Seasonal Effects of Treated Sewage Effluents upon the Reproduction and Development of European Freshwater Molluscs

A thesis submitted for the degree of Doctor of Philosophy

by

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Declaration

The work described in this thesis was carried out between 2003 and 2006 at Brunel University, Uxbridge, Middlesex, and Essex and Suffolk Water, Hanningfield Treatment Works, South Hanningfield, Chelmsford, Essex. This work was done independently and has not been submitted for any other degree.

ABSTRACT

The most widespread evidence of environmental endocrine disruption in aquatic wildlife is from the feminising effects of oestrogenic endocrine disrupting compounds. However, very little is known of the effects of these chemicals (and others) upon freshwater molluscs found in our river and lakes. This thesis aimed at evaluating the effects of treated sewage effluent upon the reproductive and developmental cycle of a range of commonly found European freshwater gastropod molluscs. Initial mesocosm experiments were undertaken to test a range of mollusc species for their the suitability to the experimental system, and to test adult snails for their reproductive and developmental responses during spring to summer time. With suitable species chosen, *P.corneus*, a pulmonate species (sequential hermaphrodite), and *V. Viviparus* a prosobranch species (dioeious; separate sexes), full reproductive output was assessed over summertime and into autumn, along with developmental responses amongst the F1 generation of snails.

My results suggest that the affects of effluent upon the reproductive and developmental cycle of *P. corneus* are strongly mitigated by both day length and water temperature (day length is most important with *V. viviparus*); results are sensitive to seasonal effects. However, at the peak of reproduction mid summer, *P. corneus* produced significantly more egg masses in effluent (100% effluent particularly), and more than one parameter of reproduction was affected. Egg masses were significantly smaller in effluent and contained significantly fewer eggs per mass. Further, there were indications that total reproductive output was increased (100% effluent significantly) in effluent compared to the river water control. In the prosobranch species *V. viviparus* results were less convincing, however, in 100% effluent a second reproductive peak occurred that was not seen in river water. Further, in both species there was a failure of certain reproductive parameters to observe the normal seasonal decline towards winter. In *P. corneus* there was a failure to stop producing egg masses in effluent, in *V. viviparus* the second reproductive peak in effluent could also threaten their survival with winter approaching. Developmental effects in the F1 generation were the subject of preliminary investigations, however, F1 *V. viviparus* demonstrated a higher than normal incidence of intersex (male and female developmental features) in effluent, and *P. corneus* appeared to have disturbed reproductive function (disturbance of both male and female reproductive function in the ovotestis).

Therefore, both of these species of molluscs demonstrated that they are sensitive to the effects of effluent in mesocosm studies. However, we need to understand much more about their responses to effluent; in particular whether these effects could have repercussions for wild mollusc populations, and whether these effects could occur over more than one generation of snail threatening the survival of wild populations of molluscs.

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Chapter One: Introduction

1.1 What is Endocrine Disruption?

To answer, "What is endocrine disruption?" it is necessary to understand what an endocrine system is, and how they are set-up to work correctly. Only then is it possible to understand how and by what these systems may be perturbed. The following sections are written to answer these questions, so that a full understanding of what endocrine disruption is will follow. They are written from the point of view of vertebrate (mammalian) biology since our present knowledge of the endocrine system (and endocrine disruption) is far greater amongst vertebrates than invertebrates. However, since my project is about freshwater snails, the invertebrate endocrine system is much more relevant to my research. Differences, both in the nature and functioning, of invertebrate endocrine systems compared to that of vertebrate ones is described where known.

1.2 What are Endocrine Systems?

Endocrine systems play an essential and pervasive role in both the short- and long-term regulation of metabolic processes. Reproductive (sexual development and puberty), nutritional, and behavioural processes are intricately regulated by endocrine systems, as are growth (including bone growth/remodelling), gut, cardiovascular, and kidney function and responses to all forms of stress. Disorders of any of the endocrine systems, involving both overactive and underactive hormone secretion, result inevitably in disease; effects which may extend too many different organs and functions, and are often debilitating or life threatening.

The endocrine system originally was considered to consist only of glands that secreted hormones into the blood that travelled to distant target tissues, bound to specific cellular receptors, and produced characteristic actions. Currently, our concept of "endocrine" has been broadened by the discovery of other chemical regulators, such

as chemicals secreted into the blood by neurons, which are sometimes called neurohormones. The term "cytocrine" has been applied to numerous local or intracellular chemical regulators, including growth factors. Intercellular cytocrines that travel through the extracellular fluids to other cells in a tissue are also known as paracrine and autocrine regulators, depending on whether they affect other cells or themselves, respectively. The term "intracrine" has been suggested for intracellular regulators such as second messengers and transcription factors. Even before allowing for the increase in complexity of "endocrinology" that has resulted from recent recognition of the many cytocrine/paracrine systems that operate, it has been realised that there were numerous "classical" endocrine systems in the body that regulate processes as diverse as metabolism, blood pressure, smooth muscle contraction, fluid balance, and bone resorption. However, still the complexity of these endocrine systems is only just being touched upon by science, even amongst vertebrates, and therefore, the explanation of the action of endocrine disruptors is likely to be a lot more complicated than presently known.

1.3 What is the definition of Endocrine Disruptors?

Endocrine disruption can be defined simply as a functional change in the normal functioning of the endocrine system in wildlife (and humans) that may lead to adverse effects upon these organisms. An assessment report compiled on endocrine disruption by the International Programme on Chemical Safety (IPCS) in 2002, defines an endocrine disruptor as:

"An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism or its progeny or (sub) populations.

A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations."

Implicit in any definition of "endocrine disruption" is that the endocrine system is in some way altered or interfered with. Further, several consensus definitions of the term endocrine disruptors include the important, though frequently only implicit stipulation that the organism is not distressed or in obvious discomfort (Oberdorster and Cheek, 2000). The term "endocrine disrupting chemicals" (or EDCs) is commonly used in the current literature to describe environmental agents that can alter the endocrine system of animals, and therefore may potentially cause adverse effects upon these organisms. However, although the threat posed by environmental chemicals with endocrine activity are potentially serious to wildlife (and humans), because much depends on the level, duration, and timing of exposure a clinically manifest disturbance of the relevant endocrine system may not result.

1.4 Overview of Endocrine Axes

It is beyond the scope of any text to describe the entire endocrine system. However, it possible to describe in general terms the normal functioning and set-up of an endocrine axis (typical of mammals, and other vertebrates; for example, fish) and by describing particular aspects of this axis it is possible to illustrate the major principles of how the endocrine systems work. The general principles on which all endocrine (and probably paracrine) axes are first set up and then operate are essentially identical, and hence, most of what is described here for the reproductive axis, can be transferred in principle to other endocrine axes that are not described. Only minor attention here is paid to invertebrate endocrine systems. As the literature reveals there are many parallels between vertebrate and invertebrate endocrine systems, but there are some major differences as detailed section 5.0. The following text consists of two main parts; the first details the normal functioning of the endocrine axes and their set-up (both in adults and in the developing organism), and the second focuses on the impact of endocrine disruptors (EDs) on organ systems and disease processes.

1.5 Normal functioning of Endocrine Axes and their Set-up

1.5.1. Homeostasis

The fundamental role of all endocrine systems is to enable a dynamic, coordinated response of a distant target tissue to signals originating from another organ and, in some instances, cues originating from outside of the body. For most endocrine systems, the primary object is to maintain some form of internal balance or "homeostasis"; avoiding wild swings in hormone levels/responses that might otherwise have detrimental metabolic effects (Norman and Litwack, 1998). A good example is the role of insulin in maintaining blood glucose levels within the normal range, that is, a range that does not fall so low as to result in unconsciousness and does not rise so high that wasteful excretion/spillage into urine occurs. When insulin levels do not respond to changing blood levels of glucose, diseases such as diabetes are the result. All endocrine systems operate to a large extent on the "seesaw" principle (Figure 1.1), in which the target cells send feedback signals (usually negative feedback) to the regulating cells, with the result that secretion of the target cell-stimulating hormone is altered (usually reduced) by one or more of the products of the targets cells (Darlington and Dallman, 1995). However, in reality, there are usually elaborations or refinements of this simple archetypal endocrine system that enable all of the endocrine systems of the body to be integrated via cross talk (see section 1.5.8). The reasons for this are obvious. For example, reproduction needs to take account of age, nutritional status, and in most animals the season of the year. Similarly, stress responses, and to a lesser extent, endocrine systems regulating hunger, need to be able to override other endocrine systems when danger threatens. This cross talk is vital for a healthy life. Exposure to an oestrogenic chemical, for example, may affect not only the reproductive axis but also several other endocrine systems as well as bone, fat, and cardiovascular systems.

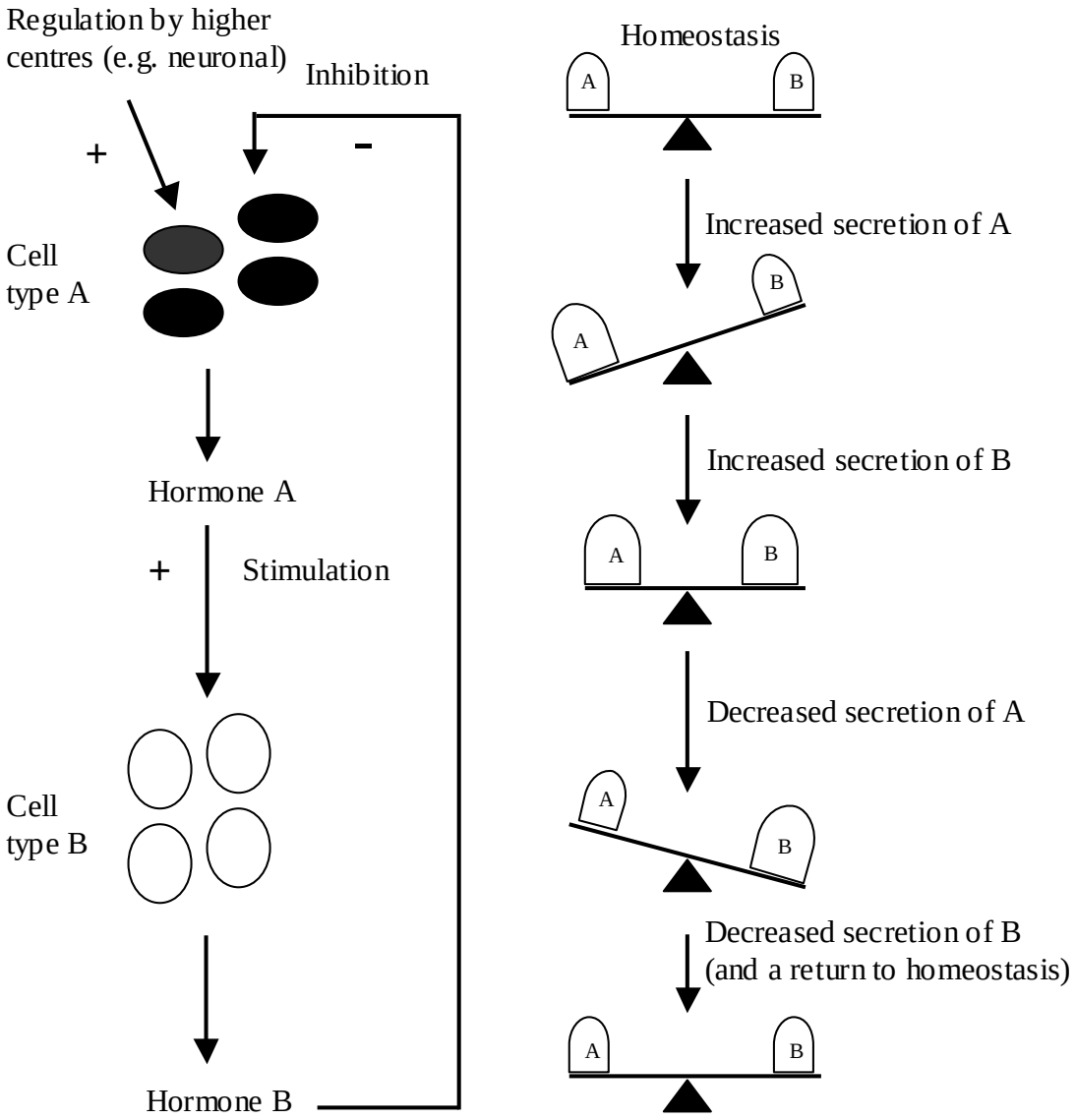


Figure 1.1 Schematic diagram illustrating the basic "seesaw" principle on which endocrine systems work. Cell type A secretes hormone A, which regulates production of hormone B by cell type B, and in turn, hormone B exerts negative feedback regulation of the secretion of hormone A. In this way, swings in secretion of hormone A or B will be compensated for to maintain homeostasis (i.e. correct levels of A and B). This general principle operates in most, if not all, endocrine and paracrine systems, although in reality there are usually additional factors that will interplay in the regulation of levels of A and B.

1.5.2 Programming of Endocrine Systems

Although homeostasis, is a central feature of all endocrine systems, it should be stressed that the balance between the two sides of the "seesaw" need to be set up or programmed before the system will work correctly. This programming determines the sensitivity of these responses (see Figure 1.1) from each side of the "seesaw", the levels at which the two sides of the seesaw will begin to respond to signals from the other side. For many endocrine systems (mammalian at least), it is established during fetal/neonatal development, and an abnormal environment at this stage of life can result in permanent misprogramming (De Kloet *et al*., 1988, Seckl, 1999).

1.5.3 The HPG Axis in Mammals

The details of this axis are an elaboration of the information in section 1.5.1 and Figure 1.1. GnRH is secreted in pulses from the terminals of GnRH hypothalamic neurons and causes pituitary gonadotropes to secrete both LH and FSH (Figure 1.1), which then act on their respective target cells in the gonad (LH on theca/Leydig cells; FSH on granulosa/Sertoli cells). As a consequence, gonadal sex steroids (stimulated by LH) and the protein hormone inhibin (stimulated by FSH) are released into the bloodstream and provide feedback to the hypothalamus and pituitary gonadotropes to reduce the secretion of GnRH, LH, and FSH, with inhibin B selectively inhibiting FSH, and the sex steroids inhibiting LH secretion. This description implies that the arrangement of stimulatory and negative feedback loops complies with the simple arrangement shown in Figure 1.1. In reality, the arrangement is more complex and sophisticated. For example, secretion of GnRH is modified by other neurons, and the actions of GnRH on gonadotropin release may be modified by other hypothalamic or pituitary peptides. Moreover, the effect of GnRH on LH and FSH secretion are radically different, with LH release being stimulated very acutely (in pulses) by the GnRH pulses, whereas the response of FSH is extremely sluggish and takes many hours. This stems from fundamental differences in GnRH-induced synthesis, packaging, and release of LH and FSH. Similarly, although the sex steroids (primarily testosterone in the male, E2 in the female) negatively regulate LH secretion via effects

on both GnRH secretion and gonadotrope function, they also exert some negative feedback on FSH secretion; in contrast, inhibin selectively inhibits FSH secretion.

1.5.4 Factors involved in the HPG axis set-up

Sensitivity of the target cell (gonadotrope cells in the pituitary, cells in the gonads) to its stimulator is regulated both acutely and chronically. For example, an abnormally high frequency of GnRH pulses or chronic exposure to GnRH results in loss or down-regulation of GnRH receptors on the gonadotropes, which serves to make them more resistant (= less sensitive) to further stimulation. In other words, each target cell in the endocrine axis also regulates its own responsiveness to stimulation. There is still further refinement of this process via cross talk between neighbouring cells, especially in the gonads. There is good evidence, for example, that Sertoli cells in the testis are able to modulate both the numbers of LH receptors expressed in neighbouring Leydig cells and their steroidogenic responsiveness via altering expression of steroid synthetic enzymes. In return, the testosterone secreted by Leydig cells exerts important paracrine regulatory effects on Sertoli cell function.

Further complexity in the component loops of the HPG axis is due to metabolism of secreted hormones. Increased or decreased catabolism, with a consequent change in half-life of a hormone, will change its effectiveness without altering its level of secretion. Of much more importance is the role of proteins that bind the sex steroids. For example, 97-98% of testosterone and E_2 that circulates in blood in humans is bound to SHBG, and only 2-3% is free and thus biologically active. Thus an indirect pathway for sex steroid regulation exists via modulation of SHBG secretion by the liver, which can potentially alter levels of bioactive sex steroids. In practice, the sex steroids themselves are the main modulators of SHBG production.

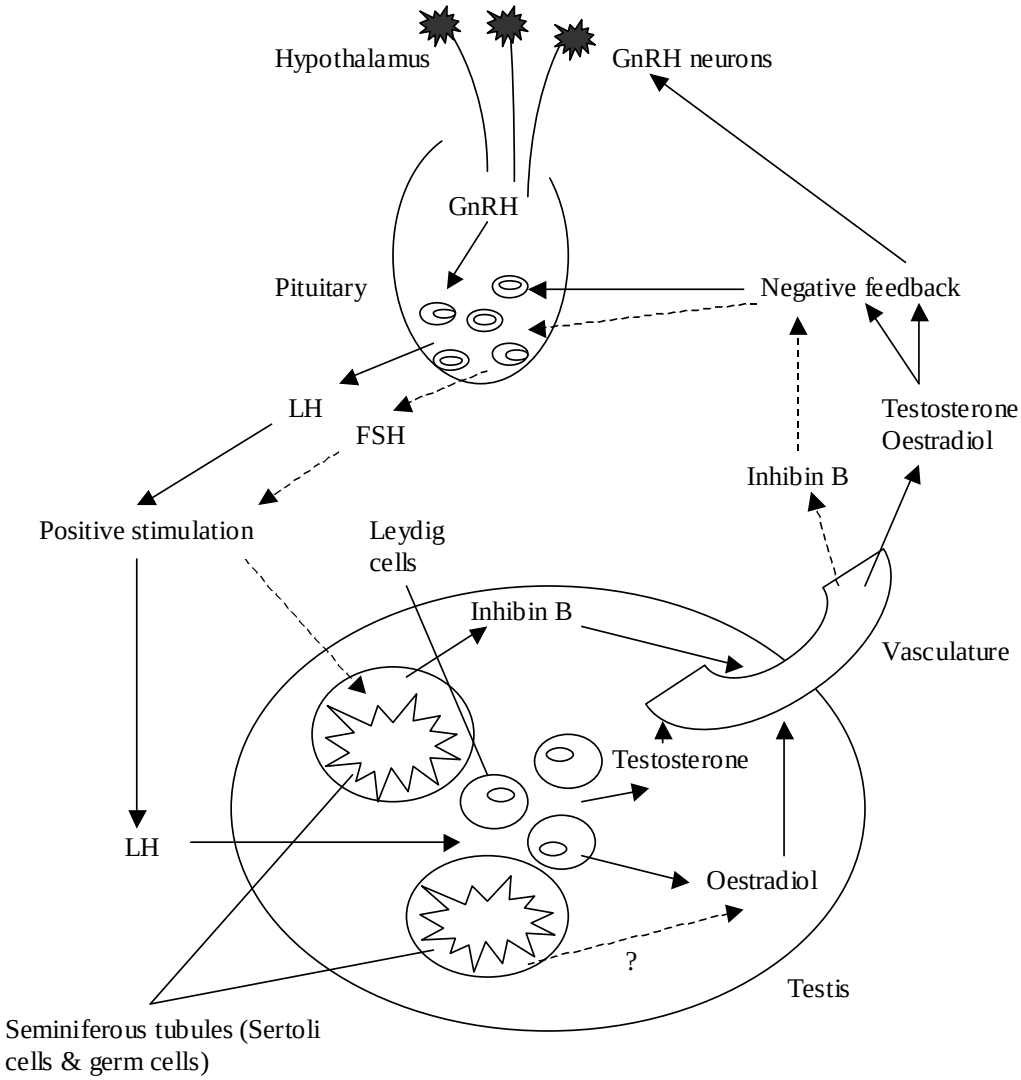


Figure 1.2. Diagrammatic representation of the main components of the mammalian HPG axis. The decapeptide GnRH is secreted from the terminals of GnRH neurons into the portal system, which delivers this "message" to gonadotrope cells in the anterior pituitary gland that express receptors for GnRH. Binding of GnRH to these receptors stimulates the synthesis and secretion into the bloodstream of the gonadotropins, LH and FSH. The gonadotropins then travel via the systemic bloodstream to reach their distant target cells in the gonad (a testis is shown for example). LH acts on Leydig cells to stimulate synthesis and secretion of testosterone, which in turn gains access to the bloodstream and via effects on the hypothalamus and anterior pituitary gland suppresses synthesis and secretion of GnRH and LH, respectively (= negative feedback). Similarly, FSH acts on Sertoli cells to drive the secretion of a protein hormone, inhibin B, which then travels via the bloodstream to the pituitary gland to suppress the synthesis and secretion of FSH (= negative feedback). Note that some of the negative feedback effects of testosterone may occur via its conversion to E2, either in the testis (by Leydig cells and/or germ cells) or in the hypothalamus/pituitary gland. Note also that testosterone and/or E2 exerts effects at many sites other than the hypothalamus and pituitary gland and that paracrine effects of these hormones, especially of testosterone within the testis, are also of vital importance. These and other refinements of the basic system illustrated here are outlined in the text (section 1.5.4).

1.5.5 Interaction of Paracrine and Endocrine Components of the HPG axis

Paracrine systems can be considered to act as local satellites of the major endocrine axis, their role being to serve local needs. For example, in the ovary, androgens produced by the theca cells exert paracrine effects on the granulosa cells in the adjacent, developing follicle (Erickson and Schreiber, 1995). The most important consequence of the exposure of granulosa cells to testosterone is that they are then able to convert this androgen to E2, which then exerts multiple endocrine effects in the uterus and elsewhere in the body, including its role in negative feedback. This conversion of testosterone to E2 also occurs at many other sites in the body, in both the male and the female (Simpson *et al*., 1997; Sharpe 1998). The ability of cells to express aromatase and/or 5 α-reductase, and thus to transform an endocrine hormone (testosterone) into a locally acting paracrine hormone (E2 or DHT; Figure 1.2), appears to be far more common (especially in the male) than was initially hypothesized. The component parts of the paracrine mechanisms illustrated in Figure 1.2 are now known to be expressed in bone, muscle, the cardiovascular system, adipose tissue, the pituitary gland, and brain as well as throughout the reproductive systems of both male and female (Simpson *et al*., 1997; Sharpe, 1998).

1.5.6 Developmental Role of the HPG axis

As noted, the setting up of endocrine axis takes place largely during fetal/neonatal development. During this period, feedback sensitivity of the hypothalamus and pituitary gonadotropes to steroids from the gonads is established, and this will determine at what level of sex steroid a reduction in GnRH and/or LH/FSH secretion will be triggered. At the same time, differences between males and female feedback centers are programmed (Dohler, 1991; Gorski, 1996). This is necessary because production of gonadal hormones is cyclical (due to oestrus or menstral cycle) in females whereas in the male it is relatively uniform, apart from special periods such as puberty and seasonal infertility. Appropriate changes to the "wiring" of the hypothalalmus of the male and female therefore have to be induced during development to ensure that the pituitary gland responds in a sex-specific manner.

Testosterone produced during fetal/neonatal life plays a role in programming the development of a "male" hypothalamus and brain, and administration of testosterone to a female during this critical programming period will result in masculinisation of hypothalamic function and consequent acyclicity and anovulation in adulthood (Dohler, 1991; Gorski, 1996).

1.5.7 The HPG Axis in Non-mammalian Species

Non-mammalian vertebrates differ greatly from mammals and one another in their modes of reproduction, with patterns of sequential and simultaneous hermaphroditism, parthenogenesis, viviparity, and gonochorism found in many major groups (van Tienhoven, 1983). Further, though the number of eggs produced may vary between as little as 100 or less up to millions per breeding season, they often produce yolky eggs. Additionally, they may be more limited in their in their breeding frequencies. Some species breed only once (semelparous), whereas others may breed two or more times (iteroprous). The time of gonadal activity may be very short, with the gonads remaining quiescent during most of the year. Dissociated reproduction where testicular and ovarian development actually occurs at different times of the year also is known for numerous species (e.g., Houck and Woodley, 1994). However, the HPG axes of these animals are surprisingly similar in their operation, in the pattern of feedback mechanisms, and in the hormones involved to that described for mammals, and in the hormones involved to that described for mammals (for a review, see Norris, 1997).

1.5.8 Endocrine Cross Talk and Endocrine Disruptors

It should be emphasized that the prediction of the reproductive consequences of a given chemical from its known sex steroid hormone activity or inactivity is far from straightforward. Even when an environmental chemical is shown to have (weak) steroid hormone activity, it can possess other relevant activities. Thus, the thyroidal activities of PCBs may be as important or even more important than the weak oestrogenic/antioestrogenic effects of these compounds when considering their potential impact on the reproductive system. Other environmental chemicals may

have antiandrogenic as well as oestrogenic effects (e.g. DDT isomers, certain phthalates), which may confuse interpretation of potency *in vivo*. For example, administration of antiandrogens is likely to elevate endogenous oestrogen levels. This occurs because antiandrogens block negative feedback loops, which leads to elevation of LH levels (Figures 1.1 and 1.2) and thus to supranormal elevation of testosterone levels, with consequent increase in availability of substrate for aromatisation (into oestrogens).

Based on the considerations detailed above, it is clear that chemicals should be tested for their "reproductive" activity (i.e. the ability to alter the development or the function of the reproductive system *in vivo*) rather than just relying solely on sex steroid activity in *in vitro* test systems. Although the endocrine systems of invertebrates differ considerably to those of vertebrates, the increasing understanding of the commonality in at least the function of both invertebrate and vertebrate endocrine systems means this last comment is likely to hold true for invertebrates also.

1.5.9 Mode of action`s (MOA`s) of EDCs

As expected from the normal functioning of endocrine systems, the cellular and molecular mechanisms of endocrine disruption are not limited to receptor binding and include, for example, inhibition of hormone synthesis, metabolism, and transport. The following list describes some of the mechanisms of steroid hormone action, as an example, and provides key steps in the process where EDCs have been shown to alter endocrine function:

i) Steroid hormones such as E2, testosterone, and progesterone are synthesized in the gonadal cells. Inhibitors of CYP450 enzymes, including drugs and pesticides, act here.

ii) Hormones are secreted into the blood from the gonad and are available to the cell through diffusion or may be transported bound to SHBG. EDs could alter SHBG levels; some hormone mimics do not bind SHBG as well as the natural ligands do, making them more available both to the target cell and for liver metabolism.

iii) The hormone diffuses into the cell where unoccupied receptors are located; the hormone or hormone mimic binds the receptor. Many xenobiotics have been shown to bind to ER or AR. Some EDCs inhibit the activation of prohormone in the target tissue (e.g. the prohormone testosterone is metabolised to E2).

iv) The receptor, now bound to a natural or synthetic ligand, undergoes a conformational change. Chemicals can cause receptors to undergo conformational changes that alter their ability to recruit co-activators. Forming homodimers, these molecules accumulate transcriptional factors, forming a transcriptional complex, which binds to specific sequences on the DNA of hormone-dependant genes, known as hormone response elements (HRE). The transcriptional complex then initiates mRNA synthesis (mRNA). Some antihormones interfere with DNA binding.

v) The end product of this synthesis is normally a protein, a marker of endocrine action, could be an enzyme, a protein hormone or growth factor, or a structural component of the cell. An example of a hormone dependant marker is vitellogenin, an oestrogen-sensitive protein produced by oviparous vertebrates.

vi) In some cases, toxicants disrupt endocrine function, either increasing or decreasing metabolism of the hormone, such that serum levels are altered. For example, some PCBs stimulate metabolism of T_4, dramatically reducing serum T_4 levels. Several pesticides have been shown to stimulate the liver and reduce serum steroid hormone levels.

Other sites in the signal transduction process are also likely to be susceptible to disruption by anthropogenic chemicals. This following section highlights the cellular and molecular mechanisms of action and toxicological impact on the developing reproductive system using selected examples of steroid receptor agonists and antagonists, steroid synthesis modifiers, and AhR agonists. EDCs typically alter reproductive development by more than one mechanism such that target organs are impacted, although not necessarily at the same dose or stage of life. *In vitro* tests can be critically limited as the multiple endocrine and nonendocrine effects of an endocrine disruptor can only be interpreted in a meaningful fashion *in vivo*. However, for the purpose of screening or prioritising chemicals *in vitro* testing is useful.

Considerable homology exists in the endocrinology of vertebrates, although the hormones, hormone synthesis, and their receptors are highly conserved, the role of specific hormones in reproductive function and development can vary greatly between vertebrate species. Additionally, significant differences in metabolism of EDCs can result in marked species differences in responses to these chemicals.

1.6 Specific MOA`s and Physiological Processes

1.6.1 Steroidal Hormone Receptors

Most of the chemicals known to disrupt endocrine function to date are able to bind to nuclear steroid receptors. In general, xenosteroids bind to steroid receptors with much weaker affinities than the natural ligand, making them probably less potent *in vivo*, often by many orders of magnitude. Some EDCs inhibit the binding of the endogenous steroid to its receptor by blocking the receptor-binding site, rendering the receptors unable to respond to natural ligand. For example, metabolites (M1 and M2) of the dicarboximide fungicide vinclozolin competitively inhibit the binding of androgens to the mammalian androgen receptor (AH).

A number EDCs can interact with both the oestrogen and androgen receptor and therefore are capable of affecting physiological processes that are controlled by both oestrogens and androgens. For example, the principle isomer of DDT (o,p′DDT) binds to the oestrogen receptor and acts as an oestrogen mimic (though is approximately 100, 000 times less potent than E2). However, o,p′DDT also interacts with the androgen receptor; o,p′DDT (and nonylphenol) has been shown to exert rapid oestrogenic (agonist) actions on rat smooth muscle cells and on croaker testicular androgen production (Ruehlmann *et al*., 1998; Loomis and Thomas, 2000). This chemical, o,p′DDT, was found to be as potent an anti-androgen as the clinical anti-androgen flutamide (Sohoni and Sumpter, 1998) . These findings illustrate that a single chemical, or isomer, can interact with more than one steroid receptor. Because a particular chemical (e.g. DDT) or mixture of isomers that interact with different steroid receptors, it is intuitive to expect that exposure to such a chemical could result

in a variety of very different responses in different tissues and at different times in the life cycle. Chemicals (or isomers) that interact with different receptors, however, may in fact cause similar biological effects.

For example, some dioxins, such as 2,3,7, 8-tetrachloro-dibenzo-p-dioxin (TCDD) can decrease the expression of the oestrogen receptor (Chaffin *et al*., 1996), but other effects of TCCD are not mediated through the oestrogen receptor but through the cytosolic aryl hydrocarbon (Ah) receptor (e.g., some dioxins, such as 2,3,7, 8-tetrachloro-dibenzo-p-dioxin (TCDD), which then induce the cytochrome P450A1 enzyme system (to hydroxylate and metabolise oestrogen). Male rats exposed *in utero* to a single dose of TCDD during gestation displayed reduced fertility, as well as delayed puberty and altered reproductive organ weights (Gray *et al*., 1997).

1.6.2 Non-Steroidal Hormone Receptor Systems

Whilst some endocrine-disrupting chemicals exert their effects via steroidal hormone receptor systems (e.g. oestrogen-like receptors), there are also non-steroidal hormone receptor systems, such as thyroid, or retinoic acid hormone receptors (Tyler *et al*., 1998). As an example, certain hydroxylated PCBs (which are oestrogenic) have also been shown to bind to thyroid hormone receptors (Mckinney & Waller, 1994), thereby blocking the binding sites for T_4 that causes its enhanced clearance from serum and decreased availability to tissues (Brouwer and Van den Berg, 1986). Chemicals that affect the production or transport of thyroxine may also affect reproduction. Further, while the function and ligands of many receptors are known (nuclear receptor gene family), there are a large and growing number of receptors with no known function or ligand- the so-called orphan receptors. The orphan receptor SXR (steroid/xenobiotic receptor) or PXR recognizes many classes of EDCs (including nonylphenol), and activates a response that results in expression of xenobiotic metabolizing enzymes (Masuyama, *et al*., 2000).

1.6.3 Non-receptor mediated pathways

Although many papers have focused on receptor-mediated systems, there are also a number of non-receptor mediated pathways through which endocrine modulators can exert their effects (Tyler *et al*., 1998). One such pathway includes alteration in the number and/or affinity of the receptors. Overall, the evidence supports the hypothesis that most TCDD effects are mediated through the Ah receptor (AhR) (Okey, *et al*., 1994; Hankinson 1995), however, it can also act to increase or decrease the expression of the oestrogen receptor (Chaffin *et al*., 1996; Romkes *et al*., 1987; Romkes and Safe, 1988; DeVito *et al*., 1992). Other, non-receptor-mediated mechanisms of action of endocrine disruptors include interference with the synthesis or degradation of endogenous hormones and disruption of their transport in the body. Examples include the phytoestrogen, β-sitosterol, which is able to reduce the biosynthetic capacity for gonadal steroids by either affecting the availability of the steroid precursor (cholesterol) or by altering the activity of P450 enzymes, resulting in a hormone imbalance and masculinisation (MacLatchy & van der kraak 1995, Denton, *et al*., 1985). Moreover, this is an example of a plant signalling molecule developed for one communication system that may be functionally misinterpreted by another system (vertebrate or invertebrate animals). TCCD may, amongst other modes of action already noted here, induce cytochrome P4501A1 that then hydroxylates and metabolises oestrogen. It is clear that TCCD does not simply act as a hormone mimic, but instead as a hormonal modulator of a multiplicity of hormone receptor-mediated (as well as non-receptor mediated) processes. Further, as testing of the many different chemicals that maybe found in the environment continues, it is likely that each chemical will be shown to have many mechanisms of action, some modes of action that as yet are not understood by science. Therefore, environmental endocrine disrupting chemicals are capable of not only mimicking, or disrupting hormone function, and interfering with other molecular pathways involved in endocrine axes, but also of fooling the endocrine system into accepting a new set of instructions that distort the normal development of an organism (McLachan, 2001).

1.7 Endocrine Disrupting Chemicals

1.7.1 Effects of EDCs in Wildife

In the last 10 years, there has been a lot of interest in the effects of contaminants (e.g. chemicals in TSE) that may interfere with reproduction and development in wildlife the so-called endocrine disrupting compounds (EDCs) (see section 1.2). Laboratories studies have shown that a variety of synthetic and natural chemicals including certain industrial intermediates, polychlorinated biphenyls (PCBs), pesticides, dioxins, and trace elements have the potential to act as EDCs and impact the endocrine system (Tyler *et al*., 1998), and other systems of not only mammals and other vertebrates, but invertebrates also.

However, TSE is a highly complicated mixture, and ill defined in terms of its endocrine disrupting potential. Known to contain about 60,000 man-made chemicals (Lester, 1990), these together with a number of naturally occurring chemicals are a potential threat to the endocrine system (and other systems) of molluscs, as well as other animals. Due to the extensive recent research into endocrine disrupting chemicals, key chemical players that are known to interfere with the endocrine system of both vertebrates and invertebrates have been identified. Although this list, importantly, includes groups of chemicals of common structure and is extensive, it is by no means inclusive; much research remains that will undoubtedly uncover more. However, TIE approaches have identified the main groups of chemicals with similar properties that have since proven to have significant endocrine disrupting properties upon vertebrates. Below are listed different chemical groups with their relevant chemical and biological properties together with evidence of their effects from *in vitro* studies, and where possible *in vivo* studies. Much of the evidence to date describing the endocrine disrupting qualities of these chemicals has come from *in vitro* studies, with fewer but significant evidence from *in vivo* field studies. These *in vivo* studies, however, have focused on overly polluted environments, close to point sources of pollution such as pulp and paper mills, sewage treatment works (STWs), or toxic waste dumps or spills, and not on environments further from these sources where "ambient" concentrations of these chemicals may occur. Therefore, although these studies may not represent the "normal" situation where ambient concentrations of chemicals may predominate, they still represent the best evidence of the effects of chemicals on wildlife populations. Many of these chemicals could be present, or have been identified in TSE. Others may yet be identified in TSE (fertilizers and pesticides)

or more pertinently, identified as significant contributors towards the endocrine disrupting capabilities of TSE in wildlife.

In summary, although there is a strong association between exposure to these chemicals and poor reproductive function, as yet there is very limited data that can directly link a reproductive abnormality with a specific hormone or anti-hormone mimic. Comparatively, most chemicals that mimic or antagonise the action of steroid hormones are only weakly active, with potencies three or more orders of magnitude less than that of natural oestrogens, such as 17β-oestradiol. Generally, therefore, most endocrine mimics have the potential to cause endocrine modulation in wildlife only if they circulate in the environment at high concentrations (e.g. "hotspots"), or their breakdown is slow and/or because they bio-accumulate, or because they are in widespread regular use and are entering the environment almost constantly. In the more likely most commonly found situations, where the concentration of chemicals is likely to representative of most stretches of rivers, these stretches of rivers have not been well studied, or alternatively, the results from such studies have not been published. Therefore, whether chemicals in these environments can cause endocrine disruption amongst wildlife populations is largely unknown.

1.7.2 Pesticides and Fungicides

In the western world, the use of DDT-pesticides is banned, due to their toxic effects in both aquatic and terrestrial animals. Nevertheless, they are still present in these environments due to their resistance to biodegradation (half-lives of some DDT metabolites of more than 50 years). Overall, concentrations of DDT-compounds and other similar organochlorine pesticides (synthetic halogenated hydrocarbons) in the general environment are at, or below the no-observed-effects-levels (NOEL) for laboratory studies, which perhaps suggests that DDT-pesticides alone are unlikely to have deleterious effects on wildlife (Tyler *et al*., 1998). However, bio-concentration factors (BCF`s) of organochlorine pesticides are well documented in aquatic animals, and they are readily bio-accumulated in fish; their BCF`s are between 1,000 and 10, 000. It is also possible that considerably lower levels may be harmful if exposure occurs over very long periods of time; equilibrium of these chemicals in animal

tissues can take many weeks to establish, or if exposure occurs at critical times of development of an egg/fetus. Exposure of eggs and fetuses by maternal transfer to very low doses of DDT-pesticides for sometimes-short periods of time have been shown to alter the development of vertebrates and marine prosobranch molluscs. Gray *et al*., 1995 showed that exposure *in utero* to DDT decreased testes size and sperm counts in male rats. Further, in a highly likely scenario, additive effects of DDT in combination with other commonly found chemicals in the environment may occur. For example, there are reports of endocrine disruption in sites heavily contaminated with DDT and its metabolites, dioxins, and/or PCBs.

The reproductive effects of the well-known organochlorine pesticide, DDT and its metabolites have been studied extensively in the laboratory. A known feminizing compound, o,p`-DDT induces dose-dependant uterotrophy in rats (Bitman *et al*., 1969), oviduct growth and feminization of male bird embryos (Fry and Toone, 1981), and synthesis of VTG in the mosquito fish (*Gambusia affinis*)(Denison *et al*., 1981) and the rainbow trout (*Oncorhynchus mykiss*)(Donohoe and Curtis, 1996). The metabolite p,p`DDE can act as a powerful anti-androgen, and it has been demonstrated that p,p`DDE delays the onset of puberty in juvenile rats and reduces androgen-dependant seminal vesicle and prostate weight in adults. Many of these effects observed *in vitro* bear a resemblance to effects observed amongst wildlife populations, however, these effects amongst wildlife populations are more likely due to additive effects (several chemicals/ groups of chemicals).

In the Baltic seas, reproductive failure of seals (both grey, *Halichoerus grypus*, and ringed, *Phoca hispida*) has been linked to with high body burdens of organochlorines (Reijnders, 1986). Reproductive success was reduced in Common seals fed on a diet of fish obtained from the Western part of the Wadden Sea which have heavy body burdens of many pollutants (Reijnders, 1986). However, it should be emphasized that it is not yet known whether these effects are manifest as a result of endocrine disruption. There are a number of studies reporting reproductive impairment in wild birds, predominantly fish-eating birds, such as gulls and terns; perhaps because of the bio-accumulative nature of these chemicals. Reproductive impairment in these birds is characterised by high embryonic and chick mortality, edema, retardation of growth, and developmental deformities (Tyler *et al*., 1998). High concentrations of DDT and

its metabolites have also been shown to cause egg thinning in many species, including glaucous winged gulls (*Larus glaucescens*) breeding in Puget Sound (Fry *et al*., 1987), peregrine falcons (*Falco peregrinus*) around the Great Lakes, and white-tailed sea eagles (*Haliaeetus albicilla*) in the Baltics (Helander *et al*., 1982). The reproductive success of the white-tailed sea eagle has been negatively correlated with residue concentrations of DDE and PCB in eggs (Helander, *et al*., 1982). Although the mechanism of eggshell thinning has never been completely deduced, suggested mechanisms have incuded: 1) limiting the supply of calcium to the shell gland (Peakall *et al*., 1975), 2) decreasing carbonate availability for shell formation (Bitman *et al*., 1970), and 3) altering steroid hormone receptors or function (Lundholm, 1985). Though egg shell thinning is one of the most cited examples of endocrine disruption in wildlife, the multitude of hypothesis regarding of the mode of action of DDE mean it cannot be stated with certainty that egg shell thinning is the result of endocrine disruption.

In reptiles, extensive evidence exists that man-made chemicals may have impaired the reproductive function of alligators in Lake Apopka in Florida. As a result of a spill of DDT, there followed a decline in the number of juvenile alligators present; female alligators had twice the concentration of plasma oestradiol and also exhibited abnormal ovarian morphology. Male alligators exhibited poorly organised testes, abnormally small phalli, and their plasma testosterone was reduced by 75% (Guillette *et al*., 1994, 1995, 1996). Hypothesized to be caused by DDT and its metabolites, concentrations of p,p`DDE in the eggs of demasculinsed alligators were as much as 90 times greater than required to exhibit anti-androgenic activity *in vitro*; gonads of alligators were permanently modified *in ovo*, altering steroidogenesis and inhibiting normal sexual maturation. The study of alligators from a pollution “hotspot” in Florida has provided some of the more convincing evidence that endocrine disruption in wildlife has resulted from exposure to hormone-disrupting mimics.

Exposure of animals to some other organochlorine pesticides, including methoxyclor, lindane, and kepone, causes similar effects to those caused by exposure to DDT compounds. Vinclozolin is a dicarboximide fungicide used widely to protect grapes, fruits, vegetables, and hops against fungal damage. It is not persistent, but rather is degraded to several metabolites (as mentioned, M1 and M2) in the soil and/or within

the plant itself. Two metabolites (but not vinclozolin itself) are anti-androgens. When vinclozolin was administered to pregnant rats during gestation and early lactation this resulted in male progeny with retained nipples, cleft phallus, hypospadias, and reduced anogenital distance, suggesting these progeny where feminized (Gray *et al*., 1994). Exposure of medaka (*Oryzias latipes*) to Lindane (contains 5 to 10% β-HCH) 24 hours after fertilisation for a 4-month period can result in the occurrence of ovo-testis/intersex (Wester, 1991).

In summary, although in the general environment in the Western world concentrations of these DDT-pesticides alone are unlikely to have deleterious effects on wildlife, at localised "hot spots" of discharge(s) into the environment, or in countries where these chemicals are still widely used there is little doubt that these chemicals can adversely affect wildlife.

1.7.3. Polychlorinated Biphenyls

(PCBs) Polychlorinated biphenyls (PCBs) are mixtures of chlorinated aromatic chemicals manufactured by the chlorination of biphenyl (209 congeners possible; 25 most relevant), they are ubiquitous and persistent environmental contaminants and are of great concern as global aquatic pollutants (Tyler *et al*., 1998). As contaminants, they are present in similar concentrations to DDT, rarely reaching 1µg/l in aquatic environments of the western world. Although most environments have concentrations of PCBs that are at or below the NOEL for laboratory studies, many ortho-substituted and hydroxylated PCBs are known to have very high bio-concentration factors. For example, substantial amounts (up to several micrograms per kilogram) of the different PCBs have been found in eggs of various species of fish even though the fish themselves were exposed to concentrations in water of only 1 to 10 ng/l. Further, 70% of the worlds production of PCBs is still in use or in stock, and therefore could enter the environment in the future.

Non-ortho-substituted PCBs can impair reproductive processes, but these effects are mediated through the Ah receptor, and are like DDT compounds in this respect. Some of the ortho-substituted PCBs have a relatively high affinity for oestrogen receptor (50

to 500 times less than oestradiol). For example, in laboratory experiments Arochlor 1221 (mixture of PCBs) has been found to induce an uterotropic response in female rats (albeit at high doses)(Gellert, 1978). However, the doses of ortho-substituted PCBs shown to induce short-term reproductive and developmental effects in animals are higher than the concentrations found in the general environment.

Some hydroxylated PCBs are structurally similar to thyroxine, and they are transferred across the placenta and may accumulate in the fetuses of rats and mice. Thyroid hormones are thought to be critical for the normal proliferation of Sertoli cells, which, in turn, orchestrate the whole process of testicular development in neonates (Cooke, 1996). A dose dependant reduction in plasma thyroxine levels was reported in the offspring of female rats exposed to Arochlor 1254 during gestation (Goldey *et al*., 1995), and a similar study showed a depression in seminal vesicle, caudal epidydimal, and pituitary gland weights (Gray *et al*., 1993). However, other authors report increasing testis weight and increased daily sperm production in response to similar doses of Arochlor 1254 (Cooke *et al*., 1996). However, some of the hydroxylated metabolites of PCBs (2´4´6´-trichloro-4-biphenylol and 2´3´4´5´-tetrachloro-4-biphenylol) are oestrogens in non-mammalian vertebreates. Eggs that were painted with a single application of 100 mg of 2´4´6´-trichloro-4-biphenylol induced 100% sex reversal in male red-eared slider turtles. The effects appeared to be dose dependant, because lower doses resulted in lower proportions of feminized hatchlings (Bergeron, *et al*., 1994).

Impaired reproduction in the male Florida panther (*Felis concolor coryi*) has been suggested to result from PCBs, resulting in males with low sperm counts, low ejaculation volume, poor sperm motility, and a high incidence of sperm malformations (Facemire *et al*., 1995). These panthers live in an area that is heavily contaminated with PCBs, but as yet, no-cause effect data are available.

In summary, PCBs and their metabolites are potentially hazardous as EDCs, although most environments have concentrations that are below the NOEL for laboratory studies, their very high bio-concentration factors are a cause for concern. Further, dose-response studies on most of the PCBs that can cause endocrine disruption have not been published.

1.7.4. Dioxins

Polychlorinated dibenzo-p-dioxins (PCCDs) can consist of up to 75 congeners and of these 2,3,7,8-tetrachloror-dibenzo-p-dioxin (TCDD) is considered the most reproductively toxic. PCCDs are chemically very stable and have low water solubility's. Because of their hydrophobicity and lipophilicity, PCCDs adsorb strongly to organic material in particulate and dissolved phases. Consequently, concentrations in the aquatic environment are generally very low, although in some areas between 0.3 and 1.4 μg/l have been reported (Tyler *et al*., 1998).

In laboratory experiments, exposure of pregnant animals to extremely low concentrations of TCDD (0.1 to 1 μg/kg/d) during the period of sexual differentiation leads to alterations in the reproductive system of the male pups (suppressed anogenital distance, sperm count, epididymus weight, and seminiferous tubule diameter), which are consistent with de-masculinisation (Mably *et al*., 1992; Gray *et al*., 1995), and which appear to be similar to the effects caused by environmental oestrogens, such as o,p`-DDT. Although known mechanistic pathways can explain many of these effects for TCDD, the way by which some of the observed effects are manifest including hormonal irregularities in the oestrus cycle, reduced litter size, and reproductive toxicity and developmental toxicity in the male offspring, remain to be determined. The ability of dioxin to act as an endocrine disruptor appears to be dependant on the target tissue.

Dioxins are part of the mixture of chemicals found in the Great Lakes basin that have affected the developmental and reproductive status of fish-eating birds (see section 2.1). Concentrations of PCCDs/Fs and PCBs are now less than those in the 1960s and 1970s, and some bird populations, such as those of double-crested cormorants and herring gulls, have made dramatic recoveries (Giesy *et al*., 1994). However, it is also true that populations of other bird species (e.g. Common and Forsters terns) continue to decline, despite falling levels of these pollutants.

Downstream of 12 bleached kraft mill effluent discharges white sucker and the Lake whitefish exhibited a number of effects upon their endocrine status including reduced

plasma sex steroid concentrations, decreased gonadal size, lower fecundity with age, absence of secondary sexual characteristics in males, and increased liver size (Munkittrick & Vanderkraak, 1994; Munkittrick *et al*., 1991; Munkittrick *et al*., 1992; Munkittrick *et al*., 1994., 1992). With reduction in gonadal size in female fish the most consistent change, the presence of 2,3,7,8-substitued chlorinated dioxin and furan congeners in the bleached-kraft mill effluent has caused concern because of the similarities between the field observations described above and results from laboratory work with dioxins (Vanenheuval *et al*., 1994).

In summary, areas heavily polluted with PCCDs such as areas receiving bleached kraft mill effluents and landfill leacheates could have relatively high concentrations of dioxins due to their persistence. Although relatively high concentrations of dioxins may persist and reproductive effects on exposed animals may occur in such areas, the complexity of the mixtures of chemicals present makes it impossible to ascribe a specific effect to a particular chemical such as TCCD (Tyler *et al*., 1998).

1.7.5 Plasticisers

Alkylphenols (APs) such as nonylphenol (NP) and octylphenol (OP), are plasticizers, and together with related compounds, are the products of microbial breakdown of non-ionic surfactants alkylphenol ethoxylates (APEs) that are used in the manufacture of plastics, elastomers and industrial detergent formulations, such as occurs during sewage treatment (Giger *et al*., 1984). Substantial amounts of alkylphenolic compounds enter the aquatic environment from wastewater discharges into rivers, domestic sewage effluents can contain up to hundreds of micrograms of alkylphenolic compounds per litre (Ahel *et al*., 1994; Ahel and Giger, 1985; Naylor, 1995). Most of the information on the fate and behaviour of APs in the environment is on NP. Although concentrations of NP in industrial effluents can exceed 100 µg/l, in most rivers studied in the UK, concentrations are typically below 1 µg/l (Backburn and Waldock, 1995). The oestrogenic activity of NP has only been known since 1938, but only recently have detailed studies into their endocrine disrupting effects been published. Alkylphenolics have been shown to possess oestrogenic activity, binding to the oestrogenic receptor with potencies of 2,000- to 100,000- fold less than oestradiol-

17β (Jobling and Sumpter, 1993), and NP and octylphenol (OP) can also interact with the androgen receptor (Gray and Kelce, 1996; Laws *et al*., 1995). These APs have also been shown to induce some of the cytochrome P450 mixed function oxidases (Lee *et al*., 1996; Lee *et al*., 1996).

Both the alkylphenolic compounds NP and octylphenol (OP) have been tested *in vivo*, and were found to be oestrogenic in fish. NP and OP (in 3-week exposure trials) induced an elevation in plasma VTG at threshold concentrations of 10 μg/l and 3 μg/l, respectively. In these experiments, doses of APs that induced VTG synthesis also significantly decreased the rate of growth of the testis (Jobling *et al*., 1995). Further, the concentrations of individual nonylphenolic compounds (OP usually lower concentrations than NP) in some UK rivers are high enough to stimulate VTG synthesis in fish (Harries *et al*., 1997).

In the UK, the oestrogenic nature of STW effluents have been confirmed with field studies using a freshwater cyprinid fish the roach (*Rutilus rutilus*). Roach placed downstream of STWs exhibit both VTG production (Purdom *et al*., 1994) and reduced growth rate of the testes (Harries *et al*., 1996). Indeed, such effects are a widespread phenomenon in rivers of low water quality, and significant stretches of some rivers receiving STW effluent have been shown to be strongly oestrogenic (Harries *et al*., 1997). In the river Aire in Yorkshire, APs were shown to be the main causative agents. APs emanating from wool-scouring plants meant that river water contained concentrations of NP alone up to 120 ug/l and 0.2 mg/kg in the bed sediments in the river; levels that are strongly oestrogenic to fish (Jobling *et al*., 1995). Therefore, studies have demonstrated, in some situations (near STWs) the concentrations of alkylphenolic chemicals in the aquatic environment were high enough to cause effects on aquatic organisms. However, in 1986, Germany instituted voluntary restrictions and Switzerland banned the use of surfactants in laundry detergents. Throughout northern Europe - England, France, Germany, and the Scandinavian countries - a voluntary ban on APE use in household cleaning products began in 1995; hence levels of these chemicals in the environment are now declining.

Bisphenol-A (BPA or 4,4´-isopropyl-idendiphenol) is a synthetic chemical used in the production of polycarbonate for the manufacture of a very wide range of plastic

products, and epoxy resins. BPA is also oestrogenic, and binds to the human oestrogen receptor (ER) with an affinity of 2,000 times less than the affinity of oestradiol-17β. Despite this weak binding and interaction with the oestrogen receptor, it is still one of the more potent known anthropogenic oestrogenic mimics (Routledge and Sumpter, 1996). Recent evidence has suggested that the reason for this could be its interaction with the oestrogen-related receptor (ERR-γ), an orphan nuclear receptor to which E2 does not bind (Okada *et al*., 2008), although the significance of the interaction between BPA and ERR-γ is presently unknown. *In vivo*, effects in rodents include (at doses of tens of μg/d) increases in uterine weight (Bond *et al*., 1980), and a stimulation of prolactin release (Steinmetz *et al*., 1997).

Theoretically BPA could contaminate areas around landfill sites due to leaching from food packaging materials and from the coatings of food and drink cans, and may be found at concentrations above 1 μg/l within aging water pipes, where it has been used for lining. However, although BPA is readily biodegraded under aerobic conditions (half-life between 4.5 and 4.7 days), BPA has limited biodegradation under anaerobic conditions, leading to concerns about BPA accumulation in anaerobics sediments (such as estuaries)(Voordeckers *et al*., 2002). Concentrations of this chemical in water samples have been found to vary greatly depending on location and time of sampling; for the majority of rivers concentrations fall below 0.1 μg/L, and very few samples contain BPA concentrations above 1.0 μg/l (Crain *et al*., 2007). BPA has been widely tested for in surface waters**.**

1.7.6 Phthalates

Phthalates are the most abundant man-made chemicals in the environment. A few are produced in large quantities and used mainly to impart flexibility in plastics. The ubiquity of phthalates in the aquatic environment is well known, and their presence has been reported in rivers, sediments, and in fish. Di-n-butyl-phthalate (DBP) is generally the most abundant phthalate commonly detected in the aquatic environment; reported concentrations in the western world range from 0.3 μg/l to 30 μg/l. Reported concentrations of phthalates are inconsistent, probably due to the contamination of laboratory equipment and standards due to their ubiquitous nature. Jobling *et al*., 1995

found that butyl-benzyl phthalate and DBP were weakly oestrogenic in fish hepatocytes at high concentrations (in vitro between 10^{-4} and 10^{-6} M), more recent epidemiological evidence suggests that prenatal phthalate exposure may reduce anogenital distance in boys (a demasculinising effect) (Swan *et al*., 2005). Reports have shown that at very high doses some (DEHP and DBP) phthalates are known to be testicular and ovarian toxicants (Gangolli, S.D., 1982; Davis *et al.*, 1994). However, the high concentration required to induce these effects have led to the assumption that phthalates are unlikely to cause adverse effects in wildlife. However, early life stages (therefore developmental stages) maybe more sensitive to the effects of these chemicals than adult stages; when tested on fish, deleterious effects on eggs and survival rate of fry occurred at concentrations in excess of 320 µg/l (Woodward, 1988).

In summary, although it is unlikely that most wild animals would be exposed to concentrations of individual phthalates known to cause effects in the laboratory, the in vivo effects of phthalate chemicals have not been investigated adequately, and therefore phthalates cannot yet be precluded from having a potential endocrine-modulating impact on wildlife (Tyler *et al*., 1998).

1.7.7 Plant Derived Oestrogens

Some natural chemicals present in the environment can mimic the effects of oestrogens, including mycooestrogens (produced by fungi), and phytooestrogens (synthesized by plants). Laboratory studies have demonstrated these chemicals may not only have oestrogenic actions, but also anti-oestrogenic actions too. For example, they have been linked to premature cessation of ovarian cyclicity, delayed vaginal opening in the female progeny (Levy *et al*., 1995), and have been shown to affect the expression of the oestrogen receptor in a dose related manner (a high dietary intake can decrease fertility in vertebrates) (Markaverich *et al*., 1995; Burroughs, 1995). Phytooestrogens also have oestrogenic activity in fish, both *in vitro* (Pelissero *et al*., 1993) and *in vivo* (Pelissero *et al*., 1991).

In Florida, discharges from paper and pulp mills (phytosterols; sitosterol and stigmastanol) have caused masculinization and behavioural changes in female mosquito fish (*Gambusia affinis*) (Bortone *et al*., 1989). The fish exhibited modification of the anal fin into a gonopodium-like structure (typical of male fish). These effects on mosquito fish have been suggested to result from exposure to the large amounts of phytosterols present in the effluent discharges at these sites (Howell and Denton, 1989).

For aquatic animals, the routes of exposure to phytoestrogens (and indeed to most endocrine-disrupting chemicals) could also include uptake from the water, in addition to dietry uptake. Though there is very little information on the concentrations of phyto- and myco-oestrogens in the aquatic environment a single paper states that concentrations of β-sitosterol are between 20 and 60 ng/l in various river water samples from Germany (Strumpf *et al*., 1996). As with other natural oestrogens, the significance of their presence in the environment is unknown; we know nothing about their availability to aquatic organisms once they have entered the river.

1.7.8 Steroidal Hormone Compounds

1.7.8.1 Synthetic Oestrogens

Synthetic pharmaceutical oestrogens are often very potent, and in some cases more potent than the natural oestrogen oestradiol-17β. Of potential concern to wildlife is the potent synthetic oestrogen, 17α-ethinyl-oestradiol (EE2), which is widely used in the contraceptive pill and enters the aquatic environment via sewage treatment works (STW) discharges. The concentration of EE2 in STW effluents is variable, but has been found to range from non-detectable to between 0.2 and 7 ng/l (Desbrow *et al*., 1998). Entering STW plants as inactive conjugates, it is thought β-glucuronidases found in many species of bacteria (including the most common bacteria *E. coli*) in sewage biotransform EE_2 back to its active form (Tyler *et al*., 1998). EE_2 is more stable than the conjugated compounds and may persist in the environment for longer.

In mammals, EE_2 administered neonatally can induce abnormalities in reproductive development in the offspring (Brown-grant *et al*., 1975). EE2 is also a very potent inducer of vitellogenesis and exposure of male trout to EE2 in the water at concentrations of only 0.1 ng/l and above causes a rapid and pronounced synthesis of VTG (Purdom *et al*., 1994). Little is known about the reproductive consequences of exposure of oviparous animals to EE2. However, dosed into water it can retard the growth and development of testes (single dose of 2 ng EE2/l; maturing male trout), and reduce the number of eggs deposited (10 ng/l for 4 weeks; fathead minnows)(Jobling *et al*., 1995). Further, reproductive dysfunction can occur even in fish with no observed gross gonadal abnormalities; juvenile three-spined sticklebacks exposed to 1.75 ng/l EE2/l for 4 weeks post hatch (reared in pristine water to adulthood) went on to build significantly fewer nests (Maunder *et al*., 2007).

The concentration of EE2 in sewage treatment works effluent and waters receiving this effluent are variable (Richardson and Bowron, 1985). Of seven different sewage treatment plants studied in the UK, in three of these effluents, EE2 was measured at concentrations ranging between 0.2 and 7 ng/l (Desbrow *et al*., 1998), but in the remaining four effluents, EE2, was non-detectable. Thus, although the concentrations of EE2 present in the environment are likely to be very low (ng/l, or less) and maybe variable, the extreme potency of this synthetic oestrogen means that even these concentrations can cause adverse biological effects in wildlife. Further, a wild population of fathead minnows has been driven to near extinction by chronic exposure (7-years) to low concentrations (5-6 ng/l) of EE2, demonstrating both the potency of this chemical, and a population level effect.

1.7.8.2 Natural Oestrogens

Recently, there has been a focus on 17β-oestradiol (E2), estrone (E1), estriol (E3), and their possible involvement in endocrine disruption in wildlife. These natural steroid oestrogens are very potent hormones and have biological activity at very low concentrations in the blood (2 to 3 pg/ml in many mammals). This focus has been due to the findings that STW effluents contain 17β-oestradiol and estrone at concentrations ranging from just above 1 ng/l up to almost 80 ng/l (Harries *et al*.,

1996); concentrations sufficient to explain the induction of vitellogenesis in caged fish placed close to effluent discharges in these rivers (Routledge *et al*., 1998). The consistency of the findings suggests that the common source of the oestrogenic compounds is domestic sewage, and the likely primary sources are women. Indeed, women excrete between 10μg and 100μg of oestrogen daily, depending on the phase of their menstral cycle (Aldercreutz *et al*., 1986). Pregnant women may secrete up to 30 mg of oestrogen a day.

All vertebrates, including humans, produce and excrete conjugated steroid hormones, including 17β-oestradiol, estrone, and estriol. Though excreted in conjugated forms (like EE_2) these hormones are also found as free oestrogens, de-conjugated by bacteria in the environment (see section 1.7.8.1.). The relevance of intensive animal farming practices as regards to the productions of these hormones is currently unknown, as is the significance of these oestrogens in the environment; as with other oestrogens (EE_2, and plant derived oestrogens), we know nothing of the availability of the oestrogens to aquatic organisms once they have entered rivers.

However, in studies with male salmonid fish, dietary exposure to high doses of oestradiol had deleterious effects on spermatogenesis and even complete regression of the testes (Billard *et al*., 1981). Responses varied according to the dose administered and stage of testicular development at the time of treatment. Similarly, oestradiol implants caused sex reversal in tadpoles over a 4-month exposure period (Chang *et al*., 1996). As yet, there are no reports on the long-term effects in fish of exposure to natural oestrogens (and androgens) via the water.

In summary, there is now quite strong evidence that these natural hormones are involved in mediating the oestrogenic effects (in rivers at point sources close to STW`s) observed in wildlife, as documented largely through laboratory fish experiments, but also in modelling studies.

1.7.9 Organotin Compounds

Most examples of endocrine disruption in wildlife are those of vertebrates. However, amongst invertebrates, the best known and probably the most convincing example of endocrine disruption amongst any wild animal population are found amongst the molluscs. This example is the effects of tin compounds upon marine molluscs that have affected populations worldwide.

Tributyltin (TBT) and related compounds are used as biocides, notably on the hulls of ships. Measurements in seawater taken prior to restrictions on TBT use in antifouling paints (1982) had shown levels generally ranging between 50 and 500 ngl^{-1} in European marinas, reaching peak values, that would have at the time been thought of as high –TBT pollution, in the range of 1 μgl^{-1} in "highly contaminated" areas (Alzieu, 2000). Today, maximum concentrations in marina waters rarely exceed 100 ng/l^{-1} along the English Channel and Atlantic coasts (average 42 and 22 ng/l^{-1} respectively)(Alzieu, 2000). Concentrations of TBT in the proximity of shipping lanes can reach 30 ng/l and higher (Langston *et al*., 1987). Both TBT and triphenyltin (TPT) have been shown to be only slightly soluble in water; and will readily absorb to particulate matter (Tyler *et al*., 1998) and accumulate in animals due to its high bioaccumulation potential. Comparatively high bio-concentration figures (BCFs) of TBT in marine molluscs range 1 x 10^3 -10^4 TBT-Sn compared to 100-500 TBT-Sn in fish.

In marine gastropods, imposex (the imposition of male characteristics upon females; including a penis and vas deferens) is now been reported worldwide, and is known that tin biocides are the causative agents. Imposex is an irreversible condition, which can effectively castrate female molluscs are therefore causing reproductive failure amongst whole populations of many molluscs' species and extinction of populations in certain areas. Affected animals exhibit an enhanced testosterone titre (altered testosterone oestrogen ratio), observed after the development of a penis and/or vas deferens, and prostate tissue in affected females. TBT can affect the aromatization of androgens to oestrogens (hence altered sex hormone ratios), and also inhibits the conjugation of testosterone. Recently, the MOA via which TBT may cause imposex in these molluscs has been further elucidated; retinoid X receptor (RXR) gene expression has been demonstrated to be significantly higher in imposex –exhibiting females (in the penis forming areas)(the rock shell; Horiguchi *et al*., 2007; dog whelk;

Castro *et al.*, 2007), and the male penis had the highest content of RXR protein amongst the tissues of normal males and females (Horiguchi *et al.*, 2007). The RXR could be involved in the mechanism of induction of male-type genetalia (penis and vas deferens) by organotin compounds in at least two species of marine mollusc. It seems reasonable to expect that other species of marine molluscs may also express this receptor in a similar manner. However, although more than 100 species of gastropod molluscs have been reported to suffer imposex the reproductive consequences of exposure to TBT varies amongst gastropod species (Gooding & Le Blanc, 2001); in some species, reproduction is seemingly unaffected. Therefore, not only does the sensitivity to tin biocide compounds vary between marine prososbranch species, but the reproductive effects also.

