The Effects of the Natural Vertebrate Steroid 17β-Oestradiol and the Xeno-Biotic Vertebrate Oestrogen Receptor Agonist Bisphenol-A on Reproduction in Selected Temperate Freshwater Gastropods: The Potential for (Neuro-) Endocrine Disruption.

A thesis submitted for the degree of
Doctor of Philosophy

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ABSTRACT

Evidence of feminising effects, including additional or enlarged female organs, have been reported in the (sub)-tropical freshwater prosobranch *Marisa cornuarietis* exposed to vertebrate estrogen receptor agonists. The primary symptom is an increase in the number of eggs laid, but this is only observed when exposure occurs at relatively low temperatures. This research project exposed temperate freshwater prosobranchs and a pulmonate to 17β-oestradiol (10-200 ngL^{-1}, nominal) in an outdoor mesocosm subject to natural seasons to determine whether similar effects occur in European native temperate freshwater gastropods. Laboratory exposures to 17β-oestradiol (1-100 ngL^{-1}, nominal) and Bisphenol-A (0.2-20 ngL^{-1}, nominal) were also carried out over a range of different temperatures and photoperiods to simulate natural seasons.

In the mesocosm exposures, significant increases in reproduction were measured in *Viviparus viviparus*, *Bithynia tentaculata* and, if the mortality rate was not significantly increased, *Planorbarius corneus*. It was observed that increases only occurred after the onset of autumn. In the laboratory, the oviposition rate in *P. corneus* was constant at 20^{o}C with a 16h photoperiod, but declined significantly at 15^{o}C with a 12h photoperiod, except at 100 ngL^{-1} 17β-oestradiol, when the rate remained constant. There were no similar effects from Bisphenol-A exposure. Small increases in reproduction were observed in all the prosobranch exposures to both compounds, but the interpretation of the data was confounded by several factors (test chemical degradation, high mortality rates and parasitized organisms) and there were no significant differences. In conclusion, there were indications that all of the assessed species were capable of increased reproduction, and in *P. corneus* this appears to be a perpetuation of summer oviposition rates in autumnal conditions. The consequences of this effect in annual semelparous pulmonates may not be detrimental at the population level, but the long-term fitness of iteroparous prosobranchs that practice 'restrained reproduction' in early breeding seasons may be adversely affected.

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Philosophical Inclusion

The year is at the spring, and day is at the morn,
Morning is at seven, the hill-side is dew-pearled,
The lark is on the wing, the snail is on the thorn,
God is in his heaven, and all is right with the world.

Robert Browning (1812 – 1889): Pippa's Song Part 1 'Morning', 1841.

I don't suppose that including philosophy in a doctoral thesis is fashionable any more, but I am a mature student (getting more so all the time, I hope!), so I will. This verse, by arguably the greatest romantic poet, seems to be an apt inclusion, not least because of its reference to snails and to seasons. The overall sentiment is that everything is ok; that ecology is in balance, unaffected by any adversity. It is interesting that this judgement is made by visual indications, much as it is in mainstream biological assessment today. However, endocrine disruption has taught us that this approach is not always reliable, that exposure to very low levels of endocrine active compounds can have subtle but insidious effects on reproduction in birds, fish and invertebrates. To effectively conserve wild-life, we need to develop ways of identifying these sometimes complex effects that may only be apparent if exposure occurs during critical life-stages or in certain seasonal conditions. The question is, how closely can we afford (and I mean that word quite literally) to look?

CHAPTER 1
INTRODUCTION, LITERATURE REVIEW AND OBJECTIVES.

1.1 Exogenous Oestrogens as Endocrine Disruptors in Aquatic Organisms.

A commonly promulgated definition of an Endocrine Disruptor is 'an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations' (World Health Organisation, 2002). This means that for a compound to be an 'endocrine disrupter', or for an effect to be termed 'endocrine disruption', two criteria must be met. Firstly a change must be measured in the normal endocrine function of an organism following exposure to a given chemical, i.e. the normal endocrinology must be described, and then a dose-response relationship as a function of exposure must be demonstrated. Secondly, the effect of such a change must be demonstrated to be adverse to the health of the organism, i.e. the normal variation in the affected processes must be understood, and a significant change measured that can be conceptualised as adverse to the organism. In the case of reproductive effects, this can be extended to a reduction in the number of, or viability of, the off-spring.

By far the most well documented case of endocrine disruption in the aquatic environment is that of the feminisation of male fish by exogenous oestrogens. The primary symptom is a condition termed 'intersex' (a descriptive term for the phenomenon of both spermatogenesis and oogenesis occurring within the gonad of a single individual from a gonochoristic species, in this case the presence of oocytes in the testes of males) and the elevation of the levels of the egg yolk protein precursor, vitellogenin, in the blood plasma of male fish to that normally observed in females (Sumpter and Jobling, 1995). Male fish can also exhibit female-like ovarian cavities and vestigial sperm ducts (Nolan et al., 2001). These effects are reported in multiple fish species (Tyler et al., 1998) and in the wild model species *Rutilus rutilus* (roach), intersex was found to be widespread in UK rivers, and associated with treated sewage effluent outfalls (Jobling et al., 1998).

The intersex condition also interferes with the reproductive health of male fish. Intersex affected roach living in rivers receiving large volumes of treated sewage effluent were shown to have a reduced milt volume and sperm density (Jobling et al., 2002a). While sewage effluent exposure has not yet been directly related to intersex, juvenile roach in long-term effluent exposures have developed oviducts which may be the first stages of intersex (Rodgers-Gray et al., 2001 and Environment Agency, 2004). Some severely intersex males may not in fact be reproductively functional, and exposure of roach embryos to environmental concentrations of an exogenous steroid (17α-ethinylestradiol) until 112 days post hatch skews the population ratio towards fish with newly differentiated gonads of characteristic female morphology (Lange et al., 2008) . Treated sewage effluents can also affect female reproductive ability, as has been shown in laboratory exposures of the Fathead Minnow (*Pimephales promelas*). Egg production was significantly reduced in breeding pairs exposed to two out of three final effluents (Thorpe et al., 2009 and Environment Agency, 2007).

Although sewage effluents may have a component of reproductive toxicity (immunotoxic and genotoxic effects adversely affecting fish health are reported by Liney et al., 2006), the synthesis of vitellogenin is under hormonal control via the activation of oestrogen receptors by endogenous steroid estrogens. Activation of the receptors initiates transcription of the gene responsible for vitellogenin production that is present in both males and females. As these receptors can also be activated by exogenous steroids taken up from the aquatic environment, vitellogenin induction can be considered as a biomarker of endocrine disruption. It has been correlated with the degree of intersex in male fish (Jobling et al., 2002b), and there is also a concentration-related response to steroid exposure at environmentally relevant levels (Routledge et al., 1998; Thorpe et al., 2003a and Environment Agency, 2005). Exogenous steroids (both natural and synthetic) have been identified as the major causative agent of vitellogenin induction in treated sewage effluent (Desbrow et al., 1998), although other estrogenic

xenobiotics such as alkylphenolic compounds are also present (Tyler and Routledge, 1998) and can have similar effects in male fish (Jobling et al., 1996).

Overall, the evidence gathered on feminizing effects in male fish is such that the WHO definition of endocrine disruption is clearly met. Oestrogenic compounds can interact with steroid receptor signalling, causing a diagnostic induction of blood plasma vitellogenin, and exposures to steroids and steroid-mimicking compounds can adversely affect both male and female reproductive potential. It is not clear whether exposure to exogenous steroids can have population level effects, because information on the population biology of wild freshwater fish populations is limited, and it is not known what level of recruitment is required to maintain population viability (Jobling and Tyler, 2003a). However the long-term exposure of a wild population of *P. promelas* to 17α-ethinylestradiol (mean = 5.3 ngL^{-1}) in an experimental lake in Canada caused an almost complete population extinction (Kidd et al., 2007), although the concentrations measured were rather higher than might be expected in sewage effluent. Consequently it might be expected that the symptoms of endocrine disruption recorded in UK field populations are indicative of potential population declines. Further data and modelling are needed to gain a better understanding of this risk, but this may be difficult to achieve.

This is due, in part, to the ability of fish to migrate, both as a natural aspect of the life history of many species, and as an avoidance response to pollution. This makes attempts to quantify wild fish populations challenging, and it tends to confound the demonstration of correlations between degree of exposure to oestrogenic compounds and reductions in those populations. At the individual level, there are also ethical concerns against making large-scale experiments sufficient to generate data in order to model extrapolations to population level effects. In the UK, wild fish fall under the protection of the Home Office and the Animals (Scientific Procedures) Act 1986. Each experiment must be licensed, and the tenet of reducing numbers of organisms used wherever possible applies. This can make proper quality control and statistical analysis difficult to justify,

which in turn can compromise the power of the experiment and therefore the robustness of the risk assessment.

There is little evidence that oestrogenic compounds affect other aquatic organisms. The invertebrate fauna of freshwater ecosystems were, until recently, not expected to be affected by exposure to the oestrogenic component of treated sewage effluent as no oestrogen receptors had been identified in the genomes of any of this clade. However, oestrogen receptor orthologs have now been sequenced in the cephalopod *Octopus vulgaris* (Keay et al., 2006), the bivalve *Crassostrea gigas* (Matsumoto et al., 2007), the opisthobranch gastropod *Aplysia californica* (Thornton et al., 2003), and the prosobranch gastropods *Thais clavigera* (Kajiwara et al., 2006) and *Marisa cornuarietis* (Bannister et al., 2007). All these species belong to the phylum Mollusca, and while the first four are marine, *M. cornuarietis* is a freshwater, albeit (sub)-tropical, species.

This raises the concern that steroid-laden discharges may also be adversely affecting the molluscan wildlife of UK rivers. Representatives of the International Union for the Conservation of Nature (IUCN), the Freshwater Mollusk Conservation Society and Unitas Malacologia consider that non-marine molluscs are 'arguably one of the most imperilled groups of animals'. There are 708 species of freshwater mollusc on the IUCN Red List (Lydeard et al., 2004). In the UK, six freshwater species are considered sufficiently threatened to be included on Biodiversity Action Plans; the bi-valves *Margaritifera margaritifera* (Pearl Mussel), *Pseudoanodonta complanata* (Depressed River Mussel) and *Pisidium tenuilineatum* (Fine-lined Pea Mussel), and the gastropods *Myxas glutinosa* (Glutinous Snail), *Aniscus vorticulus* (Little Whirlpool Ramshorn Snail) and *Segmentina nitida* (Shining Ramshorn Snail). There are many possible reasons for molluscan declines including predation, habitat destruction, unsustainable water use, pearl fishing and parasitism, but most of the Action Plans for these species cite water pollution from farm fertilizers, cattle slurry and sewage effluent as a potential cause of ecological deterioration (Willing, 1997). It is therefore a possibility that oestrogenic compounds in these forms of pollution

may be playing a role in a more generalised molluscan decline. While this is worthy of investigation in itself, there is also the possibility that an invertebrate model that is similarly sensitive to fish may be available amongst the molluscs, that could be used in place of fish in order to reduce the numbers of vertebrates sacrificed for risk assessments. Jobling et al. (2004) performed a series of experiments comparing the responses of fish to the freshwater prosobranch *Potamopyrgus antipodarum*, both to steroid mimics in the laboratory and to sewage effluents in outdoor mesocosms. In laboratory exposures to 17α-ethinylestradiol (1-100 ngL^{-1} nominal), the number of new (unshelled) embryos in the brood pouch of *P. antipodarum* was significantly increased. A dose-response curve in the form an 'inverted U shape' was recorded, with a peak response at 25 ngL^{-1} (Figure 1a) A similar effect was observed in the treated sewage effluent exposure, where 12.5% and 25% effluent showed a significant induction after 14 days, whereas 50% and 100% effluent caused an inhibition in reproduction. Trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) were exposed to the sewage effluent along with *P. antipodarum* in the mesocosm, and showed significantly increased levels of plasma vitellogenin in a dose-dependant manner, demonstrating that the effluent was indeed oestrogenic. However, the apparent effects in gastropods may be confounded by the potential for the effluent to act as a food source and it may also contain other unknown toxicants.

Jobling et al. (2004) compared their results in *P.antipodarum* to a recrudescence assay using *P. promelas*, in which single sex groups of adult fish were brought from conditions unsuitable for reproduction into a 3 week exposure to 17α-ethinylestradiol (0.1-100 ngL^{-1}) with optimal conditions for reproduction, before being returned to culture conditions in breeding pairs and allowed to spawn (after Pawlowski et al., 2004). The eggs laid were then counted, and again an 'inverted U-shaped' dose-response curve was recorded, but with a peak response at 1 ngL^{-1} (Figure 1b). It appears that the responses to oestrogens are broadly similar between fish and *P. antipodarum*, but with the gastropod having less sensitivity.

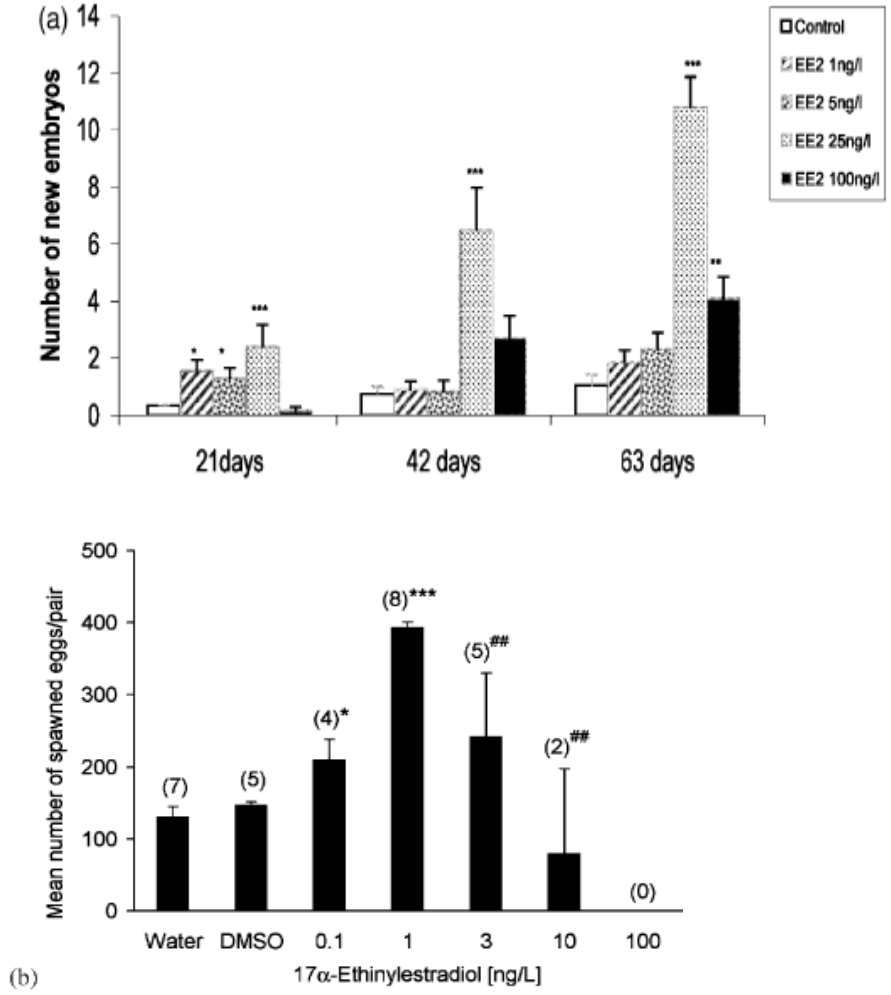


Figure 1 – (a) the number of new embryos produced by *Potamopyrgus antipodarum* following exposure to 17α-ethinylestradiol, and (b) the number of eggs produced by *P. promelas* following 14 days exposure (after Jobling et al., 2004 and Pawlowski et al., 2004). * = significant difference from control at p<0.05, ** = p<0.01 and *** = p<0.001.

1.2 Potential Endocrine Disruption by Exogenous Oestrogens in Invertebrates

A far greater proportion of the metazoans are made up of organisms loosely termed as ‘invertebrates’ as opposed to Chordates. As vertebrates consist of only about 5% of animals in terms of number of recorded species (Ruppert and Barnes, 1994), it is perhaps surprising that there are not many more adverse effects associated with endocrine disrupting compounds in invertebrates than those described in the reviews of the last decade (DeFur et al., 1999; Environment Agency, 1999; Oetken et al., 2004). These reviewers tended to focus on two case studies; the disruption of growth and moulting hormones in arthropods by pesticides, and the means by which organotins disrupt gastropod reproduction. These effects came to scientific attention in very different ways, the former because the mechanism was understood and indeed manipulated, so that the effects were actively researched, whereas the latter was discovered by diligent environmental monitoring, with the mechanisms of action studied as a consequence. These circumstances lead the reviewers to the opinion that the paucity of examples is due to insufficient targeted field monitoring and an inadequate understanding of invertebrate endocrinology.

Nevertheless, in recent years advances have been made in certain invertebrate groups (Weltje and Schulte-Oehlmann 2007), with additional effects of compounds considered to be endocrine active described and likely mechanisms proposed. LaFont (2000) made a general review of the endocrinology of invertebrates, and describes several pathways for chemical signalling in invertebrates. The most widespread is the management of nervous co-ordination using neuro-transmitters over the short distances across synapses and the lower invertebrate phyla appear to be confined to this approach. This was followed by the development of neuro-hormones to transmit chemical messages over longer distances, made by endocrine cells within the nervous system. These are generally peptide hormones, first appearing in the Phylum Annelida. Finally, lipophilic hormones developed, being synthesised by endocrine glands. There are several orders of endocrine systems within the invertebrates, classified depending

on the number of glands occurring between the neuro-transmitter and the receptor, with the culmination found in the Phylum Arthropoda.

The lipophilic hormones include steroids derived from sterols such as cholesterol. There are three types occurring in invertebrates as reviewed by Lafont and Mathieu (2007); ecdysteroids (involved in growth and moulting along with terpenoid hormones), corticosteroids (used in secreted alarm substances) and vertebrate type sex steroids. The latter are often associated with gonadal development and reproduction, and the reviewers indicate that they are highly conserved, there being evidence of either the presence of steroids or their biosynthetic pathways in diverse groups including the basal cnidarians. However, whether very early metazoans are capable of responding to exogenous hormonally active compounds, and thereby experiencing endocrine disruption, is doubtful.

Toxic effects are observed in poriferan gemmules only when exposed to Nonylphenol or Bisphenol-A in mgL^{-1} concentrations (Hill et al., 2002), although the number of functional testes and oocytes was significantly reduced in the cnidarian *Hydra vulgaris* when exposed to high levels (500 ngL^{-1}) of 17α-ethinylestradiol (Pascoe et al., 2002). This suggests that the ability to interact with vertebrate-type sex steroids, albeit to a limited extent, may fall between the divergence of these groups. There is no evidence for steroid endocrine disruption in the other major acelomate group, flatworms, although the parasitic platyhelminth shistosome *Trichobilarzia ocellata* is thought to contain progesterone and testosterone at the cercarial stage (Schallig et al., 1992), and has transcripts for steroid biosynthetic enzymes (Verjovshi-Almeida et al., 2003). However, being parasitic, it will not be straight-forward to determine whether the functions of these organisms can be disrupted by exogenous steroids or other steroid receptor agonists.

The potential that endocrine disruption may occur from exposure to exogenous vertebrate type sex steroids is more convincing in the higher invertebrates. Hoss

and Weltje (2007) reviewed the evidence in the (pseudo-coelomate) nematode *Caenorhabditis elegans* and noted that although the genome has been sequenced and many nuclear receptors are present, there are no sex steroid receptors, and that nematodes need dietary sterols for their development. However, physiological concentrations of 17β-oestradiol and environmentally relevant concentrations of Bisphenol-A significantly increase reproduction in this species (Hoshi et al., 2003), an effect that can be conceptually linked with oestrogen interference. In the true coelomates, the evidence is further improved by the presence of estrogen receptor orthologs in the polychaete annelids *Platynereis dumerilii* and *Capitella capitata* (Keay and Thornton, 2009), and 17β-oestradiol-treated eleocytes of *Nereis virens* show an increased immune response to antibodies raised against yolk proteins (Garcia-Alonso, 2006). As already mentioned, oestrogen receptor orthologs are also found in molluscs and many species also respond to oestrogen exposure (see Section 1.3 below).

It is possible then, that these groups could be adversely affected by exposure to exogenous oestrogenic compounds in the environment. However it seems less likely in the arthropods. Soin and Smagghe (2007) reviewed the evidence for endocrine disruption in insects, as did LeBlanc (2007) in the crustacea. In both cases, it seems that their endocrinology is restricted to peptides, ecdysteroids and either sesquiterpenes (juvenile hormones) in insects or methyl farnesoate in crustacea. The balance between the latter secretions and 20-hydroecdysone appear to control the development in these classes, and can be disrupted by pesticides designed to arrest growth.

In the crustacea, sex differentiation is controlled by a peptide termed ‘androgenic gland hormone’ produced in the androgenic gland on the posterior male gamete ducts. It also regulates vitellogenin synthesis in the fat bodies. In insects, the differentiation mechanism is poorly understood, although it may be related to high concentrations of ecdysone (De Loof, 2008; De Loof and Huybrechts 1998). Exposure to oestrogenic compounds certainly seems to have little effect on the reproduction of either class; in the chironomid *Chironomus riparius*, exposure to

17α-ethinylestradiol and Bisphenol-A alters the emergence time of adults in sediment tests, but this is attributed to toxicity (Watts et al., 2001). In the barnacle *Balanus amphitrite*, exposure to 17β-oestradiol and Nonylphenol increased the density of staining in a naupliar protein corresponding to Cypris Major Protein which has a similar structure to vitellogenin and can affect settlement success, but this was considered only as a potential biomarker of exposure (Billinghurst et al., 2000).

It is possible that although the genome of *Drosophila* contains an Estrogen Related Receptor (ERR) ortholog (Enmark and Gustafsson, 2001; the ERR is an orphan receptor, i.e. with no known ligand), the arthropods have lost a function for vertebrate-type sex steroids that exists within the lophotrochozoa before the putative ecdyzoa split within the protostomes (Keay and Thornton, 2009; Graham, 2000). The deuterostomes, which evolved from an earlier split with the protostomes and contain the chordates, should conceptually therefore also use vertebrate-type sex steroids unless the non-chordates have lost the ability in a separate event.

The earliest vertebrates have an estrogen receptor as demonstrated in the sea lamprey, *Petromyzon marinus* (Thornton, 2001), and appear to use 17β-oestradiol in the reproductive cycle (although not androgens, Sower et al., 1985). The other major deuterostome group, echinoderms, were reviewed by Sugni et al., (2007), and endogenous progesterone, testosterone, androstenedione, oestrone and 17β-oestradiol were recorded in this group, along with steroid biosynthetic enzymes and aromatase activity. The reviewers also report their own research, indicating that skeletal abnormalities are associated with disturbed 17β-oestradiol levels, which has a parallel in chordates as this steroid has a regulatory role in vertebrate skeletal density. Overall, it seems that the potential for endocrine disruption in invertebrates caused by vertebrate-type sex steroids is widespread, with the possible exclusion of the arthropods. Figure 2 is a composite summary of the evidence outlined above.

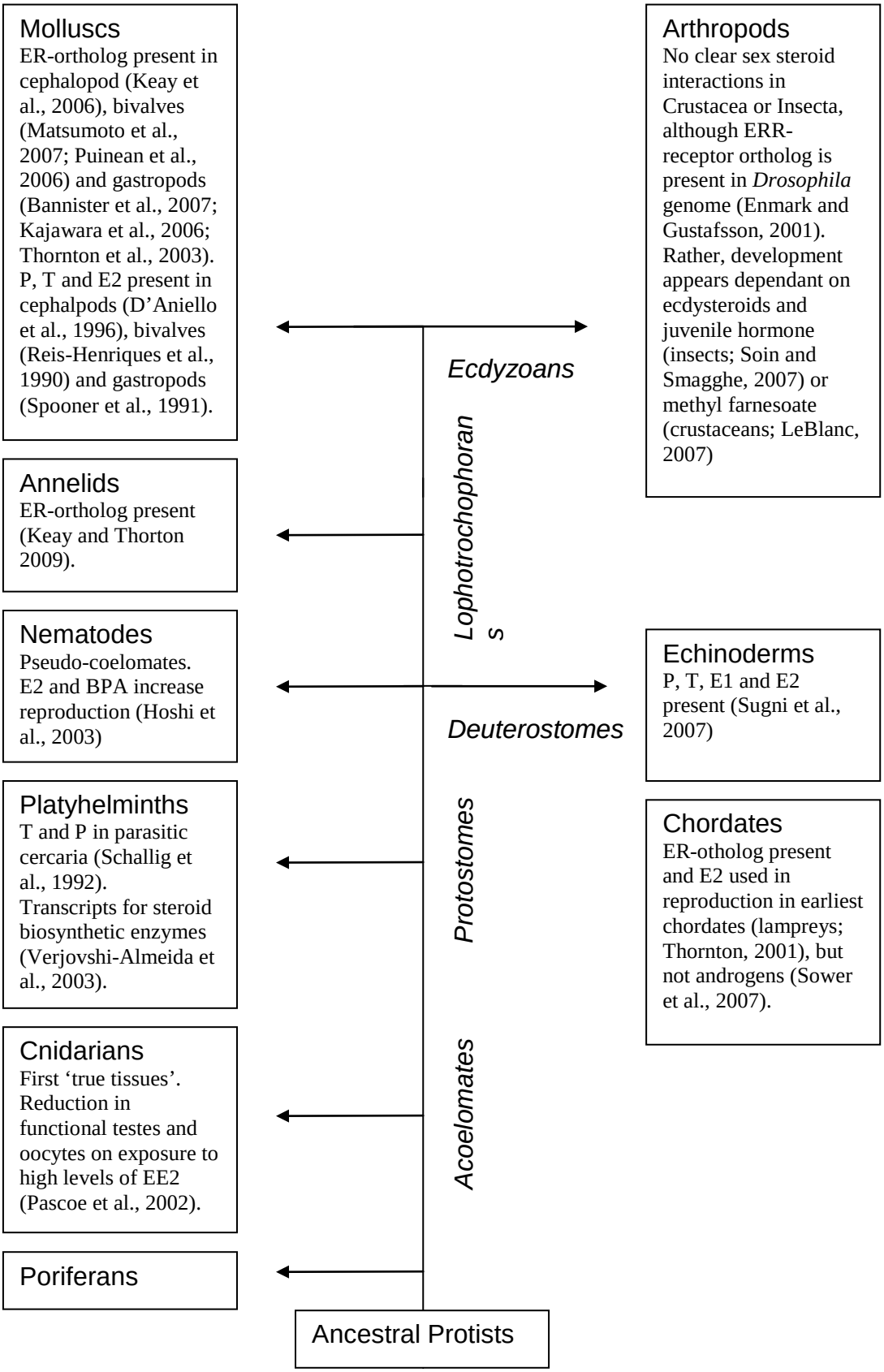


Figure 2 – A schematic summary of the current evidence for vertebrate-type sex steroid interactions in the 'tree of life' (ER; oestrogen receptor, P; progesterone, T; testosterone, E2; 17β-oestradiol, BPA; Bisphenol-A, EE2; 17α-ethinylestradiol, EER; oestrogen-related receptor).

1.3 Sex Steroids in Molluscs and the Effects of Exogenous Oestrogens

At phylum level, molluscs are extremely diverse. It is the second largest invertebrate phylum following the arthropods, with up to 200,000 living species and a long geological history stretching back to the Cambrian (Ponder and Lindberg, 2008). At least seven classes are recognised, including the Aplacophora (spinule worms), Monoplacophora (single shelled deep sea organisms), Polyplacophora (chitons) and Scaphopoda (tusk shells), but by far the most well described groups are the Cephalopoda (about 1000 species including octopus, squid and cuttlefish), the Bivalvia (about 20,000 species including scallops, oysters, mussels, cockles and clams) and the gastropods (about 100,000 species including sea slugs and sea hares, abalone, limpets, nerites, periwinkles and whelks, freshwater snails, land snails and slugs).

The basic anatomy of the ancestral mollusc is deceptively simple. Ruppert and Barnes (1994) describe the ancestral mollusc as ‘an animal that moves over and grazes on a hard substratum’. The ventral surface is a creeping sole referred to as the foot. The dorsal surface is a shell used as a shield. The shell is secreted by a mantle epidermis that overlies the visceral mass. At the rear of the animal the shell and mantle extend beyond the visceral mass to create a mantle cavity which houses the gills. At the front, the mouth opens into the buccal cavity which houses a thickened mass of cartilage called the odontophore. This is covered by a belt of teeth called the radula which continuously grows and which the animal uses to scrape or graze matter. The stomach is simply a sorting chamber which passes particles of the correct size to the digestive gland, where food is digested intracellularly. Excess particles are expelled as faecal strings of mucus. The pericardium (a coelomic cavity) houses the heart which pumps oxygen-loaded haemocyanin into the sinuses of the haemocoel, from which it migrates back to the heart via the gills. Waste products from the haemocoel pass through the walls of the heart into the pericardial coelom, which drains into the nephridia. Here salts are recovered before fluids drain into the mantle cavity via nephridiopores.

The Gastropoda are still essentially as described above, however their shells have generally become more conical and have become coiled in a variety of ways. They have retained their mobility, and the majority graze on organic matter. The Bivalvia however have become sessile, and have two shells or valves which can close over the animal completely. They have often lost almost all of the foot, and feed by means of filtering organic particles from water. Both classes have invaded freshwater. The Cephalopods are generally much larger and the shell is vestigial. They are exclusively marine and usually predatory. These three groups are the earliest protostomes subject to research in sex steroid signalling, and the only molluscan classes studied to date (Kohler et al., 2007). They will be reviewed in more detail, along with observations made of the effects of exposure to oestrogenic and other potentially hormonally active compounds.

1.3.1 Cephalopod Review

The common octopus (*O. vulgaris*) has been widely used as a model organism, and the presence of sex steroids (progesterone, testosterone, 17β-oestradiol) in the reproductive tract of this organism was recorded over a decade ago (D'Aniello et al., 1996). More recently, binding components with a high affinity for 17β-oestradiol (but which also bound other steroids) were identified in the cytosol of ovarian extracts (Di Cosmo et al., 2002), and an estrogen receptor ortholog was sequenced (Keay et al., 2006). Until this point, estrogen receptors were thought to be a vertebrate novelty. However the octopus receptor is constitutively active, meaning that it does not need a bound ligand to activate gene transcription. It is therefore not clear what is binding to 17β-oestradiol in the ovary of the octopus, nor the role of the steroid in reproduction. There does, however, appear to be a role, as both progesterone and 17β-oestradiol peak at up to 400% of basal levels when the synthesis of yolk proteins begins prior to the single spawning event in octopus life history, and steroid biosynthetic enzymes have also been reported (Di Cosmo, 2001). Finally, a novel gonadotrophin-releasing hormone receptor has been sequenced in *O. vulgaris* which has a high similarity to the chordate counterpart, known to play a role in the release of sex steroids. When it is

applied to octopus follicles and sperm, there is a dose-dependant increase in steroidogenesis (Kanda et al., 2006). Overall, the role of steroids, and particularly 17β-oestradiol, appears to be related to vitellogenesis and gamete maturation in this group, which parallels the role of this hormone in vertebrates.

1.3.2 Bivalve Review

Much of the work involving the effects of endocrine disrupting chemicals on molluscs has focussed on marine bivalves including the oysters *Crassostrea sp.* the mussels *Mytilus sp.* and the clams *Mya sp.* because they are commercial crop organisms. Researchers are concerned with means to protect valuable shell-fisheries from pollutants and also with developing ways to increase spat production and manipulate the sex ratio of breeding stocks. For example, it is common practice to inject 17β-oestradiol into the gonad of the sequential hermaphrodite *C. gigas,* and this can double the female to male sex ratio (Mori et al., 1969). However, bivalves also have two orders in the freshwater habitat in Europe, namely the Veneroidea, which include the Zebra Mussel *Dreissenia polymorpha*, and the Unioniodea, which include the Swan Mussel *Anodonta cygnea*. Members of the latter freshwater order, the freshwater mussels *Elliptio buckleyi* (Florida Shiny Spike) and *Lampsilis teres* (Yellow Sandshell), also spawn on exposure to exogenous 17β-oestradiol (Kernaghan et al., 2002), therefore it appears that a response to steroid oestrogens are wide-spread in this group.

Steroid biosynthesis was demonstrated in bivalves 35 years ago by de Longcamp et al. (1974) in the mussel *Mytilus edulis*. They also showed that gonad extract from this species could convert androstenedione to testosterone and estrone to 17β-oestradiol, implying the presence of 17β-hydroxysteroid dehydrogenase (17β-HSD). Following this, Reis-Henriques et al. (1990) identified the presence of progesterone, androstenedione, testosterone, 5α-dihydrotestosterone, 17β-oestradiol, and oestrone by Mass Spectrometry in the same species, and showed that concentrations varied during the annual reproductive cycle (Reis-Henriques

and Coimbra, 1990). Le Curieux-Belfond et al. (2001) incubated homogenates of *C. gigas* with steroid pre-cursor substrates. After incubation with androstenedione, thin layer chromatography showed metabolites which manifested at the same co-ordinates as standards of 17β-oestradiol and oestrone. They repeated the experiment with the addition of 4-androsten-4-ol-3,17-dione (4OHA), a known steroidal aromatase inhibitor and found that the substrate metabolism was blocked. They concluded that the organism can aromatise androgen into oestrogen-like compounds. Consequently, it seems clear that bivalves both make and use vertebrate-type steroids, although their roles are not understood.

Blaise et al. (1999) developed an assay to look for proteins of a similar weight to the egg-yolk precursor vitellogenin, with a view to using changes in heamolymph levels as an indication of exposure to endocrine disrupting compounds. Vitellogenin is a glycolipophosphoprotein that contains one zinc atom and two calcium atoms per molecule. They extracted haemolymph from the adductor muscle of the Soft-Shelled clam *Mya arenaria* and subjected it to treatment with alkali to hydrolyse the bound phosphates of the lipophosphoproteins and make them labile to the standard colourimetric phosphomolybdenum method for the measurement of total phosphate. This 'alkali-labile phosphate (ALP) assay' correlated with a trout immunoassay for vitellogenin, and also when Vg-like proteins were elevated, there was a concomitant rise in calcium and zinc as expected. ALP levels in wild clams tended to increase as gonadal maturation and spawning proceeded and higher levels were found in ripe females over those that had finished spawning. Males contained very little in comparison.

Injections of 17β-oestradiol and Nonylphenol were found to promote ALP levels in the clams after 48 hours. The researchers suggested that the production of Vg-like proteins could therefore be under the control of an oestrogen receptor. They went on to assess the effects of injected coprostanol (a substance that is bacterially converted from the high levels of cholesterol found in sewage effluents; Gagne et al., 2001a) and an extract of treated sewage effluent (Gagne et

al., 2001b) in the freshwater mussel *Elliptio complanata*. They also measured the ALP levels in these mussels downstream of a sewage effluent outfall (Gagne et al., 2001c). They found an increased ALP response (above control; solvent injected or upstream organisms) in all cases. However, while 17β-oestradiol and the treated sewage effluent extract were shown to bind to gonadal cytosol fractions, the molecules involved in binding them were not specified and therefore the assumption that an oestrogen receptor was signalling for the changes in Vg-like proteins expressed in the ALP assay is currently unfounded.

Gauthier-Clerc et al. (2002) also found elevated glycogen levels in *M. arenaria* downstream of sewage treatment works. At one site the clams were found to be experiencing a delay in the onset of gametogenesis. They concluded that this was due to a dysfunction in the gonadal consumption of glycogen with consequent perturbed vitellogenesis. Additionally, Quinn et al. (2004) exposed *D. polymorpha* in cages downstream of a sewage treatment works and also found that ALP levels were increased. They reported that the histology of the gonads of exposed males showed a large increase in interstitial tissue between the seminiferous tubules, suggesting that less spermatogenesis was occurring, when compared to 'control' animals held in the laboratory. However, any of these effects could be due to stressors other than exposure to oestrogens and certainly in the latter case the exposed animals had experienced many stresses from which the control organisms were exempt (high flow, freezing, effluent toxicity).

Overall, although the correlation between ALP level and oestrogen exposure has some merits, there are also many confounding factors, including the compound stresses associated with effluent and environmental exposures. Importantly, there is also an underlying uncertainty that Vg-like proteins are actually vitellin precursors. Riffeser and Hock (2002) looked for evidence of changing levels of vitellogenin itself in bivalve haemolymph, by using gel-electrophoresis techniques in *M. edulis* and the freshwater mussel *A. cygnea*. They exposed the mussels for 3 weeks to 2 μgL^{-1} 17β-oestradiol in the water and also by injection, and compared the response to that in fish. The fish plasma showed induction of

the vitellogenin molecule at 160-170dKa, as expected. They found no similar molecule induced in the heamolymph of either species of bivalve exposed by either method. The only strongly up-regulated molecules were at about 35dKa, suggesting a histidine-rich glycoprotein that is involved in heavy metal binding. Consequently they concluded that there is no vitellogenin in mussel heamolymph.

The relationship between 17β-oestradiol and vitellogenesis in bivalves or any other potential role of steroids is therefore not clear. Gagne et al. (2004) analysed levels of metallothionein-like compounds in mussels (*E. complanata)* caged downstream of an effluent (because metallothionein levels are depressed during vitellogenesis in fish), and also serotonin/dopamine levels and transport. They found that caged mussels downstream of an effluent had elevated metallothionein-like proteins, probably as a response to heavy metal bioaccummulation. Serotonin and dopamine levels were both depressed, but while the dopamine transport system was uninhibited, the serotonin was not being effectively cleared, implying serotonergia. Exogenous serotonin causes gamete release (spawning) in commercial oysters (*Crassostrea virginica*) and this is enhanced following exposure to 17β-oestradiol (Quintanna et al., 2004). Serotonergia may therefore have contributed to the perturbations in reproductive timing.

Wang and Croll (2003) also studied a potential role of steroid hormones in spawning, using the Scallop *Placopecten megellanicus*. They induced gamete release *in vitro* using serotonin. Pre-incubation with 17β-oestradiol, progesterone and testosterone all significantly increased sperm release by 200-300%. The oestrogens also caused increased egg release. Each experiment gave very similar 'inverted U-shaped' dose-response curves with peak responses at $10x^{-6}$ to $10x^{-7}$ M. The increase in egg release was abolished by the anti-oestrogen Tamoxifen, as was sperm release. The latter was also abolished by the anti-androgen Flutamide and the anti-progesteren RU486. Inexplicably, all the inhibitors when tested alone proved to be stimulatory.

However, the observation that steroids can affect gamete release and that this effect can be blocked forms part of the evidence for the existence of steroid receptors. The hypothesis that any such receptors might not be specific, instead binding to any molecules with structural similarity, is conceivable. Tying in with this, it was found that injections of all of these steroids altered the male to female ratio in juvenile clams in favour of males (Wang and Croll, 2004). The evidence for steroids determining gender is therefore contradictory, although the doses used were high (1 μg/clam) and may therefore have caused extreme perturbations. However, histological analysis showed that juveniles females treated with 17β-oestradiol had large, morphologically mature oocytes as would be expected in mature adults. When treated with testosterone, the juvenile females had degenerating oocytes in all individuals. This suggests that 17β-oestradiol at least plays a role in ovarian maturation.

The mechanism by which 17β-oestradiol exerts influence on gonad development is not yet clear. Suzuki et al. (1992) identified a female-specific protein in the oocytes of *C. gigas*, and Li et al. (1998) found that the oocytes were significantly larger and contained significantly more of this protein 40 days post-injection with 17β-oestradiol. Matsumoto et al. (1997) found that increasing concentrations of endogenous 17β-oestradiol correlated with annual ovarian maturation in the scallop *Pantinopecten yesseonsis* and the oyster *C. gigas.* Osada et al. (1998) proposed that 17β-oestradiol induces the formation of serotonin receptors on the surface of oocytes, as the onset of gonadal maturation also correlates with a sharp increase in serotonin binding at this time. They also identified putative vitellin proteins in the ovary of the scallop (by molecular weight) and observed that these were significantly increased following injections of 17β-oestradiol (Osada et al., 2003). These vitellin-like proteins were immunolocalised in the follicle cells, and no similar proteins were found in the haemolymph, indicating that there is no transport occurring from elsewhere. Therefore it was considered that vitellogenesis occurs within the ovary, and that it is controlled by 17β-oestradiol.

The same group went on to sequence genes that were expressed only in ovaries of *C. gigas* that contained oocytes, including cDNA encoding the putative vitellogenin (Matsumoto et al., 2003). They did the same for *P. yesseonsis*, finding a fragment sharing 20-35% identity with other oviparous species that was associated with growing oocytes (Osada et al., 2004). However, whereas transcription of this mRNA was promoted by 17β-oestradiol in this species, it was not the case for the corresponding sequence found in *M. edulis* when exposed to 200 ngL^{-1} (Puinean and Rotchell., 2006; Puinean et al., 2006), although it is possible that the exposure time of 7 days was too short.

Although complete or partial fragments of estrogen receptors have also been sequenced in *C. gigas* (Matsumoto et al., 2007) and *M. edulis* (Puinean et al., 2006), the former at least is constitutively active and neither are responsive to oestrogens, as is the case with the cephalopod estrogen receptor (Keay et al., 2006). The mechanism by which 17β-oestradiol could regulate vitellogenesis is therefore yet to be understood. It is possible that regulation may simply be driven by the concentration of 17β-oestradiol in the tissue, or by the relative concentration of other hormones (e.g. testosterone). Janer et al. (2004) studied the metabolism of 17β-oestradiol in *Mytilus galloprovincialis* following exposures and found large dose-dependant increases in the body burden of this steroid once extracts had been saponified to release the esterified component. They measured P450 aromatase and acyl-CoA:oestradiol acyltransferase (esterification enzyme) and showed that at physiological levels, 17β-oestradiol reduced the activity of the former and increased the latter, suggesting that the mussel is able to both down-regulate aromatisation in the presence of sufficient oestrogen and may 'de-activate' surpluses by esterification (i.e. rendering them unavailable). At higher concentrations (up to 2 μgL^{-1}), esterification rates increase dose-dependently, until it appears that the esterification process is overcome, and the levels of free 17β-oestradiol increase (Janer et al., 2005a). Aromatisation also increases, and it may be that the steroid metabolism is generally disrupted by the presence of extreme amounts of exogenous steroid.

Zhu et al. (2003) separated two isoforms of 17β-oestradiol from *M. edulis* gonad, one in the aqueous phase and one in the solvent phase, and both were immunoreactive to 17β-oestradiol rabbit antiserum. They suggest that the lipophillic isoform is likely to be a genomic signal, being relatively stable, and the hydrophilic form may be an intracellular signalling molecule. They also infer that this may be an evolutionary early role for 17β-oestradiol. Stefano et al. (2003a) sequenced a fragment of an estrogen receptor from the membrane and cytosolic fraction of the pedal ganglia with 100% identity to the human ERβ. They showed that ganglia incubated with 17β-oestradiol released nitric oxide. By coupling the 17β-oestradiol to BSA, making it too large to pass through cell membranes, they were also able to demonstrate that this effect occurred due to interaction with cell surface oestrogen receptors as opposed to a nuclear receptor. Neither testosterone nor progesterone has the same effect, so it appears to be a female response. They hypothesise that this process down-regulates immune responses to allow reproduction to proceed (Stefano et al., 2003b), although this may be illogical in a species that spawns rather than mates.

Nonetheless, Canesi et al. (2004a) studied the effects of 17β-oestradiol on *M. galloprovincialis* haemocytes *in vitro* and found a stimulation of bacteriocidal activity, and a significant decrease in lysosomal membrane stability suggesting an activation of enzyme release. Other responses included a significant increase in cytosolic calcium ions and transient increases in the phosphorylation state of components of the tyrosine kinase-mediated signal transduction pathways involving mitogen activated protein kinases (MAPK-like proteins) and signal transducing and transcription activated proteins (STAT-like proteins). All these responses were rapid (within 60 minutes), and they concluded that rapid cell signalling by 17β-oestradiol in molluscs and vertebrates is conserved. They found similar effects when 17β-oestradiol was injected *in vivo*; there were significant decreases in lysosomal membrane stability and neutral lipid accumulation assays, and also increased transcription of catalase and metallothionein gene expression suggesting an increase in anti-oxidant defences (Canesi et al., 2007a). The group also identified a putative estrogen receptor

protein responsive to human estrogen receptor antibodies in whole protein extracts, although gene transcription was not affected by 17β-oestradiol exposure *in vivo*.

With regard to the potential for xeno-oestrogen agonist mediated endocrine disruption in bivalves, Canesi et al. (2004b) also established that Diethylstilbestrol (DES) had similar effects on the phosphorylation state of protein kinases as 17β-oestradiol, although Bisphenol-A and Nonylphenol had the reverse effect (decrease of phosphorylation). All of these compounds also affected the lysosomal membrane stability assay, but at much higher concentrations than 17β-oestradiol, as might be expected for weak agonists. *In vivo*, injected Bisphenol-A (up to 5 μg/mussel) caused significant decreases in the phosphorylation of the MPAK-like proteins, suggesting a reduction in bacteriocidal ability (Canesi, 2005) and alteration in functional parameters (catalase, glutathione-S-transferase, glutathione reductases) along with up-regulation of gene fragment expression of the estrogen receptor (Canesi et al., 2007b). They also found that a synthetic mixture of compounds including 17β-oestradiol, 17α-ethinylestradiol, Bisphenol-A, Nonylphenol, Nonylphenol ethoxylate, Mestranol and Benzophenone did not have effects on the biomarkers outlined above at an environmentally relevant concentration, unless it was injected directly (Canesi et al., 2007c; Canesi et al., 2008). They concluded that the *in vitro* biomarkers are more sensitive. There was also some transient down-regulation of the *M. edulis* estrogen receptor gene fragments in *M. galloprovincialis* digestive gland extracts, but this was not conclusive.

Taken as a whole, there is good evidence that bivalves are able to synthesize vertebrate-type sex steroids, and it is becoming increasingly convincing that the apical hormone, 17β-oestradiol, is involved in female reproduction, most likely during the initiation of vitellogenesis and gonad maturation. There is no clear role for androgens or progesterone, although they cannot be excluded. The mode of action of 17β-oestradiol is not understood, and may involve a form of rapid cell-signalling rather than a genomic receptor. There is also no strong evidence

that environmental concentrations of endocrine disruptors affect populations of bivalves, with the biomarkers suggested to date only responding at high concentrations or under artificial (*in vitro* or injected) conditions. There are however a few cases that may indicate that disruption may be occurring that is not yet fully researched.

Binelli et al. (2004) report on potential effects on the population of *D. polymorpha* in the period after a spill of DDT (known to be endocrine active and to disturb reproduction in birds) in Lake Maggiore, and reported a delay in oocyte maturation that may be due to a disturbance in serotonin synthesis pre-spawning. Aarab et al. (2006) indicated an increase in the ALP assay and a disturbed spawning pattern. When exposing *M. edulis* to 50 μgL^{-1} Bisphenol-A, Ortiz-Zarragiota et al. (2006) found no effect in the same assay. Nice et al. (2003) exposed embryos of *C. gigas* to <100 ngL^{-1} of nonylphenol for 48 hours, before growing on the spat to sexual maturity. They found that there were a greater proportion of females in the exposed group, where a preponderance of males is usual in this species. They also performed crosses between control and treated parents, and found that embryo survival to 48 hour post hatch was significantly greater between control male and females than any other cross; an apparent transgenerational effect.

Finally, Chesman and Langston (2006) have reported intersex in the clam *Scrobicularia plana*, and surveyed the population in the Avon estuary. This species is putatively gonochoristic, but they found significantly less males and more intersex individuals in the summer (mean = 21%, maximum 48%, although the baseline level of intersex occurrence is not known). Following exposure of groups of undifferentiated adults to sediments contaminated with high levels of 17β-oestradiol, 17α-ethinylestradiol (100 ug/kg each), Octylphenol and Nonylphenol (1000 ug/kg each) early in the season, the rate of intersex was similar at 44%, suggesting that oestrogens could be the cause (Langston et al., 2007). The apparent reduction in the proportion of males suggests that the intersex individuals are feminised males, and the exposed females also showed an

increased mean oocyte size, suggesting ‘superfeminisation’ is occurring. The conceptual linkage is therefore valid, however the concentrations used were extreme, and further work is needed to understand the observations made in the wild populations.

1.3.3 Gastropod Review

The potential for endocrine disruption in gastropods came to attention because marine species from this group were found to be in population decline or locally extinct due to the adverse reproductive effects of Tributyltin (TBT) in ship anti-fouling paints. The gastropod class is traditionally divided into three subclasses, the prosobranchia, the pulmonata and the opisthobranchia (Hyman, 1967). The latter are the sea hares and sea slugs, and as their name suggests, are exclusively marine. The other two groups have both invaded freshwater. The pulmonates are distinguished from prosobranchs by respiration using air rather than water. The edge of the mantle cavity is sealed down to the body, leaving only a pneumostome with which to take in air at the water surface. However, taxonomists are currently reclassifying the gastropods, and mRNA gene sequences indicate that opisthobranchs and pulmonates are more closely related than previously thought (Vonnemann et al., 2005). Bouchet and Rocroi (2005) now place them together in the clade Heterobranchia, and refer to both as ‘informal groups’.

The pulmonates have only one group in the freshwater environment called the Basommatophora, which originate from a single invasion event in the Jurassic, making them a relatively recent addition to the freshwater mollusca (Dillon, 2000). They have either detorted or did not evolve torsion. The roof of the mantle cavity is highly vascularized and ventilation occurs by arching and flattening the body. They have lighter, more delicate shells, presumably to ease the passage to the water surface to respire, and as such are confined to little or no flow environments. The families present in UK freshwaters are the Lymnaeidae (shell held on the right as is usual), the Physidae (shell is on left and whorls are

sinistral), the Ancylidae (shell is simple cone) and the Planorbidae (shell is planospiral and carried in the vertical). All of these groups are now placed into the clade Hygrophila (Bouchet and Rocroi, 2005). The shell morphology is the overt distinguishing character between the families, but the difference between the latter group and the others is greater as the planorbids use heamoglobin as the oxygen vehicle as opposed to haemocyanin.

The prosobranch group is defined by having an opercula (closure for the shell) carried on the dorsal surface of the animal during locomotion. It has invaded freshwater in many waves (Dillon, 2000). Because they respire using gills and must have water flowing through the mantle cavity to ventilate them, prosobranchs are restricted to environments with a moderate to high flow, and their shells are suitably thick and heavy in order that they are not easily washed away. It may no longer be possible to define prosobranchs as a group, and indeed it appeared to the non-taxonomist as an amalgam of species that were not pulmonate. Most of the species occurring in UK freshwaters are now placed in the clade Caenogastropoda (Bouchet and Rocroi, 2005), excepting the nerite, *Theodoxus fluviatilis*, which is placed in the clade Neritimorpha (previously classified as Archaeogastropoda). The others, which had been placed together in the Mesogastropoda, are now divided between an informal group called the Architaenioglossa and the clade Hypsogastropoda. The Architaenioglossa contains the Viviparidae (and also the sub-tropical Ampullidae, including *M. cornuarietis*), and the Hypsogastropoda is divided into the Neogastropoda (a carnivorous marine group) and the Littorinimorpha which contains the Bithynidae and Hydrobiidae. However, the term prosobranch will continue to be used for the purpose of this review and project. The only remaining group in UK freshwaters is the Valvatidae, which was previously thought to be the only hermaphrodite in the prosobranch group, but is now reclassified as a lower Heterobranch.

The breadth of diversity exhibited in gastropod reproductive strategies is striking. The pulmonates are exclusively simultaneous hermaphrodites, and the

prosobranchs are generally putative gonochorists. While most prosobranch families have morphological males, it is not certain whether chromosomal differences control the sex characteristics, or whether there is another determining factor, possibly environmental or hormonal (Dillon, 2000). All pulmonates and most prosobranchs are oviparous (laying small clutches of eggs on a solid substrate), excepting the Viviparidae, which are live-bearing (viviparus). There are also three non-native viviparus parthenogenetic prosobranchs; *Campelona sp.* (range is in Americas) *Thiarid sp.* (Indian sub-continent) and *P. antipodarum* (originates in New Zealand). The latter is also present in the UK.

An understanding of the mechanism involved in any chronic reproductive effects observed in gastropods that have a potential endocrine disruptive action depends on developing an understanding of the (neuro-) endocrinology of the group. In vertebrates, the endocrine control of reproduction follows the hypothalmic-pituatary-gonadal axis, with the hypothalamus secreting gonadotrophin releasing hormone, which stimulates the pituitary gland to secrete gonadotrophin (both peptidic hormones) which then causes the gonad to synthesise relevant steroid hormones.. These then bind to nuclear receptors in the target tissue (gonad) and initiate the genetic processes of gamete development. It is not known whether gastropods have any true endocrine systems (i.e. use hormones that can signal in a diffuse manner through the blood or lymph). In general, gastropods have only a simple series of paired ganglia. The cerebral ganglia form a ring that lies over the oesophagus and pairs of buccal ganglia extend from it to enervate the radula complex, pedal ganglia to enervate the foot and pleural ganglia to enervate the mantle. The pleural ganglia branch into perietal ganglia to enervate the gills, and visceral (abdominal) ganglia to enervate the digestive and reproductive organs (Hyman, 1967).

Much of the research effort to understand the neuro-peptide signalling system in gastropods was undertaken several decades ago on the model pulmonate species *Lymnaea stagnalis*. Like all pulmonates, this is a hermaphrodite species, and

there is therefore an additional need to appreciate the mating and breeding system employed in order to understand the neuro-endocrine control of reproduction in gonochorists. The neuro-endocrine pathways involved are reasonably well-described (Figure 3), although there is comparatively little evidence as yet for the nature of the pepterdic (or otherwise) transmitters (Lagadic et al., 2007). Briefly, *L. stagnalis* is able to both self-fertilise the eggs using autosperm, or mate and cross-fertilise with allosperm, but it is considered to be a preferential out-crosser. It is a slightly protandrous species, becoming mature as a male at 7-8 weeks, with the onset of egg laying 2-3 weeks later, providing mating has occurred (van Duivenboden et al., 1985). If not, virgin snails exhibit a 'selfing delay' (van Duivenboden et al., 1983).

(neuro) endocrine control of reproduction in Basommatophora. *AG* albumen gland, *ALc* cells of the cerebral ganglia anterior lobes, *APGW* Ala-Pro-Gly-Trp-NH_2 tetrapeptide, *BC* bursa copulatrix, *CDC* caudodorsal cells, *CDCH* caudodorsal cell hormone, *CG* cerebral ganglia, *DBH* dorsal body hormone, *FP* fertilization pocket, *GnRH* gonadotropin-releasing hormone, *HD* hermaphrodite duct, *LDB* lateral dorsal bodies, *L-EGF Lymnaea* epidermal growth factor, *LGC* light green cells, *LL* lateral lobes, *LTI Lymnaea* trypsin inhibitor, *MDB* median dorsal bodies, *MG* muciparous gland, *MIP* molluscan insulin-like peptide, *OG* oothecal gland, *OT* ovotestis, *PC* pars contorta, *PE* penis, *PG* prostate gland, *SD* sperm duct, *VA* vagina, *VD* vas deferens, and *VS* vesiculae seminales

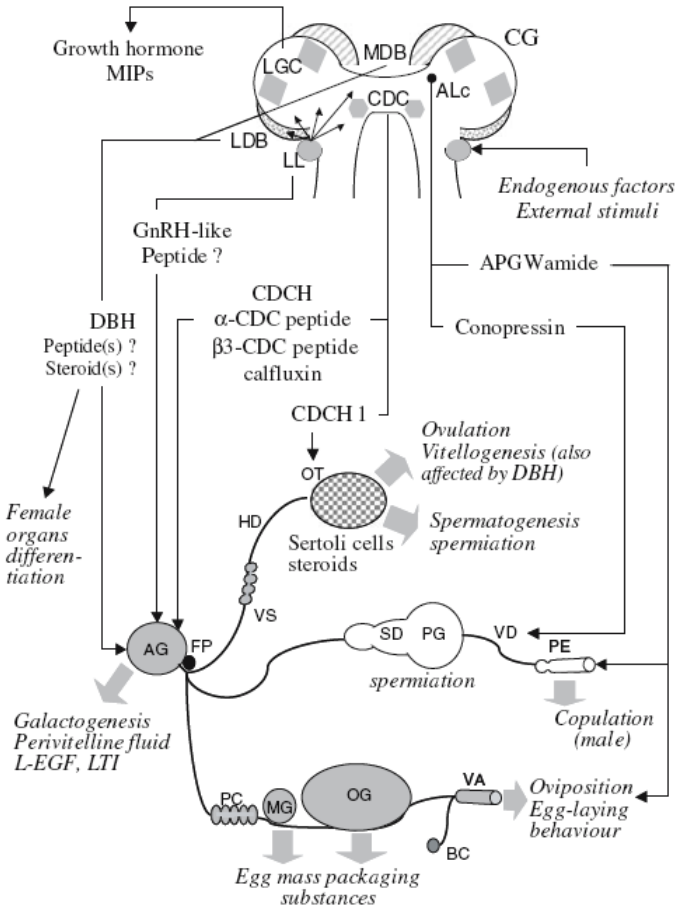


Figure 3 – A schematic view of the (neuro-)endocrine control mechanisms in *L. stagnalis*, after Lagadic et al., (2007).

The female is considered to be always receptive to mating, but the drive to mate as a male is increased when the individual has experienced a period of isolation (van Duivenboden and ter Maat, 1985), and this is linked to an increase in size of the prostate gland (de Boer et al., 1997). A gene encoding the peptide prohomone APGWamide has been isolated from the cerebral ganglia, and peptides derived from this are thought to control male mating behaviour (Smit et al., 1992). No other endocrine function has as yet been identified in the male reproductive system, with the Sertoli cells being negative for steroid biosynthetic enzymes and having no membrane-limited granules usually associated with secretion activity (de Jong-Brink et al., 1977).

The maturation of the female reproductive system is controlled by a hormone secreted by the dorsal bodies of the cerebral ganglia. Excision of the dorsal bodies in juvenile *L. stagnalis* causes the cessation of growth of the accessory sex organs. The dorsal body hormone (DBH) may have a truly endocrine mode of action, since when the ganglia are re-implanted loose into the haemolymph, the effects are reversed, and when juvenile accessory sex organs are transplanted into the haemocoel of dorsal body deprived snails they do not develop as in control implants (Geraerts and Algera, 1975). Vitellogenesis is also under the control of DBH. Oogenesis continues in dorsal body deprived snails as indicated by the presence of pre-vitellogenic oocytes, but the number of vitellogenic oocytes is decreased (Geraerts and Joosse, 1975). This role for DBH has also been indicated in terrestrial pulmonates, where infantile gonads were cultured with or without the presence of dorsal bodies in the culture fluid, and oocyte diameter only increase with the dorsal bodies (Vincent et al., 1984), and in the prosobranch *V. viviparus*, where excised female gonads remained healthy in culture but vitellogenesis did not progress (Griffond and Gomot, 1974).

The process of vitellogenesis in freshwater snails is not fully elucidated. The follicle cells do not appear to be involved in oocyte nutrition, and the ooytes have only moderate amounts of yolk granules that contain ferritin (de Jong-Brink et al., 1976). These granules are lysosomal in character, and may have a role in

utilizing the extracellular perivitelline fluid that is the source of embryonic nutrition (de Jong-Brink and Geraerts, 1982). The ferritin is secreted into the heamolymph by the digestive gland and finally sequestered into oocyte vesicles (Bottke et al., 1988). Perivitelline fluid is supplied by the albumin gland and does not circulate in the haemolymph. In the prosobranch *Pomacea canaliculata*, the major peri-vitelline lipoprotein in ovorubin (Dreon et al., 2002). These authors do not believe it pertinent to consider homologies between perivitelline lipoproteins and [vertebrate] yolk proteins on their functional analogy, as they have no structural similarity.

It may not therefore be relevant to consider a role for steroid hormones in gastropod vitellogenesis (as appears to be the case in bivalves). Indeed DBH is thought to be a peptide as cDNA encoding a single novel cytochrome P450 protein has been sequenced from the dorsal bodies of *L. stagnalis*, whereas steroid synthesis usually requires several cytochrome P450 mediated processes (Teuissen et al., 1992). However, it is known that the freshwater pulmonate *Helisoma trivolvis* has a peptide similar to vertebrate gonadotrophin-releasing hormone that can activate gonadotrophin release from goldfish pituitary glands (Goldberg et al., 1993), and that this peptide is localised in the lateral lobes of the cerebral ganglia, adjacent to the dorsal bodies (Young et al., 1999). It is possible that the reproductive axis encompasses these two peptides.

The regulation of the egg laying activity is under the control of a different mechanism. Cauterisation of the caudo-dorsal cells of *L. stagnalis* results in the cessation of oviposition (Geraerts and Bohlken, 1976). At ovulation, there is a release of secretion referred to as Caudo-Dorsal Cell Hormone (CDCH) into the intercerebral commissure. Extracts of this commissure injected into adult snails causes ovulation and oviposition (Dogterom et al., 1983). Ovulation follows a series of rapid firing of the caudo-dorsal cells following stimulation, either artificial or from transfer of the snail to clean water (Kits, 1980). Oviposition then occurs within two hours. This ‘afterdischarge’ can only occur 8 hours after the previous occasion. Egg-laying behaviour consists of three phases following

the afterdischarge; resting (40 min), turning (60 min) and oviposition (10 min). The number of eggs laid is determined by the length of the turning phase (Ferguson et al., 1993), and the time since the previous egg mass was laid (ter Maat et al., 1983).

CDCH injection induced oviposition does not induce the turning phase, and bisecting the intestinal nerve also disrupts turning. The activity of the pedal motor neurones that control turning is also correlated with CDCH excitement (Hermann et al., 1994). It appears that this highly complex and time bound process is under neuro-endocrine control only as a diffuse endocrine response could not be as precise. As with DBH, the nature of CDCH is not clear, although Geraerts et al. (1983) made partial purification from *L. stagnalis* and consider it a stable polypeptide. However there is genus specificity in the CDCH of pulmonates (Dogterom and van Loenhout, 1983), and Vreugdenhil et al. (1988) predict that 11 peptides cleave from the CDCH prohormone, suggesting that the neuro-endocrine peptide sequence controlling egg-laying will not be straight-forward to decipher.

Given this description of reproductive development and control in *L. stagnalis*, which it may be possible to apply more generally to the bassomatophora at least in part, it is not clear that steroid hormones have any role to play and it is therefore difficult to conceive of any predominantly likely modes of endocrine mediated disruption. However, as mentioned earlier, gastropods are subject to a disruption of the reproductive process clearly relatable to adverse population level effects, caused by exposure to organotin compounds in anti-fouling paints. The potentially (neuro-)endocrine mechanisms involved in this effect are reviewed in the next section.

1.4 Endocrine Disruption of Gastropod Reproduction by Organotins

Populations of marine gastropods worldwide are known to be affected by a condition that involves the females developing male reproductive organs, sometimes only vestigial approximations, but often to the same extent as the male, such that the two genders become difficult to distinguish. It was first observed in the dogwhelk, *Nucella lapillus*, in South-West England (Blaber, 1970), and it has since been recorded in over 150 species (Matthiessen and Gibbs, 1998). Smith (1971) defined the term 'imposex' as the super-imposition of male characters onto females. This includes the development of a penis and vas deferens, which can block the opening of the oviduct and cause the egg capsules, which continue to be produced, to fill the capsule gland and oviduct, rupturing the tract and resulting in sterilisation and eventual mortality. These females are not easily replaced in the population, as gastropods have limited mobility and consequently can only re-colonise slowly. Bryan et al. (1986) demonstrated that populations of *N. lapillus* in the South West were in decline and even extinct in certain localities. Where all the females in the population were masculinised, these researchers developed a Relative Penis Size Index (RPSI) against male penis size from the location to distinguish the degree of effect, so that a score of 100% implies that all females have fully developed male characteristics.

They found sites with 50% RPSI on the south coast of Devon and Cornwall where shipping movements are high. On the Atlantic coast, although most females had a penis, the RPSI was generally less than 2%. They measured metal concentrations in sampled animals and found that tin levels correlated with the degree of observed imposex. This lead to the hypothesis that TBT anti-fouling paints were the cause. They tested this by transplanting animals from the least affected site to Plymouth Sound where high levels of TBT had been reported and recorded an RSI of 50% within a year. In the reverse transplant, the RPSI became higher, but these animals already had high body burdens of TBT which may have had a prolonged effect.

The researchers went on to make 120 day laboratory exposures of *N. lapillus* to TBT-based anti-fouling paints, giving a seawater concentration of 10-20 ngL^{-1} TBT (as Sn), and the RPSI increased from background to 40%. Bryan et al. (1987) attempted further confirmation by making exposures to TBT directly dissolved in water for 52 days. This did not have an affect on penis length, although further exposures to anti-fouling paint for up to 15 months showed significant increases with concentrations as low 3.4 ngL^{-1} TBT. Even the control females, exposed to natural sea water only, showed a significant increase. It was therefore considered that a long-term exposure is required to elicit imposex. They also developed an index to indicate the degree of imposex that could be expected to sterilise the affected females. The Vas Deferens Sequence Index (VDSI) is subjective; it scores a '0' when no male characteristics are present, '1' at the first sign of a vas deferens, '2' at the occurrence of a penis, '3-4' as development of these apparatus become complete, '5' when the genital papillae of the female are overgrown by vas deferens tissue and '6' when aborted eggs are found in the capsule gland.

At stage 5 the female can no longer deposit egg capsules due to the physical blockage of the female opening. This causes a build up of material in the oviduct (Stage 6); rupture and mortality are likely to follow. The experimental organisms were predominantly Stage 3 at the start of the exposure, and most individuals were Stage 4 at the end (including the controls). A single stage 5 animal was found in the highest concentration (107 ngL^{-1}), giving an indication of the time frame and concentration required for sterility to occur. The researchers then made a field-based mark and recapture experiment over 18 months with a mean exposure concentration of 28 ngL^{-1} and recorded a further two cases of stage 5 sterility. They comment that populations of *N. lapillus* which are in apparent decline are characterised by the presence of females that have reached this stage. Finally, Gibbs et al. (1987; 1988) made long-term (2 year) laboratory exposures to demonstrate that imposex and sterility can be induced by seawater concentrations of TBT as low as 1-2 ngL^{-1}. They also observed that juvenile females are sensitive when exposed for a relatively short period, expressing the

condition following exposures to 20 ngL^{-1} in less than 12 months. They advocated the use of female *N. lapillus* as a sentinel of TBT pollution as they were abundant, hardy and extremely sensitive.

Unfortunately the sensitivity of *N. lapillus* is such that it became absent in TBT polluted areas, and it became necessary to consider the potential of other species as bio-indicators. Imposex has been found in many other marine prosobranchs, but with varying degrees of effect. For example, the rock shells (*Thais* species) are similarly effected, experiencing total sterility in areas of high shipping intensity in East Asia (Evans et al., 2001), and have also been used in field surveys (Horiguchi et al., 1995;1997a;1998). In these very sensitive species, it is not only TBT that induces imposex. Bryan et al. (1988) exposed *N .lapillus* to other organotin compounds and found that Tripropyltin also induced imposex, although not to the same extent as TBT, whereas Horiguchi et al., (1997b) discovered that Triphenyltin (TPT; not effective in *N. lapillus*) was an equally potent cause of imposex in *T. clavigera*.

Alternatively, the reticulated snail *Hinia (Nassarius) reticulata* is only moderately sensitive to TBT. Although it expresses imposex, it is not susceptible to sterilization due to its particular morphology (Stroben et al., 1992). It has also been used as an effective indicator species (Barroso et al., 2002). Similarly, a scoring system has been developed for the mud snail *Hydrobia ulvae*, which is sterilised only sporadically, and this too has been used in surveys (Schulte-Oehlmann et al., 1997). Of lesser sensitivity are *Buccinium undatum*, which only develops imposex in juvenile females following long-term exposures to 10 ngL^{-1} or more, and is not functionally sterilized (Mensink et al., 2002), and the periwinkle *Littorina littorea*, with a similar effect threshold (Bauer et al., 1997). However, the latter species does exhibit sterility and is still subject to population level impacts (Bauer et al., 1995), and it is currently used in coastal surveys where *N. lapillus* is absent (van den Broeck, 2009). A few species appear not to show any imposex response at all, e.g. *Collumbella rustica* (Gibbs et al., 1997), but this has not been confirmed with laboratory exposures.

Although the degree of imposex development and consequent sterility may simply depend on the structure of the reproductive organs in any given species, or their breeding status for the time of year (Stroben et al, 1996), the mechanism by which the male characteristics develop in the female has been the focus of much research. Since it is a masculinisation process, it is credible to assess the levels of androgens in affected females. Spooner et al. (1991) identified the presence of progesterone, testosterone and 17β-oestradiol in *N. lapillus*. Following exposure to TBT, there was no change in the progesterone or 17β-oestradiol tissue levels, but there was a significant increase in testosterone for limited periods. This was thought to be either due to an increase in testosterone synthesis, or a failure in aromatisation to 17β-oestradiol. Injections of testosterone also significantly increased penis length, although the response was not entirely dose-dependant.

Bettin et al. (1996) also studied the testosterone levels in *N. lapillus* and *H. reticulata*. They showed that testosterone concentration and imposex stage are correlated and dose-dependant with TBT exposure. They too demonstrated that imposex is expressed on exposure to testosterone ($500ngL^{-1}$). They then made exposures to TBT and cyproterone acetate (an anti-androgen) together, and found that imposex expression was blocked, indicating that gastropods have similar androgen receptors to vertebrates. It is also inferred that testosterone receptor binding initiates the development of male characteristics. The researchers considered that co-exposure of 17β-oestradiol with TBT should also mitigate the effect if the hormonal balance is a critical factor, and indeed imposex was suppressed as expected. This also infers that 17β-oestradiol has a feminising role in gastropods. Finally they make a co-exposure of 17β-oestradiol with an aromatase inhibitor (1-methyl-1,4-androstadiene-3,17-dione), hypothesising that this should have the same effect, which in fact it did. Overall, it is apparent that the expression of sexual characteristics in gastropods are dependent on vertebrate-type steroids, and TBT exposure causes imbalances in synthesis and aromatisation causing elevated testosterone levels and consequently imposex. This theory has been supported by other research including decreased 17β-

oestradiol levels in *Bolinus brandaris* in TBT contaminated sites (although without altered aromatisation rates; Morcillo and Porte, 1999) and reduced aromatise activity in *B. undatum* from shipping lanes (Santos et al., 2002).

Additionally, another proposal is that the testosterone titre builds up in the organism due to interference with its efficient excretion. Ronis and Mason (1996) exposed *L. littorea* to high levels of TBT (29 mgL^{-1}) in seawater for 42 hours and then analysed the seawater by radioimmunoassay. In the control, they detected little testosterone, but if the water was then incubated with enzymes (sulphatase and glucodase) to cleave the testosterone metabolites, free testosterone was present of which the greater proportion had been sulphur conjugatated. In the TBT exposure, they found that the sulphur conjugated testosterone levels were significantly less and the body burden of testosterone in *L. littorea* was elevated. They concluded that the sulphur-conjugation of testosterone was inhibited. However, this needs confirmation at environmental concentrations.

A third theory for the action of testosterone is proposed by Gooding et al. (2003) using *Ilyanassa obsoleta*. They consider that free steroid levels can be regulated by esterification to fatty acid esters, thereby removing them from the bioactive pool. They exposed organisms to up to $10ngL^{-1}$ TBT for at least three months and then saponified half of them to break down the esters prior to radioimmunoassay. They found that exposure TBT did not change the total levels of testosterone present (including that which had been esterified), therefore the balance between uptake and elimination rates must also remain unchanged. However the free testosterone levels were elevated as expected. The acyl-coenzyme A:testosterone aclytransferase (ATAT) that catalyses the conversion of testosterone to the ester was unaffected by TBT exposure, although it does differ according to the reproductive season (LeBlanc et al., 2003), and so the mechanism is not fully elucidated.

An alternative mechanism for the action of TBT is a disruption of the neurohormonal factors that control sexual maturation in males. The neuropeptide responsible for the development of male characteristics is referred to as Penis Morphogenic Factor (PMF). It is thought to be secreted by the right pedal ganglia, and TBT localises in the same place. Oberdorster and McClellan-Green (2002) consider that the PMF in *I. obsoleta* is APGWamide (which also controls male mating behaviour in *L. stagnalis).* When this was injected into female *I. obsoleta,* it induced imposex. They hypothesise that TBT stimulates male characters in females along the same pathway, and it is then exacerbated by a positive feedback loop of increasing testosterone levels. However they found that they could not suppress APGWamide-induced imposex with either an oestrogen or an anti-androgen as is possible with TBT induced imposex, so it may not be the same mode of action.

More recently, Nishikawa et al. (2004) discovered that TBT and TPT interacted strongly with the human Retinoid X Receptor (RXR), even to the same extent as the putative natural ligand, 9-cis-retinoic acid. They sequenced an RXR ortholog from *T. clavigera* that had close identity with the human RXR. These snails developed significant imposex following injections with 9-cis-retinoic acid, which is strong evidence that this nuclear receptor pathway is both binding and transcripting the genes for male character development. The group localised the RXR gene expression in the male and female penis (Horiguchi et al., 2007). 9-cis-retinoic acid also induced imposex in *N. lapillus*, as does Methoprene acid (another RXR ligand), although not to the same extent (Castro et al., 2007a). They have also sequenced the RXR for this species. These researchers consider it likely that imposex is caused by an interplay between neuroendocrine, steroid and retinoic pathways, although it appears that the initial signal is almost certainly via the RXR. Sternberg et al. (2008a) cloned the RXR for *I. obsoleta* and studied the expression of RXR mRNA over a breeding season. They found that the mRNA is at a relatively low level during reproductive senescence, and rose sharply in both genders during recrudescence. The males recrudesce in August and the females in November, and they hypothesise that the RXR is up-regulated in both

sexes in August, but that 9-cis-retinoic acid is up-regulated only in males in August, leaving females in a position vulnerable to other ligand activators until they up-regulate 9-cis-retinoic acid in November. This assumes that both genders use the same signalling pathway and that the response is environmentally determined, which requires confirmation.

Finally, Kanayama et al. (2005) also found that TPT interacts with the Peroxisome Proliferator Activated Receptor gamma (PPARγ), again as strongly as the natural ligand Rosiglitazone. PPARγ is abundantly expressed in adipocytes, and it occurs as a heterodimer with the RXR in humans. TBT is a potent inducer of adipogenesis in vertebrates, and increases the expression of genes that promote fatty acid storage (Iguchi and Katsu, 2008). It is therefore tempting to make a connection between this pathway and fatty acid esterification in gastropods. However, the PPARγ appears to have been a late nuclear receptor to evolve, and therefore may not be present in gastropods (Nishikawa, 2006). Although the mechanism of action of TBT is not fully elucidated, it is likely to be a cascade effect, interacting and conflicting with the many different processes that are required to occur in timed sequences to control mating and reproduction. The strongest evidence for imposex signalling is via the RXR, but it may also have effects through the multi-function oxygenase system that both debutylates and drives the cytochrome P450 reaction on which the aromatase and the steroid conjugation enzymes depend, altering the 'trade-off' of available energetics.

It is also of interest that *N. lapillus* seems to be conferred some protection from the effects of imposex by a condition referred to as 'Dumpton Syndrome'. A proportion of the males from Dumpton do not develop a penis, and where normal females are unaffected, the effects of imposex on TBT exposed Dumpton females are mediated (Gibbs, 1993). They have limited vas deferens development and consequently remain reproductively active (Quintela et al., 2002). Males with this Syndrome also have lower testosterone titres than normal males. A further study of the differences within the Dumpton population may be key to the full

elucidation of the imposex pathway, such as whether these organisms have limited RXR signalling ability.

The imposex phenomenon can clearly be regarded as adverse in many species, those that are reproductively inhibited by the imposition of male characters on the female, causing population declines. It is now also ostensible that a nuclear receptor is involved in the process, albeit not a steroid receptor. That the RXR is functional and the signal is agonised by exogenous ligands including TBT, make the case for a true endocrine disruption in gastropods according to the WHO definition. However, there is also strong evidence for a role of steroids in the masculinisation of gastropods that cannot be overlooked. TBT exposure has effects other than stimulating the growth of male secondary sexual characters in the female. Intersex also occurs in some species, i.e. spermiogenesis can occur in the ovary. Gibbs et al. (1988) showed that in long-term exposures of *N. lapillus* to 3-5 ngL^{-1} TBT, spawning was prevented, but gametogenesis was unhindered. At 20-100 ngL^{-1}, oogenesis had all but ceased, with pre-vitelline oocytes found rarely. Seminiferous tubules and Sertoli cells were present, and early stage spermatogenesis was observed. In the most extreme cases, it was possible only to distinguish intersex females from males by the immaturity of the testicular tissue.

These researchers consider that the imposition of the male tract and of spermiogenesis in females are separate phenomena and represent different responses to TBT exposure (see Figure 4 for a suggested overview). Similar effects have also been recorded in *L. littorea* (Bauer et al., 1995) and the abalones *Haliotis madaka* (Horiguchi et al., 2000; 2005a) and *Haliotis gigantea* (Horiguchi et al., 2005b). *H. reticulata* exposed to TPT also shows a depressed rate of maturation of the oogonia (Schulte-Oehlmann, 2000). Horiguchi et al. (2007) found only trace levels of RXR in the ovaries of both normal and imposex female *T. clavigera*, implying that the RXR does not signal for ovarian effects, although this is not conclusive since there is no evidence for intersex in imposex-affected individuals of this species (Cheng and Liu, 2004). As a concept however, it seems unlikely that the RXR pathway is involved in intersex development. It is

associated with appendage regeneration in urchins and crinoids (Sugni et al., 2007) and therefore seems to be more generally employed for signalling morphological processes. It seems more likely that steroid hormone titres are involved in the formation of ovarian/testicular tissue, as there is evidence that steroids are present in gastropods and consequently are likely to follow a similar and conserved evolutionary role to that in vertebrates.

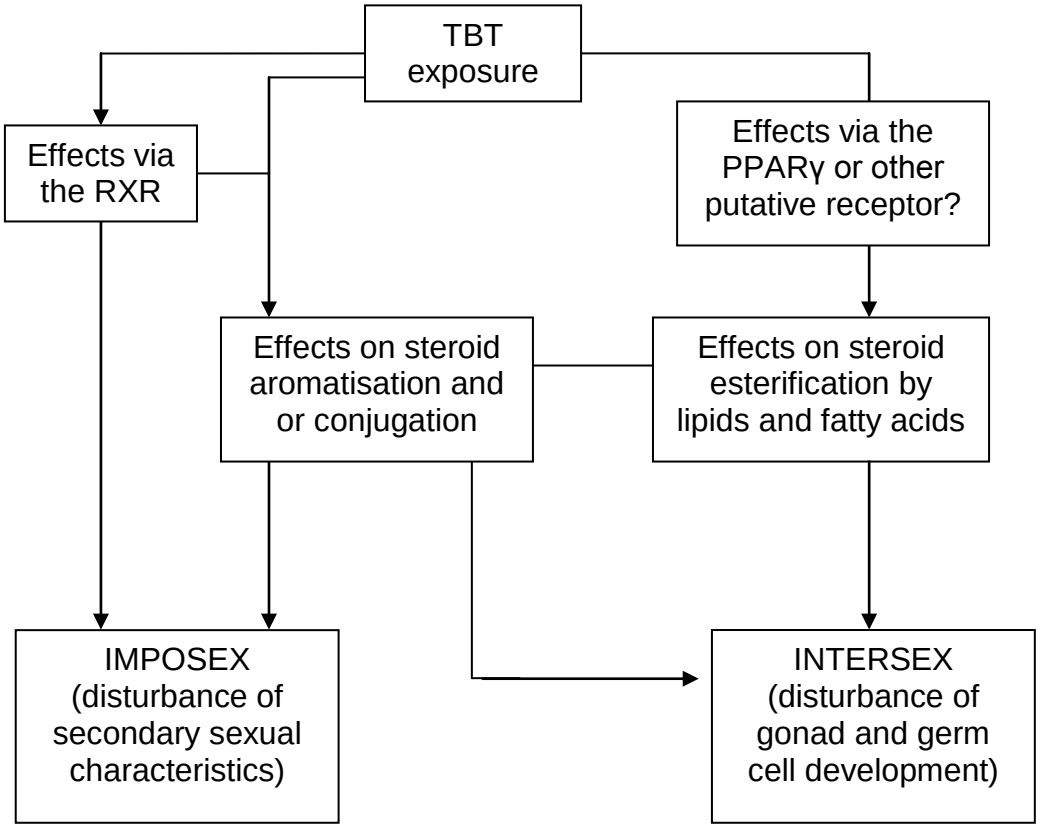


Figure 4 – The potential pathways of imposex and intersex development in gastropods exposed to TBT.

The consequences of TBT exposure for marine gastropods has lead to a global ban on its use in anti-fouling paints being imposed by the International Maritime Organisation on 17 September 2008, following ratification of the Convention on the Control of Harmful Anti-Fouling Systems on Ships by 53% of nations using at least 25% of the global tonnage. There is much less research on the effects of organotins in freshwater gastropods, but Schulte-Oehlmann et al. (1995) assessed the use of *M. cornuarietis* as a potential bioindicator of TBT in freshwaters, and

found it not notably sensitive (threshold for imposex = 50-85 ngL^{-1}). They also note that this species naturally exhibits a minor degree of imposex (as do other ampullidae; Takeda, 2000). After a three month exposure, the testosterone titres were significantly higher than the controls. 17β-oestradiol levels did not change, but the hormonal balance was moved towards androgenicity, and the researchers concluded that imposex is expressed when the steroid ratio reaches 0.3-0.4. Schulte-Oehlmann et al. (2000) also studied the effects of TPT, and found *M. cornuarietis* to be even less sensitive to this compound (threshold for imposex = 75-500 ngL^{-1}), but there was an almost complete cessation of egg-laying at 150 ngL^{-1}. Oogenesis, however, was not affected. Males were sterile after exposure to TPT, having azoospermia, which may account for the reduction in egg laying by the females. This is an unexpected effect when considering a putatively androgenic compound.

Janer et al. (2005b; 2006a) found a sexually dimorphic pathway for androstenedione metabolism in *M. cornuarietis*, with females mainly converting only to 5α-dihydroandrostenedione (DHA), and males then converting most of this to 5α-dihydrotestosterone (DHT), which is a more androgenic substance in vertebrates. *In vitro* exposures of digestive gland fractions to TBT and TPT significantly inhibited androstenedione metabolism, and the ratio of products suggested that the 5α-reductase enzyme step to produce DHA was more inhibited than the 17β-HSD step producing DHT, and *in vivo*, DHA in TPT induced imposex females was significantly reduced. Although this change only affects females, it is difficult to interpret it as masculinising; particularly as the levels of free testosterone remained unaltered.

The same research group then went on to study the quantities of esterified steroids in *M. cornuarietis*. They found that TBT exposed females had significantly less esterified steroid (both testosterone and 17β-oestradiol), but the levels of free steroids did not change. The ATAT activity levels, which were markedly higher in males than females and during spawning than non-spawning, were also not affected by TBT exposure (Janer et al., 2006b). Males exposed to

methyltestosterone did however have significantly increased ATAT activity, suggesting increased fatty acid conjugation to compensate for the exogenously derived steroid. The researchers hypothesized that TBT might affect the fatty acid levels in gastropods via a putative receptor that can dimerise with the RXR, as PPARγ does in vertebrates. They measured the total lipid levels in TBT exposed *M. cornuarietis* and found them to be significantly increased in females after 100 days, but only at high concentrations (500 ngL^{-1}; Janer et al., 2007). The fatty acid profile was variously altered, but not in any way that clearly related to the ability of the organism to esterify steroids.

In contrast, Lyssimachou et al. (2008; 2009) found that the total lipid content was significantly decreased in females after a short (7 day) exposure to TPT, and the activity of acyl-CoA oxidase enzymes (breaking long chain fatty acids) increased, although again males were not affected. The quantities of esterified testosterone in females increased, and the esterified 17β-oestradiol decreased. These differences may be due to the different compounds tested or the different exposure durations but it is suggested that the decreased pool of esterified 17β-oestradiol that occurs in both cases could have a masculinising effect. They conclude that fatty acids have a multi-functional role including energy metabolism and storage, receptor signalling and synthesis of many compounds involved in physiological regulation, and all authors suggest that there is a linkage between altered lipid and fatty acid accumulation and steroid hormone titres that is affected by TBT exposure, but the full mechanism remains to be elucidated. However, if steroid hormones are signalling in sexual development (imposex) and gametogenesis (intersex) as the evidence suggests, and the mechanisms (genomic, neuro-endocrine, cell membrane mediated or otherwise) can be masculinised by interference from organotins, then it is also possible that they can be feminised by xenobiotic compounds too. This is discussed in the final section of this review.

1.5 Endocrine Disruption of Gastropod Reproduction by Bisphenol-A

Bisphenol-A is used mainly in the production of epoxy resins and polycarbonate plastics, but also in the manufacture of flame retardants, adhesives, coatings, paints, compact discs, thermal paper and electrical parts (Staples et al., 1998). The Government of Canada has become the first to take regulatory action to control this compound, proposing a ban on the sale of polycarbonate baby bottles (Health Canada, 2008). They have also proposed that Bisphenol-A be added to the Schedule 1 list of Toxic Substances of the Canadian Environmental Protection Act (1999) due to effects in fish (Health Canada, 2009b). Jobling et al. (2004) made exposures of the gastropod *P. antipodarum* to Bisphenol-A (1-100 μgL^{-1} nominal). Analogous 'inverted U-shape' response curves were recorded to those observed for 17α-ethinylestradiol, with the response peaking between 5-25 μgL^{-1}. These observations are supported by Duft et al. (2003a) when the route of exposure was uptake from sediment. In this case the LOEC was 1$\mu g\ Kg^{-1}$, so the effective concentrations are in broad agreement although the two routes of uptake are not necessarily directly comparable. These values for Bisphenol-A are much lower than the Lowest Observable Effective Concentration (LOEC) of 1280 μgL^{-1} for the inhibition of egg production in Fathead Minnows (Sohoni et al., 2001), suggesting that gastropods may be more sensitive to xeno-oestrogens than vertebrates in terms of offspring production. However spermatogenesis in the Fathead Minnow is inhibited at similarly low concentrations as are effective in the gastropod. If this is translated into the ability of the male fish to fertilise eggs, it may be that this compound is equally detrimental to both vertebrate and invertebrate.

Further, more extensive evidence for the sensitivity of gastropods to Bisphenol-A has been provided by Oehlmann et al. over the last decade (2000a). These researchers made controlled exposures of *N. lapillus* and *M. cornuarietis* to Bisphenol-A and Octylphenol and were able to demonstrate an increase in egg production in both species. In the case of *M. cornuarietis*, this was three to four fold in the highest nominal concentrations (25-100 ngL^{-1}). Gross malformations

in the oviduct of this species, including second vaginas, enlarged pallial accessory sex glands and rupture of the oviduct due to blockage with egg masses were also recorded at the highest concentrations, and the affected individuals are referred to as 'superfemales'. Oviduct blockages did not occur in *N. lapillus*, possibly due to differences in the morphology of the reproductive tract, although the weight of the accessory pallial gland complex was significantly increased, as it was in an additional species *H. reticulata*, exposed to Bisphenol-A via sediments albeit at higher concentrations (Schulte-Oehlmann et al., 2001).

However, the observed increase in the number of eggs laid by *M. cornuarietis* is reported to only occur in exposures made under certain test conditions (Oehlmann et al., 2006). At 20°C there was a significant effect at concentrations between 0.106 and 2.17 μgL^{-1}. However at 27°C the effect was not discernable, although the number of eggs laid by the snails exposed to the higher concentrations was still elevated (Figure 5). It was speculated that not only is this effect mediated through an oestrogen receptor because co-exposure with anti-oestrogenic compounds quashes it, but also that there may be a seasonal aspect to the response as it can only be observed at relatively low temperatures and in periods when the females are not at the peak of reproductive activity. Therefore it is possible that this 'superfeminisation' effect has a seasonal component, albeit in a non-European, (sub-)tropical species.

