = 0.0001), and the percentage of females remained at ~40 %, whereas the percentage of undifferentiated, intersex, and male individuals differed (data not shown). At 20', a high proportion of intersex was observed, both in laboratory-reared and caged individuals (Table 2.5). No difference in sex ratio was observed between laboratory-reared and caged individuals at this site (Chi^2 p = 0.5), but there was a significant change over time in caged individuals (Figure 2.32, A, Chi^2 p = 0.005), but not in laboratory-reared individuals (Figure 2.32, B, Chi^2 p = 0.05). The proportion of intersex and male individuals increased over time, while the proportion of females decreased at both sites, and no females were observed in caged individuals at TP 5 (Figure 2.32, B). If intersex individuals were classified as female, and undifferentiated individuals as male, the comparison between laboratory-reared and caged individuals at 20' was very similar (Table 2.5, Chi^2 p = 0.7).

		YT					PYL					20’				
	TP	**F**	**I**	**M**	**U**	**T**	**F**	**I**	**M**	**U**	**T**	**F**	**I**	**M**	**U**	**T**
L	**3**	5	0	1	7	**13**	6	1	4	2	**13**	12	2	4	3	**21**
	4	4	0	1	0	**5**	1	0	8	0	**9**	5	4	6	0	**15**
	5	1	2	2	0	**5**	3	0	2	0	**5**	3	4	3	0	**10**
Total		**10**	**2**	**4**	**7**	**23**	**10**	**1**	**14**	**2**	**27**	**20**	**10**	**13**	**3**	**46**
Raw %		**44**	**9**	**17**	**30**	**100**	**37**	**4**	**52**	**7**	**100**	**43**	**22**	**28**	**7**	**100**
Adj. %		**53**		**47**		**100**	**41**		**59**		**100**	**65**		**35**		**100**
C	**3**	2	0	0	2	**4**	0	0	0	9	**9**	8	2	1	2	**13**
	4	1	0	0	1	**2**	0	0	0	0	**0**	3	4	3	0	**10**
	5	2	0	0	0	**2**	0	0	0	0	**0**	0	4	5	0	**9**
Total		**5**	**0**	**0**	**3**	**8**	**0**	**0**	**0**	**9**	**9**	**11**	**10**	**9**	**2**	**32**
Raw %		**63**	**0**	**0**	**37**	**100**	**0**	**0**	**0**	**100**	**100**	**34**	**32**	**28**	**6**	**100**
Adj. %		**63**		**37**		**100**	**0**		**100**		**100**	**66**		**34**		**100**
W	**3**	0	0	0	9	**9**	0	0	1	9	**10**	3	0	1	1	**5**
	4	0	0	1	4	**5**	0	0	0	0	**0**	0	0	0	0	**0**
	5	0	0	0	0	**0**	1	0	4	0	**5**	0	0	0	0	**0**
Total		**0**	**0**	**1**	**13**	**14**	**1**	**0**	**5**	**9**	**15**	**3**	**0**	**1**	**1**	**5**
Raw %		**0**	**0**	**7**	**93**	**100**	**7**	**0**	**33**	**60**	**100**	**60**	**0**	**20**	**20**	**100**
Adj. %		**0**		**100**		**100**	**7**		**93**		**100**	**60**		**40**		**100**

Table 2.5. *N* values and percentages of each sex at YT, PYL and 20’, in laboratory-reared (L), caged (C), and wild-caught (W) individuals, sampled at time points (TP) 3, 4, & 5. Raw % = percentage of each sex; Adj. % = adjusted percentage (female + intersex or male + undifferentiated). T = total number, F = female, M = male, I = intersex, U = undifferentiated.

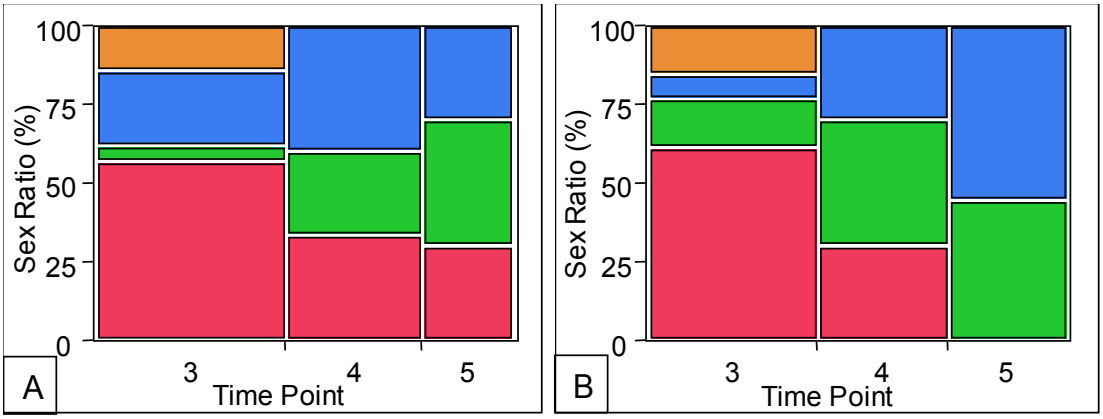


Figure 2.32. Sex ratio of individuals from 20’, at different time points (3, 4, or 5), in laboratory-reared (Panel A) and caged (Panel B) individuals. A significant difference over time was observed in caged individuals, but not laboratory-reared individuals. ■ = F, ■ = I, ■ = M, ■ = U.

2.4.3.7 Cytometric Analysis

2.4.3.7.1 Bidder’s Organ

Sex-specific: There were no sex-specific differences in BSI, number of bidderian oocytes, or size of bidderian FGP oocytes within sites in laboratory-reared

individuals, except BSI of undifferentiated individuals were larger than intersex individuals at 20' (*t*-test p = 0.037). Due to the minimal effect of sex on BSI, bidderian FGP number, or bidderian FGP size within sites, BO data was pooled between sexes for further analysis (data not shown).

Laboratory-reared: In laboratory-reared individuals, there were differences in BO measurements between sites. The BSI was larger in individuals from PYL & 20', compared to YT (Figure 2.33, non-significant), and the number of bidderian FGP oocytes was larger in PYL & 20', compared to YT (ANOVA p = 0.002, Figure 2.35, A). In contrast, the size of bidderian FGP oocytes was larger in individuals from YT compared to PYL & 20' (ANOVA p = 0.01, Figure 2.36, A).

Site comparison: At YT, BSI was significantly smaller in the caged individuals, compared to their laboratory-reared counterparts (Figure 2.33, Dunnett's $p = 0.001$), and this difference was observed at all three TP (Figure 2.34, ANOVA $p < 0.04$), however, this effect was not observed in wild-caught individuals. BSI was also reduced in both caged and wild caught individuals at TP 3 from PYL (ANOVA $p < 0.002$), and 20' (ANOVA $p < 0.0001$), but no effect was observed at other TPs (data not shown. The number of bidderian FGP oocytes from pooled TPs (3, 4, & 5) was significantly higher in caged and wild-caught individuals from YT compared to laboratory-reared individuals (Figure 2.35, A, Kruskal-Wallis p = 0.002, Dunnett's p = 0.0008 and 0.0004, respectively), and this difference became less pronounced over time (Figure 2.35, B). At PYL (Figure 2.35, C) and 20' (Figure 2.35, D), significantly more bidderian FGP oocytes were observed in caged compared to laboratory-reared individuals at TP3 only (Kruskal-Wallis p = 0.03, Dunnett's p = 0.037 & Kruskal-Wallis p = 0.005, Dunnett's p = 0.001). The size of bidderian FGP was smaller in caged and wild-caught individuals from YT (Figure 2.36, B, Dunnett's $p < 0.002$), and in caged individuals from 20' (Figure 2.36, D, Dunnett's p = 0.029), at TP3 compared to their laboratory-reared counterparts.

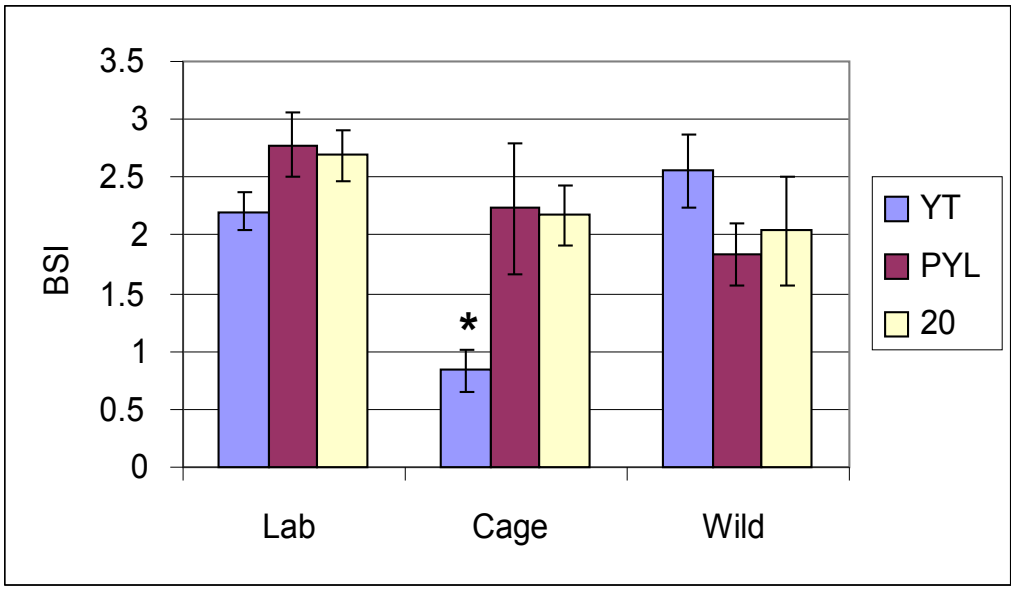


Figure 2.33. Pooled BSI values from each time point (3, 4, & 5) at YT, PYL and 20'. Lab = laboratory-reared, Cage = caged, Wild = wild caught. Values are mean ± se, for *n* values, see Table 2.5. * indicates statistically significant difference compared to their laboratory-reared counterpart

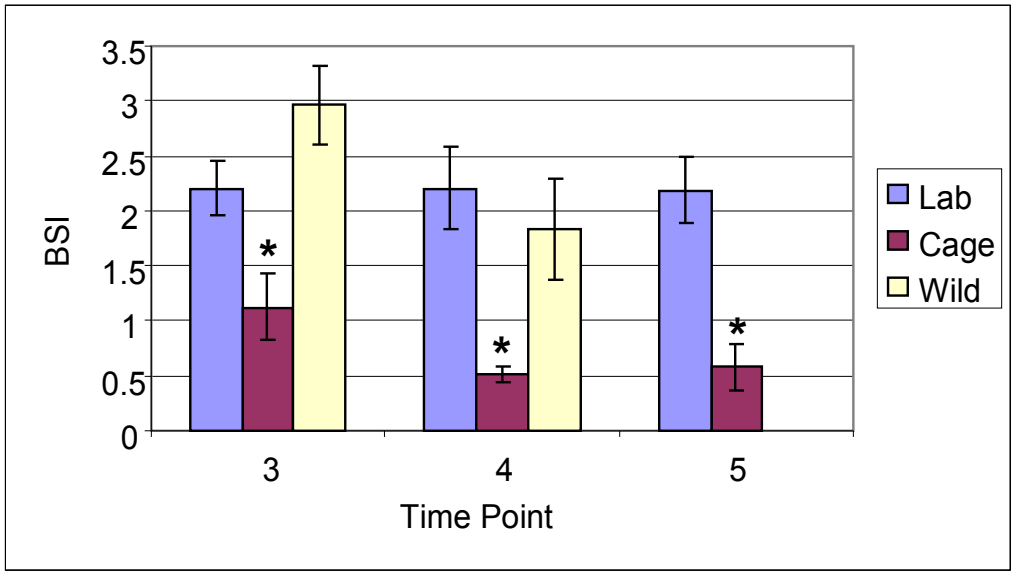


Figure 2.34. Change in BSI over time (TP 3, 4, & 5) at YT. Lab = laboratory-reared, Cage = caged, Wild = wild caught. Values are mean ± se, for *n* values, see Table 2.5. * indicates statistically significant difference compared to their laboratory-reared counterpart.

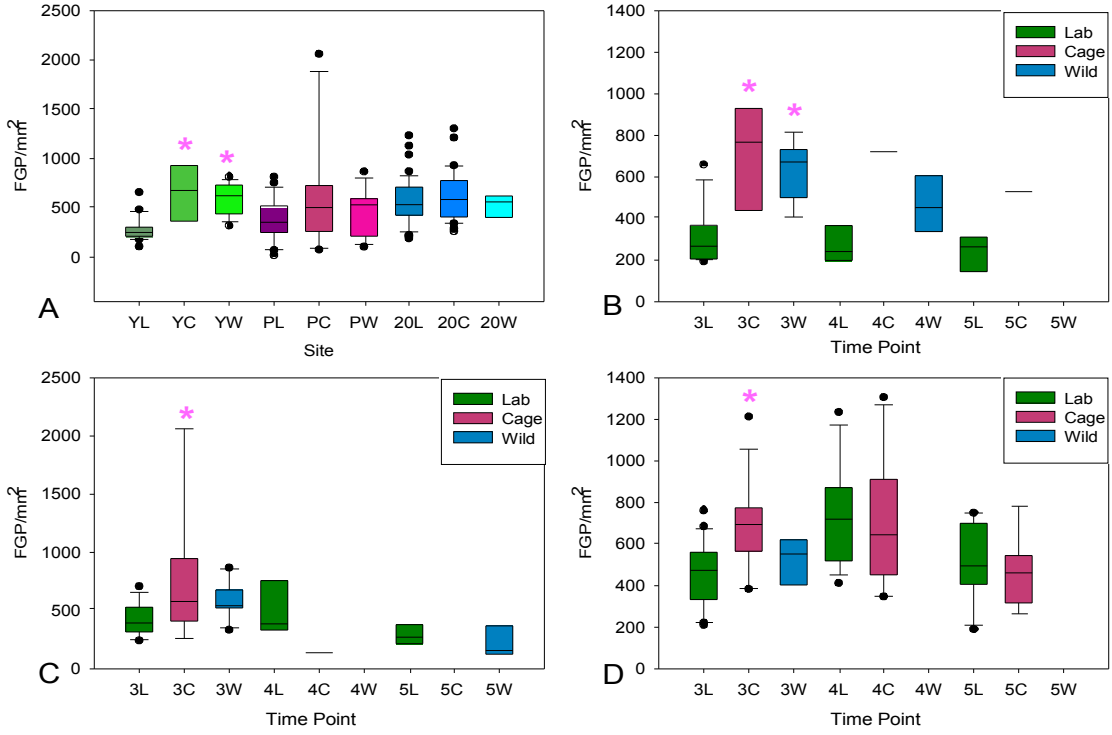


Figure 2.35. The number of bidderian FGP oocytes at YT (Y), PYL (P), and 20' (20), in laboratory-reared (L), caged (C), and wild caught (W) individuals at different time points (3, 4, or 5). Panel A = pooled timepoints; Panel B = YT; Panel C = PYL; Panel D = 20'. Values are medians (line in box), and interquartile ranges. For *n* values see Table 2.5. * indicates significant difference compared to their laboratory-reared counterpart.

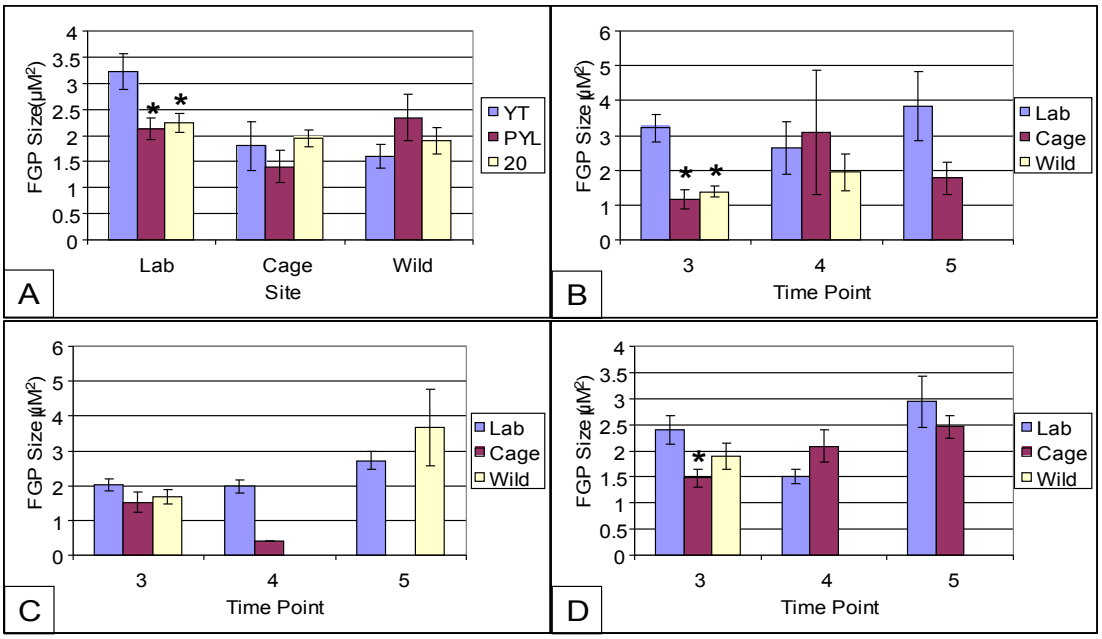


Figure 2.36. The size of bidderian FGP oocytes at YT, PYL, and 20', in laboratory-reared (L), caged (C), and wild caught (W) individuals. Panel A = laboratory-reared comparison (pooled time points 3, 4, & 5), Panel B = YT, Panel C = PYL, Panel D = 20'. Values are means ± se. For *n* values see Table 2.5. * indicates significant difference compared to their laboratory-reared counterpart.

2.4.3.7.2 Ovary

The ovary increased in size compared to the body weight over time at all sites, and expecially between TP 4 & 5 (Figure 2.37). Sex was not differentiated in caged individuals from PYL, and only one female was observed in wild caught individuals, therefore ovarian development at this site could not be analysed. The OSI was not affected by the BSI or environmental conditions (i.e. laboratory-reared or caged) at YT (ANCOVA p = 0.37 & 0.67), however, both variables affected the OSI at 20' (ANCOVA p = 0.07 & 0.05), and the OSI was smaller in caged individuals than laboratory-reared. The size of ovarian FGP oocytes in caged individuals were also 2-fold smaller than those from laboratory-reared individuals (Wilcoxon p = 0.016, data not shown).

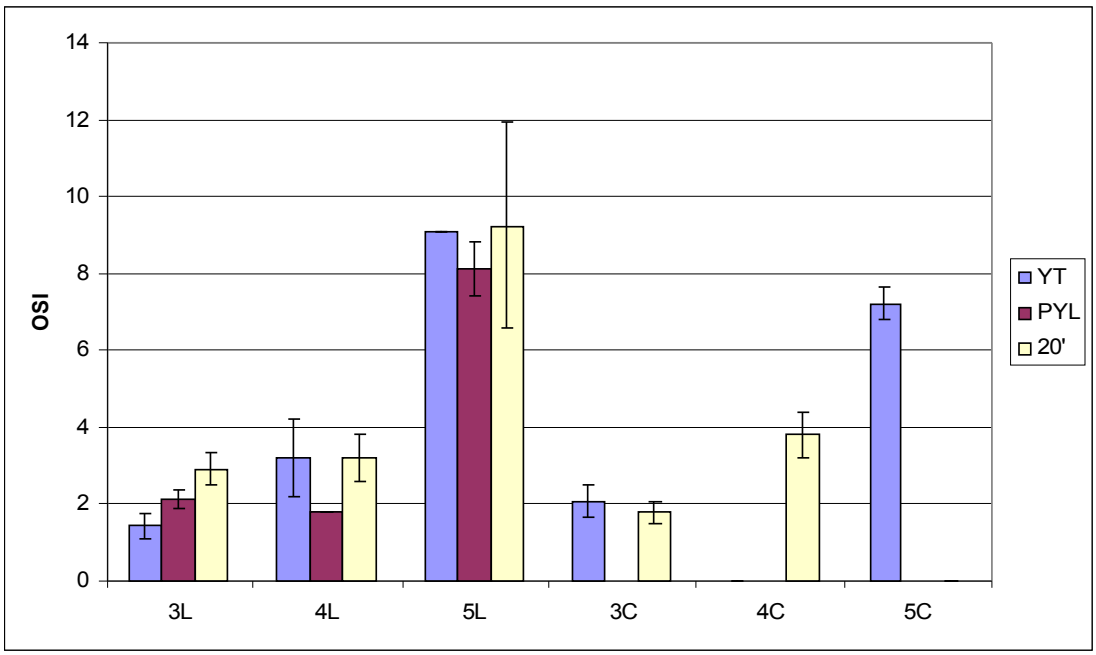


Figure 2.37. OSI at YT, PYL, and 20' from laboratory-reared (L) and caged (C) individuals at TP 3, 4, and 5. Values are mean ± se, for *n* values see Table 2.5.

2.4.3.7.3 Teste

No site specific differences were observed in TSI of laboratory-reared individuals (Figure 2.38, A). However, the trend differed over time, whereby, at YT, TSI stayed the same over time, at PYL, TSI decreased over time, and at 20', TSI decreased and then increased over time (Figure 2.38, A). TSI was significantly smaller in caged individuals compared to their laboratory-reared counterparts in pooled TPs (3, 4, & 5) from 20' (Figure 2.38, B, Kruskal-Wallis $p = 0.0006$, Dunnett's $p < 0.0001$). When separated by time point, this reduction was observed at time points 3 (non-significant) & 5 (ANOVA $p = 0.0045$), but not at time point 4 (Figure 2.38, C). TSI was affected by environmental condititions (i.e caged or laboratory-reared), and BSI (Figure 2.39, ANCOVA $p = 0.049$ & 0.0059), and was also less variable than BSI (Figure 2.39).

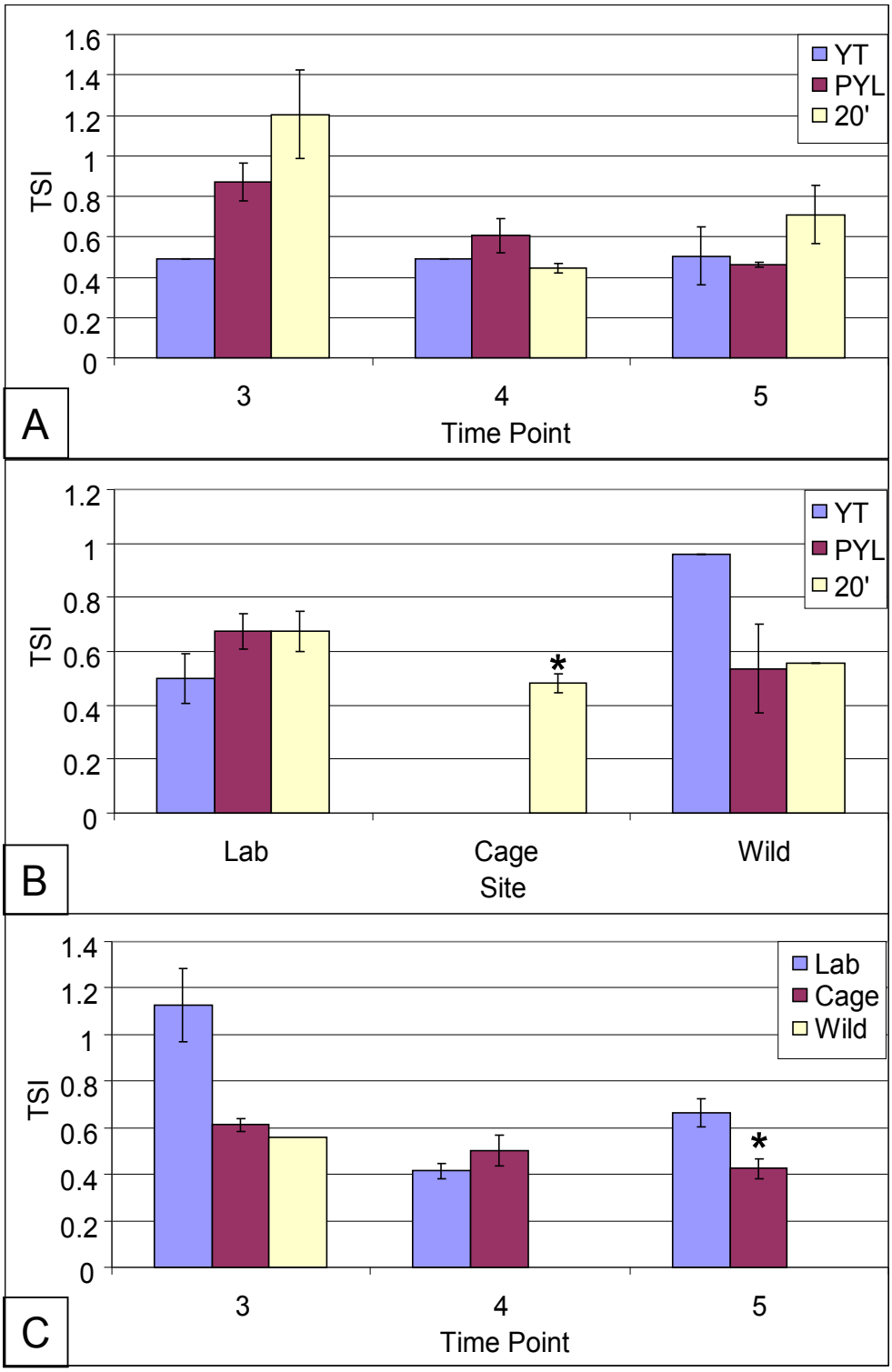


Figure 2.38. TSI at YT, PYL, and 20' from laboratory (Lab), caged (Cage), and wild-caught (Wild) individuals. Panel A = TSI of laboratory-reared individuals from YT, PYL, & 20' over time; Panel B = TSI of laboratory-reared, caged and wild-caught individuals at YT, PYL & 20' (pooled TPs 3, 4, & 5); Panel C = TSI of laboratory-reared, caged and wild-caught individuals at 20' over time. Values are mean ± se, for *n* values see Table 2.5. * indicates significant difference compared to laboratory-reared counterparts.

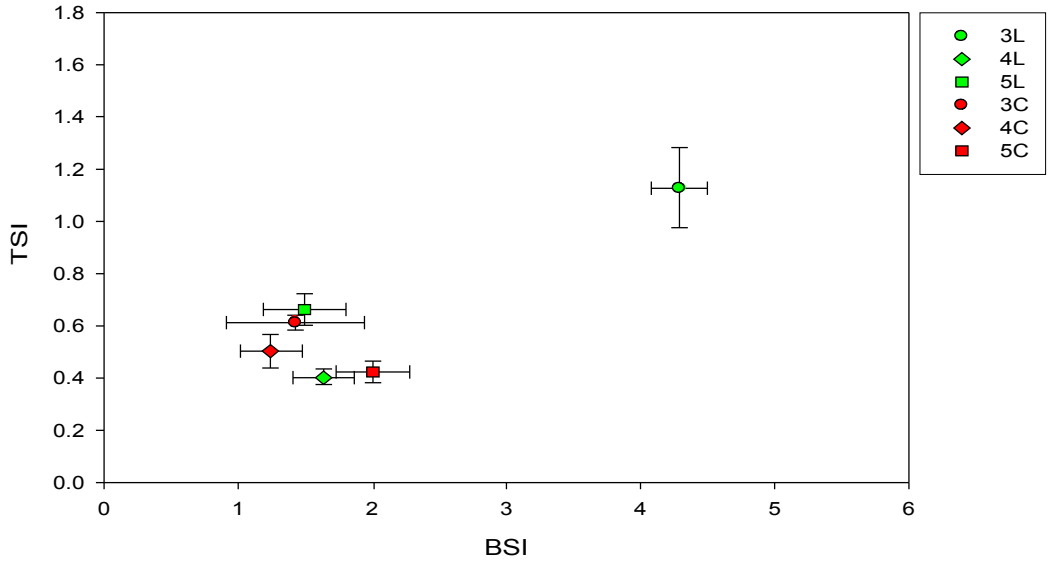


Figure 2.39. Relationship between TSI and BSI at 20', in laboratory-reared (L) and caged individuals (C) at TP 3, 4, and 5. TSI was relatively smaller compared to BSI in caged individuals, than in laboratory-reared individuals. Each data point represents mean ± bidirectional error, for *n* values see Table 2.5.

2.4.3.7.4 Intersexuality

Intersex individuals were rarely observed at YT (2 laboratory-reared individuals) or PYL (1 laboratory-reared individual), but were common at 20', therefore gonadal differentiation of intersex individuals was only analysed from the latter site. BSI and TSI values were the same in male or intersex individuals, from laboratory-reared or caged conditions (data not shown). There were no significant differences in the incidence of testicular oocytes in laboratory-reared and caged individuals (i.e. percentage of intersex individuals, see section 2.4.3.6), however, by TP5 58% of laboratory-reared and 100% of caged females were intersex (Figure 2.40). The severity of TOs appeared to be more pronounced in laboratory-reared individuals, but this difference was not statistically significant (Figure 2.40, Wilcoxon p = 0.098). The BSI decreased over time in laboratory-reared individuals, but increased over time in caged individuals (Figure 2.41, A & B). BSI was significantly affected by the interaction between site (i.e. laboratory-reared or caged) and TP, though by neither parameter alone in both male (Figure 2.41, A, ANCOVA p = 0.016) and intersex (Figure 2.41, B, ANCOVA p = 0.0004) individuals.

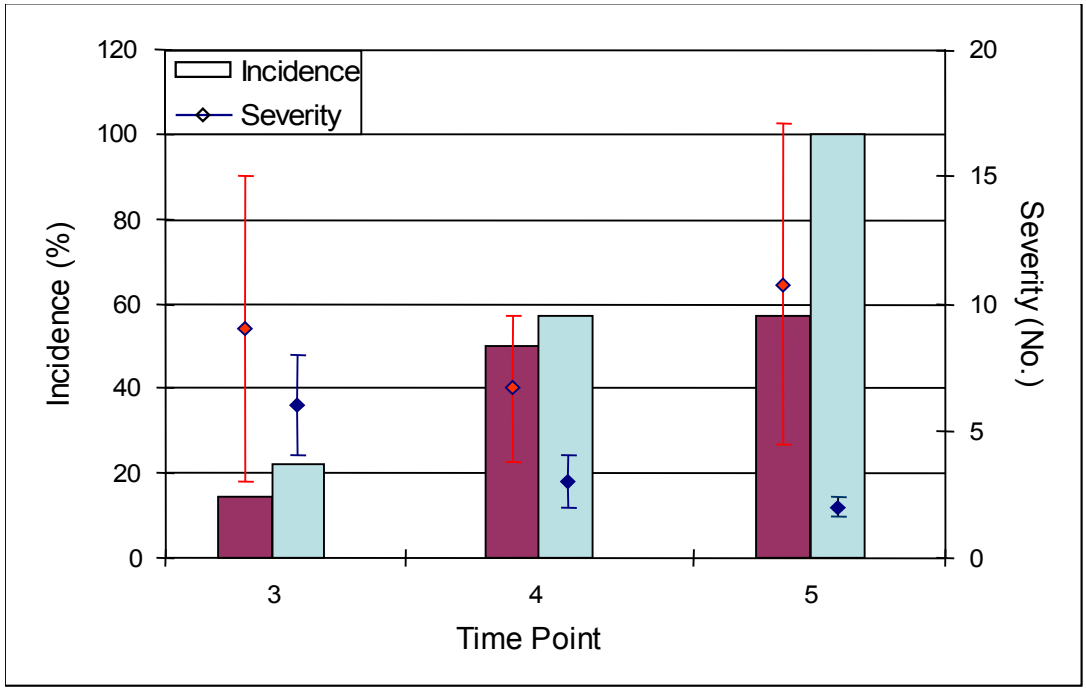


Figure 2.40. Incidence and severity of testicular oocytes observed at each time point, in laboratory-reared (red bar) and caged (blue bar) individuals at 20'. Incidence is expressed as a percentage of the total number of females observed, and severity as the number of testicular oocytes observed per intersex individual. In the latter case, values are mean ± se, see Table 2.5 for *n* values.

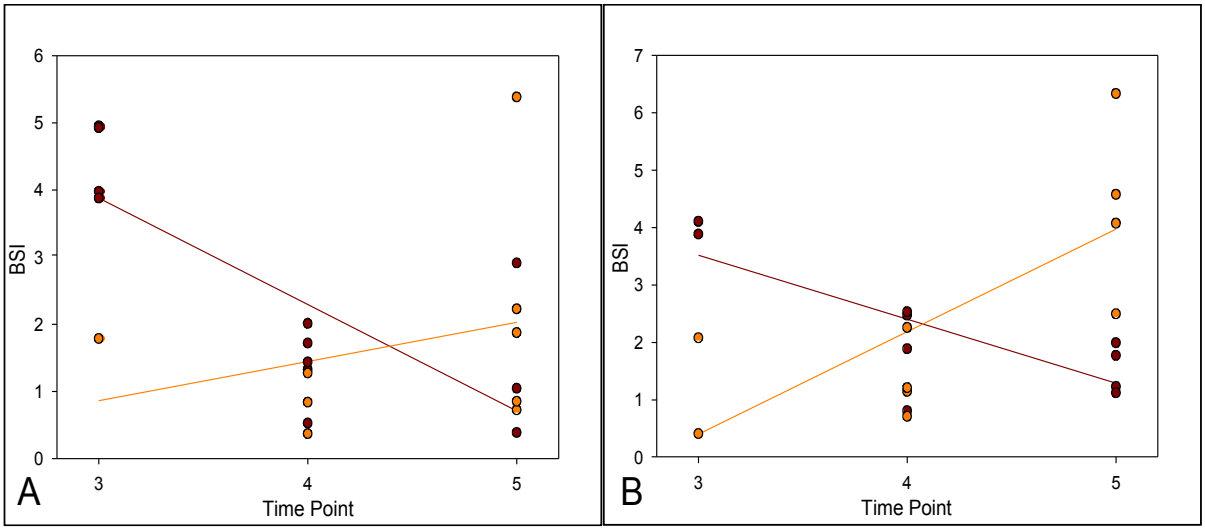


Figure 2.41. Change in BSI of male (Panel A) and intersex (Panel B) individuals over time (TP 3, 4, 5) in individuals from 20'. ● = laboratory-reared, ● = caged individuals). Each data point represents one individual. Lines are linear regressions, male: $r^2 = 0.42$, $p = 0.02$ (Lab: $r^2 = 0.58$, $p = 0.004$; Cage: $r^2 = 0.08$, $p = 0.46$); intersex: $r^2 = 0.57$, $p = 0.003$ (Lab: $r^2 = 0.63$, $p = 0.006$; Cage: $r^2 = 0.54$, $p = 0.016$).

2.4.4 Discussion

2.4.4.1 Mortality and morphology

Mortality over the experimental period was high in laboratory-reared and caged individuals from all sites, although this is not unusual for toad species. During the larval stage, mortality was less than 20 % in laboratory-reared individuals, which was lower than previous reports for *B.bufo* (37 %: Petrini and Zaccanti, 1998; 60 % Zaccanti *et al*., 1969), but slightly higher than mortality reported in *B.vulgaris formosus* . Mortality was very high during the post-larval stage, and no previous reports were available for comparison. However, in laboratory-reared individuals, this may be an artefact due to drowning, which occurred to greater or lesser extents in most tanks. In addition, many laboratory-reared individuals died when placed in compost filled vivariums, prior to changing the compost for sphagnum moss. These factors did not cause mortality in caged individuals as they metamorphosed later, and therefore, tanks were drained earlier in development to prevent drowning, and by this time compost had been replaced with sphagnum moss in the vivariums. In cages, larval mortality (typically around 80%) may have been partly due to predation, as invertebrates were observed in cages at YT and PYL, and Cooke reported that invertebrates can quickly decimate a population of tadpoles in cages. Escapism may have also occurred as newt and frog larvae were observed in a cage from PYL. In addition, caged individuals were significantly smaller than laboratory-reared individuals, and so mortality may have been caused by food scarcity and subsequent cannibalism by remaining tadpoles. Previous studies have reported loss of tadpole populations from field deployment of cages. In one case tadpole samples could not be retrieved from one third of the cages deployed , and in another mortality was generally low (exposure period over larval stage only), but all tadpoles disappeared from a few cages . There were also animals that ‘disappeared’ from the vivariums, which may have been due to cannibalism or escapism, as bodies were not observed. Under natural conditions, it is thought that a small proportion of the 1000’s of eggs laid survive to adulthood, so perhaps it is not surprising that mortality was high. However, changes in the experimental design, such as ensuring removal of invertebrates and supplementing cages with food, may have increased survival rates.

On the other hand, it could be argued that cages mimicked natural conditions, where pressures of competition and predation ensure only the fittest individuals survive.

2.4.4.4.1 Embryogenesis

Hatching and embryogenesis are very sensitive stages of development, and the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) is an established in vitro technique for assessing toxicity using these endpoints . In caged individuals, hatching success was very high at PYL (84 %), compared to YT, 20', and LP (~20 %), suggesting that early life stage toxicity was lower at the 'reference' site compared to the 'polluted' sites. A negative correlation between hatching success and increasing industrial contaminant exposure in caged *Rana pipiens* and *Rana clamitans-melanota* has also been reported . Overall mortality in laboratory-reared individuals from LP was comparable to other sites, however, it had the lowest hatching rate and also significantly higher larval abnormalities than other sites. In addition, no larvae were observed in cages or in the wild at this site after hatching, suggesting that lethal toxic effects may have occurred. It is not known what contaminants were present at LP, however, the pond was bordered by pasture on one side and arable land on the other. Toadspawn was reasonably abundant at this site, and according to the landowners toads were a common sight, although tadpoles were also not observed at breeding season two. Due to the high fidelity of toads to their breeding site, the toxicity observed may be a relatively recent phenomenon. The pesticide azinphos-methyl (active ingredient of Guthion ®) decreased hatching and increased abnormalities in FETAX at mg/L levels , but is unlikely to be causing effects observed here as its concentration range in the U.K. is 0.01-0.18 µg/L (see Appendix 1). The more commonly used herbicides glyphosate , atrazine and 2,4,-D have also been found to cause malformations in the FETAX assay. Morgan *et al*. (1996) reported that the EC50's for atrazine and 2,4-D were close to their maximum solubility values in water (33 mg/L and 245 mg/L, respectively), and much higher than levels found in the environment (maximum detected in 2004/2005: 1.96 µg/L and 18.6 mg/L, respectively). In the case of glyphosate, Perkins *et al.* (2000) reported that the surfactant POEA, which is found in the commercial formulation of glyphosate Roundup ®, was the toxic component rather than the glyphosate itself (LC50's of 5,407 mg/L for glyphosate, compared to 9.4 mg/L for POEA). Roundup ® is the most

common formulation of glyphosate, but glyphosate was still only detected up to 1.6 mg/L in the UK. Therefore, none of these pesticides are likely to be causing the effects observed here alone. However, equimolar mixtures of pesticides have been reported to cause mortality (35 %) compared to each pesticide alone (0-7.8 %) in *Rana pipiens* larvae . In addition, pesticide loading near to LP was higher than other sites (Appendix 2 pp 297-319), and the pesticides detected at the highest concentrations included 2,4-D (max: 236 ng/L, percentage of times detected: 13%), atrazine (163 ng/L, 18%), diuron (840 ng/L, 50%), isoproturon (1980 ng/L, 20%), MCPA (221 ng/L, 40%), mecoprop (563 ng/L, 53%), and PCP (2740 ng/L, 30%). Atrazine was the only pesticide that was detected near to LP and formed part of the mix tested by Hayes *et al*. (2006), however, the concentration of pesticides used in his mix was lower than measured levels here (0.1 µg/L). In addition, PCP was found at high concentrations nearby, and is highly toxic to fish, with LC50 values of between 32 µg/L – 205 µg/L . Therefore, although the toxic effects on tadpoles are unknown in response to PCP, or the other pesticides detected nearby, mixtures of these compounds, and the cause of toxicity at this site, warrant further investigation. Since no caged or wild individuals could be collected from LP for comparison to laboratory-reared individuals, samples were not analysed further.

2.4.4.4.2 Larval Stage

Between hatching and metamorphosis (larval stage), mortality rates in laboratory-reared individuals were low. The same was true in caged individuals from YT and 20', despite high initial mortality rates at hatching. The opposite trend was observed at PYL, whereby the highest mortality was observed during larval stages compared to other stages. In addition, caged metamorphs from PYL were extremely small compared to laboratory-reared and wild-caught individuals, and despite abundant food provided in the vivariums post TP 3, they decreased in weight between TP 3 and 4, and none were left by time point 5. The reason for this is unclear, but may suggest starvation of the metamorphs (despite food availability), caused by a lack of food availability during larval stages. Poor tadpole growth and small size at metamorphosis are correlated with decreased survival of metamorphs , which may explain the continuing mortality despite food availability at TPs 3, 4, & 5. Wild caught individuals from TP 3 and 5 (they were not observed at other TP) were the same size

as laboratory-reared individuals, suggesting a specific effect of the caged environment on larval growth and development. Cooke reported that clear water containing little algal growth (conditions similar to those of PYL) resulted in poor tadpole growth, which was presumably due to the lack of food. It is a turlough, so is not linked to surface or ground water inflow, except via run-off, and may therefore have a lower mineral and nutrient input. Indeed, the ambient water at PYL had low conductivity, hardness, nitrate, and phosphate values compared to other sites in breeding season two (April-May, 2005). A contributing factor to the slow growth and small size at metamorphosis observed may have been increased tadpole competition at this site, due to the high hatching rate , which then exacerbated the low food availability. Indeed, Karasov *et al*. (2005) reported that final size of metamorphs was inversely proportional to caged tadpole density. Interestingly, survivorship of caged individuals was similar at all sites (except LP) at the end of the larval period (15-20 %), perhaps suggesting that cages were initially overstocked, and mortality occurred until enough resources were available for the remaining tadpoles. In the present study 100 eggs were placed in a cage of 35 x 35 x 25 cm size, in contrast, Karasov *et al*. (2005) placed 100 eggs in a field enclosure of 102 cm height and 61 cm diameter (~ 10x larger). In the latter study, enclosures were also supplemented with food, and although one third of the cages they deployed were lost, survivorship in the remaining cages was much higher than observed here (55 % for green frogs (*Rana clamitans melanota*) and 80 % for leopard frogs (*Rana pipiens*)). In addition, Cooke put 40 tadpoles in a cage with 30 x 30 x 45 cm dimensions (~ 3x larger) and no mortality was observed over the 14 day period, although this study was of short duration. Therefore, the morphological effects at PYL were likely related to the experimental design rather than any toxic effect at the site.

Wild-caught individuals were found at PYL at TP 3 & 5 only, whereas they were found at TPs 2-4 at YT and 20'. Time point 5 was approximately 2 months post-metamorphosis, therefore, it may be expected that metamorphs would have left the area immediately adjacent to the pond at this stage, and thus would be difficult to find. However, it was surprising that no wild individuals could be found at PYL at time point 2, as they are tadpoles at this stage, or time point 4, when they are young metamorphs. It is a large deep pond surrounded by woodland, and so this finding could be an artefact of the environmental conditions, however, it was reported that

since 2000 there has been a drastic decrease in toad populations at PYL (Barney Gill pers. comm.), to the extent that there is no longer a toad crossing organised for this site by Froglife. Since minimal toxicity was observed at this site and it is known that tadpoles need salts for healthy development , it was hypothesised that the low conductivity of this site may have contributed to tadpole mortality. However, Fort *et al*. reported that conductivity values of 42-1790 µS/cm had no effect on hatching success or abnormality rates in a FETAX assay (212 µS/cm measured at PYL in 2005). Since the experimental design may have been largely responsible for morphological effects observed here, and hatching success was high, the reason for the apparent population decline at this site warrants further investigation.

2.4.4.4.3 Growth

Laboratory-reared individuals were significantly larger than caged and wild-caught individuals at all sites. Caged tadpoles were also often smaller than wild-caught individuals, however, this difference was only significant at PYL (discussed above). Therefore, although the cage had a negative impact on growth of individuals, it was less pronounced than the positive effect of feeding on laboratory-reared individuals from YT and 20'. Furthermore, the snout-vent length of all individuals was higher than those reported for *B.bufo* metamorphs sacrificed immediately following completion of metamorphosis (Petrini and Zaccanti, 1998). Interestingly, there were significant differences in size of laboratory-reared individuals from different sites, whereby those from YT were the largest, 20' were the smallest, and those from PYL were in between. Moreover, these differences persisted until TP 4. The reason for this is unknown, however it may be a function of distinct genetic groups, or a result of maternal transfer of compounds that affected growth. Interestingly, hatching success and developmental abnormalities of narrow-mouth toad (*Gastrophyrne carolinensis*) embryos were correlated with levels of trace metals (associated with coal combustion waste) in adults . Although similar studies using pesticides have not been reported in amphibians, *in ovo* exposure to the pesticides atrazine (0.2 µg/L) or endosulfan (2 or 20 µg/L) reduced hatchling size of *Caiman latirostris* , and Karasov *et al*. (2005) reported that tadpole growth was negatively correlated with contaminant loading of sediment by industrial pollutants. At YT, laboratory-reared individuals were 2 to 4-fold bigger than caged (and wild caught) individuals, and this difference persisted for

the duration of the experimental period, despite the same feeding regime for approximately half of this time. This did not occur at 20', where caged, but not wild-caught, individuals were only slightly smaller than laboratory-reared individuals, and this difference did not persist after TP 3. Since hatching rate and larval survival were similar at the two sites, this suggests that environmental factors at YT may have affected tadpole growth, which could not be overcome by approximately 8 weeks of feeding in the laboratory. A smaller size at metamorphosis may have population level consequences, as it is believed to result in a higher risk of predation, and reduced fecundity due to a smaller size at first reproduction .

2.4.4.2 Thyroidal Development

As discussed above, there were large differences in general growth between experimental groups, and these are likely to affect thyroidal endpoints. Metamorphosis can be affected by temperature, pond drying, predation, and nutrition (Rose, 2005). Temperature did not differ between experimental conditions, and there was no evidence of decreased water levels during the experimental period. Predation may have occurred, as invertebrates were observed in some cages (YT & PYL), which have been reported to decimate populations of caged tadpoles . Differences in nutrition are also thought to have occurred (see above). No data were available on the effect of food availability on the growth and development of the thyroid gland specifically, however, nutrition has been reported to be an important factor in determining timing of metamorphosis (for review see Rose, 2005). For the developing tadpole, there is a trade-off between capitalising on an abundant food supply to attain a large size at metamorphosis, and the risk of staying in a potentially dangerous habitat. Therefore, good nutrition results in larger metamorphs, which complete metamorphosis at a younger age . In contrast, Laugen *et al.* (2002) reported that food affected size but not age at metamorphosis, perhaps due to sufficient food for complete metamorphosis in both low and high food groups in this study. Therefore, due to possible nutritional differences in experimental groups, the differences observed in thyroid sensitive endpoints in larvae and metamorphs must be treated with caution.

Metamorphosis was inhibited in laboratory-reared individuals from PYL (earlier developmental stage at time point 2, smaller HLL at TP 2 & 3, decreased FLE), compared to laboratory-reared individuals from YT or 20', and this appeared to be independent of growth, as weight and SVL were lowest at 20'. As in growth endpoints, differences in thyroidal endpoints in laboratory-reared individuals were unexpected, as they developed under the same feeding, temperature, and water quality regime. Interestingly, a similar pattern of development was observed in all individuals from PYL, whereby laboratory-reared, caged, and wild caught individuals from PYL developed very slowly compared to laboratory-reared, caged, or wild caught individuals from other sites. This suggests inherent developmental rates of distinct groups of tadpoles, or a latent effect of the pond environment. It has been reported that different races of *Rana temporaria* display different age and size at metamorphosis in the laboratory, depending on latitude of the parent frogspawn , although the range of sampling sites in the latter study was ~ 14^0N (or ~1500 km) compared to 1-2^0N latitude (240 km) here. Maternal and genetic input have also been shown to affect age and size at metamorphosis . Only the male origin had a significant effect on the age at metamorphosis, whereas there was a lot more plasticity in the size at metamorphosis, and female origin, male origin, and food abundance all affected this parameter. If the same were true in *B.bufo*, it may be expected that the possible lack of food at PYL did not delay metamorphosis, but contributed to the small size observed at metamorphosis, and therefore, a non-nutrient factor may have also affected metamorphosis. As mentioned, the only reported study to test maternal transfer was in relation to coal combustion waste, and thyroid sensitive endpoints were not reported

Despite significant differences in thyroid sensitive morphological endpoints in laboratory-reared individuals between sites, the ThSI, thyroid gland activity and colloid quality were very similar between sites, suggesting the thyroid gland is less susceptible to perturbation than morphological endpoints. Effects observed on the thyroid histology were minimal in caged and wild-caught individuals, suggesting that the morphological effects on thyroid sensitive endpoints observed were largely caused by external factors rather than thyroid disruption per se. Characteristic effects of thyroid disruption are increased thyroid gland size, accompanied by an increased activity (hyperplasia and hypertrophy), and in severe cases, decreased colloid . This is

caused by decreased thyroid hormone production, leading to increased stimulation of epithelial cells by thyroid stimulating hormone. At the same time, thyroglobulin is released from the colloid, which in turn decreases colloid due to the inability of epithelial cells to replenish the thyroglobulin. In contrast, the most common effect observed in caged and wild-caught individuals in the present study was a decrease in ThSI, accompanied by a decrease in activity and decreased colloid quality. Effects were most often observed in prometamorphic tadpoles, which is not surprising as this is a stage of intense thyroid system stimulation. The mechanism responsible for a decrease in the ThSI is unknown, but it was observed in caged and wild-caught prometamorphic tadpoles at all sites, suggesting it may be a function of nutrition. Alternatively, it may have been an artefact of the earlier stage of caged and wild caught tadpoles compared to laboratory-reared individuals within groups. Indeed, in a regression analysis, only individuals at 20' were different from their laboratory-reared counterparts, and this was also the only site where reduced ThSI was observed at other TPs. In addition, colloid degeneration was severe in prometamorphic tadpoles, and no forelimb emergence was observed in caged individuals at TP 2 in caged individuals (compared to 24% at YT, and 0 % at PYL). The effects of nutrition were less marked at 20' than the other sites, and FLE was high in laboratory-reared individuals (64 %), suggesting additional environmental factors may have contributed to specific thyroidal effects observed. Pesticides identified in the highest levels near to 20' (Appendix 2, pp 288-297) included, 2,4-D (max: 60 ng/L, percentage of times detected: 14%), atrazine (45 ng/L, 86%), bentazone (200 ng/L, 14%), chloropropham (2140 ng/L, 100%), diuron (1008 ng/L, 100%), mecoprop (120 ng/L, 43%), simazine (235 ng/L, 100%), however no data on the effects of these pesticides on thyroid function were available, therefore, it is difficult to hypothesise what may have been causing these effects. However, similarly to effects of pesticide mixtures on growth, Hayes *et al.* (2006) reported increased time to FLE in response to the mixture compared to each compound alone. At YT/PYL little effect was observed on thyroid histology, suggesting that the inhibition of development at these sites were largely due to nutrition, competition, and/or other factors.