Marine molluscan populations have been extensively studied (unlike their freshwater counterparts) for well over 20 years. During the mid-1980s imposex in *N. lapillus* was investigated in U.K. waters through field studies and experiments (e.g.transplantations) augmented by laboratory studies (Matthiessen and Gibbs, 1998). Neogastropods are gonochoristic (sexes are separate) and it was observed that many female dogwhelks (*Nucella lapillus*) had a penis-like structure behind the right tentacle and treatment of larval or juveniles with concentrations of TBT as low as 1 ng/l^{-1} TBT induced imposex. In the case of the dogwhelk (*Nucella lapillus*), it was shown that the degree of severity of imposex is a graded response dependant on level of TBT exposure, as it is in some other prosobranchs. The ubiquitous distribution of the dogwhelk in the northern hemisphere led to their use as bio-monitor of TBT contamination. In wild populations of dogwhelks, imposex has been observed at 1 ng/l TBT, whereas at 6-8 ng/l TBT complete reproductive failure and local extinction due to sterility of females has resulted (Matthiessen and Gibbs, 1998). These characteristics led to several scientific groups to classify the stages of imposex through which the dogwhelk passes with exposure to increasing organotin compound concentration. Gibbs *et al.*, 1987 described six successive stages; formation of a vas deferens (stage 1), penis development (stage 2 to 4; 1-3 ng/l^{-1} of TBT in water), and blockage of the oviduct (stage 5 and 6) thereby leading to sterility as the vulva becomes blocked preventing egg capsules from being laid. Further, although anatomically sterilized, these females still continue to produce egg capsules, which accumulate within the oviduct wall, causing it to rupture, leading to death of the

animal. This phenomenon can explain the predominance of males in affected areas. Consequently, this system answered an urgent need for a simple, but reliable, early detectable morphological parameter to assess TBT concentrations in the environment, eliminating the need for water analysis. Though the implementation of laws prohibiting the use of organotin biocides on smaller vessels under 25 metres has yielded positive results, TBT is still widely used as an anti-foulant in shipping and it has not been possible to find a population of whelks in Europe that have not shown signs of exposure to this chemical.

In summary, TBT acts as an endocrine disruptor in some marine prosobranch mollusc species, and is still present in the marine environment at concentrations high enough to cause imposex in these animals.

1.8 Possible Causes of Molluscan Declines

1.8.1 Ecological needs for Freshwater Molluscs

The reasons behind the current loss of molluscan species globally and more local declines in populations is complicated by our relative lack of knowledge of their ecological needs. Indeed, at present we know only the approximate habitat preferences of many freshwater species (Table 1.1), but understanding of the precise environmental parameters and life histories of many taxa is lamentably slight (Willing, 1997). This is because, in many cases, ecological knowledge has increased little since Boycotts` classical paper on molluscan habitats (1936). This implies that we currently do not know, and cannot predict with any high degree of accuracy, the precise reasons for current declines in many snail populations. Consequently separation of the effects upon populations of known important ecological factors, such as eutrophication, from other factors (the effects of which are less well understood), is beyond our present capabilities.

This gap in our knowledge, due to lack of research is particularly surprising in view of the ecological importance of molluscs to aquatic systems. Molluscs, like other invertebrates, are key components of all ecosystems and are an important part of the

food chain in limnic systems, in their role as primary producers (Czech *et al*., 2001). Therefore, endocrine disruptive effects of TSE (containing many chemicals) on molluscs, if present, may have far reaching adverse consequences for biodiversity and the sustainability of natural ecosystems (Depledge and Billinghurst, 1999).

Table 1.1 Habitats and preferred conditions of some common freshwater gastropods found in European freshwaters, those species chosen for initial study.

Species	Main Habitats	Occurrence	Conservation listing?
Theodoxus fluviatilis	R^H, S	W	No
Bithynia tentaculata	R^H, S, L, P, C, G	W	No
Physa fontinalis	U	W	No
Lymnaea stagnalis	L, P, C, G	W	No
Planorbarius corneus	R, L, P, C, G,	W	No
Potamopygrus antipodium	U	W	No
Viviparus viviparus	R^H, C	W	No

Key

Main habitats

R^H = hard water
L = lake
S = stream
P = pond
C = canal

U = ubiquitous
G = lowland grazing
W= widespread

Adapted from Willing, 1997.

1.8.2 The recent threat of extinction to Non-Marine Molluscs and their causes

Non-marine species of molluscs, both land and freshwater, are facing an unprecedented survival crisis on a global scale which may in large part be due to the activities of man. This group includes nearly 40% of all known animal extinctions that have occurred in the last 400 hundred years, which is more than all land vertebrates together (Bouchet *et al.*, 1999). While the IUCN (The World Conservation Union) Red List of Threatened Animals lists 641 species of molluscs that have become extinct in the wild since 1600 (IUCN, 1996), conservation biologists consider four mechanisms (not mutually exclusive) currently operating that are thought to be responsible for driving species to extinction (Bouchet, 1997). These four mechanisms are fragmentation and loss of habitat, over-exploitation of commercial species, introduction of alien species, and chains of extinctions. Fragmentation, degradation, and loss of habitat are certainly the primary cause of loss of global biodiversity (Bouchet *et al.,* 1999), and these factors have clearly played a role in population decreases of European land and freshwater molluscs. Loss of habitat can be "absolute" (for instance when an area of natural land is replaced by housing or industry), but more but is more often qualitative (for example, through changes in agricultural uses, and/or point and non-point pollutions like effluents). Further, in terms of the Red List, as most data on habitat and population decline is retrospective due to the time taken to collect all relevant data, it is hard to evaluate the current status of widespread but declining species (Bouchet *et al.,* 1999). Therefore, both globally and more locally (Europe) gaps in our current knowledge of the status of molluscan populations and their habitats make it difficult to tease apart factors that may currently be causing these extinctions; at best we can only cite possible causes for population declines.

1.8.3 The Threat to European Freshwater Molluscs

Some of the most vulnerable and threatened European freshwater species like those chosen for this study have shown dramatic declines, particularly in the last 30 to 40 years (Willing, 1997). Recent studies of the declines of freshwater molluscs across

Europe (e.g. Wells and Chatfield 1992) confirm a number of common problems (often involving fragmentation, degradation, and loss of habitat) including:

1. nutrient over enrichment of waters (eutrophication) by fertilisers and sewage;
2. falling or erratic water levels (often due to changes in farming practices, over-abstraction for a water supply and hydro-electric schemes);
3. inappropriate and damaging channel management (such as mechanical ditch clearance and the canalisation of river courses);
4. an increase in suspended solids as a result of soil run-off from arable fields and livestock-trampled watercourse banks;
5. acidification of waters in soft-water areas due to acid rainfall (often accentuated by conifer plantations immediately adjacent to streams in the upper catchments);
6. the removal of small ponds, ditches and marshes for agricultural and other reasons;
7. the commercial collection of a number of larger species of snail.

Although these common problems (both global and European) may help explain the decline of freshwater molluscs, there could also be other unknown factors, combination of factors, or specific pollutants, involved. For example, sewage is listed here because it is known to contain nutrient enhancing compounds (nitrogen and phosphorus), and because of its complex biologically/endocrinologically active endocrine disrupting abilities. One of the problems with aquatic compared with terrestrial habitats is that environmental deterioration and pollution originating at one place can cause adverse effects over areas well beyond the source of the pollution (Willing, 1997). This situation is further complicated by the fact that there can be many point sources of TSE along the length of a river. With over 711 sewage-treatment plants in England and Wales serving population equivalents of at least 10, 000, TSE can be a major contributor to the flow and chemical content of British rivers. Therefore, the presence of many chemical pollutants in U.K waters is currently poorly documented. However, what is known is that in some areas, it is thought that local pollution of water bodies by organic and inorganic waste (by inference from industrial or domestic sewage and/or fertilisers) has reached such a degree as to turn rivers into “sewers” in which these snails cannot survive. It has been reported by at least one eminent scientist that in these areas, some commonly found snails are locally extinct or nearly extinct (Ulrike Schulte-0ehlmann pers com.). It has long been known, contrary to popular beliefs, that freshwater snails are indicators of clean

environments (Mouthon, 1996), and therefore freshwater snails, including common ubiquitous snails, may be under a more serious and imminent threat not as yet revealed by the literature. Part of the stimulus for this project was the knowledge that European freshwater molluscs like other aquatic species, will be exposed to treated sewage effluent (TSE); a source of (chemical) pollution already known to cause reproductive and developmental abnormalities in other wildlife species in our rivers.

1.8.4 The Link between Chemical Pollution and Population Declines

Examples in the literature of population declines in freshwater molluscs linked directly to pollution from treated sewage effluents (TSE`s) are absent. This may be because TSEs are not the cause, that this work has yet to be done, or because of the difficulties of attributing declines specifically to chemicals in the water. However, a degree of intolerance to polluted water is commonly cited in the literature for some mollusc species (Anon, 1995; Young 1995). An empirical estimation of the tolerance of several mollusc species to organic pollution using the saprobic system was proposed long ago (Sladecek, 1973). In this system, the assemblage of organisms present (where species present are counted) is used to characterize water quality. Here, the greater the number of species present the higher the water quality is proposed to be. Mouthon, 1981A also presented an outline of a range of pollution sensitivities for different freshwater mollusc species based on a limited number of degraded situations. However, studies dealing with molluscs and physico-chemical variables liable to be pollution indicators are rare (Mouthon, 1996). Therefore, though observation has shown that decreases in water quality (by observations of community structure) are associated with declines in mollusc populations, the precise reasons for this (in terms of physico-chemical properties of the water) is usually unknown. A single author, Mouthon (1996), has gone a step further. Using 48 freshwater molluscan species from five catchments in central Europe, this author was able to describe in general terms the degree of biodegradable pollution or the extremes of conditions (physico-chemical) that each species of snail (groups exhibiting commonality separated by hierarchical ascending classification) would be expected to find optimum, or inhibitory. For each species observed, threshold values for sensitivities to biodegradable pollution that could inhibit or cause the elimination of

different species was given. Further, six variables, dissolved oxygen, BOD_5, NH_4^+, NO_2^-, Kjeldhal nitrogen and PO_4^- correlated well with degraded situations, and were used to classify these snails species. However a decline in water quality or habitat enrichment is generally cited as a possible reason for observed declines amongst several other possibilities (turbidity, acidification, river-channel work, water abstraction, commercial mollusc fishing, etc.). As Mouthon (1996) noted, although these six variables are clearly indicators of biodegradable pollution, the presence of toxic substances (likely from treated sewage effluent) is also probable in most degraded situations. Science, therefore, has progressed little recently in deciphering the real causes of localised freshwater molluscan declines. No advancement past biodegradable pollution or enrichment as causes of population declines has followed after these earlier studies. Nonetheless, EDCs are contained in TSE, and although some of the more recent literature suggests that individually some of the chemicals are not overtly toxic (listed in Table 1.2 below), also their combined effects in TSE remain largely unknown to science at present.

1.9 Evidence for Effects of EDCs on Freshwater Molluscs

1.9.1 Chemicals present in TSE

A part of the stimulus for this study came from the knowledge that local declines of certain freshwater molluscan populations may not be isolated incidences but instead indicative of a more widespread problem. Due to the relatively recent and large rises in the human population, especially of industrialised countries, rivers and estuaries have become repositories for enormous amounts of industrial and domestic waste. Sewage treatment works (STWs) have been built to accommodate this waste from an increasing human population, together with advances in civilization this has led to an increased need to treat and recycle finite water resources (Routledge *et al.*, 1998). Moreover, it is estimated that 30 % of all UK water is of a recycled nature (Lester, 1990). Following reuse, this water is then returned to the aquatic environment via STW`s, of varying processes and performances, which improve greatly its quality, but to date are not capable of removing all chemical contaminates. Treated sewage effluents (TSE) have a major influence on both the flow rate and chemical content of

rivers all over the world. It is estimated that in the UK, Europe and other countries with a high population density, the relative volume of effluent discharged from STW`s to rivers can be considerable, sometimes contributing 50% of the flow of the river, a figure that can rise to 90% or more in summers with low rainfall (Routledge *et al.*, 1998). Most easily illustrated in a global context, in densely populated westernised countries (typically European), the human population has roughly trebled over the last century; it took from the beginning of time until about 1927 to put the first 2 billion people on the planet, and only a further 75 years to add 4 billion more (Cohen, 2003).

STWs receive domestic and industrial waste, and as an inevitable consequence of the treatment of this waste (despite improving considerably the quality of the final effluent) discharge a highly complex mixture of different chemicals back into the environment. The consequence of modern water management strategies to wildlife has not yet been established, with TSE discharges known to contain many chemicals with endocrine disrupting capabilities (e.g., alkylphenols and natural steroids) and chemicals that presently have unknown endocrine disrupting capabilities. Examples of endocrine-active chemicals that can be found in natural waters are listed in Table 1.2. However, as illustrated by Table 1.2, not all chemicals found in natural waters originate from TSE. For example, fertilisers and pesticides originate from agricultural practises, but still end up as part of a complex mixture of chemicals found in these natural waters. Further, all of these chemicals (either alone or in combination) have been found to cause endocrine disruption in wildlife species, although the relative importance of each chemical that has been identified in TSE, in contributing to the effects of TSE, in particular, is at present unknown. Recently the effects of a mixture of five oestrogenic chemicals that have the same mode of action (E_2, EE_2, NP, OP, and Bisphenol-A) were tested at low concentrations for VTG induction in the fathead minnow (*Pimephales promelas*). Although the concentration of each individual component in this mixture had no effect, when combined an oestrogenic effect was observed; known as the "something from nothing" phenomenon (Silva *et al*., 2002). Furthermore, this effect could be predicted using a simple mathematical model, known as concentration addition (Brian *et al*., 2005). However, this assessment of the endocrine disrupting potential of a relatively simple mixture of chemicals *in vivo* (by comparison to TSE) is one of the first such attempts to do so.

Table 1.2 Chemicals that can be found in the freshwater aquatic environment of developed regions around the world.

Class/Chemical	Use/Source	Environmental concentration*	Reference
Industrial Chemicals/Byproducts			
Alkylphenol ethoxylates (APEs)	Non-ionic surfactants; plastics and detergents formulations	Domestic sewage effluent up to 100s ugl^{-1}	ref 2
4-Nonylphenol (4-NP)	Breakdown product of APEs	U.K. rivers < 10μgl^{-1}, up to 42 µgl-1 industrial	ref 20, ref 284
Octylphenol (OP)	Breakdown product	Generally lower than for NP	ref 100
Bisphenol-A (BPA)	Used in manufacture of plastics	0.0005-0.41µgl-1in surface water	ref 100
Polychlorinated biphenyls (PCB`s)	Coolants and lubricants	Rarely reaches 1 ugl^{-1} in developing world	ref 201
Di-n-butyl phthalate (DBP)	Plasticizer or softening agent	Range from 0.3 ugl^{-1} to 30 ugl^{-1}	ref 93
Dioxins	Industrial incineration byproducts	Generally very low, some areas 0.3-1.4ugl^{-1}	ref 52
Pesticides			
Lindane	Organochlorine insecticide	At or below NOEL for laboratory studies	ref 277
DDT	Organochlorine insecticide	At or below NOEL for laboratory sutides	ref 277
Methoxychlor	Organochlorine insecticide	At or below NOEL for laboratory sutides	ref 277
Organotins e.g. Tributyltin (TBT)	Biocide	Secondary effluent 7-47 ngl^{-1}	ref 94
Metals			
Zinc	Industry (e.g., chloralkali plants)	U.K. rivers 8.5-29.8 μgl^{-1}	ref 218
Lead	Industry (e.g. battery production)	U.K. rivers 0.3-3.9 μgl^{-1}	ref 218
Natural Products			
17 β-oestradiol	Steroid oestrogen	1ngl^{-1} to almost 80 ngl-1 (STW effluents)	ref 100
Oestrone	Steroid oestrogen	From 15-220 ngl^{-1} UK study (STW effluents)	ref 94
b-Sitosterol	Phytooestrogen	20-60 ngl^{-1} in various German rivers	ref 277
Synthetic Products			
17 α -ethinyl-oestradiol (EE_2)	Pharmaceutical oestrogen	0.2-7 ngL^{-1} (STW effluents)	ref 252

* all entries assumed for western world countries unless otherwise stated.

Although the precise nature and number of chemicals contained within treated sewage effluent discharged to rivers that might cause endocrine disruption (ED) is unknown, there are some classical endocrine disrupting chemicals (EDCs) in effluent that have been shown to have effects in *in vitro* experiments in the laboratory, and therefore could have effects upon wildlife in our rivers and other waters (see section 1.9.2). However, the combined effects of all these chemicals (and others) in TSE (a highly complex mixture) on freshwater fish species (and some estuarine species) hasonly been studied since the 1970s. This research has progressed due to the discovery of an increased rate of intersex in a fish population (the Roach) immediately downstream of a TSE works (Purdom *et al*., 1994).

This research has led to considerable (and increasing) evidence for endocrine disruption in freshwater fish populations living in stretches of rivers downstream of treated sewage effluent discharges in Europe (Jobling and Tyler, 2003). In the original work, conducted in the UK, it was established that these effluents from treated sewage effluents were oestrogenic to fish (Purdom *et al*., 1994, Harries *et al*., 1996, Harries *et al.*, 1997), inducing the production of vitellogenin (egg protein precursor), in male fish. In these fish, histological examination of the gonads revealed that a larger than expected proportion of the males were in fact intersex, as defined by the simultaneous presence of both male and female gonadal characteristics. Freshwater fish species in which the occurrence of intersex has been reported and deemed to be abnormal include the roach (*Rutilus rutilus*), bream (*Abramis abramis*), the chub, the gudgeon (*Gobio gobio*), the barbel (*Barbus plebejus*), the perch (*Perca fluviatilis*), the stickleback (*Gasterosterus aculeatus*) and the shovelnose sturgeon (*Scaphirrhychus platyorynchus*) (Jobling and Tyler, 2003).

However, intersex as a consequence of exposure to effluent has been most intensively studied in the roach (*Rutilus rutilus*), a lowland fish of the UK and Europe. In 1998, Jobling *et al*., 1998 published the first strong evidence of widespread sexual disruption in the roach in which macroscopic examination of all the fish collected from eight sites appeared to show that the fish were either male or female. However, with histopathological examination, the proportion of intersex fish was shown to range from 16% to 100% between different sites, whereas from “clean” control sites 4% of males collected were intersex. This low level of intersex from clean sites

maybe “natural” as intersex in the closely related cyprinid fish the carp (*Cyprinus carpio*) is reported up to 5% (a rate also cited for other species; both vertebrate and invertebrate)(Komen *et al.,* 1989). The study concluded that the concentration of sewage effluent in a river was a major causal factor in the development of intersexuality in fish (further downstream from hotspots effects decreased), and the oestrogenic constituents were responsible for this phenomenon. This abnormal reproductive development in male roach occurs with concurrent reductions in fertility and fecundity in males and females, respectively (Jobling *et al*., 1998, Jobling *et al*., 2002b, Jobling and Tyler, 2003). Further studies by Jobling *et al.,* 2002 have further shown that intersex roach, even those mildly affected, are compromised in their reproductive capacity and may therefore produce fewer offspring than fish from uncontaminated sites. However, though the effects of oestrogenic chemicals present in effluent (for example natural and synthetic oestrogenic chemicals: EE_2, E_2, E_1, NP, OP and BPA) have been tested in aquatic vertebrates (mainly fish) in *in vitro* tests, and occasionally *in vivo,* many other chemicals including various heavy metals and many pesticides (to name a few known EDCs) are also present. However, as 60,000 man made chemicals alone are known to be present, albeit in very small quantities in effluent, their combined effects in effluent upon invertebrate species to date remain unknown. Further, to the authors’ knowledge, only one paper has studied the effects of TSE upon a freshwater molluscan population (see Watton and Hawkes, 1984).

1.9.2 Effects of EDCs on Molluscs

Although there is also a paucity of field evidence to demonstrate reproductive and developmental effects of TSE upon freshwater molluscs, there are numerous reports in the literature that demonstrate reproductive and developmental disturbances due to exposure to single chemicals in laboratory exposures.

When exposed to TBT (or TPT) the freshwater tropical Prosobranch *Marisa cornuarietis* snail demonstrated the formation of imposex, as occurs in marine molluscs (see section 1.7.9). Imposex had occurred when exposed to concentrations of 50 and 200 ngl^{-1} (Schulte-Oehlmann *et al*., 1995), which would be considered to be relatively high concentrations of tin biocides in European freshwaters (Fent & Muller,

1991; 10`s of nanograms per litre), and for imposex formation and reproductive failure in marine prosobranchs. However, fecundity was reduced at lower concentrations (EC_{10} 4 months 5.59 ngl^{-1} TPT), with a complete inhibition of spawning at nominal concentrations > 250 ngl^{-1} TPT. When *Potamopyrgus antipodarum*, was exposed to TBT (or TPT) via spiked sediments at environmentally relevant concentrations, the number of new embryos in the brood pouch declined in a time and concentration dependant manner; reproductive effects, therefore, occurred in a similar manner in both of these freshwater snail species. Therefore, both these snail species have been proven to be highly sensitive to the effects of both TBT and TPT at environmentally relevant concentrations, with a similar degree of sensitivity to tin biocide concentration (that can reduce fecundity) as their marine prosobranch cousins.

However, the European model species *Lymnaea stagnalis*, Czech *et al*., (2001) appears not to be as sensitive to organotins as some of their marine (or freshwater) cousins; exposure to 100 ngl/L TBT (nominal) for seven weeks had only slight effects on egg production in adults and hatching rate of eggs, clearly associated with this comparatively high concentration only. However, *in Lymnaea stagnalis* the associated marked degenerative effects on epithelial tissues of the lung and foot characterized by an extreme inflammatory response were thought due to toxicity effects rather than endocrine disruption, though no histopathological effects were found on the sex organs at any of the concentrations tested (1,10 and 100 ngl/L TBT). In contrast, in *Marisa* where the effects (of TBT) observed were thought to be due to endocrine disruption, histopathological analysis demonstrated only a marked impairment of spermatogenesis in males. Further, the occurrence of superfemales in *Marisa* is a very striking parallel case to the example of deleterious effects of TBT (imposex) in female marine muricid prosobranch molluscs (Oehlmann *et al*., 2000). Disruption of steroid synthesis has led to population level effects (reproductive failure) in many marine prosobranch populations, where TBT can affect P450 aromatase activity; disruption of steroid synthesis (and/or metabolism) may also play a part in abnormal sexual development and reproduction in *Marisa* (Schulte-0ehlmann *et al*., 1995).

When *Lymnaea stagnalis* was exposed to other known androgenic chemicals t-methyl testosterone or ß-sitosterol (at 100 ngl^{-1}) there were no reproductive disturbances,

snails presented only with atrophy of the albumen gland (Czech *et al*., 2001). This species appears, by comparison to *Marisa* or *Potamopyrgus antipodarum* (or marine prosobranchs) at least, insensitive to TBT (and other androgenic compounds) and unlikely to be adversely affected at environmentally relevant concentrations. As with marine species, different freshwater species or sub-classes of the class Gastropoda may be at higher risk than others of the same class (Czech *et al*., 2001), due to differences in their sensitivities to these organotin compounds. Sensitivity to tin compounds, however, has been tested in a few very freshwater gastropod species. Nevertheless, freshwaters may contain TBT at concentrations that could cause toxicity, and disturbances to reproduction and development in marine molluscs, and by influence, possibly freshwater molluscs also.

Exposure of *Lymnaea stagnalis* to 4-NP at environmentally relevant concentrations caused no observable effects upon reproduction, and only slight adverse effects on egg production of adults (and hatching rate of eggs), at high concentrations only (Czech *et al*., 2001; 100µgl^{-1} 4-NP). However, histopathological changes were observed in epithelial tissues of adults, typical of an extreme inflammatory response perhaps indicating toxicity. Exposure of *Potamopyrgus antipodarum* to BPA or OP for 63 days was found to stimulate new embryo production at 5 µgl^{-1} and EE2 was effective at 25 ngl^{-1} EE_2 (Jobling *et al*., 2003), but caused inhibitory effects at higher doses (however, 1 ngl^{-1} EE_2 is high in the aquatic environment, Carol Kelly, pers comm.). Similarly, when *Marisa* was exposed to BPA or OP in the low microgram per litre range both compounds induced a complex syndrome of alterations in female *Marisa* referred to as "superfeminisation". This was characterized by massive oocyte stimulation, an increase in pallial accessory sex organ size (albumen, and capsule gland), and blockages of the oviduct that led to mortalities. This maybe a particular worry since rivers in Switzerland have been found to contain tens of ug/L of a wide range of alkylphenolic compounds (NP, OP) (Ahel *et al*., 1994), though concentrations of these chemicals have been found to be lower in UK rivers (see Table 1.2; <10 µgl^{-1} 4-NP). The occurrence of superfemales in *Marisa* is a very striking parallel case to development of imposex in TBT exposed marine prosobranch molluscs (Oehlmann *et al.*, 2000). However, as the functions of steroids remains equivocal in many molluscs (see section 1.5), especially freshwater molluscs, other mechanistic pathways maybe involved. Unlike some of the other examples of

exposure to xeno-oestrogens, *Marisa* exhibited no histopathological changes (no effects on sperm/oocyte production) or signs of acute toxicity, and therefore the described effects might be due to true endocrine disruption (see section 1.1 onwards).

As "superfeminisation" in *Marisa* causes mortalities and could alter sex ratios (Oehlmann *et al*., 2000), this potentially could be only the second known example of true endocrine disruption that could lead to population level effects in aquatic molluscs. The presence of oestrogens and xeno-oestrogens (previously characterised in Chelmsford STW TSE) are relevant to my own exposure studies. However nobody, to my knowledge, has tested the effects of these chemicals on the species of molluscs I used in my studies.

Anti-androgens also seem to affect reproduction and development in some molluscs. However, the anti-androgens cyprotene acetate (CPA) and vinclozolin (VZ) induced effects in immature *Marisa* only, and not in mature animals. Penis and accessory male sex organ length (e.g. penis sheath, prostate) in exposed animals were significantly reduced; an effect which was reversible as the males' attained puberty. Typical androgen-mediated responses in females (imposex development) were partially or totally suppressed by simultaneous administration of the anti-androgen CPA. Compared with oestrogens and androgens, the anti androgenic responses seemed less drastic and, in contrast to the other two disruptor classes, might have no biologically significant effects at the population level, although other effects cannot be ruled out. As with xenooestrogens, antiandrogens caused advanced sexual repose in adult males. As sperm storage is a feature amongst molluscan species (Fretter, 1965; *Viviparus*; Dillon 2000; pulmonates) females might encounter fewer breeding males towards the end of the breeding season. Therefore, on balance, the heterozygosity of offspring could be affected, producing offspring on average more genetically convergent (see section 1.9.4).

1.9.3 Other Chemicals in TSE capable of eliciting an Endocrine Response in Molluscs

Many other chemicals found in treated sewage effluents, besides known (xeno) oestrogens, androgens, or (anti) androgens, could have developmental or reproductive effects upon freshwater molluscs. These include heavy metals, pesticides and herbicides.

When *Potamopyrgus* clones were exposed to sediment containing bound Cadmium, there was a significant negative effect upon reproductive output and growth (Jenson *et al*., 2001). Juvenile survival was negatively correlated with increasing Cadmium exposure, and the babies were smaller at all Cadmium concentrations (Jenson *et al*., 2001)(Moller *et al*., 1996; 200 ug/l^{-1} in water). As with many EDC`s, the route by which heavy metals (such as Cadmium) exert their effects is not fully understood. However, the toxicity of heavy metals (including, but not only Cadmium) to *P. antipodarum* has been demonstrated in TSE (Watton and Hawkes, 1984), and therefore, an element of reproductive toxicity (via CNS) cannot be ruled out. When *Lymnaea stagnalis* was exposed to relatively high concentrations (400 μgl^{-1}) of Cadmium, egg production ceased together with considerable snail mortality (Gomot, 1998). Further, hatching was reduced to 0.4% with 200μgl^{-1} Cadmium with very low mortality. However, at lower concentrations other mechanistic pathways (besides reproductive toxicity) could affect reproduction. Cadmium has been found to interact with the ligand-binding domain of the oestrogen receptor (ERα) of human MCF-7 cells (Garcia Morales *et al*., 1994)(Stoica *et al*., 2000). Therefore, if Cadmium binds to the oestrogen like-receptor or analogues (see section 1.5.4) of freshwater snails, reproduction could in theory be altered. As the function of the ERα receptor in freshwater snails is as yet unknown, or what chemicals bind to it, the effects of EDC`s binding to this receptor are also as yet unknown. Therefore, we cannot predict the effects upon reproduction. Further, Gomot, 1998, confirmed that juvenile stages of this snail were the most sensitive to the effects of endocrine disrupting or potentially toxic chemicals, as were the juvenile stages of marine prosobranch molluscs to organotin compounds. However, little work has been done on the reproductive capacity of developmentally exposed (as eggs or/and juveniles) snails.

Cuppen *et al*., 2000 exposed an aquatic freshwater snail community to the fungicide carbendazim at concentrations of 0-1000 μgl^{-1} in order to study abundance (reproductive output). This fungicide does not strongly adsorb to sediment (log K_{ow}

1.49), and is known to inhibit the activity of the enzyme acetylcholinestase, thus exhibiting direct toxicity (via CNS). Interestingly, they found variable affects upon different trophic groups of molluscan species. The sediment feeding snail species *Bithynia tentaculata* and *Bithynia leachi*, decreased in adult numbers at higher concentrations, but were the only mollusc species to do so and were the most sensitive species. The number of limnic or herbivorous snails (non-sediment specialist feeders) significantly increased in abundance at high concentrations, as did the abundance of *Lymnaea stagnalis* juveniles. Though the behaviour of sediment feeding species suggested direct toxicity (withdrew into their shells) this was not the case with herbivorous species. It is possible that a different mechanism of action in operation occured in herbivorous snails; stimulation of the cerebral ganglia being the most obvious target organs (viz CDC and DB cells) to cause an increase in reproduction. Alternatively these herbivorous snails could have been less sensitive to the toxicity of carbendazim and/or could have received a smaller dose of carbendazim due differences in absorption routes (sediment versus water). Similarly, when another limnic snail species, *Lymnaea palustris*, was exposed to hexachlorobenzene (HCB; a chlorinated fungicide of very low acute toxicity, which is highly bio-accumulated by aquatic invertebrates), an inhibition of body growth and stimulation of egg production occurred whilst adult mortality was unchanged (Baturo *et al*., 1995). When Presing (1993), exposed *Lymnaea stagnalis* to K-othrine an insecticide (active ingredient deltamethrin; 10 and 100 μgl^{-1}) egg production was significantly stimulated (though appetite was suppressed), and hatched juveniles showed a significantly lower survival rate and delay to reach maturity. However, in contrast to the chlorinated fungicide HCB, deltamethrin is a synthetic pyrethroid derivative known to be both very toxic (CNS) and oestrogenic, which could explain both the increase in reproduction and toxic effects. Though one author states the detoxification system of pulmonate snails means they are very resistant to the effects of this insecticide (Hansen *et al*., 1972), this may not be the case (see section 1.9.6). Though effects on the central nervous system were obvious (increased reproduction in exposed groups possibly due to stimulation the DB`s and CDC`s), the mechanism by which feeding was suppressed is unknown. This data implies a complex mode of action of these chemicals, where toxicity does not necessarily imply a negative effect upon all developmental and reproductive parameters. Clearly, research into the endocrine disrupting potential

(modes of action) of fungicides (herbicides and pesticides similarly) on molluscan species is at an early stage and their effects at present are not predictable.

Conversely when Wilbrink *et al*. 1987 exposed *Lymnaea stagnalis* to dihalogenated biphenols (2 analogues tested individually), that are known to have an affinity for the oestrogen receptor (ER) and very high BCF`S (highly lipophilic) (see section 1.7.3), production of egg masses and eggs were inhibited and there was an increased latency of oviposition (interfered with CDCH pathways), whilst no effect on growth or food consumption was found. However, PCB`s have been implicated in multiple actions upon the reproductive system (Tyler *et al.,* 1998) as well as affects on growth, and perhaps significantly, inhibition of xenobiotic-metabolising enzymes in mammals (see section 1.7.9; effects of TBT on marine prosobranch molluscs) (Gupta *et al*., 1981, Ueng *et al*., 1980). In this study, a single analogue reduced egg production (both increased latency of egg mass laying) whilst the other did not. Though the effects of these analogues (and their biotransformation products) are thought to be structure related (Wilbrink *et al*., 1987) all the mechanism/s of action are by no means clear or known to science. Interestingly, significant reproductive effects were only found during a peak of reproduction, and not as reproduction decreased thereafter. Therefore, important reproductive effects could be missed at certain times during the seasonal reproductive cycle, and perhaps most notably, this peak of reproduction should not be missed.

1.9.4 Individual to Population Level Effects

All of the chemicals discussed have in common the ability to affect single parameter(s) of the lifecycle (such as growth, time to first reproduction, fecundity, adult and juvenile survival, etc.). However, of supreme importance to science is the question whether effects at the level of the individual animal translate into population level effects. Jenson *et al*., 2001 used population growth rate as an ecotoxicological endpoint with four clones of *P. antipodarum*. These authors found that even large changes in both reproduction and time to first reproduction resulted in relatively small changes in population growth rate. Adult survival did not contribute notably to changes in population growth rate; feeding rate did not contribute to the negative

effects of Cadmium at all. Importantly, rather large impacts on individual life-history traits translated into smaller impacts on population growth rates. Though only a single study, these results refute the assignation made by some authors that even small changes in fecundity of a mollusc that reproduces by producing large numbers of offspring could have population level effects (see Czech *et al*., 2001). However, the study of Watton and Hawkes (1984) described a reduction in mortality rate that led to greater reproduction, and therefore, a greater density of snails in effluent compared to river water control, demonstrating a large effect upon population numbers by changing a single life cycle parameter. Further, the effects of TBT (and TPT) upon adult males of *Marisa* (see section 1.9.2) could lead to a genetic bottleneck amongst the whole snail population with fewer males breeding, and a new environmental pressure (e.g. viruses, parasites, change in climatic conditions e.g. global warming) could threaten the whole population. Similarly, where the whole population consists of clones, such as would be the case with *P. antipodarum* in Europe, the same problem of a genetic bottleneck amongst a snail population could arise. However, despite these few examples to the contrary, there is little evidence to support the contention that small changes (decreases) in the fecundity of a mollusc that produces large numbers of offspring would have population level effects.

1.9.5 Previous studies on the effects of a Complex Mixture

Though, there is a paucity of field evidence to demonstrate the effects of treated sewage effluents (TSE) upon freshwater molluscs, there are to my knowledge, two studies where freshwater molluscs have been exposed to a complex mixture, in the form of a treated sewage effluent. Jobling *et al*., (2003) exposed *Potamopyrgus antipodarum* to a graded concentration of treated sewage effluent known to contain a number of oestrogenic EDC`s. Initially all doses of effluent were stimulatory to new embryo production (only 100% significantly). However, this initial stimulation was followed by a reduction in reproduction to levels similar to controls, and TSE was inhibitory in 100% effluent. There were no effects of TSE upon growth or mortality. A similar pattern of embryo production occurred when *Potamopyrgus* was exposed to EE_2 and BPA, again survival was unaffected, however, by the end of the experiment exposure to environmentally relevant doses of BPA enhanced growth rates (Jobling *et*

al., 2003). Similarly, when *Lymnaea peregra* was exposed to a TSE (Blithe Valley STW) source, effects were time and dose dependant (Watton and Hawkes, 1984). Exposed over an entire two-year period, seasonal effects were observed including an increase in adult numbers by late summer (due to a decreased mortality rate), and therefore, increased reproduction in effluent, and an extension of the breeding season in effluent into autumn. As no chemical analysis of this effluent was undertaken, it is not possible to compare likely causative chemicals (or groups of chemicals e.g. (xeno) oestrogens) between these two TSE exposures. Nevertheless, taken together, the results of these studies suggest that chemicals present in treated sewage effluents may influence developmental and reproductive parameters in freshwater molluscs, both prosobranch, and pulmonate snails. There have, however, been no other studies to date exposing freshwater European molluscs to TSE, to determine whether or not this is the case.

1.9.6 Molluscan Detoxification Systems

Like other species, molluscs respond to xenobiotic exposure by producing a variety of enzymes and other proteins (Snyder, 2000). Together these proteins comprise the MFO (mixed function oxygenase) system; cytochrome P450 enzymes are functionally related, ubiquitous enzymes (Teunissen *et al*., 1992) and are important detoxification enzymes found in aquatic organisms (Wilbrink *et al*., 1991). These enzymes metabolise a wide range of substrates including endogenous molecules (e.g. fatty acids, steroids) and xenobiotics (e.g. hydrocarbons, pesticides, drugs) (Snyder, 2000). There is confusion in the literature as to whether these systems are capable of xenobiotic metabolism and excretion in molluscs. Their activities are much lower than in mammalian systems (Livingstone, 1989) and the level of biotransformation products are very low (Baturo *et al.*, 1995). When *Lymnaea palustris* was exposed to concentrations of HCB at concentrations high enough to affect both fecundity and growth (Baturo *et al.*, 1995), there was no observable effect upon benzo(*a*) pyrene hydroxylase (B*a*PH) activity. This enzyme is an important member of the MFO system, often cited to increase its activity when organisms are exposed to xenobiotics, thus confirming the low inducibility of mollusc xenobiotic-metabolizing enzymes by chlorinated compounds (Baturo and Lagadic, 1996). Perhaps significantly, Oehlmann

et al., (1995) working with *Marisa* and several marine species, demonstrated that molluscs accumulate more TBT (200% more than *H. reticulata*; 60% more than *N. lapillus*) than marine molluscs; a finding that was corroborated in Tsuda *et al*. (1990), and which was probably due to differences in the MFO system (Tsuda *et al*., 1990). Therefore, the consequences of differences in MFO system (including P450) upon bioaccumulation of other chemical contaminants from the environment are also yet to be established.

1.10 The Molluscan Endocrine System

1.10.1 The Role of Vertebrate Sex Steroids in Gastropod Molluscs

Until recently, most studies into EDCs in the aquatic environment were carried out in fish, because of the similarities of their endocrine system (steroidal hormone based) to that of higher vertebrates, the ease of which they can be experimented upon, and because of their commercial importance. By comparison, the endocrine system of invertebrates, in particular molluscs, is poorly understood. Consequently they have been seldom studied in spite of the fact that invertebrates (shellfish, worms, insects, sea urchins etc.) constitute 95% of all animal species. Invertebrate reproduction is known to be peptide hormone and ecdysteroid dominated (Oberdorster and Cheek, 2000; Le Blanc, 1999). Although the presence of steroidal hormones has been demonstrated in many invertebrate species (and in particular the marine molluscs), the roles of vertebrate sex steroids in these species have not been well defined (Le Blanc *et al*., 1999).

Though a seasonal cycling of steroid hormones has occasionally been demonstrated in marine and prosobranch and freshwater gonochorist snail species (Gauthier *et al*., 2006; Jobling *et al*., 2003; Schulte-Oehlmann *et al*., 1995), and suggested amongst freshwater pulmonate molluscs (Tompa *et al*., 1984), with hormone levels peaking during summer (a vertebrate “oestreus” like phenomenon), a definitive link between the presence of these steroidal hormones and the maturation of the gonad (and accessory sex organs), is currently lacking. Instead, peptidal hormones (DBH, CDCH, APGW-amide and others) are thought to be responsible for maturation of the gonad

and other accessory organs involved in reproduction, released by the cerebral ganglion or primitive brain of molluscs. Though a link between the release of peptidal hormones, steroidal hormones, and sexual maturation is often suggested by authors, steroidal hormones have only been demonstrated to control the development of the accessory sex organs (ASO`S; reproductive tract, albumen gland, etc.) where they are produced locally in the gonad of terrestrial pulmonate slugs (Takeda, 1979). However, tantalisingly, steroids are sometimes associated with sexual maturation in marine prosobranch species, though such evidence is rare in the literature. For example, Matsumoto *et al*., (1997) demonstrated an increased oestrogen profile during summer, these steroids were demonstrated to be synthesized locally in the gonad of both the scallop (*Pactinopecten yessoensis*) and oyster (*Crassostrea gigas*); 17β-oestradiol appeared responsible for sexual maturation in *C. gigas*.

The work of Laufer and Downer (1988) detailed and summarised the then current breath and depth of research into molluscan endocrinology; research that has been improved upon little since. This work included snail groups relevant to my project, found amongst the subclass Pulmonata, represented by two orders; the Basommatophora, living in freshwater habitats, and the Stylommatophora, which are terrestrial snails and slugs. It also included the prosobranchs, both marine and freshwater. Their work describes a high degree of variability in the endocrinological control (metabolic pathways) of reproduction between these different molluscan groups. Therefore, any endocrinological link that can be made between the presence of hormones (steroidal) and reproduction is limited intuitively to specific species, and the same link cannot be assumed to hold true amongst other snail groups. Therefore, currently the precise mechanisms of control of sexual development and reproduction, in particular their endocrine control, is only known to be peptide hormone based, with relatively very little knowledge of the function (if any) of steroidal hormones or their roles.

However, an ever increasing number of reports in the literature detailing the effects of EDC`s, particularly oestrogenic chemicals (natural steroidal compounds or their synthetic mimics), upon molluscs do suggest that these chemicals impact the reproductive systems of molluscs (with a consequent increase in reproduction). Whether the mechanism of action of oestrogenic (steroidal) chemicals is the same in

some invertebrates to that of vertebrates (a direct effect upon the gonad and/or affecting peptide hormone production in invertebrates), is unknown. Further, though the relevance of this class of endocrine disrupting chemicals to freshwater molluscs in particular is at yet undetermined, they do appear to be clearly relevant as endocrine disruptors in molluscs, and perhaps enhancers of reproduction in gastropod molluscs (Jobling *et al*., 2003)

1.10.2 Peptidal Hormone based control of Molluscan Reproduction

The idea that peptide based hormones control molluscan reproduction has long been known and understood (Laufer and Downer, 1988), and has been extensively researched. The first signs of hormones in molluscs concerned the endocrine control of reproductive activity (Joosse, 1988), as far back as 1943; castration techniques (with the giant garden slug *Limax maximus*) resulted in regression of female accessory sex organs and pointed towards an endocrine role of the gonad (Laufer and Downer, 1988). There soon followed the first reports suggesting the presence of neurohormones (cf 1935) and endocrine organs (cf 1957) in molluscs; these endocrine organs turned out to be the dorsal bodies (DB) of pulmonate snails.

Active investigations for 25 years to around 1988 have focused on the endocrine control of reproduction in pulmonates and sex reversal in prosobranchs (as animal (neuro) endocrine model systems) (Joosse, 1972; Le Gall and Streiff, 1975; Joosse, 1988). Hormones have been shown to control reproduction in molluscs (for reviews see Geraerts and Joosse, 1984; Joosse and Geraerts, 1983), and they also control growth, energy metabolism, blood circulation, and water and ionic metabolism (Joosse, 1988). As a consequence of these studies, the endocrine control of reproduction is relatively well researched in gastropods (and cephalopods), which are amongst the snails groups relevant to my project.

Although many details of the endocrine control of reproduction in basommatophoran pulmonates have already been have studied (Bohlken and Joosse, 1982), the endocrine control of reproduction in freshwater prosobranchs is largely unknown. In fact, in prosobranchs, only the control of the release of gametes is well known, and

great attention has been given to the phenomenon of sex reversal and its endocrine control in a few marine species (Joosse, 1988). Most studies in pulmonates have focused on the endocrinology of female phase of reproduction leading to oviposition (Wayne, 2001), with little or no work on the hormonal events leading to male copulation.

Molluscan central nervous systems are relatively simple compared to vertebrates (see Figure 1.3). Hormones that control reproduction and growth in gastropod molluscs originate in the region of the cerebral ganglia, which are part of the brain of molluscs (Wayne, 2001). In hermaphrodite basommatophoran snails, the dorsal bodies (DB`s) are peripheral endocrine glands attached to the cerebral ganglia. DB`s produce a peptide hormone, dorsal body hormone (DBH), which stimulates vitellogenesis in the oocytes and growth, cellular differentiation, and activity of the female accessory sex organs (ASO`s)(Geraerts and Joosse, 1975). In effect, DBH prepares the female reproductive system for egg laying. Importantly, DB`s have been found in every pulmonate species studied so far (Laufer and Downer 1988). The final step in ovulation or egg laying is induced by the hormone caudodorsal cell hormone (CDCH) from the neuroendocrine caudodorsal cells (CDC) of the cerebral ganglia (see Dogterom *et al*., 1984). These cells produce multiple peptides involved in induction and integrated control of ovulation, egg-mass formation and egg laying behaviour in freshwater pulmonates (Joosse, 1988). The DB`s and CDC`s are at least partly controlled by a neuroendocrine centre in the lateral lobes (LL`s) of the cerebral ganglia, which in a complex and integrated way also control growth. Geraerts, 1976 has demonstrated that these lobes contains neurosecretory cells and are involved in the control of reproductive activity and body growth and in *Lymnaea stagnalis*, apposing processes in gastropods whereby strong growth inhibits reproduction. The LL`s can exert an inhibiting effect on the light green cells (LGC), and, via these, on body growth as would be the case in winter under conditions of starvation. When the reproductive season begins (more food is available) the LL`S no longer inhibit the LGC`s which then produce a growth hormone like neuropeptide (GH). Body growth is very favourable in gastropods during springtime before summer reproduction begins, especially in maturing juveniles. For growth of the female sex organs, the presence of the gonadotropin DBH in addition to GH is needed. When the summer breeding season begins the LL`s again inhibit the light green cells, energy obtained

from food is switched from growth towards reproduction (from growth of ASO`s toward egg laying).

Due to the significance of DB`s in pulmonate snail reproduction, prosobranchs were also investigated for equivalent structures (see Joosse, 1975). In prosobranchs, extracts of cerebral ganglia were found to stimulate vitellogenesis (Streiff, 1967; and Choquet, 1969). Indeed, extracts of cerebral ganglia obtained from an active female prosobranch *Viviparus viviparus* was shown to contain a factor capable of stimulating vitellogeneis in a stylommatophoran hermaphrodite *Helix aspersa* (Joosse, 1972) suggesting a common endocrine pathway of vitellogenesis induction in both pulmonates and prosobranchs. As such, there is reasonable evidence to suggest that one type of endocrine organ (that can stimulate vitellogenesis) occurs in all gastropods, an endocrine organ of common structure called "organs juxta-ganglionires" in the older literature (Joosse, 1972), viz the DB`s. The DB`s together with the CDC`s and the LL`s control the regulation of reproduction (egg laying during the summer months in the field), growth, and mortality in pulmonate snails.

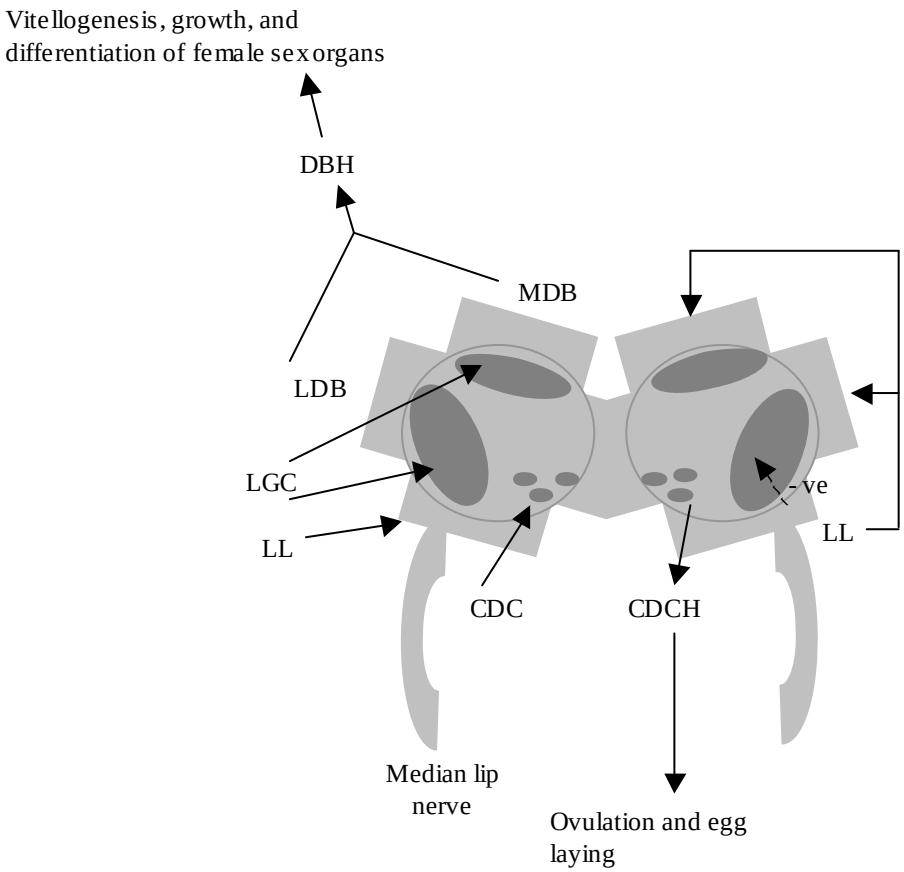


Figure 1.3 Diagrammatic representation of the transverse section through the cerebral ganglia of *L. stagnalis*, showing the location of the various neuroendocrine and endocrine centers. When the breeding season begins, neurons in the lateral lobes inhibit the light green cells (energy is switched to reproduction). Neurons in the lateral lobes also activate the endocrine dorsal bodies and caudodordal cells. Dorsal body hormone, secreted from the lateral and medial bodies, stimulates vitellogenesis and growth and differentiation of the female accessory sex organs. Caudodorsal cell hormone, secreted by the caudodorsal cells, stimulates ovulation and egg-laying behaviours. LL = lateral lobes; LGC = light green cells; LDB = lateral dorsal body; MDB = medial dorsal body; DBH = dorsal body hormone; CDC = caudodorsal cells; CDCH = caudodorsal cell hormone.

1.10.3 Steroidogenic Enzymes and Esterases

Steroidal sex hormones have been identified in the tissues of many marine gastropod species, often as part of research into the effects of TBT in marine environments (Morcillo and Porte, 1999; Bettin *et al*., 1996; Joosse, 1984; Matsumoto *et al*., 1997). Further, investigations have demonstrated that steroidogenesis takes place by biosynthetic pathways that are similar to those found in vertebrates (Matthiessen and Gibbs, 1998). Indeed marine bivalves like the scallop (*Patinopecten yessoensis*), the Pacific oyster (*Crassostrea gigas*), as well as basommatophoran pulmonates posses at least one of the enzymes involved in vertebrate steroid biosynthesis. This enzyme, 17ß- Hydroxysteroid dehydrogenase (17β-HSD), which converts 17ß- oestradiol (E2) into estrone (E1), is present in the gonad of *Lymnaea stagnalis*, the terrestrial snail *Euhadra peliomphala*, as well as the gonad of slugs (Takeda, 1980). Variation of 17β-HSD activities in the ovary of the scallop, at least, is supposed to reflect the dynamics of steroid metabolism (including 3β-HSD and aromatose activities; also essential to steroid biosynthesis), and associated with the reproductive cycle. Reports detailing the presence of the steroidal hormones in freshwater molluscs are fewer by comparison compared to marine prosobranchs. Nevertheless, their presence has been confirmed in some species (Schulte-0ehlmann *et al*., 1995). De Jong-Brink and co-workers (1981) reported not only the steroidogenic activity of the ovotestis but also of the digestive gland of *L. stagnalis*; gastropod molluscs appear able to metabolise vertebrate steroidal sex hormones partly via the digestive gland. However, importantly, at least one study has, tantalisingly, suggested a function for these steroids in the gonad of pulmonates; when either *Lymnaea stagnalis* or *Helix aspersa* were treated with steroid hormones histological changes took place (Takeda, 1979). Testosterone stimulated the male phase and inhibits the female phase; oestradiol has the reverse effect, while progesterone stimulates both phases (Aubrey, 1961). Yet surprisingly, though the importance to science of a possible link between steroidal sex hormones and reproduction (and therefore endocrine disruption) is obvious, this area of work remains neglected since the late 1980`s; no elucidation of this tentative link has been forthcoming. The function of steroidal sex hormones (particularly female sex hormones) in both marine and freshwater gastropods remains equivocal.

Of the steroidal hormones reported to be present in molluscs, the function of androgens (i.e. testosterone in particular) is best understood. Testosterone appears to be important for the development of accessory sex glands and spermatogenesis. Castrated slugs (*Euhadra prelionphala*) administered testosterone regenerated a penis (Takeda, 1980). Testosterone was also found to accelerate spermatogenesis in the land snail (*Theba pisana*) and led to a decrease in the number of mature ova (Sakr *et al.*, 1992). In basmatophorans, De Jong-Brink *et al.*, 1981 presented circumstantial evidence suggesting that Sertoli cells produce steroids, which probably control maintenance and completion of spermatogenesis and/or spermiation. As discussed (see section 1.7.9), female dogwhelks (*Nucella lapillus*) develop a penis and/or vas deferens when exposed to TBT, and this process is associated with the presence of testosterone (identified in the tissues of gastropods) (Spooner *et al*., 1991). As part of an alternative hypothesis to explain imposex, there may be an interaction between RXR and TBT in tissue (area from where penis may develop in imposex females) behind the right tentacle (Horiguchi *et al*., 2007), which maybe of importance. A speculative suggestion could be that this interaction alters steroidogenesis (via aromatse); the RAR: RXR heterodimer has been shown to induce aromatase activity in human MCF-7 cells (Mu *et al*., 2000). Since peptide hormones are known to be key in molluscan sexual development, it cannot be ruled out that TBT may cause unknown neuropeptide secretions (from cerebral ganglia) that can then affect steroidogenesis and this may also in part help explain imposex formation in these molluscs. However, other than testosterones association with the development of male reproductive tissues and organs (penis and vas deferens), and a single paper, to the authors' knowledge, describing an increased rate of spermatogenesis due to the presence of testosterone little is known of the function of testosterone (and other steroidal androgens) in molluscs.

Another explanation for the effects of TBT upon gastropods could involve the regulation of the metabolism and/or excretion of steroidal hormones, and this could help explain further the function of sex steroids in molluscs. More than one mechanism has been proposed that could explain the hormone titres found (raised testosterone and/or altered testosterone oestrogen ratios, and/or low oestrogen titres) in affected gastropods, including alteration in the rates the aromatisation of testosterone to oestrogen by cytochrome P450-dependant aromatase (Morcillo and

Porte, 1999; Ronis and Mason, 1996). Cytochrome P450 enzymes are functionally related, ubiquitous enzymes, involved in the oxidative metabolism of endogenous compounds such as steroids (responsible for converting androgens to oestrogens (Betttin *et al*., 1996), prostaglandins, vitamin D, and of numerous exogenous compounds (Ortis de Montellano, 1996). However, due to low rates of P450-dependent aromatisation of testosterone to oestrogen (Morcillo and Porte, 1999; Ronis and Mason, 1996), an alteration in the rate of excretion of testosterone may be a more likely explanation for the hormone titres observed. Fatty acid conjugates of testosterone have been described in gastropods due to the effects of TBT. The esterification of testosterone to fatty acids might be a mechanism where by steroid titres in marine gastropods are regulated and could represent a target of TBT toxicity. Due to a build up of testosterone in the haemolymph of affected snails (Ronis and Mason, 1996), excretion mechanisms could fail to remove this excess and instead testosterone (as fatty acid conjugates) is stored in the tissues of snails (Global assessment, 2002). In addition, or possibly as part of the same phenomena, TBT is thought to inhibit sulphur conjugation of testosterone and its phase I metabolites leading to the build up of testosterone. This excess pool of testosterone is thought to at least in part responsible for observed masculinisation of female marine gastropod molluscs. An alternative hypothesis, is that the masculisation of female gastropods with low concentrations of TBT could be mediated by a neuropeptide found normally in males, APGWamide, rather than a steroidal hormone (Oberdorster, McClellan Green, 2000) in response to stress. TBT is a known CNS neurotoxin that may cause the release of neurohormones (importantly APGWamide) from the pedal ganglia in females; which causes the growth of a penis and vas deferens tissue. This tissue, in theory, then goes on to produce the subsequent testosterone observed (via Sertoli cells); this is a particularly attractive hypothesis as normally the penis and vas deferens are formed before testosterone titres increase. However, this extensive research that has continued since the mid 1970s details the formation of male gonadal tissue (penis and vas deferens), and appears not to detail spermatogenesis or related processes (or female reproductive processes), and therefore, does little to further elucidate the function of male sex steroids at least in the gonads of male or female gastropod molluscs.

Though little is known about the function of male steroidal sex hormones in molluscs, even less is known about the function of female steroidal sex hormones (e.g. E2, E1, and E3) despite increases in titres during summer months in marine gastropods (LeGall and Streiff, 1975). It has been reported that oestradiol may play an important role in the synthesis of neurohormonal (peptide) compounds implicated in steroidogenesis in both male and female molluscs (Lubet and Mathieu, 1990); hence, differences between males and females in oestradiol levels are reported to be less evident, even in sexually mature individuals. However, more recently there has been some circumstantial evidence for a function of female steroidal sex hormones in the gonad of marine gastropods. Previous studies have reported the presence of E2 in the gonads of a bivalve (*Mytilus edulis*), identified by mass spectrometry (Zhu *et al*., 2003), and E1, E2, and E3 (identified by HPLC), produced by cells in the gonad were reported to be involved with egg yolk production in the Pacific oyster (*Crassostrea gigas*) (Matsumoto *et al*., 1997). There was evidence of high17β-HSD activities particularly in the gonad of this mollusc when, as part of an exposure experiment, it was placed in an environment contaminated with high levels of E2; E2 was converted to E1 almost immediately (Le Curieux-Belfond, 2005). In the freshwater Mussel (*Elliptio complanata*) vitellogenin production was reported to increase in quantity only in the maturing gonads of females, although production of this protein could not be induced with steroid hormone exposure (Seung-Jae Won, 2005). This evidence suggests the presence of vitellogenin in more than one group of aquatic mollusc. However, rising levels of 17β-oestradiol during the reproductive cycle have been reported to increase vitellogenin synthesis in the scallop (*Pactinopecten yessoensis*)(Matsumoto *et al*., 1997), and in the Pacific Oyster (*Crassostrea gigas*) when 17β-oestradiol was added to seawater (Li *et al*., 1998). Together, these results suggest that oestrogens can be synthesized in the gonads of some marine prosobranch mollusc species, that their levels may vary with the reproductive cycle, and that, they may have a role in the development of gametes. The results described here should be interpreted with caution until a direct link between the presence of female sex hormones and the maturation of the gonad can be made (or the production of egg cells). Nonetheless, they tentatively suggest the convergence of vertebrate and invertebrate endocrinology, which promises exciting new directions for the investigation into the effects of EDCs in molluscs (and humans) in the future.

1.10.4 Oestrogen-like and other Receptor Types

Steroid receptors are believed to be extremely ancient and widespread, having diversified before the origin of bilaterally symmetric animals (Thornton *et al*., 2003). The ancestral steroid receptor had oestrogen receptor-like functionality, but loss of steroid- dependant activation may have occurred in the lineage leading to *Aplysia* (Sea Hare) and other molluscs. Indeed, the apparent reproductive role of oestrogens in molluscs is more likely to take place through ancient non-oestrogen receptor (ER) mediated pathways (Keay *et al*., 2006). Although ER-orthologues in molluscs do not bind oestrogen, the fact that relatives of the vertebrate ER are found in the sea hare (*Aplysia californica)* (Thornton *et al*., 2003), octopus (*Octopus vulgaris)* (Keay *et al*., 2006), rock shell (*Thais clavigera)* (Kajiwara *et al*., 2006), and the freshwater snail (*Marisa Cornuarietis)* (Bannister *et al*., 2007) suggests a role for these receptors in molluscs.

However, alternatively, oestrogens could have effects on molluscan body tissues via other nuclear or non-nuclear steroid receptors that are as yet undiscovered or not well known at present. The retinoid-X-receptor (RXR) has been found to increase its expression in association with normal male reproductive tract recrudescence (sexual differentiation) in the eastern mud snail (*Ilyanessa obsoleta*)(Sternberg *et al*., 2008), its natural ligand being 9-cis-retinoic acid (9cisRA). Indeed, 9cisRA induces imposex in females of *Nucella lapillus* to the same degree as TBT (Castro *et al*., 2007), and forms part of another hypothesis that may help explain the imposex phenomenon in molluscs. In the rock shell, *Thais clavigera*, RXR gene expression was significantly higher in imposex-exhibiting females (RXR also binds TBT), specifically in the penis forming areas of normal females. However, none of these details regarding the functions of other nuclear receptors in molluscs helps further elucidate the suggested non-steroidal receptor pathway interaction of xenobiotics (such oestrogen or other steroids) with molluscan tissues that may play a part in a vertebrate-type reproductive sex cycle phenomenon.

1.11 Aims

When this study was initiated, it was already known that European freshwater molluscs, even common varieties, were under considerable threat from a number of known detrimental environmental factors that could include pollution. However, to date, it has not been possible to identify which of these factors are likely to be most responsible for the demise of local molluscan populations. What we do know is that TSE containing oestrogenic chemicals can affect reproduction in some freshwater molluscs in a similar way to fish, although currently only two studies have investigated this.

However, the way in which TSE affects reproduction in molluscs is presently unknown, and its direct association with oestrogenic chemicals, both natural and man-made, which are known to cause feminisation and reproductive abnormalities of native fish populations, are unproven. Therefore, the aim of this work was to study the effects of TSE on commonly found European freshwater molluscs, including both pulmonates and prosobranchs with different life cycle types; both hermaphrodites (egg layers) and gonochorists (live bearers). The normal reproductive response of the chosen species under "normal conditions" in river water over the season was established. This set-up could then be used, in parallel, to assess the reproductive effects of TSE upon adults, and developmental effects upon the offspring of snails. In short, the purpose of this study is to investigate the seasonal effects of TSE on the reproduction and development of common native species of gastropod molluscs present in European rivers and other freshwater bodies. The hypothesis to be tested is that TSE has reproductive and developmental effects upon European freshwater molluscs.

Chapter Two; General Materials and Methods

The following text describes the materials and methods used in my experiments. For each experiment, any deviations from these materials and methods are described in the individual sections for each experiment in detail.

2.1 Physical Origin of Snails

After an initial literature search, seven suitable species of freshwater snail were chosen for the first study (see sections 3.2 and 3.3). A search for these species employed local knowledge, in order to find a suitably large number of snails of each species. However, as this was the first search of this nature, its success varied, though eventually four of the seven species used in my first experiment were collected from the wild.

Bithynia tentaculata and *Physa fontinalis* were sourced locally to Chelmsford STW from below Cutton lock (573500E, 207500N) on the river Chelmer. *Viviparus viviparus* were obtained from near Brunel University on the Grand Union canal (513144N, 02850W). *Lymnaea stagnalis* were laboratory-bred animals from Cardiff University courtesy of Daire Casey (Cardiff University). *Theodoxus fluviatilis* were from the banks of the River Thames, a shallow stony site (513358N, 04014W) near Cookham. *Planorbarius corneus* were purchased from Blades biological, and were established to be wild animals collected from a single site, a Rife (or lowland ditch) near Worthing (514704E, 103103N) Sussex that has no inputs into it from any STWs. *Potamopyrgus antipodarum* were the laboratory stock of Professor Joerg Oehlmann of Frankfurt University Germany. All snails were transferred to tanks at Langford STW as quickly as possible (same day) and acclimated carefully to river Chelmer water.

2.2 Experimental Design

Field-based studies using the various species were conducted using adults and a single graded STW effluent source, from Chelmsford STW. A flow through system that received a continuous supply of both river water and treated sewage effluent served large plastic tanks (685 L) in which the snails were held (Figure 2.1). Snails were acclimated in river Chelmer water for a period of at least one week before commencement of the experiment. River Chelmer water was used as the diluent river water throughout the experiment. Morphometric measurements of shell length, width, and aperture height, and snail weight where appropriate (for details see section 2.5) were taken from all live snails to be used in the experiment (for growth estimates) at the beginning of the experiment, and snails of each species were placed into replicate groups (in enclosures (week –2)). All snails were held in river water in their own tanks for a further two weeks during which time baseline measurements of reproductive output in egg laying species, and fecundity was assessed in ovoviviparous species. Following this period, 30 snails (held in separate enclosures) of each species were euthanized (by freezing in liquid nitrogen at – 80 ^{o}C for 30 seconds) and stored immediately on dry ice (remained frozen at –70 ^{o}C) before transport back to the laboratory, and stored (at –80 ^{o}C) for baseline biochemical measurements. Throughout acclimation, baseline and exposure periods, snails were fed organic lettuce (chemical free food source), temperature and flow rates (river water and effluent) were monitored daily for each tank.

Six replicate groups of snails per species (n= 12-18 depending on reproductive mode and availability) were used in each treatment tank. Although a replicate within a tank was a form of pseudo replication, constraints upon the number of available tanks meant this was the only practical option available (though six tanks per treatment would have represented an ideal situation). After baseline measurements (reproductive output) were taken at the end of the two week period, at time zero, all replicate snails groups were then exposed to either treated sewage effluent or river water for the period of each experiment (Figure 2.2). Each tank received river water diluted with treated sewage effluent to nominal concentrations of 100%, 50%, 25% effluent, and 0% (river water only) as a control. Reproductive parameters were

assessed every two weeks throughout the length of each exposure for egg laying species. For Biochemical analyses, snails were removed prior to exposure (n=30), 3 replicate groups per treatment were analysed at week 6 (i.e. half of the snails), and the remaining 3 replicates were analysed at week 12 during experiment 1 only. During experiments 2 and 3, snails were removed prior to exposure (n=30), and the remaining 6 groups of snails per treatment were analysed at the end of the experiment. Exposed snails were also used to assess reproductive parameters in ovoviviparous species; snails were dissected and embryos counted (see section 2.2.4.2). Endpoints measured included mortality, growth (weight, length, width, and height of aperture of snails shell), number and weight of egg masses, number of eggs per mass (collected fortnightly), or number and weight of embryos in the brood pouch in ovoviviparous species (end of baseline and end of exposure period only, and also week 6 in expt. 1). Carbohydrate metabolism (glycogen phosphorylase activity) and protein content of body tissues were also only analysed during experiment 1.