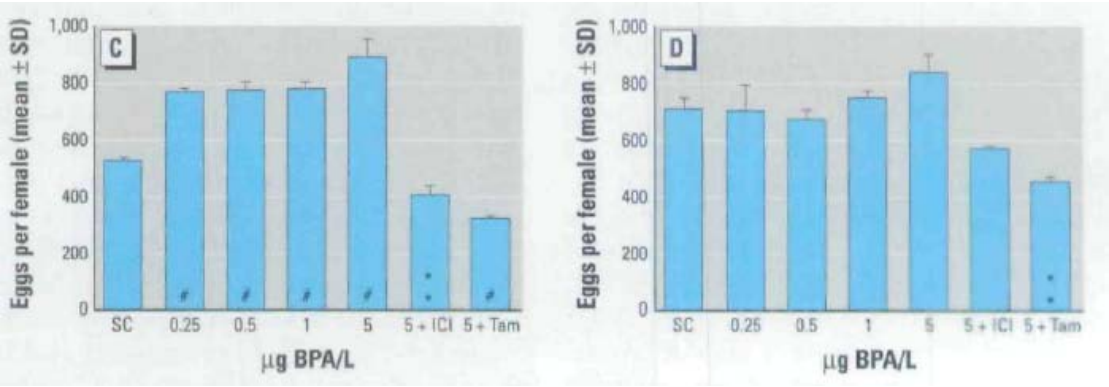


Figure 5 – The mean number of eggs per female *M. cornuarietis* exposed to Bisphenol-A (BPA) at 20°C (c) and 27°C (d) After Oehlmann et al. (2006). ICI and TAM are anti-oestrogenic compounds. # = significant difference from control at p<0.001 and ** = at p<0.01.

Also, this effect occurs at low concentrations of the compound; the No Observed Effect Concentration (NOEC) was 7.9 ngL^{-1}, the Lowest Observed Effect Concentration (LOEC) was 48.3 ngL^{-1}, and a 6-month Effective Concentration for 10% of the test population (EC_{10}) of 13.9 ngL^{-1} was calculated from the measured concentrations (Oehlmann et al., 2006). This is much lower than the Predicted No Effect Concentration (PNEC) for the freshwater ecosystems of 1.6 μgL^{-1} derived from a Fathead Minnow Full Life Cycle test by the EU Risk Assessment for Bisphenol-A (EU, 2003). The difference between these 'safe' concentrations (>x200) was cause for the Member State leading on the Risk Assessment to request that the plastics industry make a close examination of the effect and the research. Bisphenol-A is known to be a weak oestrogen in vertebrates; it binds to both human estrogen receptors (α and β) and activates reporter gene transcription (Matthews et al., 2001), and induces near maximal vitellogenin production in male Fathead Minnow at 160 μgL^{-1} after 164 days (Sohoni et al., 2001). It also appeared to increase the egg production of the female fish in this study, although not significantly. Additionally, Bannister et al. (2007) identified an estrogen receptor ortholog in *M. cornuarietis*, and it was therefore conceptually feasible that it may be an oestrogenic compound in this gastropod too.

The effects observed in *M. cornuarietis* are unusual in that it is the female that is superfeminised rather than any feminisation of the male. Indeed, at the highest concentration of Bisphenol-A (100 μgL^{-1}) imposex intensity in the female was significantly increased. Neither spermatogenesis nor oogenesis were affected (Oehlmann et al., 2000a). However the increase in the number of eggs laid per female in this study was dramatic (x3) at 5 ngL^{-1} (nominal), and it was supported by the further evidence of similar effects with a second compound at the same concentration (Octylphenol, also known to be oestrogenic in vertebrates, (White et al., 1994), and also by similar effects in a second species, *N. lapillus*. However, these studies did not include replication in the experimental design and had no analytical measurements of the test compound and were therefore criticized and considered invalid (Staples et al., 2002; Dietrich et al., 2006).

Oehlmann et al. (2006) repeated the Bisphenol-A exposures with *M. cornuarietis* and incorporated replication and analysis of the test compound into the experimental design and re-observed the superfeminisation effect, deriving the low test statistics mentioned, and demonstrating that the effect occurred only at lower temperatures. The authors concluded that if exposures are made at warmer temperatures or at the peak of the spawning period, the induction of eggs is masked by the overall increase in productivity. They also demonstrated 17β-oestradiol binding in *M. cornuarietis* gonad extracts, and Bisphenol-A binding (x100 < 17β-oestradiol, as expected), and showed that the induction of eggs at the lower temperature is quashed on co-exposure of Bisphenol-A with anti-oestrogens. This is evidence that the superfeminisation effect is mediated by an oestrogenic pathway. Oehlmann et al. (2005) proposed that these effects are sufficiently patent to potentially indicate a risk to prosobranch snail populations at environmental concentration of Bisphenol-A and that the EU Risk Assessment PNEC should be reduced accordingly.

Where Bisphenol-A is detected in environmental samples, the concentrations are below 0.1 μgL^{-1} in the majority of cases, with the highest value reported being 1.29 μgL^{-1} from European sampling programmes in the EU Risk Assessment (EU, 2003). Crain et al. (2007) argue that the maximum value should be considered when assessing the consequences of exposure because 'organisational disruptors' can have long-term effects if exposure occurs in a critical window. They quote a highest measured concentration of 19 μgL^{-1} in the Netherlands. Finally, an update to the EU Risk Assessment (EU, 2008) quotes a maximum value of 43 μgL^{-1} in Norway, but an overall mean of 0.13 μgL^{-1} and median of 0.01 μgL^{-1}. This implies that aside from the two very high values quoted, a PNEC of 1.6 μgL^{-1} indicates that aquatic ecosystems in Europe are not at risk. However if the PNEC were to be reduced to 0.0079 μgL^{-1} to accommodate the risk posed to *M. cornuarietis* by Bisphenol-A, then the majority of the aquatic ecosystem would be considered at risk. Consequently the EU Risk Assessment Rapporteur (UK) requested that the Bisphenol-A industry undertake to make further assessment of the superfeminisation effect in of this species.

Aufderheide et al. (2006) and Selck et al. (2006) made initial assessments of the optimum culture conditions for *M. cornuarietis* as a new test organisms for regulatory use. They found that there were no significant differences in the fecundity of females between 22^{o}C and 25^{o}C, or between 25^{o}C and 28^{o}C. However, juvenile growth was significantly higher at 28^{o}C than 25^{o}C, in a 16 hour photoperiod than in a 12 hour photoperiod at 25^{o}C, and at 25^{o}C than 22^{o}C. They estimate that the time to maturity would be twice as long at 22^{o}C than 25^{o}C. Hatching success of the eggs was also significantly higher at 25^{o}C than 22^{o}C, although this was a time-bound trial and it is likely that embryo development was simply slower at 22^{o}C. They also assessed the effect of increasing snail stocking density, and found that fecundity was significantly inhibited at 2 snail per litre compared to 0.8 snails per litre, and juvenile growth was also significantly slower at 2 snails per litre. Overall, they concluded that 22^{o}C is sub-optimal due to reduced juvenile growth, and that 25^{o}C to 28^{o}C lies centrally within the tolerance range of *M. cornuarietis*. They recommended a temperature of 25^{o}C, a photoperiod of 12 hours light and a stocking density of 0.8 snails per litre as favourable husbandry conditions for maintaining multi-generational populations.

Accordingly, Forbes et al (2007a; 2007b; 2008a) made full life-cycle exposures of *M. cornuarietis* to Bisphenol-A in these conditions at concentrations between 0.1 and 640 μgL^{-1} in three laboratories, and also at 22^{o}C in one laboratory. They found no effects on reproduction in any exposure, although the productivity of the snails did decline over the course of the six months, and the groups held at 22^{o}C laid significantly less eggs. They also note that there was no evidence for an inherent seasonality in fecundity in year-long observations. There were no effects on hatching success or time to first hatch, but in one exposure (Forbes et al., 2008a) the growth rate of F1 males was significantly higher at 1 μgL^{-1} and the wet weight of F1 females was also significantly lower at 640 μgL^{-1}, after 60 days, although this effect was not repeatable. They conclude that Bisphenol-A exposure is unlikely to have population level consequences in this species.

This conclusion was accepted and reported in the update to the EU Risk Assessment (EU, 2008), but with the recommendation that the potential for freshwater gastropods to exhibit effects on a seasonal basis be further researched. This is because it is possible that superfeminisation as observed by Oehlmann et al. (2006) may only occur when *M. cornuarietis* is exposed to Bisphenol-A in conditions that simulate an environment in which reproductive activity is reduced, or at times when the females are in a state of 'reproductive repose'. Oehlmann et al (2008a; 2008b) also consider it possible that the populations used in these studies had sufficiently different origins that they were separate genetic strains or cryptic sub-species, which may affect the outcome. They did not support the development of a regulatory test that used organisms that were potentially less sensitive in conditions designed for maximum reproductive activity, arguing that test models should be based on responsiveness to endocrine active agents of concern. However, it is usual to minimise all sources of stress in regulatory tests and to make exposures in conditions that promote maximum fitness in test organisms (Forbes et al., 2008b) in order that any negative deviation (adverse effect) can be assessed. It is yet to be ascertained which approach is the most appropriate in the circumstances.

1.6 Aims and Rationale of the Project.

The primary aim of this project was to establish whether a range of freshwater gastropods exhibited elevated female reproduction (i.e. 'superfeminization') when exposed to model oestrogenic compounds in the same manner as that reported for *M. cornuarietis*. Secondary aims included further determination of any seasonal aspect apparent within this effect, and to assess the possibility of deriving a standardised test method for using the response of freshwater gastropod(s) to identify oestrogen-active compounds. To this end, the work was centred on the development of apical test end-points (mortality, growth, reproduction). The elucidation of the mechanisms of action of the oestrogenic compounds on gastropod (neuro-) endocrinology was therefore considered outside of the scope of the project.

In order to best understand any seasonal component of the effect of oestrogens on gastropod reproduction, only European-native, temperate species were employed. Unfortunately, very few such species have been sufficiently studied that the optimal culture and testing conditions can be established. The only exception to this is the pulmonate *L. stagnalis* that has been widely used in neurological research. This species has previously been exposed to 17α-ethiynlestradiol, and was found to be insensitive in terms of reproductive effects (Casey, 2004). Rather than expend effort establishing cultures of other species with unknown sensitivity, the preliminary exposures were made in an outdoor mesocosm facility, with the objective of identifying those species that responded with changes in reproductive rate during summer to autumn exposure to the natural steroid oestrogen, 17β-oestradiol.

1.6.1 The Mesocosm Exposures

The mesocosm system, supplied with river water and sited outside, is designed to afford an environment as close a possible to the wild habitat, while maintaining a degree of control over the test organisms and chemicals present. It was accepted that there would be many influencing factors in the experimental design, such as changing temperature, day length, influent quality and food availability, and also difficulties with maintaining chemical and microfaunal stability. However, the rationale is that this natural state will be beneficial for gastropod survival and would allow the seasonal reproduction of the test organisms to be observed. By providing river water, and supplementing this with an additional food supply, the chance of meeting the unknown requirements of the organisms are maximised.

Although the mesocosm system discharged to the river via an activated carbon filter, the only addition of oestrogenic compounds was such that the combined system effluent resembled a treated sewage effluent. This was to ensure that should the activated carbon become overwhelmed, the discharge environment was not placed at an appreciable additional risk. Only the natural steroid 17β-

oestradiol was used, and the aim was to subject one tank to an environmentally relevant concentration and one to a higher concentration (x10, to be diluted by the discharge of nine other, non-dosed tanks present in the system). The species of gastropod selected in the first instance were the pulmonate *Planorbarius corneus* and the prosobranch *Viviparus viviparus* (see Chapter 2 for further description). The reason for this choice was that these organisms are both of a large size which made for ease of handling and captivity control in semi-field conditions. Both were also known to survive well in the mesocosm. *Bithynia tentaculata* was also added as an alternative prosobranch that was considered better suited for later transfer into laboratory conditions.

1.6.2 Laboratory Exposures to 17β-Oestradiol

The next objectives were to formulate the optimum conditions for culturing the species that had shown potential to be affected by steroid exposure in the mesocosms, and to establish breeding populations for use in controlled laboratory testing. This included the establishment of appropriate food sources and quantities, water quality, photoperiod and temperature ranges and any density dependent characteristics of the species concerned. These are necessary pre-cursors for the development of a robust test design. Also, in the case of assessments for seasonally influenced effects, it was desirable to consider any potential intrinsic circadean or circannual rhythms.

Having established suitable test conditions, the aim was to make exposures to 17β-oestradiol under controlled, laboratory conditions to confirm whether the effects observed were reproducible and to better define them as the consequences of interaction with the chemical rather than any confounding environmental factor. It was also desirable to develop sensitive tests using representatives of both the pulmonate and prosobranch sub-classes. In the selected pulmonate species, the potential seasonal factor in the response was addressed by making concurrent exposures in two differing regimes; simulated summertime (20^{o}C and 16 hours light / 8 hours dark) and simulated autumn (15^{o}C and 12 hours light / 12 hours dark). Prior to making the exposures it was intended that an assessment of

the reproductive rate for each group of snails used would be established in a pre-exposure period. These data could then be used to determine the between-group variation in reproductive activity. The concentration range of 17β-oestradiol selected for the long-term chronic exposure aimed to encompass that recorded in treated sewage effluents and in rivers, but also cover higher concentrations without causing acute toxicity.

For the prosobranchs, it was considered advantageous to select a putatively gonochoristic and oviparous species from the hydrobiidae, this being the most widely and abundantly represented prosobranch family in the UK (Kearney, 1976). Gonochorism is particularly advantageous in that any effects on reproduction that arise from disruption of the male or via the process of mating can be considered separately. To best capitalise on this, the aim was to expose male and female pairs in order that any morphological effects on the male could be observed, and that the degree of variation in reproductive activity between individual females could be assessed. Also, to fulfil the secondary objective of taking into account any seasonal aspect to the observed effects, it was determined to expose the snails in conditions as similar as possible to that which might be experienced over the annual changes in temperature and daylength. Consequently, the exposure was planned to begin in the early spring, and to expose the pairs of snails in laboratory controlled temperature and photoperiods that would be altered during the exposure to reflect a whole 'real-time' spring to autumn period of reproductive activity.

1.6.3 Laboratory Exposures to Bisphenol-A

The aim of these experiments was to make exposures of gastropods to a compound known to be a xeno-biotic oestrogen receptor agonist in vertebrates, to measure and compare any effects on reproductive performance against the response to the natural steroid. Two compounds were initially considered, namely Nonylphenol and Bisphenol-A. The latter has been shown to have stimulatory effects on the reproductive rate of *M. cornuarietis* (Oehlmann et al.,

2000a; 2006), and was therefore preferred in this regard. However it is also advantageous to test a compound with sufficient stability in order that fluctuations in concentration did not occur during the exposure. Nonylphenol is the most oestrogenically active alkylphenol and it is commonly found in the final effluents of UK sewage treatment works. It has a measured half-life of 20 days (Staples et al., 1999). Bisphenol-A has been Risk Assessed as a 'compound of equivalent concern to Persistent, Bio-accumulative or Toxic (PBT) compounds' by the European Union (EU) due to its endocrine disruptive properties, with the UK as the lead Member State (EU, 2003). It is also found in treated sewage effluent. It has a measured half-life of 2.5 to 4 days (Staples et al., 1998), and is therefore considered less stable than Nonylphenol. However, a preliminary stability study conducted in the intended exposure system showed Bisphenol-A to be more stable in these conditions, and so it was selected for further testing (see Chapter 6, Section 6.1.1).

Bisphenol-A concentrations were selected to be similar to those measured in UK treated sewage effluents, and to bracket the 'No Observed Effect Concentration (NOEC) currently proposed by the EU Risk Assessment Task Group (1.6 μgL^{-1}). A high concentration was also included to establish any adverse effects. Additionally, because the objective was to assess not only the effects of the test compound on the reproduction of pulmonate and prosobranch gastropods, but also to assess the seasonal aspects of the response, various test designs were considered in order to best capture any effects. These included the use of constant conditions at a temperature and photoperiod typical of autumn or spring, and also simulations of the onset of summer from spring and the onset of autumn from summer. A final aim was to generate data to assess a Standard Operating Procedure for gastropod exposures to endocrine active substances that has been proposed to the OECD (Schmitt et al., 2006; Duft et al., 2007).

1.6.4 Wider Considerations

The overall aim of this project was to address questions such as whether the superfeminisation effect is relevant in the context of the European Risk Assessment, given that *M. cornuarietis* is not resident in Europe, or indeed, does this effect have the potential to occur in other gastropods, specifically a range of European native species. Additionally, in a wider context of risk assessment for oestrogenically active compounds, and the protection of native freshwater mollusc populations, the project aims to consider whether the development of regulatory test methods for reproductive effects in gastropods can be sufficiently informative if standard approaches are used, or whether it is feasible / appropriate to tailor test conditions that potentially reduce the fitness of test organisms in order that a positive effect (i.e. an increase in fecundity) is measurable. Finally, this enquiry highlights the subject of whether a positive effect (i.e. increased survival, fecundity, growth) can be considered adverse, or whether it is likely that an adverse effect will occur at the population level as a consequence. This last question may underpin all of the preceding considerations, since the purpose of risk assessment is to predict population level consequences such as those occurring following the widespread exposure of gastropod molluscs to TBT.

CHAPTER 2
SELECTED TEST ORGANISMS: LIFE HISTORY AND CULTURE.

2.1 The Ecology of the Selected Test Organisms

The species trialled in the mesocosm system included the pulmonate *P. corneus* and the prosobranchs *V. viviparus* and *B. tentaculata*. *P. corneus* and *B. tentaculata* were also used in laboratory exposures, as was an additional prosobranch, *P. antipodarum*. A short description of the life history and ecology of each of these species follows.

2.1.5 *Planorbarius corneus*

Boycott (1936) describes *P. corneus* (Figure 6) as an 'exacting' species, meaning that it requires a large locus with abundant vegetation well exposed to sunlight with clear calcareous water. Generally confined to the south east in England, it often occurs in closed ponds and slow moving water bodies. It subsists on large quantities of plant material, but will also actively seek out protein sources and general detritus. Berrie (1963) infers from surveys that the juveniles hatch in May and over-winter as sub-adults before breeding the following spring and dying in July, giving a life span of 14 months. Calow (1978) agrees, listing *P. corneus* as a 'Type A' semelparous species (i.e. breeding only once per generation per year). However, the largest specimens Berrie sampled were only 8.4mm in diameter, when *P. corneus* is known to reach much larger sizes (up to 38mm, personal observation). Costil and Bailey (1998) raised individuals in a laboratory over the full-life span, and found that they could live for up to 4 years, in optimum conditions (mean = 2.64 years at 15^{o}C, 1.96 years at 20^{o}C and 1.26 years at 25^{o}C). Berrie suggests that larger wild specimens than those found in her samples may have lived an additional year, and it is therefore possible that a sub-section of the population is iteroparous (i.e. breeds in a second spring).

Figure 6 – *Planorbarius corneus* (approximately x3 life size; image from World Wide Web).

P. corneus, like all the pulmonates, is a simultaneous hermaphrodite; the gonad is an ovo-testis with both oogenesis and spermatogenesis occurring simultaneously in each acini (Figure 7), and both male and female secondary sexual organs developing on the reproductive tract (Figure 8). It is a preferential out-crosser, i.e. although it can self-fertilise, it will mate where the opportunity arises, and lays fewer egg masses when isolated (Costil and Daguzan, 1995). These researchers observed that female sexual maturity (which generally occurs slightly after male maturity in pulmonates) is reached at between 10.5 and 13mm in diameter, which occurs at the age of 15 weeks where snails are raised at 25^{o}C, and 49 weeks at 15^{o}C. Costil (1994) reported the optimum temperature for *P. corneus* is 20.5^{o}C, based on modelled growth rates, but that the temperature range for maximum longevity is 10-20^{o}C. Up to 2500 eggs can be laid per snail in a natural breeding season (personal observation), but juvenile mortality is high (up to 60%; Costil, 1994). *P. corneus*, like most pulmonates, is therefore considered to be 'r-selected' and 'weedy' (many viable embryos are produced but relatively

few juveniles survive to adulthood, unless resource availability markedly increases; Dillon, 2000).

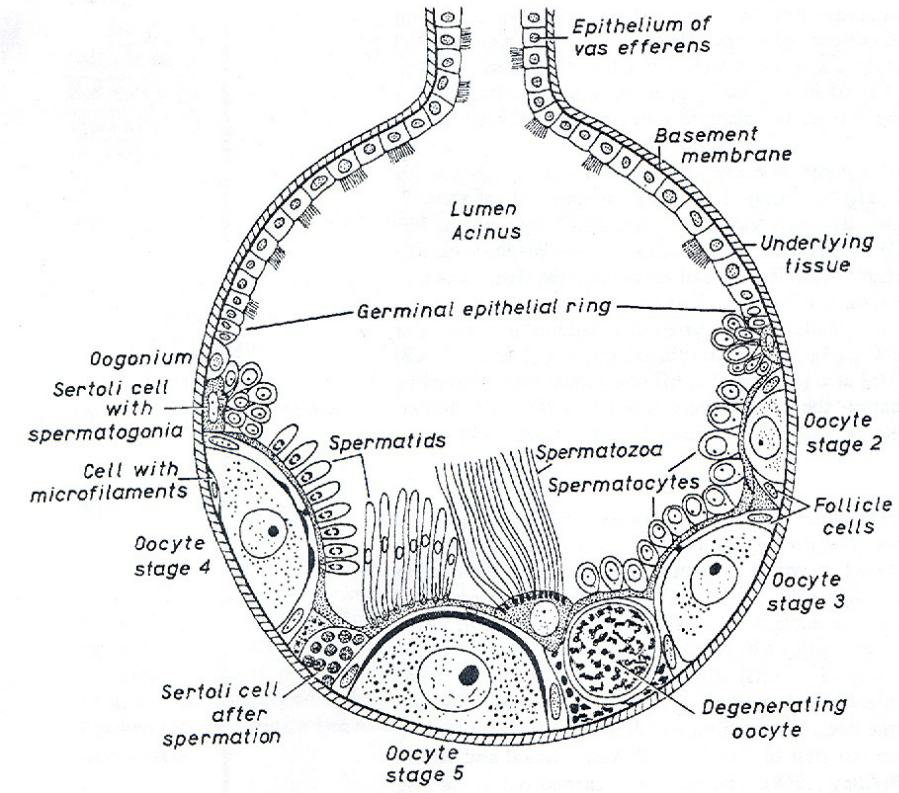


Figure 7 – A diagram of the pulmonate acinus showing increasingly mature male and female germ cell development towards the base (after de Jong-Brink, 1976).

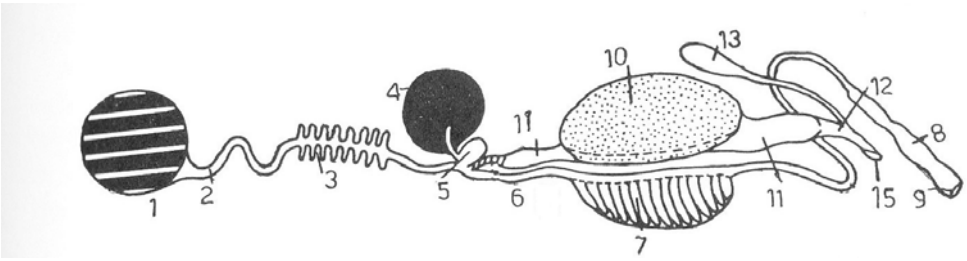


Figure 8 – The reproductive tract of *P. corneus* (after Hyman, 1967). 1, ovotestis; 2,hermaphroditic duct; 3, seminal vesicles; 4, albumin gland; 5, fertilization pouch; 6, sperm duct; 7,prostate; 8, penial complex; 9, male gonopore; 10, oothecal gland; 11, oviduct; 12, vagina; 13, spermatheca; 15, female gonopore.

2.1.6 *Viviparus viviparus*

Cook (1949) observes that the prosobranch *V. viviparus* (Figure 9) occurs in slow running rivers, and is confined to areas with some hard substratum alongside mud. They tend to lie on the substrate with the aperture uppermost and the foot slightly protruding and can remain inert for long periods. In this position, the mantle forms a 'neck-veil' to create inhalant and exhalent siphons, but it is very sensitive to any jarring, and will close the opercula. It is an inefficient grazer, having only a short radula with few teeth. In contrast, the form of the ctenidial filaments suggest that it is an efficient filter feeder. The epithelium has many mucus glands to secrete mucus which catches food particles that are moved along a food groove in the mantle towards the mouth. *V. viviparus* is able to remove seston at a rate of up to 14 mg g^{-1} dry weight of the organism, and this is the main feeding mechanism during the summer (Hoeckelmann and Pusch, 2000). Boycott (1936) notes that viviparids do not occur in stagnant or closed ponds, and Eleutheriadis and Lazaridou-Dimitriadou (1995) indicated by principle component analysis that the population size of *Viviparus contectus* is correlated to the level of dissolved oxygen in water bodies.

V. viviparus has two genders with the male having a shorter and thicker right tentacle that incorporates the penis. The female is viviparous; the embryos develop in a brood pouch (Figure 10), and are present at all stages of development at any one time. Fully independent juveniles are produced at a rate of up to one per day (personal observation). In the Worcester-Birmingham Canal, Young (1975) observed that juveniles are released continuously throughout the summer, and surviving individuals reach 8mm in length by the first winter. They migrate into the mud to over-winter but continue to grow, and the females become sexually mature by 12mm (Jakubik, 2007), containing embryos in the brood pouch by the following spring. Many survive a second winter and reproduce in the second summer, reaching 38mm in size. Calow (1978) considers *V. viviparus* to be iteroparous (breeds over several seasons).

Figure 9 – Male *Viviparus viviparus* (approximately x3 life size; image from World Wide Web).

The population tends to be skewed towards females in *V. viviparus*, with over 70% being female for most of the year in the Zegrzynski reservoir (Jukubik, 2006), although this ratio fell back to unity in the autumn (possibly due to mortalities amongst reproductively exhausted females). She estimates from surveys that *V. viviparus* can live for four years or more, and reports mean numbers of embryos per female being up to 10, although the largest number recorded was 63. In another viviparid, *Viviparus ater*, the mean annual fecundity is reported as 19.6 in Lake Zurich and 14.7 in Lake Maggiore (Ribi and Gebhardt, 1986). These low rates of reproduction indicate that viviparids are K-selected (i.e. individuals make a large proportional contribution to a population that is close to carrying capacity; Begon and Mortimer, 1986).

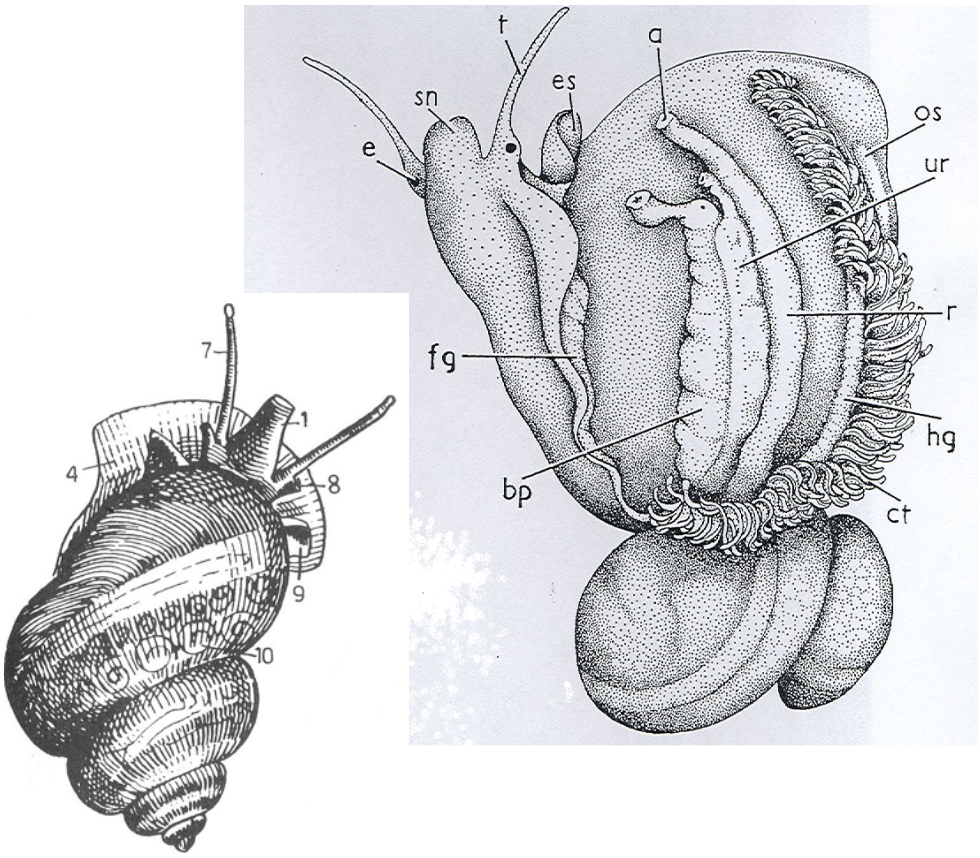


Figure 10 – Sketch of embryos in the brood pouch of *V. viviparus* (left; after Hyman, 1967), and a diagram of a dorsally dissected female (right; after Fretter and Graham, 1994). 1 and sn, snout; 4, foot; 2 and t, tentacle; 8 and e, eye; 9, nuchal lobes; 10, embryos; es, exhalent siphon, a, anus; os, osphradium; ur, ureter, r, rectum; hg, hypobranchial gland; ct, ctenidium; bp, brood pouch; fg, food groove.

2.1.7 *Bithynia tentaculata*

The prosobranch *B. tentaculata* (Figure 11) is characteristic of running water, although it can inhabit large still water bodies, and it occurs widely in the UK (Boycott, 1936). However, it is restricted to medium and hard water (>125 mgL^{-1} $CaCO_3$ equivalent; Dussart, 1979). It is a preferential filter-feeder, and comes to rest, elevating the shell and exhibiting inhalant and exhalent siphons as soon as suspended matter is encountered (Brendelburger and Jurgens, 1993). Nevertheless, *B. tentaculata* can survive and grow when restricted to grazing (Tashiro, 1982), but filter-feeding individuals have a higher net gain of carbon and nitrogen and lay more eggs (Tashiro and Colman, 1982). This is in

accordance with the hypothesis of Barnese and Lowe (1995), suggesting that prosobranchs are inefficient grazers when compared to pulmonates. However, even filter-feeding in freshwater prosobranchs is relatively inefficient in comparison to marine filter-feeding gastropods such as *Crepidula fornicata*, these having elonagated ctenidium lamellae where *B. tentaculata* does not (Declerk, 1995). He considers that filter feeding in freshwater gastropods is a recent adaptation, and observes that it occurs only in viviparids and bithinids.

Figure 11 – *Bithynia tentaculata* (approximately x10 life size; image from World Wide Web)

B. tentaculata is a dioecious gonochorist, with the male having a forked penis carried on the dorsal surface inside the mantle cavity (Figure 12). The female is ovoparus and can lay up to 900 eggs in a breeding season (mean = 400; Richter, 2001). Most of the eggs are laid from mid-May to late June, although a low proportion continue to be laid through to September (Kozminsky, 2003a). Reproduction can also begin earlier, if the water temperature reaches 12^{o}C (Richter, 2001). Embryo development time to hatching is three weeks at 16-

17°C, and two weeks at 23°C (Kozminsky, 2003a). The juveniles reach 5mm in length by the first winter, and continue to grow slowly, reaching 6mm the following April when the males are sexually mature (Lilly, 1953). The females mature slightly later, at 6.8mm (Dussart, 1979; Richter, 2001). Adults continue to grow, reaching 10mm by the second winter, and breed again the following spring (Dussart, 1979). A proportion of the population over-winters again, and can breed a third time, continuing to grow slowly over a final summer (Negus, 1998), so that three generations are usually present in the population (Dussart, 1979). Individuals can reach up to 13mm in length (personal observation).

The sex ratio of *B. tentaculata* populations is close to unity at all stages (Lilly, 1953; Richter, 2001; Kozminsky 2003b). The females tend to lay only a few eggs in the first breeding season, and seem able to curb early reproduction if the food quality is poor (Tashiro, 1982). Reproductive effort then increases with increasing age (Negus 1998). Calow (1978) considers *B. tentaculata* to be iteroparous in the UK, although bithinids can be semelparous in the north (Montreal; Pinel-Alloul and Magnin, 1971) and south (Greece; Eleutheriadis and Lazaridou-Dimitriadou, 2001) of their range. Negus (1998) describes *B. tentaculata* as 'marginally r-selected' due to a high juvenile mortality rate (90%). This agrees with Gilbert et al. (1986), who recorded a maximum of 41.7 surviving juveniles per field-caged adult after one summer.

Over-wintering in *B. tentaculata* begins in November, when snails descend into the roots of plant material to avoid wash-out, and where they continue to feed on detritus (Lilly, 1953). Interestingly, the females may require a winter period as an environmental cue for breeding, as Andrews (1968) could not induce reproduction in a laboratory without outdoor over-wintering. Hahn (2005) also noted that growth ceased at 7mm in laboratory conditions independent of temperature, and attributes this to the presence of an endogenous rhythm. Brendelberger and Jurgens (1993) recorded that the filtration rate for feeding was also reduced in October even in constant laboratory conditions, and concluded that *B .tentaculata* has an innate circannual rhythm.

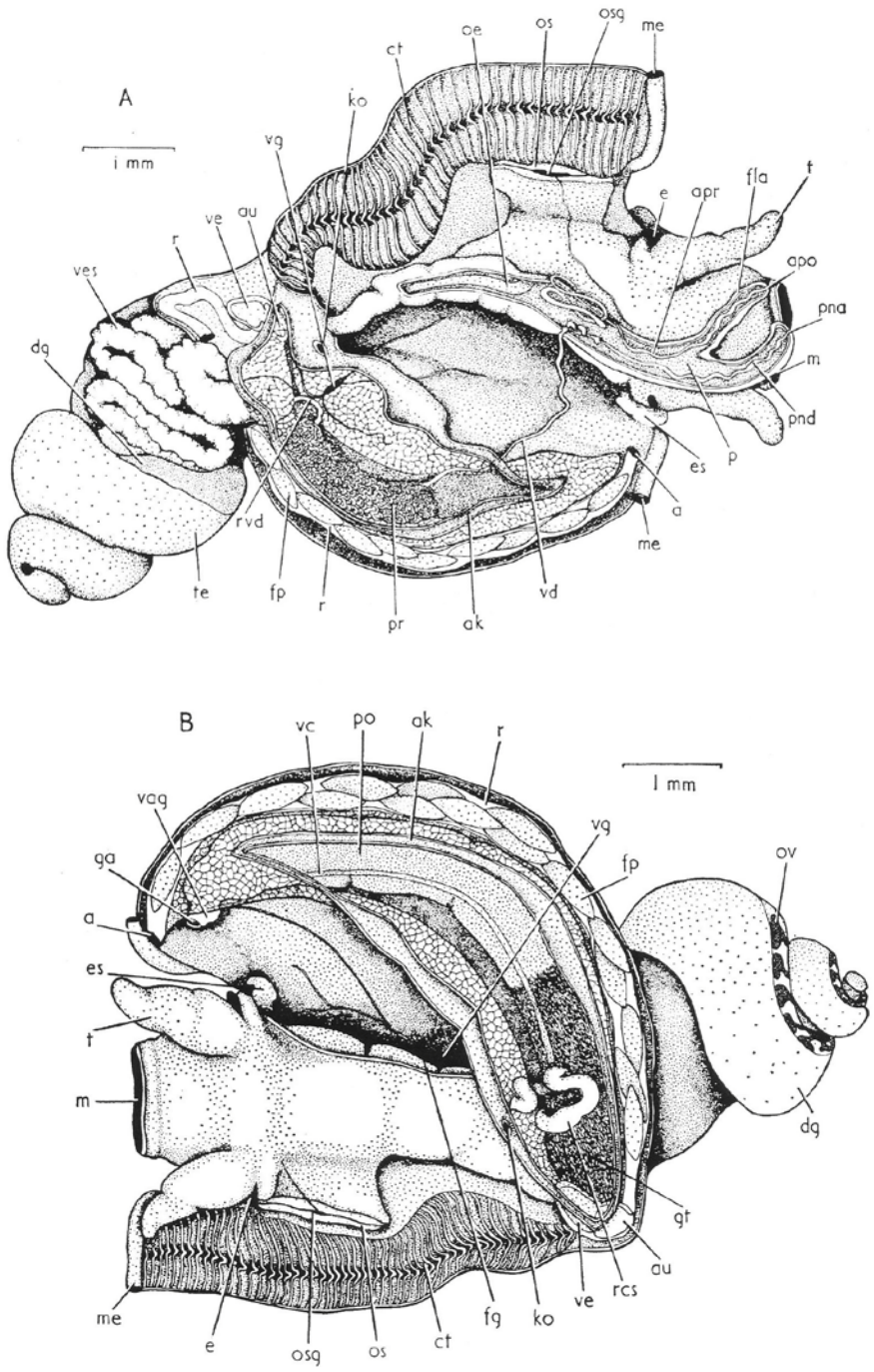


Figure 12 – diagrams of dissected male (a) and female (b) *B. tentaculata* (after Fretter and Graham, 1994). A, anus; ak, anterior kidney; apo/apr, accessory prostate gland; au, auricle; ct, ctendium; dg, digestive gland; e, eye; es, exhalent siphon; fg, food groove; fla, flagellum of penis; fp, faecal pellet; ga, genital aperture; gt, glandular tubules; ko, kidney aperture; m, mouth; me, mantle; oe, oesophagus; os, osphradium; osg, osphradial ganglion; ov, ovary; p, penis; pna, penial aperture; pnd, penial duct; po, pallial oviduct; pr, prostate; r, rectum; rcs, receptical seminilis; rvd, renal vas deferens; t, tentacle; te, testes; vag, vagina; vc, ventral channel of oviduct; vd, vas deferens; ve, ventricle; ves, vesicular seminilis; vg, visceral ganglion.

2.1.8 *Potamopyrgus antipodarum*

Ponder (1988) renamed the prosobranch *Potamopyrgus (Hydrobia) jenkinsii* as *P. antipodarum* (Figure 13) after establishing its New Zealand origins. It is not therefore a species native to the UK, but an invader that has successfully bred in European freshwaters for over 100 years (Daniel, 1894). It occurs widely in England and Wales (Kerney, 1976), although it is positively correlated with harder waters (Dussart, 1977). It requires flowing water, but this can be 'the meanest trickle' (Boycott, 1936), and it also thrives in brackish water (Todd, 1964). It generally inhabits the surface of mud, and consumes finely divided detritus and mud (Dussart, 1977). It has a low rate of carbon assimilation (4%), and produces 25% of its own flesh weight in frasse per hour (Heywood, 1962).

Figure 13 – *Potamopyrgus antipodarum* (approximately x20 life size)

P. antipodarum reproduces parthenogenetically in Europe, where very few males are found (Wallace, 1985). It is viviparous, with embryos of all stages being brooded in an undifferentiated brood pouch (Figure 14). The embryos take 30 to 35 days to develop sufficiently for release at 15^{o}C, and the growth rate can be up to 0.28mm per week with an optimum food supply. Sexual maturity is then reached at 3.1mm in three to six months (Dorgelo, 1991). Females continue to grow, and can reach almost 6mm (personal observation). Dorgelo (1991) gives mean numbers of embryos in the brood pouch between 12 and 16, but it can be as high as 30 (personal observation). Juveniles are released every few days over the reproductive period, and two generations of adult plus recent juveniles are present most months of the year (Dussart, 1977).

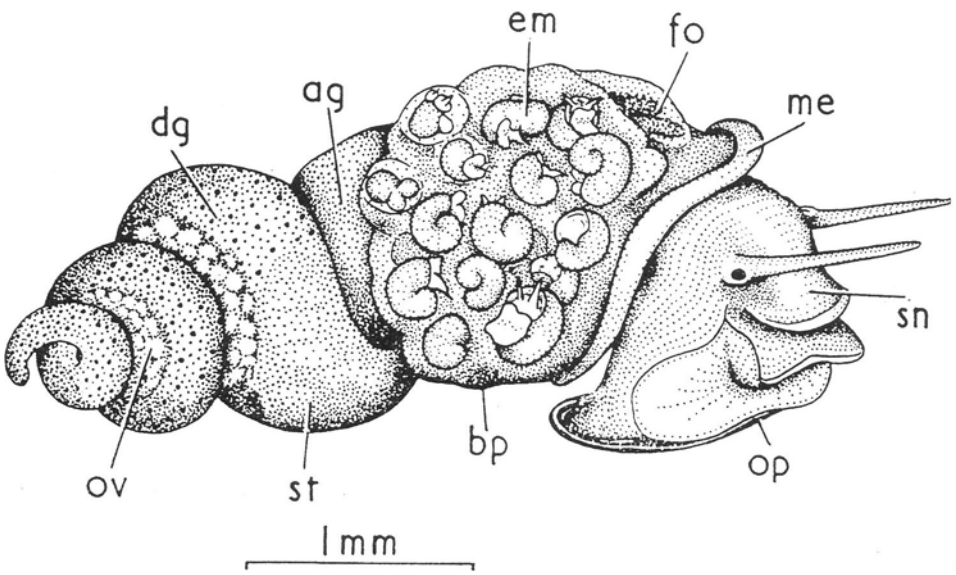


Figure 14 – Diagram of *P. antipodarum* removed from the shell (after Fretter and Graham, 1994). Ag; albumin gland, bp; brood pouch, dg; digestive gland, em; embryo, fo; female aperture, me; mantle, op; operculum; ov, ovary, st; stomach.

2.2 Culture Trials

While it was preferable to either capture organisms directly from the field for use as test subjects in the outdoor mesocosm exposures, especially where they were abundant upstream of the river intake (as in the case of *B. tentaculata*) or to

purchase wild caught test subjects where they were not, it is not an ideal approach to obtaining organisms for use in laboratory studies. A better practice is to use organisms that have been raised in similar conditions to those described in the test methods (e.g. as in the *Daphnia magna* Reproduction Test, OECD, 2008). Consequently, trials were made to establish cultures of the freshwater gastropod species employed in the mesocosm experiments with a view to providing test organisms for use in laboratory exposures.

2.2.1 *Planorbarius corneus*

Pulmonate species have been extensively cultured in the context of basic neurological research, particularly *L. stagnalis*. While *P. corneus* has not often been used for this purpose, it has been cultured on occasion for ecological studies (e.g. Moriarty, 1977; Costil, 1994; Costil and Daguzan, 1995). However, the species has not to date been cultured for use in ecotoxicological testing. The F0 organisms used to establish this culture were obtained from 'Blades' Biological Supply, and were originally captured from wild stocks in the River Rife, near Worthing. On arrival at the laboratory, they were initially held in dechlorinated tap water with a selection of pond weed from the same supplier. They were acclimated to a temperature of 20^{o}C under constant light for at least 2 weeks. They were also sorted by size into three groups <15 mm, 15 – 18 mm and >18mm in the longest axis.

Following this, groups of snails were transferred into 10 litre tanks (15cm width x 30cm length x 30cm height). The tanks contained 8 litres of Artificial Pond Water (APW) made to the following recipe:

Stock solution 1: 294 g L^{-1} $CaCl_2.2H_20$
Stock solution 2: 123.25 g L^{-1} $MgSO_4.7H_2O$
Stock solution 3: 64.75 g L^{-1} $NaHCO_3$
Stock solution 4: 5.75 g L^{-1} KCl

APW is made by adding 1ml of each stock solution per litre of reverse osmosis and activated carbon filtered tap water. However for the purposes of gastropod culture and testing, the added volume of Stock Solution 1 was doubled to 2ml L^{-1} to increase the amount of calcium available in an effort to reduce possible shell thinning that can occur over time (Bohlken and Joosse, 1982). The tanks were aerated via a glass pipette, with a flow rate of 200 mls/min of air filtered through a 0.2 um bacterial filter vent. The media was changed weekly and the snails were fed with 0.25 g/snail of fish flake (King British, Sinclair, UK), and 3-4 pellets of calcium enhanced food (Crab Cuisine, Hikari, Kyorin, Japan) after each media change.

Stocking density

The effect of two alternative stocking densities on the rate of oviposition was assessed at 20^{o}C; 1.5L/snail (6 snails in 9L) and 2L/snail (4 snails in 8L). Organism >18 mm in the longest axis were used as it had been observed that although those >15 mm occasionally laid egg masses, it was at a lesser rate than those that had reached 18mm. Egg masses were counted, recorded and removed weekly, prior to each water change, and any mortalities were recorded and replaced.

The experiment at the stocking density of 1.5L/snail was then repeated with the frequency of media changes being increased to every 48 hours, with the egg masses being counted, recorded and removed at this interval. This was also performed at 15^{o}C. A higher stocking density of 1L/snail (9 snails in 9L) was also assessed at 15^{o}C with 48 hour media changes, with the egg masses being counted, recorded and removed at either 48 hour or weekly intervals.

A summary of the oviposition rates of *P. corneus* is shown in Table 1. It can be seen that at 20^{o}C, approximately 4 egg masses are laid per snail per week at either 1.5 or 2L/snail (no significant difference, t-test, two-tailed, $p>0.05$). When the media change rate is increased to every 48 hours, the oviposition rate is slightly lower at 20^{o}C (no significant difference, t-test, two-tailed, $p>0.05$), but when the

temperature is reduced to 15°C, the oviposition rate is significantly lower, as expected (t-test, two-tailed, $p<0.001$). When the stocking density is increased to 1L/snail at 15°C the oviposition rate is slightly lower still (no significant difference from 1.5L/ snail, t-test, two-tailed, $p>0.05$), but if media changes are made only weekly in these conditions, the rate is significantly reduced by more than half (t-test, two-tailed, log-transformed, $p<0.001$). When the highest stocking density (1L/snail) was attempted at 20°C, the adult mortality rate increased sharply (not recorded).

Table 1 – Summary of Oviposition rates with varying stocking densities, temperatures and media change rates.

Stocking Density	Temperature (°C)	Media Change Rate	Masses/Snail/Week +/- SEM
1.5 L/snail	20	Weekly	3.96 ± 0.50
2.0 L/snail	20	Weekly	4.04 ± 0.09
1.5 L/snail	20	48 h	3.78 ± 0.34
1.5 L/snail	15	48 h	2.46 ± 0.46
1.0 L/snail	15	48 h	1.99 ± 0.07
1.0 L/snail	15	Weekly	0.84 ± 0.06

Feed rate

An assessment of the optimum feed rate was also undertaken using a stocking density of 4L/snail (2 snails in 8L) at 20°C. The snails were fed fish flake only at different rates on a logarithmic scale (0.032, 0.1, 0.32, 1, 3.2 and 10g/snail at each 72h media change) for 21 days. The pH, dissolved oxygen (percentage air saturated value; %ASV) and conductivity (uS/cm) of the water were measured daily to determine the influence of any excess food on the water quality

Over a 72 hour period, the pH of the water was broadly unaffected by increasing quantities of food up to 3.2 g/snail (7.50 to 7.25; a 3% reduction), but at 10 gL^{-1} it was reduced by 15% (to 6.36). However, the dissolved oxygen levels were more

seriously affected above a feed rate of 1 g/snail (a reduction from a mean ASV of 101% at 1g/snail to 28% ASV at 3.2 g/snail, and to 1% ASV at 10 g/snail), as were the conductivity values (from a mean value of 534 uS/cm at 1g/snail to 696 uS/cm at 3.2 gL^{-1} and again to 920 uS/cm at 10 g/snail).

The two highest feed rates were consequently abandoned and the oviposition rates of the snails fed up to 1g/snail were assessed. At this feed rate, the deviation of the water quality parameters over 72 hours was 14% for both dissolved oxygen (to 87% ASV) and conductivity (to 611 uS/cm). The snails fed 0.032 g/snail showed no oviposition activity. However the snail fed on all the remaining feed rates (0.1, 0.32 and 1g/snail) laid egg masses at rates of 4.42, 5.16 and 4.05 masses/snail/week respectively. This suggested that feed rates between 0.1 and 1g/snail are all equally suitable for maximal oviposition in the conditions (Table 1, values derived at 20^{o}C), without causing unacceptable media pollution.

Hatchlings

The egg masses were allowed to hatch out in batches of 20 over two weeks at 20^{o}C, in crystallising dishes containing approximately 100 ml of APW. The media was replaced every 48 hours. When hatching began, 2mls of concentrated algal cells (*Chlorella vulgaris*, cultured in the laboratory under sterile conditions in 2L volumes of Bold's Basal Medium 1, from stock cultures obtained from the Culture Collection of Algae and Protozoa (CCAP), Dunstaffnage Marine Laboratory, Oban; see Appendix 1 for details) and a two drops of Live Bearer Fish Food (Liquifry, Interpet, Dorking, UK) were added at each media change. This practice continued for four weeks, when the juveniles were 2-3 mm in diameter.

At 4 weeks post-hatch, the juvenile snails were gently washed into 2L of APW groups of approximately 50, and fed weekly with 20mls of concentrated *C. vulgaris* and approximately 5g of very well crumbled fish flake. No aeration was provided as the animals were too delicate to resist the flow. Instead a couple of

branches of *Elodea crispa* were provided to facilitate juvenile movement to the surface for respiration.

After 2-3 months when the juveniles had reached a diameter of 5-10mm they were transferred into aerated 20l tanks and fed weekly on fish flake at a rate of 0.05 g/snail and 3-4 pellets of calcium enhanced food. At about 6 months of age they were transferred to aerated 40 litre tanks of APW and fed weekly at a rate of 0.1-0.2 g/snail. At approximately 8 months old (15-18mm), the F1 generation began laying egg masses in these tanks. The F2 generation was reared in the same manner.

Optimum Test Conditions for P. corneus

Overall, it is concluded that the optimum stock density is 1.5L/snail in a semi-static system at 20^{o}C, or 1L/snail at 15^{o}C. It appears necessary to remove egg masses at less than weekly intervals, as these data suggest that the egg mass production rate is slowed when more than 10-12 masses are present on the surface area provided (approximately 0.25 m^2). Whether this is due to a chemical cue released by the egg masses or whether it is due to the rate of encountering egg masses as the snails crawl is not known, but could be established using a flow-though system. Optimum feed rates are between 0.1 and 1g/snail of fish flake for adult snails, dependant on the stocking density and the frequency of media changes. For animals in culture, it may be advantageous to supply calcium enriched pellets to reduce the likelihood of shell-thinning over the long-term, but this would not be required during chemical exposures and it is preferable to reduce feed variety and quantity as much as possible to reduce the possibility of interactions with the test substance. For feeding juveniles, a more accessible food supply than fish flake is necessary, and both an algal concentrate and Live Bearer Liquid Feed are acceptable.

The maintenance of *P. corneus* in culture was relatively straight-forward, and the full life-cycle was observed under laboratory conditions. The limiting factor is the low stocking density required as the snails matured. It is estimated that a

culture of approximately 1000 adult or near adult individuals would be required to provide organisms for chemical testing, and it was established that the Environment Agency Waterlooville facility did not have the footprint available to culture *P. corneus* in sufficient numbers. Consequently, while a small culture was maintained that provided some animals for testing, the majority used were purchased from biological suppliers and held in the facility during acclimation.

2.2.2 *Viviparus viviparus*

Prosobranchs are not commonly cultured for research and no references could be found on culturing *V. viviparus*; the first species of prosobranch deployed in the mesocosm experiments. Pairs of adults collected from the Grand Union Canal at Cowley were established in 10L tanks of aerated APW. The media was replaced weekly and the organisms were fed at a rate of 5ml of concentrated algal cells/snail (*C .vulgaris*, see Appendix 1 for details) at each media change. A selection of other food sources was also offered (fish flake, calcium enriched pellets, live-bearer fish food, carrot, dried milk), but the snails were not observed to move towards any of these options, apparently preferring to filter-feed *in situ*. The females produced juveniles, but all the adults failed to survive in the laboratory for a period of more than 12 weeks. The juveniles also filtered the algae provided to the adults, and grew up to 10mm in the longest axis, but mortality then occurred in all cases.

It was apparent that *V. viviparus* has an essentially sedentary mode of life, selecting filter feeding over any form of searching and grazing. However, while *C. vulgaris* was an acceptable food source, it may not have provided sufficient substrate for the long-term survival of the adults or for maturation of the juveniles. An increased rate of concentrated algal feed tended to cloud the media and caused super-saturation of dissolved oxygen levels which may contribute to gastropod tissue damage. In the natural riparian environment, *V. viviparus* may spread a mucoid ‘net’ to catch particulate matter and fine organic colloids as is the case with other large prosobranchs (e.g. *C. fornicata*, Jorgensen et al., 1984).

It was not possible to replicate suitable conditions for this feeding strategy in the Environment Agency Waterlooville facility, and so the attempt to establish a culture of this species was unsuccessful. Because the adults could not be consistently maintained and the rates of reproduction may also be compromised by an inadequate food supply, *V. viviparus* was not considered to be a suitable test organism for laboratory studies.

2.2.3 *Bithynia tentaculata*

Bithynia species have occasionally been cultured for use as hosts in the study of parasites (e.g. Kruetrachue et al., 1982a and 1982b), however this has always necessitated the use of sediments. It would be preferable to exclude this if possible, as it can adsorb steroid compounds (Yu et al., 2003). *B. tentaculata* were collected from the River Chelmer, upstream of the confluence with the River Blackwater at NGR TL835090. Groups of 50 organisms were established in 10L tanks of aerated APW. The media was replaced weekly and the organisms were fed at a rate of 1ml of concentrated algal cells/snail (*C. vulgaris*, see Appendix 1 for details) at each media change. This was cleared quickly, enabling similar amounts of algal cells to be added every 2 to 3 days. Sliced organic carrot was also supplemented *ad libitum*. Adults lived for periods of up to six months in the laboratory and laid egg masses consistently (data not collected).

However the newly hatched juveniles (approximately 1mm in diameter) seemed unable to access the algal cells. This was also observed by Bredelberger (1995). Declerk (1995) noted that no filter-feeding gastropod can filter-feed when just hatched, possibly because the energy required to draw a flow through the mantle is too great. Eleutheriadis and Lazaridou-Dimitriadou (1996) reported that the food requirement (aufwuchs) for growth of newly hatched *Bithynia graeca* was x3-4 that of adults, and this demand may initially preclude filtering activity. The juvenile *B. tentaculata* did not take any form of highly ground particulate or liquid fish food either. It was therefore not possible to establish an F1 generation of this species. It is probable that both the juveniles and the adults graze to some

extent on bacterial films. It was noted that in tanks that were not cleaned for up to a month where presumptive biofilms could become established, the snails laid visibly more eggs than those being maintained in cultures fed on algal cells. However this is not a viable option during the assessment of chemical compounds as biofilms are known to be effective in degrading organic compounds. Sumethanurugkul (1980) devised a food substrate for rearing *Bithynia* sp., but the recipe contains a high proportion of powdered milk, which is likely to have an oestrogenic component making it unsuitable for use in this research.

Overall, while it was not possible to establish cultures of *B. tentaculata* to provide a source of test organisms, it was considered that because consistantly reproducing adults could be held in culture conditions for six months with a mortality rate of <10%, snails collected from the field and held in the facility during acclimation could be employed in limited preliminary laboratory tests.

2.2.4 *Potamopyrgus antipodarum*

This species is currently under consideration by the OECD Endocrine Disruptors Testing and Assessment Group (EDTA) as a tool for assessing the effects of potentially endocrine disrupting compounds. As such it is in routine culture in the participating laboratories, and a Standard Operating Procedure (SOP) is in development both for culturing the organism and for conducting tests (Schmitt et al., 2006; see Appendix 2). The culture held at the Environment Agency Waterlooville facility was established using adults collected from a stream in Great Dunmow, Essex, NGR TL 595220. Approximately 200 adults were held in 10L tanks of aerated APW, containing a layer of previously dried alder leaves (*Alnus glutinosa).* The media was changed weekly and the culture was additionally fed with concentrated algal cells (*C. vulgaris*, see Appendix 1 for details) fed at a rate of approximately 0.5ml/snail at each media change. The cultures were periodically thinned through sieves (1mm mesh), with the excess being used to either establish further cultures or to provide adults (>3.5mm in the longest axis) for laboratory tests.

CHAPTER 3
METHODS AND EXPERIMENTAL DESIGNS

3.1 Mesocosm Exposures

3.1.1 Mesocosm Experiment 1 (2004)

The mesocosm tanks were sited within the Langford Water Treatment Works (Langford, Essex), that are owned and operated by Essex and Suffolk Water Company. Three tanks were used, each having an approximate working volume of 700 litres and a surface area of approximately $1m^2$. The tanks were supplied with river water from the River Blackwater immediately upstream from the confluence with the River Chelmer at NGR TL835090. The river water was pumped through a communal activated carbon filter to remove trace organic compounds before being fed to the tanks at a flow rate of approximately 6 L/min. The tank retention time was approximately 2 hours, and the water exited the system via overflows, and drained through a second gravity fed activated carbon filter before discharging to a settlement lagoon (see Figures 15 and 16 for a schematic and photograph).

One tank was allocated as the reference tank and the other two test tanks were treated with 17ß-oestradiol (CAS no. 50-28-2, >98% purity, Sigma, Poole). This was achieved by preparing a stock solution of 1 gL^{-1} in ethanol which was diluted into two mixing tanks of standing river water. The stock solution was stored at 4^oC in the dark and replaced monthly. The mixing tanks had a volume of 12 litres, to which either 172.8 µl or 1728 µl of the stock solution was added. A peristaltic pump was used to continually dose the treatment tanks from the mixing tanks at a rate of 8.33 mls/min, to attain nominal concentrations of 20 and 200 ngL^{-1} (in $<2.0x10^{-7}$µl L^{-1} ethanol vehicle). The river water flow rate to the tanks was checked daily and the pump rate checked fortnightly. The mixing tanks were manually emptied and re-filled every 48 hours.

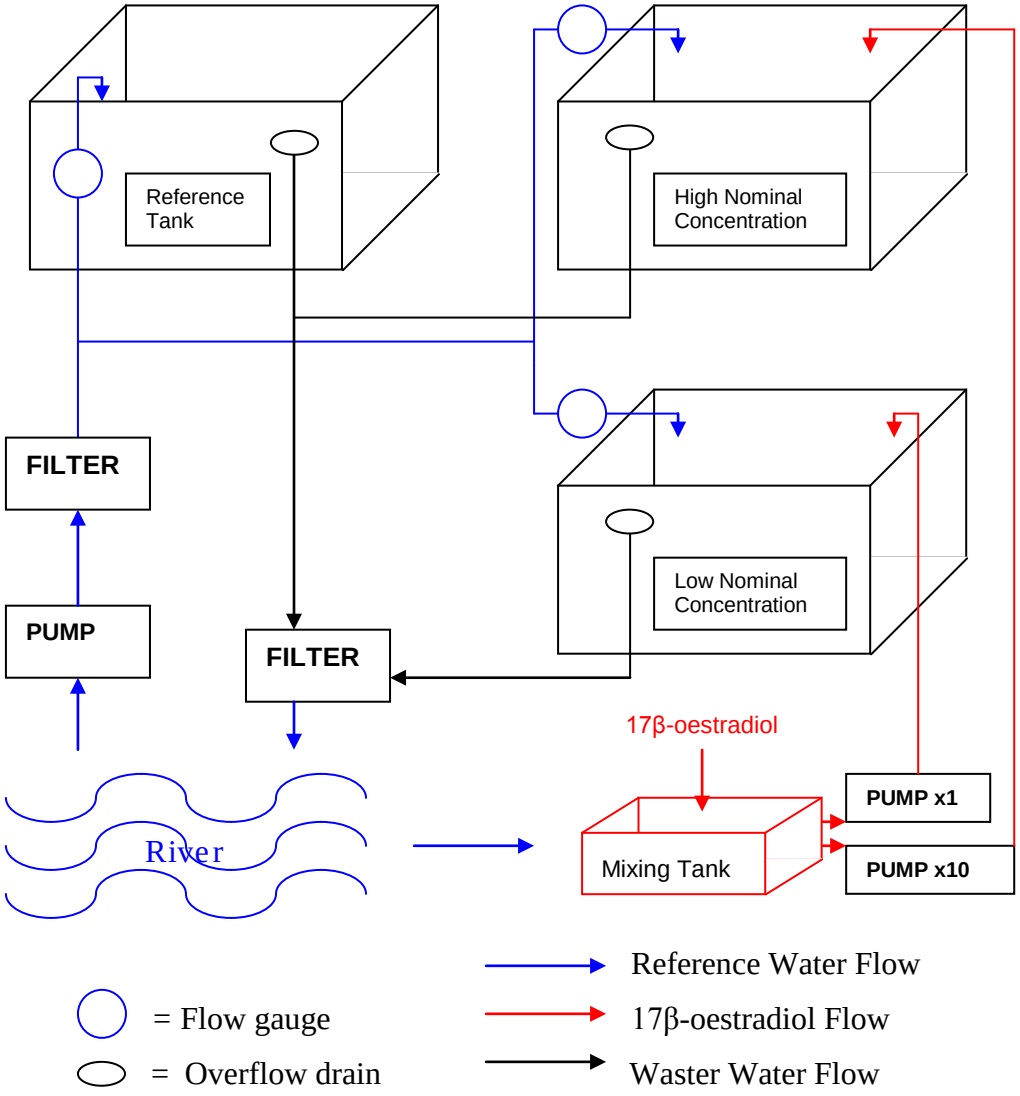


Figure 15 – A schematic view of the mesocosm exposure system

Water chemistry

Monthly samples were taken from each tank for steroid analysis (17ß-oestradiol and oestrone). 1L samples were taken in new, methanol-rinsed glass bottles that were packed on ice in insulated boxes and delivered by an overnight courier to Environment Agency National Laboratory Service (NLS), Nottingham, where they were immediately extracted onto Solid Phase Extraction (SPE) columns. Further samples were occasionally taken for the same analytes by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Burnham-on-Crouch.

These were 2L taken into amber glass bottles containing solvent. Where samples were taken for analysis at both laboratories, a 5L sample was initially taken in a clean steel bucket before being split into the relevant sample vessels.

Figure 16 – The mesocosm at Langford (Essex)

P. corneus

The test organisms were obtained from 'Blades' Biological Supply, and were originally captured from wild stocks in the River Rife, near Worthing. At the start of the experiment (24th May) nine individuals (±1) were randomly drawn from the test population and allocated to one of six groups in each treatment. Each snail was dried gently with a tissue, weighed, and measured in the longest axis with callipers. Individuals <15 mm were excluded to ensure sexual maturity. The groups were housed in cylindrical enclosures made from plastic mesh (height = 70 cm, diameter = 15 cm, mesh size = 0.5 cm). Each enclosure contained four crossed plates of glass (height = 50cm long, each arm width = 7cm; see Figure 17 for schematic). These provided both structure for the enclosures and a surface for oviposition. The enclosures were suspended from wooden rods laid across the top of each tank, and were submerged to a depth of approximately 50cm. The *P.*

corneus were fed three times per week with approximately 10 leaves of organic lettuce and 4 g of fish flakes (TetraMin Tropical, Tetra UK, Southampton) per group.

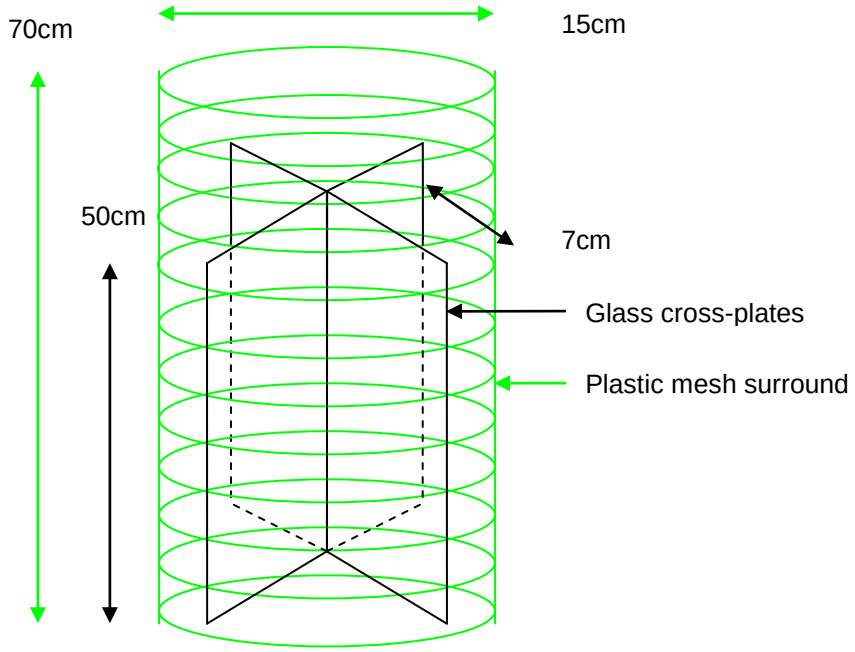


Figure 17 – A schematic view of a mesocosm enclosure

V. viviparus

The test organisms were collected from the Grand Union Canal in Cowley, at NGR TQ055810. One week after the deployment of *P. corneus* (31st May), eleven individuals (±1) were randomly drawn from the test population and allocated to one of six groups in each treatment. The same procedure was used as that for *P. corneus*, with the enclosures being inter-dispersed in the tank. Each mesocosm tank was then covered with a wire mesh to prevent interference from birds. Unlike *P. corneus*, the *V. viviparus* were not fed, as they were expected to filter feed on the algal cells growing in the tanks.

End-points

The exposure to 17ß-oestradiol began 2 weeks after the start of the experiment (7th June). Immediately prior to this, the *P. corneus* enclosures were removed

from the tanks, and all egg masses were carefully lifted from the glass surfaces using a cut-throat razor blade. The egg masses from each enclosure were transferred to glass jars containing water from the relevant tank, labelled, and taken to the Environment Agency Laboratory (Waterlooville). Each mass was photographed and the number of eggs counted. The total wet weight of egg masses from each group was also measured. When the exposure began, this process was repeated fortnightly for twelve weeks. The *V. viviparus* enclosures were also removed fortnightly in the alternate weeks and the juveniles were removed, counted and the total wet weight measured (first sampling event; 14th June) for a period of 12 weeks. At each sampling event any dead snails were removed, and the shell measured in the longest axis.

At the end of the exposure period all the remaining snails were placed in a 5% solution of magnesium chloride for at least two hours to relax the tissues. All individuals were re-weighed and measured in the longest axis. The shells were then cracked and removed, and the soft parts weighed. For *P. corneus*, the albumin gland was also dissected out and weighed. For *V. viviparus*, all the embryos in the reproductive tract were dissected out, and those that were hatched were counted separately from those still within the egg. The remaining tissues were then re-weighed.

3.1.2 Mesocosm Experiment 2 (2006)

The mesocosm system and the method of exposing the snails was essentially the same as that used in Experiment 1, but with the following alterations [rationale indicated in brackets]:

a) The river water that fed the mesocosm system was taken from the River Chelmer rather than the River Blackwater [as the latter had shown high levels of measured steroid concentrations on the previous occasion].

b) The influent river water was pre-filtered through a course filter rather than granulated carbon [because while this step had been unsuccessful in removing the background steroid levels, it did remove much of the other organic matter (algae and detritus) that contributed to the food sources of the filter feeding *V. viviparus*].

c) The nominal concentration of 17ß-oestradiol was reduced by half, to 10 and 100 ngL^{-1} [because the trend in Experiment 1 had been for increasing levels over the course of the exposure, and there was concern over the potential toxic effects of a build up of steroid hormones].

d) The *P. corneus* were fed with fish pellets (TetraPond floating sticks, Tetra, Melle, Germany) at a rate of 3-4 pellets per snail and slices of organic carrot (approximately one 5 mm slice per snail), rather than fish flake and lettuce [in order to reduce the flush out rate of the food by the flow-through of water and because the carrot rotted more slowly than lettuce].

e) The access opening of the *V. viviparus* enclosures were sealed with clips and the enclosures were lowered to the bottom of the mesocosm tanks [to allow the snails access to the sediment that collected there as an additional food source and to better shade them. The various weed species that grew in the tanks (the blanket weed *Cladophera sp.*, the gutweed *Enteromorpha sp.* and the duckweed *Lemna sp.*) were also allowed to grow in a limited manner and provide further protection for the enclosures].

f) The number of replicate groups for *P. corneus* and *V. viviparus* was reduced to three to allow sufficient space in the mesocosm for the deployment of a third species (*B. tentaculata*, see section below). [A power analysis of the data from the previous exposure suggested that this number of replicates was sufficient to identify the degree of change observed for *P. corneus* in Experiment 1 as significant (based on one-way ANOVA, 3 levels, alpha = 0.05, value of the maximum difference between the mean number of eggs laid per adult over a 12

week exposure = 1286, assumed standard deviation of reference group = 293: 6 replicates gives power = 1.000, 3 replicates gives power = 0.959). For *V. viviparus* the indicative power was further reduced (one-way ANOVA, 3 levels, alpha = 0.05, value of the maximum difference between the mean number of juveniles produced per adult over a 12 week exposure = 4.74, assumed standard deviation of reference group = 1.30: 6 replicates gives power = 0.999, 3 replicates gives power = 0.868), but this was considered sufficient to determine whether the same trend was prevalent].

B. tentaculata

The test organisms were collected from the River Chelmer, immediately upstream of the mesocosm intake. At the start of the experiment (5th May) 16 individuals (±1) were randomly drawn from the test population and allocated to one of six groups in each treatment. Each snail was dried gently with a tissue, weighed, and measured in the longest axis with callipers. Individuals <6 mm were excluded. The groups were housed in cylindrical enclosures made from plastic mesh (height = 70 cm, diameter = 20 cm, mesh size = 0.5 cm). Each enclosure contained four crossed plates of glass (height = 50cm long, each arm width = 10cm). The enclosures were inserted into nylon mesh bags (mesh size = 0.2 cm) to prevent this small species from escaping, and were submerged to a depth of approximately 50cm.

End-points

These were as for Experiment 1 for *P. corneus* (excepting that the number of egg masses and the albumin gland weights were not recorded) and for *V. viviparus*. For *B. tentaculata*, the enclosures were removed from the tanks, and all egg masses were carefully lifted from the glass surfaces using a cut-throat razor blade. The number of adults present was counted and any dead snails were removed and the shells were measured in the longest axis The egg masses from each enclosure were transferred to glass jars containing water from the relevant tank, labelled, and taken to the Environment Agency Laboratory (Waterlooville). The number of eggs in each mass counted using a light microscope (x4

magnification). The total wet weight of egg masses from each group was also measured. At the end of the exposure, all surviving snails were removed from the enclosures and transferred to labelled vessels containing approximately 500 ml of water from the relevant tank. These were also taken to the Environment Agency Laboratory. On arrival, the snails were weighed and measured in the longest axis. The shells were then cracked and the gender determined using a low-power microscope (x4 magnification). Any obvious parasite infections were also noted.

3.2 Laboratory Exposures

3.2.1 Experiment 3 – A laboratory exposure of *P. corneus* to 17ß-oestradiol in 'simulated summer' and 'simulated autumn' conditions.

Experimental Design

Two air-conditioned rooms with insulated walls and doors and without windows were programmed to hold temperatures of 15^{o}C and 20^{o}C, respectively. Vessels containing 200ml of water were spaced around the room and after a period of equilibration, measured with a platinum resistance thermometer with a factory-determined error of 0.2^{o}C, to ensure that there were no zones that varied from pre-set temperature by more than 2^{o}C. One of these vessels was then measured continually with an 'indoor/outdoor' thermometer that was calibrated against the platinum resistance thermometer and the extremes of temperature recorded daily. The tolerance of the extremes was 0.8^{o}C from the nominal temperature to account for the combined errors of the 'indoor/outdoor' thermometer (1^{o}C) and the platinum resistance thermometer.

The areas in the rooms designated for the exposures were two shelves lit by strip fluorescence light bulbs. Each shelf was fitted with two Phillips 'Fluotone' strip bulbs emitting cool white light with a measured lux value of 4000 at the height of the water surface. The photoperiod was automatically controlled to be 12 hours of light and 12 hours of dark in the room set at 15 ^{o}C and 16 hours of light and 8

hours of dark in the room set at 20 ^{o}C. The functioning of the photoperiod was checked monthly by manual forwarding of the control clock to ensure switching.

The snails were housed in groups of six in plate glass tanks with a working volume of 9 litres (1.5L/snail). The dimensions of the tanks were 15cm wide by 30cm long by 30cm high, with a water depth of approximately 24cm. The tanks were constructed using silicon sealant, and there is an outlet tap approximately 8cm from the bottom, which was made from ABS and polythene plastics. Each tank was allocated a consecutive number, and supplied with a correspondingly numbered airline consisting of a glass pipette supplied with 200 ml/min of air pumped through silicon tubing and a bacterial vent, and all tanks were covered (see Figure 18 and 19 for a schematic view of an exposure tank, and a photograph of the experimental rig).

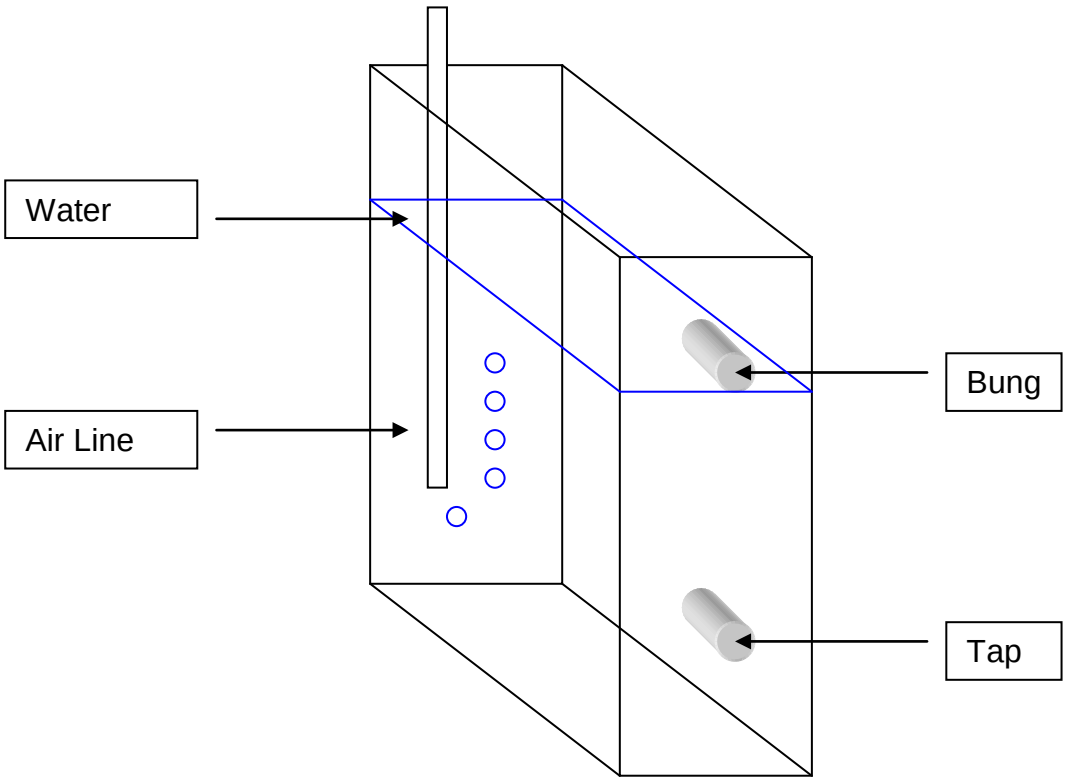


Figure 18 – A schematic view of an exposure tank.

Figure 19 – A laboratory Exposure of *P. corneus* (Experiment 5)

The tanks were filled with Artificial Pond Water (APW) made using the recipe detailed in Chapter 2, Section 2.1.1. The water used to make the media was allowed to equilibrate to the appropriate temperature for 24 hours prior to the addition of the Stock Solutions. At 48-hour intervals, the media in the tanks was drained to the level of the outlet tap to leave the animals in approximately 3 litres. This was in order that the snails were not subjected regularly to the potential shock of a complete media change. Faeces and excess food were then carefully removed by vacuum siphon, before 6 litres of fresh media was returned.

This process was divided into fortnightly 'cycles', during which algae and bacterial films tended to appear on the plate glass. This was tolerated to avoid frequent over-disturbance of the behaviour of the snails. However, at the end of each cycle, the snails were removed into correspondingly numbered vessels containing approximately 1 litre of water from the respective tank, while the media was completely drained. The tanks were then washed using chlorinated tap water and a clean cloth before refilling with fresh media. The shells of the

snails were gently cleaned with fingertips if necessary prior to their return, to avoid seeding the fresh media with an appreciable amount of algal and bacterial growth.

Prior to and after each complete or partial water change, a 100-200ml sample was taken from each control tank and the pH, dissolved oxygen and conductivity measured using WTW electrical potential meters. Samples were not taken from any of the 17β-oestradiol concentrations to avoid potential cross-contamination of any other laboratory applications via use of the anode/cathode probes. The hardness of the media was not expected to alter appreciably during the exposure, so a sample of each new batch of media only was taken and measured by means of a titration against EDTA in the presence of excess ammonia. At the end of each cycle, the hardness of all control tanks was also measured in this way to ensure variation had not become excessive.

Preparation of 17β-Oestradiol concentrations

10 mg 17β-oestradiol (Sigma, Poole) was dissolved into 1L high purity methanol and the entire vessel weighed accurately (to 0.001g). The stock solution was stored for the duration of the exposure at 4 (±2) ^{o}C in the dark. To make the media from this stock, a 1 ml aliquot was removed and added to a 1-litre glass conical flask containing a volume of approximately 250ml of 5mm diameter glass beads. The stock solution vessel was then re-weighed and the weight accurately recorded before being returned to storage. Prior to any further aliquots of the stock solution being taken, the vessel was weighed once again, and any shortfall of weight from the last recorded reading (due to evaporative loss) was made good with high purity methanol.

The methanol added to the conical flask was spread over the glass beads by means of vigorous shaking, and then allowed to evaporate under the flow of air from a pump via a bacterial vent for approximately 30 minutes. Following this, a measured volume of 1 litre of reverse osmosis filtered water was divided into 4-6 aliquots, and each aliquot in turn added to the beads, shaken vigorously and

tipped off into a collection vessel. This procedure (an adaptation of a chemical saturation method; Kahl et al., 1999) provided a working solution of 10 μgL^{-1} 17β-oestradiol. Volumes of either 1, 10 or 100 ml of this solution could then be added to 10 litres of media to make the nominal test concentrations of either 1, 10 or 100 ngL^{-1} of 17β-oestradiol for use in each tank.

The numbered tanks were allocated as either control or 1, 10 or 100 ngL^{-1} nominal 17β-oestradiol concentration by means of a random number table. There were 5 replicates for the control and each test concentration at 20°C and 4 for each at 15°C. The final layout of the exposure tanks in shown in Figure 20. At each partial media change, 1, 10 or 100 mls of the working solution was added to 6L fresh media according to the nominal concentration of 17β-oestradiol. It was assumed that the 3 litres of media remaining in the tanks during each partial media change had little or no 17β-oestradiol present as an initial stability study using the 20°C test conditions suggested that it would almost entirely degrade in 48 hours (see Chapter 4 for details).

Chemical sampling

At fortnightly intervals, at the mid-point of each cycle, equally sized aliquots were taken from each exposure tank and pooled into a 500 ml sample for each concentration. This was performed at both temperatures, immediately prior to and immediately after a partial water change. Each pooled sample was then passed through a C18 SPE column at a rate of approximately 2 mls/min under vacuum, and the column then dried completely using a flow of air. The dried columns were then immediately frozen, packaged on dry ice and shipped to the Environment Agency NLS laboratory for analysis.

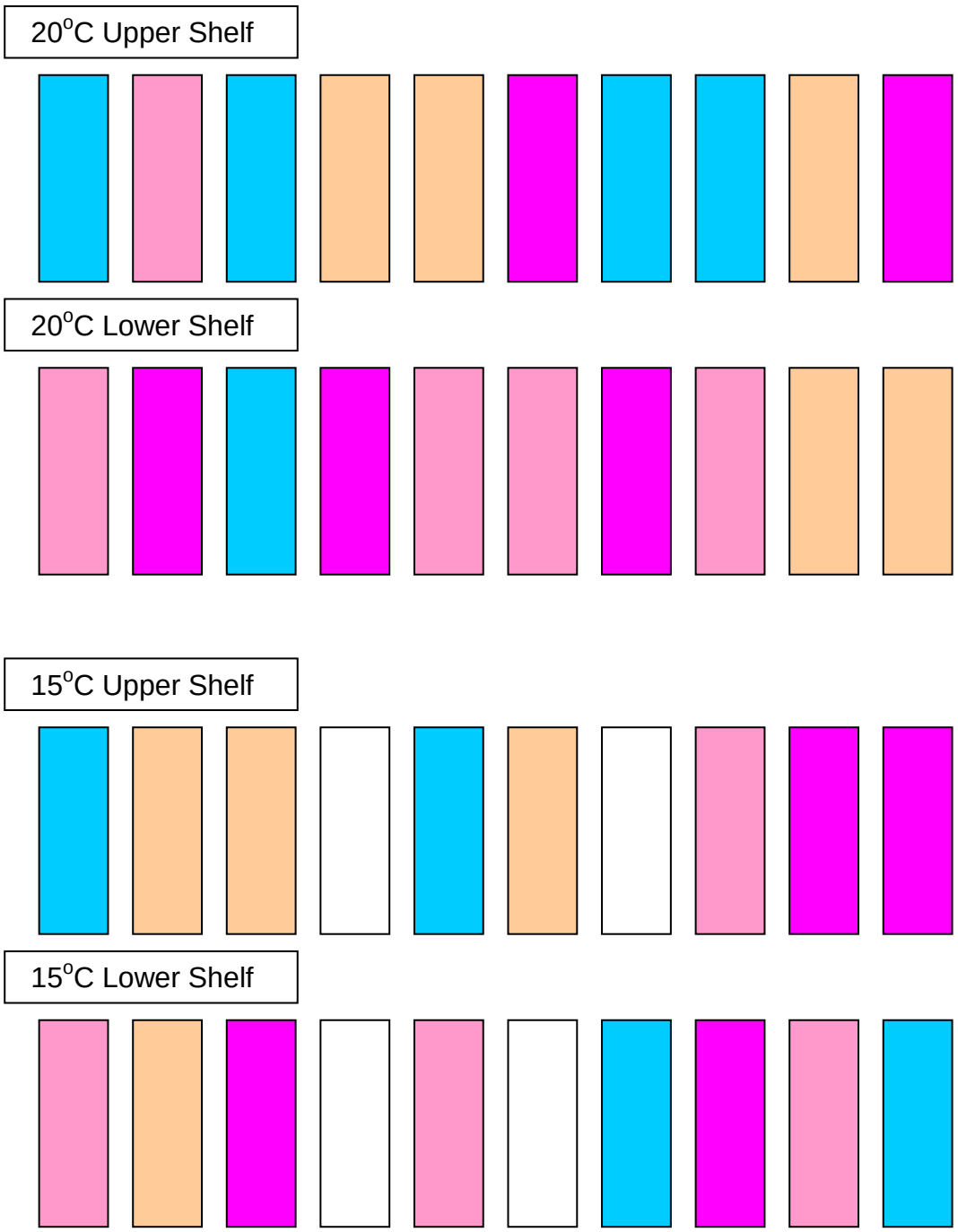


Figure 20 – A schematic layout of the control and nominal 17β-oestradiol replicates in Experiment 3 (blue = control, salmon = 1 ngL^{-1}, pink = 10 ngL^{-1} and magenta = 100 ngL^{-1}). The white tanks were not used in the exposure.

Test Organisms

Approximately half of the *P. corneus* used were obtained from 'Sciento' Biological Supply, Manchester, and half from Queensborough Tropical Fish Farm, Staines, UK. Each delivery from either source was divided on arrival into two groups of roughly equal size and placed into 100-litre tanks containing APW, one of which was sited in the 15^{o}C and one in the 20^{o}C constant temperature room. All deliveries were received over a two-week period in late April/early May, and the snails were allowed to intermix. Each tank contained approximately 150 organisms, and these were fed on fish flake food (King British, UK) *ad libitum* and allowed to acclimate to the relevant temperature for a further week.

At the start of the experiment on the 11th May 2005, snails were drawn from the stock tanks and individuals were weighed and measured in the longest axis, before being allocated to the exposure tanks using a random number table, until each tank contained 6 individuals. No *P. corneus* less than 18mm long was used in the exposure. The snails were fed on fish flake food only, at a rate of 0.25g / snail / 48 hours. The food was replaced each partial media change. The first 28 days of the experiment was conducted without the addition of 17β-oestradiol to any of the exposure tanks, in order to assess the oviposition rates of the replicate groups (the baseline period). The exposure began on the 8th June, and continued for 8 weeks.

End-points

At each 48-hour partial water change all of the egg masses laid on the plate glass of the tanks were carefully removed using a cut-throat razor. The number of masses was recorded and the eggs within each egg mass were then counted under a microscope at x8 magnification, and any egg abnormalities noted. The egg masses from each tank were then allowed to air dry for 2-5 days before being collectively weighed. On one occasion during each cycle, the egg masses were counted and transferred to 100ml crystallising dishes containing media of the appropriate 17β-oestradiol concentration. The media was changed at 48 hour

intervals and the egg masses were allowed to hatch for 14 days at 20°C. At the end of this period, the number of eggs hatching and those remaining were counted.

At the end of the exposure, all the surviving snails were placed in a 5% solution of magnesium chloride for at least two hours to relax the tissues. All individuals were re-weighed and measured in the longest axis. The shells were then cracked and removed, and the soft parts weighed. The reproductive tract was then dissected away from the gut and muscular parts in one piece wherever possible, to include the ovo-testis, the hermaphroditic ducts, the albumin gland, the prostate, sperm ducts and penile complex. The remaining tissues were then re-weighed and the condition factor (total soft tissue weight / whole organism weight) and repro-somatic index (RSI; reproductive tract weight / total soft tissue weight) were calculated. The presence of any parasites was noted.

3.2.2 Experiment 4 – An exposure of *B. tentaculata* to 17ß-oestradiol in changing conditions to simulate the onset of 'spring'.

Experimental Design

The exposure was performed in an air-conditioned room with insulated walls and without windows. The test vessels were placed on two shelves lit by strip fluorescence light bulbs. Each shelf was fitted with two Phillips 'Fluotone' strip bulbs emitting cool white light at approximately 3000 lux at the shelf surface. The photoperiod was automatically controlled, and was set to be 12 hours of light and 12 hours of dark at the start of the exposure, with lights on at 6.15am, to mimic the vernal equinox at Manchester, where the test organisms originated. The functioning of the photoperiod was checked monthly by manual forwarding of the control clock to ensure switching. As the natural season progressed, the photoperiod was adjusted in 'real-time', using the rise and set times for the sun at this location (2°13'W, 53°30'N).

The room was programmed to hold an initial temperature of 10°C, commensurate with outdoor water temperatures at this latitude for the vernal equinox when the exposure was planned to begin. The Environment Agency Water Information Management System (WIMS) database of current water temperatures for this area was referred to, and the temperature of the room adjusted accordingly until the end of May, when it became apparent that the likelihood of a generally cool and wet summer (the summer period July-August 2008 inclusive was one of the wettest on British record; Met Office, 2008) meant that the water temperature in the north-west was unlikely to rise much higher than 16°C. From this point, the temperature of the room was raised gradually independent of outdoor conditions, to reach 20°C by July (see Figure 21 for a representation of the controlled facility temperature and photo-periods settings for the 18-week exposure).

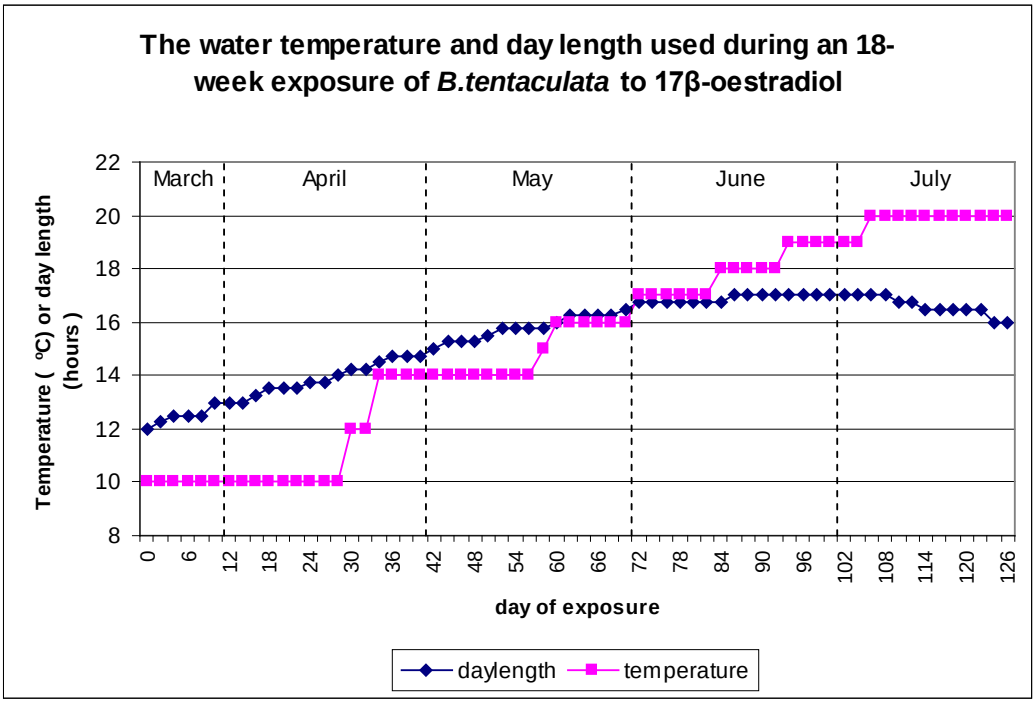


Figure 21 – The controlled facility temperature and photoperiod settings used in the 18-week exposure of *B. tentaculata* to 17β-Oestradiol (Experiment 4).