2.4.4.3 Bidderian and Gonadal Differentiation

Bidderian and gonadal development were similar to that previously reported for *B.bufo* , except the previously reported mesogonad in males was rarely observed in

this study. In addition, sex specific differences in size of BO, number of FGP oocytes, and size of FGP oocytes in the BO were previously reported (Petrini and Zaccanti, 1998), but no such effect was observed here. As previously reported for Bufonidae species, in the present study sex differentiation appeared to be of the semi-differentiated type (all develop as females first), as the percentage of females remained constant over time (~40 %), but the ratio of undifferentiated, male, and intersex differed. However, since the first samples were taken post-metamorphosis, it is not known if all gonads were originally female, therefore perhaps it is more accurate to say that ovaries developed at an earlier stage than the testes (also reported by Petrini and Zaccanti, 1998). Various investigators have reported that the relationship between gonadal development and somatic growth in amphibians to be weak. For example, in an early publication, *B.lentiginosus* tadpoles were starved throughout development, and no effect on sex ratio was observed . More recently, Ogielska & Kotusz reported that ovarian development was only slightly affected by somatic growth in *Rana lessonae* and *Rana temporaria* tadpoles, and that age was a more important determining factor in development. Similarly, Gruca and Michalowski investigated the rate of gonadal development in *Xenopus laevis* tadpoles kept under various feeding conditions, and reported that feeding conditions influenced somatic growth considerably more strongly that gonad differentiation, although decreased food availability also contributed to the inhibitory effect on gonadal development. Lastly, Chang and Hsu reported a strong correlation between age and ovarian differentiation, but no correlation to size in *Rana catesbeiana* tadpoles. In the present study, the presence of the fat body, which reflects the nutritional status of the animal , did not appear to be associated with gonadal differentiation (personal observation). Therefore, histological differences observed in gonadal development are likely to be largely independent of nutritional status. However, due to the high mortality in caged individuals, it cannot be discounted that skewed sex ratios were a function of sex-specific mortality.

2.4.4.3.1 Laboratory-reared Individuals

Sample sizes were often too small to determine statistical differences in sex ratios between laboratory-reared, caged, and wild-caught individuals within each site. If gonadal development is largely determined by age in this species, it would be

expected that similar patterns of gonadal development would occur in laboratory-reared, caged, and wild caught individuals. However, similarly to effects observed in morphological endpoints, there were significant differences in sex ratio of laboratory-reared individuals between sites, and these differences followed similar trends in caged and wild caught individuals. This could be observed in the ratio of undifferentiated individuals, as more were observed in laboratory-reared, caged, and wild caught individuals from YT compared to corresponding individuals from other sites. In addition to inhibited gonadal differentiation at YT, the BSI was smaller, the number of bidderian FGP oocytes was greater, and the size of FGP oocytes were smaller, compared to laboratory-reared individuals from PYL or 20'. More intersex individuals were also observed in laboratory-reared individuals from 20' compared to other sites (YT – 9 %, PYL – 4 %, 20' – 22 %), and this was mirrored in caged individuals (YT – 0 %, PYL – 0 %, 20' – 32 %). These findings suggest that in addition to possible effects of the ambient water, genetic, maternal, and/or a latent effect of the pond environment may have contributed to differences observed in gonadal differentiation. The differences between laboratory-reared individuals, both in growth and development, and in gonadal differentiation, highlight differences that may be observed in the 'control' group in laboratory exposures. Furthermore, these findings may partially explain differences reported in the literature, where the same compounds are tested using the same species, and experimental setup, but different responses are reported.

2.4.4.3.2 Retarded Differentiation

All caged individuals from PYL were undifferentiated at TPs 3 & 4, although since growth contributes slightly to gonadal differentiation, the retarded gonadal development at PYL may have been partially caused by starvation of these individuals. However, wild-caught individuals from PYL (TP3) were of a similar developmental stage and weight to laboratory-reared individuals, but only 10 % were differentiated, compared to 76 % differentiated in the laboratory. In addition, in wild caught individuals from PYL (TP5), the singly ovary was immature, with small oocytes and no ovarian cavity, and the testes of one of the males had a dense patch of cells in the medulla, which may be indicative of recent colonisation of this area . This suggests there may have been an endocrine disrupting effect on the gonads at this site.

However, little activity was observed in PADs in breeding season two, and only TBT (0.03 μg/L) was detected near to this site (Appendix 2 pp 281-288). Therefore, the reason for this effect is unknown, but due to the reported population decline at this site, warrants further investigation.

Undifferentiated gonads were often observed in individuals from YT, and the effects were more marked in field collected individuals than those raised in the laboratory. The *n* values for caged individuals (*n* = 8) and wild caught individuals (*n* = 14) was low, nevertheless, retarded development was observed in histological sections and it was the only site where an undifferentiated individual were observed at TP4. *B.bufo* tadpoles treated with an aromatase inhibitor (OHA: 0.1 & 1 mg/L), testosterone (1 mg/L), or DHT (1 mg/L) displayed retarded gonadal differentiation (Petrini and Zaccanti, 1998), and a similar effect was observed in larval *B.americanus* exposed to estradiol (1 mg/L) or methyltestosterone (500 μg/L) (Chang, 1955). In contrast, the anti-androgenic compounds cyproterone acetate (receptor mediated) and a 5α-R inhibitor (17βC), stimulated gonadal development in both sexes (Petrini and Zaccanti, 1998). Using this rationale, it may be expected that compounds that increased androgen or estrogen levels may lead to an increase in undifferentiated gonads. Interestingly, another consequence of increased androgen levels in toads is a decrease in BO size (see section 2.3.1), which was observed in caged, but not wild-caught, individuals at YT. In addition, FGP bidderian oocytes were smaller in caged and wild-caught individuals, compared to laboratory reared individuals from YT, and exposure of *B.bufo* tadpoles to OHA (0.1 or 1 mg/L), estradiol (0.1 mg), testosterone (1 mg/L) or dihydrotestosterone (1 mg/L), was previously reported to reduce the diameter of diplotenic oocytes (Petrini and Zaccanti, 1998). Since both estrogens and androgens seem to have a similar effect on BO, perhaps a central control mechanism is implicated. Extracts from YT at breeding season two were strongly anti-estrogenic and anti-androgenic, and the model anti-androgens flutamide (0.5 μM) and cyproterone acetate (CPA: 0.5 μM) feminised larval *Rana rugosa* , whereas CPA (0.24 μM) had no effect on sex ratios in larval *B.bufo* (Petrini and Zaccanti, 1998). In addition, the anti-estrogen ICI182780 (0.017 μM) significantly affected the sex ratio in larval *Rana pipiens*, indicated by an decrease in the proportion of males, and an increase in proportion of intersex . Therefore, it is not known what caused retarded differentiation, and extracts were not tested for other properties (such as effects on

steroidogenesis), but despite the low *n* value at this site, these findings warrant further investigation.

2.4.4.3.3 Masculinisation

A high proportion of intersex individuals were observed at 20' in laboratory-reared and caged individuals. Despite estrogenic activity of water extracts from breeding season one and two, intersex individuals were classified as masculinised females; rather than feminised males. This was primarily due to the change in sex ratios over time, which was indicative of masculinisation (TP5: laboratory-reared: M – 30 %, F – 30 %, I – 40 %; caged: M – 55 %, I – 45 %), as well as the increased severity of testicular oocytes in laboratory-reared individuals. In addition, treatment of mink (*Rana septentrionalis*) or green (*Rana clamitans*) frogs with the potent estrogen EE2 (3.3 to 9.1 ng/L) resulted in maximum intersex levels of 28 % , which is much lower than levels observed here. Since ovarian development occurred earlier than testicular development, undifferentiated individuals from TP 3 were classified as genetic males. If intersex are classified as female and undifferentiated as male, presumed genetic sex ratios in laboratory-reared and caged individuals were almost identical (Female = 64 or 65 %, respectively), indicating the higher percentage of females in both cases may be of genetic origin. Interestingly, in Bufonidae species the female is thought to be the heterogametic sex , and it has been reported that breeding of a masculinised female (zw) with a normal female (zw) in *Ambystoma mexicanum* and *A.tigrinum* results in 25 % male (zz) and 75 % female (zw & ww) offspring . Therefore, in order to test this hypothesis, it would be interesting to test the genetic sex ratio of toads from this site.

Masculinisation was more marked in caged than laboratory-reared individuals, possibly suggesting an additional effect of the ambient pond environment on sex differentiation. Masculinisation in response to xenobiotics has been linked with compounds that inhibit aromatase activity such as the effects of fadrozole (a pharmaceutical aromatase inhibitor) on fish , and TBT on marine gastropods . TBT has also been shown to cause masculinisation in Zebrafish and to damage sperm at concentrations between 0.1-100 ng/L , and to inhibit steroidogenesis in *Xenopus* ovarian explants at the lowest concentration tested . Surprisingly, TBT was detected 115 times over the range of 10-7900 ng/L in 2004/2005 in U.K. freshwaters (see

Appendix 1) and was detected once (0.02 µg/L) at a site close to 20' (Appendix 2, pp 287-296). Therefore, TBT may have contributed to the effects observed. Another pesticides that inhibits aromatase *in vitro* and is found in the UK is cypermethrin , which was found 11 times over a concentration range of 12-93 ng/L (see Appendix 1), however, it was not found in this area (Appendix 2). To the author's knowledge, no exposure studies of effects of aromatase inhibiting pesticides on amphibians have been reported, however, aromatase is thought to be important for sex differentiation in vertebrates (see Chapter 1, section 1.3.3). Furthermore, it was reported in a newt species (*Pleurodeles waltl*), that aromatase expression was down regulated during sex reversal of females to males, as a result of incubating individuals at a masculinising temperature . Similarly, fadrozole (500 µg/g in food) decreased aromatase expression, and caused complete masculinisation of developing zebrafish, which persisted after 90 days of control conditions . However, treatment of larval *B.bufo* with an OHA (an aromatase inhibitor) resulted in development of female (F), male (M) and undifferentiated (U) individuals, (1 mg/L = F0:M3:U4; 0.1 mg/L = F4:M1:U7; Petrini and Zaccanti, 1998), and results were inconclusive, perhaps due to the low *n* values in this study. Therefore, the role of aromatase inhibiting compounds in gonadal differentiation in amphibians, and the effect of pesticides on aromatase activity, both warrant further investigation.

In addition to effects on steroidogenesis, there may have also been effects on the hypothalamo-pituitary-gonadal axis. Androgenic and estrogenic hormones affect gonadotropin release via the negative feedback mechanism of the hypothalamo-pituitary-gonadal axis (see Chapter 1, section 1.3.3). Removal of the pituitary inhibited testicular development of *B.americanus*, but had no effect on ovarian development (Chang, 1955), suggesting gonadotropins are less important in ovarian growth (see Chapter 1, section 1.3.3 for details). Therefore, it is hypothesised that females develop first due to immaturity of the hypothalamo-pituitary-gonadal system in the developing tadpole, and at a later stage of development, genetic males develop testes as levels of gonadotropins increase. Therefore, any EDs that decrease circulating androgens or estrogens may therefore cause an increase in gonadotropin levels, leading to normal testicular development in genetic males, and masculinisation of genetic females. Conversely, any EDs that increased circulating levels of androgens or estrogens, and thereby decreased gonadotropin levels, may result in

undifferentiated individuals. In agreement with this hypothesis, treatment of larval *B.vulgaris* with FSH caused masculinisation , and no masculinising effect was observed after treatment of larval *B.bufo* with testosterone (1 mg/L), or DHT (1 mg/L), but instead, the incidence of undifferentiated gonads increased (Petrini and Zaccanti 1998). This effect was especially potent with DHT treatment (88% undifferentiated, n = 8), which also dramatically inhibited the growth of the undifferentiated gonad (8-fold compared to control ovary, & 4-fold compared to control teste), and DHT is known to be a potent inhibitor of gonadotropin release in larval and adult *Rana esculenta* . A similar explanation could be applied to the paradoxical effect of the receptor mediated anti-androgen CPA, which causes masculinisation in developing amphibians, and may be due to its inhibitory effect on 3β-HSD activity . This would presumably decrease hormone production by the gonad, leading to increased gonadotropin levels, and subsequent masculinisation. In addition to altered sex ratios, the BSI increased in size over time in caged male and intersex individuals, but decreased over time in laboratory-reared individuals. In addition, ovarian and testicular growth was inhibited in relation to BSI (OSI and TSI were smaller compared to the BSI), in caged individuals compared to laboratory-reared individuals. It is not known why gonadal growth was inhibited, but there is a dynamic relationship between teste size and BO size in adults (Calisi, 2005), whereby presence of the teste inhibits BO growth independently of gonadotropin levels . Therefore, perhaps the increasing size of BO inhibited both ovarian and testicular growth.

In addition to possible causes of masculinisation discussed above, many other factors could have contributed to the effects observed. For example, corticosterone levels were higher in *B.boreas* raised at a greater tadpole density , and cortisone was shown to cause masculinisation in *Rana sylvatica* tadpoles (Witschi and Chang, 1950); therefore the results should be treated with caution. Nevertheless, to the author's knowledge, *in situ* masculinisation of an aquatic species has only previously been reported in fish, in relation to paper and pulp mill effluent . Lastly, extracts from 20' from breeding season two were moderately estrogenic, weakly anti-estrogenic, and only slightly anti-androgenic. According to present understanding described here, these properties would not be expected to cause masculinisation in amphibians. However, due to the uncertainty of the cause(s) of masculinisation, unknown possible

additional properties of the extracts, and the time lapse of 1 year, perhaps this discrepancy is not surprising.

2.5 Conclusions

Interpretation of results obtained in the field work was difficult due to various factors. Firstly, due to endocrine activity in field blanks, and unknown uptake rates, the reliability of activity observed from the PADs is uncertain; secondly, very little is known about the endocrine activity of commonly used pesticides, and nothing about mixture effects of these pesticides; thirdly, growth and development were more advanced in laboratory-reared individuals compared to cage individuals; fourthly, mortality was very high, leading to low *n* values, reducing reliability of observed effects; and finally, the mechanism of gonadal differentiation in toads is largely unknown. It is also not known how these factors interact with each other, or their environmental importance. However, in spite of these limitations, preliminary results observed were interesting, and warrant further investigation.

Extracts from every site tested in breeding season two were active in at least one screen, and toads collected from all sites in breeding season three were affected in different ways. YT had very high anti-estrogenic and anti-androgenic activity, and inhibited gonadal development, little endocrine activity was observed at PYL, but populations are apparently decreasing. At 20', estrogenic and weak anti-estrogenic and anti-androgenic effects were observed, as well as various effects on gonadal and thyroid histology. Finally, at LP, high anti-estrogenic and anti-androgenic activity was observed and 100 % mortality occurred in field specimens. In addition, the activity observed at each site did not seem to have any bearing on the effects observed in the toads. This was especially true in the fens, where estrogenic activity was observed at breeding season one & two, but toads were masculinised. Interestingly, more males are consistently reported by Froglife volunteers as part of the Toads on Roads scheme. Although this is probably due to the discrepancy in the age of sexual maturity between males (2-3 years) and females (3-6 years), it may be prudent to monitor sex ratios in sites run by Froglife, to try and establish long term trends. Finally, the suitability of toads as a test species is questionable due to the high mortality observed.

However, they also have potential as biomonitoring species in the agricultural environment, due to their presence in agricultural ponds and ditches, high site fidelity, and presence of the BO, which may assist in elucidating gonadal effects. In conclusion, further investigation at each site is warranted, as well as wild sex ratios of amphibians, and effects of environmentally relevant pesticides.

Chapter 3

Assay Optimisation

3.1 Overview

There are few reported effects of endocrine disrupting compounds on amphibian tissue *in vitro*, and extrapolation from mammalian tissue may not be representative of effects in amphibians. Therefore, in this study, 3 endocrine sensitive assays based on amphibian tissue were investigated for suitability as ED screens. It was proposed that the hepatocyte assay, which was used for samples collected in breeding season one, could be used for PAD extracts; and comparison to effects observed in YES would assist in elucidating relative effects of parent compounds and metabolites. However, despite successful use in Towa-Kagaku (Japan), it could not be optimised for use at Brunel University. The germinal vesicle breakdown assay (GVBD) was developed to identify receptor-mediated anti-androgenic activity, and it was proposed that comparison to the YAS would assist in comparison of the human AR and the receptor of the oocyte. It was successfully developed for use with the relevant positive and negative control, but testing of pesticides resulted in unreliable results that were highly variable. The ovulation assay was developed to test for inhibition or stimulation of ovarian steroidogenic enzymes through measuring tissue production of progesterone, testosterone, and estradiol. In addition, the ovulatory response is ecologically relevant as inhibition or stimulation of ovulation could affect reproductive fitness. This test was successfully developed, and proved to be a sensitive and informative test for pesticides (see Chapter 4).

3.2 Introduction

3.2.1. Liver Cell Preparations

Details of the basis and rationale for using the hepatocyte monolayer assay were described in Chapter 2 (section 2.2.1.1), as this assay was used to test water extracts in Japan (Towa-Kagaku, Japan) as part of the field work aspect of this project. Training in the experimental procedure was also completed at this time. However, attempts to establish the same procedure in the UK were unsuccessful due to poor viability of the hepatocytes, and lack of VTG production when stimulated with E2. Therefore,

changes to the original hepatocyte monolayer assay protocol were implemented, including culture of whole liver slices .

3.2.2. Germinal Vesicle Breakdown (GVBD)

In the female reproductive system, the maturation of oocytes is arrested in prophase of the first meiotic division, where development is suspended (immature oocytes) until stimulated to re-enter the meiotic cycle and complete the first meiotic division (Rugh, 1951). Maturation normally occurs just before the spring breeding period , and causes morphological changes in the oocytes including breakdown of the germinal vesicle, which is characterised by swelling of the oocyte and the presence of a white spot near the centre of the animal pole, which can be observed using a dissecting microscope (Figure 3.1, Rugh, 1951).

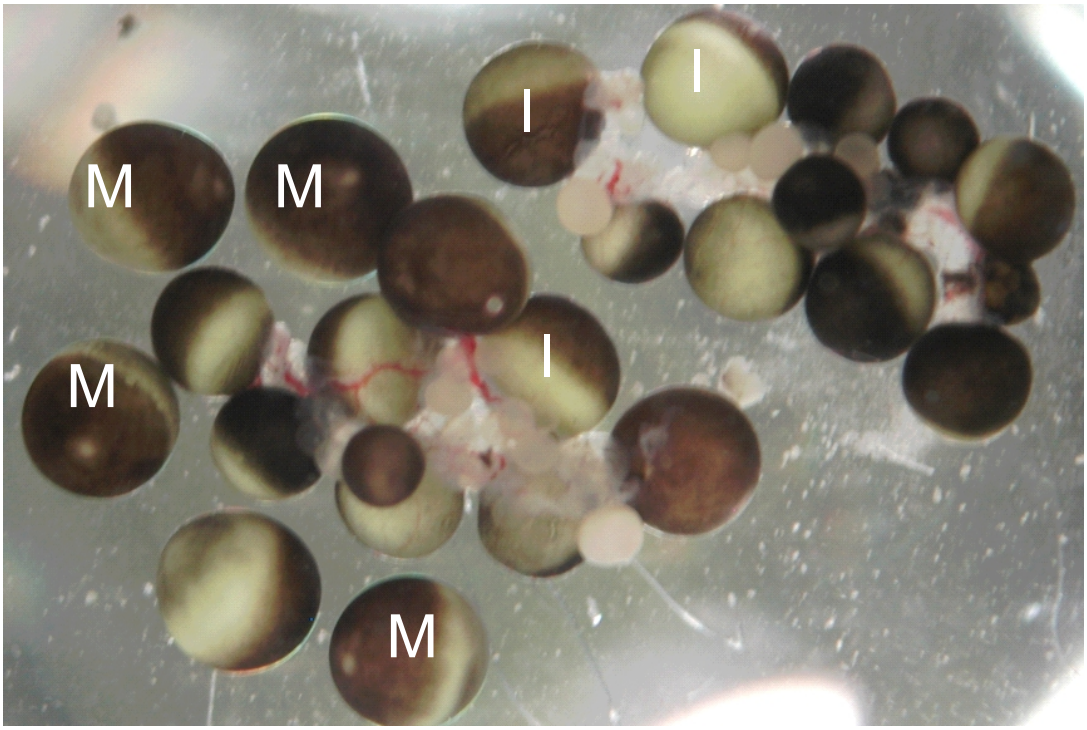


Figure 3.1 Cultured oocytes of *Xenopus laevis*, showing mature (M) and immature (I) oocytes. Mature oocytes have undergone GVBD (white spot) and detached from the connective tissue of the ovary (ovulation). Immature oocytes remain attached to the ovary tissue, and no white spot can be observed. Photo taken at x 10 magnification.

The immature oocyte is surrounded by a layer of follicle cells that synthesise steroid hormones in response to gonadotropins, resulting in maturation of the oocyte . Progesterone was thought to be the initiating factor produced by follicle cells , as it was shown to be one of the most potent inducers of GVBD , and *in vivo* treatment of *Xenopus* with gonadotropins stimulated ovarian production of progesterone . However, later researchers reported that testosterone was produced at much higher concentrations than progesterone if ovarian fragments were incubated with frog pituitary homogenate or HCG . In addition, in spite of research in this area, the progesterone receptor (PR) has not been identified in the oocyte . The receptor-initiated response is distinct from other steroid receptors in a number of ways. Firstly, steroid receptors normally induce effects via a transcription mediated response, but actinomycin D (a RNA synthesis inhibitor) does not inhibit maturation, indicating it occurs independently of transcription . Secondly, the receptor is thought to be on the cell surface rather than located nuclearly, as steroids covalently bound to polymers (and thus unable to enter the oocyte) still induced maturation . Finally, the EC_{50} for GVBD is much higher than that required for transcriptional activation by the nuclear receptor (200nM compared with 1nM). In addition the PR receptor antagonist RU486 does not block GVBD induction by progesterone, but instead acts as a weak agonist (Maller, 2001). It has been suggested that the conventional (intracellular) receptor may be located on the cell membrane of amphibian oocytes, and exert its effect via a novel mechanism of action . However, although an increase in PR expression was accompanied with an increase in GVBD, the PR could not be identified at the cell membrane where the progesterone was present, so direct interaction between progesterone and the PR may not have occurred (Bayaa *et al*., 2000).

In addition to progesterone, androgens have also long been recognised as potent inducers of maturation . It was suggested that they could be the primary inducers of maturation *in vivo* as they were found in much higher levels than progesterone (>10-fold) in amphibian ovaries *in vitro* . In addition, Lutz *et al*. reported that progesterone may be converted to androstenedione (testosterone precursor) and testosterone within the oocyte, as androgen levels in the *Xenopus* oocyte *in vitro* and *in vivo* were much higher compared to progesterone. Furthermore, Lutz *et al*. (2001; 2003) have provided evidence for the presence of the classical AR, and it has been shown both

biochemically and immuno-histochemically to be associated with the plasma membrane of *Xenopus* oocytes.

Other steroid hormones are also able to induce maturation (Table 3.1), indicating that the nature of the receptor is not hormone specific. Indeed, flutamide, and its hydroxylated metabolite 2-hydroxyflutamide, bind with high affinity to the classical androgen receptor *in vitro* , and also inhibit androgen mediated maturation *in vitro* and *in vivo* . The known agonist of AR-mediated transcription methyltrienolone (R1881), which is one of the most potent activators of AR-mediated transcription, was unable to promote nongenomic signalling and maturation in oocytes when incubated at 1 μM alone (Lutz *et al*., 2001). Instead, it inhibited testosterone-induced maturation (co-incubation of R1881 and testosterone), presumably by binding to the receptor without stimulating the cascade of events leading to maturation (Lutz *et al*., 2003). In contrast, androstenediol was a poor promoter of AR-mediated transcription, but a strong mediator of maturation and nongenomic signalling in oocytes (Lutz *et al*., 2003). Interestingly, most of the steroids which were able to induce maturation by incubation have a ketone group in the 23 position, and those that were not effective have a hydroxyl group in the same position (Jacobelli *et al*., 1974). The absence of any enhanced activity of the more water soluble sulphated steroids suggests that the differential effectiveness of the steroids is not due to their solubility characteristics . In addition, injected testosterone and hydrocortisone both induced maturation, but progesterone and aldosterone (Jacobelli *et al*., 1974) did not (Table 3.1).

	Incubation - % maturation											*Injection*
Reference	Schorderet-			Jacobelli *et al*., 1974				Smith and		Schuetz,		Jacobelli *et*
Source	Slatkine, 1972							Ecker, 1971		1967		*al*., 1974
Hormone (ug/L)	*0.01*	*0.1*	*1*	*0.01*	*0.1*	*1*	*5*	*0.01*	*0.1*	*0.67*	*6.7*	*0.013*
Progesterone	100	97	94	14	93	97	100	87	-	100	100	0
Pregnenolone	-	27	100	0	100	100	100	-	85	0	50	-
Testosterone	-	-	-	38	100	100	100	-	100	0	83	72 (*0.003*)
Aldosterone	-	-	-	100	100	100	77	-	5	0	0	0
Hydrocortisone	19	100	89	0	0	100	100	-	100	0	50	100

Deoxycortic*	-	-	-	-	-	-	-	-	-	100	100	-
Cortisone	38	100	90	-	-	-	-	-	-	-	-	-
Estrogen	-	-	-	0	0	0	0	-	0	0	0	0

Table 3.1 – The effects of various hormones on ooctye maturation in *Xenopus laevis.*
*Deoxycortic. = deoxycorticosterone

In conclusion, the relative roles of androgens and progesterone in oocyte maturation, and the nature of their corresponding receptors, remain unclear, as they do not induce gene transcription like conventional nuclear receptors, and can be stimulated by diverse hormones. However, testosterone induces maturation, and this response can be inhibited with AR binding compounds (flutamide and R1881), and therefore GVBD has potential for development as an amphibian specific anti-androgenic screen. Exposure to progesterone for as little as 5 minutes, followed by washing, initiates the cascade of events that lead to maturation (Schuetz, 1967). Therefore, both flutamide and R1881 were tested for their effectiveness as anti-androgens, both with and without pre-incubation periods, to elucidate the optimum conditions for testing unknown compounds for inhibitory activity on GVBD.

3.2.3. Ovulation Assay

Oocyte maturation (GVBD) and ovulation occurs more or less synchronously in response to gonadotropin stimulation *in vivo* and *in vitro*, and the two events are closely linked to each other . Induction of maturation and ovulation can be stimulated by crude pituitary homogenates , and purified gonadotropins . Removal of follicle cells from the oocyte results in inhibition of this response (Masui, 1967; Smith, 1968), but it can be ameliorated by co-incubation with follicle cells that have been excised from the oocyte (Masui, 1967). This is due to simulation of steroid production , which in turn causes maturation and ovulation of the oocyte . Steroidogenic enzymes are responsible for the biosynthesis of various steroid hormones, including progesterone, testosterone, and estradiol (Figure 3.2). Enzymes involved in ovulation include specific cytochrome P450 enzymes, hydroxysteroid dehydrogenases, and steroid reductases . Steroid synthesis begins with the conversion of cholesterol to pregnenolone by CYP450scc, and subsequent conversion to progesterone by 3β-HSD, which together are the precursors of all other steroid hormones .

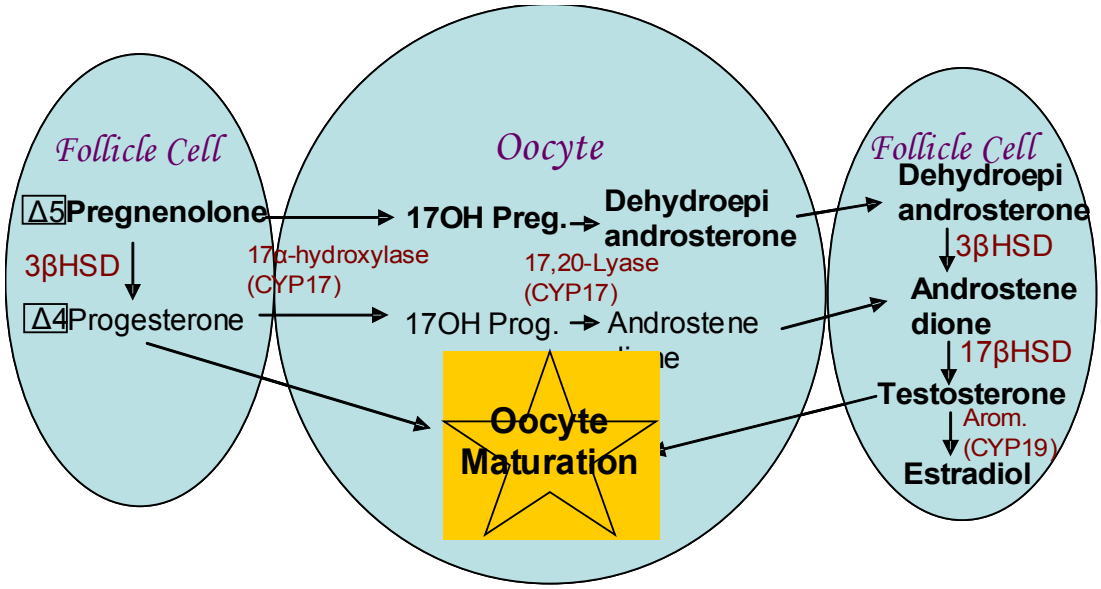


Figure 3.2. Proposed steroidogenic pathways in the amphibian oocyte, see text for details. Taken (modified) from Lutz *et al.* and Yang *et al.* CYP = cytochrome P450 enzyme; 17OH Preg. = 17-hydroxypregnenolone; 17OH Prog. = 17-hydroxyprogesterone.

In vitro, stimulation of frog ovarian follicles with gonadotropin results in production of progestins and androgens, and maturation of the oocyte . Progesterone and testosterone concentrations increased markedly after stimulation with LH, and then decreased after approximately 10 hours, and ovulation only occured when concentrations of both were elevated (El-Zein *et al*., 1988). In contrast, estradiol levels increased more slowly and then remained stable over the experimental period (El-Zein *et al*., 1988), and estradiol has been shown to have an inhibitory effect on ovulation . In addition, simultaneous treatment of *Xenopus* ovarian explants with frog pituitary homogenate and cyanoketone (3β-HSD inhibitor) inhibited the conversion of pregnenolone to progesterone, and also inhibited ovulation . Similarly, co-treatment of Indian Major Carp oocytes with LH and the 3β-HSD inhibitor epostane , also inhibited ovulation , though hormone levels were not measured. Therefore, it is anticipated that inhibition or stimulation of enzymes involved in steroid hormone production, resulting in corresponding changes in hormone levels, will in turn affect maturation and ovulation of the oocyte. Therefore, the ovulation assay was designed to measure effects of xenobiotics on steroidogenesis by measuring the ovulatory response (i.e. release of eggs from ovarian connective tissue), and hormone

production by the follicles. In addition, co-administration with epostane inhibited the ovulatory response, and thus acted as the positive control for inhibition of ovulation.

3.3 Methods & Results

The methods and results sections are grouped due to the nature of assay optimisation, whereby methods were altered by results obtained from each experiment.

3.3.1 Liver Cell Preparations

Initially, methods were followed as described previously (see Chapter 2, section 2.2.2.2), however, despite a total of 13 attempts, hepatocytes did not form a monolayer and remain viable. Therefore, liver cells were later cultured as tissue fragments, instead of dissociated cells (see below).

3.3.1.1 Hepatocyte Monolayer

The first four times, the original protocol was followed, but the cells did not survive, and this was not due to contamination of the cell culture. The fifth time, cells were incubated at 22^0C and 27^0C, however, cells were not viable. The ELISA protocol was verified using VTG and ALB standards, and response to standards was similar to those obtained in Japan. Therefore, the lack of VTG and ALB production by the hepatocytes was not due to a fault in the ELISA. In the sixth experiment, a small amount of VTG (~ 9 ng/ml) was produced in hepatocytes exposed to 1.2-9.4 nM E2, but was slightly below the detection limit of the ELISA (detection range 11-300 ng/ml VTG). For comparison, hepatocytes cultured in Japan produced around 1000 ng/ml of VTG when exposed to 1.11 nM E2.

It was discovered that the cell culture plates used in Japan were of a different brand than the ones used in the UK, therefore a comparison between the two different types was made. In addition, media was supplemented with varying concentrations of HEPES (a buffer) as it was observed that the media was becoming acidic during the cell culture. Therefore, cells were incubated in NUNC (UK) or Sumiton (Japan) plates, and with 0, 5, or 10 mM HEPES, and exposed to E2. No VTG was produced in cells cultured in either plastic plate, but ALB was produced by cells cultured in 5 and 10 mM HEPES, with higher cell viability (i.e. ALB production) at the highest concentration of HEPES (Figure 3.3), and no difference in plate type was observed. However, even under our best condition, the levels of ALB in the media were still approximately 10-fold lower than those observed in Japan (1000 ng/ml compared to 10000 ng/ml). Due to the results of this experiment, NUNC plates, and media supplemented with 10 mM HEPES, were used in following experiments.

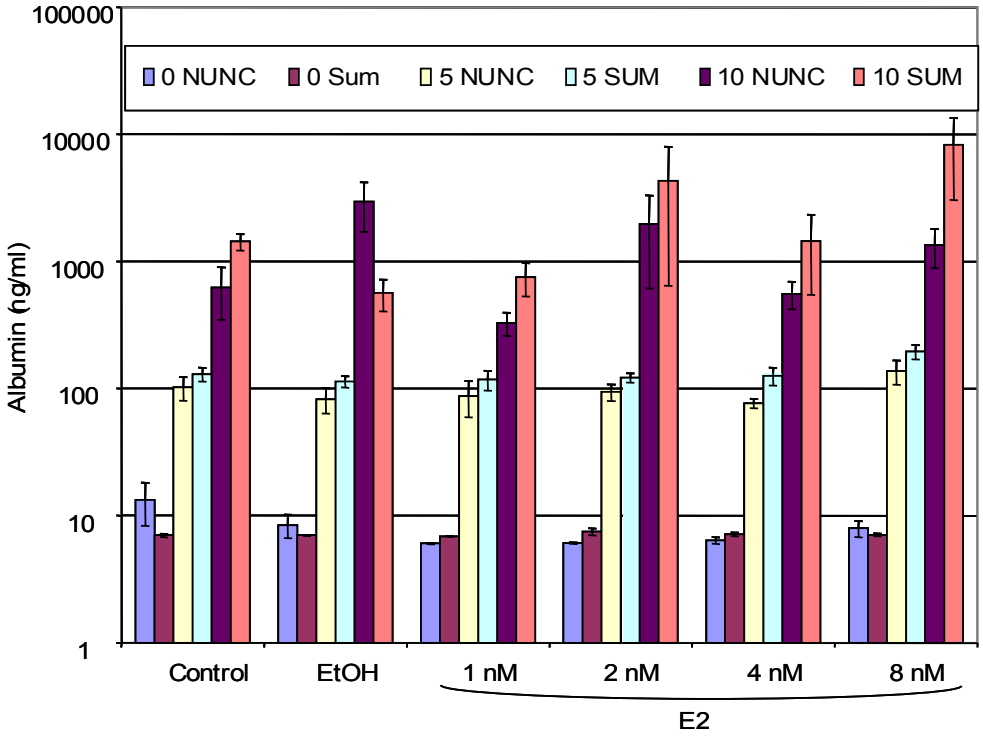


Figure 3.3. Albumin produced by hepatocytes exposed to a range of estradiol (E2) concentrations (1-8 nM), in either NUNC or Sumiton (SUM) 96 well plates, and in media without HEPES, or containing 5 or 10 mM HEPES. Values are mean ± SE based on 3 replicate wells.

Due to previous experiments, the viability of the hepatocytes was slightly improved, however, VTG production was still very low or absent. Therefore, the effects of cell composition and seeding density were assessed. Cell preparations derived from centrifugation of liver cells at 20g (original), 50g & 80g were seeded at 1 x 10^5 cells/ml (original), 0.5 x 10^5 cells/ml, and 2 x 10^5 cells/ml. The cells were exposed to a range of estradiol concentrations (0.125, 0.25, 0.5, 1, 2 nM), or a control (control, ethanol control). VTG was produced in low concentrations (15-21 ng/ml) under four conditions (20g 1 x 10^5 cells/ml, 50g 1 x 10^5 cells/ml, 50g 2 x 10^5 cells/ml, & 80g 2 x 10^5 cells/ml), but not in a dose-dependant manner, indeed control cells also produced VTG at a similar level. Therefore, data from all conditions were pooled (Figure 3.4). ALB production by cells was also measured, which was different between experimental conditions (Figure 3.5), but lower than in the previous experiment. Neither the spin speed nor the cell density clearly resulted in healthier hepatocytes, however when combined with the VTG data, it was decided that 50g, 1 x 10^5 cells/ml was the optimum condition for the hepatocytes.

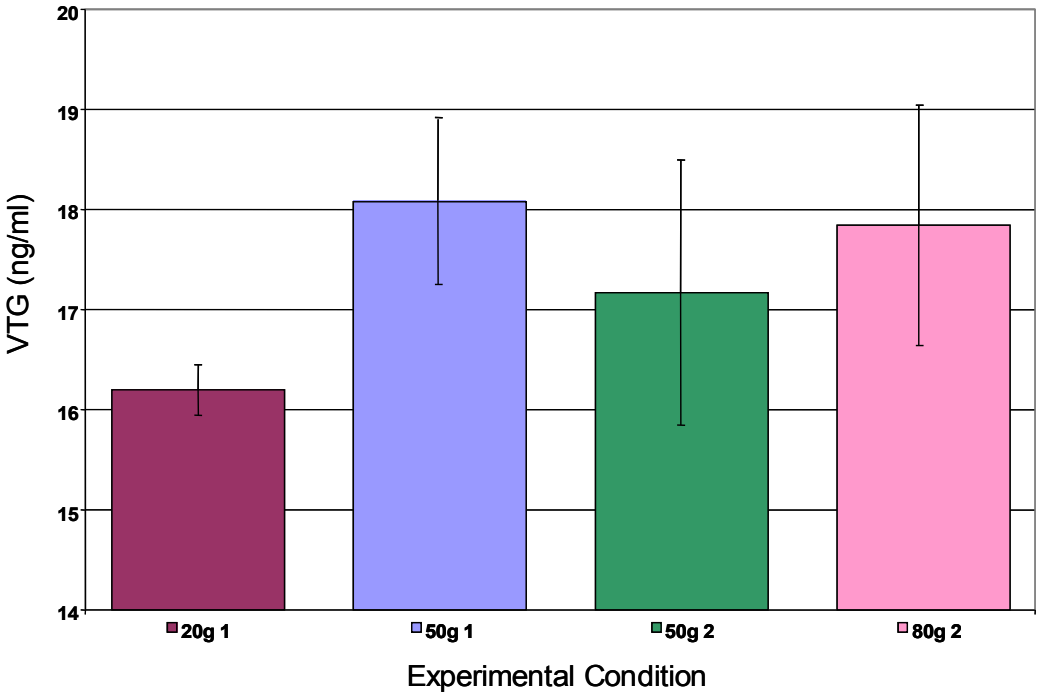


Figure 3.4. VTG produced by hepatocytes exposed to 0.125, 0.5, 1 or 2 nM E2 (pooled). Cells centrifuged at different speeds (20g, 50g or 80g), and inoculated at different densities (1 & 2 x 10^5 cells/ml). Mean ± SE based on 12 replicate wells (2 wells from each E2 concentration).

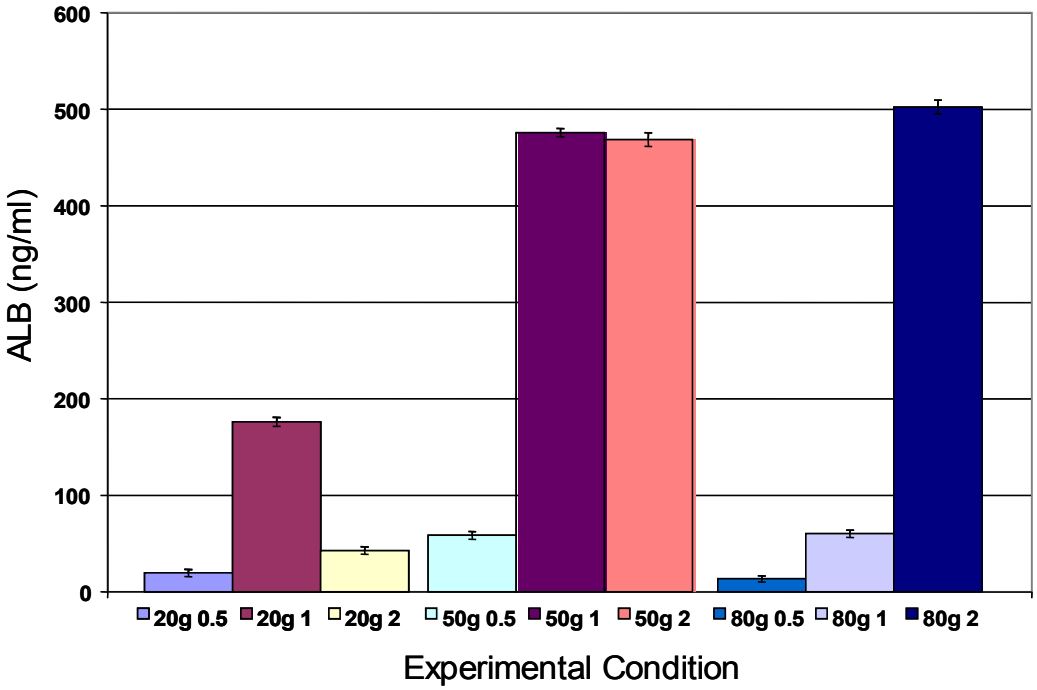


Figure 3.5. Albumin produced by hepatocytes exposed to 0.125, 0.5, 1 or 2 nM E2, or a control (pooled). Cells centrifuged at different speeds (20g, 50g or 80g), and inoculated at different densities (0.5, 1 & 2 x 10^5 cells/ml). Mean ± SE based on 12 replicate wells (2 wells from each E2 concentration, control, and EtOH).

As a result of these experiments, the experimental protocol was modified as described. However, despite 5 more attempts, the cells never produced VTG in appreciable concentrations. Therefore, it was decided that using whole liver tissue, instead of isolated hepatocytes, may result in better survival and responsiveness of hepatocytes.

3.3.1.2 Liver Slice

Adult male *Xenopus* were immersed in MS-222 until reflexes ceased. The body cavity was opened, the liver removed and placed in ice cold media. It was cut into approximately 50 mg pieces, and each was placed into a well (24-well plate) containing 500 µl media. The media was changed on day 3, by removal of 400 µl, and replacement with fresh media. On day 6, the media was removed, and VTG and ALB were measured. The liver slice assay was performed a total of 6 times. The first time VTG was produced in response to estradiol (Figure 3.6). However, the second time, only 11 wells (out of 48) produced detectable levels of VTG, and even then only in very low concentrations (max. – 16 ng/ml, data not shown).

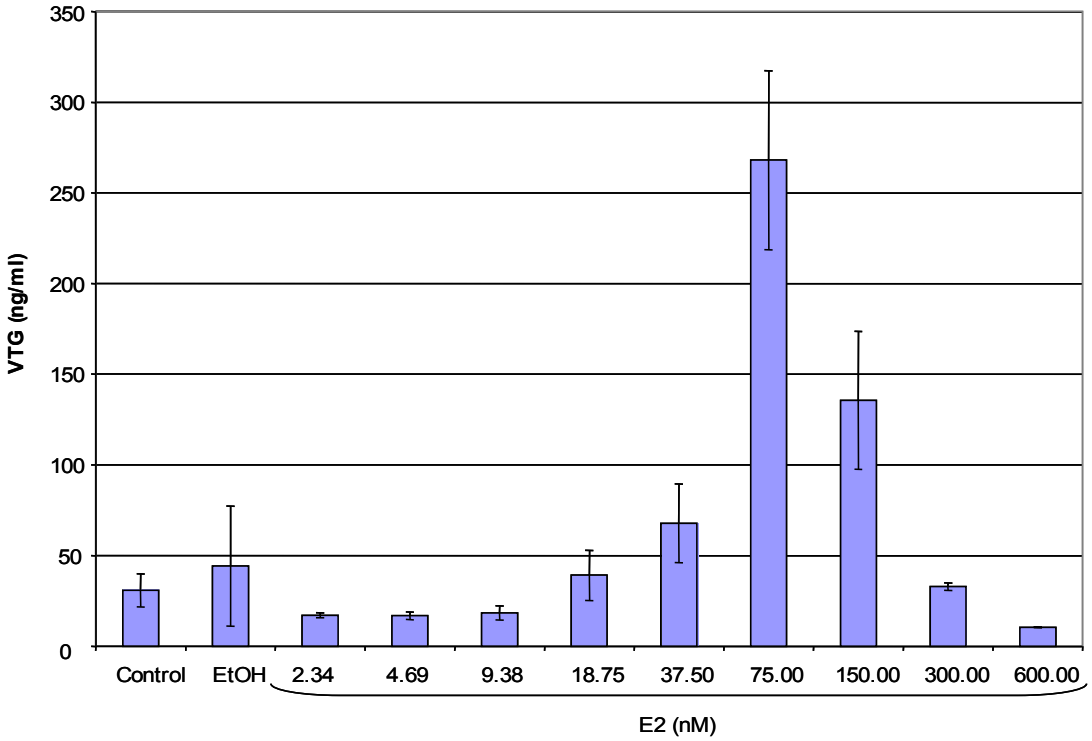


Figure 3.6. VTG production by liver slices when exposed to a range of estradiol concentrations (2.34-600 nM) or a control (control, ethanol control). Mean ± SE based on 3 replicate wells per experimental condition.

The third time, VTG was produced in a dose-dependant manner, although the control and ethanol wells also produced high levels of VTG (Figure 3.7, A). Normalisation of VTG by ALB (Figure 3.7, B) or by weight (Figure 3.7, C), did not reduce variability of the data. The relationship between the amount of albumin produced and the weight of the tissue was also weak (Figure 3.7, D). Due to the positive response caused by the control and ethanol control, glassware was solvent rinsed, and the experiment was repeated. In this case VTG produced in the control and ethanol wells was minimal, and dose-response curve was observed (Figure 3.8).