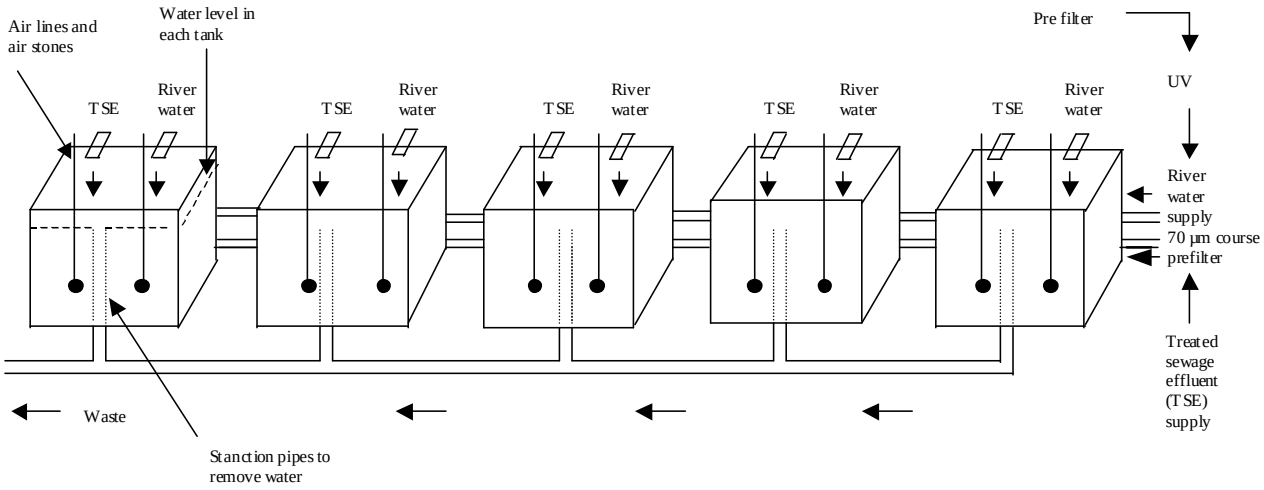


Figure 2.1 Flow diagram to illustrate the basic design of the experimental system to include the layout of the tanks, flow of both river and treated sewage effluent through the system, and illustrates how both supplies were treated prior to their introduction to the system, how river water and treated sewage effluent became mixed, and how tank water was run to waste.

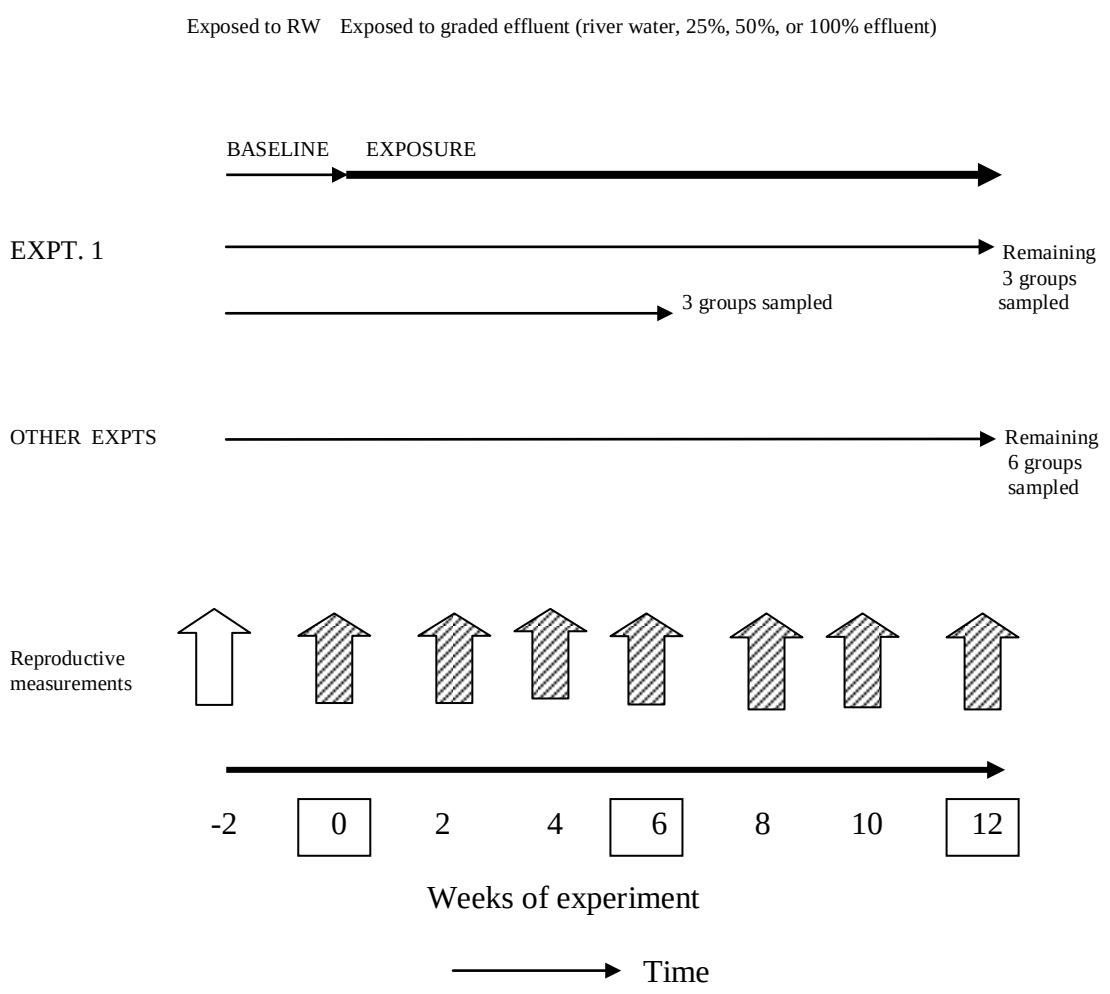


Figure 2.2 Flow diagram to illustrate the basic experimental design used in experiment 1 including treatments, sampling points, and time line for each replicate within a treatment. Adult snails were kept in graded concentrations of treated sewage effluent, or river water as a control, from week 0 to week 12, and sampled for snail mortality and reproductive parameters fortnightly. Morphometric and biochemical analyses were carried out at weeks 0, 6 (expt. 1 only) and week 12 at termination of the experiment. In subsequent experiments the 6 replicate groups of snails were not sampled until the end of the experiment. Details of the sampling protocols are in the text.

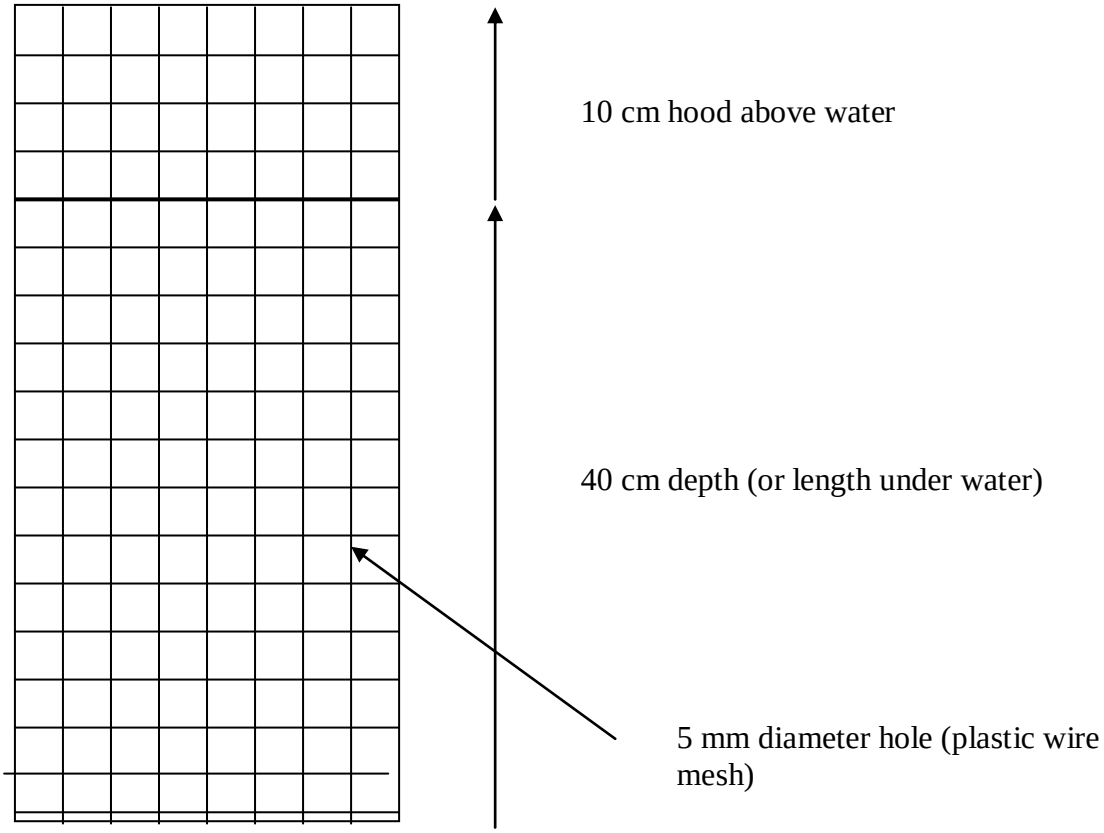
2.2.1 Treated Sewage Effluent

The treated sewage effluent was derived from Chelmsford Sewage Treatment Works (STW), Chelmsford, Essex, UK. This treated sewage effluent was chosen because it has been well-characterised with respect to its induction of oestrogenic responses in fish (Rodgers-Gray *et al*., 2000; 1999; Rodgers-Gray *et al*., 2001) and its oestrogenic steroid and nonylphenolic content (Harries *et al*., 1999). Chelmsford STW receives influent with a population equivalent of 138, 000 and the STW has both activated sludge and biological filter secondary treatments. The influent load is primarily domestic, although industrial inputs contribute 14% of the load (Rodgers-Gray *et al*., 2001).

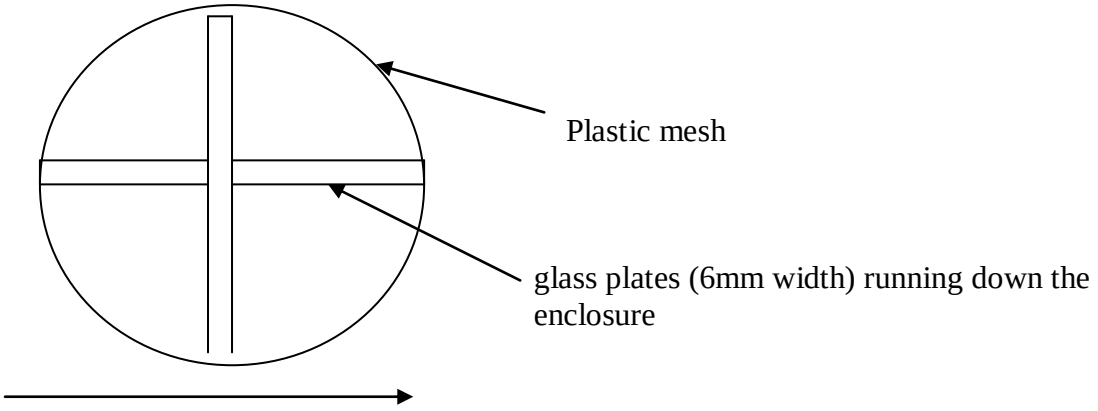
2.2.2 Housing; Design of Snail Enclosures

Each cage, that I designed, consisted of a plastic wire netting cylinder (40 cm high) of differing diameter depending on size of each species (Table 2.1), and contained glass plates (40cm high by the same diameter for each species) to allow a surface area on which the snails could move and lay egg masses (Figure 2.3). Each enclosure had a 10 cm high hood that extends above water level to prevent snails escaping the enclosure. For smaller species (Table 2.1) a1.6 mm diameter nylon mesh covered the outside of the enclosures to prevent snails escaping.

Side view:



Top view:



12 cm diameter for *Lymnaea stagnalis*

Figure 2.3 Design of snail enclosures to show the most important features: depth, diameter, and construction materials. These enclosures were used in each of the three field based experiments.

Table 2.1 Relative dimensions of snail enclosures.

Species	Diameter
Large	
Lymnaea	12 cm
Planorbarius	12
Viviparus	14.7
Small	
Bithynia	10.2
Physa	10.2
Theodoxus	9.5
Potamopyrgus	9.5

2.2.3 Morphometric Analyses

At the end of the two-week period used to estimate baseline reproductive parameters (see Figure 2.2), all snails used in the experiment were weighed and their shells measured using TESA IP65 electronic callipers. For the larger species (*Lymnaea, Viviparus, Planorbarius)* length, width, and aperture height of the shell opening were measured. For smaller *Physa* and *Bithynia* the length and width only was measured and in *Theodoxus* shell height replaced a measure of the aperture. *Potamopyrgus* was the smallest species at ~ 3.8 mm, and only shell length could be measured in live animals. Snail enclosures were numbered and identified as 1-6 for each species in each tank from the beginning of the experiment to enable identification of each snail enclosure, and therefore, group of snails.

2.2.4 Mortality and Reproductive Measurements

2.2.4.1 Snail Mortality

Snail mortality was assessed at the same time as reproductive parameters. All snails were gently removed from their enclosures (as internal glass plates were lifted out) and the number of live snails noted. Live snails were put into plastic bowls for their safety, before reproductive parameters were assessed, enclosures were then reassembled and returned to their original positions in tanks, and finally, live snails (and their lettuce) returned to their enclosures.

2.2.4.2 Reproductive Parameters

Ovoviviparus Species

For *Viviparus viviparus*, after morphometric analysis (length, width, aperture diameter) snail shells were cracked (with pliers) and shells removed. Once the whole snail minus shell had been weighed, the brood pouch was dissected and the number of embryos contained within were counted and weighed. For *Viviparus viviparus*, the comparatively large size of this snail allowed the counting and weighing (after their removal from the brood pouch) of individual shelled embryos, and the counting of

unshelled embryos whilst still in the brood pouch; a thin membrane surrounding unshelled embryos did not allow them to be removed intact from the brood pouch, and therefore, could not be weighed. In *Potamopygrus antipodarum,* as snails and embryos were small by comparison, snails were dissected under water in Petri dishes; embryo production was assessed after cracking the snails` shells (with a clamp) to release all embryos, and an artists fine paint brush was used to brush embryos out of the snail. Shelled and unshelled embryos could then be counted (but were too small to be weighed).

Egg Mass and Egg Case Laying Species

At all sampling points, from week zero onwards, egg masses were collected from enclosures for all egg laying species at fortnightly intervals. Egg cases were collected from *Theodoxus fluviatilis*. For egg layers, snails and lettuce were gently removed from each numbered enclosure in turn, and the number of live snails noted (see 2.2.4.1 Snail mortality). Each enclosure was disassembled into its component parts, by removing the glass plates from within each enclosure (see Figure 2.3), and by removing the nylon netting from the outside of the smaller enclosures. A new razor blade was used to gently lift egg masses off surfaces (glass or plastic) within the enclosure. Egg masses were placed into 20ml universal containers (Sterilin U.K.) containing treatment water, which were immediately transported back to the laboratory and stored on the bench in a laboratory (see 3.3 Egg Hatching Method Development and Validation). The enclosures were then reassembled and returned to their original positions in the tank; live snails and lettuce were then replaced back into enclosures. In the laboratory, in all cases the number of egg masses per replicate was counted and recorded. Where possible, the number of eggs per mass was recorded for the first 40 intact masses. The first twenty egg masses per replicate were weighed and then used to assess their hatchability. Each egg mass was placed under a stereomicroscope and the number of eggs counted manually, each egg mass was then placed into a separate labelled well (Cell culture plate: NUNC^{TM}) containing 1.5 ml of treatment water. Numbered for further identification, micro titre plates where stored in a room with a minimum-maximum temperature range of 18- 22 ^{o}C. Treatment water was replaced every other day in all wells containing egg masses. Every two

days, each micro titre plate was placed under a stereomicroscope and the number of un-hatched eggs counted and recorded for each mass, and therefore, the percentage hatched could be calculated using the total number of eggs per mass. This procedure was repeated every other day until no further eggs hatched, and a constant number of un-hatched eggs were obtained. Enough treatment water was transported back to the laboratory every two weeks (stored at 18- 22 ^{o}C in a laboratory; ready for water changes), when sampling reproductive parameters, to carry out all water changes necessary. In the laboratory treatment water, effluent or river water, were mixed in the correct ratio to give the correct nominal percentage of TSE or river water for water changing every two days.

Egg capsules (from *Theodoxus fluviatilis*) were collected in the same manner as egg masses, and enclosures handled in the same way, egg capsules were then placed into 20ml universal containers (Sterilin U.K). In the laboratory, in all cases the number of egg capsules were counted and stored in a minimum-maximum temperature room with a range of 18-22 °C. Capsules were checked at least twice a week to see if they had hatched; few capsules were laid and egg capsule to water volume ratio was large, therefore, water changes were unnecessary. Further, due to the extended but unknown gestation period of *Theodoxus* capsules, hatching was unpredictable in its nature, and therefore, the observation of their capsules continued for up to 16-weeks (from time zero collection) after returning them to the laboratory.

2.2.5 Biochemical Analyses: Measurement of Glycogen Phosphorylase activity and Protein Content

Approximately 30 snails of each species were sampled (at time zero) before exposure to assess biochemical parameters, protein content and glycogen phosphorylase activity of mantle tissue. Analyses required dissection of tissues. In the laboratory, individual snails were placed in labelled Petri dishes containing crushed ice and allowed to warm to 0 ^{o}C over a 30 minute period. At this temperature it was physically possible to carry out the dissection (~0 ^{o}C after 30 minutes). In experiment 1 only, at weeks 6 and 12 three replicate enclosures were terminated and all live snails remaining were used for biochemical analyses. In addition, for *viviparus* species, once the number of shelled and unshelled embryos in the pallial oviduct had been counted

and weighed, shelled embryos were dried on tissue, and frozen in the same manner as whole snails.

2.2.5.1 Measurement of Glycogen Phosphorylase

Glycogen phosphorylase activities of mantle tissues were measured in the direction of glycogen breakdown using a coupled enzyme system (Childress and Sacktor, 1970), adapted for use with my snail species (see section 3.4). Briefly, mantles were homogenised in 50 mM sodium phosphate buffer (pH 7.2), 5 mM EDTA, 20 mM sodium fluoride (NaF) (1:20, W/V) in an ice-cold homogeniser. The homogenates were centrifuged at 3,000 g for 90 min at 4 ^{o}C, and the supernatants used for glycogen phosphorylase assay. Supernatants were divided into aliquots, one for later protein determination.

The assay for total phosphorylase activity was performed in the presence of 2mM adenosine-5'-monophosphate (5-AMP). In a final volume of 1 ml, the reaction medium contained 40 mM sodium phosphate buffer (pH 7.0), 5 mM imidazole, 5 mM magnesium acetate, 1.4 mM DL-Dithriothreitol (DTT), 2 mM EDTA, 50 µl enzyme extract, 0.6 mM NADP, 2 mg glycogen, 4 µM glucose-1,6-diphosphate, and 4 units of phosphoglucomutase (PGlum). After incubation at room temperature for 8 min, adding 0.8 units of glucose-6-phosphate dehydrogenase (G6P-DH) initiated the reaction. Formulation of NADPH was recorded at 340 nm for 2 mins using double beam spectrophotometer and calculated using least squares regression using between 0.5 and 1.5 min. Phosphorylase activity was expressed as µmole glucose-1-phosphate/min/mg protein.

2.2.5.2 Protein Determination

Protein determination was analysed as described by Bradford (1976). Protein solution was diluted to give a concentration range between 10 - 100 µg/ 5-mls, and the weight of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in unknown samples.

2.2.6 Albumen Gland Weight

In the laboratory, individual snails were placed in labelled Petri dishes containing crushed ice and allowed to warm to 0 ^{o}C over a 30 minute period (see section 2.2.5 Biochemical analyses). The shell of each snail was cracked with pliers and all the shell removed. Whilst under water, the mantle skin was cut along the ventral surface of the snail and the albumen gland cut free and gently removed from the body, blotted to remove excess water, prior to weighing (Precisa 125 balance, Switzerland).

2.2.7 River Water and TSE Sampling

Samples, 2.5L bottles (supplied by CEFAS Fisheries Laboratory, Burnham-on-Crouch) of river water or TSE were filled from different positions in each tank. The neck of the empty sampling bottle was plunged approximately 6 inches below the surface of the water; approximately one fifth the volume of the bottle was allowed to enter. The sampling bottle was then quickly removed from the tank. This sampling procedure was repeated in several random sites around the surface of the tank until the bottle was completely full, after which the bottle top was tightly secured. This same procedure was followed for all the tanks sampled. Sample bottles were immediately transferred to the CEFAS Fisheries Laboratory (Burnham-on-Crouch, Essex) and stored at 4 ^{o}C until analyses.

2.2.8 Measurement of Natural and Synthetic Oestrogens in TSE

Samples collected were analysed for the presence of natural steroids 17β-oestradiol (E2) and estrone (E1), the synthetic steroid 17α-ethinylestradiol (EE2), and for the alkylphenolic chemicals BPA, 4-OP, 4-nonylphenol (NP), and the nonylphenol mono- and diethoxylates (NPIEO and NP2EIO). The methodologies used to measure these chemicals are described in Rodgers-Gray, *et al.*, 2000. Briefly, the oestrogenic chemicals were immobilised on a C_{18} silica-bonded solid-phase extraction column, eluted, and analyzed using gas chromatography-mass spectrometry (GC/MS).

2.2.9 Statistical Analyses

A Shapiro-Wilk W test and Bartlett`s test were employed to test the normality of the data distribution and the homogeneity of the variances, respectively. In many cases, data was not normal and could not be transformed into a data set suitable for parametric analysis. Transformation of data sets was attempted, for example, using the number squared, square root, 1 divided by the square root, $\log_{10}$, ln, etc. However, on occasions the data tended towards being bimodal, for example, size of adult snails or the number of eggs within an egg mass, and therefore, could not be transformed into a normal distribution. Similarly, where it was attempted to transform data sets to allow non-parametric 2-way analysis of variance, Friedman's analysis of variance, it was found in each case that the data sets were not suitable for this form of statistical analysis. If data was not normally distributed, differences in morphological, egg or embryo production parameters were determined using a Wilcoxon rank sum test with comparisons to respective controls. On the rare occasions where the data was normal, and showed homogeneity of variance, an analysis of variance (ANOVA) analysis of the standardised means of each group was used.

2.3 Individual Experimental Methodology

The environmental requirements of most freshwater gastropods are very similar (see section 3.2. The objective of the first experiment was to compare the development and reproductive responses of a variety of snail species chosen, to a graded treated sewage effluent from a single source. Secondly, with this data analysed, to select suitable species for further study.

For general Experimental Design features see section 2.2. In experiment 1, a 12-week study was conducted using adults from 5 of the 7 different species chosen; only *Bithynia tentaculata* and *Physa fontinalis* were not used (snails collected died during acclimation). Six replicate groups of snail per species were used in each treatment (*Planorbarius corneus*; n =11, *Lymnaea stagnalis*; n =6, *Viviparus viviparus*; n = 12, *Potamopyrgus antipodarum*; n =10, *Theodoxus fluviatilis*; n =10), and one tank per treatment was available for each treatment. The origins of the snails used in experiment 1 are described in section 2.1, in this chapter. The specifics of the experimental design for experiment 1 are described in Figure 2.4 below.

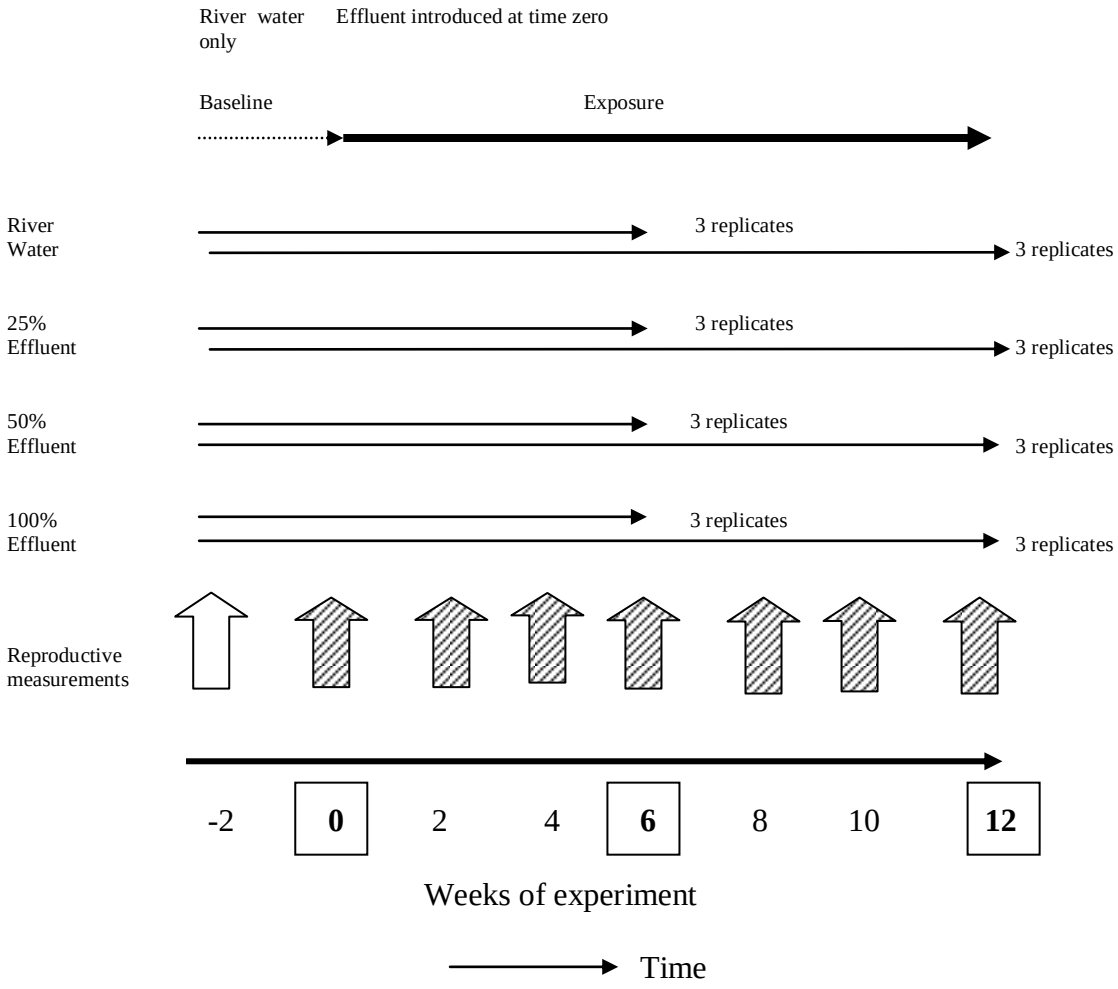


Figure 2.4 Flow diagram to illustrate the experimental design for experiment 1 including treatments, sampling points, and time line for each replicate within a treatment. Adult snails were kept in graded concentrations of treated sewage effluent, or river water as a control, from week 0 to week 12, and sampled for reproductive parameters biweekly. Morphometric and biochemical analyses (30 snails) were carried out at weeks 0, 6 and week 12 at termination of the experiment, by sampling 3 replicate groups of snails of each species. Details of the sampling protocols are in the text.

The source and chemical characteristics of the treated sewage effluent used in experiment 1 are described in section 2.2.1. The snail enclosures used did not vary from those described in section 2.2.2. Snails were measured and weighed as described in section 2.2.3. Of the six numbered and labelled enclosures, three were chosen randomly at week 6, and the same numbered enclosures (numbers 1, 2 and 5) were sampled for each species. The remaining three enclosures for each species were sampled at week 12. As part of baseline morphometric analyses, each snail (except *Potamopyrgus*, due to there small size) was individually numbered to increase accuracy of the growth data. Each shell was dried and nail vanish applied using a fine artists brush. The methodology used for recording snail mortalities did not vary from section 2.2.4.1. For *Viviparus viviparus* and *Potamopyrgus antipodarum* embryo production was assessed as described in section 2.2.4.2. Egg mass and egg case collection, their handling and processing in the laboratory did not vary from the method described in section 2.2.4.2. *Lymnaea stagnalis* also produced egg masses in Experiment 1, but these egg masses were collected by Daire Casey as part of his PhD thesis (Cardiff University). In order to obtain a measure of the energy content of the snails and to ensure that any differences in reproductive output of the snails were not due to differences in the available energy stores between the exposure groups, the protein content and glycogen phosphorylase activity of mantle tissue were measured at weeks 0, 6 and 12 only (see section 2.2.5). From the same samples, albumen gland weight was also used as an index of reproductive activity, as ASO weight is closely linked to both snail growth and reproductive activity (see section 4.3.8). Analysis required dissection of snails` tissues, to remove the albumen gland (gonad had been removed previously) and leave the main body of the snail for analysis of energy (carbohydrate) content. *P. corneus* was the first snail species to be analysed, and due to time constraints was the only species analysed for protein content and glycogen phosphorylase activity. The remaining body of the snail (mainly mantle and other tissues) was then blotted dry, weighed, and placed in a 5-ml glass test tube prior to homogenising, and the correct volume of ice-cold homogenising fluid was added immediately. The snails tissues remained in an ice bucket at 0 ^{o}C to prevent the action of enzymes (proteases) on the snails tissues (denaturing enzymes); the glass homogeniser was kept in the ice bucket with reagents so that all equipment and reagents remained at 0^{o}C. The methodology used for assessing glycogen phosphorylase activities of mantle tissues did not vary from the methodology

described in section 2.2.5.1. Protein content of snail samples was analysed by the method described in section 2.2.5.2. Albumen glands were dissected from snails and weighed by the method described in section 2.2.6. Samples (2.5L) were collected by the method described in section 2.2.7 from 100% TSE and river water tanks on two occasions during the months of June and September during experiment 1. Samples collected were analysed on both occasions for the presence of natural steroids 17β-oestradiol (E2) and estrone (E1), the synthetic steroid 17β-ethinylestradiol (EE2), and for the alkylphenolic chemicals BPA, 4-OP, 4-nonylphenol (NP), and the nonylphenol mono- and diethoxylates (NPIEO and NP2EIO). Sample bottles were immediately transferred to the CEFAS Fisheries Laboratory Burnham-on-Crouch, Essex, and stored at 4 ^{o}C until analyses.

2.4 Experiment 2

The aim of this study was to investigate further the effects of graded concentrations of TSE upon the development and reproductive responses of *P. corneus.* In experiment 1, significant reproductive effects of TSE were observed at the peak of reproduction mid summer. This experiment investigated whether such an effect would occur during the autumn winter time period. The concentrations of TSE were chosen as those most likely to demonstrate reproductive effects after analyses of experiment 1 data.

For general Experimental Design features see section 2.2. The specifics of the experimental design for experiment 2 are described in Figure 2.5 below. In experiment 2, an 8-week study was conducted using adults of *P. corneus*. The *P. corneus* snails used in experiment 2 were purchased from Blades biological as described in section 2.1. At the beginning of the experiment, each tank contained 6 groups of *P. corneus* containing 9 snails in each group.

Due to the late time of year no baseline measurements of reproductive output were undertaken. Baseline morphometric measurements were taken at time zero; snails were exposed to a graded concentration of TSE (25%, or 50%) or to river water alone.

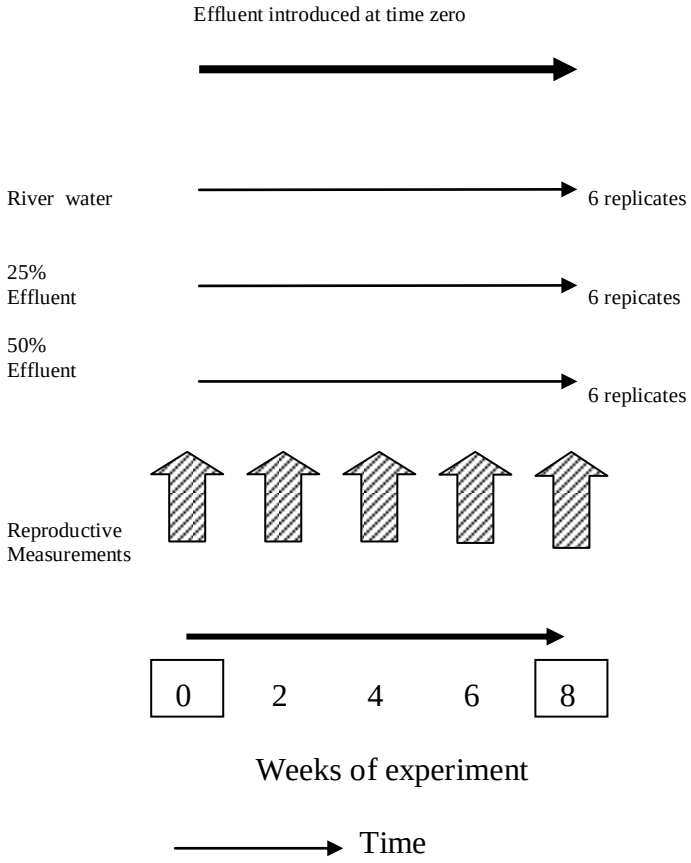


Figure 2.5 Flow diagram to illustrate the experimental design for experiment 2 including treatments, sampling points, and time line for each replicate within a treatment. Adult snails were kept in graded concentrations of treated sewage effluent, or river water as a control, from week 0 to week 8, and sampled for reproductive parameters biweekly. Morphometric and biochemical analyses were carried out at weeks 0, and week 8 at termination of the experiment, by sampling 6 replicate groups of snails of each species. Details of the sampling protocols are in the text.

The source and chemical characteristics of the treated sewage effluent used in experiment 2 is described in section 2.2.1. The snail enclosures did not vary from those described in section 2.2.2. The methodologies used for morphometric analyses of snails did not change from experiment 1; see section 2.2.3. The methods used to assess snail mortality did not vary from section 2.2.4.1. The method of egg mass collection and handling of snails did not vary from experiment 1; see section 2.2.4.2. No biochemical measurements (glycogen phosphorylase, or protein content) of adult snails from experiment 2 were attempted due to time constraints. Albumen glands of stored snails were dissected and weighed by the method described in section 2.2.6. No water samples were taken during experiment 2, and therefore no analysis of water chemistry was undertaken. Data from this experiment was analysed as described in section 2.2.9.

2.5 Experiment 3

Experiment 1 was repeated with *Viviparus viviparus* and *P. corneus* in order to determine whether the responses observed in both snail species was a repeatable phenomenon. Therefore, experiment 3 was essentially a repeat of experiment 1 but with a full measurement of reproductive output. In order to obtain cumulative reproductive output, it was necessary for *P. corneus* to count eggs in all egg masses and to weigh each egg mass. For *V. viviparus*, all baby snails born into enclosures were collected, counted and weighed. This experiment therefore represented a much more detailed study, that includes total reproductive output of snails.

For general Experimental Design features of experiment 3 see section 2.2. In experiment 3, a 14-week study was conducted using adults of *Planorbarius corneus* and V*iviparus viviparus.* The physical origins of the snails used in experiment 3 are described in section 2.1. Six replicate groups of snails per species were used in each treatment (*Planorbarius corneus*; n =9, *Viviparus viviparus*; n = 11), and one tank per treatment was available for each treatment. The specifics of the experimental design for experiment 3 are described in Figure 2.6 below. The following text describes the experimental design differences between experiments 1 and 3. During experiment 3, time zero was staggered for each species so that reproductive parameters (and therefore mortality) could be measured for one species only each week; each species was sampled on alternate weeks. In addition, the length of dead snail shells, those snails found to have died at each two weekly sampling period was also measured (length only); by the method described to measure live snails in section 2.2.3.

All adult *P. corneus* were held in river water (all 6 tanks) for 2 weeks for baseline measurement of reproductive parameters (weeks –2 to 0). All *P. corneus* snails (6 replicate groups per treatment) were measured and weighed at the beginning of the experiment (week 0). At time zero, after collection of egg masses, the effluent was turned on and diluted with river water to give the appropriate concentration of effluent in each treatment tank (river water, 25%, 50%, and 100% effluent). In experiment 3, the diet of *P. corneus* was supplemented with tetramin flake food (oestrogen free) once per week (3.6 g per enclosure).

All adult viviparids were held in the river water tank for a further week (without measurement); 6 replicate groups were used for baseline measurement of reproductive parameters one week later and placed in the river water tank only (end of week –2 to -1 of *P. corneus* experiment). This was the beginning of experiment 3 for *V. viviparus* (week –2). The remaining viviparids remained free in the river water tank. At time zero (week 0) for *V. viviparus*, all snails were measured and weighed for river water, 25%, 50%, and 100% effluent treatments (*P. corneus* were at the end of week 1 by this time). Therefore, a baseline measurement of reproductive parameters (release of mature embryos) in viviparids was only measured in the 6 replicate groups in the river water tank (as TSE was turned on for *P. corneus* in the effluent exposed tanks).

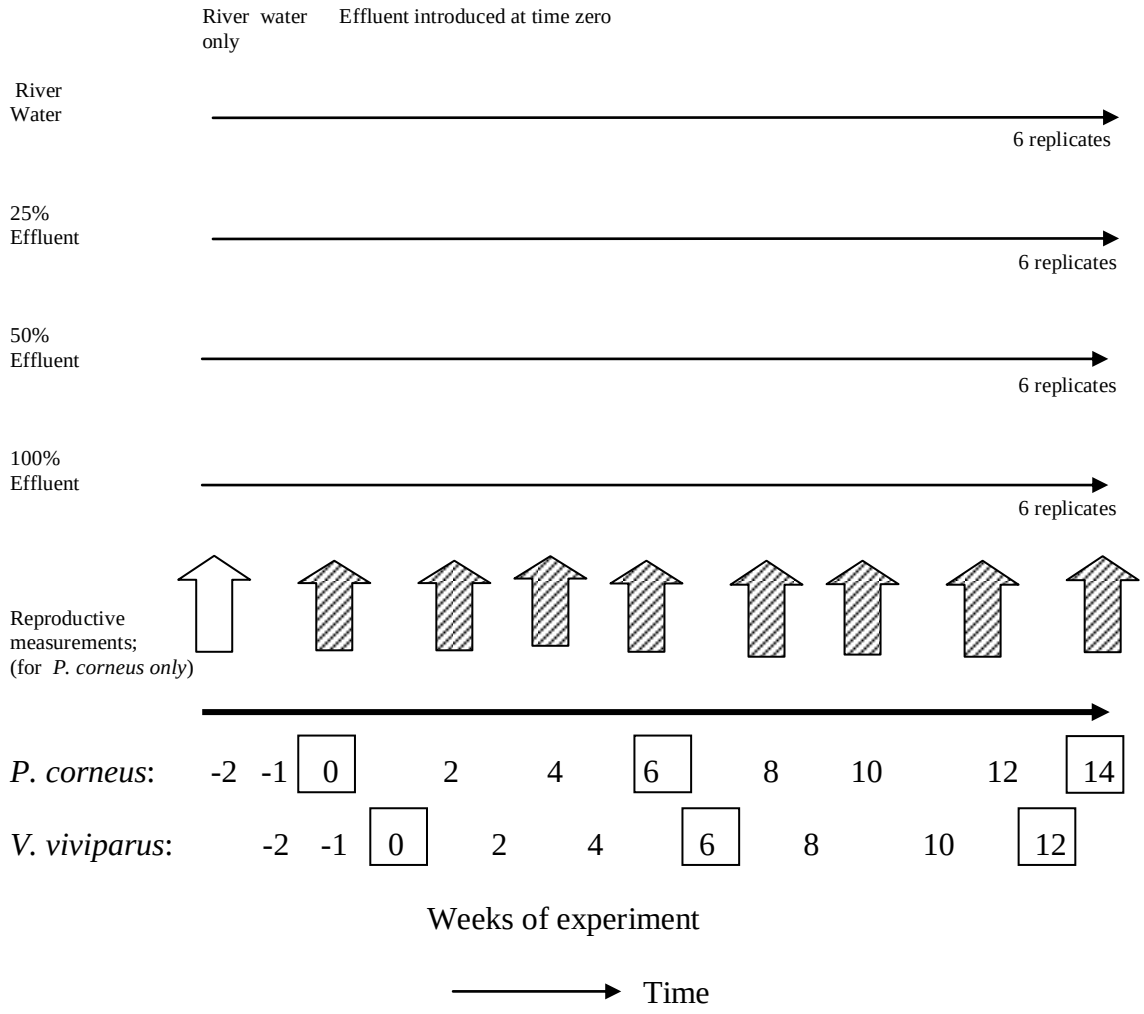


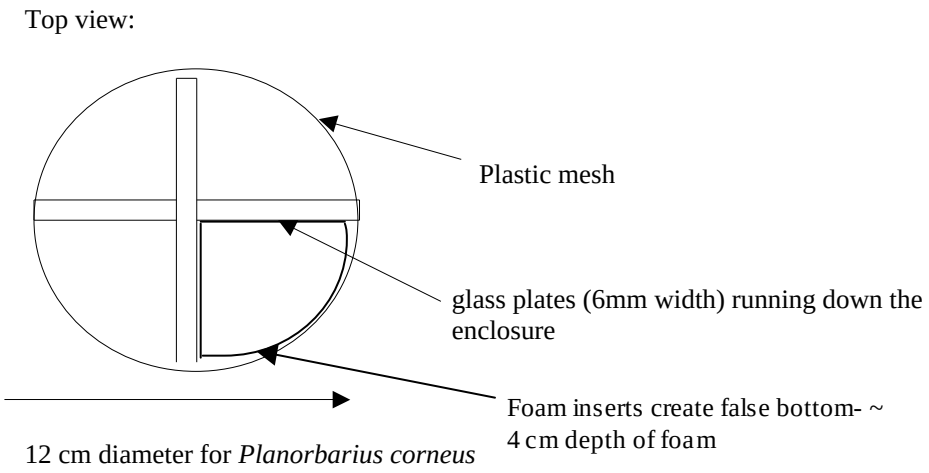
Figure 2.6 Flow diagram to illustrate the experimental designs for experiment 3 including treatments, sampling points (for each species), and time line for each replicate within a treatment. Adult snails were kept in graded concentrations of treated sewage effluent, or river water as a control, from week 0 to week 14 (to week 12 for V. viviparus), and sampled for reproductive parameters biweekly. The source and chemical characteristics of the treated sewage effluent used in experiment 3 is described in section 2.2.1. Due to maintenance procedures being carried out at Langford STW, who provided both river water supplies (and was where all experiments were carried out), river Chelmer water was unavailable for the whole period of experiment 3, therefore, river Blackwater water was used as the only available alternative. Morphometric analysis was carried out at the beginning of the experiment for each species (week 0), and at termination of the experiment for each species as detailed in section 2.2.3. Baseline reproductive measurements were taken for 6 groups of snails (*V. viviparus*) between –2 and 0 weeks, after which time the remaining *V. viviparus* were placed in groups and assigned to TSE exposure tanks. Details of how adult *V. viviparus* were kept in river water whilst the experiment with *P. corneus* proceeded, and the sampling protocols are in the text.

The text below describes the common methodologies used for both snail species during experiment 3. Details of the methods used for morphological analyses of snails during experiment 3 are in section 2.2.3. The methodology used to assess snail mortalities did not vary from the general method cited in section 2.2.4.1. No biochemical analysis of snails` tissues from experiment 3 was attempted. The methodology used to dissect and weigh albumen glands did not vary from section 2.2.6. Samples (2.5L) were collected by the method described in section 2.2.7, and were collected from 100% TSE and river water tanks on three occasions at the end of weeks 7, 9, and 13 during experiment 3. Samples were analysed on all three occasions for the presence of natural steroids 17β-oestradiol (E2) and estrone (E1), the synthetic steroid 17α-ethinylestradiol (EE2), and on two occasions for the alkylphenolic chemicals BPA, 4-OP, 4-nonylphenol (NP), and the nonylphenol mono- and diethoxylates (NPIEO and NP2EIO). A separate 1L sample of TSE or river water was collected at the same time as steroid and alkylphenolic chemicals samples and analysed for Di- and Tri-butyltin (DBT and TBT, respectively). Sample bottles were immediately transferred to the CEFAS Fisheries Laboratory Burnham-on-Crouch, Essex, and stored at 4 ^{o}C until analyses. Data from this experiment was analysed as described in section 2.28.

2.5.1 Constant Density of Snails in Enclosures

In experiment 3, snail density in all enclosures was kept equal despite snail mortalities experienced. In each enclosure, a false bottom was created using a triangular-shaped foam inserts that fitted tightly into each quarter of the enclosure (Figure 2.7A). By dividing the 40 cm height of the water column in each enclosure by the total number of snails contained within (40cm/9= 4.4cm per snail), it was possible to move the false bottom up when mortalities occurred (at two weekly intervals when reproduction was assessed) so that the volume each live snail had remained constant.

A)



B)

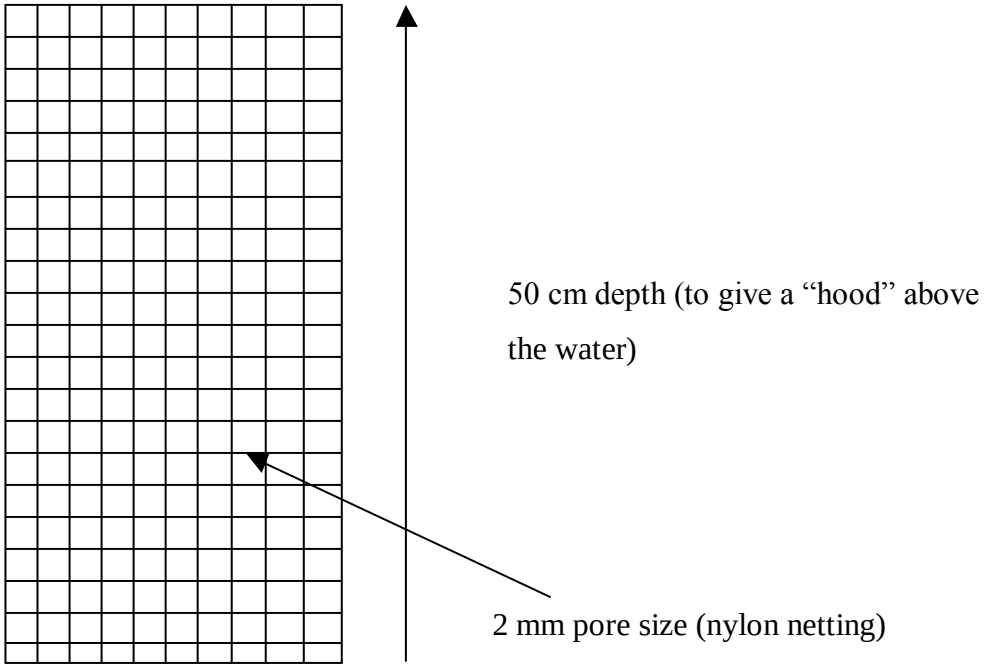


Figure 2.7A Design of A) triangular-shaped foam inserts, and B) nylon socks used in experiment 3 to show the most important features: shape of inserts, depth, diameter, and construction materials of both. These additional features were used in experiment 3 only, in part of a regime to detail total reproductive output, details are in the text.

2.5.2 *P. corneus*

The text in sections 2.5.2 and 2.5.3 below describes the specific methods used to assess the reproductive parameters for each of the two snail species used in experiment 3. The basic methodology used to assess reproductive parameters did not vary from section 2.2.4.2. In brief, to obtain cumulative data, the number of eggs per mass and weight of individual egg masses per enclosure was recorded for all egg masses (where possible) collected in experiment 3. Where time constraints (during peak of reproduction) limited the number of egg masses in which the number of eggs per mass could be counted (only a few were not counted) still the total number of egg masses in each replicate was counted. All egg masses were weighed.

The method by which egg masses were collected, transported, stored, and then hatched in the laboratory was considerably improved as described in section 3.3.1.6. Egg masses were collected into 500-ml polypropylene flasks containing treatment water for transport back to the laboratory. In the laboratory, for storage of egg masses (prior to analysis), each flask had its own airline to achieve water movement and gas exchange. After masses had been counted, weighed, and eggs/mass counted, the first 10 from each enclosure were placed into 500-ml volume glass beakers, each with its own airline. Treatment water was changed every 5 days, or when the water became cloudy during hot weather. In experiment 3, room (and therefore water) temperature was not controlled, and varied more by comparison to the minimum-maximum temperature controlled room used previously. It was thought that the considerable change in ratio of egg masses to diluent water volume, with the addition of aeration should represent a considerable improvement in hatching conditions. Due to workload, hatchability was assessed time permitting during this exposure experiment.

2.5.3 *V. viviparus*

The basic methodology used to assess reproductive parameters did not vary from section 2.2.4.2. In addition, to obtain cumulative data on the mature embryos released, a sock with a 2 mm mesh size was placed around the outside of *Viviparus* enclosures

(Figure 2.7B). This mesh was just small enough to retain even the smallest of the mature embryos released from brood pouches of female snails. This sock could be placed over the enclosures (from the bottom up), and was tight enough such that it did not fall off. In brief, every two weeks the sock was removed, and the number of baby snails released collected into 500-ml polypropylene flasks. The emptied sock was then replaced around an enclosure before enclosures were placed back into their respective tanks. String was used to tie the sock over the top of enclosures as added protection against them slipping or falling off. These baby snails were returned to the laboratory, here they were counted and weighed.

2.5.4 Testing food

Prior to supplementing the food given (in addition to lettuce) to the snails in experiment 3, a leading brand of flaked fish food (Tetra GmbH; TetraMin Flake Food) was tested so that a non- oestrogenic brand could be chosen; as an oestrogenic food would have complicated the interpretation of the results. A 2.5kg tub was purchased and tested in order that a single batch of food could be used throughout the experiment, and only a single test for oestrogenic activity would be necessary. It was decided to use the yeast screen for this purpose, because it has been shown to be specific for compounds that exhibit oestrogenic activity via the oestrogen receptor (Routledge and Sumpter, 1996), and was available in our laboratory.

2.5.4.1 Extraction of powdered food

A gram of the fish food was ground down to a fine powder using a pestle and mortar and then placed in a 50ml propylene tube. 25ml of purite water was added to this powder and the tubes were shaken by hand for several minutes to provide a fine suspension. To this suspension, 10ml of ethyl acetate was added and mixed well by shaking. A “control” sample (tube without any flaked fish food) was also treated in this way, to make sure the solvent did not extract any oestrogenic chemicals from the plastic tube. The samples were periodically mixed (at least twice) whilst being stored for 24 hours at 4 ^{o}C. After 24 hours, the contents of the tubes were mixed well and centrifuged at 700 rpm at 4 ^{o}C for an hour. The layer of ethyl acetate from both

samples was then removed and placed into 15ml falcon tubes. The volume that was taken off was recorded. Using a gyrovap, the extracts were evaporated to dryness. The oily droplet formed was re-suspended in 1ml of absolute ethanol and stored at –20 ^{o}C for analysis of its oestrogenicity using the yeast oestrogen screen.

2.5.5 Histopathology of Snail Tissues

At the end of experiment 1, after analysis of all results of this experiment, it was decided to continue with two species of snail, these were *Planorbarius corneus* and *Viviparus viviparus*. Therefore, as part of the investigation into possible developmental effects of TSE, analysis of babies born (F1) to *Viviparus viviparus* females (and of *Planorbarius corneus* snails) during experiment 3 was undertaken. As *Viviparus viviparus* are dioecous (separate males and females) and therefore these effects, if any, could be more easily observable, I analysed this species first. To investigate for the presence of developmental abnormalities (such as intersex), a morphological and histopathological method was developed (by manipulation of relevant tissues) and used. Figure 2.8 describes the system I developed and used to analyse the baby viviparid snails collected.

Histopathological analysis of the F1 generation of *P. corneus* born in the experimental system was not attempted as this snail is a hermaphrodite, and would be expected to contain both male and female sexual organs and structures, and therefore, developmental effects would be much harder to identify. However, gonads of the parent generation (F0) of *P. corneus* snails used in experiment 3, and whole bodies of F1 babies born (from F0 snails) in the experimental system were sent to a histopathological expert (Burkard Waterman, LimnoMar, Hamburg, Germany), for analysis. Time constraints also meant only a single species could be analysed. The results from this laboratories work are also presented in chapter 6.

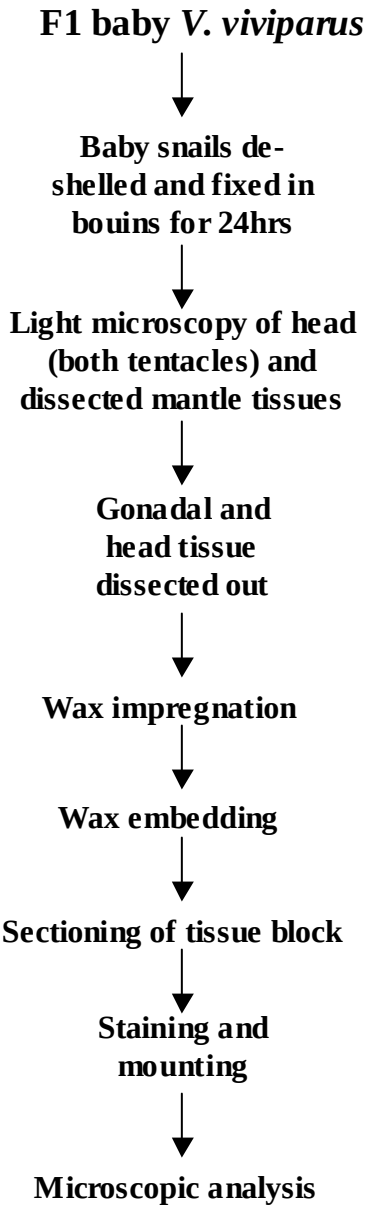


Figure 2.8 Flow diagram of the histology protocol used to assess gonadal, head, and mantle tissue abnormalities in F1 baby *V. viviparus*.

2.5.5.1 Preparation of Snails for Histological Analysis

The size of baby F1 *Viviparus viviparus* snails that underwent fixation ranged from 6.8-19.7 mm (6.6-21.1 mm for F1 *P. corneus*) in length and 0.12-2.0 g in weight (including shells)(0.13-1.9 g for *P. corneus*). All baby snails were measured for shell length and weighed before further analyses. Pliers were used to crack snail shells and all of the shell was removed to leave only the soft body parts. This was important, as any shell would remain hard, and if present when sectioning would damage the sharp edge of the microtone blade and ruin the sectioning process.

2.5.5.2 Fixation

Once euthanised, whole body samples (de-shelled) of F1 *V. Viviparus* were fixed in bouins solution (Sigma)(750ml Picric acid saturated aqueous solution, 250ml 37-40% formalin (formaldehyde), and 50ml glacial acid for 24-hours, and then washed twice with 70% ethanol, and finally stored in 70% ethanol at room temperature until analyses. This washing was done to remove the picric acid and prevent excessive hardening of the tissue. Head and mantle tissues were then analysed by light microscopy.

2.5.5.3 Post-fixation Preparation and Morphological Analysis of tissues

In the laboratory, on removal from 70% ethanol the foot was dissected away, as this was not part of further analysis (does not contain any sexual organs or structures). Macroscopic investigation was then made to observe whether the baby snails` right tentacle was male in form, or female. Males have the penis incorporated into the right tentacle, and the "hook" or pore through which this appendage can be extended can often be seen under the light microscope. The nose and both tentacles were removed in one piece from each snail. The mantle edge and surrounding tissue, essentially a thin flap of tissue, was cut away from the rest of the body; in females, the openings present are the "female opening" (*viz.* vagina) if the snail is old enough for this to develop, anus, and ureter. These structures develop on the inner surface of this part of

the mantle tissue, and if present, they were observed and recorded under a light microscope. In males, there is no female opening. The number and description of openings was recorded for each snail. Due to the small size and delicate nature of this piece of tissue, a picture was not always possible. Representative pictures of the structures found during the sexual development of baby viviparid snails born in my experimental system are displayed in chapter 6. After light microscope analysis tissues were stored again until further analysis. The third histological sample taken for each snail was the gonad, which was dissected away from the rest of the body before wax impregnation.

2.5.5.4 Wax Impregnation

Whole snail bodies (including head, gonad, and mantle tissues) were then wax impregnated. The fixed tissue samples were placed inside numbered plastic cassettes, and put into the processor (a Shanon autoembedder). The processor runs on a 20-hour cycle, first dehydrating the tissue with increasing IMS concentrations (Table 2.2) and then clearing it using Histoclear (which is miscible with IMS and wax). The system, therefore, allows complete impregnation of the tissue with wax.

Table 2.2 Tissue processor protocol.

Solution	Immersion time (hours)
70% IMS	3
90%IMS	2.5
95% IMS	1.5
100% IMS	1.5
100% IMS	1.5
100% IMS	1.5
Histoclear	1.5
Histoclear	1.5
Histoclear	1.5
Parafin wax	1.25
Parafin wax	1.25
Total	**20**

2.5.5.5 Wax Embedding

After impregnation, tissue was embedded in a wax block using an Embedder (Raymond A. Lamb, Laboratory supplies, London). For gonads, vertical sections were taken, with occasional longitudinal sections. Gonads were placed centrally in the cassette. For mantle tissue, sections were taken across the ureter, female opening (if present) and anus in cross section, to look for their presence under the microscope. With a small amount of hot molten wax in a cassette, the mantle tissue was placed such that it "stood up" in the cassette, once steady, molten wax was added to fill the cassette. In this way, cross sections of sexual openings could be achieved. For the nose and two tentacles, longitudinal sections were taken that would intersect across the newly formed penal tissue (if present) in the right tentacle. Nerve tissue ran up both tentacles in all snails (see Figure 2.9). The nose and tentacles were placed centrally in the cassette; the lower edge of the tissue was dissected to form a flat surface in order to aid a complete horizontal section to be taken across all three structures (both tentacles with "nose" in between).

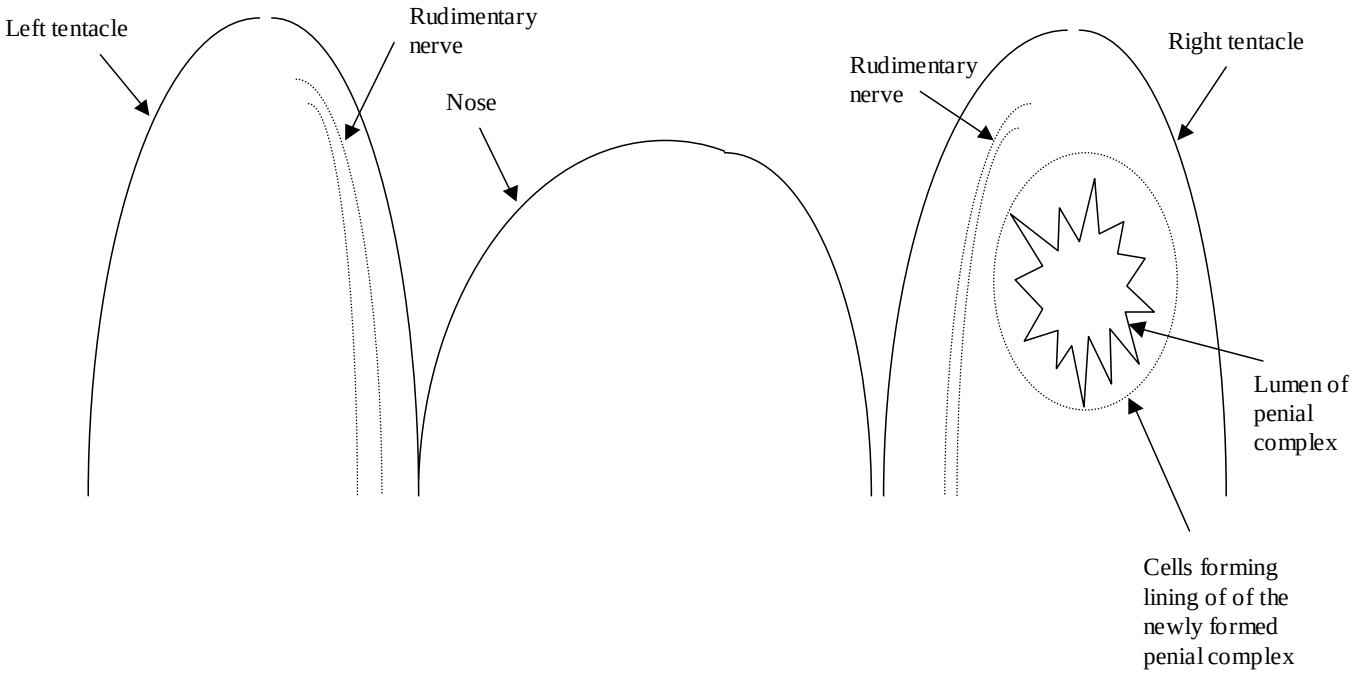


Figure 2.9 Diagrammatic illustration of a horizontal section through the nose and both tentacles of baby *V. viviparus*. Drawn to illustrate the relative position of the features described, and the position of the newly forming penal tissues in putative male snails (not present in females). Nerve tissue running the lengths of both tentacles are found in the same relative position regardless of the sex of the snail analysed.

2.5.5.6 Sectioning

An Anglia Scientific rotary microtome was used to cut sections from each block. A fresh part of the disposable steel microtome blade (Thermo Shandon) was used as necessary, to ensure that it was sharp and reduce "chattering" across sections. The clearance angle used was 5-8 degrees. Ribbons of wax and tissue were placed in a bath of 30% IMS to remove any creases and the ribbons were "floated out" on a water bath maintained at 50 °C. Consecutive sections were attached to each slide (to provide replacements if any were faulty). Sections were taken at various positions through the mantle tissue, nose and tentacle blocks, as it was necessary to check for the various features I was looking for throughout the tissue. The exact position of the sexual organs was an unknown, and to my knowledge has not been documented before. Slides with adhered sections uppermost were placed on a hot bed at 45 °C and left overnight until the wax had melted and stretched out the sections. Vectabond (Vector Laboratories) pre-coated slides were used for all sections taken in order to increase adherence of sections to the glass slides.

2.5.5.7 Staining

To stain the sections, Haematoxylin (Harris BDH) was used and these were then counter stained with eosin (Gurr BDH). Alum haematoxylin gives particularly clear nuclear staining and is generally used regressively in routine histology. It was stored in the dark to prolong shelf life, and was replaced after 11 or 12 uses to ensure consistent staining.

Sections were resolved using 1% solution of hydrochloric acid in 70% IMS. Reduction in pH causes the stain to lighten in colour and become a pinky red, but lithium carbonate raises the pH, allowing the stain to return to its previous colour.

Staining was carried out by hand using the protocol shown in Table 2.3.

Table 2.3 Staining protocol.

Solution	Duration	Comments
Histoclear	15 mins	Clearing agent- removes
Histoclear	15 mins	wax
100% IMS	2 mins	Rehydration. Haematoxylin
100% IMS	2 mins	functions in aqueous soln
90%IMS	2 mins	
70% IMS	2 mins	
Running tap water	2 mins	
Mayes Haematoxylin	15 mins	Stains nuclear material
Running tap water	15 mins	Wash
1% acid alcohol and agitation	20 secs	Stain resolution
Running tap water	20 secs	Wash
1% saturated lithium carbonate	10 secs	Raises pH and removes yellow stain from Bouins
Running tap water	20 secs	Wash
Eosin	10 secs	Cytoplasm stain
Running tap water	5 mins	Wash
70% IMS	2 mins	
90%IMS	2 mins	Dehydration
100% IMS	5 mins	
Histoclear	5 mins	Allows applicant of mountant

The slides were put through the staining protocol described above and then mounted with cover slips using DPX mountant (BDH) and left to dry for 24 hours in a fume hood prior to analysis using a light microscope (Olympus, London.).

Chapter Three: Method Validation

3.1 Aims of Experiment One

To address the aims of this thesis, it was first necessary to assess the survival, reproduction and development of a variety of snail species in the experimental set up, and therefore, the ecological and physico-chemical robustness of these species to river water (and TSE). The findings of this experiment were be used to determine the choice of species that would be used for further studies.

3.2 Snail Species Chosen for Study

A literature search was conducted to identify potential species for use in my studies. From this, representative species of commonly found European freshwater snails were chosen from different taxa. The main life cycle characteristics of interest were size at maturity, ability to be handled (morphological measurements and histological assessments) and caged, age at maturity, life span, reproductive mode, feeding niche, and habitat characteristics (substrate, water chemistry, depth, and flow).

Of the 6 Classes of mollusc, only the Gastropoda can provide suitable candidate species, of the three Sub-classes within this Class, the Opistobranchs are exclusively marine, leaving only the Prosobranchs and Pulmonates to be considered here. Though the environmental requirements of both these gastropods groups are similar, and this may not be surprising given that all classes of molluscs evolved in the sea and share the same broad body plan (Dillon, 2000), they may still be divided to demonstrate a basic difference. A quote and generalisation from Boycott (1936) is apt, though there are exceptions; "The 10 operculates (or prosobranchs)(of England) live almost exclusively in running water; being gill breathers and unable to come to the surface and gulp in air as the pulmonates do, they presumably need water which is fairly well oxygenated and also free from particles which might choke their gills". Therefore, TSE containing heavy particulate matter colud directly affect the prosobranchs species chosen in my experimental system, as it may clog their gills and cause ill health.