The snails were allocated as male / female pairs, with each pair being housed in a 250ml glass beaker containing 200 ±10 ml of APW. Each beaker was numbered

consecutively and covered with a watch glass to reduce evaporation. No aeration was provided as it would cause turbulence in the test vessels and it was considered that the surface area to volume ratio was high enough to allow diffusion of oxygen into the media sufficient for two snails. All of the media was replaced at 48 hour intervals by tipping off; the snails tended to simply close their opercula for the period that they were out of the water (less than 1 minute).

Any beakers that had visible amounts of algae growing on the glass were replaced during the exposure. These beakers were washed with hot tap water (soaked at approximately 60^{o}C) and rinsed with methanol only between uses. Following each media change, the temperature, pH, dissolved oxygen and conductivity was measured in the discarded water from each control beaker using WTW meters. The contents of the beakers were then pooled and the hardness of the discarded media was measured by means of a titration against EDTA in the presence of excess ammonia, as was each new batch of media prepared.

Preparation of 17β-Oestradiol concentrations

10 mg of 17β-oestradiol (Sigma, Poole) was dissolved into 100 ml high purity methanol and the entire vessel weighed accurately (to 0.001g). From this, a 1ml aliquot was taken and added to a further 100 ml of high purity methanol to make a 1 mgL^{-1} Working Solution. After preparation of the Working Solution, the Stock Solution vessel was then weighed once again and the weight recorded. The Working Solution was replaced fortnightly from the Stock Solution, which was re-weighed before the 1ml aliquot was taken, and any shortfall of weight from the last recorded reading being made good with high purity methanol. Both the Stock and Working Solutions were stored at 4 (±2) ^{o}C in the dark.

To make the test concentrations from the working solution, a 1.2 ml aliquot was removed and added to a dry 5-litre glass bottle and spread over the internal surfaces as a ‘solvent window’ by tipping and revolving the bottle. The solvent was then allowed to evaporate for approximately 15 minutes. Once dry, a measured volume of 120 mls of reverse osmosis and activated carbon filtered

water was added to the bottle and swirled thoroughly, to make a 10 μgL^{-1} 17β-oestradiol solution. Volumes of either 0.1, 1 or 10 mls of this solution were then added to 1L of media to make the nominal test concentrations of either 1, 10 or 100 ngL^{-1} 17β-oestradiol for use in five replicate 200 ml beakers.

The numbered beakers were allocated as either control or 1, 10 or 100 ngL^{-1} nominal 17β-oestradiol concentration in groups of five, by means of a random number table. The groups of beakers were filled using the same 1-litre aliquot of solution and positioned in rows parallel to each other. There were 10 groups of 5 beakers (50 independent pairs of snails per treatment) for the control and each test concentration. The final layout of the exposure vessels in shown in Figure 22.

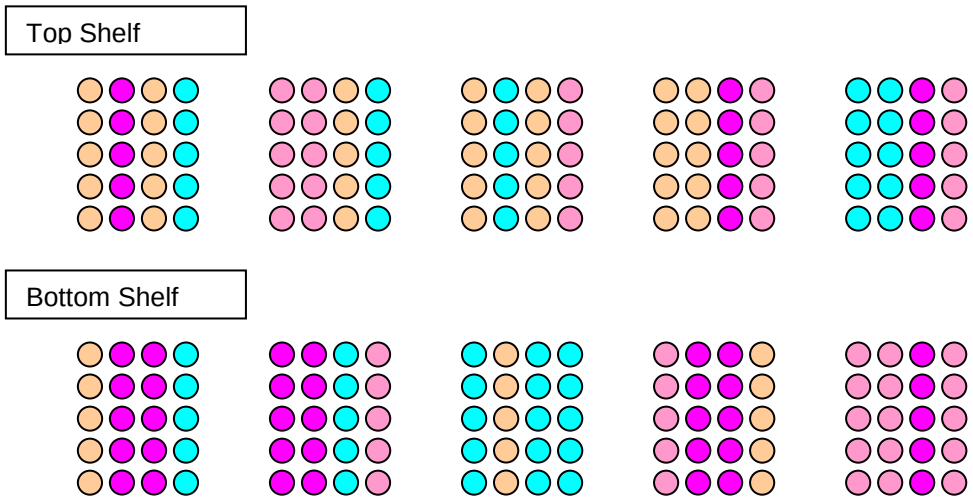


Figure 22 – A schematic layout of the control and nominal 17β-oestradiol replicates in Experiment 4 (blue = control, salmon = 1 ngL^{-1}, pink = 10 ngL^{-1} and magenta = 100 ngL^{-1}).

Chemical sampling

It was recognised that the working volume of the exposure vessels was insufficient to allow aliquots to be taken for chemical analysis between media changes without appreciably affecting the volume of media available to the test organisms. Consequently five further replicate beakers for each concentration of 17β-oestradiol and the control were maintained in the same conditions and with

the same density of snails present. The performance of these snails was not recorded in the end-points of the test, indeed the sex of the snails was not ascertained, but the media in these vessels was periodically taken for analysis, with the contents of all five 200 ml beakers making up the 1-litre sample required.

Samples were taken from these vessels for chemical analysis at six-weekly intervals. On the first occasion, the media was sampled immediately after replacement, replaced again and then re-sampled after 24 hours, then replaced again and re-sampled after 48 hours. On the subsequent occasions, the media was sampled after 24 hours exposure only (the mid-point between media changes in the test). The pooled, 1L samples were passed through a C18 SPE column at a rate of approximately 2 mls/min under vacuum, and the column then dried completely using a flow of air. The dried columns were then immediately frozen, packaged on dry ice and shipped to the Environment Agency NLS laboratory for analysis.

Test Organisms

All animals were obtained from 'Sciento' Biological Supply House, Manchester, having been collected from the inlet to a reservoir of a local drinking water provider. On delivery, the snails were held in a 100-litre tank containing APW at 10^{o}C with a 12 hour light / 12 hour dark photoperiod for two weeks, and fed on an indeterminate amount of concentrated algal cells (*C. vulgaris,* see Appendix 1 for details). The snails had been collected as they arose from aestivation during late February and early March, and were received in the laboratory in the first week of March. Egg laying activity begins immediately in *B. tentaculata* (Richter, 2001), and indeed the first few masses were laid in the holding tank.

Before the start of the experiment, snails were drawn from the stock tank and individuals were weighed and measured in the longest axis, before the sex of each animal was determined. This was done by wedging the organism in a position such that the opercula is uppermost, but tipped slightly to the right so as to

encourage the snail to reach downwards as it emerges from the shell and attempts to place the foot on the substrate surface. As full extension of the snail body is reached, the forked structure that incorporates the penis can be seen on the dorsal surface. This procedure must be performed in a vibration free area or the snail cannot be encouraged to protrude from the shell to the full extent.

Having been separated into two groups of separate sexes, the snails were randomly assigned to the beakers in male and female pairs using a random number table at the start of the exposure on the 21st March 2007 (vernal equinox). A record was kept of the statistics of each animal allocated to each beaker. No *B. tentaculata* less than 8mm long was used in the exposure to ensure sexual maturity. The snails were fed on concentrated algal cells (*C. vulgaris*, see Appendix 1 for details) constituting 0.3 mg of total organic carbon (TOC) / snail / day delivered in a 2 ml aliquot to each beaker after each media change.

End-points

At each 48-hour media change, the number of egg masses laid in each beaker and the number of eggs in each mass was counted by eye against a bright light. The egg masses were recorded, dated and left *in situ* until the beaker was changed (approximately weekly). The number of masses laid by this species is low in comparison with pulmonates, and so it was rare that more than one or two egg masses were present in a beaker at any time. On no occasion did any egg mass hatch out. It is not possible to remove the egg masses of *B. tentaculata* without damaging the eggs, and so no hatching studies were undertaken. Where one member of a pair died during the exposure, the other was sacrificed. The shells of both animals were cracked and removed, and the genders confirmed and recorded. At the end of the exposure, all the remaining snails were re-weighed and the aperture lengths measured before being sacrificed and the gender determined in the same manner. The presence of any parasites was noted.

3.2.3 Experiment 5 – An exposure of *P. corneus* to Bisphenol-A in constant 'simulated autumn' conditions.

Experimental Design

The design of Experiment 5 was essentially the same as that used in Experiment 3 for the exposure made at 15^{o}C, but with the following alterations [rationale indicated in brackets]:

a) The area in the room designated for the exposure incorporated three shelves lit by strip fluorescence light bulbs [because a further 8 exposure tanks were included, but the light intensity was otherwise the same].

b) The snails were housed in groups of nine (1L/snail) [as previous observations had indicated that this did not adversely affect the mortality rate at 15^{o}C, perhaps because the cooler conditions reduced the locomotory activity and therefore the number of encounters occurring between individuals].

c) The feed rate was reduced to 0.1g / snail [because the organisms tend to consume less at the cooler temperature; higher food rates began to pollute the media during acclimation].

d) The baseline period was reduced to one fortnight long cycle [this was considered sufficient to make an assessment of the oviposition rate of the replicate groups].

Preparation of Bisphenol-A concentrations

10 mg of Bisphenol-A (4,4'-Isopropylidenediphenol, CAS number 80-05-7, >97% purity, Acros Organics) was dissolved in 1L of water that had been filtered through activated carbon and reverse osmosis filters. The initial suspension was sonicated for approximately 2 hours to break up the pellets before being stirred for 24 hours on a magnetic stirrer. It was then filtered through a 0.2 um membrane to remove any solid residue. This Stock Solution was stored at 4^{o}C

(±2) in the dark. It was replaced fortnightly with the excess being disposed via a chemical disposal route.

Volumes of 0.2, 2 or 20 mls of the Bisphenol-A stock solution were added to 10L of media to make nominal exposure concentrations of 0.2, 2 or 20 μgL^{-1}. The numbered exposure tanks were allocated as either control, 0.2, 2 or 20 μgL^{-1} Bisphenol-A by means of a random number table. The final layout of the exposure tanks is shown in Figure 23. At each partial media change, 0.12, 1.2 or 12 mls of the stock solution was added to the 6L of fresh media that was used to make the replacement. An assumption was made that the 3 litres of media remaining in the tanks would not experience an appreciable amount of degradation. This was indicated by a stability study that preceded the exposure (where the slightly higher concentrations of 2.5 and 25 μgL^{-1} were selected; see Chapter 6 for details).

However, it was noted that measured concentrations were lower than expected at the end of the cycles, perhaps due to the adaptation of the microbial presence to the use of Bisphenol-A as a substrate. Therefore at the half-way point of the exposure (4 weeks) the volume of Stock Solution added at each partial media change was increased by 25% to 0.15, 1.5 or 15 mls to 6L. The concentration of Bisphenol-A remaining in the discarded media was considered acceptable for disposal to the foul drain once diluted with the control media, except in the case of the highest concentration (20 μgL^{-1} nominal) and this media was passed over an activated carbon filter to remove it (according to Bautista-Toledo et al., 2005) before being similarly disposed.

Chemical sampling

Equally sized aliquots were taken from each exposure tank prior to, and after, media changes and pooled to make a single 1L sample from each concentration. This procedure was performed at the start, mid-point and end of each exposure cycle. The samples were preserved by the addition of 1ml of a 250 gL^{-1} copper sulphate solution and 1ml of concentrated hydrochloric acid to halt biological

degradation, before being shipped on ice to the Environment Agency NLS laboratory for analysis.

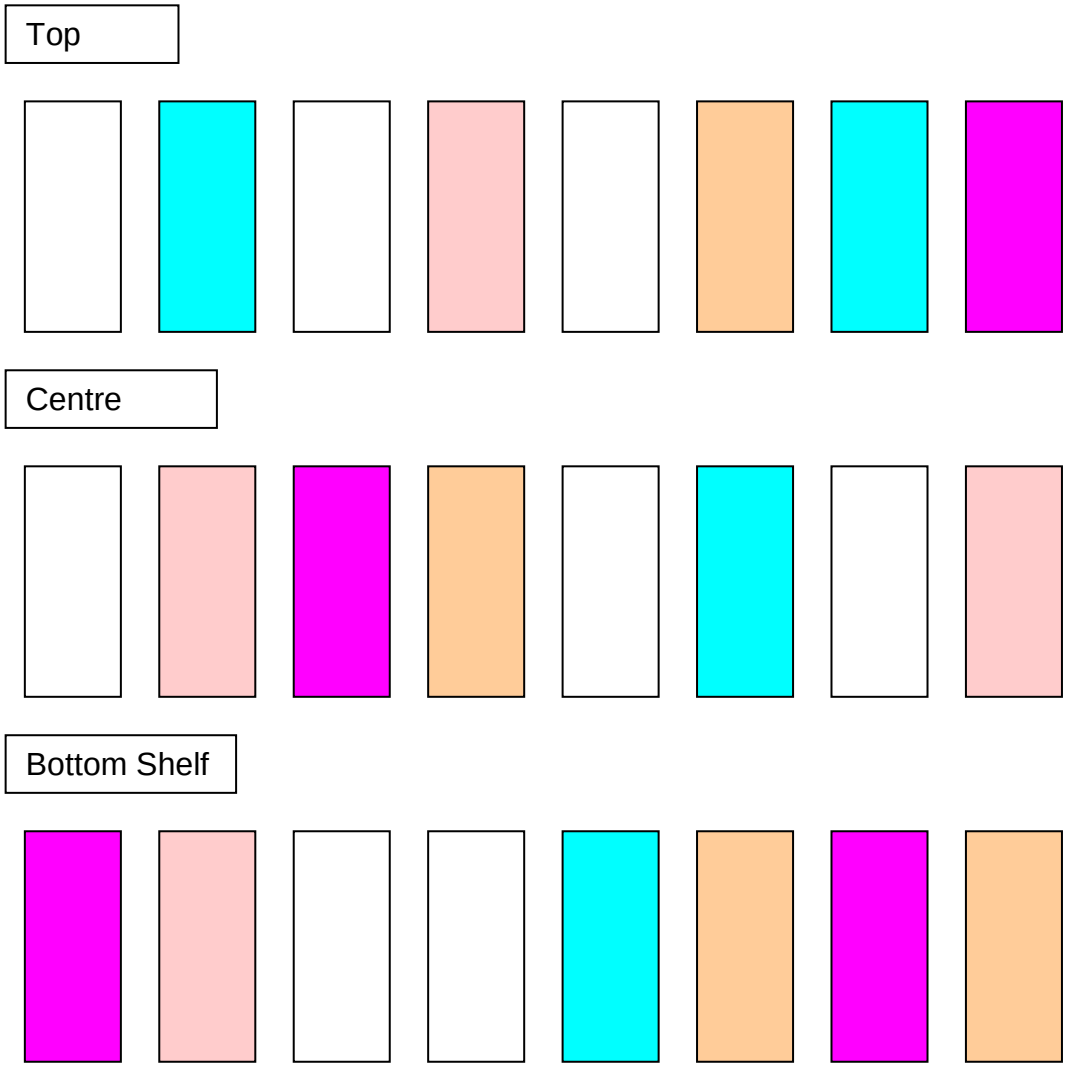


Figure 23 – A schematic layout of the control and nominal Bisphenol-A replicates in Experiment 5 (blue = control, salmon = 0.2 µgL^{-1}, pink = 2 µgL^{-1} and magenta = 20µgL^{-1}). The white tanks were not used in the exposure.

Test Organisms

Approximately one quarter of the test organisms were F1 snails drawn from the laboratory culture. One further quarter was obtained from 'Blades' Biological Supply (Kent) and the remaining half from 'Sciento' Biological Supply (Manchester). All of the organisms were allowed to intermix in a 100-litre holding tank filled with APW. It should be noted that a malfunction of the

photoperiod controls in the constant-temperature room and a period of operator sickness contributed to an unplanned acclimation period of approximately 10 weeks at 15^{o}C. At the start of the experiment on the 3rd April 2006, individuals were drawn out, weighed and measured in the longest axis, and randomly allocated to the exposure tanks using a random number table. No *P. corneus* less than 18mm long was used in the experiment and a record was kept of the statistics of each animal allocated.

End-points

The end-points of this exposure were identical to those in Experiment 3.

3.2.4 Experiment 6 – An exposure of *P. corneus* to Bisphenol-A in changing conditions to simulate the onset of 'autumn'.

Experimental Design

The design of Experiment 6 was essentially the same as that used in Experiment 3, but with the following alterations [rationale indicated in brackets]:

a) Two air-conditioned rooms were programmed to hold the same temperature. This was set at 19^{o}C for the 4 week baseline period. Once the exposure began the temperature was reduced by 1^{o}C per week over 4 weeks. The final temperature of 15^{o}C was then held for a further 4 weeks.

b) Similarly, the photoperiod in both rooms was set at 16 hours light / 8 hours dark for the duration of the 4 week baseline period. Once the exposure began the light phase was reduce by 1 hour per week over 4 weeks. The final photoperiod of 12 hours light/12 hours dark was then held for a further 4 weeks.

c) The water temperature in each test tank was measured with an externally calibrated platinum resistance thermometer every 48 hours prior to each media change [rather than monitor the room temperature in a separate vessel].

d) The snails were housed in groups of 9 (1L/snail) as in Experiment 5. The feed rate was 0.25g / snail / 48 hours [as in Experiment 3] for the duration of the baseline period, and was then reduced to 0.1g / snail / 48 hours [as in Experiment 5] for the duration of the exposure.

e) The number of replicates used for the control and at each test concentration was increased to 10 [in an effort to reduce the high between-replicate variation previously observed].

Preparation of Bisphenol-A concentrations

A Stock Solution of 12 mgL^{-1} of Bisphenol-A was made in the same way as that described in Experiment 5. Volumes of 0.15, 1.5 or 15 mls of the stock solution were added to 9L of media to make the nominal exposure concentrations of 0.2, 2 or 20 μgL^{-1}, this being reduced to 0.1, 1 or 10 mls in 6L when the media was only partially changed. After the baseline phase of the experiment was complete, the exposure tanks were allocated as either control, 0.2, 2 or 20 μgL^{-1} Bisphenol-A (nominal) following seven different allocations on paper by means of a random number table. The allocation providing the highest equality of variance between treatment groups was selected for the final layout of the exposure tanks (Bartlett's test, $p = 0.869$), and this is shown in Figure 24.

Chemical sampling

Samples were taken for chemical analysis weekly, either by sampling a 100ml aliquot from each exposure tank 24 hours after a partial media change (i.e. the midpoint between partial media changes) or by sampling a 50ml aliquot from each tank immediately prior to, and after, the partial media change. The method of sampling depended on the day of the week on which the samples were taken. All aliquots within each concentration were pooled for analysis except on two occasions, when 500 ml samples were taken from each tank for individual analyses to assess the between-replicate variability. Each 1L sample was preserved by the addition of 1ml of a 250 gL^{-1} copper sulphate solution and 1ml

of concentrated hydrochloric acid, before being shipped to the Environment Agency NLS laboratory for analysis.

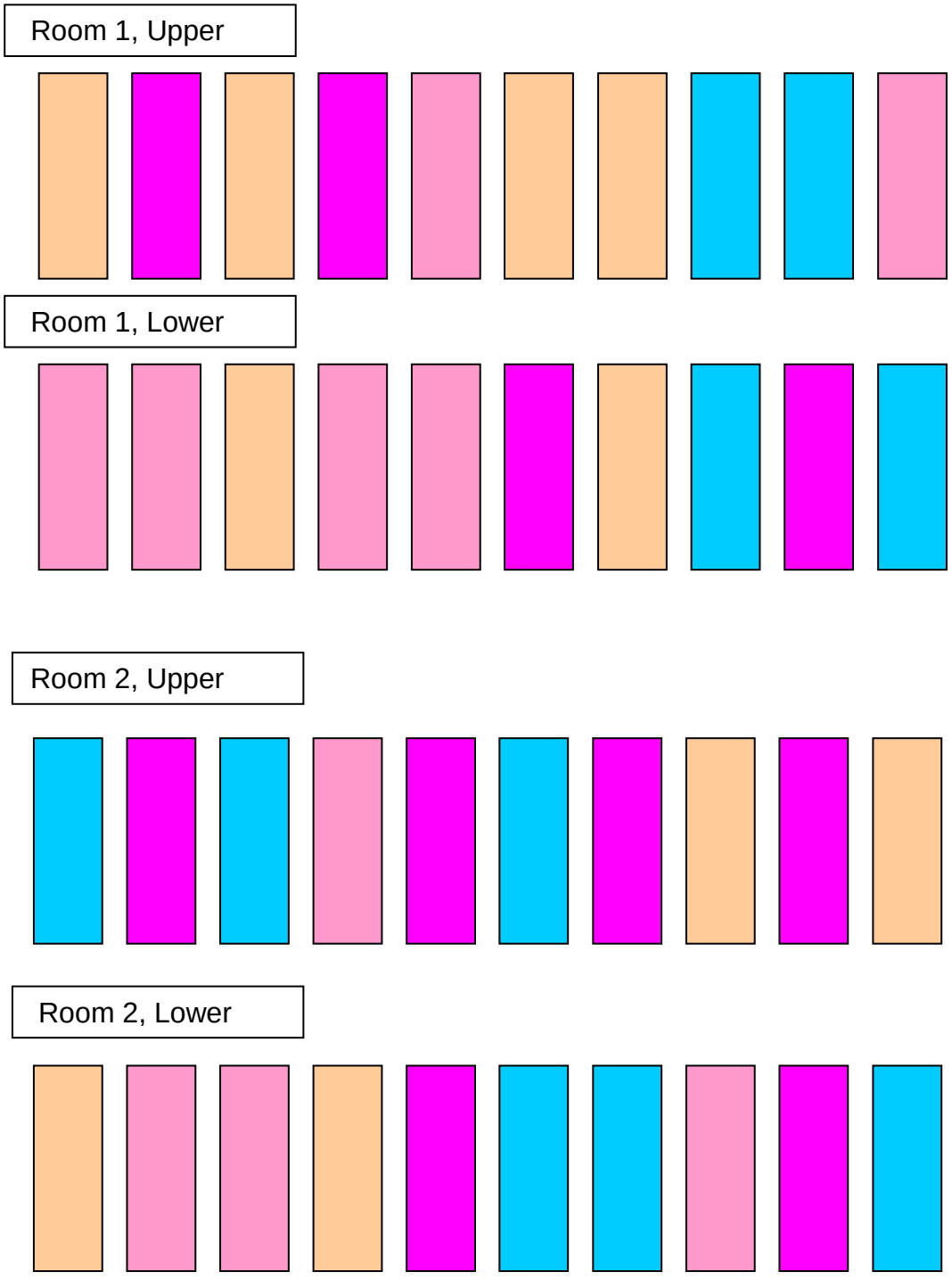


Figure 24 – A schematic layout of the control and nominal Bisphenol-A replicates in Experiment 6 (blue = control, salmon = 0.2 μgL^{-1}, pink = 2 μgL^{-1} and magenta = 20μgL^{-1}).

Test Organisms

All of the test organisms were obtained from 'Sciento' Biological Supply (Manchester). All were allowed to intermix in a 100-litre holding tank at 15^{o}C with a 12 hour light / 12 hour dark photoperiod (commensurate with outdoor conditions) prior to individuals being drawn out, weighed and measured in the longest axis, and allocated to the exposure tanks using a random number table until each tank contained nine organisms. No *P. corneus* less than 18mm long was used in the experiment and a record was kept of the statistics of each animal allocated. After a two-week period, the temperature was raised to 19^{o}C and the photoperiod was extended to 16 hours light / 8 hours dark, and the experiment began on 11th October 2007.

End-points

The end-points of this exposure were identical to those in Experiments 3 and 5, excepting that no assessment of egg hatching success was made.

3.2.5 Experiment 7 – An exposure of *P. antipodarum* to Bisphenol-A in changing conditions to simulate the onset of 'summer'.

Experimental Design

The first 8 weeks of this experiment was as far as possible performed in accordance with the draft OECD SOP entitled 'Reproduction Test with the Prosobranch Snail *P. antipodarum* for Testing Endocrine Active Chemicals' (Schmitt et al., 2006). This specifies an 8-week exposure at 16^{o}C. Therefore an air-conditioned room with insulated walls and doors and without windows was set at 16^{o}C for the first 8 weeks. After this period, the temperature was raised by 1^{o}C per week over another 4 weeks to end at 20^{o}C to simulate the onset of summer. The area in the room designated for the exposure was a shelf lit by Phillips 'Fluotone' strip bulbs emitting cool white light with a measured lux value of 1000 at the height of the water surface. The photoperiod was automatically controlled to be 16 hours of light and 8 hours of dark as specified by the SOP,

and the functioning of the photoperiod was checked monthly by manual forwarding of the control clock to ensure switching.

The snails were housed in groups of 80 in beakers with a working volume of 1L as specified in the SOP. However, the medium used was 800ml of APW per vessel rather than the reconstituted medium recommended in the SOP (reconstituted 'Tropic Marin Sea Salts'; $0.15gL^{-1}$ and $NaHCO_3$; $0.09gL^{-1}$). Each beaker was numbered consecutively and covered with a watch glass. All the media was replaced at 48 hour intervals by tipping off; the snails tended to simply close their opercula for the period that they were out of water. The vessels were rinsed with chlorinated tap water and wiped with a clean cloth in the early part of the experiment, but required scrubbing with paper towels to remove the biofilm as the exposure progressed. The water temperature in each control vessel was measured with a mercury thermometer every 48 hours prior to the media being changed, and the replacement media was stored in the same constant temperature facility for 24 hours prior to use. Also prior to each media change, a 100-200ml sample was taken from each control vessel and the pH, dissolved oxygen, conductivity and hardness was measured as previously described.

Preparation of Bisphenol-A concentrations

16 mg of Bisphenol-A (Acros Organics) was dissolved into 1L of water that had been filtered through activated carbon and reverse osmosis filters. The solution was sonicated for approximately 2 hours to break up the pellets before being stirred for 24 hours. It was replaced weekly with the excess being disposed via a chemical disposal route. Volumes of 37.5ul, 375ul or 3.75ml of the stock solution were added to 3L of media to make the nominal exposure concentrations of 0.2, 2 or 20 μgL^{-1} (only three concentrations were tested in contravention of the minimum of 5 concentrations specified in the SOP). There were 3 replicate vessels for the control and each of the test concentrations (one more than the minimum of 2 replicates specified by the SOP). The numbered exposure vessels were allocated as either control, 0.2, 2 or 20 μgL^{-1} Bisphenol-A (nominal), as shown in Figure 25.

Chemical sampling

Weekly samples were taken for analysis, following a media change. The discarded media was pooled at each nominal concentration and a 1L sample was taken (48 hours after the Bisphenol-A was added). This was because it would have been necessary to take a 333ml aliquot from each vessel had samples been taken between the media changes, and as this comprises a large proportion of the test media volume (>40%), it was not considered an acceptable approach due to the effect on stocking density. The samples were preserved by the addition of 1 ml of a 250 gL^{-1} copper sulphate solution and 1ml of concentrated hydrochloric acid to halt biological degradation, before being shipped to the Environment Agency NLS laboratory for analysis.

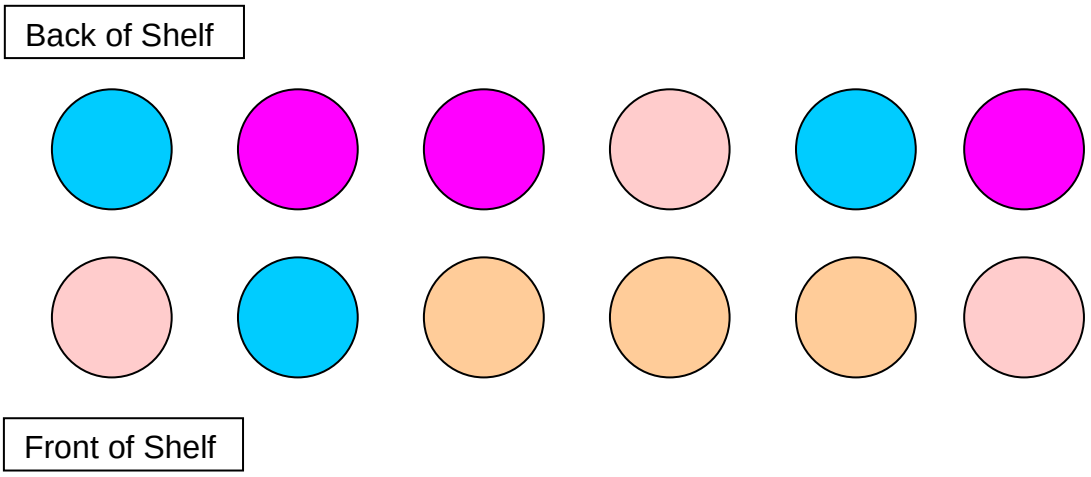


Figure 25 – A schematic layout of the control and nominal Bisphenol-A replicates in Experiment 8 (blue = control, salmon = 0.2 μgL^{-1}, pink = 2 μgL^{-1} and magenta = 20μgL^{-1}).

Test Organisms

The test organisms were taken from laboratory cultures established 5 years previously. The original animals were collected from a stream in Great Dunmow, Essex (NGR TL595220). The length of the organisms used ranged from 3.6 to 4.6mm in the longest axis (measured at x4 magnification under a low power stereo-microscope and using a micrometer), which is slightly wider range than that specified in the SOP (3.7 to 4.3mm). The snails were fed a finely ground

mixture of dried algal discs (Algal Wafers, Hikari, Kyorin, Japan) and calcium enriched pellets (Crab Cuisine, Hikari, Kyorin, Japan) at a ratio of 1 disc to 1 pellet, as a replacement for 'Tetraphyll' suggested by the SOP. The feed rate was 0.4 mg mixture/snail following 48 hour each media change, not 2 ug/snail/day as specified in the SOP (later found to be an error in the SOP).

End-points

A sub-sample of 20 snails was randomly taken from each replicate at 2, 4, and 8 weeks after the exposure began. These snails were narcotised for 45 to 90 minutes in a 2.5% solution of magnesium chloride. They were measured in the longest axis at x4 magnification under a low power stereo-microscope. The shells were then carefully cracked with tweezers and the embryos were dissected out of the brood pouch under x8 magnification, and counted. Shelled and unshelled embryos were not distinguished as advised by the SOP because the dissection process caused the shell of some of the embryos to break open. At 12 weeks after the start of the exposure and once the temperature had been raised to 20^{o}C, the remaining animals were dissected in the same manner.

3.2.6 Experiment 8 – An exposure of *B. tentaculata* to Bisphenol-A in changing conditions to simulate the onset of 'summer'.

Experimental Design

This experiment was performed concurrently with Experiment 7, in the same air-conditioned room set at 16^{o}C, with a 16 hour light / 8 hour dark photoperiod. These conditions were held for the first 4 weeks of the exposure which began at Week 5 of Experiment 7, and after this period the temperature was raised by 1^{o}C per week over the following 4 weeks, ending at 20^{o}C. The area in the room designated for the exposure were two shelves lit by a Phillips 'Fluotone' strip bulb emitting cool white light with a measured lux value of 750 at the height of the water surface. For the second 4 week period, another Phillips 'Fluotone' strip bulb was fitted to each shelf to increase the lux value at the water surface to 1000.

The snails were housed in groups of 40 in beakers containing APW with a working volume of 2L. Each beaker was numbered consecutively and covered with a watch glass. The media was completely replaced at 48 hour intervals by tipping off, and all the aeration, media monitoring arrangements and the vessel cleaning procedures were as for Experiment 7.

Preparation of Bisphenol-A concentrations

A 16 mgL^{-1} solution was made in the same way as for Experiment 7, and volumes of 25ul, 250ul or 2.5ml of the stock solution were added to 2L of media to make the nominal exposure concentrations of 0.2, 2 or 20 μgL^{-1} Bisphenol-A. There were 5 replicate vessels for the control and each of the test concentrations. The numbered exposure vessels were randomly allocated to either control, 0.2, 2 or 20 μgL^{-1} Bisphenol-A (nominal) treatments, as shown in Figure 26.

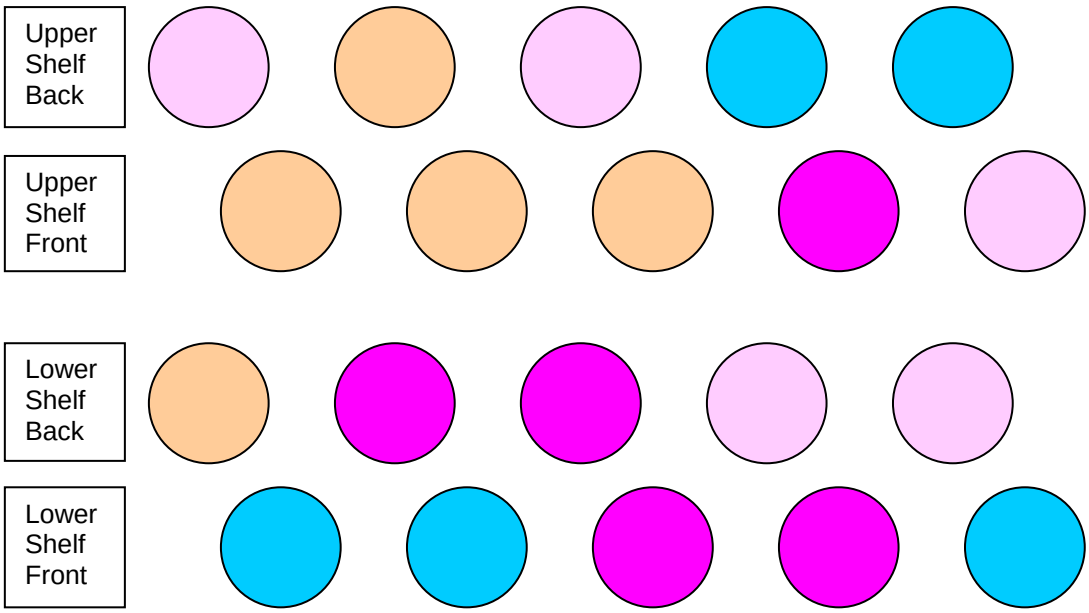


Figure 26 – A schematic layout of the control and nominal Bisphenol-A replicates in Experiment 7 (blue = control, salmon = 0.2 μgL^{-1}, pink = 2 μgL^{-1} and magenta = 20μgL^{-1}).

Chemical sampling

Weekly samples were taken for chemical analysis, in the same manner as for Experiment 7, and indeed because Experiments 7 and 8 ran concurrently for part of the exposure period (from Week 5 of Experiment 7), the discarded media from both exposures were pooled prior to taking a sub-sample for chemical analysis.

Test Organisms

All animals were collected from the Lewes Brooks, Lewes, W.Sussex (NGR TQ419106) in late February and early March. All the animals were allowed to intermix in a 100-litre holding tank before individuals were drawn out, weighed and measured in the longest axis, and randomly allocated to the exposure vessels using a random number table. No *B. tentaculata* less than 7mm long was used in the experiment and a record was kept of the statistics of each animal allocated. The snails were fed on concentrated algal cells (*C. vulgaris*, see Appendix 1 for details) constituting 0.15 mg of total organic carbon (TOC) / snail / day delivered in 20mls of media, after each media change, during the two-week acclimation period. The exposure began on the 27th March 2008 and this feed rate was continued for the first 4 weeks. Over the second 4 weeks, this was increased by 0.0375 mg TOC / snail / day each week in an additional 5mls to culminate in double the original feed rate (0.3 mg TOC / snail / day in 40mls). At the end of the exposure period, up to 16 litres of algal culture was being produced per week prior to being concentrated by centrifuge to the required density.

End-points

The end-points of this exposure were identical to those in Experiment 4, except that any mortalities occurring were simply removed at each partial media change.

3.3 Analytical Chemistry

17β-Oestradiol and Oestrone

All analysis was performed by the Environment Agency NLS (Nottingham). The steroids were then desorbed from the SPE columns with dichloromethane. Extracts were then concentrated and cleaned-up using gel permeation chromatography fractionation followed by a solvent exchange to 95/5v/v isohexane/propan-2-ol and then cleaned up again using normal phase chromatography in an amino LC column. The extract was then evaporated to incipient dryness, re-dissolved in methanol and analysed using High Performance Liquid Chromatography with negative ion atmospheric photo-ionisation interface and Mass Spectrometry with time-of-flight detection. The mean recovery from river water was 87.7% ±2.9 and the stated uncertainty was 29.2%.

Bisphenol-A

The compound was desorbed from the SPE columns with dichloromethane. Extracts were then concentrated and derivatised using trifluroacetic anhydride before analysis using Gas Chromatography with Mass Spectrometry Electron Ionisation in secondary ion mass spectrometry mode. The mean recovery from river water was 92.0% ±5.4 and the stated uncertainty was 30.5%.

3.5 Statistical Analysis

Where the data met the assumptions of normality and the variances were found to be homogeneous, means were compared using One Way Analysis of Variance (ANOVA) followed by a Dunnett's test for comparison with the reference data. Where the effects of a co-variable such as snail size was assessed, a One Way Anaysis of Co-Variance (ANCOVA) was used. Where two means only were being compared, a two-tailed Student's t-test was employed. Where two means were compared from pre-exposure (baseline) and exposure periods in the same

replicate, paired Student t-tests (two-tailed) were used following a Repeated Measures ANOVA.

Where data was not normally distributed or the variances were heterogeneous, a Kruskal-Wallis One Way Analysis of Variance on Ranks was used, followed by a Steel's Many-One Rank test for comparison with the reference data. Where only two means were compared, a Mann-Whitney U-test was used.

To assess the strength of association between dose and response, or to test any other correlations, a Pearson Product Moment Correlation Co-efficient was employed.

CHAPTER 4
REPRODUCTIVE EFFECTS OF EXPOSURE TO 17β-OESTRADIOL IN THE PULMONATE GASTROPOD *P. corneus.*

4.1 Introduction and Methodology

This chapter presents the results of two outdoor mesocosm-based exposures of the pulmonate gastropod *P. corneus* to 17β-oestradiol (Experiments 1 and 2) and also a laboratory exposure of this steroid, made under two different temperature an photoperiod regimes intended to simulate summer and autumn conditions (Experiment 3).

The methods and experimental design used for the mesocosm exposures are set out in Chapter 3, Section 3.1. The first mesocosm exposure (Experiment 1; 2004) had several unexpected outcomes; the measured concentrations of 17β-oestradiol were much higher than expected as was the test organism mortality rate. Consequently, for the second exposure (Experiment 2; 2006), the 17β-oestradiol concentrations were reduced by half. In Experiment 1, the influent water for the mesocosm system was passed though an activated carbon pre-filter to remove any oestrogenic background content, but this may have also removed nutrients and contributed to the poor survival of the test organisms, so the filter was not used in Experiment 2. The hypothesis for both of the mesocosm exposures is: 'Exposure to 17β-oestradiol will significantly alter the reproductive rate of the gastropod *P. corneus*, causing deviation from the seasonal pattern of reproduction observed in the reference groups'.

The method and experimental design used in the laboratory exposure (Experiment 3) is set out in Chapter 3, Section 3.2.1. The hypothesis for this experiment is: 'Exposure to 17β-oestradiol under controlled temperature and photoperiod conditions that represent a simulated summer and/or autumn will significantly alter the number of eggs produced by *P. corneus* relative to the control'.

4.2 Results and Discussion

4.2.1 Mesocosm Experiment 1 (2004)

4.2.1.1 Steroid Analysis

Table 2 shows the results of the 17β-oestradiol analysis for the samples taken from the three mesocosm tanks (control, 20 ngL^{-1} and 200 ngL^{-1}, nominal). The results of the oestrone analysis are also given, as this is the primary biotransformation product of 17β-oestradiol and is also known to be oestrogenic in vertebrates (e.g. Thorpe et al., 2003b). Measurements of 17α-ethinylestradiol were additionally performed as part of the routine analysis suite, and this was not found on any occasion.

Table 2 – The results of the 17β-oestradiol and oestrone analysis of samples taken from the mesocosm tanks, 2004 (LOD = 1 ngL^{-1}).

Date of Sampling	17β-oestradiol (ngL^{-1} nominal)			Oestrone (ngL^{-1} nominal)		
	0	20	200	0	20	200
14 June 2004	6.14	89.9	104	2.44	39.5	67.3
20 July 2004	14.9	23.1	259	3.40	3.56	52.0
17 August 2004	10.0	51.2	228	6.81	14.1	70.6
13 September 2004	20.3	28.1	889	<1.0	<1.0	242
Mean	**12.8**	**48.1**	**370**	**4.22**	**19.1**	**108**
Standard Deviation	6.13	30.5	352	2.30	18.5	90.0

The levels of 17β-oestradiol and oestrone found in the reference tank was unexpected, but was confirmed by additional analysis undertaken by the Centre for Fisheries, Environment and Aquaculture Sciences (CEFAS) on three sampling occasions at least two weeks apart. The results of the CEFAS analysis were 3.7, 5.2 and 9.2 ngL^{-1}.

The result of the 17β-oestradiol analysis from the higher nominal exposure tank (200 ngL^{-1}) on the last sampling occasion was also unexpectedly high (889 ngL^{-1}), but this too was confirmed by CEFAS in a sub-sample taken from the same pool as that sent to the NLS. The results of the CEFAS analysis was 1120 ngL^{-1}.

Unfortunately the high level of 17β-oestradiol measured in the lower nominal exposure tank (20 ngL^{-1}) on the first sampling occasion (89.9 ngL^{-1}) was not confirmed by a second laboratory. High levels was also measured in this tank on the third sampling occasion (51.2 ngL^{-1}), confirmed by CEFAS, again from the same sample pool. The CEFAS result was 53 ngL^{-1}.

The mean measured concentrations of 17β-oestradiol were significantly different to one another (Mann-Whitney U-tests between 12.8 ngL^{-1} (reference) and 48.1 ngL^{-1} (nominal 20 ngL^{-1}), p= 0.03, and between 48.1 ngL^{-1} (nominal 20 ngL^{-1}) and 370 ngL^{-1} (nominal 200 ngL^{-1}); p=0.03). This means that the three measured concentrations can be considered as discreet from one another.

However as 17β-oestradiol was measured in the reference tank at concentrations up to 20.3 ngL^{-1}, an absolute control is not available for intra-comparison in this experiment, On two occasions CEFAS also measured appreciable levels of 17α-ethinylestradiol in this tank (results were 0.9 and 0.7 ngL^{-1}). The measured oestrone levels more or less tracked the 17β-oestradiol results, always being lower as might be expected for a biotransformation product of the parent compound.

4.2.1.2 Water Quality

Figure 27 shows the weekly mean measured water temperatures in the mesocosm tanks, together with the minimum and maximum temperatures recorded in that week, and the minutes of daylight between sunrise and sunset (U.S. Navy data for London). The exposure of *P. corneus* ran from Weeks 1 to 12 inclusive and the

pre-exposure period was Weeks -2 and -1. No measurements were taken in Week 4, due to difficulty in travelling to the site.

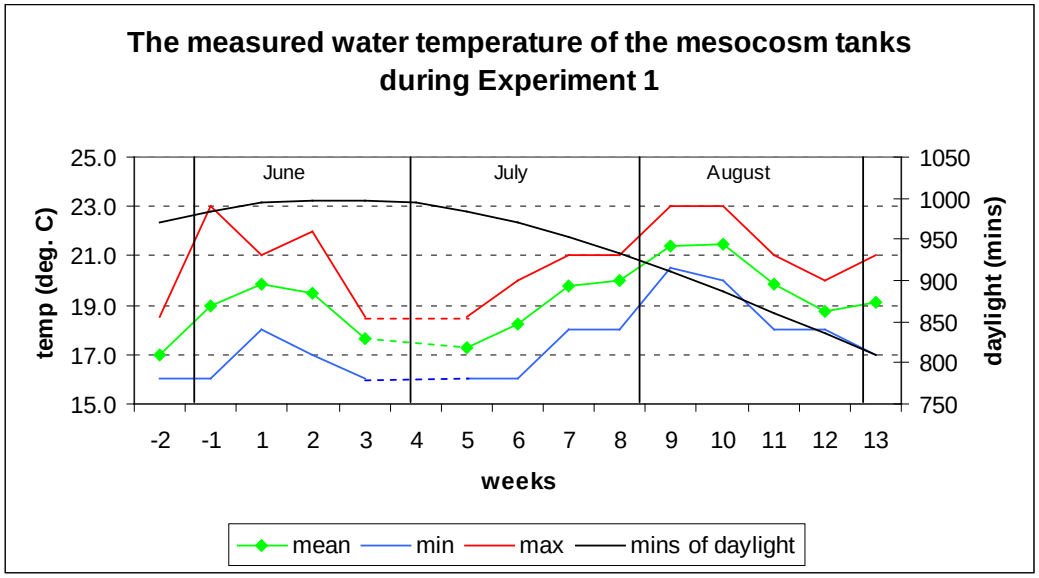


Figure 27 – The mean, minimum and maximum measured water temperatures recorded in the mesocosm tanks and the minutes of daylight during Experiment 1.

4.2.1.3 Test Organism Survivorship

At the beginning of the exposure (Week 0), the mortality in the population allocated to become the higher nominal concentration of 17β-oestradiol had already reached 11.1%, but this was not significantly different from the reference population (ANOVA, $p>0.05$).

At the end of the exposure, the rate of mortality in the reference population had reached 20.4%. A mortality rate of 20% among control organisms is usually considered acceptable in invertebrate exposures (e.g. OECD, 2008). The group exposed to the lower concentration of 17β-oestradiol (20 ngL^{-1}, nominal) had similar (slightly lower) rates of mortality over the course of the exposure. However the group exposed to the higher concentration of 17β-oestradiol (200 ngL^{-1}, nominal) showed a significantly higher rate of mortality than the reference population by Week 6 of the exposure (ANOVA, $p = 0.012$), and this persisted

through to the end of the exposure (ANOVA, Week 8, p = 0.028; Week 10, p = 0.013; Week 12, p = 0.010; Figure 28). The mortality rate of this group had reached 44.4% at the end of the exposure. The extrapolated first quartile percentage lethal dose of 17β-oestradiol (LC_{25}) over the twelve-week exposure based on the mean measured concentrations of 17β-oestradiol was 319 ngL^{-1} (linear interpolation, data insufficient to generate confidence interval).

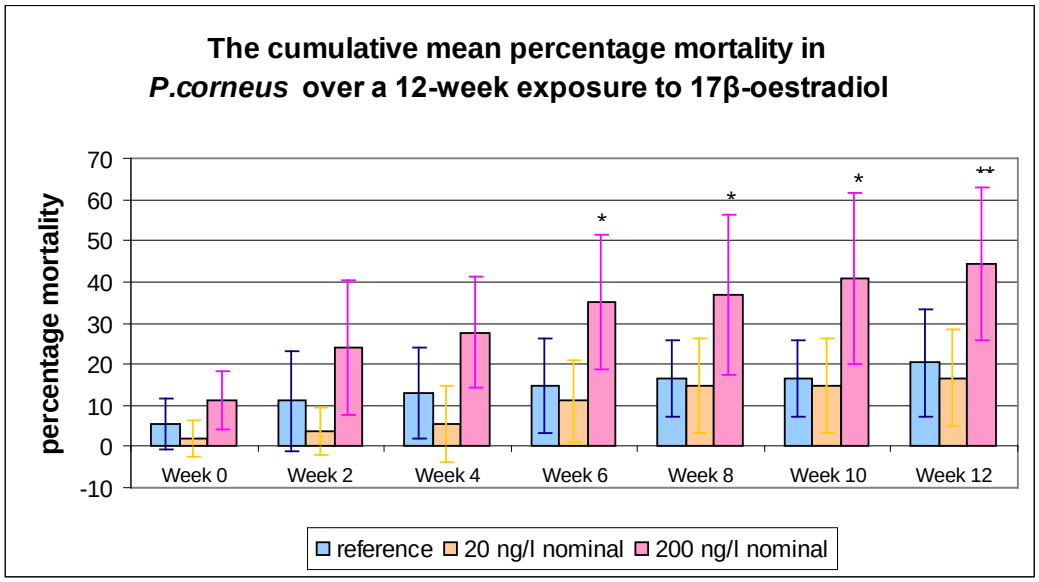


Figure 28 – The cumulative mean mortality rate of *P. corneus* over a 12-week exposure to 17β-oestradiol (error bars represent the standard deviation, asterisks indicate a significant difference from the reference group *p<0.05, **p<0.01).

4.2.1.4 Test Organism Morphology and Growth

There were no significant differences in the mean length in the longest axis of the snails allocated to the three mesocosm tanks at the start of the experiment (ANOVA, p>0.05). However there was a significant difference in the mean weight of the snails, with the weight of the group randomly allocated to the higher concentration of 17β-oestradiol (2.05g ± 0.18) being significantly heavier than the reference population (1.68g ± 0.20; ANOVA, p = 0.004).

Figure 29 shows the mean percentage increase in shell diameter (in the longest axis) and weight during the 12-week exposure. The mean increase in the shell diameter of the reference population was 17.8%. This was significantly higher than that of the group exposed to the lower concentration of 17β-oestradiol (10.6%, ANOVA, p = 0.012). The mean growth of the group exposed to the higher concentration was slightly higher (20.3%), but not significantly so. The mean increase in weight was 60.8% in the reference population. It was lower in the two 17β-oestradiol treatments (38.8% in the lower concentration and 51.5% in the higher concentration), but there were no significant differences (ANOVA, p>0.05).

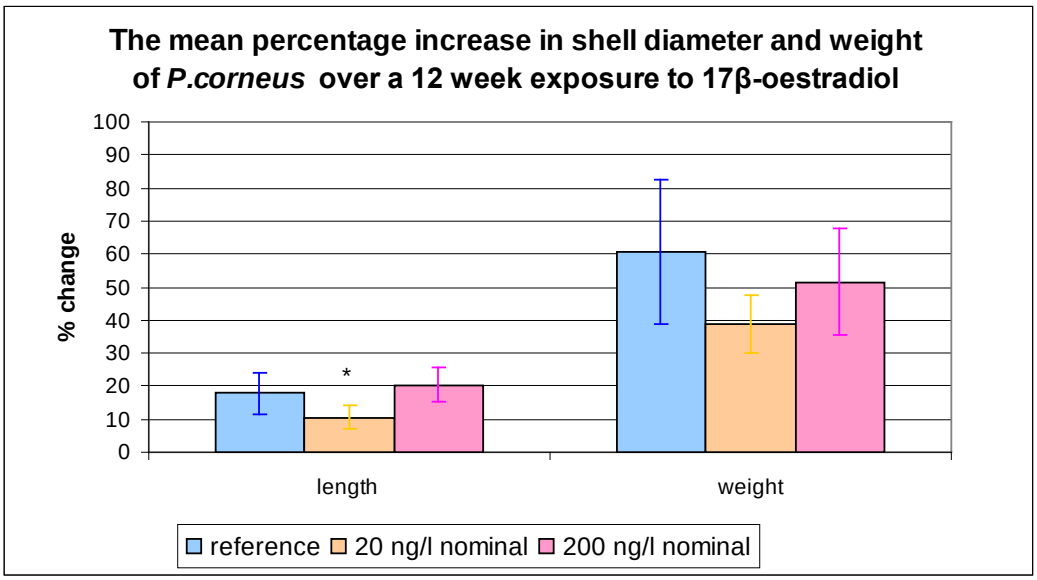


Figure 29 – The mean percentage increase in *P. corneus* length and weight during a 12-week exposure to 17β-oestradiol (error bars represent the standard error of the mean, asterisk indicates a significant difference from the reference group at *p<0.05).

At the end of the experiment, once the shells were cracked and removed, it was determined that there was no significant difference in the ratio of flesh weight to shell weight between the reference and 17β-oestradiol treatments (ANOVA, p>0.05), and that there was also no significant difference in ratio of albumin

gland weight to flesh weight between the reference and 17β-oestradiol treatments (ANOVA, $p>0.05$).

4.2.1.5 Test Organism Reproduction

As the group allocated to the higher concentration of 17β-oestradiol were significantly heavier than the other groups, it might be expected that these snails would have a higher rate of reproduction, but there was no significant difference in the mean number of egg masses or eggs per snail produced between this group and the reference population during the pre-exposure (baseline) period (ANOVA, $p>0.05$). In fact this group was slightly less productive at this time (see Figure 30). Interestingly though, the mean weight of the egg masses produced was significantly greater in this tank (ANOVA, $p = 0.018$).

Figure 30 shows that at the first sampling point (Week 2), the number of egg masses laid per surviving snail was significantly reduced in both 17β-oestradiol treatments compared to the reference population (ANOVA, $p<0.001$). At this time, the effect appeared to be dose-dependant. Two weeks later (Week 4) the reduction in the oviposition rate had become even more strongly pronounced (ANOVA $p<0.001$), with the group allocated to the lower concentration of 17β-oestradiol (20 ngL^{-1} nominal) laying fewer eggs than those exposed to the higher concentration (200 ngL^{-1} nominal). At Week 6, the snails exposed to the lower concentration of 17β-oestradiol had almost stopped laying egg masses altogether (1 egg mass in 2 weeks between the whole group). The rate of oviposition in the group exposed to the higher concentration of 17β-oestradiol was not reduced to the same extent, but both treatments were still significantly different from the reference population (Kruskall Wallis, $p = 0.001$, Steel's Many-One Rank test, rank sum < critical value for both treatments).

The group exposed to the lower concentration laid slightly more eggs at the next sampling point (Week 8), but still laid less than one egg mass per surviving snail per week (significantly different from that of the reference group, ANOVA, $p =$

0.001). However, because the number of egg masses produced by the snails in the reference tank was falling at each sampling point with the onset of autumn, the oviposition rates of the group exposed to the higher concentration of 17β-oestradiol was by now statistically indistinguishable from the reference group. For the final 4 weeks of the exposure, all the remaining snails had almost ceased reproductive activity (laying less than 0.5 egg masses / snail / week on average in all the mesocosm tanks) and the oviposition rates of the reference and treatment groups were not significantly different (ANOVA, $p>0.05$ at Weeks 10 and 12).

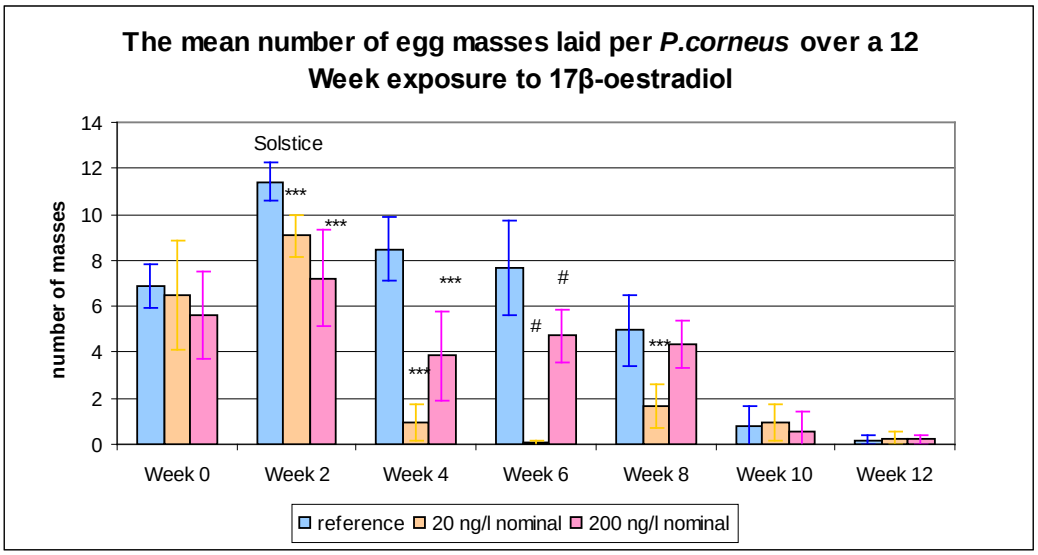


Figure 30 – The mean number of egg masses laid per *P. corneus* during a 12-week exposure to 17β-oestradiol (error bars represent the standard deviation, asterisks indicate a significant difference from the reference group at ***p<0.001, hash indicates a significant difference from the reference group using Steel's Ranking).

Figure 31 shows that despite the decreasing rates of oviposition and irrespective of 17β-oestradiol exposure, the number of eggs deposited within each egg mass remained similar throughout the exposure, at an average of 63.1 eggs per mass in the reference group, 52.0 in the lower 17β-oestradiol treatment (20 ngL^{-1} nominal) and 64.1 in the higher 17β-oestradiol treatment (200 ngL^{-1} nominal).

There are no significant differences between the reference and treatment populations at any sampling point (ANOVA, $p>0.05$ for all weeks except Week 6 when the single egg mass laid in the lower 17β-oestradiol concentration was excluded, t-test between reference and higher 17β-oestradiol concentration, $p>0.05$).

The number of eggs per mass was lower in all three mesocosm tanks during the pre-exposure (baseline) period at an average of 48.7, but this is possibly an artefact of acclimation to mesocosm conditions.

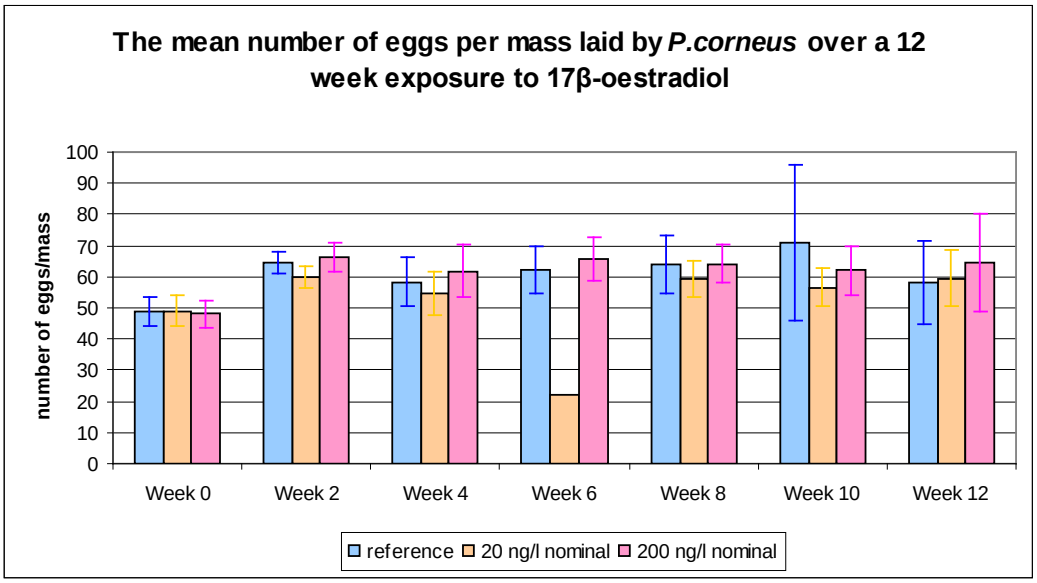


Figure 31 – The mean number of eggs per egg mass laid by *P. corneus* during a 12-week exposure to 17β-oestradiol (error bars represent the standard deviation).

Given that the number of eggs per egg mass was unaffected by exposure to 17β-oestradiol, the mean the number of eggs produced per adult (a more definitive measure of reproduction) follows a very similar pattern of response to that for the number of masses produced per adult (see Figure 32).

At the first sampling point (Week 2), the number of eggs per surviving snail was significantly reduced in both treatment tanks when compared to the reference

population (ANOVA, p = 0.007). This effect was even more highly significant at Week 4 (ANOVA, p<0.001).

At Week 6 both treatments were still significantly different from the reference population (Kruskall Wallis, p = 0.001, Steel's Many-One Rank test, rank sum =/< critical for both treatments). At Week 8 only the group exposed to the lower concentration of 17β-oestradiol (20 ngL^{-1} nominal) laid significantly fewer eggs per snail than the reference group (ANOVA, p<0.001), because the number of eggs laid per snail was declining in the reference group due to the onset of autumn. There were no significant differences between the reference and treatments at Week 10 or Week 12 (ANOVA, p>0.05 in both cases).

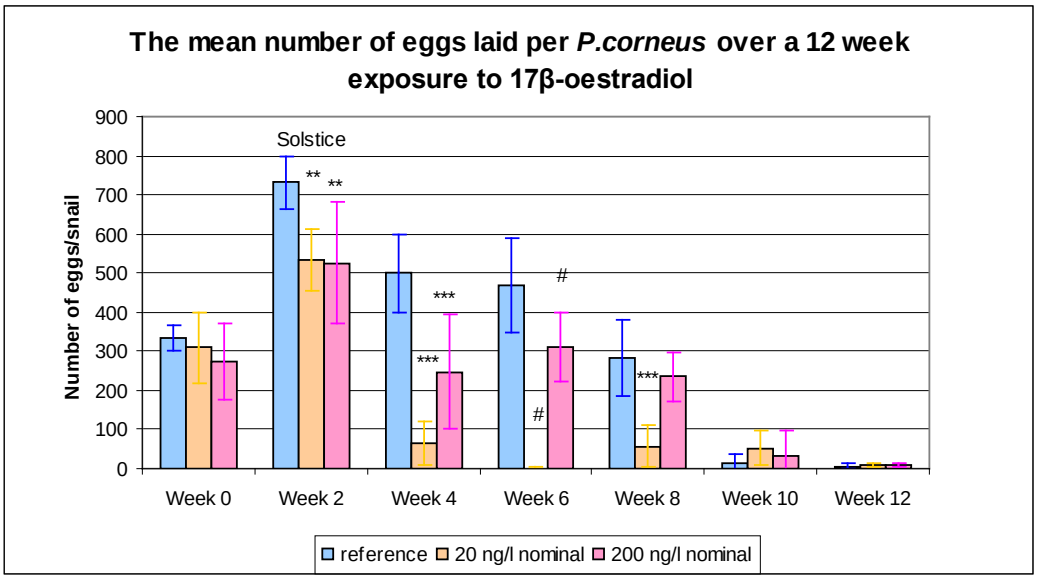


Figure 32 – The mean number of eggs laid per snail during a 12-week exposure to 17β-oestradiol (error bars represent the standard deviation, asterisks indicate a significant difference from the reference group at **p<0.01, ***p<0.001, hash# indicates a significant difference from the reference group using Steel's Ranking).

Figure 33 shows that the mean weight of the egg masses produced was also consistent over the course of the exposure in the main. This was as expected given that the mean number of eggs per mass was consistent. The exception was at the Week 4 sampling point, when the egg masses produced by the group

exposed to the higher concentration of 17β-oestradiol were significantly heavier than those produced by the reference population (Kruskall-Wallis, p = 0.008). The number of eggs per mass did not significantly increase at this time point in this group, so it appears that the increase in weight is due to an increase in egg packaging material. The snails in this group were also producing significantly heavier masses during the pre-exposure (baseline) period. This might be expected given that the snails allocated to this group were significantly heavier than those in the other groups, and this suggests that larger animals may tend to produce heavier masses, notwithstanding exposure to 17β-oestradiol.

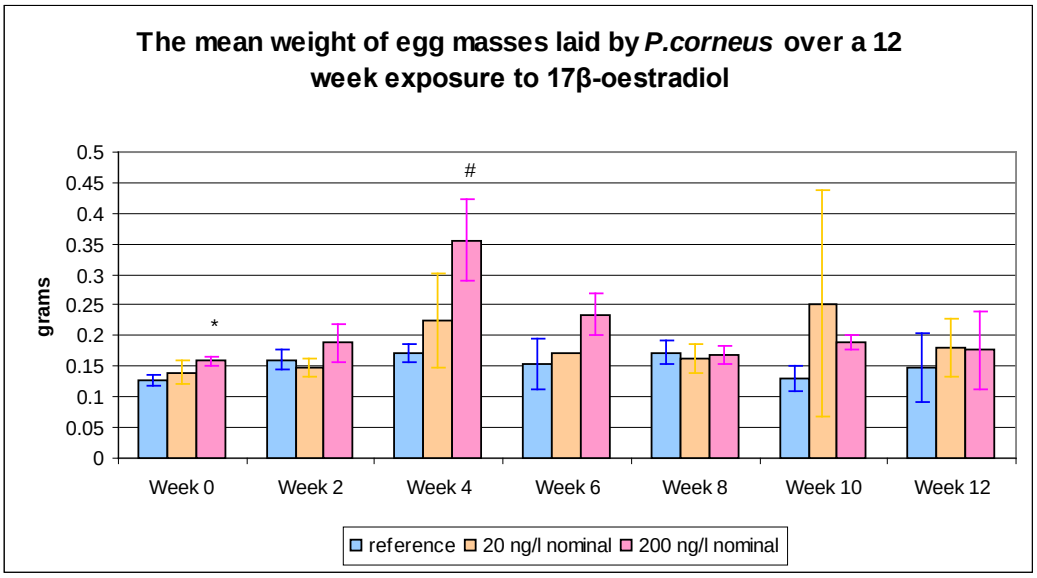


Figure 33 – The mean weight egg masses laid by *P. corneus* during a 12-week exposure to 17β-oestradiol (error bars represent the standard deviation, asterisk indicates a significant difference from the reference group at *p<0.05, hash# indicates a significant difference from the reference group using Steel's Ranking).

As egg mass weight was often consistent, the mean total weight of the egg masses produced per snail follows a similar pattern to that for the number of egg masses and the number of eggs produced per snail (see Figure 34). At the first sampling point (Week 2), the total weight of egg masses per surviving snail was significantly reduced in both treatment tanks compared to the reference population (ANOVA, p = 0.015). This effect was even more highly significant at

Week 4 in the group exposed to the lower concentration of 17β-oestradiol (20 ngL^{-1} nominal, ANOVA, $p<0.001$), but due to the heavier egg masses produced by the group exposed to the higher concentration (200 ngL^{-1} nominal), there was no significant difference between this group and the reference group (i.e. there were less egg masses, but each egg mass was heavier). At Week 6 both treatments were still significantly different from the reference population (Kruskall Wallis, $p = 0.001$, Steel's Many-One Rank test, rank sum =/< critical for both treatments). At Week 8 only the group exposed to the lower concentration of 17β-oestradiol (20 ngL^{-1} nominal) laid a significantly lighter overall weight of egg masses per snail than the reference group (ANOVA, $p = 0.002$) because the number of egg masses laid per snail in the reference group was declining in this group due to the onset of autumn. There were no significant differences between the reference and treatments at Week 10 or Week 12 (ANOVA, $p>0.05$ in both cases).

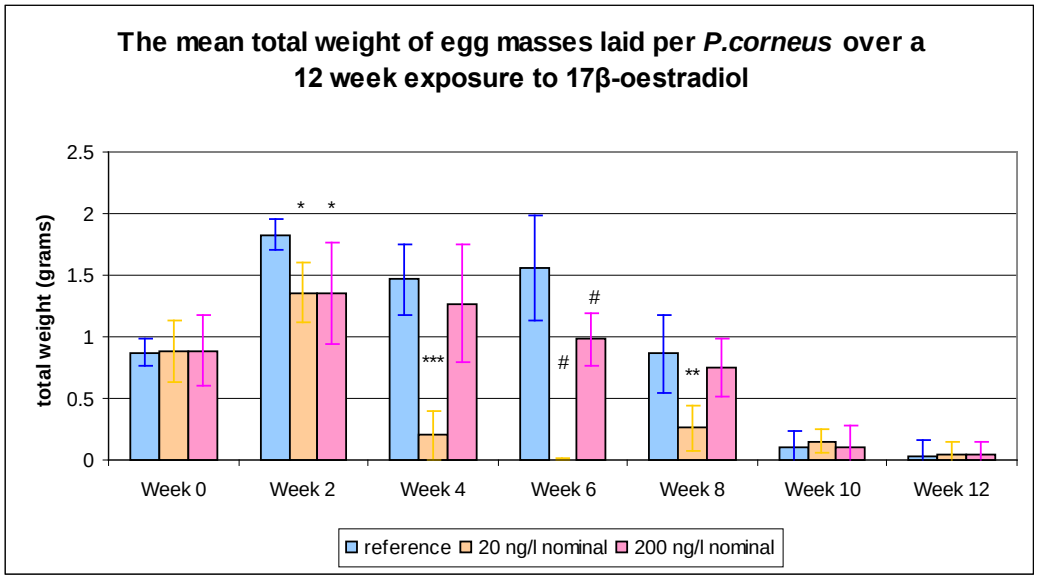


Figure 34 – The mean total weight egg masses laid per *P. corneus* during a 12-week exposure to 17β-oestradiol (error bars represent the standard deviation, asterisk indicates a significant difference from the reference group at *p<0.05, **p<0.01, ***p<0.001, hash# indicates a significant difference from the reference group using Steel's Ranking).

4.2.2 Mesocosm Experiment 2 (2006)

4.2.2.1 Steroid Analysis

The analysis of 17ß-oestradiol and oestrone was performed by the Environment Agency NLS at 4 week intervals, in the same manner as for Experiment 1. The results are set out in Table 3 (where the result was less than the LOD, half of this value is taken to calculate the mean).

Table 3 – The results of the 17β-oestradiol and oestrone analysis of samples taken from the mesocosm tanks, 2006 (LOD = 0.3 ngL^{-1} for 17β-oestradiol and 1 ngL^{-1} for oestrone).

Date of Sampling	17ß-oestradiol (ngL^{-1} nominal)			Oestrone (ngL^{-1} nominal)		
	0	10	100	0	10	100
02 June 2006	5.71	ND	232	<1.0	ND	0.6
05 July 2006	<0.3	28.6	41.7	1.05	15.0	5.70
04 August 2006	<0.3	1.59	6.38	<1.0	1.30	5.20
04 September 2006	<0.3	2.84	1.25	<1.0	1.46	<1.0
Mean	**1.54**	**11.0**	**70.3**	**0.638**	**5.92**	**10.5**
Standard Deviation	2.78	15.2	109	0.275	7.86	13.6

This table shows that the 17ß-oestradiol levels in the reference tank were approximately the same at the beginning of this exposure as had been measured at the start of Experiment 1, despite changing the supply river. The first measured value in the higher 17ß-oestradiol exposure tank (100 ngL^{-1} nominal) was more than twice that expected, but unfortunately the tube delivering the 17ß-oestradiol solution to the lower exposure tank (10 ngL^{-1} nominal) had become blocked the day prior to the samples being taken and therefore no result was recorded on this occasion. However the measured value in this tank was also twice that expected at the second sampling event.

After this time, the measured concentrations of 17ß-oestradiol declined sharply. Indeed in the higher exposure tank this process began before the Week 8 sampling point, with only 41.7% of the nominal value being measured in this sample. On the third sampling occasion (Week 12), only 15.9% and 6.38% of the nominal concentrations were measured in the lower and higher exposure tanks respectively, and on the fourth occasion, just prior to the cessation of the experiment (Week 16), the measured values in the two exposure tanks were as low as to be effectively indistinguishable from one another.

This trend is the opposite of that observed in the previous mesocosm exposure, where increasing concentrations of 17ß-oestradiol were measured over time. The steroid load reduction is likely to have been caused by adsorption to the increasing amounts of organic material building up in the tanks (as described in Yu et al., 2003) following the removal of the carbon pre-filters. Indeed, in the second half of the exposure (Weeks 8 to 16), the rate of weed growth was quite exceptional, indicating the degree of eutrophication occurring and necessitating the manual removal of large quantities of plant and algal material.

The decline in the measured concentration of 17ß-oestradiol was such that mean values over the whole exposure became almost meaningless, as is demonstrated by all the coefficients of variation (COV) being above 100%. In the first half of the exposure (up to Week 8 and prior to the mid-summer solstice), the treatments are comparable to levels of 17ß-oestradiol that might be expected in sewage effluents and in the second half of the exposure (Weeks 8 to 16), lower concentrations of 17ß-oestradiol and higher general organic loads are experienced, similar to the characteristics of polluted lowland water courses.

In addition, measurable levels of 17α-ethinylestradiol were recorded in the river water from the reference tank on each sample occasion, and also in both of the treatment tanks. The values ranged between 0.370 ngL^{-1} and 0.659 ngL^{-1} over the first two sampling occasions with no pattern evident. The presence of 17α-ethinylestradiol may have occurred due to the change in the supply river, or it

may be because the analytical LOD for the compound was reduced from 1 ngL^{-1} in Experiment 1 (2004) to 0.1 ngL^{-1} on this occasion (2006). However in the latter half of the experiment, it was measured at concentrations up to 2.34 ngL^{-1}, and it was also found in the influent river water at concentrations up to 3.73 ngL^{-1}.

4.2.2.2 Water Quality

Figure 35 shows the weekly mean measured water temperatures in the mesocosm tanks, together with the minimum and maximum temperatures recorded in that week, and the minutes of daylight between sunrise and sunset (U.S. Navy data for London).

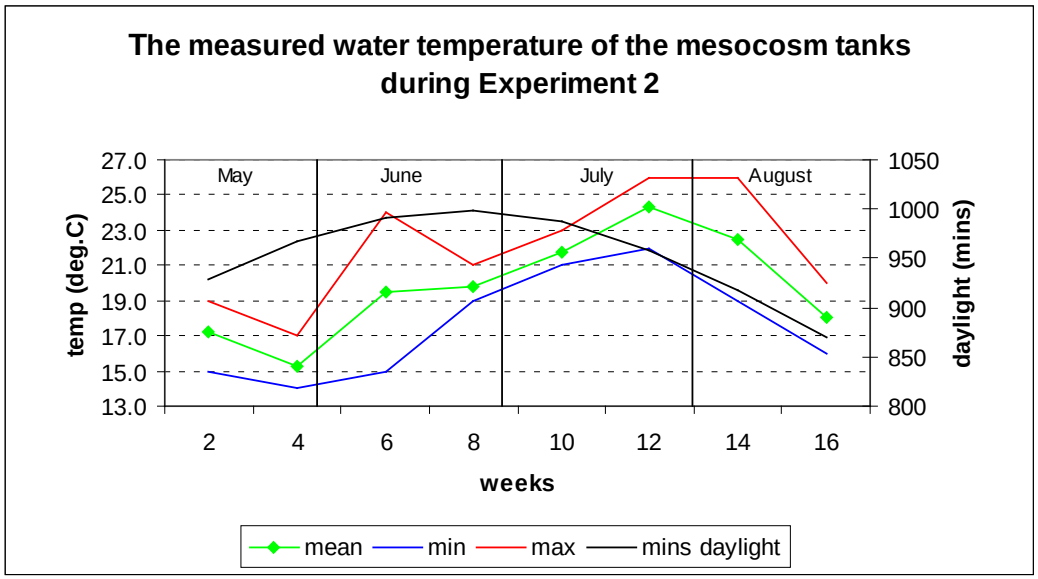


Figure 35 – The mean, minimum and maximum measured water temperatures recorded in the mesocosm tanks and the minutes of daylight during Experiment 2.

4.2.2.3 Test Organism Survivorship

The mortality rate of the reference population in this exposure was very similar to that observed in Experiment 1. The maximum cumulative mortality was 22.2% compared to 20.4% in Experiment 1 (however the second experiment was two weeks longer than the first).

In both experiments, there was a significantly increased mortality in the higher exposure group (100 ngL^{-1} nominal in this case) compared to the reference group after 12 weeks; it was 44.4% at 12 Weeks in Experiment 1, and 51.8% at the same time in this exposure (ANOVA, p=0.002), despite the nominal and measured levels of 17ß-oestradiol being lower. This second exposure continued for a further 4 weeks, and as Figure 36 shows, the mortality rate in this group continued to increase until the final sampling point (Week 16), when the mortality rate reached 74.0% (ANOVA, p = 0.001).

Also as in Experiment 1, the mortality rate occurring in the lower exposure group (10 ngL^{-1} nominal in this case) was never significantly different from the reference population (ANOVA, p>0.05).

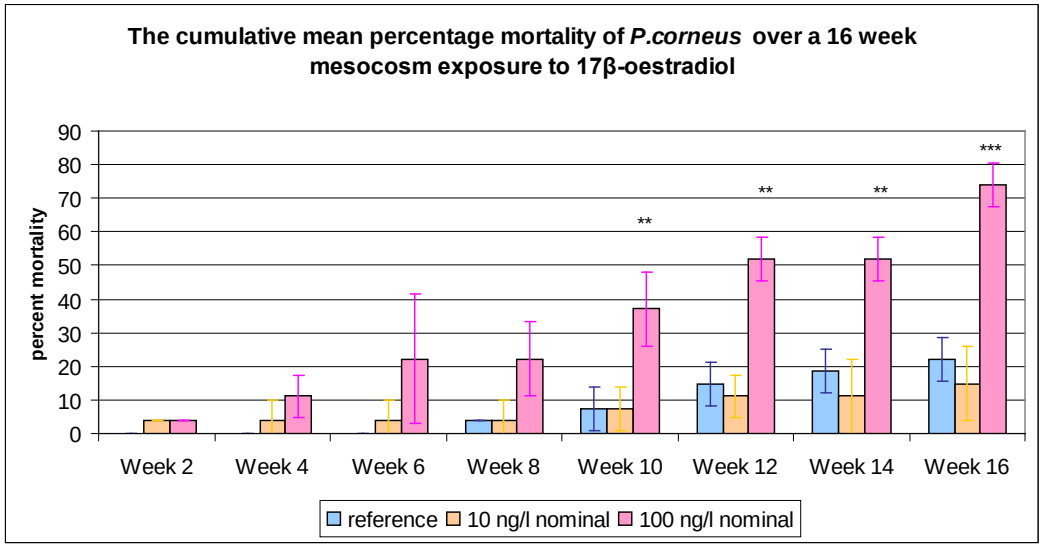


Figure 36 – The cumulative mean mortality rate of *P. corneus* over a 16-week exposure to 17β-oestradiol (error bars represent the standard deviation, asterisks indicate a significant difference from the reference group **p<0.01, ***p<0.001).

4.2.2.4 Test Organism Morphology and Growth

There were no significant differences between the reference group and the exposure groups in the length in the longest axis or in weight at the start of the experiment (ANOVA, $p>0.05$ in both cases).

The growth of the reference population was almost exactly the same in this experiment as that in Experiment 1, despite being 2 weeks longer. This group showed a mean increase in the length of the longest axis of 21.0%, and the mean weight increased by 62.2% (see Figure 37), compared to 17.8% and 60.8% respectively, in Experiment 1.

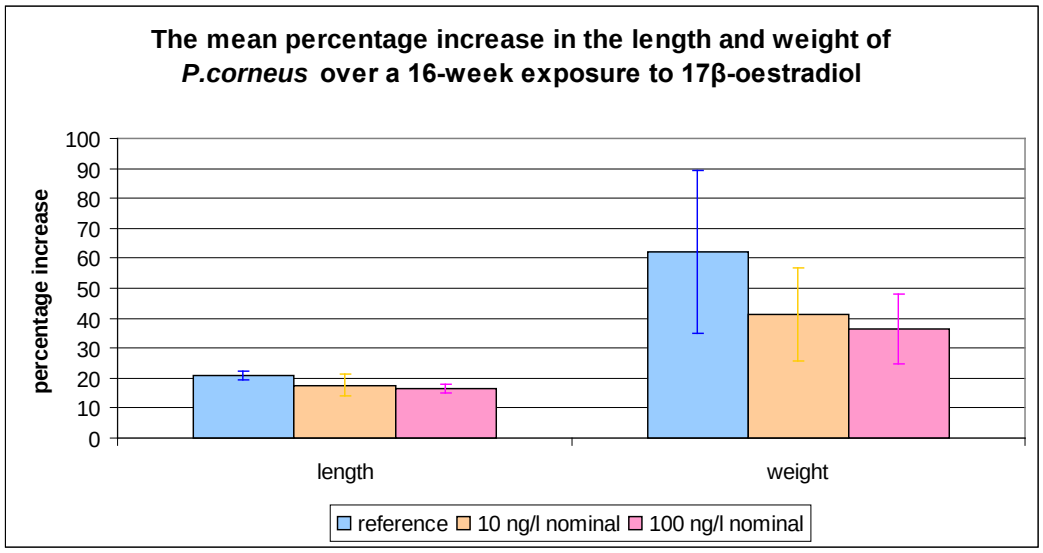


Figure 37 – The mean percentage increase in *P. corneus* length and weight during a 16-week exposure to 17β-oestradiol (error bars represent the standard error of the means).

The mean gain in weight was also suppressed in both exposure groups as it was in Experiment 1, but in this exposure there was an apparent dose-dependency, although this was not significant (Pearson's Product Moment Coefficient, $p>0.05$). Nor were there any significant differences between the mean weight gain of the exposed groups and the reference population (ANOVA, $p>0.05$).

There was also an apparent dose-dependency of the suppression in the increase in length of the longest axis, although again this is not significant (Pearson's Product Moment Coefficient, $p>0.05$). Previously there had been no suppression of growth in this parameter in the group exposed to the higher concentration of 17ß-oestradiol.

Also as in Experiment 1, the condition factor (flesh weight as a proportion of the overall weight) of the exposed groups were not significantly different to the reference population, with mean values for the groups varying between 45.0% and 46.0% only.

4.2.2.5 Test Organism Reproduction

Figure 38 shows the number of eggs produced per surviving adult at each fortnightly sampling point. On this occasion, this was the only parameter of reproduction assessed, because Experiment 1 demonstrated that there was no effect of exposure to 17ß-oestradiol on the number of eggs laid per egg mass. The egg masses were not therefore photographed immediately, and were often partially degraded prior to being assessed. The eggs have a very much more robust membrane than that of the gelatinous mass, and once the packaging had degraded they were counted using a cell-counting chamber.

In the first half of the exposure (up to Week 8) the reference population produced the largest number of eggs per adult, reaching a total 534 eggs per individual in the fortnight ending at the Week 6 sampling point in mid-June. The measured concentration of 17ß-oestradiol in the treated tanks was relatively high over this period, and yet there were no significant reductions in the number of eggs laid (ANOVA, $p>0.05$) until Week 8, when the group exposed to the higher concentration of 17ß-oestradiol (100 ngL^{-1} nominal) laid significantly less eggs (ANOVA, $p = 0.011$). The effect appears to be dose-dependant at this time in a

similar manner to that observed in Experiment 1, and the correlation is indeed significant (Pearson's Product Moment, $p<0.01$, $r^2 = 0.77$).

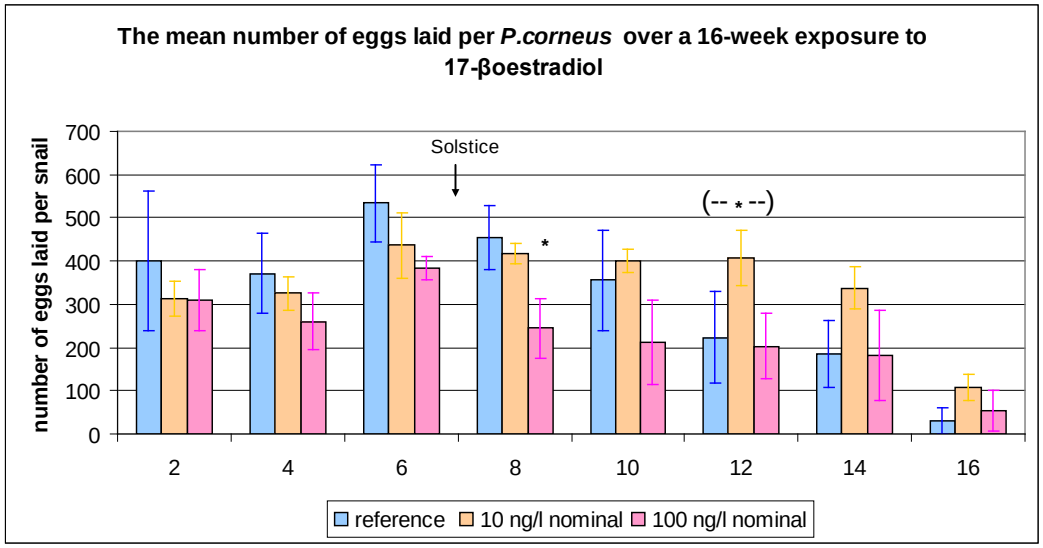


Figure 38 – The mean number of eggs laid per *P. corneus* during a 16-week exposure to 17β-oestradiol (error bars represent the standard deviation, asterisk indicates a significant difference from the reference group at *p<0.05, dashed asterisk (--*--) indicate a significant difference between the groups at p<0.05).

After this time, the number of eggs laid by the reference population begins to decline in the second half of the exposure (Weeks 9 to 16), falling to 29.2 eggs per snail at Week 16. The two groups exposed to 17ß-oestradiol do not decline in the same manner. The mean number of eggs laid by the group exposed to the higher concentration was already suppressed to approximately 200 eggs per snail per fortnight, and because this is maintained, the number of eggs laid by this group appears to be similar to that of the reference group as the latter declines at Weeks 12 and 14.

However, the numbers of eggs produced by the group exposed to the lower concentration of 17ß-oestradiol (10 ngL^{-1} nominal) also maintain production of higher mean numbers of eggs being laid per individual (over 300 eggs per snail until the last sampling point at the end of the 16-week exposure). As the mean number of eggs laid by the reference group declines, the difference between these

two groups becomes more pronounced until at Week 12 there is a significant difference between the groups (ANOVA, p = 0.045), although there are no significant differences between the reference and treatment groups (Dunnett's comparison). However the shape of this response persists through to the end of the exposure. When the total number of eggs laid over the second half of the exposure (post solstice; Weeks 9 to 16) is calculated, the group exposed to the lower concentration of 17ß-oestradiol (10 ngL^{-1}, nominal) laid an average of 58.4% more eggs per individual than the reference group (mean = 1256 eggs/snail compared to 793 eggs per snail in the reference group), although this difference is also not significant (ANOVA, p = 0.06).

4.2.3 Experiment 3 – An exposure of *P. corneus* to 17ß-oestradiol in 'simulated summer' and 'simulated autumn' conditions.

4.2.3.1 17β-Oestradiol Stability Study

A 17β-oestradiol stability study was performed before the laboratory experiments began. A single exposure tank containing 6 snails was prepared for each concentration tested (1, 10, 100 and 1000 ngL^{-1} nominal), with the animals being fed and the media changed as described for the exposure. The study was conducted at 20^{o}C with 16 hours of light and 8 hours of dark as this was considered most likely to encourage bacterial degradation. Samples were taken from each tank in the manner described for the exposure at time zero (immediately following a partial media change), and at 3, 9, 21 and 45 hours, which provided degradation time spans of 3, 6, 12 and 24 hours between sampling.

The results of the analysis demonstrated that degradation of 17β-oestradiol did indeed occur in the test conditions, and the model that best fitted the degradation rate was an exponential curve for all concentrations (r^2 is above 0.9 in all cases, see Table 4 and Figure 39). This is presumably due to the exponential nature of

population increase in the bacteria responsible for the degradation. The half-life of the compound was notably shorter at the higher concentrations, presumably due to a more rapid adaptation of the bacteria to the steroid substrate where it was more plentiful. However, the half-lives in all cases were too short to feasibly dictate the frequency of water changes, and so it was accepted that the test organisms would experience peaks and troughs in the concentration of 17β-oestradiol even using the shortest practicable frequency of media changes.

Table 4 – The r^2 values, half-lives and mean concentration of 17β-oestradiol calculated from measured concentrations in the stability study.