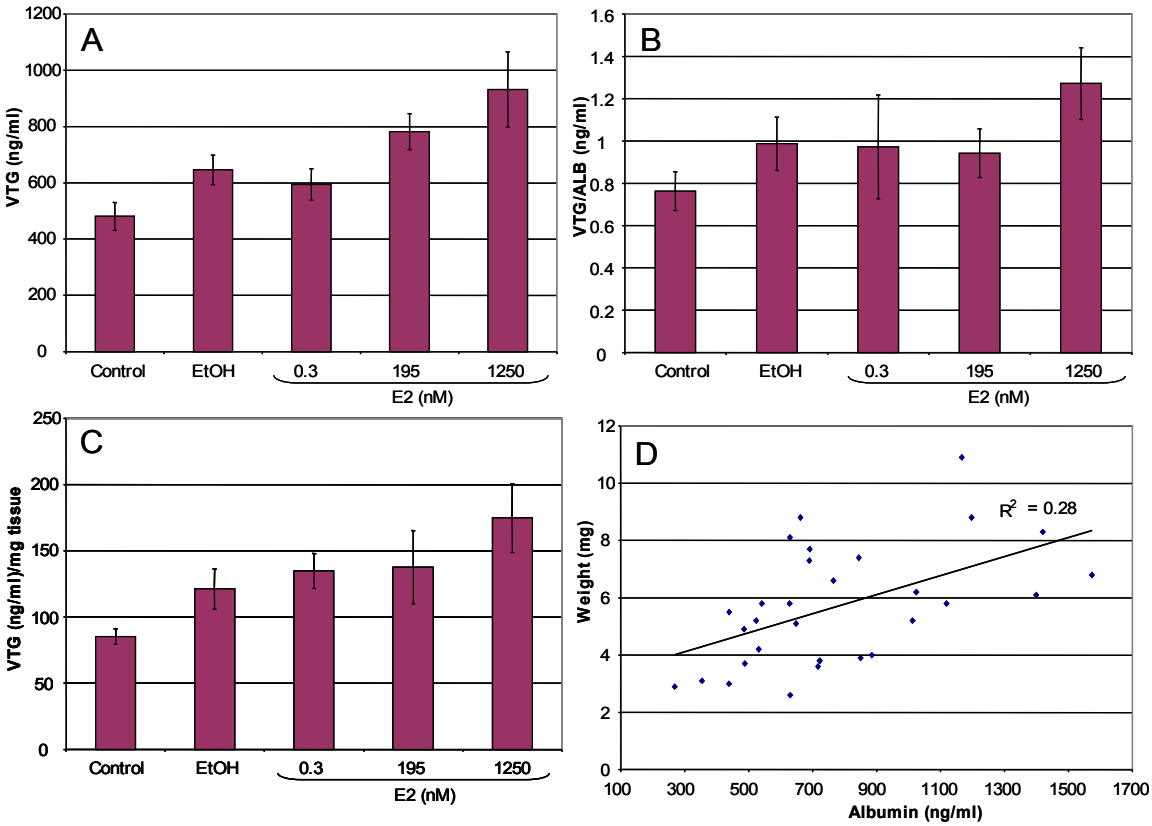


Figure 3.7. VTG production by liver slices when exposed to a range of estradiol (E2) concentrations (0.3, 195, 1250 nM) or a control (control, ethanol (EtOH) control). Values are mean ± SE based on 6 replicate wells. Panel A = VTG produced in response to estradiol; Panel B = VTG produced per ng/ml of albumin (ALB); Panel C = VTG produced per mg tissue; Panel D = relationship between ALB (ng/ml) and tissue weight (mg), each point represents one value.

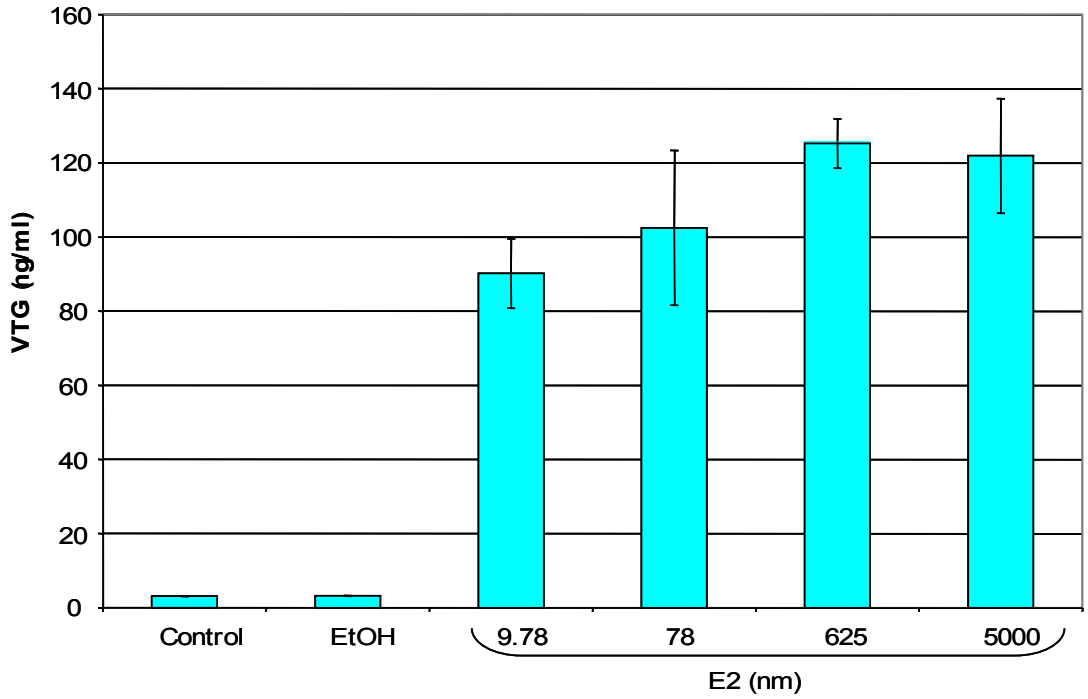


Figure 3.8. VTG production by liver slices when exposed to a range of estradiol concentrations (9.78-5000 nM) or a control (control, EtOH control) for 6 days. Values are mean ± SE, based on 4 replicate wells per treatment.

As a result of limited success in the optimisation experiments, environmental samples were tested three times, however, VTG was only produced at very low levels (< 6 ng/ml) in response to E2 in each experiment. Therefore, no further tests with this assay were undertaken.

3.3.2 GVBD

There were two distinct phases of optimisation; firstly, different media, collagenase, and methods were tested for increasing oocyte responsiveness and viability, and secondly, anti-androgenic compounds were tested for their effectiveness in inhibiting maturation both with and without pre-incubation steps.

3.3.2.1 Reagents

- Calcium Free Hanks O Medium (Hanks O: D.Pickford):

- Ca^{2+} -free, phenol red-free Hanks balanced salts solution diluted in 1.3L distilled water, buffered with HEPES 10 mM, pH 7.6 and supplemented with Polyvinylpyrolidone (1g/L) and Penicillin/streptomycin solution (5000 U/50 mg per ml). Sterilised by filtration (0.22 μm)

- Calcium Free Ringers Solution (RO: Jane Kirk, Cancer Research Institute, U.K.):
  - 825 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, buffered 10 mM HEPES, pH 7.6, penicillin/streptomycin solution (5000 U/50 mg per ml), sterilised by filtration (0.22 μm).

- Collagenase Solution:
  - Hanks O or RO supplemented with 0.2% collagenase (Type A, Roche Diagnostics, or Type II, Worthington Biochemical Corporation), sterilised by filtration (0.22 μm)

- Modified Barths Culture Medium (MBS: Jane Kirk, Cancer Research, UK):
  - 88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM Ca $(NO_3)_2$, buffered 10 mM HEPES, pH 7.6, Penicillin/Streptomycin (5000 U/50 mg per ml), sterilised by filtration (0.22 μm)

- Defined Nutrient Oocyte Media :
  - Amino Acids:
    - L-tryptophan (20 mg/L), L-lysine·HCl (280 mg/L), L-histidine·HCl (60 mg/L), L-arginine·HCl (140 mg/L), L-aspartic acid (Mg salt, 450 mg/L), L-threonine (70 mg/L), L-serine (240 mg/L), L-glutamic acid·HCl (1200 mg/L), L-proline (60 mg/L), glycine (60 mg/L) L-alanine (130 mg/L), L-valine (60 mg/L), L-methionine (50 mg/L), L-isoleucine (50 mg/L), L-leucine (80 mg/L), L-phenylalanine (50 mg/L), L-cysteine·HCl hydrate (20 mg/L), L-tyrosine (40 mg/L), L-cystine (10 mg/L), L-glutamine (80 mg/L).

- Salts:
    - NaCl (2820 mg/L), KCl (186 mg/L), Na_2HPO_4 (142 mg/L), $CaCl_2$ (111 mg/L), $MgCl_2 \cdot 6\ H_20$ (203 mg/L)
- Others:
    - Pyruvic acid (110 mg/L), oxaloacetic acid (152 mg/L), polyvinylpyrrolidone (500 mg/L), phenol red (10 mg/L), gentamicin (100 mg/L), D-Ca pantothenate (1 mg/L), choline chloride (1 mg/L), folic acid (1 mg/L), i-Inositol (2 mg/L), nicotinamide (1 mg/L), pyridoxal·HCl (1 mg/L), riboflavin (1 mg/L), thiamine·HCl (1 mg/L).
- Sterilised by filtration (0.22 μM), pH 7.6.

- Trichloroacetic Acid (TCA):
    - TCA – 5% in water.

- Methyl Green solution:
    - 0.4 % methyl green, 6 % Acetic Acid, 2 mM $CaCl_2$, in water

- Ethyl 3-aminobenzoate sulfonate salt (MS222):
    - MS222 (2g/L), buffered with $NaHCO_3$ (0.5 M), pH 7.4

3.3.2.2 Phase 1

3.3.2.2.1. Oocyte Preparation

Sexually mature female *Xenopus laevis* (Blades Biological, UK) were anaesthetised by submersion in MS222 until reflexes ceased, and were then sacrificed by pithing. The body cavity was opened, the ovaries were removed, and were placed in Hanks O or RO. They were cut into thin strips, rinsed several times, and placed into a collagenase solution for digestion of follicle cells. Follicle cells were removed to avoid confounding effects due to steroidogenic activity of follicle cells. The ovarian tissue was incubated on a gentle shaker at 22^0C, until follicle cells were no longer present (60-120 mins). The presence of follicle cells was tested with methyl green

solution, which stains the follicle cells but not the oocyte surface epithelium. Large, banded, preovulatory, stage VI oocytes, were then selected by hand, using a sterile Pasteur pipette, under a stereo dissecting microscope. After selection, they were placed in sterile 24-well polystyrene culture plates (20 oocytes per well) containing DNOM. Oocytes were incubated for in the presence or absence of steroids and/or antagonists for 20 hours. At the end of the incubation period, oocytes were fixed with TCA for observation of maturation and abnormality, which were calculated as a percentage of the total number of oocytes for each well.

3.3.2.2.2 Standard Curve

Oocytes were exposed to progesterone or testosterone (10, 100, 320, 1000 nM), media only, or an EtOH control. Therefore, hormone stocks were prepared in ethanol at a concentration of: 0.022 mM for 10 nM, 0.22 mM for 100 nM, 0.71 mM for 320 nM, and 2.22 mM for 1000 nM, and they were diluted 1000-fold in DNOM/MBS. 900 µl of this dilution was added to each well, along with 900 µl of DNOM/MBS, to make 1.8 ml per well, and a further 0.2 ml was added to the wells, along with oocytes from the petri dish, resulting in a final volume of 2 mls. This resulted in a final dilution of x2222 of each hormone stock, and 0.05 % EtOH in each well. Four frogs were tested at these concentrations of progesterone or testosterone (Figure 3.9). Testosterone was always a more potent initiator of maturation than progesterone within each frog. According to published literature, close to 100 % GVBD would be expected in response to 1000 nM of testosterone or progesterone (see Table 3.1), therefore, even at the highest concentration of hormone, only a sub-maximal response was observed (testosterone = 55%, progesterone = 40%).

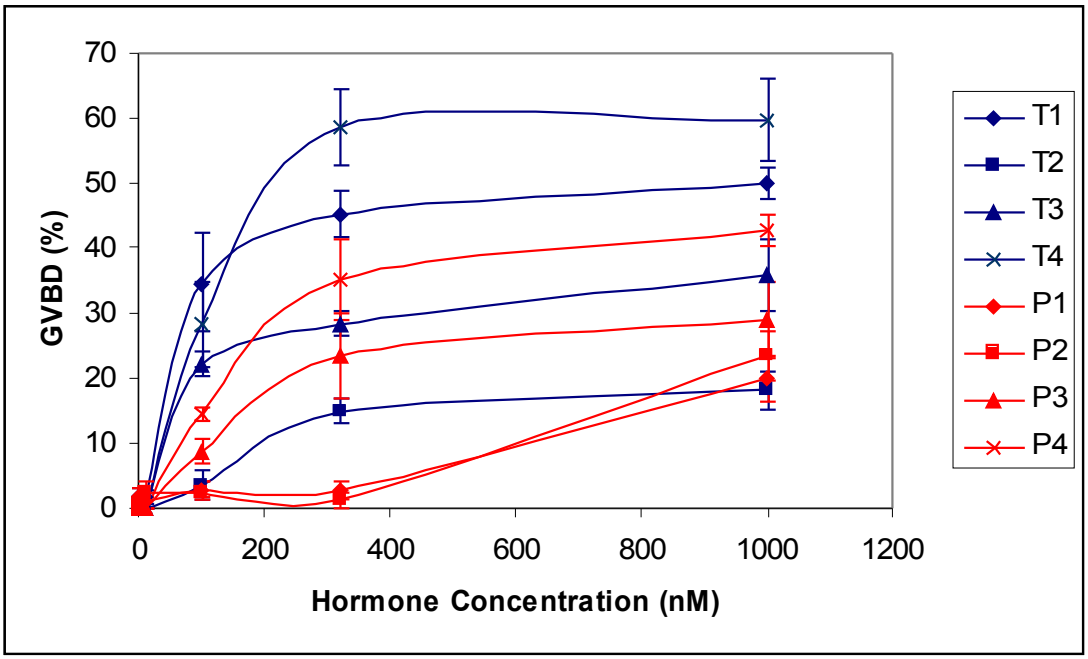


Figure 3.9. GVBD response of oocytes exposed to testosterone (T) or progesterone (P) over a range of concentrations (10, 100, 320 or 1000 nM), or a media only/EtOH control. Four frogs were used in four separate experiments, indicated by the number following either T or P (1, 2, 3 or 4). Values are mean ± SE, n = 6.

3.3.2.2.3 Assay Optimisation

Due to submaximal response, and in some cases, low viability (data not shown), the incubation temperature, and the type of collagenase (A, B, D & dispase – Roche Diagnostics, U.K.), used for digestion was altered. Dispase did not digest the ovary tissue sufficiently to remove follicle cells, even after 4 hours, so these oocytes were discarded. There was little difference in the percent maturation using collagenase A, B or D, but the percentage of abnormal oocytes differed (Figure 3.10). Based on these results, collagenase D was used in further experiments. Initially the digestion step was conducted at 18^0C, but it was observed that digestion occurred more quickly when the temperature was increased to room temperature (23^0C), and no adverse effects on the oocytes was observed (data not shown).

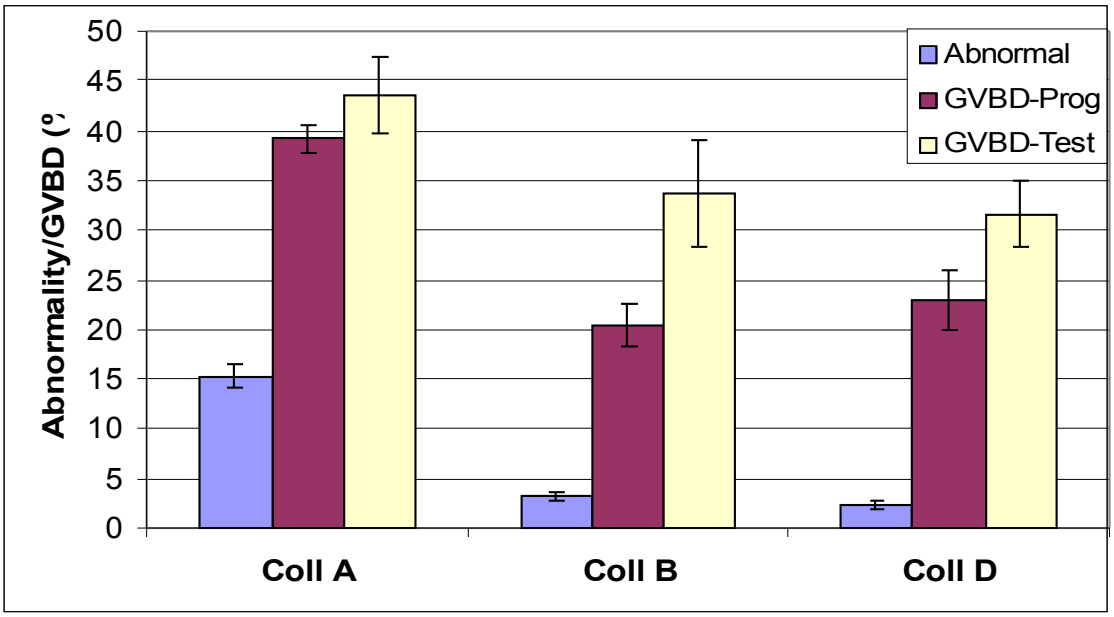


Figure 3.10. Effect of different collagenase (Coll) treatments (A, B, or D) on incidence of GVBD and abnormality in cultured *Xenopus* oocytes. GVBD response to progesterone (Prog) or testosterone (Test) at 1000 nM, and pooled abnormality values over a range of progesterone/testosterone concentrations (control, EtOH, and progesterone/testosterone at: 10, 100, 320 & 1000 nM). Values are mean ± SE, $n = 6$ for hormone treatments, $n = 60$ for abnormality values.

Due to low maturation and high abnormality, I contacted an expert on *Xenopus* oocyte maturation (Dr. Jane Kirk, Cancer Research U.K. (CRUK), Clare Hall Laboratories), and visited her laboratory to resolve experimental difficulties. A comparison of methods and reagents using oocytes from the same frog resulted in higher abnormality and lower maturation in original conditions compared to those used at CRUK (abnormality: ~50% compared to ~5%; GVBD: ~40% compared to ~90%). Various differences in methods were observed. Firstly, the preparation of the oocytes was distinct. At CRUK, glass petri dishes and rounded glass pasteur pipettes were used for tissue preparation, whereas in the original experimental design, plastic was used in both cases, which may have damaged the oocytes. Secondly, to reduce time spent in preparation of the oocytes for digestion with collagenase, a method of tearing ovarian lobes with forceps was employed at CRUK, whereas in the original experimental design, lobes were cut with microscissors, which resulted in more handling time, and possibly damage to the oocyte with the scissors. Lastly, at CRUK, the collagenase solution was replaced every hour, reducing possible impact of cell debris, whereas in

the original design they were left in the same solution for the entire incubation period. These aspects of the experimental design were altered in favour of methods used at CRUK, and reagents were then compared. The Ca^{2+}-free media (collagenase media) used at CRUK was RO, and that used originally was Hanks O solution. The type of collagenase was also distinct (CRUK – Type II; original – Type D), as was the incubating media (CRUK – MBS; original – DNOM). The experimental design for testing anti-androgenic effects involved maintaining viability of oocytes over 2 days, therefore the various media, and collagenase were tested on day 1 and day 2 (Table 3.2). The Ca^{2+}-free media Hanks O caused high abnormality in the oocytes and low maturation (Table 3.2), and collagenase Type D also caused higher abnormality than collagenase type II (Table 3.2). However, the effects of the incubation media were similar. Under optimal conditions (collagenase type II, RO, and MBS or DNOM), there was little difference in viability (abnormality) or maturation on different days. DNOM is a media designed for the long-term maintenance of *Xenopus* oocytes, which is not necessary in this case, therefore, it was decided that type II collagenase, RO, and MBS would be used in further experiments with *Xenopus* oocytes.

Day	Coll.	Ca^{+} -Free	Incubating	T. Conc.	Ab. (%)	GVBD (%)
1	D	Hanks	DNOM	0	6	0
				100	5	0
				1000	8	5.4
			MBS	0	8	0
				100	14	1.8
				1000	9	17.3
	II	Ringers	DNOM	0	0	0
				100	0	2.5
				1000	1	69.6
			MBS	0	0	0
				100	3	24.6
				1000	7	69.6
2	D	Hanks	DNOM	0	24	0
				100	27	0
				1000	19	14.0
			MBS	0	47	0
				100	49	9.1
				1000	46	28.2
	D	Ringers	MBS	0	0	0
				100	12	29.9
				1000	6	53.0
	½ D	Hanks	DNOM	0	1	0
				100	6	60.8
				1000	14	62.8
		Ringers	MBS	0	1	0
				100	1	23.2
				1000	5	65.4
	II	Ringers	DNOM	0	0	0
				100	2	2.6
				1000	1	76.2
			MBS	0	2	0
				100	2	9.1
				1000	2	63.9
		Hanks	DNOM	0	37	0
				100	43	3.6
				1000	44	0

Table 3.2. Comparison between reagents used for *Xenopus* oocyte culture on 2 days. Coll. = collagenase; T Conc. = testosterone concentration; Ab. = abnormality.

3.3.2.3 Phase 2

3.3.2.3.1 Anti-Androgen Treatments

The experimental design was based on first testing the responsiveness and viability of the oocytes by exposing them to a range of testosterone concentrations (10, 100, 320, 1000 nM), or a control (control, EtOH control) on day 1. Providing the abnormality observed was less than 5%, a submaximal concentration of testosterone was chosen to raise the background of GVBD, so that anti-androgenic effects could be observed. Two anti-androgens were possible candidates to be used as the positive control;

flutamide and R1881. In order to elucidate the optimal anti-androgen and testosterone concentrations, two R1881 and flutamide concentrations (0.5 and 1 µM) were co-incubated with a range of testosterone concentrations (10, 100, 320, & 1000 nM). Two pre-incubation times were tested for each concentration of anti-androgen, 1 hour (Figure 3.11) and 24 hours (Figure 3.12). After 1 hour pre-incubation, inhibition was observed when co-incubated with 100 µM of testosterone, but this effect was largely lost at higher concentrations. However, after pre-incubation for 24 hours, all the anti-androgenic treatments caused inhibition of GVBD at all testosterone concentrations. Flutamide was the strongest inhibitor of GVBD at 1 µM, and therefore was used as the positive control for GVBD inhibition in further experiments.

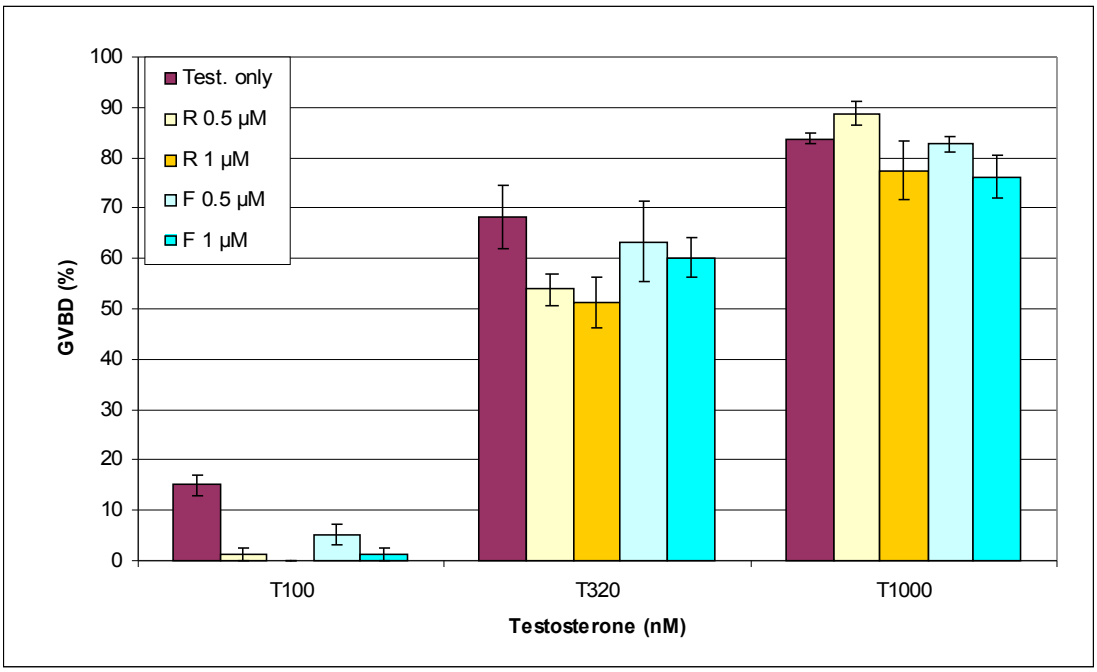


Figure 3.11. Pre-incubation with anti-androgens (R1881 (R) or flutamide (F)) for 1 hour, prior to co-incubation with testosterone (T: 100, 320 or 1000 nM). Test. only = testosterone only. Values are mean ± SE based on 4 replicate wells.

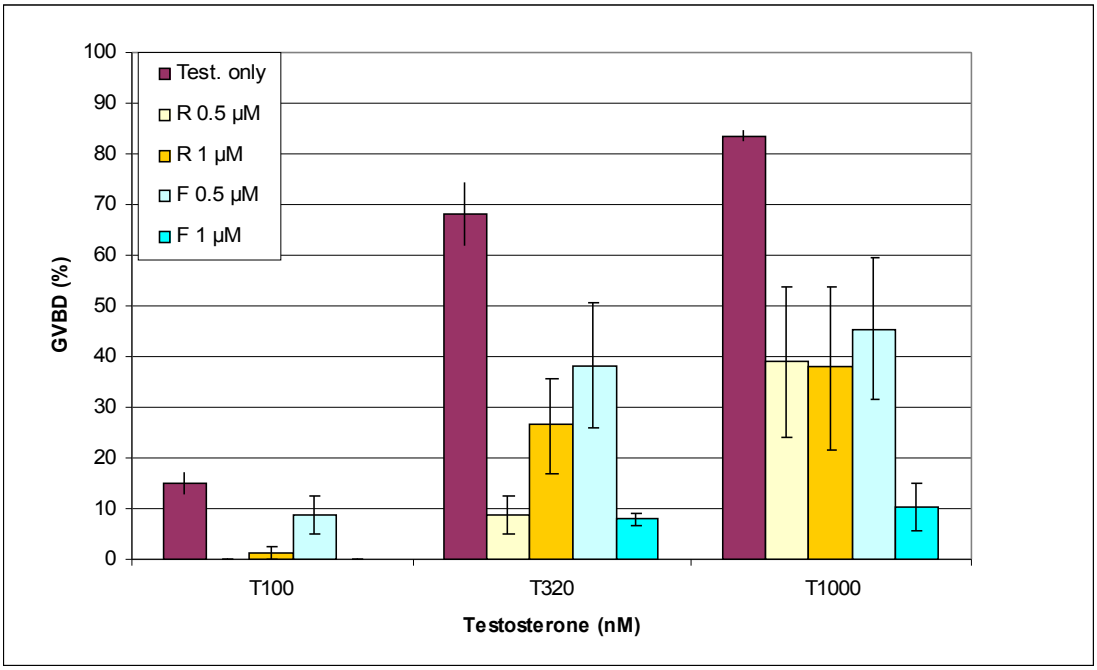


Figure 3.12. Pre-incubation with anti-androgens (R1881 (R) or flutamide (F)) for 24 hours, prior to co-incubation with testosterone (T: 100, 320 or 1000 nM). Test. only = testosterone only. Values are mean ± SE based on 4 replicate wells.

3.3.2.3.2 Flutamide Inhibition

Oocytes were exposed to a range of testosterone concentrations (10, 100, 320, & 1000 nM) for 20 hours on day 1. GVBD was observed in a dose-dependant manner (Figure 3.13), and abnormality was below 5% (data not shown). Therefore, unexposed oocytes of the same batch were exposed to a range of flutamide concentrations (5, 1, 0.1, 0.01 µM) on day 2, and were incubated for 24 hours. Since 100 nM (54% of full response) and 320 nM (90% of full response) both displayed submaximal responses, they were tested by co-incubation with each flutamide concentration on day 3. The background GVBD was higher in oocytes exposed to 320 nM testosterone, than 100 nM, and this resulted in a larger dynamic range of the flutamide dose-response curve (Figure 3.14).

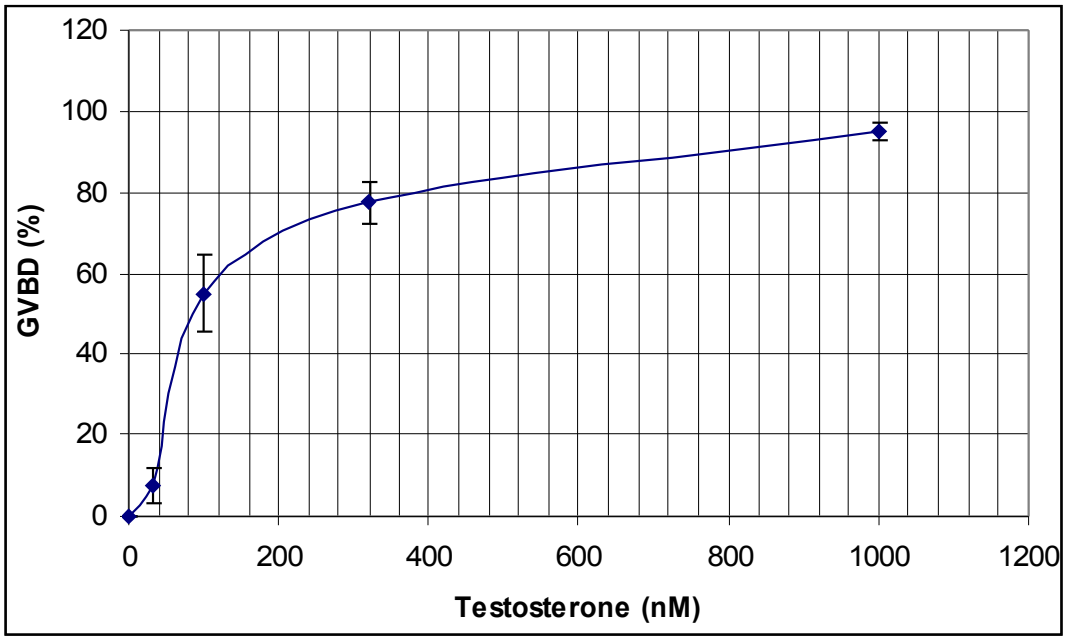


Figure 3.13. GVBD response of oocytes exposed to a range of testosterone concentrations (0, 10, 100, 320 & 1000 nM) for 20 hours (on day 1). Values are mean ± SE based on 4 replicate wells.

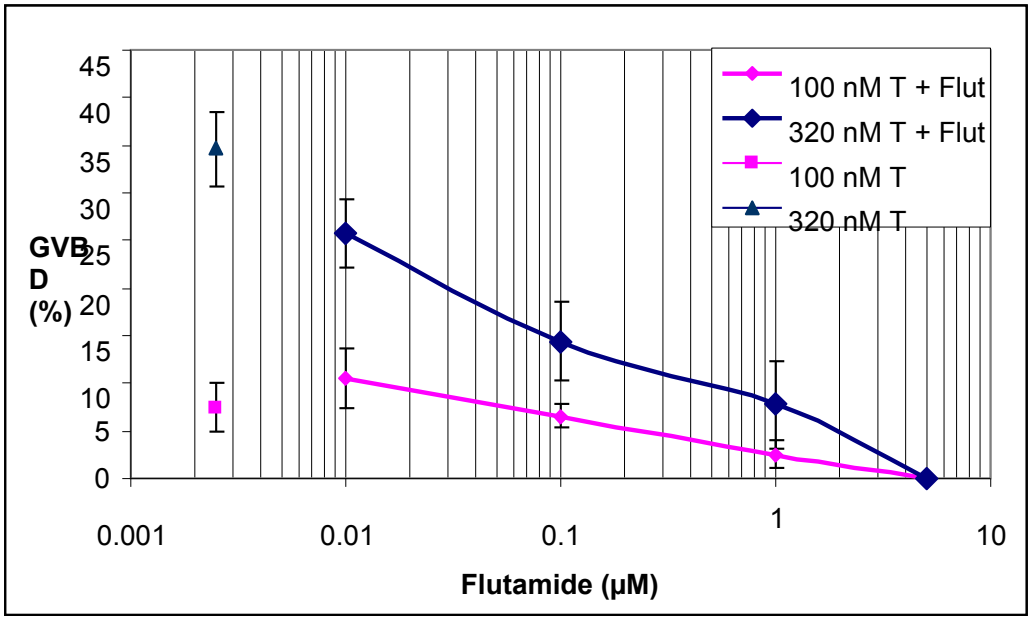


Figure 3.14. GVBD response of oocytes to a range of flutamide (Flut) concentrations (0.01, 0.1, 1, & 5 µM), pre-incubated for 24 hours on day 2, and co-incubated with either 100 or 320 nM testosterone (T) on day 3. Oocytes were also pre-incubated in media only, followed by addition of testosterone on day 3 for maximal response values (100 or 320 nM T). Values are mean ± SE based on 4 replicate wells.

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3.3.3 Ovulation

3.3.3.1 Oocyte Preparation

Sexually mature female *Xenopus laevis* (Blades Biological, U.K) were anaesthetised by submersion in MS222 until reflexes ceased, and were then sacrificed by pithing. The ovaries were removed and placed in a glass petri dish containing MBS and cut into pieces, the size of which was altered during the optimisation process. Ovarian tissue was incubated with human chorionic gonadotropin (HCG: 6.25, 12.5, 25, 50 I.U.), or media only, for 20 hours. At the end of incubation, media was extracted, frozen on dry ice, and stored at -80 until hormone analysis by radioimmunoassay (RIA: for description of methods see Chapter 4, section 4.2.5). In addition, the oocytes were tested for viability with Trypan blue (0.2%), fixed with TCA, and the number of ovulated oocytes were counted (i.e. the number of oocytes released from ovarian tissue). Ovulated oocytes were checked for GVBD to ensure that released oocytes had undergone maturation.

3.3.3.2 Optimisation

The proposed experimental design comprised of two steps: 1. a sensitivity-test of ovarian tissue; and 2. co-incubation with HCG and the test compound. The optimisation procedure comprised to 3 phases, the viability and responsiveness of ovarian tissue was tested over 2 days (phase 1), the optimal size of the tissue fragments was determined (phase 2), and the proposed positive control agent was tested (phase 3). In addition, the effectiveness of weight as a normalising factor on incidence of ovulation by tissue fragments was tested.

3.3.3.2.1 Phase 1

Ovarian fragments were exposed to a range of HCG concentrations (6.25, 12.5, 25, & 20 I.U.), or a media only control, for 20 hours, on day 1 or 2. Oocytes remained viable during the exposure time, as measured with Trypan Blue solution, and the ovulatory response was similar on days 1 & 2, although, it was slightly less on day 2 (Figure

3.15, A). In addition, weight was not effective at reducing variability of the data (Figure 3.15, B).

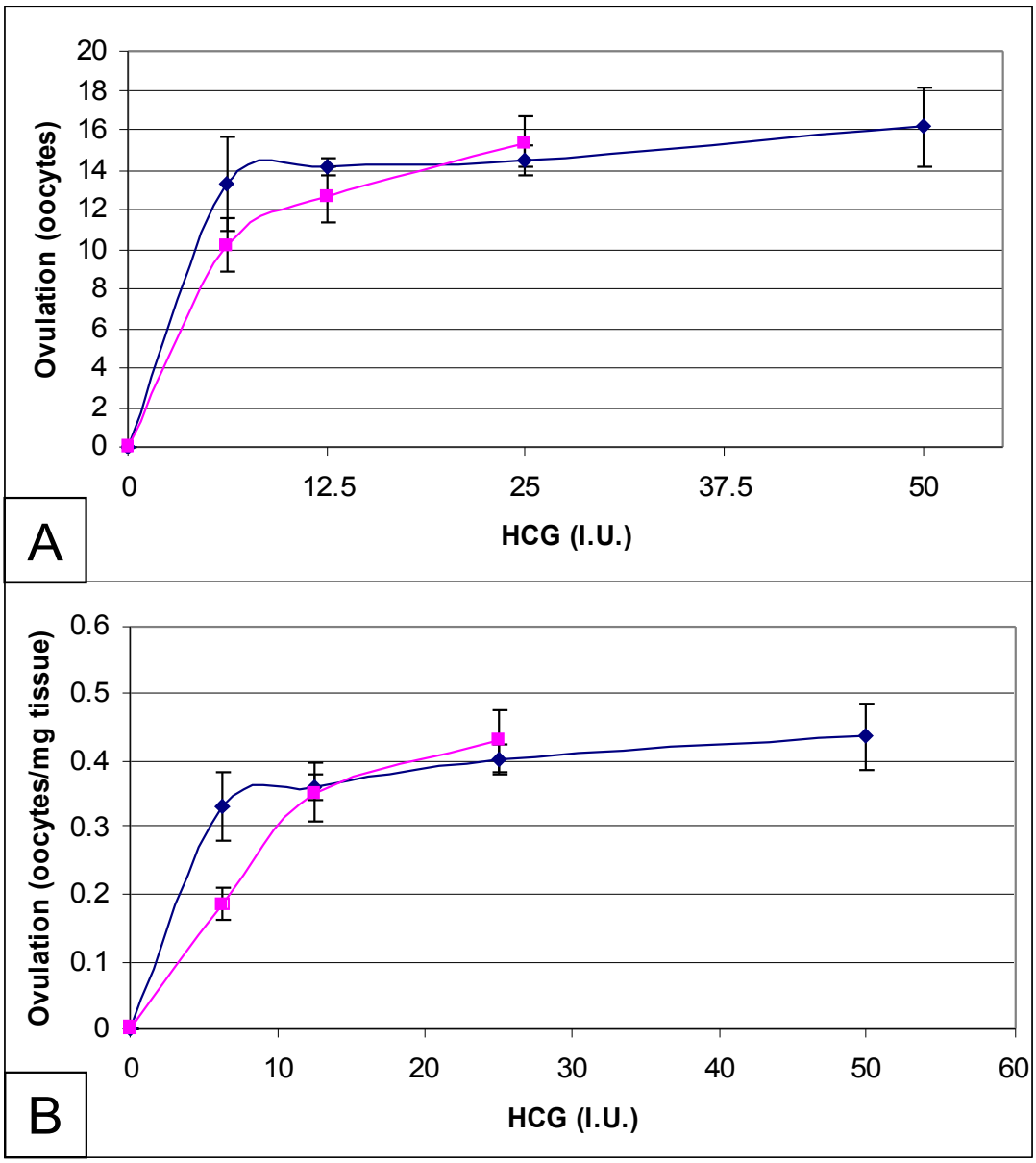

Figure 3.15. Ovulation of oocytes following exposure to HCG (6.25, 12.5, 25, or 50 I.U.) in MBS media for 20 hours, beginning exposure on day 1 or 2. Either ovulation values alone (Panel A), or per mg of tissue (Panel B). Tissue fragment size was 40-60 mg. Values are mean ± SE based on 6 replicate wells. ◆ = day 1, ■ = day 2.

3.3.3.2.2 Phase 2

The optimal tissue size, for hormone production and decreased variability, was assessed by identifying the straight portion of dose response curves of ovulation and

hormone production. The ovary was cut into pieces of tissue ranging from 10-113 mg, incubated with 3 concentrations of HCG (12.5, 25, and 50 I.U.) or a media only control, and ovulation (Figure 3.16) and hormone production (Figure 3.17-3.19), were measured. Ovulation (released oocytes) per mg of tissue decreased with increasing tissue size, irrespective of the HCG concentration used, as did testosterone (Figure 3.17) and progesterone (Figure 3.18) production. However, hormone production of the smallest tissue fragments was near the detection limit of the RIA. Ovulation was never observed in unstimulated wells, and hormone levels were not detectable. The straight portion of the dose-response curve occurred in tissue sizes < 60 mg in the ovulation (Figure 3.16), and testosterone (Figure 3.18) production, when oocytes were exposed to 12.5 or 25 I.U. HCG (but not 50 I.U.). For progesterone, the dose-response relationship was linear over the range of tissue sizes tested at all concentrations of HCG. The production of estradiol (Figure 3.19) followed the opposite pattern to the other parameters measured whereby estradiol production increased per mg of tissue as the size of the tissue increased. However, the straight portion of the dose-response curve also occurred in tissue size < 60 mg. Due to these results, two pieces of tissue of 30-40 mg per well were cultured in 62.5, 12.5, or 25 I.U. HCG, or in media only. The ovulatory response was measured, and progesterone, testosterone, and estradiol were measured in the culture media. Variability was decreased in ovulation, progesterone and testosterone, but not estradiol production (Figure 3.20). Hormone production was also sufficiently elevated for detection in the RIA, and values fell in the mid-range of the standard curve.

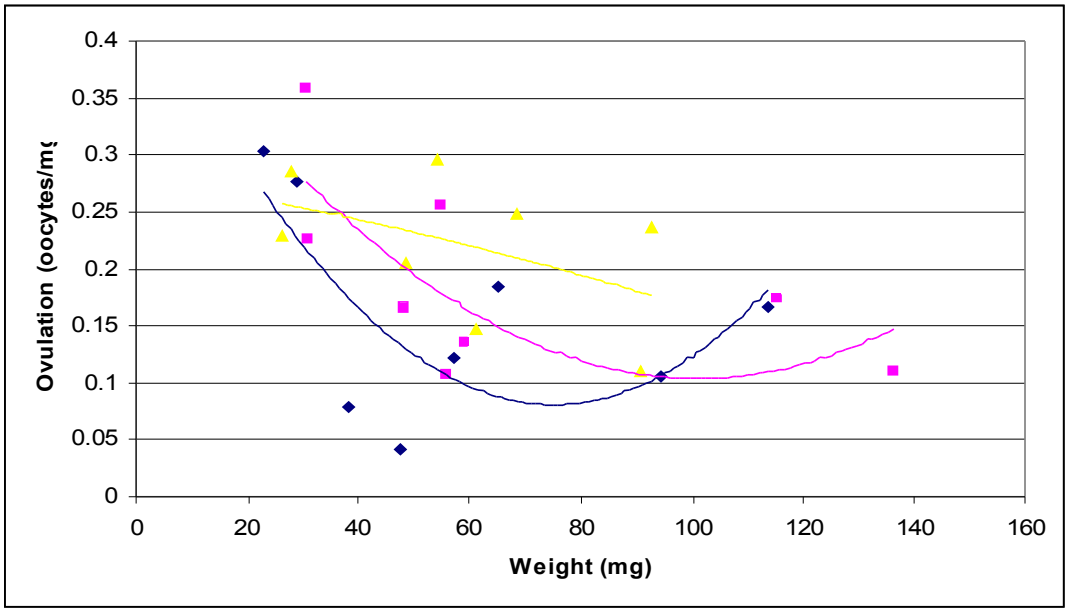

Figure 3.16. Ovulation (oocytes) per mg ovarian tissue in different tissue fragment sizes, exposed to a range of HCG concentrations (12.5, 25 or 50). Each data point represents one tissue fragment, 8 tissue fragments of ascending size were used per HCG concentration, and lines' are polynominal regressions. ♦ = 12.5 I.U., ■ = 25 I.U., ▲ = 50 I.U., of HCG

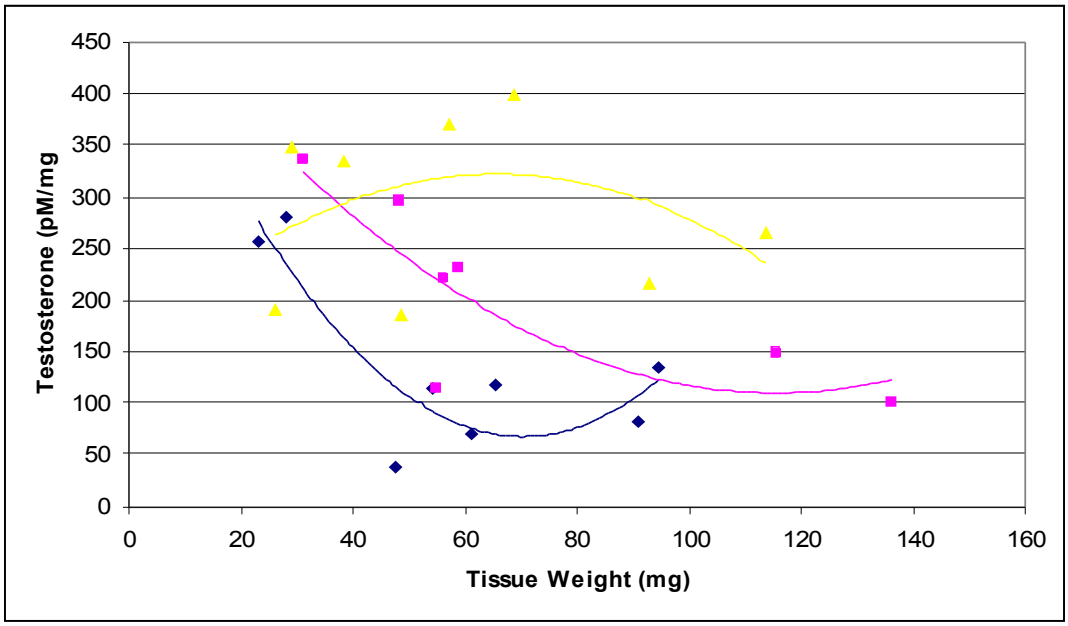

Figure 3.17. Testosterone production per mg ovarian tissue in different tissue fragment sizes, exposed to a range of HCG concentrations (12.5, 25 or 50). Each data point represents one tissue fragment, 8 tissue fragments of ascending size were used per HCG concentration, and lines' are polynominal regressions. ♦ = 12.5 I.U., ■ = 25 I.U., ▲ = 50 I.U., of HCG.

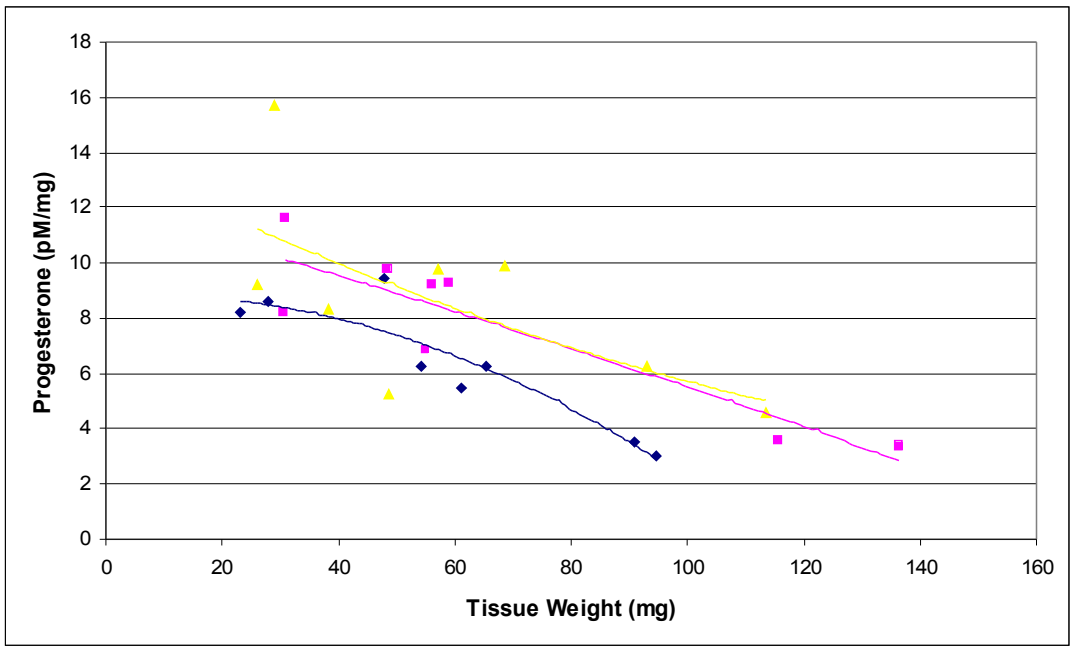

Figure 3.18. Progesterone production per mg ovarian tissue in different tissue fragment sizes, exposed to a range of HCG concentrations (12.5, 25 or 50). Each data point represents one tissue fragment, 8 tissue fragments of ascending size were used per HCG concentration, and lines' are polynominal regressions. ♦ = 12.5 I.U., ■ = 25 I.U., ▲ = 50 I.U., of HCG

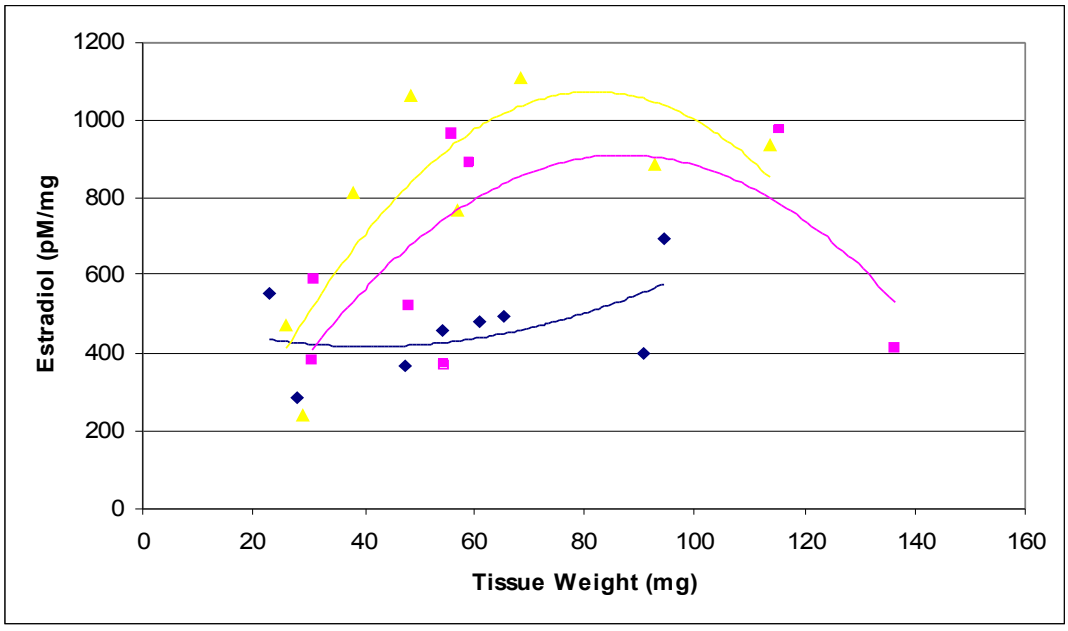

Figure 3.19. Estradiol production per mg ovarian tissue in different tissue fragment sizes, exposed to a range of HCG concentrations (12.5, 25 or 50). Each data point represents one tissue fragment, 8 tissue fragments of ascending size were used per HCG concentration, lines' are polynominal regressions. ♦ = 12.5 I.U., ■ = 25 I.U., ▲ = 50 I.U., of HCG.