The eight families of prosobranchs are the most primitive gastropods and are diverse in their evolutionary origin having invaded fresh waters (from marine waters) on many separate occasions. Fresh water prosobranchs are consequently varied in their reproductive biology as well as in other features. Some generalisations are however possible as they share a few (probably ancestral) characters; most freshwater prosobranchs are dioecious (separate males and females) and occasionally parthenogenic, possess opercula, and have typical ctenidia, or gills through which they breathe. Brooding is widespread among freshwater prosobranchs, enhancing the protection of young embryos by retention in a modified pallial oviduct until they are small snails. Oviparity is also a common strategy; eggs are released and the parent pays no further attention to them. Fewer eggs are produced by prosobranchs compared to pulmonates. Of the eight prosobranch families, two the Hydrobidae and Viviparidae, are represented by 10 British operculates that live in freshwater. Prosobranchs grow more slowly than pulomnates and while 39% of prosbranch populations have generation times of 24 months or more, and can reproduce during more than one season, this phenomenon is rare in pulmonates (Dillon, 2000).

Considered the most advanced or highest Basomatophoran freshwater molluscs (Geraerts and Joose, 1984), freshwater pulmonates are commonly represented by species from five families, the Physidae, Lymaeidae, Planorbidae, Acroloxidae and Ancylidae. Aquatic pulmonates are 100% monoecious (adults are simultaneous hermaphrodites), have no opercula, have lost their ctenidia and replaced them with highly vascularized mantle cavities. These snails lay egg masses that vary from the more loosely packed, irregular, and convex spawns of physids and lymnaeids to the rather tough, flat, ovoid masses of planorbids and ancylids (Dillon, 2000). In general, living to reproduce during a single period (semelparity), is much more common amongst the Pulmonates, with a high fecundity (Schmude *et al.*, 1997). Breeding in late spring or early summer, they exhibit an annual reproductive life cycle (natural duration of life usually 9-15 months or less). There are many variations of this basic pattern (Geraerts and Joosse, 1984); including two generations per year (bivoltine pattern), three successive generations (one each in spring, summer, and autumn); all patterns can occur with or without complete replacement of one generation with another (see Chapter 4 Introduction). The ecology and biology of each of these two sub-classes of molluscs illustrates their basic differences, and how my choice of

species allowed a full range of life cycle characteristics and reproductive modes to be chosen and species to be tested in experiment 1.

3.3 Egg Hatching Method Development and Validation

To my knowledge, the hatchability of *P. corneus* eggs in intact egg masses had not been assessed before in the laboratoty. The method by which I hatched these eggs (in egg masses) in experiment 1 was the subject of on going method development. The structure of *P. corneus* egg masses (ovoid, flattened, with "jelly like" filling) meant that individual eggs could not be removed from egg masses. As a consequence, a laboratory method was developed that allowed eggs to hatch out from intact egg masses.

3.3.1 Development of egg mass collection, storage, and hatching during Experiment 1

For details of the initial methods employed to collect and store egg masses before hatching rate could be assessed see section 2.2.4.2.

Lymnaeid eggs masses are much more ovoid than those of planorbids, and consequently eggs are much less tightly packed, enabling researchers to remove individual eggs and hatch these one egg at a time from masses (see PhD Daire Casey). Planorbid egg masses are considerably more flattened and compact; the surrounding medium and egg cases make it impossible to remove the eggs, without damaging them.

3.3.1.1 Hatchability of Eggs over weeks –2 to 0, and 0 to 2

For the first month, the practical constraints of running the experiment meant that the time taken to deal with egg masses collected in the field were extended compared to following weeks. Consequently, the condition of egg masses in 20ml universal containers (Sterilin U.K) had deteriorated such that their ability to hatch would have been unreliable. Universal containers were stored in a room that was not temperature

controlled; rising temperatures during the heat of early summer caused masses to deteriorate within 3-4 days. Consequently, there were no results for egg hatchability during this first month. However over time, such constraints were overcome and all consequent time periods produced reliable results.

3.3.1.2 Hatchability of Eggs over weeks 2 to 4

In addition to the initial egg mass collection and hatchability method (see section 2.2.4.2), the egg masses were transferred from 20ml universal containers (Sterilin U.K) into Petri dishes to avoid the egg masses deteriorating before their hatchability could be assessed.

3.3.1.3 Hatchability of Eggs over weeks 4 to 6

There were no methodological changes relative to section 3.3.1.2 during this time period. Egg masses collected at the end of weeks 4 to 6 revealed a slight deterioration in condition (after 2-3 days as ambient temperatures were high) that did not affect hatching; egg masses that previously could support their own weight became floppy (but remained intact). As this deterioration occurred while egg masses where stored and before their hatchability could be assessed, it was necessary to further refine the collection and storage process.

3.3.1.4 Hatchability of Eggs over weeks 6 to 8

Egg masses collected from each replicate group of snails in the field were split equally into two universals (total volume 50-mls), instead of one, before returning them to the laboratory. In the laboratory, each universal was emptied into a labelled Petri dish and topped up with treatment water. In this way egg mass density was halved throughout collection and storage. If hatching rate did not improve it was hypothesised that the volume of treatment water each egg mass had previously in each Petri dish maybe not be the cause of the perceived low hatching rates. If hatching rates improved, then lowering egg mass density during collection and storage had improved the condition of egg masses, and therefore, egg hatching rate.

3.3.1.5 Hatchability of Eggs over weeks 8 to 10, and 10 to 12

There were no methodological changes during this time period relative to section 3.3.1.4.

3.3.2 Development of Egg mass collection, storage, and hatching method during Experiment 3

The method by which egg masses were collected, transported, stored, and then hatched in the laboratory was considerably improved by experiment 3 (`04) compared to experiment 1 (`03). Egg masses from each enclosure were collected into a labelled 500-ml polypropylene flask for transport back to the laboratory. In the laboratory, for storage of egg masses, each flask had its own airline to achieve sufficient water movement for gas exchange. After egg masses had been counted, weighed, and eggs/mass counted, the first 10 from each enclosure were placed into 500-ml volume glass beakers, each with its own airline. Treatment water was changed every 5 days, or when the water became cloudy during hot weather. In experiment 3, room and therefore water temperature was not controlled, however, a cooler room with less extreme temperature extremes was used. It was thought that the considerable change in ratio of egg masses to diluent water volume, with the addition of aeration should represent a considerable improvement in hatching conditions. Due to workload, hatchability was assessed time permitting during this exposure experiment.

3.4 Glycogen Phosphorylase Method Validation

3.4.1 Stability of Glycogen Phosphorlyase enzyme in homogenates

The methodology adopted to analyse snail tissues was that of Childress and Sacktor, 1970. As this method was not specific to snail tissues, application of the method to snail tissues had to be tested. In particular, the way in which tissues were prepared prior to adding to the reaction mixture at the beginning of the assay was different (i.e. Whole insects in the case of Childress and Sacktor, and frozen snail mantle tissue from my own experiments). Therefore, method validation was necessary to ensure a

suitable homogenate (with still active glycogen phosporylase) could be prepared from frozen stored snail mantle tissues for this assay.

Firstly, I needed to test the stability of the glycogen phosporylase enzyme in defrosted snail mantle tissue. This enzyme is found in the mantle tissues of pulmonate snails (Joosse,1988, Baturo *et al*., 1995, Jumel *et al*., 2002), where glycogen is broken down to glucose and is used as energy supply in eggs. As the practical work involved in the methodology (Childress and Sacktor, 1970) must be carried out on ice and takes some time to complete, there was concern about whether enzyme activity could be lost during this time period. The stabilty of the glycogen phosphorylase enzyme in the snail tissue homogenate was tested after spinning (to produce a clear supernatant), as this is the main time consuming part of the assay, where enzyme activity could be lost.

In each case the preparation of a snail body homogenate proved relatively simple. An ice cold hand held homogeniser with an appropriatley calculated volume of ice cold buffer was used to homogenise the snail body until all the tissue had been reduced to a small size (approximately pieces of 2 mm or smaller). Then, in the first test of the methodology, the homogenate that had been kept on ice was spun for 1.5 hours at 3, 000g as the methodology of Childress and Sacktor, 1970 suggests (see section 2.2.5.1) and then activity of the supernatant was tested for the following 270 minutes (therefore, time zero was 1.5 hours after homogenation), with activity of the glycogen phosphorylase enzyme in the supernatant tested at regular time intervals (see Figure 5.15A).This methodology was then compared with a quick but harder spin where the supernatant was spun for 20 minutes at 13,100 g, and the activity of the glycogen phosphorylase enzyme in the supernatant was tested throughout the next 400 minutes at regular time intervals (therefore, time zero was 20 minutes after homogenation) (Figure 5.15B). In so doing, enzyme activity of homogenates were assessed for 390 mins and 420 minutes by each of these methods respectively (after the homogenates were made from snail tissues).

3.4.2 Rigour of Spinning; repeatabilty of sampling supernatant

The process of homogenation of snail tissues should produce a clear supernatant (without "bits" of snail tissue) that can then be pippeted accurately into the reaction mixture after centrifugation. If a volume other than 50 µl was pippetted (or the volume pippetted was variable due to suspended tissue), error would be introduced into the assay. To test the repeatability of testing a single snail homogenate, a sample was prepared by the following methodology: homogenated snail tissue was spun for 90 minutes at 3, 000g at 4 ^{o}C and a 50 µl aliquot was tested for glycogen phosphorylase enyme activity sequentially 20 times as quickly as was possible after centrifugation. The results were analysed statistically to see if the results were consistent enough to indicate sufficient rigour of this part of the assay.

Chapter Four: The Seasonal Reproductive and Development Cycle of Gastropod Molluscs in River Water

4.1 Introduction

4.1 Seasonality in Gastropod Molluscs

Gastropod molluscs, especially those living in temperate regions, generally exhibit a well defined seasonal reproductive activity which is controlled by environmental (external) parameters, such as photoperiod, temperature, and availability of food (see reviews of Joosse and Geraerts, 1983; Geraerts and Joosse, 1984). In general, molluscs display seasonal cycles not just in reproduction, but also in growth, and storage and mobilisation of the energy reserves; processes that appear to be interdependent (Hemminga, *et al.*, 1985). Evidence suggests that long-term internal regulation of reproduction can be dramatically altered by these environmental factors (referred to as "ultimate factors") (Bohlken and Joosse, 1982). Several aspects of the relationship between these environmental factors, and the endocrine system and reproduction system have been investigated by endocrinological, neurophysiological, and histological methods (Dogterom *et al.*, 1984). These external factors are thought to exert their influence via the endocrine system to affect reproductive organs (Geraerts and Joosse, 1984; Joosse and Geraerts, 1983; Bohlken and Joosse, 1982), although the precise mechanisms at play remain largely unknown. The effects of external environmental factors upon reproduction are mediated by changing seasons (or seasonality). To illustrate, increasing daylength, water temperature or food quantity influences the activity of the female gonadotropic centres, including LL`s, (and therefore the DB`s and CDC`s), resulting in increased production of both DBH and CDCH. This signals the start of reproduction in spring or can increase reproductive rate (as does clean water, lack of parasitism, and changes in atmospheric pressure). This increase in DBH not only stimulates vitellogenesis in the ovotestis, but

also growth, differentiation, and synthetic activity in the female accessory sex organs (ASO`s) (Geraerts and Joosse, 1975).

Seasonality (or seasonal cycles) in organisms result, therefore, from annual oscillations of a number of environmental cues (Hemminga *et al.*, 1985). As a result gastropod molluscs living in temperate regions where changes in the environment are more profound (than in tropical regions) with the changing season, exhibit well defined seasonal reproductive periods. These environmental factors affect reproduction (and mortality), growth, energy balance, but also blood circulation, and water and ionic metabolism (Laufer and Downer, 1988). There are also short-term environmental factors (proximate factors) that may affect reproduction via non-endocrinological routes, such as copulation (snail density) (Van Duivenboden *et al.*, 1985), atmospheric pressure (Bohlken and Joosse, 1982)**,** and water quality (Ter Maat, 1983).

Environmental signals therefore profoundly influence the life cycle of gastropod molluscs including reproductive pattern, and total reproductive output during their lifespan. However, life cycle patterns of aquatic gastropods are complicated and highly variable. Several patterns can be distinguished in the freshwater gastropods of temperate zones (Tompa *et al*., 1984), and the "life cycle pattern" of these molluscs is described within the context of differences in the timing of reproduction through life, survivorship, age and size at reproduction, and reproductive strategy (semelparity vs. iteroparity). Cohorts are semelparous when they undergo (when mature) a single reproductive event (usually through summer), and iteroparous when they reproduce over two or more time periods (same or different summers), at some point death follows the final act of spawning (Dillon, 2000). Experiments with gastropod molluscs in the laboratory have demonstrated that under controlled conditions (usually temperature, day length, and feeding), snails can be made to breed all year round; demonstrating the considerable effects of these environmental factors. In the laboratory (medium day length (12 hours day/12 hours dark), fed fresh lettuce *ad libitum* at 15 ^{o}C, *P. corneus* can breed all year round, but continuously for 44 weeks corresponding to 76% of its maximum longevity (Costil and Daguzan, 1995). In the final part of its lifespan *P. corneus*, lays no egg masses possibly due to either gamete exhaustion and/or damage to the reproductive system (Costil and Daguzan, 1995).

The Lymnaeidae, Physidae, Planorbidae, and Ancylidae are considered the most advanced or highest limnic Basommatophora that typically show an annual lifecycle (Wilbur, 1984). These major freshwater pulmonate families evolved from a single invasion of freshwater (from land), and now respire over the inner surface of their mantle (Dillon, 2000). The parental generations reproduce over a single period and then die (i.e. are semelparous), having produced enough offspring to replace the adult population. Studies of snail populations (mostly in Europe) indicate that around 1/3 of pulmonate populations display generation times of <1 year and generation times exceeding 2 years are rare. In contrast, 39% of prosobranch populations have generation times of 24 months or more, and only two populations of prosobranchs are known where generation times are less than 1 year (Dillon, 2000). For example, the prosobranch snail *V. georgianus* needs 36-47 months to sexually mature, and it is reported that males mate and die at age 2 years and have a shorter lifespans (by 6 to 12 months) than females, which reproduce semelparously at age three (Jokinen *et al*., 1982). Spoel (1958) found that *V. viviparus* commonly lives to an age of 6 years, and sometimes to 11, and females live a year and a half longer than males (Fretter, 1965). In French ponds Costil and Daguzan (1995b) reported that the pulmonate snail *P. corneus* reproduces intermittently, habitually in Spring and less often in Summer. Some cohorts were iteroparous and others semelparous; lifespan of different cohorts ranged between 15-21 months and were therefore close to being biennial. The reproductive strategy of young-of-the-year snails (and 1+ year olds) in any one year was determined by environmental conditions, with adverse environmental conditions (storms, floods, extreme water temperature changes, and therefore lack of food) preventing reproduction at these times of the year (typically mid summer). Conversely, favourable environmental conditions were taken advantage of with greater reproductive activity. The literature frequently demonstrates that local environmental conditions controls the plasticity of response of molluscan populations, to a large extent determining life cycle parameters.

The different reproductive strategies of pulmonates and prosobranchs are striking given that both classes may, and often do, cohabit the same environment. Each employs a different solution to the same problem; how to replace themselves to ensure survival of their species. Pulmonates produce many small eggs during a single

breeding season that contain relatively little energy (egg yolk) per egg, and provide no care to their offspring. Prosobranchs reproduce over several seasons, but produce far fewer larger eggs, each having a greater relative input of parental energy resources, and tend to provide more care to their offspring (as with the ovoviviparus viviparids).

Russell-Hunter documented great interpopulation variability in freshwater gastropod life cycles, and produced their first formal classification based primarily on the number of breeding events per year (iteroparity or semelparity), with the possibility of snails producing more than one age-class (i.e. cohort) or generation of snails per year, and the survival of the adults after egg-laying (Dillon, 2000). Though he reviewed only several dozen populations of snails, mostly European pulmonates and a few prosobranchs, he recognized seven lifecycles; (1) one generation (or age class) per year, these maturing adults reproducing semelparously in the spring, (2) one generation per year, maturing adults reproducing semelparously in the late summer, (3) two generations or cohorts per year, the spring-born cohort iteroparous and the summer-born cohort semelparous, (4) two generations or cohorts per year, both maturing cohorts semelparous, (5) three semelparous generations or cohorts per year, (6) one generation or cohort per year, maturing adults reproducing iteroparously, and (7) a two-year generation time to maturation (Dillon, 2000).

British populations of *Lymnaea peregra*, for example, may display five of the seven categories of this pattern (1-4 and 6). He stated "In most cases, these interpopulation variations could be determined phenotypically by trophic or temperature differences in the environment". Russell-Hunter`s primary conclusion in 1961 was that "selection has produced genotypes which can show phenotypic variation" (Dillon, 2000). However, reciprocal transplant experiments have shown there to be a role for genetics in determining these lifecycle patterns. Indeed, snails can become genetically adapted to their local environment over long periods of time so that when snails are moved to a different environment, they may respond in terms of their growth and reproductive patterns in a similar manner to their "home" environment, as they are "programmed" to do. Adaptive plasticity allows snail populations to have maximum productivity under normal environmental conditions (to compensate for bad years) and retain the ability to modify their response in a changing environment (Costil and Daguzan, 1995B). When Costil and Daguzan (1995A) moved *P. corneus* into a laboratory

environment their near continual reproduction at several steady temperatures (and other conditions) demonstrated this snails over riding phenotypic plasticity in spite of any pre determined "genetic programme".

Reports in the literature regarding the effects of external factors upon reproduction and life cycles are relatively few. Of these, most are laboratory studies, and a precious few are field studies. Under natural seasonal conditions the activities of both the DB-cells and the CDCs are also shown to have an annual cycle, the increased activity of which coincides with the onset of ovipository activity. Several authors (Bohlken and Joosse, 1982, Dogterom *et al*., 1984b, Dogterom *et al*., 1985) have noted that for snails living in temperate climatic conditions there is a threshold that must be passed before egg laying or oviposition can begin in spring. The parameters studied to this regard have been photoperiod, temperature and food quality and quantity.

In winter, temperature is low and snails live under conditions of starvation due to lack of food availability. External environmental factors act synergistically on the snails endocrine system, the accessory sex organs (ASO`s) are in a regressed state. The membrane potential of the caudodorsal cells are more negative (hyperpolarized) and ovulation hormone (CDCH) is not released (Ter Maat, 1982), and the secretion of DBH ceases (Wilbur, 1984). Under these conditions the gonad and ASO`s do not respond to ovulation hormone (CDCH), even if present, indicating that the target organs are insensitive to the hormone. This regressed state of the ASO`s may be caused by long periods of inhibition of the gonadotropins, especially of the dorsal-body hormone (Wilbur, 1984).

In springtime or early summer, day length, and therefore water temperature increases**,** and food becomes more abundant with an increased growth rate of aquatic plants, macrophytes and algal blooms. At this time, the DB`s of *L. stagnalis* (and of other gastropod species also) show a clear change in their histology, and the amount of DBH increases as the critical threshold for egg laying approaches. These external environmental factors will dramatically affect the reproductive cycle once the critical threshold for egg laying has been reached.

Wilbur, 1984, described breeding condition (growth of gonads, ASO`s) as being induced in early spring only when the photoperiodic threshold (though not precisely established) of 14hours daylight per 24 hours is exceeded. In spring, the synergistic action of increasing day length, water temperature, and increasing food availability increases DBH and CDCH production, the reproductive system of gastropods matures, and once thresholds are exceeded the egg-laying season begins.

4.2 Environmental Factors affecting Reproduction

4.2.1. Photoperiod

It is widely acknowledged that for most seasonal breeders living in temperate climates, photoperiod is the primary environmental signal that regulates the timing of reproduction (Goldman, 2001). Reports regarding photoperiodic effects on reproduction in freshwater pulmonate snails are scarce (Bohlken and Joosse, 1982), but all authors suggest that long day photoperiod causes increases in egg production. Although no precise critical photoperiod for egg laying has been established (Dogterom, *et al*., 1985), several authors have estimated the photoperiodic threshold for oviposition to be between 12 and 16 hrs of daylight (Bohlken and Joosse, 1982).

Long Day (LD) photoperiods induce a relatively early maturation of the female reproductive system (viz DBH production and release) and a "high rate" of egg production in *Lymnaea stagnalis* held in laboratory conditions (Bohlken *et al.*, 1978)(Bohlken and Joosse, 1982). *Lymnaea stagnalis* (16h L/ 8H D) produced 4.0 egg masses/snail/fortnight fed bemax (wheat germ product) and lettuce on alternate days at 20 °C (Bohlken and Joosse, 1982), though snail density could not be determined from their methods. At the end of the experimental 24 weeks the total number of egg masses produced had been 6 and 10 times higher than that of the MD (12 h light, 12 h dark) and the SD (8 h light, 16 h dark) animals, respectively. The mean size (eggs/mass) of the egg masses of the LD snails was only 30% and 10% larger than those egg masses laid by snails under SD and MD conditions (Bohlken and Joosse, 1982). Similarly, Dogterom *et al.*, 1984 kept *Lymnaea stagnalis* in individual beakers at 20 °C under MD (12h light/12 h dark) with lettuce fed *ad*

libitum with continuous water supply and these snails produced approximately 4.2 egg masses/ fortnight, a remarkably similar figure. Again, in the laboratory, under constant conditions of daylight (MD; 12 h light/ 12 h dark), temperature (20 ^{o}C) and nutrition *P. corneus* snails reproduced over 34 weeks averaging approximately 3.0 egg masses per snail per fortnight (Costil and Daguzan 1995). Further, in an earlier laboratory study with *Lymnaea stagnalis* Bohlken *et al.,* (1978) found that at the same LD photoperiod (16 h light, 8 h dark) snails produced 10-20 times more eggs than other groups (MD or SD groups), however mortality increased markedly towards the end of the 25 week experiment. LD`s (usually defined as 16 hrs daylight for laboratory experiments) therefore appear to be a strong stimulus for egg production compared to MD`s or SD`s.

4.2.2 Temperature

The minimum threshold temperature for reproduction of freshwater snails appears to be between 7 °C and 12 °C (Costil and Daguzan, 1995B); at these temperatures the reproductive system is able to produce egg masses albeit at a low rate. Laboratory experiments have demonstrated that, in *L. stagnalis* the critical water temperature for egg laying is about 8 °C (Dogterom *et al.,* 1984b). At low temperatures a low DB activity may be the cause of reduced oocyte maturation, low synthetic activity of the albumen gland and low responsiveness of the reproductive organs (Dogterom *et al.*, 1984). Transferring snails to higher temperatures results in decreased oviposition latency, as egg masses are produced more often, suggesting a rapid increase in DB and CDC activities. At an optimal 20 °C in the laboratory, isolated fed LD specimens of *L. stagnalis* produce about 7 egg masses/ snail / fortnight (Costil and Daguzan, 1995A). It was concluded that temperature has a direct effect on the rate of egg mass formation (and existence of a threshold) in the female tract, as well as an indirect effect on general development and growth (Costil and Daguzan, 1995A).

In French ponds, *P. corneus* began to reproduce above the minimum threshold at 15-16 °C (Costil and Daguzan 1995A), although Precht (1936) suggested 12 °C was sufficient for this species. Costil and Daguzan, 1995 state that temperature strongly affects reproduction in *P. corneus.* Again, in the laboratory, under constant conditions

of daylight (MD; 12 h light, 12 h dark) temperature (20 °C) and nutrition, maturing *P. corneus* produced a short reproductive peak and the maximum number of egg masses reached 8.5 per snail per fortnight averaging 5.0 per snail per fortnight for 10 weeks (Costil and Daguzan 1995). At 15 ^{o}C there was an egg laying rhythm of 44 weeks, but with no peak to reproduction, snails laid approximately 2.0 egg masses per snail fortnight, at 25 ^{o}C snails produced a constant number of masses between 0.5-1.0 egg masses per snail fortnight. Laboratory studies suggest that at 20 ^{o}C the number of egg masses produced at the peak of reproduction in *Lymnaea stagnalis* can be 4.0 to 7.0 egg masses per snail per fortnight (Bohlken *et al*., 1978; Bohlken and Joosse, 1982; Seuge and Bluzat, 1983; Bohlken and Joosse, 1984; Dogterom *et al*., 1984). Van der Schalie and Berry (1973) from their studies deduced that planorbids reproduced and thrived best in warmer water (22 °C to 25 °C) than lymnaeids (19 °C to 22 °C). Unfortunately no such detailed information is available for prosobranch molluscs.

4.2.3 Food

It is difficult, especially in field studies, to separate out the continued effects of day length, temperature, and food/feeding upon reproduction. However, food maybe the most important environmental factor affecting reproduction as it determines both the timing of the beginning of the egg laying season and, to the greatest extent, the number of egg masses laid (i.e. frequency), and number of eggs per mass. Therefore, female reproductive activity is strongly affected by food quantity and quality. In natural systems, it is the quantity of assimilated food that triggers the onset of the egg-laying season (Dogterom, *et al.*, 1985). It is also noteworthy that with increasing food supply the quantity of assimilated food reaches an upper level, determined by the intake capacity of the digestive gland (Scheerboom, 1978). However, the importance of food quantity and quality may determine to a high degree the reproductive capacity snail species with a high reproductive output, such as the pulmonates.

In Autumn, the snails become progressively less sensitive to CDCH which indicates that DB activity gradually decreases during this period. DB activities are controlled by the synergistic actions of environmental factors (Dogterom *et al.,* 1985), of which food, becomes in short supply towards winter. Evidence has demonstrated that

starvation lowers the membrane potential of the CDC, which also inhibits egg laying (Ter Maat, 1982). Interestingly, at the end of the egg-laying season, these three factors (day length, water temperature, and food quality and quantity) were above critical threshold values for egg laying, and for a long time (Dogterom, *et al.*, 1985). This indicates that the termination (and onset) of the egg-laying season is not controlled by a single factor; but by the synergistic actions of a number of external and internal factors.

In *L. stagnalis*, the critical water temperature for egg laying is about 8 °C (Dogterom *et al.*, 1984b), and female reproductive activity only occurs at critical assimilation values >6 mg of food (dry weight of lettuce) per day (Scheerboom, 1978). From field observations it has been noted that where photoperiod exceeded 12 hr, and winter temperature was above 8 °C, this situation existed well before the onset of the egg-laying season (Dogterom *et al.*, 1985). On the basis of previous experiments it seems very likely that it is not until May that food assimilation increases above the critical value for egg laying (Scheerboom, 1978; Scheerboom and van Elk, 1978). Hence, these authors concluded that the quantity of assimilated food is probably the crucial factor that triggers the onset of the egg-laying season (Dogterom *et al.*, 1985). Further, reproduction, the production of egg masses and eggs, is linearly related to the quantity of lettuce assimilated in laboratory fed *Lymnaea stagnalis* (Scheerboom, 1978). Fed to excess, individual snails in beakers at 20 °C (MD; 12 h light, 12 h dark) produced 3.6 masses / snail / fortnight fed lettuce and 5.7 masses / snail / fortnight (x1.6) fed bemax (wheat germ product) thereby demonstrating that quality of food is also important.

4.2.4 Water quality

Although day length and water temperature are external environmental factors and ultimate cues that can dramatically affect reproduction, proximate cues such as water quality, can also have effects upon reproduction. Dirty water inhibits (precise mechanism unknown) egg laying but without affecting the membrane potential of the CDC (Ter Maat, 1982). Snails in their natural habitat, particularly lymnaeids, feed near the bottom where water is “dirty”, low in oxygen and high in organics but choose

not to lay egg masses here. *Lymnaea stagnalis*, instead, travel near to the surface of the water and lay egg masses on the underside of leaves. By doing this they choose the best microenvironment for their offspring, partly also as protection from predation. Further, the natural habitats of both lymnaeids and planorbids are freshwater lakes, ponds, and ditches, and fast flow rates may, therefore, discourage them from egg laying. In contrast, prosobranchs such as *Viviparus viviparus* live on the bottom of relatively large slow flowing (compared to smaller rivers and streams) lowland rivers and canals where dissolved organ carbon content (DOC) maybe naturally lower. Though viviparids have been observed to travel to shallower waters to give birth in spring (Van Cleave and Lederer, 1932), the reasons for this and indeed whether all prosobranchs (viviparids in particular) do this is not documented. Therefore, flow rates in experimental test systems may not be suited to the prosobranchs, and may negatively affect their reproductive rate.

4.2.5 Grouping

A laboratory study demonstrated that the grouping of isolated *L. stagnalis* snails reduces the rate of egg mass production by half. This reduction was due to increased mating effort (van Duivenboden *et al.*, 1985), which effectively reduced the amount of time available to lay egg masses. Freshwater pulmonates live in environments that are transient in time and exhibit a high degree of small-scale, short- term isolation (Russel-Hunter 1978). At high densities they may take the opportunity to mate with several partners while the opportunity arises. There is some evidence that semelparity and hermaphroditism (the capacity for self-fertilisation) and a high degree of fecundity have evolved together, enabling a rapid population growth to cope with the uncertainty of freshwater habitats (Calow, 1978). Prosobranchs have evolved a different reproductive strategy to cope with the transient freshwater habitat, a lower rate of egg production and viviparity (Calow, 1978), and are often found in groups at certain times of year (Van Cleave and Lederer, 1932), suggesting that grouping may have less pronounced effects on viviparids.

4.3 The Seasonal Developmental cycle of snails

4.3.1 Growth

The growth of freshwater pulmonates is continuous until death (indeterminate growth), and occurs seasonally (Costil and Daguzan, 1995b). Spring is very favourable for the growth of *P. corneus* and other pulmonate species (Costil and Daguzan, 1995b). Since shell weight is known to be a highly variable parameter (Laufer and Downer, 1988), an increase in shell length might be better indicator of growth. However, shell growth (increase in length) and weight increase are under the control of the same endocrinological hormonal control system and these parameters are, therefore, intrinsically linked (Joosse, 1985). Contributing to this growth, is the particularly fast growth of ASO`s which occurs concurrently in spring, before the onset of the egg-laying season (Berrie, 1966). In summer (July-August, high reproductive rate) and in winter (December-March; lack of food, low temperatures), a lower rate of shell growth was noticed for *L. peregra* (Lambert, 1990) and also for *P. corneus* in a French pond (Costil and Daguzan, 1995b). This reduced growth rate, in summer, was due to the antagonism (partition of energy towards reproduction) between reproduction and growth (Bohlken and Joosse, 1982). Low growth rates in summer could also be due to excessively high temperatures, as temperatures in French ponds can reach 26 ^{o}C (Costil and Daguzan, 1995b), whereas optimal maximum growth rates in the laboratory occur around 20 ^{o}C (Costil, 1994). During Autumn, Costil and Daguzan, 1995b noted *P. corneus* snails grew with intermediate rates.

4.3.2 Energy Balance

In laboratory studies, Hemminga and colleagues (1985) reported depletion of glycogen (bodies energy stores) in mantle tissue of *L. stagnalis* at LD photoperiods (inversely related to ovipository activity), and deposition of glycogen at SD photoperiods. These authors reported some evidence for a seasonal decline in glycogen from winter to late summer in the glycogen stores (mantles) of field specimens of *L. stagnalis.* At LD photoperiods glycogen metabolism in *L. stagnalis,* and other pulmonates, is highly directed towards the glucose-consuming process of female reproductive activity at the expense of the glycogen reserves (Wijsman, 1989). Described as “reproduction in the face of adversity” (Dillon, 2000, Calow, 1978) this

process leads to lethal exhaustion of the snails` energy reserves, and results in a greatly increased mortality rate after several weeks (Bohlken, *et al.,* 1978; Bohlken and Joosse, 1982). Therefore, energy content of mantle tissues can indicate whether food was in excess during periods of high reproduction or whether available energy could have been a limiting factor.

4.3.3 Albumen Gland Weights

Several authors have observed that accessory sex organ (ASO) weights also change seasonally and during reproduction. In egg-layers a commonly measured ASO is the albumen gland (responsible for the manufacture albumen an important protein constituent of eggs). As winter conditions predominate, growth stops immediately at the beginning of a period of starvation; this includes growth of the ASO`s (including therefore albumen glands). The rapid decrease in weight of the female accessory sex organs (albumen gland, nidamental gland, oviduct) during the first 2 weeks of starvation is mainly due to the continued production of egg masses; gametogenesis is not interrupted by the absence of food (growth stops), energy released by their reduction in weight is transferred to egg mass production (Joosse *et al.,* 1968). In the pond snails ripe sex cells (sperm and oocytes) are resorbed in such quantities that their products may play an important role in their metabolism; helping to maintain (somatic) body tissues when no more food is available (autumn into winter)(Joosse *et al.,* 1968).

Conversely, in the field the albumen glands of *L. stagnalis* increased in size March to April, and at the same time the snails were growing rapidly (Berrie, 1966). Further, their gonads grew rapidly April to May, and the number and density of oocytes present also increased greatly. By mid June, at the beginning of the egg-laying season, albumen glands showed a considerable reduction in size, after which they remained a constant size until September. Therefore, at onset of reproductive activity, the weight of ASO`s falls as energy is diverted into egg production (Berrie, 1966). However, the degree to which this occurs is related to available food quantities (Joosse *et al*., 1968). In *Lymnaea stagnalis*, most authors report albumen gland weights to average between

30-50 mgs (well fed; Dogterom *et al.*, 1984; Boer and Cornelisse, 1968), and only 10 mg after 2 weeks of starvation.

Bohlken and Joosse, 1982 reported the proportional wet weight (10 snails from each treatment) of the albumen gland compared to total weight under different light regimes. Under LDs it was1.82 +/- 0.61 mg (means +/- S.D.) and this proportion was significantly bigger than MDs (1.29 +/- 0.52 mg) but not SDs (1.66 +/- 0.42 mg) proportions. In the LD and SD animals the proportional weights of these organs were 30-40% and 20-30% larger, respectively, than in MD snails. After spontaneous oviposition, there is an immediate linear increase in galactogen in the albumen gland from approximately 0.96 to 5.1mg/gland after about 32 hours (Wijsman, 1989), secretions lost to an egg mass are quickly replaced.

Therefore, evidence suggests that albumen gland weight is highly variable and closely linked to the metabolism of snails, and a combination of food quantity and season are the most important factors that affect their weight. Unfortunately, in only a single paper has the size of albumen glands in a prosobranch mollusc been reported. Indeed, Oehlmann *et al.*, 2000 reported a greater volume of albumen glands as part of a response to the presence of a xeno-oestogen (e.g.4-nonylphenol) in their experiment. Therefore, increased albumen gland volume could, in theory, result from an oestrogenic response in this prosobranch species.

4.3.4 Mortality

Bohlken and Joosse (1982) stated that the effects of a LD photoperiod on female reproductive activity and metabolism may be responsible for the high mortality rates observed amongst pulmonates during summer under natural conditions. In their laboratory bred *L. stagnalis*, during the last 2 weeks of a 24 week experiment on photoperiod the total mortality of the LD group increased rapidly to 29% of the original number, while that of the MD and SD groups remained low at 6% and 9%, respectively. However, their snails were also near the end of their natural life in the laboratory, and both factors could have contributed to the observed high mortality rates. This work followed that of Bohlken *et al.*, 1978 who found that under LDs

conditions *L. stagnalis* produced 10-20 times more eggs than either MD or SD groups, and towards the end of this 25-week experiment, again, mortality of LD snails increased markedly. Therefore, a high mortality rate compared to other experiments, even those involving invertebrate species, might be expected amongst pulmonte snails egg-laying during summer under long day conditions, and water temperatures (near) suitable for maximal reproduction. Further, although mortality (and the timing of it) is a natural part of any life cycle, environmental factors (like photoperiod) can influence this, particularly in pulmonate molluscs. A single paper by Ribi and Gebhardt, 1986, demonstrated a very much lower mortality rare (3.9%-4.5%) over the course of a 27 week long experiment with the caged prosobranch *V. ater* in two Swiss Lakes. Though not comparable to a laboratory experiment using a pulmonate mollusc, the large difference in mortality rates is likely due to the life cycle differences of these two different snail groups.

It is clear from this evidence that the environmental parameters to which snails, whether pulmonate or prosobranch, are exposed to will greatly affect their response both in terms of their development (including mortality), and reproduction. There is adequate information to suggest that both the reproductive pattern and output amongst the pulmonate snails could be greatly influenced by the semi natural conditions of our mesocosm experiments, though much less can be known about the response of the viviparids. The extent to which these parameters will differ compared to the laboratory values cited is difficult to know as there are large differences in the conditions to which the snails were exposed, and we know, for example, even changing a single parameter, such as water temperature, can have large effects upon reproduction. Therefore, a seasonal combination of changing environmental factors in river water exposed snails could have even larger effects upon the snails life cycle parameters, especially reproductive output as the literature suggests. In this chapter, I will therefore consider the "natural" seasonal cycle of *P. corneus* and *V. viviparus* in a semi-natural mesocosm set-up, and will relate this to environmental cues.

4.4 Results

4.4 Brief Materials and Materials

Over the course of two seasons (during 2003 and 2004), three experiments of 10-14 weeks duration were undertakenwhich, together, covered the entire reproductive and developmental season of snails from spring through to winter. Experiment 1 in 2003, was 12-weeks in duration from spring to mid summer, experiment 2 in the same year, lasted 10-weeks, from late summer into winter. Experiment 3 was the only experiment to be run in 2004, this experiment lasted 14-weeks, from late spring into early autumn. There was only a single weeks gap between experiments 2 and 3, although they were run in different years.

A detailed description of all three experiments, including detailed experimental design can be found in Chapter 2, "Methods and Materials". Briefly, in each of experiments 1 and 3, river water, 25%, 50% and 100% TSE were used as treatments (river water, 25% and 50% TSE in experiment 2); only the results of snail groups in river water are described in this chapter in order to establish the response of snails to river water. One tank was available for each treatment, six replicate groups of snails were used in each treatment, and each group or replicate contained between 9 and 11 snails; dependant upon on the numbers of snails available for each experiment. Baseline morphological measurements were taken at the beginning of each experiment (time zero), and at week 6 during (experiment 1 only), and again at the end of each experiment. Biological measurements, estimation of reproductive output; number of egg masses laid or number of embryos in pallial oviducts and babies released (experiment 3 only), were taken every 2-weeks. In each experiment, at the end of the baseline period (2-weeks) when all snails had been in river water only, TSE was introduced at time zero (mixed with river water diluent to obtain correct dilution of TSE), biological measurements were taken at this time, and every two weeks thereafter to the end of each experiment. In this chapter, the developmental and reproductive responses of different snail species to river water are described.

4.4.1 Seasonal changes in Daylength and Water Temperatures

Common to all field or semi-field experiments are the seasonal changes in day length and water temperature that occurred as each of my three experiments progressed. The text below describes these changes over the period during which my experiments took place, and Figure 4.1 illustrates these changes.

For illustrative purposes in my thesis, where this figure is displayed, water temperatures measured in river water and all treatment tanks (25%, 50%, and 100% effluent) were used to calculate the mean water temperatures displayed. River water temperatures measured are described in section 4.2 of this chapter, with temperatures in effluent dosed tanks described in section 5.2.1 of chapter 5.

Over the first month of experiment 1 in spring day length increased by 1 hour 23 minutes (from 14 hours 42 minutes to 16 hours 5 minutes; a 9.4% increase) during this period, water temperature remained relatively constant between 14.5°C- 14.7°C. During the next four 4 weeks of experiment 1, from the 25th May to early Summer, day length increased by only a further 35 minutes (3.6% increase) to a maximum on the 22nd June (16 hours and 37 minutes), water temperature increased by 5.7 °C (from 14.3 °C to 20.0 °C), and by 0.6 °C over the final two weeks of this period during experiment 3 (from 18.4 °C to 19.0 °C). Over the next calendar month, common to both experiments 1 (2003) and 3 (2004), from early towards mid Summer, from the 22nd June to 21st July, day length decreased by 42 minutes (4.2% decrease), and water temperature increased by a further 1.1 °C (experiment 1; from 20.0°C to 21.1 °C), and was remarkably similar in both years. In experiment 3, between 21st June and 19th July water temperature dropped slightly from 19.0 °C to 18.3°C.

The last 8 weeks of experiment 3 were autumnal in their nature. Day length on the 21st July was 15 hours 53 minutes and 12 hours and 45 minutes by the 13th September, representing a drop of 3 hours 8 minutes (19.8 %) in day length. During this period, water temperature did not decrease but varied between 19.4 °C and 21.4°C.

The 8 weeks of experiment 2 were autumnal in nature, however, this experiment ran until the14th November, day length on the 19th September was 12 hours and 6 minutes and 9 hours 0 minutes by the end of the experiment, representing a drop of 3 hours 6 minutes (25.8%) in day length. From the end of week 2 (3rd October), to the end of the experiment on the14th November 6 weeks` later, water temperatures fell sharply from 18.1 ℃ to 9.0 ℃, a decrease of 9.1 ℃.

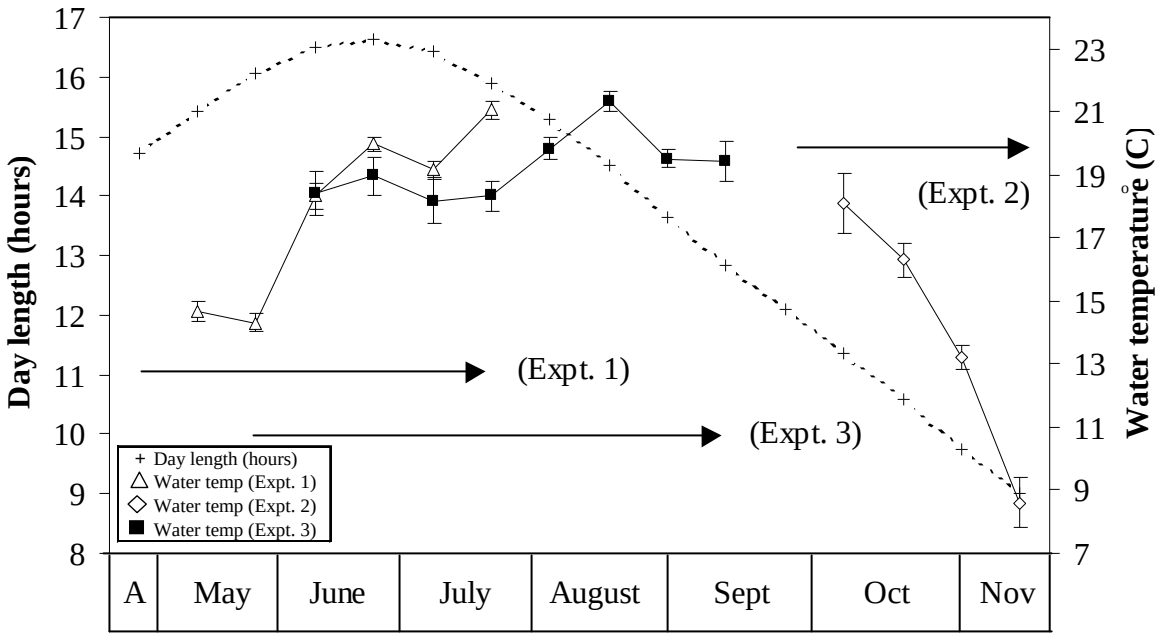


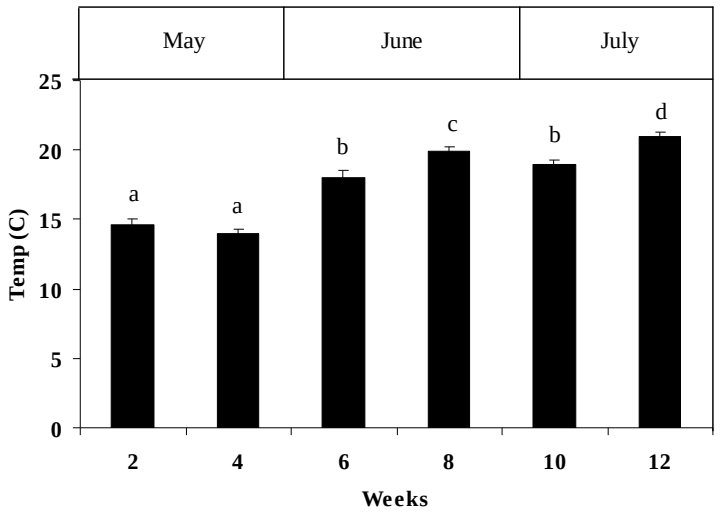
Figure 4.1 Change in day length and mean water temperatures (± SE) over the season during experiments 1, 2 and 3. Experiment 1, 14th April- 21st July 2003; Experiment 3, 25th May- 13th September 2004; Experiment 2; 19th September-14th November 2003.

4.4.2 Seasonal Water Temperature

Figure 4.2A-C shows the mean river water temperatures from experiments 1, 2, and 3.

During experiment 1 (spring-summer), there were no significant ($P>0.05$) differences in water temperature by week 4 (no temperature measurements were taken over the baseline period), there were significant ($P<0.05$) increases of water temperature over the following four weeks (to week 8), and further significant increases by the end of the experiment in mid-summer (Figure 4.2A). In experiment 2 (autumn-winter), water temperatures fell significantly ($P<0.05$) over the first six weeks of the experiment, but increased significantly over the final two weeks of the experiment (Figure 4.2B). In experiment 3 (early summer- autumn), overall, there were high summer water temperatures from the beginning to the end of the experiment; however, there were significant ($P<0.05$) differences in water temperature during the experiment (Figure 4.2C).

A)



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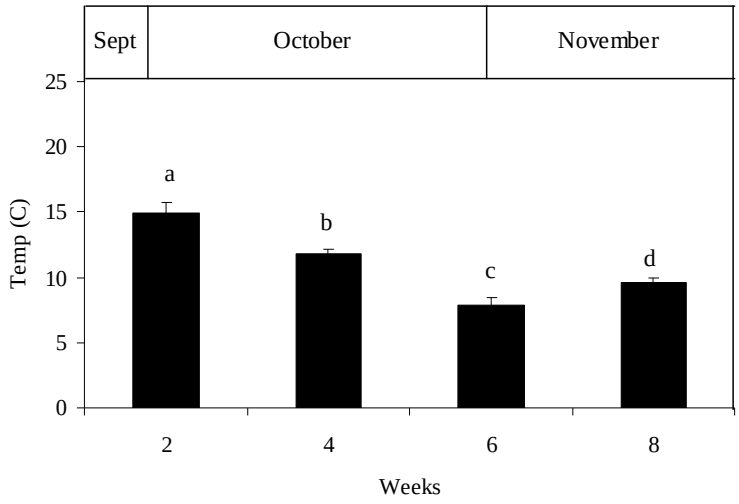


C)

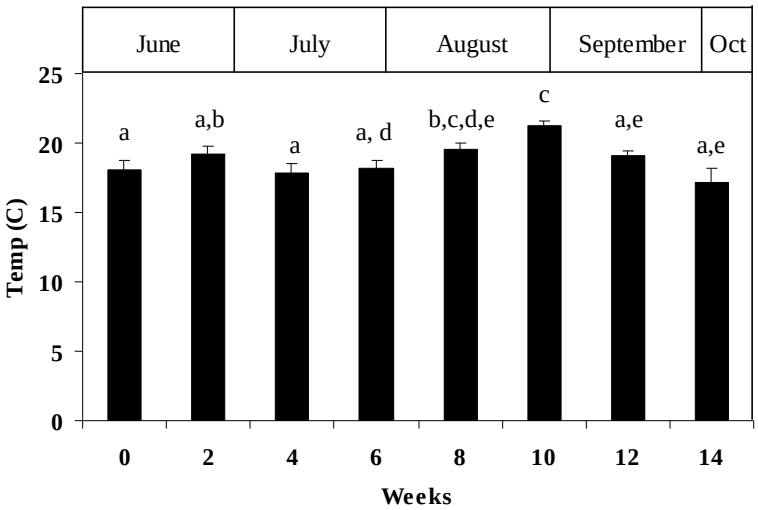


Figure 4.2.A-C Mean river water temperatures (± SE) over the course of the three experiments A) Experiment 1, 14th April- 21st July 2003; B) Experiment 2; 19th September-14th November 2003; C) Experiment 3, 25th May- 13th September 2004. Different letters indicate statistical differences between time periods $^{a,b,c,d,e}P< 0.05$.

4.5 Seasonal Effects of River Water upon the Mortality and Growth of *Planorbarius corneus*

A single period was found in spring when snails grew better than during the summer. The beginning of both experiments 1 and 3 was associated with initially higher mortality rates, which then decreased as both experiments progressed. Particularly high mortality in spring was associated with a rapid increase in reproduction (and water temperatures). However, during summer, when reproduction was higher still, the mortality rate was lower compared to the early weeks of the springtime experiment.

4.5.1 Mortality of snails in river water

Table 4.1 shows the percent mean survival from experiments 1, 2, and 3 of *Planorbarius corneus* maintained in river water. Figure 4.3 shows the seasonal mean % survival per enclosure per two weekly periods in each of the experiments.

There was an 8-week overlap in the timing of Experiment 1 (Spring to mid Summer 2003) and Experiment 3 (early Summer to early Autumn 2004) with a single weeks gap between Experiment 1 and Experiment 2 (Autumn into winter 2003), enabling a composite picture of seasonal mortality to be produced over a seven calendar month period. Mortality was continuous over the course of all experiments. Overall mean mortality in experiment 1 (65.2 % of the snails survived over 14 weeks) was almost equivalent to mean mortality in experiment 3 (55.6 % of the snails survived over 16 weeks). Although experiment 2 was shorter in duration (10 weeks), overall mean survival during this time was 88.6%, and mortality was considerably lower than in both experiments 1 and 3. In Experiment 1 half the snails were sampled after week 6, which results in an apparent increase in survival on week 8 (Fig. 4.3). During the first 6 weeks, mortality was greatest in snails placed in river water in late spring to early summer (58.0% survival) in experiment 1 and decreased in snails placed in river water in early Summer to Autumn (66.7% survival) in experiment 3, and Autumn to winter (92.7% survival) experiment 2 (Table 4.1).

Seasonally the highest mortality rates (calculated over a period of two weeks) were in Spring during May (experiment 1) with an average of 1 snail per enclosure perishing every 82 days. By contrast, mortality rates were lower in Summer during both experiments 1 and 3 with an average of 1 snail perishing every 583 days (Exp. 1) or 291 days (Exp. 3) by July. These mortality rates continued to decrease for the rest of Summer during both experiments, but increased substantially from Autumn into winter with an average of 1 snails per enclosure perishing every 172 days by the 14th November (Exp. 2).

Table 4.1 Percentage of snails remaining alive (+/-SE) in River Water (A) Experiment 1, 14th April- 21st July 2003, (B) Experiment 2, 3rd October-14th November 2003, (C) Experiment 3, 25th May- 13th September 2004. Weeks –2-0 was a 2-week baseline period prior to commencement of each experiment (effluent exposure). There was no baseline period prior to the commencement of experiment 2.

(A) Experiment 1- 2003

Date	28th April	11th May	25th May	8th June	*22nd June	*6th July	*21st July
Weeks	-2-0	0-2	2-4	4-6	6-8	8-10	10-12
River water	100+/-0.0%	76.9+/-10.0%	76.9+/-10.0%	58+/-10.9%	71.2+/-10.6%	68.2+/-9.5%	65.2+/-7.6%

(B) Experiment 2- 2003

Date	3rd Oct	17th Oct	31st Oct	14th Nov
Weeks	0-2	2-4	4-6	6-8
River water	100+/-0.0%	95.3+/-3.2%	92+/-2.9%	88.6+/-1.4%

(C) Experiment 3-2004

Date	8th June	22nd June	6th July	21st July	2nd August	16th August	30th August	13th Sept
Weeks	-2-0	0-2	2-4	4-6	6-8	8-10	10-12	12-14
River water	96.3+/-3.7%	85.2+/-6.2%	74.1+/-7.9%	66.7+/-12.5%	61.1+/-11.0%	61.1+/-11.0%	57.4+/-10.1%	55.6+/-9.5%

* 3 replicates per treatment remained after sampling on the 8th June 2003

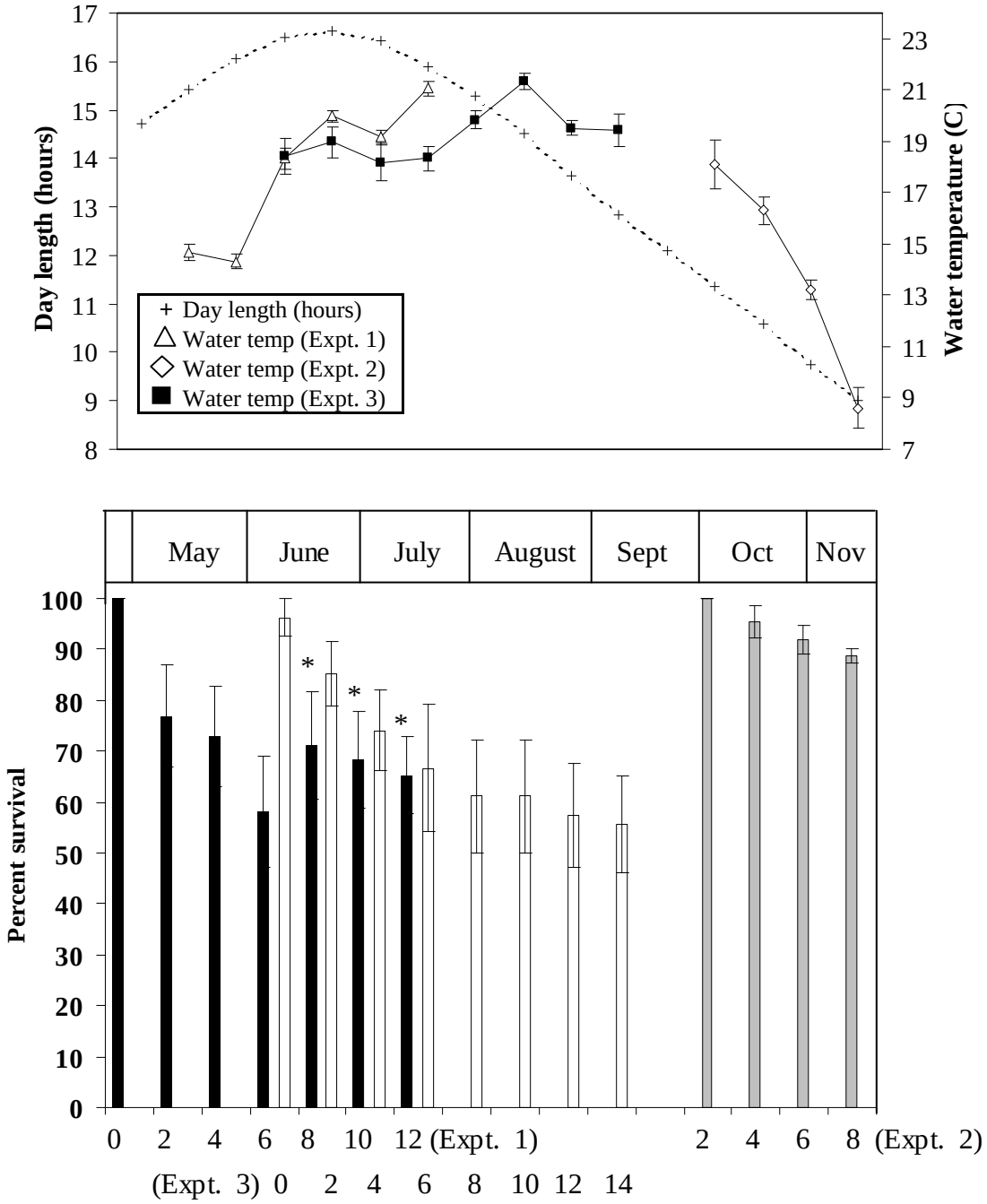


Figure 4.3 Seasonal mean number of surviving snails (± SE) per enclosure from groups of *P. corneus* snails in river water (Expt 1, 11 snails/enclosure; Expt 2, 10 snails/enclosure; Expt 3, 9 snails/enclosure). Experiment 1, 14th April- 21st July 2003 (black bars); Experiment 3, 25th May- 13th September 2004 (white bars); Experiment 2; 19th October-14th November 2003 (grey bars). * 3 replicates per treatment remained after sampling on week 6.

4.5.2 Seasonal Effects of River Water on Growth of *P. corneus*

Figure 4.4 shows the mean increase in A) shell length and B) weight (with shells) of the surviving *Planorbarius corneus* maintained in river water during experiments 1, 2, and 3.

Surviving snails grew well throughout the duration of both experiments 1 (Spring-mid Summer) and 3 (Summer-Autumn) and attained a mean shell length and weight of 25.2mm and 2.86 g (Expt. 1), and 23.1 mm and 2.49 g (Expt. 3) respectively (Fig. 4.4), which represents an overall increase of 32.5% and 102.8% (Expt. 1), and16.8% and 54.6% (Expt. 3) relative to their original lengths and weights following introduction into the experimental system, respectively. The surviving snails in experiment 2 (Autumn-Winter) grew to a moderate extent, and attained a mean shell length of 21.6 mm (an increase of 1.4mm) and weight of 1.99 g (an increase of 0.41 g), which represents an overall increase of 7.0% and 25.9 %. Further, the increase in snails' shell length and weight (with shells) between sampling points within each experiment through the seasons were significant ($P<0.05$).

During late spring to early summer (between 28th April and 8th June) there was a comparatively large (51.1%) increase in the mean weight of snails. The mean rate of weight increase during late spring to early Summer (28th April to 8th June) of 0.017g/day +/-0.0009 confirmed the high growth rates observed at this time relative to the 7.9% increase in shell length. From early to mid summer (between 8th June and 21st July) the increase in shell length (22.6%) and weight (34.3% increase) was equivalent. From early summer into Autumn (between 8th June and 13th September), the 54.6% increase in weight considerably exceeded the 16.6% increase in shell length; there was a 54.6% increase (weeks 0 to 14; from 1.61 g to 2.49 g) in the mean weight (with shells) of snails in Experiment 3.

From Autumn into Winter (between 19th September and 14th November) the increase in weight (25.9%) continued to exceed the increase in shell length (6.9%). During this period, increases in shell length and weight (with shells) indicated growth generally slowed from Autumn into winter. Indeed, from Autumn into winter the mean rate of weight increase fell to 0.007+/-0.001mg/day.

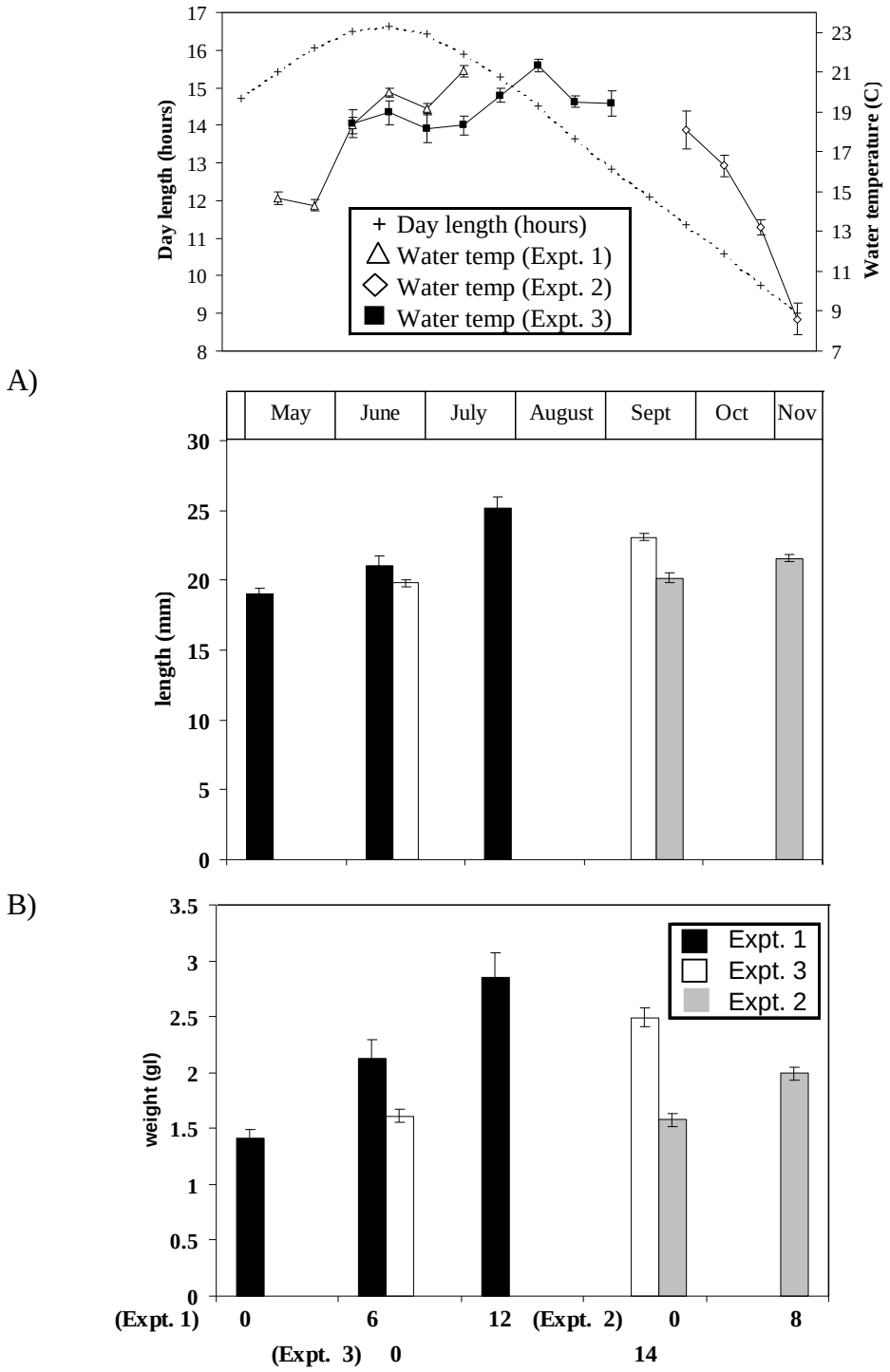


Figure 4.4 Seasonal mean increases in A) shell length (mm) and B) weight (g) (with shells) of snails (±SE) from groups of *P. corneus* snails in river water (Experiment 1; 11 snails, Experiment 2; 10 snails, Experiment 3; 9 snails). Spring to mid-Summer time (black bars); Experiment 1, 14th April- 21st July 2003, early Summer to Autumn (white bars); Experiment 3; 25th May- 13th September 2004, and Autumn to Winter (grey bars); Experiment 2; 19th September-14th November 2003.

Figure 4.5 shows the composite picture of the seasonal mean fold increase in A) snail shell length and B) weight (with shells) using data from experiments 1, 2, and 3. There were significant ($p>0.05$) differences in the baseline mean values of shell length between experiments 1 and 2 (longer), experiments 1 and 3 (longer), but not between experiments 2 and 3. There was no significant ($p>0.05$) difference in the baseline mean values of weight (with shells) of snails between experiments though snails were collected on three different occasions (from the wild) at different times of the season.

During Spring to early Summer between 28th April- 8th June there was a small but significant fold increase ($p<0.05$) in mean snail shell length and snail weight with shells compared to baseline values in river water. During early to mid Summer between 8th June- 21st July (comparison of week 6 and week 12 values) there was an apparently large but non-significant ($p= 0.18$) fold increase in mean snail shell length and a small non-significant ($p=0.51$) increase in the mean fold increase in weight of shelled snails, however, this was to a great extent due to poor growth of snails in a single enclosure. Without this enclosure included in the analysis, this difference in growth would likely have been a significant increase, however, due to the small number of enclosures remaining ($n=2$), this statistical analysis was not possible. Though the mean fold increase in snail shell length appeared greater during this period (than weight increase), this was due mainly to the comparatively fast growth of snails in a single enclosure that had been small at time zero, and therefore, gave a misleading impression of growth overall.

Comparison of the fold increase in mean shell length values between early to mid summer (8th June to 21st July) and early summer to Autumn (8th June to 13th September) revealed a significant decrease (Wilcoxon, $P<0.05$), though again this was largely due to the fast growth of snails a single enclosure in early to mid summer. There was, however, a small non-significant decrease ($p>0.05$) in the mean fold increase in the weight of snails over the same seasonal period.

During Autumn to Winter between 19th September to 14th November (comparison of week 0 to week 8) there was a further significant ($p<0.05$) decrease in both the mean

fold increase of snail shell lengths and weights compared to shell growth and weight increase from early summer to Autumn, snails grew comparatively less during this period.

The rate of increase of length (mm) indicated growth was greatest early to mid Summer (0.070 mm/day), and exceeded the rate of increase in length of measured between late spring and early Summer (0.056mm/day)(data not shown). The rate of increase of length in early Summer to Autumn (0.032mm/day) confirmed that growth decreased considerably compared to maximum rates observed, and decreased still further from Autumn into winter (0.025mm/day).

However, the rate of increase of weight (mg/day) was greatest between late spring to early Summer (0.017mg/day) and decreased considerably between early to mid Summer (0.010g/day), and still further between Summer to Autumn (between 8th June and 13th September) to 0.009mg/day. Between Autumn and winter (between 19th September and 14th November) growth rates reached their lowest at 0.007mg/day.