Nominal concentration (ngL^{-1}):	**1**	**10**	**100**	**1000**
r^2 of the regression	0.988	0.913	0.987	0.923
Half-life (hours)	18	16	10	8
Mean (ngL^{-1})	1.23	6.80	82.9	864

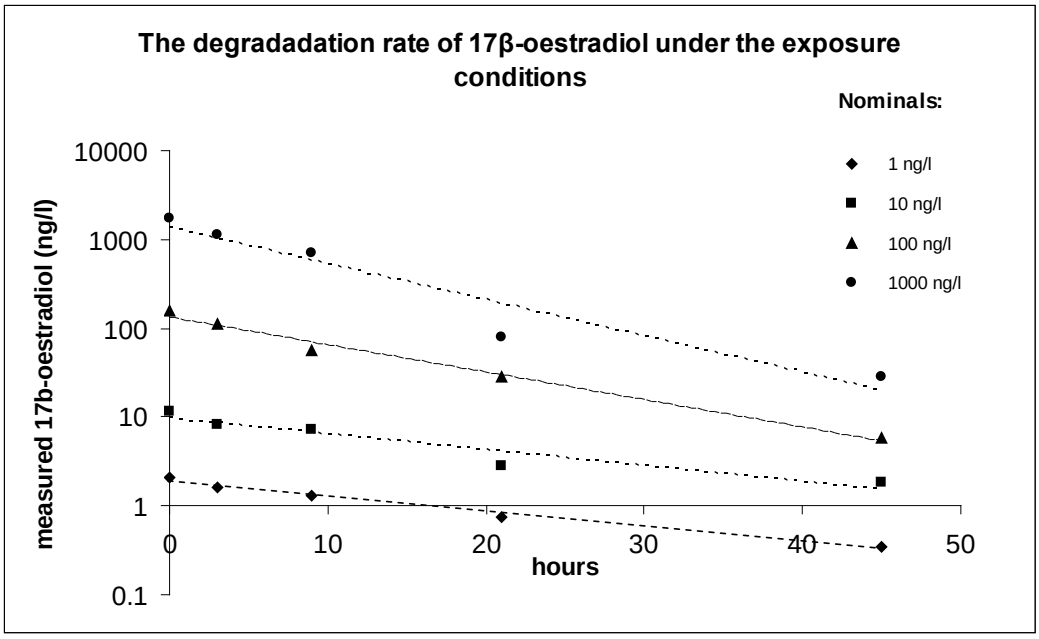


Figure 39 – The degradation rate of 17β-oestradiol at 20°C under the exposure conditions with test organisms present.

Although the degradation rate of 17β-oestradiol was high, the concentrations selected for the exposure (1, 10 and 100 ngL^{-1} nominal) did not cross over. Therefore, the organisms in the test concentrations would experience increasing levels of 17β-oestradiol with increasing nominal concentrations, although they become lower than the nominal value after the initial addition. In the case of 1 ngL^{-1} nominal, it appeared likely that the steroid would be lost entirely between water changes, and the test organisms would be exposed to pulses of 17β-oestradiol. The 1000 ngL^{-1} nominal test concentration was not adopted for the exposure, due to the likelihood of causing toxic effects in the test organisms.

4.2.3.2 Steroid Analysis

The results of the fortnightly analysis performed immediately before a partial media change and immediately after the addition of the steroid during the 8-week exposure are set out in Table 5, with the calculated mean concentrations. Values below the limit of detection (<0.2 ngL^{-1}) are treated as 0.1 ngL^{-1}. There are several occasions where no data (ND) are available. In the first cycle, the SPE columns used for the samples taken from the nominal 10 ngL^{-1} tanks at 15^{o}C eluted a cloudy, yellow substance in the solvent that interfered with analysis. In the second cycle, the 17β-oestradiol results given for the nominal 1 ngL^{-1} sample at 20^{o}C were a factor of 10 too high, and without correspondingly high oestrone results, were considered incredible and removed from the dataset (mean 17β-oestradiol = 11.2 ngL^{-1}. mean oestrone = 0.632 ngL^{-1}, compare with result for 10 ngL^{-1} nominal at the same temperature). Finally, in the third cycle, the control results at both temperatures were high, and this was traced back to the glassware the samples were taken in not having being solvent-washed following the previous sampling event (mean at 15^{o}C = 0.963 ngL^{-1}, and at 20^{o}C = 1.02 ngL^{-1}).

For each concentration, the 17β-oestradiol results between the two temperatures were compared, and no significant differences were found (1 tailed t-tests, $p>0.05$ in all cases). Therefore it was concluded that the steroid did not degrade more slowly at the lower temperature. When the results for both temperatures are

pooled for each nominal concentration, the measured concentrations are significantly different to one another (control and 1 ngL^{-1} nominal values, 1 tailed t-test, p = 0.021; 1 ngL^{-1} and 10 ngL^{-1} nominal values, Mann-Whitney test, p = 0.003; 10 ngL^{-1} and 100 ngL^{-1} nominal values, Mann-Whitney test, p = 0.002). This indicates that each treatment was sufficiently different from the neighbouring treatment to be assessed independently in relation to any effects observed in the test organisms. It can be seen from Table 5 that in most cases, the level of 17β-oestradiol measured in the sample taken after the partial media change was higher than that in the sample taken prior to the partial media change as expected. The reverse is generally true for oestrone, as being the biotransformation product of 17β-oestradiol, it tends to build up while 17β-oestradiol breaks down. While it is recognised that the oestrone will contribute some of the oestrogenic activity in this exposure, 17β-oestradiol is approximately x10 more potent in the fathead minnow (*P. promelas)* than oestrone (Thorpe et al., 2003), and it may therefore have exerted a lesser effect.

Table 5 – The measured 17β-oestradiol and oestrone concentrations performed prior to (PRE) and after (POST) a partial media change and the calculated mean (LOD = 0.2 ngL^{-1}).

	17β-oestradiol (ngL^{-1})			oestrone (ngL^{-1})		
	PRE	POST	**Mean**	PRE	POST	**Mean**
1st Exposure Cycle:	*(8th – 21st June)*					
15°C, Control	<0.2	<0.2	**0.1**	0.392	<0.2	**0.246**
15°C, 1 ngL^{-1} nominal	0.390	0.896	**0.643**	1.55	0.442	**0.996**
15°C, 10 ngL^{-1} nominal	ND	ND	**-**	ND	ND	**-**
15°C, 100 ngL^{-1} nominal	24.0	122	**73.0**	151	4.8	**97.4**
20°C, Control	<0.2	<0.2	**0.1**	0.598	<0.2	**0.349**
20°C, 1 ngL^{-1} nominal	0.372	1.72	**1.05**	1.39	0.818	**1.10**
20°C, 10 ngL^{-1} nominal	2.20	11.4	**6.80**	9.54	3.3	**6.42**
20°C, 100 ngL^{-1} nominal	21.0	122	**71.5**	106	41.3	**73.7**

2nd Exposure Cycle:	*(22nd June – 5th July)*					
15°C, Control	0.744	0.204	**0.474**	0.432	<0.2	**0.266**
15°C, 1 ngL^{-1} nominal	0.826	0.952	**0.889**	0.908	0.356	**0.785**
15°C, 10 ngL^{-1} nominal	12.8	14.7	**13.8**	6.32	2.58	**4.45**
15°C, 100 ngL^{-1} nominal	150	160	**155**	77.6	27.2	**52.4**
20°C, Control	0.690	0.718	**0.704**	0.502	0.262	**0.382**
20°C, 1 ngL^{-1} nominal	ND	ND	**-**	ND	ND	**-**
20°C, 10 ngL^{-1} nominal	12.6	16.9	**14.8**	9.06	2.52	**5.79**
20°C, 100 ngL^{-1} nominal	117	178	**148**	66.2	33.6	**49.9**
3rd Exposure Cycle:	*(6th – 19th July)*					
15°C, Control	ND	ND	**-**	ND	ND	**-**
15°C, 1 ngL^{-1} nominal	0.716	1.06	**0.888**	0.970	0.364	**0.667**
15°C, 10 ngL^{-1} nominal	0.606	1.58	**1.09**	4.80	2.64	**3.72**
15°C, 100 ngL^{-1} nominal	23.2	38.4	**30.8**	64.6	29.8	**47.2**
20°C, Control	ND	ND	**-**	ND	ND	**-**
20°C, 1 ngL^{-1} nominal	2.38	1.26	**1.82**	1.07	0.414	**0.742**
20°C, 10 ngL^{-1} nominal	3.38	3.86	**3.62**	5.52	2.94	**4.23**
20°C, 100 ngL^{-1} nominal	21.2	43.2	**32.2**	44.8	20.8	**32.8**
4th Exposure Cycle:	*(20th July – 2nd August)*					
15°C, Control	<0.2	<0.2	**0.1**	<0.2	2.76	**1.43**
15°C, 1 ngL^{-1} nominal	<0.2	0.514	**0.307**	1.14	0.454	**0.797**
15°C, 10 ngL^{-1} nominal	0.816	5.14	**2.98**	5.62	2.48	**4.05**
15°C, 100 ngL^{-1} nominal	24.8	100	**62.4**	58.2	23	**40.6**
20°C, Control	<0.2	0.288	**0.190**	<0.2	0.362	**0.181**
20°C, 1 ngL^{-1} nominal	0.542	0.318	**0.430**	0.332	0.750	**0.541**
20°C, 10 ngL^{-1} nominal	1.59	7.26	**4.43**	5.38	2.44	**3.91**
20°C, 100 ngL^{-1} nominal	13.8	90.0	**51.9**	59.4	19.3	**48.9**

It is not clear why both the steroid compounds were detected in the control media. It may be due to low levels of cross-contamination despite every effort to avoid this occurrence. All control tanks were serviced and covered with clear perspex before the treatment tanks were serviced, air-lines were numbered to ensure they were not returned to the wrong tanks, no sampling vessels were introduced to exposure tanks, back-filling was performed slowly to avoid splashes and surfaces were frequently wiped down with methanol. It is also possible that the steroids are synthesised de-novo by the test organisms, as 17b-oestradiol has been recorded in the gonad tissue of the bi-valve *M. edulis* (Zhu et al., 2003). Whichever is the case, the maximum measured concentration of 17β-oestradiol in the control media was 0.744 ngL^{-1}, which is below the proposed Predicted No Effect Concentration (PNEC) for fish (1 ngL^{-1}, Environment Agency, 2002).

4.2.3.3 Media Physico-Chemical Characteristics

Temperature

Table 6 shows the minimum and maximum temperatures recorded for each cycle of the pre-exposure (baseline) and exposure periods measured by the continuous data capture of an 'indoor/outdoor' thermometer. While this is indicative of the range of temperatures the exposure tanks experienced, it is not possible to generate a mean value. However the temperature in the tanks was also measured manually using a mercury thermometer with an error margin of 0.5^{o}C and a mean temperature for each period from these readings is also given. It can be seen that this mean value does not always fall within the automatically recorded minimum and maximum values, due to the different error margins of the instruments involved.

Table 6 – The mean, minimum and maximum temperatures recorded in each constant temperature room, for each cycle of the experiment.

	Temperature (^{o}C)		
Experimental Cycles at 20^{o}C:	**Mean (mercury)**	**Minimum (automatic)**	**Maximum**
1st Pre-exposure Cycle *(11th - 24th May)*	**21.1**	20.1	21
2nd Pre-exposure Cycle *(25th May-7th June)*	**20**	19.9	20.9
1st Exposure Cycle *(8th – 21st June)*	**19.7**	20.1	21
2nd Exposure Cycle *(22nd June – 5th July)*	**20**	20.3	21.1
3rd Exposure Cycle *(6th – 19th July)*	**20.6**	19.9	21.3
4th Exposure Cycle *(20th July – 2nd August)*	**20.2**	19.2	21
Experimental Cycles at 15^{o}C:	**Mean**	**Minimum**	**Maximum**
1st Pre-exposure Cycle *(11th - 24th May)*	**15.7**	13.9	15
2nd Pre-exposure Cycle *(25th May-7th June)*	**15.1**	14.1	15
1st Exposure Cycle *(8th – 21st June)*	**15.3**	14.4	15.1
2nd Exposure Cycle *(22nd June – 5th July)*	**15.4**	14.4	15
3rd Exposure Cycle *(6th – 19th July)*	**15.7**	14.2	15.6
4th Exposure Cycle *(20th July – 2nd August)*	**15.4**	14.2	15.1

The data show that the temperature did not deviate more than 2^{o}C from nominal in either of the constant temperature rooms. However there were in fact a few minor deviations from the tolerated range (0.8^{o}C in combination). At 20^{o}C, the exposure tanks were outside of the upper tolerance limit by 0.2^{o}C on one occasion in the 3rd exposure cycle, and out of the lower tolerance limit by 0.3^{o}C on one occasion in the 4th exposure cycle. Also, on one occasion in the 3rd exposure cycle the switching mechanism in the room set to 15^{o}C failed, and the temperature of the room was rapidly elevated to 24.5^{o}C. The malfunction was noticed and rectified within a couple of hours, and the behaviour of the snails appeared unaffected. The automatically recorded temperature data from this

event have been omitted. Apart from this occasion the temperature of the two constant temperature rooms did not come closer than 3.6°C of each other.

pH

Table 7 shows the mean and standard deviation of pH measurements from aliquots taken from the tanks before and after media changes. It can be seen that the addition of fresh media brought the pH of the exposure tanks close to pH 7 in most cases, and that the pH rose by approximately 0.5 units over the 48-hour period between media changes. It was considered that this degree of pH change was not adverse to the test organisms.

Table 7 – The measured pH and standard deviation (SD) of aliquots of media taken from the tanks before and after media changes.

	pH Before		**pH After**	
Experimental Cycles at 20°C:	**Mean**	**SD**	**Mean**	**SD**
1st Baseline Cycle *(11th - 24th May)*	**7.38**	0.17	**7.13**	0.14
2nd Baseline Cycle *(25th May - 7th June)*	**7.33**	0.11	**7.06**	0.05
1st Exposure Cycle *(8th – 21st June)*	**7.38**	0.15	**7.28**	0.15
2nd Exposure Cycle *(22nd June – 5th July)*	**7.49**	0.12	**7.49**	0.14
3rd Exposure Cycle *(6th – 19th July)*	**7.39**	0.07	**7.28**	0.16
4th Exposure Cycle *(20th July – 2nd August)*	**7.41**	0.21	**7.22**	0.13
Experimental Cycles at 15°C:	**Mean**	**SD**	**Mean**	**SD**
1st Pre-exposure Cycle *(11th - 24th May)*	**7.36**	0.14	**7.15**	0.11
2nd Pre-exposure Cycle *(25th May-7th June)*	**7.30**	0.16	**7.08**	0.07
1st Exposure Cycle *(8th – 21st June)*	**7.41**	0.10	**7.34**	0.14
2nd Exposure Cycle *(22nd June – 5th July)*	**7.50**	0.11	**7.54**	0.14
3rd Exposure Cycle *(6th – 19th July)*	**7.39**	0.08	**7.24**	0.15
4th Exposure Cycle *(20th July – 2nd August)*	**7.41**	0.20	**7.20**	0.08

Dissolved Oxygen

The dissolved oxygen levels were measured as a percentage of the air saturation value (%ASV) giving an indication of the oxygen available to the test organisms. The measurements are set out in Table 8 with standard deviations. The behaviour of *P. corneus* had indicated that the species was tolerant of relatively low levels of dissolved oxygen, which is expected as they are pulmonate and move to the surface to respire. In the event, the mean dissolved oxygen levels only generally dropped by approximately 10% over the 48-hour periods between media changes.

Table 8 – The dissolved oxygen (DO) and standard deviation (SD) of aliquots of media taken from the tanks before and after media changes.

	DO Before (% ASV)		**DO After (% ASV)**	
Experimental Cycles at 20°C:	**Mean**	**SD**	**Mean**	**SD**
1st Pre-exposure Cycle *(11th - 24th May)*	**84**	9.82	**85**	12.9
2nd Pre-exposure Cycle *(25th May-7th June)*	**87**	6.00	**93**	3.07
1st Exposure Cycle *(8th – 21st June)*	**89**	5.16	**95**	2.79
2nd Exposure Cycle *(22nd June – 5th July)*	**87**	8.41	**96**	3.93
3rd Exposure Cycle *(6th – 19th July)*	**86**	5.19	**97**	1.96
4th Exposure Cycle *(20th July – 2nd August)*	**85**	11.9	**94**	3.92
Experimental Cycles at 15°C:	**Mean**	**SD**	**Mean**	**SD**
1st Pre-exposure Cycle *(11th - 24th May)*	**79**	12.3	**86**	11.5
2nd Pre-exposure Cycle *(25th May-7th June)*	**89**	6.74	**93**	6.30
1st Exposure Cycle *(8th – 21st June)*	**92**	4.22	**96**	2.59
2nd Exposure Cycle *(22nd June – 5th July)*	**92**	4.83	**96**	3.61
3rd Exposure Cycle *(6th – 19th July)*	**92**	4.20	**99**	2.74
4th Exposure Cycle *(20th July – 2nd August)*	**90**	7.35	**97**	5.40

The standard deviations are higher in some cases because, on occasion, the dissolved oxygen in a single tank dropped rapidly due to blockages in the air-line. This did not appear to affect the snails for the reasons described. Low dissolved oxygen levels may be detrimental to egg mass hatching success if allowed to continue into the longer term. However the super-saturation of dissolved oxygen levels would have been of more concern, because it was observed that mortality rates increased markedly in the culture if this was allowed to occur when appreciable quantities of algae were present.

Conductivity

The food and faecal strings present in the tanks did not dissolve to any great extent, and the conductivity of the media remained relatively low, as can be seen from Table 9. The conductivity did generally decrease slightly with each media change, but when the standard deviations are taken into account, this was barely perceptible.

Table 9 – The conductivity (CND) and standard deviation (SD) of aliquots of media taken from the tanks before and after media changes.

	CND Before µS/cm		**CND After µS/cm**	
Experimental Cycles at 20°C:	**Mean**	**SD**	**Mean**	**SD**
1st Pre-exposure Cycle *(11th - 24th May)*	**1004**	150	**927**	90
2nd Pre-exposure Cycle *(25th May-7th June)*	**954**	96	**907**	63
1st Exposure Cycle *(8th – 21st June)*	**985**	69	**936**	164
2nd Exposure Cycle *(22nd June – 5th July)*	**993**	113	**973**	111
3rd Exposure Cycle *(6th – 19th July)*	**1052**	153	**1008**	139
4th Exposure Cycle *(20th July – 2nd August)*	**1017**	272	**1012**	207

Experimental Cycles at 15°C:	**Mean**	**SD**	**Mean**	**SD**
1st Pre-exposure Cycle *(11th - 24th May)*	**926**	92	**889**	88
2nd Pre-exposure Cycle *(25th May-7th June)*	**972**	124	**968**	157
1st Exposure Cycle *(8th – 21st June)*	**900**	79	**881**	103
2nd Exposure Cycle *(22nd June – 5th July)*	**932**	87	**908**	81
3rd Exposure Cycle *(6th – 19th July)*	**1067**	146	**998**	139
4th Exposure Cycle *(20th July – 2nd August)*	**1041**	160	**1050**	158

Hardness

The mean hardness ($CaCO_3$ equivalents) of batches of fresh media used over the duration of the exposure was 401.5 mgL^{-1}. This was very close to the calculated value of 394 mgL^{-1} $CaCO_3$ equivalents for the amount of salts added to the reverse osmosis filtered water. The mean hardness of aliquots taken from the tanks during the exposure are shown in Table 10. Unfortunately the samples taken during the third exposure cycle were disposed of in error.

Table 10 – The mean hardness and standard deviation (SD) of aliquots of media taken from the tanks before media changes.

	Hardness (mgL^{-1} $CaCO_3$ equivalents)			
	15 °C		**20 °C**	
Experimental Cycles:	**Mean**	**SD**	**Mean**	**SD**
1st Pre-exposure Cycle *(11th - 24th May)*	**437.4**	39.1	**422.4**	64.9
2nd Pre-exposure Cycle *(25th May-7th June)*	**418.8**	37.9	**387.2**	27.9
1st Exposure Cycle *(8th – 21st June)*	**428.5**	59.6	**421.2**	24.4
2nd Exposure Cycle *(22nd June – 5th July)*	**408.5**	35.0	**423.6**	35.9
3rd Exposure Cycle *(6th – 19th July)*	**ND**	ND	**ND**	ND
4th Exposure Cycle *(20th July – 2nd August)*	**393.5**	46.4	**417.6**	90.0

4.2.3.4 Test Organisms Survivorship

In the experiment performed at 15^{o}C, the mortality over the duration of the exposure was negligible, with only one death occurring across all of the control replicate groups, and also one at each test concentration. In the experiment performed at 20^{o}C, the mortality rate was more appreciable, although still low until the last week of the exposure (three control organisms and three at each test concentration, constituting 10% of the test organisms). However in the last week of the exposure at this temperature (Week 8), three further control animals died, increasing the mortality rate of the control group to 20%. This is usually considered acceptable in chronic invertebrates exposures (e.g. OECD, 2008), and there were no significant differences between the treatment groups (ANOVA, $p>0.05$).

4.2.3.5 Test Organism Morphology and Growth

At the start of the experiment, the mean diameter and weight of each group was compared between treatments within each exposure temperature. There were no significant differences between the control groups and any of the treatment groups at either temperature (ANOVA, $p>0.05$). At the end of the exposures, the growth of the organisms were calculated in terms of the mean percentage increase in length and weight, and these are shown in Figure 40. All of the groups grew between 5.12 and 8.88% in mean length and between 11.7 and 20.2% in mean weight, but there were no significant differences between the control and treatments at either temperature (ANOVA, $p>0.05$ in both cases).

At the end of the exposures, the shells of the animals were removed and the soft flesh weighed, and the ratio between the mean weight of each group with and without the shell was calculated to give an indication of body condition factor. The mean condition factors ranged between 41.2 and 46.8% flesh to shell for the groups exposed at 15^{o}C, and between 36.7 and 40.9% at 20^{o}C. There were no significant differences between the control and treatment groups at either

temperature (ANOVA, $p>0.05$ in both cases), although the animals exposed at 15^{o}C had significantly more body condition at the end of the exposure than those exposed at 20^{o}C (ANOVA, $p<0.001$).

Once the reproductive organs were dissected out, a ratio between the mean total body weight and mean reproductive organ weight for each group was calculated, to give a repro-somatic index (RSI). The mean RSI ranged between 52.5 and 54.5% reproductive organ weight to somatic weight at 15^{o}C, and between 51.5 and 57.6% at 20^{o}C. There were no significant differences between the control and treatment groups at either temperature, nor between temperatures (ANOVA, $p>0.05$ in all cases).

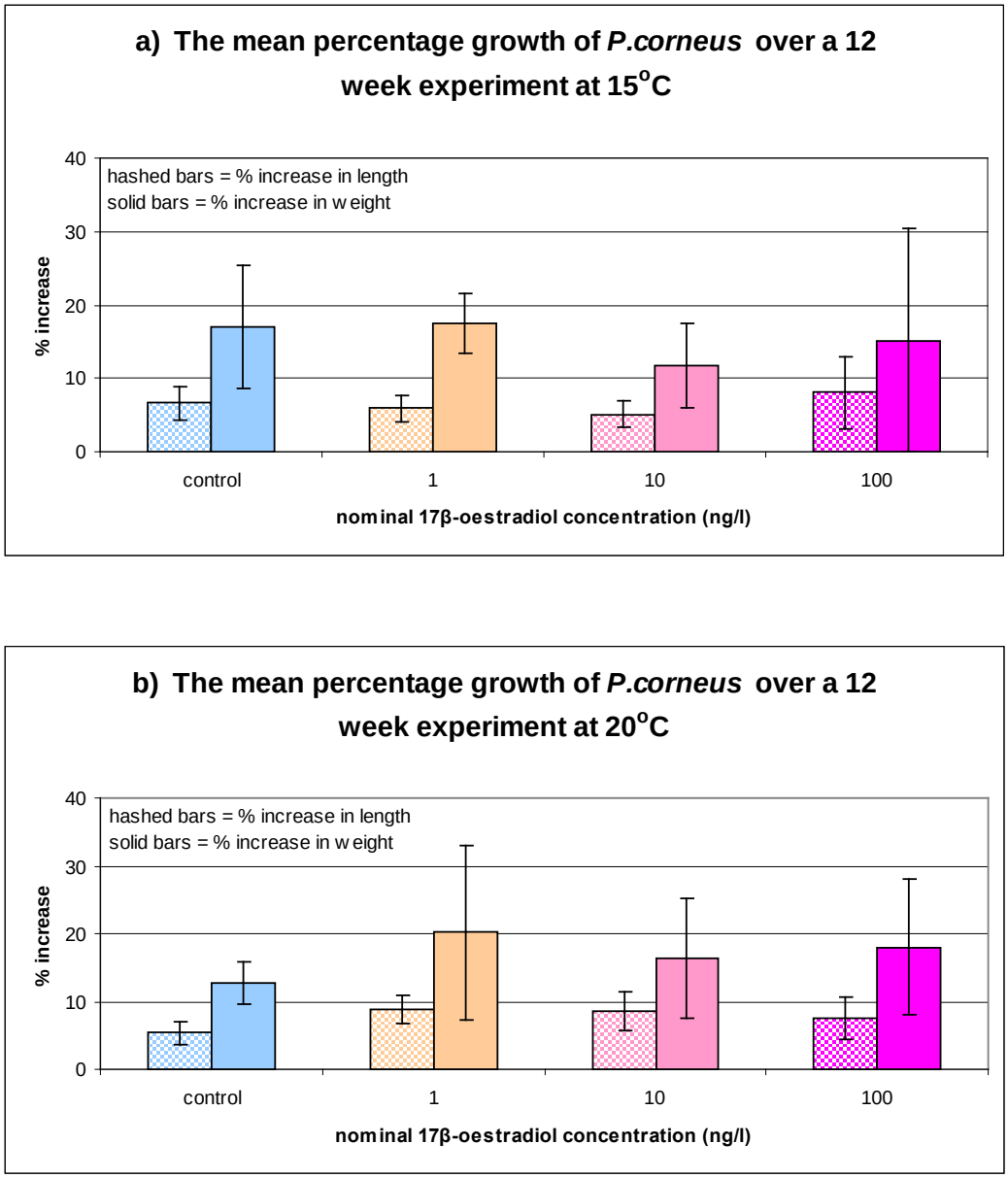


Figure 40 – The mean percentage increase in length and weight of *P. corneus* over a 12 week long experiment, at (a) 15°C and (b) 20°C (error bars represent the standard error of the means).

4.2.3.6 Test Organism Reproduction

During the pre-exposure (baseline) phase of the experiment, all of the groups were maintained at the respective temperatures and photoperiods but without the addition of 17β-oestradiol. The number of egg masses were counted at each 48-hour partial media change and a mean was calculated for the entire 4-week

period. At 20^{o}C, the mean number of egg masses laid per snail per day was 0.58 (SEM = 0.11, COV = 19.8%). At 15^{o}C, the mean number of egg masses laid per snail per day across the original number of tanks (5 replicates for control and each treatment = 20) was lower at 0.38 (SEM = 0.12, COV = 31.2%). While a lower number of masses per snail was expected at the lower temperature, a higher COV was likely to confound the results, and so the four tanks producing the lowest number of eggs per snail per day (0.14, 0.18, 0.23 and 0.24) were excluded. Over the 16 remaining tanks (4 to be allocated to control and to each treatment) the number of masses laid per snail per day was 0.42 (SEM = 0.08, COV = 18.6%). This process indicated that a minimum of 0.25 egg masses per snail per day might be an acceptable quality criterion for minimising inter-replicate variation.

Because the degree of variation in the number of egg masses produced between replicates was still appreciable (more than double the number of masses were laid between the least and most productive replicates at each temperature; i.e. between 0.32 and 0.73 masses/snail/day at 20^{o}C and 0.30 and 0.61 at 15^{o}C), the data were used to determine whether reproductive output might be adjusted by taking into account the size differences between the snails in each replicate. Koene et al. (2007) suggest that in many hermaphrodite snails, egg production is positively correlated with body size. Figure 41 shows the correlation between the total number of eggs produced per tank in the pre-exposure period and the total length or weight of the snails present. All of these relationships are significant (Pearson's Product Moment Correlation; $p = 0.021$ and $p = 0.022$ for weight and length respectively at 20^{o}C, and $p<0.001$ and $p = 0.001$ respectively at 15^{o}C), but the model fit is poor in all cases ($r^2 = 0.26$, except between weight and egg production at 15^{o}C when $r^2 = 0.34$).

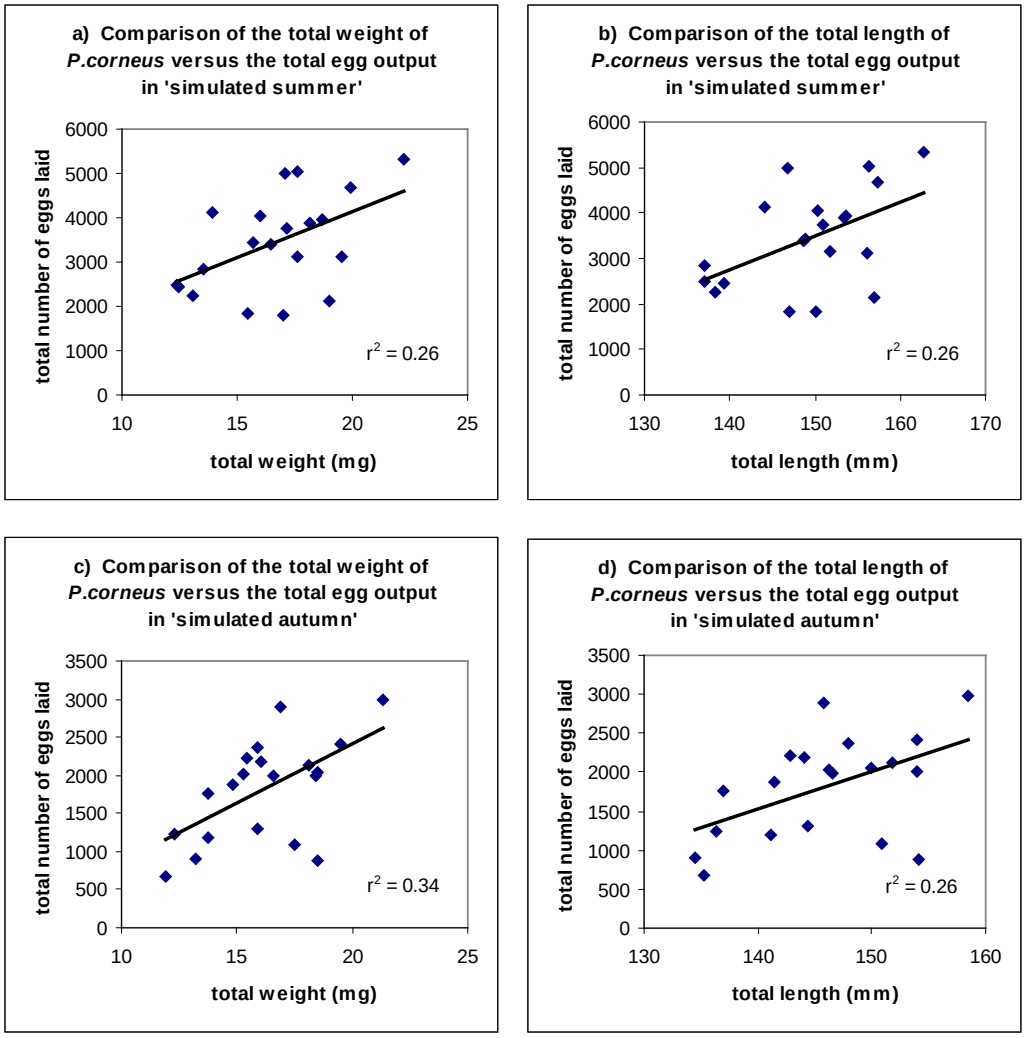


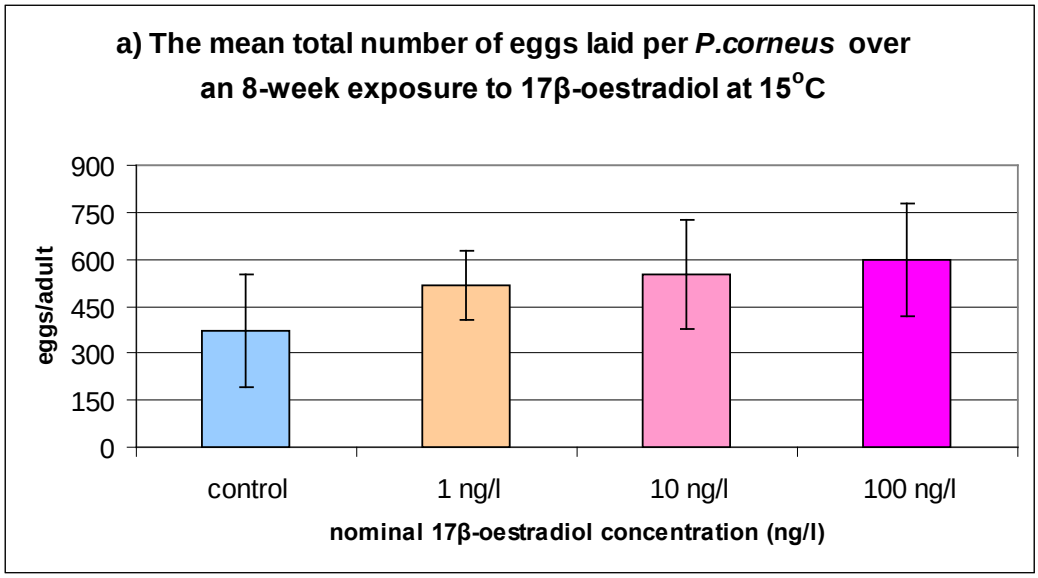
Figure 41 – The relationship between the total number of eggs laid in the pre-exposure period and the total weight and length at 20°C (a and b) and 15°C (c and d) of 40 replicate groups of 6 *P. corneus*.

It is apparent that the remaining variation is contributed by an unknown factor at the individual level. At the end of the experiment it was observed on dissection of the test subjects, that a few snails were heavily parasitized with digenean trematodes (6.3% at 15°C and 6.7% at 20°C). The degree of parasitisation in these snails was to the extent that the membranes enclosing the gonad and upper reaches of the reproductive tract was entirely filled with larvae. It is considered likely that these individuals were virtually inactive in terms of egg production (in a similar manner to the parasitic castration recorded in *Lymnaea truncatula,* Wilson and Denison, 1980) and that this was the possible cause of the presence of

egg masses that were devoid of eggs. For this reason, the number of egg masses laid per snail was not a suitable reflection of reproductive potential, and only the number of eggs laid per snail was used as a measure of reproduction in this experiment. Those snails that were parasitized were excluded from the analysis, and the data were adjusted in the same manner as for mortalities occurring.

The total number of eggs laid per snail at each exposure regime are shown in Figure 42. The trends of the response to increasing 17β-oestradiol concentration in 'simulated summer' (20^{o}C, 16h light / 8h dark photoperiod) and 'simulated autumn (15^{o}C, 12h light / 12h dark photoperiod) are opposed. In the simulated summer exposure there is a trend towards inhibition in egg production as the concentration of 17β-oestradiol increases, and in the simulated autumn exposure there is a trend towards induction of egg production with increasing concentration of 17β-oestradiol. Neither outcome was entirely unexpected as the outdoor seasonal mesocosm experiments showed that exposure to 17β-oestradiol can cause significant changes in the reproductive rate of *P. corneus* in both directions.

These laboratory exposures show that the direction of change is influenced by the conditions of the exposure, and not only by the action of overt toxicity, as may have been the case in the mesocosm studies. However, neither the induction nor the inhibition of the total number of eggs laid per snail in these experiments was significantly different from the control at any of the concentrations of 17β-oestradiol tested (ANOVA, $p>0.05$ in all cases). When the mean size of the snails (wet weight) was used as a co-variable, this did not change (ANCOVA, $p>0.05$ in both cases). Also, although the trends described appear to be dose-dependant, there was no significant correlation between the total number of eggs laid per snail and the nominal concentration of 17β-oestradiol under either exposure regime (Pearson's Product Moment Correlation Co-efficients, $p>0.05$ in both cases).



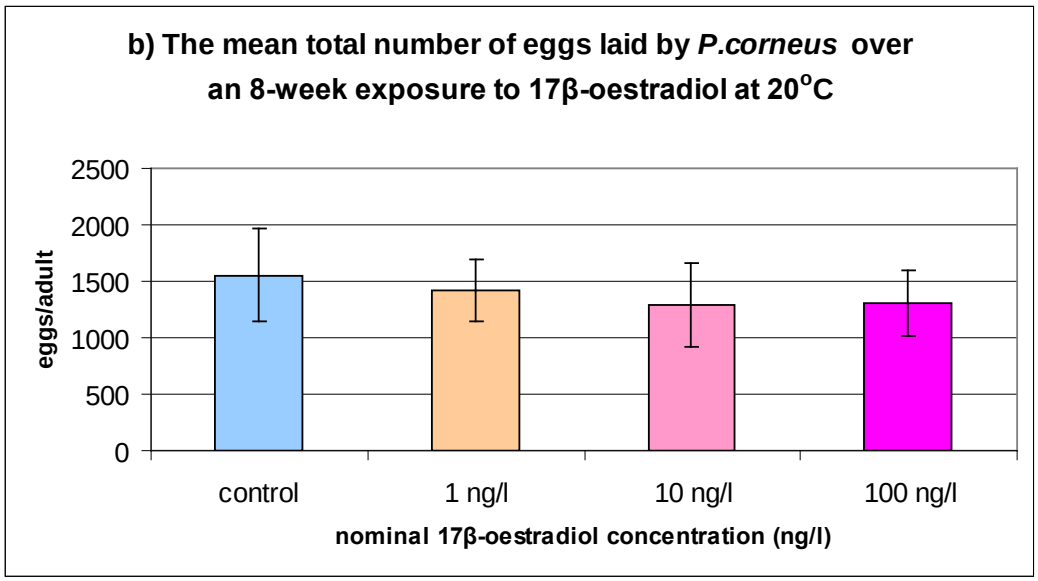


Figure 42 – The mean total number of eggs produced per *P. corneus* over an 8-week exposure to 17β-oestradiol at (a) 15°C and (b) 20°C (error bars represent the standard error of the means).

The test has a low power to detect significant differences from the control due to the high degree of variation between the replicate groups. A retrospective power analysis showed that in the simulated autumn exposure, the power of the test was only 0.214 (based on one-way ANOVA, 4 levels, 4 replicates, alpha = 0.05, value of the maximum difference between the mean number of eggs laid per adult over

the 8 week exposure = 226.6, standard deviation of control group = 182.4), and in the simulated summer exposure it was only 0.102 (based on one-way ANOVA, 4 levels, 5 replicates, alpha = 0.05, value of the maximum difference between the mean number of eggs laid per adult over the 8 week exposure = 262.5, standard deviation of control group = 412.1).

However the power to detect change is better when comparing the reproductive rates of the replicate groups during the pre-exposure and exposure phases, as described by Harries et al. (2000) in a test design for a Fathead Minnow (*P. promelas*) Pair Breeding Assay. In this assay, certain fish pairings perform consistently better or worse than others, and the same appears to be the case with groups of reproducing *P. corneus*. Table 11 shows the mean number of eggs laid per snail per 48 hours in each replicate over the duration of the 28 day pre-exposure (baseline) phase, the first 28 day period of the exposure and over the whole exposure. The reproductive rate of each replicate group is compared only with itself before and after exposure using paired 2-tailed t-tests, and the results for comparisons between the pre-exposure and the first 28 days of the exposure, and between the pre-exposure and the whole exposure period are also given. There was an overall significant difference between the mean number of eggs laid per snail per 48 hours in the pre-exposure period and exposure periods at 15^{o}C (Repeated Measures ANOVA, $p<0.001$ both after 28 days and in the whole exposure), and also at 20^{o}C, but after 28 days only (Repeated Measures ANOVA, $p = 0.001$).

Table 11 – The mean number of eggs laid per *P. corneus* in each replicate (in the same order in each case) per 48 hours in the 28 day pre-exposure (baseline) period, the first half (28 days) of the exposure and the whole (56 day) exposure at (a)15°C and at (b) 20°C, and the results of paired t-tests.

a) 15°C	17β-oestradiol concentration			
	Control	1 ngL^{-1} nominal	10 ngL^{-1} nominal	100 ngL^{-1} nominal
Mean number of eggs laid in the pre-exposure phase	30.8 26.8 37.1 15.5	28.2 25.9 23.5 26.2	25.3 26.0 38.8 28.4	29.5 16.2 22.8 12.3
Mean number of eggs laid in the first 28 days of exposure	19.0 5.10 22.7 7.67	22.9 19.7 12.6 22.5	24.0 7.17 29.8 21.3	33.7 18.3 17.9 12.6
Paired t-test between pre-exposure and first half of exposure	Significantly lower p = 0.018	Significantly lower p = 0.024	No significant difference p>0.05	No significant difference, p>0.05
Mean number of eggs laid in the whole exposure period	18.7 5.35 18.5 10.6	22.4 17.1 13.7 21.0	18.8 8.76 24.1 21.6	25.8 14.0 16.2 14.7
Paired t-test between pre-exposure and the whole exposure	Significantly lower, p = 0.031	Significantly lower, p = 0.007	Significantly lower, p = 0.026	No significant difference, p>0.05

b) 20°C	17β-oestradiol concentration			
	Control	1 ngL^{-1} nominal	10 ngL^{-1} nominal	100 ngL^{-1} nominal
Mean number of eggs laid in the pre-exposure phase	28.9 53.8 54.8 28.1 38.0	51.4 48.9 32.5 43.6 69.3	31.2 43.9 66.2 50.1 64.1	64.4 24.2 40.3 25.1 44.1
Mean number of eggs laid in the first 28 days of exposure	33.2 60.6 72.1 38.7 41.2	52.9 43.7 45.1 62.6 77.2	36.6 41.4 71.7 65.0 60.8	74.8 33.2 38.1 39.8 46.1
Paired t-test between pre-exposure and first half of exposure	Significantly higher, p = 0.030	No significant difference p>0.05	No significant difference p>0.05	No significant difference, p>0.05
Mean number of eggs laid in the whole exposure period	36.2 51.3 62.9 33.2 41.3	46.9 44.9 36.9 50.8 68.2	33.4 30.3 60.1 55.2 51.6	60.6 31.7 31.9 37.3 44.1
Paired t-test between pre-exposure and the whole exposure	No significant difference, p>0.05	No significant difference, p>0.05	No significant difference, p>0.05	No significant difference, p>0.05

Table 11 shows that in the simulated autumn exposure (a), the control group lays a significantly lower number of eggs per snail per 48 hours in the 'exposure' period than in the 'pre-exposure' period. Of course, no 17β-oestradiol was added at any time, and the rate of reproduction can be expected to fall over time due to the relatively cool temperature and short day length. The same is true for the groups exposed to 1 ngL^{-1} and 10 ngL^{-1} 17β-oestradiol (nominal), with the number of eggs laid in the exposure period being significantly lower than in the pre-exposure period. However, this was not the case for the groups exposed to 100 ngL^{-1} 17β-oestradiol (nominal), as there is no significant difference in the number of eggs laid per snail per 48 hours in the pre-exposure and exposure phases.

Figure 43 (d) shows that the cumulative reproductive rate of this group remained close to constant for the first 42 days of the exposure, reducing only slightly in the final 14 days. In the control group (a), the overall reduction was 52.9%. The intermediate treatment groups (1 and 10 ngL^{-1} 17β-oestradiol nominal; b and c) are also reduced, but to a lesser degree. It appears that exposure to 100 ngL^{-1} 17β-oestradiol (nominal) perpetuates the rate of reproduction that was observed at the onset of autumn, into the period when the reproductive rate of un-exposed snails begins to decline. There may also be some evidence of dose-dependency in this effect, as in the 10 ngL^{-1} (nominal) treatment group there is no significant decline from the rate of reproduction in the pre-exposure phase over the first 28 days of the exposure phase, suggesting that there may have been a more transient perpetuating effect.

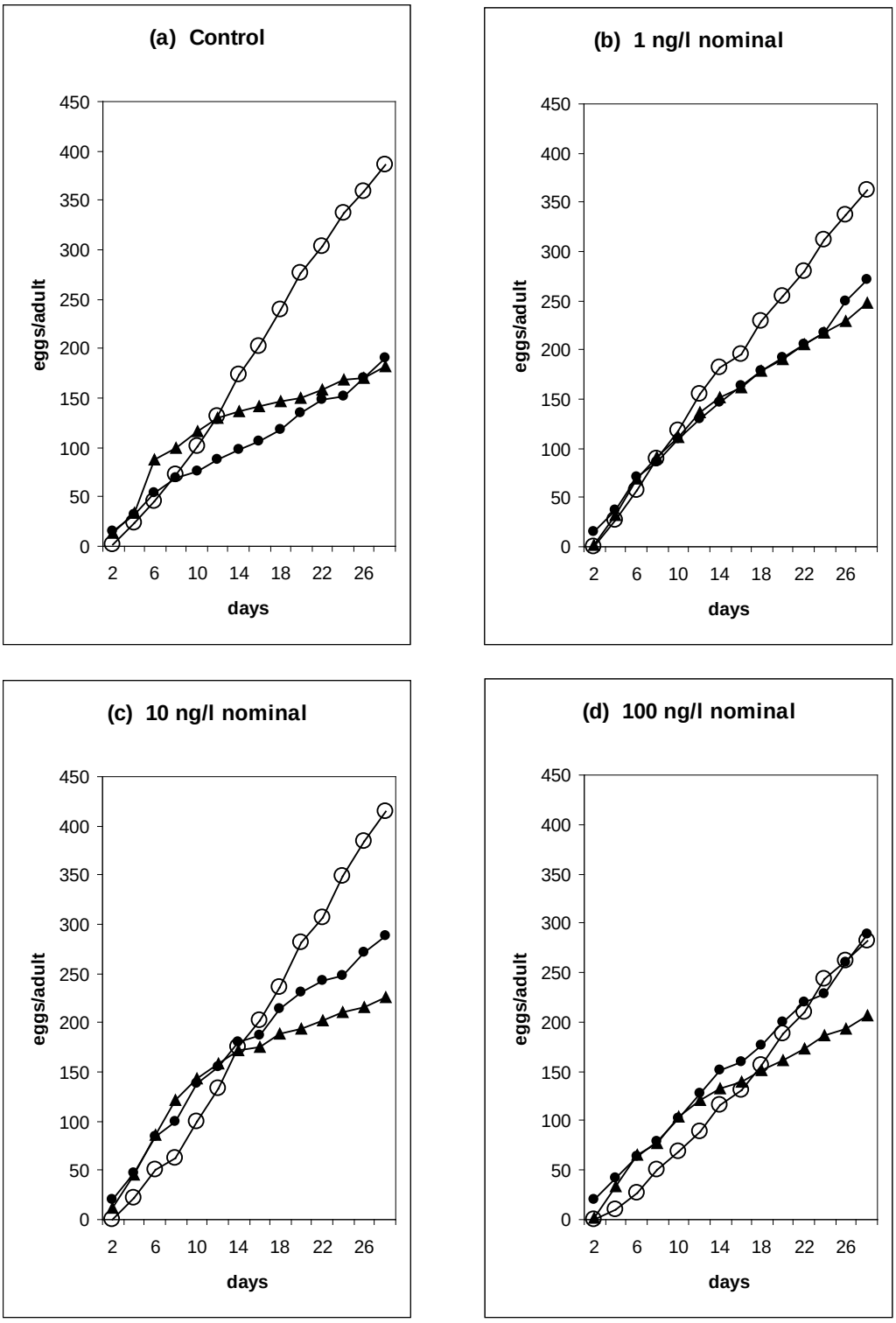


Figure 43: Cumulative mean number of eggs laid per *P. corneus* at 15°C, 12hL/12hD, during the pre-exposure period (open circles), and the first (black circles) and second (black triangles) 28 days of the exposure period.

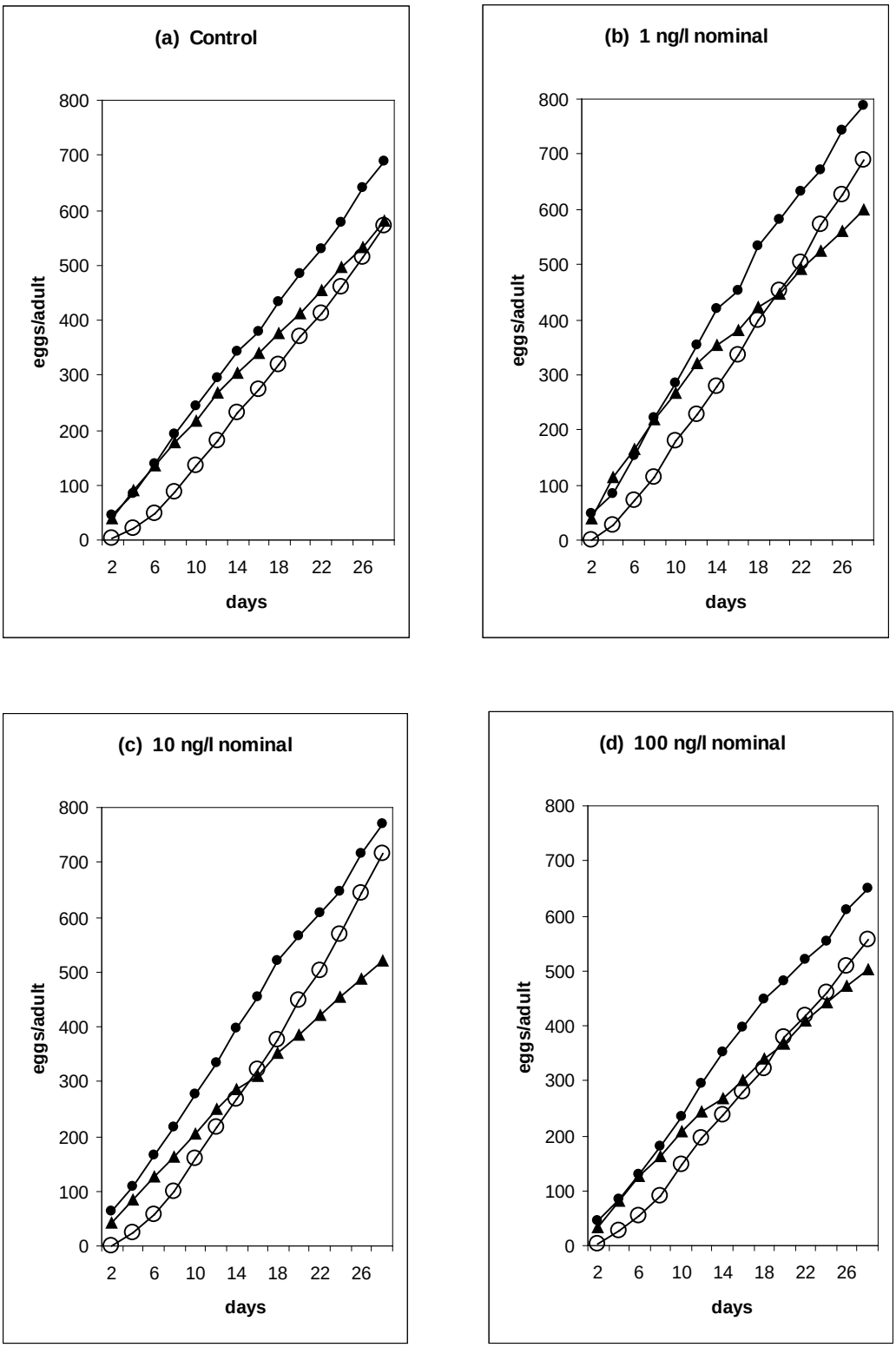


Figure 44 - Cumulative mean number of eggs laid per *P. corneus* at 20°C, 16hL/8hD, during the pre-exposure period (open circles), and the first (black dots) and second (black triangles) 28 days of the exposure period.

These results make it clear that rather than exposure to17β-oestradiol causing an induction in the egg laying behaviour of *P. corneus* (i.e. an increase in the number of eggs produced over that expected for the conditions), it is a perpetuation of the summer time reproductive rates after the onset of autumn that cause the overall number of eggs laid per snail to appear greater. The opposite was the case at 20^{o}C with a 16 hour day length. Table 11 (b) and Figure 44 show that while the number of eggs laid per snail increases slightly during the first 28 days of the exposure in all the treatment groups under these conditions, this is significant only in the control group. It is possible that exposure to 17β-oestradiol slightly inhibited the increase in egg laying behaviour that occurred in the control group during the prolonged simulated summer conditions. However the significance of this increase in the control group was lost over the course of the whole exposure, and there are no other significant differences between the pre-exposure and exposure phases in any of the 17β-oestradiol treatments.

4.2.3.7 Egg Mass and Egg Effects

Number of Eggs per Mass

Figure 45 shows the mean number of eggs counted in each mass in the simulated summer and autumn exposure regimes during the pre-exposure phase (including all tanks), and the control and each 17β-oestradiol treatment in the exposure phase. There are no significant differences between the mean number of eggs per mass in the control group and any treatment group in either exposure (ANOVA, $p>0.05$ in both cases). In simulated summer conditions (20^{o}C) the mean number of eggs per mass in the control and treatment groups during the exposure phase was 36.8, and in simulated autumn conditions (15^{o}C) it was very similar at 37.8. There is no significant difference between the mean number of eggs per mass in these two exposure regimes (2-tailed t-test, $p>0.05$).

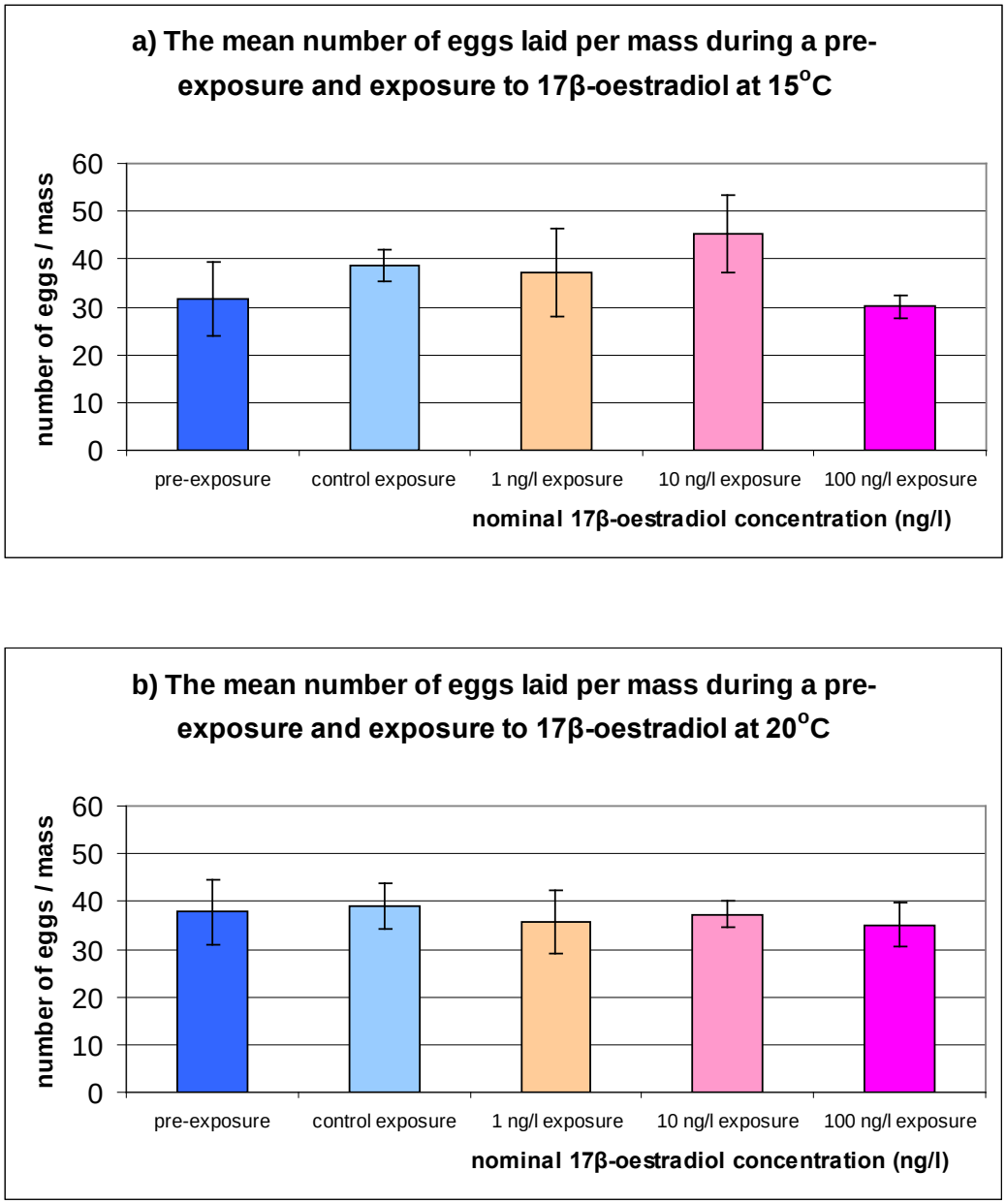


Figure 45 - The mean number of eggs per mass in egg masses laid by *P. corneus* at 15°C (a) and 20°C (b) during a pre-exposure and exposure to 17β-oestradiol (error bars represent the standard error of the means).

Egg Mass Dry Weight

Figure 46 shows the mean dry weight of egg masses (total dry weight divided by the number of masses) in the simulated summer and autumn exposure regimes during the pre-exposure phase (including all tanks), and for the control and each 17β-oestradiol treatment in the exposure phase. There are no significant differences between the mean dry weight of the egg masses laid by the control group and those in any treatment group in either exposure (ANOVA, $p>0.05$ in both cases). However, in simulated summer conditions ($20^{o}C$) the mean dry weight of the egg masses in the control and treatment groups during the exposure phase was 6.31 mg, but in simulated autumn conditions ($15^{o}C$) the mean weight of the egg masses was significantly heavier (exactly 1 mg heavier per mass at 7.31 mg, 2-tailed t-test, $p = 0.015$). Because the egg masses laid in the simulated summer exposure regime are lighter than those laid in the simulated autumn exposure regime, but the number of eggs per mass was similar in both exposures, the eggs laid in simulated summer must be smaller and / or the amount of packaging material (the gelatinous substance surrounding the eggs within the egg mass) must be less.

Egg Dry Weight

Figure 47 shows the mean dry weight of the egg masses divided by the mean number of eggs per mass in the simulated summer and autumn exposure regimes during the pre-exposure phase (including all tanks), and for the control and each 17β-oestradiol treatment in the exposure phase. In simulated summer exposure ($20^{o}C$) the mean dry weight of each egg and its relative proportion of packing material was 0.175 mg, and in simulated autumn exposure ($15^{o}C$) it was significantly more at 0.196 mg (2-tailed t-test, $p = 0.001$). Again there are no significant differences between the dry weight of the eggs and their proportion of the packing material in the control group and any treatment group in either exposure (ANOVA, $p>0.05$ in both cases).

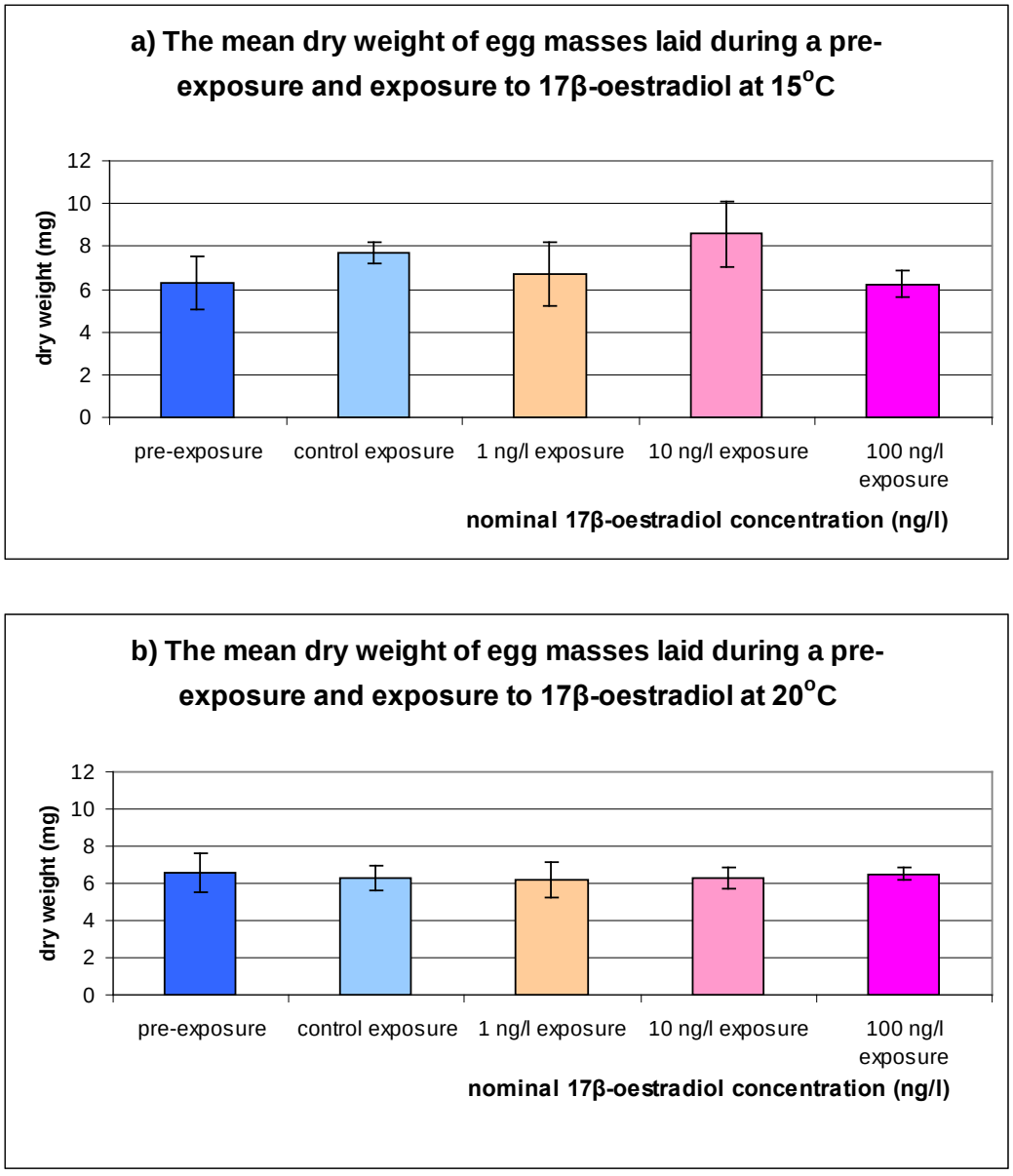
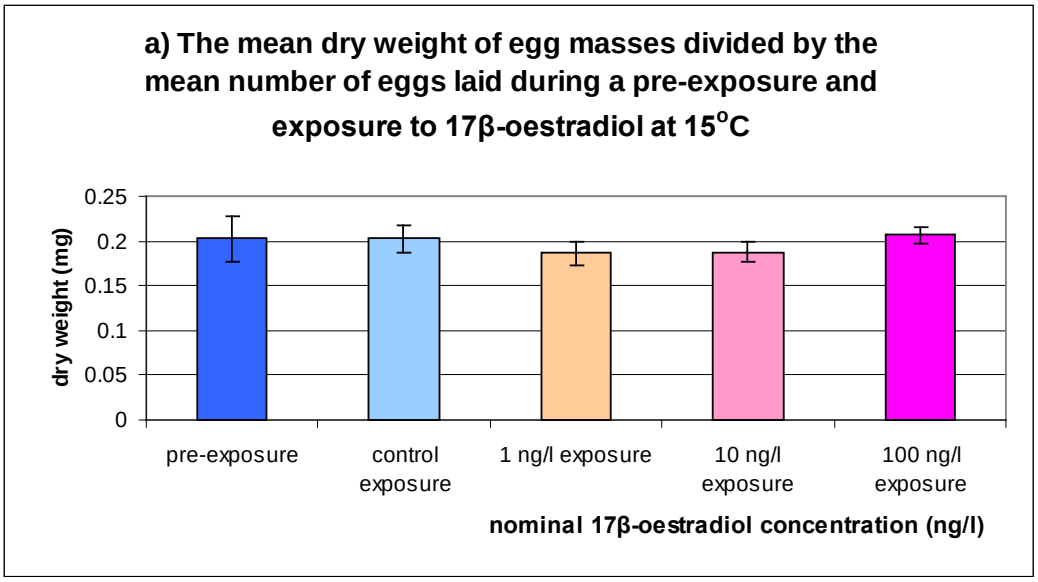


Figure 46 - The mean dry weight of egg masses laid by *P. corneus* at 15°C (a) and 20°C (b) during a pre-exposure and exposure to 17β-oestradiol (error bars represent the standard error of the means).



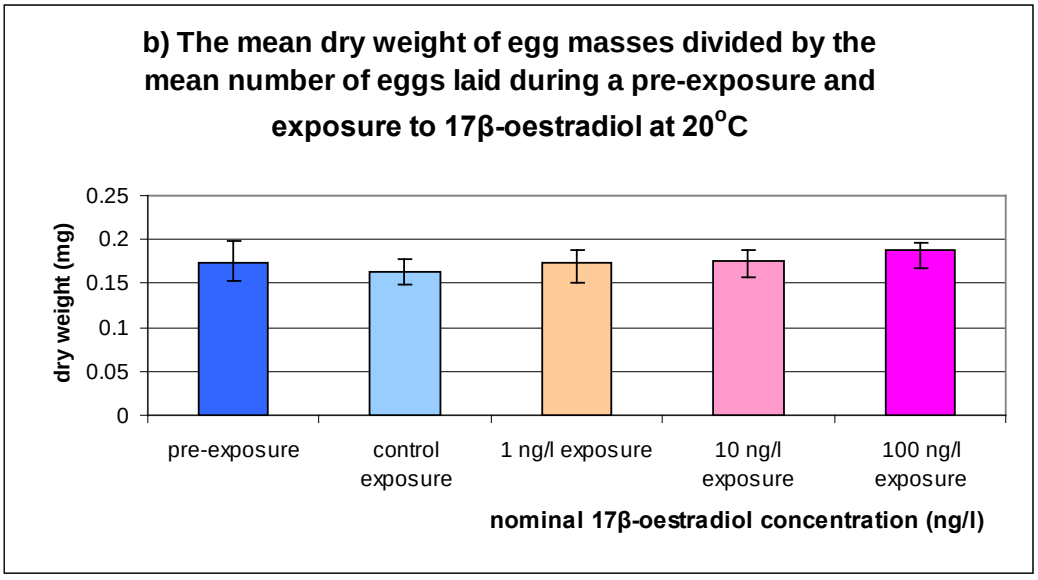


Figure 47 - The mean dry weight of the eggs and their relative proportion of packaging material in masses laid by *P. corneus* at 15°C (a) and 20°C (b) during a pre-exposure and exposure to of 17β-oestradiol (error bars represent the standard error of the means).

Egg Abnormality rate

During the egg counting process, many types of abnormalities associated with the mass or the eggs themselves were recorded. These included the presence of striated gelatinous material between the eggs giving the egg mass a cloudy appearance, discoloured albumin (red or brown instead of yellow) within the eggs, misshapen (dwarf or giant) eggs and conjoined pairs of eggs. While these observations were of interest, none of these symptoms are ascertained as preclusive to the ability of the embryo to hatch successfully, and so these abnormalities are not included in the assessment of the ratio of 'normal' to 'abnormal' eggs. Only abnormalities considered likely to affect the hatching success of the egg are taken into account. These include eggs without an embryo or with the embryo outside of the egg capsule, or those with multiple embryos inside the egg, as previous observations had shown that the development of these embryos is arrested when the area inside the egg is fully utilised (Costil, 1994). The ratio was therefore derived by dividing the number of abnormal eggs (those without or with multiple embryos) by the total number of unbroken eggs (broken eggs were excluded as it was not possible to know their status) and multiplying by 100.

Figure 48 shows the mean percentage of egg abnormalities occurring in the simulated summer and autumn exposure regimes during the pre-exposure phase (including all tanks), and for the control and each 17β-oestradiol treatment in the exposure phase. On first assessment, it appears that exposure to 17β-oestradiol increases the abnormality rate, as the mean abnormality rate in the control group is lower than all the exposed groups in both exposures. However, the rate of egg abnormality observed in the pre-exposure phase was relatively high in both exposures, although this may have been an artefact of acclimation to test conditions. It is therefore possible that exposure to 17β-oestradiol was an additional stressor, but there were no significant differences between abnormality rates recorded in the eggs laid by the control group and those laid by any treatment group in either exposure (Kruskall-Wallis, $p>0.05$ in both cases). In the simulated summer exposure ($20^{o}C$), the overall mean percentage abnormality

was 1.12%, but in simulated autumn conditions (15°C) it was significantly less, at 0.50% (Mann-Whitney test, p = 0.013). This suggests that the slower rate of reproduction in autumn contributes to a lower rate of egg abnormalities.

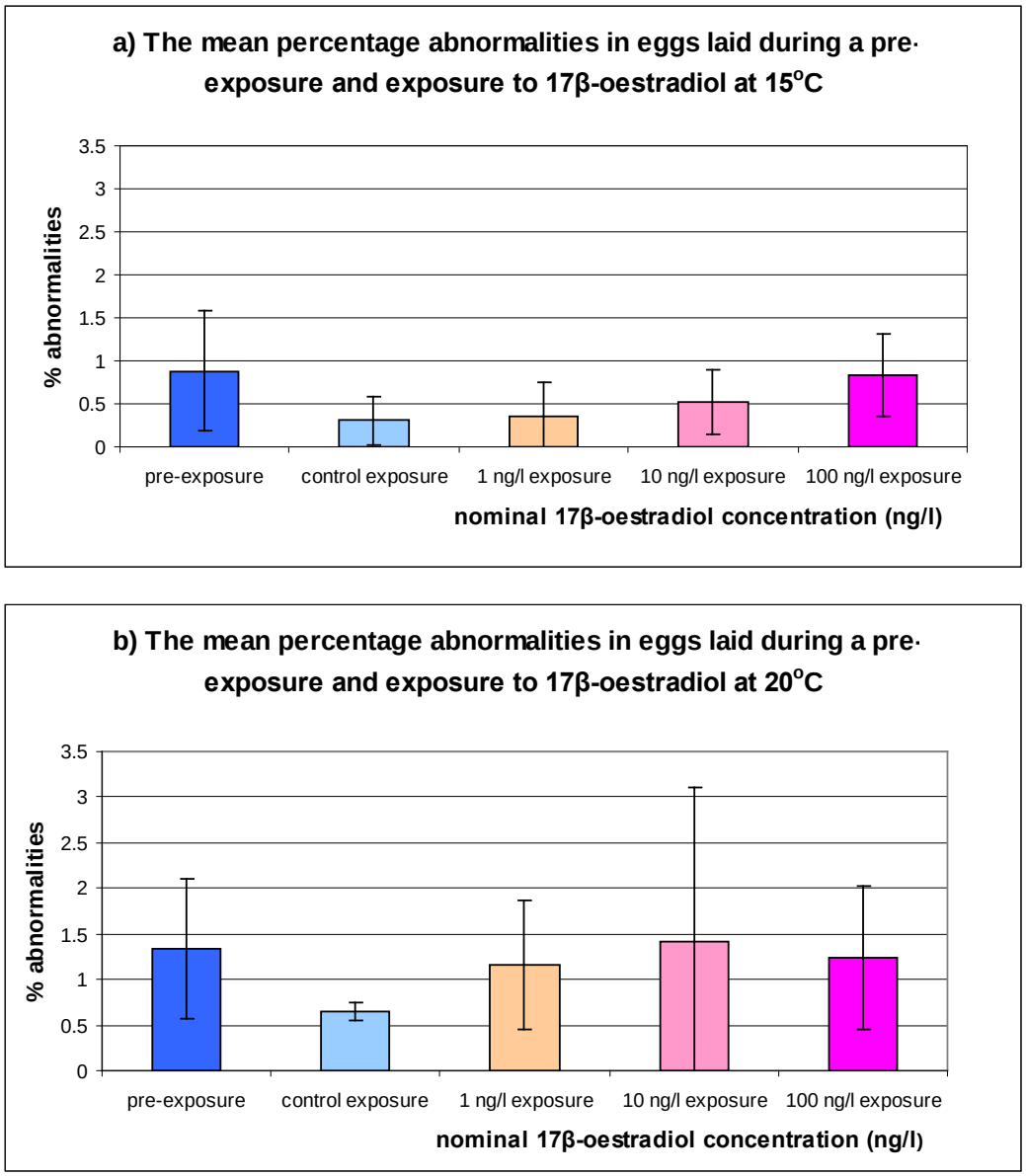


Figure 48 - The mean percentage abnormality in eggs laid by *P. corneus* at 15°C (a) and 20°C (b) during a pre-exposure and exposure to 17β-oestradiol (error bars represent the standard error of the means).

Egg Hatching Success

On one occasion during each cycle of the exposure the eggs laid in one 48-hour period were counted and eggs that were broken, without an embryo or with multiple embryos were excluded from the count. Of the remaining eggs, the number that successfully hatched within 14 days (incubated at 20^{o}C) was counted and a percentage successful hatching rate calculated. No assessment was made during the fourth cycle as it overran the end of the exposure and incubation facilities were not available.

Figure 49 shows the mean percentage of successful hatching in eggs laid in the simulated summer and autumn exposure regimes during the pre-exposure phase (including all tanks), and for the control and each 17β-oestradiol treatment in the exposure phase. The results were highly variable and unexpectedly low in all the trials, including those laid in the pre-exposure period. The highest percentage successful hatch was recorded in the first pre-exposure trial in simulated summer, and at 67.4%, is comparable to that recorded for *Planorbis carinatus* (Pounds et al., 2008). It is possible that the lower percentage successful hatches were attributable to the technique of lifting the egg masses from the site of oviposition, causing imperceptible damage. On the third occasion, eggs from the group exposed to 100 ngL^{-1} 17β-oestradiol (nominal) in the simulated autumn exposure failed to hatch at all, although this is unlikely to be an effect of the steroid as the highest percentage successful hatch in this exposure occurred in eggs laid by the group exposed to 10 ngL^{-1} 17β-oestradiol (nominal) on the second occasion (66.1%). There are no significant differences between the mean percentage of successful egg hatching in the control group and in any treatment group in either exposure (Kruskall-Wallis, $p>0.05$ in both cases).

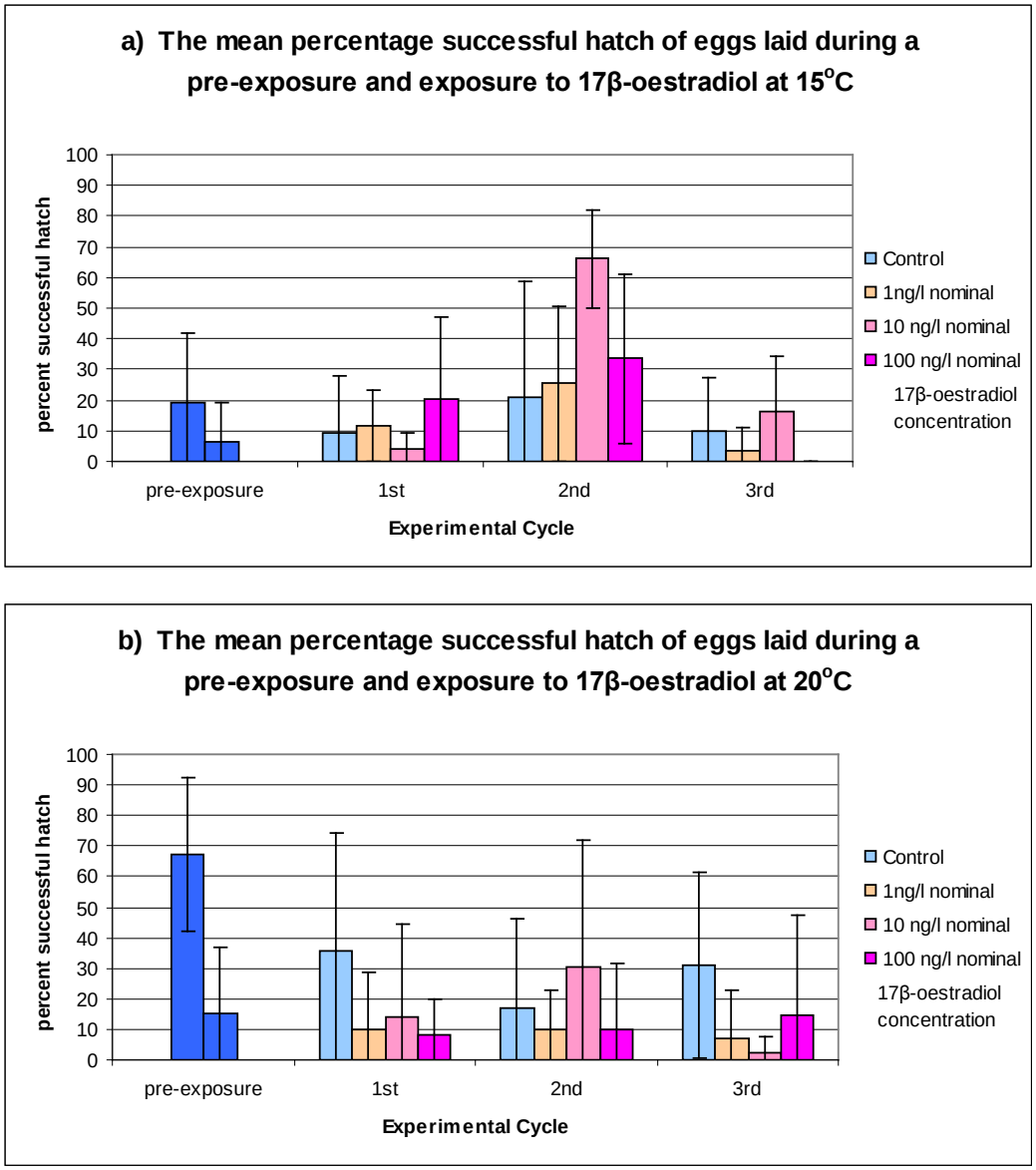


Figure 49 - The mean percentage of successful hatching in eggs laid by *P. corneus* at 15°C (a) and 20°C (b) during a pre-exposure and exposure to 17β-oestradiol (error bars represent the standard error of the means).

4.3 Comparative responses of *P. corneus* to 17β-oestradiol.

4.3.1 Physico-chemical conditions

While it is possible to make comparisons between the 17β-oestradiol treatments in the exposures described, it is more difficult to attribute the observed effects to defined steroid concentrations for a hazard assessment, because the results of the chemical analyses were more variable than expected. Perhaps this was not surprising in the mesocosm exposures, since pump performances were variable over time and filters and flow gauges frequently became blocked while unattended. In the first exposure, the measured concentrations in the 17β-oestradiol dosed tanks were within the expected order of magnitude at each sampling event, but were also intermittently up to x4 times higher than planned. However, the data collected from monthly samples show that the treatment tanks were significantly different to each other and to the reference tank. This is considered indicative only, as four spot samples from a long-term exposure can not be used to determine an entirely reliable mean, but overall it appears that 17β-oestradiol dosing was broadly successful.

In the second mesocosm exposure, the results from the highest treatment tank were initially similar, despite the concentration of the dosed steroid having been reduced by half. The measured levels of 17β-oestradiol then fell over the course of the exposure, until the treatments became indistinguishable from the reference in the autumn. On this occasion the influent filter had been removed to allow the snails to feed on the algae and detritus in the river water. The falling concentrations are therefore likely to be due to adhesion of the steroid to the layer of sediment and detritus that built up on the bottom of the tanks over time. Again, these analyses are only indicative of the treatment conditions; nonetheless it appears that the treatments were sufficiently differentiated at the outset, but that the general pattern was of declining exposure over the summer.

The quality of the influent river water used in the mesocosm exposures was of greater concern. 17β-oestradiol was repeatedly measured in the reference tank on both occasions, and the reason for this is far from clear. On the first occasion, the measured concentrations in the reference tank ranged between 6.14 and 20.3 ngL^{-1}, which is approximately x10 that which might normally be expected in rivers that receive treated sewage effluent. The water supply to this tank had also been pumped though an activated carbon filter. These levels therefore seemed implausible, but the confirmatory laboratory also measured levels from 3.7 to 9.2 ngL^{-1} in independent samples taken on the same day. However, there are no large sewage treatment works entering the River Blackwater upstream of the abstraction point, the nearest being Chelmsford Sewage Treatment Works which discharges into the River Chelmer / Blackwater estuary below a barrage. The source of this steroid load is unlikely to be operator contamination, as it was repeatedly recorded in samples taken from several tanks. The only other potential sources were contamination from the 17β-oestradiol treated tanks, or from another unknown upstream contamination point on the river.

After this mesocosm exposure had been completed and all the measured concentrations had been received from the analysing laboratory, the tank system was dosed with sufficient fluorescein to generate visible colour in the dosed tanks and a Relative Fluorescence Unit (RFU) of approximately 200,000 above background for 24 hours of normal operating procedure. This would allow any cross-contamination between the tanks via drain backflows to be identified. The mean detected levels in the treatments tanks was 90,170 and 187,380 RFU. Given that the reference tank contained approximately x10 less 17β-oestradiol that the lower treatment and x100 less than the higher treatment, an approximate RFU of between 2000 and 20,000 would be expected if cross-contamination was occurring. However, the mean RFU value from the reference tank was only 245, and instrumental background was 227, therefore the possibility of cross-contamination can be excluded.

The influent water used in the second mesocosm exposure was taken from the River Chelmer in an attempt to avoid any unknown sources of contamination on the River Blackwater. However, a high level of 17β-oestradiol was again measured in the reference tanks early in the exposure (5.71 ngL^{-1}). An upstream section of the River Chelmer was surveyed for other potential sources of contamination, and several dairy cow wallows were identified. Hanselman et al. (2003) recorded that 17β-oestradiol levels in cattle liquid waste can reach 239 ug/kg so it is possible that cattle farming was a source of contamination in the area where the two rivers converge. However 17β-oestradiol was not recorded in the reference tank after this time, or in the river above 0.428 ngL^{-1}. Nonetheless, it is clear that there were no 17β-oestradiol unexposed gastropods in the mesocosm experiments.

It also became apparent that 17α-ethinylestradiol was also present in the second mesocosm exposure, as it was repeatedly recorded at concentrations of up to 2.34 ngL^{-1} in the reference and treatment tanks. This chemical was not used in these exposures, but it was used in a parallel and concurrent experiment. As cross-contamination had been excluded and the highest concentration was found in the influent river water (3.73 ngL^{-1}), it appeared that the source was again upstream. However, there are no known sources of environmental 17α-ethinylestradiol other than treated sewage effluent and this value is higher even than might be expected in the discharge from a large urban treatment works. It is therefore implausible that this chemical should be present in this quantity, and casts the analysis into doubt. 17α-ethinylestradiol was not used at all in the mesocosm system before this time, and it was also not found by the laboratory during the first mesocosm exposure, but the limit of detection was x10 higher on this occasion ($1ngL^{-1}$). However it was recorded as present by the confirmatory laboratory (CEFAS) at concentrations below this, which suggests that either two laboratories can mis-analyse this compound or that there is indeed an unknown source. Neither of these explanations are credible, but the former at least is possible, since analytical methods for this compound were in their infancy at this

time. Nonetheless, within a vertebrate context, it remains possible that both of the mesocosm exposures were set against a background of potent oestrogenicity.

Overall, the use of a mesocosm system to make the preliminary exposures of native freshwater gastropods to an exogenous vertebrate steroid was of value, and it was accepted that in these circumstances, the steroid concentrations would not be dependable. Also, due to the scale of the mesocosm tanks, the only practical approach to generating appropriate information for statistical analysis was a form of pseudo-replication. This increases the potential for tank effects to be the cause of any differences observed because each tank is an individual mesocosm and liable to develop differing habitats. However, it was a suitable approach to make a general assessment of the responses of gastropods without the need to prepare for large-scale laboratory exposures using species for which little is known about housing and feeding requirements. Although the mesocosm exposure have been 'repeated' to some degree, a controlled repeat is not possible in this format of experiment. Consequently the next objective was to undertake controlled exposures using independent replication in laboratory conditions.

The choice of test vessels used in the *P. corneus* exposures worked well; the mean pH remained between 7 – 7.5 and the dissolved oxygen levels were above 80% ASV on all except a very few occasions when the air lines became blocked. Being pulmonate, the test organisms did not appear to be affected. The mean hardness of the media was close to the calculated level of 394 mgL^{-1} $CaCO_3$ equivalents and the excess food and faeces levels were not polluting, as the conductivity remaining around 1000 us/cm throughout. Apart from an occasion when a constant temperature facility malfunctioned for a brief interval (less than 2 hours), the two test temperatures were constant and remained distinct from one another by at least $3.5^{o}C$, allowing for the error of measurement.

However, as the preliminary exposure study made before the *P. corneus* exposures demonstrated, 17β-oestradiol degrades rapidly, and therefore in a semi-static system the exposure concentrations will by necessity fluctuate. Also, the

analytical costs dictate that only a certain number of samples can be analysed, so once again the analytical data can only give an indication of the exposure conditions. Nonetheless, aside from a number of methodological errors made during the analysis of the samples, the measured concentrations indicated that the exposures had progressed as expected. The predicted means at 24 hours post media change (calculated from the stability study) were 1.23, 6.8 and 82.9 ngL^{-1}, and the measured means were 0.853, 6.8 and 78 ngL^{-1} respectively.

While every effort was made to ensure the control media was not contaminated with 17β-oestradiol, a mean value of 0.215 ngL^{-1} was recorded during the exposures. The quoted analytical uncertainly in river water was 29.2% over a range of 4-16 ngL^{-1}, and it is likely to be greater below 4 ngL^{-1}. Even in ultra-high purity water blanks, the uncertainty is 13.1% and the highest recorded value was 0.1368 ngL^{-1}. This implies that some very low measurements are spurious and indeed the laboratory prefers to use a pragmatic minimum reportable value of 0.3 ngL^{-1}. However, the highest measured concentrations were above this value, and were found both before (0.744 ngL^{-1}) and after (0.718 ngL^{-1}) media changes, so are not attributable to excretion by the snails. As these values are similar and from the same (second) sampling occasion, it is more likely to be due to insufficient cleaning of the vessels that the sample media was pooled into. Indeed this error was discovered with certainty on the third sampling occasion, and consequently the values from this event (ranging between 0.746 and 1.18 ngL^{-1}) are not included. There was almost no detectable 17β-oestradiol in the first and fourth sets of samples, and it is therefore considered likely that the control tanks were at worst only marginally exposed.

4.3.2 Mortality

A significant increase in the mortality rate of *P. corneus* was observed in the highest test concentration of both mesocosm exposures, the mean and maximum measured concentrations being 370 ngL^{-1} and 889 ngL^{-1} 17β-oestradiol on the first occasion and 70.3 ngL^{-1} and 232 ngL^{-1} on the second. Although these

concentrations are different, the response is consistent and therefore unlikely to be a random tank-effect as different tanks were used for these two exposures. As the mortality rate at these concentrations was 44.4% after 12 weeks exposure on the first occasion and 74.1% after 16 weeks exposure on the second, it is possible that the long-term LC_{50} for this species lies at a concentration approximately x10 above environmental levels. However, there was no significant increase in mortality at the highest concentration of 17β-oestradiol in the 8-week laboratory exposures (mean = 78.0 ngL^{-1} and maximum = 178 ngL^{-1}), so it is probable that the observed mortality in the mesocosm was caused by a combination of steroid exposure and environmental stress.

4.3.3 Growth

The surviving *P. corneus* grew well in the mesocosm experiments, with the reference group increasing in mean diameter by 17.8% and 21.0% and in mean wet weight by 60.8% and 62.2% on the first and second occasions respectively. On the first occasion, the groups exposed to the lower concentration of 17β-oestradiol (mean = 48.1 ngL^{-1}) increased in length significantly less, but there was no concentration-response relationship and the group exposed to the higher concentration grew more than the reference group (20.3%). On the second occasion both of the exposed groups grew less than the reference group and there was a concentration-response relationship, but this was not significant. There were no effects on the condition factor. The animals in the laboratory exposures also grew well, although not to the extent of that observed in the mesocosm exposures (increasing in mean diameter by 6.6% and 5.4% and in mean wet weight by 17.0% and 12.8% at 15 and 20^{o}C respectively over the 12 week experiment). There were no effects of 17β-oestradiol on growth, and indeed a greater mean increase in size and weight was observed in all groups exposed to 17β-oestradiol at 20^{o}C.

4.3.4 Reproduction

In the first mesocosm exposure, reproduction in *P. corneus* was significantly inhibited in both 17β-oestradiol treatments at every sampling event during the exposure excepting the last two, when egg-laying had almost ceased in all the groups. The high mortality observed in the group exposed to the highest concentration of 17β-oestradiol is a clear indication of chronic toxicity, and it is possible that the group exposed to the lower concentration also experienced toxicity as the measured concentrations were unintentionally high. Whereas the reference group showed a clear peak of oviposition at Week 2 (summer solstice) and a gradual decline from this point until the end of the exposure (mid-August, onset of autumn), the 17β-oestradiol treated groups experienced much steeper declines. This was most pronounced in the group exposed to the lower concentration, with the mean number of eggs per adult dropping from over 500 to below 100 between Weeks 2 and 4. The number of eggs laid by the group exposed to the higher concentration was reduced by approximately half at this time (to a mean of 248 eggs / snail).