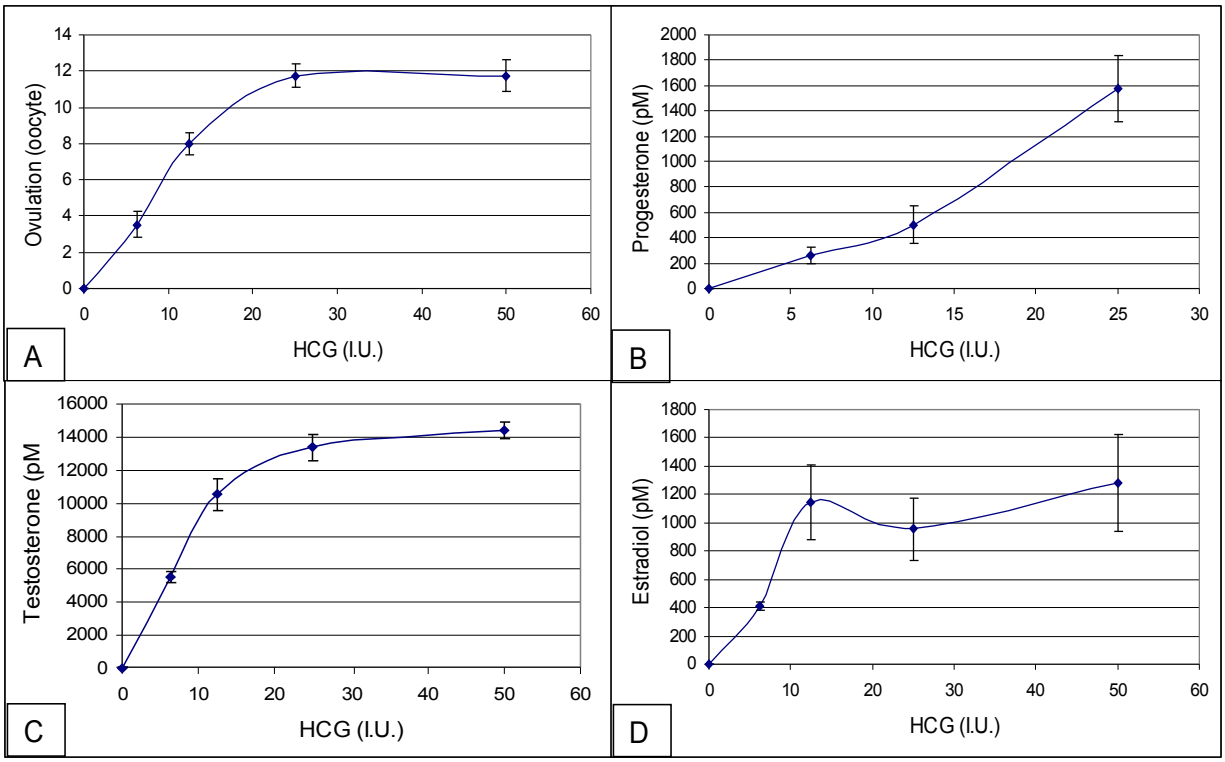

Figure 3.20. Ovulatory response (Panel A), and progesterone (Panel B), testosterone (Panel C), and estradiol (Panel D) production, by ovarian tissue (two fragments of 30-40 mg each) exposed to HCG (6.25, 12.5, 25, or 50) or a media only control (20 hours). NB: Progesterone production in response to 50 I.U. samples was lost. Values are mean ± SE based on 6 replicate wells.

3.3.3.2.3 Phase 3

The effectiveness of epostane as an ovulatory inhibitor was tested to determine it's applicability as a positive control for inhibition of ovulation. Two pieces of ovarian tissue (30-50 mg) were co-incubated with HCG, to induce ovulation, and a range of epostane concentrations (0.1, 1, 10, 100, 1000 and 5000 nM), to inhibit ovulation. A full dose-response curve was observed when exposed to 12.5 and 25 I.U., and a partial dose-response curve when exposed to 6.25 I.U. In addition, complete inhibition of ovulation was observed when tissue was exposed to 5000 and 1000 nM epostane (Figure 3.21). Variability was not reduced by normalisation with weight values, and therefore, these were not used in further experiments.

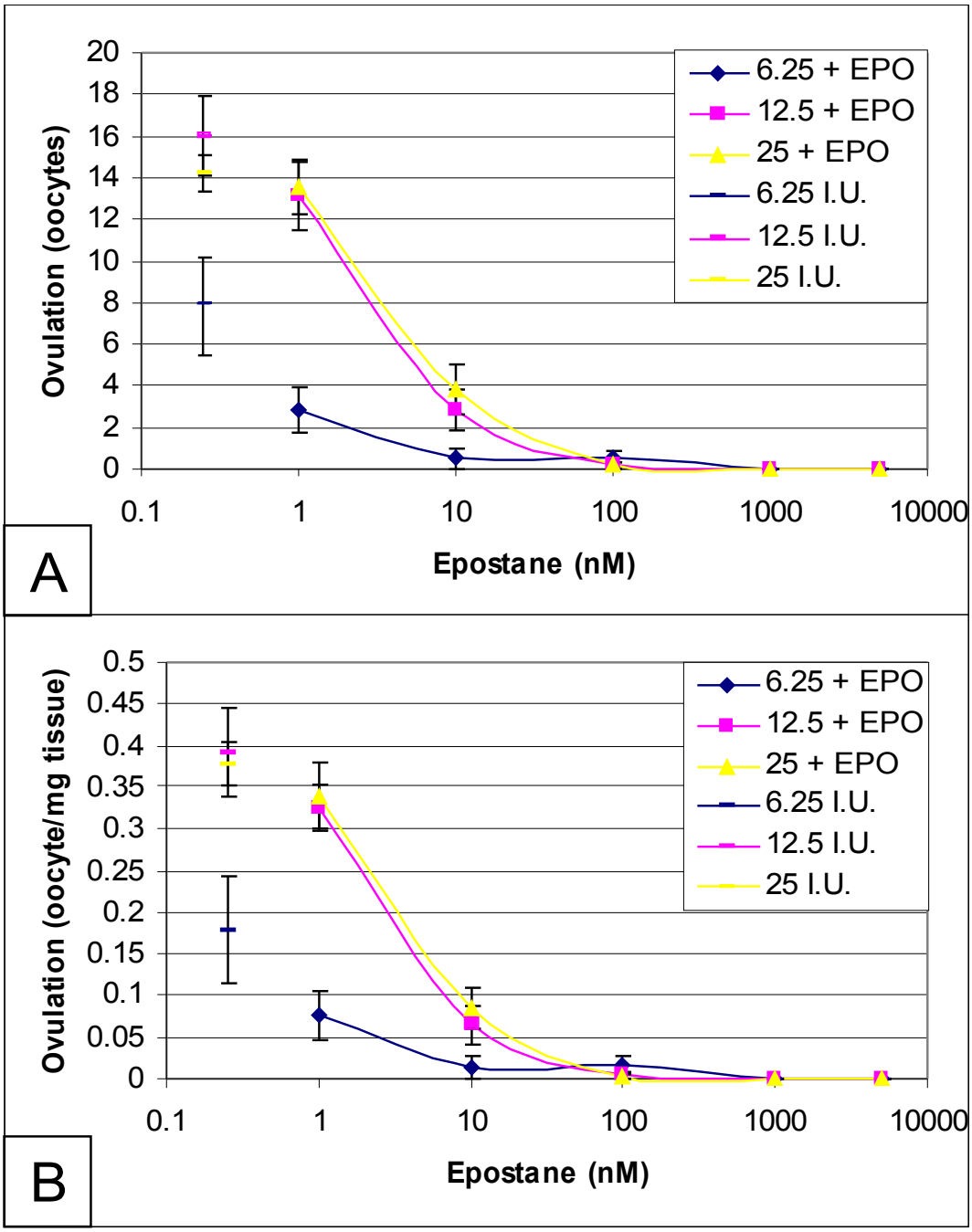

Figure 3.21. Ovulatory response of ovarian *Xenopus* tissue incubated with HCG alone (6.25, 12.5, or 25 I.U.), or co-incubated with a range of epostane (EPO) concentrations (1, 0, 100, 1000, or 5000 nM). Data was expressed as the number of oocytes ovulated (Panel A), or the number ovulated per mg ovarian tissue (Panel B). Values are mean ± SE, based on 6 replicate wells.

3.4 Discussion

Two novel assays for testing ED based on amphibian tissues were successfully developed and optimised for use (GVBD and ovulation), however, optimisation of liver cell preparations for use at Brunel University was not successful. In addition, despite successful optimisation of the GVBD assay as described here, when used for measuring effects of pesticides, results were highly variable and unreliable (2 replicate assays). Therefore, GVBD was not used for further analysis of compounds.

3.4.1 Optimisation Procedure

The problems encountered in use of the hepatocyte assay were not anticipated, as the author had performed this assay successfully during training with N.Mitsui (Towa-Kagaku, Japan). The reasons for failure of hepatocyte culture are unknown, but short initial training and a lack of on-hand expertise impeded the problem solving process. Indeed, frequent and detailed communication between myself and N.Mitsui did not isolate the cause of failure, and it became clear that it was not possible to problem-solve effectively without further training in the laboratory environment. In retrospect, it would have been better to take the test compounds to Japan for testing, where the assay was already set up for testing estrogenic and anti-estrogenic compounds. Alternatively, an expert at Brunel University, or longer initial training, may have resulted in successful hepatocyte culture. The rationale for addition of HEPES was to prevent acidosis of the incubating media, which did improve viability of hepatocytes. However, acidosis did not occur during training, indicating that an essential part of the hepatocyte preparation procedure was altered in some way. In addition, different centrifugation speeds were implemented, resulting in different cell compositions, as co-incubation of rat hepatocytes with parenchymal cells increased their viability . In addition, cells were seeded at different densities, as contact between cells is necessary to form a monolayer (N.Mitsui pers comm), but there is also a finite amount of nutrients and oxygen available to cells in culture. However, neither of these changes to the protocol appreciably altered the sensitivity of the hepatocytes to E2. The liver slice assay was based on methods used by Hurter *et al*., , and was initially more successful, but it was not reliable; and no results from the water extracts could be

obtained. Similar problems occurred initially when optimising the GVBD assay, whereby viability and responsiveness of oocytes was low. However, due to the experience with the liver cell preparations, laboratory training in the method was obtained, and this resulted in effective problem-solving. The cell culture conditions of ovarian fragments (for the ovulation assay) was based on findings from the GVBD optimisation procedure, and resulted in viable and responsive cells; and therefore no further optimisation of basic cell culture conditions was necessary. The assays are presented here in chronological order, and therefore, there was probably an influence of the authors' laboratory experience and training, on the success of the optimisation procedure. Although it is also true that the assays decreased in complexity over time, and it may have been prudent to begin with the assay requiring the least laboratory skill (ovulation), and proceeding to the GVBD assay, and finally the hepatocyte assay.

3.4.2 GVBD Optimisation

After initial optimisation, which was concerned with basic cell culture conditions, the assay was optimised for detecting anti-androgenic activity. Two anti-androgenic compounds were tested for suitability as the inhibitory control, and different pre-incubation exposure periods were investigated. Both R1881 and flutamide were previously shown to inhibit testosterone induced GVBD (Lutz *et al*., 2003), and were tested at two concentrations. Testosterone has higher affinity for the AR than R1881 or flutamide (Lutz *et al*., 2003), and once maturation has been initiated, steroid stimulation is no longer necessary, which can be achieved in as little as five minutes incubation with the initiator (Schuetz, 1967). Therefore, it was decided that incubation with the anti-androgen prior to addition of testosterone would result in a more effective inhibition of GVBD, and therefore make the assay more sensitive. Both the anti-androgens were more potent inhibitors of GVBD after 24 hours than 1 hour, but flutamide was more effective than R1881. The increased potency of flutamide after 24 hours pre-incubation was likely due to testosterone binding with higher affinity than flutamide to the receptor, and effectively out-competing flutamide when only 1 hour pre-incubation occurred (Ankley *et al*., 2004). In addition, the anti-androgen was most effective if the background GVBD was raised to 80% (320 nM testosterone) of the maximal response (1000 nM testosterone). Therefore, to ensure a sufficient dynamic

range of the assay, and to maintain it's sensitivity to weak anti-androgens, it was decided that a concentration of 70% of the maximal response on day 1 would be optimal for testing xenobiotics. Unfortunately, as mentioned above, testing of pesticide resulted in highly variable and unreliable results. Perhaps repeating assays at an earlier stage of optimisation would have identified this problem prior to testing pesticides

3.4.3 Ovulation Optimisation

Due to optimisation of culture conditions for GVBD, no alteration to cell culture conditions was necessary for the ovulation assay. However, response of ovarian fragments was highly variable. Although this would be expected between individuals due to the endogenous hormone environment , high variability was observed between wells from the same frog. In an attempt to reduce this variability, data were initially normalised using the weight of each individual fragment; however, no difference in variability could be observed. Therefore, the effect of fragment size on ovulation and hormone production was investigated. The original fragment size (50-60 mg) was discovered to reside in the curved section of the dose response curve. That may have led to variable results, as marginally different fragment sizes would have resulted in different fragments falling on different standard curves. Therefore, fragments were sized to lie on the first half of the graph (30-40 mg), as this was the steeper slope, and more hormone was being produced per mg of tissue. Two fragments were also placed in each well, to ensure sufficient hormone levels were produced to allow detection if inhibition of steroidogenesis occurred. This resulted in substantially decreased variability. In addition, the 3β-HSD inhibitor, epostane, was shown to be an effective inhibitor of ovulation and was used as the inhibitory control.

3.4.4 The role of Androgens in the Ovary

Data presented here provides supporting evidence of a prominent role of testosterone in GVBD and ovulation of the amphibian oocyte. Firstly, testosterone was always a more potent initiator of GVBD than progesterone. Secondly, flutamide and R1881 effectively inhibited testosterone induced GVBD (as previously reported in Lutz *et*

al., 2003), indicating that testosterone binds to a receptor on the oocyte, which initiates ovulation. Thirdly, patterns of ovulation and testosterone production by ovarian fragments were similar over two different experiments (see Figures 3.16/3.17 & 3.20). The pattern of production was also suggestive of conversion of progesterone to testosterone, as progesterone levels were low when testosterone was increasing, and increased sharply when testosterone levels reached a plateau (see Figure 3.20). Interestingly, it was previously reported that stimulation of ovarian tissue with frog pituitary homogenate increased metabolism of progesterone to other products (Snyder and Schuetz, 1973), although the authors did not identify these compounds. Finally, testosterone production was approximately 10-fold higher than progesterone production (as previously reported in El-Zein *et al*., 1998), and Jacobelli *et al*. (1974). Furthermore, Masui and Mackert reported that injected progesterone did not induce maturation, whereas testosterone injection did (Jacobelli *et al*., 1974). Interestingly, exposure of ovarian fragments to 12.5 I.U. HCG resulted in production of 10 nM testosterone, 0.5 nM progesterone, and ovulation/GVBD of oocytes. The level of testosterone produced was approximately 10-fold lower than levels required to initiate GVBD in denuded oocytes by exogenous administration (100 nM testosterone), indicating that other factors may be involved. Both progesterone and testosterone levels are elevated after stimulation and El-Zein *et al*. (1988) reported that it was necessary for both progesterone and testosterone to be elevated to stimulate ovulation. Therefore, since various steroid hormones are capable of initiating maturation at high concentrations, and progesterone is a precursor of testosterone, the optimal condition for GVBD and ovulation may be a combination of these hormones.

Chapter 4

Pesticide-induced Endocrine Disruption

4.1 Overview

The potential for agricultural chemicals to cause ED is an increasing concern, both in humans and in wildlife . The organochlorine DDT was the first pesticide to be identified as an ED , and to date, the majority of published research on pesticides and ED concerns organochlorine pesticides. However, although these chemicals are environmentally important because of their persistence and ability to bioaccumulate, their use has been largely superseded by less persistent pesticides (see Appendix 1), of which much less is known. Indeed, plant growth regulators/herbicides form the largest group of pesticides, and made up 44 % of the world market in agricultural pesticide use in 2000 and 2001. In comparison, 28 % were insecticides, 19% were fungicides, and 9 % were other pesticides . To reflect this pattern of usage, eleven herbicides (MCPA (4-chloro-2-methylphenoxy acetic acid), mecoprop, 2,4,-D (2,4-dichlorophenoxy acetic acid), bentazone, isoproturon, diuron, linuron, simazine, atrazine, trifluralin, chlorpropham), two insecticides (sulcofuron and flucofuron), one fungicide (tecnazene), and one general use biocide (PCP: pentachlorophenol) were tested for endocrine disrupting activity. EDs have many potential targets in the organism (see Chapter 1, section 1.3), however, current evidence primarily supports agonism/ antagonism of nuclear hormone binding to receptors, and to a lesser extent stimulation/inhibition of enzymes involved in steroidogenesis. Therefore, estrogenic/androgenic and anti-estrogenic/ androgenic activity was tested in a recombinant yeast assay, and disruption of the steroidogenic pathway was tested using cultured *Xenopus* oocytes. Although *in vitro* tests are limited in predicting effects *in vivo*, they can be useful for indicating which compounds require further testing, and can also assist in elucidating mechanism of action . Therefore, the predictive ability of these screens was tested with the most active pesticide identified *in vitro* (PCP), by means of a short *in vivo* test using adult *Xenopus*.

4.2 Introduction

To date, most *in vitro* tests for endocrine disruption have focused on agonism/antagonism of hormone receptors, with estrogen receptor agonism and androgen receptor antagonism being the most common (Ashby, 2000), and agrochemicals and industrial chemicals are often demonstrated to possess receptor mediated estrogenic and/or anti-estrogenic and/or anti-androgenic activity . Some of the pesticides used here have previously been tested for these activities (see Chapter 1, section 1.5), however, for the majority it is the first time they have been tested for androgenic and anti-estrogenic activity. The recombinant yeast used here have been transfected with the human estrogen receptor or the human androgen receptor, and the screen is highly sensitive, reproducible and rapid (Routledge and Sumpter, 1996). Disruption of the steroidogenic pathway is a less well researched area, but is increasingly gaining attention as an important target for EDs . Ovulation is an environmentally relevant endpoint, and has the potential to have direct effects on the reproductive capability of individuals. It has also previously been shown to be a sensitive endpoint to pesticide exposure . Many steroid hormones can induce ovulation in amphibians, but progesterone and testosterone are generally recognised as being the most potent (Jacobelli *et al*., 1974; Smith and Ecker, 1971), whereas estradiol inhibits maturation . Therefore, it is expected that alterations in steroidogenic enzyme activity and the corresponding changes in hormone levels, will in turn affect maturation and ovulation of the oocyte (Masui, 1967; Snyder and Schuetz, 1973). Furthermore, measurement of hormone levels in the ovulation media can assist in identifying which enzymes are being affected by the treatment (e.g. Ahn *et al*., 2007).

In the UK, herbicides were widely used and were found frequently at low concentrations in the environment, whereas other types of pesticides tended to be found less frequently, but at higher concentrations (see Appendix 1). The phenoxy herbicides MCPA, 2,4,-D, and mecoprop, and the substituted-urea herbicides diuron, linuron and isoproturon, were the highest ranked pesticides in the U.K. (Mecoprop-1st, MCPA-4th, 2,4,-D-5th, diuron-2nd; linuron-24th; isoproturon-3rd). In addition, 2,4,-D was the 5th most commonly used pesticide in the U.S. in 2001 . The former are synthetic auxins, which are used for post-emergence control of weeds in a wide variety of applications, including in cereals, maize, grassland, under fruit trees, on roadside verges and in the aquatic environment (Tomlin, 2006). The latter function by inhibiting photosynthetic electron transport and are used for pre- and post- emergence

control in vegetable and wheat crops (Tomlin, 2006). Despite the widespread use of these pesticides, information pertaining to their endocrine effects, if any, is scarce. MCPA caused a loss of maturing spermatids, and slight testicular degeneration in the seminiferous tubules of rats dosed with 112 mg/kg/day . In addition, newts exposed to 800 mg/L displayed increased hepatic enzyme activity in females but not males , although, these exposure levels and concentrations are much higher than those found in the environment. Mecoprop had a slightly toxic effect on murine embryos exposed to 0.5 µg/L , but was not reported to have estrogenic or anti-estrogenic activity in a reporter cell line . 2,4,-D was reported to decrease circulating thyroxine levels in ewes exposed to 30 mg/kg/week for 5 weeks, although estradiol, cortisol and LH levels were unaffected . In addition, rats exposed to a chemical formulation containing 2,4,-D (Tordon 75D®) displayed testicular germ cell depletion, shrunken tubules, and a decrease in teste weight, but only in animals exposed to the highest dose (150 mg/kg body weight) for 9 weeks . Circulating testosterone levels were not depleted, possibly suggesting a direct toxic effect on the teste, rather than interference with the hypothalamo-pituitary-gonadal axis. *In vitro*, *Xenopus* oocyte maturation was irreversibly inhibited by exposure to 10 mM 2,4,-D , although this is an extremely high concentration (corresponds to 2.2 g/L). Diuron had no effect on fish testicular or ovarian steroidogenesis *in vitro* at 0.1 or 1 mM . In addition, no effect was observed on prostate 5α-R activity up to 100 µM , or placental aromatase activity up to 50 µM , in human tissue homogenates. It also did not interact with the estrogen receptor , but was weakly anti-androgenic in a reporter gene assay (8.7 µM caused 20 % inhibition of the maximal androgenic response; Kojima *et al*., 2004). Considering its weak/absent endocrine effects *in vitro*, perhaps it is not surprising that diuron had minimal effects on reproductive parameters in rats exposed *in vivo* . At similar concentrations, linuron also did not interact with the estrogen receptor (Vingaard *et al*., 1999; Kojima *et al*., 2004), and did not inhibit placental aromatase acitivity (Vingaard *et al*., 2000). However, linuron was approximately 3 times more anti-androgenic than diuron , and inhibited prostate 5α-R activity by 50% at 86 µM (Lo *et al*., 2007). Several studies have shown anti-androgenic activity of linuron on reproductive parameters in rats *in vivo*, and the mechanism of action is hypothesised to be at least partly via antagonism of the androgen receptor . Anti-androgenic activity has also been demonstrated in an aquatic exposure study, whereby inhibition of spiggin production in female Stickleback co-exposed to 150 µg/L linuron and

methyltestosterone was reported . However, in the latter study, no effect was observed at 15 µg/L nominal levels, although measured values were lower (3.4-12 µg/L), they were still higher than the maximum detected concentration in the UK in 2004/2005 (1.4 µg/L). Isoproturon is the least well studied of the phenoxy herbicides, and to the author's knowledge, in just one publication has this chemical been tested for endocrine effects *in vitro*. In this study, no estrogenic or anti-estrogenic activity was observed in a reporter cell line up to 30 µM . Isoproturon has been reported to inhibit spermatogenesis at 400-800 mg/kg , and to reduce activity of testicular 3α-HSD (Sarkar *et al*., 1997), and liver detoxification enzymes in rats. Moreover, in *Bombina bombina* (fire-bellied toad) tadpoles exposed to 0.1-100 µg/L isoproturon, it has been shown to cause mortality and developmental deformities .

The triazine herbicides, atrazine and simazine, were the next most highly ranked pesticides in the U.K. (simazine-6th, atrazine-7th), and atrazine was the second most commonly used pesticide in the US in 2001 (Kiely, 2004). They also function by inhibiting photosynthetic electron transport and are used for pre- and post- emergence control in maize, sugar cane, pineapples, grassland and for industrial weed control (Tomlin, 2006). Atrazine and simazine do not interact with the estrogen or androgen receptor , but they have been shown to affect steroidogenesis . Aromatase upregulation was observed in human adrenocortical carcinoma, and placental choriocarcinoma cells at between 0.3-30 µM . However, upregulation could not be detected in amphibians or reptiles *in vivo* . Atrazine also significantly inhibited 5α-R, 3αHSD, and 17βHSD activity in cultured rat pituitary cells when they were exposed to 0.92 µM, and a similar effect was observed in the hypothalamus of rats exposed *in vivo* (12mg/100g b.w./day for 7 days, Babic-Gojmerac *et al*., 1989). Furthermore, 5α-R activity was inhibited in fish testicular homogenate exposed to 100 µM atrazine (Thibaut and Porte, 2004), and atrazine inhibited adrenocorticotropin hormone (ACTH) stimulated cortisol secretion in adrenocortical cells of *Onchorhynchus mykiss* at 0.005-5 µM (Bisson and Hontela, 2003) and *Rana catesbeiana* at 10-100 µM (but not *Xenopus laevis,* Goulet and Hontela, 2003). Atrazine has also been reported to cause endocrine disruption in amphibians via analysis of gonadal histology at environmentally relevant concentrations . The diazinone herbicide bentazone also inhibits photosynthetic electron transport, and is used on cereals, maize, and peas (Tomlin, 2006). It was ranked 15th in the UK (see Appendix 1), and was the pesticide

most commonly found above 0.1 µg/L in groundwater in 2005 . However, no information related to its EDg potential was available.

The dinitroaniline herbicide trifluralin and the carbamate herbicide chlorpropham both function by inhibiting cellular microtubule assembly. Trifluralin is used for pre-emergence weed control in many vegetables, and is often used in combination with linuron or isoproturon in winter cereals (Tomlin, 2006). It was the 12th most commonly used pesticide in the US in 2001 (Kiely, 2004), and was ranked 28th in the UK (see Appendix 1). Trifluralin decreased thyroxine, increased estradiol, and decreased LH concentrations in ewes exposed to 35 mg/kg/week for 5 weeks (Rawlings *et al*., 1998). However, it did not interact with the estrogen or androgen receptor *in vitro* , therefore, the mechanism of action is unknown. Chlorpropham constituted 91% of the total tonnage used to prevent sprouting in stored potatoes in 2002 , and was ranked 16th in the U.K. (see Appendix 1). Chlorpropham was not active on the estrogen or androgen receptor , and had little effect on reproductive parameters in rats . The fungicide tecnazene inhibits lipid peroxidation and is also used as a sprout suppressant on stored potatoes (Tomlin, 2006). It was found rarely (4 times), but at up to 0.21 µg/L, which was the highest concentration of the fungicides (see Appendix 1). To the author's knowledge, no information pertaining to its ED effects have been published.

The urea insecticides sulcofuron and flucofuron (mitins) inhibit digestion in wool-feeding insect larvae, and are used as mothproofing agents (Tomlin, 2006). Discharge of mothproofing chemicals from the textile industry are frequently the cause of water quality failures in the UK , and although the mitins are reportedly not used in moth-proofing at present, they were detected in freshwaters. Indeed, they were among the highest ranked insecticides (33rd & 41th), and although detected rarely (4 & 6 times in 2004/2005), they had the highest median concentration of all insecticides (0.545 & 0.255 µg/L). To the author's knowledge, no reports pertaining to ED activity of these insecticides was available.

The general use biocide PCP functions by uncoupling oxidative phosphorylation. It was the most potent pesticide tested *in vitro*, and therefore, was also tested *in vivo*. It is used as an insecticide, fungicide, and herbicide and thus has a wide range of

applications, agriculturally, and industrially, but its primary use is to protect timber from wood-boring insects and fungal rots (Tomlin, 2006). In spite of it's restricted use in Europe since 1991 , it was ranked 12th in the U.K., and was detected 93 times at a median concentration of 0.16 µg/L, and the maximum detected concentration was 2.74 µg/L. Assuming use of PCP is minimal, it's high ranking may have been partly due to metabolism of hexachlorobenzene and hexachlorocyclohexane isomers (e.g. lindane), resulting in environmental PCP . Alternatively, it may have been via improper disposal of left-over stocks. PCP has previously been reported to have anti-estrogenic activity *in vitro* (50% inhibition at 1.6 µM-Jung *et al*., 2004, 80% inhibition at 3 µM-Lemaire *et al*., 2006), although estrogenic and anti-androgenic activity were not observed , and it had no effect on aromatase activity (Vinggaard *et al*., 2000). In a series of publications, Beard and Rawlings have demonstrated various reproductive effects in mammals, for example, decreased whelping rate in mink , increased severity of oviductal intraepithelial cysts in adult ewes , and seminiferous tubule atrophy in rams . Interestingly, serum hormone and gonadotropin levels were unaffected in these studies, suggesting a direct toxic effect on reproductive tissues. Indeed PCP has been shown to inhibit ovulation of Zebrafish oocytes *in vitro* at > 0.6 µM (Tokumoto *et al*., 2005), and to cause toxicity in rat sertoli cells at 10 nM, which was the lowest concentration tested . Furthermore, PCP reduced the number of eggs laid, and their subsequent hatching rates in Japanese medaka, and induced formation of testis-ova . Finally, a link between PCP concentration and follicle stimulating hormone in women with gynaecological dysfunction has been reported .

4.2 Methods

4.2.1 Chemicals

17β-estradiol (> 98% pure), testosterone (> 98% pure), progesterone (> 99% pure), flutamide (98% pure), 4-hydroxytamoxifen (> 98% pure), and all pesticides (> 97% pure) except sulcofuron and flucofuron were obtained from Sigma Chemical Company Ltd. (Dorset, UK). Sulcofuron (> 99% pure) and flucofuron (> 97% pure) were obtained from Greyhound Chromatography and Allied Chemicals (Wirral, UK). Cell culture media components, radioimmunoassay buffer components, and human

chorionic gonadotropin (HCG) were purchased from Sigma (Dorset, UK). Epostane was gifted from V. Luu-Thé (Oncology and Molecular Endocrinology Research Center, CHUQ pavillon CHUL, 2705 Laurier Boulevard, Ste-Foy, Quebec, G1V 4G2, Canada). Hormones and epostane were dissolved in ethanol to make a stock solution of 10 mM. Pesticides were dissolved in ethanol to make a stock solution of 20 mM, except simazine, which was dissolved in methanol at a concentration of 2 mM.

4.2.2 Yeast screen

The methods for (anti-) YES and (anti-) YAS assays were described previously (Chapter 2, section 2.3.2.4), therefore, only specific methods related to pesticide exposure will be described here. Initially, pesticides were added to wells over the range of 1000 – 0.49 μM, but turbidity readings were unacceptably low for some pesticides, indicating a toxic effect on the yeast cells. Therefore, isoproturon, flucofuron, and trifluralin were subsequently tested over the range of 125-0.06 μM, diuron, linuron, chlorpropham, sulcofuron, and tecnazene over the range of 15.6-0.008 μM, PCP over the range of 7.8-0.004 μM, and MCPA, mecoprop, 2,4,-D, atrazine, simazine, and bentazone were re-tested at the same concentrations. Where cell turbidity was significantly reduced compared to the EtOH or media only controls, data was omitted from statistical analysis. After evaporation, 200 μL of medium containing CPRG and yeast (8 X 10^5 cells/ml) were added to each well. For the anti-estrogenic/androgenic screens, 0.25 nM of estradiol, or 2.5 nM of testosterone, was added to the media prior to addition to the wells. Ethanol and media only controls were also run in each assay. Pesticides were tested in triplicate over three plates, and over two experiments. Absorbance was measured after 2-5 days, depending on the assay.

4.2.3 Ovulation

Sexually mature female *Xenopus laevis* (gifted by Jane Kirk, Cancer Research Institute, UK) were anaesthetised by submersion in MS222 until reflexes ceased, and were then sacrificed by pithing. The ovaries were removed and placed in a glass Petri

dish containing MBS (see Chapter 3, section 3.3.2.1). They were cut into ~ 30 mg tissue fragments (~ 10 stage VI oocytes), and two sections were cultured per well in 24-well plates. After 20 hours incubation, media was extracted, frozen on dry ice, and stored at -80 until hormone analysis (by RIA). In addition, the oocytes were tested for viability with Trypan blue (0.2%), fixed with TCA (5%), and the number of ovulated oocytes were counted. Oocytes were initially incubated with 50, 25, 12.5, 6.25 I.U. HCG or control media, for 20 hours. On day two a submaximal concentration of HCG (~ 60% of maximum ovulatory response) was chosen for co-incubation with pesticides, and oocytes were cultured in control media, HCG alone, or co-incubated with a pesticide, in sextuplicate wells. The ovulation assay was repeated three times with modifications to pesticide concentrations. Initially, all pesticides were tested at 62.5 and 6.25 μM, and pesticides that had no effect or only an effect at 62.5 μM, were re-tested at the same concentrations. Those that had an effect at 62.5 & 6.25 μM were additionally tested at 0.625, 0.0625, & 0.00625 μM (PCP, sulcofuron, chlorpropham, atrazine). The complete range was then repeated for each pesticide, therefore, each concentration of each pesticide was tested twice or three times. Exceptions were PCP and sulcofuran, which were only tested at the top concentration once, due to complete inhibition at this concentration. In addition, the 3β-HSD inhibitor epostane was tested at 1, 0.1, 0.01, and 0.001 μM, to verify inhibition of ovulation via inhibition of steroid hormone synthesis . The GVBD assay was also used to test selected pesticides, however, flutamide was not an effective inhibitor over two assays, and data was very variable (data not shown).

4.2.4 *In vivo* Exposure

Fifty-four adult female *Xenopus laevis* (4 years old) were taken from the breeding stock of the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB, Berlin). The frogs were fed twice per week prior to exposure and the light:dark cycle was 12:12 hours. They were placed in 9 x 12 L glass aquaria containing 10 L reconstituted tap water (distilled water supplemented with 2.5 g marine salt, Tropic Marin Meersalz, Tagis, Dreieich, Germany), at a loading density of 6 animals/tank, and were not fed during the exposure period. PCP has a log Kow of 5.18 and has been shown to bioaccumulate . Thus, it was tested for effects *in vivo* at environmentally

relevant concentrations (1 & 0.1 µg/L = 0.375 nM & 3.75 nM), with the assumption that it would be bioaccumulated to higher levels within the animal. A PCP stock solution of 1 mg/L was prepared in distilled water at the beginning of exposure, and was stored at 4^0C during the exposure period. This stock was diluted x 10000 for the 0.1 µg/L treatment, and x 1000 for the 1 µg/L treatment, and tanks were re-dosed every 48 hours during water change (100 %). Temperature and pH were also measured prior to and following each water change, and water samples were taken from each tank at the same time for chemical analysis of PCP. At the end of the exposure period, animals were bled, and total weight, liver weight and ovary weight were recorded. Blood was centrifuged at 6000 g for 1 minute, and plasma was removed and snap frozen in liquid nitrogen. The liver and ovary were removed and weighed, and ovarian follicles were cultered in MBS supplemented with 20 I.U. HCG in sextuplicate wells. Media was removed after 20 hours, frozen, and stored at -20^0C.

4.2.5 Radioimmunoassays

4.2.5.1 Media

Unextracted media samples were tested for progesterone, testosterone, and estradiol concentration. Assay tubes contained 300 µL test media (for testosterone, 200 µL of test media was diluted with 100 µL of culture media), 100 µL of antibody, and 100 µL of radiolabelled steroid, for a total assay volume of 500 µL. Antibodies (AbD Serotec, Morphosys AG, Germany) were added at final concentrations of 1:500 for progesterone (cross reactivity: 0.02% cortisone only), 1:4000 for testosterone (cross reactivity: 11β-hydroxy testosterone 3.3%, 5α di-hydroxy testosterone 2%), and 1:2000 for estradiol (cross reactivity: oestrone 14%, oestriol 5%). Radiolabelled steroids, [1,2,6,7-^{3}H] progesterone, [1,2,6,7-^{3}H] testosterone, and [2,4,6,7-^{3}H] estradiol (Perkin Elmer, Massachusetts, USA), were added at 120000 counts/ml. Antibodies and radiolabel were diluted in 0.2 M phosphate buffered saline, supplemented with 0.02% sodium azide and 0.5% bovine serum albumin (sPBS) (Sigma, Dorset, UK). After addition of all components, tubes were vortexed and incubated overnight at 4^0C. To separate bound-free hormone, 500 µL of activated charcoal slurry (0.5% charcoal and 0.05% dextran in sPBS), was added to each tube.

Tubes were incubated on ice (10 minutes) and centrifuged (2000 g, 15 minutes), the supernatant was added to scintillation vials. Scintillation cocktail (5 ml) was added to the vials, they were vortexed, and radioactivity was measured (Tri-Carb, Packard Instrument Company, CT, USA). Samples were tested singly over two assays, therefore they were frozen and thawed twice. In order to minimise error, standards were diluted in culture media at the time of test media extraction, and freeze-thawed for use in the two assays. The detection range for estradiol and progesterone was 109–3500 pM, and for testosterone was 875–7000 pM, and the interassay coefficients of variance were 22% for progesterone, 8% for testosterone, and 17% for estradiol for *in vitro* pesticide testing. Media samples from the *in vivo* exposure were run in one assay, so no interassay variability values were available.

4.2.5.2 Plasma

The same reagents used for the media RIA were also used for the plasma RIA. For hormone extraction of plasma, ethyl acetate was added (~ 3:1 ratio of ethyl acetate to plasma), and tubes were shaken vigorously for 10 minutes. Tubes were then centrifuged at 2000 g for 2 minutes to separate the aqueous and solvent phases, and the top layer (ethyl acetate) was removed. This step was repeated, and combined ethyl acetate was then dried with purified nitrogen gas. 650 µL MBS was then added to each dry tube, and they were vortexed vigorously. 100 µL of extracted sample was added to each assay tube in duplicate (progesterone, testosterone & estradiol), and 100 µL of antibody and radiolabel were also added (total = 300 µL). Tubes were incubated overnight at 4^0C, and charcoal dextran slurry was used to separate the bound from the unbound fraction, as described above. Samples were run in one assay, and intrassay variability was 22 % for progesterone, 18 % for testosterone, and 12 % for estradiol. Extraction efficiencies were 68 % for progesterone, 86 % for testosterone, and 73 % for estradiol.

4.2.6 Statistics

Data was tested for normality using the Shapiro-Wilk W test. For normally distributed data, ANOVA was used to find differences between groups. For non-parametric data,

Kruskal-Wallis test was used to find differences between groups. Dunnett's test was used as a post-hoc test in both cases, to test differences from the control. Yeast data and ovulation data were converted into percentages of the control value to allow for variation between experiments prior to statistical analysis. Data were pooled from different experiments, resulting in *n* values of 3 or 6 for yeast data, 6, 12, or 18 for ovulation. Media hormone data were analysed without conversion, and correlations between hormone levels and ovulatory response were calculated using Pearson's correlation co-efficient. For the *in vivo* exposure, liver and ovary weight were converted to hepatic somatic index (HSI) and gonadal somatic index (GSI), by dividing values by the total weight of the individual, and these values were analysed statistically. Plasma hormone data, ovulation data, and abnormality data were log transformed for normalisation, prior to statistical analysis.

4.3 Results

4.3.1 Yeast Screen

All yeast screen data was analysed parametrically. None of the pesticides tested were agonistic in the estrogen or androgen screen, however, seven pesticides were anti-estrogenic, and eleven were anti-androgenic. Cytotoxicity was often observed. Therefore, the top concentrations reported below refer to the highest concentration eliciting an effect on the receptor, without affecting turbidity readings (pesticides were cytotoxic above these concentrations unless otherwise stated).

PCP was the most potent compound tested and was anti-estrogenic from 0.015-7.8 μM ($p < 0.004$). Anti-estrogenic activity was also observed from 0.9-31.5 μM for diuron ($p < 0.004$), from 1.9-31.5 μM for linuron ($p < 0.02$), and from 15.3-250 μM for isoproturon ($p < 0.02$) (Figure 4.1, A). Atrazine, and flucofuron were also weakly anti-estrogenic (1000-125 & 1000-500 μM, respectively, Figure 4.1 A & B). Flucofuron also displayed agonistic activity from 62.5-3.9 μM ($p < 0.04$), although no agonistic response was observed in the estrogen screen (Figure 4.1, B). Lastly, sulcofuron was antagonistic from 15-3.9 μM ($p < 0.02$), though no effect was observed at other concentrations (Figure 4.1, B).

PCP was also a potent anti-androgen, and displayed activity from 0.015-3.9 μM ($p < 0.02$, Figure 4.1, D). Anti-androgenic activity was also observed from 15.6-31.25 μM for diuron ($p < 0.04$), from 0.97-62.5 μM for linuron ($p < 0.03$), and from 125-250 μM for isoproturon ($p < 0.02$) (Figure 4.1, C). Flucofuron was anti-androgenic from 0.5-1000 μM ($p < 0.01$), and sulcofuron from 0.9-62.5 μM ($p < 0.03$) (Figure 4.1, E). In addition, trifluralin was anti androgenic from 15.6-1000 μM ($p = 0.03$), and chlorpropham from 0.5-15.6 μM ($p < 0.02$) (Figure 4.1, D). Bentazone was a very weak anti-androgen, and only exerted an effect at 500 & 1000 μM (data not shown). Agonistic and antagonistic activity was observed in response to atrazine (androgenic: 3.9 – 31.25 μM, $p < 0.03$; anti-androgenic: 125-1000 μM, $p < 0.04$), and androgenic activity was observed in response to simazine (125 μM, $p < 0.03$) when co-incubated with testosterone (Figure 4.1, F). Indeed, no agonistic response was observed with atrazine or simazine in the androgen screen (data not shown).

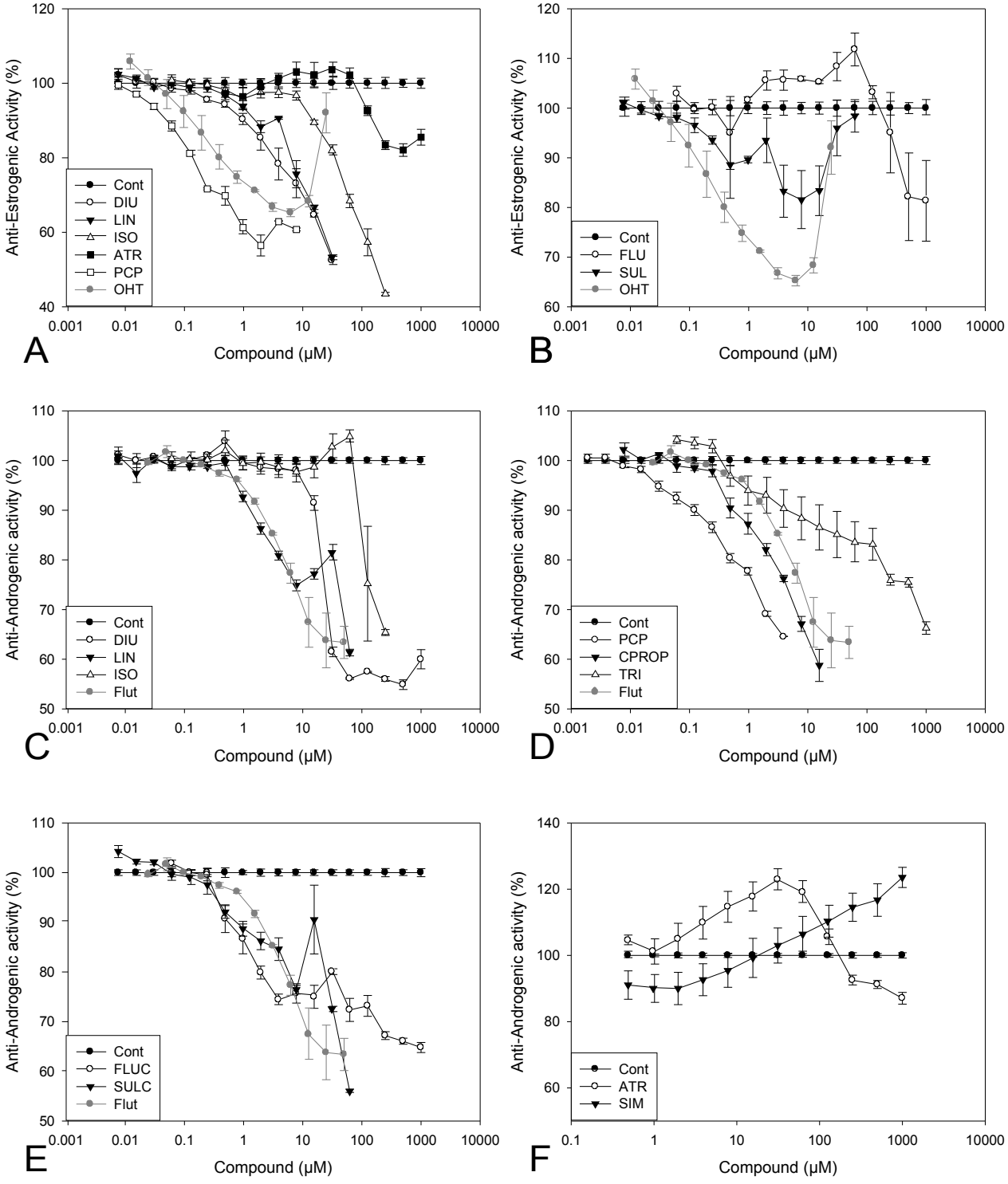

Figure 4.1. Activity of standards (grey line) and pesticides in the yeast anti-estrogen (A & B) and anti-androgen screen (C-F). Values are mean ± SE, *n* = 3 or 6 for the pesticides, and 4 for the standards. Cont = ethanol control, OHT = hydroxy-tamoxifen, Flut = flutamide, Diu = diuron, Lin = linuron, Iso = isoproturon, Atr = atrazine, Sim = simazine, PCP = pentachlorophenol, Flu = flucofuron, Sul = sulcofuron, Cprop = chlorpropham, Tri = trifluralin.

4.3.2 Ovulation

Ovulation data were analysed non-parametrically. The negative control epostane effectively inhibited ovulation in a dose-dependant manner (Figure 4.2, A). Seven pesticides tested had a significant effect on ovulation (Figure 4.2, B, C, D). PCP (Figure 4.2, C) and sulcofuron (Figure 4.2, D) were the strongest inhibitors of ovulation, and inhibition was observed at 62.5, 6.25, and 0.625 µM. Isoproturon (Figure 4.2, B), diuron (Figure 4.2, B), chlorpropham (Figure 4.2, C), and flucofuron (Figure 4.2, D) inhibited ovulation at 62.5 µM, but not at 6.25 µM. Atrazine had both a stimulatory and inhibitory effect on ovulation at different concentrations (Figure 4.2, D), with stimulation observed at 6.2 µM and inhibition at 0.0625 µM.

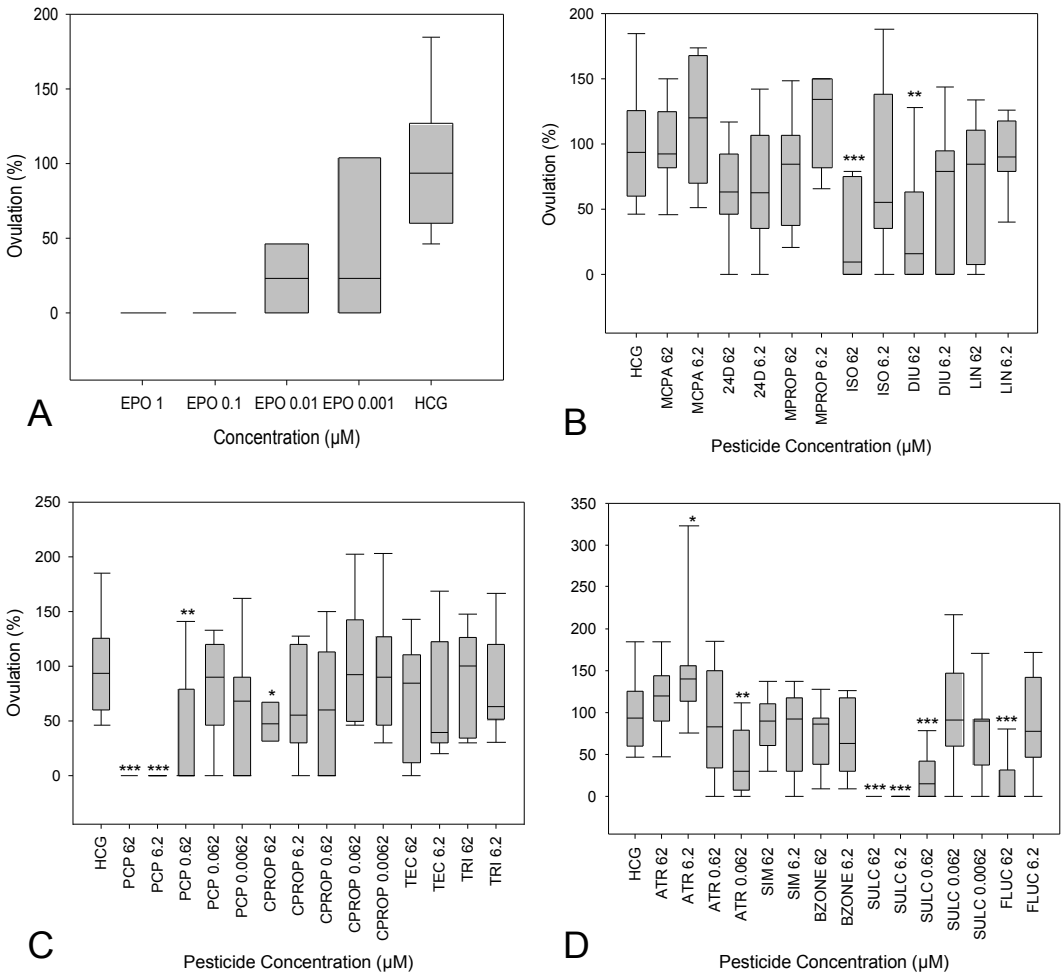

Figure 4.2. Effects of pesticides on *in vitro* ovulation. Values are medians (line in box), interquartile ranges (box) and 25th & 75th percentiles ('whiskers'), and *n* vlaues were 6, 12, or 18. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. HCG = human chorionic gonadotropin, EPO = epostane, TEC = tecnazene, BZONE = bentazone, see Figure 4.1 for other abbreviations.