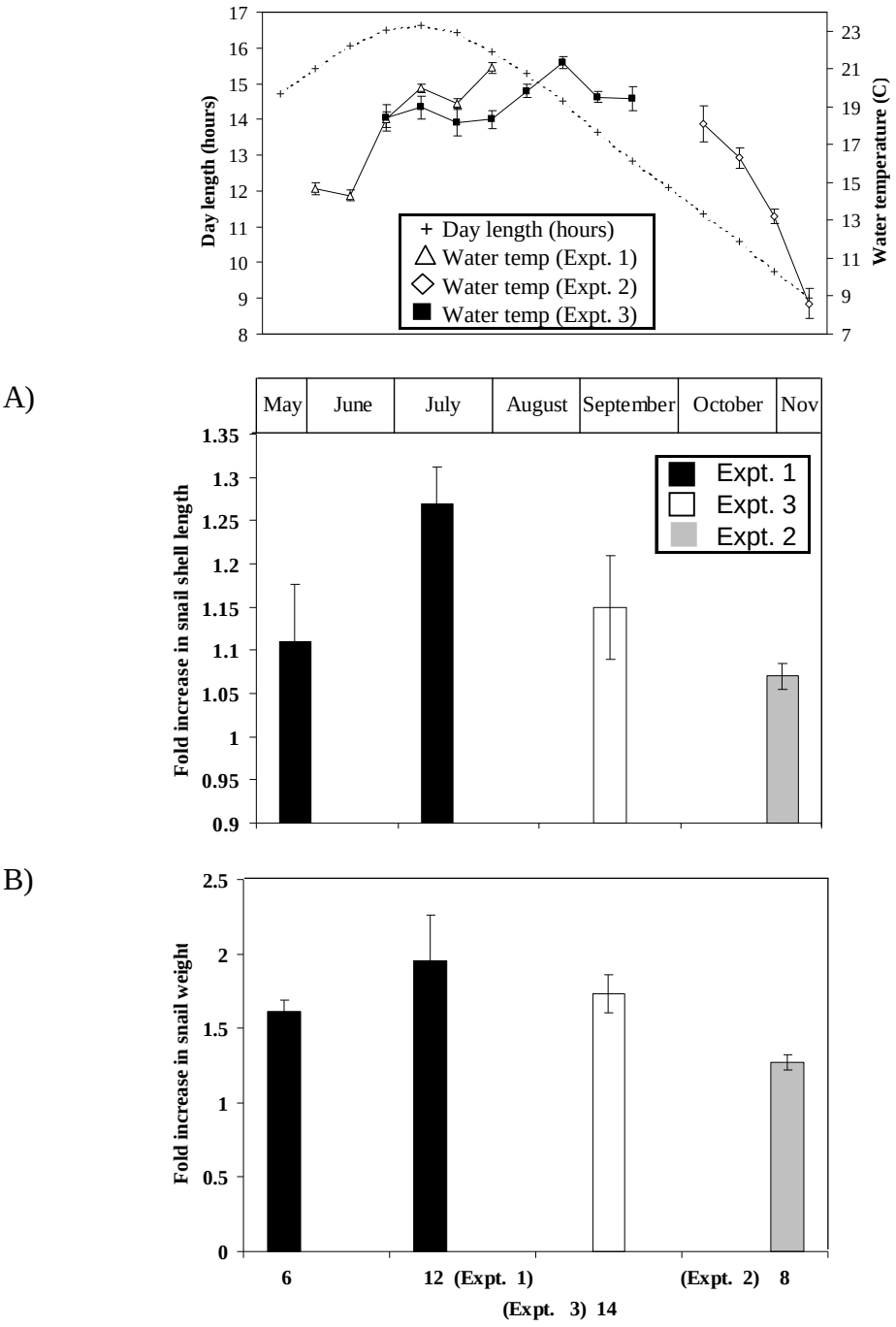


Figure 4.5 Seasonal mean fold increases in A) shell length (mm) and B) weight (g) (with shells) (±SE) from groups of *P. corneus* snails in river water (Experiment 1; 11 snails, Experiment 2; 10 snails, Experiment 3; 9 snails). In experiment 1, snails in each of 6 enclosures were measured at time zero when baseline measurements were taken, and also on week 6 (3 enclosures sampled) and on week 12 (when remaining 3 enclosures were sampled). Measurements were used to calculate mean fold increase values by comparison to baseline mean shell lengths for each enclosure. In experiments 2 and 3, all 6 enclosures were measured at the beginning and end of each experiment.

Figure 4.6 shows the seasonal increase in snail weight (without shells) during experiment 3, measured between early Summer and Autumn (8th June to 13th September).

There was a significant ($p<0.05$) 65.8% increase (from 0.81 g to 1.34 g) in the mean weight of unshelled snails during this period, compared to a 54.6% increase (from 1.61 g to 2.49 g) in the mean weight of the snails taken with their shells over the same period.

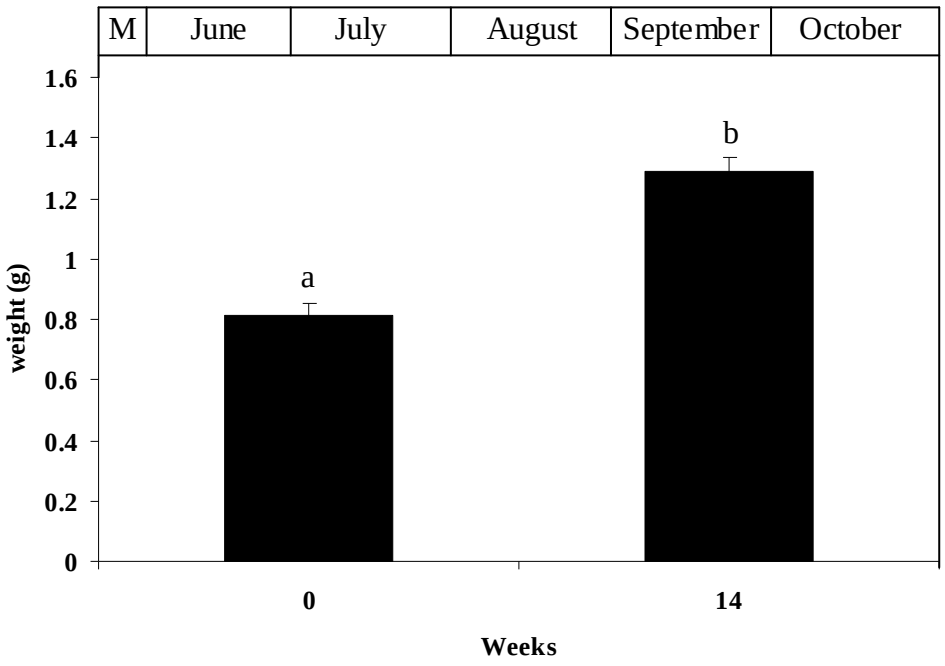


Figure 4.6 Seasonal increase in snail weight (without shells) (±SE) from groups (Experiment 3; 9 snails) of *P. corneus* snails in river water from early Summer to Autumn; 8th June- 13th September.

4.5.3 Seasonal Changes in Reproduction of *P. corneus* in River Water

The seasonal reproductive response of *P. corneus* exposed to river water was assessed over two consecutive years to establish the "normal pattern" of reproductive output. Two reproductive periods were found, between 25/5/03 and 2/8/04 a large reproductive peak when output was maximal, and a second smaller peak of reproduction between 3/9/03 and 17/9/03.

Figure 4.7 shows the combined seasonal trend in egg mass production from Experiments 1, 2 and 3 in *Planorbarius corneus* maintained in river water between May and November in 2-week intervals together with seasonal photoperiod and water temperature.

Overall, there was an increase in the mean number of egg masses laid per snail over the course of Spring-Summer, followed by a sharp decrease in Summer-Autumn, with a smaller reproductive peak during Autumn-Winter.

In springtime, there was a near doubling of the mean number of masses per snail from 3.5 to 6.0 egg masses per snail per fortnight between 28th April and 25th May. During the following two weeks to the 8th June, egg mass production was especially high, doubling again to 12.0 egg masses per snail per fortnight. From this date in early summer, to mid-summer on the 21st July, egg mass production was relatively stable at between 7.1 and 9.5 mean egg masses per snail per fortnight. The same pattern was seen between early to mid summer the following year, where egg mass production was equally as high, ranging between 7.3 and 10.5 mean egg masses per snail. Indeed mean egg mass production per snail during this common time period in both experiments was not statistically different ($p > 0.05$). From mid Summer into Autumn (13th September), mean egg mass production dropped sharply from 8.2 to 0.23 egg masses per snail every 2 weeks. However, in the two weeks between the 19th September and 3rd October in the Autumn of that year, egg mass production briefly increased again to 2.0, and 3.7 egg masses per snail on the 17th October, but egg mass production ceased thereafter as winter approached.

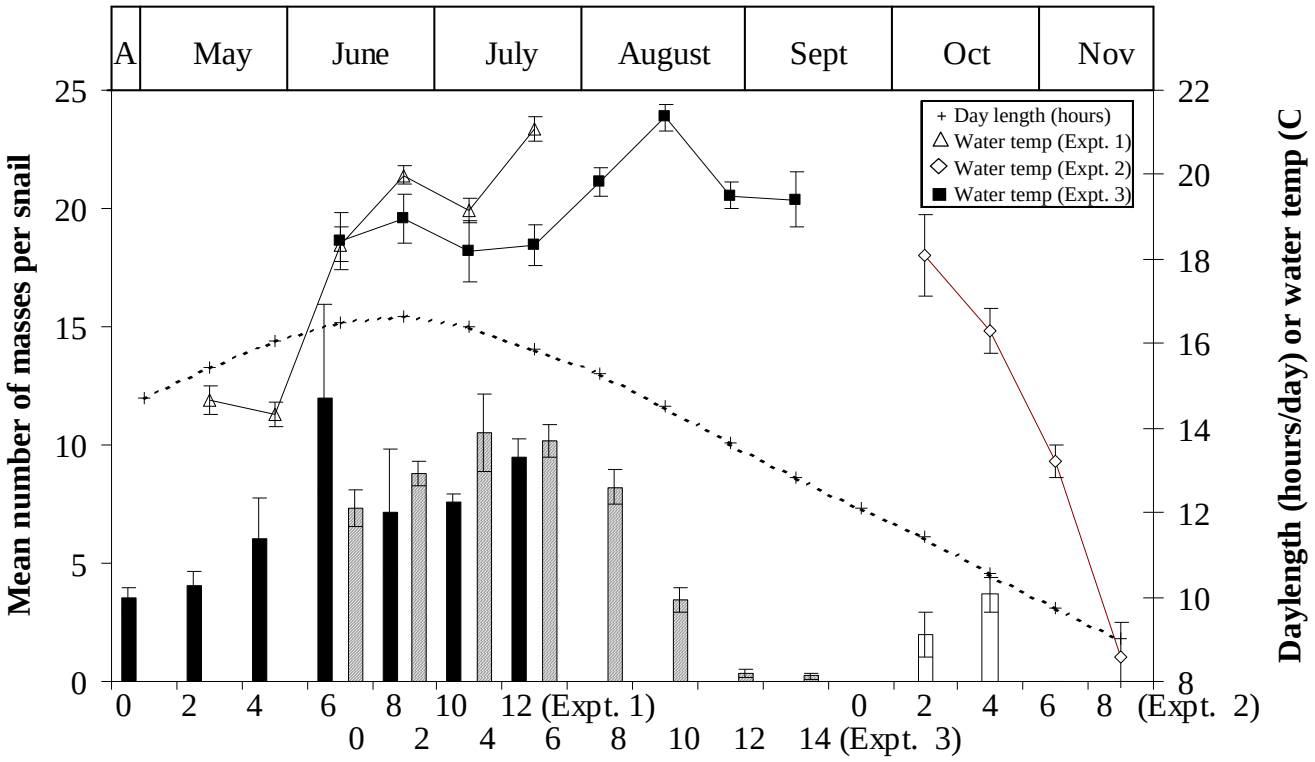


Figure 4.7 Mean number of egg masses per snail (+/- SE) produced by groups of *P. corneus* snails in river water (Experiment 1; 11 snails (6 groups 0-6 weeks; 3 groups 8-12 weeks), Experiment 2; 10 snails (6 groups in Expt 2 and 3), Experiment 3; 9 snails). Day length and tank water temperature (+/- SE) are plotted on all graphs to illustrate change in season. Spring to mid-Summer time (black bars); Experiment 1, 14th April- 21st July 2003, early Summer to Autumn (black and white stripped bars); Experiment 3; 25th May- 13th September 2004, and Autumn to Winter (white bars); Experiment 2; 19th September-14th November 2003.

Figure 4.8 shows the combined seasonal trend in eggs per mass from Experiments 1, 2 and 3 in *P. corneus* maintained in river water between May and November in 2-week intervals.

Concomitant with the increased number of masses, there was also an increase in the mean number of eggs per mass between the 28th April and 21st July from an initial 27 to a peak of 68 (2.5 times more) from spring into mid-summer 2003. The mean number of eggs per mass peaked one month earlier in 2004 at 63 eggs/mass on the 22nd June, the longest day of summer. Comparison between the two experiments revealed that the mean number of eggs per mass on corresponding dates in 2003 and 2004 were statistically very similar ($p=1.0$), giving a mean during this common mid-summer period of 55 eggs/mass (2.0 times more than initial values). From the peak in July 2004, the mean number of eggs/mass fell by 10 eggs/mass but then remained constant into Autumn (13th September) at 53 eggs/mass (1.9 times greater than the initial value of 27 on the 28th April 2003.

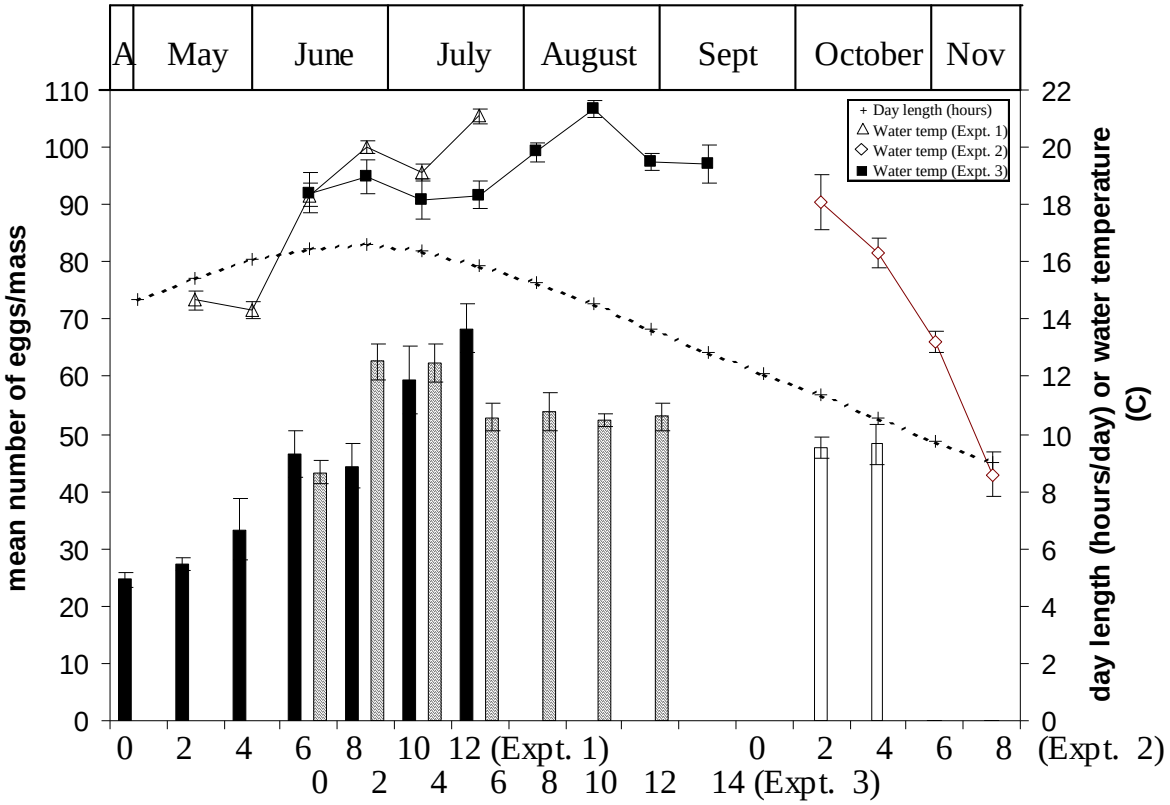


Figure 4.8 Mean number of eggs/mass (± SE) produced by groups of *P. corneus* snails in river water. The eggs were counted from the masses presented in Figure 4.7. Day length and tank water temperature (+/- SE) are plotted on all graphs to illustrate change in season. Spring to mid-Summer time (black bars); Experiment 1, 14th April-21st July 2003, early Summer to Autumn (black and white stripped bars); Experiment 3; 25th May- 13th September 2004, and Autumn to Winter (white bars); Experiment 2; 19th September-14th November 2003.

Figure 4.9 shows the combined seasonal trend in the mean weight of egg masses from Experiments 1, 2 and 3 in *P. corneus* maintained in river water between May and November in 2-week intervals.

There was a steady increase in the mean weight of individual egg masses in spring on the 28th April 2003 to mid-summer on the 21st July 2003 reaching a peak of 0.1480g. This represented a 22% increase over a period of 6 weeks from early summer on the 8th June (from 0.1213g). On the equivalent date, the 8th June of the following year, the mean weight of individual egg masses was 0.1209g and nearly identical. However, within 6 weeks from the start of the experiment, the mean individual egg mass weight had increased to 0.1739 g (43.8% increase), peaking 2 weeks earlier than the previous year on the 6th July. From this peak, the mean weight of individual egg masses fell steadily to 0.062 g (64.3 % weight decrease from 6th July) over a period of 10 weeks into Autumn on the 13th September.

However, during the short second reproductive peak in Autumn, the mean individual weight of egg masses was 0.196 g and 0.198 g; values similar but increased compared to those of the summer peak.

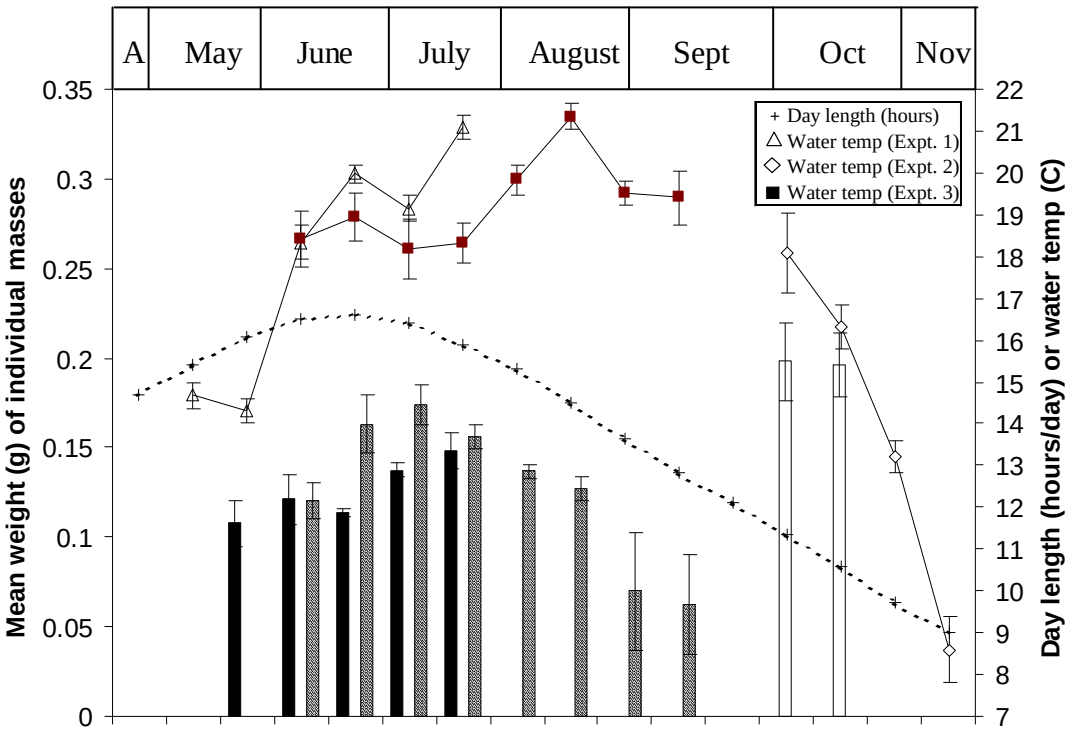


Figure 4.9 Mean weight of individual eggs masses (± SE) produced by groups of *P. corneus* snails in river water showing day length and tank water temperature (± SE). The egg masses weighed were from the masses collected for Figure 3.8. Experiment 1, 14th April- 21st July 2003, Experiment 2, 19th September-31st- 14th November 2003, Experiment 3, 24th May- 13th September 2004. Data from weeks –2-0 and 0-2 of Experiment 1 was lost due to degradation of egg masses whilst in storage.

4.5.3.1 The Effects of Methodology on the ability of eggs to hatch

Table 4.2A and B illustrates the hatchability of eggs from egg masses collected from each of the enclosures during Experiments 1 and 3.

Although not tested statistically (as only a single figure of hatchability was produced from each enclosure or replicate group of snails) the data appears to show no clear pattern to egg hatchability dependant on the time taken to process and distribute egg masses into cell culture plates from universals during either experiment 1 or 3.

During Experiment 1, hatching decreased from 80.7% to 49.0% during this process (weeks 4 to 6), and from 87.5% to 60.8% (weeks 6 to 8) by the time the final replicate group of masses had been processed (Table 4.2A). However, eggs in egg masses hatched as well on the first day they were processed and distributed into cell culture plates (which could be up to approximately 5 days later for the final replicate), as on the last occasion this procedure was followed during other time periods, for example, masses collected at the end of week 4 (Table 4.2A).

Though not tested statistically, there appeared to be less variability (always high hatchability rates), due to time taken to process and distribute egg masses, in the ability of eggs in egg masses to hatch during experiment 3 (Table 4.2B). The methodologies used to hatch eggs from egg masses can be found in sections 3.3.1-3.3.2; a single methodological approach was used to hatch eggs from egg masses during experiment 3.

During Experiment 1, eggs hatched significantly less ($P<0.03$) during weeks 4-6 than during weeks 2-4, though there were indications they may also have hatched less during weeks 6-8 ($P=0.1$), 8-10 ($P=0.05$), and 10-12 ($P=0.05$) than during weeks 2-4 (Table 4.2A). During experiment 3, there were no significant ($P>0.05$) differences in the hatching of eggs in egg masses between any of the time period (Table 4.2B).

Tables 4.2A and B Percentage mean hatchability of eggs in egg masses from each enclosure (or replicate) over the time taken to process and distribute egg masses into cell culture plates during A) Experiment 1 and B) Experiment 3. Egg masses were collected from groups of *P. corneus* in river water. (Experiment 1; 11 snails (6 groups 0-6 weeks; 3 groups 8-12 weeks), Experiment 3; 9 snails). A) Experiment 1 – 14th April to 21st July 2003. B) Experiment 3, 24th May- 13th September 2004.

A)

	Enclosure:					
	1st	2nd	3rd	4th	5th	mean
			% Hatch:			
Weeks:						
4	85.6%	99.7%	90.9%	92.7%	92.7%	92.3%[a]
6	80.7%	36.3%	49%			55.3%[b]
8	87.5%	86.5%	60.8%			78.3%[a,b]
10	79.4%	89.2%	82%			83.5%[a,b]
12	66.8%	53.5%	88.3%			69.5%[a,b]

Different letters indicate between time period statistical differences [a,b]$P < 0.05$.

B)

	Enclosure:						
	1st	2nd	3rd	4th	5th	6th	mean
			% Hatch:				
Weeks:							
0	97.4%	98.4%	96.1%	100%	99.8%	90.6%	97.1%
2	100%	99.1%	99.3%	100%	98.7%	99.8%	99.5%
14	100%	97.9%	97.8%				98.6%

Different letters indicate between time period statistical differences [a,b]$P < 0.05$.

4.6 Seasonal Changes in Growth and Mortality of *Viviparus viviparus* in River Water

A single period of growth was found, from mid to late Summer which was also associated with medium mortality rates common to both spring and summer and increased fecundity (production of new embryos in the brood pouch). Snails most probably grew in springtime also as there was no weight loss following the release of mature embryos, carried over from the previous season. Towards autumn, snails lost weight and mortality rates increased greatly.

4.6.1 Seasonal Mortality of *V. viviparus* in River Water

Table 4.3 shows the percent survival from Experiments 1 and 3 in *V. viviparus* maintained in river water during both experiments. Mortality was continuous over the course of both Experiment 1 (Spring-Summer) and Experiment 3 (Summer-Autumn) (Table 4.5). From the 21st July 2004 mid-Summer onwards, snail mortality rate increased towards Autumn. Overall mean mortality in Experiment 3 was more than twice (x2.45) mean mortality in experiment 1; 71.2 % of the snails survived in experiment 1 compared with 28.8% in experiment 3. In Experiment 3, over half (57.6%) the snails died between mid Summer and Autumn, when mortality was at its highest.

Table 4.3 Percentage of snails remaining alive in River Water. (A) Experiment 1, 14th April- 21st July 2003, (B) Experiment 3, 14th June- 6th September 2004.

A) Experiment 1- 2003

Date	28th April	8th June	21st July
Weeks	-2-0	0-6	6-12
R/w	100+/-0.0%	85.1+/-3.3%	71.0+/-10.1%

B) Experiment 3-2004

Date	14th June	28th June	12th July	26th July	9th Aug	23rd Aug	6th Sept
Weeks	-2-0	0-2	2-4	4-6	6-8	8-10	10-12
R/w	98.5+/-1.5%	97.0+/-1.9%	92.0+/-1.5%	86.4+/-3.9%	65.2+/-7.6%	54.5+/-7.8%	28.8+/-3.7%

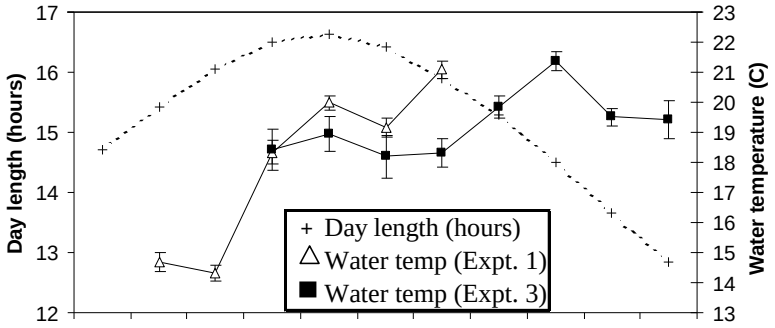
4.6.2 Seasonal Effects of River Water on Growth of *V. viviparus*

Figure 4.10 shows the seasonal increase in A) mean shell length and B) weight (with shells) of *V. viviparus* maintained in river water from Experiments 1 and 3. All surviving snails in river water were measured at each sampling point and were used for analyses of the increase in snails' shell length or change in snails' weight (with shells).

The surviving snails in Experiment 1 (Spring to mid-Summer) grew well and increased their mean lengths by 2.7mm (10.5%), and reached a final weight of 5.92 g, which represents a non-significant ($p>0.05$) increase of 17.9% relative to their original mean weights following introduction into the experimental system. From early Summer into Autumn (between 15th June and 6th September) growth was negative, there was a non-significant ($p>0.05$) –2.1% decrease (weeks 0 to 12; from 33.0mm to 32.1mm) in the mean length of snails, and a significant ($p<0.0001$) 10.5% decrease (from 8.7 g to 7.8 g) in the mean weight of shelled snails over this period (Expt. 3).

During late Spring to early Summer (between 28th April and 8th June) growth was slow, resulting in a non-significant ($p>0.05$) 3.8% increase (weeks 0 to 6; from 25.6mm to 26.6mm) in the mean length, and non-significant ($p>0.05$) 0.2% increase in the mean weight (with shells)(from 5.02 to 5.03 g) of snails. From early to mid Summer (between 8th June and 21st July) growth rate increased, resulting in a further non-significant ($p=0.15$) 6.5% increase (weeks 6 to 12; from 26.6mm to 28.3mm) in the mean length, and 17.7% increase (from 5.03g -5.92 g) in the mean weight (with shells) of snails.

A)



B)

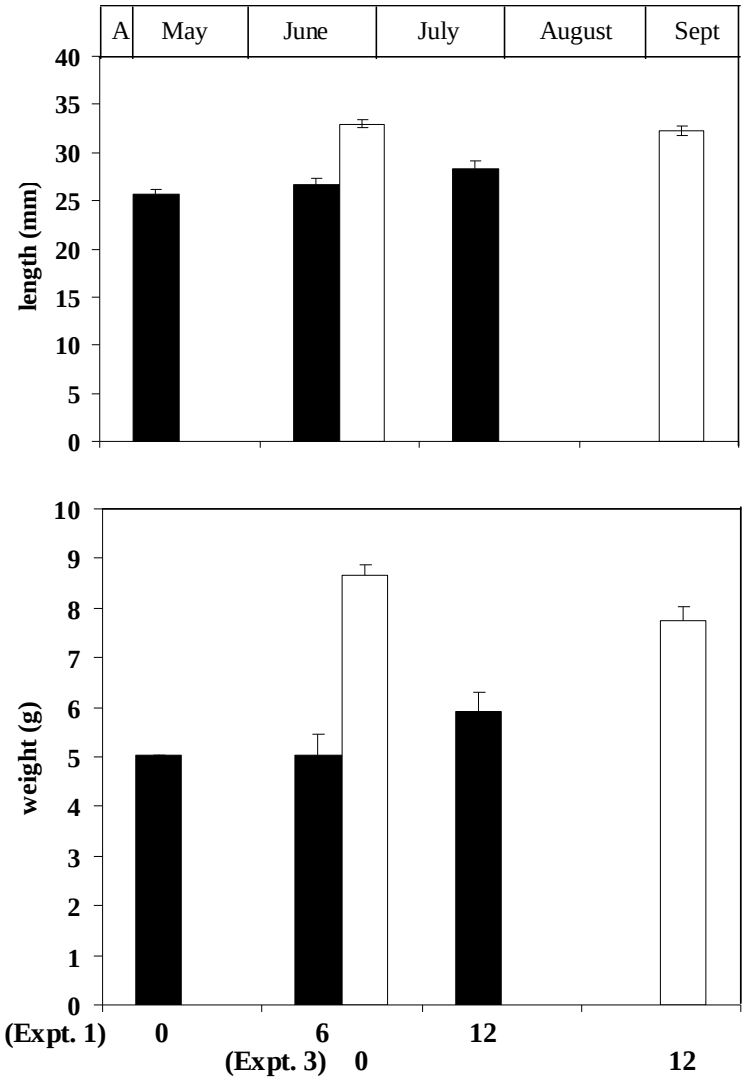


Figure 4.10 Seasonal mean increases in A) mean shell length and B) weight (with shells) of snails (±SE) from groups of *V. viviparus* snails in river water (Experiment 1 and 3; 11 snails per group). Spring to mid-Summer time (black bars); Experiment 1, 14th April- 21st July 2003; early Summer to Autumn (white bars); Experiment 3; 1st June- 6th September 2004.

Seasonal changes in snail shell length and weight (with shells) were analysed using individual enclosure means. The mean shell lengths and weights (with shells) of all snails was established in all enclosures during baseline measurements, and all snails in the appropriate enclosures were measured again at subsequent sampling points to calculate the fold increase in snail shell length or weight (with shells) per enclosure. These values were then averaged across enclosures. There was a significant ($p<0.05$) difference in the baseline mean values of length of snails between experiments; where snails in Experiment 3 were larger than those in Experiment 1. However, there was no significant ($p>0.05$) difference in the baseline mean values of weight (with shells) of snails between experiments.

Overall from Spring into mid-Summer of Experiment 1 (between 28th April and 21st July) there was a significant ($p<0.05$) 17% increase in the mean shell lengths, and 30.0% increase in weight of shelled snails averaged across all enclosures over Experiment 1. From early Summer into Autumn of experiment 3 (between 1st June and 6th September) there was a small but significant ($p<0.05$) –2.3% fall in shell lengths across the enclosures, and a non significant ($p>0.05$) - 4 % decrease in the weight of shelled snails (data not shown).

Snails without shells were weighed at the beginning and end of Experiment 3. From early Summer into Autumn between (15th June to 6th September) there was a non-significant ($p>0.05$) 3.2% decrease (weeks 0 to 12; from 2.47 g to 2.39 g) in the mean weight of unshelled snails (data not shown).

Snails without shells or embryos were also weighed at the beginning and end of experiment 3 alone. In Experiment 3, mean weight without shells or embryos of snails was 1.69 g by Autumn on the 6th September 2004, which was 41.4% less than the mean weight of unshelled snails over the same period (2.39 g). Therefore, contents of the pallial oviduct (i.e. shelled and unshelled embryos) constituted 29.3% of the total weight at this time (data not shown).

4.6.3 Mean shell lengths of Deceased *V. viviparus*

Figure 4.11 shows the mean shell lengths of deceased snails from *V. viviparus* snails maintained in river water during Experiment 3. Deceased snail shells were collected at each sampling point from all enclosures and were used for analyses. The increase or decrease in mean shell length of dead snails was obtained by comparison to the mean value obtained for weeks 2 to 4; no dead shells were collected before this time period, therefore, no data is available for weeks 0 to 2.

There was a 3.2% increase in the mean length of dead snail shells by summer (between 13th July and 27th July; weeks 4 to 6) from 34.6 mm to 35.7 mm. Thereafter, there were successive changes in the mean shell length of snail mortalities of –9.2%, +2.7%, and -1.8% during weeks 6-8, 8-10, and 10-12, respectively. There appeared to be no clear trend in the length of shells from mid-summer (13th July) towards Autumn (6th September).

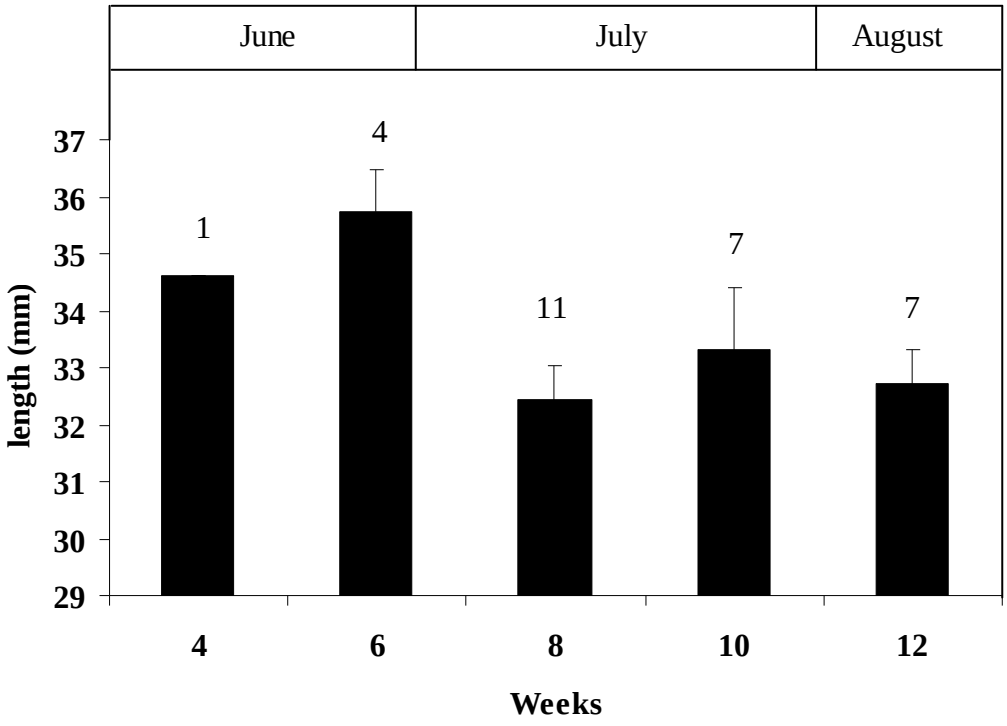


Figure 4.11 Mean shell lengths of deceased *V. viviparus* (±SE) collected from enclosures in Expt 3. Weeks 4 to 12; 13th July- 6th September 2004. Numbers above bars represent the number of deceased *V. viviparus* over the previous two-week period (N=11 snails per enclosure). Numbers above bars represents the number of dead shells collected from all enclosures at the end of each two weekly period; and was used to calculate the mean for that time period.

4.6.4 Seasonal Reproduction

There was an overlap in the sampling between experiments 1 (2003) and 3 (2004) that enabled a composite picture of both fecundity and seasonal reproduction to be produced (despite slightly different sample days) over a 5-month period. Statistical analyses were carried out data permitting. Where too few replicate points were available no statistics are cited.

Figure 4.12 shows the combined seasonal trend in unshelled (new), shelled (oldest), and total embryo production from experiments 1 and 3 in *V. viviparus* maintained in river water between April and September in approximate 6-week intervals.

In Experiment 1, there was a 0.92-fold decrease in the mean total number of embryos present in the brood pouches of female snails (from 15.8 to 14.6 embryos per snail) between 28th April and 8th June (spring to early Summer). During this time, there was a 1.67-fold increase of the mean number of unshelled embryos per snail from 5.2 to 8.7 and a 0.55-fold decrease of the mean number of shelled embryos per snail from 10.7 to 5.9. Day length increased by 1 hour 48 minutes (from 14 hours 42 minutes to 16 hours 30 minutes; 12.2% increase) and water temperature also increased from 14.7 C-18.3 ^{o}C (Figure 3.15). Maximum day length occurred on the 22nd June (16 hours and 37 minutes) two weeks later. A further four weeks later mid Summer (week 12; 21st July) mean total embryo production per snail was significantly higher, increasing 2.43-fold. At this time, there was a statistically non-significant 0.69-fold fall ($P>0.05$) in the mean number of shelled embryos to a seasonal low of 4.1. In contrast, the mean number of unshelled embryos per snail increased 3.6-fold ($P<0.05$) to 31.4 embryos per snail. Day length decreased by 45 minutes during this time, whilst water temperature increased further from 18.3 ^{o}C to 21.1 ^{o}C. The final reproductive sampling point was week 12 of experiment 3, which was approximately 8 calendar weeks later than the end of Expt 1 in early Autumn on the 6th September. This final sampling point was autumnal in nature. During this time, mean total embryo production was 0.43-fold lower ($P<0.05$) than it was in mid Summer of the previous year (Experiment 1). The mean number of unshelled embryos per snail was significantly ($P<0.05$) lower (31.4 to 5.4 embryos per snail) than mid Summer,

whereas there was a non-significant (P=0.06) increase in the mean number of shelled embryos per snail from 4.1 to 10.0 (a 2.45-fold increase compared to mid Summer of Experiment 1. Day length on the 21st July (Exp 1; week 10) was 15 hours 53 minutes and 12 hours and 45 minutes by the end of Experiment 3, representing a decrease in day length of 3 hours 8 minutes in day length. Water temperature on the 21st July was 18.3 ^{o}C and rose to 19.4 ^{o}C (a rise of 1.1 ^{o}C) by 13th September at the end of Experiment 3.

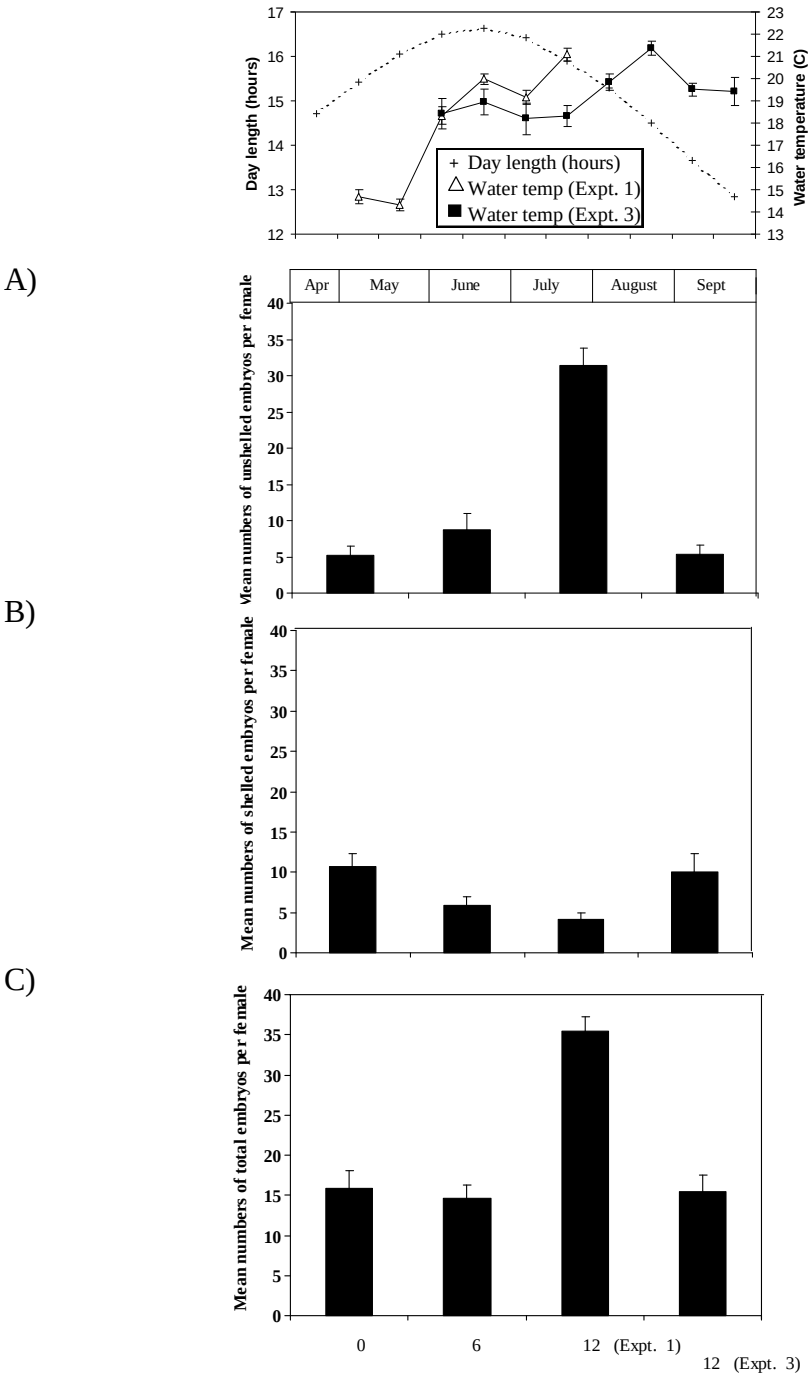


Figure 4.12 Mean number of A) Unshelled, B) Shelled, C) Total embryos in the brood pouches of female *V. viviparus* snails (+/- SE) produced throughout the season in river water (Experiment 1 and 3; 11 snails per group). Experiment 1, 14th April- 21st July 2003; 3 sampling points, Experiment 3, 1st June- 6th September 2004; single sampling point. In experiment 1, a group (n=13) of acclimated snails were sampled at time zero when baseline measurements were taken. Snails in each of 3 enclosures were sampled on week 6, and on week12 the 3 remaining enclosures were sampled. In experiment 3, snails in each of the 6 remaining enclosures were sampled.

Figure 4.13 shows the seasonal trend in the A) mean number and B) weight of mature embryos released during experiment 3 by *V. viviparus* maintained in river water.

Overall, there was a 7.8-fold decrease (from 5.7 to 0.75) in the mean number of mature embryos released per snail over the season from early Summer to Autumn between the 1st June and 6th September (Figure 3.14A). There was a rapid fall in the mean number of babies released per snail over two weekly periods from 5.7 +/-0.55 SEM (week –2-0) in Spring to a mid Summer low of 0.25+/- 0.09 SEM (week 6-8) on the 9th August. After this time, there was a 2.9-fold increase (to 0.72 +/-0.36 SEM) in the mean number of babies released per snail during weeks 8-10, and identical mean numbers released over the final two weeks of experiment 3 (mid-Summer to Autumn).

Overall there was a 3.5-fold decrease (from 0.060 g +/-0.001 SEM to 0.017 g +/-0.008 SEM g) in the mean weight of baby snails released from female *V. viviparus* over the season from the 1st June to 6th September from early Summer into Autumn (Figure 3.15B). The weight of baby snails between early and mid-Summer between 15th June and 27th July varied, but decreased significantly compared to baseline values as Autumn approached. From mid-Summer into Autumn (between 27th July and 6th September) there were successive decreases in the mean weight of baby snails collected in river water, which were significantly lighter in August and September than baby snails born in June and July.

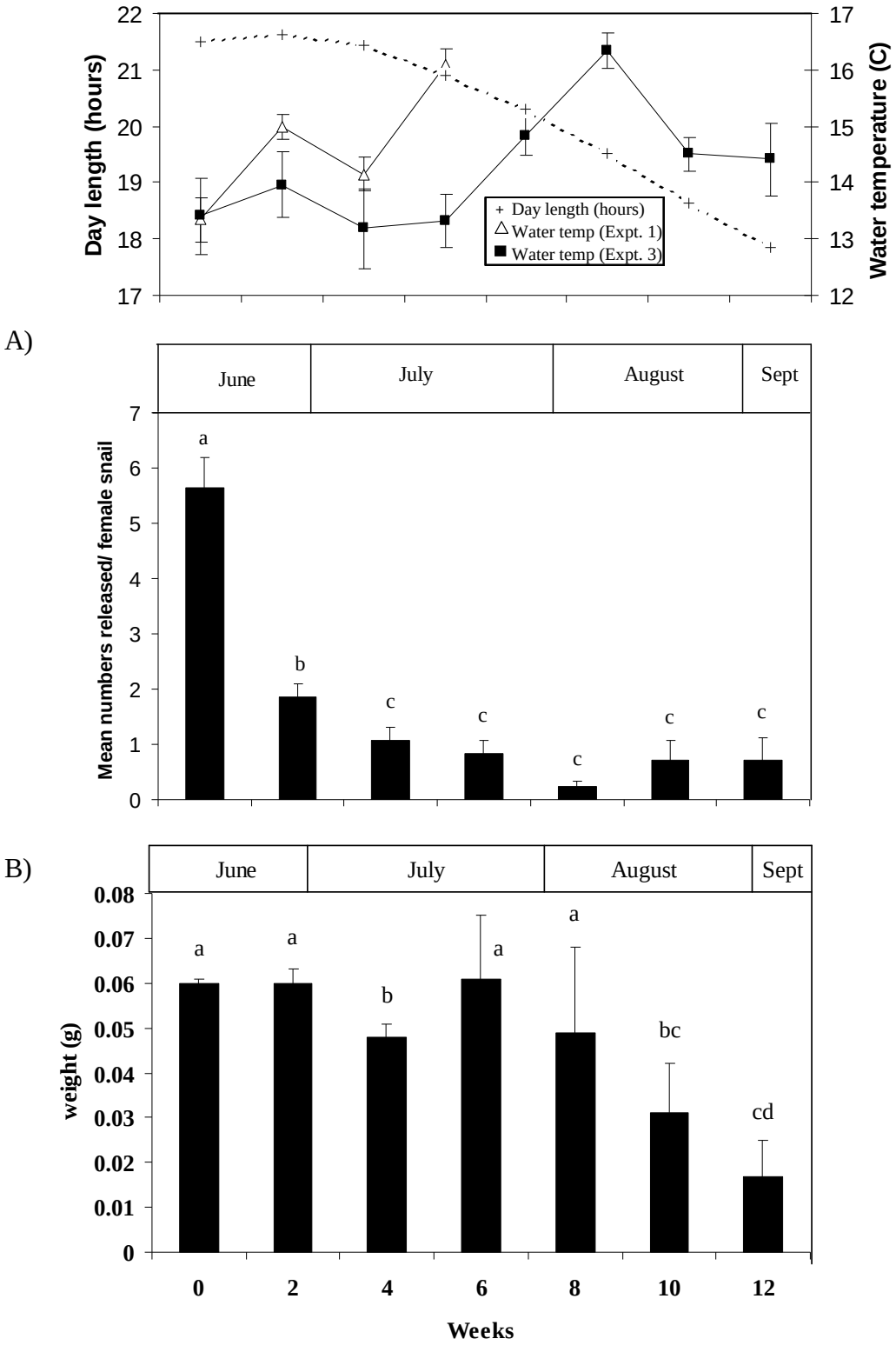


Figure 4.13 A) Mean number and B) weight of mature embryos released per female snail (+/- SE) produced by groups (Experiment 3; 11 snails per group) of *V. viviparus* snails in river water. Experiment 3, 1st June- 6th September 2004; sampled every two weeks.

Figure 4.14 shows the combined seasonal trend in number of mature embryos released by female snails, and new embryo production from experiments 1 and 3 in *V. viviparus* maintained in river water between April and September.

Clearly, the peak in the number of mature embryos released coincided to some degree with the peak in day length. However, as the first recorded observation of the number of mature embryos released was not until early summer, on the 15th June (maximum day length 21st June), we cannot predict with any certainty when numbers released began to rise or where they peaked.

The seasonal peak in the number of unshelled embryos in the pallial oviducts of female snails appeared to be around the 21st July, although destructive sampling allowed only a single sampling of snails. Nevertheless, this peak appears to be approximately one month after the longest day of the season, and perhaps more importantly, the rate of new embryo production increases rapidly just after the peak of mature embryo release. This graph illustrates that new embryo production is most likely responsible for the replacement of mature embryos that have been released.

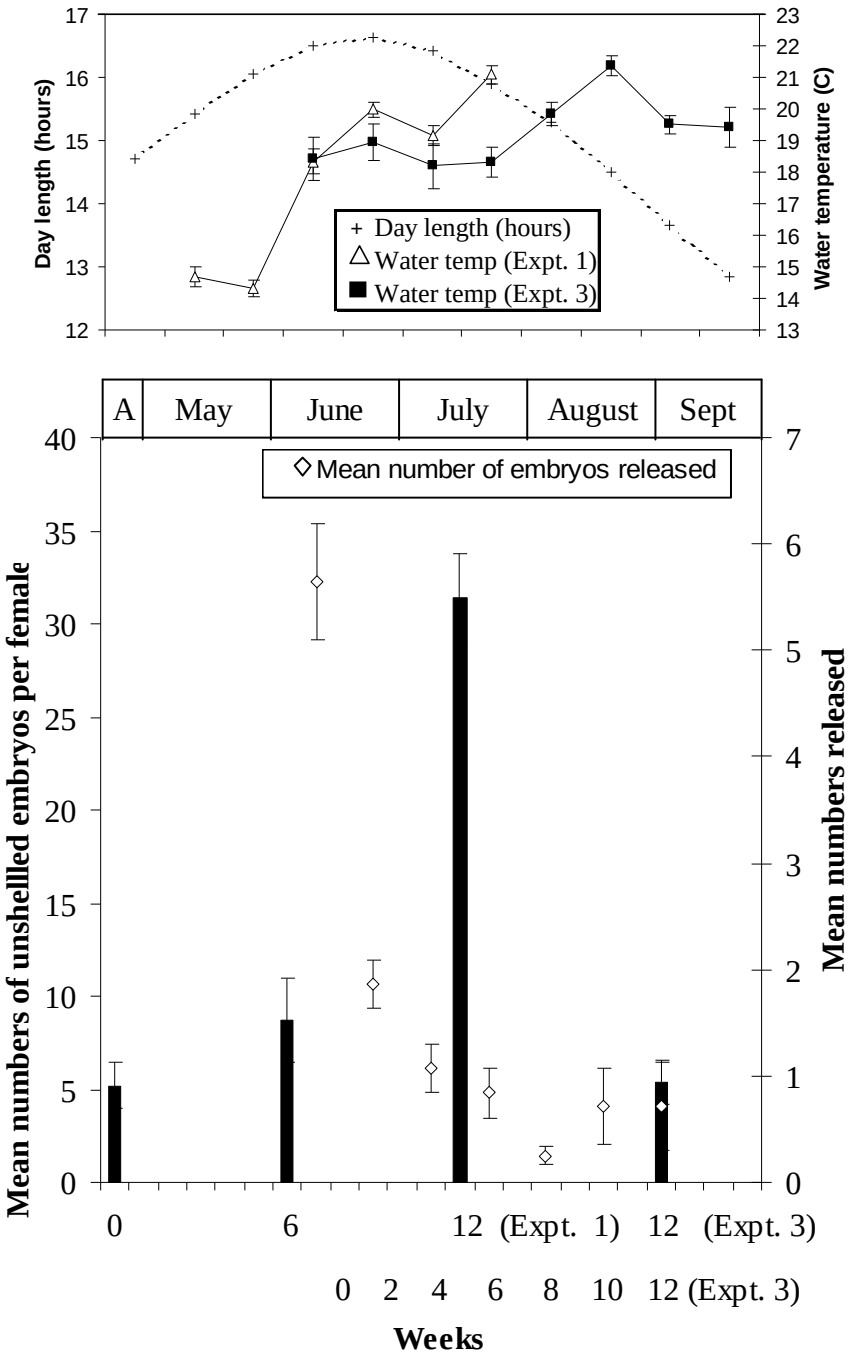


Figure 4.14 Mean number of mature embryos released per female snail (+/- SE) measured in 2-week intervals, and mean number of unshelled embryos (black bars) in the brood pouches of female *V. viviparus* snails (+/- SE) (Experiment 1 and 3; 11 snails per group) in river water. Experiment 1, 14th April- 21st July 2003; New embryos; 3 sampling points, Experiment 3, 1st June- 6th September 2004; single sampling point; Mature embryos released; Experiment 3, 1st June- 6th September 2004; sampled every two weeks.

Figure 4.15 shows the combined seasonal trend in the mean weight of shelled embryos within brood pouches of female *V. viviparus* maintained in river water from experiments 1 and 3.

Overall there was a 1.2-fold increase in the mean weight of shelled embryos sampled from brood pouches from 28th April 2003 to 6th September 2004. From Spring to early Summer (between 28th April and 8th June) the mean weight of shelled embryos within brood pouches of female snails did not change (0.024 g), between early to mid-summer from 8th June to 21st July there was a non significant ($p>0.05$) 0.9-fold decrease (6 weeks; 0.024 g to 0.021 g) in the mean weight of shelled embryos within brood pouches. From this seasonal low in the mean weight of shelled embryos, from mid-Summer into Autumn between 21st July and 6th September there was a non significant ($p>0.05$) 1.4-fold increase (approximate 12-weeks; 0.021 g to 0.029 g) in the mean weight of shelled embryos within brood pouches by the end of experiment 3 the following year.

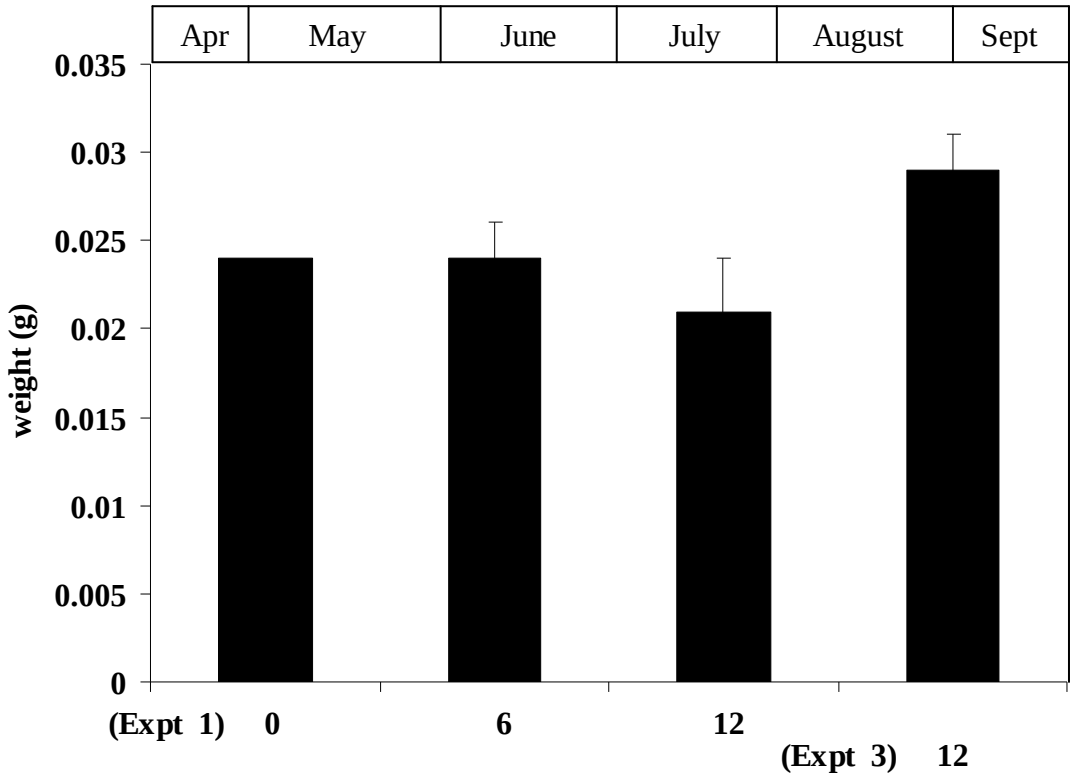


Figure 4.15 Mean weight of shelled embryos within brood pouches per female snail (+/- SE) produced by groups (Experiment 1 and 3; 11 snails per group) of *V. viviparus* snails in river water. Experiment 1, 14th April- 21st July 2003; three 6 weekly sampling points, Experiment 3, 1st June- 6th September 2004; single sampling point after 12 weeks.

4.7 Discussion

4.7.1 *Planorbarius corneus*

4.7.1.1 Egg Hatchability

This study, to my knowledge, is the first to report on the hatchability of *P. corneus* eggs exposed to river water. In experiment 1, there was a single time period (weeks 4-6) during which the methodology employed had a significant inhibitory effect upon hatching that was not concurrent with effects upon other reproductive parameters. During this period, universals containing egg masses were left in a laboratory without temperature control. With the prevailing hot weather, room temperatures were high at this time (Neil Clarke pers. obs.). It is possible that temperature effects, and secondary oxygen deprivation (though not tested) were the cause of the comparatively low hatching rates at this time (see Table 4.2A). Therefore, the methodology employed at this time could be the cause of the results observed, rather than any other effect, such an effect of river water. The consistently high hatching rate during Experiment 3 demonstrated that the methodology employed at this time was superior to that of Experiment 1 (see Table 4.B, and section 3.3.2).

Though there are few examples in the literature where the hatching rates of the eggs of pulmonate freshwater snails were measured, my figures (97.1%-99.6% in river water in experiment 3) exceed those reported elsewhere. There are to my knowledge, no examples in the literature of studies where planorbid eggs were hatched, and therefore, comparison is only possible with other species of the same subclass (Basomatophorans). In a study with *Lymnaea stagnalis*, Czech *et al*., (2001) classified a successful hatch rate as being >50% of eggs hatching in a mass. In control tap water, <20% of masses were designated as unhatched. This implies that in 0% to 20% of masses less than 50% of eggs hatch. Therefore, my hatching rate was, by comparison, favourable. This author hatched egg masses in an aquaria equivalent in size to the one containing parent snails; diluent volume for egg masses was therefore far in excess of my own. Furthermore, egg masses were transferred without apparent delay from aquaria to aquaria (not directly stated in methods), and therefore should

not have suffered stress due to oxygen deprivation or temperature extremes. Further, a 75%-90% hatching success was reported in a similar study with *Lymnaea stagnalis* where individual eggs where hatched each in its own cell culture plate well in tap water (Daire Casey; PhD thesis (Cardiff University)). Percentage of eggs hatching in this study was comparable, but slightly lower than results obtained in my own study. As both authors used *Lymnaea stagnalis* in the laboratory, the comparatively low hatch rate by the method of Czech *et al*., 2001 with egg masses is not easily explained. Therefore, my method, especially the methodology employed during Experiment 3, has been proven to hatch a high percentage of eggs; higher than by methods employed by other authors with similar pulmonate snail species.

4.7.2 Seasonal Growth

As stated (see section 4.3.1), the growth of freshwater pulmonates is continuous until death (indeterminate growth), and occurs seasonally (Costil and Daguzan, 1995b). Further, under natural conditions, spring is a time for fast snail growth, as water temperatures rise and more food becomes available before reproduction begins. In spring, the shell growth of *Lymnaea humilis* (Say) was very fast reaching 7% per day (MacCraw, 1961). Berrie 1966 observed the fast growth of ASO`s (therefore increase in weight) amongst *L. stagnalis* in springtime before the onset of reproduction, followed by comparatively little growth or null growth during the summer reproductive period. For a population of *P. corneus* in England, Berrie (1963) recorded a mean size increase of 4% between July and August (therefore, whilst reproducing maximally), and increases of 70% and 33%, respectively, between June-July and August-September. Climate was very important for growth in *P. corneus* in French ponds (Costil and Daguzan, 1995a). Adult *P. corneus* snails increased shell diameters by approximately 2-3 mm in only 2 weeks, from approximately 19 to 21 mm in one cohort, and 21 to 24mm in another (5^{th} May- 2^{nd} June). Therefore, it would not be surprising if *P. corneus* snails in our own experiments underwent a period of fast growth during springtime before increased reproduction mid summer. However, to my knowledge there is no evidence in the literature suggesting significant mortality amongst adult snails accompanying this strong growth during springtime. All snails used in my experiments had over wintered, were adult size before the experiments began (and were therefore ready to reproduce season allowing). Snails` shell length

measured 19.0 mm at the beginning of experiment 1, and snails could have potentially grown to > 26 mm as they did in French ponds (Costil and Daguzan, 1995b).

Shell length increased most in my snails over late Spring- early summer period (section 4.5.2: 4.4 weeks 6-12; 1.0 mm/fortnight) greater than over the summer (weeks 0-12; 0.45 mm/fortnight) of Experiment 3 and springtime (weeks 0-6; 0.78 mm/fortnight). In agreement with the literature, increase in weight (with shell), however, peaked earlier during Spring-early Summer (28^{th} April – 8^{th} June; weeks 0-6) and growth rate per day was almost double (0.017 g/day compared to 0.009 g/day) that over Summer weeks. This may well be due to the growth of ASO`s that increase in size before the beginning of the egg laying season, allowing growth as measured by increase in weight (with shell) to outstrip increase in length during springtime. The comparatively high reproductive output during Spring as temperatures increased to 14.3 ^{o}C on 25^{th} May by week 4, and in particular the doubling of reproduction between weeks 0-4, could have been growth limiting due to limited energy available for both processes (and therefore explain the slow down in growth into summer). However, this seems not to have been the case as growth was greatest during the springtime.

Growth as measured by increase in weight then slowed towards summer, growth rate per day of snails during summer weeks was almost equal to that of the Autumn-Winter period (average 0.007 g/day) during the period where reproductive rate was highest. The greater reproduction in Summer weeks could have been achieved (from an energetic standpoint) by a switching of metabolic resources from growth to reproduction as has been previously documented with *L. stagnalis* under natural conditions (Berrie, 1966). Though energy may have been thought to be in excess, growth was lower in the summer time in my experiments and might indicate that the availability of energy was limited by higher reproductive rates mid summer only. Further, as in nature there is switch of energy away from growth to reproduction, this may be an endogenously regulated and programmed (possibly genetic) switch that may have occurred my own snails. Therefore, the possible decrease of growth mid summer may in part be due to such phenomena. Therefore, my experiments are in agreement with the literature, snails grew by far the most (though reproducing comparatively well) in springtime with rising and highly variable water temperatures,

and less well in summer (higher, more stable water temperatures) whilst reproducing far in expectation of theoretical values in the literature.

4.7.3 Reproductive Pattern

The reproductive pattern demonstrated by *Planorbarius corneus* in these experiments appeared to show two distinctive reproductive peaks, a single large reproductive peak during spring-summer months and a much smaller reproductive peak in autumn. Literature suggests that the reproductive period in pulmonates commences in Spring (Costil and Daguzan, 1985A; Dogterom *et al*., 1984; Dogterom *et al*., 1985), although in our experiments egg laying had already commenced by the week 0 (28th April) of experiment 1, albeit not maximally. My experiments show a large peak of reproductive activity between the 8th June (week 6 of experiment 1) and the 21st July (week 12 of experiment 1) over the summer months (Figure 4.7), followed by a sharp fall in reproductive output between the 21st July and the end of Experiment 3 on the 13th September. There was a one-week gap with no overlap between Experiments 2 and 3, which were conducted in different years. A much smaller reproductive peak was observed between weeks 0-4 of experiment 2, and reproduction fully ceased in the last 4 weeks of Experiment 2 (17th October-14th November).

Though several other basommatophoran species exhibit similar seasonal reproductive profiles to the observed *Planorbarius corneus* (see Geraerts and Joosse, 1984), the possible nature of this profile and overall reproductive output (e.g. egg masses per snail per time period) under natural conditions has been poorly documented. Berrie, 1966 observed a single reproductive period between May and August, with a peak in June in *L. stagnalis* living in a small pond in Scotland. Dogterom *et al.*, 1985, observed this species in a long ditch 20km from Amsterdam and reported a well-defined egg-laying season between mid-May and mid-September. In *L. catascopium*, maturing members of a spring cohort reproduced as long as water temperatures allowed (Pinel Alloul and Magnin, 1979). Similarly, De Coster and Persoone (1970) sampled baby (0-2mm) *P. corneus* (Ghent, Belgium), from April to September indicating they bred over a similar period to snails in my own experiments.

In the wild, the length of the egg-laying period in individual *P. corneus* may be due to inter-individual growth variation (Costil and Daguzan 1995B), suggesting that reproduction could occur if members of a cohort were mature to reproduce. Cohorts are semelparus when they are mature for a single period of reproduction, iteroparus if they are mature to reproduce over two or more periods of reproduction (Dillon, 2000). However, of four cohorts observed in French ponds at least one was likely to be iteroparous, reproducing intermittently throughout their reproductive life, while other cohorts reproduced habitually in Spring over an approximately 4-6 week period, and less often in Summer over an approximately 16 week period, with a complete cessation of reproduction at other times. Taken together, this evidence would suggest that prevailing environmental conditions would determine the reproductive pattern observed in *Planorbarius corneus*. Further, this evidence suggests that the timing of the main reproductive period in our experiments, and indeed the smaller autumnal peak of reproduction both fit with the pattern of reproduction observed in *P. corneus* and other freshwater pulmonates detailed in the available literature.