This pattern forms a 'U-shaped' concentration-response curve, the reason for which should be explicable by an additional underlying mechanism mediating an otherwise normal dose-response (Davis and Svendsgaard, 1990). In this case, the secondary mechanism is not clear, but it may be a re-routing of the remaining energy budget of the more severely affected organisms to reproduction, much as a stressed plant will bloom vigorously before succumbing. This is described by Calow (1979) as a 'reckless reproductive strategy'. A scattergram of the mean number of eggs laid divided by the mean mortality to give a birth rate versus death rate ratio for each treatment supports this theory (Figure 50). The 'mortality per unit of reproductive effort' between Weeks 2 and 4 in the reference group was relatively high; 500 eggs are laid in lieu of each snail death. This is markedly reduced in both of the exposed groups (to between 100 and 200 eggs per snail death).

While the number of eggs laid per snail mortality declines over time in the reference group (possibly due to an exhaustion of effort or resource), the additional suppression in the two exposed groups is consistently similar, suggesting that both of these groups were experiencing toxicity to the same degree. It appears that while the group exposed to the lower concentration of 17β-oestradiol exhibited the toxicity as a strong inhibition of reproduction, the group exposed to the higher concentration experienced a greater mortality, but the surviving individuals invested more effort 'recklessly' into reproduction. However, the reference group always laid the greatest number of eggs, so there is no evidence for any steroid-mediated induction of reproduction, as reported in *M. cornuarietis* by Oehlmann et al. (2006).

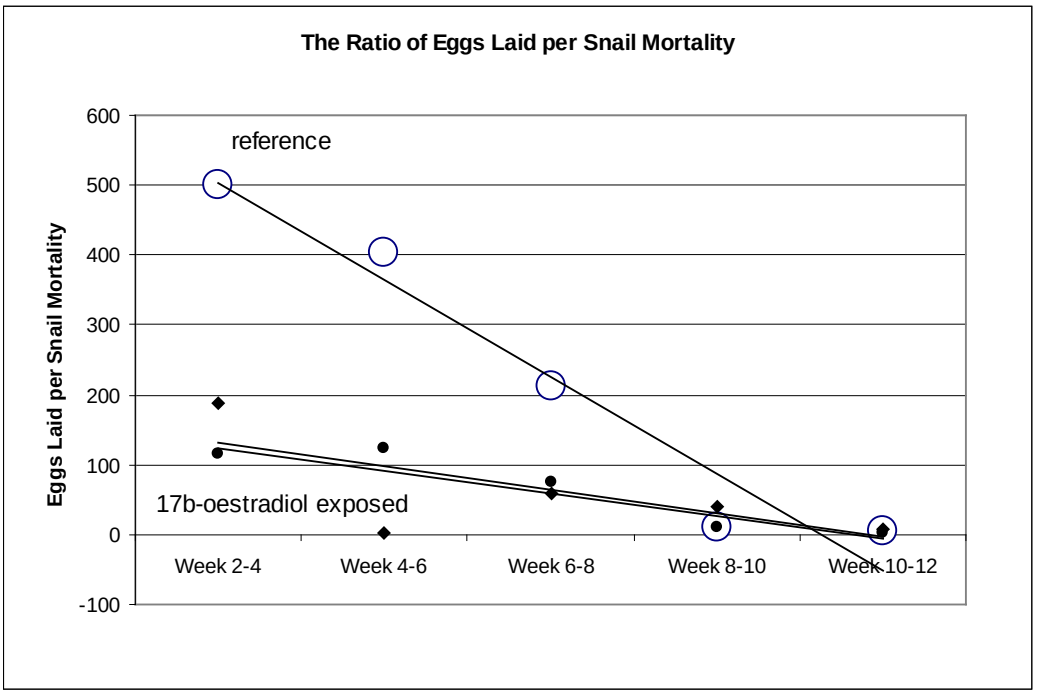


Figure 50 – The ratio between the number of eggs laid and the mortality of *P. corneus* over a 12 week mesocosm exposure to 17β-oestradiol (open circles = reference group, black dots = exposed groups).

Despite the potential toxic effects, the mean number of eggs laid per mass remained relatively constant. This was also the case in laboratory exposures employing *P. corneus*. It is possible that this species can alter the size of egg

masses in response to environmental conditions e.g. higher flow rates or poor adhesion surfaces, however it appears that 17β-oestradiol exposure has no effect. The egg masses laid by the group exposed to the highest concentration in the mesocosm exposure were significantly heavier at Week 4 only. This may be an effect of 17β-oestradiol exposure as this was the first sampling event where toxic effects were manifest, but this group also laid significantly heavier egg masses before the exposure began, and may therefore already be disposed to providing the masses with more packaging material. The effect did not occur on any subsequent sampling events, nor in later laboratory exposures, and these end-points are therefore not considered further.

In the second mesocosm experiment, the number of eggs laid per snail in the reference group again peaked at the summer solstice (Week 6), and declined over the course of the remainder of the exposure. In the first 8 weeks of the experiment, the number of eggs laid in the exposed groups was again reduced, but less so. Although this reduction was dose-dependant, it was not significant until Week 8, the first sampling event after the solstice. Only the reproduction of the group exposed to the highest concentration of 17β-oestradiol was significantly inhibited. In combination with the significantly increased mortality observed in this group, this suggests that overt toxicity was once again an influencing factor. The number of eggs laid per snail in the group exposed to the lower concentration of 17β-oestradiol was only marginally inhibited at Week 8 (not significantly different to the reference group), and interestingly, the reproduction in neither of the exposed groups notably declined over the following 6 weeks, in contrast with the reference group.

Because the number of eggs laid by the group exposed to the highest concentration of 17β-oestradiol was already inhibited by Week 8, and by Week 12 the reproduction of the reference group had also similarly declined, the number of eggs laid by the lower exposure group was approximately double that of either of the other groups at this point, forming an ‘inverted U-shaped’ concentration response curve. The two mechanisms involved on this occasion

appear to be overt toxicity (and consequent increased mortality and reproductive inhibition) in the higher 17β-oestradiol treatment, and a perpetuation of peak reproductive rates in the lower treatment group. There were no significant differences between the treatment groups at any time after Week 8, excepting at Week 12 when ANOVA detected a significant difference but Dunnett's tests did not.

However, conventional statistical tests are not designed to assess differences in non-monotonic curves (Davis and Svendsgaard, 1990). It could be argued that any exposure concentration causing appreciable toxicity to test organisms should be excluded from the interpretation of chronic end-points such as the potential for endocrine disruption (Barata et al., 2004), making the higher test concentration in both the first and second mesocosm exposures of *P. corneus* invalid. If they are excluded, and comparisons made between the reproduction of the lower treatment group and the reference group only, then there are significant inhibitions of reproduction in the first exposure from the outset and almost through to the end of the experiment at a mean measured concentration of 48.1 ngL^{-1} 17β-oestradiol, and significant inductions at the end of the second exposure (onset of autumn) at the much lower mean measured concentration of 11.0 ngL^{-1} (2-tailed t-tests, $p = 0.043$ at Week 14, $p = 0.034$ at Week 16).

Of course, the alternative interpretation is that there were no significant differences in the reproduction of 17β-oestradiol exposed *P. corneus* in the second exposure, excepting an inhibition of the highest treatment group at Week 8. However the advantage of having several sampling events over the course of the exposure is that trends can be observed, and more thorough explanations considered. Nonetheless, the potential for this species to perpetuate high reproductive rates into the autumn needed further exploration and confirmation in controlled laboratory conditions. Hence, two concurrent laboratory exposures of *P. corneus* to 17β-oestradiol were made in 'simulated summer' and 'simulated autumn' conditions. The chosen test conditions were kept constant both previous to and during the exposure, and an assessment the reproductive performance of

each group of snails was made before the steroid was added. This was compared with the reproduction observed during the exposure, in order that the between-group variation could be taken into account. This meant that there were several levels of control; an unexposed group in each test condition and an unexposed period for every group prior to the commencement of the exposure.

In 'simulated summer', the mean pre-exposure reproductive rate was constant and high in all groups, culminating in between 556 and 715 eggs per adult over the 4-week period. When exposed to 17β-oestradiol, there were no significant differences from this in any of the groups excepting the control group, when reproduction was significantly higher over the first 28 days of the exposure (although not over the whole exposure period). In 'simulated autumn', the mean pre-exposure reproductive rate of the groups was also constant, but lower, culminating in between 261 and 415 eggs per adult. In contrast to that observed in 'simulated summer', the reproductive rate of the control group declined significantly over the course of the exposure. This was expected in view of the declining reproduction of the reference group with the onset of autumn in the mesocosm exposures. The groups exposed to 1 and 10 ngL^{-1} 17β-oestradiol (nominal) were also significantly reduced, but the group exposed to the highest 17β-oestradiol concentration (100 ngL^{-1} nominal) was not, implying that the reproductive rate in this group was indeed perpetuated in 'simulated autumn'. The reproductive rate of the group exposed to 10 ngL^{-1} (nominal) was also maintained for the first 28 days of the exposure, suggesting a concentration-dependency in this effect.

There were no indications of toxic effects in the laboratory exposures, and the effect observed at 10 ngL^{-1} (nominal) 17β-oestradiol (mean measured = 6.77 ngL^{-1}) broadly tallies with the concentration used in the mesocosm at which elevated levels of reproduction were observed at the onset of autumn (also 10 ngL^{-1} nominal, mean measured = 11.0 ngL^{-1}). In the laboratory exposure, the perpetuated high levels of reproduction were more pronounced at the highest exposure concentration (mean measured = 78.0 ngL^{-1}), but a similar concentration

caused apparent toxicity in the second mesocosm exposure (mean measured = 70.3 ngL^{-l}). This may have been a compound effect with environmental stresses. In the laboratory exposures, there were no effects on the number of eggs per egg mass, the dry weight of the masses or the partitioning of the eggs and the egg mass packaging for either test condition. The rate of egg abnormalities was also unaffected, being similar in the pre-exposure phase and in the 17β-oestradiol exposed groups. There were no effects on egg hatching success, although the approach used did not appear to be suited to successful hatching as less than 25% of the eggs hatched in the sub-sample of egg masses from the control group.

Overall, it is considered that of the original hypotheses, that 'exposure to 17β-oestradiol will significantly alter the reproductive rate of *P. corneus* in the mesocosm exposures, causing deviation from the seasonal pattern of reproduction in the reference groups' is tentatively supported, with the additional indications that concentrations above environmental levels can cause toxicity, and that the observed inductions in reproduction occur at the onset of autumn. There seems to be little value in further repeating the mesocosm exposures, given the lack of control inherent in the system, and that the objective to make initial assessments of the responses of a range of freshwater gastropods has been fulfilled. Similarly, the second hypothesis, that 'exposure to 17β-oestradiol under controlled temperature and photoperiod conditions that represent a simulated summer and/or autumn will significantly alter the number of eggs produced by *P. corneus* relative to the control' is supported in the case of a simulated autumn but not in the case of a simulated summer, adding credence to the results from the mesocosm exposures.

4.3.5 Parasitisation

A small proportion of the wild-caught animals used in the laboratory exposures were parasitized with digenean trematodes (6.5%). It was assumed that they were incapable of contributing to the reproduction of the group, and they were removed from the data analysis. This may not be entirely the case, but it is likely

given that a high proportion of the gonadal tissue was destroyed. No parasitized animals were found at the end of either mesocosm exposures. Since the organisms used in the laboratory exposures were from multiple sources, it is possible that one of the supplies was taken from an unusually highly parasitized population, but the proportion does not indicate either supplier. It is more likely that this species is widely parasitized at low levels, and parasitized animals were not observed in the mesocosm exposures simply because they were sampled later in the year, allowing the parasitized animals to die naturally before the remaining population was sacrificed and dissected.

CHAPTER 5
REPRODUCTIVE EFFECTS OF EXPOSURE TO 17β-OESTRADIOL IN THE PROSOBRANCH GASTROPODS *V. viviparus* AND *B. tentaculata.*

5.1 Introduction and Methodology

This chapter presents the results of two outdoor mesocosm-based exposures of the prosobranch gastropod *V. viviparus* to 17β-oestradiol (Experiments 1 and 2), and both a mesocosm-based exposure (Experiment 2) and a laboratory exposure (Experiment 4) of the prosobranch gastropod *B. tentaculata* to this steroid.

The mesocosm experiments are the same experiments as those described in Chapter 4 as the pulmonates and the prosobranchs were co-exposed in the same mesocosm system. Consequently, the method and experiment design set out in Chapter 3, Section 3.1, and the alterations that were made between Experiment 1 (2004) and Experiment 2 (2006) also apply to these exposures. The additional prosobranch species (*B. tentaculata*) was deployed in Experiment 2 because it had not been possible to successfully raise *V. viviparus* in laboratory cultured conditions. The hypothesis for the mesocosm exposures is: 'Exposure to 17β-oestradiol will significantly alter the reproductive rate of the gastropods *V. viviparus* and/or *B. tentaculata*, causing deviation from the seasonal patterns of reproduction observed in the reference groups'.

The method and experimental design used in the laboratory exposure (Experiment 4) is set out in Chapter 3, Section 3.2.2. The conditions of this exposure were manipulated to mimic a real-time natural spring and summer. The hypothesis for this experiment is: 'Exposure to 17ß-oestradiol under controlled temperature and photoperiod conditions that represent a natural spring and summer will significantly alter the rate of egg production in *B. tentaculata* relative to the control.

5.2 Results and Discussion

5.2.1 Mesocosm Experiment 1 (2004) – *V. viviparus*

5.2.1.1 Water Chemistry and Water Quality

This experiment was conducted concurrently with the 2004 *P. corneus* exposure described in Chapter 3, Section 3.1.1. The snails were co-deployed in the same mesocosm tanks, and therefore the 17β-oestradiol treatment concentrations (both nominal and measured) are the same. The reference conditions and the water quality parameters are also as previously described.

5.2.1.2 Test Organism Survivorship

This species experienced high levels of mortality over the 12-week exposure, with the greater proportion of deaths occurring in the second 6 week period. Due to the 1 week acclimation period, the animals had in fact been in the mesocosm for three weeks at the Week 2 sampling point, although the treated groups had only been exposed to 17β-oestradiol for 2 weeks at this time. The mean mortality rate in the in all three tanks remained below 10% until the Week 6 sampling point, but Figure 51 shows that a dose-dependency was already becoming apparent. At Week 6, mean mortality of the group exposed to 200 ngL^{-1} 17β-oestradiol (nominal) was significantly higher than that of the reference population (ANOVA, $p = 0.022$). The dose-dependant effect persisted through to the end of the exposure, although there were no other occasions when significant differences occurred (ANOVA, $p>0.05$ in all cases).

The mean mortality rate in the reference population was 21.8% at Week 10, which is usually considered acceptable in chronic invertebrate exposures (e.g. OECD 2008), but by Week 12 it had reached 34.0%, which is less so, and the experiment was terminated. At this time, the mean mortality rate in the exposure groups (20 and 200 ngL^{-1} nominal) was 40.1% and 57.6% respectively. The

extrapolated first quartile percentage lethal dose of 17β-oestradiol (LC_{25}) over the twelve-week exposure was 224 ngL^{-1} (linear interpolation, data insufficient to generate confidence interval).

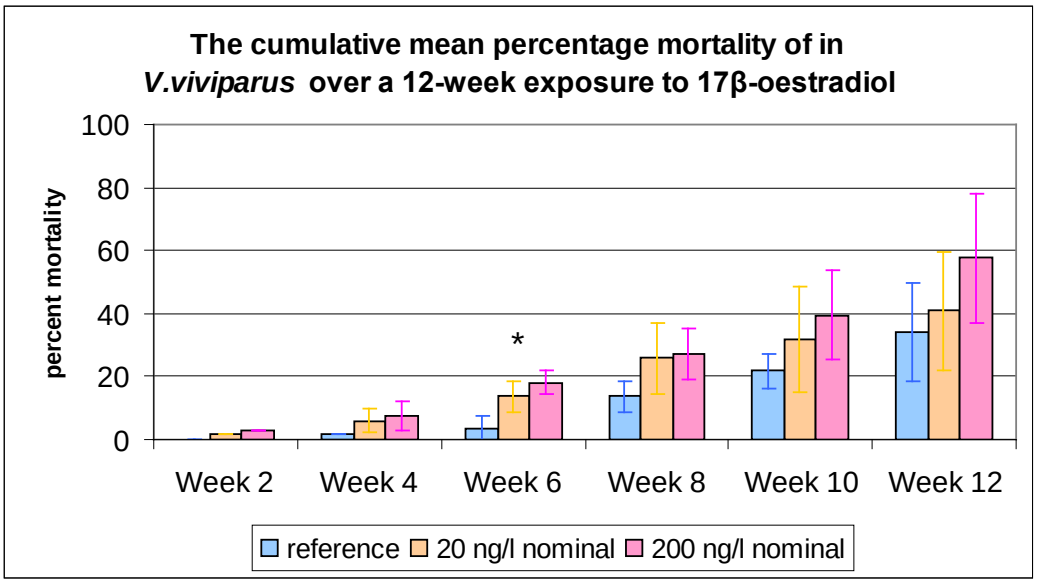


Figure 51 – The cumulative mean mortality rate of *V. viviparus* over a 12-week exposure to 17β-oestradiol (error bars represent the standard deviation, asterisks indicate a significant difference from the reference group *p<0.05).

5.2.1.3 Test Organism Morphology and Growth

There were no significant differences between the three tanks in the mean length (in the longest axis) or weight of the snails allocated to the three mesocosm tanks at the start of the experiment (ANOVA, $p>0.05$ in both cases).

During the 12-week exposure, almost all the groups showed a mean decrease in shell length. The greatest decrease occurred in the reference population but this was minimal at 1.5%. This was considered to be due to handling wear on the spire tip during sampling events, and so was not considered for further analysis. More importantly, the mean weight of this group decreased by 9.56%. The mean weight of the treated groups was also reduced, but less so, with the groups exposed to 20 and 200 ngL^{-1} 17β-oestradiol (nominal) decreasing by 4.11% and

4.73% respectively. However there was no significant differences between the tanks (ANOVA, $p>0.05$).

At the end of the experiment, once the shells of the remaining animals were cracked and removed and the embryos dissected from the brood pouch, it was determined that there was no significant difference in the condition factor of the females (the percentage weight of the flesh of the snails in relation to the total weight minus the embryos) between the reference and treatment groups (26.2%, 25.1% and 25.6% in the reference group, 20 and 200 ngL^{-1} exposed groups respectively, ANOVA, $p>0.05$).

5.2.1.4 Test Organism Gender Ratio

It was observed that in the reference group, 83.2% of the remaining snails were female. Of the groups exposed to 20 and 200 ngL^{-1} 17β-oestradiol (nominal), 88.3% and 95.8% were female, respectively. There was no significant difference in the gender ratio between the reference and treatment groups (ANOVA, $p>0.05$), but there was a significant correlation between the gender ratio and the nominal concentration of 17β-oestradiol (Pearson's Product Moment Co-efficient, $p<0.05$). However, as the data are quantal, the regression relationship is poor ($r^2 = 0.219$).

5.2.1.5 Test Organism Reproduction

The *V. viviparus* were allocated to the tanks 1 week prior to the start of the exposure in order to acclimate to the conditions in the mesocosm. No reproduction data were collected in this period.

During the course of the exposure, the number of neonates collected was adjusted for the total number of adults surviving, as the gender of the adults could not be established in the circumstances. Figure 52 shows that there was no apparent pattern in the number of neonates produced per adult; the level of variation was

high relative to the low productivity, making meaningful interpretation of the results difficult. The mean number of neonates produced by the treated adults was significantly higher than the reference population on only two sampling occasions. At Week 4, the group exposed to 20 ngL^{-1} 17β-oestradiol (nominal) laid 2.73-fold more neonates per adult than the reference group (0.964 compared to 0.356 neonates per adult, ANOVA, $p = 0.043$), and at Week 12, the group exposed to 200 ngL^{-1} 17β-oestradiol (nominal) laid 6.08-fold more neonates per adult than the reference group (2.44 compared to 0.402 neonates per adult, Kruskall-Wallis, $p = 0.048$). However, the mean total number of neonates produced per adult over the whole exposure was significantly higher in the group exposed to 200 ngL^{-1} 17β-oestradiol (nominal) than that in the reference group (Kruskall-Wallis, $p = 0.007$, see Figure 52), and there was a significant concentration-response relationship (Pearson's Product Moment Correlation, $p<0.01$) with a moderate model fit ($r^2 = 0.495$). There was no significant difference in the mean weight of the neonates (total weight of neonates divided by the total number) between the reference and exposed groups (ANOVA, $p>0.05$).

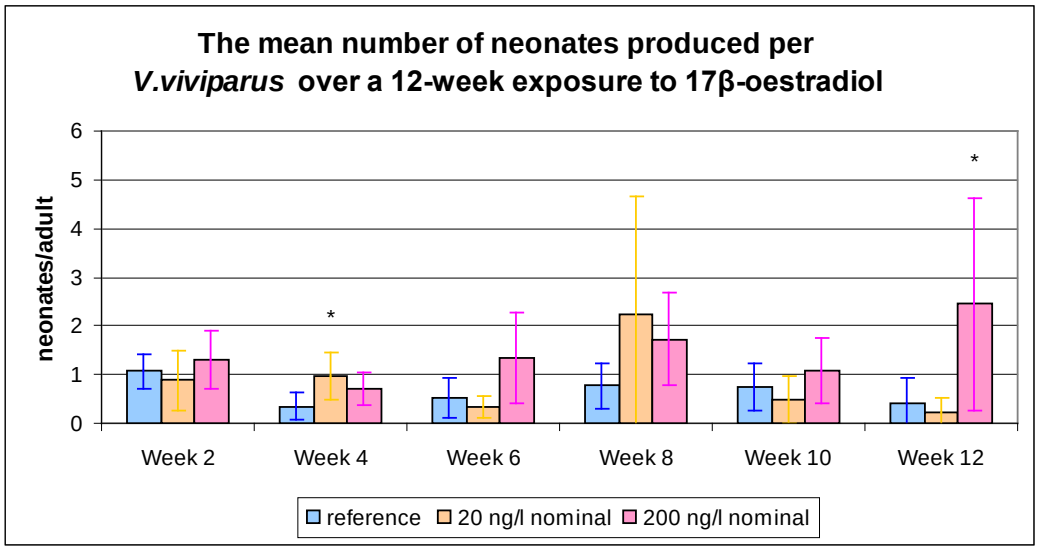


Figure 52 – The mean number of neonates produced per *V. viviparus* during a 12-week exposure to 17β-oestradiol (error bars represent the standard deviation, asterisks indicate a significant difference from the reference group at *p<0.05).

At the end of the exposure, the embryos were dissected out of the brood pouches of all the surviving females. The embryos were divided into those still within the eggshell and those that had hatched. Figure 53 shows the embryos per female ('unhatched embryos' and 'hatched embryos' respectively). There were no significant differences in the mean number of unhatched embryos within the females between the three tanks (ANOVA, $p>0.05$), the mean number of hatched embryos with shells within the females (Kruskal-Wallis, $p>0.05$), or the mean total number of embryos within the females (ANOVA, $p>0.05$).

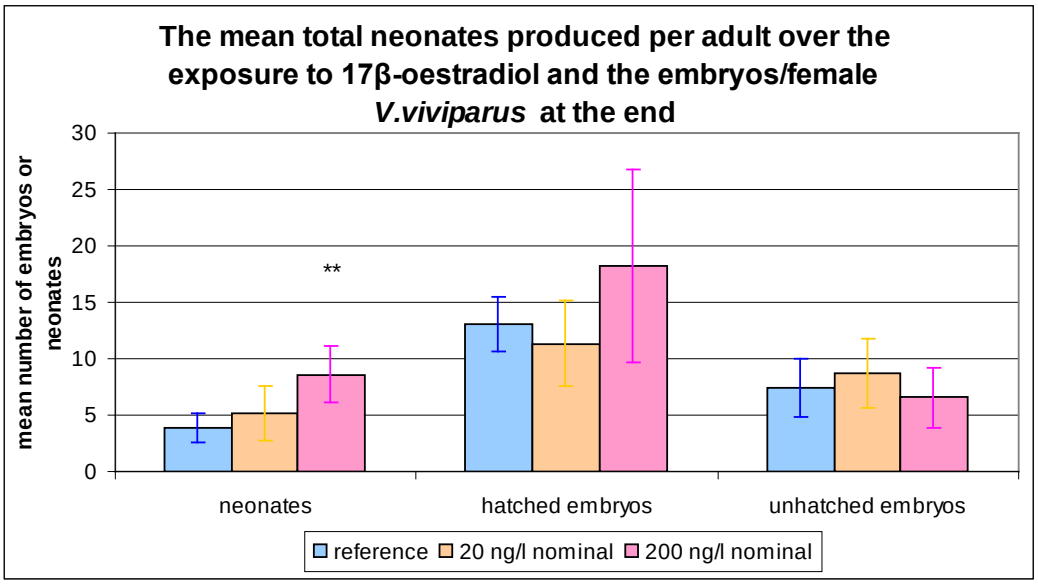


Figure 53 – The mean total number of neonates produced per *V. viviparus* during a 12-week exposure to 17β-oestradiol, and the mean number of hatched and unhatched embryos in the brood pouches of females at the end of the exposure (error bars represent the standard deviation for neonates, or standard error of the mean for embryos; asterisks indicate a significant difference from the reference group at **p<0.01).

5.2.2 Mesocosm Experiment 2 (2006) – *V. viviparus*

5.2.2.1 Water Chemistry and Water Quality

This experiment was conducted concurrently with the 2006 *P. corneus* exposure described in Chapter 3, Section 3.1.2. The snails were co-deployed in the same mesocosm tanks, and therefore the 17β-oestradiol treatment concentrations (both nominal and measured) are the same. The reference conditions and the water quality parameters were also as previously described in Chapter 4, Section 4.2.1.

5.2.2.2 Test Organism Survivorship

The mortality rates recorded in this mesocosm exposure are very similar to that on the first occasion, with a mean total percent mortality of 30.3% in the reference population over the 16-week exposure, and 58.5% in the group exposed to 100 ngL^{-1} 17β-oestradiol (nominal), although this concentration was lower (nominal and actual) and the duration of the exposure longer. The mean total percent mortality in the group exposed to 10 ngL^{-1} 17β-oestradiol (nominal) was slightly less at 18.2% (Figure 54).

The difference between the two exposures lies in the observation that there was an apparent dose-response relationship through-out the first exposure, but on the second there was little or no increase in mortality in the exposed groups until the end of the experiment at the last sampling point. At this time (Week 16), the mortality of the group exposed to the highest test concentration doubled, and six snails were found to be dead in one replicate. It is unlikely that the death of these animals had been missed beforehand, as the decomposing bodies of this species are extremely and distinctively odiferous. It is possible that the tank had become deoxygenated near the sediment layer, causing or contributing to the sudden rise in the observed mortality. Overall, however, the level of cumulative percentage mortality of the test organisms was as expected given the outcome of the first exposure, but on this occasion the reduced number of replicates and the quantal nature of the data are confounding, and there are no significant differences

between the reference and treatment groups on any sampling event (ANOVA, p>0.05 in all cases).

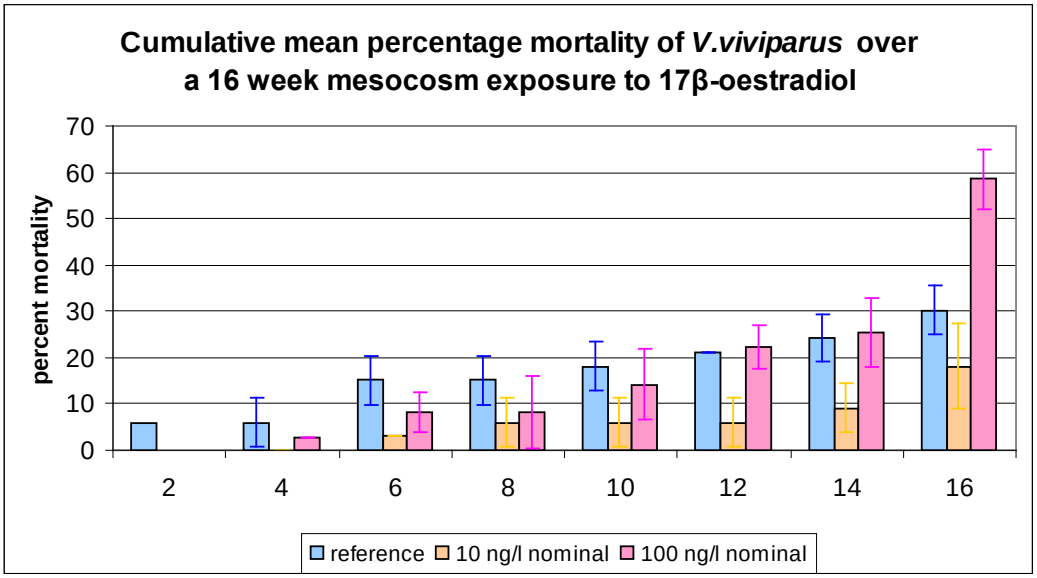


Figure 54 – The cumulative mean mortality rate of *V. viviparus* over a 16-week exposure to 17β-oestradiol (error bars represent the standard deviation).

5.2.2.3 Test Organism Morphology and Growth

At the start of the experiment, there were no significant differences in the mean length (in the longest axis) or the mean weight between the reference group and the exposure groups in the length at the start of the experiment (Kruskall-Wallis for length, ANOVA for weight, p>0.05). As on the first occasion, the snails exposed to 10 ngL^{-1} 17β-oestradiol (nominal) showed no mean increase in the length during the course of the exposure, appearing to shrink by 1.39% due to the loss of the spire during handling. The other groups increased in mean length slightly (reference group, 1.09%; 100 ngL^{-1} nominal treatment group, 0.18%), although this too was within conceivable margins of error.

However, as on the first occasion, all the groups lost weight, and the proportion of weight loss was similar (3.0%, 8.61% and 3.49% in the reference group, 10 ngL^{-1} and 100 ngL^{-1} exposed groups respectively). This continued weight loss

suggests that the increased food availability due to the removal of the carbon pre-filters did not ameliorate the starvation endured by this species over the course of the exposure. It is possible that the energy budget expended by the snail over the course of the summer reproductive period always exceeds that which is ingested. 17ß-oestradiol exposure does not appear to exacerbate this, as there are no significant differences between the reference and exposed groups in either the mean percentage weight loss of the snails, or in the condition factor of the females (proportion of flesh weight to the total weight minus the embryos, mean values are 26.6%, 30.6% and 28.2% in the reference group, 10 ngL^{-1} and 100 ngL^{-1} exposed groups respectively), at the end of the experiment (ANOVA, $p>0.05$ in both cases).

5.2.2.4 Test Organism Gender Ratio

On this occasion 90.5% of the reference group were female. Of the groups exposed to 20 and 200 ngL^{-1} 17β-oestradiol (nominal), 83.3% and 94.4% were female respectively. There were insufficient data to assess whether this quantal, not normally distributed data had any significance, but as the reference ratio falls between that of the two treatment groups, it is unlikely that there is an effect.

5.2.2.5 Test Organism Reproduction

The mean number of neonates produced by *V. viviparus* was in the main similar to that produced on the previous occasion, at up to 1.5 neonates per adult per week. The exception to this was the first sampling point (Week 2), when close to x3 this number was produced by all the groups (see Figure 55). A possible reason for this could be that this second exposure was begun slightly earlier in the year, and was therefore more likely to have captured the peak of neonate production which occurs early in the year in iteroparous prosobranchs ('Type G' lifecycle; one generation per year, perennial, after Calow, 1978). However, it is clear that there is little or no effect of 17ß-oestradiol for the first half of the exposure (ANOVA, $p>0.05$ at Week 2 to 6), except at Week 8, when ANOVA

detects a significant difference between the groups (p = 0.045), but a Dunnett's test cannot distinguish where the difference lies.

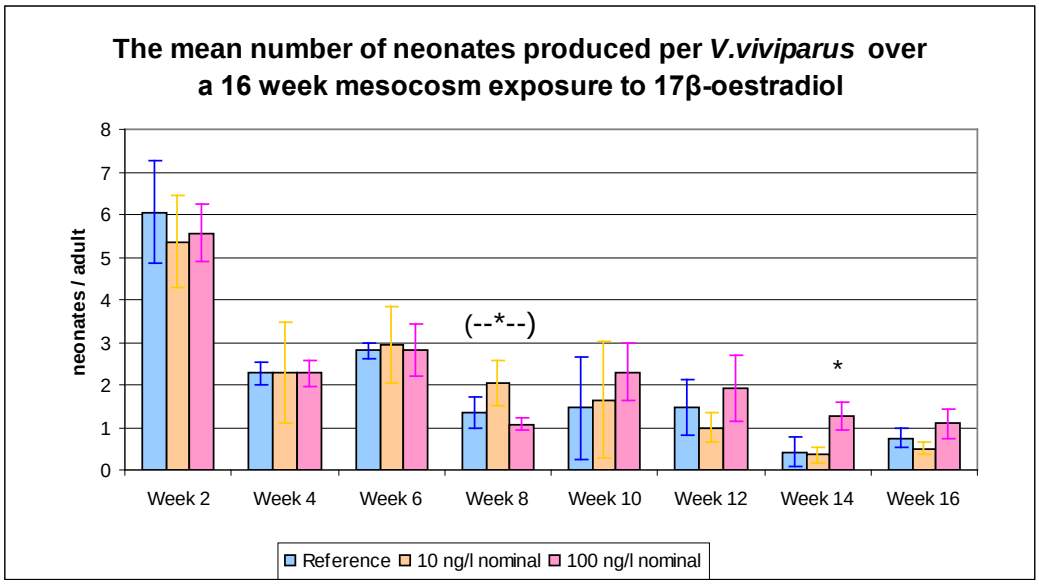


Figure 55 – The mean number of neonates produced per *V. viviparus* during a 16-week exposure to 17β-oestradiol (error bars represent the standard deviation, asterisk indicates a significant difference from the reference group at *p<0.05, or between groups (--*--) at p<0.05.

In the second half of the exposure, after the summer solstice, the group exposed to 100 ngL^{-1} 17ß-oestradiol (nominal) consistently produced more neonates per adult than the reference group, but this was only significant at the Week 14 sampling point (ANOVA, p = 0.016). This effect is a similar to that observed in the first exposure, when the group exposed to 200 ngL^{-1} 17ß-oestradiol (nominal) also produced significantly more neonates at the last sampling point. This appears to confirm that exposure to relatively high levels of 17ß-oestradiol stimulate *V. viviparus* to produce more neonates than expected in the late summer and autumn. Indeed, Figure 56 shows the mean total number of neonates per adult for the whole exposure, the second half (Weeks 8-16) and the last quarter (Weeks 12-16). In the second half, ANOVA detects a significant difference between the groups (p = 0.039), but a Dunnett's test does not distinguish where the difference lies. However in the last quarter, when the autumn response was

established, the group exposed to 200 ngL^{-1} 17ß-oestradiol (nominal) produced significantly more neonates than the reference group (ANOVA, p = 0.005).

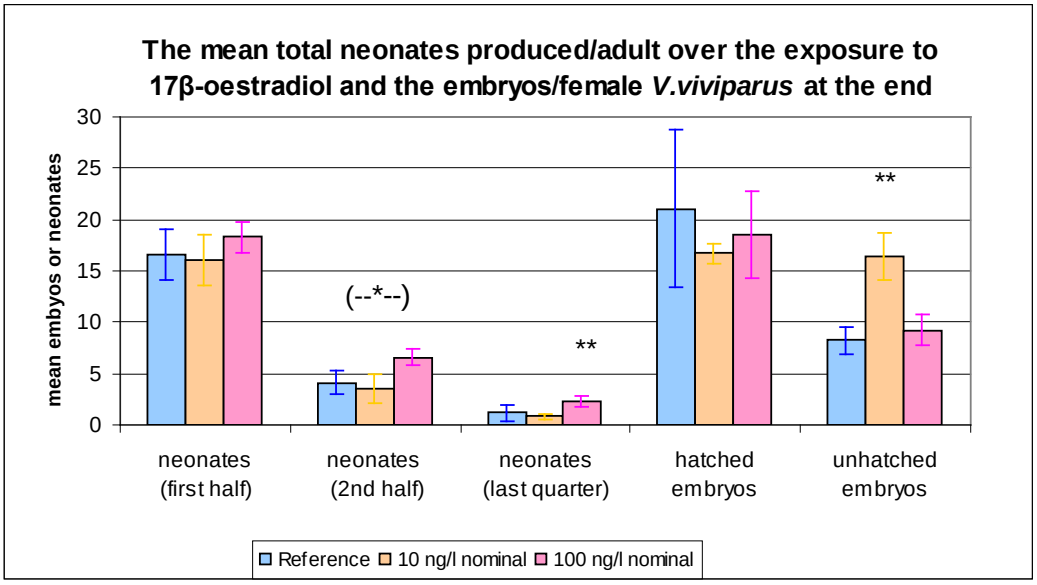


Figure 56 – The mean total number of neonates produced per *V. viviparus* during a 16-week exposure to 17β-oestradiol, the second half (Weeks 8 to 16) and the last quarter (Weeks 12-16), and the mean number of hatched and unhatched embryos in the brood pouches of females at the end of the exposure (error bars represent the standard deviation for neonates, or standard error of the mean for embryos; asterisks indicate a significant difference from the reference group at **p<0.01, or between groups (--*--) at p<0.05).

On dissection of the females at the end of the exposure, the mean number of hatched embryos found within the brood pouch was on average slightly higher than on the first occasion (18.8 per female in this exposure, previously 14.2 per female, see Figure 56). The brood pouch often appeared distended; it is likely that the female continues to hold as many neonates as possible during winter rather than release them in the autumn. Also, the mean number of unhatched embryos found in the brood pouches of the reference group was similar to that recorded on the first occasion (8.21 per female in this exposure, previously 7.43).

There were no significant differences in the number of hatched embryos found in the brood pouch. However, there were x2.0 more unhatched eggs in the females exposed to 10 ngL^{-1} 17ß-oestradiol (nominal) than the reference group, which is a significant increase (ANOVA, $p<0.003$). It is possible that the females in this groups were stimulated to continue production of eggs in the autumn period. The same effect does not occur in the group exposed to 100 ngL^{-1} 17ß-oestradiol (nominal). However the mortality rate in this group was greater, and it is possible that general toxicity and metabolic decline may have suppressed the production of new neonates in the surviving snails.

5.2.3 Mesocosm Experiment 2 (2006) – *B. tentaculata*

5.2.3.1 Water Chemistry and Water Quality

This experiment was conducted concurrently with the 2006 *P. corneus* exposure (Chapter 3, Section 3.1.2) and with the *V. viviparus* exposure described above (Section 5.2.2). The snails were co-deployed in the same mesocosm tanks, and therefore the 17β-oestradiol treatment concentrations (both nominal and measured) are the same. The reference conditions and the water quality parameters are also as previously described.

5.2.3.2 Test Organism Survivorship

Figure 57 shows the cumulative percentage mortality at each sampling event. It is an expression of those animals known to have died as a proportion of those deployed. Adult *B. tentaculata* are much smaller than adult *P. corneus* or *V. viviparus* and despite the use of an additional barrier with a smaller mesh size, an appreciable proportion escaped during the exposure (22.1%, 25.0% and 32.2% in the reference group, 10 and 100 ngL^{-1} (nominal) 17β-oestradiol exposure groups respectively). The cumulative mortality of the reference group reached 17.8% in the first 8 weeks of the exposure, which is usually considered acceptable in

chronic invertebrate exposures (e.g. OECD 2008), but by Week 16 it had reached 29.4%, which is less so, and the experiment was terminated. Although there was an apparent reduction in the mortality with increasing 17ß-oestradiol exposure, there are no significant differences between the groups at any time point (ANOVA, $p>0.05$ in all cases). It is notable that as in the co-deployed *V. viviparus*, the mean mortality of the *B. tentaculata* exposed 100 ngL^{-1} 17ß-oestradiol (nominal) increased sharply from 15.0% to 26.3% in the last two weeks of the exposure, and this may also be due to de-oxygenation as both species are gill-respiring. Overall, however, it is considered that the mortality rate of *B. tentaculata* is unaffected by exposure to 17ß-oestradiol in these concentrations.

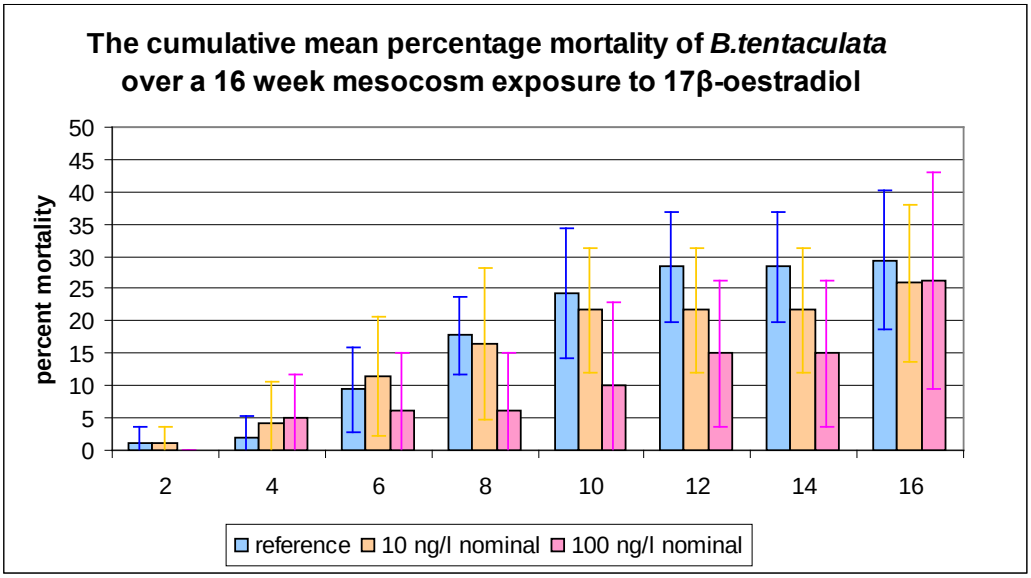


Figure 57 – The cumulative mean mortality rate of *B. tentaculata* over a 16-week exposure to 17β-oestradiol (error bars represent the standard deviation).

5.2.3.3 Test Organism Morphology and Growth

There were no significant differences in mean length (in the longest axis) or mean weight of the test organism allocated to the reference group and the exposure groups at the start of the experiment (Kruskall-Wallis, $p>0.05$ in both cases). Rather surprisingly for a prosobranch species, the snails grew well over the 16-

week exposure period, with the reference population increasing in mean length by 16.5% and in mean weight by 38.6% (see Figure 58). Both of the 17ß-oestradiol treated groups grew slightly more (increasing in mean length by 20.6% and 17.4%, and in mean weight by 53.8% and 51.8% in the 10 and 100 ngL^{-1} (nominal) exposure groups respectively). However, none of these differences were significant between any of the groups (ANOVA, $p>0.05$ in both cases). Due to the small size of the animals, no further analysis of the morphology was undertaken.

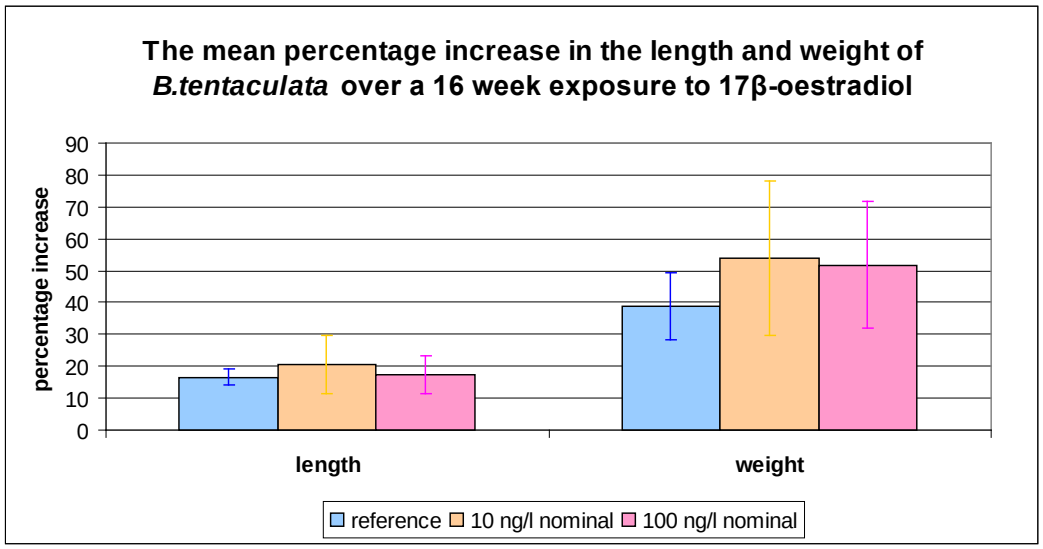


Figure 58 – The mean percentage increase in *B. tentaculata* length and weight during a 16-week exposure to 17β-oestradiol (error bars represent the standard error of the means).

5.2.3.4 Test Organism Gender Ratio

At the end of the exposure, the gender of the remaining animals was established destructively. The proportion of females in the reference population was 59.3% and it was 62.4% and 67.8% in the 10 ngL^{-1} and 100 ngL^{-1} 17ß-oestradiol (nominal) exposure groups. There is therefore a slight dose-dependant skew towards females. This was unexpected, as unlike *V. viviparus*, that tend towards strongly female skewed wild populations (e.g. Jakubik, 2006), *B. tentaculata*

populations are more likely to exhibit a 50/50 gender ratio in natural populations (e.g. Kozminsky, 2003b). It is possible that the males made more escapes and / or experienced a greater rate of mortality during the exposure. However, there was no significant difference in the proportion of females between the groups, and the dose-dependant trend does not have a significant correlation (Pearson's Product Moment Coefficient, $p>0.05$).

5.2.3.5 Test Organism Reproduction

Figure 59 shows the mean number of eggs laid per adult surviving at each sampling point. No effort has been made to refine these data to correct for the number of females present in each replicate at the end of the exposure, as the rate of losses (mortality and escape) was such that this is likely to be misleading. An equal sex ratio could be assumed, in which case the data should simply be halved. As a pragmatic precaution against any serious skew in the gender ratio, any replicate groups that were found to be either entirely females (one replicate in each of the 10 and 100 ngL^{-1} 17ß-oestradiol nominal exposure groups), or effectively incompetent, i.e. composed of males and parasitized females (one replicate in the 100 ngL^{-1} 17ß-oestradiol nominal exposure group) at the end of the exposure were removed from the data analysis.

The reproductive rate of the reference group was higher than might be expected for this slow-breeding prosobranch (*B. tentaculata* allocates only 5% of total absorbed energy to egg production, where it is closer to 20% in pulmonates; Calow, 1979). At the first sampling point (Week 2) an average of 57.7 eggs per snail had been laid by the overall test population. There was a slight dose-dependant increase, but the levels of variation were high, no doubt due in part to an inability to determine the number of live females present in each replicate. As in *V. viviparus*, this number of eggs per adult appears to again represent spring peak of reproductive activity common for prosobranchs ('Type G' lifecycle, perennial, iteroparous, one generation per year; Calow, 1978). After this time and until the mid-point of the exposure (summer solstice), the overall number of

eggs laid was lower and any dose-dependency is lost, with the 10 ngL^{-1} 17ß-oestradiol (nominal) exposure group laying slightly less eggs than the reference group and the 100 ngL^{-1} (nominal) exposure group laying slightly more. There are no significant differences between the reference and any treatment group at any of these sampling events (ANOVA, $p>0.05$ in all cases).

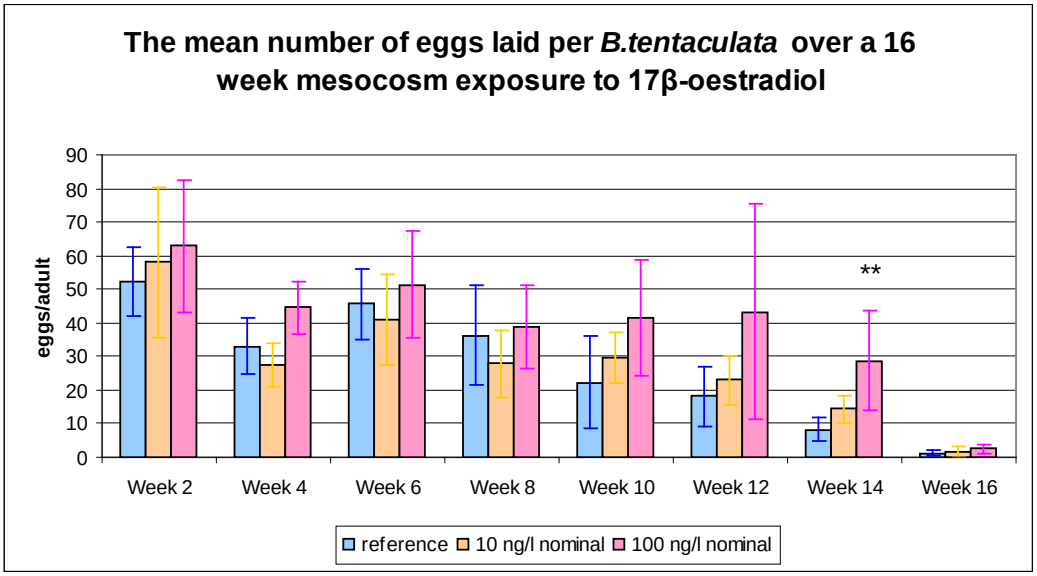


Figure 59 – The mean number of eggs laid per *B. tentaculata* during a 16-week exposure to 17β-oestradiol (error bars represent the standard deviation, asterisks indicate a significant difference from the reference group at **p<0.01).

However, in the second half of the exposure (after summer solstice, Week 10 onwards) it can be seen that the dose-dependency appears to become re-established. The egg laying behaviour of the reference group declines quite sharply, and the 17ß-oestradiol treated groups less so. This pattern culminates in a significant difference between the reference group and the 100 ngL^{-1} (nominal) exposure group at Week 14 (Kruskall-Wallis, $p = 0.007$). At Week 16, all the groups have almost ceased to lay eggs completely, and there are no other significant differences at any sampling point (ANOVA; Weeks 10 and 16, Kruskall-Wallis; Week 12, $p>0.05$ in all cases). The mean total number of eggs laid per adult for the second half of the exposure is significantly correlated with the nominal concentration of 17ß-oestradiol (Pearson's Product Moment

Coefficient, $p<0.05$), although the variation between replicates is high so the regression relationship is only moderate ($r^2 = 0.391$).

5.2.4 Experiment 4 – A laboratory exposure of *B. tentaculata* to 17ß-oestradiol in changing conditions to simulate the onset of 'spring'.

5.2.4.1 Steroid Analysis

The results of the steroid analysis are presented in Table 12. On the first sampling occasion (Week 6, Time 0), the recovery of 17β-oestradiol from the freshly spiked media was satisfactory (mean percentage recovery = 83.5% of nominal). This demonstrated that the preparation of the working solution of 17β-oestradiol and the spiking procedure was adequate.

As expected, the degradation rate of 17β-oestradiol over 48 hours was appreciable. After 24 hours, 62.9%, 66.3% and 63.8% of the Time 0 measured concentration remained in the 1, 10 and 100 ngL^{-1} nominal media respectively, and after 48 hours this had further reduced to 43.6%, 47.7% and 45.4% respectively. Therefore the half-lives in this exposure system were calculated to be 40.0, 43.6 and 41.3 hours, respectively, which is in fact much better than the half-lives for 17β-oestradiol recorded in the previous exposure of *P. corneus* (10 to 18 hours). This is possibly due to there being less organic material (food and faeces) present in the media encouraging bacterial growth.

Although no 17β-oestradiol was recorded in the fresh control media (although the LOD is rather high), it was present in increasing amounts at 24 and 48 hours. This suggests that either the test organisms were secreting steroids or that contamination occurred, but the latter does not seem likely to increase over time in undisturbed vessels. A similar value was seen in the control media on the second sampling occasion (12 weeks).

Table 12 - The measured 17β-oestradiol and oestrone concentrations (ngL^{-1}) performed during an 18 week exposure of *B. tentaculata* (LOD = 0.15 ngL^{-1} for 17β-oestradiol and 0.5 ngL^{-1} for oestrone).

		control	1 ngL^{-1} nominal	10 ngL^{-1} nominal	100 ngL^{-1} nominal
First occasion (6 weeks)					
0 hours	*17β-oestradiol*	<0.15	0.784	8.57	86.4
	Oestrone	<0.5	<0.5	<0.5	<0.5
24 hours	*17β-oestradiol*	0.169	0.493	5.68	55.1
	Oestrone	<0.5	<0.5	0.578	7.22
48 hours	*17β-oestradiol*	0.257	0.342	4.09	39.2
	Oestrone	<0.5	<0.5	<0.5	5.72
Second occasion (12 weeks)					
24 hours	*17β-oestradiol*	0.271	0.189	0.355	4.09
	Oestrone	<0.5	<0.5	<0.5	<0.5
Third occasion (18 weeks)					
24 hours	*17β-oestradiol*	1.18	1.91	0.964	19.7
	Oestrone	ND	1.46	3.84	30.7

On the second and third sampling occasions, samples were only taken 24 hours after a media change. It appears that by this point (12 weeks), most of the 17β-oestradiol had degraded, possibly due to an adapted bacterial population. Indeed, only the highest concentration (100 ngL^{-1} nominal) was x10 above the others, which are essentially indistinguishable from each other. This was also true at the third sampling occasion (18 weeks), although all the measured concentrations were higher, including in the control media, where 17β-oestradiol was measured at over 1 ngL^{-1}. The reason for this is unknown.

The mean measured concentrations over the three 24 hour sampling points are 0.54 ngL^{-1} (sd = 0.557) in the control media, 0.864 ngL^{-1} (sd = 0.919) in the 1 ngL^{-1} (nominal) concentration, 2.33 ngL^{-1} (sd = 2.91) in the 10 ngL^{-1} (nominal) concentration and 26.3 ngL^{-1} (sd = 26.1) in the 100 ngL^{-1} (nominal) concentration. There was no fourth sampling occasion because the experiment was terminated earlier than originally planned. While another sampling point may have reduced the very high co-efficients of variation (COV = 103% for the control, and 106%, 125% and 99.2% for the 1, 10 and 100 ngL^{-1} nominal concentrations respectively), the reduction is unlikely to have been of value given the spread of the existing three data points.

These COV, being close to or above 100%, are such that a mean concentration becomes meaningless, and it might be considered that only the first 6 weeks of the experiment is valid for further interpretation. However, there may be merit in considering the 100 ngL^{-1} (nominal) 17β-oestradiol exposure group as distinct from the other groups as the concentrations measured were on average x10.5 higher than the recorded for any other group within each sampling occasion. However after the first six weeks, the other treatment groups (1 and 10 ngL^{-1} nominal) can only be described as being exposed to similarly low levels of 17β-oestradiol. There is also no experimental control after this time, as similar concentrations of 17β-oestradiol are measured in the untreated media, making it indistinguishable from these treatments.

5.2.4.2 Media Physico-Chemical Characteristics

Temperature

Table 13 shows the mean, minimum and maximum temperatures measured manually in the discarded media following each media change, using a mercury thermometer with an error margin of 0.5^{o}C. The temperature was gradually raised according to the scheme described in Figure 21 (Section 3.2.3, Chapter 3). The constant temperature room operated within the laboratory tolerances of +/- 2^{o}C over the whole exposure, with no faults being recorded.

Table 13 – The mean, minimum and maximum temperatures recorded for each fortnight of the experiment.

	Temperature (^{o}C)		
Exposure Period	**Mean**	**Minimum**	**Maximum**
1. *21st March to 4th April*	10.07	10.0	10.5
2. *5th April to 18th April*	10.00	10.0	10.0
3. *19th April to 2nd May*	13.36	12.0	14.0
4. *3rd May to 16th May*	14.07	14.0	14.5
5. *17th May to 31st May*	15.86	15.0	16.0
6. *1st June to 13th June*	16.93	16.5	17.5
7. *14th June to 27th June*	18.29	17.5	18.5
8. *28th June to 11th July*	19.58	19.0	20.0
9 *12th July to 25th July*	20.00	20.0	20.0

pH

Table 14 shows the mean and standard deviation of pH measurements from the discarded media following each media change. It can be seen that the mean pH varies between 7.22 and 7.50, and that there was a slight trend to increasing pH over the course of the exposure. The overall mean pH of fresh media was 7.49 (sd = 0.17). The range of the pH values and the standard deviations are small, and this degree of pH change was unlikely to be adverse to the test organisms.

Table 14 – The mean pH and standard deviation (SD) recorded for each fortnight of the exposure.

	pH units	
Exposure Period	**Mean**	**SD**
1. *21st March to 4th April*	7.22	0.12
2. *5th April to 18th April*	7.30	0.14
3. *19th April to 2nd May*	7.43	0.11
4. *3rd May to 16th May*	7.37	0.11
5. *17th May to 31st May*	7.35	0.17
6. *1st June to 13th June*	7.32	0.12
7. *14th June to 27th June*	7.37	0.15
8. *28th June to 11th July*	7.50	0.30
9 *12th July to 25th July*	7.46	0.26

Dissolved Oxygen

The mean Percentage Air Saturation Values (%ASV) and standard deviations for each fortnight of the exposure are given in Table 15. It can be seen that the mean dissolved oxygen levels range between 59 and 76.6%, which are adequate levels for the respiration of gill dependant organisms when it is considered that these measurements are taken in the discarded media. The mean dissolved oxygen level of the new media over the course of the exposure was 101.6% (sd = 3.4). However the often high standard deviation in the discarded media reflects the fact that the dissolved oxygen level recorded in some of the individual test vessels

was depressed to between 40 and 50% (in approximately 10% of measurements) and between 30 and 40% (in approximately 3% of measurements), with the lowest recorded value being 32%. Some of the lowest values were associated with recent snail deaths, where the decomposing tissue has exerted a biological oxygen demand, but other occasions cannot be explained.

Table 15 – The mean dissolved oxygen levels (%ASV) and standard deviation (SD) recorded for each fortnight of the exposure.

	Dissolved Oxygen (% ASV)	
Exposure Period	**Mean**	**SD**
1. *21st March to 4th April*	70.3	17.7
2. *5th April to 18th April*	62.7	14.6
3. *19th April to 2nd May*	59.0	14.1
4. *3rd May to 16th May*	63.0	14.1
5. *17th May to 31st May*	73.2	14.6
6. *1st June to 13th June*	67.6	13.2
7. *14th June to 27th June*	66.1	14.2
8. *28th June to 11th July*	75.6	16.5
9 *12th July to 25th July*	76.6	15.1

Conductivity

Table 16 shows the mean and standard deviation of the conductivity values (uS/cm) from the discarded media following each media change. It can be seen that there was an apparent increase in conductivity over the course of the exposure, and the same trend was also present to a lesser extent in the fresh media, suggesting an increasing load of conductive material may be present in the influent the tap water as the summer progressed. The values do not reach excessive levels and are not likely to have affected the test organisms.

Table 16 – The mean conductivity (uS/cm) and standard deviation (SD) recorded for each fortnight of the exposure.

	Conductivity (uS/cm)	
Exposure Period	**Mean**	**SD**
1. *21st March to 4th April*	779	66
2. *5th April to 18th April*	795	67
3. *19th April to 2nd May*	846	60
4. *3rd May to 16th May*	842	54
5. *17th May to 31st May*	894	79
6. *1st June to 13th June*	926	69
7. *14th June to 27th June*	941	71
8. *28th June to 11th July*	1000	100
9 *12th July to 25th July*	1004	121

Hardness

The mean hardness and standard deviations measured in the discarded media from the exposure vessels are given in Table 17. They are in good agreement with the calculated value (394 mgL^{-1} $CaCO_3$ equivalents) for the salts added to the reverse osmosis filtered water. The mean hardness the samples of fresh media was 424 mgL^{-1} $CaCO_3$ equivalents (sd = 44). This standard deviation is rather high, due to two occasions when the conductivity was measured at 500 mgL^{-1} $CaCO_3$ equivalents, probably due to errors made when preparing the fresh media. This is not considered likely to have affected the exposure, as gastropods generally prefer a hard water environment to facilitate the extraction of calcium for shell-building (Boycott, 1936).

Table 17 – The mean hardness and standard deviation (SD) recorded for each fortnight of the exposure.

	Hardness (mgL^{-1} $CaCO_3$ equivalents)	
Exposure Period	**Mean**	**SD**
1. *21st March to 4th April*	418	20.9
2. *5th April to 18th April*	419	22.8
3. *19th April to 2nd May*	433	9.10
4. *3rd May to 16th May*	416	17.6
5. *17th May to 31st May*	433	31.9
6. *1st June to 13th June*	434	23.1
7. *14th June to 27th June*	426	13.4
8. *28th June to 11th July*	419	27.8
9 *12th July to 25th July*	400	22.1

5.2.4.3 Test Organism Mortality

The mean cumulative mortality of the control group reached 26% over the course of the exposure, which is generally considered rather high for a chronic exposure (e.g. OECD, 2008). Figure 60 shows that there was a slightly higher mortality amongst the males than the females in the control and all the treatment groups, this being most pronounced in the group exposed to 10 ngL^{-1} 17β-oestradiol (nominal), where 65% of the recorded deaths were male. Overall, there was a significantly higher rate of mortality in the group exposed to 1 ngL^{-1} (nominal) than in the control group (ANOVA, $p = 0.036$), but the reason for this is not clear. While a similar level of effect was also observed in the group exposed to 10 ngL^{-1} (nominal), it was not seen in the highest concentration (100 ngL^{-1} nominal). There are no significant differences between the control group or any of the exposure groups for the rates of male or female mortality alone (ANOVA, $p>0.05$ in both cases).

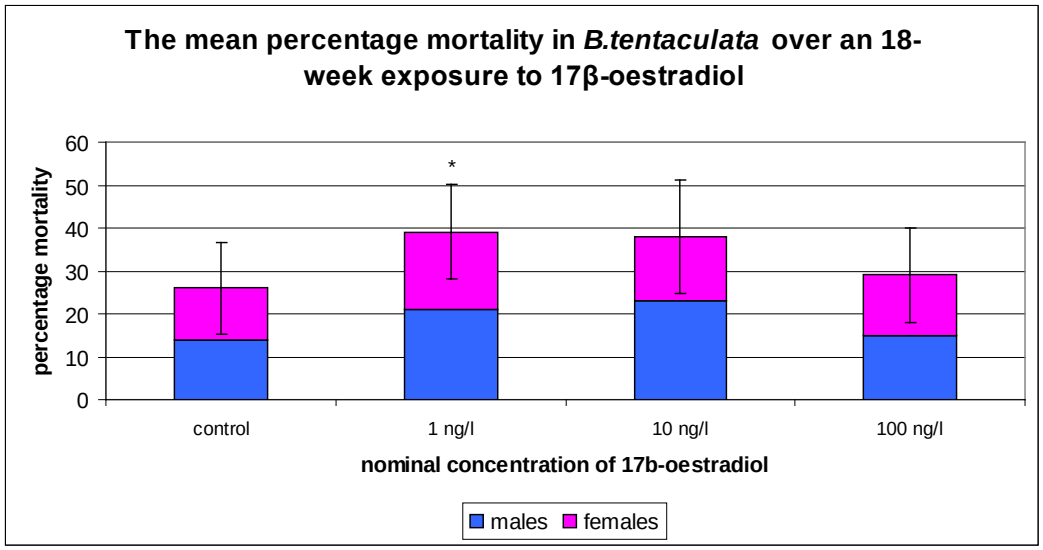


Figure 60 – The mean percentage mortality in *B. tentaculata* over an 18-week exposure to 17β-oestradiol (error bars represent the standard deviation, asterisks indicate a significant difference from the reference group at *p<0.05).

Because each death represented the loss of pair of test organisms, over half the replicate pairs from the control and each treatment group were removed by the end of the exposure. Two potential contributors to the overall mortality rate are identifiable; firstly, it may be that the working volume of the exposure vessels (200 ml) was an insufficient volume to maintain acceptable water quality parameters for *B. tentaculata*, and the low dissolved oxygen levels that sometimes occurred, along with the large degree of difference in the oxygenation status of old and new media, was a stressor in this gill-respiring species.

Alternatively, the rate of mortality may have been due to the almost endemic levels of parasitism found in the wild stock of this species. Visible parasites were found in 35% of the surviving partners (following a mortality within a pair), and in 50% of the snails remaining at the end of the exposure. Richter (2001) also found up to 50% of wild-caught *B. tentaculata* to be parasitized. However in this exposure, the first parasitized animals were not seen until early May, when the water temperature had reached 14^{o}C. Given the increasing incidence of visible parasites without a transfer vector present, it is possible that these test organisms were parasitized from the outset; or indeed that all the test organisms were

parasitized but that some did not reach a sufficiently heavy or late-stage infection during the exposure for it to be visible on generalised microscopic inspection.

The pattern of cumulative mortality over time can be seen in Figure 61. This shows that while the number of female mortalities increased steadily in the control and all the exposure concentrations, the pattern of male mortality was less straight-forward. In the control group and in the group exposed to the highest concentration (100 ngL^{-1} 17β-oestradiol nominal), the number of male deaths remained low for the first 10 weeks of the exposure (until early June, when the temperature had reached 16°C). After this, the mortality rate increased sharply to culminate in similar levels to that observed in the females. In contrast, the number of male deaths recorded in the other treatments (1 and 10 ngL^{-1} nominal) was accelerated above that occurring in the females earlier in the exposure (Week 6, early May, when the temperature had reached 14°C). This rate continued to the end of the exposure, culminating in a higher overall percentage mortality than that of the females. However, as there is no indication of a dose-response relationship, it appears that this is not an effect of exposure to 17β-oestradiol.

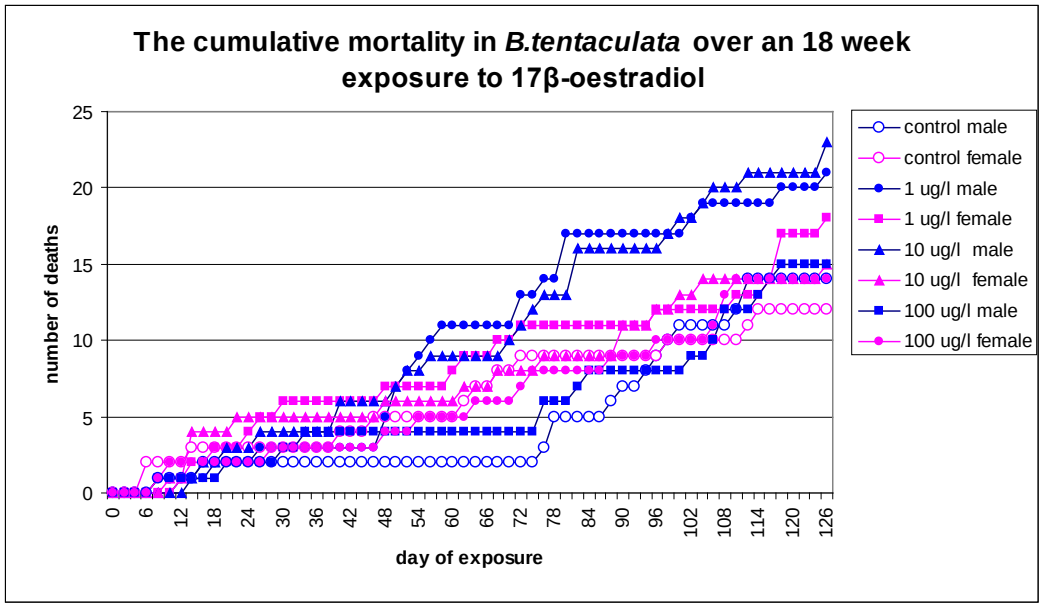


Figure 61 – The cumulative mortality of *B. tentaculata* over an 18-week exposure to 17β-oestradiol.

5.2.4.4 Test Organism Morphology and Growth

The mean length (in the longest axis) of the overall test population at the start of the experiment was 11.0mm and the mean weight was 0.217 mg. There were no significant differences between any of the groups ($p>0.05$ in both cases, ANOVA for length analysis, Kruskall-Wallis for weight analysis). The measurement of length was abandoned after the initial observations were recorded, as it had been noted that there is a tendency for the spire to be rubbed off in this species, which causes a false impression of the body size of the snail. Therefore the length of the aperture in the longest axis (longitudinally) was also measured, and this was used to compare the size of the organisms at the start, during (those that died or were sacrificed), and at the end of the exposure. The results are set out in Table 18, along with the mean weights for the same sampling points (weights relate to live animals only as dead animals absorb water, skewing the mean).

It can be seen that the growth of the test organisms over the whole exposure period (18 weeks) was barely perceptible, with the control group showing an increase in aperture size of 0.56%, and groups exposed to 1 and 10 ngL^{-1} 17β-oestradiol (nominal) by 1.67% and 1.5%. This is possibly because all the snails used in the experiment were already close to full size. Certainly no growth ring arising from the change in environment from wild to laboratory stock was observable as it is in pulmonates. It is interesting to note that the group exposed to the highest test concentration (100 ngL^{-1} nominal) did not appear to grow at all, indeed appearing to 'shrink' because the larger animals suffered a greater mortality rate than the smaller ones as indicated by the large mean size of the animals that died during the exposure. The mean weight of control and exposure groups did not alter to any marked degree, becoming only very slightly heavier in all cases except the group exposed to 100 ngL^{-1} 17β-oestradiol (nominal), indicating that at least the test organisms did not lose condition over the course of the exposure.

Table 18 – The mean length of the aperture and the mean weight of test organisms with standard deviations (sd) at the start, during (died or sacrificed), and end of an 18-week exposure of *B. tentaculata* to 17β-oestradiol (nominal).

	Aperture length (mm)			**Weight (g)**		
	At start	Died during	At end	At start	Sacrificed during	At end
Control	5.37	5.42	5.40	0.21	0.23	0.22
(sd)	(0.34)	(0.32)	(0.36)	(0.04)	(0.03)	(0.05)
1 ngL^{-1}	5.40	5.51	5.49	0.22	0.22	0.25
(sd)	(0.37)	(0.40)	(0.38)	(0.05)	(0.04)	(0.06)
10 ngL^{-1}	5.39	5.43	5.47	0.22	0.22	0.23
(sd)	(0.37)	(0.43)	(0.34)	(0.05)	(0.05)	(0.06)
100 ngL^{-1}	5.40	5.56	5.36	0.22	0.22	0.22
(sd)	(0.34)	(0.30)	(0.34)	(0.04)	(0.04)	(0.04)

5.2.4.5 Test Organism Gender Ratio

The snails were monitored for activity (ventral surface of the foot adhered to the glass vessel and tentacles fully unfurled) every 48 hours during the exposure, and where individuals were observed to be quiescent (opercula closed) over 5 consecutive days, a needle was carefully inserted under the edge of the opercula to assess the response of the snail. Live animals rapidly withdraw the opercula further within the rim of the aperture on this assault, whereas dead animals are easily withdrawn from the shell entirely. Due to the frequency of this monitoring, the gender of any test organisms that died during the test could be established before decomposition became too extensive (the mantle and penis were remarkably resistant to decay in comparison to the other species employed).

The other member of the pair was sacrificed and the gender determined at the same time. At the end of the exposure, all the remaining snails were sacrificed and the gender established. The overall sex ratio of the population of test organisms was 49% females and 51% males. There were 4 homogeneous pairs (both males, where a male was assessed as a female due to an inadequate extension of the body from the shell during live gender determination), which once discovered were retrospectively excluded from all subsequent analysis of reproductive effort. This equates to an error rate of only 1% for live gender determination, which indicates that this technique, although laborious, has a high degree of accuracy.

5.2.4.6 Test Organism Reproduction

Only 21 of the 200 pairs deployed in the experiment proved to be viable in terms of successful oviposition behaviour. Of the 50 pairs established in the control group, 5 pairs were successful, and of the same number established for each treatment, 4 were successful at 1 ngL^{-1} 17β-oestradiol (nominal), 5 at 10 ngL^{-1} (nominal) and 7 at 100 ngL^{-1} (nominal). The incompetent pairs were removed from the analysis of the results, on the grounds of an inability to reproduce. This is possibly due to the high levels of parasitism observed in the population and the consequent onset of parasitic castration (see Wilson and Denison, 1980), although it was observed that 38% of the successfully breeding females were host to varying degrees of parasitisation, and also that 43% of the unsuccessful pairs had no observable parasites even after the onset of microscopically visible parasitisation in the test population.

Figure 62a shows the mean number of eggs laid by the successful pairs per day, adjusted for the number of days that both members of the pair survived. The measured concentrations of 17β-oestradiol suggest that the control group and the groups exposed to 1 and 10 ngL^{-1} (nominal) were all exposed to 'low' levels the steroid. The mean number of eggs laid per pair per day across these groups was 0.510. In the group exposed to 100 ngL^{-1} 17β-oestradiol (nominal), for which the

measured concentrations were x10 higher, the mean number of eggs laid per pair per day rises to 0.848. However the variability between the pairs also markedly increases in this group, and there are no significant differences between any of the groups (Kruskall-Wallis, $p>0.05$). The high variability in the group exposed to 100 ngL^{-1} 17β-oestradiol (nominal) arises because three of the seven pairs lay more than 1 egg per day, which is a higher rate of egg production than any other successfully ovipositing pair in the experiment. These three pairs lay 3.76, 2.67 and 2.40-fold more eggs than the mean of the control group, whereas the other four pairs lay a number of eggs that is equal to or below that of the control group (see Figure 62b). It is possible that these three females have responded to the additional 17β-oestradiol exposure (above that experienced by the control and other treatment groups) by increasing their egg output to a level above the expected production rate. Indeed, these three results are statistical outliers against the overall productivity of the 21 successfully ovipositing pairs (i.e. they are >x1.5 of the inter-quartile range).

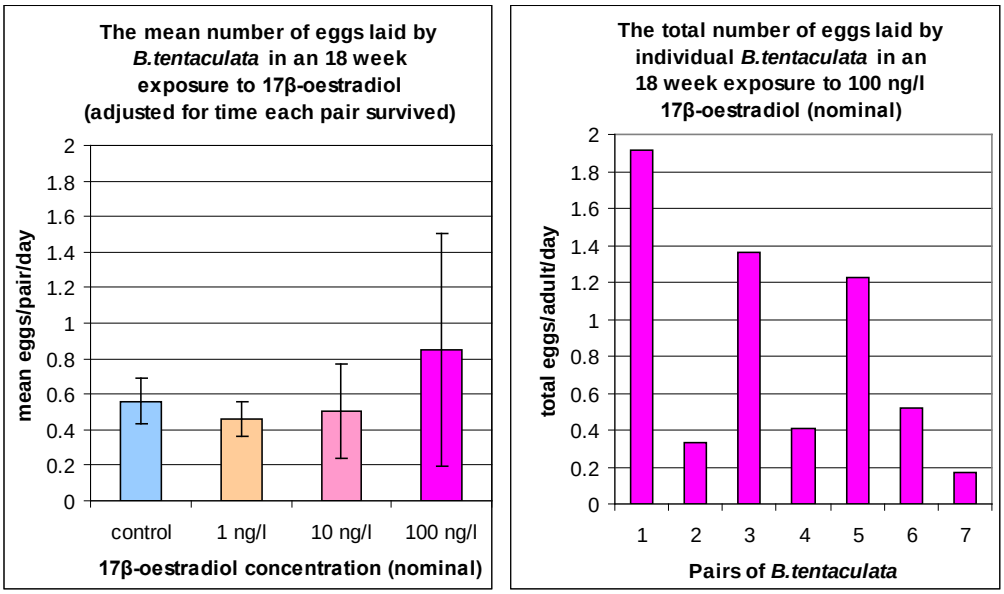


Figure 62 - (a) the mean number of eggs laid / pair / day, adjusted for survival, over an 18-week exposure of *B. tentaculata* to 17β-oestradiol (error bars represent the standard deviation), and (b) the total number of eggs laid by individual pairs exposed to 100 ngL^{-1} 17β-oestradiol (nominal) during this period.

Figure 63 shows the cumulative mean number of eggs laid per pair over the course of the exposure. The mean egg production of the treated groups does not fall outside the standard deviation of the control group at any time point, and the means of the groups exposed to 1 and 10 ngL^{-1} at each time point follow that of the control quite closely for the duration of the exposure. However, the cumulative mean of the group exposed to 100 ngL^{-1} (nominal) deviate most widely from that of the control group in the early (spring) and late (autumn) phases of the exposure. This group was the first to begin laying eggs in the experiment, and for the first two weeks of the test (at approximately 10^{o}C with a 12/12hr light / dark photoperiod), the mean was notably above that of the others. It reaches 20.1 eggs / pair in 10 days where the other groups take longer, with decreasing nominal 17β-oestradiol exposure, to reach the same level of productivity (18 days at 10 ngL^{-1} nominal, 26 days at 1 ngL^{-1} nominal and 34 days in the control group).

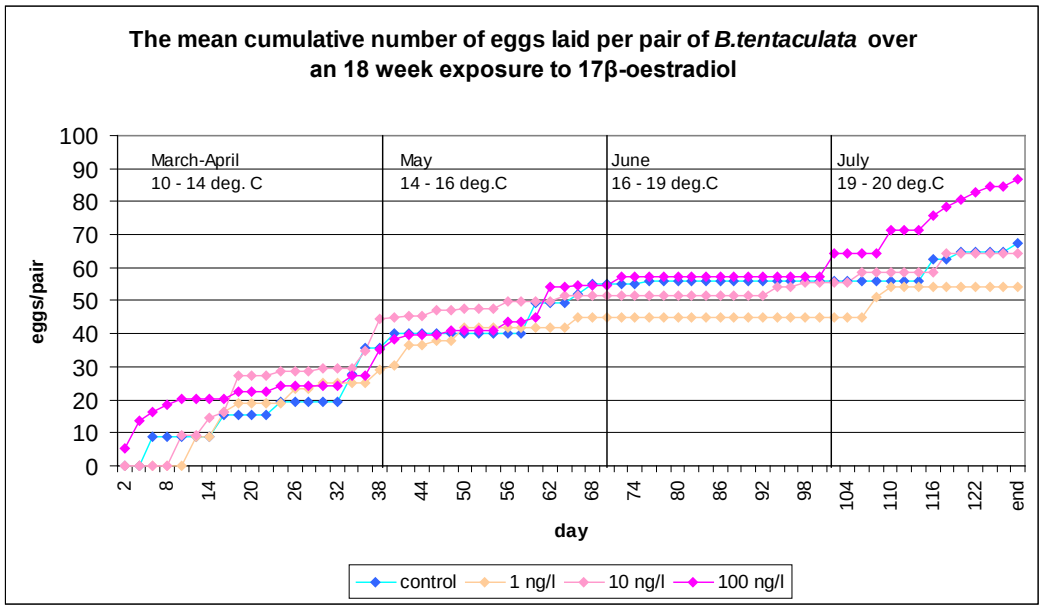


Figure 63 – The mean cumulative number of eggs laid per pair of *B. tentaculata* over the course of an 18-week exposure to 17β-oestradiol.

The cumulative mean of all the groups then remained similar and close to constant until early in July, when the temperature has reached approximately 20^{o}C and the 16/8 light/dark photoperiod begins to reduce. At this time (Day 100), the mean cumulative total across all of the groups was 53.5 eggs per pair. By the end of the exposure (Day 128), the mean cumulative total of the control group has increased by 20.3% to 67.2 eggs/pair, and the groups exposed to 1 and 10 ngL^{-1} (nominal) are below this at 54.1 and 64.1 eggs per pair respectively. However, the group exposed to 100 ngL^{-1} has increased by 50.7% to 86.1 eggs/pair. It is of note that there are therefore two periods of increased productivity, and that both are more pronounced in the group exposed to 100 ngL^{-1} 17β-oestradiol (nominal). Dillon (2000) suggests that for some iteroparous species two generations per year may be usual, and these data may be a reflection of this.

5.3 Comparative responses of the prosobranchs *V. viviparus* and *B. tentaculata* to 17β-oestradiol.

5.3.1 Physico-chemical conditions

The mesocosm exposures were made concurrently and in the same tanks as those described for *P. corneus*, and therefore the same observations regarding the physico-chemical conditions apply (see Chapter 4, Section 4.4).

The 17β-oestradiol analysis in the laboratory exposure of *B. tentaculata* was much more limited than those taken during the laboratory exposures of *P. corneus* (due to cost constraints) and not as successful. However the mini-stability study undertaken at the first sampling event (6 weeks) indicated that the spiking procedure was acceptable (78.4-86.4% recovery) and that the 17β-oestradiol was degrading rather less than expected. However, on the subsequent occasion the degradation rate was much greater, reaching over 95% in 24 hours. Perhaps this is not surprising since the first four weeks of the experiment were

performed at 10°C, rising to 14°C at the time that the first set of samples was taken. Following this, the temperature was then incrementally increased to 18°C when the second set was taken. The volume of the water to internal surface area ratio of the small (200 ml) glass vessels was much higher than in the 10L exposure tanks, and it was observed that an appreciable biofilm was building up on the glass surfaces. It did not appear that *B. tentaculata* grazed on this to any extent, preferring to remain stationary and filter-feed. Consequently it is likely that that the microbial activity increased in the warmer conditions, favouring an accelerated 17β-oestradiol degradation rate.

After the potential for biofilm development was recognised, the vessels were more rigorously cleaned, but on the final sampling occasion the degradation rate in the highest nominal concentration (100 ngL^{-1}) was still approximately 80%. Also, whereas in the first two sampling occasions only marginal concentrations of 17β-oestradiol had been measured in the control vessels, a higher level of contamination was recorded on the final occasion (1.18 ngL^{-1}). The source of this contamination is not known, and may represent an inadvertent cross-contamination. While it was not severe, being similar to environmental concentrations, the increased degradation of the spike in the treated media meant that the control and the two lower treatments became indistinguishable, so that there is no experimental control after Week 6. If this analysis represents the exposure conditions, it can be argued that the experiment is invalid. However, the analysis for the highest test concentration (100 ngL^{-1} nominal) remained above the others by a factor of 10 on both the subsequent sampling occasions, therefore there may still be value in a comparison between this treatment (approximately equivalent to a sewage effluent) and the three groups exposed to low levels of 17β-oestradiol (similar to river waters).

The small size of the test vessels and the growth of biofilms may also have contributed to additional water quality concerns. The dissolved oxygen levels in some beakers dropped below 50% on frequent occasions, but remained above 80% in others, possibly due to differences in the microbial communities present,

since algae were added as a food supply. However, oxygen levels below 50% are not acceptable for gill-respiring organisms such as *B. tentaculata*, and the benefit of exposing independent pairs was out-weighed by the unsuitability of the housing provided. A better approach would be to use flow-though systems, but because *B. tentaculata* predominantly filter-feed on algae (Brendelburger and Jurgens, 1993), it may be difficult to feed this species in flow-though. Another alternative is to use vessels large enough to be aerated without stressing the snails.

5.3.2 Mortality

The mortality rates in the *V. viviparus* mesocosm exposures were consistently elevated at the highest concentration of 17β-oestradiol, although not significantly so, except at Week 6 on the first occasion. On the second occasion, the majority of the snails in one of the cages exposed to the highest concentration died during the last weeks (Weeks 14-16), but this is likely to be attributable to a cage-effect following the death and degradation of one or more snails in the relatively warm conditions. *B. tentaculata*, while experiencing an appreciable mortality rate overall (reference group mortality over 16 weeks = 29.4%), was not further affected by exposure to 17β-oestradiol in the mesocosm exposure. A similar mortality rate was also observed in the laboratory exposure (control group mortality over 18 weeks = 26.0%), and while it was significantly increased in the 1 ngL^{-1} (nominal) exposure group, there were no other significant differences in the higher exposure concentrations. Overall, this suggests that the survivorship of *B. tentaculata* is not affected by exposure to 17β-oestradiol at environmental concentrations or above, but *V. viviparus* may exhibit toxicity at concentrations approximately x10 of environmental levels, although further confirmation is required.

5.3.3 Growth

The *B. tentaculata* grew well in the mesocosm exposure, with the reference group increasing in mean length by 16.5% and mean wet weight by 38.6%. The 17β-oestradiol exposed groups grew more than this, but there were no significant differences. In contrast, the *B. tentaculata* used in the laboratory exposure did not perceptibly grow in any treatment group, perhaps because they were larger at the start of the experiment. However they did not lose weight, suggesting that they were not starving. The *V. viviparus* also showed no increase in mean length during the mesocosm exposures, but the mean weight of the reference group decreased by 9.56% on the first occasion, suggesting this species did starve. However, there was no additional effect in the 17β-oestradiol exposed groups, in fact they lost less weight. On the second occasion, when the activated carbon pre-filter was removed and weed allowed to grow, the snail still lost a similar proportion of weight across all groups (6.13% on the first occasion, 5.03% on the second), indicating that the starving was not ameliorated. It is not known whether a weight reduction is usual for this species following a reproductive period, but again there was no additional effect of 17β-oestradiol exposure, and the condition factor was also unaffected on either occasion. Overall it appears that 17β-oestradiol exposure does not affect the growth or weight gain of the gastropod species tested.

5.3.4 Gender Ratio

Due to time constraints, it was not possible to pre-determine the gender of the prosobranchs prior to the mesocosm exposures, so the allocation of males and females was random. However in all cases (two exposures of *V. viviparus* and one of *B. tentaculata*), the proportion of females at the end of the exposure was greatest in the groups exposed to the highest concentration of 17β-oestradiol. This may be a random occurrence that arose during the allocation, or it may be an adverse effect of the steroid exposure on males, but there were no significant differences from the reference groups in any case. There were dose-response

relationships in the first *V. viviparus* exposure and the *B. tentaculata* exposure, although only the former was significant and the model fit was poor. Further evidence for a greater effect on the males than the females was noted in the laboratory exposure of *B. tentaculata* when a bias towards increased male mortality was recorded in the groups exposed to 1 and 10 ngL^{-1} (nominal, Figure 61). However this too was not significant, and there was no similar effect in the group exposed to the highest concentration of 17β-oestradiol (100 ngL^{-1} nominal). Taken together, this evidence for intensified male prosobranch mortality is disturbing, but insufficient to conclude that it is caused by steroid exposure, particularly as *V. viviparus* populations are tendent to being skewed towards a greater proportion of females (Jakubik, 2006).

5.3.5 Reproduction

The observed effects of steroid exposure on reproduction in the prosobranchs were more consistent and more convincing of a female effect. In the first mesocosm exposure, there was a significant concentration-response relationship between 17β-oestradiol exposure and the number of neonates produced per adult, and significantly more *V. viviparus* neonates were produced by the group exposed to the highest concentration. This increase in reproduction is not the expected effect were the response to be mediated through toxicity (an inhibition of reproduction would be more likely). Moreover, when the data are examined at each time point, it can be determined that the largest release of neonates from the group exposed to the highest 17β-oestradiol concentration occurred in the last week of the experiment when the onset of autumn had begun (mid-August). This effect was not as clear in the second mesocosm exposure of *V. viviparus* which began earlier in the year (5^{th} May instead of 24^{th} May), consequently capturing the initial spring release of neonates. The number of neonates released at this time was 2 to 3-fold more than during the summer, and obscures more subtle changes in neonate production when the data is viewed as a whole. Nonetheless, more neonates were released per adult at each sampling event for the second half of the exposure, and this was significant in the penultimate event (Week 14).

This pattern was repeated with remarkable similarity in the reproduction of *B. tentaculata* in the same mesocosm exposure. Again, the early spring peak in reproduction was present, with up to 2-fold more eggs laid per adult compared to the rest of the summer. The number of eggs laid by the group exposed to the highest concentration was greater than that of the reference group or the lower treatment group at every sampling event, and again this was significant at Week 14. A concentration-response was also apparent at each sampling event during the second half of the exposure, until egg-laying behaviour ceases almost entirely at Week 16. Taken together, the evidence suggests that exposure to concentrations of 17β-oestradiol above environmental levels can cause an increase in the reproductive output of these prosobranch species, but only at certain times and in certain seasonal conditions, i.e. at the onset of autumn.