4.3.2.1 Hormone concentrations.

Hormone data were analysed non-parametrically. Nine pesticides tested had an efffect on progesterone, testosterone, and/or estradiol concentrations (Table 4.1), and epostane inhibited testosterone and estradiol concentrations in a dose-dependant manner. Testosterone concentrations were the most sensitive to perturbation, estradiol was the least, and the most common effect was elevation of progesterone and depression of testosterone. Progesterone concentrations were elevated by linuron (62.5 μM, $p = 0.0005$), atrazine (62.5 μM, $p < 0.0001$; 6.25 μM, $p = 0.0002$,), and chlorpropham (0.00625 μM, $p = 0.002$), and were depressed by PCP (62.5, $p = 0.009$), and sulcofuron (62.5, $p = 0.018$). Testosterone concentrations were elevated by MCPA (62.5, $p = 0.01$), mecoprop (62.5, $p = 0.036$), atrazine (6.25, $p = 0.003$), and simazine (62.5, $p = 0.004$). Testosterone concentrations were depressed by diuron (62.5, $p < 0.0001$), PCP (62.5, not detectable, 6.25, $p < 0.001$), chlorpropham (62.5, $p = 0.008$), sulcofuron (62.5 and 6.25, $p < 0.001$), and flucofuron (62.5, $p < 0.001$). Estradiol concentrations were elevated by mecoprop (6.25, $p < 0.001$), and depressed by PCP (62.5, $p = 0.003$, 6.25, $p = 0.02$) and sulcofuron (62.5, $p < 0.0001$).

4.3.2.2. Correlations

Using Pearson's correlation co-efficient, all combinations of percentage ovulation, progesterone, testosterone, and estradiol were statistically significant ($p < 0.0002$). Percentage ovulation was most closely correlated with testosterone ($R^2 = 0.63$, correlation co-efficient = 0.79), followed by progesterone ($R^2 = 0.45$, correlation co-efficient = 0.67), and estradiol ($R^2 = 0.32$, correlation co-efficient = 0.57). In comparison of hormone concentrations, estradiol was most closely correlated with testosterone ($R^2 = 0.54$, correlation co-efficient = 0.73), followed by progesterone with testosterone ($R^2 = 0.41$, correlation co-efficient = 0.64), and progesterone with estradiol ($R^2 = 0.27$, correlation co-efficient = 0.52).

Treatment	Conc. (μM)	Prog. (pM)	Test. (pM)	E2 (pM)
HCG	N/A	374 ± 28	7646 ± 443	819 ± 61
EPO	1	531 ± 32 ↑	2325 ± 108 ↓↓↓	377 ± 19 ↓↓↓
	0.1	400 ± 30	2934 ± 371 ↓↓↓	382 ± 41 ↓↓
	0.01	355 ± 23	2819 ± 368 ↓↓↓	491 ± 41 ↓
	0.001	472 ± 61	7585 ± 556	556 ± 75
ISO	62	394 ± 37	7567 ± 619	592 ± 46
	6.2	515 ± 93	7927 ± 619	800 ± 65
DIU	62	372 ± 30	4102 ± 522 ↓↓↓	728 ± 65
	6.2	416 ± 64	7021 ± 648	933 ± 73
LIN	62	634 ± 58 ↑↑↑	6715 ± 400	751 ± 94
	6.2	396 ± 43	7657 ± 717	921 ± 94
MCPA	62	493 ± 48	9966 ± 583 ↑	937 ± 79
	6.2	399 ± 38	9410 ± 398	946 ± 85
MPROP	62	363 ± 41	10047 ± 322 ↑	985 ± 103
	6.2	430 ± 53	9685 ± 334	1296 ± 115 ↑↑↑
2,4,-D	62	424 ± 47	7417 ± 610	759 ± 55
	6.2	451 ± 50	8447 ± 703	918 ± 64
ATR	62	762 ± 68 ↑↑↑	9103 ± 505	795 ± 50
	6.2	609 ± 58 ↑↑↑	9514 ± 353 ↑↑	939 ± 71
	0.62	419 ± 50	7118 ± 711	657 ± 67
	0.062	389 ± 13	6616 ± 443	953 ± 71
SIM	62	398 ± 22	9835 ± 341 ↑	779 ± 93
	6.2	512 ± 87	9121 ± 653	923 ± 155
PCP	62	< 109 ↓↓↓	< 875 ↓↓↓	267 ± 59 ↓↓
	6.2	351 ± 30	2701 ± 117 ↓↓↓	483 ± 43 ↓
	0.62	387 ± 56	5960 ± 570	714 ± 65
	0.062	335 ± 18	8096 ± 621	846 ± 100
	0.0062	341 ± 25	7743 ± 728	775 ± 63
FLUC	62	253 ± 28	3640 ± 431 ↓↓↓	692 ± 51
	6.2	386 ± 46	6036 ± 459	809 ± 62
SULC	62	115 ± 3 ↓	2320 ± 64 ↓↓↓	225 ± 20 ↓↓↓
	6.2	248 ± 26	5198 ± 534 ↓↓↓	812 ± 98
	0.62	430 ± 36	7581 ± 675	1007 ± 103
	0.062	468 ± 48	9283 ± 607	890 ±103
	0.0062	486 ± 32	8093 ± 678	845 ± 94
TRI	62	342 ± 25	8098 ± 563	770 ± 97
	6.2	343 ± 35	7760 ± 455	1007 ± 101
CPROP	62	246 ± 22	4192 ± 448 ↓↓	583 ± 54
	6.2	375 ± 27	7335 ± 428	784 ± 74
	0.62	397 ± 28	6538 ± 555	799 ± 58
	0.062	414 ± 32	6372 ± 821	735 ± 99
	0.0062	634 ± 92 ↑↑	8844 ± 862	968 ± 93
BZONE	62	349 ± 39	8383 ± 573	721 ± 91
	6.2	498 ± 101	9354 ± 608	857 ± 71
TEC	62	339 ± 19	8050 ± 641	813 ± 119
	6.2	376 ± 54	9158 ± 583	673 ± 97

Table 4.1. Hormone concentrations in oocyte incubation media after incubation with pesticides or epostane. Values are mean ± SE, and *n* values were 6, 12, or 18. Arrows (↑ = elevation compared to HCG, ↓ = depression compared to HCG) indicate significant differences from HCG only treated oocytes (↑↑↑/↓↓↓ = $p < 0.001$, ↑↑/↓↓ = $p < 0.01$, ↑/↓ = $p < 0.05$). Prog = progesterone, Test = testosterone, E2 = estradiol, N/A = Not Applicable, for list of other abbreviations see Figure 4.2.

4.3.4 *In vivo* Exposure

Water temperature was 18.9^{0}C ± 0.11 and pH was 6.13 ± 0.08 over the exposure period, and there were no differences between tanks. PCP concentration was similar to nominal values in the high treatment after each water change (0.89 ± 0.08 µg/L), however, had decreased markedly after 48 hours (0.29 ± 0.06 µg/L). No PCP was found in the control tanks (detection limit 0.2 µg/L), and water from the low treatment group was not analysed. No difference in total weight, HSI, or GSI was observed between treatments, however, morphological differences were observed (Figure 4.3). Normal ovarian sacs are filled with large, banded stage VI oocytes (Panel A), which undergo maturation upon stimulation with HCG (Panel B). One individual from the low treatment contained a large fluid-filled cyst in place of the ovary (Panel C), which contained just a few oocytes (Panel D). Another individual from the low treatment group had an ovary containing very few stage VI oocytes, and instead had many stage I/II oocytes (Panel E & F). One individual from the high treatment had an almost empty ovarian sac (Panel G), and others contained many abnormal oocytes post HCG treatment (Panel H). Abnormal oocytes were also observed in control individuals after HCG treatment, however the degenerative effects observed in the whole ovary of treated individuals were never observed. In addition, more abnormal oocytes were observed in low (log transformed value, mean ± SE: 1.19 ± 0.08), and high (1.24 ± 0.07) treatments, compared to control (1.06 ± 0.07), though this difference was not statistically significant (Dunnett's: control & low $p = 0.37$, control & high $p = 0.16$). There was no difference in plasma testosterone (ANOVA $p = 0.97$) or estradiol (ANOVA $p = 0.4$) between treatments, however progesterone concentrations approached significance (ANOVA $p = 0.09$). They were elevated in individuals from the low treatment (log transformed value, mean ± SE: 3.2 ± 0.03; Dunnett's $p = 0.06$), and the high treatment (3.16 ± 0.05; Dunnett's $p = 0.21$), compared to the control (3.05 ± 0.06). If progesterone values from low and high treatment values were pooled (i.e. 'treated'), they were significantly different from the control (ANOVA $p = 0.036$). Progesterone and testosterone production by cultured ovarian tissue differed between treatments ($p = 0.06$ & 0.04 Kruskal Wallis, respectively), and both were depressed in the low treatment compared to the high treatment (progesterone: Dunnett's $p = 0.04$; testosterone: Dunnett's $p = 0.14$), but neither were different from the control ($p > 0.7$).

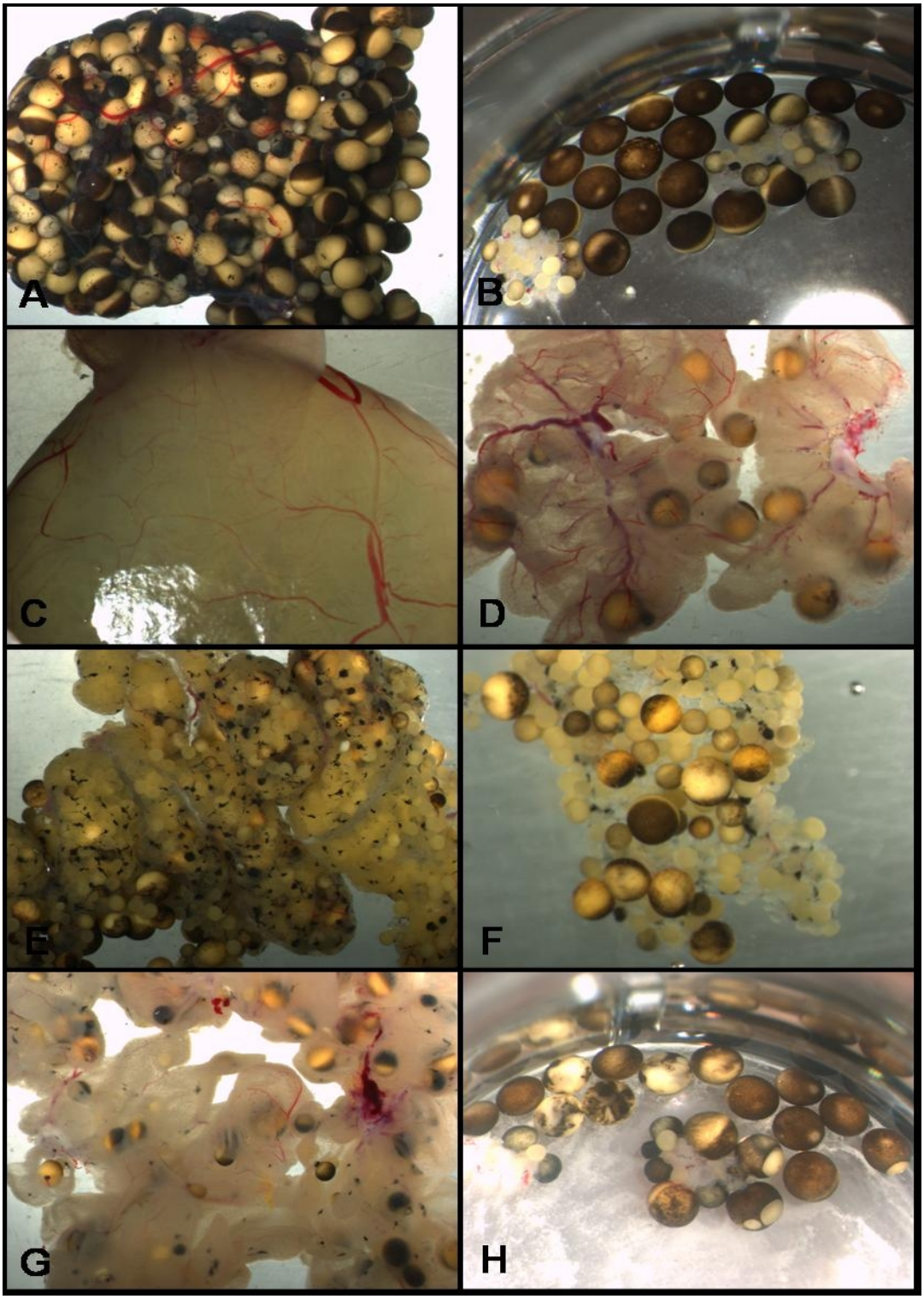

Figure 4.3. Ovaries taken from experimental treatments. Panel A & B show a control ovary, both before (A) and after (B) treatment with HCG. Note that oocytes with a visible white spot on the animal pole are matured. Panel C & D show a cyst found in a low dose treated individual (C), and the oocytes it contained (D). Panels E & F show an undeveloped ovary (E) containing few oocytes (F), from the high dose treatment. Panel G shows an almost empty ovarian sac from a high dose treated individual, and Panel H shows many abnormal oocytes (marbled appearance) observed in HCG treated oocytes taken from a high dose exposure individual. Note there are also a few normally matured oocytes.

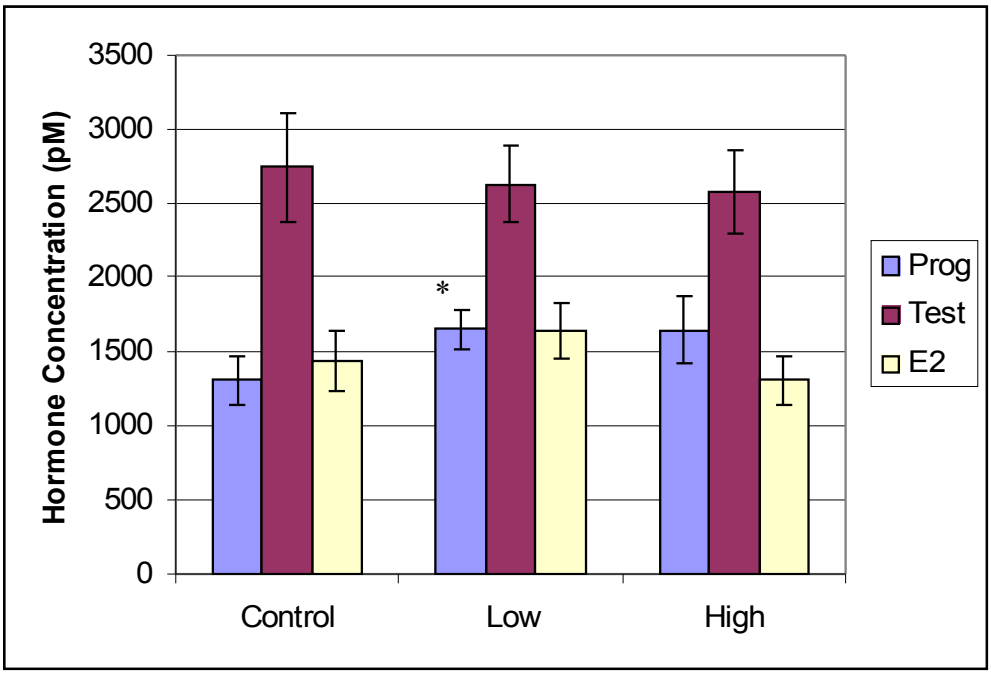

Figure 4.4. Hormone concentrations in plasma from control, low dose, and high dose individuals. Values are mean ± SE, *n* = 18. Prog = progesterone, Test = testosterone, and E2 = 17β-estradiol. * denotes significant difference from control.

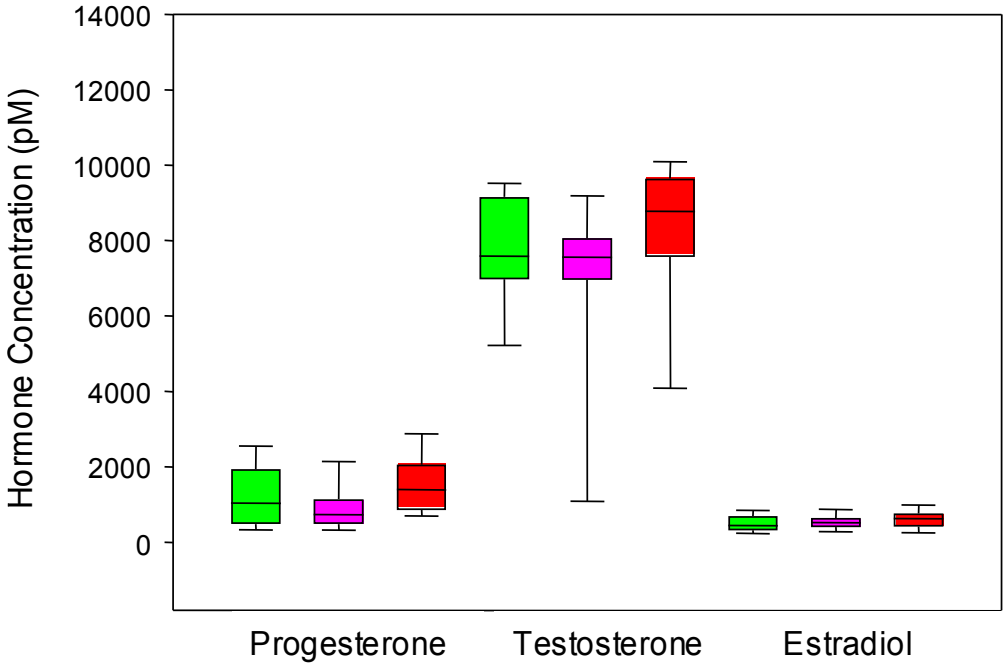

Figure 4.5. Hormone concentrations in ovulation media from control (green), low dose (pink), and high dose (red) individuals. Values are medians (line in box) and interquartile ranges (box) and 25th & 75th percentiles ('whiskers'), *n* = 18 (oocytes plated in sextuplicate wells for each individual).

4.4 Discussion

None of the pesticides were estrogenic at any concentration tested. In agreement, Nishihara *et al*., (2000) tested 118 pesticides (including linuron, atrazine, simazine, chlorpropham, PCP, & trifluralin), and found that none were estrogenic. Indeed, pesticides reported as having estrogenic activity are mainly organochlorines and organophosphates (Kojima *et al*., 2004), which are generally not environmentally relevant to the UK. Twelve out of fifteen pesticides tested were active in at least one assay (2,4,-D, bentazone, & tecnazene had no effect) and classes of pesticides tended to behave similarly, especially in the yeast screen. There did not appear to be a correlation between mode of action of the pesticide and ED activity in either screen. More pesticides were anti-androgenic (10) than anti-estrogenic (7), which may be partially due to the higher specificity of the estrogen receptor compared with the androgen receptor (Sohoni and Sumpter, 1998). In addition, many of the pesticides had an effect on either ovulation or media hormone levels (11). Receptor-mediated anti-androgenic activity and decreased testosterone levels appeared to be linked to inhibited ovulation. There is some evidence that androgens, rather than progesterone, are the physiological initiators of ovulation *in vivo* . In agreement, testosterone was more closely correlated to ovulation than progesterone, and was found at approximately 23x higher levels.

4.4.1. *In vitro* effects

Isoproturon, diuron, and linuron had similar effects in the yeast screen, all were anti-estrogenic and anti-androgenic, although isoproturon was less potent than diuron and linuron. In addition, isoproturon and diuron inhibited ovulation, whereas no effect was observed in response to linuron. Progesterone and testosterone are known inducers of ovulation (Jacobelli *et al*., 1974; Smith and Ecker, 1971), and anti-androgenic compounds are known to inhibit testosterone induced maturation of the oocyte (Lutz *et al*., 2001). Therefore, perhaps the anti-ovulatory response was caused by anti-androgenic activity at the receptor on the oocyte. In the case of linuron, progesterone concentrations in the media were double control values, so perhaps the receptor-mediated anti-androgenic effect was ameliorated by increased progesterone

concentrations. In addition, atrazine had a similar effect in the anti-androgen screen and on the ovulatory response, and testosterone levels, whereby, it had both stimulatory and inhibitory effects over the concentration range tested. Furthermore, sulcofuron, flucofuron, chlorpropham, and PCP were all anti-androgenic, and also inhibited ovulation (although trifluralin was anti-androgenic, without affecting ovulation), suggesting that the testosterone produced by ovarian tissue *in vitro* may have an important role in initiating ovulation (see Chapter 3, section 3.4.4).

Diuron and linuron were previously shown to possess anti-androgenic activity *in vitro* (Kojima *et al*., 2004; linuron – Lambright *et al*., 2000), and *in vivo* (Linuron – Lambright *et al*., 2000), but this is the first time their anti-estrogenic activity has been reported. In fact, both were more potent anti-estrogens than anti-androgens in the yeast assays (LOEC's were 0.98 μM and 31.25 μM for diuron, and 0.98 μM and 7.8 μM for linuron). Linuron has previously been identified as a more potent endocrine disruptor than diuron, however, the LOEC for anti-estrogenic activity of diuron was close to environmental levels (maximum detected concentrations: 28 μg/L (0.12μM)), whereas this was not the case for linuron (maximum detected concentration: 1.4 μg/L (0.0056 μM)). In addition to effects at the receptor, diuron also inhibited ovulation and testosterone concentration at 62.5 μM, indicating disruption of steroidogenic pathways. Diuron has previously been reported to have no effect on 17β-HSD activity in fish ovarian microsomes at 0.1 or 1 mM (Thibaut and Porte, 2004), or placental aromatase activity up to 50 μM in human tissue homogenates (Vinggaard *et al*., 2000), or rats exposed *in vivo* (Fernandes *et al*., 2007). However, its effects on amphibians have not been tested, and it is ubiquitous in the UK environment (detected > 0.01 μg/L 785 times). Linuron caused an increase in progesterone concentration at 62.5 μM, and a corresponding decrease in testosterone concentrations (non-significant). It was previously reported to inhibit 5α-R activity (50% inhibition, 86 μM) in human prostate tissue homogenate (Lo *et al*., 2007). Therefore, it may also be inhibiting enzymes involved in the conversion of progesterone to testosterone. Isoproturon caused a decrease in ovulation without changing hormone levels, but was anti-androgenic, indicating it may have competed at the receptor site but did not affect steroidogenic enzymes. It is the first time it has been tested for anti-androgenic activity *in vitro*, but was shown to cause testicular degeneration and retardation of spermatogenesis in rats (Sarkar *et al*., 1995). However, reprotoxic effects were in

conjunction with hepatic and renal toxicity, and effective levels were close to the LC_{50} for rats (800 mg/kg compared with 1826 mg/kg), suggesting they were possibly a by-product of general toxicity. Isoproturon was also weakly anti-estrogenic (LOEC 15 μM), but had previously been reported to have no anti-estrogenic activity in a reporter cell line (HELN) at 10 μM (Lemaire *et al*., 2006). The effects observed here occurred at concentrations higher than those found in the environment (maximum detected concentration – 29.5 μg/L (0.14 μM); LOEC's: anti-YES – 15 μM, anti-YAS – 125 μM; ovulation assay – 62.5 μM), however, it has been shown to bioaccumulate in tadpoles , and freshwater macrophytes . Therefore, in addition to aquatic exposure, isoproturon may enter the tadpole through diet, and has been shown to be toxic to embryos and young tadpoles at levels as low as 0.1 μg/L (Greulich *et al*., 2002). Furthermore, it is persistent in the aquatic environment, with only 9 % of the isoproturon removed from an aquatic microcosm in 21 days (Bottcher and Schroll, 2007), and is ubiquitous in the UK environment (found above 0.01 μg/L 635 times). Therefore, although diuron and isoproturon were not as potent as linuron, their effects may be greater, and more research is needed to elucidate risk to amphibians.

Mecoprop and MCPA were not active in receptor binding assays, which has previously been reported (Kojima *et al*., 2004, Lemaire *et al*., 2006), however, they had a stimulatory effect on steroidogenesis. Both increased testosterone and estradiol levels, and were the only compounds to have this effect. Indeed, mecoprop was the only compound to significantly increase estradiol concentration (at 6.25 μM only), and both significantly increased testosterone concentration (at 62.5 μM only). Since both testosterone and estradiol were affected, but progesterone was not, it seems that stimulation occurred at some point in the steroidogenic pathway between progesterone and testosterone. No information could be found on the endocrine effects of these two compounds, but they are ubiquitous in the environment (detected > 0.01 μg/L 1444 & 615 times, respectively) and are found at high concentrations (maximum measured concentrations: 6180 μg/L (28.8 μM) & 4700 μg/L (23.4 μM), respectively). 2,4,-D, bentazone (except for very weak anti-androgenic activity), and tecnazene were not active in any of the assays. Kojima *et al*. (2004) also found no estrogenic or anti-androgenic activity in a receptor binding assay for 2,4,-D, and bentazone. 2,4,-D was previously demonstrated to block maturation of defolliculated *Xenopus* oocytes (LaChapelle *et al*., 2007), and thus was expected to inhibit

maturation and ovulation in this study. Ovulation was depressed, but not significantly ($p = 0.07$), which may be explained by the higher concentrations used previously (10 mM compared to 62.5 μM), or by limited entry into the oocyte due to the presence of follicle cells in this study.

Atrazine was weakly anti-estrogenic, which has previously been reported , but was not anti-androgenic, which has also previously been reported for atrazine and simazine . However, both atrazine and simazine stimulated androgenic activity in the anti-androgen screen, but were not agonists in the androgen screen. In addition, atrazine was weakly anti-androgenic at higher concentrations (250-1000 μM). Atrazine affected ovulation in a similar way, and caused stimulation at an intermediate concentration (6.25 μM), but inhibition at a lower concentration (0.0625 μM). The mechanism of action of triazines is unknown, but this effect could potentially be explained by upregulation of the AR receptor, leading to higher sensitivity to testosterone. Xenoestrogens are known to upregulate the ERβ *in vitro* , and research into prostate cancer treatment has shown that anti-androgens can upregulate the androgen receptor . This is a relatively untested route of endocrine disruption, and may explain the effects observed on ovulation. However, it is unlikely to be causing the observed effect in the yeast screen as the AR is upregulated by a copper-dependant promoter in this test system, and therefore upregulation would have to affect this promoter (E.Routledge, pers. comm.). Alternatively, atrazine and simazine may have altered the behaviour of the ligand bound receptor (i.e. AR-testosterone) on the promoter of the reporter gene. Stimulated ovulation was accompanied by increased progesterone and testosterone levels, but no change in hormone levels were observed when ovulation was inhibited, and it was not anti-androgenic at the receptor at the lowest concentration tested (0.9 μM). In addition, Kojima *et al*., (2004) reported no anti-androgenic activity of atrazine at any concentration (0.01-10 μM). Therefore, the mechanism by which ovulation was inhibited is unknown, but occurred at environmentally relevant concentrations (0.0625 μM = 1.29 μg/L). It is also unclear whether the potency of atrazine would have increased as concentrations decreased, and at what point inhibition would have ceased. Atrazine was the only compound to exert such an effect, but this pattern has previously been reported in cortisol secretion by adrenocortical cells in response to atrazine . Furthermore, Hayes *et al*. (2003) reported higher levels of

hermaphoroditism in frogs exposed to 0.1 µg/L than 25 µg/L atrazine. The 'inverted-U' dose-response curve has recently gained wider recognition and clearly requires further investigation. An increase in steroid concentrations in response to atrazine is in contrast to previous reports of atrazine inhibition of 17β-HSD and 3α-HSD in rat pituitary homogenate (Babic-Gojmerac *et al*., 1989), which would result in decreased hormone levels in the ovulation assay (as occurred with the 3β-HSD inhibitor epostane). However, perhaps this is due to the test system used as atrazine had no effect on 17β-HSD levels in carp testicular microsomes (Thibaut and Porte, 2004). Finally, atrazine upregulates aromatase in the human adrenocorticocarcinoma cell line H295R , and the mechanism of action is thought to be via the "orphan" steroid receptor, steroidogenic factor-1 . Amphibians are known to possess this receptor , but there was no evidence of stimulatory effects on aromatase, as estradiol did not increase, and testosterone did not decrease.

Trifluralin was moderately anti-androgenic (LOEC: 15.6 µM), and chlorpropham was strongly anti-androgenic (LOEC: 0.5 µM), but both had previously been found to have no activity in an anti-androgen screen over a similar concentration range (Kojima *et al*., 2004). This discrepancy was unusual, as all the other receptor-mediated effects reported here were generally in agreement with previously published data. In addition, trifluralin was also the only anti-androgenic compound that did not affect ovulation. Both compounds act by inhibiting cell division, and therefore it is possible that anti-androgenic responses could have been artefacts of cell growth. However, since results with decreased turbidity readings were omitted from the statistical analysis, it is unlikely that this would have affected the result. Therefore, the anti-androgenic nature of these compounds should be treated with caution, and further testing in a different system is needed to verify their anti-androgenic nature. Levels eliciting a response for trifluralin were 4 orders of magnitude higher than levels found in the environment (maximum measured concentration: 3.06 µg/L (0.009 µM)), so it may not be environmentally relevant alone, whereas, effective levels were similar to levels found in the environment for chlorpropham (maximum measured concentration: 269 µg/L (1.26 µM)). Chlorpropham also inhibited ovulation at 62.5 µM, and an accompanying decrease in testosterone concentrations was also observed, however, it was previously reported to have no effect on reproductive parameters in rats , though, specific anti-androgenic parameters, such as reduction in male

anogenital distance and male sex gland reduction , were not measured. It also had an unexpected effect of increasing progesterone concentrations as its concentration decreased, and this effect was significant at 0.000625 μM. It is found fairly frequently in the U.K. environment (104 times), and more research is warranted on its effects in aquatic organisms.

No previous information on effects to the endocrine system of sulcofuron and flucofuron could be found. They were strongly anti-androgenic (LOEC's: 0.9 & 0.5 μM), and had variable effects in the anti-estrogen screen. Sulcofuron was a potent inhibitor of ovulation (> 0.625 μM), and also inhibited progesterone (62.5 μM), testosterone (62.5 & 6.25 μM), and estradiol (62.5 μM) concentrations. Flucofuron had a similar effect, but was less potent than sulcofuron. They exert their toxic effect through enzyme inhibition in moth larvae (Tomlin, 2006), therefore it is not surprising that they caused enzyme inhibition in this experiment. Although they are not often found in the environment, aquatic point-source levels from industrial discharge can be relatively high (maximum observed concentration: 0.75 μg/L (0.0014 μM) & 0.52 μg/L (0.0012 μM)), and therefore more research into their effect *in vivo* is warranted.

4.4.2. Pentachlorophenol

PCP was the strongest inhibitor tested across all assays, it was anti-estrogenic and anti-androgenic at concentrations as low as 0.01 μM. In addition, it completely abolished the ovulatory response and decreased hormone levels at 62.5 and 6.25 μM, and partially inhibited ovulation at 0.625 μM. The concentrations that elicited an effect *in vitro* approached those found in the environment (maximum measured concentration: 2.74 μg/L (0.01 μM)). Anti-estrogenic activity has previously reported in response to PCP , though it was previously reported to have no anti-androgenic activity at 0.1-10 μM (Hamster ovary cells: Kojima *et al*., 2004) or 0.01-10 μM (monkey kidney CV-1 cell line: Sun *et al*., 2006), in reporter gene assays in . It was shown to inhibit DHT binding to the AR by 30 % at 100 μM . In addition, inhibition of ovulation has also previously been reported in zebrafish oocytes *in vitro*, over a similar concentration range, which persisted after washout and re-stimulation (Tokumoto *et al*., 2005). The inhibition of ovulation observed here was accompanied

by a decrease in hormone concentrations, and testosterone concentrations were particularly sensitive. *In vivo*, it has previously been shown to cause seminiferous tubule atrophy and a decrease in sperm density in rams , which are characteristic of anti-androgenic activity (Gray *et al*., 2001), and could potentially be explained by both antagonistic effects at the receptor and inhibition of steroidogenesis as reported here. Furthermore, although testosterone concentrations were not affected *in vivo* in the present study, plasma progesterone levels were increased in the treated individuals. This could be explained by inhibition of conversion to testosterone in the ovary, as testosterone levels were inhibited at 62.5, 6.25, and 0.625 μM *in vitro*. A reprotoxic effect on the ovary was also observed *in vivo*, which has also previously been reported in relation to (testicular) sertoli cells *in vitro* at 10 nM . Furthermore, dose-dependant inhibition of testicular development, and degeneration of the ovary, was observed in medaka exposed to 20-200 μg/L (Zha *et al*., 2006), and increased severity of oviductal intraepithelial cysts was observed in adult ewes (Rawlings *et al*., 1998). Although PCP levels in the frogs were not measured, it was greatly decreased in the water after 48 hours. It is known to have considerable ability to bioaccumulate , and when Goldfish were exposed to 5 μg/L PCP for 96 hours at pH 7, it was rapidly bioaccumulated by the fish, as it was removed from the water (the bioconcentration factor was 607; Stehly and Hayton, 1990). This may also explain effects observed in cultured oocytes, where progesterone and testosterone levels were decreased in the low treated individuals (only in relation to high dose animals, and neither were different than the control), as a latent effect of *in vivo* exposure. Although, it is not known why only low dose animals were affected by *in vivo* treatment.

4.5 Conclusions

The ovulation assay has been shown to be a sensitive, informative, and environmentally relevant test for steroidogenic endocrine disruption. Although it was not possible to elucidate specific enzyme inhibition/stimulation in the present study, this has recently been achieved by measuring additional steroids in the media (Ahn *et*

al., 2007). In addition, the majority of research concerned with steroidogenic enzymes has focused on aromatase, and to a lesser extent 5α-R. DHT levels in the media were not tested, therefore effects on 5α-R are unknown, however, there were no obvious effects on aromatase in response to any pesticides. Instead it appeared that the hydroxy steroid dehydrogenases were affected, as the most common effect was decreased testosterone levels in relation to progesterone levels. The effect of PCP *in vitro* was broadly indicative of endocrine disruption *in vivo*, and although the effects *in vivo* were weak, considering the low concentration (0.1 & 1 µg/L) and short exposure duration (6 days), the presence of this compound in the environment is a cause for concern.

Approximately half of the pesticides tested disrupted endocrinological endpoints at or near to levels observed in the environment, and if possible bioaccumulation and mixture effects are taken into consideration, the effects of these pesticides are a cause for concern for wildlife, and specifically amphibians, which inhabit agricultural ponds. Furthermore, information pertaining to the effects of these pesticides is sparse, and there is continuing focus of resources on pesticides about which much is known, and use is restricted (e.g. DDT), despite the dearth of information about environmentally relevant pesticides.

Chapter 5

Discussion

The aim of this study was to assess whether there is evidence for alteration in thyroid function and reproductive parameters in native amphibian populations, specifically in relation to agrochemicals. To achieve this objective, *in vitro* tests and population data were used to select suitable field sites, and toad specimens from a subsample of these sites were used to compare morphology, thyroidal, and gonadal development of caged and wild-caught tadpoles/metamorphs to their laboratory-raised counterparts. In addition, the endocrine disrupting potential of environmentally relevant pesticides was assessed *in vitro*, and a short-term *in vivo* exposure was used to assess the predictive ability of the *in vitro* screens. Biological activities of water sample extracts, together with indications of delayed metamorphosis, skewed sex ratios, and altered gonadal differentiation suggest there may be endocrine disrupting effects in native amphibians in the agricultural landscape. Further investigation is needed to confirm these findings due to limitations in the field work. Interpretation of results was hindered by various factors, including: unknown contaminants present at field sites; unknown effects of mixtures of environmentally relevant pesticides in amphibians; high mortality of toad specimens; unknown impact of genetics and maternal transfer on measured endpoints; and lastly, lack of reliable baseline data concerning the effects of model endocrine disrupting compounds in toads, for comparison to effects observed. However, findings reported in this study also filled previous gaps in knowledge, primarily in relation to *in vitro* effects of environmentally relevant pesticides. In addition, during the project, major gaps in the literature were identified, which must be addressed if the risk of the agricultural environment to amphibians, and indeed other wildlife, can be assessed.

5.1 Assays

The assay development aspect of this project highlighted the difficulties involved in optimising experimental procedures. In total five assays were used, 4 *in vitro* screens (yeast screen, GVBD, hepatocyte, ovulation), and 1 short-term *in vivo* screen (transgenic assay). The yeast screen was previously optimised for use, however, the other screens were developed in this study with varying success, indeed, only the ovulation assay proved to be useful for detecting EDg compounds. It was shown to be sensitive, informative, and of ecological relevance due to the role of ovulation in

reproduction, and could be modified to elucidate specific enzyme inhibition/ stimulation by testing additional steroids, and their intermediate compounds, in the culture media (Ahn *et al*., 2007). The majority of published reports concerning perturbations to steroidogenesis have focused on aromatase and 5α-R, due to their importance in catalyzing production of the highly active hormones E2 and DHT. Despite the important role of these enzymes *in vivo*, there were no obvious effects on aromatase in response to any pesticides, although the effect of the pesticides on 5α-R is not known as DHT was not measured. Instead it appeared that the hydroxy steroid dehydrogenases (HSDs) were affected, as testosterone levels were often decreased in relation to progesterone levels. Therefore, in addition to testing aromatase and 5α-R, my research suggests that the effects of pesticides and water contaminants on HSDs warrant further investigation. Furthermore, considering that many pesticides function by inhibiting active processes in the target organism, such as electron transport or enzyme activity, this route of ED is underrepresented in the literature. Data presented here have partially filled this gap in the literature, and demonstrated the importance of enzymatic endpoints on a physiological response (ovulation). Although activity of liver biotransformation has been measured in field-collected reptiles , it would also be interesting to measure gonadal enzyme expression in amphibians *in situ*. Since short-term *in vivo* exposure to environmentally relevant concentrations of PCP (slightly) affected hormone production of stimulated ovarian tissue *in vitro*, perhaps culture of ovarian tissue of collected individuals would be informative. Lastly, in the fens, estrogenicity was observed in hepatocytes exposed to C_{18} and OASIS extracts (breeding season one) and in YES in response to PADs (breeding season two), suggesting that these extraction methods and assays were comparable.

5.2 Pesticides

During this investigation, it became clear that very little is known about the effects of pesticides that are currently used in the UK and the USA, and thus probably the western world in general, on the endocrine system of wildlife. Paradoxically, in relative terms, much more is known about pesticides that are banned or have restricted use (e.g. DDT), and there is a negative correlation between published data of the effects of pesticides, and their presence in the UK environment. It precludes

explanation that banned pesticides have been found to have adverse effects on wildlife. These compounds may still be affecting wildlife, however, if the aim of environmental research is to protect the environment by assessing risk to wildlife and humans, it is essential that such research is environmentally relevant. Therefore, testing of compounds that are present in the environment and which have continued use, should have a higher priority than elucidating fine details, such as precise mechanisms of action, of redundant compounds. Furthermore, although many pesticides that have now been banned in the western world due to their EDg nature (e.g. DDT) are still used in developing countries, the issue is arguably one of a cultural nature, rather than a scientific one. On the other hand, since the majority of pesticides that were detected here (Appendix 1) are not new compounds, perhaps the fact that highly toxic effects have not been reported in recent history indicates that they are not having a large impact on wildlife. However, it is arguable that dramatic declines in amphibians (and other species) are indeed occurring, and these may be at least partially related to pesticides. Therefore, in contrast to the extreme toxic effects of pesticides reported in the 1960's, the effects we see presently are more subtle due to improved environmental quality, which are characteristic effects of ED.

The question of the effects of environmentally relevant pesticides was partially addressed in this study, and it was surprising to find that 80 % of the pesticides tested had a measurable response in at least one test, and approximately half at concentrations close to those found in the environment. In addition, effects of PCP observed *in vitro* were partially corroborated by effects observed *in vivo*, suggesting that other pesticides active in the yeast screen and ovulation assay may also affect endocrine endpoints *in vivo.* Furthermore, since pesticide mixtures have been reported to have an increased effect compared to single compounds (Hayes *et al.*, 2006), it would be informative to test the other pesticides that were active *in vitro* in exposure studies, both singly and in combination. Chemical analysis of the water extracts would have also assisted in interpretation of endocrine effects in relation to pesticides present in the extract. Nevertheless, it was interesting that the predominant receptor-mediated effects in response to water extracts were anti-estrogenic and anti-androgenic activity, which were also the effects observed in response to environmentally relevant pesticides, which were purchased and tested separately. In contrast, none of the pesticides tested were estrogenic, and estrogenic activity of water

extracts was only observed from one site, which may have been related to sewage treatment works effluent. Very little is known about the effects of model anti-estrogens/anti-androgens, or anti-estrogenic/anti-androgenic pesticides on amphibians. Therefore, in light of data presented here, the importance of anti-estrogenic/anti-androgenic pesticides and model compounds on amphibians *in vivo* warrants further investigation. Furthermore, comparison of these findings to *in vitro* data would assist in assessing the predictive value of the ovulation and yeast screen assays, on endocrinological effects *in vivo*. Finally, *in vitro* and *in vivo* tests could also be modified to increase environmental relevance by testing mixtures as well as single compounds.

5.3 Toads

Extracts from every field site tested were active in the yeast screens, and effects were observed in amphibians from a subsample of these sites, however, no correlation between ED effects of extracts and on toads *in vivo* could be observed. This was especially true at 20', which was highly estrogenic, but apparent masculinisation in toads occurred. However the low *n* values of specimens at all sites hindered the interpretation of results. Indeed, the field work aspect of this study highlighted the difficulties in doing field studies with amphibians. Due to previous reports of high mortality during larval stages, it could be argued that toads are not a good test species. However, toads are widespread in agricultural water bodies, and they may be declining in the UK . Furthermore, the presence of BO may assist to elucidating effects. Therefore, if the problems of mortality could be overcome by draining tanks earlier to prevent drowning, increasing cage area, as well as supplementing cages with food, they may be a useful test species in further experiments.

An unexpected finding from the field work was the substantial and prolonged differences in the laboratory-reared individuals collected at the various sites, in spite of the same rearing conditions. The eggs collected were of a very early stage in development, so whatever the cause of these effects, they occurred early in the life of the individual. Possible candidates include: a latent effect of the pond environment, genetic factors, or maternal transfer of contaminants. To the author's knowledge,

there are no reports in relation to latent effects of the pond environment. This could perhaps be studied by exposure of cloned embryos to test substances for varying periods (e.g. 48, 72, 96 hrs) and observing morphological and/or thyroidal and/or gonadal development. Maternal transfer of TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) administered to adult Zebrafish via the food, has been reported to cause early life stage toxicity of eggs . In this study, TCDD levels in the eggs were approximately 100-fold lower than the levels present in the food (40 or 100 ng/g) except at the lowest concentration (10 ng/g) after 20 days. In contrast, in a frog species (*Gastrophryne carolinensis*), approximately 50 % of the maternal burden of selenium, was transferred to the eggs, and levels were correlated with tadpole abnormalities in the FETAX assay . Therefore, it seems that adult exposure can affect offspring (e.g. DES in humans), and this may help to explain the high incidence of intersex observed in both laboratory-reared and caged individuals from 20'. Using this rationale, perhaps a combination of early life exposure and maternal transfer affected the individuals in this study. In contrast, it has been shown in *Rana temporaria* that there are effects of nutritional status of the female on size of eggs laid and corresponding tadpole and metamorph size. In addition, genetic factors such as male origin, affected the age at metamorphosis , indicating that differences observed in the lab may not have been related to contaminants. Furthermore, larval survival rate was correlated with genetic diversity and was lower in smaller, isolated urban, habitats in comparison to larger rural habitats . Therefore, care must be taken with interpretation of results of the field study, and the factors outlined above need to be taken into account. Finally, whatever the cause, this finding has implications for comparison of laboratory studies, where different effects have sometimes been reported in response to the same compounds under similar experimental conditions.

5.4 Conclusions & Further Direction

There is preliminary evidence that native amphibians may be affected by agricultural compounds, however, this requires further investigation. Furthermore, data presented here suggests that previously untested pesticides are EDs *in vitro*, but that the interactions between pesticides and amphibians are complex. Due to the work of Froglife, the UK is in a unique situation concerning long-term qualitative population

data, however, sex ratios are rarely recorded. Although, males are always more prevalent than females at crossing sites, it would be interesting to see if the sex ratio changes over time. In addition, it would be relatively easy to obtain these data by requesting that current toads on roads groups note the sex of individuals at the time of carrying across the road. In relation to specific sites and pesticides, in the first instance it would be beneficial to repeat the caged study (breeding season three) with modifications to try and decrease mortality, to see if results were reproducible. Water samples could be collected at these sites by grab samplings and PADs to verify anti-estrogenic/anti-androgenic/estrogenic results observed previously. Furthermore, analysis of genetic sex ratio and fitness (Hitchings and Beebee, 1998) could be tested in caged and laboratory-reared individuals at euthanisation. If necessary, further samples of adults from the populations could also be taken for genetic analysis, without killing the animals. In relation to the effects of pesticides, it would be informative to test the active pesticides reported in this study, *in vivo*, including various mixtures of these pesticides. These pesticides and mixtures could also be tested in relation to maternal transfer of compounds and their effects on offspring after short-term egg exposure. Finally, it may be informative to transplant and cage spawn from a relatively unpolluted site (e.g. PYL) as a 'reference' to other sites, which would provide a baseline of development, against which caged spawn from the native sites could be tested.

In conclusion, data reported here are preliminary in nature, but effects were observed in every endpoint tested, suggesting pesticides may be affecting amphibian populations, but further research is needed to substantiate reported effects.

6 Bibliography

Appendix 1

Levels of agrochemicals measured in U.K. freshwaters in 2004/2005 (Environment Agency Copyright 2007), and expenditure in the U.S.A. in 2001 (Kiely, 2004).