Our experimental conditions (e.g. food, temperature, safety from predation) were, however, more favourable compared to these natural ponds, and these factors may have influenced the snails` reproductive pattern and increased overall reproductive output. The adaptive plasticity typically displayed by pulmonate snails allows populations to have maximum productivity under favourable conditions to compensate for bad years (Russell-Hunter, 1961). Although good environmental conditions could therefore booster reproduction, it is also possible that the way in which snails perceive their environment (for example, unpredictable or imminent hostile conditions) could also influence their reproduction. For example, under natural conditions the drying up of ponds (or rife) in summer may increase snail reproduction in anticipation of their own impending death. The same may be true for our mesocosm set-up, that is, "R-adapted" strategy; see Dillon, 2000. Therefore, a potential increase in reproduction may have been caused by relatively large and sudden temperature fluctuations in our experimental setup, or other unknown factors being interpreted by the snails as an imminent drying up event. However, in French ponds observed by Costil and Daguzan, 1985b there was an equally variable temperature regime and yet snails reproduced in two reproductive peaks. This reproductive pattern in French ponds was more likely due to food shortages or

perhaps bad weather, such as storms, that these natural ponds would typically endure. Therefore, the process of moving snails from the wild to a new environment (i.e. the experimental set up) could have affected (increased) their reproductive output due to better environmental conditions (e.g. excess food, protection from predation), the environmental nature of the experimental setup, and/or its unpredictable "nature mimicking" conditions which are also found in potentially unstable non-permanent water bodies (e.g. ponds or rifes).

The second smaller reproductive peak could be likened to an Autumnal phenomenon that has been documented in Viviparids (Ribi and Gebhardt, 1986), though to my knowledge this has only been reported once before in a pulmonate population, in which reproduction did not end until 17th November after a 16 week period (Costil and Daguzan, 1985b; *P. corneus*). This smaller Autumnal reproductive peak observed in my experiment could be due to favourable environmental conditions provided by the mesocosm. However, in Autumn snails become progressively more insensitive to CDCH as dorsal body activity gradually decreases during this period (Dogterom *et al.*, 1985). Usually in the field, photoperiods of 12 hours or shorter occur in Autumn, winter, and early spring, when little food is available (Scheerboom and van Elk, 1978). Further, the cessation of reproduction during these seasons (Joosse, 1964; Berrie, 1965) may ensure that the reproductive effort of adult snails is restricted to those periods when food is available and when the rapid growth and survival of any offspring are maximal (Dogterom *et al.*, 1984). Since food was readily available in the experimental set-up, day length (ultimate factor) and water temperature (ultimate and proximate factor) are likely to be the most important environmental factors that influence the timing of reproduction. However, during the last month of reproduction in my experiment water temperature dropped by 5 ^{o}C (to <9 ^{o}C) and day length from 12 hours to less than 10 hours per day. With winter approaching, the available literature would suggest that DBH activity would fall because of the synergistic action of environmental factors. However, the snails still reproduced in the experimental setup. Nevertheless, it is not inconceivable that reproduction occurred in Autumn, given that it has been reported to occur under natural conditions; conditions that are likely to be less favourable than my experimental setup, and notably lacking food towards winter. However, the possibility that exogenous chemicals present in the river

water inhibited, in part, the natural cessation of reproduction towards winter cannot be ruled out.

4.7.4 Reproductive Output

Over a 28-week calendar period reproduction in river water observed for *P. corneus* in Experiments 1 (Spring-Summer 2003), 2 (Autumn-Winter 2003), and 3 (early Summer to Autumn 2004) gave means of 7.1, 1.4 and 6.4 egg masses per two weeks, respectively, over the course of each experiment. Therefore, my snails in the mesocosm produced both a higher number of embryos at the reproductive peak compared to *P. corneus* in the laboratory and were reproductively active over a longer time period.

To my knowledge, there are no reports in the literature detailing the reproductive output of *P. corneus* under natural conditions. Unfortunately, laboratory experiments are usually conducted under artificial conditions and the results are rarely directly applicable to field conditions (Watton and Hawkes, 1984). Further, parasitism in wild populations of *P. corneus* could in theory have given a false impression of reproduction with only some or even few snails reproducing. My own observations during the dissection of snails, and histological analyses of snail tissues led me to believe my snails were parasite free, and therefore, able to reproduce to their full potential. Therefore still, however, an idea of how much the snails might have reproduced in my own mesocosm experiments can be gained from laboratory studies. Precht (1936) reported that *P. corneus* brought into the laboratory during Autumn or Winter did not reproduce indicating that reproduction may have an endogenously (day length, temperature) driven component. The difference in fecundity between basommatophoran (freshwater pulmonate) species is difficult to compare because it is strongly affected by experimental conditions, of which combinations (day length, water temperature, feeding, snail density and water exchange) vary widely in the literature (Costil and Daguzan 1995). Further, one author has stated that in his opinion the reproductive rate of pulmonates at the height of the reproductive season is sub-maximal, and easily capable of further increases if required (Tompa *et al.*, 1984). A comparison is possible, however, by analysing the effects of various combinations of

environmental parameters upon reproduction in freshwater pulmonate snails under laboratory experimental conditions.

4.7.5 Effects of Season on Reproduction

Photoperiod is stated to be the primary environmental signal that regulates the timing of reproduction (Goldman, 2001), although other authors suggest temperature is equally important (Bohlken and Joose, 1992). Further, even with adequate photoperiod and water temperature, it is the quantity of food assimilated that triggers the onset of the egg-laying season (Dogterom *et al.,* 1985). The short (approximately one month) seasonal growth of the reproductive system (Berrie, 1966) prior to the onset of egg laying is also likely due to a combination of these environmental factors. Reports of photoperiodic effects on reproduction in freshwater pulmonate snails are scarce (Bohlken and Joosse, 1982; Bohlken, 1978)(see section 4.2.1); however, all authors suggest that long day photoperiod causes increases in egg production.

During spring (weeks 0-6; to 8th June) in my first experiment, *P. corneus* snails averaged 6.4 egg masses per snail fortnight, mean water temperature was 15.8^{o}C and day length averaged in excess of 15 hours. During the common period of highest reproduction (6 weeks between 8th June and 21st July; experiments 1 and 3) our *P. corneus* snails averaged 9.1 and 9.2 egg masses per snail per fortnight. These figures were greater than could be expected from the water temperature data alone (18.4 ^{o}C-20.1 ^{o}C, day length 15.5 –16.3 hrs), as water temperatures were sub-optimal for maximal reproduction in this species (22 ^{o}C to 25 ^{o}C required for maximal reproduction in planorbids) (Costil and Daguzan 1995a). This data suggests that the reasons for the reproductive output observed are complex; day length in combination with water temperature, excess nutrients (compared to spring time algal growth under natural conditions) and other environmental conditions to a large extent determined reproductive rate in our own experiments, which exceeded reproductive for similar conditions from laboratory studies cited in the literature.

In the terrestrial pulmonate *Limax maximus* long day (LD) conditions trigger the onset of sexual maturation (Bohlken and Joosse, 1982). In the laboratory LD (16 h light, 8 h

dark) photoperiod induces a relatively early maturation of the female reproductive system (viz DBH production and release) and a "high rate" of egg production (Bohlken *et al.*, 1978)(Bohlken and Joosse, 1982). In my own experiments, clearly the most important climatic factor influencing the reproductive output of the snails appears to be day length, rather than temperature, since the rise and fall in reproductive output of my snails closely mirrored the seasonal increase and decrease in day length. Indeed, by springtime on the 25th May my *P. corneus* snails were averaging 6.0 egg masses per snail/ fortnight when there were 16 hours of daylight, and egg mass production had clearly increased greatly (doubling from one month earlier) along with day length. Since food was given *ad libitum* food quantity or quality was probably not a factor that limited egg mass production during the experiments, though actual food assimilation values are unknown. Whether high reproductive rates and the amount of food given limited growth is more difficult to determine as snails in the summer experiment overall grew less than in the spring-summer experiment, suggesting that less energy was available for growth in the summer. Most probably under natural conditions the effects of LD photoperiod on reproductive activity and metabolism will cause high mortality during summer, and was found to be the case under laboratory conditions (Bohlken and Joosse, 1982). It is well known that temperature is a very important factor that determines reproductive rate in freshwater pulmonate snails (see section 4.2.2.). The minimum threshold temperature for reproduction of freshwater snails appears to be between 7 °C and 12 °C (Costil and Daguzan, 1995B); above this temperature the reproductive system is able to produce egg masses albeit at a low rate (as demonstrated in Autumn in Experiment 2). In French ponds, *P. corneus* began to reproduce above the minimum threshold at 15-16 °C, although Precht (1936) suggested 12 °C was sufficient for this species. Costil and Daguzan, 1995 state that temperature strongly affects reproduction in *P. corneus*. Indeed, in my own experiments by 25th May, water temperature had reached 14.3 ^{o}C, although it had risen to 18.3 ^{o}C two weeks later and remained at least this high well into autumn.

The main peak of reproduction in my own experiments lasted approximately 12-weeks (>6.0 egg masses/ 2 weeks), when day length was long (>16 hours) and when water temperature remained above 18 ^{o}C, confirming the importance of these two

environmental factors upon reproduction in my own experiments that was suggested by the literature.

4.7.6 Seasonal Mortality

Associated with the reproduction during weeks 0-4 of Spring-Summer there was a particularly high mortality rate. Of the 34.8% of snail mortality by the end of Experiment 1, 27.1% of the original numbers had died by the end of week 4, and only 7.7% more died over the last 8 weeks (25th May; see Table 4.1 and Figure 4.3). Similarly, over the first 6 weeks of experiment 3 between 8th June and 21st July there was a high rate of mortality (a common period of time with Experiment 1 during which mortality was low) that decreased and was much lower over the final 8 summer weeks of this experiment. Taken together, this data would suggest that mortalities occurred at the beginning of both experiments that began at seasonally different times, and mortality in my experiments, therefore, appears not related directly to season (and not at times of high reproduction).

As the first few weeks of Experiment 1 were relatively early in the season, DBH and CDCH production would be expected to be low, the capacity of the accessory sex organs (ASO`s) to produce eggs and egg masses would also be low. As the reproductive rate in springtime was almost as high as some maximum rates of reproduction recorded amongst both lymnaeids and planorbids in the laboratory, reproductive stress could explain the high mortality rates recorded at this time, which was considerably higher over the first weeks of the spring–summer experiment than over summer or autumn. The utilisation of excess food not normally available in spring under natural conditions might explain the high reproductive rates in springtime. Further, the lack of significant mortalities during mid summer in either experiment would suggest the high reproductive rate generally did not cause exhaustion of the snails` energy reserves leading to their death. This possible early season reproductive stress might occur together with other stressors experienced by the snails at this time, including both increasing and unstable water temperatures in the mesocosms brought about by springtime climatic conditions resulting in increased mortality. Moreover, handling stress at the beginning of experiment 1, could have

been an additional cause of mortalities. The presence of other unknown stressor/s (chemicals in the water) cannot be ruled out, which may have also enhanced semelparity in my snails leading to mortalities. At the beginning of Experiment 3 great care was taken to reduce handling stress yet the same pattern to mortality was observed; higher mortality in the first few weeks of the experiment followed by greatly reduced mortality rates. Mortality was higher at the beginning (weeks 0-2) of Experiment 1 than Experiment 3, though reproduction was higher in Experiment 3. When taken together, this data might suggest that handling stress may be responsible, in part, for mortalities in Experiments 1 and 3 in the first few weeks of each experiment. There were no mortalities over the first 4 weeks of the autumnal experiment, where water temperatures and reproductive rate were much lower. This may suggest that overall stress (environmental, reproductive), in addition to handling stress were lower at the reduced temperatures and reproductive rates of Autumn. Therefore conversely, the effects of handling stress could have been magnified by higher reproductive rates both during spring and summer time experiments.

Mortality rates were much lower over the second half of both the spring and summer time experiments, suggesting no apparent exhaustion of the snails` energy reserves leading to their death. Growth rate did not decrease in a cohort of *P. corneus* in French ponds that did not reproduce at all during summer (Costil and Daguzan, 1995b), suggesting that available energy was perhaps limited mid summer in my experiments; enough to limit (but not stop) growth, without causing significant mortalities. Significant mortalities were observed in the Autumnal experiment over the last few weeks of the experiment as winter approached. Such a phenomenon is expected under natural conditions, and is thought to be due to deteriorating environmental conditions. However, an alternative suggestion to explain the lack of mortalities in the Autumnal experiment could include that when these snails were taken from the wild (by Blades), already the weakest snails had died off; leaving only the strongest for my experiment, and therefore, little mortality occurred during Experiment 2 by comparison to my other experiments. This data also suggests that the autumnal die off of algae observed had little or no effects upon the mortality of these snails. Further, these stronger snails may not have been parasitized; snails chosen for my other experiments could have, by comparison, suffered from a higher incidence or developed more fully parasitism, and therefore, died more easily during

my experiments. In nature, the birth of newborn snails is sometimes associated with the death of the parental generation (occurs at the exact same time of the year) in freshwater annual pulmonate snails, replacement of the parental generation with the following generation (see Watton and Hawkes, 1984). However, in my experiments adult snails continued to survive from summer right into Autumn, and therefore, complete replacement of the adult generation with newborn snails did not occur in my experiments, and therefore, could not explain significant mortalities amongst *P. corneus* adults in either of my Experiments 1 or 3.

In conclusion, the reproductive pattern, growth, and mortality pattern observed in my experiments generally followed the seasonal patterns described for pulmonate snails in the literature. However, the environmental conditions in which *P. corneus* snails were held allowed for a much greater and extended peak of reproduction, a possibly greater growth rate mid summer and lack of significant mortalities due to length of this snail species life cycle late season.

4.8 *Viviparus viviparus* in River Water

4.8.1 Seasonal Growth

In dioecious freshwater prosobranchs (families Viviparidae, Bithyniidae, Hydrobiidae, and Ampullariidae) it is commonplace for females to reach a larger size than males. Whether females grow faster than males, or whether only females continue to grow after the first reproductive season, to attain a greater size, is not known (Estebenet and Cazzaniga, 1998). Therefore, we do not know whether all snails used in my experiments were capable of reproduction. Snails collected for Experiments 1 (5.02 +/- 0.24g SE) and 3 (8.7 +/- 0.19g SE) were a different size at the beginning of each experiment. This implies, therefore, that they could also have been of a different age, although this cannot be known for certain as no attempt to age snails was made. It follows, therefore, that both their reproductive capability and ability to grow could also have differed between experiments.

Increases in shell lengths of adult *Viviparus* snails in my experiment were greatest (x1.7) in summer (weeks 6-12) than in the spring (week 0-6: 28th April and 8th June). Similarly, weight (with shells) increased very slightly over spring-summer, but by far more (x 5.5 more) over summer weeks. The contents of the pallial oviduct were around 1/3 of the total weight (without shell) of snails by the end of Experiment 3. Since the numbers of embryos in the brood pouches of females in spring and Autumn appeared to be approximately the same, we could, reasonably expect embryos to be approximately 1/3 the weight of female snails in spring also (though the weight of embryos can vary).

It follows therefore, that a greater weight loss from the release of mature embryos may have occurred in springtime when embryo release was high, compared to the summer. Snails did not loose weight between spring-summer (between 28th April and 8th June, weeks 0-6 of Experiment 1) despite actively giving birth suggesting that a strong period of growth also occurred in which they replaced the weight of the embryos they had given birth to, and at least began to replace these young with new embryos in the brood pouch. However, snails underwent apparent weight loss from summer into Autumn when reproductive rate was low, perhaps therefore suggesting poor health at this time. Poor health of snails towards the end of Experiment 3 would most probably have had a greater effect on reproduction than the difference in size of female sails between Experiments 1 and 3, as stated (see section 4.8.2; Reproductive pattern).

In only one study, has the growth of a viviparid been documented in a natural population. Ribi and Gebhardt, 1987 recorded the growth of *V. ater* in experimental cages over annual increments in snails aged 0+ to 6+ years of age. The observed growth pattern of these snails included slow growth over winter, as well as faster growth in spring and summer. Female snails 0+ and 1+-year-old showed an average averaged an increase in weight of 0.88 g per 12 weeks. Amongst snails of all year classes (ages 0+ to 6+) the average increase in weight was 0.12 g per 12 weeks. The mean weight of year 2+ snails was 6.82 g compared with 8.13 g in the year 6+ snails; suggesting that growth occurred rapidly in order to obtain a relatively large size after which growth rate reduced dramatically. Therefore, the age mix of snails in our experiments could have greatly influenced the overall mean growth rate. If a greater

proportion of the snails were young, then a higher growth rate could be expected. In Experiment 1, late spring growth was almost null (weeks 0-6), but summer growth was much faster, averaging 0.9 g per 12 weeks. Our snails therefore, increased in weight at a rate almost identical to fast growing 0+ and 1+ *V. ater* snails (averaged winter and summer growth)**.** By summer, our snails in Experiment 1 were 57.8 % of the weight of the largest group of *V. viviparus* recorded (Experiment 3; week 0 summer 04`), and therefore it is reasonable to assume that our snails were reasonably young (2+ or older) and reproducing, but importantly, were growing at a reasonably fast rate for a viviparid species. As it has been shown in several species that viviparids will vary their growth according to food availability (Van Cleave and Lederer, 1932), a good supply of food in our experimental set up is indicated. Furthermore, the high mortality rate is likely not to be due to starvation during this summertime period, or therefore, at any other time.

4.8.2 Reproductive pattern

The reproductive pattern of embryo release demonstrated by *Viviparus viviparus* in my experiments showed a single distinctive reproductive peak during springtime. The precise timing and size of this peak is unknown since reproduction had already started by the beginning of Experiment 3. Numbers of embryos released were comparatively large compared to summer months. However, in Experiment 3, females released most mature embryos during early spring; numbers released then decreased rapidly and reached a minimum by the end of May. The numbers of babies released remained low to the end of Experiment 3 (Figure 4.13)**.** Also during May, brood pouches of female snails contained the highest number of shelled (oldest) embryos with few unshelled (newest) embryos. By mid-summer (21st July), the inverse was true; the least number of shelled embryos, but highest number of unshelled embryos, were found in the brood pouches of female *Viviparus viviparus* snails. Snails returned to their springtime compliment of embryos (total, shelled, and unshelled) by Autumn.

Only Ribi and Gebhardt, 1986 have been able to maintain a viviparid (*V. ater)* in the laboratory, for an unknown period of time. For this reason, field studies alone can be used to establish the normal reproductive pattern and output, and development of *V. viviparus*; a species that has proven difficult to maintain in the laboratory (pers.

comm. Rachel Benstead). To my knowledge, there are only two papers in the literature describing the reproductive pattern and output of a viviparid snail under natural conditions. Ribi & Gebhardt, 1986 & Gebhardt & Ribi, 1987 observed *V. ater* in two European Lakes (Lake Zurich and Lake Maggiore) supplied by different drainage systems; in Lake Zurich, by far the largest peak to reproduction produced approximately 2.25 mature embryos released per female per week during springtime. A second smaller reproductive peak followed in August, and females released approximately 1.0 mature embryos per female per week. In lake Maggiore, there was no peak of reproduction, instead a plateau with 1.25 mature embryos released per female per week from early June to mid July. Females in Lake Zurich produced more numerous and larger offspring than females in Lake Maggiore (Ribi and Gebhardt, 1986). Overall, in Lake Zurich the mean number of offspring born to each female over the season was 19.6 versus 14.7 in Lake Maggiore, and the mean weight of offspring was 0.41 g in Lake Zurich versus 0.20 g in Lake Maggiore. The size of offspring at birth was correlated with the size of the females in both Lakes, with larger females giving birth to more numerous and larger offspring than the smaller females. The authors stated this difference in the seasonal distribution of the number and size of offspring might reflect generally better climatic and environmental conditions in Lake Zurich. The water temperature was 2°C to 5°C higher throughout the period of observation, and more food (both algae and detritus) accumulated in the Lake Zurich experimental cages that housed the snails between weekly cleaning. Decalcification of shells and resultant stress negatively affected *V. ater* in Lake Maggiore that had relatively lower water calcium levels. Thus, more favourable environmental conditions may have allowed two reproductive peaks per season rather than a single plateau of reproduction.

A further paper in the literature has described the reproductive pattern of another member of the viviparid family (Van Cleave and Lederer, 1932). *V. contectoides* in New York State, shows a single period of reproduction, which peaks between March and June. Females were spent by the early months of summer, none bore fully (shelled) formed young by June. In central Illinois, females of *V. contectoides* released most of their young over the months of March and April. Again, there was a single period of reproduction.

These authors estimated the development time from egg to time of birth of mature embryos to be between 8 and 10 months, and stated for 6-7 months of this time the embryos had a distinct external shell. This shell had colour bands for at least the final 3 months before birth. However, females by late summer contained some shelled embryos that had completed their development (Van Cleave and Lederer, 1932). These shelled embryos remain in the brrod pouch over winter, and are released in March to April the following year. Indeed, the authors observed females in winter fully gravid, packed full of fully developed shelled embryos. These observations in general seem to fit well with the reproductive pattern and output observed in my own experiments.

Therefore, the general pattern to the reproductive profile of a few members of the family Viviparidae appears common between both species and location, with over wintered mature embryos being released in spring and early Summer with a second peak of reproduction at the end of summer in some locations, perhaps determined by environmental conditions such as water temperature and food availability. Therefore, it seems unusual that only a single peak of reproduction was observed in Experiment 3, if environmental conditions were favourable to the snails (safe from predation in enclosures). The peak we observed (late spring-early summer) was, most likely, the birth of mature embryos already fully developed before the snails were introduced to the experimental setup, conceived the previous summer. Whether the snails produced, due to the timing of experiments, mature embryos and gave birth to these embryos under our experimental conditions is unknown. There could have been 6-month old embryos in pallial oviducts as snails were held in the experimental system at least this long, however the gestation of embryos is usually longer than this period (8-10 months). However, whether there is any flexibility in gestation times amongst viviparids (or other similar snails) is not documented to my knowledge. It is possible that environmental conditions were not ideal for *V. viviparus* in our experimental setup (including flow rate, temperature, water chemistry, or nutrition) resulting in an absence of autumnal reproduction in this instance.

In only a single study has, the reproductive capability of *V. ater* (viviparid that has a similar maximum size and life-span to our own *V. viviparus*) been studied over successive age classes in natural cages (Gebhardt and Ribi, 1987). These authors

observed that year 2+ female snails from Lake Zurich (more favourable conditions) slightly increased their reproductive effort as they grew older (including year 6+ snails), where as in Lake Maggiore (less favourable conditions), there was a significant decrease in annual reproductive effort with increasing age. This suggests the ecological suitability of the mesocosms may have influenced the reproductive output of our snails. Whether the larger (and therefore most probably older) snails would reproduce more (or less) is difficult to know because of their unknown health status. However, the slightly larger offspring in pallial oviducts at the end of experiment 3 might suggest a greater reproductive effort from these larger snails (see Figure 4.15), though this is speculative due to lack of significant evidence. Further, as stated, these snails may have diverted more energy towards producing more mature embryos (therefore, larger offspring), at the expense of new embryo production (due to environmental stressers). Therefore, the ecological suitability of the mesocosms and seasonal effects upon health may have had a larger effect on the reproductive output of female snails than age alone. However, size (age) and health status potentially confound one another in terms of their effects upon reproduction. I would conclude from this data that the size of snails was probably not an important factor determining reproduction particularly in snails during Experiment 3 towards Autumn.

The full compliment of embryos in our own *Viviparus viviparus* experiments had formed by September, suggesting there were enough males to copulate with females. However, the increasing mortality rate towards Autumn suggests that the snails may have been stressed. As viviparid snails can potentially live 6-8 years, only senescent snails in the final year of life should normally die during Autumn or, more likely, during winter. Since neither a low reproductive rate in spring or a narrow age range was suggested by the data the possibility that we collected a population of uniformly old snails (by chance) for use in my experiments seems an unlikely explanation for the pattern of mortality observed towards Autumn. The numbers (or timing) of the release of mature embryos that would have occurred in the absence of a stressor is unknown, but suggests that healthy snails might have released more embryos towards late summer or Autumn. Further, Ribi and Gebhardt, 1986 demonstrated that stressed *V. ater* in Lake Maggiore had reduced reproductive effort, possibly in order to survive and produce more offspring in the future. These snails invested more energy into developing embryos, and less into new eggs when the mortality risk was high. With

increasing age they produced larger offspring (to increase the chances of offsprings survival) suggesting that the snails allocated less energy into new egg production, and more energy into already maturing embryos. Likewise, mature embryos in my own experiments were bigger (x1.4 fold increase) in Autumn than in summer, which may indicate our snails were stressed. Therefore, the snails in Experiment 3 may have been alive but reproducing less (notably late summer). Further, in Lake Maggiore these stressful conditions caused earlier senescence (snails lived longer in Lake Zurich), and this may have occurred in our snails also. Since experiments 1 and 3 were run in different years, previous growing conditions could have differed (i.e. quality of food, water temperature or chemistry). A poor growing season in either year could affect the subsequent reproductive cycle (number of embryos formed and/or babies released), and therefore a direct comparison between years may not be possible.

4.8.3 Reproductive output

Although *V. viviparus* and *V. ater* belong to the same family, there is no information available, to my knowledge, on the reproductive output of *V. viviparus* or of the effects of different environmental conditions (such as latitude, water chemistry, temperature, food) on the reproduction of populations of *V. viviparus.* As viviparids can be either lake or canal dwelling species restricted to large, deep bodies of still or slowly moving lowland rivers or canals (Kerney, 1999), the relatively rapid speed of water flow in the experimental setup may have affected their health and reproduction. If the water flow was too fast this may have reduced habitat suitability, stressing the snails thereby reducing reproductive output.

According to the only available literature on *V.ater*, *V. viviparus* might be expected to release between 2 and 4.5 mature embryos per female per fortnight during the spring reproductive peak depending on environmental conditions (Ribi and Gebhardt, 1986). In Experiment 3, the maximum mean number of mature embryos released per female over weeks -2-0 in late Spring between 25th May and 8th June was 5.7 (2.9 week/ snail) which is comparable. However, whether this was the peak of reproduction is not known (Figure 4.14A), because a very large number of babies were released before the beginning of Experiment 3 (during the acclimation period), which were not

quantified. Reproduction decreased quickly over the following month into summer; the mean number of mature embryos released was 0.6 per female per fortnight over this period. The rise in reproductive output of viviparid snails (irrespective of species or location) appears keyed to springtime, suggesting that the most important environmental factor influencing the reproductive pattern of our viviparid snails was day length. However, of the possible influences upon reproductive output, intuitively food would seem likely to be the most important, as was observed in Lakes Zurich and Maggiore (Ribi and Gebhardt, 1986). Since food consumed by snails in my experiments was most probably either filamentous algae growing on cages, or detritus accumulating inside cages, or filtered suspended solids (e.g. algae, detritus, etc), it is not known whether food was a limiting factor during the spring time reproductive period.

As a full brood of mature embryos would most likely have been present over winter (van Cleave and Lederer, 1932), food availability is unlikely to have affected reproduction in spring as much as it would have affected new embryo production and their development in summer and autumn. However, stressed female viviparids may have released their young prematurely in springtime, due to both handling stress from being moved from the local canal to the mesocosms (during acclimation), and/or excessive flow rate in the tanks. However, Ribi and Gebhardt, 1986 compared reproductive rate between natural populations and *V. ater* snails in their cages and proved statistically that their snails in cages neither released embryos prematurely nor retained them longer than they did naturally. Therefore, it is possible that our own snails were not affected in terms of their reproductive output in spring by moving them to our mesocosms.

4.8.4 Age at Reproduction and reproductive capability

Since adult *V. viviparus* were not sexed before experimentation, the sex ratio or number of reproductive females per enclosure at the beginning of each experiment is unknown. From my own histological observations of *V. viviparus* (born and taken from our mescosms at the of Experiment 3), a female snail born in the experimental system as small as approximately 15mm (less than one year old) was capable of

reproduction, as indicated by mature fully coloured shelled embryos in the pallial oviduct and mature female openings. This is much earlier than V. *georgianus*, which need 36-47 months to sexually mature (Jokinen *et al.*, 1982) or *V. ater* in which adults do not reproduce before the age of two (Ribi and Gebhardt, 1986). Snails in both experiments were considerably larger than this. However, from the examination of pallial oviducts from females remaining alive in river water at the end of both Experiments 1 and 3, only 11% (2 of 18 snails in both experiments) were carrying fewer embryos compared to the others. Furthermore, although snails in experiment 1 were smaller than those in Experiment 3, it was not consistently the smallest females in Experiment 1 that had relatively empty pallial oviducts. Therefore, smaller size of snails in Experiment 1 does not appear to have affected the females' ability to reproduce.

Since females used in my experiments would have been gravid over winter, a lack of males is springtime would not have affected reproduction at this time. However, a lack of males mid summer could have prevented or limited copulation and influenced the likelihood of a second reproductive peak at the end of summer (formation of a new brood of embryos). However, whether male availability had an influence on reproduction at this time cannot be directly addressed. Further, pulmonate snails are known to be able to store sperm for up to approximately 8 months, but whether female viviparids are able to do this is unknown. Therefore, the availability of males, and its possible effects upon fecundity and reproduction later in the season, are also largely unknown, though full pallial oviducts (equal number of embryos in pallial oviduct compared to springtime) in females by Autumn, clearly suggests that sperm was available to females.

4.8.5 Seasonal Mortality

During Experiments 1 and 3, the mortality rate was equal between spring-summer and summer weeks, indicating that reproduction during springtime was not directly associated with mortality. However, the 12-week duration of these experiments represents only a small part of the total lifespan of these viviparid snails and, therefore, relatively low mortality rates (compared to pulmonate snails) might have

been expected. Ribi and Gebhardt, 1986 reported that the placement of *V. ater* in experimental cages during springtime did not cause their snails to give birth prematurely or retain mature embryos (nor caused mortalities). Therefore, moving the snails to our mescosms is unlikely to be the cause of the demonstrated mortalities. Most importantly, Ribi and Gebhardt demonstrated with caged snails in Lake Zurich and Maggiore that the overall mortality rate observed between March and October was only 4.5% (60 of 1344 caged snails died). Mortality was higher in the less favourable Lake Maggiore (53 died; 3.9%) than in Lake Zurich (7 died; 0.52%)(Ribi and Gebhardt, 1986). In natural populations of *V. georgianus* mortality rates of between 22% and 78% during the first year of life have been reported (Browne, 1978; Jokinen *et al.*, 1982), which may suggest that in *V. ater* the mortality rate in the natural environment is probably much higher than in cages (due to predation by birds and fish), and would probably be highest amongst young (small) snails. However, the caged snails of Ribi and Gebhardt (1986) were adults like those used in my experiments, and therefore may provide a better guide to mortality rates we could expect amongst snails in my experiments. Mortality rates in my experiments were consistent apart from the final 8-weeks of Experiment 3. There was an approximate 15% mortality rate amongst snails per 6 weeks, apart from Autumn when 58% of snails died over a final 8-week period. This spring and summer time mortality rate was far greater than in the experiments of Ribi and Gebhardt (1986), and the autumnal mortality rate far greater again. When taken together, this evidence suggests that snails grew well overall, but grew especially well in summer. The mortality rate throughout our experiments was probably higher than could be expected, and was especially high in Autumn.

I can only speculate as to the possible reasons for the mortality of the *V. viviparus* snails in river water during our experiments. However, most, or all, explanations would include environmental factors. Lack of water depth is unlikely to have been a problem since viviparids have been observed to migrate to shallow water to give birth in spring (van Cleave and Lederer, 1932; Ribi and Gebhardt, 1986). Indeed, the movement of snails to shallow water in spring was observed to occur in my local viviparid populations during collection also. Incorrect temperature ranges, too fast a flow rate, or the effects of chemicals in the river water are all possible causes of this high mortality (see section 4.7.6; causes of *P. corneus* deaths). Further, the growth

data appears to suggest that a lack of suitable food or feeding quantity was not the cause of the mortalities observed.

From week 6 of Experiment 3 on the 27th July onwards there was a decrease in day length; the last 8 weeks of this experiment were Autumnal in nature as day length shortened by 19.8% by the end of the experiment. Water temperature varied between 19.4 °C and 21.4°C over weeks 6-12 but did not fall over this period. In Experiment 3, from early Summer into Autumn (between the 15th June and 6th September) snails appeared not to grow, in fact there was an apparent weight loss. This might suggest that the biggest snails were dieing at this time indicating a particular environmental stressor (e.g. lack of oxygen). However, the length of dead snails shells compared to live snails during experiment 3 indicated that the biggest female snails were not dieing at any specific time point in the experiment over which measurements were taken (Figure 4.11). Only during weeks 4-6 (4 snails died in river water) were the length of dead shells significantly ($P < 0.05$) greater than those of live snails measured at the end of the experiment. This suggests that the largest snails were not dieing earlier in the experiment, leaving the smallest left alive at the end. Notwithstanding this, if the largest snails had died this would not have affected the pattern to reproduction, but could have affected the growth data as younger snails grow faster than old. However, no snails grew towards Autumn indicating that stress upon snails was probably an important factor at this time. Therefore, death of snails in Experiment 3 appears to have affected the growth data towards Autumn, and may have influenced the reproductive pattern, as dieing snails may fail to reproduce. Further, this stress may have affected the number of embryos recruited to the pallial oviducts of gravid females. However, as the number of embryos present in pallial oviducts was equivalent to numbers present in springtime, this suggests recruitment was complete before any significant stress levels affected the snails` health.

As there is no evidence to my knowledge of such a pattern to mortality amongst viviparids occurring under natural conditions, though little literature is available on this subject. The observation that mortalities increased dramatically towards autumn might suggest a connection with season. Further, shortening day length may have precipitated the effects of other stressors to bring about the mortalities observed. The observation of an autumnal die off of algae could have led to a contributing factor;

toxic effects of this dead algae leading to the mortalities observed in autumn. However, no such negative effects of this die off of algae was observed with *P. corneus*, and is likely therefore, not to have had a significant effect on the mortality of this viviparid. Therefore, the data would suggest a particularly high mortality towards autumn associated with shortening day length and possibly environmental conditions. Although a genetic component cannot be ruled out, it seems likely that the experimental set up was in some way responsible.

There are a number of possibilities that might help explain the lack of mortalities associated with high reproduction during spring and summer in our viviparid snails, possibilities that might help explain increasing mortality towards autumn. In autumn, it might be expected that with increasing mortality (decreasing health), snails would reproduce less. However, reproduction appears not to decrease towards autumn; reproduction continued despite the declining health of snails. Factors leading to declining health of snails may have included the combined effects of a lack of nutrition, and/or toxicity (chemicals in the water), or flow rate, resulting in a lack of resources devoted to body maintenance, that may have led to a form of semelparity observed in pulmonate snails, starvation leading to increased reproduction before the snails death during autumn. Interestingly, as discussed, it has been shown that for several species of viviparids that they vary their growth rate according to food availability (Van Cleave and Lederer, 1932). Therefore, weight gain during and especially after partuition in Experiment 1 (see section 4.6.2) together with a lack of significantly high mortality rate during the first 6 weeks of either experiment (Table 4.3), would suggest that sufficient and suitable food was available. Furthermore, after sufficient feeding during spring and summer, starvation would seem an unlikely explanation for snail mortality in autumn as food resources, (also stored in body tissues) would still have been available. Perhaps importantly, the high number of number embryos in the pallial oviducts in autumn (return to a full compliment) suggests both sufficient food resources and that mating did occur during summer in most female snails at least (or sperm storage had taken place). Buckley, 1986 found no differences in survival between reproductive and non-reproductive females in *V. georgianus*, and as nearly all females in my experiments had full pallial oviducts by autumn, failure to mate seems an unlikely explanation for the mortalities observed. However, as a period of senescence followed by death has been recorded in the oldest

age class of female viviparids (Ribi and Gebhardt, 1986), and these snails were observed to have empty pallial oviducts, there is still the possibility that a few females may have been senescent. As senescence in the wild is gradual process that occurs in the final year of life (Ribi and Gebhardt, 1986), we could reasonably expect that mortality rates should have been low during my experiments as in caged viviparid snail experiments, but this was not the case. The possibility remains that environmental factors are likely, in part, to explain the pattern to mortality observed. However, a full compliment of embryos by Autumn and strong growth into summer suggests complicated reasons for the pattern to mortality observed.

Taken together, this evidence suggests an abnormally high mortality rate at all times during my experiments, but particularly high towards Autumn due to unknown environmental stressor/s. In my view, this is almost certainly due to environmental conditions in the mesocosms, with excessive flow rate and/or the release of chemical toxins from decaying body tissues contributing to the stress suffered by the snails. Further, algal death (mainly filamentous) has been observed in the mesocosms in Autumn (Alice Baines *pers comm*.); that may cause a deterioration of conditions including the release of algal toxins. This decomposition of organic material (part of eutrophication process) could also release other chemicals that may affect the health of snails (e.g. nitrate and phosphate); however, since no measurements were taken, the results of the process of decomposition of algae in particular are not known. However, the actual cause/s remain unknown due to a lack of definitive evidence.

4.9 Conclusions

The development and reproduction data in river water suggests that these two species differed in their ecological suitability to the experimental setup. *P. corneus* thrived in terms of reproductive output and grew well in springtime, and indeed there is little suggestion that the mortality rates associated with this species was in any way abnormal. The reproductive rate of *V. viviparus* in spring appeared normal, and was similar to another species in the Genus, although the rate of reproduction in this particular species is unknown. The growth rate of *V. viviparus* through the spring and summer appeared to be good, and might indicate good health had other life cycle parameters such as reproductive and mortality rates been in good agreement.

However, the lack of reproduction of *V. viviparus* towards Autumn, combined with abnormally high mortality rates and lack of growth in late Summer and Autumn indicates a poor health status during this time period. Clearly then, the experimental system with river water flowing through the system as a control (for effluent experiments), was not entirely suited to this viviparid species.

Pulmonates and prosobranchs follow different strategies to combat the same environmental problems encountered in freshwater systems despite being found in the same environment (Dillon, 2000). Pulmonates produce many more offspring and devote comparatively less energy and parental care to each over a relatively short period of time (approximately one year). In doing so, they try to put their own genes (via their progeny) into the next generation before their own mortality intervenes ("R-related" strategy). Prosobranchs produce far fewer offspring over a season, but reproduce over several seasons spreading their reproductive effort over a greater number of seasons to combat unfavourable environmental conditions that may occur in the short term (during seasons). There are no references in the literature indicating that viviparids adopt an "R-related" strategy in response to unfavourable conditions. Therefore, the notion of an "R-related" strategy amongst viviparids would be purely hypothetical, and is, therefore, not suggested. In fact viviparids have been demonstrated to do the theoretical opposite; to reproduce less to preserve their own lives and spread their reproductive effort over a greater period (Ribi and Gebhardt, 1986). The extensive reproductive profile of *P. corneus* in my experimental system indicated this snail species was capable of a high reproductive rate when placed in this system, though the reasons for this are complex but include both favourable environmental conditions and an "R-related" strategy. Factors such as food availability and protection from predation might indicate favourable conditions compared to natural conditions. Freshwater pulmonate snails are known to be able to compensate for bad years by taking advantage of favourable conditions (part of "R-related" strategy)(Costil and Daguzan, 1995b), as they appeared to have done in our mesocosm experiments. Stressful conditions including changing and highly variable water temperatures, moving snails to a new environment, and manual handling at the beginning and during the experiments, or chemicals in the water may also boost reproduction via "R-related" strategies, resulting in an enhanced form of semelparity. However, this environmental stress does not necessarily indicate poor health status of

the snails. Instead, it might indicate that the result of "stress" was an attempt, via phenotypic plasticity, to replace the parental generation of snails in a perceived stressful environment before their own mortality intervenes. Therefore, it is unclear whether the health status of *P. corneus* was compromised in our experimental set-up; the reproductive, growth and mortality patterns observed could have been part of their own phenotypic plasticity, characteristic in particular of freshwater pulmonate snails. Pulmonate snails are able to adapt their lifecycle to suit local conditions, in order to survive and reproduce appropriately; their ability to reproduce "recklessly" in the face of adversity being a particular and distinctive feature. Whether for example, the mortality rates are higher than could be expected (indicating stress and poor health) is difficult to establish since no similar experiment or natural conditions have replicated the conditions in our own mesocosm experiments, therefore, no direct comparison can be made.

In contrast, the health of *V. viviparus* was most likely compromised as indicated by the unusually high mortality rates during the experiments and towards autumn compared to other caged viviparid experiments. With the viviparids in my own experiments, mature embryos released in spring were most probably conceived the previous summer. Conversely, approximately 5 months of an 8-10 month gestational period from new to mature embryos was observed whilst these snails were in our experimental setup. Whether some eggs where retained from the previous summer, or perhaps more likely a new brood was conceived early summer (around May or June time), means development of (new) embryos was likely to have occurred between June and September. Further, the number of maturing embryos in the pallial oviduct at this time was the probably the best indication of the effects of the experimental set up upon reproduction in these snails as this entire process occurred whilst the snails were in our setup. Replenishment of a full springtime compliment of embryos might indicate good health status, although the death of snails towards Autumn might contradict this idea. Alternatively, the replenishment of embryos may have been the cause of their death; replenished inspite of poor health status, further lowering their chances of survival. A large allocation of energy to reproduction in the face of high-energy costs of body maintenance in a stressful environment could help explain high snail mortality rates. True senescence did not occur in my viviparid snails as the pallial oviducts of females were full of embryos and not empty as found in senescent

snails in natural systems. Therefore, flow rates, endocrine disruption or any combination of environmental factors are possible reasons for their decline of these snails in an apparently unfavourable environment. One further possibility could be that *V. viviparus* snails were less tolerant of their chemical environment than *P. corneus* (that thrived by comparison in my mesocosms), however, how much of the poor health status of my viviparids was because of their chemical environment is unknown. Such high mortality rates would not have been anticipated in such a long-lived species, as was indicated by the available literature.

Chapter Five: The Seasonal Effects of Treated Sewage Effluent Upon Development and Reproduction in freshwater molluscs

5.1 Introduction

Reports in the literature describing the effects of a treated sewage effluent (TSE) upon the development and reproductive cycle of freshwater gastropod molluscs are few. Of those, Watton and Hawkes, 1984 documented significant and relevant information to my own exposure experiments, as they simultaneously exposed the pulmonate *Lymnaea peregra* and a prosobranch *Potamopyrgus antipodarum* to a graded TSE source. Further, Jobling *et al.*, 2003 exposed the same prosobranch species to a different TSE source. Therefore, both within and between taxa differences in the response of molluscs to TSE are largely unresearched, but are likely to exist. To illustrate differences are known to occur in the response of marine prosobranchs to organotin compounds (Blaber, 1970, *Nucella lapillus;* Smith 1971, *Ilyanasssa obsolete;* Stroben *et al*. 1995, *Hinia reticulata*; Schulte-Oehlmann *et al*., 1997, *Hydrobia ulvae*; Morcello *et al*., 1998, *Ruditapes desussata*). As a consequence, over the last two decades, the phenomenon of imposex (the term used to describe the range of abnormalities seen) has been described in more than 120 species of marine molluscs worldwide (Morcello and Porte, 1999). The pronounced seasonality of European freshwater molluscs compared to tropical species might also affect their response to TSE due to seasonal differences in their reproductive cycle and, indeed, their reproductive strategy (if any). This report is the first to describe the seasonal response of different freshwater molluscan species to a possible endocrine disrupting mixture, namely TSE.

Watton and Hawkes, 1984 exposed these two snail species to a graded concentration of TSE (river water, 25%, and 50%) from January 1979 to October 1980 in three raceways in Birmingham, England (overall length 300m, depth 0.2-0.4 m; substratum pebbles, gravel and accumulated silt); a natural system like our own mesocosms. These authors observed the reproductive output from the F1`s of *L. peregra* since the

generation time for this snail species was almost exactly one year. There was complete replacement of the adult (F0) population by young (F1) over a 3-month period (June onwards); which were monitored until October 1980. The prosobranch studied was *P. antipodarum*; a non-native species, introduced to the UK around 1897 from New Zealand (Fretter &Graham, 1962). *P. antipodarum* is a pathenogenetic live bearer (females only in the UK) and exhibits a generation time of between a few months to three years (depending on their survival during winter under natural conditions), which is typically shorter than that of European viviparids (Fretter & Graham, 1962). Therefore, the study was effectively a multi-generation test (as cohorts could not be separated) for this species in graded concentrations of effluent. The effluent used in this study was from the Blithe valley STW and was characterized by its physiochemical parameters rather than for its chemical content.

In both cases, exposure of *L. peregra* to STW (25% and 50%) resulted in an increase in reproductive output relative to river water. Egg production and egg mass production was greatest in 25% effluent (7.4 fold above river water). The lower reproductive output in the 50% treatment (1.7 fold above river water) may have been due to the higher density of snails in this treatment. Greater reproduction over the season was the result of both production of a greater number of egg masses each month, as well as a longer breeding season in TSE. However, during the peak of reproduction in June, more eggs per snail per month were laid in river water (20.9/snail/m^{-2}) compared to 25% effluent (9.9/snail/m^{-2}) or 50% effluent (3.5/snail/m^{-2}); population densities during this month were inversely proportional to egg production per snail. Although there were significant between-treatment differences in the number of eggs per mass in every month sampled, there was no discernable seasonal pattern over the duration of the experiment. The number of eggs per mass peaked during spring and summer in all treatments at different times and at least once, after which the number of eggs per mass decreased towards autumn in all treatments. Reproduction ceased in river water during July**,** but continued in both effluent treatments, albeit at decreasing rates, for at least a further two months until the end of the experiment.

According to Watton and Hawkes (1984), reproduction in *P. antipodarum* was continuous and occurred in waves throughout all seasons. In river water, total

embryos in the brood pouches of snails increased to a peak in May, but it was only in June that more mature (shelled) embryos appeared in the brood pouches of females than at other times of the year, suggesting higher fecundity at this time. However, direct evidence of a reproductive peak was lacking, as the number of mature embryos released from brood pouches was not counted. There were many more *P. antipodarum* in 25% effluent compared to numbers present in river water, despite no significant differences in the total number of embryos in the brood pouches of female snails over the period of the experiment. *P. antipodarum* did not survive well in the 50% effluent treatment (though a few were counted on two occasions), and this was presumed to be due the toxicity of heavy metals present in the effluent.

As well as increased reproduction, there was both a greater number and biomass (adults and offspring) of *L. peregra* in TSE compared to river water over the period of the experiment (no significant difference between effluent doses). There was no significant difference in the growth rate between treatments; adult *L. peregra* achieved similar weights and sizes in all three channels. However, the lifecycle of *L. peregra* in 50% effluent appeared to lag slightly behind those in other channels. The greatest single population peaks recorded during the approximately two years of the study were 1486 snails/m^2 (May 1979), 1173 snails/m^2 (August 1979), and 160 snails/m^2 (May 1980), were recorded in 50% and 25% TSE, and river water, respectively. Standing biomasses (snails sampled monthly over 0.1 m^{-2} of substrate; soft and hard parts dried at 105 ^{o}C) for the year (June 1979 to June 1980) were 0.83 g m^{-2} in the river water channel, 2.89 g m^{-2} in the 25% effluent channel, and 8.04 g m^{-2} in the 50% effluent channel. This data would suggest that TSE was not toxic to this snail species; indeed, it suggests that it was in some way advantageous causing increases in both snail numbers and biomass.

Vastly more *P. antipodarum* were present in river water where peak numbers reached 15,933 per m^2 compared with a peak of 286 per m^2 in 25% effluent, and too few to quantify in 50% effluent. Standing biomasses for the year were 11.96 g m^2 in river water and 0.13 g m^2 in the 25% effluent, and 0.01 g m^{-2} in 50% effluent. Due to overlapping cohorts and low biomasses at times, growth rates, adult size and weights could not be compared between treatments in this case. Watton and Hawkes, (1984) sampled water from their three raceways during winter into spring (January-March)

and summer into winter (August-November). In winter, river water contained approximately 96 ugl^{-1} of total heavy metals (Cd, Cr, Pb, Ni, and Zn) and 33 ugl^{-1} in summer. Whereas, 25% effluent contained 170 ugl^{-1} in winter and 130 ugl^{-1} in summer, and 50% effluent contained 215 ugl^{-1} in winter and 200 ugl^{-1} total heavy metals in summer. In light of this information it is likely that heavy metals could be responsible for the reduced growth (and survival) of *P. antipodarum* in this TSE. Indeed, it has been demonstrated that combined concentrations of chromium and copper (at concentrations less than those recorded from the channels which contained effluent) killed *P. antipodarum* both in the laboratory and in a caged experiment on the river Holme in Yorkshire (Brown, 1980). Further, Extence (1980) observed that total copper concentrations as low as 25 ugl^{-1} suppressed reproduction in the same species. In the study by Jobling *et al*., (2003), lower concentrations of metal ions could be suspected in this TSE as only 100% effluent had a negative effect on growth whereas 25% effluent was toxic in the study of Watton and Hawkes.

Jobling *et al.,* 2003 carried out a set of experiments the same mesocosm set-up and effluent source (Chelmsford STW) that was used in my own experiments. In contrast to the study of Watton and Hawkes (1984), Jobling *et al*., (2003) reported a stimulatory effect upon new (unshelled) embryo production with *P. antipodarum* after 7 days exposure to 100% effluent compared to both a tap water and river water control. After 14 days, the stimulatory effect was more pronounced with river water, 12.5% and 50% treatments showing enhanced embryo production compared to tap water control, with 12.5% and 50% effluent. In contrast, 100% effluent appeared to have a negative effect on embryo production compared to river water. The initial increases in river water, 12.5% and 50% TSE was then followed by a decrease in embryo production compared to the tap water control (from 14 to 42 days exposure). In contrast, embryo production in tap water increased with the reproductive cycle (or possibly acclimation) to day 14, then remained constant for the rest of the exposure period to day 42. The reason/s for the stimulatory effect of river water compared to a tap water control is unclear, though the authors questioned whether low concentrations of oestrogens (both natural and/or synthetic), though not measured during the study, and several other studies with this river water source, could be responsible. Also in contrast to Watton and Hawkes (1984) the *P. antipodarum* experienced no mortality over the course of the experiment. Snails grew equally well

in all effluent concentrations except in 100% effluent where snails were smaller than in other treatments by the end of the experiment (perhaps suggesting some chronic toxicity).

Therefore, the small number of studies in both freshwater pulmonates and prosobranchs, indicate that TSE may increase fecundity (of the individual, or population) at certain times of the year, resulting in both an increase in eggs and egg masses in pulmonates (Watton and Hawkes, 1984) and of new (unshelled) embryos in prosobranchs (Jobling *et al.* 2003). Watton and Hawkes (1984) suggest that increased food supply with increasing effluent concentration was the reason for the increase in reproduction in *L. peregra.* The amount of filamentous algae, a favourite food (as organic material), increased with increasing effluent concentration (as it did in our own mesocosms), but an association between food supply and snail fecundity could not be proven statistically. These authors did not infer that chemicals within the effluent might have been responsible for the observed effects. In contrast**,** Jobling *et al.,* 2003 inferred that oestrogens in TSE could be responsible for the effects observed in those treatments also. In the study of Jobling *et al*., 2003, the involvement of endocrine disrupting chemicals was suggested, chemicals proven to affect reproduction in other taxa, for example, oestrogenic chemicals demonstrated to cause abnormal reproductive development in vertebrate fish species (e.g. roach (*Rutilus rutilus;* Jobling *et al*., 1998, Jobling *et al*., 2002b, Jobling and Tyler, 2003).

As physicochemical and chemical analyses were not carried out in both studies it is difficult to draw any comparative conclusions about the causes of the effects seen in *P. antipodarum.* The effects described by Watton and Hawkes (1984) with both a pulmonate and prosobranch species demonstrate the possible taxon differences in the response of different snail species to a TSE (or even river water), though both the physico-chemical and chemical composition of an effluent could affect greatly the different effects each effluent could have.

Overall, in the study of Watton and Hawkes, 1984 the cause of the effects seen with this TSE was unknown, although different mortality rates (and therefore the numbers of snails present) lead to greater reproduction in effluent overall. However, the possible mechanisms by which oestrogenic chemicals could affect reproduction in

freshwater snails species are largely unknown. In particular, the presence of endogenously produced oestrogens in the gonad of temperate freshwater snails (known to occur in vertebrates) has been largely unproven, although their presence has been suggested in at least one study (Jumel *et al.*, 2002). In this study, the presence of both oestrogen-like and testosterone-like hormones (by RIA methodology) in mantle homogenates of *Lymnaea stagnalis* were reported, and indicated a hormonal cycle peaking early summer. However, where attempted, most studies on freshwater gastropods have identified progesterones and androgens only in the tissues of freshwater snails (Joosse, 1972, Joosse, 1984)**.** Therefore, the way in which TSE could affect reproduction in a freshwater pulmonate (or prosobranch) snail, or which chemicals, or combinations of chemicals, might be responsible is almost entirely unknown.

In this chapter, I will therefore consider the effects of graded TSE on two species of gastropod molluscs over the seasonal reproductive cycle; in particular to study their reproductive and developmental responses over this period.

5.2 Results of Seasonal effects of TSE on P. corneus

5.2 *P. corneus*: Brief Materials and Materials

Over the course of two seasons (2003-2004), three experiments of 8-16 weeks duration were undertaken which, together, covered the reproductive and developmental season from spring through to winter. Experiment 1 was run in 2003, and its duration (14-weeks; 12 weeks with TSE) covered spring to mid summer. Experiment 2 in the same year, lasted 8-weeks, and covered from late summer into winter. Experiment 3 was the only experiment to be run in 2004, and lasted 16-weeks (14-weeks with TSE), and covered early summer into early autumn. There was only a single weeks gap in time between experiments 2 and 3 though they were run in different years.

A detailed description of all three experiments, including experimental design can be found in Chapter 2. Briefly, in Experiments 1 and 3, river water, 25%, 50% and 100% TSE were used as treatments. In experiment 2, treatment included river water, 25% and 50% TSE. Six replicate groups of snails were used in each treatment, and each group consisted of between 9 and 11 snails (dependant upon on the numbers of snails available for each experiment). Baseline morphological measurements were taken at the beginning of each experiment (at time zero), and at the end of week 6 (Experiment 1 only), and again at the end of each experiment. In each experiment, at the end of the 2-week baseline period in which all snails had been exposed to river water only, TSE was introduced at time zero (mixed with river water diluent to obtain correct dilution of TSE), biological measurements were taken at this time, and every two weeks thereafter until the end of each experiment.

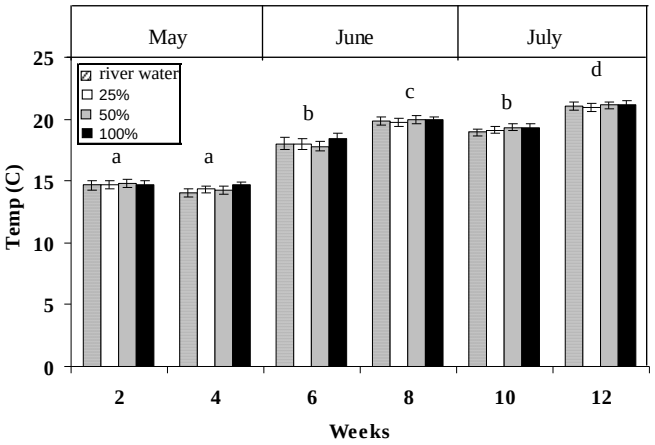
5.2.1 Seasonal Changes in Water Temperature

Figure 5.1 A-C shows the analysis of mean temperatures in river water and TSE (25%, 50%, and 100%) dosed tanks from Experiments 1, 2, and 3.

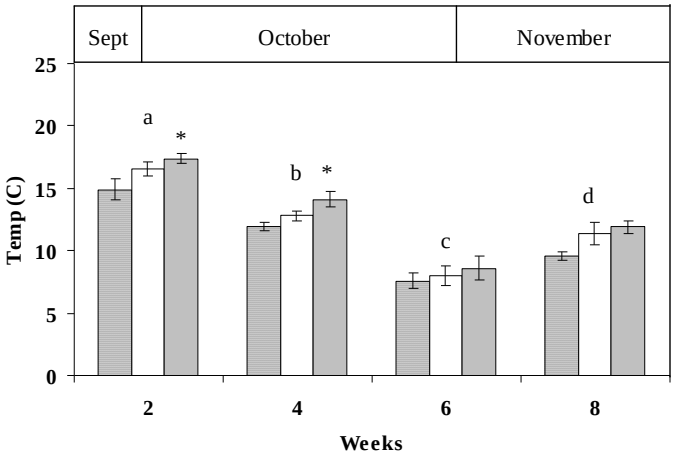
Seasonal changes in the mean temperature of the water and TSE during the experiments occurred (Fig. 5.1) which suggest a peak in temperature between July and September.

There were no significant ($P<0.05$) differences in water temperature between river water and effluent dosed tanks during any 2-weekly time period during either of Experiments 1 and 3 (Figures 5.1A and C). During Experiment 2, there were significant differences in temperature between river water and the 50% effluent dosed tank (weeks 0-2; $P<0.05$; weeks 2-4; $P<0.01$), with effluent dosed tanks warmer on each occasion (Figure 5.1B).

A)



B)



C)

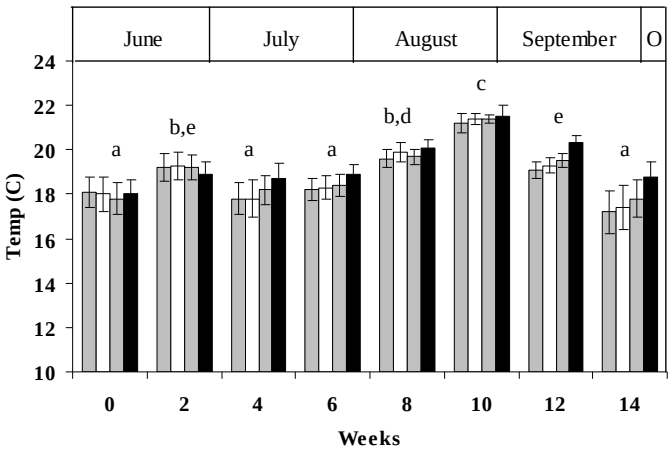


Figure 5.1.A-C. Mean water temperatures (±SE) over the course of the three experiments. A) Experiment 1, 14th April- 21st July 2003; B) Experiment 2; 19th September-14th November 2003; C) Experiment 3, 25th May- 13th September 2004. Different letters indicate statistical differences between time periods $^{a,b,c,d,e}P< 0.05$. Asterisks indicate statistical differences from the river water control $^{*}P < 0.05$. During experiment 2, only river water, 25%, and 50% effluent treatments were used.

5.2.2 Measured Concentrations of EDC`s

The results of the chemical analyses of known endocrine active components from water samples taken during Experiments 1 and 3 are summarized in Tables 5.1A-C. No water samples were taken during experiment 2.

During Experiment 1, all chemicals measured were found in full strength effluent (100%) on both sample occasions with the exception of 17α-ethynylestradiol which was measured at 1.5 ng/L in September only (Table 5.1A). None of these chemicals were found in river water at any time during Experiment 1. The mean concentration of E2 during this experiment was 109 ng/L and 1.7 fold higher than the mean concentration of E1 that was 65 ng/L in effluent. NP and ethoxylates were present in the low µg/L range, Bis-A at around 0.2 µg/L and OP ten-fold lower again.

During Experiment 3, the natural estrogens, 17β-estradiol (E2), and estrone (E1), were detected in all treatments (Table 5.1B) at all sampling points. The mean concentration of E2 in effluent was 73.0 ng/L; 2.8 fold higher than the mean concentration of E_1 (26.5 ng/L). The mean concentration of E2 in river water was 20.7 ng/L; 8.6 fold higher than the mean concentration of E_1 (26.5 ng/L). The synthetic oestrogen 17α-ethynylestradiol was detected in the effluent only (detection limit <1 ng/L) and was 0.9 ng/L on 3/8/4 and 2- fold higher on the 13/9/4. Of the organotin compounds analysed, TBT was measured at 125 ng/L in full effluent only, and on a single occasion only.

Table 5.1A Measured concentrations of Estrone (E1), 17β-estradiol (E2), 17α-ethynylestradiol (EE2), of Nonylphenol (NP), Nonylphenol mono- and di-ethoxylate (NP1EO and NP2EO), Octylphenol (OP) and Bisphenol A (Bis-A) in river water and 100% Treated Sewage Effluent during experiment 1. Experiment 1, 14th April-28th April 2003. Details of sampling protocols are in the text.

Sample Type	Month	E1 (ng/L)	E2 (ng/L)	EE2 (ng/L)	E2 equivalents (ng/L)
100% effluent	**June**	**60**	**207**	**<0.5**	**3.6**
	September	**70**	**10**	**1.5**	***NT**
	mean	**65**	**109**	***NS**	***NS**
River water	**June**	**<0.5**	**<0.5**	**<0.5**	**< detection limit**
	September	**<0.5**	**<0.5**	**<0.5**	***NT**
	mean	**<0.5**	**<0.5**	**<0.5**	***NS**
		NP (µg/L)	**NP1EO/2EO (µg/L)**	**OP (µg/L)**	**Bis-A (µg/L)**
100% effluent	**June**	**1.1**	**11**	**0.01**	**0.13**
	September	**0.92**	**4.2**	**0.03**	**0.23**
	mean	**1.04**	**7.6**	**0.02**	**0.18**
River water	**June**	**<0.02**	**<0.05**	**<0.02**	**<0.02**
	September	**<0.02**	**<0.05**	**<0.02**	**<0.02**
	mean	**<0.02**	**<0.05**	**<0.02**	**<0.02**

*NT; Not tested for. *NS; Not suitable for analysis

Table 5.1B Measured concentrations of 17β-estradiol (E2), Estrone (E1), 17α-ethynylestradiol (EE2), Dibutyltin (DBT) and Tributyltin (TBT) in river water and 100% Treated Sewage Effluent during experiment 3. Experiment 3, 25th May-8th June 2004.

Sample Type	Month	E1 (ng/L)	E2 (ng/L)	EE2 (ng/L)	DBT (ng/L)	TBT (ng/L)
River water	03/08/2004	2.7	19.0	<0.5	*NT	*NT
	17/08/2004	2.6	20.0	<0.5	<7.1	<8.2
	13/09/2004	1.8	23.0	<0.5	<20.0	<24.0
	mean	2.4	20.7	<0.5	*NS	*NS
100% effluent	17/08/2004	15.0	17.0	0.9	<13.0	125
	13/09/2004	38.0	129.0	1.8	<42.0	<52.0
	mean	26.5	73.0	1.4	*NS	*NS

*NT; Not tested for. *NS; Not suitable for analysis

Table 5.1C Measured concentrations of Nonylphenol (NP), Nonylphenol mono- and di-ethoxylate (NP1EO and NP2EO), Octylphenol (OP) and Bisphenol A (Bis-A) in river water and 100% Treated Sewage Effluent during experiment 3. Experiment 3, 25th May-8th June 2004.

Sample Type	Month	NP (ng/L)	NP1EO (ng/L)	NP2E0 (ng/L)	OP (ng/L)	Bis-A (ng/L)
River water	17/08/2004	<0.2	<1.3	1.3	<0.2	<0.2
100% effluent	17/08/2004	395.0	<0.2	647.0	7.0	1.4
River water	13/09/2004	0.4	<0.2	0.4	<0.2	<0.2
100% effluent	13/09/2004	18.0	<0.2	56.0	0.3	<0.2

5.2.3 Oestrogenicity of TetraMin Flake Food (Tetra GmbH)

The 2.5 kg tub of flaked fish food was tested using the yeast screen assay (see sections 2.5.4 and 2.5.4.1) was demonstrated to exhibit negligible oestrogenic activity by this methodology.

5.2.4 Mortality of *P. corneus* in TSE

Table 5.2 shows the mean percent survival from Experiments 1, 2, and 3 of *P. corneus* maintained in river water, 25%, 50%, or 100% effluent. Figure 5.2 shows the mean mortality rate per enclosure per day in each of the experiments.

Overall mean mortality in effluent dosed tanks was higher in spring-summer where between 40.3%-48.5% of the original number of effluent dosed snails remained alive at the end of the Experiment 1, compared with early to late summer where 57.4%-75.9% survived in Experiment 3 (Table 5.2). Mortality in effluent doses between autumn and winter (Expt 2) was very low compared to other seasons. Indeed, not until weeks 6-8 (31st October-14th November) did any mortality occur in 50% effluent. During experiment 1, between spring and mid-summer there were no significant differences in the number of surviving snails between river water and effluent dosed groups during any time period (Table 5.2). In Experiment 3, significantly ($P<0.05$) more snails survived in 25% effluent ($P<0.05$) compared to river water during mid-summer between weeks 6-8, 8-10, and 10-12. There were no other significant differences in the number of surviving snails between river water and effluent dosed groups during experiment 3. During experiment 2, (between Autumn and into winter) there were no significant ($P>0.05$) differences in the number of surviving snails between river water and effluent exposed snail groups.

Table 5.2 Percentage of snails remaining alive in River Water, 25%, 50% or 100% effluent. (A) Experiment 1, 14th April- 21st July 2003, (B) Experiment 2, 5th September-14th November 2003, (C) Experiment 3, 25th May- 13th September 2004. All snails were in river water in their own treatment tanks for a baseline period to assess reproductive output; (A) Experiment 1, 14th April-28th April 2003, (B) Experiment 2, 19th September-19th September 2003, (C) Experiment 3, 25th May-8th June 2004.