In *V. viviparus*, it was also possible to assess the number of embryos in the brood pouch of the females at the end of the mesocosm experiments. While there was no effect on the first occasion, on the second occasion there was a significant increase in the number of embryos still within the egg in the females from the lower exposure concentration (mean measured = 11.0 ngL^{-1} 17β-oestradiol). There was no effect at the higher concentration. It is possible that both the rate of development and the rate of release is increased at the higher exposure concentration, so that more neonates are produced but the number in the brood pouch remains the same, while at the lower concentration only the rate of (early) development is increased, which increases the number of embryos present in the brood pouch. This theory needs further experimentation with dissections made after different exposure times for confirmation.

Unfortunately it was not possible to make any assessment of *V. viviparus* reproduction in laboratory conditions as it was not possible to adequately feed the adults and a high mortality rate occurred. However it was possible to maintain adult *B. tentaculata* in the laboratory for extended periods of time in conditions that successfully allowed egg-laying and hatching, and therefore an initial

assessment of the effects of 17β-oestradiol on reproduction in this species could be undertaken. The experimental design for this study was innovative in that the only pair breeding study known to be undertaken prior to this employed the (sub) tropical prosobranch species *M. cornuarietis* (Forbes et al, 2007b). In that study, the snails were not housed as independent pairs, rather they were housed in tanks that were sub-divided with steel mesh into a segment for each pair, forming pseudo-replicates. While this is helpful in making an assessment of reproduction in each female, it also has the potential to affect the egg-laying behaviour of the females via signalling chemicals emitted by neighbours.

Instead, the snails were housed as completely independent male and female pairs, in separate beakers. Determining the sex of the snails prior to allocation, while laborious, was not difficult or subjective, and proved to be correct in 99% of cases on subsequent dissection. The temperature and the photoperiod of the controlled facility were altered in real-time to simulate a 'spring' and 'summer' period. In the event, only 21 of the 200 pairs deployed successfully laid eggs, and there were no significant differences between treatments in the number of eggs laid by the remaining pairs. However it was of interest to note that three of the seven successful pairs exposed to the highest concentration of 17β-oestradiol (100 ngL^{-1}, nominal) showed a marked increase in egg production, and were statistical outliers from the other 18 successful pairs in the experiment. Also, this increase occurred only early in the exposure ('simulated spring'; $10^{o}C$, photoperiod increasing from 12 to 13 hours) and at the end (onset of 'autumn'; $20^{o}C$, photoperiod decreasing from 17 to 16 hours). This evidence, while weak, provides a further indication that these prosobranchs are affected by exposure to 17β-oestradiol, but only under certain seasonal conditions. Richter (2001) observed the reproductive strategy of *B. tentaculata* is to lay a large number of eggs in the early spring, before reducing output over the summer whilst rebuilding energy reserves, and then increasing productivity again in late summer or early autumn. The results of this research suggest that exposure to 17β-oestradiol in a real-time simulated seasonal cycle may be able to stimulate additional egg laying behaviour at these times.

Overall, the original hypotheses, that ‘exposure to 17β-oestradiol will significantly alter the reproductive rate of the prosobranch gastropods *V. viviparus* and/or *B. tentaculata*, causing deviation from the seasonal pattern of reproduction in the reference groups’ is tentatively supported in both species, with the additional indication that that the observed inductions in reproduction occur at the onset of autumn. The hypothesis that ‘exposure to 17ß-oestradiol under controlled temperature and photoperiod conditions that represent a natural spring and summer will significantly alter the rate of egg production in *B. tentaculata* relative to the control’ is not supported, although the exposure provided weak evidence that at the highest concentration of 17β-oestradiol tested, this species can also show reproductive increases during simulations of seasonal change. However, these experiments have indicated that the prosobranchs assessed may be less sensitive to 17β-oestradiol exposure than the pulmonate (*P. corneus*). The latter showed an elevation of reproductive rates at 10 ngL^{-1} (nominal; mean measured = 6.77 ngL^{-1}) in the second mesocosm exposure, and also at 10 ngL^{-1} (nominal; mean measured = 11.0 ngL^{-1}) in the simulated autumnal laboratory exposure. Similar effects occurred in the prosobranchs only at the highest concentrations tested in the mesocosm exposures (nominal = 100 and 200 ngL^{-1}, mean measured = 70.3 and 370 ngL^{-1}), and in the laboratory exposure of *B. tentaculata*, indications of increased reproduction were also only observed at the highest concentration tested (100 ngL^{-1} nominal, mean measured = 26.3 ngL^{-1}).

5.3.6 Parasitisation

No parasites were observed in the *V. viviparus* deployed in the mesocosm, and the rate of parasitisation recorded in the *B. tentaculata* was low at 7.2%. This does not necessarily indicate that the rest of the test organisms were not parasitized, as a light infection or the presence of encysted parasites may not have been observable. It is also not clear whether the presence of a heavy infestation of parasites inhibits reproduction in *B. tentaculata*. The parasitized individuals were not accounted for in the mesocosm excepting the exclusion of the group that

reproduced minimally and consisted of males and parasitized females. In the laboratory exposure of *B. tentaculata*, the infection rate was unexpectedly much higher at 44%, and the parasites were associated with pairs in the majority of cases (33 pairs with both partners being infected, 11 with only one infected partner at the end of the test). Some infected pairs reproduced successfully (10 of the 21 successful pairings were parasitized), and were included in the data analysis. There were also 62 pairs without overt parasites that did not lay eggs for the duration of the exposure. The degree to which parasitisation affects reproduction and the causes of reproductive failure are therefore not clear in this species.

CHAPTER 6
REPRODUCTIVE EFFECTS OF EXPOSURE TO BISPHENOL-A IN THE PULMONATE GASTROPOD *P. corneus*

6.1 Introduction and Methodology

This Chapter presents the results of two laboratory exposures of the pulmonate gastropod *P. corneus* to Bisphenol-A (Experiments 5 and 6). The methodology and design of these experiments was similar to that used for the exposure of this species to 17β-oestradiol, but with some amendments. These are set out in Chapter 3, Sections 3.2.3 and 3.2.4 respectively.

The first exposure (Experiment 5) was made at 15^{o}C with a 12 hour photoperiod only, as the effects of 17β-oestradiol on reproduction were observed in this 'simulated autumn' condition. Experience with *P. corneus* in culture indicated that at this relatively cool temperature, the stocking density could also be increased. This was exploited with the objective of reducing the between-group variation. The second exposure (Experiment 6) was made to determine whether acclimation in the pre-exposure conditions was influencing the outcome of the test. This experiment assessed the reproductive rate in the groups of snails in simulated summer (19^{o}C with a 16-hour light / 8-hour dark photoperiod) in the pre-exposure period, and then both the temperature and the photoperiod were reduced during the course of the exposure to simulate the onset of autumn. Some additional modifications were also made to the exposure system and media handling in order to reduce the contact time with plastics to reduce the background levels of Bisphenol-A. The number or replicates per concentration were also increased to as many as was practically feasible, in order to increase the power of the test. The hypothesis for these experiments is: 'Exposure to Bisphenol-A under controlled temperature and photoperiod conditions that represent a simulated autumn will significantly alter the number of eggs produced by *P. corneus* relative to the control'.

6.2 Results and Discussion

6.2.1 Experiment 5 – An exposure of *P. corneus* to Bisphenol-A in constant 'simulated autumn' conditions.

6.2.1.1 Bisphenol-A Stability Study

Stability studies were performed using both Bisphenol-A and Nonylphenol for comparative purposes before an exposure was planned, as both compounds have the potential to degrade (Staples et al., 1998 and 1999 respectively). A single exposure tank was populated with snails for each concentration tested (2.5 and 25 μgL^{-1} for each chemical). The stability studies were performed under the conditions planned for the exposure ($15^{o}C$, 12 hours light/12 hours dark) with the animals being fed and the media changed as described for the exposure. Samples were taken from each tank and preserved in the manner described at time zero (immediately following a partial media change), and at 3, 9, 21 and 45 hours, which provided degradation time spans of 3, 6, 12 and 24 hours between samples.

Figure 64 shows that Bisphenol-A was markedly more stable than Nonylphenol in this exposure system. The degradation rate, half-life and mean measured concentration of the two compounds are given in Table 19. Although the regression relationship for the measured concentration over time is weak in the 25 μgL^{-1} Bisphenol-A (nominal) media due to a small degree of variation at the earlier sampling points, the percentage degradation is <20% and it is clear that the half-life of the compound is >5 days in this exposure system. In contrast, Nonylphenol experienced >50% degradation over the study, and the half-lives were more similar to those observed for 17β-oestradiol in Experiment 4 (*B. tentaculata* exposure, see Chapter 4, Section 4.2.3.1).

Bisphenol-A was therefore considered to be a suitable candidate xenobiotic for use in the gastropod exposures. The slow degradation rate of the compound in this exposure system was advantageous, as it implied that the fluctuations in

concentration over time and with media changes can be considered as almost negligible. Indeed, the calculated mean concentrations in this stability study were very close to the nominal concentrations.

Table 19 – The r^2 values, half-lives and mean concentration of Bisphenol-A and Nonylphenol calculated from measured concentrations in the stability study.

	Bisphenol-A		Nonylphenol	
Nominal concentration (μgL^{-1}):	**2.5**	**25**	**2.5**	**25**
r^2 of the regression	0.934	0.336	0.931	0.786
Half-life (hours)	194	128	44	34
Mean (μgL^{-1})	2.39	25.7	1.17	9.24

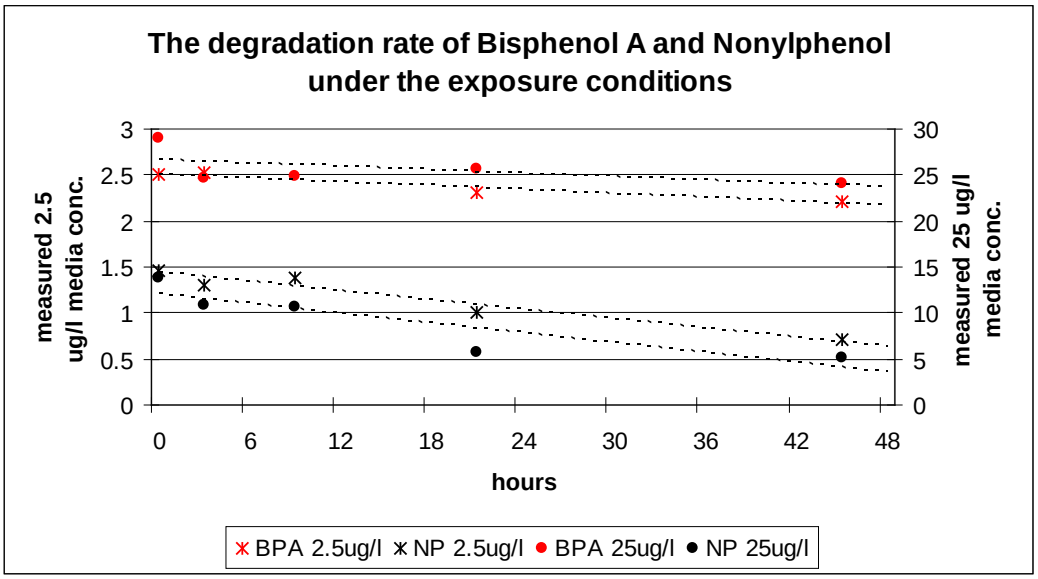


Figure 64 – The degradation rate of Bisphenol-A and Nonylphenol at 15°C under the exposure conditions with test organisms present.

6.2.1.2 Bisphenol-A Analysis

The results of the Bisphenol-A analysis performed at the start, midpoint and end of each fortnight-long cycle of the 8-week exposure are set out in Table 20. No difficulties arose with the preservation, shipping or analysis of the samples and so the data set is complete. An overall mean and standard deviation is also given for each nominal concentration (values below the LOD of 0.04 μgL^{-1} are treated as 0.02 μgL^{-1} to calculate the mean). Although some standard deviations are rather high, when the results between neighbouring concentration are compared, they are all significantly different to each other (Mann Whitney U-tests; $p<0.001$ in all cases). This is a satisfactory indication that each treatment concentration is sufficiently different from the others to be assessed independently in relation to any effects observed in the organisms.

At the first sampling event, the calculated degradation rate in the highest concentrations was 31%, which while higher than expected from the stability study, was not excessive. The result from the 0.2 μgL^{-1} (nominal) sample was high (0.694 μgL^{-1}), and it was surmised that a tank was spiked with 2 μgL^{-1} on one occasion rather than 0.2 μgL^{-1}. It was not possible to identify this tank, and it remained in the experiment. In most cases, the measured Bisphenol-A concentration decreased during each exposure cycle, but recovered following each complete media change (when the tanks are cleaned thoroughly). This suggests that the Bisphenol-A was to some extent degraded by the algal slime that accumulated on the sides of the tanks over the two-week cycle. At the end of the second cycle, the calculated degradation reached 86%, and the spike was therefore increased by 25%. Low levels of Bisphenol-A were also recorded in the control tanks, and it is possible that this originated from the plastic components involved in handling and storing the water used to prepare the test media.

Table 20 – The measured Bisphenol-A concentrations at the start, midpoint and end of each exposure cycle and the calculated mean and standard deviation (LOD = 0.04 μgL^{-1}).

		Bisphenol-A concentration			
		Control	0.2 μgL^{-1} (nominal)	2 μgL^{-1} (nominal)	20 μgL^{-1} (nominal)
1st Exposure Cycle *(3rd – 16th April)*	Start	0.054	0.694	1.59	15.8
	Mid-point	0.104	0.171	1.36	10.7
	End	<0.04	0.119	1.24	9.22
2nd Exposure Cycle *(17th –30th April)*	Start	<0.04	0.106	1.28	11.6
	Mid-point	0.089	0.210	1.23	9.55
	End	0.114	0.119	0.93	8.53
3rd Exposure Cycle *(1st – 14th May)*	Start	0.056	0.330	1.36	15.1
	Mid-point	0.132	0.287	1.56	14.1
	End	0.060	0.138	1.49	15.3
4th Exposure Cycle *(15th – 28th May)*	Start	0.043	0.251	2.44	21.3
	Mid-point	<0.04	0.208	1.85	19.8
	End	<0.04	0.150	1.16	12.2
Mean		**0.061**	**0.232**	**1.46**	**13.6**
(Standard Deviation)		**(0.040)**	**(0.162)**	**(0.389)**	**(4.06)**

6.2.1.3 Media Physico-Chemical Characteristics

Temperature

The constant temperature room used for this exposure was set to 16^{o}C (1^{o}C above the exposure made using 17β-oestradiol because its performance record showed it tended to slightly overcool before switching to heat). Table 21 shows the minimum and maximum temperatures measured during each cycle of the baseline

and exposure periods by the continuous data capture of an 'indoor/outdoor' thermometer (error margin of 1^{o}C). It can be seen that in the event, the room performed within the tolerances during the baseline period, but the minimum recorded temperatures varied from the tolerated range by up to 1.2^{o}C during the exposure period. However these 'cool troughs' occurred only occasionally; on two days in the first exposure cycle (exceeding the tolerated range by 1.2 ^{o}C and 0.8 ^{o}C); one day in the third cycle (by 0.4^{o}C) and two days in the last cycle (by 0.3^{o}C and 0.2^{o}C).

While this is indicative of the range of temperatures the exposure tanks experienced, it is not possible to generate a mean from this information. The temperature was also measured manually using a mercury thermometer (error margin of 0.5^{o}C) in sub-samples taken from the exposure tanks. A mean temperature is also given from these readings. The mean value does not always fall within the automatically recorded minimum and maximum values, due to the different error margins of the instruments involved, but it is clear that the mean temperature is within 1^{o}C of that expected.

Table 21 – The mean, minimum and maximum temperatures recorded for each cycle of the experiment.

	Temperature (^{o}C)		
Experimental Cycles	**Mean**	**Minimum**	**Maximum**
Baseline Cycle *(20th March – 2nd April)*	16.1	15	16.6
1st Exposure Cycle *(3rd – 16th April)*	16.3	13.8	16.5
2nd Exposure Cycle *(17th – 30th April)*	16.5	15.1	16.5
3rd Exposure Cycle *(1st – 14th May)*	16.7	14.6	16.5
4th Exposure Cycle *(15th – 28th May)*	16.6	14.7	16.5

pH

Table 22 shows the mean and standard deviation of pH measurements in aliquots taken from the exposure tanks before and after each media change. It can be seen that the mean pH rose by less than 0.3 units over the 48-hour period between

media changes, and that the addition of fresh media returned the mean pH to between 7.04 and 7.23. The standard deviations are small, and it was considered that this degree of pH change was not adverse to the test organisms.

Table 22 – The mean measured pH and standard deviation (SD) of aliquots of media taken from the tanks before and after media changes.

	pH Pre-Change		pH Post-Change	
Experimental Cycles	**Mean**	**SD**	**Mean**	**SD**
Baseline Cycle *(20th March – 2nd April)*	7.31	0.13	7.04	0.07
1st Exposure Cycle *(3rd – 16th April)*	7.37	0.11	7.14	0.09
2nd Exposure Cycle *(17th – 30th April)*	7.28	0.15	7.18	0.16
3rd Exposure Cycle *(1st – 14th May)*	7.51	0.10	7.22	0.13
4th Exposure Cycle *(15th – 28th May)*	7.45	0.25	7.23	0.10

Dissolved Oxygen

The mean Percentage Air Saturation Values (%ASV) and standard deviations for each cycle of the experiment are given in Table 23. It can be seen that the mean %ASV dropped by up to 20% over the 48-hour period between media changes. The dissolved oxygen level of the fresh media are higher than those recorded in the previous exposure made at a similar temperature, possibly due to the cooler storage conditions of the reverse osmosis filtered water (at the earlier time of year). However the levels in the discarded media were similar. The standard deviations are higher in the discarded media for the second and fourth exposure cycles because the %ASV dropped on one occasion to 18.2% and 15.1% respectively in a single exposure tank, because the air-line had become blocked. This did not appear to affect the snails as being pulmonate, they simply rose to the surface to satisfy their oxygen requirement.

Table 23 – The mean dissolved oxygen (DO) and standard deviation (SD) of aliquots of media taken from the tanks before and after media changes.

	DO Pre-Change % ASV		DO Post-Change % ASV	
Experimental Cycles	**Mean**	**SD**	**Mean**	**SD**
Baseline Cycle *(20th March – 2nd April)*	88	2.96	96	7.82
1st Exposure Cycle *(3rd – 16th April)*	88	4.05	101	6.52
2nd Exposure Cycle *(17th – 30th April)*	85	13.56	98	5.25
3rd Exposure Cycle *(1st – 14th May)*	89	4.43	97	7.49
4th Exposure Cycle *(15th – 28th May)*	82	18.33	101	7.03

Conductivity

Table 24 shows that the conductivity remained low over the duration of the experiment, apparently being unaffected by the presence of food or faecal matter. The levels did rise slightly between partial media changes, but when the standard deviations were taken into account, this was barely perceptible.

Table 24 – The mean conductivity (CND) and standard deviation (SD) of aliquots of media taken from the tanks before and after media changes.

	CND Pre-Change µS/cm		CND Post-Change µS/cm	
Experimental Cycles	**Mean**	**SD**	**Mean**	**SD**
Baseline Cycle *(20th March – 2nd April)*	860	146	846	140
1st Exposure Cycle *(3rd – 16th April)*	848	151	827	152
2nd Exposure Cycle *(17th – 30th April)*	902	117	883	115
3rd Exposure Cycle *(1st – 14th May)*	928	130	920	126
4th Exposure Cycle *(15th – 28th May)*	914	101	899	87

Hardness

The mean hardness of the samples of fresh media taken over the duration of the experiment was 468.2 mgL^{-1} $CaCO_3$ equivalents (standard deviation = 33.0). This was high relative to the calculated value of 394 mgL^{-1} $CaCO_3$ equivalents. The mean results from the discarded media (given with standard deviations in Table 25), are also slightly higher than those recorded in the previous exposure. It is possible that one or more of the stock solutions of salts was prepared incorrectly. However the increased hardness was considered unlikely to have affected the snails, as gastropods in general prefer a hard water environment to facilitate the extraction of calcium for shell-building (Boycott, 1936). Indeed, it had previously been observed from the culture of *P. corneus* that if the hardness was allowed to decline, the snails would tend to rasp the shells of others, possibly to acquire additional calcium.

Table 25 – The mean hardness and standard deviation (SD) of aliquots of media taken from the tanks before media changes.

	Hardness (mgL^{-1} $CaCO_3$ equivalents)	
Experimental Cycles	**Mean**	**SD**
Baseline Cycle *(20th March – 2nd April)*	418.5	35.9
1st Exposure Cycle *(3rd – 16th April)*	433.5	38.1
2nd Exposure Cycle *(17th – 30th April)*	410.5	47.4
3rd Exposure Cycle *(1st – 14th May)*	447.0	18.7
4th Exposure Cycle *(15th – 28th May)*	436.5	41.6

6.2.1.4 Test Organism Survivorship

Three snails died during the baseline period (2.1%), before the exposure tanks were allocated to control or test concentrations. Of the groups reduced to eight snails, one was then randomly allocated as 0.2 μgL^{-1} Bisphenol-A (nominal), and two as 2 μgL^{-1} (nominal). During the exposure period, a further animal died at

0.2 μgL^{-1} (nominal) in the first cycle of the experiment, and a control animal died in the last cycle. The control mortality was therefore 2.78% and the total mortality was 3.5% (5 out of the 144 animals used). This was considered an acceptable level of mortality for a chronic experiment (e.g. OECD, 2008), and there was no pattern in the mortality rate over time or concentration, and therefore no indication of overt toxicity from the test compound. Indeed, there were no deaths at all in the highest concentration of Bisphenol-A (20 μgL^{-1}, nominal) over the whole exposure period.

6.2.1.5 Test Organism Morphology and Growth

At the start of the experiment, the mean length and weight of each group was compared between treatments, and there were no significant differences between the control groups and any of the treatment groups (ANOVA, $p>0.05$). At the end of the exposure, the growth of the organisms was calculated in terms of percentage increase in length and weight, and these are shown in Figure 65. All of the groups exposed to Bisphenol-A exhibited retarded growth for both mean length and weight increase compared to the control group. In the previous exposure (to 17β-oestradiol), all the groups grew at least 10% in the longest axis and put on at least 5% mean weight at a similar temperature. In this exposure the control group grew slightly less well, with a mean increase of 3.64% in length and 7.66% in weight. However, this could be attributable to the experiment being two weeks shorter. The Bisphenol-A exposed groups increased even less in both mean length and weight (up to 3.18% and 5.46% respectively); however this suppressed growth was not significantly different to the control group (ANOVA, $p>0.05$ for both length and weight).

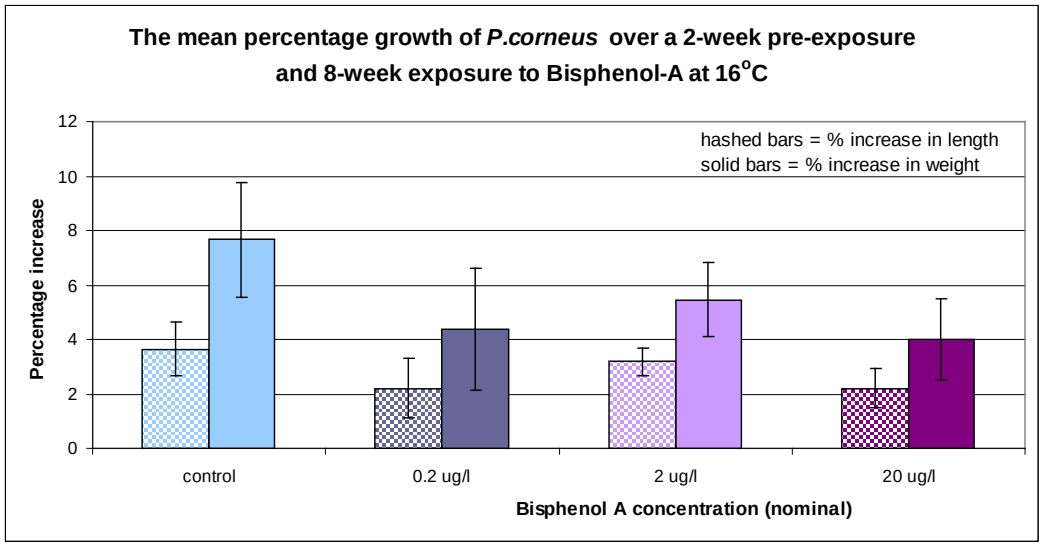


Figure 65 – The mean percentage increase in length and weight of *P. corneus* over a 10 week long experiment at 16°C (error bars represent the standard error of the means).

At the end of the exposure, there was no significant difference in the mean condition factor between the control and exposure groups (a ratio of total weight to flesh weight, giving an impression of the body condition of the animal inside the shell, ANOVA, $p>0.05$). However there was a significant difference in the mean repro-somatic index (RSI; the ratio of the flesh weight to the weight of the reproductive organs, ANOVA, $p = 0.036$). This difference was not between the control and exposed groups, rather it was between the two highest Bisphenol-A exposure concentrations (2 and 20 μgL^{-1} nominal). Figure 66 shows that the mean RSI increases with increasing nominal concentration of Bisphenol-A except at the highest concentration (20 μgL^{-1} nominal), when it falls to less than that of the control group.

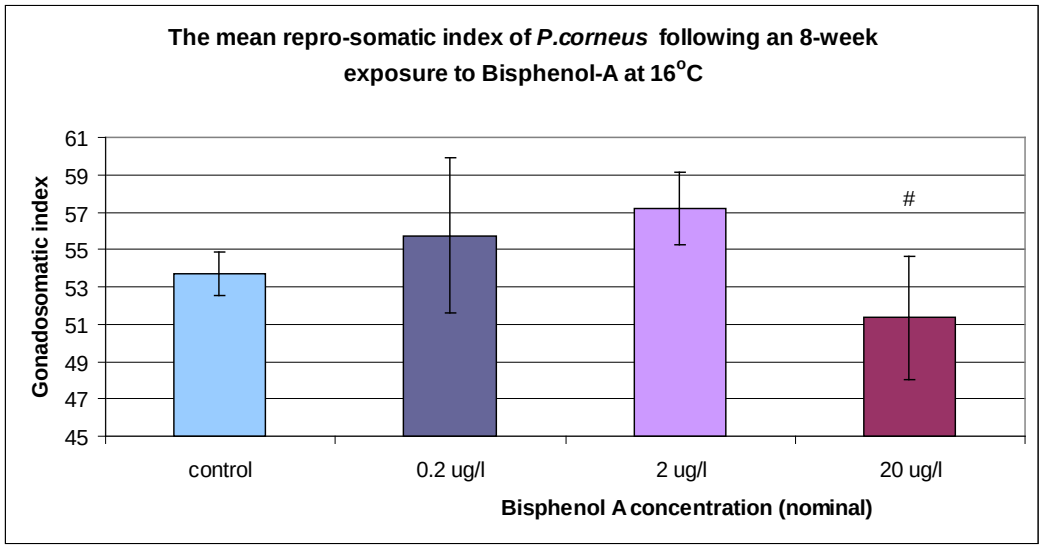


Figure 66 – The mean RSI of *P. corneus* following an 8 week long exposure to Bisphenol-A at 16°C (error bars represent the standard error of the means, hash indicates a significant difference from the 2 μgL^{-1} (nominal) exposure group at #p<0.05).

6.2.1.6 Test Organism Reproduction

The mean total number of eggs laid per snail over the 2 week pre-exposure period was 78.8. This was less than 50% of the number of eggs laid per snail in the same period during the previous 17β-oestradiol exposure at a slightly cooler temperature (161.8 at 15°C), suggesting that reproduction in the test population had already begun to decline. However, the COV between the number of masses laid per snail per 48 hours was 23.7% (mean = 0.372, SEM = 0.088), which was comparable with that of the previous exposure (COV = 18.6%). A further 8 groups had been assessed during the baseline period, but the number of masses laid by these groups was low, so that the overall COV would have been 44.5% (mean = 0.302, SEM = 0.134), which was not considered acceptable, and they were therefore excluded from the exposure.

Figure 67 shows the mean total number of eggs laid per snail during the exposure period. The control group laid a mean total of 106.6 eggs per snail during the 8-week exposure, which was 3-fold fewer than during the previous 17β-oestradiol

exposure of the same length (372.0). The group exposed to 20 μgL^{-1} (nominal) Bisphenol-A laid almost exactly the same number of eggs per snail as the control group (107.1), and there were no significant differences between any of the groups (ANOVA, $p>0.05$).

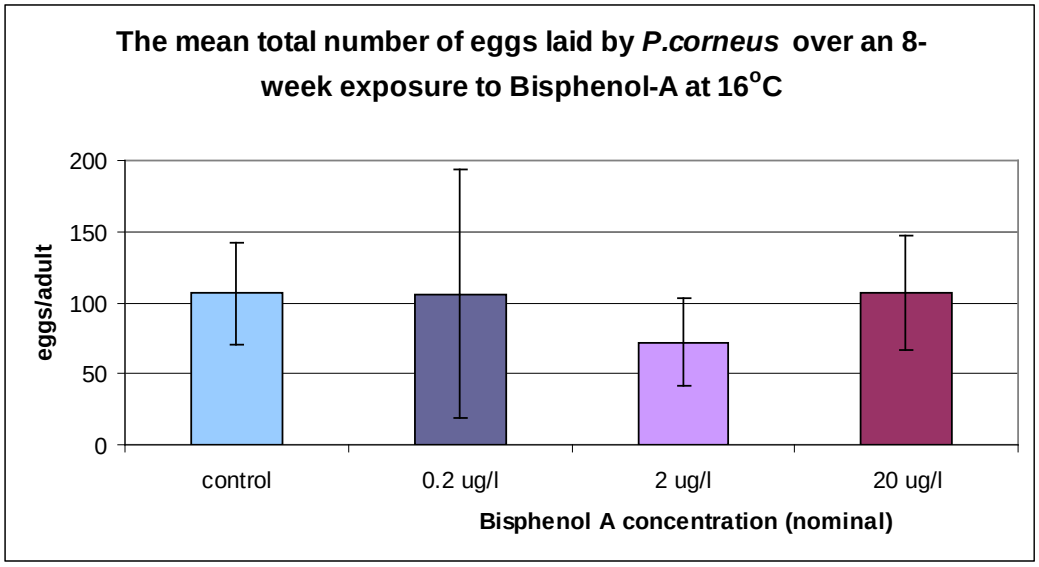


Figure 67 – The mean total number of eggs produced per *P. corneus* over an 8-week exposure to Bisphenol-A at 16°C (error bars represent the standard error of the means).

However, the between group variability was once again high (particularly so in the group exposed to 0.2 μgL^{-1} nominal Bisphenol-A). Therefore, comparisons are again made with the relevant pre-exposure oviposition rate in the manner of Harries et al. (2000), and the results of paired 2-tailed t-tests between the pre-exposure and the first 14 days of the exposure, and between the pre-exposure and the whole exposure period are given in Table 26. There was an overall significant difference between the mean number of eggs laid per snail per 48 hours in the pre-exposure period and exposure periods (Repeated Measures ANOVA, $p<0.001$ both after 14 days and in the whole exposure).

Table 26 – The mean number of eggs laid per *P. corneus* by each replicate (in the same order in each case) per 48 hours in the 14 day pre-exposure period, the first 14 days of the exposure and the whole (56 day) exposure at 16°C, and the results of paired t-tests.

		Bisphenol-A concentration		
	Control	0.2 µgL^{-1} nominal	2 µgL^{-1} nominal	20 µgL^{-1} nominal
Mean number of eggs laid in the pre-exposure phase	19.2 14.6 14.4 4.19	5.90 11.8 13.2 7.51	6.81 16.2 7.67 13.6	14.2 9.41 6.86 14.1
Mean number of eggs laid in the first 28 days of the exposure	8.14 3.67 7.59 4.70	3.38 8.37 12.9 2.81	1.54 7.14 3.48 8.73	5.98 5.17 8.57 2.43
Paired t-test between pre-exposure and first half of exposure	No significant difference p>0.05	No significant difference p>0.05	Significantly lower, p = 0.013	No significant difference, p>0.05
Mean number of eggs laid in the whole exposure period	2.91 2.83 5.59 3.90	1.85 4.12 8.05 1.13	1.25 3.62 2.14 3.32	3.48 4.37 5.43 2.02
Paired t-test between pre-exposure and the whole exposure	No significant difference p>0.05	Significantly lower p = 0.005	Significantly lower, p = 0.017	No significant difference, p>0.05

This table shows that although the mean number of eggs per snail per 48 hours was reduced in three out of the four control groups over the first 14 days of the exposure period compared to the pre-exposure period, this reduction is not

significant ($p = 0.08$). Nor is it significantly different over the whole exposure period ($p = 0.07$). This suggests that the oviposition activity rate of the control group had effectively already reached a basal level for 'simulated autumn' conditions when the experiment began. However, Figure 68a shows the mean cumulative number of eggs laid per snail over the pre-exposure and exposure periods (divided into each 14-day exposure cycle). A reduction in oviposition activity over each successive exposure cycle can be seen, that culminates in a 77.0% reduction overall. It is likely that this decline is not significantly different from the pre-exposure phase because the fourth control group laid a lower number of eggs per snail per 48 hours than the other groups from the outset, and continued at this low rate of oviposition during the whole exposure period (even increasing slightly in the first 14 days of the exposure). It is therefore likely that this one replicate group had reached the basal level of egg production for 'simulated autumn' conditions at the start of the experiment.

Figures 68b and c show the same graph for the groups exposed to 0.2 and 2 μgL^{-1} Bisphenol-A (nominal) respectively. These graphs also clearly show the decline in oviposition activity over each successive exposure cycle, culminating in respective 79.9% and 88.4% reductions, overall. Table 26 shows that these are significant reductions in the number of eggs laid per snail per 48 hours between the pre-exposure and exposure periods in both groups. The graphs appear to be very similar to Figure 68a (the control group), which suggests that there was no effect of Bisphenol-A at these nominal concentrations. However, the response of the group exposed to 20 μgL^{-1} (nominal) was slightly different. Figure 68d shows that the number of eggs laid per snail per 48 hours declines in the expected manner over the first and second exposure cycles (culminating in an 80.4% reduction), but also that there seems to be a degree of recovery in the third and fourth exposure cycles that was not observed in any of the other groups (final overall reduction was only 61.1%). This recovery is the cause of there being no significant reduction in the number of eggs per snail per 48 hours between the pre-exposure and the exposure period overall.

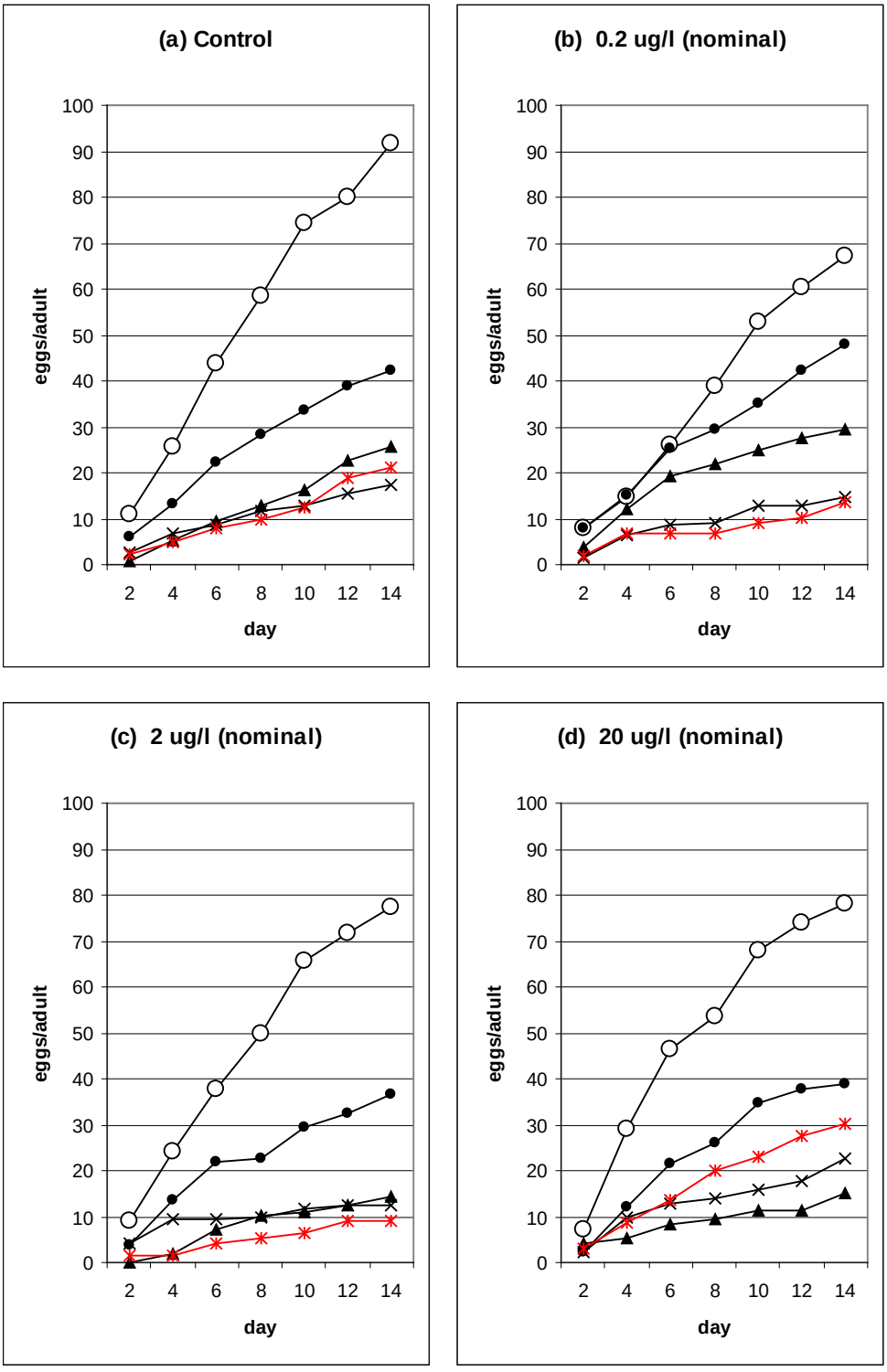


Figure 68: Cumulative mean number of eggs laid per *P. corneus* at 16°C, 12hL/12hD, during the pre-exposure period (open circles), and the first (black circles), second (black triangles), third (black crosses) and fourth (red asterisks) fortnight of the exposure period.

6.2.1.7 Egg Mass and Egg Effects

Number of Eggs per Mass

The mean number of eggs per mass was 30.1 (±9.87) in the pre-exposure phase of the experiment, and this is comparable to the mean number of eggs per mass recorded in the pre-exposure phase of the 17β-oestradiol exposure at 15°C (mean = 31.6). The mean number of eggs per mass laid by the control group was also very similar (mean = 30.0 ±10.9). There appears to be a slight dose-dependant increase in the number of eggs per mass within increasing Bisphenol-A concentration (see Figure 69), but this is not significant (Pearson's Product Moment Correlation Coefficient, $p>0.05$), and there was no significant difference from the control group for either method of data interpretation in any exposure group (ANOVA, $p>0.05$).

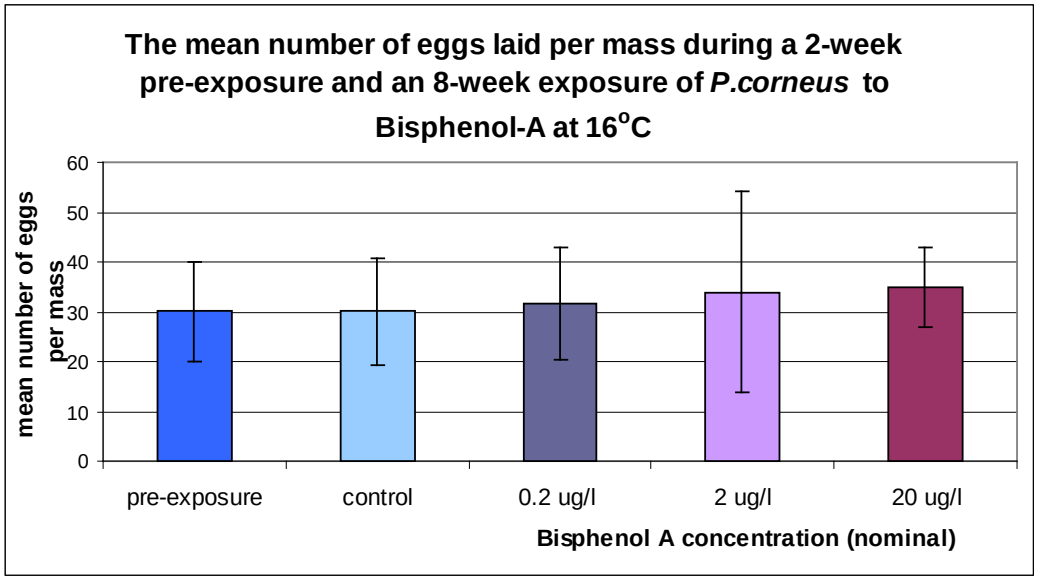


Figure 69 - The mean number of eggs per mass in egg masses laid by *P. corneus* at 16°C during a pre-exposure and exposure to Bisphenol-A (error bars represent the standard error of the means).

Egg Mass Dry Weight

The mean dry weight of the egg masses was 5.63 mg in the pre-exposure phase of the experiment. Again this is comparable to the mean dry weight of the masses

laid in the pre-exposure phase of the 17β-oestradiol exposure at 15°C (mean = 6.28 mg). The mean dry weight of masses laid by the control group during the exposure was also similar (mean = 6.01 mg). Figure 70 shows that the mean dry weight of the masses laid by groups exposed to Bisphenol-A were all slightly heavier (up to 7.29 mg for the group exposed to 2 μgL^{-1}, nominal). However the response was not dose-dependant and there were no significant differences between those of the control and any exposure group (ANOVA, $p>0.05$).

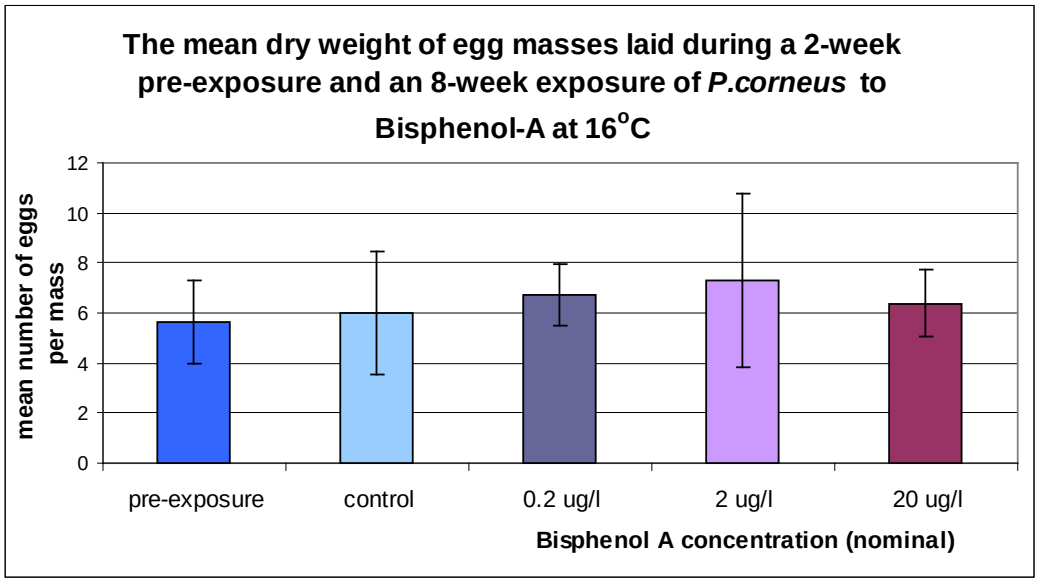


Figure 70 - The mean dry weight of egg masses laid by *P. corneus* at 16°C during a pre-exposure and exposure to Bisphenol-A (error bars represent the standard error of the means).

Egg Abnormality Rate

The mean percentage of eggs observed to have abnormalities likely to adversely affect hatching success (either no embryo, multiple embryos or the embryo outside of the egg) was 0.769% in the pre-exposure phase of the experiment, and this is comparable to the mean abnormality rate observed in the pre-exposure phase of the 17β-oestradiol exposure at 15°C (mean = 0.885%). However the mean abnormality rate in the control group during the exposure was higher (1.08%), and there was an apparent dose-dependant decrease in the mean percentage of egg abnormalities with increasing Bisphenol-A concentration (see

Figure 71). This is conceptually unlikely, and indeed the correlation is not significant (Pearson's Product Moment Correlation Coefficient, $p>0.05$), and the abnormality rate in the control group was not significantly different from any of the Bisphenol-A exposed groups (Kruskall-Wallis, $p>0.05$).

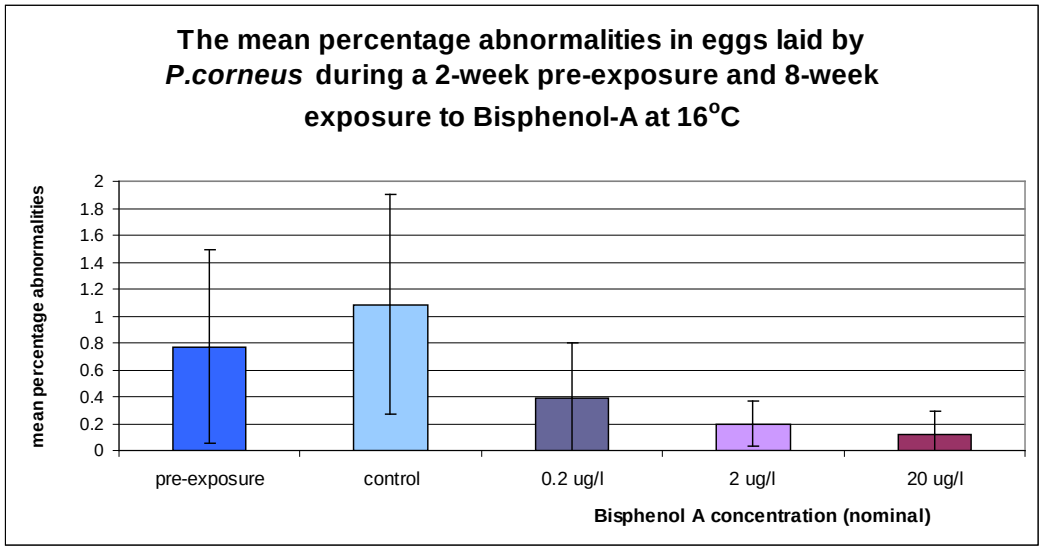


Figure 71 - The mean percentage abnormality in eggs laid by *P. corneus* at 16°C during a pre-exposure and exposure to Bisphenol-A (error bars represent the standard error of the means).

Egg Hatching Success

The hatching success of eggs laid during the exposure was as variable as that observed during the previous exposure to 17β-oestradiol. Figure 72 shows that there was a complete failure of eggs laid by some groups to hatch on several occasions (no eggs were laid by the group exposed to 0.2 μgL^{-1} nominal Bisphenol-A in the samples taken from the third and forth exposure cycles), but that the success rate was as high as 62.0% in the group exposed to 2 μgL^{-1} (nominal) in the first cycle. In the 17β-oestradiol exposure, the success rate ranged between 0 and 66.1%. There is no discernable pattern to the hatching rate during either exposure, and the success or failure of egg hatching may depend on whether the mass is damaged during sampling.

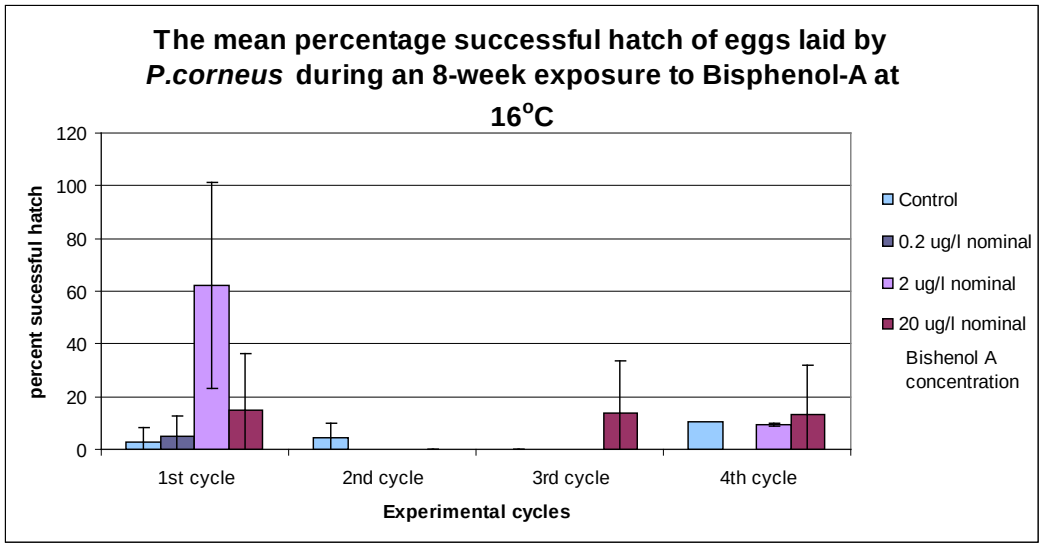


Figure 72 - The mean percentage of successful hatching in eggs laid by *P. corneus* at 16°C during an exposure to Bisphenol-A (error bars represent the standard error of the means).

6.2.2 Experiment 6 – An exposure of *P. corneus* to Bisphenol-A in changing conditions to simulate the onset of 'autumn'.

6.2.2.1 Bisphenol-A Analysis

The results of the Bisphenol-A analysis for each week of the exposure are set out in Table 27, together with the overall mean and standard deviations (values below the LOD of 0.04 μgL^{-1} are treated as 0.02 μgL^{-1} when calculating the mean, unless a lower value was reported by the NLS Laboratory). The mean percentage recoveries are 90.4%, 71.5% and 65.8% for the 0.2, 2 and 20 μgL^{-1} (nominal) concentrations, respectively. This was lower than expected for the upper two test concentrations, compared to the results of the initial stability study. However, the analysis results from the control and each treatment concentration are significantly different to each other in all cases (Mann Whitney U-tests, $p<0.001$ between each neighbouring concentration). This is a satisfactory indication that each treatment concentration is sufficiently different from control and the other

treatments to be assessed independently in relation to any effects observed in the organisms.

Table 27 – The measured Bisphenol-A concentrations from samples taken from each replicate and pooled for each treatment (Weeks 1, 2, 4 to 6 and 8, from each exposure tank (Weeks 3 and 7) and the calculated mean and standard deviation (LOD = 0.04 µgL^{-1}).

	Bisphenol-A concentration (µgL^{-1})			
	Control	**0.2 µgL^{-1} (nominal)**	**2 µgL^{-1} (nominal)**	**20 µgL^{-1} (nominal)**
Week 1 *(8th Nov.)*	0.056	0.212	1.68	16.0
Week 2 *(15th Nov.)*	---	0.230	1.83	16.7
Week 3 *(22nd Nov.)*	<0.04	0.208	1.60	15.0
	0.058	0.183	1.42	6.45
	---	0.228	1.74	14.9
	---	---	1.72	15.7
	0.049	0.189	1.56	15.4
	0.086	0.144	1.43	14.3
	0.054	0.220	1.44	---
	<0.04	0.206	1.41	16.6
	0.049	0.191	1.66	15.7
	0.059	0.217	1.51	16.4
Week 4 *(29th Nov)*	0.037	0.207	1.80	17.8
Week 5 *(6th Dec.)*	0.030	0.170	1.68	---
Week 6 *(13th Dec.)*	0.050	---	---	12.8
Week 7 *(20th Dec.)*	0.046	---	1.16	10.8
	0.043	0.200	1.22	4.95
	---	0.128	---	12.0
	---	---	1.14	10.3
	0.098	0.172	1.16	10.1
	0.054	0.112	0.876	11.8
	<0.04	0.166	1.25	---
	0.075	0.137	1.19	11.7
	0.043	0.135	0.971	13.5
	<0.04	---	1.51	10.2
Week 8 *(27th Dec).*	0.030	0.140	1.36	---
Mean	0.047	0.181	1.43	13.1
SD	0.021	0.036	0.264	3.38

There were however several concerns with the control dataset. The pooled control sample from Week 2 was high (0.570 μgL^{-1}). It was calculated that only an erroneous aliquot pooled from an exposure tank containing the highest nominal Bisphenol-A treatment concentration (20 μgL^{-1}) in error could have been the cause of this degree of elevation. At Week 3, when the tanks were individually analysed, it was observed that one of the control tanks remained elevated (0.686 μgL^{-1}) and one of the tanks allocated to 20 μgL^{-1} (nominal) Bisphenol-A was low (6.45 μgL^{-1}) following the three intervening partial media changes. It was therefore likely that this control tank had been spiked with Bisphenol-A in error. Consequently it was decided to exclude the individuals in this control tank from all further biological assessment, and the pooled chemical analysis result from Week 2 was also discarded.

There were also various errors of sample identification within the two spatial sampling events (sent for analysis together). There were two tanks that had two results for Week 3 and none for Week 7, and three tanks appeared to have become switched within the week 7 sample set. These seven results all fell within the expected range for the control and treatments (values reported were <0.04, <0.04, 0.232, 0.236, 1.93, 11.1 and 11.5 μgL^{-1}), but because the original identity of the samples could not be ascertained, all results cast into doubt in this manner have been excluded. Because one of the tanks involved was a control tank, this was also removed from further analysis to ensure that a contaminated tank was not inadvertently used as a comparison for any effects of Bisphenol-A.

There were several other problems encountered within the dataset from the treated tanks. At Week 6 the results were also identified ambiguously, with two values recorded as being representative of 2 μgL^{-1} (nominal) treatment, and none representing the 0.2 μgL^{-1} (nominal) treatment. Therefore these two values (0.17 and 13.7 μgL^{-1}) were excluded. On two occasions, (Weeks 5 and 8) the results from the 20 μgL^{-1} (nominal) concentration were high (169 and 133 μgL^{-1} respectively). These samples were composed of pooled aliquots from each replicate exposure tank in this treatment, and so the high values are more likely to

be due to a dilution error made at the analytical laboratory. Consequently these values were also excluded. Finally, one sample from the 0.2 μgL^{-1} (nominal) treatment was broken (Week 7).

6.2.2.2 Media Physico-Chemical Characteristics

Temperature

Figure 73 describes the mean temperature of the exposure tanks prior to each media change, along with the temperature of the coolest tank (minimum) and the warmest tank (maximum) on each occasion. The temperature did not differ by more than 2°C at any time across the tanks. The mean temperature during the pre-exposure period was 18.7°C. During the first 28 days of the exposure, the temperature of the two rooms was gradually decreased by approximately 1°C per week to simulate the onset of autumn. After this, the mean temperature was 14.9°C for a further 28 days.

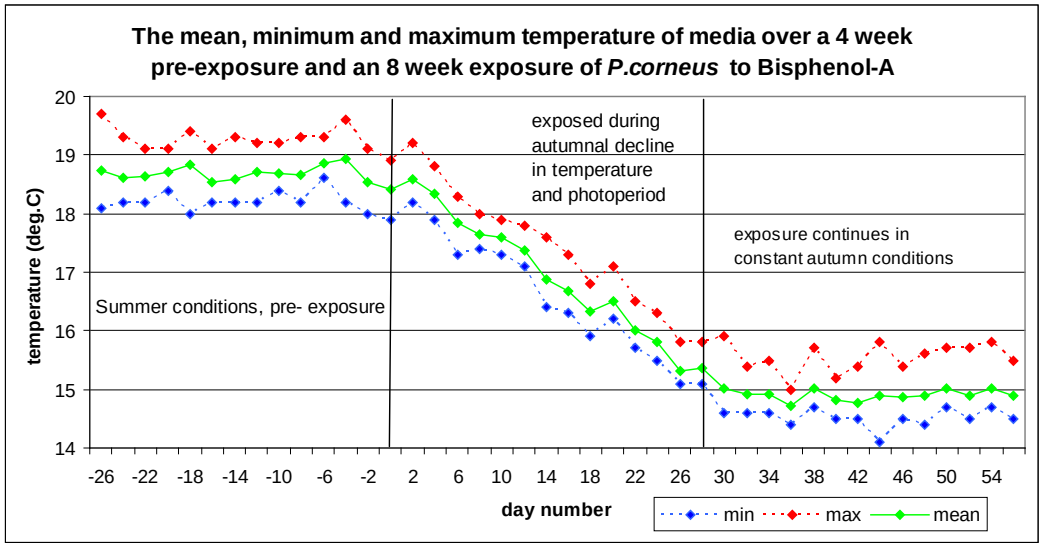


Figure 73 – The mean, minimum and maximum temperature measured in the exposure tanks over a 4 week pre-exposure and an 8 week exposure of *P. corneus* to Bisphenol-A.

pH

Table 28 shows the mean and standard deviation of pH measurements from aliquots taken from the exposure tanks before and after each media change. It can be seen that the mean pH rose by less than 0.3 units over the 48-hour period between media changes, and that the addition of fresh media returned the mean pH to between 7.23 and 7.39 (this was slightly more alkaline than the previous exposure to Bisphenol-A, but by <0.2 pH units). The standard deviations are small, and it was considered that this degree of pH change was not adverse to the test organisms.

Table 28 – The mean measured pH and standard deviation (SD) of aliquots of media taken from the tanks before and after media changes.

	pH Pre-Change		pH Post-Change	
Experimental Cycles	**Mean**	**SD**	**Mean**	**SD**
1st Pre-Exposure Cycle *(11th Oct. – 24th Oct.)*	7.39	0.170	7.30	0.153
2nd Pre-Exposure Cycle *(25th Oct. - 7th Nov.)*	7.44	0.145	7.32	0.101
1st Exposure Cycle *(8th Nov. - 21st Nov.)*	7.46	0.122	7.23	0.159
2nd Exposure Cycle *(22nd Nov. - 5th Dec.)*	7.51	0.185	7.35	0.123
3rd Exposure Cycle *(6th Dec. – 19th Dec.)*	7.46	0.146	7.30	0.216
4th Exposure Cycle *(20th Dec. – 3rd Jan.)*	7.58	0.147	7.39	0.163

Dissolved Oxygen

The mean Percentage Air Saturation Values (%ASV) for each fortnight long cycle of the experiment are set out along with the standard deviations in Table 29. It can be seen that the mean %ASV dropped by up to 20% over the 48-hour period between media changes in the pre-exposure period, but that the levels of dissolved oxygen in the fresh media are high. It is also notable that these levels were lower during the exposure period, which was not expected since it was considered that the %ASV would rise as the temperature decreased. However the drop during the 48-hours between media changes was also reduced to <10%. The

standard deviations are again occasionally high in the discarded media due to blockages in individual air-lines. This did not appear to affect the snails.

Table 29 – The mean dissolved oxygen (DO) and standard deviation (SD) of aliquots of media taken from the tanks before and after media changes.

	DO Pre-Change (% ASV)		DO Post-Change (%ASV)	
Experimental Cycles	**Mean**	**SD**	**Mean**	**SD**
1st Pre-Exposure Cycle *(11th – 24th Oct.)*	88	17.5	108	10.1
2nd Pre-Exposure Cycle *(25th Oct. - 7th Nov.)*	80	15.0	91	13.8
1st Exposure Cycle *(8th Nov. - 21st Nov.)*	79	9.20	87	8.00
2nd Exposure Cycle *(22nd Nov. - 5th Dec.)*	79	7.43	85	10.9
3rd Exposure Cycle *(6th Dec. – 19th Dec.)*	80	10.2	87	8.34
4th Exposure Cycle *(20th Dec. – 3rd Jan.)*	76	12.4	83	8.30

Conductivity

Table 30 shows that the mean conductivity of the sub-samples taken from the exposure tanks was slightly elevated in the pre-exposure period due to the higher feed rate. Once this was reduced, the mean conductivity fell to below 1000 uS/cm and remained low for the duration of the exposure period.

Hardness

The mean hardness of the samples of fresh media taken over the duration of the experiment was 454.1 mgL^{-1} $CaCO_3$ equivalents (standard deviation = 35.9). This was again high relative to the calculated value of 394 mgL^{-1}. The mean results from the aliquots taken from the exposure tanks at the end of each cycle are set out in Table 31, and these are also high. The reason for this is not clear, but it was considered unlikely to have affected the snails.

Table 30 – The mean conductivity (CND) and standard deviation (SD) of aliquots of media taken from the tanks before and after media changes.

Experimental Cycles	CND Pre-Change µS/cm		CND Post-Change µS/cm	
	Mean	SD	Mean	SD
1st Pre-Exposure Cycle *(11th Oct. – 24th Oct.)*	1142	266	1170	314
2nd Pre-Exposure Cycle *(25th Oct. - 7th Nov.)*	904	134	942	123
1st Exposure Cycle *(8th Nov. - 21st Nov.)*	946	130	932	130
2nd Exposure Cycle *(22nd Nov. - 5th Dec.)*	895	69.3	897	62.4
3rd Exposure Cycle *(6th Dec. – 19th Dec.)*	903	41.3	894	44.2
4th Exposure Cycle *(20th Dec. – 3rd Jan.)*	903	56.7	897	55.8

Table 31 – The mean hardness and standard deviation (SD) of aliquots of media taken from the tanks before media changes.

Experimental Cycles	Hardness (mgL^{-1} $CaCO_3$ equivalents)	
	Mean	SD
1st Pre-Exposure Cycle *(11th Oct. – 24th Oct.)*	482	44.5
2nd Pre-Exposure Cycle *(25th Oct. - 7th Nov.)*	403	47.1
1st Exposure Cycle *(8th Nov. - 21st Nov.)*	467	15.3
2nd Exposure Cycle *(22nd Nov. - 5th Dec.)*	441	12.6
3rd Exposure Cycle *(6th Dec. – 19th Dec.)*	459	16.6
4th Exposure Cycle *(20th Dec. – 3rd Jan.)*	465	16.5

6.2.2.3 Test Organisms Survivorship

The exposure tanks were not allocated to control or test concentrations for the pre-exposure period, during which 21 of the 360 animals died (5.8%). Once allocation to control or treatment groups was completed, the control group consisted of 67 snails distributed over 8 replicate groups (2 replicates excluded due to potential contamination), and the groups exposed to Bisphenol-A consisted of 88, 81 and 85 snails over 10 replicate groups at the 0.2, 2 and 20 μgL^{-1} (nominal) concentrations respectively.

The total mean control mortality (including the animals that died in the pre-exposure period) was 20.5%, which is usually considered acceptable in chronic invertebrates exposures (e.g. OECD, 2008). It was a higher rate of mortality than that observed during the first Bisphenol-A exposure (2.8%), but this was to some extent expected as a stocking density of 1 snail per litre had been noted to cause an increased mortality rate at the warmer temperature used during the pre-exposure phase (up to 20^{o}C, see Chapter 2, Section 2.2.1).

The mean percentage mortality that occurred during the exposure is shown in Figure 74, divided into the first 28 day period (changing conditions; temperature cooling and the light period being reduced) and second 28 day period (constant cool conditions with short day length). The mean mortality rate in all of the Bisphenol-A exposed groups was higher than that of the control group, but there was no evidence of a dose-dependant relationship, and there were no significant differences between the control and any Bisphenol-A exposed group (ANOVA, $p>0.05$). Overall, the rate of mortality increased over the course of the experiment; 7.6% of the snails died during the first 28 days of the exposure (changing conditions), and 11.7% died during the second 28 days of the exposure (constant cool conditions). This latter rate was unexpected as the overall mortality rate during the previous exposure to Bisphenol-A had been lower in similarly constant cool conditions (3.5% over 8 weeks).

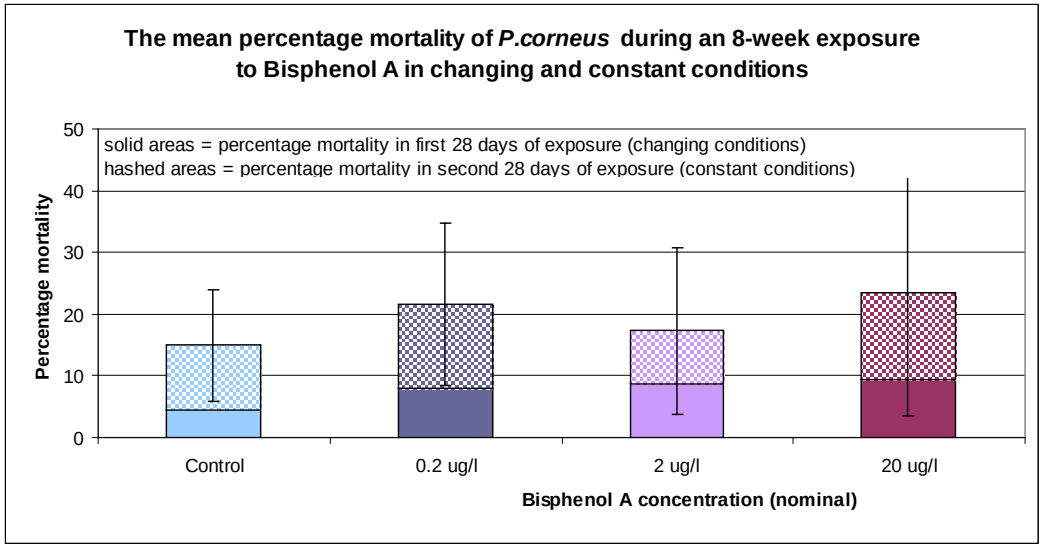


Figure 74 – The mean percentage mortality of *P. corneus* over an 8-week exposure to Bisphenol-A in changing conditions (temperature reduced from 18.7°C (mean) to 14.9°C (mean) and photoperiod reduced from 16h to 12h light, and then constant conditions; 14.9°C (mean) and 12h light; error bars represent the standard error of the means for the total percentage mortality).

6.2.2.4 Test Organism Morphology and Growth

At the start of the experiment, the mean length and weight of each group was compared between treatments, and it was found that the group allocated for exposure to 2 μgL^{-1} (nominal) Bisphenol-A was significantly smaller and lighter than the group allocated for exposure to 20 μgL^{-1} (nominal), although neither group was significantly different from the allocated control group (ANOVA, $p = 0.021$ for mean length and $p = 0.013$ for mean weight). At the end of the exposure, the growth of the organisms was calculated in terms of percentage increase in length and weight and the results are shown in Figure 75. The mean length of the control group increased by 3.62% and the mean weight by 9.03%, which is comparable to the previous exposure (3.64% and 7.66% respectively.

However, the relatively high rate of mortality confound the analysis of growth to some extent. The shell of dead animals can be measured which allows an

assessment of growth up to time of death, and ensures that the exposed groups did not appear smaller overall, as the larger, older animals died more frequently. This was not possible for the weight at time of death as the tissue rapidly degrades, which meant that some groups were artificially lighter than at the start of the test, and this also caused a very high error rate (standard error of the means). However, on average the groups exposed to Bisphenol-A did increase in length and weight, suggesting that the test conditions and food supply were adequate.

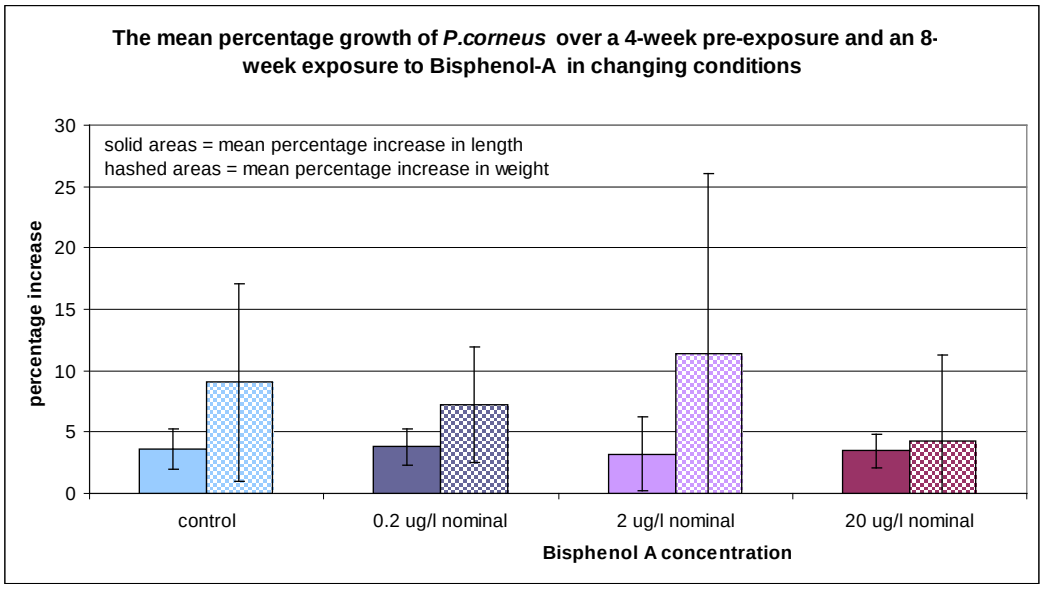


Figure 75 – The mean percentage increase in length and weight of *P. corneus* over a 12 week long experiment, in changing conditions (temperature held at 18.7°C (mean) with 16h light for a 4-week pre-exposure period, then reduced to 14.9°C (mean) and 12h light over the following 4 weeks of exposure, then held at 14.9°C (mean) and 12h light for final 4 weeks of exposure; **error bars represent the standard error of the means).**

On dissection of the surviving animals at the end of the exposure, there was no significant difference in the mean condition factor (ratio of total weight to flesh weight) between the groups (ANOVA, $p>0.05$). The overall mean condition factor was 40.2%, which was slightly less than the previous exposure (44.0%) and more similar to that recorded in the 17β-oestradiol exposure made at 20°C

(39.2%). This suggests that the organisms lose body condition at warmer temperatures. The pattern of response in the RSI was the same as that seen in the previous experiment (see Figure 76); the proportional weight of the reproductive organs increased with increasing nominal Bisphenol-A concentration, except in the group exposed to 20 μgL^{-1} (nominal), where a reduction was again observed. However on this occasion there were no significant differences between the control and any of the treatment groups (Kruskall-Wallis, $p>0.05$).

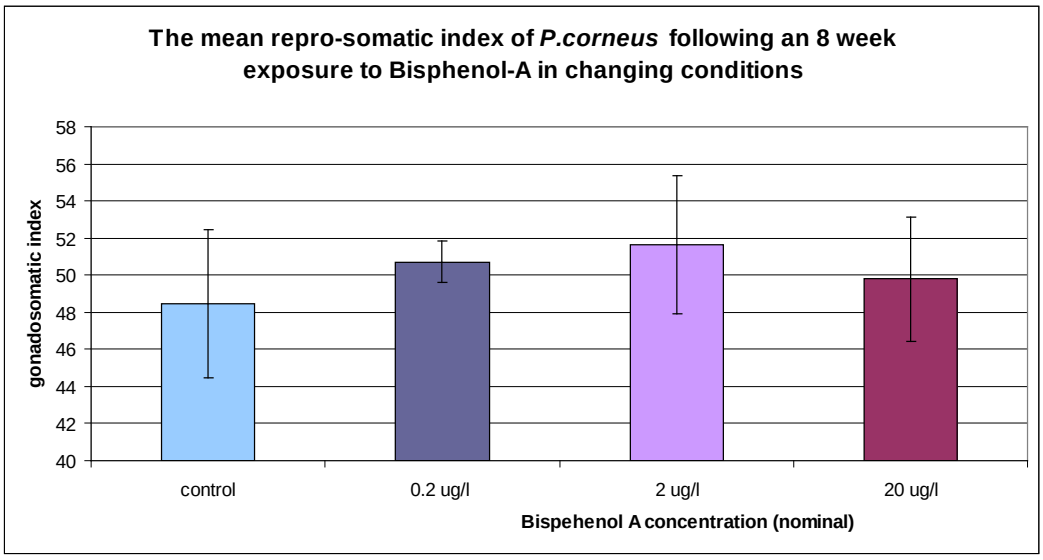


Figure 76 – The mean RSI of *P. corneus* following an 8 week long exposure to Bisphenol-A in changing conditions (temperature reduced from 18.7°C (mean) to 14.9°C (mean) and photoperiod reduced from 16h to 12h light over 4 weeks, and then constant conditions; 14.9°C (mean) and 12h light; error bars represent the standard error of the means**).**

6.2.2.5 Test Organism Reproduction

The mean number of eggs laid per snail over all of the replicate groups in the 28 day pre-exposure period was 417, which was 72% of the number of eggs laid by snails during the same period in the 17β-oestradiol exposure at 20°C (572). Moderately less oviposition activity was not unexpected as the mean measured temperature was slightly cooler in this pre-exposure period compared to that measured in the previous study (18.7°C compared to 20.3°C). The mean number

of egg masses laid per snail during the pre-exposure period was 0.60, which is x2.4 the criterion suggested in the 17β-oestradiol study (0.25) and was therefore sufficiently high to allow any decline in oviposition rates in 'simulated autumn' to be observed. The COV over all of the replicate groups in the pre-exposure period was 19.8% (S.E.M. = 86.9) and following allocation into control and treatments, this was broken down into 18.3% for the control group, and 19.3%, 22.6% and 16.1% groups allocated to 0.2, 2 and 20 μgL^{-1} (nominal) Bisphenol-A treatments, respectively.

Figure 77 shows the mean total number of eggs laid per snail over the 8-week exposure. It can be seen that all of the groups exposed to Bisphenol-A laid fewer eggs than that of the control group. However there were no significant differences between any of the groups for the total number of eggs laid (ANOVA, $p>0.05$). There were also no significant differences if the mean weights of the snail groups were included as a co-variable (ANCOVA, $p>0.05$).

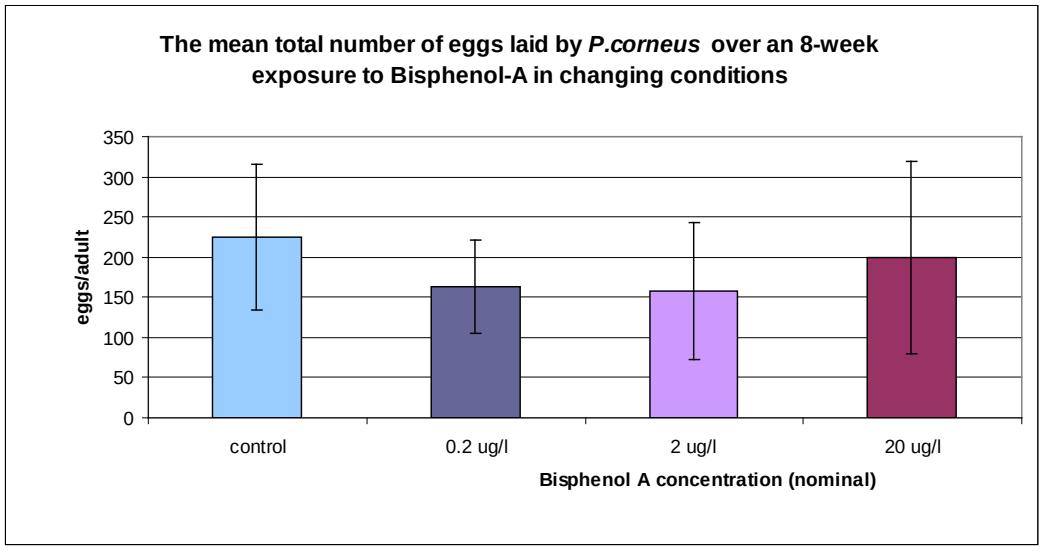


Figure 77 – The mean total number of eggs laid per snail following an 8 week long exposure to Bisphenol-A in changing conditions (temperature reduced from 18.7°C (mean) to 14.9°C (mean) and photoperiod reduced from 16h to 12h light, and then constant conditions; 14.9°C (mean) and 12h light; error bars represent the standard error of the means**).**

Despite the increased number of replicate groups employed in this exposure (10 replicates per treatment instead of 4), the between group variation in the number of eggs laid per snail was little improved (mean COV is 47.2%, compared to 49.0% in the previous Bisphenol-A exposure). Therefore, the number of eggs laid per snail in the control group and in each treatment group were once again compared with the number of eggs laid per snail in the respective pre-exposure period, in the manner of Harries et al., (2000). Table 32 gives the mean number of eggs laid per snail per 48 hours in the 28 day pre-exposure period, the first 28 days of the exposure period and the whole exposure period, with the results of paired 2-tailed t-tests between them. There was an overall significant difference between the mean number of eggs laid per snail per 48 hours in the pre-exposure period and exposure periods (Repeated Measures ANOVA, $p<0.001$ both after 28 days and in the whole exposure).

The manipulation of the test temperature and photoperiod to simulate the onset of 'autumn' was successful, in that the mean number of eggs per snail per 48 hours in the control group declined as expected. Table 32 shows that this decline was strongly significant both for the whole exposure and also within the first 28 days of the exposure when compared to the pre-exposure period. This was also the case for all of the groups exposed to Bisphenol-A, and therefore there is no apparent effect on *P. corneus* reproduction at these test concentrations (up to 20 μgL^{-1} nominal, 13.6 μgL^{-1} mean measured).

Figure 78a shows the mean cumulative number of eggs laid per snail in the pre-exposure period and the 'exposure period' (divided into two 28 day periods) for the control group. The mean number of eggs per snail declines by 49.8% in the first 28 days of the exposure compared to the pre-exposure period. However, Figures 78b-d show that this same decline in the Bisphenol-A groups is consistently greater, at 66.2, 67.9 and 63.3% in the groups exposed to 0.2, 2 and 20 μgL^{-1} (nominal). Therefore there is a mean additional decrease of 16.0% across all of the Bisphenol-A exposed groups when compared to the control.

Table 32 – The mean number of eggs laid per *P. corneus* in each replicate (in the same order in each case) per 48 hours in the 28 day pre-exposure period (temperature held at 18.7°C (mean) with 16h light)**, the first 28 days of the exposure (temperature** reduced to 14.9°C (mean) and light reduced to 12h)**, and the whole exposure, with the results of paired t-tests.**

		Bisphenol-A concentration		
	Control	0.2 µgL^{-1} nominal	2 µgL^{-1} nominal	20 µgL^{-1} nominal
Mean number of eggs laid in the pre-exposure phase	33.6	36.7	18.9	29.0
	25.4	29.1	25.6	31.9
	30.8	22.8	39.8	35.8
	31.2	26.7	15.8	36.5
	29.3	38.6	25.1	33.2
	29.7	32.9	29.5	43.1
	16.9	26.8	24.3	28.9
	29.0	20.7	35.3	33.9
		24.6	27.3	22.2
		26.6	27.4	30.9
Mean number of eggs laid in the first 28 days of the exposure	12.6	18.3	11.5	6.44
	16.1	11.5	8.13	6.40
	17.0	4.48	15.7	23.0
	21.2	11.7	6.42	23.0
	9.35	7.58	5.84	10.6
	6.22	12.1	5.07	20.5
	9.02	12.4	4.57	12.8
	9.33	5.97	15.9	4.14
		5.96	10.6	6.18
		6.55	2.64	6.20
Paired t-test between pre-exposure and first half of exposure	Significantly lower, p<0.001	Significantly lower, p<0.001	Significantly lower, p<0.001	Significantly lower, p<0.001

Mean number of eggs laid in the whole exposure period	**7.26** **10.7** **11.4** **13.1** **5.75** **4.72** **6.27** **5.04**	**9.63** **5.99** **3.11** **6.03** **3.79** **6.53** **8.04** **3.26** **6.67** **5.41**	**6.03** **4.08** **10.4** **4.22** **4.20** **3.14** **2.99** **10.8** **7.59** **3.09**	**3.80** **3.32** **14.1** **13.2** **5.3** **12.0** **6.54** **3.11** **4.85** **4.92**
Paired t-test between pre-exposure and first half of exposure	**Significantly lower, p<0.001**	**Significantly lower, p<0.001**	**Significantly lower, p<0.001**	**Significantly lower, p<0.001**

This trend towards an increased reduction in the number of eggs laid in the Bisphenol-A exposed groups is less discernable at the end of the exposure, with the overall decline being 86.2% in the control group, and 92.8, 90.1 and 92.9% in the groups exposed to 0.2, 2 and 20 μgL^{-1} (nominal). The mean difference was therefore reduced to 5.7%. However, as Figure 78 shows, <50 eggs per snail (mean, all groups = 37.0) was accumulated in the second half of the exposure, which is comparable to that accumulated in the same period in the first Bisphenol-A exposure (mean, all groups = 35.4). This suggests that a basal level of oviposition activity may have been reached by all of the groups by the end of both of these exposures.

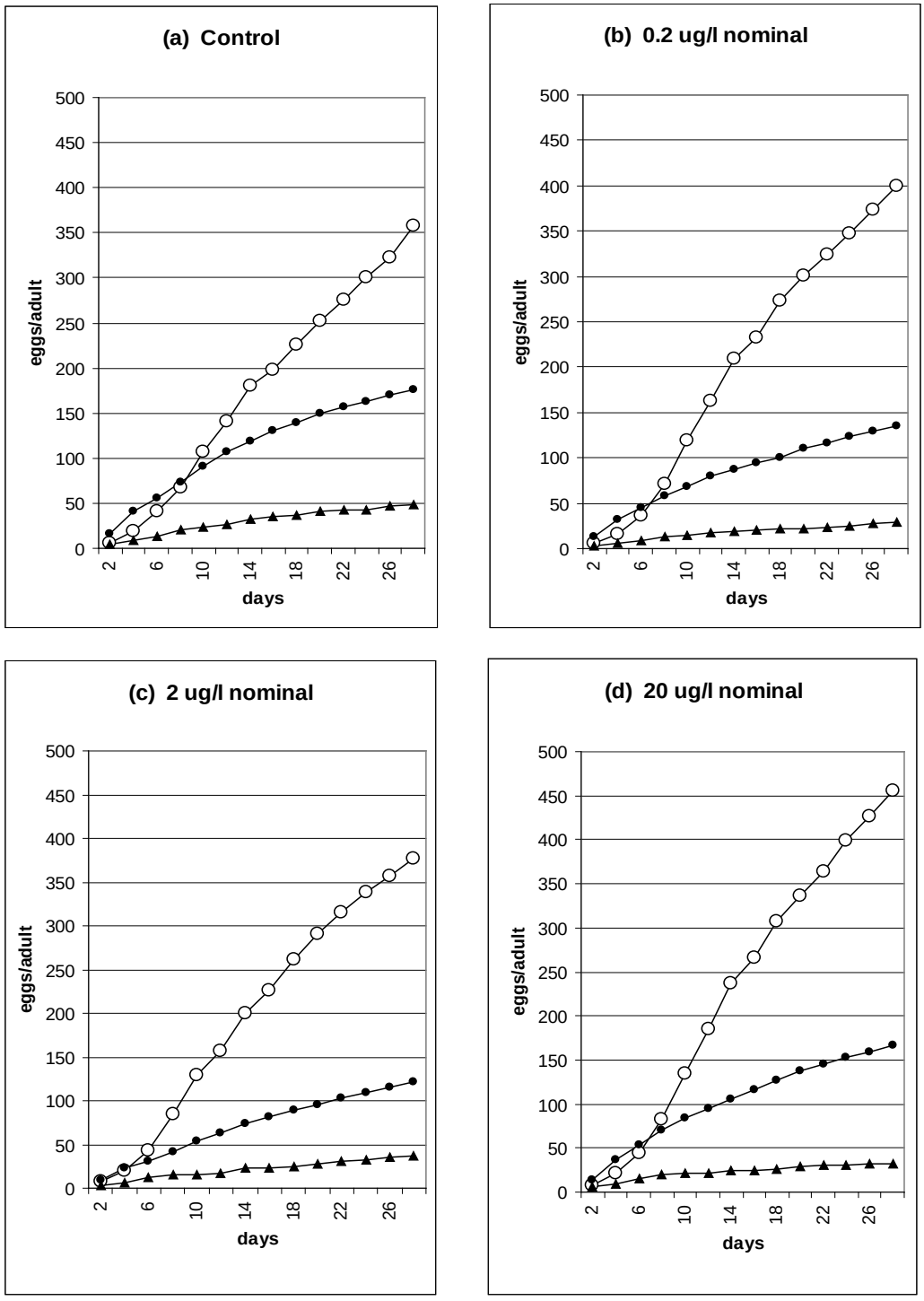


Figure 78: Cumulative mean number of eggs laid per *P. corneus* during the pre-exposure period with the temperature held at 18.7°C (mean) with 16h light **(open circles), in the first exposure period when the** temperature was gradually reduced to 14.9°C (mean) and the photoperiod gradually reduced to 12h light **(black circles), and the second exposure period when the temperature was** held at 14.9°C (mean) with 12h light **(black triangles).**

This subtle inhibitory effect was re-analysed by two independent statisticians. van der Hoeven (2008 and Appendix 2) concurred that there was no significant difference in oviposition activity between the control and any of the Bisphenol-A exposed groups (opting to include the control tank for which an ambiguously labelled sample had been returned from the analytical laboratory, and using the pre-exposure data as a co-variate; ANCOVA, p = 0.12, 0.10 and 0.13 for the groups exposed to 0.2, 2 and 20 μgL^{-1}, nominal, respectively). However she also recorded that the data collected from each Bisphenol-A treated group deviated from the control in a similar way, and when all of the data from these groups were pooled and compared to the control, the 'Bisphenol-A treatment' differed significantly (ANCOVA, p = 0.049). She concluded that a new experiment, designed to test this hypothesis, would be required to confirm this.

Barnes (2010 and Appendix 3) re-analysed the data using a Linear Trend Model (also including the ambiguous control and accounting for the variation in the per-exposure period), and also found a significant dose-response relationship between the control group and the pooled 'Bisphenol-A-treated' group for the number of egg masses laid per snail (p = 0.035). However, he also found a significant difference between all groups for the number of eggs laid per snail (p = 0.026 for the nominal concentrations, but only for the first 28 days of the exposure), lending further support to the concept of a subtle inhibitory effect of the Bisphenol-A treatment on reproduction in *P. corneus*.

6.2.2.6 Egg Mass and Egg Effects

Number of Eggs per Mass

Figure 79 shows the mean number of eggs per mass in the pre-exposure period and for the control and treatment groups in the exposure period. The mean number eggs laid per mass in the pre-exposure period was 23.6, which is a lower number than for any other pre-exposure period (mean = 37.8 in the 17β-oestradiol exposure at a similar temperature). The mean number of eggs per mass was slightly lower yet in the control group (mean = 20.2, compared to 39.2 in the 17β-

oestradiol exposure). The reason for the reduced number of eggs per mass is not clear. However, although the number of eggs per mass is even further reduced in the groups exposed to 2 and 20 μgL^{-1} Bisphenol-A (nominal), there are no significant differences between the control and any treatment group (ANOVA, $p>0.05$).

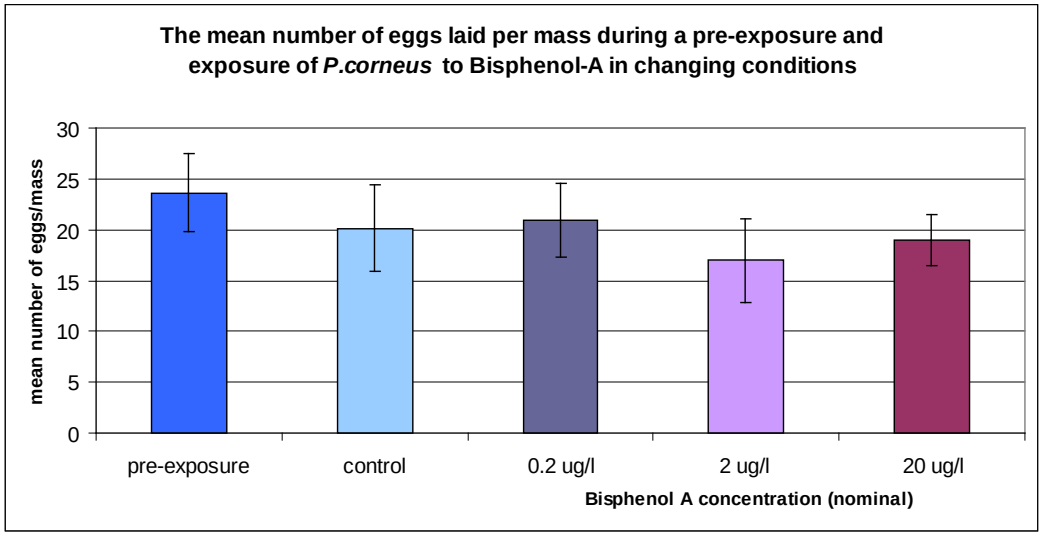


Figure 79 - The mean number of eggs per mass in egg masses laid by *P. corneus* at during a pre-exposure period (temperature held at 18.7°C (mean) with 16h light), **and exposure to Bisphenol-A** (temperature reduced from 18.7°C (mean) to 14.9°C (mean) and photoperiod reduced from 16h to 12h light over 4 weeks, and then constant conditions; 14.9°C (mean) and 12h light **for a further 4 weeks; error bars represent the standard error of the means).**

Egg Mass Dry Weight

The mean dry weight of the egg masses laid by the control and treatment groups during the Bisphenol-A exposure is shown in Figure 80 (data not collected during the pre-exposure period). The mean dry weight of the mass laid by the control group was 7.7 mg, which is comparable to that measured in the control group during the 17β-oestradiol exposure at 15°C (also 7.7 mg), although heavier than the masses laid in the first Bisphenol-A exposure (6.0 mg). However the mean weight of the masses laid by the group exposed to 0.2 μgL^{-1} (nominal) was 10.2 mg, which was heavier than that recorded for any group in any of the earlier

exposures. This treatment group and the control group laid a similar number of eggs per mass (see Figure 80), which suggests that the egg masses were heavier due to additional packaging material. However, the mean dry weight of the egg masses laid by the groups exposed to 2 and 20 μgL^{-1} Bisphenol-A (nominal) was closer to the mean control dry weight, and there were no significant differences between of the groups (Kruskall-Wallis, $p<0.05$).