Herbicides/Plant Growth Regulators

Mode of Action	Type	Compound	Mean (µg/L)	Range (µg/L)	Median (µg/L)	Incidence (> 0.01 µg/L)	Median x Incidence	Rank UK	US
Synthetic auxin	Phenoxy- acids	Mecoprop	6.593	0.028-6180	0.1	1444	144.4	1	
		MCPA	12.052	0.04-4700	0.152	615	93.48	4	
		2,4-D	40.348	0.04-18600	0.140	488	68.32	5	5
		Dichlorprop	5.112	0.04-561	0.200	120	24	9	
		2,4-DB	0.315	0.04-1.4	0.200	35	7	17	
		MCPB	0.296	0.04-1.81	0.1	65	6.5	19	
		2,4,5-T	0.077	0.02-0.352	0.059	24	1.416	44	
		4-CPA	0.165	0.04-0.995	0.075	12	0.9	49	
	Benzoic Acid	Dicamba	89.617	0.035-5090	0.1	57	5.7	21	24
		2,3,6-TBA	0.814	0.01-4.4	0.21	19	3.99	26	
	Pyridinecarboxylic acid	Fluroxypyr	0.315	0.044-4.54	0.084	26	2.184	32	
		Triclopyr	0.097	0.03-0.34	0.061	27	1.647	39	
		Clopyralid	0.093	0.04-0.37	0.05	12	0.6	53	
	Unknown	Benazolin	0.908	0.05-4.150	0.585	21	12.285	14	
Photosynthetic electron transport inhibitor	Urea	Diuron	0.652	0.016-28	0.131	785	102.835	2	
		Isoproturon	0.443	0.02-29.5	0.150	635	95.25	3	
		Chlorotoluron	0.271	0.021-5.87	0.077	217	16.709	10	
		Linuron	0.123	0.2-1.4	0.057	74	4.218	24	
		Monuron	0.346	0.04-0.94	0.196	9	1.764	36	
		Fenuron	0.129	0.07-0.213	0.124	10	1.24	46	
		Neburon	0.055	0.04-0.089	0.053	7	0.371	54	
	Triazine	Simazine	0.067	0.01-2.24	0.038	1444	54.872	6	23

		Atrazine	0.053	0.01-1.96	0.024	1464	35.136	7	2
		Trietazine	1.559	0.01-5.91	0.925	18	16.65	11	
		Terbutryn	0.134	0.01-4	0.027	148	3.996	25	
		Propazine	1.075	0.5-2.76	0.567	5	2.835	30	
	Benzothiadiazinone	Bentazone	0.121	0.04-1.75	0.07	137	9.59	15	
	Hydroxybenzonitrile	Bromoxynil	0.838	0.04-6.5	0.35	18	6.3	20	
	Pyridazinone	Chloridazon	0.573	0.04-1.73	0.32	7	2.24	31	
Uncoupler of oxidative phosphorylation	Dinitrophenol	DNOC (insecticide)	0.274	0.04-1.8	0.1	17	1.7	38	
	Unknown	Pentachlorophenol (biocide)	0.304	0.016-2.74	0.163	93	15.159	12	
Inhibits cell division (blocks microtubule function)	Carbamate	Chlorpropham	4.816	0.01-269.6	0.089	103	9.167	16	
		Carbetamide	0.264	0.05-0.744	0.297	17	5.049	23	
	Benzamide	Propyzamide	2.55	0.01-310	0.042	124	5.208	22	
	Dinitroaniline	Trifluralin	0.038	0.01-3.06	0.018	183	3.294	28	12
Inhibits cell division (blocks protein synthesis)	Chloroacetamide	Metazochlor	0.048	0.01-0.588	0.019	82	1.558	40	
Inhibts cell division (blocks sterol synthesis)	Triazole	Paclobutrazol	0.021	0.01-0.038	0.02	9	0.18	62	
Inhibiton of cell wall synthesis	Benzonitrile	Dichlobenil	0.168	0.01-10.6	0.026	122	3.172	29	
Chitin synthesis inhibitor	Benzoylurea	Diflubenzuron	0.11	0.04-0.87	0.058	21	1.218	47	
Inhibits lipid synthesis	Benzofuron	Ethofunesate	0.573	0.01-13.3	0.023	76	1.748	37	
	Thiocarbamate	Tri-allate	0.016	0.01-0.035	0.012	13	0.156	63	
Inhibits synthesis of essential amino acids	Glycine Derivative	Glyphosate	27.95	0.11-1600	0.229	149	34.121	8	1

Insecticides & Fungicides (f)

Mode of Action	Type	Compound	Mean (µg/L)	Range (µg/L)	Median (µg/L)	Incidence (> 0.01 µg/L)	Median x Incidence	Rank UK	US

Nerve Poison (affects sodium balance in nerves)	Organochlorine	DDT	0.025	0.01-0.094	0.02	21	0.42	53	
		TDE (DDD)	0.027	0.014-0.11	0.018	11	0.198	61	
Antagonist of the GABA receptor	Cyclodiene organochlorine	Dieldrin	5.647	0.01-153.4	0.096	69	6.624	18	
		Aldrin	0.05	0.011-0.312	0.024	15	0.36	55	
		HCH	0.019	0.01-0.055	0.017	14	0.238	60	
Cholinestersae inhibition	Organophosphate	Diazinon	0.046	0.01-0.535	0.03	451	13.53	13	
		Parathion	0.754	0.012-3	0.085	17	1.445	43	
		Mevinphos	0.33	0.01-2.53	0.03	29	0.87	50	
		Dimethoate	0.055	0.011-0.367	0.024	29	0.696	51	
		Azinphos-methyl	0.053	0.01-0.178	0.032	11	0.352	56	
		Malathion	0.03	0.01-0.109	0.019	17	0.323	57	6
		Fenitrothion	0.068	0.013-0.137	0.06	4	0.24	59	
		Propetamphos	0.019	0.01-0.034	0.016	7	0.112	64	
		Chlorfenvinphos	0.032	0.015-0.073	0.023	4	0.092	66	
		Triazophos	0.013	0.01-0.02	0.01	6	0.06	67	
	Carbamate	Pirimicarb	0.328	0.011-8.86	0.085	46	3.91	27	
Nerve poison (blocks sodium transport in nerves)	Pyrethroid	Permethrin	1166.8	0.01-26400	0.029	30	0.87	50	
		Cypermethrin	0.033	0.012-0.093	0.024	11	0.264	58	
Inhibition of digestive enzymes	Mitin	Sulcofuron	0.523	0.25-0.75	0.545	4	2.18	33	
		Flucofuron	0.293	0.18-0.52	0.255	6	1.53	41	
	Unknown	Eulan	0.107	0.02-0.7	0.07	14	0.98	48	
Uncoupler of oxidative phosphorylation	Organotin	Tributyltin	0.089	0.01-7.9	0.016	115	1.84	34	
		Dibutyltin	0.034	0.01-0.4	0.017	87	1.479	42	
Inhibition of beta-tubulin synthesis	Benzimadazole	Carbendazim (f)	0.11	0.023-0.516	0.069	20	1.38	45	
Inhibtion of ergosterol synthesis	Triazole	Flutriafol (f)	0.072	0.012-0.162	0.068	26	1.768	35	
	Morpholine	Fenpropimorph (f)	0.03	0.018-0.064	0.02	5	0.1	65	
Lipid peroxidation	Chlorphenyl/nitroaniline	Tecnazene (f)	0.013	0.01-0.21	0.011	4	0.044	68	

(f) refers to fungicide

Appendix 2

Detected Pesticides at Sampling Sites

(Environment Agency)

2.1 Yatton:

- Co-ordinates: x,y = 342549, 175219

Date	Pesticide	Less than	Conc. (ng/L)	x	y
15/08/2005	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	10	347815	195172
25/07/2005	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	10	347815	195172
19/09/2005	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	1	347815	195172
10/10/2005	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	40	347815	195172
17/10/2005	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	40	347815	195172
13/06/2005	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	10	347815	195172
24/05/2005	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	10	347815	195172
12/12/2005	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	40	347815	195172
07/11/2005	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	40	347815	195172
10/02/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	366163	169000
17/03/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	366163	169000
13/05/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	366163	169000
17/06/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	366163	169000
15/04/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	60	366163	169000
15/08/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347815	195172
25/07/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347815	195172
10/10/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347815	195172
19/09/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347815	195172
17/10/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347815	195172
21/03/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347815	195172
18/01/2005	(2,4-DICHLOROPHENOXY)ETHANOIC	<	50	347815	195172

	ACID				
21/02/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347815	195172
24/05/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347815	195172
13/06/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347815	195172
18/04/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347815	195172
12/12/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347815	195172
07/11/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347815	195172
17/08/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347820	195220
11/10/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347820	195220
21/09/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347820	195220
22/11/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347820	195220
25/10/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347820	195220
08/11/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347820	195220
09/03/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347820	195220
13/04/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347820	195220
20/01/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347820	195220
24/02/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347820	195220
21/06/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347820	195220
19/07/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347820	195220
18/05/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	347820	195220
21/03/2005	2,4,5-TRICHLOROPHENOL	<	10	347815	195172
18/01/2005	2,4,5-TRICHLOROPHENOL	<	10	347815	195172
21/02/2005	2,4,5-TRICHLOROPHENOL	<	10	347815	195172
18/04/2005	2,4,5-TRICHLOROPHENOL	<	10	347815	195172
11/10/2004	2,4,5-TRICHLOROPHENOL	<	10	347820	195220
17/08/2004	2,4,5-TRICHLOROPHENOL	<	10	347820	195220
21/09/2004	2,4,5-TRICHLOROPHENOL	<	10	347820	195220
22/11/2004	2,4,5-TRICHLOROPHENOL	<	10	347820	195220
25/10/2004	2,4,5-TRICHLOROPHENOL	<	10	347820	195220
08/11/2004	2,4,5-TRICHLOROPHENOL	<	10	347820	195220
20/01/2004	2,4,5-TRICHLOROPHENOL	<	10	347820	195220
09/03/2004	2,4,5-TRICHLOROPHENOL	<	10	347820	195220
13/04/2004	2,4,5-TRICHLOROPHENOL	<	10	347820	195220
24/02/2004	2,4,5-TRICHLOROPHENOL	<	10	347820	195220
21/06/2004	2,4,5-TRICHLOROPHENOL	<	10	347820	195220
19/07/2004	2,4,5-TRICHLOROPHENOL	<	10	347820	195220
18/05/2004	2,4,5-TRICHLOROPHENOL	<	10	347820	195220

15/08/2005	2,4-DB	<	100	347815	195172
25/07/2005	2,4-DB	<	100	347815	195172
19/09/2005	2,4-DB	<	100	347815	195172
17/10/2005	2,4-DB	<	40	347815	195172
10/10/2005	2,4-DB	<	40	347815	195172
21/03/2005	2,4-DB	<	100	347815	195172
18/01/2005	2,4-DB	<	100	347815	195172
21/02/2005	2,4-DB	<	100	347815	195172
24/05/2005	2,4-DB	<	100	347815	195172
13/06/2005	2,4-DB	<	100	347815	195172
18/04/2005	2,4-DB	<	100	347815	195172
12/12/2005	2,4-DB	<	40	347815	195172
07/11/2005	2,4-DB	<	40	347815	195172
11/10/2004	2,4-DB	<	100	347820	195220
17/08/2004	2,4-DB	<	100	347820	195220
21/09/2004	2,4-DB	<	100	347820	195220
22/11/2004	2,4-DB	<	100	347820	195220
25/10/2004	2,4-DB	<	100	347820	195220
08/11/2004	2,4-DB	<	100	347820	195220
09/03/2004	2,4-DB	<	100	347820	195220
13/04/2004	2,4-DB	<	100	347820	195220
20/01/2004	2,4-DB	<	100	347820	195220
24/02/2004	2,4-DB	<	100	347820	195220
21/06/2004	2,4-DB	<	100	347820	195220
19/07/2004	2,4-DB	<	100	347820	195220
18/05/2004	2,4-DB	<	100	347820	195220
17/03/2004	ALDRIN	<	1	366163	169000
17/06/2004	ALDRIN	<	1	366163	169000
12/08/2004	ALDRIN	<	1	366163	169000
15/02/2005	ALDRIN	<	1	366163	169000
17/03/2005	ALDRIN	<	1	366163	169000
14/04/2005	ALDRIN	<	1	366163	169000
13/05/2005	ALDRIN	<	1	366163	169000
14/06/2005	ALDRIN	<	1	366163	169000
16/08/2005	ALDRIN	<	1	366163	169000
11/07/2005	ALDRIN	<	2	366163	169000
10/02/2004	ATRAZINE	<	3	366163	169000
17/03/2005	ATRAZINE	<	3	366163	169000
14/04/2005	ATRAZINE		8	366163	169000
15/02/2005	ATRAZINE		9	366163	169000
17/03/2004	ATRAZINE		11	366163	169000
15/04/2004	ATRAZINE		12	366163	169000
13/05/2004	ATRAZINE		16	366163	169000
17/06/2004	ATRAZINE	<	18	366163	169000
16/08/2005	ATRAZINE		19	366163	169000
13/05/2005	ATRAZINE		23	366163	169000
12/08/2004	ATRAZINE		36	366163	169000
14/06/2005	ATRAZINE		86	366163	169000
15/07/2004	ATRAZINE		129	366163	169000
11/07/2005	ATRAZINE		148	366163	169000
15/08/2005	ATRAZINE	<	10	347815	195172

25/07/2005	ATRAZINE	<	10	347815	195172
17/10/2005	ATRAZINE	<	10	347815	195172
19/09/2005	ATRAZINE	<	10	347815	195172
10/10/2005	ATRAZINE	<	10	347815	195172
21/03/2005	ATRAZINE	<	10	347815	195172
18/01/2005	ATRAZINE	<	10	347815	195172
21/02/2005	ATRAZINE	<	10	347815	195172
24/05/2005	ATRAZINE	<	10	347815	195172
13/06/2005	ATRAZINE	<	10	347815	195172
18/04/2005	ATRAZINE	<	10	347815	195172
12/12/2005	ATRAZINE	<	10	347815	195172
07/11/2005	ATRAZINE	<	10	347815	195172
11/10/2004	ATRAZINE	<	10	347820	195220
17/08/2004	ATRAZINE	<	10	347820	195220
21/09/2004	ATRAZINE	<	10	347820	195220
22/11/2004	ATRAZINE	<	10	347820	195220
25/10/2004	ATRAZINE	<	10	347820	195220
08/11/2004	ATRAZINE	<	10	347820	195220
09/03/2004	ATRAZINE	<	10	347820	195220
13/04/2004	ATRAZINE	<	10	347820	195220
20/01/2004	ATRAZINE	<	10	347820	195220
24/02/2004	ATRAZINE	<	10	347820	195220
21/06/2004	ATRAZINE	<	10	347820	195220
19/07/2004	ATRAZINE	<	10	347820	195220
18/05/2004	ATRAZINE	<	10	347820	195220
10/02/2004	AZINPHOS-ETHYL	<	6	366163	169000
17/03/2004	AZINPHOS-ETHYL	<	6	366163	169000
15/04/2004	AZINPHOS-ETHYL	<	6	366163	169000
13/05/2004	AZINPHOS-ETHYL	<	6	366163	169000
17/06/2004	AZINPHOS-ETHYL	<	6	366163	169000
15/07/2004	AZINPHOS-ETHYL	<	6	366163	169000
12/08/2004	AZINPHOS-ETHYL	<	6	366163	169000
15/02/2005	AZINPHOS-ETHYL	<	6	366163	169000
17/03/2005	AZINPHOS-ETHYL	<	6	366163	169000
14/04/2005	AZINPHOS-ETHYL	<	6	366163	169000
14/06/2005	AZINPHOS-ETHYL	<	6	366163	169000
11/07/2005	AZINPHOS-ETHYL	<	6	366163	169000
13/05/2005	AZINPHOS-ETHYL	<	7	366163	169000
16/08/2005	AZINPHOS-ETHYL	<	7	366163	169000
10/02/2004	AZINPHOS-METHYL	<	3	366163	169000
17/03/2004	AZINPHOS-METHYL	<	3	366163	169000
15/04/2004	AZINPHOS-METHYL	<	3	366163	169000
13/05/2004	AZINPHOS-METHYL	<	3	366163	169000
17/06/2004	AZINPHOS-METHYL	<	3	366163	169000
15/07/2004	AZINPHOS-METHYL	<	3	366163	169000
12/08/2004	AZINPHOS-METHYL	<	3	366163	169000
15/02/2005	AZINPHOS-METHYL	<	3	366163	169000
17/03/2005	AZINPHOS-METHYL	<	3	366163	169000
14/04/2005	AZINPHOS-METHYL	<	3	366163	169000
13/05/2005	AZINPHOS-METHYL	<	3	366163	169000
14/06/2005	AZINPHOS-METHYL	<	3	366163	169000

11/07/2005	AZINPHOS-METHYL	<	3	366163	169000
16/08/2005	AZINPHOS-METHYL	<	3	366163	169000
15/08/2005	BENAZOLIN	<	25	347815	195172
25/07/2005	BENAZOLIN	<	25	347815	195172
19/09/2005	BENAZOLIN	<	25	347815	195172
17/10/2005	BENAZOLIN	<	40	347815	195172
10/10/2005	BENAZOLIN	<	40	347815	195172
21/03/2005	BENAZOLIN	<	25	347815	195172
18/01/2005	BENAZOLIN	<	25	347815	195172
21/02/2005	BENAZOLIN	<	25	347815	195172
24/05/2005	BENAZOLIN	<	25	347815	195172
13/06/2005	BENAZOLIN	<	25	347815	195172
18/04/2005	BENAZOLIN	<	25	347815	195172
12/12/2005	BENAZOLIN	<	40	347815	195172
07/11/2005	BENAZOLIN	<	40	347815	195172
21/09/2004	BENAZOLIN	<	25	347820	195220
11/10/2004	BENAZOLIN	<	25	347820	195220
17/08/2004	BENAZOLIN	<	25	347820	195220
22/11/2004	BENAZOLIN	<	25	347820	195220
25/10/2004	BENAZOLIN	<	25	347820	195220
08/11/2004	BENAZOLIN	<	25	347820	195220
13/04/2004	BENAZOLIN	<	25	347820	195220
09/03/2004	BENAZOLIN	<	25	347820	195220
20/01/2004	BENAZOLIN	<	25	347820	195220
24/02/2004	BENAZOLIN	<	25	347820	195220
21/06/2004	BENAZOLIN	<	25	347820	195220
19/07/2004	BENAZOLIN	<	25	347820	195220
18/05/2004	BENAZOLIN	<	25	347820	195220
10/02/2004	BENTAZONE	<	40	366163	169000
17/03/2004	BENTAZONE	<	40	366163	169000
13/05/2004	BENTAZONE	<	40	366163	169000
17/06/2004	BENTAZONE	<	40	366163	169000
15/04/2004	BENTAZONE	<	50	366163	169000
15/08/2005	BENTAZONE	<	100	347815	195172
25/07/2005	BENTAZONE	<	100	347815	195172
17/10/2005	BENTAZONE	<	40	347815	195172
19/09/2005	BENTAZONE	<	100	347815	195172
10/10/2005	BENTAZONE	<	40	347815	195172
21/02/2005	BENTAZONE	<	100	347815	195172
21/03/2005	BENTAZONE	<	100	347815	195172
18/01/2005	BENTAZONE	<	100	347815	195172
24/05/2005	BENTAZONE	<	100	347815	195172
18/04/2005	BENTAZONE	<	100	347815	195172
13/06/2005	BENTAZONE	<	100	347815	195172
12/12/2005	BENTAZONE	<	40	347815	195172
07/11/2005	BENTAZONE	<	40	347815	195172
21/09/2004	BENTAZONE	<	100	347820	195220
11/10/2004	BENTAZONE	<	100	347820	195220
17/08/2004	BENTAZONE	<	100	347820	195220
22/11/2004	BENTAZONE	<	100	347820	195220
25/10/2004	BENTAZONE	<	100	347820	195220

08/11/2004	BENTAZONE	<	100	347820	195220
09/03/2004	BENTAZONE	<	100	347820	195220
20/01/2004	BENTAZONE	<	100	347820	195220
24/02/2004	BENTAZONE	<	100	347820	195220
21/06/2004	BENTAZONE	<	100	347820	195220
13/04/2004	BENTAZONE	<	100	347820	195220
19/07/2004	BENTAZONE	<	100	347820	195220
18/05/2004	BENTAZONE	<	100	347820	195220
17/10/2005	CHLORFENVINPHOS	<	1	347815	195172
15/08/2005	CHLORFENVINPHOS	<	1	347815	195172
25/07/2005	CHLORFENVINPHOS	<	1	347815	195172
13/06/2005	CHLORFENVINPHOS	<	1	347815	195172
19/09/2005	CHLORFENVINPHOS	<	1	347815	195172
10/10/2005	CHLORFENVINPHOS	<	1	347815	195172
21/02/2005	CHLORFENVINPHOS	<	1	347815	195172
21/03/2005	CHLORFENVINPHOS	<	1	347815	195172
18/01/2005	CHLORFENVINPHOS	<	1	347815	195172
24/05/2005	CHLORFENVINPHOS	<	1	347815	195172
18/04/2005	CHLORFENVINPHOS	<	1	347815	195172
12/12/2005	CHLORFENVINPHOS	<	1	347815	195172
07/11/2005	CHLORFENVINPHOS	<	1	347815	195172
21/09/2004	CHLORFENVINPHOS	<	1	347820	195220
11/10/2004	CHLORFENVINPHOS	<	1	347820	195220
17/08/2004	CHLORFENVINPHOS	<	1	347820	195220
22/11/2004	CHLORFENVINPHOS	<	1	347820	195220
19/07/2004	CHLORFENVINPHOS	<	1	347820	195220
25/10/2004	CHLORFENVINPHOS	<	1	347820	195220
08/11/2004	CHLORFENVINPHOS	<	1	347820	195220
09/03/2004	CHLORFENVINPHOS	<	1	347820	195220
20/01/2004	CHLORFENVINPHOS	<	1	347820	195220
24/02/2004	CHLORFENVINPHOS	<	1	347820	195220
21/06/2004	CHLORFENVINPHOS	<	1	347820	195220
13/04/2004	CHLORFENVINPHOS	<	1	347820	195220
18/05/2004	CHLORFENVINPHOS	<	1	347820	195220
17/03/2004	CYFLUTHRIN	<	1	366163	169000
17/06/2004	CYFLUTHRIN	<	1	366163	169000
12/08/2004	CYFLUTHRIN	<	1	366163	169000
17/03/2005	CYFLUTHRIN	<	1	366163	169000
17/10/2005	CYPERMETHRIN	<	1	347815	195172
15/08/2005	CYPERMETHRIN	<	1	347815	195172
13/06/2005	CYPERMETHRIN	<	1	347815	195172
25/07/2005	CYPERMETHRIN	<	1	347815	195172
19/09/2005	CYPERMETHRIN	<	1	347815	195172
10/10/2005	CYPERMETHRIN	<	1	347815	195172
21/03/2005	CYPERMETHRIN	<	1	347815	195172
18/01/2005	CYPERMETHRIN	<	1	347815	195172
21/02/2005	CYPERMETHRIN	<	1	347815	195172
24/05/2005	CYPERMETHRIN	<	1	347815	195172
18/04/2005	CYPERMETHRIN	<	1	347815	195172
12/12/2005	CYPERMETHRIN	<	1	347815	195172
07/11/2005	CYPERMETHRIN	<	1	347815	195172

21/09/2004	CYPERMETHRIN	<	1	347820	195220
11/10/2004	CYPERMETHRIN	<	1	347820	195220
17/08/2004	CYPERMETHRIN	<	1	347820	195220
22/11/2004	CYPERMETHRIN	<	1	347820	195220
25/10/2004	CYPERMETHRIN	<	1	347820	195220
08/11/2004	CYPERMETHRIN	<	1	347820	195220
09/03/2004	CYPERMETHRIN	<	1	347820	195220
20/01/2004	CYPERMETHRIN	<	1	347820	195220
24/02/2004	CYPERMETHRIN	<	1	347820	195220
21/06/2004	CYPERMETHRIN	<	1	347820	195220
19/07/2004	CYPERMETHRIN	<	1	347820	195220
13/04/2004	CYPERMETHRIN	<	1	347820	195220
18/05/2004	CYPERMETHRIN	<	1	347820	195220
17/03/2004	DDE (PP)	<	1	366163	169000
17/06/2004	DDE (PP)	<	1	366163	169000
12/08/2004	DDE (PP)	<	1	366163	169000
15/02/2005	DDE (PP)	<	1	366163	169000
17/03/2005	DDE (PP)	<	1	366163	169000
14/04/2005	DDE (PP)	<	1	366163	169000
13/05/2005	DDE (PP)	<	1	366163	169000
14/06/2005	DDE (PP)	<	1	366163	169000
16/08/2005	DDE (PP)	<	1	366163	169000
11/07/2005	DDE (PP)	<	2	366163	169000
17/03/2004	DDT (OP)	<	3	366163	169000
17/06/2004	DDT (OP)	<	3	366163	169000
15/02/2005	DDT (OP)	<	3	366163	169000
17/03/2005	DDT (OP)	<	3	366163	169000
14/04/2005	DDT (OP)	<	3	366163	169000
13/05/2005	DDT (OP)	<	3	366163	169000
16/08/2005	DDT (OP)	<	3	366163	169000
14/06/2005	DDT (OP)	<	4	366163	169000
11/07/2005	DDT (OP)	<	5	366163	169000
17/03/2004	DDT (PP)	<	1	366163	169000
17/06/2004	DDT (PP)	<	1	366163	169000
12/08/2004	DDT (PP)	<	1	366163	169000
15/02/2005	DDT (PP)	<	1	366163	169000
17/03/2005	DDT (PP)	<	1	366163	169000
14/04/2005	DDT (PP)	<	1	366163	169000
13/05/2005	DDT (PP)	<	1	366163	169000
14/06/2005	DDT (PP)	<	1	366163	169000
16/08/2005	DDT (PP)	<	1	366163	169000
11/07/2005	DDT (PP)	<	2	366163	169000
15/08/2005	DIAZINON	<	1	347815	195172
13/06/2005	DIAZINON	<	1	347815	195172
25/07/2005	DIAZINON	<	1	347815	195172
19/09/2005	DIAZINON	<	1	347815	195172
10/10/2005	DIAZINON	<	1	347815	195172
21/02/2005	DIAZINON	<	1	347815	195172
21/03/2005	DIAZINON	<	1	347815	195172
18/01/2005	DIAZINON	<	1	347815	195172
24/05/2005	DIAZINON	<	1	347815	195172

18/04/2005	DIAZINON	<	1	347815	195172
17/10/2005	DIAZINON	<	1	347815	195172
12/12/2005	DIAZINON	<	1	347815	195172
07/11/2005	DIAZINON	<	1	347815	195172
21/09/2004	DIAZINON	<	1	347820	195220
11/10/2004	DIAZINON	<	1	347820	195220
17/08/2004	DIAZINON	<	1	347820	195220
08/11/2004	DIAZINON	<	1	347820	195220
22/11/2004	DIAZINON	<	1	347820	195220
25/10/2004	DIAZINON		17.3	347820	195220
09/03/2004	DIAZINON	<	1	347820	195220
20/01/2004	DIAZINON	<	1	347820	195220
19/07/2004	DIAZINON	<	1	347820	195220
24/02/2004	DIAZINON	<	1	347820	195220
21/06/2004	DIAZINON	<	1	347820	195220
13/04/2004	DIAZINON	<	1	347820	195220
18/05/2004	DIAZINON	<	1	347820	195220
15/08/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347815	195172
13/06/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347815	195172
25/07/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347815	195172
19/09/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347815	195172
10/10/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	40	347815	195172
21/02/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347815	195172
21/03/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347815	195172
18/01/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347815	195172
24/05/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347815	195172
18/04/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347815	195172
12/12/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	40	347815	195172
17/10/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	40	347815	195172
07/11/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	40	347815	195172
11/10/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347820	195220
21/09/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347820	195220
17/08/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347820	195220
08/11/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347820	195220
22/11/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347820	195220
25/10/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347820	195220
09/03/2004	DICAMBA {3,6-DICHLORO(O-	<	25	347820	195220

	METHOXYBENZOIC ACID)}				
20/01/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347820	195220
24/02/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347820	195220
21/06/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347820	195220
19/07/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347820	195220
13/04/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347820	195220
18/05/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	25	347820	195220
10/02/2004	DICHLORVOS	<	4	366163	169000
17/03/2004	DICHLORVOS	<	4	366163	169000
15/04/2004	DICHLORVOS	<	4	366163	169000
13/05/2004	DICHLORVOS	<	4	366163	169000
17/06/2004	DICHLORVOS	<	4	366163	169000
15/07/2004	DICHLORVOS	<	4	366163	169000
12/08/2004	DICHLORVOS	<	4	366163	169000
15/02/2005	DICHLORVOS	<	4	366163	169000
17/03/2005	DICHLORVOS	<	4	366163	169000
14/04/2005	DICHLORVOS	<	4	366163	169000
14/06/2005	DICHLORVOS	<	4	366163	169000
11/07/2005	DICHLORVOS	<	4	366163	169000
16/08/2005	DICHLORVOS	<	4	366163	169000
13/05/2005	DICHLORVOS	<	5	366163	169000
10/02/2004	DIELDRIN	<	1	366163	169000
17/03/2004	DIELDRIN	<	1	366163	169000
15/04/2004	DIELDRIN	<	1	366163	169000
17/06/2004	DIELDRIN	<	1	366163	169000
15/07/2004	DIELDRIN	<	1	366163	169000
12/08/2004	DIELDRIN	<	1	366163	169000
15/02/2005	DIELDRIN	<	1	366163	169000
17/03/2005	DIELDRIN	<	1	366163	169000
14/04/2005	DIELDRIN	<	1	366163	169000
13/05/2005	DIELDRIN	<	1	366163	169000
14/06/2005	DIELDRIN	<	1	366163	169000
16/08/2005	DIELDRIN	<	1	366163	169000
11/07/2005	DIELDRIN	<	2	366163	169000
13/05/2004	DIELDRIN	<	10	366163	169000
10/02/2004	DIMETHOATE	<	6	366163	169000
17/03/2004	DIMETHOATE	<	6	366163	169000
15/04/2004	DIMETHOATE	<	6	366163	169000
13/05/2004	DIMETHOATE	<	6	366163	169000
17/06/2004	DIMETHOATE	<	6	366163	169000
15/07/2004	DIMETHOATE	<	6	366163	169000
14/06/2005	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)	<	6	366163	169000
16/08/2005	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)	<	6	366163	169000
11/07/2005	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)	<	11	366163	169000
10/02/2004	ENDOSULPHAN ALPHA	<	1	366163	169000
17/03/2004	ENDOSULPHAN ALPHA	<	1	366163	169000
15/04/2004	ENDOSULPHAN ALPHA	<	1	366163	169000

17/06/2004	ENDOSULPHAN ALPHA	<	1	366163	169000
15/07/2004	ENDOSULPHAN ALPHA	<	1	366163	169000
13/05/2004	ENDOSULPHAN ALPHA	<	10	366163	169000
10/02/2004	ENDOSULPHAN BETA	<	2	366163	169000
17/03/2004	ENDOSULPHAN BETA	<	2	366163	169000
15/04/2004	ENDOSULPHAN BETA	<	2	366163	169000
17/06/2004	ENDOSULPHAN BETA	<	2	366163	169000
15/07/2004	ENDOSULPHAN BETA	<	2	366163	169000
12/08/2004	ENDOSULPHAN BETA	<	2	366163	169000
15/02/2005	ENDOSULPHAN BETA	<	2	366163	169000
17/03/2005	ENDOSULPHAN BETA	<	2	366163	169000
14/04/2005	ENDOSULPHAN BETA	<	2	366163	169000
13/05/2005	ENDOSULPHAN BETA	<	2	366163	169000
14/06/2005	ENDOSULPHAN BETA	<	2	366163	169000
16/08/2005	ENDOSULPHAN BETA	<	2	366163	169000
11/07/2005	ENDOSULPHAN BETA	<	4	366163	169000
13/05/2004	ENDOSULPHAN BETA	<	20	366163	169000
17/03/2004	ENDRIN	<	1	366163	169000
17/06/2004	ENDRIN	<	1	366163	169000
12/08/2004	ENDRIN	<	1	366163	169000
15/02/2005	ENDRIN	<	1	366163	169000
17/03/2005	ENDRIN	<	1	366163	169000
14/04/2005	ENDRIN	<	1	366163	169000
13/05/2005	ENDRIN	<	3	366163	169000
14/06/2005	ENDRIN	<	3	366163	169000
16/08/2005	ENDRIN	<	3	366163	169000
11/07/2005	ENDRIN	<	5	366163	169000
10/02/2004	FENITROTHION	<	1	366163	169000
17/03/2004	FENITROTHION	<	1	366163	169000
15/04/2004	FENITROTHION	<	1	366163	169000
13/05/2004	FENITROTHION	<	1	366163	169000
17/06/2004	FENITROTHION	<	1	366163	169000
15/07/2004	FENITROTHION	<	1	366163	169000
12/08/2004	FENITROTHION	<	1	366163	169000
15/02/2005	FENITROTHION	<	1	366163	169000
17/03/2005	FENITROTHION	<	1	366163	169000
14/04/2005	FENITROTHION	<	1	366163	169000
13/05/2005	FENITROTHION	<	1	366163	169000
14/06/2005	FENITROTHION	<	1	366163	169000
11/07/2005	FENITROTHION	<	1	366163	169000
16/08/2005	FENITROTHION	<	1	366163	169000
25/07/2005	FENPROPIMORPH	<	10	347815	195172
15/08/2005	FENPROPIMORPH	<	10	347815	195172
13/06/2005	FENPROPIMORPH	<	10	347815	195172
19/09/2005	FENPROPIMORPH	<	10	347815	195172
10/10/2005	FENPROPIMORPH	<	10	347815	195172
21/02/2005	FENPROPIMORPH	<	10	347815	195172
21/03/2005	FENPROPIMORPH	<	10	347815	195172
18/01/2005	FENPROPIMORPH	<	10	347815	195172
24/05/2005	FENPROPIMORPH	<	10	347815	195172
18/04/2005	FENPROPIMORPH	<	10	347815	195172

12/12/2005	FENPROPIMORPH	<	10	347815	195172
17/10/2005	FENPROPIMORPH	<	10	347815	195172
07/11/2005	FENPROPIMORPH	<	10	347815	195172
21/09/2004	FENPROPIMORPH	<	10	347820	195220
11/10/2004	FENPROPIMORPH	<	10	347820	195220
17/08/2004	FENPROPIMORPH	<	10	347820	195220
22/11/2004	FENPROPIMORPH	<	10	347820	195220
08/11/2004	FENPROPIMORPH	<	10	347820	195220
25/10/2004	FENPROPIMORPH	<	10	347820	195220
24/02/2004	FENPROPIMORPH	<	10	347820	195220
09/03/2004	FENPROPIMORPH	<	10	347820	195220
20/01/2004	FENPROPIMORPH	<	10	347820	195220
21/06/2004	FENPROPIMORPH	<	10	347820	195220
19/07/2004	FENPROPIMORPH	<	10	347820	195220
13/04/2004	FENPROPIMORPH	<	10	347820	195220
18/05/2004	FENPROPIMORPH	<	10	347820	195220
10/02/2004	FENTHION	<	1	366163	169000
17/03/2004	FENTHION	<	1	366163	169000
15/04/2004	FENTHION	<	8	366163	169000
13/05/2004	FENTHION	<	8	366163	169000
17/06/2004	FENTHION	<	8	366163	169000
15/07/2004	FENTHION	<	8	366163	169000
15/02/2005	FENTHION	<	8	366163	169000
14/04/2005	FENTHION	<	8	366163	169000
14/06/2005	FENTHION	<	8	366163	169000
11/07/2005	FENTHION	<	8	366163	169000
13/05/2005	FENTHION	<	9	366163	169000
16/08/2005	FENTHION	<	9	366163	169000
17/03/2004	FLUCOFURON	<	100	366163	169000
17/06/2004	FLUCOFURON	<	100	366163	169000
12/08/2004	FLUCOFURON	<	100	366163	169000
17/03/2005	FLUCOFURON	<	100	366163	169000
15/08/2005	FLUMETHRIN	<	1	347815	195172
13/06/2005	FLUMETHRIN	<	1	347815	195172
25/07/2005	FLUMETHRIN	<	1	347815	195172
19/09/2005	FLUMETHRIN	<	1	347815	195172
10/10/2005	FLUMETHRIN	<	1	347815	195172
21/02/2005	FLUMETHRIN	<	1	347815	195172
21/03/2005	FLUMETHRIN	<	1	347815	195172
18/01/2005	FLUMETHRIN	<	1	347815	195172
24/05/2005	FLUMETHRIN	<	1	347815	195172
18/04/2005	FLUMETHRIN	<	1	347815	195172
12/12/2005	FLUMETHRIN	<	1	347815	195172
17/10/2005	FLUMETHRIN	<	1	347815	195172
07/11/2005	FLUMETHRIN	<	1	347815	195172
21/09/2004	FLUMETHRIN	<	1	347820	195220
11/10/2004	FLUMETHRIN	<	1	347820	195220
17/08/2004	FLUMETHRIN	<	1	347820	195220
08/11/2004	FLUMETHRIN	<	1	347820	195220
22/11/2004	FLUMETHRIN	<	1	347820	195220
25/10/2004	FLUMETHRIN	<	1	347820	195220

09/03/2004	FLUMETHRIN	<	1	347820	195220
20/01/2004	FLUMETHRIN	<	1	347820	195220
19/07/2004	FLUMETHRIN	<	1	347820	195220
24/02/2004	FLUMETHRIN	<	1	347820	195220
21/06/2004	FLUMETHRIN	<	1	347820	195220
13/04/2004	FLUMETHRIN	<	1	347820	195220
18/05/2004	FLUMETHRIN	<	1	347820	195220
15/08/2005	FLUROXYPYR	<	25	347815	195172
13/06/2005	FLUROXYPYR	<	25	347815	195172
25/07/2005	FLUROXYPYR	<	25	347815	195172
19/09/2005	FLUROXYPYR	<	25	347815	195172
10/10/2005	FLUROXYPYR	<	50	347815	195172
21/02/2005	FLUROXYPYR	<	25	347815	195172
18/01/2005	FLUROXYPYR	<	25	347815	195172
21/03/2005	FLUROXYPYR	<	25	347815	195172
24/05/2005	FLUROXYPYR	<	25	347815	195172
18/04/2005	FLUROXYPYR	<	25	347815	195172
12/12/2005	FLUROXYPYR	<	50	347815	195172
17/10/2005	FLUROXYPYR		57	347815	195172
07/11/2005	FLUROXYPYR	<	50	347815	195172
21/09/2004	FLUROXYPYR	<	25	347820	195220
11/10/2004	FLUROXYPYR	<	25	347820	195220
17/08/2004	FLUROXYPYR	<	25	347820	195220
08/11/2004	FLUROXYPYR	<	25	347820	195220
19/07/2004	FLUROXYPYR	<	25	347820	195220
22/11/2004	FLUROXYPYR	<	25	347820	195220
25/10/2004	FLUROXYPYR	<	25	347820	195220
09/03/2004	FLUROXYPYR	<	25	347820	195220
20/01/2004	FLUROXYPYR	<	25	347820	195220
24/02/2004	FLUROXYPYR	<	25	347820	195220
21/06/2004	FLUROXYPYR	<	25	347820	195220
13/04/2004	FLUROXYPYR	<	25	347820	195220
18/05/2004	FLUROXYPYR	<	25	347820	195220
17/03/2004	HCH ALPHA	<	2	366163	169000
17/06/2004	HCH ALPHA	<	2	366163	169000
12/08/2004	HCH ALPHA	<	2	366163	169000
15/02/2005	HCH ALPHA	<	2	366163	169000
17/03/2005	HCH ALPHA	<	2.1	366163	169000
14/04/2005	HCH ALPHA	<	2.1	366163	169000
13/05/2005	HCH ALPHA	<	2.1	366163	169000
16/08/2005	HCH ALPHA	<	2.1	366163	169000
14/06/2005	HCH ALPHA	<	3	366163	169000
11/07/2005	HCH ALPHA	<	4	366163	169000
17/03/2004	HCH BETA	<	2	366163	169000
17/06/2004	HCH BETA	<	2	366163	169000
12/08/2004	HCH BETA	<	2	366163	169000
15/02/2005	HCH BETA	<	2	366163	169000
17/03/2005	HCH BETA	<	2.1	366163	169000
14/04/2005	HCH BETA	<	2.1	366163	169000
13/05/2005	HCH BETA	<	2.1	366163	169000
16/08/2005	HCH BETA	<	2.1	366163	169000

14/06/2005	HCH BETA	<	3	366163	169000
11/07/2005	HCH BETA	<	4	366163	169000
17/03/2004	HCH GAMMA	<	2	366163	169000
17/06/2004	HCH GAMMA	<	2	366163	169000
12/08/2004	HCH GAMMA	<	2	366163	169000
15/02/2005	HCH GAMMA	<	2	366163	169000
17/03/2005	HCH GAMMA	<	2.2	366163	169000
14/04/2005	HCH GAMMA	<	2.2	366163	169000
13/05/2005	HCH GAMMA	<	2.2	366163	169000
16/08/2005	HCH GAMMA	<	2.2	366163	169000
14/06/2005	HCH GAMMA	<	3	366163	169000
11/07/2005	HCH GAMMA	<	4	366163	169000
16/08/2005	HCH TOTAL (ALPHA, BETA & GAMMA)	<	6.4	366163	169000
14/06/2005	HCH TOTAL (ALPHA, BETA & GAMMA)	<	9	366163	169000
11/07/2005	HCH TOTAL (ALPHA, BETA & GAMMA)	<	12	366163	169000
17/03/2004	HEXACHLOROBENZENE	<	1	366163	169000
17/06/2004	HEXACHLOROBENZENE	<	1	366163	169000
12/08/2004	HEXACHLOROBENZENE	<	1	366163	169000
15/02/2005	HEXACHLOROBENZENE	<	1	366163	169000
17/03/2005	HEXACHLOROBENZENE	<	1	366163	169000
13/05/2005	HEXACHLOROBENZENE	<	1	366163	169000
14/06/2005	HEXACHLOROBENZENE	<	1	366163	169000
16/08/2005	HEXACHLOROBENZENE	<	1	366163	169000
11/07/2005	HEXACHLOROBENZENE	<	2	366163	169000
17/03/2004	ISODRIN	<	1	366163	169000
17/06/2004	ISODRIN	<	1	366163	169000
12/08/2004	ISODRIN	<	1	366163	169000
15/02/2005	ISODRIN	<	1	366163	169000
17/03/2005	ISODRIN	<	1	366163	169000
14/04/2005	ISODRIN	<	1	366163	169000
13/05/2005	ISODRIN	<	1	366163	169000
14/06/2005	ISODRIN	<	1	366163	169000
16/08/2005	ISODRIN	<	1	366163	169000
11/07/2005	ISODRIN	<	2	366163	169000
10/02/2004	LINURON	<	50	366163	169000
17/03/2004	LINURON	<	50	366163	169000
15/04/2004	LINURON	<	50	366163	169000
13/05/2004	LINURON	<	50	366163	169000
17/06/2004	LINURON	<	50	366163	169000
15/07/2004	LINURON	<	50	366163	169000
10/02/2004	MALATHION	<	2	366163	169000
17/03/2004	MALATHION	<	2	366163	169000
15/04/2004	MALATHION	<	2	366163	169000
13/05/2004	MALATHION	<	2	366163	169000
17/06/2004	MALATHION	<	2	366163	169000
15/07/2004	MALATHION	<	2	366163	169000
12/08/2004	MALATHION	<	2	366163	169000
15/02/2005	MALATHION	<	2	366163	169000
17/03/2005	MALATHION	<	2	366163	169000
14/04/2005	MALATHION	<	2	366163	169000
13/05/2005	MALATHION	<	2	366163	169000

14/06/2005	MALATHION	<	2	366163	169000
11/07/2005	MALATHION	<	2	366163	169000
16/08/2005	MALATHION	<	2	366163	169000
10/02/2004	MCPA	<	40	366163	169000
17/03/2004	MCPA	<	40	366163	169000
17/06/2004	MCPA	<	40	366163	169000
15/04/2004	MCPA	<	50	366163	169000
13/05/2004	MCPA		80	366163	169000
15/08/2005	MCPA	<	25	347815	195172
13/06/2005	MCPA	<	25	347815	195172
25/07/2005	MCPA	<	25	347815	195172
19/09/2005	MCPA	<	25	347815	195172
10/10/2005	MCPA	<	40	347815	195172
24/05/2005	MCPA	<	25	347815	195172
12/12/2005	MCPA	<	40	347815	195172
17/10/2005	MCPA	<	40	347815	195172
07/11/2005	MCPA	<	40	347815	195172
25/07/2005	MCPB	<	100	347815	195172
13/06/2005	MCPB	<	100	347815	195172
15/08/2005	MCPB	<	100	347815	195172
19/09/2005	MCPB	<	100	347815	195172
10/10/2005	MCPB	<	60	347815	195172
21/02/2005	MCPB	<	100	347815	195172
18/01/2005	MCPB	<	100	347815	195172
24/05/2005	MCPB	<	100	347815	195172
21/03/2005	MCPB	<	100	347815	195172
18/04/2005	MCPB	<	100	347815	195172
12/12/2005	MCPB	<	60	347815	195172
17/10/2005	MCPB	<	60	347815	195172
07/11/2005	MCPB	<	60	347815	195172
21/09/2004	MCPB	<	100	347820	195220
11/10/2004	MCPB	<	100	347820	195220
19/07/2004	MCPB	<	100	347820	195220
17/08/2004	MCPB	<	100	347820	195220
08/11/2004	MCPB	<	100	347820	195220
22/11/2004	MCPB	<	100	347820	195220
25/10/2004	MCPB	<	100	347820	195220
24/02/2004	MCPB	<	100	347820	195220
09/03/2004	MCPB	<	100	347820	195220
20/01/2004	MCPB	<	100	347820	195220
21/06/2004	MCPB	<	100	347820	195220
13/04/2004	MCPB	<	100	347820	195220
18/05/2004	MCPB	<	100	347820	195220
10/02/2004	MECOPROP	<	40	366163	169000
13/05/2004	MECOPROP	<	40	366163	169000
17/06/2004	MECOPROP	<	40	366163	169000
15/04/2004	MECOPROP	<	50	366163	169000
17/03/2004	MECOPROP		80	366163	169000
25/07/2005	MECOPROP	<	25	347815	195172
13/06/2005	MECOPROP	<	25	347815	195172
19/09/2005	MECOPROP	<	25	347815	195172

10/10/2005	MECOPROP	<	40	347815	195172
15/08/2005	MECOPROP	<	25	347815	195172
21/02/2005	MECOPROP	<	25	347815	195172
18/01/2005	MECOPROP	<	25	347815	195172
24/05/2005	MECOPROP	<	25	347815	195172
21/03/2005	MECOPROP	<	25	347815	195172
18/04/2005	MECOPROP		1025	347815	195172
12/12/2005	MECOPROP	<	40	347815	195172
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21/09/2004	MECOPROP	<	25	347820	195220
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22/11/2004	MECOPROP	<	25	347820	195220
25/10/2004	MECOPROP	<	25	347820	195220
24/02/2004	MECOPROP	<	25	347820	195220
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13/04/2004	MECOPROP	<	25	347820	195220
18/05/2004	MECOPROP	<	25	347820	195220
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17/03/2004	MEVINPHOS	<	2	366163	169000
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17/06/2004	MEVINPHOS	<	8	366163	169000
15/07/2004	MEVINPHOS	<	8	366163	169000
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17/03/2004	PARATHION {PARATHION ETHYL}	<	4	366163	169000
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16/08/2005	PARATHION {PARATHION ETHYL}	<	4	366163	169000
13/05/2005	PARATHION {PARATHION ETHYL}	<	5	366163	169000
25/07/2005	PARATHION {PARATHION ETHYL}	<	5	347815	195172
13/06/2005	PARATHION {PARATHION ETHYL}	<	5	347815	195172
18/04/2005	PARATHION {PARATHION ETHYL}	<	5	347815	195172
19/09/2005	PARATHION {PARATHION ETHYL}	<	5	347815	195172
15/08/2005	PARATHION {PARATHION ETHYL}	<	5	347815	195172
10/10/2005	PARATHION {PARATHION ETHYL}	<	5	347815	195172
21/02/2005	PARATHION {PARATHION ETHYL}	<	5	347815	195172
18/01/2005	PARATHION {PARATHION ETHYL}	<	5	347815	195172

24/05/2005	PARATHION {PARATHION ETHYL}	<	5	347815	195172
21/03/2005	PARATHION {PARATHION ETHYL}	<	5	347815	195172
07/11/2005	PARATHION {PARATHION ETHYL}	<	5	347815	195172
17/10/2005	PARATHION {PARATHION ETHYL}	<	5	347815	195172
12/12/2005	PARATHION {PARATHION ETHYL}	<	5	347815	195172
21/09/2004	PARATHION {PARATHION ETHYL}	<	5	347820	195220
11/10/2004	PARATHION {PARATHION ETHYL}	<	5	347820	195220
19/07/2004	PARATHION {PARATHION ETHYL}	<	5	347820	195220
17/08/2004	PARATHION {PARATHION ETHYL}	<	5	347820	195220
08/11/2004	PARATHION {PARATHION ETHYL}	<	5	347820	195220
22/11/2004	PARATHION {PARATHION ETHYL}	<	5	347820	195220
25/10/2004	PARATHION {PARATHION ETHYL}	<	5	347820	195220
24/02/2004	PARATHION {PARATHION ETHYL}	<	5	347820	195220
09/03/2004	PARATHION {PARATHION ETHYL}	<	5	347820	195220
20/01/2004	PARATHION {PARATHION ETHYL}	<	5	347820	195220
21/06/2004	PARATHION {PARATHION ETHYL}	<	5	347820	195220
13/04/2004	PARATHION {PARATHION ETHYL}	<	5	347820	195220
18/05/2004	PARATHION {PARATHION ETHYL}	<	5	347820	195220
10/02/2004	PARATHION-METHYL	<	3	366163	169000
17/03/2004	PARATHION-METHYL	<	3	366163	169000
15/04/2004	PARATHION-METHYL	<	3	366163	169000
13/05/2004	PARATHION-METHYL	<	3	366163	169000
17/06/2004	PARATHION-METHYL	<	3	366163	169000
15/07/2004	PARATHION-METHYL	<	3	366163	169000
12/08/2004	PARATHION-METHYL	<	3	366163	169000
15/02/2005	PARATHION-METHYL	<	3	366163	169000
17/03/2005	PARATHION-METHYL	<	3	366163	169000
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11/07/2005	PARATHION-METHYL	<	3	366163	169000
16/08/2005	PARATHION-METHYL	<	3	366163	169000
17/03/2004	PCSD'S (CHLORPHENYLID)	<	2	366163	169000
17/06/2004	PCSD'S (CHLORPHENYLID)	<	2	366163	169000
12/08/2004	PCSD'S (CHLORPHENYLID)	<	4	366163	169000
17/03/2005	PENTACHLOROPHENOL	<	20	366163	169000
14/04/2005	PENTACHLOROPHENOL	<	20	366163	169000
13/05/2005	PENTACHLOROPHENOL	<	20	366163	169000
14/06/2005	PENTACHLOROPHENOL	<	20	366163	169000
11/07/2005	PENTACHLOROPHENOL	<	20	366163	169000
17/03/2004	PENTACHLOROPHENOL	<	40	366163	169000
17/06/2004	PENTACHLOROPHENOL	<	40	366163	169000
12/08/2004	PENTACHLOROPHENOL	<	40	366163	169000
16/08/2005	PENTACHLOROPHENOL	<	125	366163	169000
17/03/2004	PERMETHRIN, CIS	<	1	366163	169000
17/06/2004	PERMETHRIN, CIS	<	1	366163	169000
12/08/2004	PERMETHRIN, CIS	<	1	366163	169000
17/03/2005	PERMETHRIN, CIS	<	2	366163	169000
17/03/2004	PERMETHRIN, TRANS	<	1	366163	169000
17/06/2004	PERMETHRIN, TRANS	<	1	366163	169000
12/08/2004	PERMETHRIN, TRANS	<	1	366163	169000