Experiment 1- 2003

Date	28th April	11th May	25th May	8th June	*22nd June	*6th July	*21st July
Weeks	-2-0	0-2	2-4	4-6	6-8	8-10	10-12
River water	100+/-0.0%	76.9+/-10.0%	72.9+/-10.0%	58.0+/-10.9%	71.2+/-10.6%	68.2+/-9.5%	65.2+/-7.6%
25%	100+/-0.0%	89.9+/-6.4%	88.4+/-6.1%	69.7+/-10.4%	69.7+/-18.4%	60.6+/-16.9%	48.5+/-13.2%
50%	100+/-0.0%	92.3+/-4.4%	87.7+/-3.8%	77.0+/-6.5%	66.8+/-4.5%	53.3+/-4.2%	47.3+/-6.9%
100%	100+/-0.0%	86.4+/-8.3%	81.8+/-8.3%	63.6+/-9.0%	45.5+/-10.1%	40.0+/-3.8%	40.3+/-3.8%

Experiment 2-2003

Date	19th Sept	3rd Oct	17th Oct	31st Oct	14th Nov
Weeks	-2-0	0-2	2-4	4-6	6-8
River water	100+/-0.0%	100+/-0.0%	95.3+/-3.2%	91.7+/-2.9%	88.6+/-1.4%
25%	100+/-0.0%	98.3+/-1.7%	96.7+/-2.1%	95.0+/-2.2%	91.7+/-3.1%
50%	100+/-0.0%	100+/-0.0%	100+/-0.0%	100+/-0.0%	96+/-2.2%

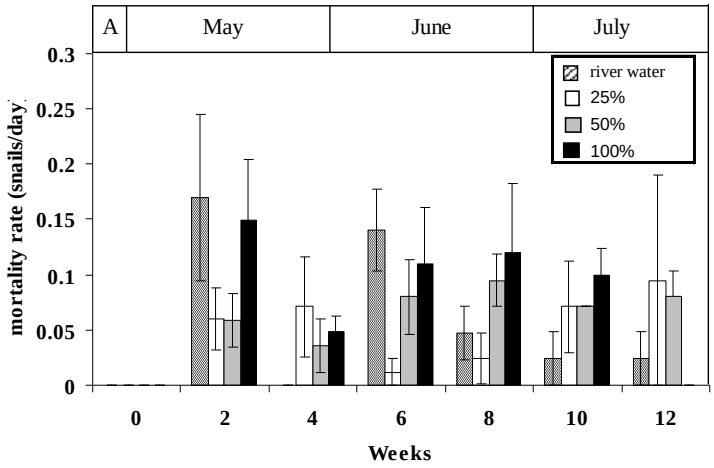
Experiment 3-2004

Date	8th June	22nd June	6th July	21st July	2nd August	16th August	30th August	13th Sept
Weeks	-2-0	0-2	2-4	4-6	6-8	8-10	10-12	12-14
River water	96.3+/-2.5%	85.2+/-5.0%	74.1+/-4.5%	66.7+/-4.5%	61.1+/-3.7%	61.1+/-3.8%	57.4+/-5.3%	55.6+/-5.3%
25%	98.0+/-1.9%	88.9+/-2.9%	83.3+/-2.5%	83.3+/-2.5%	83.3+/-2.5% †	83.3+/-2.5% †	79.6+/-3.4% †	75.9+/-5.3%
50%	90.7+/-3.4%	87.0+/-1.9%	79.6+/-3.4%	77.8+/-4.1%	66.7+/-5.0%	61.1+/-3.8%	57.4+/-4.5%	57.4+/-4.5%
100%	90.7+/-5.3%	87.0+/-5.3%	79.6+/-5.3%	72.2+/-5.8%	66.7+/-4.1%	63+/-3.7%	61.1+/-3.8%	57.4+/-5.3%

* 3 replicates per treatment remained after sampling on the 8th June 2003. † Indicates significantly different from river water.

The highest seasonal mortality rates in effluent and river water occurred in the Spring (May) of Experiment 1, with an average of between 0.06-0.15 snails per enclosure dieing in effluent dosed tanks daily over a period of 2 weeks (Fig 5.2A). By contrast, mortality rates in effluent were also lower in both Summer (Expt 3) and Autumn-winter (Expt 2) experiments, as they were in river water with means between 0.012-0.06 (experiment 3) and 0.012-0.024 (experiment 2) snails per enclosure dieing per 2 weeks (Fig.5.2C and B). Mortality rates in effluent, as in river water, decreased during both spring-summer and summer-autumn experiments, but increased from Autumn into winter with an average of 0.02 snails per enclosure dieing daily over a period of 2 weeks by the 14th November (experiment 2); a mortality rate still substantially lower than those recorded in effluent during springtime.

A)



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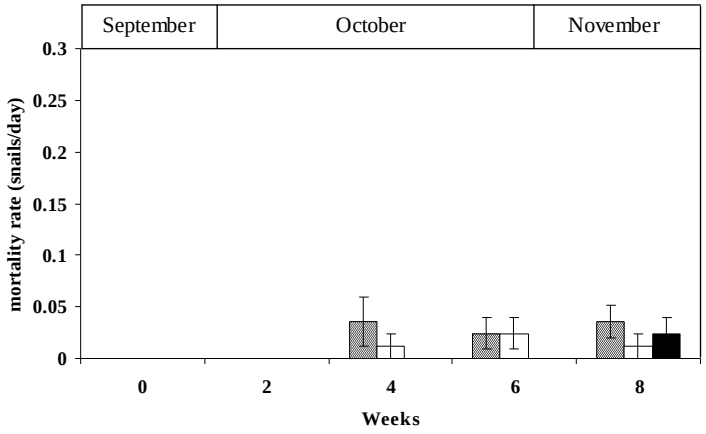

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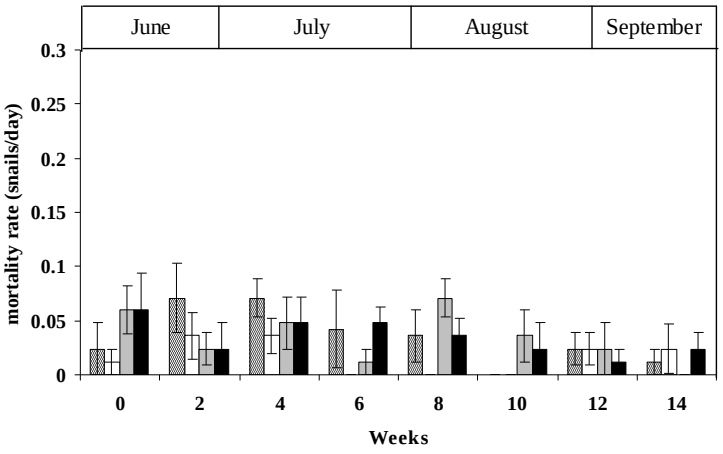


Figure 5.2 Mean mortality rate (+/- SE), expressed as the number of snails that died daily, averaged across replicate enclosures sampled at 2-weekly intervals from groups of *P. corneus* snails in river water, 25%, 50%, or 100% effluent (Experiment 1; 11 snails, Experiment 2; 10 snails, Experiment 3; 9 snails). A) Experiment 1; 14th April-21st July 2003, B) Experiment 2; 19th September-14th November 2003, and C) Experiment 3; 25th May- 13th September 2004.

5.2.5 Seasonal Effects of TSE on Growth of Snails

Figure 5.3 A-F shows the increase in mean shell length (mm) and weight (g) (with shells) of *P. corneus* maintained in river water, 25%, 50%, or 100% effluent during Experiments 1, 2, and 3.

In Experiment 1, from spring to mid-summer the mean size of snails (shell length) in 25% effluent during baseline measurements (28th April) was statistically ($P<0.05$) smaller than snails in river water. However, there were no differences in the mean size of snails (shell length) between river water and effluent exposed groups of snails at the end of the experiment (25.2 mm compared to 23.6-24.8 mm) (Figure 5.3.A.). However, conversely, the mean size of snails (weight with shells) in 25% effluent during baseline measurements (28th April) were almost significantly ($P=0.05$) larger than snails in river water. But by early summer (week 6) on the 8th June snails in 25% effluent was significantly ($P<0.05$) smaller than those in river water.There were differences in the mean weight of snails (weight with shells) between river water and effluent dosed groups at the end of experiment by mid summer on the 21st July (Figure 5.3.B.).

By the end of the Experiment 3, from early summer into Autumn, however, the shells of snails in effluent doses were significantly ($P<0.05$) larger than those in river water (24.1-24.9 mm compared to 23.1 mm) (Figure 5.3.E), and the mean weight of snails with shells in both 50% and 100% effluent doses were significantly ($p<0.05$) greater the mean weight of snails with shells in river water (Figure 4.3.F).

In Experiment 2, from autumn into winter the mean weight of snails with shells in 50% effluent during baseline measurements (19th September) were significantly ($P<0.05$) larger than snails in river water. By the time winter had arrived (14th November, 8-weeks later), the shells of snails in both effluent doses studied (25% and 50% effluent) were significantly ($P<0.05$) larger (22.2 mm and 24.3 mm, respectively) than those in river water (21.6 mm) (Figure 5.3C), and their weight with shells was also significantly larger than snails in river water (Figure 5.3D).

Experiment 1 Experiment 2 Experiment 3

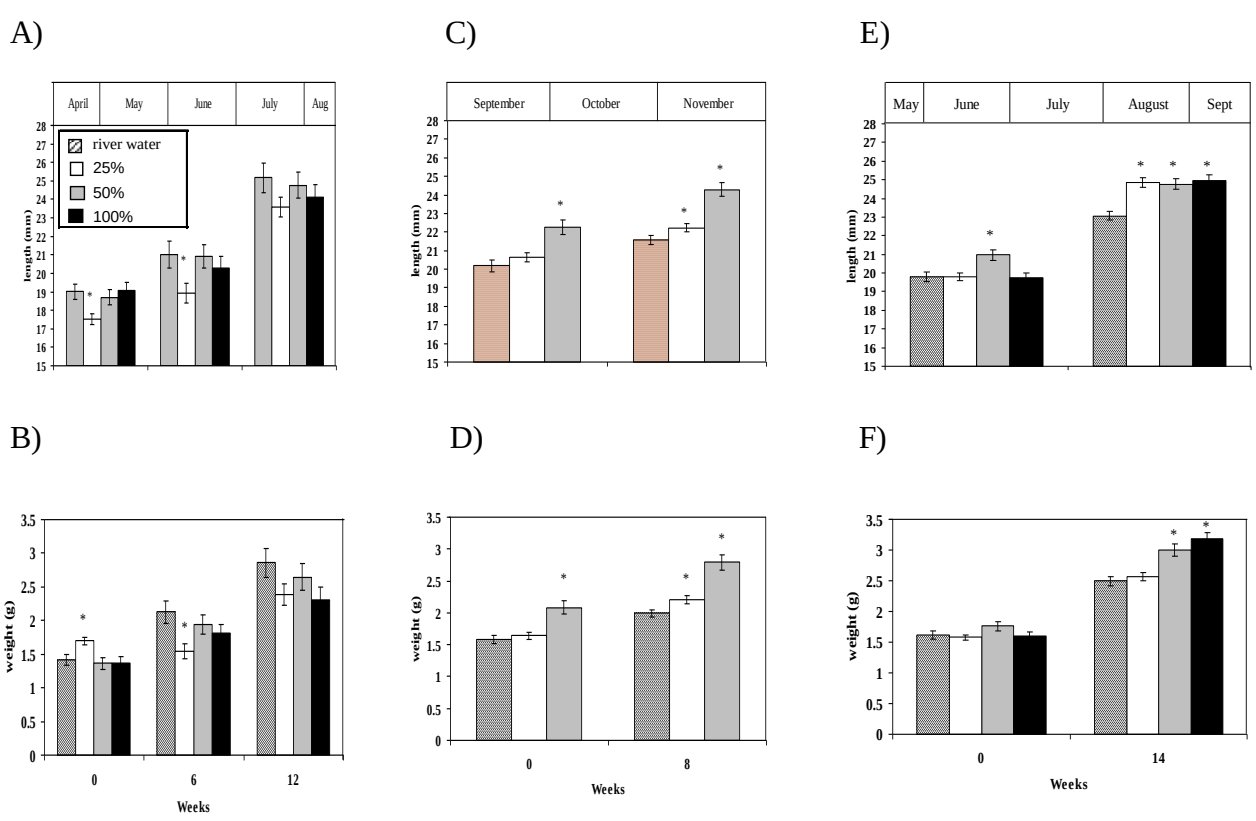


Figure 5.3 Increase in mean (±SE) shell length (A, C, E) and weight (B, D, F) of snails from groups (Experiment 1; 11 snails, Experiment 2; 10 snails, Experiment 3; 9 snails) of *P. corneus* snails in river water, 25%, 50%, or 100% effluent. Spring to mid-Summer time; Experiment 1, 14th April- 21st July 2003, Experiment 2, Autumn to Winter; 19th September-14th November 2003, and Experiment 3, early Summer to Autumn; 25th May- 13th September 2004. Asterisks indicate statistical differences from the river water control $^{*}P < 0.05$

Figure 5.4 shows the seasonal mean fold increase in shell length and weight (with shells) in river water, 25%, 50%, or 100% effluent across the enclosures throughout Experiments 1, 2, and 3.

In Experiment 1, in early summer by the 8th June (week 6) the mean fold-increase in weight gain of snails sampled from 25% effluent was significantly less ($P<0.05$) than the mean weight gain of snails from the river water tank. Overall, there were significant differences ($P<0.05$) in the mean fold increase in shell lengths between river water and effluent dosed (25% and 100%) tanks from snails measured at the end of experiment 3; an experiment that ran from early summer into Autumn (8th June-13th September). Despite this, there were no other significant differences in the overall mean fold-increase in shell lengths or weight gain of effluent-exposed snails compared with river water-exposed snails at any other time.

The seasonal pattern to growth seen in river water and effluent exposed groups of snails in which growth peaked by mid summer (increase in length and weight (with shell)), and then decreased towards winter, and was confirmed by the daily rate (mm/day) of increase in shell lengths, however, in effluent (particularly 50% and 100% effluent) snails failed to undergo the seasonal decrease in daily growth rate (g/day) seen in river exposed snails (data not shown). Statistical analysis was not appropriate, as only a single figure was calculated for each time period.

Overall, the rate of increase of length (mm/day) of snails shells` in effluent appears to have exceeded that in river water at all times of the season, with the possible exception of late spring to early summer (weeks 0-6), where growth of snails shells` in river water exceeded that in effluent (data not shown).

Seasonal increases in weight (with shells) (g/day) in all effluent exposed snails appeared to be less than in river water exposed snails until mid-summer, but greater in all effluent exposed snails from mid-summer onwards (data not shown).

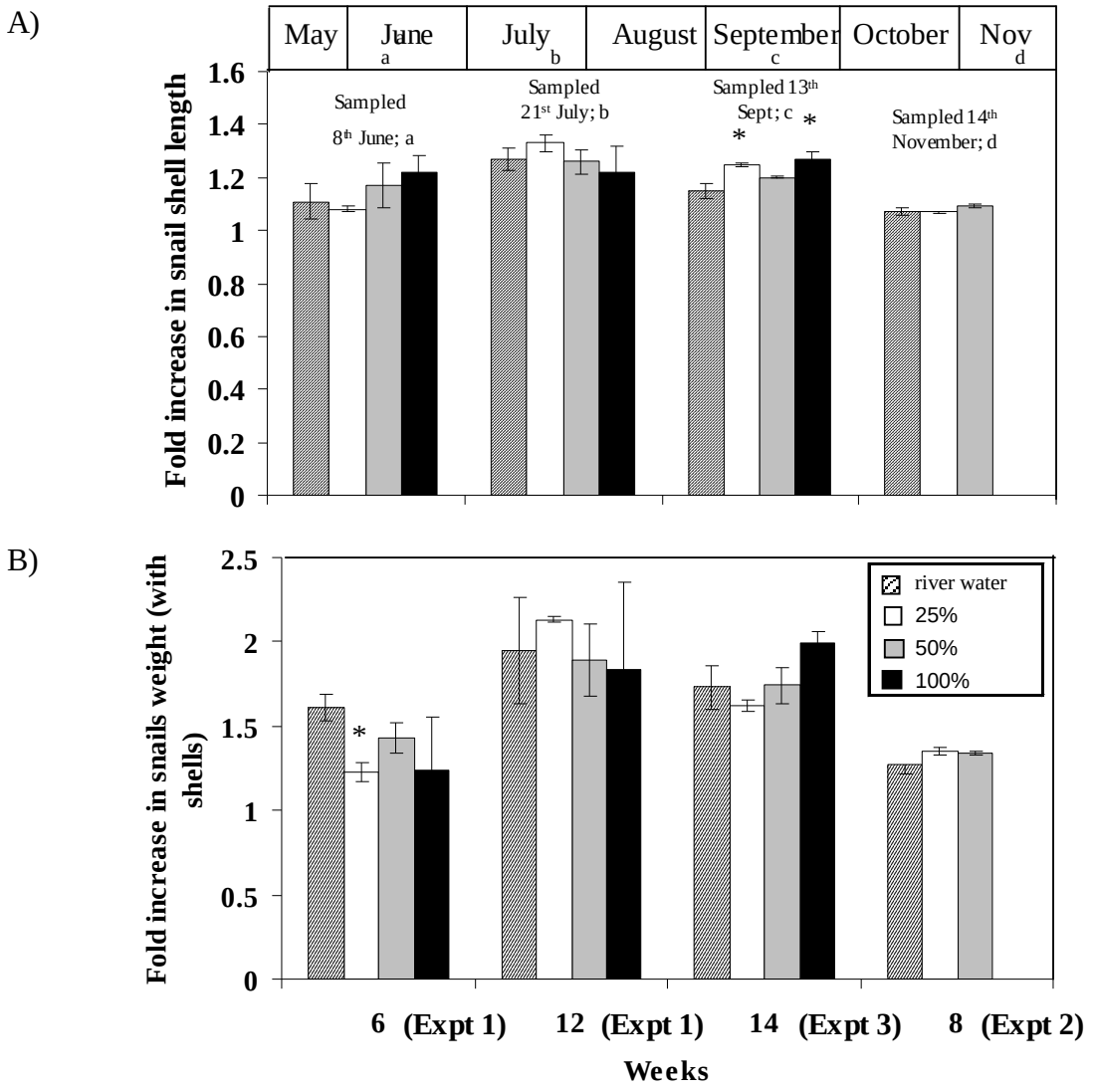


Figure 5.4 Seasonal mean fold increase in A) shell length (mm) and B) weight (mg) (with shells) from groups (Experiment 1; 11 snails, Experiment 2; 10 snails, Experiment 3; 9 snails) of *P. corneus* snails (±SE) in river water, 25%, 50%, or 100% effluent. In experiment 1, snails in each of 6 enclosures were measured at time zero and baseline measurements taken, at weeks 6 and 12, 3 enclosures were measured again to calculate mean fold increase values by comparison to mean baseline shell lengths for each enclosure. In experiments 2 and 3, replicate groups (each enclosure) were measured at the beginning and end of each experiment. Spring to mid-Summer time; Experiment 1, 12 weeks; 14th April- 21st July 2003, Experiment 2; Autumn to Winter; 8 weeks; 19th September-14th November 2003, and Experiment 3; early Summer to Autumn; 14 weeks; 25th May- 13th September 2004. Asterisks indicate statistical differences from the river water control $^{*}P < 0.05$.

Figure 5.5 shows the seasonal increase in snail weight (without shells) in river water, 25%, 50%, or 100% effluent during experiment 3.

In experiment 3, by Autumn on the 13th September there were significant ($p<0.05$) 87.6%, 81.5%, 95.1% increases (from 0.81 g to 1.52 g, 1.47 g, and 1.58 g respectively) in the mean weight (without shells) of snails in 25%, 50%, 100% effluent during this period, respectively, compared to a 54.6% (from 0.81 g to 1.34 g) in the mean weight (without shells) of snails in river water over the same period.

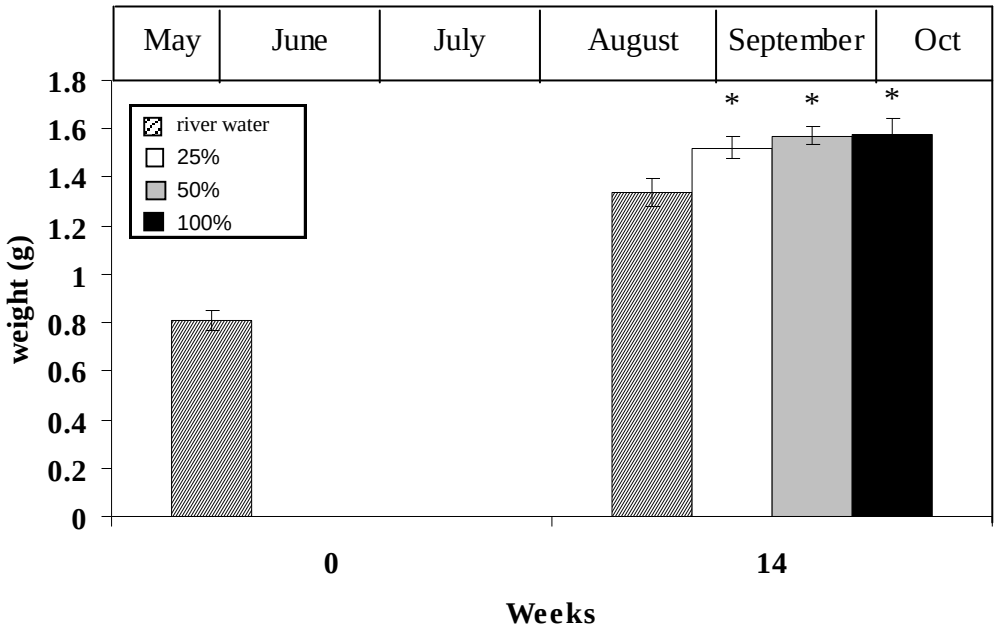


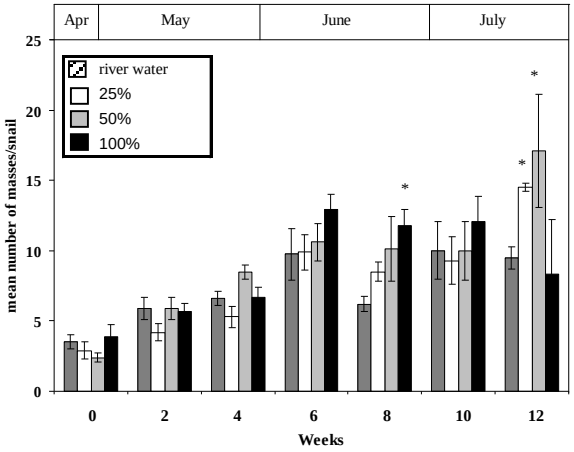
Figure 5.5 Seasonal increase in snail weight (without shells) from groups (Experiment 3; 9 snails) of *P. corneus* snails (±SE) in river water, 25%, 50%, or 100% effluent from early Summer to Autumn; 8th June- 13th September. Asterisks indicate statistical differences from the river water control $^{*}P < 0.05$.

5.2.6 Seasonal Effects upon Reproduction of TSE

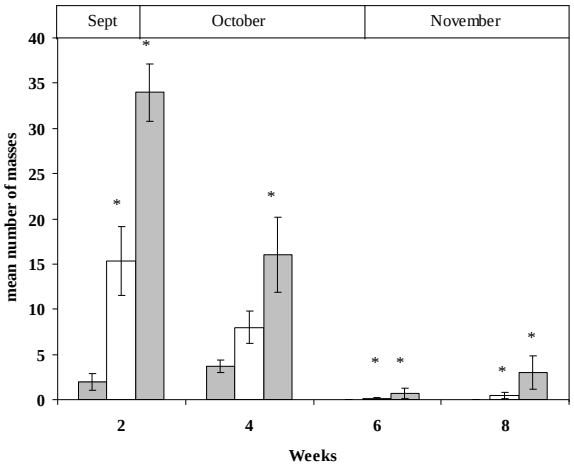
In Experiment 1, exposure to treated sewage effluent significantly increased mean egg mass production in the snails exposed to 100% effluent relative to snails in river water during early Summer (between 8th and 22nd June or weeks 6-8). Significant increases ($p<0.05$) were also seen in effluent by mid-Summer (10-12)(Figure 5.6A).

Similar effects were seen in Experiment 3 (Figure 5.6C), where exposure to 50 % effluent doses significantly increased egg mass production compared to snails in river water from early Summer into Autumn. Further, stimulatory effects were also observed in Experiment 2, during Autumn into mid-September (Figure 5.6B), where exposure to 25% and 50% (all weeks) effluent doses significantly increased egg mass production compared to snails in river water during a time period which followed on from Experiment 3 (one week gap between experiments; 13th September to 19th September). Indeed, effluent continued to be stimulatory into winter on the 14th November.

A)



B)



C)

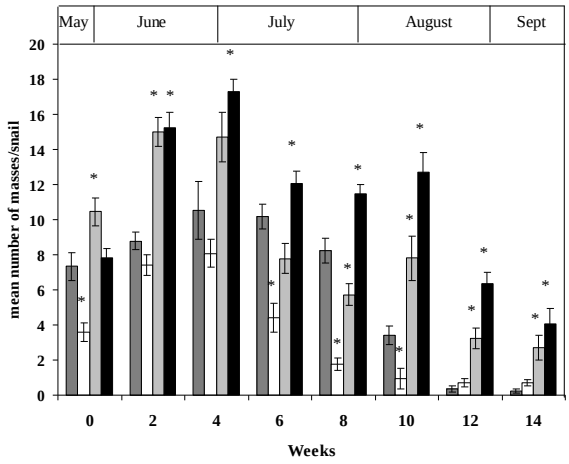


Figure 5.6 Mean number of masses per snail (±SE) per enclosure from groups of *P. corneus* snails in river water, 25%, 50%, and 100% effluent (Experiment 1; 11 snails per group, Experiment 2; 10 snails per group, Experiment 3; 9 snails per group). Spring to mid-Summer time; (A) Experiment 1, 14th April- 21st July 2003, Autumn to Winter; (B) Experiment 2; 19th September-14th November 2003; (C) and early Summer to Autumn; Experiment 3; 25th May- 13th September 2004. Asterisks indicate statistical differences from the river water control $^{*}P < 0.05$.

The cumulative mean number of masses produced over the course of the Experiment 3 (from 8th June and 13th September) was only statistically significantly different ($P<0.05$) from the reference river water tank in the highest dose tested (100% effluent) by the end of the experiment (Figure 5.7). The dose-response curve was non-monotonic, with the 50% effluent dose causing a small (but statistically non-significant) decrease in mean egg mass production relative to the river water, and the 25% and 100% effluent doses showing an increase in mean egg mass production relative to the river water control.

This trend was apparent after 2 weeks exposure (during early Summer between the 8th and 22nd June) when the 25% and 100% effluent treatments caused non-significant ($P>0.05$) stimulatory effects (3.2-fold and 3.0-fold, respectively) on mean egg mass production relative to the river water and 50% effluent doses; a pattern that remained for a further 4 weeks to mid-Summer on the 21st July. After 8 weeks exposure (2nd August) the 50% effluent dose appeared inhibitory whereas the relationship between river water and 25% effluent remained unchanged (continued to the end of the experiment). The 100% effluent dose, however, continued to cause a stimulatory effect on mean egg mass production per snail right through to the end of the experiment in Autumn on the 13th September (week 14), by which time egg mass production in the other treatments had largely stopped. Indeed, the cumulative fold increase in mean egg masses produced per snail had increased by >11fold above original baseline values and 1.6-fold ($P<0.05$) relative to the river water.

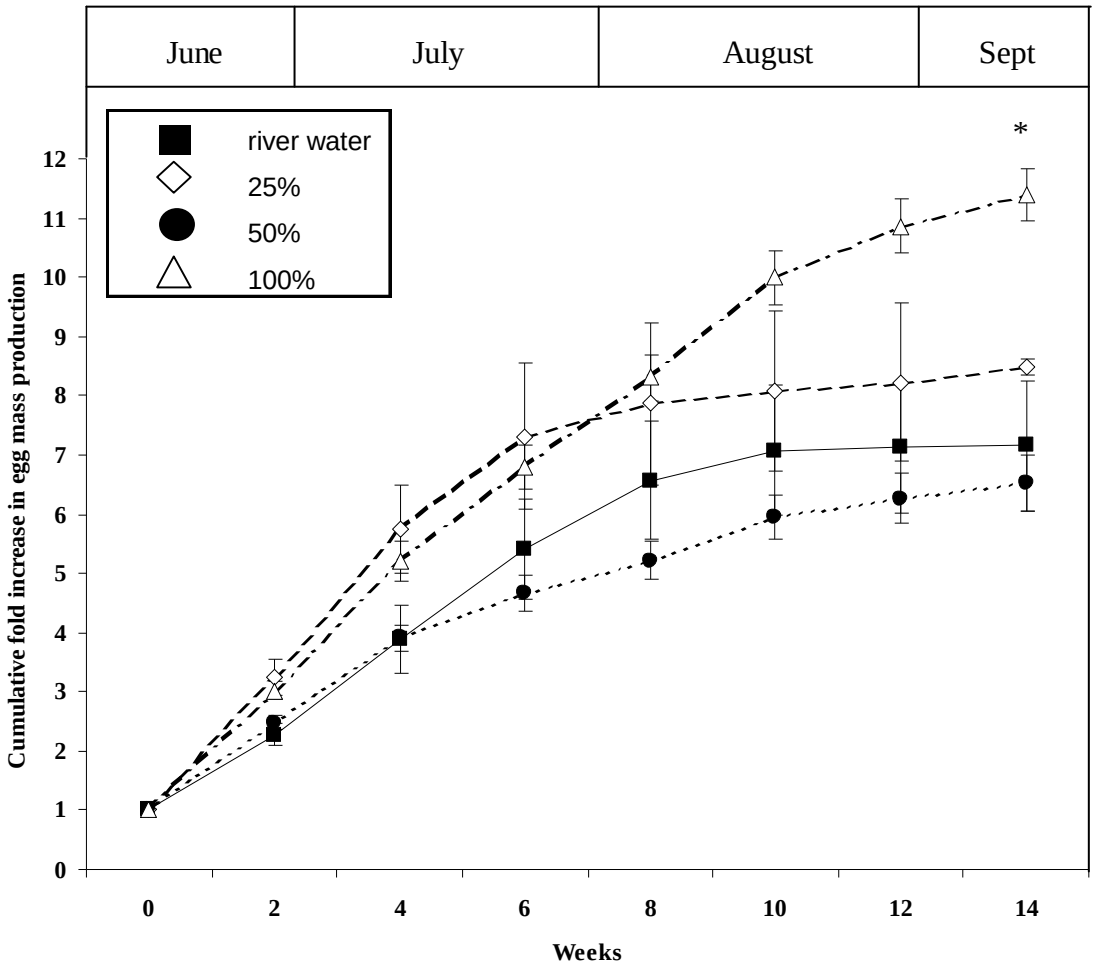
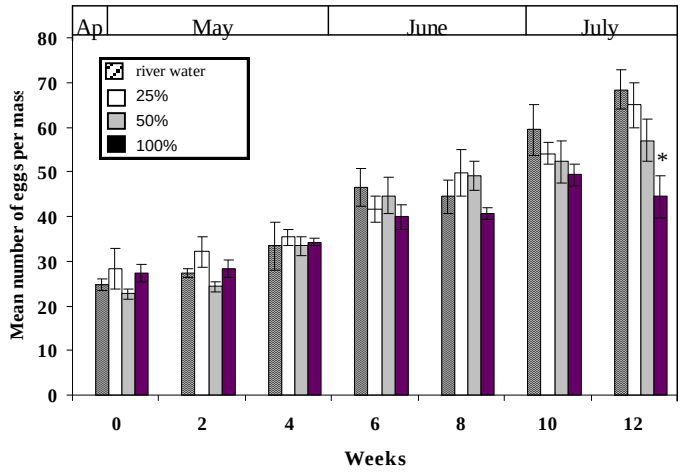


Figure 5.7 Cumulative mean number of egg masses per snail (+/- SE) produced by groups of *P. corneus* exposed to river water, 25 %, 50%, or 100% treated sewage effluent. (Experiment 3, 8th June- 13th September 2004) Values are expressed as fold induction above baseline values where all snails where in river water before commencement of effluent treatments (weeks –2 to 0; 24th May-8th June). Asterisks indicate statistical differences from river water control $^{*}P < 0.05$.

Figure 5.8 shows the effect of effluent on the number of eggs per mass during Experiments 1, 2, and 3. Overall, there were significant ($P<0.05$) decreases in the mean number of eggs per mass in snails exposed to 100% effluent relative to river water during both early and mid-Summer, with a two-week period in Autumn where the opposite effect with 50% effluent was observed.

During Experiment 1, exposure to treated sewage effluent significantly ($P<0.05$) reduced the mean number of eggs per mass in snails exposed to 100% effluent relative to river water during mid-Summer (between the 6th and 21st July or weeks 10-12). This time period almost coincided with the significant decreases in the mean number of eggs per mass observed in the 100% effluent exposed in Experiment 3 between the 8th June and 21st July (Figure 5.8; A and C). In contrast, the opposite effect on egg number per was observed during the first 2 weeks of Experiment 3, where there was a significant ($P<0.05$) increase in the number of eggs per mass in both 50% and 100% effluent doses compared to the river water. In Autumn, between the 3rd and 17th October exposure to 50% treated sewage effluent significantly ($P<0.05$) increased the mean number of eggs per mass relative to egg masses in river water (Figure 5.8; B).

A)



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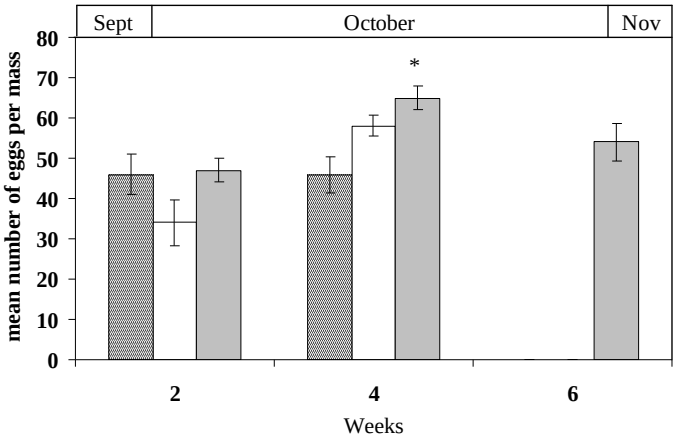


C)

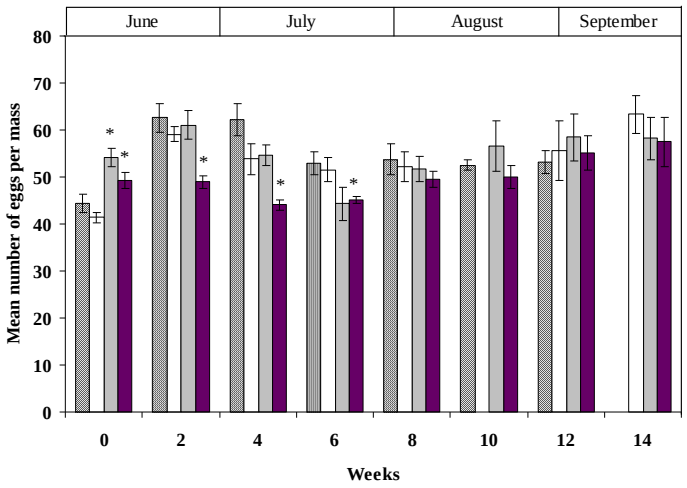
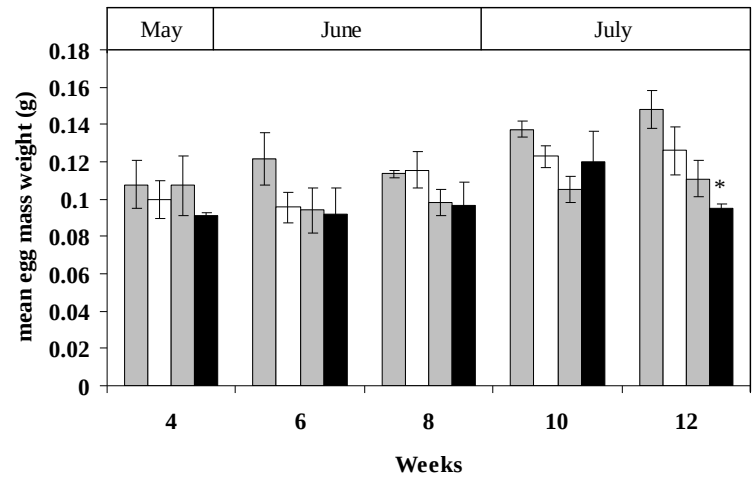


Figure 5.8 Mean number of eggs per mass (±SE) per enclosure from groups of *P. corneus* snails in river water, 25%, 50%, and 100% effluent (Experiment 1; 11 snails per group, Experiment 2; 10 snails per group, Experiment 3; 9 snails per group). The eggs were counted from the masses presented in Figure 4.8 (A) Experiment 1, 14th April- 21st July 2003, (B) Experiment 2, 19th September-14th November 2003, (C) Experiment 3, 25th May- 13th September 2004. Asterisks indicate statistical differences from the river water control $^{*}P < 0.05$.

Figure 5.9 shows the combined seasonal trend in the mean weight of egg masses from Experiments 1, 2, and 3 in *P. corneus* maintained in river water or a graded concentration (25%, 50%, or 100%) of TSE between May and November in 2-week intervals.

In all experiments, exposure to effluent resulted in differential effects on weight of individual egg masses produced by snails that was time dependant. During Experiment 1, there were no statistically significant differences in the weight of individual egg masses between river water and effluent exposed groups of snails until mid Summer between the 6th and 21st July (Figure 5.9; A). At this time, there was a dose related decrease in individual egg mass weight compared to those in river water (100% effluent significant; $P < 0.05$). In Experiment 3, there were significant ($P < 0.05$) decreases in mean weight of individual egg masses of effluent exposed snails compared to river water during consecutive two weekly periods from the early summer on 22nd June and mid Summer on the 2nd August (Figure 5.9; C). From mid Summer (on the 16th August into Autumn on the 13th September) the mean weight of individual egg masses in all effluent doses increased relative to those in the river water, which underwent a seasonal decrease. These differences, however, were not statistically significant. In contrast, during Experiment 2, there was a significant ($p<0.05$) decrease in individual egg mass weight in 25% and 50% effluent compared to those in river water in autumn between the 19th September and 3rd October (Figure 5.9; B).

A)



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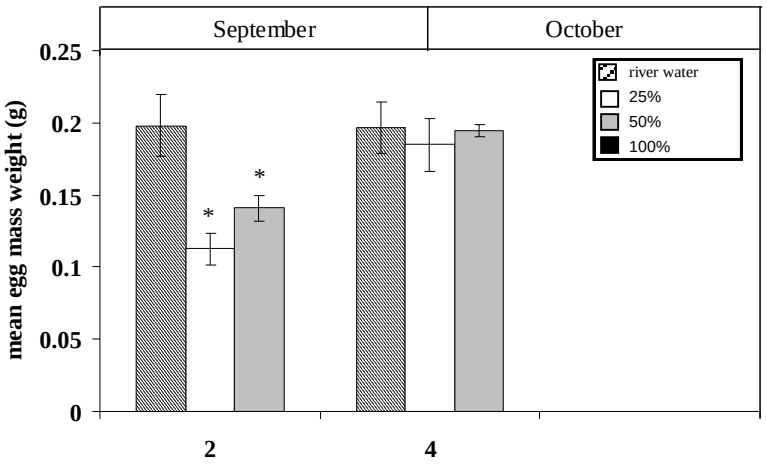

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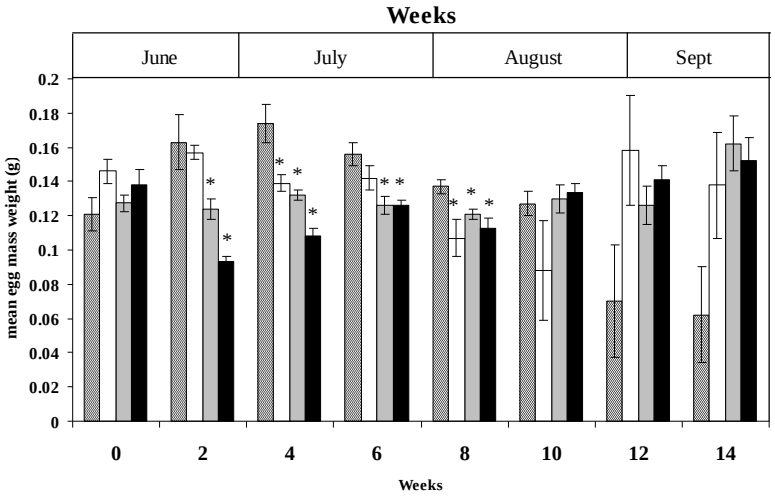


Figure 5.9 Mean weight of egg masses (±SE) per enclosure from groups (Experiment 1; 11 snails per group, Experiment 2; 10 snails per group, Experiment 3; 9 snails per group) of *P. corneus* snails in river water, 25%, 50%, and 100% effluent. (A) Experiment 1, 14th April- 21st July 2003, (B) Experiment 2, 19th September-14th November 2003, (C) Experiment 3, 25th May- 13th September 2004. Asterisks indicate statistical differences from the river water control $^{*}P < 0.05$.

Figure 5.10 shows the combined seasonal trend in the cumulative mean weight of egg masses from Experiment 3 in *P. corneus* maintained in river water or a graded concentration (25%, 50%, or 100%) of TSE between June and November in 2-week intervals.

Exposure to treated sewage effluent had slight stimulatory effects on the cumulative mean total weight of egg masses produced per snail in the 100% effluent treatment from mid Summer to Autumn (2nd July to13th September or weeks 8 to 14). This increase in the mean total weight of egg masses was not significant at the 95% level however (P=0.14), even though the mean total egg mass weight produced per snail in the 100% effluent dose had increased 1.2-fold relative to the river water by the end of Experiment 3. Both the 25% and 50% effluent showed nearly identical but non-significant decreases in the mean total egg mass weight in the same experiment, from mid Summer on the 21st July (week 6) to the end of the experiment.

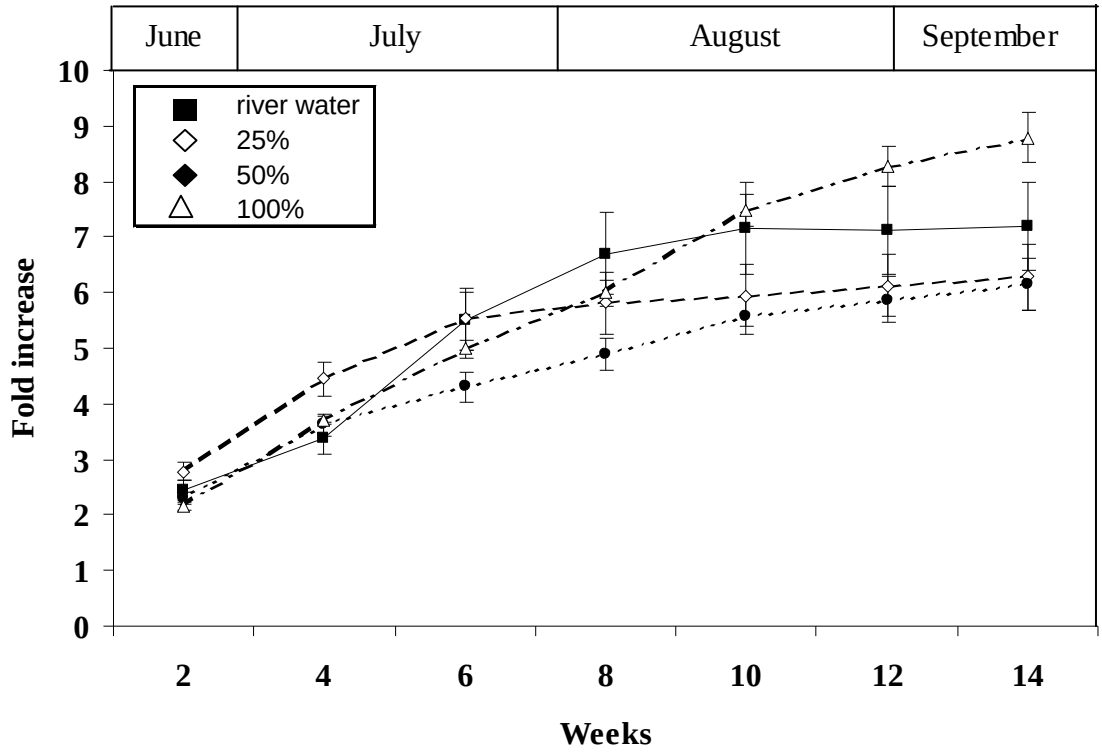


Figure 5.10 Cumulative mean weight of egg masses (±SE) per enclosure from groups of *P. corneus* snails in river water, 25%, 50%, or 100% effluent (Experiment 3; 9 snails per group). Experiment 3, 25th May- 13th September 2004. Asterisks indicate statistical differences from the river water control $^{*}P < 0.05$.

5.2.6.1 The Effects of Methodology on the ability of Eggs to Hatch, and Hatchability of eggs

Tables 5.3A-E and 5.4A-C illustrate the hatchability of eggs from egg masses collected from each of the enclosures during experiments 1 and 3.

Although hatching was highly variable between enclosures (for example, week 2-4; ranging between 12.5%-99.7% hatch), at all time periods, there appeared to be no pattern to the ability of eggs to hatch due to the time taken to process and distribute egg masses into cell culture plates.

Further, when analysed by parametric correlation analysis (though data was non-parametric), no relationship between the number of masses (data not shown) in a replicate (one 20 ml universal) and the ability of eggs to hatch using my methods at any time was observed.

In Experiment 1, eggs in all treatments during weeks 4-6 hatched significantly ($P<0.05$) less than those in weeks 2-4, but not significantly ($P>0.05$) less well than in any of the following fortnightly periods (Tables 5.3A-E). In weeks 2-4, eggs in effluent (all effluent doses tested) hatched significantly ($P<0.05$) less than those in river water; their ability to hatch was inversely proportionally to effluent concentation (Table 5.3A).

During Experiment 3, where assessed, eggs in all treatments hatched significantly ($P<0.01$) more during the experiment than during the baseline time period (Tables 5.4A-C). However, there were no significant ($P>0.05$) differences in the ability of eggs to hatch in river water compared to effluent doses tested during any time period tested (Tables 5.4A-C).

Tables 5.3A-E Percentage hatchability of eggs in egg masses from each of the enclosures over the time taken to process and distribute egg masses from universals into cell culture plates; egg masses were those collected from groups of *P. corneus* (11 snails per group) in river water, 25%, 50%, and 100% effluent during Experiment 1. Spring to mid-Summer time; 12 weeks- 14th April- 21st July 2003.

A) Weeks 2-4[b]

	Enclosure:					
	1st	2nd	3rd	4th	5th	mean
Treatment	% Hatch:					
River water	85.6%	99.7%	90.9%	92.7%	92.7%	92.3%
25% Effluent	89.8%	77.9%	81.1%	57.9%		76.7%*
50% Effluent	12.5%	69.8%	59.5%	33.3%	70.1%	49%*
100% Effluent	25.1%	51.6%	85.9%	18.0%		45.2%*

B) Weeks 4-6[a,c]

	Enclosure:					
	1st	2nd	3rd	4th	5th	mean
Treatment	% Hatch:					
River water	80.7%	36.3%	49.0%			55.3%
25% Effluent	95.0%	61.0%	86.3%	19.0%		65.3%
50% Effluent	81.8%	19.2%	22.5%	8.5%		33.0%
100% Effluent	74.8%	21.9%	38.3%	10.4%	17.2%	32.5%

Different letters indicate statistical between time period differences [a,b]P< 0.05. Asterisks indicate statistical differences from the control *P < 0.05.

C) Weeks 6-8[b,c]

	Enclosure:			
	1st	2nd	3rd	mean
Treatment	%Hatch:			
River water	79.4%	89.2%	82.0%	83.5%
25% Effluent	76.5%	65.7%	86.6%	76.3%
50% Effluent	95.3%	26.7%	84.9%	69.0%
100% Effluent	49.9%	81.7%	91.4%	74.3%

D) Weeks 8-10[b,c]

	Enclosure:			
	1st	2nd	3rd	mean
Treatment	% Hatch:			
River water	87.5%	86.5%	60.8%	58.6%
25% Effluent	80.5%	89.3%	85.9%	65.5%
50% Effluent	55.9%	71.5%	65.7%	89.2%
100% Effluent	58.6%	65.5%	89.2%	71.1%

E) Weeks 10-12[b,c]

	Enclosure:			mean
	1st	2nd	3rd	
Treatment	% Hatch:			
River water	66.8%	53.5%	88.3%	69.5%
25% Effluent	73.8%	65.1%	51.9%	63.6%
50% Effluent	74.2%	57.3%	94.5%	75.3%
100% Effluent	91.6%	74.9%	83.2%	83.2%

Tables 5.4A-C Percentage hatchability of eggs in egg masses from each of the enclosures over the time taken to process and distribute egg masses from universals into cell culture plates; egg masses were those collected from groups of *P. corneus* (9 snails per group) in river water, 25%, 50%, and 100% effluent during Experiment 3. Early summer to autumn; 14 weeks - 25th May- 13th September 2004.

A) Weeks –2-0[a]

			Enclosure:				mean
	1st	2nd	3rd	4th	5th	6th	
Treatment			% Hatch:				
River water	97.4%	98.4%	96.1%	100.0%	99.8%	90.6%	97.1%
25% effluent	96.7%	93.8%	75.1%	89.5%	96.2%	97.1%	91.4%
50% effluent	95.2%	98.8%	95.3%	98.9%	98.1%	100.0%	97.7%
100% effluent	99.5%	99.5%	91.7%	99.3%	94.1%		96.8%

Different letters indicate between time period statistical differences [a,b]$P < 0.05$.

Asterisks indicate statistical differences from the control $^{*}P < 0.05$.

B) Weeks 0-2[b]

			Enclosure:				mean
	1st	2nd	3rd	4th	5th	6th	
Treatment			% Hatch:				
River water	100.0%	99.1%	99.3%	100.0%	98.7%	99.8%	99.5%
25% Effluent	99.0%	100.0%	96.6%	100.0%	99.2%	99.3%	99.0%
50% Effluent	99.8%	100.0%	99.0%	100.0%	99.2%		99.6%
100% Effluent	100.0%	99.3%	98.1%	97.6%	93.9%		97.8%

C) Weeks 12-14[b]

			Enclosure:				mean
	1st	2nd	3rd	4th	5th	6th	
Treatment			% Hatch:				
River water	100.0%	97.9%	97.8%				98.6%
25% Effluent	100.0%	99.2%	99.5%	99.5%			99.6%
50% Effluent	99.3%	100.0%	99.3%	100.0%	100.0%	94.1%	98.7%
100% Effluent	99.1%	95.4%	99.5%	98.9%	97.0%	99.5%	98.2%

5.2.6.2 Seasonal change in Size of Albumen Glands

Figure 5.11 shows the trend in the mean weight of albumen glands from Experiments 1 and 3 in *P. corneus* maintained in river water or a graded concentration (25%, 50%, or 100%) of TSE between June and September.

A clear relationship between the weight of the albumen gland and total body weight of snails was not found. However, on one sampling occasion following a near doubling of reproduction in 25% and 50% effluent dosed snails (see Figure 5.7A; week 12) albumen gland weight decreased in 25% and 50% effluent dosed snails compared to snails from other treatments. Albumen gland weight generally increased inline with total body weight of the snails as they grew throughout the season. However, the weight of an albumen gland was highly variable in snails of any size class when expressed as a proportion of its total weight.

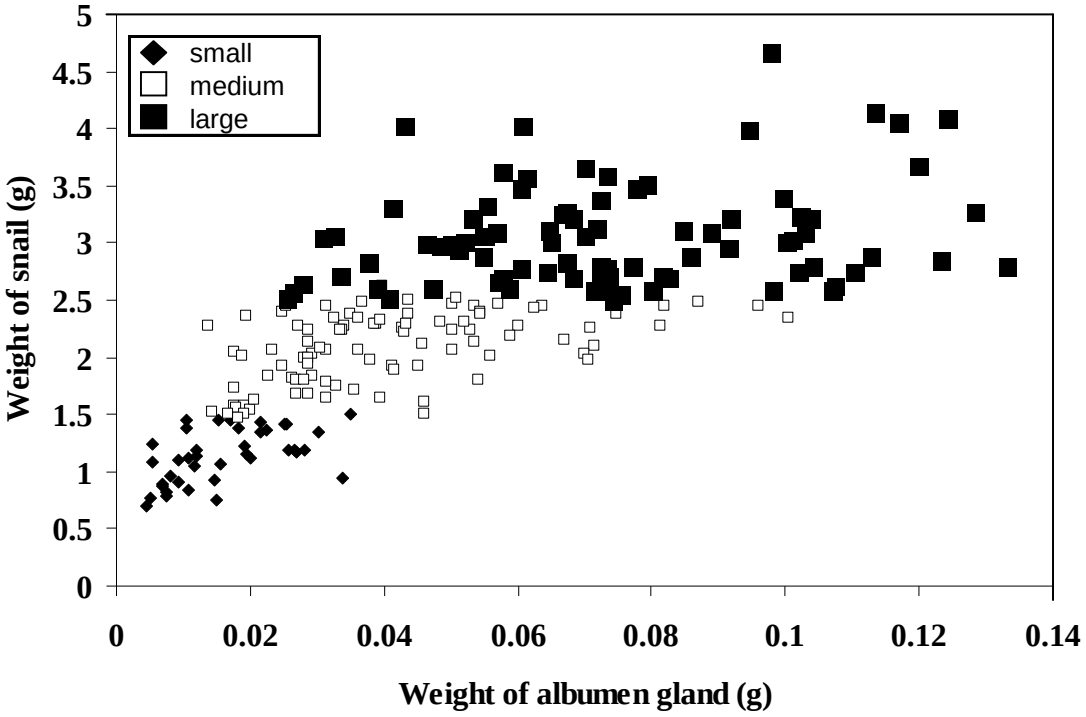


Figure 5.11 Individual snails total body weight and weight of its albumen gland from *P. corneus* in river water, 25%, 50%, or 100% effluent extracted from small (0.5-1.5 g), medium (1.5-2.5 g), or large (>2.5g) sized snails. Snails from all treatment groups and from all time periods from experiments 1 (14th April- 21st July 2003) and 3 (25th May- 13th September 2004) were used in analysis.

5.2.7 Glycogen Phosphorylase assay validation

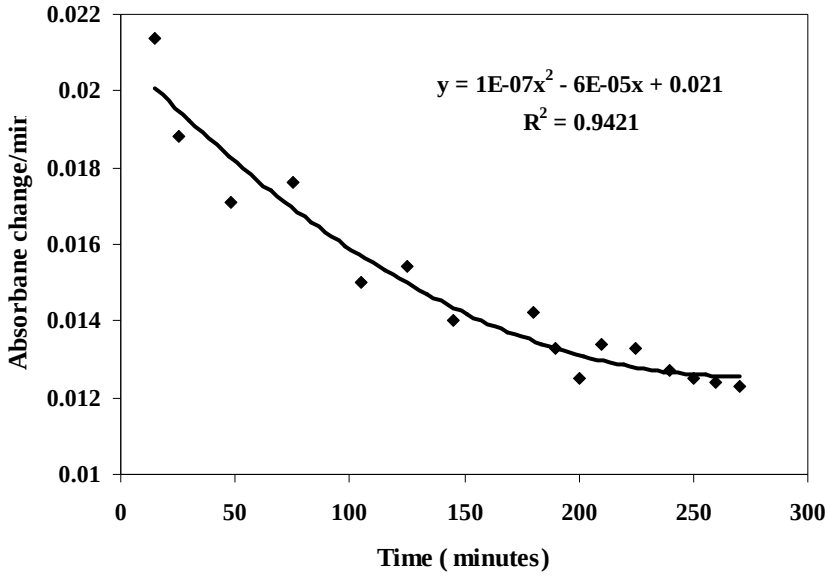
5.2.7.1 Stability of Glycogen Phosphorylase Enzyme in Homogenates

Figure 5.12A and B illustrates the degradation of glycogen phosphorylase enzyme activity from a single snail (*P. corneus*) homogenate sample after spinning.

The initial absorbance change per minute given by 50 µl of supernatant in this assay system was 0.021 units; this value was obtained 15 minutes after centrifugation was complete (Figure 5.12A). The change in absorbance of the supernatant was observed for a total of 300 minutes after centrifugation was complete by this method. The final absorbance change per minute value was 0.012, or 57.4% of the initial value.

The initial absorbance change per minute given by 50 µl of supernatant by the second the method of centrifugation was 0.012 units, this value was obtained just 7 minutes after centrifugstion was complete (Figure 5.12B). The change in absorbance of the supernatant was observed for a total of 400 minutes after centrifugation was complete by this method. The final absorbance change per minute value was 0.004, or 19.0% of the initial value.

A)



B)

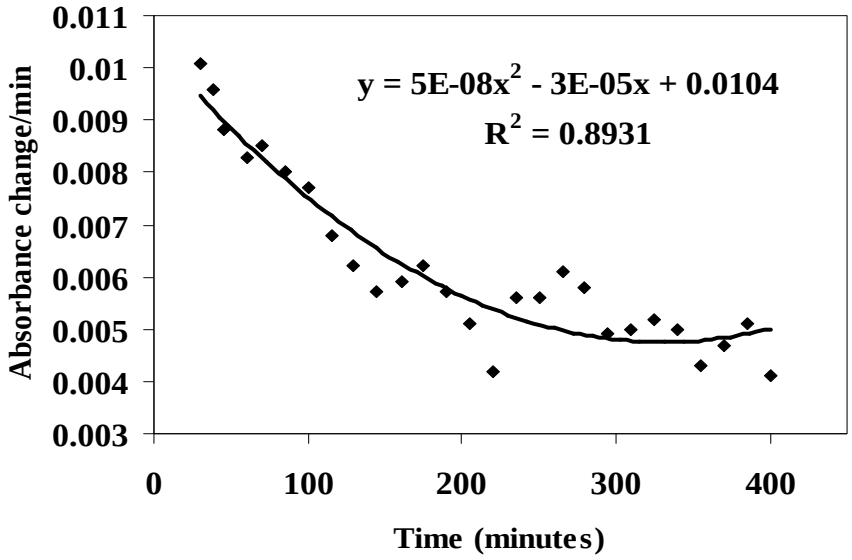


Figure 5.12 Degradation of glycogen phosphorylase enzyme activity from a single snail (*P. corneus*) homogenate sample A) after spinning at 3, 000g for 90 minutes, and B) after spinning for 13, 100 g for 20 minutes. Time zero represents when the snail homogenate sample had completed centrifugation. The snail homogenate was prepared on ice at º0 C, as described in section 3.2.1.

5.2.7.2 Rigour of Spinning; Repeatabilty of Sampling Supernatant

Figure 5.13 illustrates the repeatability of sampling from a preparation of snail homogenate spun to produce a clear supernatant (see section 3.2.2).

The repeated sampling of a single spun homogenate (90 minutes at 3, 000 g at 4 C) produced a mean change in absorbance of 0.0501 +/-0.0003 units per 2 minutes. The first value obtained was 0.0495 units, and final value obtained was a 0.0502 change in absorbance units, with no general trend in change of absorbance units per 2 minutes, indicating no loss of enzyme activity during this period of testing the supernatant.

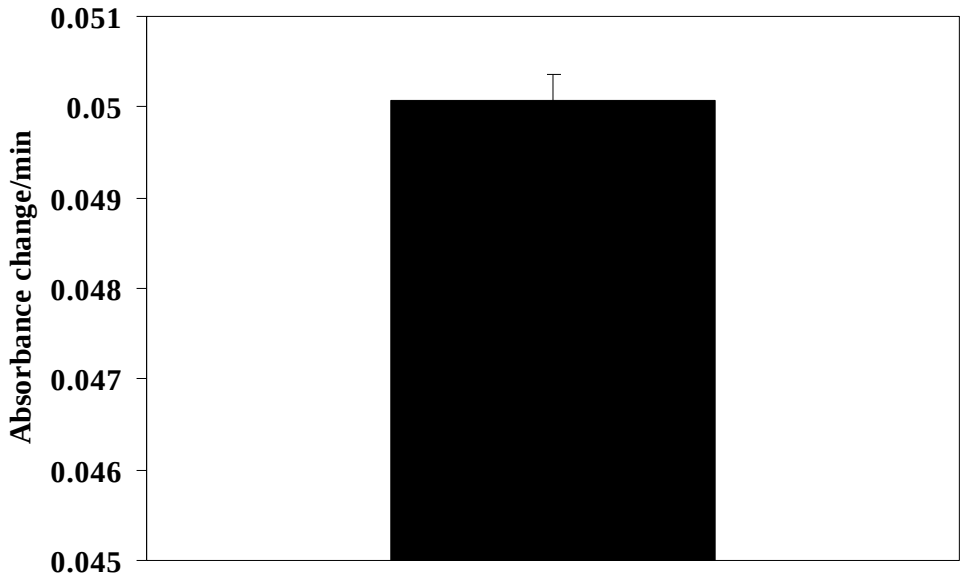


Figure 5.13 Repeatability of sampling from snail supernatant produced by a standard methodology to produce a clear supernatant from a snail body homogenate, the supernatant was sampled 20 times and the activity of the glycogen phosphorylase enzyme contained within used to assess accuracy of pipetting of supernatant.

5.2.8 Glycogen Phosphorylase activity of mantle tissue

Figure 5.14 shows the seasonal trend in the glycogen phosphorylase activity of the mantle tissues from Experiment 1 in *Planorbarius corneus* maintained in river water or 100% TSE between Spring on the 28th April and mid Summer on the 21st July in 6-week intervals. Overall, in Experiment 1, exposure to treated sewage effluent resulted in differential effects on glycogen phosphorylase activity that was time dependant.

There was a sharp increase in the activity of this enzyme between the start of the experiment in late Spring on the 11th May and early Summer on the 8th June in river water exposed snails from 1.9 x 10^{-5} to 3.0 x 10^{-5} µmole G1P/min/mg protein, a 1.6 fold increase. Activity in 100% effluent treated snails was almost exactly equal (2.9 x 10^{-5} µmole G1P/min/mg protein) to those in river water at this time in early Summer. By late Summer on the 21st July, enzyme activity from snails in river water had not significantly changed from those sampled on the 8th June (3.2 x 10^{-5} µmole G1P/min/mg protein) but was significantly less ($P<0.02$) in snails sampled from the 100% effluent treatment. Indeed, enzyme activity was 2.47 x 10^{-5} µmole G1P/min/mg protein in snails sampled from the 100% effluent treatment; a drop in activity of 23% compared to snails sampled from river water.

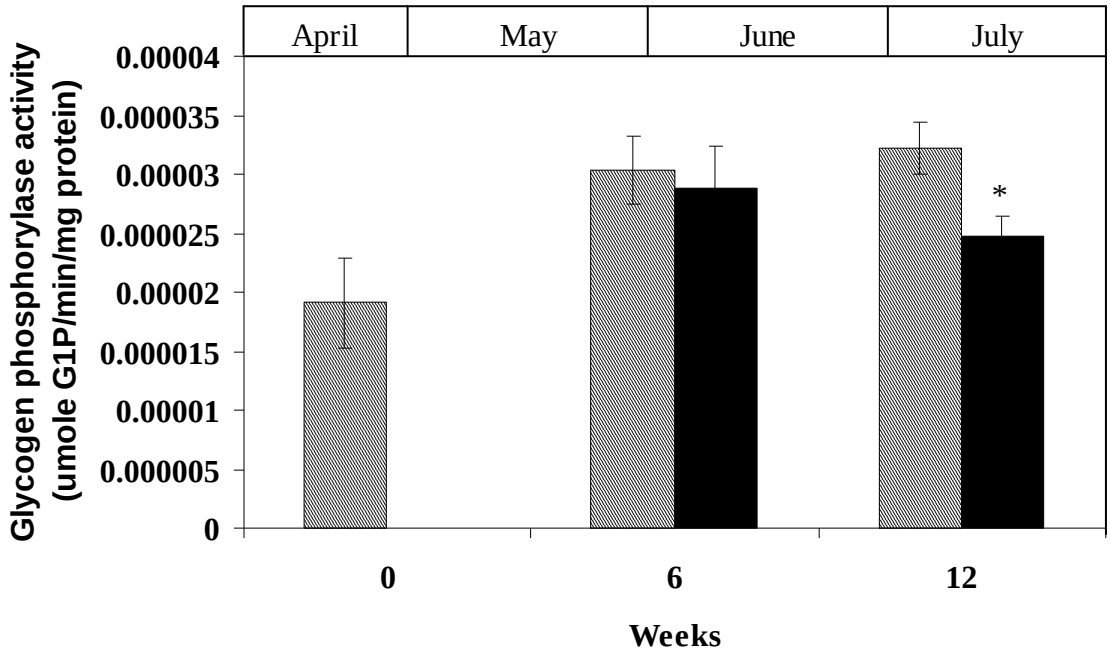


Figure 5.14 Individual snails glycogen phosphorylase activity from *P. corneus* in river water (black and white stripped bars) or 100% (black bars) effluent extracted from the bodies of each snail. Snails from experiment 1 only were used for analysis (14th April- 21st July 2003). Asterisks indicate statistical differences from the river water control $^{*}P < 0.05$.

5.2.9 Protein content of snail mantle tissue

Figure 5.15 shows the seasonal trend in the protein content of the mantle tissues from Experiment 1 in *P. corneus* maintained in river water or 100% TSE between Spring on the 28th April and mid Summer on the 21st July in 6-week intervals. Overall in river water, there was a significant decline in the protein content of snail mantle tissue between late Spring time on the 28th April and snails sampled mid Summer on the 21st July at the end of the experiment. In river water, between late Spring on the 28th April and early Summer on the 8th June there was a slight but non-significant decrease in protein content of snail mantle tissue from 4.6 to 4.3 mg protein/ gm body weight; a 6% decrease in protein content. By mid Summer on the 21st July, the protein content of snail mantle tissue in river water had decreased to 3.3 mg protein/ gm body ($P<0.007$); a 27.8% fall from the beginning of the experiment. However, the protein content of snails in 100% effluent treatment did not significantly differ from those in river water at any time during the experiment.