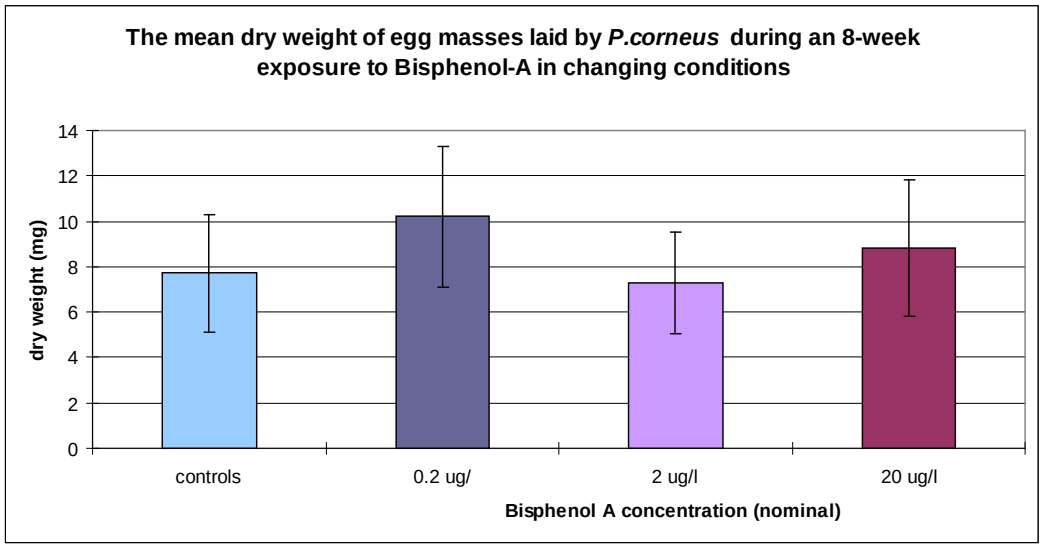


Figure 80 - The dry weight of egg masses laid by *P. corneus* at during an exposure to Bisphenol-A (temperature reduced from 18.7°C (mean) to 14.9°C (mean) and photoperiod reduced from 16h to 12h light over 4 weeks, and then constant conditions; 14.9°C (mean) and 12h light **for a further 4 weeks; error bars represent the standard error of the means).**

Egg Abnormality Rate

Figure 81 shows the mean percentage of abnormal eggs laid during the pre-exposure and exposure periods. The mean abnormality rate in the pre-exposure period was 1.06%, which was similar to that observed in the previous exposures (1.29% in the 17β-oestradiol exposure at 20°C, and 0.77% in the first Bisphenol-A exposure). However, unlike the first Bisphenol-A exposure, when the observed trend was towards a decreasing abnormality rate with increasing Bisphenol-A concentration (see Figure 71), on this occasion the trend was in general towards an increasing abnormality rate with increasing Bisphenol-A

concentration. The mean percentage abnormality in eggs laid by the groups exposed to 2 and 20 μgL^{-1} (nominal) was more than double that observed for any group in any of the previous exposures (3.76 and 3.25% respectively). However the variability between replicate groups also tends to increase in the Bisphenol-A exposed groups, and there was therefore no significant difference between any treatment group and the control during the exposure (Kruskall-Wallis, $p>0.05$).

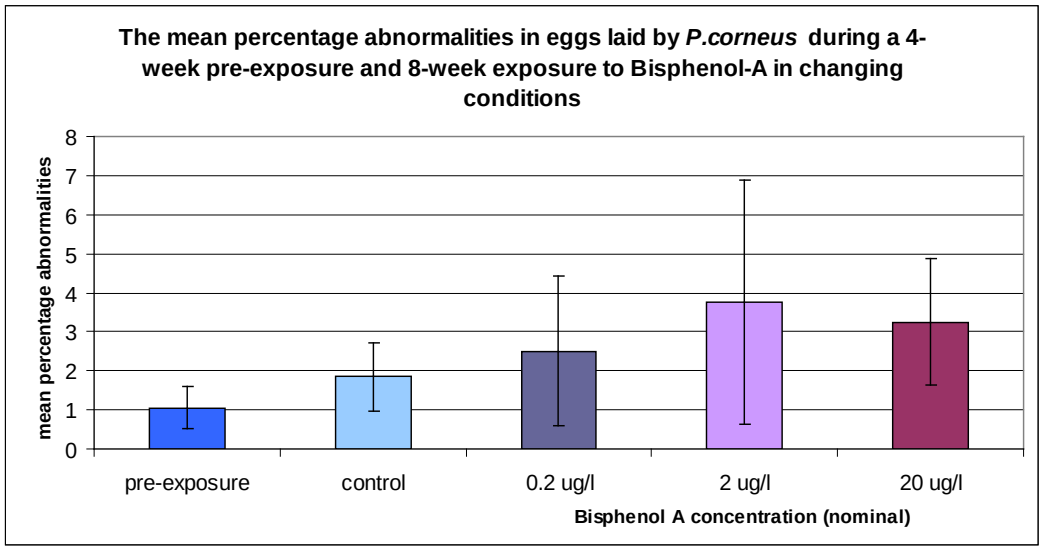


Figure 81 - The mean percentage abnormality in eggs laid by *P. corneus* at during a pre-exposure period (temperature held at 18.7°C (mean) with 16h light), **and exposure to Bisphenol-A** (temperature reduced from 18.7°C (mean) to 14.9°C (mean) and photoperiod reduced from 16h to 12h light over 4 weeks, and then constant conditions; 14.9°C (mean) and 12h light **for a further 4 weeks; error bars represent the standard error of the means).**

6.3 Comparative responses of *P. corneus* to Bisphenol-A

6.3.1 Physico-chemical conditions.

The same test tanks were used in the two *P. corneus* exposures to Bisphenol-A as were used in the 17β-oestradiol exposures, and the measured water quality parameters were very similar on both occasions to those recorded previously. The mean hardness was overall slightly higher, but this was not considered detrimental. The Bisphenol-A analysis from the first exposure indicated that the degradation rate was greater than predicted by the results of the stability study, and the spike was increased at the mid-point of the exposure in an effort to compensate for this. The degradation rate was less marked in the second exposure, being approximately 30% after 24 hours in the highest concentration at the Week 3 sampling event. This did increase over the duration of the exposure however, being approximately 50% after 24 hours at Week 7, and it was also variable, reaching 75% in one tank. The lower concentrations were remarkably stable.

It was also apparent that a tank in the lowest treatment group was probably over-dosed at the start of the first exposure, and in the second exposure, a control tank was almost certainly spiked with Bisphenol-A in error. The latter group was excluded along with another control tank brought into doubt by an error made at the analytical laboratory. Of greater concern was the repeated finding of notable levels of Bisphenol-A in the other samples taken from the control vessels in the first exposure (mean = 0.061 μgL^{-1}, maximum = 0.132 μgL^{-1}). Being a regular occurrence, this is unlikely to be cross-contamination; a more probable source is contamination from the plastics used in the exposure system, which consequently were removed as far as was practically possible. This action did reduce the concentrations measured in the control media in the second exposure, but did not eliminate the occurrence of Bisphenol-A entirely (mean = 0.047, maximum = 0.098). It is likely that some of the detections occur due to the analytical uncertainty of the method (30.5% in river water), but it may be that media contact

with plastics must be completely eradicated to fully combat this problem. The overall implication is that neither of the *P. corneus* exposures to Bisphenol-A had an unaffected control. Oehlmann et al. (2006) found the LOEC for increased egg productivity in *M. cornuarietis* to be 0.048 μgL^{-1} (mean measured). Therefore it cannot be concluded that the organisms used to control these exposures were not subject to a similar induction, or, given the overall trend at the higher concentrations, an inhibition of reproduction.

6.3.2 Mortality

At the concentrations selected for testing (0.2, 2 and 20 μgL^{-1}), there was no appreciable toxicity associated with Bisphenol-A observed in any of the test organisms. In the first *P. corneus* exposure, the overall test organism mortality rate was only 3.5%, and there was complete survivorship at the highest test concentration. In the second exposure, the test organism mortality was higher at 23.6% overall, but this exposure began at a warmer temperature (19^oC) than the first (16^oC) while being stocked at the same density (1 snail/litre), and an increase in mortality at this temperature had been expected from culture experience with this species. The control group mortality was 20.5%, and there were no significant differences from this in the Bisphenol-A exposed groups.

6.3.3 Growth

The *P. corneus* also grew well during the exposures, with the control groups increasing in mean diameter by approximately 4% and in mean weight by approximately 8% in both experiments. In the first exposure, the Bisphenol-A exposed snails did not grow quite as much, but there were no significant differences from control. In the second exposure, the estimates of growth were not as reliable due to the higher mortality rate, but again there were no notable differences, and there were no effects on the condition factor of the organisms at the end of the exposures in either case. More interestingly, there was a significant difference in the RSI between treatments in the first exposure,

however, the response was in the form of an inverted 'U-shape' with increasing relative reproductive organs weights recorded with increasing Bisphenol-A concentration until the highest treatment group (20 μgL^{-1} nominal), in which the mean reproductive organ weight was less than the control group. On the second occasion, the same pattern was observed, although in this case there were no significant differences. Nonetheless, the repeatability of the effect suggests that Bisphenol-A affects the amount of resource allocated to the reproductive organs in both a positive and negative manner according to the exposure concentration.

6.3.4 Reproduction

In isolation, it might be considered that an increase in reproductive organ weight would be indicative of increased reproductive activity, and therefore more eggs per adult might be expected in the 0.2 and 2 μgL^{-1} (nominal) Bisphenol-A exposed groups than the controls. In fact the reverse was true in the first exposure, with these groups showing a significant reduction in oviposition during the exposure in comparison with the pre-exposure period, whereas the control group did not. However this is likely to be a statistical artefact, since the mean number of eggs laid in the pre-exposure phase (14 days) was 91.7 per adult in the control group, and then 21.1 per adult in the last 14 days of the exposure, a reduction of 77.0%. The equivalent percentage reduction in the 0.2 μgL^{-1} (nominal) exposure group was 79.9%, suggesting there is no real difference between these two groups. The lack of a significant difference in the control group was because one of the replicates laid fewer eggs than the others in the pre-exposure phase (29.3 eggs per adult compared to more than 100 in the other groups), and it is likely that the majority of this group had already undergone the autumnal seasonal decline in reproductive activity.

The percentage reduction in the group exposed to 2 μgL^{-1} (nominal) was 88.4%, but in contrast, in the highest exposure group (20 μgL^{-1} nominal), it was only 61.2%, and this was not a significant decline from the reproductive rate observed in the pre-exposure phase. This may be an indication (albeit unreliable) that

exposure to high concentrations of Bisphenol-A (approximately x10 that expected in a treated sewage effluent) slightly perpetuates reproduction in *P. corneus*. Yet rather than maintaining a steady rate of reproduction as was observed in the highest concentration of the 17β-oestradiol exposure (100 μgL^{-1} nominal), egg-laying in the snails in the highest Bisphenol-A exposure group declined sharply, and then made a degree of 'recovery' towards the end of the exposure (see Figure 68d). This could also be interpreted as a suggestion of reproductive recklessness due to increasingly toxic effects. In combination with the decrease in the observed RSI, it might be concluded that this group experienced symptoms of reproductive toxicity. However the nature of these effects cannot be clearly elucidated because the control group did not experience a significant decline in reproductive activity either.

The reason for the low numbers of eggs laid per snail is likely to be the unintentionally long period of time (8 weeks) that the organisms were held at temperatures similar to the test conditions before the pre-exposure phase began. This followed a photoperiod malfunction during which the snails were inadvertently held in constant light for two weeks. It was initially considered that maintaining the organisms for an extended period at 16-17.5^{o}C with a 12-hour photoperiod would re-establish 'normal autumnal behaviour'. However, rather than establishing a constant rate of oviposition as might usually be expected when acclimating an organism to test conditions, this period allowed these seasonally reproducing snails to gradually reduce their oviposition activity from a 'summer' response to constant light, to the minimal level of reproductive activity that might be expected following autumn. It was recognised that the organisms used in the 17β-oestradiol exposures had been supplied in late spring, and acclimated to test conditions for one to three weeks. Consequently the mean number of eggs laid per adult per week during the pre-exposure period at 15^{o}C in a 12-hour photoperiod was 90.4, and these animals experienced an 'early autumn'. In contrast, the snails used in the Bisphenol-A exposure experienced a short, unintentional 'summer', followed by a long 'autumn', and the mean number of

eggs laid per adult per week during the pre-exposure period at the end of this process was only 39.4.

Three conclusions were drawn from these observations. Firstly, it is possible to manipulate the reproductive behaviour of this species, encouraging altered rates of oviposition by changing the temperature and photo-period, regardless of the natural season. Secondly and importantly, it is not appropriate to acclimate seasonally reproducing test organisms to the test conditions for any notable period, and indeed constant test conditions may not be an appropriate approach if seasonal changes are a desired observation. Finally, it may be necessary to take steps to ensure that the egg production rates per group of snails are similar at the outset, in order that tests and treatments are properly inter-comparable. This could include the introduction of pre-determined minimum oviposition rates (i.e. a quality criterion) to exclude those groups that are not behaving as expected given the test conditions (possibly due to inappropriate pre-exposure conditions).

Taking account of these conclusions, a second exposure of *P. corneus* was undertaken, but this experiment was split into three 4-week 'phases'; a pre-exposure phase in constant 'summer' conditions (19^{o}C with a 16 hour photo-period), followed by an exposure phase in which the temperature and photoperiod were gradually reduced, and ending in a continued exposure in constant 'autumn' conditions (15^{o}C with a 12 hour photoperiod). It was not necessary to introduce a quality criterion or exclude any tanks as the warm conditions ensured that all the groups laid at least 0.34 masses per snail per day in the pre-exposure phase. However the final experimental design was selected from a series of random allocations to reduce the between-treatment variation. Overall, this approach was successful in that the oviposition rate of each treatment group was similar in the pre-exposure phase, and the reproduction in control groups declined steadily and significantly over the course of the exposure in response to the simulation of autumn. The reproduction of all of the Bisphenol-A treated groups also declined significantly and at a slightly faster rate than the control group. These laboratory exposures of *P. corneus* were made to assess whether there was any similar

induction or perpetuation of reproduction in response to Bisphenol-A as was observed for 17β-oestradiol, as Bisphenol-A is a weak xenobiotic oestrogen in vertebrates, and a compound reported to cause superfeminisation and increased reproduction in *M. cornuarietis* (Oehlmann et al., 2000a; 2006). However, from the results of this experiment, it is concluded that Bisphenol-A does not induce a higher rate of reproduction in *P. corneus*, nor does it perpetuate the summer reproductive rate.

However, it is of concern that exposure to Bisphenol-A in all of the treatment groups caused a similar deviation from the rate of decline observed in the control group (mean additional decline of 16% after 28 days, 5.7% after 56 days). While the reproductive rate in any one treatment is not significantly different to the control, if all the treated groups are combined due to the similarity of their response, the 'Bisphenol-A treated' groups have declined significantly more than the controls. This does not suggest a conventional response to toxicity (i.e. a dose-response), but it does indicate that Bisphenol-A is interfering with the reproductive rate by a consistent means. This could be by an indirect route, e.g. the presence of Bisphenol-A makes the flaked food less available, perhaps by blocking chemo-reception, or it changes the surface qualities of the glass tanks, making effective locomotion more difficult, both of which would cause a deviation of effort away from reproduction. Alternatively, it could be a direct route, i.e. 0.2 μgL^{-1} of Bisphenol-A or less can mimic or enhance the signal that inhibits reproduction in autumnal conditions. Either are possible, but it should also be considered that the use of many 'replicate' groups can find statistical significance in a trivial effect (Ioannidis, 2005). Additional carefully designed experiments using lower concentrations are required to both confirm the effect and elucidate the mechanism.

As in the exposures of *P. corneus* to 17β-oestradiol, there were no effects on the number of eggs per mass or the dry weight of the egg masses in either of the Bisphenol-A exposures. The rate of egg abnormalities observed in the first exposure was of interest in that there appeared to be a dose-dependant reduction

in abnormalities occurring with increasing Bisphenol-A concentration, although the control group variation was high, so there were no significant differences. However, on the second occasion, the reverse trend was true, demonstrating that Bisphenol-A has no real effect on ovum assembly. A second attempt to assess effects on egg hatching success was even less successful than the previous occasion, with < 10% of the sub-samples of egg masses from the control group hatching within two weeks at 20^{o}C (despite over 60% hatching in one Bisphenol-A treated sub-sample). It was concluded that the techniques used to move the egg masses must cause damage, and the assessment of this end-point was abandoned.

Overall, the original hypotheses for these experiments; that 'exposure to Bisphenol-A under controlled temperature and photoperiod conditions representing a simulated autumn will significantly alter the number of eggs produced by *P. corneus* relative to the control' are not supported. However, the evidence is sufficient to suggest that Bisphenol-A at a concentration of 0.2 μgL^{-1} (nominal) or greater either affects the physical conditions in the test vessels, and/or causes a slight inhibition of reproduction in *P. corneus*. However, all the exposures would require repeating before any firm conclusions could be drawn.

6.3.5 Parasitisation

No parasites were observed on dissection of the test organisms at the end of either exposure to Bisphenol-A. As the majority of the animals were wild-caught, the possibility remains that parasitic infections were present but not recorded. The animals used in the first exposure were also maintained in the laboratory for a longer period, which may have allowed any parasitized individuals to die naturally before the surviving animals were sacrificed and dissected. Those used in the second exposure were collected later in the year, which may also have reduced the proportion of parasitized organisms present in the population.

CHAPTER 7
REPRODUCTIVE EFFECTS OF EXPOSURE TO BISPHENOL-A IN THE PROSOBRANCH GASTROPODS *P. antipodarum* AND *B. tentaculata.*

7.1 Introduction and Methodology

This chapter presents the results of the laboratory exposure of two prosobranch gastropods, *P. antipodarum* and *B. tentaculata* to Bisphenol-A (Experiments 7 and 8 respectively). The methodology used in the *P. antipodarum* exposure (Experiment 7) was as similar as possible to that prescribed in the draft OECD SOP (Schmitt et al., 2006). This specifies an 8-week exposure at 16^{o}C with an 16-hour light / 12-hour dark photoperiod. However the test was extended for a further 4 weeks and incorporated increasing temperatures (to 20^{o}C) to simulate the onset of summer. Full details of the methods and experimental design are set out in Chapter 3, Section 3.2.5.

The *B. tentaculata* exposure (Experiment 8) was performed concurrently with Experiment 7 and in the same test conditions, excepting that it began 4 weeks later. Therefore the test organisms were exposed at 16^{o}C with an 16-hour light / 12-hour dark photoperiod for 4 weeks, followed by the additional 4 weeks at increasing temperatures to simulate the onset of summer. The test organisms were also supplied with increasing amounts of algae, as might naturally be available in a summer algal bloom. Full details of the methods and experimental design are set out in Chapter 3, Section 3.2.6.

The hypothesis for these experiments is: 'Exposure to Bisphenol-A under controlled temperature and photoperiod conditions that represent a simulated summer will significantly alter the number of embryos in the brood pouch of *P. antipodarum* and/or the number of eggs produced by *B. tentaculata* relative to the control'.

7.2 Results and Discussion

7.2.1 Experiment 7 - An exposure of *P. antipodarum* to Bisphenol-A in changing conditions to simulate the onset of 'summer'.

7.2.1.1 Bisphenol-A Analysis

Table 33 gives the results of the Bisphenol-A analysis for each week of the exposure. There were some further set-backs encountered in the shipping and analysis of the samples. In the first four weeks of the experiment, four samples were broken in one shipment. However, the measured concentrations in the remaining samples did not overlap, and the mean values were 0.036 μgL^{-1} in the control (values below the LOD of 0.04 μgL^{-1} are treated as 0.02 μgL^{-1}) and 0.196, 1.37 and 13.0 μgL^{-1} in the 0.2, 2 and 20 μgL^{-1} (nominal) media respectively, for this period. The highest mean degradation rate was therefore 35% from nominal, which was higher than expected following the results of the earlier stability study (see Chapter 5, Section 5.2.3.1), but this was not considered excessive in discarded media.

Unfortunately, from this point in the experiment the degradation rate increased sharply, being 63%, 91% and 89% in the 0.2, 2 and 20 μgL^{-1} (nominal) media respectively at Week 5, increasing to 98% and 97% in the 2 and 20 μgL^{-1} (nominal) media respectively at Week 6. The Bisphenol-A was unexpectedly almost completely degrading over 48 hours. Indeed it was not detected at all in Weeks 7 and 8 (although the analysis laboratory made x5 dilutions of all samples in error prior to analysis). It is possible that the bacteria in the biofilms occurring in the test vessels had adapted to use Bisphenol-A as a substrate more effectively.

Due to the delay in sample turn-around time, this increased rate of degradation was not observed until towards the end of the exposure. After Week 10 the test vessels were partially sterilised using water >60^{o}C at each media change. It can be seen that at Week 11, the degradation rate in the 2 and 20 μgL^{-1} (nominal)

media had improved, being 65% and 83% respectively, and was again further improved to 43% and 37% respectively at Week 12. This suggests that the preparation of the Bisphenol-A spiked media had continued successfully over the course of the exposure.

After Week 8, the levels of Bisphenol-A in the control media had become elevated (to almost 0.2 μgL^{-1}). This is also a possible cause for the levels in the 0.2 μgL^{-1} (nominal) media rising above the nominal concentration on one occasion.

Table 33 – The measured Bisphenol-A concentrations in samples taken from the discarded media pooled from each replicate for each treatment (LOD = 0.04 μgL^{-1}, unless an x5 dilution was made, raising it to 0.2 μgL^{-1}, or a x50 dilution, raising it to 2.0 μgL^{-1}).

	Bisphenol-A concentration (ug^{-1})			
	Control	**0.2 μgL^{-1} nominal**	**2 μgL^{-1} nominal**	**20 μgL^{-1} nominal**
Week 1	**<0.04**	broken	broken	**6.86**
Week 2	broken	**0.183**	**1.32**	broken
Week 3	**0.068**	**0.236**	**1.22**	**15.0**
Week 4	**<0.04**	**0.170**	**1.58**	**17.1**
Week 5	**0.076**	**0.074**	**0.186**	**2.18**
Week 6	broken	**0.084**	**0.044**	**0.682**
Week 7	<0.2	<0.2	<0.2	<0.2
Week 8	<0.2	<0.2	<0.2	<0.2
Week 9	**0.192**	**0.081**	**0.166**	<2.0
Week 10	**0.145**	**0.149**	**0.437**	<2.0
Week 11	**0.168**	**0.348**	**0.709**	**3.42**
Week 12	**0.089**	**0.129**	**1.14**	**12.7**

7.2.1.2 Media Physico-Chemical Characteristics

Temperature

Table 34 shows the mean, minimum and maximum temperatures measured manually in the exposure vessels over each fortnight of the experiment using a mercury thermometer (error margin = 0.5^{o}C). It can be seen that during the part of the exposure when the conditions were constant (the first 8 weeks at a nominal 16^{o}C), the temperature did not vary by more that 1^{o}C. However, when the temperature was increased over the last four weeks, the vessels experienced higher temperatures than were intended. Unfortunately this was compounded at the end of the exposure when the constant temperature room malfunctioned on Day 82 and the temperature rose to 24^{o}C for up to 24 hours.

Table 34 – The mean, minimum and maximum temperatures recorded for each fortnight of the experiment.

	Temperature (^{o}C)		
Exposure Period	**Mean**	**Minimum**	**Maximum**
1. *26th Feb. to 11th March* (nominal 16^{o}C)	16.1	16.0	16.5
2. *12th to 25th March* (nominal 16^{o}C)	16.3	16.0	17.0
3. *26th March to 8th April* (nominal 16^{o}C)	16.1	15.5	16.5
4. 9th *to 22nd April* (nominal 16^{o}C)	16.1	16.0	16.6
5. *23rd April to 6th May* (nominal 16-18^{o}C)	18.3	17.5	19.0
6. *7th to 20th May* (nominal 18-20^{o}C)	20.7	19.5	24.0

pH

Table 35 gives the mean and standard deviation of the pH measurements from sub-samples taken from the exposure vessels before and after media changes over each fortnight of the experiment. It can be seen that the pH of the fresh media generally rises over the course of the exposure, possibly due to increasing algal activity in the supply water. However, the standard deviations are low, and it was not considered that this degree of pH change was adverse to the test organisms.

Table 35 – The mean pH and standard deviation (SD) recorded for each fortnight of the exposure.

	pH Pre-Change		pH Post-Change	
Exposure Period	**Mean**	**SD**	**Mean**	**SD**
1. *26th Feb. to 11th March* (nominal 16°C)	7.41	0.09	7.34	0.13
2. *12th to 25th March* (nominal 16°C)	7.78	0.05	7.92	0.08
3. *26th March to 8th April* (nominal 16°C)	7.89	0.23	7.85	0.18
4. 9*th to 22nd April* (nominal 16°C)	7.78	0.10	7.84	0.28
5. *23rd April to 6th May* (nominal 16-18°C)	7.69	0.10	8.04	0.29
6. *7th to 20th May* (nominal 18-20°C)	7.88	0.04	8.36	0.46

Dissolved Oxygen

Table 36 shows the mean dissolved oxygen levels measured as a percentage of the air saturation value (%ASV) in sub-samples taken from the exposure vessels before and after media changes over each fortnight of the experiment, with the standard deviations. It can be seen that the dissolved oxygen levels remain high over the course of the exposure. This is an important requirement of this species as individuals are known to cross the meniscus when the oxygen levels are insufficient (Schmitt et al., 2006).

Conductivity

The conductivity of the sub-samples taken from the exposure vessels before and after media changes over each fortnight of the experiment is given in Table 37 with the standard deviation. The conductivity remained low over the course of the exposure, indicating that the snails and ground food had little impact on the overall water quality as expected due to their small size and minimal food requirement.

Table 36 – The mean dissolved oxygen levels (DO) and standard deviation (SD) recorded for each fortnight of the exposure.

	DO Pre-Change (% ASV)		DO Post-Change (% ASV)	
Exposure Period	**Mean**	**SD**	**Mean**	**SD**
1. *26th Feb. to 11th March* (nominal 16°C)	95	4.96	100	1.23
2. *12th to 25th March* (nominal 16°C)	96	2.14	98	1.99
3. *26th March to 8th April* (nominal 16°C)	97	1.23	98	1.38
4. *9th to 22nd April* (nominal 16°C)	97	2.38	100	1.52
5. *23rd April to 6th May* (nominal 16-18°C)	97	3.15	96	4.83
6. *7th to 20th May* (nominal 18-20°C)	94	3.53	95	1.92

Table 37 – The mean conductivity (CND) and standard deviation (SD) recorded for each fortnight of the exposure.

	CND Pre-Change (µS/cm)		CND Post-Change (µS/cm)	
Exposure Period	**Mean**	**SD**	**Mean**	**SD**
1. *26th Feb. to 11th March* (nominal 16°C)	900	19	951	46
2. *12th to 25th March* (nominal 16°C)	948	20	925	50
3. *26th March to 8th April* (nominal 16°C)	879	52	901	129
4. *9th to 22nd April* (nominal 16°C)	885	30	865	17
5. *23rd April to 6th May* (nominal 16-18°C)	882	54	873	53
6. *7th to 20th May* (nominal 18-20°C)	909	33	854	71

Hardness

Table 38 gives the mean hardness of the test media with the standard deviation. It was consistently higher than the expected value of 394 mgL^{-1} $CaCO_3$ (calculated for the salts added to the reverse osmosis filtered water. This was not

considered likely to have affected the exposure, as gastropods generally prefer a hard water environment to facilitate the extraction of calcium for shell-building (Boycott, 1936).

Table 38 – The mean hardness and standard deviation (SD) recorded for each fortnight of the exposure.

	Hardness (mgL^{-1} $CaCO_3$ equivalent)	
Exposure Period	**mean**	**SD**
1. *26th Feb. to 11th March* (nominal 16°C)	441	11.5
2. *12th to 25th March* (nominal 16°C)	452	10.4
3. *26th March to 8th April* (nominal 16°C)	428	31.5
4. *9th to 22nd April* (nominal 16°C)	423	5.8
5. *23rd April to 6th May* (nominal 16-18°C)	427	29.7
6. *7th to 20th May* (nominal 18-20°C)	405	5.12

7.2.1.3 Test Organism Survivorship

The mean mortality rate of the control group was low at 4.17% over the whole exposure (sd = 1.91). It was also similarly low in all of the groups exposed to Bisphenol-A, at 2.92% (sd = 1.44), 5.0% (sd = 3.31) and 3.33% (sd = 2.60) in the groups exposed to 0.2, 2 and 20 μgL^{-1} (nominal) respectively. There were no significant differences between the control and any exposed group (ANOVA, $p>0.05$).

7.2.1.4 Test Organism Morphology and Growth

As groups of 20 test organisms were randomly sampled at the end of Week 2, 4 and 8 and the remaining organisms at Week 12, the relevant parameter is the relative size of the snails sampled in each group at each sampling occasion. At the Week 2 sampling point, there was a significant difference between the sizes

of the groups sampled (ANOVA, p = 0.026), but none of the Bisphenol-A treated groups were significantly different to the control. The group exposed to 20 μgL^{-1} (nominal) was significantly larger than the group exposed to 0.2 μgL^{-1} (nominal). There were no significant differences between the size of the sampled groups at Week 4 (Kruskall-Wallis p>0.05) or Week 8 (ANOVA, p>0.05). However at Week 12, the groups exposed to 2 and 20 μgL^{-1} are significantly larger than the control group (Kruskall-Wallis, p = 0.028).

Because the test organisms were sampled progressively and not identified individually, it not possible to make a reliable estimate of growth. A larger group may indicate growth had occurred, or it may indicate that the large animals had been randomly selected, at any given sampling point.

7.2.1.5 Test Organism Reproduction

Figure 82 shows the mean number of eggs in the brood pouch of the sacrificed *P. antipodarum* at each of the sampling points (Week 2, 4 and 8 at 16^{o}C, and Week 12 after increasing the temperature to 20^{o}C). The culture conditions of this species maintained breeding activity at a minimum level. The food supplied during the exposure was enriched by comparison. Therefore at Week 2 the brood pouch of the sacrificed animals held very few embryos as insufficient time had elapsed on the enriched diet to allow for many eggs to develop.

However by Week 4 the mean number of embryos in the brood pouches of the control group had more than doubled. The mean number of embryos in the brood pouches of all of the groups exposed to Bisphenol-A was even greater, more than tripling in the group exposed to 0.2 μgL^{-1} (nominal). It is of interest to note that the 'inverted U-shaped' response of the Bisphenol-A treated groups is similar to that previously observed when this species was exposed to oestrogenic compounds (Nonylphenol and Octylphenol in sediments, Duft et al., 2003; 17α-ethinylestradiol, Bisphenol-A and Octylphenol in aqueous phase, Jobling et al., 2004).

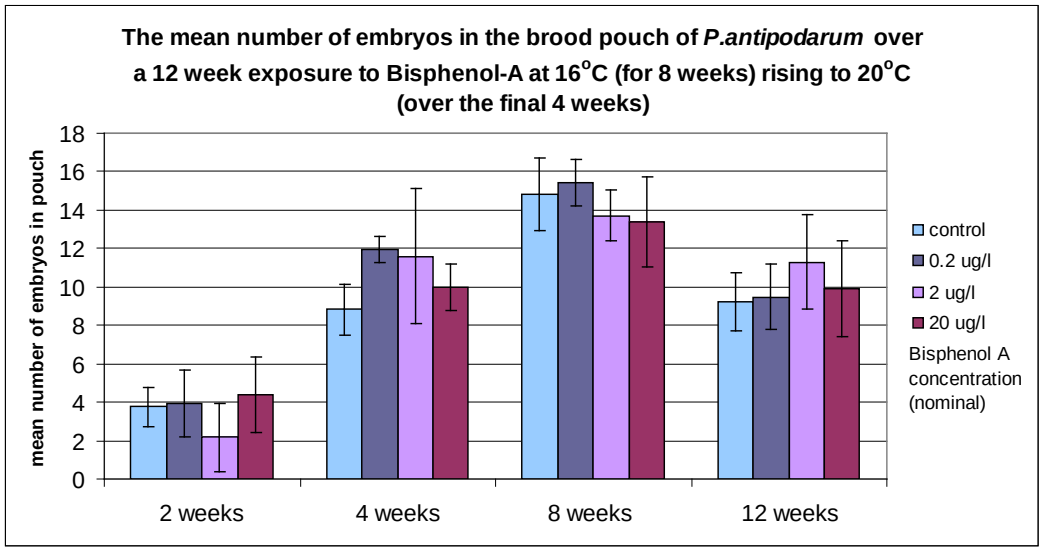


Figure 82 - The mean number of embryos in the brood pouch of *P. antipodarum* after 2, 4 and 8 weeks exposure to Bisphenol-A at 16.2°C (mean) and then with the temperature gradually increased to 20.7°C by Week 12 (error bars represent the standard deviation).

At Week 8 the mean number of embryos in the brood pouches of the control group had again increased (by a further 68%). Similar increases were also observed in the Bisphenol-A treated groups, but there was no clear pattern of response. At Week 12 the mean number of embryos in the brood pouches of the control group has declined (by 62%). This may reflect an intolerance to the increased exposure temperature. Overall however, there are no significant differences between the control and any Bisphenol-A exposed group at any sampling point (ANOVA, $p>0.05$).

7.2.2 Experiment 8 – An exposure of *B. tentaculata* to Bisphenol-A in changing conditions simulate the onset of 'summer'

7.2.2.1 Bisphenol-A Analysis

The samples taken for Bisphenol-A analysis for this experiment were pooled with that from Experiment 7. The exposure of *B. tentaculata* began at Week 5 of the *P. antipodarum* exposure, and so the Week 5 analysis shown on Table 33 equates to the Week 1 analysis for this exposure. Unfortunately, this implies that almost all of the Bisphenol-A was degrading within 48 hours (between media changes) from the beginning of this exposure, only improving with the increased cleaning and sterilising of the test vessels towards the end. This improvement suggests that the Bisphenol-A spiked media was properly prepared throughout the experiment as the procedure for this was not changed. However, the mean exposure concentrations again cannot be determined, and Bisphenol-A (up to 0.2 μgL^{-1}) was also measured in the control media.

7.2.2.2 Media Physico-Chemical Characteristics

Temperature

Table 39 shows the mean, minimum and maximum temperatures measured in the exposure vessels. It can be seen that during the constant part of the exposure (the first four weeks at a nominal 16^{o}C), the temperature did not vary by more that 1^{o}C. However, when the temperature was increased over the second four week period, the vessels experienced higher temperatures than were intended, and a temperature control malfunction on Day 56 allowed the temperature to rise to 23.5^{o}C for up to 24 hours.

Table 39 – The mean, minimum and maximum temperatures recorded for each fortnight of the experiment.

	Temperature (^{o}C)		
Exposure Period	**Mean**	**Minimum**	**Maximum**
1. *26th March to 8th April* (nominal 16^{o}C)	16.5	16.0	17.0
2. 9*th to 22nd April* (nominal 16^{o}C)	16.3	16.0	17.0
3. *23rd April to 6th May* (nominal 16-18^{o}C)	18.4	17.0	19.0
4. *7th to 20th May* (nominal 18-20^{o}C)	20.8	19.5	23.5

pH

Table 40 gives the mean and standard deviation of pH measurements from sub-samples taken from the exposure vessels before and after media changes. It can be seen that the mean pH falls between 7.65 and 8.36 over the course of the exposure. The standard deviations are low, and it was considered that this degree of pH change was not adverse to the test organisms.

Table 40 – The mean pH and standard deviation (SD) recorded for each fortnight of the exposure.

	pH Pre-Change		pH Post-Change	
Exposure Period	**Mean**	**SD**	**Mean**	**SD**
1. *26th March to 8th April* (nominal 16^{o}C)	7.69	0.16	7.81	0.32
2. 9*th to 22nd April* (nominal 16^{o}C)	7.70	0.13	7.90	0.42
3. *23rd April to 6th May* (nominal 16-18^{o}C)	7.65	0.15	8.02	0.27
4. *7th to 20th May* (nominal 18-20^{o}C)	7.69	0.10	8.32	0.34

Dissolved Oxygen

Table 41 shows the mean dissolved oxygen levels measured as a percentage of the air saturation value (%ASV) in sub-samples taken from the exposure vessels before and after media changes over each fortnight of the experiment, with the

standard deviations. It can be seen that the dissolved oxygen levels remain high over the course of the exposure.

Table 41 – The mean dissolved oxygen levels (DO) and standard deviation (SD) recorded for each fortnight of the exposure.

	DO Pre-Change (% ASV)		DO Post-Change (% ASV)	
Exposure Period	**Mean**	**SD**	**Mean**	**SD**
1. *26th March to 8th April* (nominal 16°C)	93	4.61	99	1.48
2. *9th to 22nd April* (nominal 16°C)	96	3.85	100	2.14
3. *23rd April to 6th May* (nominal 16-18°C)	96	4.96	98	3.95
4. *7th to 20th May* (nominal 18-20°C)	89	6.60	95	1.72

Conductivity

The conductivity of the sub-samples taken from the exposure vessels before and after media changes over each fortnight of the experiment is given in Table 42 with the standard deviation. The conductivity remained low over the course of the exposure, indicating that the water quality was not compromised by the high stocking density of *B. tentaculata.*

Table 42 – The mean conductivity (uS/cm) and standard deviation (SD) recorded for each fortnight of the exposure.

	CND Pre-Change (µS/cm)		CND Post-Change (µS/cm)	
Exposure Period	**Mean**	**SD**	**Mean**	**SD**
1. *26th March to 8th April* (nominal 16°C)	885	72	856	51
2. *9th to 22nd April* (nominal 16°C)	874	21	866	30
3. *23rd April to 6th May* (nominal 16-18°C)	885	41	847	64
4. *7th to 20th May* (nominal 18-20°C)	895	78	871	68

Hardness

Table 43 gives the mean hardness of the test media with the standard deviation. It was consistently higher than the expected value of 394 mgL^{-1} $CaCO_3$ (calculated for the salts added to the reverse osmosis filtered water). This was not considered likely to have affected the exposure, as gastropods generally prefer a hard water environment to facilitate the extraction of calcium for shell-building (Boycott, 1936).

Table 43 – The mean hardness and standard deviation (SD) recorded for each fortnight of the exposure.

	Hardness (mgL^{-1} $CaCO_3$ equivalent)	
Exposure Period	**mean**	**SD**
1. *26th March to 8th April* (nominal 16°C)	427	24.6
2. *9th to 22nd April* (nominal 16°C)	435	19.7
3. *23rd April to 6th May* (nominal 16-18°C)	421	19.0
4. *7th to 20th May* (nominal 18-20°C)	408	19.1

7.2.2.3 Test Organism Survivorship

Figure 83 shows the mean percentage mortality rate of the test organisms over the 8 week exposure to Bisphenol-A. The mean mortality in the control group was low (4.0%). It can be seen that there is a dose-dependant increase in the mortality rate with increasing Bisphenol-A concentration, and that the mean percentage mortality in the group exposed to 20 μgL^{-1} (nominal) is x2 that observed in the control group. However, this is largely because one of the replicates in this group experienced a mortality rate of 22.5%, causing the standard deviation to be wide. There was no significant correlation between the mortality rate and the nominal concentration of Bisphenol-A (Pearson's Product Moment Co-efficient, $p<0.05$), and there are no significant differences between the control and any of the treatment groups (Kruskal-Wallis, $p>0.05$).

The proportion of parasitized individuals observed in the surviving test organisms was 11.7%. This is much lower than that recorded in the previous laboratory exposure (50% at termination of the exposure to 17β-oestradiol, see Chapter 5, Section 5.2.3.6). However, the degree of parasitisation within the test replicates varied widely. Two control groups were highly parasitized (61.5% and 63.2% of the surviving organisms) and two had no observed parasites at all. The mean percentage of parasitized individuals in the control population was 29.8%, and in the Bisphenol-A treated groups it was lower at 5.2%, 3.0% and 8.9% in the 0.2, 2 and 20 μgL^{-1} (nominal) groups, respectively. Overall, there was no significant difference in the proportion of parasitized individuals between the control and any of the Bisphenol-A treated groups (Kruskall-Wallis, $p>0.05$).

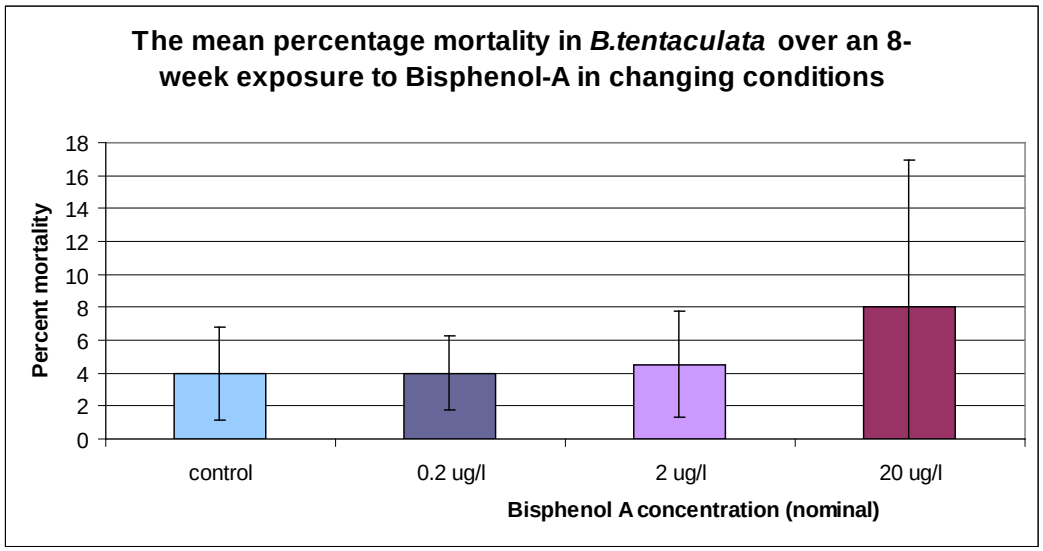


Figure 83 – The mean percentage mortality in *B. tentaculata* over a 4-week exposure to Bisphenol-A at 16.4°C (mean) and then a further 4-week exposure with the temperature gradually increased to 20.8°C by Week 8 (error bars represent the standard deviation).

7.2.2.4 Test Organism Morphology and Growth

There were no significant differences in the mean length or weight of the groups of test organisms at the start of the experiment (ANOVA, $p>0.05$ in both cases). Figure 84 shows the mean percentage increase in the weight of the groups during the 8 week exposure (length was not measured due to the likelihood of a spurious 'shrinking' effect caused by rubbing of the shell spires). All of the replicate groups increased in weight, except one in the 2 μgL^{-1} Bisphenol-A (nominal) treated group. The mean percentage increase in weight was over 3% in the control and all treatment groups, indicating that the exposure conditions were not adverse to the test organisms.

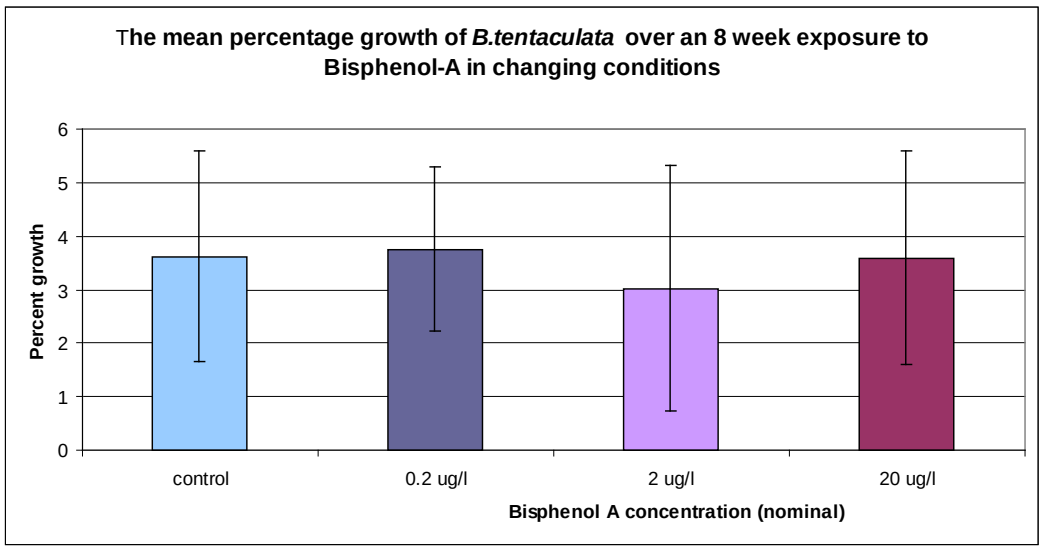


Figure 84 – The mean percentage growth of *B. tentaculata* over a 4-week exposure to Bisphenol-A at 16.4°C (mean) and then a further 4-week exposure with the temperature gradually increased to 20.8°C by Week 8 (error bars represent the standard error of the means).

7.2.2.5 Test Organism Gender Ratio

The proportion of males and females in the population of test organisms surviving at the end of the exposure was 47.5% to 52.5%, respectively. This is

close to the equal sex ratio expected for *B. tentaculata* (e.g. Kozminsy, 2003). The percentage of females in the control group was exactly representative of the test population (52.5%), and the percentage in the Bisphenol-A treated groups was 47.9%, 56.9% and 52.6% in the 0.2, 2 and 20 μgL^{-1} (nominal) groups, respectively. This variation principally occurs due to a low proportion of females in one replicate in the 0.2 μgL^{-1} exposure group (39.5%) and a high proportion of females in one replicate in the 2 μgL^{-1} exposure group (75.7%). Overall, there are no significant differences in the gender ratio between the control and any of the Bisphenol-A treated groups (ANOVA, $p>0.05$).

7.2.2.6 Test Organism Reproduction

Figure 85 shows the mean number of eggs laid per female recorded at the end of the exposure, and also the mean number of eggs laid per female without parasites. It is currently unclear whether any degree of parasitic burden reduces breeding activity or indeed whether parasites can castrate *B. tentaculata*, and therefore both datasets are shown. It can be seen that in fact there is little difference in the result of these two approaches. The control group laid 8.76 eggs per female on average, or 10.06 if parasitized females are excluded. This difference is small because the heavily parasitized groups laid fewer eggs than the relatively unparasitized groups, and indeed the most heavily parasitized group did not lay any eggs during the whole of the experiment.

The groups treated with Bisphenol-A all laid a higher mean number of eggs per female or per unparasitized female than the control group, and the group exposed to 20 μgL^{-1} (nominal) laid >x2 that of the control group. However there were no significant differences in the number of eggs laid between the control and any of the Bisphenol-A treated groups, whether parasitisation is accounted for or not (ANOVA, $p>0.05$ in either case). There is a significant correlation between the nominal Bisphenol-A concentration and the number of eggs laid per unparasitized female (Pearson's Product Moment Correlation Co-efficient, $p>0.05$), but the regression relationship is poor ($r^2 = 0.226$).

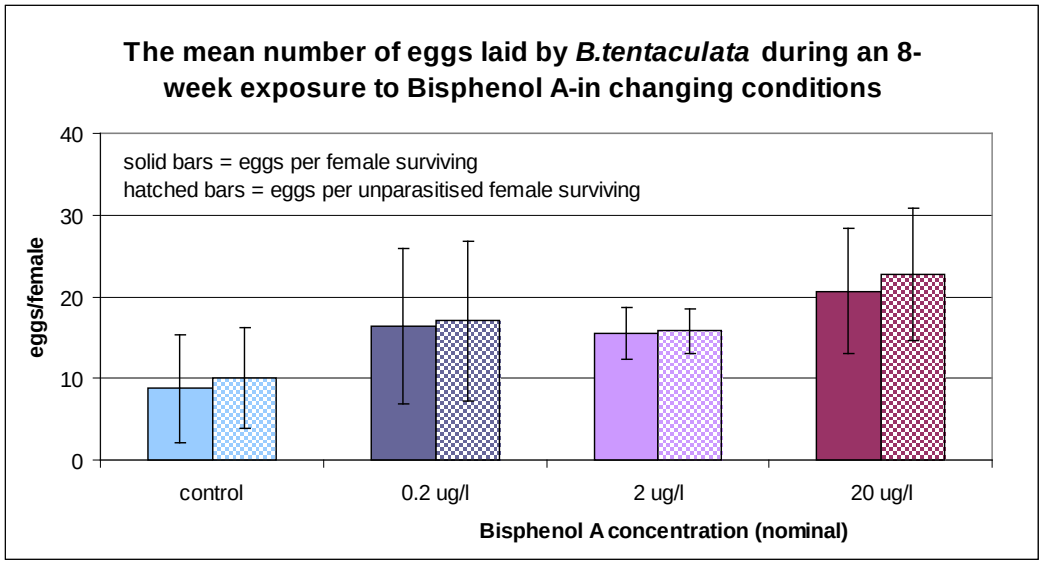


Figure 85 – The mean number of eggs laid by female *B. tentaculata* (total females and females without parasites) over a 4-week exposure to Bisphenol-A at 16.4°C (mean) and then a further 4-week exposure with the temperature gradually increased to 20.8°C by Week 8 (error bars represent the standard deviation).

However, Figure 86 shows the cumulative number of eggs laid over the course of the experiment (assuming the proportion of females remained the same as the gender of mortalities occurring could not be ascertained). It can be seen that none of the groups begin to lay eggs until the second week of the experiment (at a constant temperature of 16°C), and that the rate of egg laying increased in all groups from the fifth week of the experiment (when the temperature had begun gradually increasing). It was also observed that all of the Bisphenol-A treated groups deviate in a similar way from the control group, as was observed in the second *P. corneus* exposure to Bisphenol-A (see Chapter 6, Section 6.2.2.5), but that the deviation in this case was in the opposite direction (an increase in oviposition as opposed to a decrease). If all of the data from the Bisphenol-A treated groups are pooled and compared to the control, the overall 'Bisphenol-A treatment' differs significantly (2 tailed t-test, $p = 0.037$). Again, a new experiment, designed to test this hypothesis, is required to confirm this.

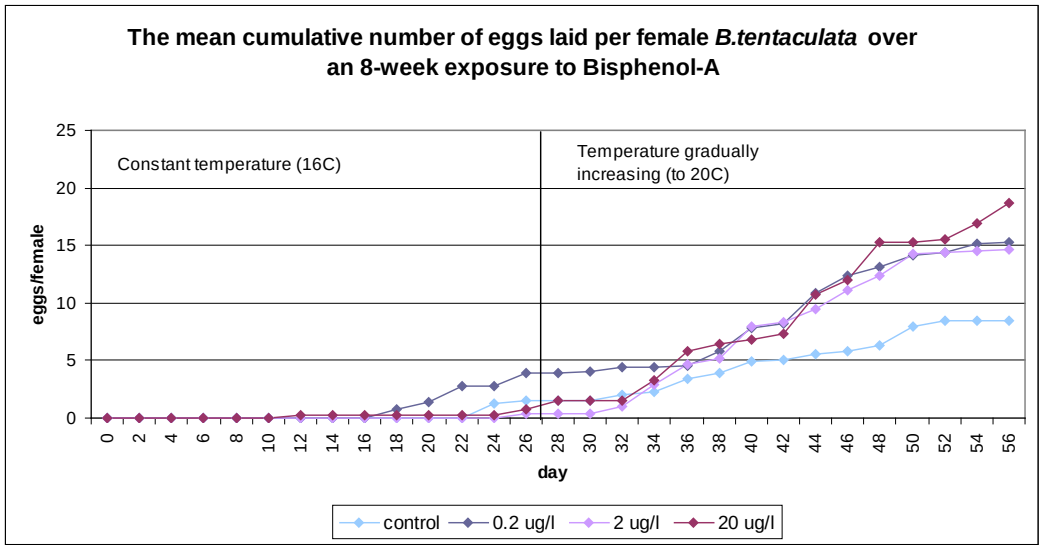


Figure 86 – The mean cumulative number of eggs laid per pair of *B. tentaculata* over the course of an 8-week exposure to Bisphenol-A.

7.3 Comparative responses of *P. antipodarum* and *B. tentaculata* to Bisphenol-A

7.3.1 Physico-chemical conditions

The number of samples taken for Bisphenol-A analysis during the prosobranch exposures was again fewer, and were combined across the two exposures to reduce the cost. This was initially considered acceptable as the exposures were made concurrently, and both used moderately sized test vessels (1-2 litres) placed in the same exposure facility. Only the *P. antipodarum* exposure proceeded for the first four weeks, and although a small amount of Bisphenol-A was again measured in a control sample, and the measured quantity in a sample from highest nominal concentration was lower than expected, the analysis did indicate that Bisphenol-A was present in the test vessels in the expected ratio, even 48 hours post-spiking. At Week 5, the *B. tentaculata* media was added to the pooled samples, and the degradation rate appeared to increase markedly. It is possible that the higher density of snail tissue per vessel (approximately 3g of *B.*

tentaculata per litre, as opposed to approximately 1g of *P. antipodarum* per litre) contributed to this effect.

Over the following 5 weeks Bisphenol-A was barely detected in the test vessels. This was probably due to a combination of over-dilution of samples by the analytical laboratory and extensive degradation of the test compound. It was again apparent that prosobranchs allow the development of a more extensive biofilm due to lack of any appreciable grazing than do pulmonates. When this was recognised, partial sterilization of the test vessels at 60^{o}C was implemented, and this appeared to redress the degradation to some extent. In the last week of the exposure the spiked Bisphenol-A levels were again measurable in the expected ratio, demonstrating that the spiking procedure was consistent. It is not known how long the compound was present in the test vessels for the period when the degradation rate was most rapid, but Oehlmann et al. (2006) reported half-lives for Bisphenol A of between 1 and 6 hours. It is therefore likely that the concentration decreased rapidly.

In the final 4 weeks of the exposures, Bisphenol-A was also measured in appreciable amounts in the control media (up to 0.192 μgL^{-1}). It is not clear why this occurred, as almost all media contact with plastics had been eliminated in these relatively small scale exposures. The aggregation of the highest values towards the end of the exposure suggests that the media storage tank had become contaminated. This is possibly because the laboratory was undergoing dismantling, and the tank had to be re-sited at this time. The alternative supply pipes may therefore have been a transient source of Bisphenol-A. The elevated values combined with the increased degradation rate made the control and the lower two treatment concentrations indistinguishable from Week 5 onwards (incorporating the whole of the *B. tentaculata* exposure). Overall, the results of the analytical chemistry indicate that the outcomes of the prosobranch exposures are not reliable. It is likely that the organisms in the higher treatment groups experienced fluctuations of Bisphenol-A exposure, but since the experimental half-life cannot be known, the results must be interpreted with extreme caution.

7.3.2 Mortality

The mortality in the *P. antipodarum* exposure was low, at 3.9% of the test organisms, and in the *B. tentaculata* exposure, the overall mortality was only slightly higher at 5.1%. In the latter exposure, the mortality observed in the group exposed to the highest test concentration (8.0%) was double that of the control group (4.0%), but again this was not a significant difference. Overall, Bisphenol-A did not appear to cause any distinguishable toxicity in the freshwater prosobranchs used in this research.

7.3.3 Growth

Unexpectedly, the *B. tentaculata* grew well during the exposure, increasing in mean weight by 3.5% overall. No such increase had been observed in the 17β-oestradiol exposure using this species. This may be because the animals were slightly smaller at the outset (mean length = 9.4mm in the Bisphenol-A exposure as opposed to 11.0mm in the 17β-oestradiol exposure), but it is more likely to be due to the additional food source supplied (organically grown carrot). All the treatment groups increased in weight to similar degrees, and there were no significant differences from the control. No meaningful assessment of growth was made in *P. antipodarum* due to the staggered adult sampling regime.

7.3.4 Reproduction

There were also no significant effects on reproduction in the prosobranch exposures to Bisphenol-A. The initial *P. antipodarum* exposure had not included any seasonal simulation, rather it was made in constant test conditions (16^{o}C, 12 hour photoperiod) for 8 weeks, as specified in the method proposed by Schmitt et al. (2006) and Duft et al (2007). However, the culture conditions used to maintain the organisms before testing was not that proscribed in this method. Instead, the test organisms were taken from a long-term culture that had been fed on minimal nutrition dry leaf litter, in order to reduce the reproductive rate.

Consequently, the increase in food quality and availability experienced by the snails on introduction to the test conditions simulated the increase in algal and plant growth expected in springtime. The effect of the increased food supply was demonstrated by the rapid increase in the mean number of embryos dissected from the sub-samples of adults in the control group. The increase was 135% between the second and fourth weeks, and by a further 68.1% between the fourth and eighth weeks. Duft et al. (2003b) stated that *P. antipodarum* embryos develop in 8 weeks or less, and this is supported by these results.

The number of embryos in the sub-sample of adults taken after 4 weeks of exposure to Bisphenol-A formed an 'inverted U-shaped' dose-response curve. This had also previously been observed in sediment exposures of this species to octylphenol and nonylphenol (Duft et al., 2003a) and 17α-ethinylestradiol and Bisphenol-A (Jobling et al., 2004). The peak of the response to Bisphenol-A was in the 0.2 μgL^{-1} exposed group (mean measured = 0.196 μgL^{-1}), which is markedly lower than that observed by Jobling et al. (2004) for this compound (5-25 μgL^{-1}, nominal). The mean magnitude of induction in embryo development in this group was 35.5% above control, but there were no significant differences from the control, although the interpretation may again be confounded by the inability of the statistical techniques to assess non-monotonic dose-response curves.

There is no clear explanation for the shape of the dose-response on this occasion, as there are no indications of toxicity in the higher exposure concentrations. Ultimately however, while this pattern persisted at the formal end of the exposure (8 weeks), the magnitude of the response was greatly reduced. The sub-sample of adults from the group exposed to 0.2 ug^{-l} (nominal) had a mean of only 4.1% more embryos than the control group at this time. This may be partially explained by an increase in the degradation rate of Bisphenol-A after the Week-4 sampling event. Overall however, the 'effect' is both transient and inexplicable, making any evidence for an induction of embryo production by Bisphenol-A in *P. antipodarum* from this research very weak. Nonetheless, it does appear to support the work of previous researchers, and as the observed maximum

induction occurs at a very low concentration (below the NOEC of 1.6 μgL^{-1} accepted by the EU Risk Assessment for Bisphenol-A), the experiment should be expanded and repeated to strengthen or refute the current information.

Finally, a seasonal aspect was added to the exposure by extending the experiment for a further 4 weeks and gradually increasing the temperature and photoperiod to $20^{o}C$ and 16 hours of light with an increased lux intensity to simulate summer. The mean number of embryos in the remaining adults at the end of this period was lower than those sampled at 8 weeks. It is not clear why this should occur, when it might be expected that the reproduction of the organisms would increase further in the favourable conditions. It is possible that the increased temperature and light was in fact stressful for this relatively cryptic mud-dwelling species. While the shape of the Bisphenol-A concentration response curve again persisted, the peak of the 'inverted U-shape' occurred in the 2 μgL^{-1} (nominal) treatment group on this occasion. However the degree of induction was small relative to the variation in embryo number between individuals, and there were no significant differences.

The *B. tentaculata* exposure was made concurrently with the *P. antipodarum* exposure but with a 4-week delay, so that the test organisms experienced a 4-week period at $16^{o}C$ with a 12 hour photoperiod, followed by the same simulated summer described above. All of the Bisphenol-A exposed groups laid a higher mean number of eggs than the control group, and the group exposed to the highest concentration (20 μgL^{-1}, nominal) laid more than double that of the control, although there were no significant differences. However, when the rate of oviposition during the course of the exposure is examined, it can be seen that the slope of the cumulative increase of mean eggs per adult in the gradually warming phase is similar in all of the Bisphenol-A exposed groups, and markedly steeper than that of the control group (see Figure 86). As in the second *P. corneus* exposure, if all the treated groups are combined due to the similarity of their response, the 'Bisphenol-A treated' groups are significantly different to the control. However, in contrast, the presence of Bisphenol-A appears to cause an

increase in oviposition rate in *B. tentaculata*, rather than the decrease observed in *P. corneus*.

This difference in response could be interpreted as a difference between these species or indeed the sub-classes, in that the prosobranch *B. tentaculata* may be more susceptible to Bisphenol-A affecting reproductive control and causing an induction in egg-laying, in a similar manner to that described in *M. cornuarietis* (Oehlmann et al., 2000a; 2006). However, it could also be due to an effect of the Bisphenol-A on the physical test conditions. For instance, since *B. tentaculata* predominantly filter-feed on algae (Tashiro and Colman, 1982), it is possible that the test compound altered the availability of the algal cells by increasing flocculation, allowing more efficient feeding and consequently providing more energy that can be used in reproduction. However on balance, the former explanation is more likely, as the *B. tentaculata* in this exposure were not fed algae *ad libitum*, and all groups tended to quickly clear the suspensions provided. The other food source was slices of carrot that were constantly available in excess, but it is difficult to postulate a means by which the presence of Bisphenol-A could affect these large objects. Again, further experiments are needed to establish whether the observed induction, particularly at the lowest test concentration (0.2 μgL^{-1} nominal, 0.133 μgL^{-1} mean measured) is repeatable and therefore whether a conclusion that very low concentrations of Bisphenol-A (below the EU accepted NOEC of 1.6 μgL^{-1}) cause an induction of egg-laying in *B. tentaculata* is supportable.

Overall, the original hypotheses for these experiments; that 'exposure to Bisphenol-A under controlled temperature and photoperiod conditions representing a simulated spring and summer will significantly alter the number of embryos in the brood pouch of *P. antipodarum* and/or the number of eggs produced by *B. tentaculata* relative to the control' are not supported. However, the evidence does tentatively suggest that Bisphenol-A at a concentration of 0.2 μgL^{-1} (nominal) or greater causes a slight induction of reproduction in both of the prosobranch species assessed. However this induction is set against a

background of contaminated control organisms, which may also be subject to a similar effect, and therefore the extent of the induction in the treatment groups may be masked. It is clear that the exposures require refining and repeating to further define and elucidate the effects before any firm conclusions can be drawn.

7.3.5 Parasitisation

While the *P. antipodarum* were laboratory bred and free from any observable parasites, the population of *B. tentaculata* used was found to have a 11.7% parasitisation rate on dissection at the end of the exposure, despite a sub-sample taken prior to the exposure being only 2.2% parasitized (n = 91). It is therefore clear that the degree of observable parasitisation increased over time. It is possible that some snails were parasitized from the outset, but that the parasites were initially unobservable. However it was also noted that the proportion of parasitized animals was much greater in certain test vessels (in the control, 2 groups were >60% parasitized, one group was 24% parasitized and two had no observable parasites), giving rise to suspicion that the parasites could transfer between hosts without the presence of an additional vector.

Also, one of the highly parasitized control groups entirely failed to reproduce, suggesting that parasitisation does inhibit reproduction in this species, although apparently unparasitized females were also present in this group. This is in contrast to the exposure of *B. tentaculata* to 17β-oestradiol, when parasitized females were observed to lay eggs. The other heavily parasitized control groups also laid markedly fewer eggs than the groups in which no parasites were observed (42.7% - 76.4% less). It is possible that the ability to continue to lay eggs depends on the degree and /or stage of parasitisation. Overall, the extent to which parasitisation affects reproduction in *B. tentaculata* remains unclear.

CHAPTER 8
GENERAL DISCUSSION AND CONCLUSIONS

8.1 Gastropod Reproductive Induction: Possible Mechanisms and Outcomes.

There are limited reports on the effects of chemicals on freshwater gastropod reproduction, and many of these are pesticide toxicity studies that aim to establish techniques for the control of vectors of human parasites or for crop protection. For example, Bhide (1998) found decreased egg laying in *L. stagnalis* after exposure to high (500 mgL^{-1}) concentrations of nuvan, thimet and methyl parathion, which is a clear case of reproductive toxicity. There are also studies that aim to provide hazard assessment data for the protection of gastropods, and they too observe inhibited reproduction, e.g. Bacchetta et al. (2002) exposed *Physa fontinalis* to environmentally relevant levels of paraquat and record reduced egg laying and increased numbers of degenerating oocytes, which is evidence of gonadal toxicity. However, other researchers have exposed gastropods to putative endocrine disruptive compounds and have also found reproductive inhibition. This has been observed in *L. stagnalis* exposed to DDT (Woin and Bronmark, 1992), TBT (Leung et al., 2007) and Fenarimol (Weltje et al., 2003). These instances are perhaps not surprising as aside from any chronic toxicity, DDT is know to adversely affect the development of female reproductive organs in vertebrates, TBT is a masculinising agent in gastropods and Fenarimol is a putative vertebrate aromatase inhibitor, all effects which could be expected to reduce female reproductive fitness.

Perhaps it is more surprising that Nonylphenol also significantly reduced the number of egg masses laid by this species over a 12 week period (Czech et al, 2001) given that alkylphenols are known to feminise male fish. However the concentration at which this occurred was moderately high (100 μgL^{-1}, nominal), and this may therefore have been a toxic effect. In contrast, Jumel et al. (2002) studied the effect of Nonylphenol polyethoxylate as an adjuvant to a herbicide exposure of *L. stagnalis*, and found that the reproductive inhibition caused by the

herbicide was negated in the presence of the adjuvant. However, it was also found that the adjuvant reduced the measured concentration of the herbicide in the exposure mesocosm and it therefore may not have been sufficiently bioavailable to cause the same adverse effects.

Interestingly, several pesticides have been shown to cause an induction of reproduction in lymnaids. Presing (1993) recorded an induction of up to 45.1% in *L. stagnalis* exposed to the insecticide K-Orthrine following repeated exposures to simulate spraying, although the effect was not dose-dependant. She also noted that the food consumption of the snails was greater while the reproductive rates were elevated. Baturo et al. (1995) also found that exposure of *Lymnaea palustris* to the fungicide hexachlorobenzene caused increase egg production for up to 12 weeks following a single application of 5 μgL^{-1} in a mesocosm. Again the effect was not dose-dependant, but the researchers also analysed the activity of polysaccharide hydrolysing enzymes and found a stimulation after 1 week, indicating that the glycogen reserves were being mobilized. Tripathi and Singh (2004) found marked, dose-dependant increases in the number of eggs laid by *L. stagnalis* exposed to 4, 8 and 12 μgL^{-1} alphamethrin and cypermethrin culminating in an almost 300% increase in both cases. However this was a very short exposure of 96 hours. The snails were not fed at all, and the tissue concentrations of protein, nucleic acids, glycogen and pyruvates were all significantly reduced, which is likely to be a result of the increased reproductive effort.

It seems then, that the exposure to chemicals can both increase and decrease the reproduction of pulmonate gastropods. Reproductive inhibition is, however generally relatable to toxicity, but it is more difficult to explain the cause of reproductive induction from pesticide exposure. There is no evidence for, nor a conceptual link to, an endocrine disruption effect. It is possible that the cases described are examples of ‘reproductive recklessness’ (Calow, 1979), where the toxicity of the compound is causing the organisms to mobilise all reserve resources into egg production before death occurs. If this is within the capability

of gastropods, it adds a layer of complexity to the interpretation of both the toxicological studies and in understanding any (neuro-) endocrine effects. However, reproductive induction also occurs in exposures where overt toxicity is not as probable.

Watton and Hawkes (1984) exposed *Lymnaea peregra* to 25 and 50% treated sewage effluent in artificial streams. They did not accurately assess the number of adults in the streams, but they did find significantly more egg masses in both treatments than in the control. They also noted the presence of an 'inverted U-shaped' dose-response curve due to the number of egg masses in the 25% effluent being greater than in the 50% effluent, which is similar to the results of the second mesocosm exposure of *P. corneus* to 17β-oestradiol. However, they also observed that the effluent was toxic to *P. antipodarum* (which was co-exposed) and considered this to be heavy metal toxicity from elevated levels of chromium, cadmium and copper. This may have contributed to the increased reproduction in *L. peregra*. It must also be considered that treated effluent can be a food source for gastropods, and additionally, can be androgenic as well as oestrogenic. Santos et al. (2008) observed that treated sewage effluent can induce imposex in *N. lapillus*. Therefore there are several possible mechanisms that may be interacting to produce this non-monotonic response curve, including increased resources, heavy metal toxicity, potential reproductive recklessness, and also any masculinising or indeed feminising compounds that may exert an endocrine effect that are present in the effluent.

Clarke et al. (2009) improved on this experiment by exposing *P. corneus* to treated sewage effluent in a mesocosm, and relating the number of masses laid to the number of snails present. They too found that the snails exposed to the effluent (at 50 and 100%) laid significantly more egg masses than the controls. On this occasion, the effect was dose-dependant, with the number of masses laid by the group exposed to 100% effluent being significantly higher than that of the control at all the fortnightly sampling events. This suggests that an aspect of treated sewage effluent is consistently inducing an elevated oviposition rate,

which may be due to the chemical content of the effluent (conceivably endocrine disrupting compounds), the increased food supply provided by the effluent or even the physical properties of the effluent, i.e. a bacterial film that encourages the organisms to lay smaller but more frequent egg masses due to poor adhesion. Perhaps of more interest is the observation that in the group exposed to 50% effluent, the induction of egg mass number was confined to the early (spring) and late (autumn) sampling events (Figure 87).

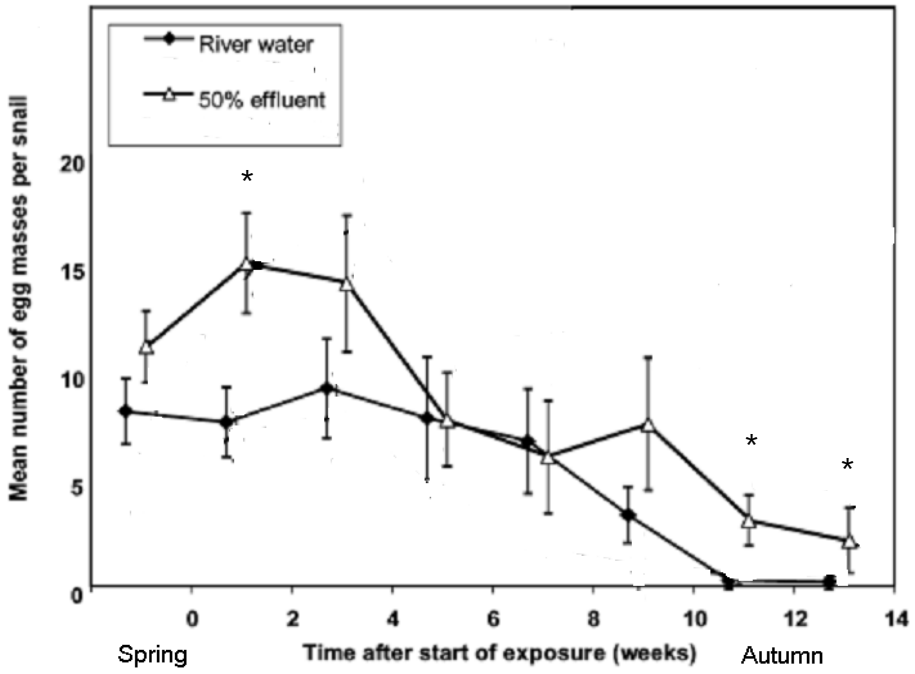


Figure 87 – The number of egg masses laid by *P. corneus* over a spring to autumn mesocosm exposure to 50% treated sewage effluent (adapted from Clarke et al., 2009). Asterisks = significant difference from river water control.

Watton and Hawkes (1984) also noted that the increase in *L. peregra* egg masses in sewage effluent streams was greatest at the July sampling event, at which point the control snails ceased to produce egg masses, while the snails in the effluent streams continued laying into August and September (Figure 88). The apparent seasonality of the effect is in agreement with the results of the exposures of both pulmonates and prosobranchs to 17β-oestradiol made in this research project, and taken together, there is a body of evidence that implies that increased egg laying

can be induced by exposure to both treated sewage effluent and 17β-oestradiol alone. The consistent nature of this response provides a conceptual linkage that excludes the physical attributes of, and additional resource provided by, sewage effluent and indicates that steroid exposure promotes the induction or perpetuation of reproduction in freshwater pulmonate gastropods. The aspect that remains unclear is whether the observed effects are a form of hormesis; Calabrese and Baldwin (2001) describe this as an over-compensation reaction below the threshold of toxicity, and this may be expressed as reproductive recklessness in gastropods responding to 17β-oestradiol as a toxin.

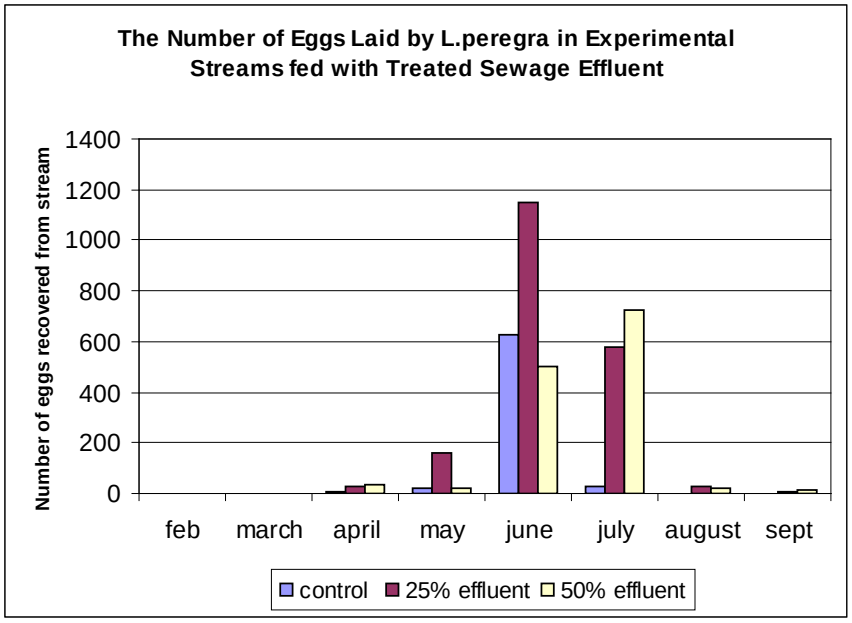


Figure 88 – The number of eggs recovered from experimental streams populated with *L. peregra* and exposed to 25 and 50% treated sewage effluent (plotted from Watton and Hawkes, 1984).

However, seasonally reproducing temperate river invertebrates have varying strategies for coping with periods of adversity (i.e. over-wintering). Ladle and Ladle (1992) recognise four classes; those living essentially one year, that grow over-winter and reproduce in large numbers in the spring, those living longer than one year that hibernate in some form and reproduce in lesser numbers the following year, those that have a resting stage (cysts or cocoons), and those that

reproduce sexually in good conditions and asexually in adverse conditions. It appears that pulmonates, being generally semelparous, fall into the first category and prosobranchs, often being iteroparous, fall into the second. Calow (1978; 1979) also considers that iteroparity is related to a longer life-span and reduced overall reproductive effort, but with the 'effort per unit off-spring' being increased. He also hypothesizes that viviparity occurs when the egg-size can no longer be increased so that adults brood the off-spring in order to provide further resources, allowing greater development before independence and increasing the chance of survival. This reproductive strategy does not allow any form of 'reproductive recklessness' as the adult must survive to brood the embryos. Browne and Russell-Hunter (1978) agree, quoting mean values of reproductive effort of 29.9% of assimilated (non-respired) energy in semelparous and 18.2% in iteroparous molluscs, and that viviparous species expend a mean of 5.25% compared with 24.2% in oviparous species. They also conclude that reproductive effort increases annually in iteroparous species with a ratio of 1:2:4, quoting values of 2.9%, 4.2% and 6.3% over the first three years in *Viviparus georgianus*.

Given that the three species used in these 17β-oestradiol exposures are therefore likely to have different levels of investment, with *P. corneus* being a semelparous pulmonate, *B. tentaculata* being an oviparous iteroparous prosobranch and *V. viviparus* being a viviparous iteroparous prosobranch, care must be afforded to the interpretation of how an induction or perpetuation of reproduction may affect wild populations. *V. viviparus* at least should not exhibit reproductive recklessness, which suggests that rather than hormesis, exposure to certain concentrations of 17β-oestradiol is more likely to be interfering with the management of reproduction in this species. The observed induction occurs only at the onset of autumn, after the summer solstice, indicating that the effect is a symptom of the natural autumnal suppression of reproduction being overcome. This in turn implies a role for 17β-oestradiol in the seasonal timing of reproduction that can be perturbed by exogenous exposure. However, in species that use a semelparous strategy, it remains possible that reproductive induction

following 17β-oestradiol exposure also has a hormetic component in response to toxicity.

Nonetheless, it is clear that all temperate species have evolved and adapted to the adverse conditions of winter in the annual seasonal cycle in order to maximise survival and successful reproduction, and it is therefore possible that any perturbation of this pattern may have population level consequences for all of the described strategies. The mechanisms of seasonal control and timing of reproduction in freshwater gastropods are dependant on a number of factors and environmental cues, including temperature, photoperiod, food availability, water quality and parasitisation status (Wayne, 2001). The process by which these translate into reproductive control are not well understood, and consequently the modes by which exogenous steroids could interfere are a matter of conjecture. However there are some evidences that can contribute to the formulation of a hypothesis, and these are discussed below.

It is probable that differing environmental aspects cue female accessory organ recrudescence and regression, and the initiation and cessation of egg-laying behaviour. This is because temperate organisms must 'predict' when to begin and cease activity to best advantage without 'risking' being active at inappropriate times. Hence, females must recrudesce in preparation for reproduction before conditions suitable for off-spring survival arises, and must cease activity before conditions become adverse in order to allow the juveniles sufficient time to mature before winter. Sastry (1971) observed that egg capsule deposition begins in *I. obsoleta* when the required environmental temperature is reached. Dogterom et al. (1984;1985) also showed that in *L. stagnalis*, water temperature is a critical factor for egg-laying, and considered that a temperature of 8^{o}C must be reached for reproduction to begin. They conclude that photoperiod is not a critical factor, but that there must be sufficient food resource available when the threshold temperature is reached for reproduction to be successful. This seems plausible as day-length begins to increase in December, making it an unsuitable cue in terms of resource availability, whereas rising

temperature is a better indication of the onset of good conditions for plant and algal growth. Costil and Bailey (1998) observed that *P. corneus* devotes much of the additional activity that occurs at warmer temperatures to feeding, irrespective of photoperiod, therefore it is likely that increasing temperatures in spring allow gastropods to feed and build the energy reserves required for reproduction.

Jess and Marks (1998) studied the effect of rearing *Helix aspersa* in continuous darkness as a cost-efficiency in this food-crop gastropod. They also found that recrudescence was unaffected. However they observed that egg-laying activity tailed-off. Bailey (1981) and Gomot et al. (1989) also found that egg-laying ceased in a short-day regime in this species. Blanc et al. (2003) recorded the presence of melatonin peaks in ganglia, this being a mediator of photoperiod information in vertebrates. Hotchkiss et al. (2008) studied the seasonal regression of the reproductive organs of *I. obsoleta* and found that animals held in constant long-day conditions continued to reproduce until they were released from phase-lock by reducing light levels. Bohlken and Joosse (1982) and Bohlken et al. (1986) observed that *L. stagnalis* continues to lay eggs in long-day conditions even in the absence of sufficient food. Overall, it is apparent that day-length controls the extent of the reproductive season, which is in agreement with the mesocosm exposures made in this research project. Decreasing day-length begins in June, which was the peak of reproductive activity in the reference groups, with oviposition and/or embryo production rates declining from this time.

It is unlikely that food resource or temperature are factors in the seasonal reproductive decline, as plant and algal material is still abundant and water temperatures often peak at this time. The organisms in the mesocosm exposures were fed at a constant rate throughout the exposure, and *P. corneus* can continuously reproduce for up to 175 weeks in constant conditions (Costil and Daguzan, 1995), so reproductive exhaustion is also unlikely. However it is probable that those gastropods that have a semelparous life-history adopt a different strategy of energy budget management to that of iteroparous gastropods when preparing to over-winter. Boyle and Yoshino (2000) consider that in the

pulmonate *Biomphalaria glabrata* the rate limiting factor for reproduction is the peri-vitelline fluid supplied by the albumin gland. Therefore this is probably the most expensive resource in female reproduction. Berrie (1966) observed that in *L. stagnalis* the albumin gland increases rapidly in size from March to May, coinciding with increasing oviposition rates. This suggests that the energy for peri-vitelline fluid production is derived from concurrent feeding rather than any stored reserves. Also, Wijsman (1989) measured the galactogen content of the albumin gland of this species and found that levels are restored 32 hours after oviposition, indicating that energy is continuously diverted to vitellogenesis. As semelparous gastropods do not 'expect' to over-winter as adults, it is likely that reproduction will proceed at a maximal rate until such time when the survival of the off-spring is likely to be compromised, i.e. at the onset of autumn. While this strategy is not intrinsically 'reckless', Bohlken et al. (1986) showed that under long-day conditions, *L. stagnalis* continues to reproduce even when entirely starved. Then the organisms do experience reproductive exhaustion and the researchers recognise that 'the price that must be paid [is a] considerable shortening of the parental life-span'. In this truly reckless reproductive strategy, day-length and consequently optimum conditions for off-spring development out-strip all other parental considerations, including survival.

It is less likely that iteroparous prosobranchs employ a reckless reproductive strategy that has the potential to drain the resources of other organs and impair successful over-wintering. Indeed, early life reproduction is often 'restrained' (Browne and Russell-Hunter, 1978; Calow, 1979). This may be because over-wintering can be more 'expensive' even than reproduction; Strohmeier et al. (2000) recorded that the bi-valve *Pecten maximus* lost an average of 27 kJ over winter, compared to 11 kJ used in gonad development. It is also likely that prosobranchs recrudesce using stored energy; Young (1975) noted that prosobranchs he observed began reproduction earlier in the spring than the pulmonates. *B. tentaculata* lays eggs immediately after rising from the mud (personal observation) and in the second mesocosm exposure, the peak of

reproduction in both this species and *V. viviparus* was at least 4 weeks earlier than *P. corneus*.

However, as observed in the mesocosm exposure of *B. tentaculata*, prosobranchs tend to maintain a lower, but steady rate of reproduction after the spring peak until the summer solstice, and this additional activity is likely to be resourced by concurrent feeding. After the solstice reproduction tails-off, and in *V. viviparus* the developing oocytes and follicle cells are shed (Griffond and Gomot, 1979). This suggests that no further energy is wasted on conceiving embryos that would be released too late in the season for over-winter survival. However, the number of off-spring produced by prosobranch populations sometimes has a second, lesser peak in the autumn. Ribi and Gebhardt (1986) report this phenomenon in the *V. ater* population of Lake Zurich, and Eleutheriadis and Lazaridou-Dimitriadou (2001) observed it in a population of *B. graeca* in Lake Kerkini (N.Greece). They considered that these eggs were laid by first summer juveniles that had matured, but it was also observed in the long-term laboratory experiment using *B. tentaculata* in this research project, and no first summer juveniles were deployed. Indeed, the majority of the snails used in this experiment were 10-12 mm in length, and were therefore likely to be 2.5 years old at the start of the study. This is generally the last summer of reproduction in this species (Dussart, 1979; Negus, 1998), and iteroparous molluscs divert more energy to reproduction at the end-of-life stage (Browne and Russell-Hunter, 1978). Therefore it is conceivable that this second peak is an expression of reproductive recklessness deriving from the oldest cohort of the population reproducing maximally using their remaining resources.

This potential difference in the reproductive control of semelparous pulmonates and iteroparous prosobranchs, and between the life-stages of the iteroparous prosobranchs, has consequences for the interpretation of reproductive induction in these groups but it may also aid in formulating a hypothesis for the mechanisms involved. It seems that female prosobranchs in particular can use a varying mechanism on an annual basis to control reproductive activity according

to their age. Taken as a whole, the evidence outlined above implies that maximal reproduction according to environmental cues or the available resource is not an inherent characteristic of iteroparous freshwater gastropods, rather reproductive effort can be 'tailored' to the circumstances. The mechanism for this can only be postulated since there is currently only a limited understanding of the control of reproduction in gastropods and this is mainly confined to the pulmonates. However, it has been demonstrated that bivalves have neurological control over the apportionment of energy to reproduction or survival/growth. Lubet and Mattieu (1978) incubated explants of *C. gigas* cerebral ganglia with gonads and storage tissues and found that gametogenesis proceeded, and the storage tissue lysed, leading to the conclusion that the energy budget (represented by the storage tissue) is neurologically controlled. Strohmeier et al. (2000) also demonstrated that annual somatic and reproductive growth are separated in time in *P. maximus*, implying a sophisticated degree of reproductive budgeting.

In the gastropod *L. stagnalis*, Geraerts (1976a; 1976b) used excision of the lateral lobes and cauterisation of the Light Green Cells (LGC) of the cerebral ganglia in *L. stagnalis* to show that the LGC produce a growth hormone and that immature snails grow until the gonad reaches maturity, and then growth slows down when oviposition begins. With the lateral lobes removed, the production of Dorsal Body Hormone (which induces maturation of the female gonad) is delayed and growth continues, causing gigantism. The lateral lobes contain three types of neurosecretory cells, and it appears that at least one of these is responsible for managing the energy budget. In *B. tentaculata*, the cerebral ganglia have large numbers of cells with cytoplasmic inclusions at the onset of reproduction suggesting neurosecretory activity, and these cells are emptied in conditions of constant light, but remain unchanged in constant darkness, also suggesting a neuro-endocrine control of reproduction (Andrews, 1968). While the nature of these secretions is not clear, it has been proposed that Molluscan Insulin-like Peptides found in the cerebral ganglia of *Helisoma duryi* are involved in growth (Khan et al., 1992) and may therefore form a part of the control mechanism. Additionally, serotonin is present in the LGC of *L. stagnalis*, and is thought to

play a role in circadean rhythmicity. A serotonin receptor has also been cloned in this species (Sugamori et al., 1993), and in *B. glabrata* it has been demonstrated that blocking this receptor with methiothepin significantly reduces egg laying (Muschamp and Fong, 2001). This is convincing evidence that serotonin is involved in reproductive control of gastropods, particularly as it also governs spawning in bivalves.

In terrestrial pulmonates, there is some evidence that steroid hormones are also able to influence reproduction. In *H. aspersa*, both photoperiod and temperature influence gonadal development, with short days inhibiting spermatogenesis (Medina et al., 1998) and low temperatures inhibiting vitellogenesis (Griffond et al., 1992). However, Csaba and Bierbauer (1979) found that injections of oestrone and progesterone administered to *Helix pomatia* 'at the autumnal period of their life cycle' caused a significant increase in the number of oocyte follicles in the gonad. Similarly, Takeda (1979) injected the slugs *Decoceras reticulatus* and *Limax flactus* with 17β-oestradiol and oestrone, and found that egg-laying was increased (Figure 89). He also notes that immature ova were oviposited, suggesting that 17β-oestradiol was overriding the control of ovum assembly. Testosterone had no effect in either case.