17/03/2005	PERMETHRIN, TRANS	<	2	366163	169000
25/07/2005	PROCHLORAZ	<	29	347815	195172
13/06/2005	PROCHLORAZ	<	29	347815	195172
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17/10/2005	PROCHLORAZ	<	29	347815	195172
21/09/2004	PROCHLORAZ	<	29	347820	195220
19/07/2004	PROCHLORAZ	<	29	347820	195220
11/10/2004	PROCHLORAZ	<	29	347820	195220
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20/01/2004	PROCHLORAZ	<	29	347820	195220
21/06/2004	PROCHLORAZ	<	29	347820	195220
13/04/2004	PROCHLORAZ	<	29	347820	195220
18/05/2004	PROCHLORAZ	<	29	347820	195220
25/07/2005	PROPETAMPHOS	<	1	347815	195172
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18/01/2005	PROPETAMPHOS	<	1	347815	195172
24/05/2005	PROPETAMPHOS	<	1	347815	195172
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25/10/2004	PROPETAMPHOS	<	1	347820	195220
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21/06/2004	PROPETAMPHOS	<	1	347820	195220

13/04/2004	PROPETAMPHOS	<	1	347820	195220
18/05/2004	PROPETAMPHOS	<	1	347820	195220
25/07/2005	PROPICONAZOLE	<	10	347815	195172
13/06/2005	PROPICONAZOLE	<	10	347815	195172
19/09/2005	PROPICONAZOLE	<	10	347815	195172
15/08/2005	PROPICONAZOLE	<	10	347815	195172
10/10/2005	PROPICONAZOLE	<	10	347815	195172
21/02/2005	PROPICONAZOLE	<	10	347815	195172
18/01/2005	PROPICONAZOLE	<	10	347815	195172
24/05/2005	PROPICONAZOLE	<	10	347815	195172
21/03/2005	PROPICONAZOLE	<	10	347815	195172
18/04/2005	PROPICONAZOLE	<	10	347815	195172
07/11/2005	PROPICONAZOLE	<	10	347815	195172
12/12/2005	PROPICONAZOLE	<	10	347815	195172
17/10/2005	PROPICONAZOLE	<	10	347815	195172
21/09/2004	PROPICONAZOLE	<	10	347820	195220
19/07/2004	PROPICONAZOLE	<	10	347820	195220
17/08/2004	PROPICONAZOLE	<	10	347820	195220
08/11/2004	PROPICONAZOLE	<	10	347820	195220
22/11/2004	PROPICONAZOLE	<	10	347820	195220
11/10/2004	PROPICONAZOLE	<	10	347820	195220
25/10/2004	PROPICONAZOLE	<	10	347820	195220
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09/03/2004	PROPICONAZOLE	<	10	347820	195220
20/01/2004	PROPICONAZOLE	<	10	347820	195220
18/05/2004	PROPICONAZOLE	<	10	347820	195220
21/06/2004	PROPICONAZOLE	<	10	347820	195220
13/04/2004	PROPICONAZOLE	<	10	347820	195220
15/07/2004	SIMAZINE	<	3	366163	169000
17/03/2005	SIMAZINE	<	3	366163	169000
15/04/2004	SIMAZINE		10	366163	169000
17/06/2004	SIMAZINE		10	366163	169000
10/02/2004	SIMAZINE		11	366163	169000
11/07/2005	SIMAZINE		12	366163	169000
13/05/2004	SIMAZINE		13	366163	169000
14/06/2005	SIMAZINE		14	366163	169000
12/08/2004	SIMAZINE		19	366163	169000
15/02/2005	SIMAZINE		23	366163	169000
13/05/2005	SIMAZINE		23	366163	169000
17/03/2004	SIMAZINE		31	366163	169000
16/08/2005	SIMAZINE		32	366163	169000
14/04/2005	SIMAZINE		59	366163	169000
25/07/2005	SIMAZINE	<	10	347815	195172
13/06/2005	SIMAZINE	<	10	347815	195172
19/09/2005	SIMAZINE	<	10	347815	195172
15/08/2005	SIMAZINE	<	10	347815	195172
21/02/2005	SIMAZINE	<	10	347815	195172
18/01/2005	SIMAZINE	<	10	347815	195172
18/04/2005	SIMAZINE		35.3	347815	195172
24/05/2005	SIMAZINE	<	10	347815	195172
21/03/2005	SIMAZINE	<	10	347815	195172

07/11/2005	SIMAZINE	<	10	347815	195172
12/12/2005	SIMAZINE	<	10	347815	195172
10/10/2005	SIMAZINE	<	10	347815	195172
17/10/2005	SIMAZINE	<	10	347815	195172
21/09/2004	SIMAZINE	<	10	347820	195220
19/07/2004	SIMAZINE	<	10	347820	195220
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08/11/2004	SIMAZINE	<	10	347820	195220
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21/06/2004	SIMAZINE	<	10	347820	195220
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10/02/2004	SULCOFURON	<	2500	366163	169000
17/03/2004	SULCOFURON	<	2500	366163	169000
15/04/2004	SULCOFURON	<	2500	366163	169000
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17/03/2005	SULCOFURON	<	2500	366163	169000
17/03/2004	TDE (PP)	<	1	366163	169000
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11/07/2005	TDE (PP)	<	2	366163	169000
25/07/2005	TERBUTRYN	<	2	347815	195172
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12/12/2005	TERBUTRYN	<	2	347815	195172
17/10/2005	TERBUTRYN	<	2	347815	195172
21/09/2004	TERBUTRYN	<	2	347820	195220
19/07/2004	TERBUTRYN	<	2	347820	195220
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08/11/2004	TERBUTRYN	<	2	347820	195220
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20/01/2004	TERBUTRYN	<	2	347820	195220
18/05/2004	TERBUTRYN	<	2	347820	195220
21/06/2004	TERBUTRYN	<	2	347820	195220
13/04/2004	TERBUTRYN	<	2	347820	195220
10/02/2004	TRIAZOPHOS	<	4	366163	169000
17/03/2004	TRIAZOPHOS	<	4	366163	169000
13/05/2004	TRIAZOPHOS	<	4	366163	169000
17/06/2004	TRIAZOPHOS	<	4	366163	169000
15/07/2004	TRIAZOPHOS	<	4	366163	169000
10/02/2004	TRIBUTYL TIN	<	2	366163	169000
17/03/2004	TRIBUTYL TIN	<	2	366163	169000
15/04/2004	TRIBUTYL TIN	<	2	366163	169000
13/05/2004	TRIBUTYL TIN	<	2	366163	169000
17/06/2004	TRIBUTYL TIN	<	2	366163	169000
15/07/2004	TRIBUTYL TIN	<	2	366163	169000
10/02/2004	TRIFLURALIN	<	11	366163	169000
17/03/2004	TRIFLURALIN	<	11	366163	169000
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16/08/2005	TRIFLURALIN	<	13	366163	169000
14/06/2005	TRIFLURALIN	<	14	366163	169000
11/07/2005	TRIFLURALIN	<	21	366163	169000
13/05/2004	TRIFLURALIN	<	114	366163	169000

2.2 Pant-y-Llyn:

- Co-ordinates: x,y = 260654,216617

Date	Pesticide	Less than	Conc. (ng/L)	x	y
15/03/2005	TRIAZOPHOS	<	1	261461	208764
14/06/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	261461	208764
15/03/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	261461	208764
06/12/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	261461	208764
13/09/2005	(2,4-DICHLOROPHENOXY)ETHANOIC	<	50	261461	208764

	ACID				
15/06/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	261461	208764
09/03/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	261461	208764
17/02/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	261461	208764
11/05/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	261461	208764
13/01/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	261461	208764
24/05/2005	ALDRIN	<	2.5	261461	208764
14/04/2005	ALDRIN	<	2.5	261461	208764
14/06/2005	ALDRIN	<	2.5	261461	208764
11/01/2005	ALDRIN	<	2.5	261461	208764
15/02/2005	ALDRIN	<	2.5	261461	208764
15/03/2005	ALDRIN	<	2.5	261461	208764
08/11/2005	ALDRIN	<	2.5	261461	208764
11/10/2005	ALDRIN	<	2.5	261461	208764
06/12/2005	ALDRIN	<	2.5	261461	208764
09/08/2005	ALDRIN	<	2.5	261461	208764
12/07/2005	ALDRIN	<	2.5	261461	208764
13/09/2005	ALDRIN	<	2.5	261461	208764
10/08/2004	ALDRIN	<	2.5	261461	208764
13/07/2004	ALDRIN	<	2.5	261461	208764
15/06/2004	ALDRIN	<	2.5	261461	208764
17/02/2004	ALDRIN	<	2.5	261461	208764
09/03/2004	ALDRIN	<	2.5	261461	208764
13/01/2004	ALDRIN	<	2.5	261461	208764
11/05/2004	ALDRIN	<	2.5	261461	208764
15/04/2004	ALDRIN	<	2.5	261461	208764
14/06/2005	ATRAZINE	<	10	261461	208764
15/03/2005	ATRAZINE	<	10	261461	208764
06/12/2005	ATRAZINE	<	10	261461	208764
13/09/2005	ATRAZINE	<	10	261461	208764
15/06/2004	ATRAZINE	<	10	261461	208764
17/02/2004	ATRAZINE	<	10	261461	208764
13/01/2004	ATRAZINE	<	10	261461	208764
09/03/2004	ATRAZINE	<	10	261461	208764
11/05/2004	ATRAZINE	<	10	261461	208764
15/04/2004	ATRAZINE	<	10	261461	208764
14/06/2005	AZINPHOS-METHYL	<	1	261461	208764
15/03/2005	AZINPHOS-METHYL	<	1	261461	208764
06/12/2005	AZINPHOS-METHYL	<	1	261461	208764
13/09/2005	AZINPHOS-METHYL	<	1	261461	208764
15/06/2004	AZINPHOS-METHYL	<	1	261461	208764
09/03/2004	AZINPHOS-METHYL	<	1	261461	208764
17/02/2004	AZINPHOS-METHYL	<	1	261461	208764
13/01/2004	AZINPHOS-METHYL	<	1	261461	208764
11/05/2004	AZINPHOS-METHYL	<	1	261461	208764
15/04/2004	AZINPHOS-METHYL	<	1	261461	208764
06/12/2005	BENTAZONE	<	40	261461	208764

14/06/2005	BENTAZONE	<	100	261461	208764
15/03/2005	BENTAZONE	<	100	261461	208764
13/09/2005	BENTAZONE	<	100	261461	208764
15/06/2004	BENTAZONE	<	100	261461	208764
17/02/2004	BENTAZONE	<	100	261461	208764
13/01/2004	BENTAZONE	<	100	261461	208764
11/05/2004	BENTAZONE	<	100	261461	208764
14/04/2005	DDE (PP)	<	1.5	261461	208764
24/05/2005	DDE (PP)	<	1.5	261461	208764
14/06/2005	DDE (PP)	<	1.5	261461	208764
15/02/2005	DDE (PP)	<	1.5	261461	208764
11/01/2005	DDE (PP)	<	1.5	261461	208764
15/03/2005	DDE (PP)	<	1.5	261461	208764
08/11/2005	DDE (PP)	<	1.5	261461	208764
11/10/2005	DDE (PP)	<	1.5	261461	208764
06/12/2005	DDE (PP)	<	1.5	261461	208764
09/08/2005	DDE (PP)	<	1.5	261461	208764
12/07/2005	DDE (PP)	<	1.5	261461	208764
13/09/2005	DDE (PP)	<	1.5	261461	208764
13/07/2004	DDE (PP)	<	1.5	261461	208764
10/08/2004	DDE (PP)	<	1.5	261461	208764
15/06/2004	DDE (PP)	<	1.5	261461	208764
17/02/2004	DDE (PP)	<	1.5	261461	208764
13/01/2004	DDE (PP)	<	1.5	261461	208764
11/05/2004	DDE (PP)	<	1.5	261461	208764
09/03/2004	DDE (PP)	<	1.5	261461	208764
15/04/2004	DDE (PP)	<	1.5	261461	208764
24/05/2005	DDT (PP)	<	1	261461	208764
14/04/2005	DDT (PP)	<	1	261461	208764
14/06/2005	DDT (PP)	<	1	261461	208764
15/02/2005	DDT (PP)	<	1	261461	208764
11/01/2005	DDT (PP)	<	1	261461	208764
15/03/2005	DDT (PP)	<	1	261461	208764
08/11/2005	DDT (PP)	<	1	261461	208764
11/10/2005	DDT (PP)	<	1	261461	208764
06/12/2005	DDT (PP)	<	1	261461	208764
09/08/2005	DDT (PP)	<	1	261461	208764
12/07/2005	DDT (PP)	<	1	261461	208764
13/09/2005	DDT (PP)	<	1	261461	208764
13/07/2004	DDT (PP)	<	1	261461	208764
10/08/2004	DDT (PP)	<	1	261461	208764
15/06/2004	DDT (PP)	<	1	261461	208764
17/02/2004	DDT (PP)	<	1	261461	208764
13/01/2004	DDT (PP)	<	1	261461	208764
11/05/2004	DDT (PP)	<	1	261461	208764
09/03/2004	DDT (PP)	<	1	261461	208764
15/04/2004	DDT (PP)	<	1	261461	208764
14/06/2005	DICHLORVOS	<	1	261461	208764
15/03/2005	DICHLORVOS	<	1	261461	208764
06/12/2005	DICHLORVOS	<	1	261461	208764
13/09/2005	DICHLORVOS	<	1	261461	208764

15/06/2004	DICHLORVOS	<	1	261461	208764
17/02/2004	DICHLORVOS	<	1	261461	208764
13/01/2004	DICHLORVOS	<	1	261461	208764
11/05/2004	DICHLORVOS	<	1	261461	208764
15/04/2004	DICHLORVOS	<	1	261461	208764
09/03/2004	DICHLORVOS	<	1	261461	208764
24/05/2005	DIELDRIN	<	2.5	261461	208764
14/04/2005	DIELDRIN	<	2.5	261461	208764
14/06/2005	DIELDRIN	<	2.5	261461	208764
15/02/2005	DIELDRIN	<	2.5	261461	208764
11/01/2005	DIELDRIN	<	2.5	261461	208764
15/03/2005	DIELDRIN	<	2.5	261461	208764
08/11/2005	DIELDRIN	<	2.5	261461	208764
11/10/2005	DIELDRIN	<	2.5	261461	208764
06/12/2005	DIELDRIN	<	2.5	261461	208764
09/08/2005	DIELDRIN	<	2.5	261461	208764
12/07/2005	DIELDRIN	<	2.5	261461	208764
13/09/2005	DIELDRIN	<	2.5	261461	208764
13/07/2004	DIELDRIN	<	2.5	261461	208764
10/08/2004	DIELDRIN	<	2.5	261461	208764
15/06/2004	DIELDRIN	<	2.5	261461	208764
17/02/2004	DIELDRIN	<	2.5	261461	208764
13/01/2004	DIELDRIN	<	2.5	261461	208764
15/04/2004	DIELDRIN	<	2.5	261461	208764
11/05/2004	DIELDRIN	<	2.5	261461	208764
09/03/2004	DIELDRIN	<	2.5	261461	208764
14/06/2005	DIMETHOATE	<	5	261461	208764
15/03/2005	DIMETHOATE	<	5	261461	208764
06/12/2005	DIMETHOATE	<	5	261461	208764
13/09/2005	DIMETHOATE	<	5	261461	208764
15/06/2004	DIMETHOATE	<	5	261461	208764
17/02/2004	DIMETHOATE	<	5	261461	208764
13/01/2004	DIMETHOATE	<	5	261461	208764
11/05/2004	DIMETHOATE	<	5	261461	208764
15/04/2004	DIMETHOATE	<	5	261461	208764
09/03/2004	DIMETHOATE	<	5	261461	208764
14/06/2005	ENDOSULPHAN ALPHA	<	1	261461	208764
15/03/2005	ENDOSULPHAN ALPHA	<	1	261461	208764
06/12/2005	ENDOSULPHAN ALPHA	<	1	261461	208764
13/09/2005	ENDOSULPHAN ALPHA	<	1	261461	208764
15/06/2004	ENDOSULPHAN ALPHA	<	1	261461	208764
17/02/2004	ENDOSULPHAN ALPHA	<	1	261461	208764
13/01/2004	ENDOSULPHAN ALPHA	<	1	261461	208764
15/04/2004	ENDOSULPHAN ALPHA	<	1	261461	208764
11/05/2004	ENDOSULPHAN ALPHA	<	1	261461	208764
09/03/2004	ENDOSULPHAN ALPHA	<	1	261461	208764
14/06/2005	ENDOSULPHAN BETA	<	1	261461	208764
15/03/2005	ENDOSULPHAN BETA	<	1	261461	208764
06/12/2005	ENDOSULPHAN BETA	<	1	261461	208764
13/09/2005	ENDOSULPHAN BETA	<	1	261461	208764
15/06/2004	ENDOSULPHAN BETA	<	1	261461	208764

17/02/2004	ENDOSULPHAN BETA	<	1	261461	208764
15/04/2004	ENDOSULPHAN BETA	<	1	261461	208764
11/05/2004	ENDOSULPHAN BETA	<	1	261461	208764
09/03/2004	ENDOSULPHAN BETA	<	1	261461	208764
24/05/2005	ENDRIN	<	2.5	261461	208764
14/04/2005	ENDRIN	<	2.5	261461	208764
14/06/2005	ENDRIN	<	2.5	261461	208764
15/02/2005	ENDRIN	<	2.5	261461	208764
11/01/2005	ENDRIN	<	2.5	261461	208764
15/03/2005	ENDRIN	<	2.5	261461	208764
08/11/2005	ENDRIN	<	2.5	261461	208764
11/10/2005	ENDRIN	<	2.5	261461	208764
06/12/2005	ENDRIN	<	2.5	261461	208764
09/08/2005	ENDRIN	<	2.5	261461	208764
12/07/2005	ENDRIN	<	2.5	261461	208764
13/09/2005	ENDRIN	<	2.5	261461	208764
13/07/2004	ENDRIN	<	2.5	261461	208764
10/08/2004	ENDRIN	<	2.5	261461	208764
15/06/2004	ENDRIN	<	2.5	261461	208764
17/02/2004	ENDRIN	<	2.5	261461	208764
15/04/2004	ENDRIN	<	2.5	261461	208764
11/05/2004	ENDRIN	<	2.5	261461	208764
09/03/2004	ENDRIN	<	2.5	261461	208764
14/06/2005	FENITROTHION	<	1	261461	208764
15/03/2005	FENITROTHION	<	1	261461	208764
06/12/2005	FENITROTHION	<	1	261461	208764
13/09/2005	FENITROTHION	<	1	261461	208764
15/06/2004	FENITROTHION	<	1	261461	208764
17/02/2004	FENITROTHION	<	1	261461	208764
15/04/2004	FENITROTHION	<	1	261461	208764
11/05/2004	FENITROTHION	<	1	261461	208764
09/03/2004	FENITROTHION	<	1	261461	208764
24/05/2005	HCH GAMMA	<	1	261461	208764
14/04/2005	HCH GAMMA	<	1	261461	208764
14/06/2005	HCH GAMMA	<	1	261461	208764
15/02/2005	HCH GAMMA	<	1	261461	208764
11/01/2005	HCH GAMMA	<	1	261461	208764
15/03/2005	HCH GAMMA	<	1	261461	208764
08/11/2005	HCH GAMMA	<	1	261461	208764
11/10/2005	HCH GAMMA	<	1	261461	208764
06/12/2005	HCH GAMMA	<	1	261461	208764
09/08/2005	HCH GAMMA	<	1	261461	208764
12/07/2005	HCH GAMMA	<	1	261461	208764
13/09/2005	HCH GAMMA	<	1	261461	208764
13/07/2004	HCH GAMMA	<	1	261461	208764
10/08/2004	HCH GAMMA	<	1	261461	208764
15/06/2004	HCH GAMMA	<	1	261461	208764
17/02/2004	HCH GAMMA	<	1	261461	208764
15/04/2004	HCH GAMMA	<	1	261461	208764
09/03/2004	HCH GAMMA	<	1	261461	208764
15/03/2005	LINURON	<	20	261461	208764

06/12/2005	LINURON	<	20	261461	208764
15/06/2004	LINURON	<	20	261461	208764
17/02/2004	LINURON	<	20	261461	208764
11/05/2004	LINURON	<	20	261461	208764
15/04/2004	LINURON	<	20	261461	208764
09/03/2004	LINURON	<	20	261461	208764
14/06/2005	LINURON	<	23	261461	208764
13/09/2005	LINURON	<	24	261461	208764
14/06/2005	MALATHION	<	5	261461	208764
15/03/2005	MALATHION	<	5	261461	208764
06/12/2005	MALATHION	<	5	261461	208764
13/09/2005	MALATHION	<	5	261461	208764
11/05/2004	MALATHION	<	5	261461	208764
15/06/2004	MALATHION	<	5	261461	208764
17/02/2004	MALATHION	<	5	261461	208764
15/04/2004	MALATHION	<	5	261461	208764
09/03/2004	MALATHION	<	5	261461	208764
14/06/2005	MECOPROP	<	25	261461	208764
15/03/2005	MECOPROP	<	25	261461	208764
13/09/2005	MECOPROP	<	25	261461	208764
11/05/2004	MECOPROP	<	25	261461	208764
15/06/2004	MECOPROP	<	25	261461	208764
17/02/2004	MECOPROP	<	25	261461	208764
09/03/2004	MECOPROP	<	25	261461	208764
06/12/2005	MECOPROP	<	40	261461	208764
14/06/2005	MEVINPHOS	<	1	261461	208764
15/03/2005	MEVINPHOS	<	1	261461	208764
06/12/2005	MEVINPHOS	<	1	261461	208764
13/09/2005	MEVINPHOS	<	1	261461	208764
11/05/2004	MEVINPHOS	<	1	261461	208764
15/06/2004	MEVINPHOS	<	1	261461	208764
17/02/2004	MEVINPHOS	<	1	261461	208764
15/04/2004	MEVINPHOS	<	1	261461	208764
09/03/2004	MEVINPHOS	<	1	261461	208764
11/05/2004	OMETHOATE	<	10	261461	208764
15/06/2004	OMETHOATE	<	10	261461	208764
17/02/2004	OMETHOATE	<	10	261461	208764
15/04/2004	OMETHOATE	<	10	261461	208764
09/03/2004	OMETHOATE	<	10	261461	208764
15/03/2005	SIMAZINE	<	10	261461	208764
14/06/2005	SIMAZINE	<	10	261461	208764
06/12/2005	SIMAZINE	<	10	261461	208764
13/09/2005	SIMAZINE	<	10	261461	208764
15/06/2004	SIMAZINE	<	10	261461	208764
11/05/2004	SIMAZINE	<	10	261461	208764
17/02/2004	SIMAZINE	<	10	261461	208764
15/04/2004	SIMAZINE	<	10	261461	208764
09/03/2004	SIMAZINE	<	10	261461	208764
14/04/2005	TDE (PP)	<	1.5	261461	208764
24/05/2005	TDE (PP)	<	1.5	261461	208764
15/02/2005	TDE (PP)	<	1.5	261461	208764

11/01/2005	TDE (PP)	<	1.5	261461	208764
15/03/2005	TDE (PP)	<	1.5	261461	208764
11/10/2005	TDE (PP)	<	1.5	261461	208764
08/11/2005	TDE (PP)	<	1.5	261461	208764
13/09/2005	TDE (PP)	<	1.5	261461	208764
06/12/2005	TDE (PP)	<	1.5	261461	208764
09/08/2005	TDE (PP)	<	1.5	261461	208764
14/06/2005	TDE (PP)	<	1.5	261461	208764
12/07/2005	TDE (PP)	<	1.5	261461	208764
13/07/2004	TDE (PP)	<	1.5	261461	208764
10/08/2004	TDE (PP)	<	1.5	261461	208764
11/05/2004	TDE (PP)	<	1.5	261461	208764
15/06/2004	TDE (PP)	<	1.5	261461	208764
17/02/2004	TDE (PP)	<	1.5	261461	208764
15/04/2004	TDE (PP)	<	1.5	261461	208764
09/03/2004	TDE (PP)	<	1.5	261461	208764
13/09/2005	TRIAZOPHOS	<	1	261461	208764
06/12/2005	TRIAZOPHOS	<	1	261461	208764
14/06/2005	TRIAZOPHOS	<	1	261461	208764
15/06/2004	TRIAZOPHOS	<	1	261461	208764
11/05/2004	TRIAZOPHOS	<	1	261461	208764
17/02/2004	TRIAZOPHOS	<	1	261461	208764
15/04/2004	TRIAZOPHOS	<	1	261461	208764
09/03/2004	TRIAZOPHOS	<	1	261461	208764
24/05/2005	TRIBUTYL TIN	<	2	261461	208764
14/04/2005	TRIBUTYL TIN	<	2	261461	208764
15/03/2005	TRIBUTYL TIN	<	2	261461	208764
15/02/2005	TRIBUTYL TIN	<	2	261461	208764
13/07/2004	TRIBUTYL TIN	<	2	261461	208764
15/06/2004	TRIBUTYL TIN	<	2	261461	208764
10/08/2004	TRIBUTYL TIN	<	2	261461	208764
11/05/2004	TRIBUTYL TIN	<	2	261461	208764
17/02/2004	TRIBUTYL TIN	<	2	261461	208764
15/04/2004	TRIBUTYL TIN	<	2	261461	208764
09/03/2004	TRIBUTYL TIN	<	2	261461	208764
11/01/2005	TRIBUTYL TIN		3.2	261461	208764
15/03/2005	TRIFLURALIN	<	5	261461	208764
13/09/2005	TRIFLURALIN	<	5	261461	208764
06/12/2005	TRIFLURALIN	<	5	261461	208764
14/06/2005	TRIFLURALIN	<	5	261461	208764
15/06/2004	TRIFLURALIN	<	5	261461	208764
11/05/2004	TRIFLURALIN	<	5	261461	208764
17/02/2004	TRIFLURALIN	<	5	261461	208764
13/01/2004	TRIFLURALIN	<	5	261461	208764
15/04/2004	TRIFLURALIN	<	5	261461	208764
09/03/2004	TRIFLURALIN	<	5	261461	208764
14/04/2005	TRIPHENYLTIN COMPOUNDS	<	2	261461	208764
15/03/2005	TRIPHENYLTIN COMPOUNDS	<	2	261461	208764
24/05/2005	TRIPHENYLTIN COMPOUNDS	<	2	261461	208764
11/01/2005	TRIPHENYLTIN COMPOUNDS	<	2	261461	208764
15/02/2005	TRIPHENYLTIN COMPOUNDS	<	2	261461	208764

13/07/2004	TRIPHENYLTIN COMPOUNDS	<	2	261461	208764
15/06/2004	TRIPHENYLTIN COMPOUNDS	<	2	261461	208764
10/08/2004	TRIPHENYLTIN COMPOUNDS	<	2	261461	208764
11/05/2004	TRIPHENYLTIN COMPOUNDS	<	2	261461	208764
17/02/2004	TRIPHENYLTIN COMPOUNDS	<	2	261461	208764
13/01/2004	TRIPHENYLTIN COMPOUNDS	<	2	261461	208764
15/04/2004	TRIPHENYLTIN COMPOUNDS	<	2	261461	208764
09/03/2004	TRIPHENYLTIN COMPOUNDS	<	2	261461	208764

2.3 20' River/Ibberson's Pump:

- 20' River co-ordinates: x,y = 532215, 297208
- Ibberson's Pump co-ordinates: x,y = 535900, 288000

Date	Pesticide	Less than	Conc. (ng/L)	x	y
04/03/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	539400	274700
14/04/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	539400	274700
12/05/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	539400	274700
04/06/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	539400	274700
11/08/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	50	539400	274700
14/04/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID		60	539400	274700
14/07/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	60	539400	274700
19/04/2004	2,3,5,6-TETRACHLOROAMINOBENZENE {2,...ANILINE}	<	1	539400	274700
14/07/2004	2,3,5,6-TETRACHLOROAMINOBENZENE {2,...ANILINE}	<	1	539400	274700
11/04/2005	2,3,5,6-TETRACHLOROAMINOBENZENE {2,...ANILINE}	<	1	539400	274700
05/05/2005	2,3,5,6-TETRACHLOROAMINOBENZENE {2,...ANILINE}	<	1	535664	293648
02/06/2005	2,3,5,6-TETRACHLOROAMINOBENZENE {2,...ANILINE}	<	1	535664	293648
03/08/2005	2,3,5,6-TETRACHLOROAMINOBENZENE {2,...ANILINE}	<	1	535664	293648
11/02/2005	2,3,5,6-TETRACHLOROAMINOBENZENE {2,...ANILINE}	<	5	535664	293648
14/03/2005	2,3,5,6-TETRACHLOROAMINOBENZENE {2,...ANILINE}	<	5	535664	293648
07/04/2005	2,3,5,6-TETRACHLOROAMINOBENZENE {2,...ANILINE}	<	10	535664	293648
05/05/2005	2,3,5,6-TETRACHLOROTHIOANISOLE	<	1	535664	293648
02/06/2005	2,3,5,6-TETRACHLOROTHIOANISOLE	<	1	535664	293648
03/08/2005	2,3,5,6-TETRACHLOROTHIOANISOLE	<	1	535664	293648
11/02/2005	2,3,5,6-TETRACHLOROTHIOANISOLE	<	5	535664	293648
14/03/2005	2,3,5,6-TETRACHLOROTHIOANISOLE	<	5	535664	293648
07/04/2005	2,3,5,6-TETRACHLOROTHIOANISOLE	<	10	535664	293648
04/03/2004	ALDRIN	<	1	539400	274700
14/04/2004	ALDRIN	<	1	539400	274700
14/04/2004	ALDRIN	<	1	539400	274700
12/05/2004	ALDRIN	<	1	539400	274700
04/06/2004	ALDRIN	<	1	539400	274700
14/07/2004	ALDRIN	<	1	539400	274700

14/07/2004	ALDRIN	<	1	539400	274700
11/08/2004	ALDRIN	<	1	539400	274700
10/02/2005	ALDRIN	<	1	539400	274700
10/03/2005	ALDRIN	<	1	539400	274700
11/04/2005	ALDRIN	<	1	539400	274700
10/05/2005	ALDRIN	<	1	539400	274700
06/06/2005	ALDRIN	<	1	539400	274700
01/08/2005	ALDRIN	<	1	539400	274700
18/02/2004	ALDRIN	<	1	544200	299200
14/04/2004	ALDRIN	<	1	544200	299200
05/05/2004	ALDRIN	<	1	544200	299200
25/05/2004	ALDRIN	<	1	544200	299200
28/07/2004	ALDRIN	<	1	544200	299200
10/08/2004	ALDRIN	<	1	544200	299200
15/02/2005	ALDRIN	<	1	544200	299200
14/03/2005	ALDRIN	<	1	544200	299200
08/04/2005	ALDRIN	<	1	544200	299200
02/06/2005	ALDRIN	<	1	544200	299200
03/08/2005	ALDRIN	<	1	544200	299200
05/05/2005	ALDRIN		2	544200	299200
11/04/2005	ATRAZINE	<	3	539400	274700
10/02/2005	ATRAZINE		6	539400	274700
10/03/2005	ATRAZINE		7	539400	274700
14/04/2004	ATRAZINE		12	539400	274700
14/04/2004	ATRAZINE	<	13	539400	274700
06/06/2005	ATRAZINE		15	539400	274700
04/06/2004	ATRAZINE		16	539400	274700
11/08/2004	ATRAZINE		18	539400	274700
10/05/2005	ATRAZINE		20	539400	274700
14/07/2004	ATRAZINE		23	539400	274700
12/05/2004	ATRAZINE		25	539400	274700
14/07/2004	ATRAZINE		26	539400	274700
18/07/2005	ATRAZINE		38	539400	274700
01/08/2005	ATRAZINE		45	539400	274700
14/04/2004	AZINPHOS-METHYL	<	3	539400	274700
14/04/2004	AZINPHOS-METHYL	<	3	539400	274700
12/05/2004	AZINPHOS-METHYL	<	3	539400	274700
04/06/2004	AZINPHOS-METHYL	<	3	539400	274700
14/07/2004	AZINPHOS-METHYL	<	3	539400	274700
11/08/2004	AZINPHOS-METHYL	<	3	539400	274700
04/03/2004	BENTAZONE	<	40	539400	274700
14/04/2004	BENTAZONE	<	40	539400	274700
14/04/2004	BENTAZONE	<	40	539400	274700
04/06/2004	BENTAZONE	<	40	539400	274700
14/07/2004	BENTAZONE	<	40	539400	274700
11/08/2004	BENTAZONE	<	40	539400	274700
12/05/2004	BENTAZONE		200	539400	274700
02/06/2005	CHLOROPROPHAM		8	535664	293648
03/08/2005	CHLOROPROPHAM		10	535664	293648
05/05/2005	CHLOROPROPHAM		17	535664	293648
07/04/2005	CHLOROPROPHAM		419	535664	293648
11/02/2005	CHLOROPROPHAM		601	535664	293648
14/03/2005	CHLOROPROPHAM		2140	535664	293648
14/07/2004	CHLOROTHALONIL	<	10	539400	274700
18/07/2005	CHLOROTHALONIL	<	40	539400	274700
11/04/2005	CIS-HEPTACHLOR EPOXIDE	<	2.3	539400	274700
19/04/2004	DDE (OP)	<	1	539400	274700
14/07/2004	DDE (OP)	<	1	539400	274700

11/04/2005	DDE (OP)	<	1	539400	274700
04/03/2004	DDE (PP)	<	1	539400	274700
14/04/2004	DDE (PP)	<	1	539400	274700
14/04/2004	DDE (PP)	<	1	539400	274700
12/05/2004	DDE (PP)	<	1	539400	274700
04/06/2004	DDE (PP)	<	1	539400	274700
14/07/2004	DDE (PP)	<	1	539400	274700
14/07/2004	DDE (PP)	<	1	539400	274700
11/08/2004	DDE (PP)	<	1	539400	274700
10/02/2005	DDE (PP)	<	1	539400	274700
10/03/2005	DDE (PP)	<	1	539400	274700
11/04/2005	DDE (PP)	<	1	539400	274700
10/05/2005	DDE (PP)	<	1	539400	274700
06/06/2005	DDE (PP)	<	1	539400	274700
01/08/2005	DDE (PP)	<	1	539400	274700
19/04/2004	DDT (OP)	<	3	539400	274700
14/07/2004	DDT (OP)	<	3	539400	274700
11/04/2005	DDT (OP)	<	3	539400	274700
04/03/2004	DDT (PP)	<	1	539400	274700
14/04/2004	DDT (PP)	<	1	539400	274700
14/04/2004	DDT (PP)	<	1	539400	274700
12/05/2004	DDT (PP)	<	1	539400	274700
04/06/2004	DDT (PP)	<	1	539400	274700
14/07/2004	DDT (PP)	<	1	539400	274700
14/07/2004	DDT (PP)	<	1	539400	274700
11/08/2004	DDT (PP)	<	1	539400	274700
10/02/2005	DDT (PP)	<	1	539400	274700
10/03/2005	DDT (PP)	<	1	539400	274700
11/04/2005	DDT (PP)	<	1	539400	274700
10/05/2005	DDT (PP)	<	1	539400	274700
06/06/2005	DDT (PP)	<	1	539400	274700
01/08/2005	DDT (PP)	<	1	539400	274700
14/04/2004	DICHLORVOS	<	4	539400	274700
14/04/2004	DICHLORVOS	<	4	539400	274700
12/05/2004	DICHLORVOS	<	4	539400	274700
04/06/2004	DICHLORVOS	<	4	539400	274700
14/07/2004	DICHLORVOS	<	4	539400	274700
11/08/2004	DICHLORVOS	<	4	539400	274700
04/03/2004	DIELDRIN	<	1	539400	274700
14/04/2004	DIELDRIN	<	1	539400	274700
14/04/2004	DIELDRIN	<	1	539400	274700
12/05/2004	DIELDRIN	<	1	539400	274700
04/06/2004	DIELDRIN	<	1	539400	274700
14/07/2004	DIELDRIN	<	1	539400	274700
14/07/2004	DIELDRIN	<	1	539400	274700
11/08/2004	DIELDRIN	<	1	539400	274700
10/02/2005	DIELDRIN	<	1	539400	274700
10/03/2005	DIELDRIN	<	1	539400	274700
11/04/2005	DIELDRIN	<	1	539400	274700
10/05/2005	DIELDRIN	<	1	539400	274700
06/06/2005	DIELDRIN	<	1	539400	274700
01/08/2005	DIELDRIN	<	1	539400	274700
18/02/2004	DIELDRIN	<	1	544200	299200
14/04/2004	DIELDRIN	<	1	544200	299200
05/05/2004	DIELDRIN	<	1	544200	299200
25/05/2004	DIELDRIN	<	1	544200	299200
28/07/2004	DIELDRIN	<	1	544200	299200
10/08/2004	DIELDRIN	<	1	544200	299200

15/02/2005	DIELDRIN	<	1	544200	299200
14/03/2005	DIELDRIN	<	1	544200	299200
08/04/2005	DIELDRIN	<	1	544200	299200
02/06/2005	DIELDRIN	<	1	544200	299200
03/08/2005	DIELDRIN	<	1	544200	299200
05/05/2005	DIELDRIN		3	544200	299200
14/04/2004	DIMETHOATE	<	6	539400	274700
14/04/2004	DIMETHOATE	<	6	539400	274700
12/05/2004	DIMETHOATE	<	6	539400	274700
04/06/2004	DIMETHOATE	<	6	539400	274700
14/07/2004	DIMETHOATE	<	6	539400	274700
11/08/2004	DIMETHOATE	<	6	539400	274700
14/04/2004	DIURON		914	539400	274700
14/04/2004	DIURON		1008	539400	274700
11/04/2005	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)	<	4	539400	274700
18/02/2004	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)	<	4	544200	299200
14/04/2004	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)	<	4	544200	299200
05/05/2004	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)	<	4	544200	299200
25/05/2004	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)	<	4	544200	299200
28/07/2004	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)	<	4	544200	299200
10/08/2004	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)	<	4	544200	299200
15/02/2005	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)	<	4	544200	299200
14/03/2005	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)	<	4	544200	299200
08/04/2005	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)	<	4	544200	299200
02/06/2005	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)	<	6	544200	299200
03/08/2005	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)	<	6	544200	299200
05/05/2005	DRINS TOTAL (AL-, DIEL-, EN- & ISO-)		9	544200	299200
04/03/2004	ENDOSULPHAN ALPHA	<	1	539400	274700
14/04/2004	ENDOSULPHAN ALPHA	<	1	539400	274700
14/04/2004	ENDOSULPHAN ALPHA	<	1	539400	274700
19/04/2004	ENDOSULPHAN ALPHA	<	1	539400	274700
12/05/2004	ENDOSULPHAN ALPHA	<	1	539400	274700
04/06/2004	ENDOSULPHAN ALPHA	<	1	539400	274700
14/07/2004	ENDOSULPHAN ALPHA	<	1	539400	274700
14/07/2004	ENDOSULPHAN ALPHA	<	1	539400	274700
11/08/2004	ENDOSULPHAN ALPHA	<	1	539400	274700
11/04/2005	ENDOSULPHAN ALPHA	<	1	539400	274700
04/03/2004	ENDOSULPHAN BETA	<	2	539400	274700
14/04/2004	ENDOSULPHAN BETA	<	2	539400	274700
14/04/2004	ENDOSULPHAN BETA	<	2	539400	274700
19/04/2004	ENDOSULPHAN BETA	<	2	539400	274700
12/05/2004	ENDOSULPHAN BETA	<	2	539400	274700
04/06/2004	ENDOSULPHAN BETA	<	2	539400	274700
14/07/2004	ENDOSULPHAN BETA	<	2	539400	274700
14/07/2004	ENDOSULPHAN BETA	<	2	539400	274700
11/08/2004	ENDOSULPHAN BETA	<	2	539400	274700
11/04/2005	ENDOSULPHAN BETA	<	2	539400	274700
04/03/2004	ENDRIN	<	1	539400	274700
14/04/2004	ENDRIN	<	1	539400	274700
14/04/2004	ENDRIN	<	1	539400	274700
12/05/2004	ENDRIN	<	1	539400	274700
04/06/2004	ENDRIN	<	1	539400	274700
14/07/2004	ENDRIN	<	1	539400	274700
14/07/2004	ENDRIN	<	1	539400	274700
11/08/2004	ENDRIN	<	1	539400	274700
10/02/2005	ENDRIN	<	1	539400	274700
10/03/2005	ENDRIN	<	1	539400	274700
11/04/2005	ENDRIN	<	1	539400	274700

10/05/2005	ENDRIN	<	3	539400	274700
06/06/2005	ENDRIN	<	3	539400	274700
01/08/2005	ENDRIN	<	3	539400	274700
18/02/2004	ENDRIN	<	1	544200	299200
14/04/2004	ENDRIN	<	1	544200	299200
05/05/2004	ENDRIN	<	1	544200	299200
25/05/2004	ENDRIN	<	1	544200	299200
28/07/2004	ENDRIN	<	1	544200	299200
10/08/2004	ENDRIN	<	1	544200	299200
15/02/2005	ENDRIN	<	1	544200	299200
14/03/2005	ENDRIN	<	1	544200	299200
08/04/2005	ENDRIN	<	1	544200	299200
05/05/2005	ENDRIN	<	3	544200	299200
02/06/2005	ENDRIN	<	3	544200	299200
03/08/2005	ENDRIN	<	3	544200	299200
14/04/2004	FENITROTHION	<	1	539400	274700
14/04/2004	FENITROTHION	<	1	539400	274700
12/05/2004	FENITROTHION	<	1	539400	274700
04/06/2004	FENITROTHION	<	1	539400	274700
14/07/2004	FENITROTHION	<	1	539400	274700
11/08/2004	FENITROTHION	<	1	539400	274700
04/03/2004	HCH ALPHA	<	2	539400	274700
14/04/2004	HCH ALPHA	<	2	539400	274700
14/04/2004	HCH ALPHA	<	2	539400	274700
19/04/2004	HCH ALPHA	<	2	539400	274700
12/05/2004	HCH ALPHA	<	2	539400	274700
04/06/2004	HCH ALPHA	<	2	539400	274700
14/07/2004	HCH ALPHA	<	2	539400	274700
14/07/2004	HCH ALPHA	<	2	539400	274700
11/08/2004	HCH ALPHA	<	2	539400	274700
10/02/2005	HCH ALPHA	<	2	539400	274700
10/03/2005	HCH ALPHA	<	2.1	539400	274700
11/04/2005	HCH ALPHA	<	2.1	539400	274700
10/05/2005	HCH ALPHA	<	2.1	539400	274700
06/06/2005	HCH ALPHA	<	2.1	539400	274700
01/08/2005	HCH ALPHA	<	2.1	539400	274700
18/02/2004	HCH ALPHA	<	2	544200	299200
14/04/2004	HCH ALPHA	<	2	544200	299200
05/05/2004	HCH ALPHA	<	2	544200	299200
25/05/2004	HCH ALPHA	<	2	544200	299200
28/07/2004	HCH ALPHA	<	2	544200	299200
10/08/2004	HCH ALPHA	<	2	544200	299200
15/02/2005	HCH ALPHA	<	2	544200	299200
14/03/2005	HCH ALPHA	<	2.1	544200	299200
08/04/2005	HCH ALPHA	<	2.1	544200	299200
02/06/2005	HCH ALPHA	<	2.1	544200	299200
03/08/2005	HCH ALPHA	<	2.1	544200	299200
05/05/2005	HCH ALPHA		3	544200	299200
04/03/2004	HCH BETA	<	2	539400	274700
14/04/2004	HCH BETA	<	2	539400	274700
14/04/2004	HCH BETA	<	2	539400	274700
19/04/2004	HCH BETA	<	2	539400	274700
12/05/2004	HCH BETA	<	2	539400	274700
04/06/2004	HCH BETA	<	2	539400	274700
14/07/2004	HCH BETA	<	2	539400	274700
14/07/2004	HCH BETA	<	2	539400	274700
11/08/2004	HCH BETA	<	2	539400	274700
10/02/2005	HCH BETA	<	2	539400	274700

10/03/2005	HCH BETA	<	2.1	539400	274700
11/04/2005	HCH BETA	<	2.1	539400	274700
10/05/2005	HCH BETA	<	2.1	539400	274700
06/06/2005	HCH BETA	<	2.1	539400	274700
01/08/2005	HCH BETA	<	2.1	539400	274700
18/02/2004	HCH BETA	<	2	544200	299200
14/04/2004	HCH BETA	<	2	544200	299200
05/05/2004	HCH BETA	<	2	544200	299200
25/05/2004	HCH BETA	<	2	544200	299200
28/07/2004	HCH BETA	<	2	544200	299200
10/08/2004	HCH BETA	<	2	544200	299200
15/02/2005	HCH BETA	<	2	544200	299200
14/03/2005	HCH BETA	<	2.1	544200	299200
08/04/2005	HCH BETA	<	2.1	544200	299200
05/05/2005	HCH BETA	<	2.1	544200	299200
02/06/2005	HCH BETA	<	2.1	544200	299200
03/08/2005	HCH BETA	<	2.1	544200	299200
04/03/2004	HCH DELTA	<	1	539400	274700
14/04/2004	HCH DELTA	<	1	539400	274700
14/04/2004	HCH DELTA	<	1	539400	274700
19/04/2004	HCH DELTA	<	1	539400	274700
12/05/2004	HCH DELTA	<	1	539400	274700
04/06/2004	HCH DELTA	<	1	539400	274700
14/07/2004	HCH DELTA	<	1	539400	274700
14/07/2004	HCH DELTA	<	1	539400	274700
11/08/2004	HCH DELTA	<	1	539400	274700
10/02/2005	HCH DELTA	<	1	539400	274700
10/03/2005	HCH DELTA	<	1	539400	274700
11/04/2005	HCH DELTA	<	1	539400	274700
10/05/2005	HCH DELTA	<	1	539400	274700
06/06/2005	HCH DELTA	<	1	539400	274700
01/08/2005	HCH DELTA	<	1	539400	274700
18/02/2004	HCH DELTA	<	1	544200	299200
14/04/2004	HCH DELTA	<	1	544200	299200
05/05/2004	HCH DELTA	<	1	544200	299200
25/05/2004	HCH DELTA	<	1	544200	299200
28/07/2004	HCH DELTA	<	1	544200	299200
10/08/2004	HCH DELTA	<	1	544200	299200
15/02/2005	HCH DELTA	<	1	544200	299200
14/03/2005	HCH DELTA	<	1	544200	299200
08/04/2005	HCH DELTA	<	1	544200	299200
05/05/2005	HCH DELTA	<	1	544200	299200
02/06/2005	HCH DELTA	<	1	544200	299200
03/08/2005	HCH DELTA	<	1	544200	299200
04/03/2004	HCH GAMMA	<	2	539400	274700
14/04/2004	HCH GAMMA	<	2	539400	274700
14/04/2004	HCH GAMMA	<	2	539400	274700
12/05/2004	HCH GAMMA	<	2	539400	274700
04/06/2004	HCH GAMMA	<	2	539400	274700
14/07/2004	HCH GAMMA	<	2	539400	274700
14/07/2004	HCH GAMMA	<	2	539400	274700
11/08/2004	HCH GAMMA	<	2	539400	274700
10/02/2005	HCH GAMMA	<	2	539400	274700
10/03/2005	HCH GAMMA	<	2.2	539400	274700
11/04/2005	HCH GAMMA	<	2.2	539400	274700
10/05/2005	HCH GAMMA	<	2.2	539400	274700
06/06/2005	HCH GAMMA	<	2.2	539400	274700
01/08/2005	HCH GAMMA	<	2.2	539400	274700

18/02/2004	HCH GAMMA	<	2	544200	299200
14/04/2004	HCH GAMMA	<	2	544200	299200
05/05/2004	HCH GAMMA	<	2	544200	299200
25/05/2004	HCH GAMMA	<	2	544200	299200
28/07/2004	HCH GAMMA	<	2	544200	299200
10/08/2004	HCH GAMMA	<	2	544200	299200
15/02/2005	HCH GAMMA	<	2	544200	299200
14/03/2005	HCH GAMMA	<	2.2	544200	299200
08/04/2005	HCH GAMMA	<	2.2	544200	299200
02/06/2005	HCH GAMMA	<	2.2	544200	299200
03/08/2005	HCH GAMMA	<	2.2	544200	299200
14/10/2005	HCH GAMMA	<	2.2	544200	299200
05/05/2005	HCH GAMMA		4	544200	299200
11/04/2005	HCH TOTAL (ALPHA, BETA & GAMMA)	<	6.4	539400	274700
18/02/2004	HCH TOTAL (ALPHA, BETA & GAMMA)	<	6	544200	299200
14/04/2004	HCH TOTAL (ALPHA, BETA & GAMMA)	<	6	544200	299200
05/05/2004	HCH TOTAL (ALPHA, BETA & GAMMA)	<	6	544200	299200
25/05/2004	HCH TOTAL (ALPHA, BETA & GAMMA)	<	6	544200	299200
28/07/2004	HCH TOTAL (ALPHA, BETA & GAMMA)	<	6	544200	299200
10/08/2004	HCH TOTAL (ALPHA, BETA & GAMMA)	<	6	544200	299200
15/02/2005	HCH TOTAL (ALPHA, BETA & GAMMA)	<	6	544200	299200
14/03/2005	HCH TOTAL (ALPHA, BETA & GAMMA)	<	6.4	544200	299200
08/04/2005	HCH TOTAL (ALPHA, BETA & GAMMA)	<	6.4	544200	299200
02/06/2005	HCH TOTAL (ALPHA, BETA & GAMMA)	<	6.4	544200	299200
03/08/2005	HCH TOTAL (ALPHA, BETA & GAMMA)	<	6.4	544200	299200
05/05/2005	HCH TOTAL (ALPHA, BETA & GAMMA)		9.1	544200	299200
19/04/2004	HEPTACHLOR	<	1	539400	274700
14/07/2004	HEPTACHLOR	<	1	539400	274700
11/04/2005	HEPTACHLOR	<	1	539400	274700
19/04/2004	HEXACHLOROBENZENE	<	1	539400	274700
14/07/2004	HEXACHLOROBENZENE	<	1	539400	274700
11/04/2005	HEXACHLOROBENZENE	<	1	539400	274700
19/04/2004	ISODRIN	<	1	539400	274700
14/07/2004	ISODRIN	<	1	539400	274700
11/04/2005	ISODRIN	<	1	539400	274700
18/02/2004	ISODRIN	<	1	544200	299200
14/04/2004	ISODRIN	<	1	544200	299200
05/05/2004	ISODRIN	<	1	544200	299200
25/05/2004	ISODRIN	<	1	544200	299200
28/07/2004	ISODRIN	<	1	544200	299200
10/08/2004	ISODRIN	<	1	544200	299200
15/02/2005	ISODRIN	<	1	544200	299200
14/03/2005	ISODRIN	<	1	544200	299200
08/04/2005	ISODRIN	<	1	544200	299200
05/05/2005	ISODRIN	<	1	544200	299200
02/06/2005	ISODRIN	<	1	544200	299200
03/08/2005	ISODRIN	<	1	544200	299200
04/03/2004	LINURON	<	50	539400	274700
14/04/2004	LINURON	<	50	539400	274700
14/04/2004	LINURON	<	50	539400	274700
12/05/2004	LINURON	<	50	539400	274700
14/07/2004	LINURON	<	50	539400	274700
11/08/2004	LINURON	<	50	539400	274700
04/06/2004	LINURON	<	60	539400	274700
14/04/2004	MALATHION	<	2	539400	274700
14/04/2004	MALATHION	<	2	539400	274700
12/05/2004	MALATHION	<	2	539400	274700
04/06/2004	MALATHION	<	2	539400	274700