Gooding and LeBlanc (2004) injected the prosobranch *I. obsoleta* with testosterone and found that the levels of esterified steroid increased in proportion to the amount injected, while free steroid levels remained constant. They proposed that esterified steroids provide an inactive steroid reservoir that can be mobilized for reproductive purposes. Sternberg et al. (2008b) went on to study the seasonal fluctuations of both testosterone and 17β-oestradiol in this species, and found that in males, total testosterone was relatively high at the start of recrudescence, and declined over the reproductive season, while it was fairly constant in females. In contrast, females had a relatively high level of 17β-oestradiol in dormancy, and low levels during reproduction, while in males the levels were higher and constant. This suggests that steroids are playing a role in the seasonal control of reproduction, but it is not clear exactly how they are

utilized. This is an important question as the seasonal variation of biological products does not necessarily equate to a role in reproduction. For instance, in bivalves there are seasonal changes in the levels of CYP1A-like enzymes (Wootton et al., 1996) anti-oxidants (glutathione, vitamin E and carotenoids) and anti-oxidant enzymes (superoxide dimutase, catalases and peroxidises) in *M. edulis* (Viarengo et al., 1991) and also in *Perna perna* (Filho et al., 2001). These activities may be related to an increased metabolic rate, which in turn may be due to an increased food supply, but this is not directly relatable to reproduction.

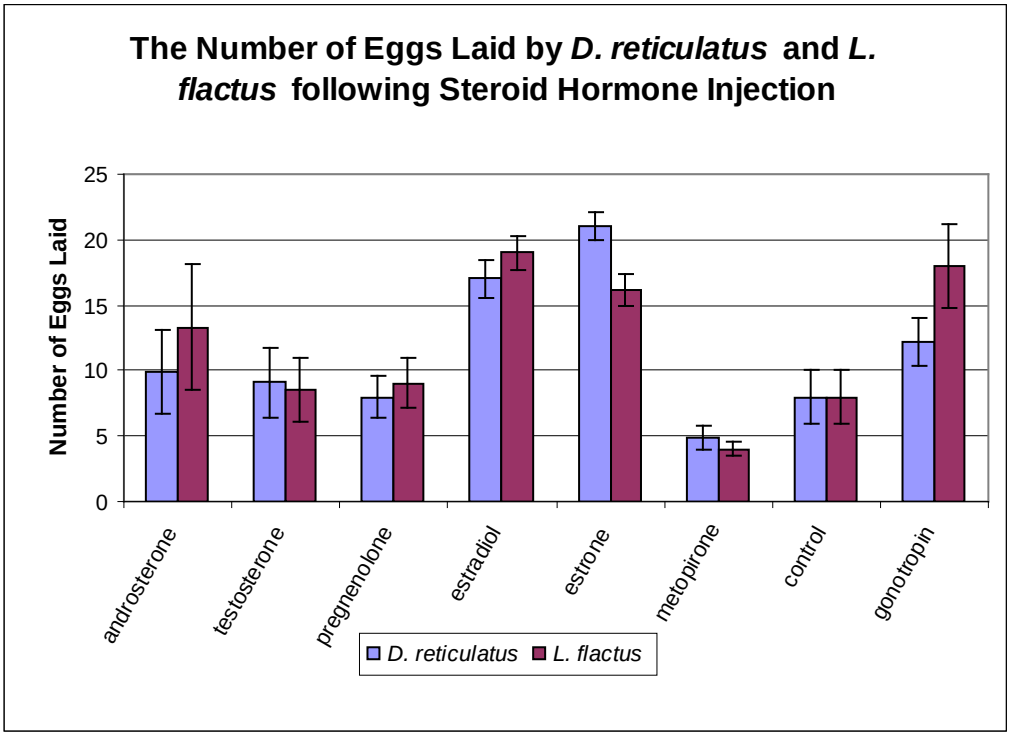


Figure 89 – Increased numbers of eggs laid by *D. reticulatus* and *L. flactus* following injection with steroid hormones in autumnal conditions (plotted from Takeda, 1979). Error bars represent the standard deviation.

Conceptually, steroid levels are better linked to reproduction, but nonetheless it is necessary to elucidate a mode of action to confirm this. Sternberg et al. (2008b) also looked for steroid receptors in *I. obsoleta*, and sequenced an estrogen receptor that was closely homologous to those sequenced for *T. clavigera* (Kajiwara et al., 2006) and *A. californica* (Thornton et al, 2003). There was no

evidence for an androgen receptor, and this is in accord with the concept that androgen receptors are a more recent vertebrate novelty (Thornton, 2001). However, all the estrogen receptors sequenced from molluscs to date appear to be constitutively active. Therefore it does not appear that either testosterone or 17β-oestradiol exert influence on reproduction via a nuclear receptor pathway. However the evolution of nuclear receptors is not fully understood and it may be that while molluscs have conserved early receptor forms (and the RXR and steroid sub-families are closely related; Laudet et al., 1992), they may not operate in the same way as 'modern' vertebrate receptors.

Escriva et al. (1997) considered a model proposing that nuclear receptors evolved as unliganded orphan receptors which suggests that the conformational change that governs the activity of the receptor can be achieved by other means. They thought the model supportable since the ligands used within the nuclear receptor families are unrelated compounds that are unlikely to have evolved from a single chemical species. Sternberg et al. (2008b) found that the estrogen receptor mRNA of male *I. obsoleta* was elevated at recrudescence, suggesting that it may be involved in signalling for reproduction, but it does not appear to need a steroid ligand to do so. Signalling may be achieved simply by the quantitative expression of the receptor in the tissues. Castro et al. (2007b) found that expression of an estrogen receptor ortholog in *N. lapillus* was elevated after exposure to low dilutions of sewage effluent, suggesting a route of steroid interaction with gastropod reproduction. However the oestrogenic load of the effluent was very low (0.014 – 0.056 ngL^{-1} 17β-oestradiol and 0.011 – 0.042 μgL^{-1} alkylphenols at the applied dilution), there was no dose-dependency, and only the lowest concentration showed significant elevation, so this was not conclusive.

The alternative hypotheses are that either there is an additional nuclear receptor other than vertebrate-type sex steroid receptors that uses the steroid hormone(s) as a ligand, or that the steroid is used for signalling in a manner unrelated to nuclear receptors. Very recent information suggests that the former is a possibility. Katsu et al. (2010) isolated nuclear receptors from the

cephalochordate *Branchiostoma belcheri*, finding not only an estrogen receptor ortholog, but also an ancestral steroid receptor that has identity with the androgen, progesterone and corticosteroid receptors. They found that while the estrogen receptor ortholog did not activate reporter genes in the presence of 17β-oestradiol and oestrone, the ancestral steroid receptor did so in a dose-dependant manner. Interestingly, the estrogen receptor otholog behaved as a negative regulator of the ancestral steroid receptor, suggesting that the early forms of nuclear receptors may have played different roles in early vertebrates, and this may also be the case in molluscs.

Finally, Thornton (2001) considers that the ancestral receptor evolved to exploit ligands that were already present in the cell, and proposes that 17β-oestradiol already had a signalling role independent of receptors, a conclusion drawn from the presence of steroids in early metazoans. The majority of steps involved in vertebrate steroid biosynthesis have been indicated in molluscs using a variety of approaches (including radioimmunoassay, enzyme-linked immunosorbent assay and high-performance liquid chromatography), although not all in the same species or even class (Janer and Porte, 2007; Figure 90). 17β-oestradiol is the ultimate steroid compound in the biosynthetic pathway and it has been shown that it has a non-genomic cell-surface signalling role in *M. edulis* (Canesi et al., 2004a). However, there is currently no evidence for a mechanism of this nature in the control of reproduction in gastropods.

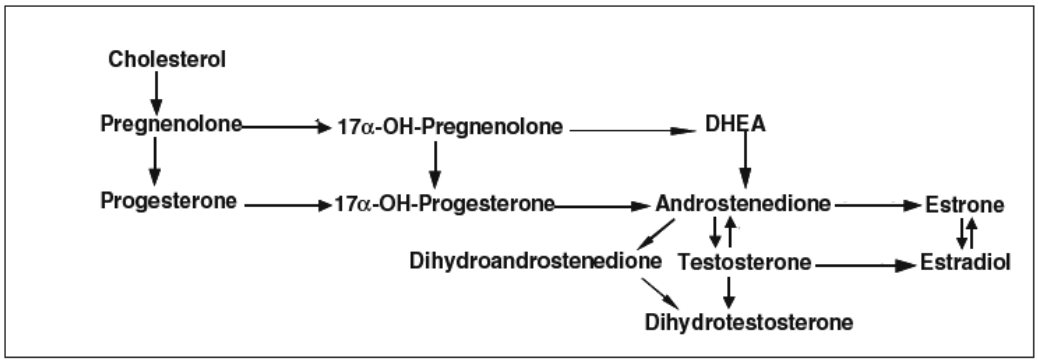


Figure 90 – The steroid compounds and biosynthetic pathways that have been indicated in molluscs (adapted from Janer and Porte, 2007).

Overall, lack of a clear mode of action means that any putative roles for 17β-oestradiol in gastropod reproduction can only be postulated from the sparse evidence outlined above. In bivalves, increasing concentrations of endogenous 17β-oestradiol is associated with ovarian maturation (Matsumoto et al., 1997) injected 17β-oestradiol significantly increases oocyte size (Li et al., 1998) and the levels of vitellin-like proteins in the follicles (Osada et al., 2003). These effects indicate a role in vitellogenesis. There is no equivalent evidence in gastropods, although 17β-oestradiol does increase follicle number (Csaba and Bierbauer, 1979) and egg number (Takeda, 1979). The difference between bivalve and gastropod oogenesis is that gastropod eggs are not heavily yolked, instead depending on peri-vitelline fluid provided by the albumin gland (de Jong-Brink and Geraerts, 1982; Dreon, 2002). Therefore in gastropods 17β-oestradiol may influence albumin gland galactogenesis as the equivalent vitellogenic pathway.

It is also possible that 17β-oestradiol is held in an inactive pool during the dormant period in females, and is mobilised for reproduction in the spring, depleting the reservoir (Sternberg et al., 2008b). However there is no evidence to suggest the mode of signalling. Osada et al. (1998) suggest that 17β-oestradiol causes an increase in serotonin receptors on the surface of oocytes in bivalves, with sertonergia subsequently triggering spawning. Serotonin has also been implicated in gastropod egg-laying (Muschamp and Fong, 2001), and so 17β-oestradiol may have a similar role in this class. It would require a rapid signal as egg-mass preparation occurs within 2 hours in *L. stagnalis* (Ferguson et al., 2003), and therefore signalling for oviposition may be non-genomic as described by Canesi et al. (2004a) in *M. edulis.* It is therefore possible that 17β-oestradiol affects the quantity, quality (peri-vitellogenic status) and delivery of gastropod ova, although none of these hypotheses are confirmed.

A final consideration is the route by which exposure to exogenous 17β-oestradiol could disturb the seasonal reproductive pattern and cause the changes observed in this research project (i.e. increased offspring production or perpetuation of egg-

laying into the autumn). It is possible that a step in the neuro-endocrine control of reproduction causes an esterified reservoir of 17β-oestradiol to be gradually converted to the free steroid in the spring and in turn signals vitellogenesis in the albumin gland. The amount of galactogen then available for egg production would therefore be determined by either the amount of 17β-oestradiol available in the free form or the amount of resource available for glycogenesis / galactogenesis. Since there is evidence that a mechanism for the curtailment of breeding activity does exist, at least in iteroparous prosobranchs (Browne and Russell-Hunter, 1978), it is unlikely that this is controlled simply by resource availability as this is often abundant even after reproductive activity has ceased.

If vitellogenesis is controlled by 17β-oestradiol signalling, the conversion from esterified to free steroid should be reduced after the summer solstice, cued by reducing day-length when reproductive activity declines. Consequently, the organisms will not be 'expecting' a signal after this time. However, further exposure to exogenous 17β-oestradiol may prolong signalling for vitellogenesis into late summer and autumn. The albumin gland may also then continue to process peri-vitelline fluid for ovum assembly, causing an induction or perpetuation of reproduction beyond the time when reproduction would normally cease. As this is probably the most costly resource in female reproduction, it is possible that there are consequent adverse repercussions from this expenditure of energy for the individual. Any prediction of population level effects would by necessity be unsubstantiated because as yet no field monitoring has been undertaken to assess natural gastropod populations that are potentially exposed to 17β-oestradiol. However, it is conceivable that in semelparous species the penalty of additional reproduction would be no greater than that of a reckless strategy, and indeed recklessness itself may be related to a signal volume that cannot be over-ruled. The individual would experience an earlier mortality than might otherwise be expected, but since these organisms have reproduced successfully in the spring and summer and are not themselves likely to survive the winter, there is no obvious deleterious outcome from the additional energy expenditure.

The consequences for iteroparous species might be considerably more severe. It is likely that individuals of this species need to conserve energy for additional growth, for over-winter survival and for reproduction early in the following spring. As in semelparous species, this may not be detrimental in the final year of reproduction, but it may impact the survival of females in the early years of reproductive activity, decreasing the chance of survival and/or continued successful reproduction in subsequent years; in short, reducing female fitness. In K-selected species that devote less overall energy to reproduction, this could cause $r < 0$ and the population to decline. Any increase in F1 mortality would also contribute to this outcome. In r-selected semelparous species, the loss of autumn-laid eggs due to the onset of adverse conditions is likely to be inconsequential provided that the more numerous spring-laid eggs developed successfully. However loss of eggs in this manner in K-selected species would represent a greater proportion of the total eggs per female, and therefore a greater impact at the population level.

In viviparid species, this 'loss per unit of reproductive effort' is greater still due to the parental energy invested in brooding the embryos. When the *V. viviparus* were dissected at the end of the mesocosm experiments (autumn), there were more embryos in the brood pouch than had been released during the spring and summer. As few further embryos are likely to be released after this time, it appears that this species harbours many embryos over winter. Viviparous gastropods should not reproduce recklessly according to Calow (1978), as survival of the adult is paramount in order that future off-spring are successfully brooded, and therefore weakened adults may also re-absorb or abort embryos over the winter, further reducing the number of juveniles contributed to the population. However there are many layers of assumption in this argument, and unless the adults are clearly adversely affected, it is difficult to demonstrate population level consequences.

Finally, the best case would be that the quality of the spring-born off-spring from all the 17β-oestradiol exposed species is unaffected. However, the worst case is that these juveniles are also adversely impacted by parental or developmental exposure. Takeda (1979) noted that slugs injected with 17β-oestradiol oviposited immature ova, which is likely to be detrimental for hatching success. Also, Baynes (2009) over-wintered samples of developmentally-exposed juvenile *P. corneus* and *V. viviparus* from the mesocosm exposures, and found that the exposed F1 *P. corneus* laid less than half the number of eggs the following year compared to the reference group, and the exposed *V. viviparus* had significantly less oocytes and a significantly lower spermatogenesis score per slide in gonadal histological analyses. Therefore, while it may not be straight-forward to identify an adverse outcome associated with reproductive induction in the affected adult, it is apparent that exposure to 17β-oestradiol can impact both semelparous and iteroparous species in the second generation.

8.2 Bisphenol-A as an Environmental Hazard for Gastropods

The oestrogenic properties of Bisphenol-A have come under increasing attention from regulatory bodies during the life of this research project, both as a human risk factor and as an environmental hazard. However many of the published effects are not without controversy, both in mammalian studies where 'low-dose effects' are recorded (effects that are apparent only at pico- or nanomolar levels that may occur through non-genomic pathways, reviewed by Myers et al., 2009), and in environmental studies where exposed populations of *Xenopus laevis* tadpoles developed skewed sex ratios on one occasion, yet not in repeat exposures by other researchers (reviewed by Crain et al., 2007). This is a parallel situation with exposures of the prosobranch *M. cornuarietis*, where Oehlmann et al. (2000a; 2006) reports the superfeminisation effects described (Chapter 1, Section 1.1), but Forbes et al. (2007a; 2007b) was unable to reproduce them.

Unfortunately the exposures of temperate freshwater gastropods made in this research project can neither fully support nor refute either of the outcomes reported for *M. cornuarietis*. The observed changes on exposure to Bisphenol-A, while not significant, were divergent between the two gastropod sub-classes; reproduction in the pulmonate *P. corneus* was marginally inhibited, while it was marginally induced in both of the prosobranch species. The results of the pulmonate exposure clearly do not support the concept of superfeminisation, yet from the species tested, *P. corneus* showed a greater sensitivity to 17β-oestradiol in terms of enhanced reproductive activity than the prosobranchs, so a similar sensitivity to oestrogen mimicking compounds might have been expected. It may that Bisphenol-A does not have the same mode of action as 17β-oestradiol; for instance, Katsu et al. (2010) found that Bisphenol-A did not induce estrogen response element-dependant transactivation in the cephalochordate *B. belcheri* as the natural steroids did. It is also possible that Bisphenol-A acted as a toxin instead of an oestrogen; Li et al. (2008) showed that in *Bellamya purificata*, concentrations of 1-1100 μgL^{-1} dose-dependently and significantly decreased gill and digestive gland glutathione levels and significantly increased glutathione-S-transferase activity, suggesting that energetic mechanisms were being activated for protective purposes. Schirling et al. (2006), showed that exposure to 100 μgL^{-1} significantly decreased the heart rate in unhatched embryos of *M. cornuarietis*. There is also some evidence for an inhibitory effect of reproduction in fish; Lahnsteiner et al., (2005) demonstrated that late pre-ovulation exposure to environmental levels of Bisphenol-A (up to 5 μgL^{-1}) delayed ovulation and the autumnal spawning dates in brown trout (*Salmo trutta f. fario*) by several weeks. It is therefore possible that in some species, exposure to Bisphenol-A can cause reduced or delayed ovipository activity despite the oestrogenic potential of this compound. However, it is not clear whether this is a toxicological or an endocrinological effect.

In contrast, the results from the prosobranch exposures tentatively support the superfeminisation effects observed in *M. cornuarietis* but this may not be surprising given the very different life histories and strategies of pulmonates and

prosobranchs. Conventionally, the lack of any significant differences between the treatment groups and the control for the end-points assessed suggest that Bisphenol-A does not affect reproduction in the species tested even at concentrations approximately x10 higher than might be expected in the environment. However the power of the tests, when reviewed retrospectively, was potentially too low to have reliably detected a biologically significant effect. The power of the *B. tentaculata* exposure to detect significant change at the highest concentration tested was 67.4%, which while not abysmal, was not as high as is usually demanded (80 – 95%; Thomas and Juannes, 1996). The power to detect a significant change in the *P. antipodarum* test at 28 days was lower still at 60.9%. It is therefore possible that biologically significant changes were not detected in these tests for lack of sufficient power. Therefore repeated exposures should be made to better understand the potential of Bisphenol-A to interfere with the normal patterns of reproduction in temperate freshwater prosobranchs.

There are three alternative interpretations of the test mostly clearly indicating a reproductive induction following Bisphenol-A exposure; this being the *B. tentaculata* test in which the group exposed to the highest concentration (20 ngL^{-1}, nominal) laid more than twice the number of eggs laid by the control group. The first is that this test, albeit low powered, did not detect any significant differences because there were no differences. While it is a relatively straight-forward matter to state that significant differences from the control have been observed and consequently that the hypothesis is supported, it is always more difficult to draw the conclusion that there is no overall effect of the test compound and determine that the null hypothesis is therefore proven. However, there is a necessity to be fair to the test compound which may have high commercial value, and indeed this may be the outcome of further testing. However there is also a pressing environmental need not to erroneously determine Bisphenol-A as a 'safe' compound especially in the light of evidence of more overt reproductive effects in other species. The other two foreseeable potential outcomes are that (a) the responses observed in the highest concentration are different to the control group, or that (b) the responses of all of

the concentrations tested are the same as each other and different to the control group, implying that the threshold of effect is lower still. If the former is true, then further testing should establish a LOEC similar to the highest tested concentration, which would suggest that these prosobranchs are not at risk from environmental concentrations of Bisphenol-A.

Finally, outcome (b) requires consideration of the reasons for the observed similarity in the responses of the Bisphenol-A treated groups. If an alteration of the physical test conditions by the compound can be excluded, this phenomena could be due to two further alternative hypotheses. Either the response can only be induced to a limited extent, perhaps because of limited available energy, or it is a response to a metabolite or degradation product that is present in a uniform amount. The latter does not seem likely if the mechanism is an ER-like receptor, as the metabolite Bisphenol-A glucuronide does not displace 17β-oestradiol in binding assays (Matthews et al., 2001), and the biodegradation products from the major (*p*-hydroxyacetophenone, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid) and minor (2,3-bis(4-hydroxyphenyl)-1,2-propanediol and *p*-hydroxyphenaycl) breakdown routes are much less oestrogenic in yeast oestrogenicity screens than Bisphenol-A (Ike et al., 2002).

Therefore the possibility remains that the lowest tested concentration of Bisphenol-A tested (0.2 μgL^{-1} nominal, 0.133 μgL^{-1} mean measured) is able to maximally induce the autumnal reproductive capacity of *B. tentaculata* according to the energy available, with the potential consequences for iteroparous species outlined in Section 8.1. This premise can only be drawn very tentatively as the effect is only significant if the mean reproduction in all of the treatment groups is combined, and this was not the *a priori* intention. Further exposures are certainly required for confirmation. However, if further evidence indicates that this is a real effect and the threshold is lower than the concentrations tested, then the superfeminisation observed in *M. cornuarietis* at very low concentrations of Bisphenol-A (LOEC = 0.048 μgL^{-1}; Oehlmann et al., 2006) may be extendable to *B. tentaculata*. This may also apply to *P. antipodarum* which demonstrated the

greatest increase in embryo production at the lowest concentration tested. If this is the case, then there is cause for concern for wild population of these species that are environmentally exposed to low levels of Bisphenol-A. As Oehlmann et al. (2007) concludes, prosobranchs may have an exceptionally high susceptibility to xeno-estrogens, and this combined with their low fecundity and limited mobility, may have population level consequences.

8.3 Gastropod Reproductive Variability and Parasitisation.

Despite the varying test conditions in the mesocosm exposures and the use of wild-caught organisms, the retrospectively calculated COV of reproduction in the reference groups was relatively low. In the *P. corneus* exposures, it was 19.0% and 24.5% in the first and second occasions respectively. It was higher in the first *V. viviparus* exposure (33.7%), but this was expected as the number of off-spring per adult is much lower than in an egg-laying species, and variation tends to increase with decreasing sample size. Also, the gender was not determinable in the organisms that died during the test. The *B. tentaculata* exposure was also confounded by an inability to pre-determine gender, which was of particular detriment as approximately 50% of the test organisms died or escaped during the exposure. Nonetheless, the control group COV was 25.0%, and in the second *V. viviparus* exposure it was only 15.3%. Overall, these values indicated that reproduction in the selected species was not excessively variable in comparison with *P. promelas* (mean control COV estimated at 21% from Harries et al., 2000) and therefore that wild-caught organisms could potentially be used in laboratory exposures given that it was not possible to successfully culture sufficient animals within the time and cost constraints of the research project.

However, the COV of the control groups in the laboratory exposures of *P. corneus* and *B. tentaculata* were higher than expected, making the identification of differences in reproduction on exposure to oestrogenic compounds less likely. In the *P. corneus* exposure made in simulated summer, the COV was tolerable at

26.6%, but in all the exposures made in simulated autumn it was higher; in the exposure to 17β-oestradiol it was 48.9%, and in the Bisphenol-A exposures it was 33.7% and 40.3% on the first and second occasions, respectively. This may not be surprising given that the number of eggs laid per adult is fewer in cooler conditions with a shorter photoperiod. Nonetheless, because increasing variation compromises the power of the test, it is necessary to consider all possible steps to reduce the potential sources of inter-individual or inter-group variation.

In these exposures, the history of the wild-caught test organisms was unknown. Factors including age, reproductive condition and/or parasitisation status of the test population are likely to account for a proportion of the observed variation. Costil and Daguzan (1995) recorded that *P. corneus* ceases to lay eggs up to 40 weeks before mortality occurs when kept in controlled laboratory conditions for the whole life-cycle. It is therefore possible that some of the test organisms used were at, or close to, this stage of life when caught, and this may have contributed to some groups being consistently less fecund than others. Clearly, an improvement would be to breed and use test organisms of a known age. Reproductive condition in hermaphrodites is more difficult to control for, and may depend on group size and familiarity. Koene (2006) reanalysed deVisser et al. (1994) to show that in *L. stagnalis*, reproduction as a male is as energetically 'expensive' as reproduction as a female. The males perform an elaborate mating behaviour and produce copious amounts of sperm on mating. He hypothesizes that snails introduced into new groups prefer to act as males, in order to maximise their genetic impact, and he suggests that snails are able to discriminate partners to whom they have previously donated sperm, becoming less willing to re-mate with them. Female reproduction may therefore be the 'default' process, and consequently group dynamics may play an important part in hermaphrodite egg-laying behaviour.

The parasitisation status of individuals has the potential to account for a large proportion of the observed variation in the reproduction of freshwater gastropods. While the proportion of parasitized *P. corneus* observed in these exposures was

not high, it would clearly have been better to exclude them from the outset, as it is likely that their reproductive performance was affected. Trematode parasites use molluscs as intermediate hosts, using the host resources to multiply before leaving the depleted host and migrating to the definitive host (Llewellyn, 1965). It is in the interest of the parasite to bring about a cessation of reproductive activity (castration) in the intermediate host in order to divert energy for its own use. This is achieved in a number of ways; in *L. truncatula* infected with *Fasciola hepatica*, castration occurs within 21 days of infection by direct consumption of the ovotestis by the rediae (Wilson and Denison, 1980). This can cause gigantism as the excess energy is directed towards growth, no doubt also in the favour of the parasite. However, Sullivan et al. (1995) noted that castration can take other forms in *I. obsoleta*; the gonads can be immature in infected adults, or present but devoid of gametes, and also castrated individuals occur that have only light infections. They hypothesise that castration can be caused by physical gonadal starvation by chemical interference with gonadotrophins or other means (see Figure 91).

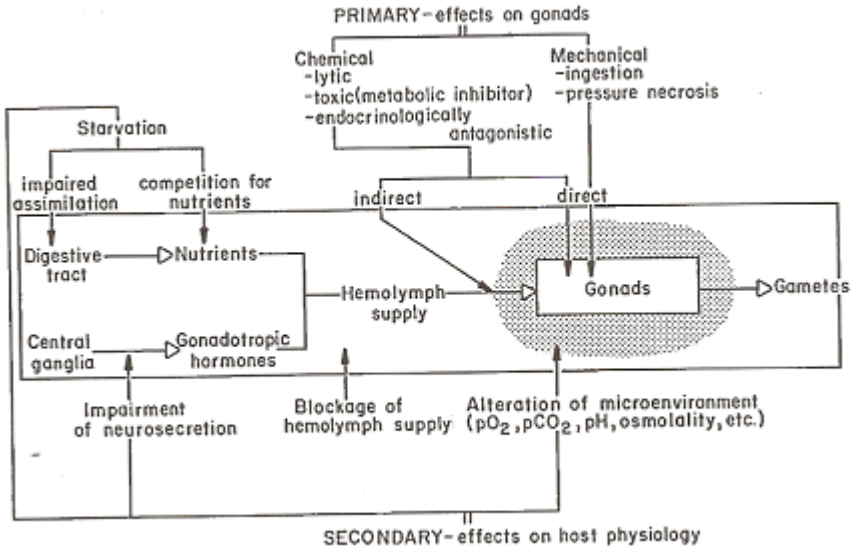


Figure 91 – A schematic of the potential pathways by which parasites may inhibit reproduction in gastropods (after Sullivan et al., 1995).

While it is relatively straight-forward to justify the exclusion of any overtly parasitized and probably castrated organisms from the analysis of these studies on

the basis that the effect is similar to a mortality, it is more difficult if the affected organisms have the potential to alter their reproductive behaviour in the early stages of infection or even just in the presence of parasites. Gastropods use chemoreception to avoid certain plants and predators (Duval et al., 1994) and to follow sexually active opposite sex individuals (Straw and Rittschof, 2004), therefore it is possible that they also discriminate and respond to the chemical characteristics of parasites. Minchella and Loverde (1981) found that *B. glabrata* exposed to *Schistosoma mansoni* showed an increased rate of oviposition, whether or not they were actually infected. Thornhill et al. (1986) went on to establish that this increase only lasted for two weeks, before castration occurred, but that the effect lasted longer in uninfected snails. It is thought to be a counter-adaptation response to the potential of parasitic infection, and while it does not appear to be at the cost of growth, it may be detrimental to future reproduction. However, this response in the presence of parasites is likely to affect the outcome of reproduction tests and as such is another reason to use cultured F1 organisms in future testing. By exclusion of the definitive host in culture, the test population is ensured as parasite free.

This may not be so straight-forward to achieve in *B. tentaculata*. This species is also infected by at least eight species of trematode (Ataev et al., 2002), and can be castrated by physical destruction of the gonads by radiae (Reader, 1971), but infection rates in populations tend to be low. Morley et al. (2004) found 4.7% infected with metacercariae and 7.7% with cercariae in a closed water body (gravel pit). In the mesocosm exposure, 7.2% of the test organisms were observed to be harbouring parasites, but the species was an oligochaete that resided under the mantle rather than in the gonad of the snail. The infection rate was unexpectedly higher in the laboratory exposures, the highest being 41% in the 17β-oestradiol exposure. Moreover, the infection pattern seemed to be associated with pairs, and in the Bisphenol-A exposure it was concentrated within certain test vessels. This lead to the suspicion that the parasites were passing between the hosts independently of another vector. Young (1974) studied the presence of the commensal sub-species *Chaetogaster limnaei limnaei* in *B.*

tentaculata, and found similar infection rates. They increased from <3% in early spring to >70% in summer, this being the same pattern as that observed in the sacrificed partners of snails that died during the 17β-oestradiol exposure. He also observed this species leaving the host, and cites between host migrations. While a positive identification of the oligochaete present in this research project was not made, it seems likely that the 'parasite' observed in *B. tentaculata* was *C. limnaei limnaei* (although there is also an endoparasitic subspecies; *C. limnaei vaghini*).

If this supposition is correct, it may be that reproduction in *B. tentaculata* is unaffected by the presence of *C. limnaei limnaei*. Indeed, some of the few successfully reproducing snails in the 17β-oestradiol study were infected. However, to exclude the oligochaetes, raising juveniles in a mixed culture will not be sufficient, rather it will be necessary to separate out and clean egg masses. The outcome of the exposures made here are not altered whether the infected animals are included or excluded, and thus the observed variation does not appear to stem from this 'affliction'. It is more probable that variability of reproduction between individuals of this species is driven by the amount of effort expended according to age. Because *B. tentaculata* is iteroparous, it will be necessary to culture organisms and develop an understanding of their life-history and consequently the comparability of reproduction in different age classes. This may also apply to *V. viviparus*, but ironically it may not be relevant in the short-lived and easily cultured *P. antipodarum*. The latter species also has no parasitism concerns, because while the related European small hydrobiids are often heavily parasitized and breed early to compensate (e.g. Sola, 1996) the asexually reproducing *P. antipodarum* is free of parasites in Europe (Jokela and Lively, 1995).

The increased reproduction of gastropods in response to potential parasitic infection may confound assessments of reproduction, but it might also provide further insight into the mechanisms by which gastropods control reproductive activity and therefore the potential for interference from endocrine active compounds. It appears that gastropods can both detect parasites and respond to

that signal, increasing the rate of oviposition accordingly. Hordijk et al. (1991a; 1991b) radiolabelled calfluxin, the female gonadotrophic neuropeptide that is responsible for stimulating secretions of proteins and galactogen in the albumin gland. They found that binding to the receptor (guanyl nucleotide binding protein) was inhibited in the presence of both haemolymph from infected *L. stagnalis* and purified schistosomin, another neuropeptide. This compound was found to both inhibit the firing pattern of the caudo-dorsal calls and excite the LGC, and therefore over-exposure to increased levels of schistosomin derived from the parasite may be a potential mechanism for parasitically controlled reproductive inhibition and gigantism (Hordijk et al, 1991c; 1992). However it was isolatable from both infected and non-infected snails, and is therefore likely to be involved in the suppression of reproduction more generally in adverse conditions. Conversely, a reduced schistosomin level is also a possible cause of reproductive induction.

de Jong-Brink et al. (2001) found that the gene encoding Caudo-dorsal Cell Hormone is up-regulated following parasite infestation, which may be part of the mechanism by which reproductive activity is initially increased. However as increased egg-laying is also recorded in uninfected snails in the presence of parasites, it seems unlikely that increased levels of schistosomin, this being inhibitory, is the signal for this process. These researchers also found that incubation of the penis tip with parasite secretions inhibited penis growth. This suggests a possible interaction with the retinoic acid and RXR signalling pathway, as this is the most likely route of signalling for penis development in male and in imposex female gastropods (Nishikawa et al. 2004; Horiguchi et al., 2007). It is also supported to some degree by Rato et al. (2009), observing that parasitized male and imposex female *N. reticulata* tend to have a smaller penis, and that this may affect the RSI score. de Mendonca et al. (2000) also sequenced the RXR receptor in *S. mansoni*, but Bouton et al. (2005), consider that although the *B. glabrata* RXR transactivates transcription in the presence of 9-cis-retinoic acid, it is unlikely that the *S. mansoni* receptor can do the same as it is poorly conserved in the ligand binding domain. Nonetheless, they thought that as this

receptor is present in both parasite and host, it could be an element of the molecular dialogue between them.

Another possibility is that sterol-based compounds are involved in host-parasite signalling. There is some sparse evidence that ecdysteroids are involved; de Jong-Brink (1989) identified ecdysteroids by radioimmunoassay in *L. stagnalis* and found a differential uptake of injected ecdysteroids by the ovotestis and digestive gland between parasitized and unparasitised snails. However, no evidence for ecdysteroid biosynthesis was found. de Mendonca (2000) also found ecdysteroid conjugates in *S. mansoni*, but again there was no evidence for biosynthesis. It is therefore not clear how these compounds might be utilised, nor why they are present in lophotrochophoran hosts and parasites. Vertebrate type sex steroids are also found in both gastropods and trematodes, but possibly only as traces of testosterone and progesterone in the latter (Schallig et al., 1992). Steroid receptors are present in both host and parasite too (elements of estrogen-related receptors and progesterone receptors have been sequenced from *S. mansoni*, Verjovski-Almeida et al., 2003). However, there is no further support for a steroidal dialogue as in the gastropod at least, the receptors seem to be ligand independent. However, a non-genomic route cannot be ruled out, and it is also possible that the steroid 'balance' of the host is altered by parasitic utilisation of stored energy reserves, affecting the amount of esterified steroids present in the tissues.

Finally, perhaps the most compelling evidence for a mechanism for reproductive responses to parasitic infection is provided by Manger et al. (1996). They exposed severely parasitized *B. glabrata* to serotonin, and found that oviposition was restored to the pre-infection rates. They also measured serotonin levels and found it was suppressed in both infected snails and in those exposed to *S. mansoni* but remaining uninfected. Exposure of uninfected snails to serotonin also increased the oviposition rate. It appears that serotonin increases and schistosomin inhibits reproductive activity, and it is therefore possible that the action of both of these compounds in combination controls the oviposition rate in

gastropods. However, how parasites might manipulate this balance to cause the cessation of laying remains unclear, as does how the counter-adaptation to parasites might increase egg laying, or indeed how endocrine active compounds might interfere. Overall, while parasitism does not appear to strongly influence the outcome of exposure to endocrine disruptive substance in fish (Jobling and Tyler, 2003b), even the presence of parasites can potentially initiate reproductive compensation responses in gastropods, and therefore it is of high importance to develop cultures of parasite free organisms that can be used in reproduction tests.

8.4 Further Work and Development of Future Test Methods.

In the past decade, the need for a variety of validated test end-points for endocrine disruption encompassing a range of invertebrate taxa has been widely recognised (De Fur et al., 1999). When progress was reviewed in 2007 (Weltje and Schulte-Oehlmann), it was acknowledged that advances were being made in understanding the effects of exposure to endocrine disrupting substances in selected groups, including Crustacea, Insecta, Nemotoda, Mollusca, Cnidaria and Echinodomata, but that formal (OECD) test development was focused on the Arthropoda. Oehlmann and Schulte-Oehlmann (2003) suggested that sentinel species from each major taxon should be identified, and considered that prosobranchs were suitable candidates within the Mollusca as vertebrate-type steroids appeared to have a functional role in this group. Oehlmann et al. (2000b) had previously proposed *M. cornuarietis* as a biological test system for hormone-mimetic xeno-biotics, including the described effects of the xeno-oestrogens Bisphenol-A and Octylphenol (Oehlmann et al., 2000a). In addition, this species demonstrates imposex on exposure to TPT, methyl-testosterone and also paradoxically 17α-ethinylestradiol. In some cases, the effects of imposex could be reversed or reduced by co-exposure with the anti-androgens cyproterone acetate and vinclozolin (Schulte-Oehlmann et al., 2000; Tillmann et al., 2001).

This wide-ranging response to vertebrate steroids and endocrine disrupting compounds is advantageous in a model invertebrate, but the inconsistency of the responses are difficult to explain and reproductive induction has not yet been repeated by other researchers. This is likely to be due to the circumstances of the exposures; those made by Oehlmann et al. (2000a; 2006) to Bisphenol-A used organisms that had been held in conditions that were cooler than 'optimum' for a (sub-) tropical species over a long period of time. These snails appear to have developed a fluctuating annual cycle of increasing and decreasing oviposition (spawning) rates resembling a seasonally controlled reproductive cycle even at a constant temperature, and the researchers have incorporated these transient changes into the exposure period of their experiments (see Figure 92).

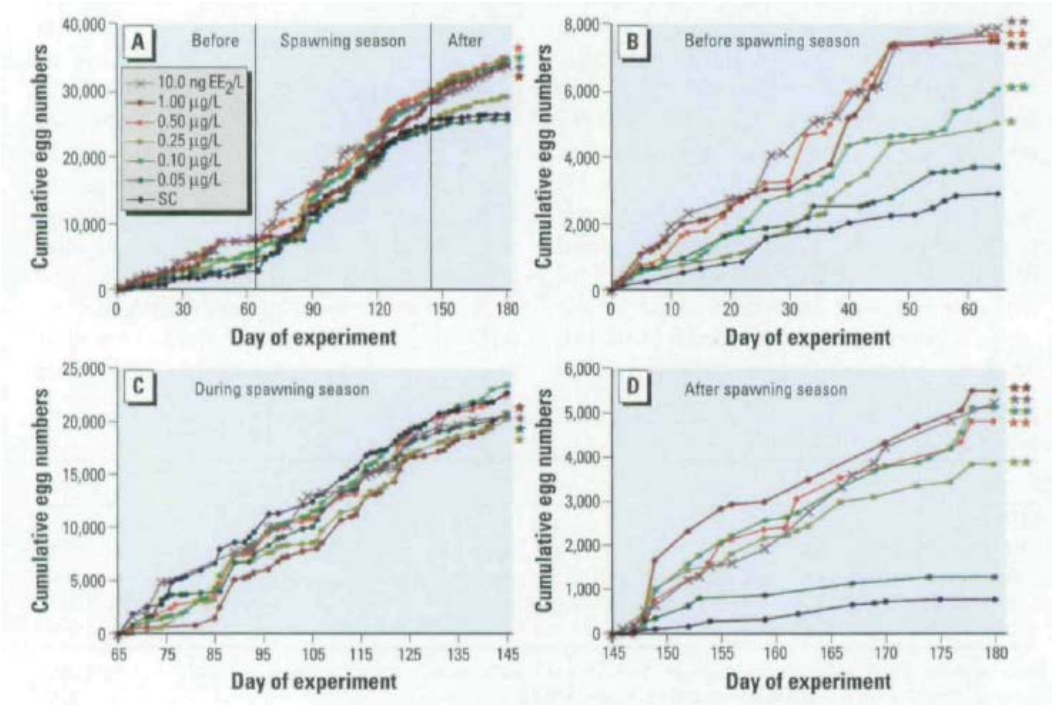


Figure 92 – The results of a 180-day exposure of *M. cornuarietis* to Bisphenol-A (a), incorporating pre-spawning (b), spawning (c) and post-spawning (d) periods (after Oehlmann et al., 2006).

In contrast, Forbes et al. (2008a) did not measure an induction even in cooler conditions, but this is likely to be explainable by the length of the period elapsed between introducing the test organisms to the test conditions and the beginning of

the test. The *M. cornuarietis* were pre-acclimated for 30 days to a temperature only 3^{o}C lower than that used in the culture of this species, allowing the reproductive rate to decline before the exposure to Bisphenol-A began. It appears that an aspect of environmentally-driven change in the reproductive rate of the control group, within the exposure period, may be necessary in order to detect an effect of the test compound on the reproductive rate of exposed gastropods.

All of the exposures of gastropods made in this research project can only be considered as preliminary, given that it was not possible to adequately describe the optimal culture conditions for reproduction and it was therefore not possible to use laboratory-bred snails as test organisms. However, the responses of the three species in the mesocosm exposures indicate that while increases in reproduction above the reference rate can be induced by steroid exposure, this effect again tends to occur only in changing conditions, i.e. at the onset of autumn. The *P. corneus* laboratory exposures also demonstrated that this apparent increase seems to be due to a prolongation of the high level of reproduction previously observed in warm conditions. These results tend to support those of Oehlmann et al. (2006), in that it is possible to observe reproductive induction in other freshwater gastropod species, and the pattern of the responses to some extent explain the reasons that Forbes et al. (2008a) did not. It appears that exposure to oestrogens does not cause reproduction to be re-elevated above control once this decline has taken place, rather the effect seems confined to an inhibition of the natural (seasonal) decline. This is problematic with regard to the development of test methods to utilize this end-point, because it implies that the organisms cannot be acclimated to constant test conditions. Instead, either the observations need to be made during the period of adjustment between different pre-exposure and exposure conditions, or the conditions used in the test must change to induce the required response.

The latter approach, while unconventional, is less likely to cause additional stress to the test organisms; pre-exposure acclimation is intended to allow stressful periods of adjustment to pass before the stress of exposure to the test chemical

begins. Conventionally this is as long as possible to allow all of the biochemical adaptations to take place, and it is generally understood that differing acclimation and test temperatures can alter the toxicity profile of test chemicals. For example, Moller et al. (1994) exposed *P. antipodarum* to cadmium and found that organisms acclimated to an optimal temperature were more susceptible to acute toxicity when exposed at higher and lower temperatures than those acclimated in sub-optimal conditions.

Deliberate pre-stress is also know to have an adverse effect on reproduction in gastropods. Coutellec and Lagadic (2006) reported that reproduction was lower in *L. stagnalis* subjected to periods of low resource, adverse environmental conditions and chemical stress even after return to optimal laboratory conditions. It could therefore be argued that the increased reproduction observed in *M. cornuarietis* and the species used in this research project do not arise from an additional stress response to the test chemical as reproduction was enhanced rather than inhibited. Barata et al (2004) consider that where an end-point such as reproduction is promoted, it is likely to be indicative of an influence on the (endocrine) control mechanisms rather than a stress response. They also conclude that this is more plausible when there are no other indications of stress, i.e. no appreciable mortality, and the health status of the organisms is otherwise uncompromised (e.g. growth and condition factor), although a putative mechanism adds further credibility.

While reproductive induction may not generally be a direct stress response, it may be a symptom of reproductive recklessness prior to mortality or a form of reproductive compensation for a 'foreseen' stressor such as parasitisation in some species. In order to exclude these possibilities, unacceptable stresses must be removed from the experimental design. It is relatively straight-forward to exclude parasites and to demonstrate that the health status of the test organisms is within acceptable parameters, i.e. minimal mortality and continuous growth. However, it remains to consider the level of environmental stresses that are 'acceptable', and this will differ depending on the species used. It is difficult to

determine the optimal temperature range for a given species and the degree of deviation from this that might be considered stressful.

Each species has a temperature tolerance zone; ‘a range of environmental temperatures that can be tolerated more or less indefinitely’ (Cairns et al., 1975). This tolerance zone determines the geographical range but it can be shifted within the genetic limits of a species by long-term acclimatization. Sastry (1970) studied latitudinally separated populations of the Bay Scallop *Aequipecten irridians* and observed that breeding only occurs at characteristic temperatures, and that where the species occurred on the edge of the geographical range, breeding was reduced to short periods of the year. Therefore these ‘fringe conditions’ might be sub-optimal. Conversely, the organisms are able to reproduce and maintain a population, so it appears that this level of stress is ‘acceptable’. However, a doctrine of ecotoxicology dictates that the employment of test organisms under sub-optimal conditions should be avoided because the responses of organisms to chemical stressors may become more variable on the outer fringes of the tolerance zone, making reliable experimental replication more difficult to achieve. This is due to the genetic variation that enables a species to evolve and optimise performance in newly available niches. For example, Schrag et al. (1994) observed that *Bulinus truncatus* is euphallic (having a fully functioning male reproductive tract) significantly more often in relatively cool conditions, whereas >90% are aphallic (only able to self-fertilise) in optimal breeding conditions. Therefore for consistent test results, species should only be employed in the centre of their tolerance zone.

However, a special case may be needed for temperate species that are highly adapted to changing climates. Precht et al. (1973) notes that a small rise in temperature after a period of cold can initiate a burst of reproductive activity in some species. It is possible that they have become dependant on this cue, and may fail to breed in constant conditions. *B. tentaculata* may be one such species; Andrews (1968) noted that this species did not reproduce in constant warm temperatures (17-20^{o}C), and the same phenomenon was also observed in the first

part of the exposure of this species to Bisphenol-A (at 16^{o}C). It is possible that *B. tentaculata* does not have an optimum temperature for breeding, rather it has an 'optimal sequence of seasonal change'. If this is the case, then conditions used to test the reproductive response of this species must be adapted to simulate the environmental cues that are required. Nonetheless, it is likely to be more advantageous to use a species that is dependant on seasonal cues, rather than one breeding at the fringe of its temperature tolerance, if the long-term outcome for the major fraction of wild populations is to be understood.

It should also be recognised that (sub-)tropical species may not use the same environmental cues as temperate species; for example, Albrecht et al. (1999) found that while egg-laying was affected by temperature in the apple snail *P. canaliculata*, it was irresponsive to photoperiod, possibly because day length varies less nearer the equator. Temperate species may also have endogenous responses to temperature or light; Kavaliers (1980) showed that *H. trivolis* snails moved between warmer and cooler temperatures daily with or without the presence of a photoperiod, suggesting an endogenous circadian rhythm, and Hahn (2005) suspects that *B. tentaculata* has an endogenous circannual rhythm as it ceases to grow on reaching 7mm in length in the first year, even when held in constant conditions.

All the aspects of experimental design will need further careful consideration when making assessments of reproduction in freshwater gastropods. Stocking density and competition for resources are also likely to strongly influence the outcome of experiments. In general, the higher the number of animals per unit, the lower the reproductive output will be. Gilbert et al. (1986) demonstrated that this is not caused by any chemical depreciation of the water quality. They caged *B. tentaculata* in natural streams and found that both the number and the size of the juveniles produced was less at higher stocking densities. However this effect was strongly related to resource. When they supplemented the cages with food, the number of juveniles increased markedly at all densities. Neiman (2006) thought that a high stocking density negatively affected embryo production in *P.*

antipodarum, but the groups were fed the same amount irrespective of the number of snails present, and the effect may therefore also be attributable to resource availability. Overall, it is important to ensure that the feed rate is adjusted on a per snail basis to ensure that competition for resources is the same between groups. *Ad libitum* feeding can also reduce competitiveness, but it is often not a practical option given that gastropods can exhibit almost continual consumption and large amounts of food can pollute the test vessels.

However, resource competition alone does not entirely account for the suppression of reproduction at high stocking densities. Brown et al. (1994) found that grazing was significantly reduced with increasing density in *Physella virgata*, although algal grazing availability was always in excess. They recorded increased numbers of shell-shaking episodes following inter-snail contact and increased respiration rates in individuals, suggesting that conspecifics can be a cause of stress to each other. Thomas and Benjamin (1974) established an optimum stocking density for *B. glabrata*, above and below which reproduction is reduced (without limited resources). The 'Allee Effect' refers to a population that increases until a non-resource-related threshold density is reached, after which further population growth is suppressed. This suppression can be due to environmental pollution or via individuals suppressing breeding activity via physical or chemical cues. It is therefore necessary to ensure that the optimum group size for the test species is established and not exceeded. However, this optimum can change at different temperatures and with altered activity levels, as observed in *P. corneus* in this research project.

In contrast, there are several additional factors that can influence the reproductive rate in low density groups. Isolated 'virgin' hermaphrodites can exhibit a selfing delay (i.e. reproduction is inhibited in the absence of cross-fertilisation; van Duivenboden, 1983). This can be misinterpreted as an effect of the test chemical, e.g. Leung et al. (2004) observed that *P. fontinalis* laid less eggs when exposed to TBT in isolation than in group exposures, and concluded that grouping alleviates the toxicity of TBT. However it is more likely that early adult hermaphrodites

were employed. The same effect was measured by Vianey-Liaud and Dussart (2002); egg laying was increased in newly mated pairs compared to isolated animals. However, in some cases, isolated hermaphrodites have greater growth and lay more eggs than those kept in groups. Bayomy and Joosse (1987) found this effect was reversible in *B. truncatus* when isolated animals are grouped, suggesting that the effort of the repeated mating in grouped animals reduces the energy available for growth and reproduction. A further layer of complexity is added by Koene et al. (2006b); they demonstrated that *L. stagnalis* is able to lay twice as many eggs if allowed to mate only once a week, being isolated for the remaining time. These researchers consider that individuals are able to identify previous partners and are less willing to re-mate with them (Koene et al., 2006a). Rupp and Woolhouse (1999) also observed that geographic strains within *Biomphalaria pfeifferi* and *B. glabrata* were twice as likely to mate with sympatric as allopatric individuals.

Overall, the necessity to understand the group dynamics of gastropods exposed in test systems is clear, particularly so for pulmonate hermaphrodites. Consequently it may be necessary to ensure that group size is consistent between exposures, to allow for effective comparison. It is also preferable to maintain the group dynamics within the replicate groups of a test. Jordeans et al. (2007) consider that the number of available partners can influence reproductive behaviour due to the number of new opportunities to express male fitness (courtship and mating) and this in turn can affect female fitness and egg-laying behaviour. Therefore it is not advisable to replace individuals that die as new additions are likely to alter this dynamic. It is probably more appropriate to reduce the volume of the test vessel accordingly so that the number of conspecific interactions is maintained. Many of the aspects discussed (group size and dynamics, age and reproductive maturity/condition, source population, resource availability/ competition) are also likely to apply to prosobranchs, but the effects on reproduction are not well researched, possibly due to the difficulties of maintaining and culturing these organisms.

This lack of research is an additional barrier to prosobranch species being effectively used for assessing the reproductive effects of endocrine disruptive compounds. Nonetheless, it may be necessary to overcome this because while there are drivers to develop tests using pulmonate species, particularly in the search for environmentally acceptable control measures for terrestrial pests (Flari and Edwards, 2003; Hall et al., 2009), unless deliberate steps are taken to develop acutely destructive control measures, it is difficult to project adverse population level outcomes for this sub-class due to their essentially annual life-history. However, the OECD simply require a test method that can be used as a screen for substances that have the potential to be endocrine active in invertebrates and that is sufficiently robust to satisfy the Mutual Acceptance of Data criteria (Gourmelon and Ahtianen, 2007), without any recourse to the consequences for wild populations. Matthiessen (2008) had addressed this question, and suggested the use of *L. stagnalis* and *P. antipodarum*, mainly because these species have well-established culture protocols.

However, pulmonates may not be sufficiently sensitive to fulfil even this role. This research project has indicated that the pulmonate *P. corneus* exhibits prolonged reproductive activity when exposed to 17β-oestradiol at concentrations found in treated sewage effluent, but it did not show a similar response when exposed to Bisphenol-A. Also, *L. stagnalis* did not respond to high concentrations of the potent and persistent oestrogen 17α-ethinylestradiol (although this exposure was made in constant 'autumnal' conditions of 15^{o}C, 12 hours light; Casey, 2004). In contrast, the prosobranch *M. cornuarietis* is reported to be highly responsive to Bisphenol-A at low concentrations (Oehlmann et al., 2000a; 2006). The two prosobranchs used in this research project also showed the potential for an increase in reproductive activity when exposed to this compound. It is therefore possible that prosobranchs are more susceptible to certain vertebrate oestrogen mimics than are pulmonates.

The prosobranch recommended by Matthiessen (2008), *P. antipodarum*, has the advantage of being very small, a trait which is recognised as beneficial for use in

routine screening tests (Hutchinson, 2007). However, while it is generally considered advantageous to establish cloned strains for ecotoxicological testing (to reduce the variation in response), this may not be the most appropriate way forward for assessing reproductive effects. *P. antipodarum* is parthenogenetic in Europe, and therefore not subject to a mating system, so that any male effects cannot be assessed. In this regard, a small dioecious species would be more suitable, and *B. tentaculata* fulfils these criteria. It is likely that an assessment of male effects would be relatively straight-forward in this species as the penis is positioned on the dorsal surface behind the head, making it easily accessible for measurement. It is also forked, with an additional prostate area on one branch that may deliver additional fluid to the ejaculate (Lilly, 1953), providing a possible method of studying mating frequency.

It may also be preferable to make assessments over complete invertebrate life-cycles, taking into account all aspects of the reproductive process, especially since it is not yet possible to identify critical periods of sensitivity to endocrine disruptors because invertebrate endocrinology in inadequately described (Segner et al., 2003). In gastropods, there is a need to comprehend the trade-offs between the factors influencing reproduction and how alterations in this balance might impact population stability (Lagadic et al., 2007). This is particularly relevant in iteroparous species that practice reproductive restraint in early breeding seasons in order to conserve energy for over-wintering and growth. To understand the population level impacts of oestrogens in freshwater prosobranchs, it may be necessary to conduct long-term experiments to assess effects in different age classes. This means that the establishment of breeding cultures of prosobranch species with currently ill-defined husbandry requirements may become unavoidable. Reproduction is the most comprehensive measure of organism fitness, and increases above normal reproductive rates may be indicative of (neuro-)endocrine effects, but to obtain unequivocal information on reproductive impacts, a satisfactory understanding of the reproductive activity of a population in culture is required, in order that individual level variability can be reduced to a minimum (Buikema and Benfield, 1979).

8.5 Conclusions

This research project has provided evidence that supports the following conclusions, with particular consideration for the issues identified at the outset (see Section 1.6):

(1) It is possible to measure increases in the reproductive rate of the native European freshwater gastropods, provided the test design is such that control groups are allowed to undergo the seasonal reproductive decline. It is also considered likely that exposure of organisms in relatively cool and constant conditions (in order to reduce reproductive activity) will be insufficient as the expected oviposition rate following an indeterminate acclimation period is difficult to ascertain, and it could also be argued that sub-optimal exposure conditions cause additional stress to the test organisms. However, it may be appropriate, and indeed necessary, to subject certain seasonally reproducing species to a cycle of temperature and photoperiod conditions emulating natural seasons to properly measure effects on reproductive activity.

(2) These measures are most likely to be necessary when exposing iteroparous prosobranchs because these species seem to be more dependant on distinct seasonal cues and/or endogenous rhythms than are pulmonates. In contrast, it seems that the reproductive activity of pulmonates can be manipulated by artificially changing the temperature and photoperiod. It is also possible that the effects are more profound in iteroparous prosobranchs because when reproductive activity is increased or prolonged in the late summer and autumn, the conserved energy intended for over-wintering, growth and later reproduction may be wastefully expended.

(3) Although *M. cornuarietis* is a (sub-)tropical species, this is not relevant to its potential use as a model organism in the European Risk Assessment of Bisphenol-A, as long as it is demonstrably representative of a range of native temperate species. The evidence provided by this research project indicates that

while an increase in reproduction may indeed occur in some European prosobranch species, the effect appears to be related to the seasonal pattern of reproductive activity. Therefore it is not clear whether *M. cornuarietis* is a worthy representative for the most vulnerable seasonally breeding gastropods, as it is capable of reproducing all year round where conditions are suitable (Forbes et al., 2007a). It therefore seems to be an opportunist, more like the r-selected pulmonates than an iteroparous prosobranch. Further information on the life history and reproductive strategy of *M. cornuarietis* is required before it can be properly evaluated as a model species for temperate prosobranchs.

(4) Finally, it remains to be established whether the observed effects on reproduction (and the effects on the survival and quality of the F1 generation) do indeed impact on wild populations of the species assessed. There are two possible ways forward, one being the establishment of large and long-term cultures of prosobranchs with specialised breeding conditions that can be difficult to emulate, followed by full-life cycle experiments that may need to extend over several years. The other is to generate detailed survey data for gastropod species in areas of good and poor water quality to determine whether wild populations can be classified as impoverished at impacted sites. Indeed, invertebrate experts have recommended to the Environment Agency that ‘gastropods and bivalves from a range of sites below sewage works and industrial effluents should be surveyed for evidence of reproductive or other morphological abnormalities’ (Environment Agency, 1999). While the former option may be unavoidable if a cause and effect linkage is to be unequivocally demonstrated, the latter option, adapted to make provision for the current state of knowledge and using existing data to look for evidence of wide-scale impacts, is likely to be of benefit for the identification of vulnerable gastropod groups before further effort is expended on laboratory testing.

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APPENDIX 1
THE SEMI-CONTINUOUS CULTURE OF FRESHWATER ALGAE

SOURCE CULTURES

Source liquid cultures of *Chlorella vulgaris* (strain 211/12) are obtained from the Culture Centre of Algae and Protozoa (CCAP), Institute of Freshwater Ecology, Windermere Laboratory, Far Sawrey, Ambleside, Cumbria. Source cultures, stored at approximately 4^{o}C in the dark to prevent excessive additional growth, will remain viable for 8-12 weeks when new cultures should be obtained. Ensure that contamination of source cultures is avoided by the use of sterile equipment (eg pipette tips) when removing aliquots of source algae. Discard and replace any source culture which displays signs of bacterial contamination, eg if the media discolours or becomes gelatinous.

NUTRIENT MEDIA

The water used in the preparation of nutrient stock solutions and media must be sterilised (autoclaved at 115-121 ^{o}C for 15 minutes) distilled, deionised or reverse osmosis water. Care must also be taken to avoid contamination of the water with inorganic or organic substances, especially copper, during preparation and storage.

The preparation of BBM1 nutrient media initially involves setting up a series of fourteen stock solutions using analytical grade reagents (see Table A over). Sterilisation of the stocks is not required. Store the solutions at ambient temperature in the dark. Freshly prepare stocks 1 to 6 at least every three months and stocks 7 to14 at least yearly.

Table A: Reagents used to Prepare BBM1 Medium

Stock solution	Nutrient	Concentration in stock solution
1	K_2HPO_4	7.5 gL^{-1}
2	KH_2PO_4	17.5 gL^{-1}
3	$MgSO_4.7H_2O$	7.5 gL^{-1}
4	$NaNO_3$	25 gL^{-1}
5	$CaCl_2.2H_2O$	2.5 gL^{-1}
6	NaCl	2.5 gL^{-1}
7	$C_{10}H_{12}N_2O_8Na_4$ + KOH	50 gL^{-1} + 31 gL^{-1}
8	$FeSO_4.7H_2O$ + H_2SO_4 (1)	4.9 gL^{-1} + 1mLL^{-1}
9	H_3BO_3	11.42 gL^{-1}
10	$ZnSO_4.7H_2O$	17.64 gL^{-1}
11	$MnCl_2.4H_2O$	2.92 gL^{-1}
12	$CuSO_4.5H_2O$	3.144 gL^{-1}
13	$Co(NO_3)_2.6H_2O$	1 gL^{-1}
14	$Na_2MoO_4.2H_2O$	2.384 gL^{-1}

(1) H_2SO_4, weight per mL = 1.84 g

Prepare batches of fresh media by adding the following amounts of the stock solutions to sterile distilled, deionised or reverse osmosis water in appropriate sterile glassware:

- stock solutions 1 to 6, add 10 mLL^{-1}
- stock solutions 7 to 9, add 1 mLL^{-1}
- stock solutions 10 to 14, add 0.5 mLL^{-1}

Allow to equilibrate after preparation by leaving to stand overnight or by continual gentle stirring for one hour. The pH of the media after equilibration must be 6.5-7.2. No adjustment of the pH should be required. If the pH of the media is outside the range 6.5-7.2 after equilibration, stir for a further 30 minutes or discard and prepare a new solution.

SMALL-SCALE BATCH CULTURE

Small scale cultures are initially inoculated from the source culture and sub-cultured to provide inoculant of sufficient density for the maturation of large scale cultures over four to five days.

Primary cultures are inoculated directly from the source culture, as follows.

- Add 50 mL nutrient media to a sterile 250 mL glass conical flask.
- Shake the source culture until no algae is present on the base of the tube and, using a sterile pipette, transfer 0.1 mL of inoculant from the source culture into the flask.
- Plug the flask opening with sterile, non-absorbent cotton wool.
- Label primary cultures sequentially as inoculated from each source culture, eg 1.01 is the first primary culture from source 96/01.
- Place in an orbital incubator under continuous fluorescent illumination (6000-10000 lux) at 21±2 ^{o}C.
- After 2-4 days incubation the primary culture should be of sufficient density to sub-culture or use as inoculant for large scale culturing.

To sub-culture, transfer 0.5 mL of the primary culture to 50 mL fresh media and incubate as described above. A line of sub-cultures is thus produced from each primary culture by transferring 0.5 mL of each sub-culture into 50 mL fresh media every 3 to 4 days. Each mature sub-culture may be used as inoculant for large scale culturing. Discard the remaining algal suspension once the inoculant has been removed.

Primary or sub-cultures must only be utilised as large scale culture inoculant or for further sub-culturing if the cell density has reached a sufficient level. This can be determined by measuring the light absorbance of the culture at 440 nm (A440) using a spectrophotometer and should be 0.8 (40 mm path length). Discard primary or sub-cultures which do not attain this minimum density after the required incubation period and set up a new line from the source culture.

Under non-axenic conditions, the process of small scale batch culture should be semi-continuous, each primary culture line being sub-cultured on no more than six occasions before a new primary culture line is set up from the source. The continual renewing of sub-culture lines prevents the accumulation of bacteria and other microorganisms and the gradual reduction in cell numbers over successive sub-cultures owing to lower nutrient availability.

Axenic culture lines may be continuous if complete sterility can be assured at all procedural steps.

LARGE SCALE BATCH PRODUCTION

- Add 1-2 L of nutrient media to a 2-3 L sterile, glass conical flask.
- Using a sterile pipette, transfer 10 mLL^{-1} of inoculant from a mature small scale sub-culture to the flask.
- Plug the opening with sterile, non-absorbent cotton wool.
- Place in an incubator or temperature controlled room under continuous fluorescent lighting (3000 -10000 lux) at 21±2 ^{o}C for four to five days.
- Vigorously aerate the culture throughout the incubation period.
- Label each large scale culture with an identification comprising the date of establishment, a sequential number indicating the number of cultures established on that date
- Record the culture identification, date of inoculation, inoculant identification and media type and pH on the relevant laboratory worksheets.
- After 4-5 days growth, cultures may be stored in the dark at 4 °C for up to 48 hours prior to concentration.

PREPARATION OF ALGAL CONCENTRATES

- Measure and record the A440 of the large-scale culture to be concentrated.
- Concentrate the culture by centrifugation (e.g. 5000 g for 20 minutes) or filtration.
- Resuspend the pellet of cells or filtrate in an appropriate volume of culture media to achieve an absorbance of 2.0 at 440 nm in a 1/10 dilution of the concentrated stock.
- For each batch of algal feed produced, record the culture identification, age of culture on concentration, A440 prior to concentration and volume of feed produced.
- Each batch of algal feed, once concentrated, may be stored in the dark at 2-6 ^{o}C for up to 1 week.

The total organic carbon concentration of standardised algal concentrates can be determined by reference to a standard curve of absorbance against total organic carbon content of algae cells.

APPENDIX 2
EXTRACTS FROM THE ECOSTAT REPORT ON THE STATISTICAL ANALYSIS OF EXPERIMENT 6 (van der Hoeven, 2008).

The statistical analysis of the reproductive end-points.

The main question was whether the average reproductive output of the snails exposed to BPA differed significantly from the reproductive output of the control snails. The complete 5% significance level was allotted to the testing of this question. An additional question was whether the decrease in egg production due to the simulated onset of autumn was significantly different in BPA exposed snails then in control snails. Since no significance was left over to test this question, the testing of this question was only to see whether this question merits further research.

Snails, which are high producers in the pre-exposure periods, are probably snails with a relatively high egg production capacity, and therefore they are expected to have a relatively high egg production in the exposure period too. On the other hand, if the egg production is high in the pre-exposure period, it can decrease more than if it was small. And if the egg production of a group of snails in the pre-exposure period is relatively small, the probability that it happens to be below the long-term average for that group of snails is higher than if the egg production in the pre-exposure period was high. Therefore, the egg production of a snail group with a high pre-exposure reproduction is expected to decrease more than the egg production of a snail group with a low pre-exposure reproduction.

To compensate for these dependencies, the egg production in the treatment period and the rate of change in the egg production were adjusted for the egg production in the pre-exposure period in the same test vessel with an Analysis of Covariance (ANCOVA). The egg production in the period before exposure was the covariance factor in this ANCOVA. Let y_{ij} be the egg production in the *j*-th vessel of the *i*-th treatment ($y_{ij}= R_{ij}(Exp1)$, $y_{ij}= R_{ij}(Exp2)$, or $y_{ij}=R_{ij}(Exp1)+R_{ij}(Exp2)$) or the slope in egg production ($y_{ij}=b_{ij}(Exp1)$, or $y_{ij}=b_{ij}(Exp2)$), then the ANCOVA model is

$$y_{ij} = \mu_i + \beta\left(R_{ij}(pe) - \overline{R}_{i.}(pe)\right) + e_{ij}$$

where μ_i is the expected (covariable-adjusted) treatment mean and e_{ij} a normal distributed error term. Using ANCOVA, the difference between the adjusted treatment means was tested, that is the treatment mean adjusted for the (linear) dependence on the co-variable (the egg production in the pre-exposure period). This adjusted treatment mean was calculated as

$$\overline{y}_{ia} = \overline{y}_{i.} - \hat{\beta}\left(\overline{R}_{i.}(pe) - \overline{R}_{..}(pe)\right)$$

where $\hat{\beta}$ is the estimate of the slope variable β in the ANCOVA model.
For each variable, the differences between the means of the observed value in the control and each of the treatments were calculated after adjustment for the pre-exposure reproduction. The standard deviation of the difference between the control and treatment adjusted mean was calculated as

$$\sqrt{MSE\left(\frac{1}{r_0}+\frac{1}{r_i}\right)\left[1+\frac{T_{xx}}{k\,E_{xx}}\right]} \quad [5]$$

where r_0 is the number of replicates in the control (r_0=9) and r_i the number per treatment (r_i=10), k the number of treatments compared with the same control (k=3), T_{xx} is the Sum of Squares of the "treatment effect" on the pre-exposure reproduction, E_{xx} the residual Sum of Squares of the ANOVA of the pre-exposure reproduction with the treatment levels as factors, and *MSE* is the mean residual SS of the ANCOVA analysis (software: S-plus). This adjusted is only applied if it is efficient to correct for the pre-treatment reproduction. This efficiency is calculated as

$$E=\frac{MSE_r}{MSE\left(\frac{1}{r_0}+\frac{1}{r_i}\right)\left[1+\frac{T_{xx}}{k\,E_{xx}}\right]} \quad [6]$$

where MSE_r is the mean square error for the ANOVA without correction for the pre-treatment reproduction. Correcting for the pre-treatment reproduction is only efficient if E>1.

The Dunnett's test was used to calculate the probability that an equal or larger difference would be observed given that the treatment does not influence that variable. For the reproductive output and for the rate of change of the reproductive output, the two-sided Dunnett's test was used (software: S-plus).

For each difference between the adjusted value in the control and each of the treatments, the 95% confidence interval (CI) is calculated as

$$\left(\overline{y}_{ia}-\overline{y}_{0a}\right)\pm d_{0.025,3,35}\sqrt{MSE\left(\frac{1}{r_0}+\frac{1}{r_i}\right)\left[1+\frac{T_{xx}}{k\,E_{xx}}\right]} \quad [7]$$

where $d_{0.025,3,35}$ is the critical value for the two-sided Dunnett test at significance level 0.05 if 3 treatments are compared with the same control and with 35 degrees of freedom.

Results

The mean reproduction per snail per treatment level in the pre-exposure period (day –27 to 0), the first exposure period (day 1 to 28) and the second exposure period (day 29 to 56) are listed in the Table B below. The means of the reproduction in the exposure period adjusted for the reproduction in the pre-exposure period are also listed in that table. The efficiency of correcting for the pre-treatment reproduction varied from 4.6 for the second period to 6.9 for the first one. The mean reproduction in the combination of all three treatment levels is also listed. For the comparison of the control with the combination of the three treatment levels, it is not efficient to adjust the reproduction for the reproduction in the pre-treatment period. The efficiency for this adjustment varied between 0.010 for period 2 to 0.016 for period 1.

Table B: The mean reproduction per exposure period for each treatment level, and the mean of the egg production in each test vessel adjusted for the reproduction in the pre-exposure period in that test vessel.

		Raw mean egg production per vessel			Mean egg production per vessel adjusted for the pre-exposure reproduction					
Treat-ment	pre-treatment period	first treatment period, day 2-28	second treatment period, day 30-56	Total treatment period	first treatment period, day 2-28		second treatment period, day 30-56		Total treatment period	
					N	% of control	N	% of control	N	% of control
Control	410	188	57.6	246	191		58.0		249	--
0.2 µg/l	400	135	28.7	164	144	75%	29.6	51%	173	70%
2 µg/l	397	121	37.4	158	131	69%	38.5	66%	170	68%
20 µg/l	455	167	32.4	199	145	76%	30.1	52%	175	70%
All exposed	417	141	32.8	174	140	75%[a]	32.8	57%[a]	173	69%[a]

[a]: % difference with control for the unadjusted egg production.

The difference between the adjusted egg production in the control and the three treatment levels was tested with the two-sided Dunnett's test (see Table C below). This test was performed for both exposure periods separately (days 2 to 28 and days 30 to 56) and for the combination of both exposure periods. For the test, the reproduction in the test vessels was adjusted for the reproduction in the pre-exposure period in each test vessel.

The observed reproduction was lower in all the three treatment levels than in the control. These differences were, however, not significant at the 5% significance level.

The difference between the reproductive output in the control and the combination of the three exposure levels is tested with a two-sided Student-*t*-test. For this test the reproductive output was not corrected for the pre-treatment reproduction because this correction was not efficient. This test gives *p*-values of 0.12, 0.036 and 0.049 for the first period, the second period and the complete exposure period, respectively. The 95% confidence intervals for the difference in reproductive output between the combined concentration levels and the control in per cent of the control were (-57% – 7%), (-83% – -3%) and (-58% to –0.1%), respectively.

Table C: The percent differences (compared to the control) between the adjusted egg production per individual in the treatment vessels and in the control. And the *p*-value for the two-sided Dunnett test comparing the adjusted egg production in the treatment levels with that in the control.

Egg production adjusted for the pre-exposure reproduction	Percent difference between mean egg production in treatment and in control			Standard deviation of the difference as percent of control value	*p*-value of the two-sided Dunnett test		
	C1-C0	C2-C0	C3-C0		C1	C2	C3
pre-treatment period	-2.5%	-3.1%	11.1%	9.4%	0.987	0.975	0.50
treatment period, day 2-28	-25%	-31%	-24%	16%	0.27	0.12	0.29
treatment period, day 30-56	-49%	-34%	-48%%	25%	0.14	0.39	0.14
Total treatment period	-30%	-32%	-30%	15%	0.12	0.10	0.13

Table D: The differences between the adjusted egg production per individual in the treatment vessels and in the control and, between brackets the 95% confidence interval for that difference.

Egg production adjusted for the pre-exposure reproduction	Difference between mean egg production in treatment and in control		
	C1-C0	C2-C0	C3-C0
pre-treatment period	-10 (-104 – 84)	-13 (-107 – 81)	46 (-49 – 140)
treatment period, day 2-28	-48 (-120 – 25)	-60 (-133 – 13)	-46 (-119 – 26)
treatment period, day 30-56	-28 (-63 – 6.8)	-19 (-55 – 16)	-28 (-63 – 7.2)
Total treatment period	-76 (-167 – 16)	-80 (-171 – 12)	-74 (-166 – 17)

The slope of the egg production as function of time was calculated for each test vessels for the two exposure periods separately. This slope is a measure for the change in the egg production rate. In Table E both the means of the slope per treatment level for each of the two exposure periods are given and the means of the slopes adjusted for the pre-exposure egg production in each test vessel. The efficiency of correcting for the pre-treatment reproduction was 3.3 for the first period and 4.8 for the second one. The fact that all mean slope values are negative indicates that the average egg production decreases in these periods in all treatment levels and the control.

Table E: The mean of the adjusted slope of the egg production in a two-days period versus time.

	Mean slope of the egg production in time ((eggs/2d).d^{-1})		Mean of the adjusted slope of the egg production in time ((eggs/2d).d^{-1})	
Treatment	first treatment period, day 2 to 28	second treatment period, day 30 to 56	first treatment period, day 2 to 28	second treatment period, day 30 to 56
Control	-0.561	-0.127	-0.570	-0.130
0.2 µg/l	-0.391	-0.074	-0.417	-0.082
2 µg/l	-0.231	-0.051	-0.260	-0.060
20 µg/l	-0.464	-0.171	-0.402	-0.151

The effect on the change in egg production rate of the treatment level in comparison to that of the control was tested with the same test as the effect on the egg production itself. The results of these tests are listed in Table F. These *p*-values can only be used as indication whether further research into the end-point "change in egg production rate" is needed for the total significance level was attributed to the test of effects on reproductive output.

Table F: The differences between the adjusted change in egg production rate in the treatment vessels and in the control. And the *p*-value for the two-sided Dunnett's test comparing the adjusted change in egg production rate in the treatment levels with that in the control.

	Difference between mean adjusted change in egg production rate in treatment and in control			Standard deviation of the difference	*p*-value of the one-sided Dunnett's test		
	C1-C0	C2-C0	C3-C0		C1	C2	C3
1st treatment period, day 2 to 28	0.154	0.310	0.168	0.143	>0.5	0.09	0.5
2nd treatment period, day 30 to 56	0.048	0.070	-0.021	0.077	>0.5	>0.5	>0.5

This test did not show any *p*-values below 0.05 during either of the two exposure periods, so in this experiment does not indicate that BPA influence the change in egg production rate, i.e. the ability to adapt to lower temperatures, at any of the test concentrations.

Discussion

In this experiment several variables were investigated. The probability that some of these would be found significant at the 5% level increases with the number of variables tested. Therefore, in this study only the end-point "total reproductive output" is formally tested. Since none of the *p*-values were less than 0.05 for the toxicological end-points "the rate of change in reproduction in the first exposure period" and "the rate of change in reproduction in the second exposure period" these end-points are not suspected to be affected by BPA at the concentrations used in this experiment.

When testing the reproductive output in the combination of all exposed test vessels with that in the control vessels, the reproduction was marginally significantly reduced in the exposed vessels in the second part of the exposure period and in the complete exposure period (p=0.049). This may be interpreted as a warning that some significant effect on reproduction might have occurred. The result should, however, be treated with caution: The decision to combine the three exposure levels would, no doubt, not have been made if the reproductive output in the lowest concentration was equal to the control reproduction, or had deviated in the opposite direction. Therefore, the test is performed conditional on that fact that an eyeball observation of the data suggested that the three exposure levels deviated in a similar way from the control. To prove that each of the three exposure levels, 0.2, 2 and 20 μg BPA per litre, have a similar effect on the reproductive output in comparison to the control, a new experiment is necessary. For that new experiment the decision to test the reproductive output in the combination of the three exposure levels against the reproductive output in the control should be taken before that experiment is performed.

APPENDIX 3
EXTRACTS FROM A REPORT ON THE STATISTICAL ANALYSIS OF EXPERIMENT 6 (Barnes, 2010).

The duration of the experiment was 84 days and observations were taken every 2 days. Days were numbered from -26 to 56 in steps of 2.

Of the 40 tanks, tank 1 was omitted from all analysis because of contamination.

A feature of the experimental design was the controlled temperature regime, which was as follows:

Period 1 (13 observations from day -26 to day -2): maintained at 19°C;

Period 2 (14 observations from day 0 to day 26): steadily decreasing to 15°C in day 26;

Period 3 (15 observations from day 28 to day 56): maintained at 15°C.

No treatments were applied during period 1. After this control period, the 40 tanks were randomly allocated to four *nominal dose* levels of BPA: 0, 0.2, 2 and 20 µg/l.

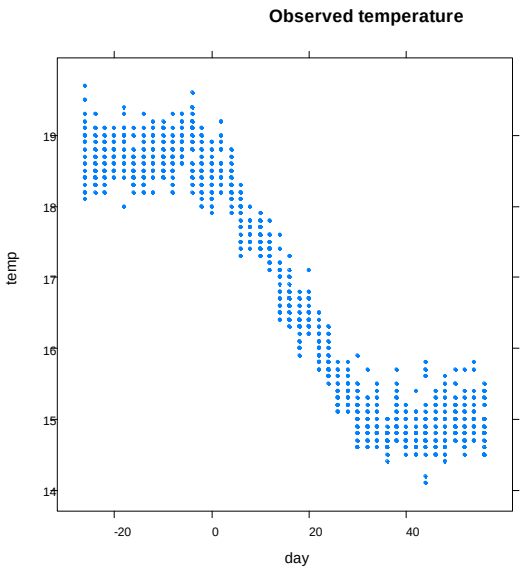

Figure 1

The *actual dose* level of concentration, in µg/l, of BPA was also measured during the experiment.

The actual temperature was measured throughout the experimental period and the data are shown in Fig. 1.

The aim of the analysis was to assess the effects of BPA dose on reproductive success of the snails, as measured by egg production. Two response variables were analysed: number of masses, and total number of eggs. Dose-response relationships were sought with both the *nominal dose* and the *actual dose* as measured in the tanks.

Exploratory analysis indicated that there was much variability in both response variables between tanks, so hierarchical linear models were used for the analysis, with random effects for tanks.

Data from period 1 (the control period) were used to derive baseline measures of level and rate of change of each response to be used as covariates in the analysis of period 2 responses.

The relationship between the response variables and temperature were modelled separately. Because of the experimental design, time and temperature were confounded, making a joint analysis difficult.

1. Response: no. of masses

Period 1: days -26 to -2

No treatments applied – this is the control period.

Data in this period were used to set baseline values for analysis of period 2 data.

The first three observations appeared to be erratic, so these three observations (days numbered -26, -24, -22) were taken as a "settling in" period and were omitted from the analysis.

In most tanks, there was a tendency for number of masses per snail to decrease over this period; but there was considerable variability between tanks.

Figure 2 shows data with separate fitted smooth (loess) for each tank. Suggests that a linear regression is a reasonable approximation for most tanks.

Separate linear regressions were fitted for each tank. The slopes and intercepts were used as covariates in the modelling for period 2. Note that the intercept (at day = 0) is the estimated starting value for period 2.

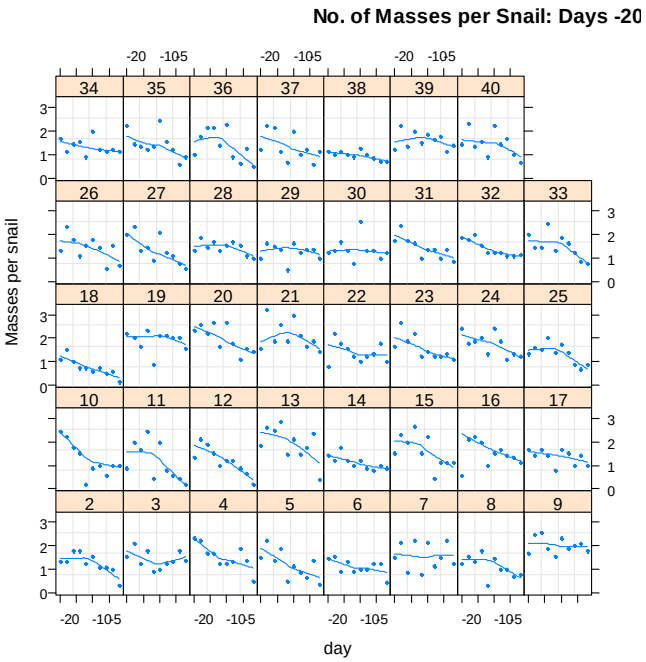

Figure 2

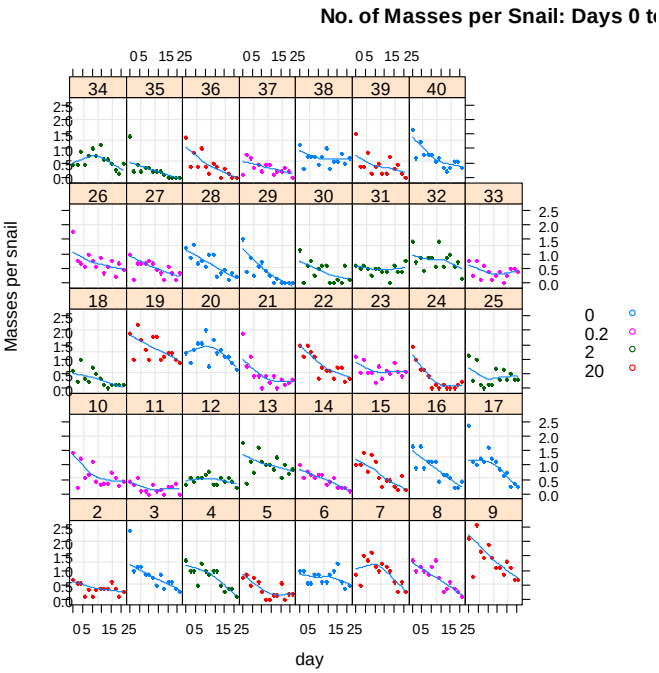

Figure 3

Period 2: days 0 to 26

The data are shown in Fig. 3 as number of masses per snail, with a loess scatterplot smoother.

Modelling approach: generalised linear hierarchical model with days at level 1 and tanks at level 2. The response variable is a count and was therefore modelled as having a Poisson distribution.

The slopes and intercepts of the separate regressions of number of masses per snail on days in period 1 (*slope0* and *int0*) were used as (level 2) baseline covariates for modelling period 2 – the idea was to account for at least some of the wide variation in response, both in mean levels and in rates of change, between tanks at the start of period 2.

The model selection strategy was to first fit a linear trend model, accounting for between-tank variation and baseline values (*slope0* and *int0*). This is the *baseline* model. By fitting BPA dose terms to this model, we test hypotheses concerning BPA effects after accounting for known sources of variation.

The baseline model fitted was ~ ( ), where

log( ) = + + + [log( ]) + + ,

where = baseline intercept (*int0*), = baseline slope (*slope0*) and = number of snails in tank , for tank , = 2, … , 40;

, , , are regression coefficients (fixed effects);

~ (,) and ~ (,) are the intercepts and slopes for tank (random effects);

= day = 2 − 2, = 1, … , 14 , and = number of masses per snail in tank on day .

Having fitted the baseline model, effects of BPA were tested by adding terms to the model to represent BPA levels. Nominal and actual measured levels of BPA were separately tested in the model.

Results

Baseline model:

- Negative linear time trend, ($p < 0.0001$);
- *int0* significant, ($p < 0.0001$), so the number of masses at the start of period 2 was important;
- The initial rate of change, *slope0*, was not significant;
- Interaction, , between *slope0* and trend was not significant.

The simplified baseline model was therefore

log( ) = + + [log( ]) +

Effect of nominal dose:

To test the effect of nominal dose, a categorical variable (factor) representing BPA dose was added term to the above model.

The 4-level dose factor (0, 0.2, 2, 20 μg/l) was not quite significant: likelihood ratio test (LRT), = 6.33 on 3 d.f., p = 0.097.

However, the contrast no BPA=0 vs BPA>0 was significant: LRT, $^{2} = 4.47$ on 1 d.f., p = 0.035.

The coefficient was negative, which implies lower number of masses per snail when BPA > 0.

The effect of nominal dose on the time trend (rate of decline) was also tested and found to be not significant.

Effect of actual dose:

The variable log(*actual dose*) was first tested by adding it to the baseline model as a covariate. The result was: : LRT, $^{2} = 0.06$ on 1 d.f., which is nowhere near significant.

The distribution of actual dose over periods 2 and 3 is shown in Fig.4 – suggests that just two levels (greater than 2.0 or not, say) are adequate to describe it.

However, the result of fitting the model with this factor was the same as with log(*actual dose*) – not significant.

There was also no significant effect of actual dose on the rate of decline of number of masses.

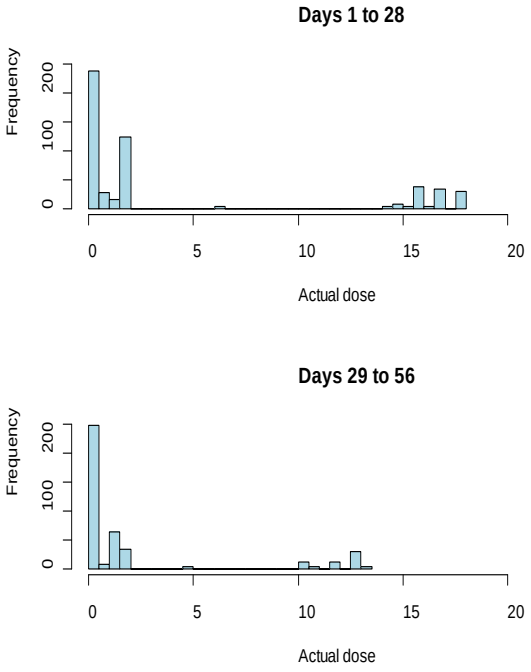

Figure 4

Conclusions for period 2 (days 0 to 26):

(1) In general the no. of masses per snail decreases in the period from day 0 to day 26.
(2) The only apparent BPA effect is a decrease (p = 0.03) in the mean number of masses per snail with BPA present, when compared with no BPA.
(3) There is no significant effect of *actual measured* BPA dose level on the means number of masses, or on the rate of decline over time.

Period 3: days 28 to 56

The predicted value at day 26 from the model fitted in period 2 was used as baseline value.

The fitted model suggests that although there was an overall general decline in number of masses per snail over time, neither the mean number nor the rate of decline was affected by the nominal dose. The result was: LRT, $^{2} = 0.61$ on 1 d.f., which is not significant.

The corresponding result the actual dose was: LRT, $^{2} = 1.88$ on 1 d.f., p = 0.17. This is again not significant.

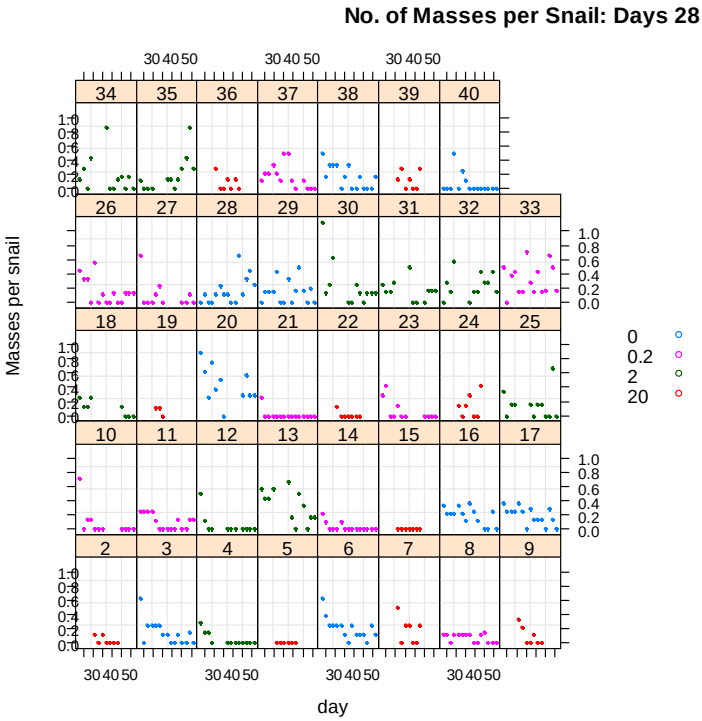

Figure 5

2. *Response: total no. of eggs*

Period 1: days -26 to 0

As before, first 3 days (-26, -24, -22) omitted to allow for "settling in".

All tanks show a decline in total no. of eggs per snail over this period, and the decline is more or less linear in most tanks.

Period 2: days 0 to 26

In general, results follow those for the number of masses very closely; the baseline model was the same.

The effect of nominal dose on mean number of eggs was significant: LRT, $^{2} = 4.94$ on 1 d.f., p = 0.026.

There was no detectable effect of actual dose. For the two-level contrast (low vs high), the LRT was $^{2} = 0.14$ on 1 d.f., p = 711.

Period 3: days 28 to 56

No significant effect of nominal dose: LRT, $^{2} = 1.41$ on 1 d.f., p = 0.235, …

… or of actual dose: LRT, $^{2} = 0.40$ on 1 d.f., p = 0.530.