14/07/2004	MALATHION	<	2	539400	274700
11/08/2004	MALATHION	<	2	539400	274700
04/03/2004	MECOPROP	<	40	539400	274700
14/04/2004	MECOPROP	<	40	539400	274700
14/04/2004	MECOPROP	<	40	539400	274700
11/08/2004	MECOPROP	<	40	539400	274700
14/07/2004	MECOPROP		50	539400	274700
04/06/2004	MECOPROP		100	539400	274700
12/05/2004	MECOPROP		120	539400	274700
14/04/2004	MEVINPHOS	<	2	539400	274700
14/04/2004	MEVINPHOS	<	8	539400	274700
12/05/2004	MEVINPHOS	<	8	539400	274700
04/06/2004	MEVINPHOS	<	8	539400	274700
14/07/2004	MEVINPHOS	<	8	539400	274700
11/08/2004	MEVINPHOS	<	8	539400	274700
10/03/2005	PENTACHLOROPHENOL	<	20	539400	274700
11/04/2005	PENTACHLOROPHENOL	<	20	539400	274700
10/05/2005	PENTACHLOROPHENOL	<	20	539400	274700
06/06/2005	PENTACHLOROPHENOL	<	20	539400	274700
18/07/2005	PENTACHLOROPHENOL	<	20	539400	274700
04/03/2004	PENTACHLOROPHENOL	<	40	539400	274700
12/05/2004	PENTACHLOROPHENOL	<	40	539400	274700
04/06/2004	PENTACHLOROPHENOL	<	40	539400	274700
14/07/2004	PENTACHLOROPHENOL	<	40	539400	274700
14/07/2004	PENTACHLOROPHENOL	<	40	539400	274700
11/08/2004	PENTACHLOROPHENOL	<	40	539400	274700
04/03/2004	PERMETHRIN, CIS	<	1	539400	274700
14/04/2004	PERMETHRIN, CIS	<	1	539400	274700
14/04/2004	PERMETHRIN, CIS	<	1	539400	274700
12/05/2004	PERMETHRIN, CIS	<	1	539400	274700
04/06/2004	PERMETHRIN, CIS	<	1	539400	274700
14/07/2004	PERMETHRIN, CIS	<	1	539400	274700
11/08/2004	PERMETHRIN, CIS	<	1	539400	274700
04/03/2004	PERMETHRIN, TRANS	<	1	539400	274700
14/04/2004	PERMETHRIN, TRANS	<	1	539400	274700
14/04/2004	PERMETHRIN, TRANS	<	1	539400	274700
12/05/2004	PERMETHRIN, TRANS	<	1	539400	274700
04/06/2004	PERMETHRIN, TRANS	<	1	539400	274700
14/07/2004	PERMETHRIN, TRANS	<	1	539400	274700
11/08/2004	PERMETHRIN, TRANS	<	1	539400	274700
11/08/2004	SIMAZINE		29	539400	274700
10/03/2005	SIMAZINE		34	539400	274700
14/07/2004	SIMAZINE		37	539400	274700
14/07/2004	SIMAZINE		40	539400	274700
01/08/2005	SIMAZINE		41	539400	274700
04/06/2004	SIMAZINE		49	539400	274700
10/05/2005	SIMAZINE		69	539400	274700
18/07/2005	SIMAZINE		72	539400	274700
14/04/2004	SIMAZINE		74	539400	274700
14/04/2004	SIMAZINE		80	539400	274700
06/06/2005	SIMAZINE		83	539400	274700
10/02/2005	SIMAZINE		132	539400	274700
12/05/2004	SIMAZINE		226	539400	274700
11/04/2005	SIMAZINE		235	539400	274700
19/04/2004	TDE (OP)	<	1	539400	274700
14/07/2004	TDE (OP)	<	1	539400	274700
11/04/2005	TDE (OP)	<	1	539400	274700
04/03/2004	TDE (PP)	<	1	539400	274700

14/04/2004	TDE (PP)	<	1	539400	274700
14/04/2004	TDE (PP)	<	1	539400	274700
12/05/2004	TDE (PP)	<	1	539400	274700
04/06/2004	TDE (PP)	<	1	539400	274700
14/07/2004	TDE (PP)	<	1	539400	274700
14/07/2004	TDE (PP)	<	1	539400	274700
11/08/2004	TDE (PP)	<	1	539400	274700
10/02/2005	TDE (PP)	<	1	539400	274700
10/03/2005	TDE (PP)	<	1	539400	274700
11/04/2005	TDE (PP)	<	1	539400	274700
10/05/2005	TDE (PP)	<	1	539400	274700
06/06/2005	TDE (PP)	<	1	539400	274700
01/08/2005	TDE (PP)	<	1	539400	274700
19/04/2004	TECNAZENE	<	10	539400	274700
11/04/2005	TECNAZENE	<	10	539400	274700
14/07/2004	TECNAZENE	<	11	539400	274700
05/05/2005	TECNAZENE	<	10	535664	293648
02/06/2005	TECNAZENE	<	10	535664	293648
03/08/2005	TECNAZENE	<	10	535664	293648
11/02/2005	TECNAZENE	<	50	535664	293648
14/03/2005	TECNAZENE	<	50	535664	293648
07/04/2005	TECNAZENE	<	100	535664	293648
19/04/2004	TRANS-HEPTACHLOR EPOXIDE	<	3	539400	274700
14/07/2004	TRANS-HEPTACHLOR EPOXIDE	<	3	539400	274700
11/04/2005	TRANS-HEPTACHLOR EPOXIDE	<	3	539400	274700
14/04/2004	TRIAZOPHOS	<	4	539400	274700
14/04/2004	TRIAZOPHOS	<	4	539400	274700
12/05/2004	TRIAZOPHOS	<	4	539400	274700
04/06/2004	TRIAZOPHOS	<	4	539400	274700
14/07/2004	TRIAZOPHOS	<	4	539400	274700
11/08/2004	TRIAZOPHOS	<	4	539400	274700
04/03/2004	TRIBUTYL TIN	<	2	539400	274700
14/04/2004	TRIBUTYL TIN	<	2	539400	274700
14/04/2004	TRIBUTYL TIN	<	2	539400	274700
19/04/2004	TRIBUTYL TIN	<	2	539400	274700
12/05/2004	TRIBUTYL TIN	<	2	539400	274700
04/06/2004	TRIBUTYL TIN	<	2	539400	274700
14/07/2004	TRIBUTYL TIN	<	2	539400	274700
14/07/2004	TRIBUTYL TIN	<	2	539400	274700
11/08/2004	TRIBUTYL TIN	<	2	539400	274700
10/02/2005	TRIBUTYL TIN	<	2	539400	274700
10/03/2005	TRIBUTYL TIN	<	2	539400	274700
11/04/2005	TRIBUTYL TIN	<	2	539400	274700
10/05/2005	TRIBUTYL TIN		2	539400	274700
06/06/2005	TRIBUTYL TIN	<	2	539400	274700
18/07/2005	TRIBUTYL TIN	<	2	539400	274700
01/08/2005	TRIBUTYL TIN	<	2	539400	274700
14/04/2004	TRIFLURALIN	<	11	539400	274700
14/04/2004	TRIFLURALIN	<	11	539400	274700
19/04/2004	TRIFLURALIN	<	11	539400	274700
12/05/2004	TRIFLURALIN	<	11	539400	274700
04/06/2004	TRIFLURALIN	<	11	539400	274700
14/07/2004	TRIFLURALIN	<	11	539400	274700
11/08/2004	TRIFLURALIN	<	11	539400	274700
11/04/2005	TRIFLURALIN	<	11	539400	274700
14/07/2004	TRIFLURALIN	<	12	539400	274700
04/03/2004	TRIFLURALIN	<	19	539400	274700
04/03/2004	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700

14/04/2004	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700
14/04/2004	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700
19/04/2004	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700
12/05/2004	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700
04/06/2004	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700
14/07/2004	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700
14/07/2004	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700
11/08/2004	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700
10/02/2005	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700
10/03/2005	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700
11/04/2005	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700
10/05/2005	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700
06/06/2005	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700
18/07/2005	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700
01/08/2005	TRIPHENYLTIN COMPOUNDS	<	2	539400	274700

2.4 Layes Pool:

- Co-ordinates: x,y = 380253, 265808

Date	Pesticide	Less than	Conc. (ng/L)	x	y
15/08/2005	DICHLORVOS	<	0.5	381350	270950
15/07/2005	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
24/05/2005	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
16/03/2005	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
09/02/2005	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
10/08/2004	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
02/07/2004	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
25/06/2004	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
03/06/2004	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
22/04/2004	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
15/03/2004	(2,4,5-TRICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
16/03/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
09/02/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
10/08/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
12/07/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
02/07/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
25/06/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
25/06/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
03/06/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
03/06/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
22/04/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
22/04/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
15/03/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
15/03/2004	(2,4-DICHLOROPHENOXY)ETHANOIC ACID	<	40	381350	270950
24/05/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID		52	381350	270950
15/07/2005	(2,4-DICHLOROPHENOXY)ETHANOIC ACID		236	381350	270950
15/07/2005	2,4-DB	<	40	381350	270950
24/05/2005	2,4-DB	<	40	381350	270950
16/03/2005	2,4-DB	<	40	381350	270950
09/02/2005	2,4-DB	<	40	381350	270950
10/08/2004	2,4-DB	<	40	381350	270950

02/07/2004	2,4-DB	<	40	381350	270950
25/06/2004	2,4-DB	<	40	381350	270950
03/06/2004	2,4-DB	<	40	381350	270950
22/04/2004	2,4-DB	<	40	381350	270950
15/03/2004	2,4-DB	<	40	381350	270950
14/07/2005	ALDRIN	<	2.5	381350	270950
14/04/2005	ALDRIN	<	2.5	381350	270950
13/04/2005	ALDRIN	<	2.5	381350	270950
15/08/2005	ALDRIN	<	5	381350	270950
15/07/2005	ALDRIN	<	5	381350	270950
10/06/2005	ALDRIN	<	5	381350	270950
24/05/2005	ALDRIN	<	5	381350	270950
16/03/2005	ALDRIN	<	5	381350	270950
09/02/2005	ALDRIN	<	5	381350	270950
10/08/2004	ALDRIN	<	5	381350	270950
12/07/2004	ALDRIN	<	5	381350	270950
25/06/2004	ALDRIN	<	5	381350	270950
03/06/2004	ALDRIN	<	5	381350	270950
22/04/2004	ALDRIN	<	5	381350	270950
15/03/2004	ALDRIN	<	5	381350	270950
15/08/2005	ATRAZINE	<	30	381350	270950
15/07/2005	ATRAZINE	<	30	381350	270950
15/07/2005	ATRAZINE	<	30	381350	270950
14/07/2005	ATRAZINE	<	30	381350	270950
10/06/2005	ATRAZINE	<	30	381350	270950
24/05/2005	ATRAZINE	<	30	381350	270950
14/04/2005	ATRAZINE	<	30	381350	270950
13/04/2005	ATRAZINE	<	30	381350	270950
16/03/2005	ATRAZINE	<	30	381350	270950
16/03/2005	ATRAZINE	<	30	381350	270950
09/02/2005	ATRAZINE	<	30	381350	270950
09/02/2005	ATRAZINE	<	30	381350	270950
10/08/2004	ATRAZINE	<	30	381350	270950
12/07/2004	ATRAZINE	<	30	381350	270950
01/07/2004	ATRAZINE	<	30	381350	270950
25/06/2004	ATRAZINE	<	30	381350	270950
15/03/2004	ATRAZINE	<	30	381350	270950
15/03/2004	ATRAZINE	<	30	381350	270950
03/06/2004	ATRAZINE		52.3	381350	270950
02/07/2004	ATRAZINE		52.6	381350	270950
03/06/2004	ATRAZINE		57.7	381350	270950
25/06/2004	ATRAZINE		163	381350	270950
15/08/2005	AZINPHOS-ETHYL	<	20	381350	270950
15/07/2005	AZINPHOS-ETHYL	<	20	381350	270950
15/07/2005	AZINPHOS-ETHYL	<	20	381350	270950
14/07/2005	AZINPHOS-ETHYL	<	20	381350	270950
10/06/2005	AZINPHOS-ETHYL	<	20	381350	270950
24/05/2005	AZINPHOS-ETHYL	<	20	381350	270950
24/05/2005	AZINPHOS-ETHYL	<	20	381350	270950
14/04/2005	AZINPHOS-ETHYL	<	20	381350	270950
13/04/2005	AZINPHOS-ETHYL	<	20	381350	270950
16/03/2005	AZINPHOS-ETHYL	<	20	381350	270950
16/03/2005	AZINPHOS-ETHYL	<	20	381350	270950
09/02/2005	AZINPHOS-ETHYL	<	20	381350	270950

09/02/2005	AZINPHOS-ETHYL	<	20	381350	270950
10/08/2004	AZINPHOS-ETHYL	<	20	381350	270950
10/08/2004	AZINPHOS-ETHYL	<	20	381350	270950
12/07/2004	AZINPHOS-ETHYL	<	20	381350	270950
02/07/2004	AZINPHOS-ETHYL	<	20	381350	270950
01/07/2004	AZINPHOS-ETHYL	<	20	381350	270950
25/06/2004	AZINPHOS-ETHYL	<	20	381350	270950
25/06/2004	AZINPHOS-ETHYL	<	20	381350	270950
03/06/2004	AZINPHOS-ETHYL	<	20	381350	270950
03/06/2004	AZINPHOS-ETHYL	<	20	381350	270950
15/03/2004	AZINPHOS-ETHYL	<	20	381350	270950
15/03/2004	AZINPHOS-ETHYL	<	20	381350	270950
15/08/2005	AZINPHOS-METHYL	<	1	381350	270950
15/07/2005	AZINPHOS-METHYL	<	1	381350	270950
15/07/2005	AZINPHOS-METHYL	<	1	381350	270950
14/07/2005	AZINPHOS-METHYL	<	1	381350	270950
10/06/2005	AZINPHOS-METHYL	<	1	381350	270950
14/04/2005	AZINPHOS-METHYL	<	1	381350	270950
13/04/2005	AZINPHOS-METHYL	<	1	381350	270950
16/03/2005	AZINPHOS-METHYL	<	1	381350	270950
16/03/2005	AZINPHOS-METHYL	<	1	381350	270950
09/02/2005	AZINPHOS-METHYL	<	1	381350	270950
09/02/2005	AZINPHOS-METHYL	<	1	381350	270950
10/08/2004	AZINPHOS-METHYL	<	1	381350	270950
10/08/2004	AZINPHOS-METHYL	<	1	381350	270950
12/07/2004	AZINPHOS-METHYL	<	1	381350	270950
02/07/2004	AZINPHOS-METHYL	<	1	381350	270950
01/07/2004	AZINPHOS-METHYL	<	1	381350	270950
25/06/2004	AZINPHOS-METHYL	<	1	381350	270950
25/06/2004	AZINPHOS-METHYL	<	1	381350	270950
03/06/2004	AZINPHOS-METHYL	<	1	381350	270950
03/06/2004	AZINPHOS-METHYL	<	1	381350	270950
15/03/2004	AZINPHOS-METHYL	<	1	381350	270950
15/03/2004	AZINPHOS-METHYL	<	1	381350	270950
15/08/2005	CARBOPHENOTHION	<	10	381350	270950
15/07/2005	CARBOPHENOTHION	<	10	381350	270950
15/07/2005	CARBOPHENOTHION	<	10	381350	270950
14/07/2005	CARBOPHENOTHION	<	10	381350	270950
10/06/2005	CARBOPHENOTHION	<	10	381350	270950
24/05/2005	CARBOPHENOTHION	<	10	381350	270950
24/05/2005	CARBOPHENOTHION	<	10	381350	270950
14/04/2005	CARBOPHENOTHION	<	10	381350	270950
13/04/2005	CARBOPHENOTHION	<	10	381350	270950
16/03/2005	CARBOPHENOTHION	<	10	381350	270950
16/03/2005	CARBOPHENOTHION	<	10	381350	270950
10/08/2004	CARBOPHENOTHION	<	10	381350	270950
10/08/2004	CARBOPHENOTHION	<	10	381350	270950
12/07/2004	CARBOPHENOTHION	<	10	381350	270950
02/07/2004	CARBOPHENOTHION	<	10	381350	270950
01/07/2004	CARBOPHENOTHION	<	10	381350	270950
25/06/2004	CARBOPHENOTHION	<	10	381350	270950
25/06/2004	CARBOPHENOTHION	<	10	381350	270950
03/06/2004	CARBOPHENOTHION	<	10	381350	270950
03/06/2004	CARBOPHENOTHION	<	10	381350	270950

15/03/2004	CARBOPHENOTHION	<	10	381350	270950
15/03/2004	CARBOPHENOTHION	<	10	381350	270950
15/08/2005	CHLORFENVINPHOS	<	1	381350	270950
15/07/2005	CHLORFENVINPHOS	<	1	381350	270950
15/07/2005	CHLORFENVINPHOS	<	1	381350	270950
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13/04/2005	CHLORFENVINPHOS	<	1	381350	270950
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10/08/2004	CHLORFENVINPHOS	<	1	381350	270950
12/07/2004	CHLORFENVINPHOS	<	1	381350	270950
02/07/2004	CHLORFENVINPHOS	<	1	381350	270950
01/07/2004	CHLORFENVINPHOS	<	1	381350	270950
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15/07/2005	CHLORTOLURON	<	40	381350	270950
24/05/2005	CHLORTOLURON	<	40	381350	270950
16/03/2005	CHLORTOLURON	<	40	381350	270950
09/02/2005	CHLORTOLURON	<	40	381350	270950
10/08/2004	CHLORTOLURON	<	40	381350	270950
02/07/2004	CHLORTOLURON	<	40	381350	270950
03/06/2004	CHLORTOLURON	<	40	381350	270950
22/04/2004	CHLORTOLURON	<	40	381350	270950
15/03/2004	CHLORTOLURON	<	40	381350	270950
25/06/2004	CHLORTOLURON		140	381350	270950
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15/08/2005	COUMAPHOS	<	1	381350	270950
15/07/2005	COUMAPHOS	<	1	381350	270950
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24/05/2005	COUMAPHOS	<	1	381350	270950
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13/04/2005	COUMAPHOS	<	1	381350	270950
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01/07/2004	COUMAPHOS	<	1	381350	270950
25/06/2004	COUMAPHOS	<	1	381350	270950
25/06/2004	COUMAPHOS	<	1	381350	270950
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15/07/2005	CYFLUTHRIN	<	10	381350	270950
14/07/2005	CYFLUTHRIN	<	10	381350	270950
10/06/2005	CYFLUTHRIN		10	381350	270950
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13/04/2005	CYFLUTHRIN	<	10	381350	270950
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25/06/2004	CYFLUTHRIN	<	10	381350	270950
03/06/2004	CYFLUTHRIN	<	10	381350	270950
22/04/2004	CYFLUTHRIN	<	10	381350	270950
15/03/2004	CYFLUTHRIN	<	10	381350	270950
14/07/2005	CYPERMETHRIN	<	10	381350	270950
14/04/2005	CYPERMETHRIN	<	10	381350	270950
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14/04/2005	DDE (OP)	<	2	381350	270950
13/04/2005	DDE (OP)	<	2	381350	270950
15/08/2005	DDE (OP)	<	5	381350	270950
15/07/2005	DDE (OP)	<	5	381350	270950
10/06/2005	DDE (OP)	<	5	381350	270950
24/05/2005	DDE (OP)	<	5	381350	270950
16/03/2005	DDE (OP)	<	5	381350	270950
09/02/2005	DDE (OP)	<	5	381350	270950
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12/07/2004	DDE (OP)	<	5	381350	270950
25/06/2004	DDE (OP)	<	5	381350	270950
03/06/2004	DDE (OP)	<	5	381350	270950
22/04/2004	DDE (OP)	<	5	381350	270950
15/03/2004	DDE (OP)	<	5	381350	270950
14/07/2005	DDE (PP)	<	1.5	381350	270950
14/04/2005	DDE (PP)	<	1.5	381350	270950
13/04/2005	DDE (PP)	<	1.5	381350	270950
15/08/2005	DDE (PP)	<	5	381350	270950
15/07/2005	DDE (PP)	<	5	381350	270950
10/06/2005	DDE (PP)	<	5	381350	270950
24/05/2005	DDE (PP)	<	5	381350	270950
16/03/2005	DDE (PP)	<	5	381350	270950
09/02/2005	DDE (PP)	<	5	381350	270950
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12/07/2004	DDE (PP)	<	5	381350	270950
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22/04/2004	DDE (PP)	<	5	381350	270950
15/03/2004	DDE (PP)	<	5	381350	270950
14/07/2005	DDT (OP)	<	1.5	381350	270950

14/04/2005	DDT (OP)	<	1.5	381350	270950
13/04/2005	DDT (OP)	<	1.5	381350	270950
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15/07/2005	DDT (OP)	<	5	381350	270950
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15/03/2004	DDT (PP)	<	5	381350	270950
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14/07/2005	DEMETON-S-METHYL	<	50	381350	270950
14/04/2005	DEMETON-S-METHYL	<	50	381350	270950
16/03/2005	DEMETON-S-METHYL	<	50	381350	270950
10/08/2004	DEMETON-S-METHYL	<	50	381350	270950
12/07/2004	DEMETON-S-METHYL	<	50	381350	270950
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15/08/2005	DIAZINON	<	1	381350	270950
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16/03/2005	DIAZINON	<	1	381350	270950
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02/07/2004	DIAZINON	<	1	381350	270950
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15/03/2004	DIAZINON	<	1	381350	270950
13/07/2005	DIBUTYL TIN	<	1	381350	270950
15/07/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	40	381350	270950
24/05/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	40	381350	270950
16/03/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	40	381350	270950
09/02/2005	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	40	381350	270950
02/07/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	40	381350	270950
25/06/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	40	381350	270950
03/06/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	40	381350	270950
22/04/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	40	381350	270950
15/03/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}	<	40	381350	270950
10/08/2004	DICAMBA {3,6-DICHLORO(O-METHOXYBENZOIC ACID)}		42.4	381350	270950
15/07/2005	DICHLOBENIL	<	20	381350	270950
14/07/2005	DICHLOBENIL	<	20	381350	270950
24/05/2005	DICHLOBENIL	<	20	381350	270950
14/04/2005	DICHLOBENIL	<	20	381350	270950
16/03/2005	DICHLOBENIL	<	20	381350	270950
09/02/2005	DICHLOBENIL	<	20	381350	270950
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02/07/2004	DICHLOBENIL	<	20	381350	270950
01/07/2004	DICHLOBENIL	<	20	381350	270950
25/06/2004	DICHLOBENIL	<	20	381350	270950
03/06/2004	DICHLOBENIL	<	20	381350	270950
15/03/2004	DICHLOBENIL	<	20	381350	270950
15/07/2005	DICHLORPROP	<	40	381350	270950
24/05/2005	DICHLORPROP	<	40	381350	270950
16/03/2005	DICHLORPROP	<	40	381350	270950
09/02/2005	DICHLORPROP	<	40	381350	270950
10/08/2004	DICHLORPROP	<	40	381350	270950
02/07/2004	DICHLORPROP	<	40	381350	270950
03/06/2004	DICHLORPROP	<	40	381350	270950
22/04/2004	DICHLORPROP	<	40	381350	270950

15/03/2004	DICHLORPROP	<	40	381350	270950
25/06/2004	DICHLORPROP	<	80	381350	270950
15/07/2005	DICHLORVOS	<	0.5	381350	270950
15/07/2005	DICHLORVOS	<	0.5	381350	270950
14/07/2005	DICHLORVOS	<	0.5	381350	270950
10/06/2005	DICHLORVOS	<	0.5	381350	270950
24/05/2005	DICHLORVOS	<	0.5	381350	270950
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14/04/2005	DICHLORVOS	<	0.5	381350	270950
13/04/2005	DICHLORVOS	<	0.5	381350	270950
16/03/2005	DICHLORVOS	<	0.5	381350	270950
16/03/2005	DICHLORVOS	<	0.5	381350	270950
10/08/2004	DICHLORVOS	<	0.5	381350	270950
10/08/2004	DICHLORVOS	<	0.5	381350	270950
12/07/2004	DICHLORVOS	<	0.5	381350	270950
02/07/2004	DICHLORVOS	<	0.5	381350	270950
01/07/2004	DICHLORVOS	<	0.5	381350	270950
25/06/2004	DICHLORVOS	<	0.5	381350	270950
25/06/2004	DICHLORVOS	<	0.5	381350	270950
03/06/2004	DICHLORVOS	<	0.5	381350	270950
03/06/2004	DICHLORVOS	<	0.5	381350	270950
15/03/2004	DICHLORVOS	<	0.5	381350	270950
15/03/2004	DICHLORVOS	<	0.5	381350	270950
14/07/2005	DIELDRIN	<	2.5	381350	270950
14/04/2005	DIELDRIN	<	2.5	381350	270950
13/04/2005	DIELDRIN	<	2.5	381350	270950
15/08/2005	DIELDRIN	<	5	381350	270950
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22/04/2004	DIELDRIN	<	5	381350	270950
15/03/2004	DIELDRIN	<	5	381350	270950
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24/05/2005	DIFLUROBENZURON	<	40	381350	270950
16/03/2005	DIFLUROBENZURON	<	40	381350	270950
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02/07/2004	DIFLUROBENZURON	<	40	381350	270950
25/06/2004	DIFLUROBENZURON	<	40	381350	270950
03/06/2004	DIFLUROBENZURON	<	40	381350	270950
22/04/2004	DIFLUROBENZURON	<	40	381350	270950
15/03/2004	DIFLUROBENZURON	<	40	381350	270950
12/07/2004	DIMETHOATE	<	10	381350	270950
03/06/2004	DIMETHOATE	<	10	381350	270950
22/04/2004	DIMETHOATE	<	10	381350	270950
15/03/2004	DIMETHOATE	<	10	381350	270950
15/07/2005	DIURON	<	50	381350	270950
09/02/2005	DIURON	<	50	381350	270950

10/08/2004	DIURON	<	50	381350	270950
22/04/2004	DIURON	<	50	381350	270950
15/03/2004	DIURON	<	50	381350	270950
16/03/2005	DIURON		55	381350	270950
02/07/2004	DIURON		190	381350	270950
24/05/2005	DIURON		310	381350	270950
03/06/2004	DIURON		503	381350	270950
25/06/2004	DIURON		840	381350	270950
14/07/2005	ENDOSULPHAN ALPHA	<	1	381350	270950
14/04/2005	ENDOSULPHAN ALPHA	<	1	381350	270950
13/04/2005	ENDOSULPHAN ALPHA	<	1	381350	270950
15/08/2005	ENDOSULPHAN ALPHA	<	5	381350	270950
15/07/2005	ENDOSULPHAN ALPHA	<	5	381350	270950
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24/05/2005	ENDOSULPHAN ALPHA	<	5	381350	270950
16/03/2005	ENDOSULPHAN ALPHA	<	5	381350	270950
09/02/2005	ENDOSULPHAN ALPHA	<	5	381350	270950
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12/07/2004	ENDOSULPHAN ALPHA	<	5	381350	270950
25/06/2004	ENDOSULPHAN ALPHA	<	5	381350	270950
03/06/2004	ENDOSULPHAN ALPHA	<	5	381350	270950
22/04/2004	ENDOSULPHAN ALPHA	<	5	381350	270950
15/03/2004	ENDOSULPHAN ALPHA	<	5	381350	270950
14/07/2005	ENDOSULPHAN BETA	<	2	381350	270950
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13/04/2005	ENDOSULPHAN BETA	<	2	381350	270950
15/08/2005	ENDOSULPHAN BETA	<	5	381350	270950
15/07/2005	ENDOSULPHAN BETA	<	5	381350	270950
10/06/2005	ENDOSULPHAN BETA	<	5	381350	270950
24/05/2005	ENDOSULPHAN BETA	<	5	381350	270950
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14/04/2005	FENCHLORPHOS {RONNEL.}	<	1	381350	270950
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09/02/2005	ISOPROTURON (DIIP1,3DITHIOLAN-2-YLIDENEMALONATE)	<	50	381350	270950
02/07/2004	ISOPROTURON (DIIP1,3DITHIOLAN-2-YLIDENEMALONATE)	<	50	381350	270950
25/06/2004	ISOPROTURON (DIIP1,3DITHIOLAN-2-YLIDENEMALONATE)	<	50	381350	270950
03/06/2004	ISOPROTURON (DIIP1,3DITHIOLAN-2-YLIDENEMALONATE)	<	50	381350	270950
22/04/2004	ISOPROTURON (DIIP1,3DITHIOLAN-2-YLIDENEMALONATE)	<	50	381350	270950
15/03/2004	ISOPROTURON (DIIP1,3DITHIOLAN-2-YLIDENEMALONATE)	<	50	381350	270950
15/07/2005	ISOPROTURON (DIIP1,3DITHIOLAN-2-YLIDENEMALONATE)	<	60	381350	270950
24/05/2005	ISOPROTURON (DIIP1,3DITHIOLAN-2-YLIDENEMALONATE)	<	60	381350	270950
16/03/2005	ISOPROTURON (DIIP1,3DITHIOLAN-2-YLIDENEMALONATE)		97	381350	270950
10/08/2004	ISOPROTURON (DIIP1,3DITHIOLAN-2-YLIDENEMALONATE)		1890	381350	270950
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12/07/2004	LINURON	<	40	381350	270950
02/07/2004	LINURON	<	40	381350	270950
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22/04/2004	MCPA	<	40	381350	270950
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15/07/2005	MCPA		119	381350	270950
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16/03/2005	MECOPROP	<	40	381350	270950
09/02/2005	MECOPROP	<	40	381350	270950
12/07/2004	MECOPROP	<	40	381350	270950
02/07/2004	MECOPROP	<	40	381350	270950
25/06/2004	MECOPROP	<	40	381350	270950
15/03/2004	MECOPROP	<	40	381350	270950
15/03/2004	MECOPROP	<	40	381350	270950
03/06/2004	MECOPROP		65	381350	270950
03/06/2004	MECOPROP		66.7	381350	270950
25/06/2004	MECOPROP		80	381350	270950
10/08/2004	MECOPROP		85.5	381350	270950
24/05/2005	MECOPROP		149	381350	270950
15/07/2005	MECOPROP		177	381350	270950
22/04/2004	MECOPROP		534	381350	270950
22/04/2004	MECOPROP		563	381350	270950
14/07/2005	METHOXYCHLOR	<	5	381350	270950
14/04/2005	METHOXYCHLOR	<	5	381350	270950
15/07/2005	MEVINPHOS	<	5	381350	270950
14/07/2005	MEVINPHOS	<	5	381350	270950
14/04/2005	MEVINPHOS	<	5	381350	270950

16/03/2005	MEVINPHOS	<	5	381350	270950
09/02/2005	MEVINPHOS	<	5	381350	270950
10/08/2004	MEVINPHOS	<	5	381350	270950
12/07/2004	MEVINPHOS	<	5	381350	270950
02/07/2004	MEVINPHOS	<	5	381350	270950
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25/06/2004	MEVINPHOS	<	5	381350	270950
25/06/2004	MEVINPHOS	<	5	381350	270950
03/06/2004	MEVINPHOS	<	5	381350	270950
03/06/2004	MEVINPHOS	<	5	381350	270950
15/07/2005	MONURON	<	40	381350	270950
24/05/2005	MONURON	<	40	381350	270950
16/03/2005	MONURON	<	40	381350	270950
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02/07/2004	MONURON	<	40	381350	270950
25/06/2004	MONURON	<	40	381350	270950
03/06/2004	MONURON	<	40	381350	270950
22/04/2004	MONURON	<	40	381350	270950
15/03/2004	MONURON	<	40	381350	270950
15/07/2005	NEBURON	<	40	381350	270950
24/05/2005	NEBURON	<	40	381350	270950
16/03/2005	NEBURON	<	40	381350	270950
09/02/2005	NEBURON	<	40	381350	270950
10/08/2004	NEBURON	<	40	381350	270950
02/07/2004	NEBURON	<	40	381350	270950
25/06/2004	NEBURON	<	40	381350	270950
03/06/2004	NEBURON	<	40	381350	270950
22/04/2004	NEBURON	<	40	381350	270950
15/03/2004	NEBURON	<	40	381350	270950
12/07/2004	OMETHOATE	<	10	381350	270950
25/06/2004	OMETHOATE	<	10	381350	270950
03/06/2004	OMETHOATE	<	10	381350	270950
22/04/2004	OMETHOATE	<	10	381350	270950
15/03/2004	OMETHOATE	<	10	381350	270950
15/08/2005	PARATHION {PARATHION ETHYL}	<	10	381350	270950
15/07/2005	PARATHION {PARATHION ETHYL}	<	10	381350	270950
15/07/2005	PARATHION {PARATHION ETHYL}	<	10	381350	270950
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10/06/2005	PARATHION {PARATHION ETHYL}	<	10	381350	270950
24/05/2005	PARATHION {PARATHION ETHYL}	<	10	381350	270950
24/05/2005	PARATHION {PARATHION ETHYL}	<	10	381350	270950
14/04/2005	PARATHION {PARATHION ETHYL}	<	10	381350	270950
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12/07/2004	PARATHION {PARATHION ETHYL}	<	10	381350	270950
02/07/2004	PARATHION {PARATHION ETHYL}	<	10	381350	270950
01/07/2004	PARATHION {PARATHION ETHYL}	<	10	381350	270950
25/06/2004	PARATHION {PARATHION ETHYL}	<	10	381350	270950

25/06/2004	PARATHION {PARATHION ETHYL}	<	10	381350	270950
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15/08/2005	PARATHION-METHYL	<	15	381350	270950
15/07/2005	PARATHION-METHYL	<	15	381350	270950
15/07/2005	PARATHION-METHYL	<	15	381350	270950
14/07/2005	PARATHION-METHYL	<	15	381350	270950
10/06/2005	PARATHION-METHYL	<	15	381350	270950
24/05/2005	PARATHION-METHYL	<	15	381350	270950
24/05/2005	PARATHION-METHYL	<	15	381350	270950
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13/04/2005	PARATHION-METHYL	<	15	381350	270950
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10/08/2004	PARATHION-METHYL	<	15	381350	270950
12/07/2004	PARATHION-METHYL	<	15	381350	270950
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01/07/2004	PARATHION-METHYL	<	15	381350	270950
25/06/2004	PARATHION-METHYL	<	15	381350	270950
25/06/2004	PARATHION-METHYL	<	15	381350	270950
03/06/2004	PARATHION-METHYL	<	15	381350	270950
03/06/2004	PARATHION-METHYL	<	15	381350	270950
15/03/2004	PARATHION-METHYL	<	15	381350	270950
15/03/2004	PARATHION-METHYL	<	15	381350	270950
15/08/2005	PCSD'S (CHLORPHENYLID)	<	50	381350	270950
15/07/2005	PCSD'S (CHLORPHENYLID)	<	50	381350	270950
10/06/2005	PCSD'S (CHLORPHENYLID)	<	50	381350	270950
24/05/2005	PCSD'S (CHLORPHENYLID)	<	50	381350	270950
13/04/2005	PCSD'S (CHLORPHENYLID)	<	50	381350	270950
16/03/2005	PCSD'S (CHLORPHENYLID)	<	50	381350	270950
09/02/2005	PCSD'S (CHLORPHENYLID)	<	50	381350	270950
10/08/2004	PCSD'S (CHLORPHENYLID)	<	50	381350	270950
12/07/2004	PCSD'S (CHLORPHENYLID)	<	50	381350	270950
25/06/2004	PCSD'S (CHLORPHENYLID)	<	50	381350	270950
03/06/2004	PCSD'S (CHLORPHENYLID)	<	50	381350	270950
22/04/2004	PCSD'S (CHLORPHENYLID)	<	50	381350	270950
15/03/2004	PCSD'S (CHLORPHENYLID)	<	50	381350	270950
15/08/2005	PENTACHLOROPHENOL	<	100	381350	270950
15/07/2005	PENTACHLOROPHENOL	<	100	381350	270950
10/06/2005	PENTACHLOROPHENOL	<	100	381350	270950
24/05/2005	PENTACHLOROPHENOL	<	100	381350	270950
13/04/2005	PENTACHLOROPHENOL	<	100	381350	270950
16/03/2005	PENTACHLOROPHENOL	<	100	381350	270950
09/02/2005	PENTACHLOROPHENOL	<	100	381350	270950
10/08/2004	PENTACHLOROPHENOL	<	100	381350	270950
12/07/2004	PENTACHLOROPHENOL	<	100	381350	270950
25/06/2004	PENTACHLOROPHENOL	<	100	381350	270950
03/06/2004	PENTACHLOROPHENOL	<	100	381350	270950
22/04/2004	PENTACHLOROPHENOL	<	100	381350	270950

15/03/2004	PENTACHLOROPHENOL	<	100	381350	270950
15/02/2005	PENTACHLOROPHENOL		730	371120	266000
02/03/2005	PENTACHLOROPHENOL	<	100	371120	266000
20/01/2005	PENTACHLOROPHENOL		952	371120	266000
12/07/2005	PENTACHLOROPHENOL	<	100	371120	266000
17/08/2005	PENTACHLOROPHENOL	<	100	371120	266000
08/09/2005	PENTACHLOROPHENOL	<	100	371120	266000
04/04/2005	PENTACHLOROPHENOL		272	371120	266000
24/06/2005	PENTACHLOROPHENOL		639	371120	266000
13/05/2005	PENTACHLOROPHENOL	<	100	371120	266000
16/07/2004	PENTACHLOROPHENOL		248	371120	266000
13/09/2004	PENTACHLOROPHENOL		2740	371120	266000
29/06/2004	PENTACHLOROPHENOL		397	371120	266000
22/11/2004	PENTACHLOROPHENOL		102	371120	266000
03/12/2004	PENTACHLOROPHENOL		581	371120	266000
22/09/2004	PENTACHLOROPHENOL	<	100	371120	266000
12/10/2004	PENTACHLOROPHENOL	<	100	371120	266000
18/02/2004	PENTACHLOROPHENOL	<	100	371120	266000
15/01/2004	PENTACHLOROPHENOL		134	371120	266000
27/05/2004	PENTACHLOROPHENOL	<	100	371120	266000
14/04/2004	PENTACHLOROPHENOL	<	100	371120	266000
15/08/2005	PERMETHRIN, CIS	<	1	381350	270950
14/07/2005	PERMETHRIN, CIS	<	1	381350	270950
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24/05/2005	PERMETHRIN, CIS	<	5	381350	270950
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09/02/2005	PERMETHRIN, CIS	<	5	381350	270950
10/08/2004	PERMETHRIN, CIS	<	5	381350	270950
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03/06/2004	PERMETHRIN, CIS	<	5	381350	270950
22/04/2004	PERMETHRIN, CIS	<	5	381350	270950
15/03/2004	PERMETHRIN, CIS	<	5	381350	270950
15/08/2005	PERMETHRIN, TRANS	<	1	381350	270950
14/07/2005	PERMETHRIN, TRANS	<	1	381350	270950
14/04/2005	PERMETHRIN, TRANS	<	1	381350	270950
15/07/2005	PERMETHRIN, TRANS	<	5	381350	270950
10/06/2005	PERMETHRIN, TRANS	<	5	381350	270950
24/05/2005	PERMETHRIN, TRANS	<	5	381350	270950
13/04/2005	PERMETHRIN, TRANS	<	5	381350	270950
09/02/2005	PERMETHRIN, TRANS	<	5	381350	270950
10/08/2004	PERMETHRIN, TRANS	<	5	381350	270950
12/07/2004	PERMETHRIN, TRANS	<	5	381350	270950
25/06/2004	PERMETHRIN, TRANS	<	5	381350	270950
03/06/2004	PERMETHRIN, TRANS	<	5	381350	270950
22/04/2004	PERMETHRIN, TRANS	<	5	381350	270950
15/03/2004	PERMETHRIN, TRANS	<	5	381350	270950
15/08/2005	PHORATE	<	10	381350	270950
15/07/2005	PHORATE	<	10	381350	270950
15/07/2005	PHORATE	<	10	381350	270950
14/07/2005	PHORATE	<	10	381350	270950
10/06/2005	PHORATE	<	10	381350	270950

24/05/2005	PHORATE	<	10	381350	270950
24/05/2005	PHORATE	<	10	381350	270950
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13/04/2005	PHORATE	<	10	381350	270950
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15/07/2005	PROPAZINE	<	14	381350	270950
14/07/2005	PROPAZINE	<	14	381350	270950
24/05/2005	PROPAZINE	<	14	381350	270950
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16/03/2005	PROPAZINE	<	14	381350	270950
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15/07/2005	PROPETAMPHOS	<	1	381350	270950
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15/08/2005	SIMAZINE	<	30	381350	270950

15/07/2005	SIMAZINE	<	30	381350	270950
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13/04/2005	SIMAZINE	<	30	381350	270950
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02/07/2004	SIMAZINE		87.5	381350	270950
15/08/2005	SULCOFURON	<	250	381350	270950
15/07/2005	SULCOFURON	<	250	381350	270950
10/06/2005	SULCOFURON	<	250	381350	270950
24/05/2005	SULCOFURON	<	250	381350	270950
13/04/2005	SULCOFURON	<	250	381350	270950
16/03/2005	SULCOFURON	<	250	381350	270950
09/02/2005	SULCOFURON	<	250	381350	270950
25/06/2004	SULCOFURON	<	250	381350	270950
03/06/2004	SULCOFURON	<	250	381350	270950
22/04/2004	SULCOFURON	<	250	381350	270950
15/03/2004	SULCOFURON	<	250	381350	270950
10/08/2004	SULCOFURON	<	2500	381350	270950
12/07/2004	SULCOFURON	<	2500	381350	270950
14/07/2005	TDE (OP)	<	2	381350	270950
14/04/2005	TDE (OP)	<	2	381350	270950
13/04/2005	TDE (OP)	<	2	381350	270950
15/08/2005	TDE (OP)	<	5	381350	270950
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24/05/2005	TDE (OP)	<	5	381350	270950
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09/02/2005	TRIAZOPHOS	<	0.5	381350	270950
10/08/2004	TRIAZOPHOS	<	0.5	381350	270950
12/07/2004	TRIAZOPHOS	<	0.5	381350	270950
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14/07/2005	TRIAZOPHOS	<	5	381350	270950
14/04/2005	TRIAZOPHOS	<	5	381350	270950
16/03/2005	TRIAZOPHOS	<	5	381350	270950
15/07/2005	TRIBUTYL TIN		1	381350	270950
10/06/2005	TRIBUTYL TIN	<	1	381350	270950
16/03/2005	TRIBUTYL TIN		1	381350	270950
25/06/2004	TRIBUTYL TIN	<	1	381350	270950
03/06/2004	TRIBUTYL TIN	<	1	381350	270950
15/03/2004	TRIBUTYL TIN		1.2	381350	270950
13/07/2005	TRIBUTYL TIN		2	381350	270950
10/08/2004	TRIBUTYL TIN		2	381350	270950
12/07/2004	TRIBUTYL TIN		2	381350	270950
22/04/2004	TRIBUTYL TIN		2	381350	270950
24/05/2005	TRIBUTYL TIN		4	381350	270950
09/02/2005	TRIBUTYL TIN		5	381350	270950
15/07/2005	TRICLOPYR	<	40	381350	270950
24/05/2005	TRICLOPYR	<	40	381350	270950
16/03/2005	TRICLOPYR	<	40	381350	270950
09/02/2005	TRICLOPYR	<	40	381350	270950
10/08/2004	TRICLOPYR	<	40	381350	270950
02/07/2004	TRICLOPYR	<	40	381350	270950
25/06/2004	TRICLOPYR	<	40	381350	270950
03/06/2004	TRICLOPYR	<	40	381350	270950
22/04/2004	TRICLOPYR	<	40	381350	270950
15/03/2004	TRICLOPYR	<	40	381350	270950
15/08/2005	TRIFLURALIN	<	10	381350	270950
15/07/2005	TRIFLURALIN	<	10	381350	270950
14/07/2005	TRIFLURALIN	<	10	381350	270950
10/06/2005	TRIFLURALIN	<	10	381350	270950
24/05/2005	TRIFLURALIN	<	10	381350	270950
14/04/2005	TRIFLURALIN	<	10	381350	270950
13/04/2005	TRIFLURALIN	<	10	381350	270950
16/03/2005	TRIFLURALIN	<	10	381350	270950
09/02/2005	TRIFLURALIN	<	10	381350	270950

10/08/2004	TRIFLURALIN	<	10	381350	270950
12/07/2004	TRIFLURALIN	<	10	381350	270950
25/06/2004	TRIFLURALIN	<	10	381350	270950
03/06/2004	TRIFLURALIN	<	10	381350	270950
22/04/2004	TRIFLURALIN	<	10	381350	270950
15/03/2004	TRIFLURALIN	<	10	381350	270950
15/08/2005	TRIPHENYLTIN COMPOUNDS	<	1	381350	270950
15/07/2005	TRIPHENYLTIN COMPOUNDS	<	1	381350	270950
13/07/2005	TRIPHENYLTIN COMPOUNDS	<	1	381350	270950
10/06/2005	TRIPHENYLTIN COMPOUNDS	<	1	381350	270950
24/05/2005	TRIPHENYLTIN COMPOUNDS	<	1	381350	270950
16/03/2005	TRIPHENYLTIN COMPOUNDS	<	1	381350	270950
09/02/2005	TRIPHENYLTIN COMPOUNDS	<	1	381350	270950
10/08/2004	TRIPHENYLTIN COMPOUNDS	<	1	381350	270950
12/07/2004	TRIPHENYLTIN COMPOUNDS	<	1	381350	270950
25/06/2004	TRIPHENYLTIN COMPOUNDS	<	1	381350	270950
03/06/2004	TRIPHENYLTIN COMPOUNDS	<	1	381350	270950
22/04/2004	TRIPHENYLTIN COMPOUNDS	<	1	381350	270950
15/03/2004	TRIPHENYLTIN COMPOUNDS	<	1	381350	270950

Appendix 3

Correction Factor

For each developmental stage, an average of values represented below was used as the correction factor. Except time points (TP) values, where TP 3 was used to correct individuals from TP3, and TP 4 was used to correct individuals from TP 4 & TP 5.

TK Stage (site)	Weight (mg)			TL (mm)			SVL (mm)			HLL (mm)		
	B	A	C	B	A	C	B	A	C	B	A	C
17 (YT)	743	446	1.67	40	35	1.14	16.9	14	1.21	9.7	9	1.08
17 (PYL)	418	257	1.63	33.7	29	1.16	13.1	12	1.09	8.6	7	1.23
18 (YT)	453	298	1.52	36	32	1.13	14	12	1.17	14.2	12	1.18
18 (PYL)	437	273	1.60	40.2	37	1.09	14.4	13	1.11	13.3	12	1.11
18 (20')	365	224	1.63	33	29	1.14	12.5	12	1.04	9.5	9	1.06
19 (PYL)	360	220	1.64	33.3	29	1.15	13	12	1.08	10.9	10	1.09
19 (20')	360	225	1.60	31.9	29	1.10	14	12	1.17	11.6	12	0.97
20 (YT)	426	268	1.59	34.6	30	1.15	13.4	12	1.12	13.5	12	1.13
20 (20')	329	216	1.52	30.6	28	1.09	12.8	12	1.07	13.4	11	1.22
21 (YT)	292	199	1.47	29.5	26	1.13	13.4	11	1.22	13.6	13	1.05
21 (PYL)	229	143	1.60	27	25	1.08	12.3	11	1.12	10.4	11	0.95
TP 3 (YT)	237	211	1.12	n/a	n/a	n/a	15.7	13.2	1.19	16.1	15.2	1.06
TP 4 (YT)	479	453	1.06	n/a	n/a	n/a	17.3	16.3	1.06	18.6	17.1	1.09