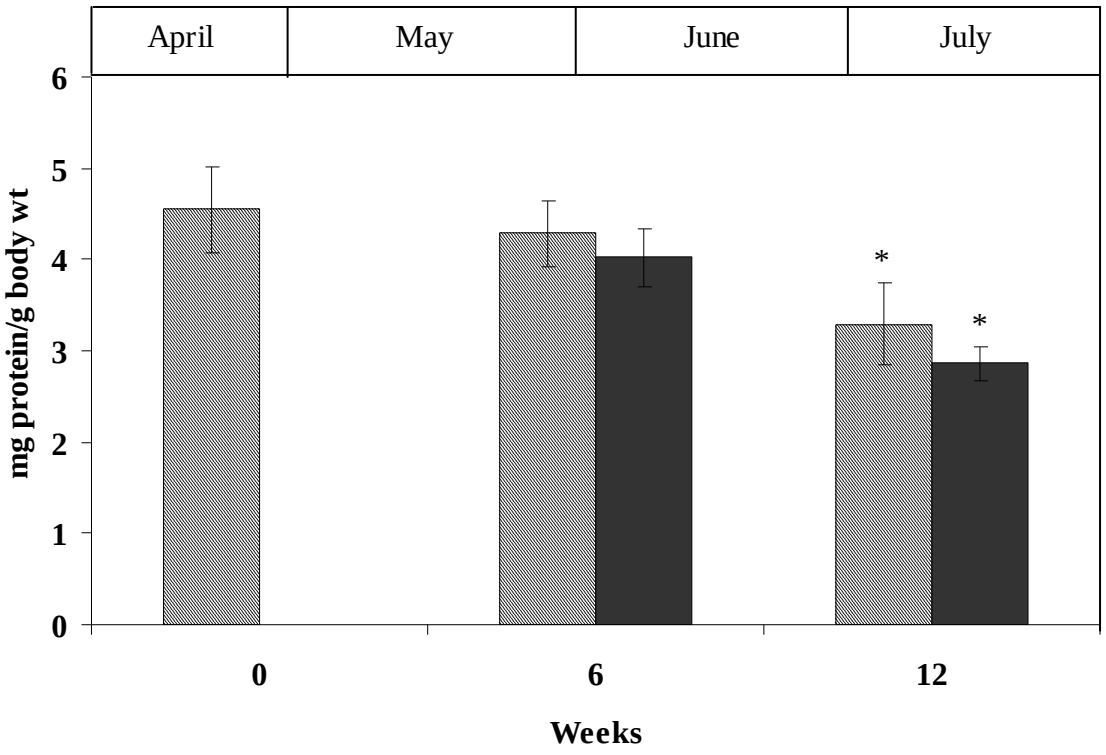


Figure 5.15 Individual snails protein content from *P. corneus* in river water (black and white stripped bars) or 100% (black bars) effluent extracted from the bodies of each snail. Snails from experiment 1 only were used for analysis (14th April- 21st July 2003). Asterisks indicate statistical differences from the river water control at week 0 $^{*}P < 0.05$.

5.3 Results of Seasonal Effects of TSE on V. viviparus

5.3 *V. viviparus*: Brief Methods and Materials

Over the course of two seasons (during 2003-2004), two experiments of 12-weeks duration were undertaken which, together, covered the reproductive and developmental season from spring through into early Autumn. Experiment 1 was run in 2003, from spring to mid summer. Experiment 3 of the following year (04`) covered late spring into early autumn. There was an around five weeks overlap in dates between Experiments 1 and 3, despite being carried out in different years.

A detailed description of both experiments, including detailed experimental design can be found in Chapter 2. Briefly, in each of Experiments 1 and 3, river water, 25%, 50% and 100% TSE were used as treatments. Six replicate groups of snails were used in each treatment, and each group contained of 11 snails; dependant upon on the numbers of snails available for each experiment. Baseline morphological measurements (and embryos in the brood pouch were counted) were taken at the beginning of each experiment (time zero), and at the end of week 6 during (during Experiment 1 only), and again at the end of both experiments. In each experiment, at the end of the baseline period (weeks –2-0) when all snails had been in river water only, TSE was introduced at time zero (mixed with river water diluent to obtain correct dilution of TSE). Biological measurements, including an estimation of fecundity (i.e. the number of embryos in the pallial oviducts; Expts 1 and 3) and reproductive output (the number of babies released: Experiment 3 only), were taken at this time. Reproductive output was then measured two weeks thereafter in Expt. 3 only to the end of the experiment.

5.3.1 Mortality of *V. viviparus* in TSE

Table 5.5 shows the percent mean survival of *V. viviparus* from Experiments 1 and 3 maintained in river water, 25%, 50%, or 100% of TSE. Mortality was continuous over the course of both experiments. However, mortality was considerably higher from late Summer into Autumn between the 27th July and 6th September over the last 6 weeks of Experiment 3.

Overall, mean mortality in effluent during experiment 3 (Summer-Autumn; 39.4-45.2%) was higher than during experiment 1 (Spring-Summer; 8.3-11.6%). In the river water control, 28.8 % of the original number of snails remained alive in experiment 3 compared with 71.0% in Experiment 1. During Experiments 1 and 3 there were no significant differences in the mean number of surviving snails between river water and effluent dosed groups during any time period.

In effluent treatments, between 88.4-91.7% of the original number of snails remained alive by mid Summer (21st July 2003) at the end of Experiment 1, compared with between 78.8-84.8% of snails that remained alive by mid Summer (week 6; 27th July 2004) during Experiment 3. Over the final 6 weeks of Experiment 3 from late Summer into Autumn (27th July to 6th September), a further 38.3-57.6% of snails in effluent treatments died (Table 5.5).

Table 5.5 Percentage of snails remaining alive (+/-SE) in river water, 25%, 50% or 100% Treated Sewage Effluent. (A) Experiment 1, 14th April- 21st July 2003, (B) Experiment 3, 1st June to 6th September.

A) Experiment 1- 2003

Date	28th April	8th June	*21st July
R/w	100+/-0.21%	85.1+/-0.7%	71+/-1.0%
25%	100+/-0.21%	97.2+/-0.3%	88.9+/-0.7%
50%	100+/-0.22%	88.1+/-0.3%	91.7+/-0.3%
100%	100+/-0.2%	86.1+/-1.2%	88.4+/-0.0%

B) Experiment 3-2004

Date	14th June	28th June	12th July	26th July	9th Aug	23rd Aug	6th Sept
R/w	98.5+/-1.52%	97+/-1.92%	92+/-1.52%	86.4+/-3.89%	65.2+/-7.58%	54.5+/-7.78%	28.8+/-3.65%
25%	100+/-0.0%	100+/-0.0%	97+/-1.92%	84.8+/-3.83%	65.2+/-5.46%	56.1+/-6.38%	39.4+/-8.99%
50%	100+/-0.0%	92.4+/-4.93%	90.9+/-4.69%	83.5+/-3.7%	64.3+/-5.06%	56.9+/-5.4%	45.2+/-7.61%
100%	100+/-0.0%	93.9+/-3.03%	86.4+/-5.63%	78.8+/-5.07%	50+/-8.05%	40.9+/-7.7%	28.8+/-8.3%

*3 replicates remained per treatment after sampling on the 8th June 2003.

5.3.2 Seasonal Effects of TSE on growth of V. viviparus

Figure 5.17A-D shows the mean shell length and weight of the surviving *V. viviparus* with their shells maintained in river water, 25%, 50%, or 100% TSE during Experiments 1 and 3. In Experiment 1, in springtime on the 28th April there were significant ($P<0.05$) differences in the mean weight of snails (with shells) between the 25 and 50 % effluent exposed and river water exposed snails prior to the start of the exposure experiment (Figure 5.17C).

Of the surviving snails in Experiment 1 (Spring-Summer), those in river water, 25%, and 50% effluent doses grew well and increased their mean lengths by 2.7mm (10.5%), 1.0 mm (3.5%), and 1.5 mm (5.8%), respectively (Figure 5.17A). In contrast, in 100% effluent (Expt. 1) the snails grew in the first 6 weeks in a similar way to those in river water, but by week 12 their mean shell length had fallen bellow those measured at week zero; though the significant ($p<0.05$) difference in the mean weight (with shells) of snails exposed to 100% effluent relative to the river water snails remained to week 6 only (Figure 5.17C). In Experiment 3 (Summer-Autumn), the mean shell length of snails in all effluent treatments decreased by 2.1%-3.7% ($P=0.003$) relative to the mean shell length of snails in river water (Figure 5.17B). However, by Autumn (on the 13th September) there was a significant ($P<0.05$) decrease in the mean weight (with shells) of all snail groups compared to baseline measurements early Summer (8th June) (Figure 5.17D). There were, however, no significant differences in the mean weight of snails (with shells) between effluent and river water exposed groups of snails during experiment 3.

Experiment 1 Experiment 3

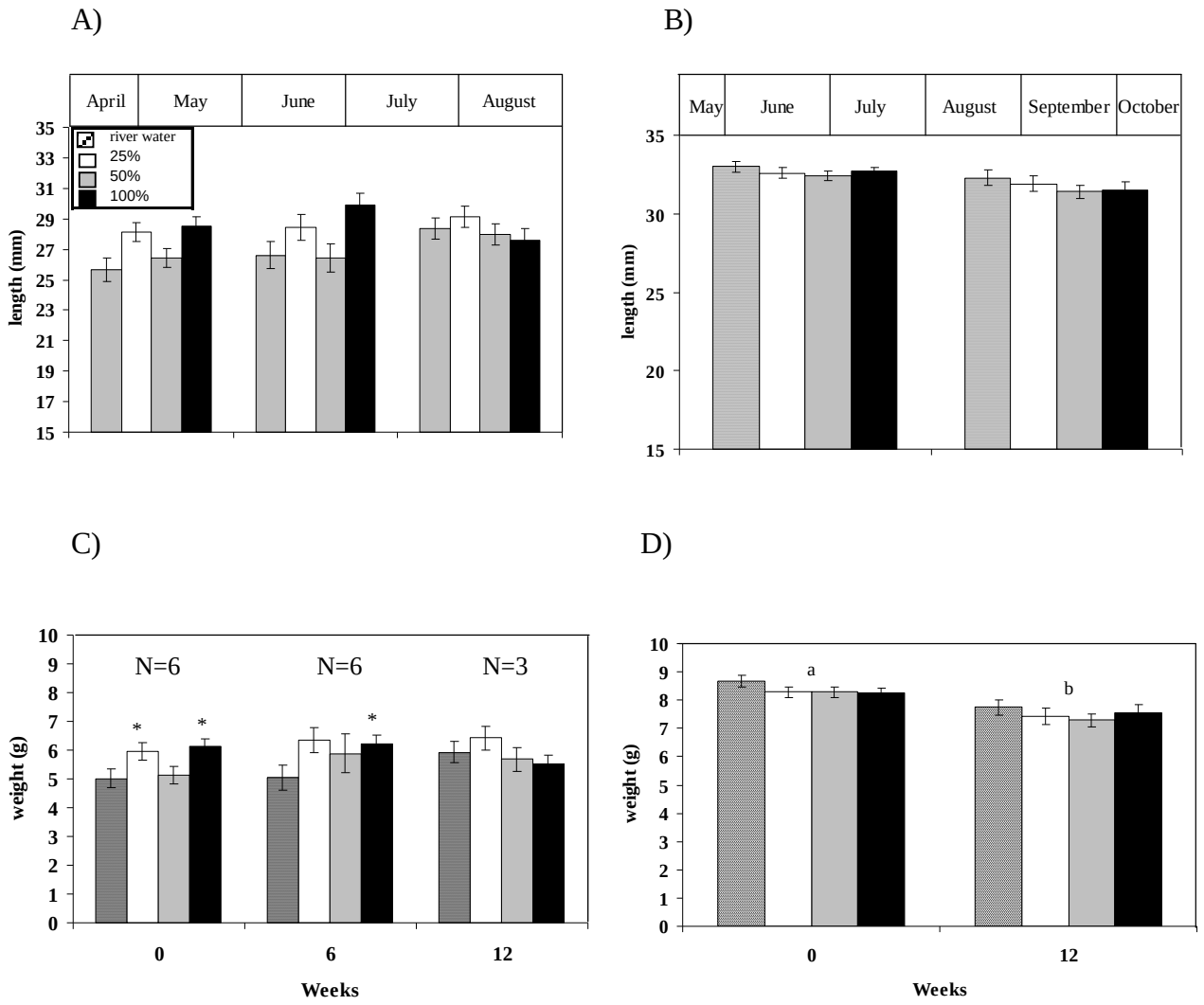


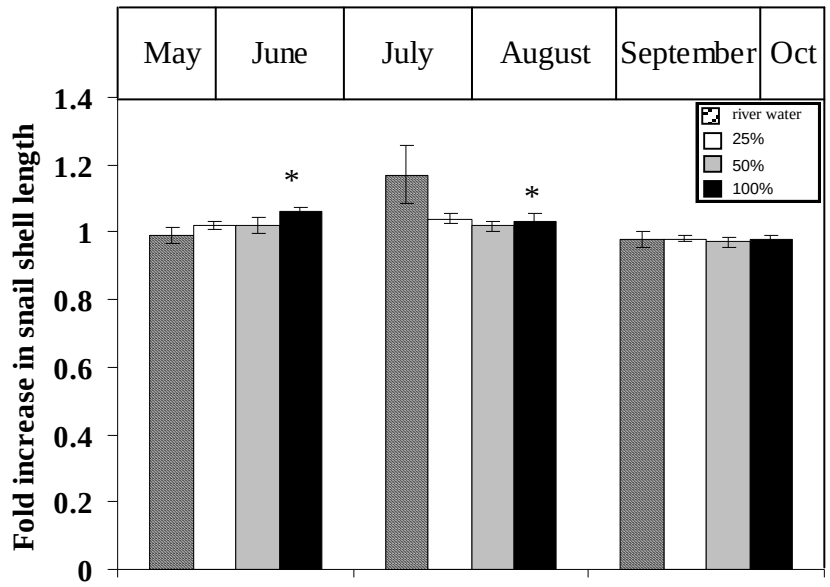
Figure 5.17 Mean change in A) and B) shell length of snails (±SE) and C) and D) weight (g) from groups (±SE) of *V. viviparus* snails in river water, 25%, 50%, or 100% effluent (Experiment 1; 12 snails, Experiment 3; 11). In Experiment 1, snails in each of the 6 enclosures were measured at time zero and baseline measurements taken. At weeks 6 and 12, 3 enclosures were measured again to calculate the increase shell lengths for each enclosure. In experiment 3, snails in 6 enclosures were measured at the beginning and end of each experiment. Spring to mid-Summer time; Experiment 1, 14th April- 21st July 2003, Experiment 3, early Summer to Autumn; 15th June to 6th September. Asterisks indicate statistical differences from the river water control $^{*}P < 0.05$. Different letters indicate between time period statistical differences $^{a,b}P< 0.05$.

Figure 5.18 shows the seasonal mean fold change in shell length and snail weight (with shells) of *V. viviparus* maintained in river water, 25%, 50%, or 100% TSE in Experiments 1 and 3. The seasonal fold change in snail shell length and weight (with shells) was calculated using individual enclosure means. Mean shell length and weight (with shells) for all snails was established in all enclosures during baseline measurements, and all remaining live snails in all enclosures were measured again at subsequent sampling points.

By early Summer on the 8th June there was a significant (week 6: $P<0.05$) increase in both snail mean shell lengths of groups exposed to 100% effluent and (week 6: $P<0.03$) in mean weight of snail groups with shells exposed to 50 % effluent compared to river water snails, followed by a significant decrease (week 12: $P<0.05$) in both snail shell length and weight with shells of the same groups compared to river water by mid Summer (21st July); though this was due to unusually high growth in a single enclosure in river water by week 12. By early Autumn on the 6th September there was no significant difference in the mean of snail shell lengths between any effluent exposed and river water exposed snail groups. There were no significant differences in the mean fold change in weight (with shells) of snail groups exposed to 25% or 50% effluent doses compared to snails in river water over this same time period. Too few snails survived in the 100% effluent dose to be included in this analysis.

Overall, the rate of increase of snails shells`length (mm/ day) and weight (with shell) (g/day) in effluent compared to river water exposed snail groups was inconsitent during experiment 3, however, overall growth (snails shells`length (mm/ day) and weight (with shell) (g/day)) in all treatments was positive until mid summer, and negative thereafter (data not shown).

A)



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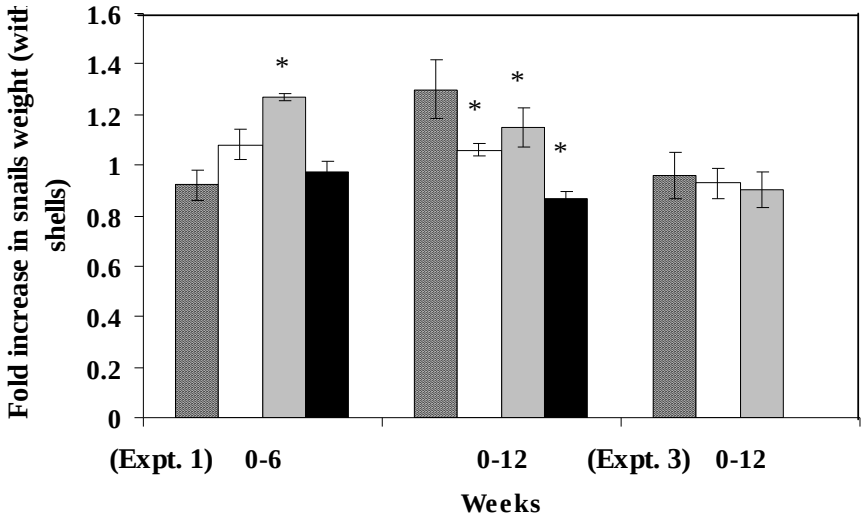


Figure 5.18 Seasonal mean fold increase in A) shell length (mm) and B) snail weight with shells (g) from groups (±SE) (Experiment 1 and 3; 11 snails per group) of *V. viviparus* snails in river water, 25%, 50%, or 100% effluent. In Experiment 1, snails in each of 6 enclosures were measured at time zero and baseline measurements taken. At weeks 6 and 12, 3 enclosures were measured again to calculate mean fold increase values by comparison to mean baseline shell lengths for each enclosure. In Experiment 3, snails in 6 enclosures were measured at the beginning and end of the experiment. Spring to mid-Summer time; Experiment 1, 12 weeks; 14th April- 21st July 2003, Experiment 3; early Summer to Autumn; 12 weeks; 15th June- 6th September 2004. Asterisks indicate statistical differences from the river water control $^{*}P < 0.05$.

Figure 5.19 shows the seasonal change in the weight (g) of *V. viviparus* without shells maintained in river water, 25%, 50%, or 100% effluent during Experiment 3. This parameter was only measured during Experiment 3. From early Summer into Autumn (15^{th} June to 6^{th} September) there were no significant ($P>0.05$) differences in the mean weight (g) of snails without shells between the baseline river water groups (week 0) and snails groups at the end of the experiment, either river water or effluent exposed. Further, in Autumn by the end of Experiment 3 (on the 6^{th} September) there were no significant ($P>0.05$) differences in the mean weight of snails without shells between river water and effluent exposed groups of snails.

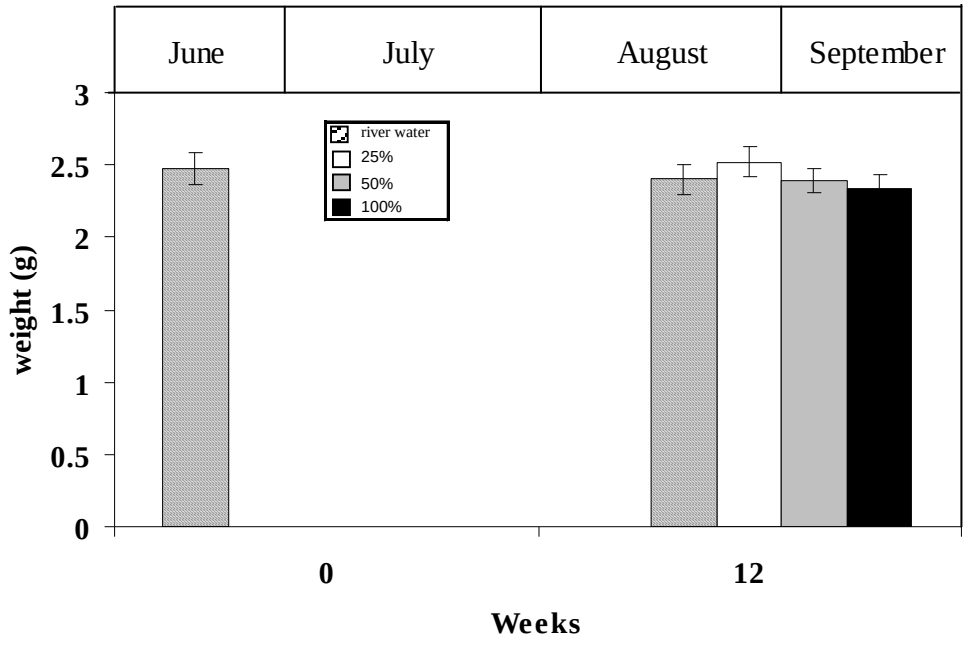


Figure 5.19 Seasonal mean change in weight without shells from groups (±SE) of *V. viviparus* snails in river water, 25%, 50%, or 100% effluent (Experiment 3; 11 snails). In experiment 3, a sample of 30 snails from river water (after acclimation) were used to establish weight without shells of snails at the beginning of the experiment, and all snails remaining alive in each of 6 enclosures in each treatment were measured at the end of experiment. Experiment 3; early Summer to Autumn; 12 weeks; 15th June- 6th September 2004.

Figure 5.20 shows the seasonal weight of *V. viviparus* without shells or embryos maintained in river water, 25%, 50%, or 100% effluent during Experiment 3. This parameter was only measured during Experiment 3 and no baseline measurements were taken. By autumn (on the 6th September) there were no significant ($P>0.05$) differences in the mean weight without either shells or embryos between river water and effluent exposed groups of snails.

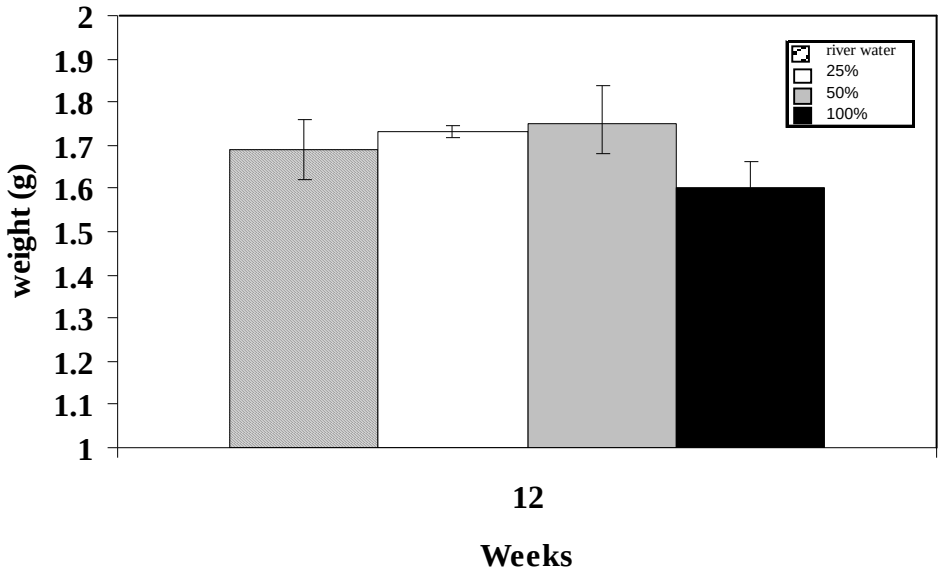


Figure 5.20 Seasonal mean weight (g) without shells or embryos from groups (±SE) of *V. viviparus* in river water, 25%, 50%, or 100% effluent (Experiment 3; 11 snails). All snails remaining alive in each of 6 enclosures in each treatment were weighed at the end of the experiment . Experiment 3; early Summer to Autumn; 12 weeks; 15th June- 6th September 2004.

Figure 5.21 shows the mean shell lengths of deceased snails from *V. viviparus* snails maintained in river water, 25%, 50%, or 100% effluent during Experiment 3. Mean shell lengths were measured from snails in all treatments at fortnightly intervals to access differences between and within time periods. There were no significant differences in the mean length of deceased snail shell lengths measured throughout the exposure between time periods with the exception of weeks 4 to 6 were shell lengths were significantly ($P<0.05$) increased compared to weeks 6 to 8, and 10 to 12. Too few deceased snails shells were available for statistical analysis at weeks 2 to 4. However, there were no significant differences in the mean length of deceased snails shells between river water and effluent exposed groups of snails during any time period when analysed for between treatment differences.

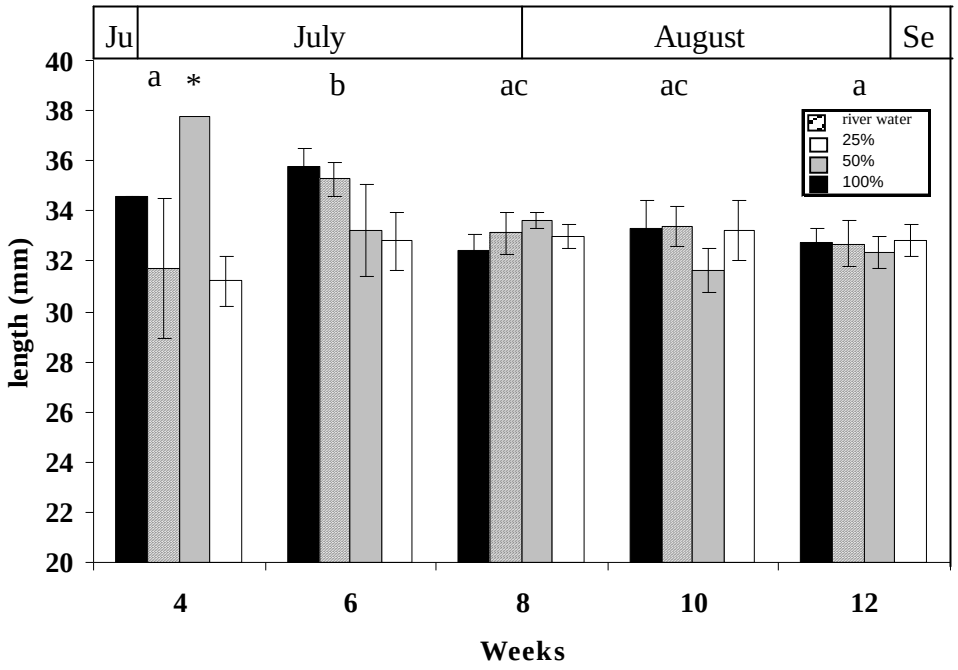


Figure 5.21 Seasonal mean length of deceased snail shells from groups (±SE) of *V. viviparus* snails maintained in river water, 25%, 50%, or 100% effluent (Experiment 3; 11 snails). Deceased snails were collected every two weeks when enclosures were emptied in order to count number of newborn snails. Deceased (and live) snails were counted and measured before live snails were returned to their enclosures. Experiment 3; early Summer to Autumn; 12 weeks; 15th June- 6th September 2004. Different letters indicate statistical between time period differences $^{a,b,c}P < 0.05$. Asterisks indicate statistical differences from the river water control $^{*}P < 0.05$.

Figure 5.22 shows the seasonal rate of increase in length (mm) and weight (g) per day of *V. viviparus* snails (with shells) in river water, 25%, 50%, or 100% effluent during Experiments 1 and 3. The daily rate of increase of length or weight was calculated using by listing the length or weight of each snail in each treatment over a specific time period, and calculating a mean value for each parameter. This procedure, to calculate a mean length or weight, was carried out over each time period stated. The difference in the measurements was divided by the number of days between sample points, and therefore, statistical analyses were not appropriate.

Overall, the rate of increase of length (mm) of snails shells` per day in effluent appears not to have followed a consistent pattern over the seasons observed and differs from that of snails in river water (Figure 5.22). However, any potential seasonal difference in the rate of increase of length (mm) of snails shells` per day in river water or effluent is difficult to access from this figure, in the absence of statistical analyses and from the very few measurements taken throughout the experiments. However, there may have been an increased growth rate (length only) in 100% effluent compared to river water early summer.

The increases in shell length appear small overall, and therefore, the differences observed may not be as significant as might otherwise be indicated. The increase in weight of snails in effluent exceeded that of river water during spring and summer apart from snails in 100% effluent, where growth was consistently negative. However, growth of snails in effluent was equivalent (in terms of negative growth) compared to river water towards winter.

Seasonal growth, as measured by the rate of increase of weight (g/day) was greater in 25% and 50% effluent than in river water early in summer (8th June). By mid-summer (21st July), snails in river water grew equally as well those in 50% effluent, snails in 25% effluent grew at an increased rate compared to both these treatments. Growth in 100% effluent was negative at all time periods measured, growth was equivalent and negative in all treatments by Autumn (6th September).

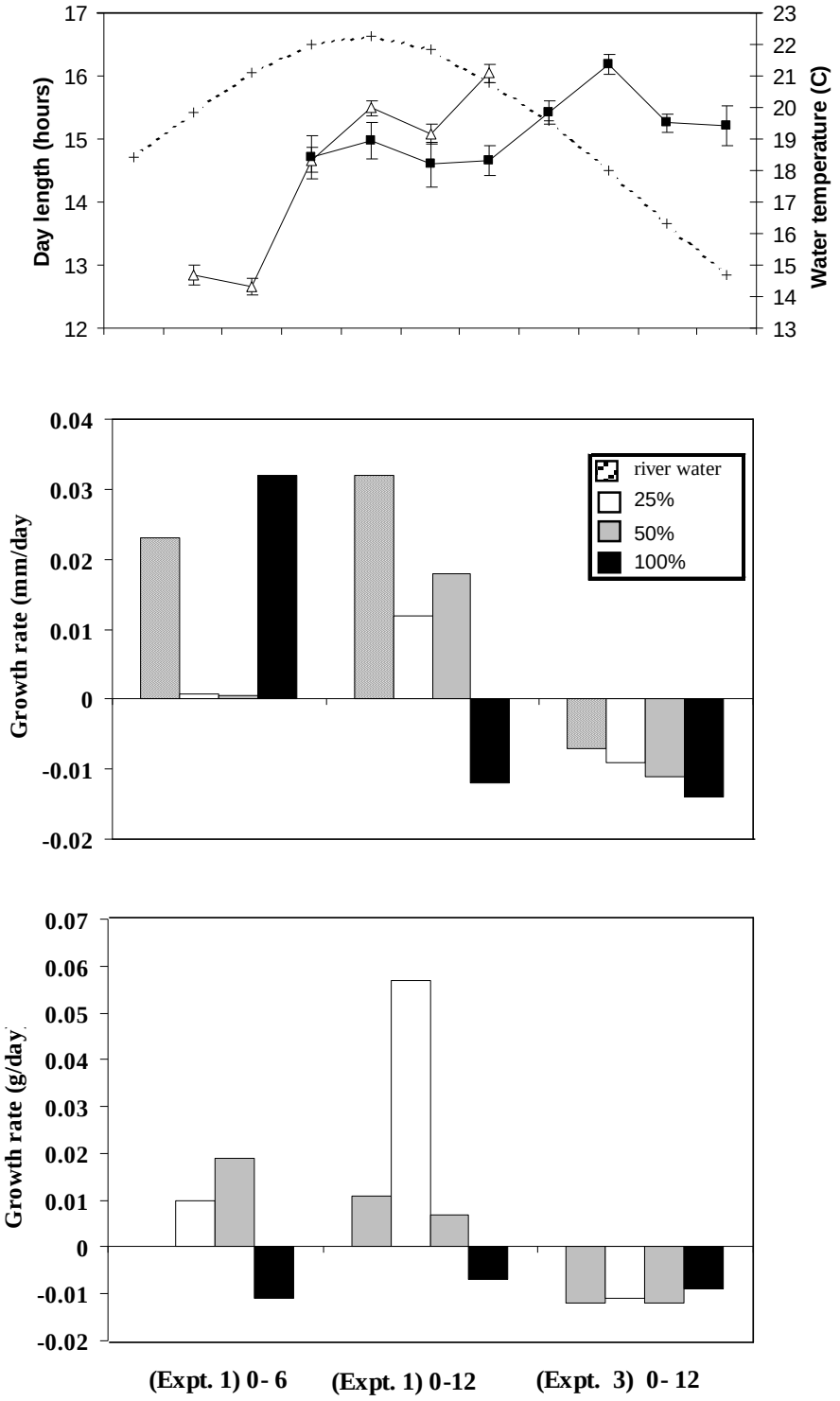


Figure 5.22 Seasonal rate of increase of length (mm) or weight (mg) from groups of *V. viviparus* snails with shells in river water, 25%, 50%, or 100% effluent (Experiment 1 and 3; 11 snails). Spring to mid-Summer time; Experiment 1, 12 weeks; 14th April- 21st July 2003, early Summer to Autumn; Experiment 3; 12 weeks; 15th June- 6th September 2004.

5.3.3 Seasonal effects of TSE upon Reproduction of Snails

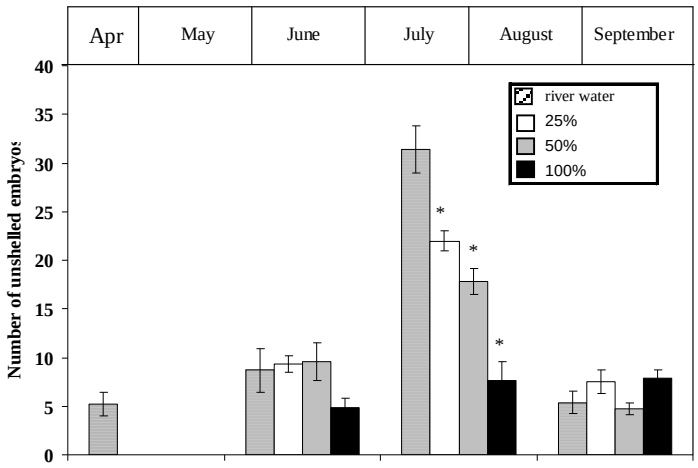
The seasonal reproductive response of snails exposed to river water was assessed over two consecutive years to establish the "normal pattern" of reproduction (see Chapter 4). In the same experiments, snails were exposed to a graded concentration of TSE to observe reproductive effects. A single reproductive period was found, between 25/5/03 and 2/8/04 where reproduction occurred in all treatments. Reproduction and fecundity were highest in early summer when most mature embryos were released and replaced by new embryos in the brood pouch of female snails.

Figure 5.23 shows the mean number of unshelled, shelled, and total embryos in the brood pouch of female viviparid snails at sample points over two consecutive years during Experiments 1 and 3. Experiment 1 took place in 2003 and experiment 3 in 2004, and therefore, results are composites from separate years of observations. There was a dose dependant decrease in the mean number of unshelled embryos produced per snail, and significant ($P<0.05$) decrease in mean number of total embryo production in the snails exposed to 50% and 100% effluent compared to snails in river water that was time dependant (Figure 5.23A and C). By mid Summer (on the 21st July 2003 or week 12) exposure to treated sewage effluent significantly decreased ($P<0.05$) the mean number of unshelled embryos in females brood pouches in a dose related manner, and mean total embryo production was significantly decreased ($P<0.05$) in the snails exposed to 50% and 100% effluent.

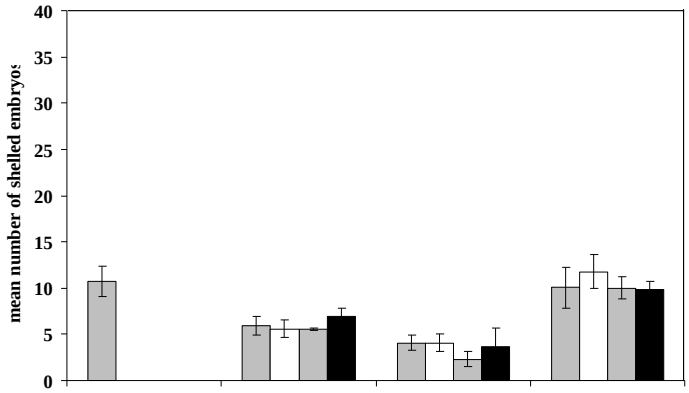
By Autumn of the following year (on the 6th September) there were no significant ($P>0.05$) effects of effluent upon either the mean number of unshelled or total number of embryos produced per snail compared to snails in river water.

From Spring into Autumn (between the 28th April and 6th September) there were no significant ($P>0.05$) differences in the mean number of shelled embryos in female brood pouches of female snails between effluent and river water exposed groups of snails at any time (Figure 5.23B). However, these observations were made from data gathered in different years.

A)



B)



C)

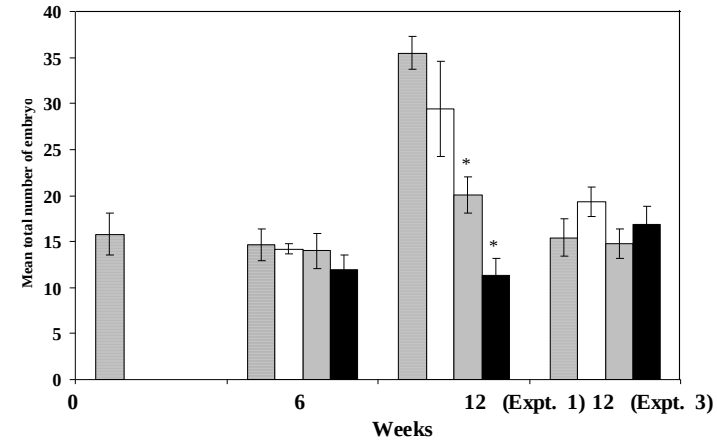
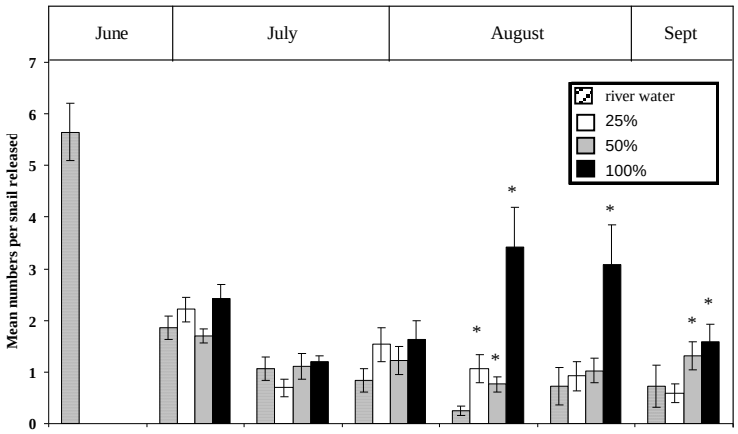


Figure 5.23A-C Mean number of (A) unshelled (B) shelled (C) and total embryos in the brood pouches of female *Viviparus viviparus* from groups (±SE) (Experiment 1 and 3; 11 snails) of snails in river water, 25%, 50%, or 100% effluent. Spring to mid-Summer time; Experiment 1, 12 weeks; 14th April- 21st July 2003, early Summer to Autumn; Experiment 3; 12 weeks; 15th June- 6th September 2004. Asterisks indicate statistical differences from the river water control $^{*}P < 0.05$.

Figure 5.24A shows the mean number of mature embryos released from the brood pouch of female viviparid snails in river water, 25%, 50%, or 100% effluent during Experiment 3. By mid to late Summer onwards (from the 9th August 2004) exposure to treated sewage effluent significantly ($P < 0.05$) increased the mean number of mature embryos released from the brood pouch of female snails (week 8; all effluent doses, week 10; 100%, week 12; 50% and 100% effluent doses) compared to river water.

Figure 5.24B shows the mean weight of mature embryos released from the brood pouch of female viviparid snails in river water, 25%, 50%, or 100% effluent during Experiment 3. For one month, from the 28th June, exposure to treated sewage effluent significantly decreased the mean weight of mature embryos released in all effluent doses (week 2; all effluent doses, week 4; 50 and 100% effluent doses) compared to river water. However, during late summer on the 23rd August (weeks 8 to 10) there was a small but non-significant ($P > 0.05$) increase in the mean weight of mature embryos released in all effluent doses compared to river water, these effects were significant only in Autumn during the final two weeks (6th September: weeks 10 to 12) of the experiment (100% effluent; $P < 0.03$).

A)



B)

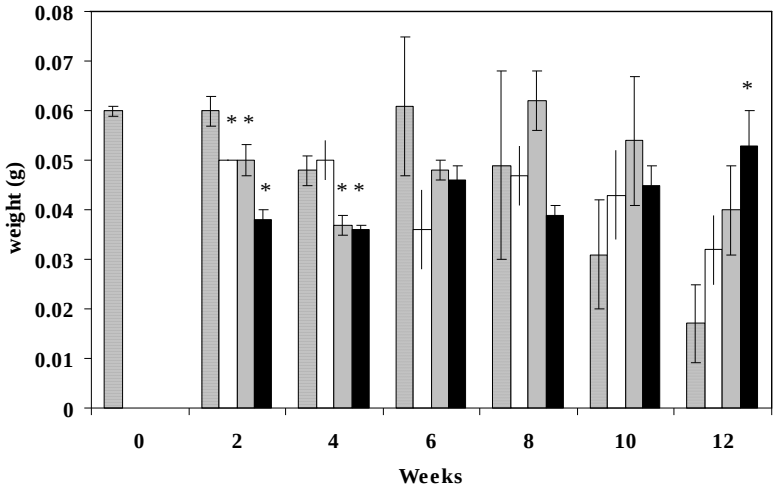


Figure 5.24 (A) Mean number of mature embryos, and (B) mean weight of mature shelled embryos released from the brood pouches of female *Viviparus viviparus* from groups (±SE) (Experiment 3; 11 snails) in river water, 25%, 50%, or 100% effluent. Early Summer to Autumn; Experiment 3; 12 weeks; 15th June- 6th September 2004. Asterisks indicate statistical differences from the river water control $^{*}P < 0.05$.

Figure 5.25 shows the mean weight of mature embryos within the brood pouches of female viviparid snails in river water, 25%, 50%, or 100% effluent during Experiment 3. During the season there were no significant ($P>0.05$) effects upon the mean weight of mature shelled embryos within the brood pouches of female snails of effluent exposed compared to in river water exposed groups of snails.

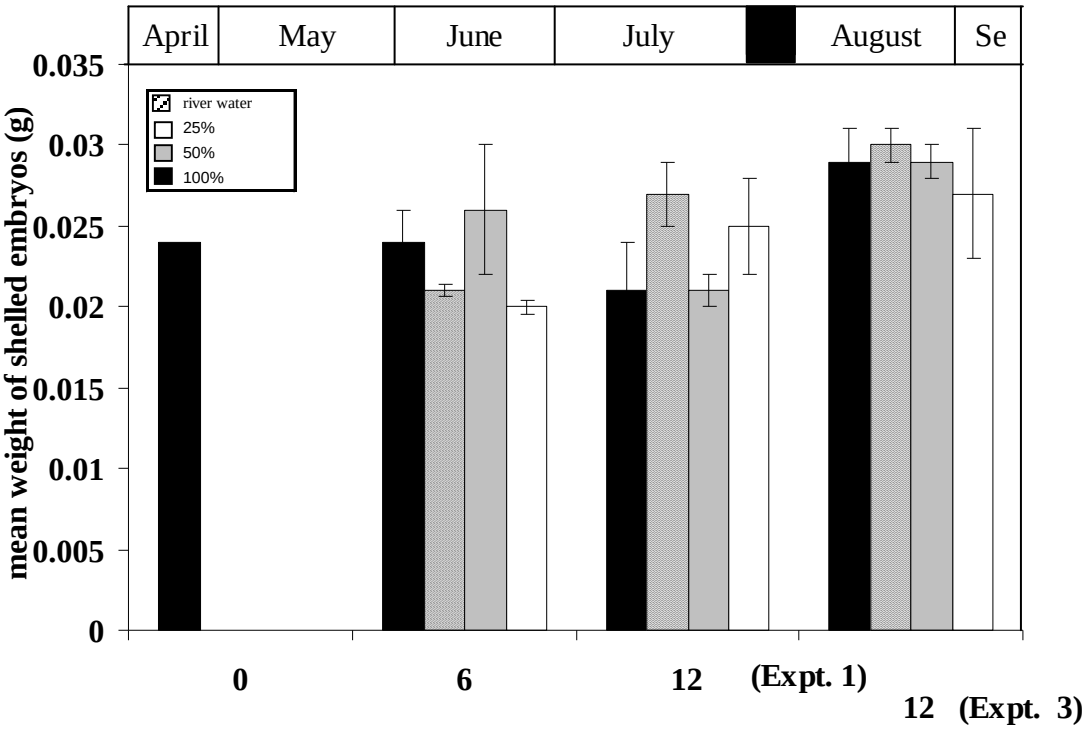


Figure 5.25 Mean weight of mature shelled embryos within the brood pouches of female *Viviparus viviparus* from groups (±SE) in river water, 25%, 50%, or 100% effluent (Experiment 1 and 3; 11 snails). Experiment 1; 12 weeks (Spring to mid-Summer time); 14th April- 21st July 2003. Experiment 3; 12 weeks (early Summer to Autumn); 15th June- 6th September 2004.

Figure 5.26 shows the mean cumulative number of baby snails released per *V. viviparus* snail in river water, 25%, 50%, or 100% effluent during Experiment 3. After 2 weeks exposure, in early Summer on the 15th June the cumulative mean number of mature embryos released per snail in all effluent doses was not different (range 1.7-2.4 fold increase) to mean numbers released in river water control (1.9 fold increase); a pattern that remained until week 8. By week 6 there was a non-significant ($P>0.05$) stimulatory effect of 100% effluent. Two weeks later on the 9th August (after 8 weeks exposure), both 25% (x5.5) and 50% (x4.8) effluent doses produced small non-significant ($P>0.05$) stimulatory effects upon cumulative mean number of mature embryos released per snail relative to river water (x 4.0); a pattern that remained unchanged to the end of the experiment. From early Summer into early Autumn (between the 15th June and 6th September) the cumulative mean number of mature embryos released per snail over the course of the experiment was significantly different from the river water tank ($p<0.002$) in the highest dose tested (100% effluent) by mid summer on the 9th August (after 8 weeks of exposure); a pattern that remained to end of the experiment. At which time release of mature embryos in the other treatments had largely stopped. Indeed, the cumulative fold increase in mature embryos released per snail had increased by 5.5-fold above original baseline values and 2.5-fold ($P<0.001$) relative to the river water. The dose-response curve was non-monotonic, with the 25% and 50% effluent doses causing a small (but statistically non-significant) almost identical stimulatory increase in mature embryos released relative to the river water control, and the 100% effluent dose showing a significant ($P<0.05$) increase in mature embryos released relative to the river water control.

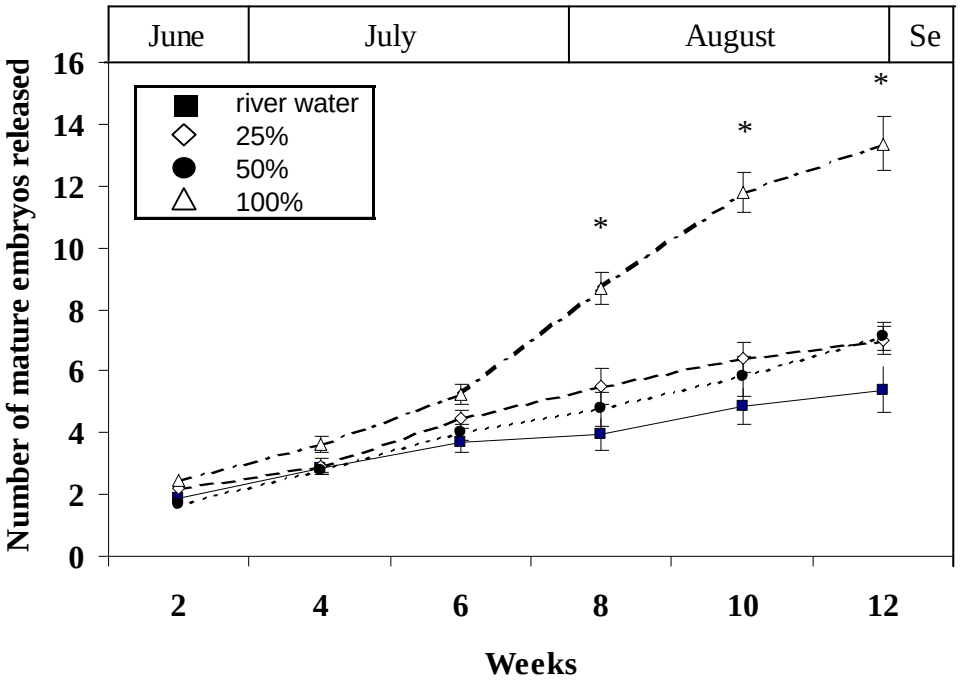


Figure 5.26 Mean cumulative number of mature embryos released per snail from groups (±SE) of *Viviparus viviparus* in river water, 25%, 50%, or 100% effluent (Experiment 3; 11 snails). Early Summer to Autumn; Experiment 3; 12 weeks; 15th June- 6th September 2004. Asterisks indicate statistical differences from the river water control $^{*}P < 0.05$.

Figure 5.27 shows the mean cumulative weight of mature embryos released per *V. viviparus* snail in river water, 25% 50%, or 100% effluent during Experiment 3.

The mean cumulative weight of mature embryos released in effluent was significantly decreased ($P<0.05$) compared to the river water in 25% effluent in early Summer on the 28th June (first 2 weeks), in 50% effluent to mid Summer on the 27th July (first 6 weeks), and in 100% effluent to mid Summer on the 9th August (first 8 weeks). After 2 weeks exposure (28th June) the cumulative mean weight of mature embryos released in 25%, 50%, and 100% effluent treatments were significantly lighter ($P<0.005$) than those released in river water (-0.78-fold, -0.77-fold, and -0.58-fold). After 4 weeks exposure (13th July), 50% (-0.79-fold) and 100% (-0.66-fold) effluent doses remained inhibitory (see fig. 5.30); a pattern that remained for a further 2 weeks to mid Summer on the 27th July. After 8 weeks exposure (9th August) only the 100% (-0.71-fold) effluent dose continued to have an inhibitory effect on cumulative mean weight of mature embryos released relative to the river water. Thereafter, in Autumn on the 6th September the cumulative mean weight of mature embryos in 25%, 50% and 100% effluent continued to increase at an unchanged rate until the end of the experiment, whereas the cumulative mean weight of embryos released in river water decreased over the final 4 weeks of the experiment. By the end of the experiment (6th September), the 50% effluent dose had a small but non-significant stimulatory effect ($P>0.05$) on cumulative mean weight of mature embryos released compared to the river water.

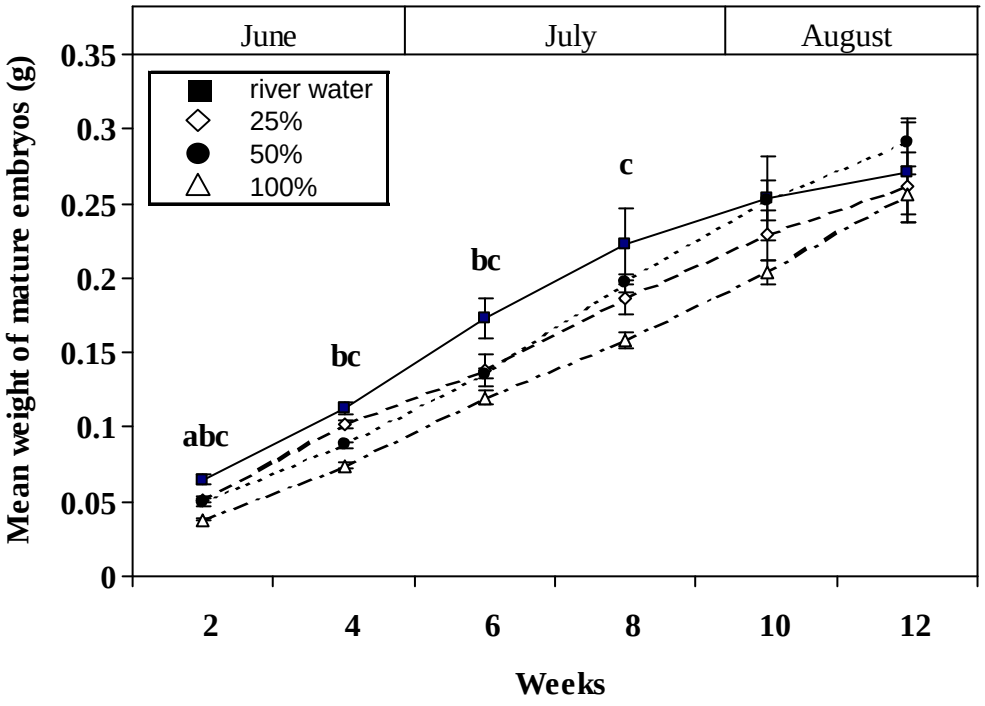


Figure 5.27 Mean cumulative weight of mature embryos released per snail from groups of *Viviparus viviparus* in river water, 25%, 50%, or 100% effluent (Experiment 3; 11 snails). Early Summer to Autumn; Experiment 3; 12 weeks; 15th June- 6th September 2004. Statistical significance from river water; a= 25% effluent statistically different to river water; b= 50% effluent statistically different to river water; c= 100% effluent statistically different to river water.

5.4 Discussion

5.4.1 *Planorbarius corneus*

5.4.1.1 Glycogen Phosphorylase assay validation

A test of this methodology involved preparation of a homogenate from thawed snail tissues that contained liberated glycogen phosphorylase enzyme (after homogenisation), spinning of this broken down snail tissue produced a clear supernatant that could, in theory, be added to a standard reaction mixture to start the reaction (if containing stable enzyme). Homogenation proved to be a simple process that appeared to process snail tissue down to an apparent suitable particle size. The stability of this enzyme in tissue samples (frozen stored snail bodies) was assessed from when snail body tissues were thawed to the adding of prepared supernatant; as this was not part of the cited method that could be used (see Childress and Sacktor, 1970). Different animal tissues to those used by Childress and Sacktor, 1970 were used in my experiments.

The spinning of homogenates by two different methods indicated that there was an initial drop in enzyme activity with time, and then a plateauing out of results by either method employed (Figs 5.12A and B). Also by either method, taking natural logs of the results produces a straight line indicating a first order reaction. Therefore, by either method of spinning, the results could be logged and the results before degradation of the enzyme calculated by following the straight line back to its origin (time zero, before degradation occurred). However, a choice was available as to how to present results. By either method of spinning reasonable results were obtained that could be used to produce reliable results, as further evidenced by analyses of my method validation procedures and analysis of thawed snail tissues.

After spinning for 90 minutes approximately 50% of the enzyme activity had been lost. By this method of spinning down, the true enzyme activity of the samples could be back calculated, as already stated. Alternatively, results could be obtained after

spinning for 1.5 hours, and not back calculated. This would not provide an estimate of the original enzyme activity of the tissue, but instead results that could be compared treatment-to-treatment, snail tissue to snail tissue. The relative activity of this enzyme in different snail tissues from different treatments was the vital information I required, and therefore, a comparison of treatment-to-treatment results was justified. Using this approach, only a direct comparison to values of glycogen phosphorylase activity found in the literature would not be possible. However, since very few other authors have analyzed glycogen phosphorylase activity in snail tissues, the importance of this loss of information was considered minimal. Therefore, I decided not to back calculate results, as no real advantage would be gained for my PhD.

As well as deciding how best to present the data, it was also necessary to decide how to appropriately spin down homogenates to obtain good results. As stated, by either method of spinning reasonable results were obtained. However, the results of the precision test (Figure 5.13), demonstrated that highly repeatable results were obtained after spinning for 90 minutes. Direct comparison of enzyme activity between the methods of spinning was not possible as different snail tissues were used on different days; and this explains the differences in enzyme activities found between methods (see Figures 5.12A and B). The difference in the activity of this enzyme between snail samples was also unknown at this time, before analysis of snail tissues from the different treatments in my experiments. Therefore, the 90 minute method of spinning homogenates was adopted for analyses of snail tissues (from different treatments) as the precision test showed that any "bits" of tissue had formed a dense pellet did not affect the volume of supernatant pipetted or the enzyme activity. A high degree of repeatability of results was obtained after spinning for 90 minutes at 3, 000 g. Therefore, any error that could have been introduced by pipetting an inconsistent volume of supernatant (due to "bits") appeared to be at least suitably limited by spinning homogenates by this method.

The degradation of snail tissue (and therefore glycogen phosphorylase enzyme) samples over time whilst were thawing could also introduce an error into the assay; as samples could have degraded at different rates (and therefore produced inaccurate results). Both tests of glycogen phosphorylase enzyme stability demonstrated that

samples degrade depending on time (Figs 5.12A and B), and both demonstrated similar shaped curves. Therefore, analysis and therefore analysis of snail homogenate samples using the spectrophotometer occurred immediately. However, possible differences in degradation of this enzyme over time between samples was not a testable phenomenon, as each snail sample could only be analysed once, and therefore no direct comparison was possible. However, by standardizing my methodology for each snail tissue sample I analyzed, I attempted to limit the effects that any timing issues might have on my results.

5.4.1.2 Method Development; Egg Hatchability

In Experiment 1, hatching during weeks 2 to 4 in TSE varied between 45.2%-76.6%, and was 92.3% in river water. It is possible that bacterial respiration (associated with organic matter) in effluent samples further deprived these samples of oxygen compared to river water samples. Further evidence for this was derived from 1L water samples collected from my tanks at Langford STW in which oxygen content was measured. Dissolved oxygen decreased with increasing concentration of TSE from 6.2 mg/l (river water), 6.2mg/l (25% effluent), 5.8mg/l (50% effluent), to 4.5 mg/l in 100% effluent (T = 22 ^{o}C at time of testing). Water left in 10 L containers for storage at 4 ^{o}C contained more oxygen; river water 9.5 mg/l; and 100% effluent 8.9 mg/l. Water, from cell culture wells of my hatching experiment removed during a water change that I had carried out contained 4.5mg/l oxygen. Taken together, this evidence supports the idea, especially in hot weather, that increasing amounts of effluent could deprive sample water of oxygen. Although there were no significant effects of effluent at weeks 4-6, overall hatching of eggs was low, possibly due to temperature effects of storage of egg masses in universals (before their distribution into cell culture plates)(Table 5.3A).

Although an inhibitory effect of effluent upon the ability of eggs to hatch was only observed during a single time period (weeks 2-4), there is a possibility this could be a real effect of effluent (and not a methodological effect). This raises the question how does TSE reach the eggs and embryos? Exposure could have occurred during oogenesis in the hermaphroditic gland, during formation of the egg in the genital tract or after egg mass laying from the water (Gomot, 1998). Gomot, 1998 exposed

Lymnaea stagnalis to Cadmium and suggested that up to concentrations of 200 µg Cd/L, the oocytes fertilized are protected from Cadmium by the maternal tissues (including albumen gland, prostate and uterus). The eggs "suffer " from exposure to Cadmium only after being laid in contaminated water. In the pond snail (*L. stagnalis)*, Cadmium is concentrated and preferentially stored in the digestive gland (S-Rozsa *et al.*, 1988). This process of bioconcentration and bioaccumulation of heavy metals has also been demonstrated to occur in other freshwater snails such as *B. glabrata* (Yager and Harry, 1963). It is probable that in most pulmonate freshwater snails the digestive gland, which completely surrounds the gonadal tissues, provides efficient protection of the gonad from Cadmium (up to concentrations of 200 µg Cd/L)(Gomot, 1998). However, unlike *Lymnaea stagnalis,* the digestive gland does not completely surround the ovotestis in *P. corneus*, and therefore, may provide less protection from xenobiotics. Therefore, bioaccumulation of xenobiotics contained in TSE could remain a route of gonadal exposure in this species.

Therefore, if bioaccumulation of xenobiotics was responsilbe for an effect upon hatching of eggs this effect could be expected to be observed over time during my exposure experiment, and maybe expected to effect other reproductive parameters such as masses/snail, eggs/mass, and egg mass weight. However, potential effects of effluent on hatching occurred during weeks 2 to 4 of Experiment 1, while effects upon other reproductive parameters (increased numbers of egg masses, decreased number of eggs/mass and weight of egg masses) occured only during weeks 10-12 of experiment 1. Effects on other reproductive parameters were associated with the peak of reproduction, and not thought specifically due to bioaccumulation and bioconcentration of compunds in maternal tissues over time (see section 5.6.1.2). Taken together, this evidence suggests bioaccumulation of xenobiotics was probably not the cause of reduced hatchability in TSE during weeks 2 to 4 of experiment 1.

Chronic exposure of *Lymnaea stagnalis* to high concentrations (100 ng/l) of phytoestrogen β-sitosterol, caused a non-significant stimulation of egg mass production with an associated cost to egg quality (decreasing hatching rate of eggs) and atrophy of the albumen gland (Czech *at al*,. 2001). The stimulation of egg mass production with concurrent reduction in eggs/mass and egg mass weight at weeks 10-12 of Experiment 1 could have been at the cost of egg quality. However, as there was

no effect of effluent on hatching at this time, the stimulation of egg mass production appeared not to be at the expense of egg quality in this case.

Conversely, these same authors demonstrated at 100 µg/l of either 4-NP or tributyltin lowered egg mass production, and in the case of tributlytin, also lowered fertility/hatching. However, these effects were at concentrations 3-4 orders of magnitude higher than these compounds are generally found in the aquatic environment. Athough there was a possible lowered hatching rate during weeks 2-4 of my Experiment 1, there was no associated decreased egg mass production or increased mortality rate at this time (see Figure 5.6 and Table 5.2). During Experiment 1, at no time did TSE reduce egg mass production compared to river water, and therefore, this possible association between reduced egg mass production and reduced fecundity/fertility was not seen.

Czech *at al*,. 2001 suggested effects on egg hatching were due not to putative endocrine disrupting capacity of theses compounds, but due to toxic effects and general lowered health staus of these snails. However, there were no associated increases in mortality when egg hatching rate was low during Experiment 1 (week 2-4 in particluar), and therefore, egg hatching rates did not suggest any toxic effects of effluent.

Taken together, evidence from this study suggests that effects on egg hatching were not due to any toxic effects of TSE, nor were toxic effects suggested by snail mortality rates or by reproductive effects upon egg masses (number of masses, weight or number of aggs per mass). Further, there was a concurrent absence of evidence for a negative effect of TSE upon reproduction of parental snails at this time; providing more evidence that the effects seen on egg hatching in Experiment 1 were likely to be methodological.

In Experiment 3, though the egg-hatching rate in all treatments exceeded those in Experiment 1, lower hatching rates were observed during the baseline time period compared to other times. With all tanks supplied with river water (weeks –2-0), one tank performed poorly in terms of hatching rate during this time period; compare the tank that became 25% effluent dosed with others (Table 5.4A). An effect was also

observed in this tank during the baseline period where the number of egg masses produced per snail was significantly reduced compared to all other tanks (Figure 5.6C). Though the number of egg masses produced per snail remained comparatively low compared to other treatments for most of this experiment (to week 8), hatching rate improved immediately. Eggs from all tanks hatched equally well during weeks 2-4 and 12-14, though their ability to hatch was not tested at other times (notably weeks 4-6, and 6-8 when egg mass production was low in 25% effluent). There are no obvious explanations for this phenomenon (or "tank effect"), however, human error, in the form of inadvertent contamination, is a possibility. Significantly, whatever the reason, a methodological explanation (as part of egg hatching) seems unlikely.

The results of Experiment 3, contrary to Experiment 1, suggest no effect of effluent upon hatching rate of eggs in egg masses. As effluent characteristics change over time in both the short- and long term, these results could be genuine for both these experiments. Equally the significant results (with TSE) found in experiment 1 could be erroneous. Due to the methodological differences, and the extreme high temperatures at the time during Experiment 1, oxygen deprivation in egg masses (alone or in combination with toxicity of TSE) could explain the results found in Experiment 1, as stated. Certainly the higher egg hatching rates in Experiment 3 demonstrated clearly an improvement in method performance, and therefore, the results of Experiment 3 are more likely to be accurate and genuine.

5.4.1.3 Effects on Reproduction and Development

In this study, the effects of a graded treated sewage effluent on the seasonal development and reproduction of the pulmonate gastropod mollusc *Planorbarius corneus* are reported. My results indicate that in river water (see Chapter 4) *P. corneus* adopted a reproductive strategy that was tightly influenced by seasonal cues such as day length and water temperature. Despite this, my results indicate that various doses of treated sewage effluent were able to alter the seasonal response of *P. corneus*. Although not clearly dose dependant, snails exposed to 100% effluent experienced a steep increase in their reproductive rate during the peak of reproduction mid summer without significantly affecting their growth. Further, snails in all effluent

doses failed to undergo the characteristic seasonal decline in egg mass production expected at the end of their reproductive season in autumn. I have also shown that the effects of effluent on reproduction were repeatable over subsequent years. Although the underlying mechanism(s) responsible for these effects are unknown, there is literature demonstrating similar effects.

In a study by Watton and Hawkes (1984) the pulmonate snail *L. peregra* displayed reproductive responses to TSE common to those found in my own experiments. These snails produced more egg masses and eggs in 25% effluent than in 50% effluent, and both effluent doses increased reproductive output. However, the greater number of snails found in effluent during summer was largely due to a decreased mortality rate with more snails surviving to reproduce in effluent (increased density of snails in effluent). Interestingly, the Watton and Hawkes study shows effluent was not stimulatory in spring months when snails started to reproduce, but was stimulatory when reproduction was maximal during summer. Further, effluent extended the snails breeding season (as was observed in my experiments); egg mass production had ceased by mid August in river water but continued to at least mid October in effluent. The Watton and Hawkes study shows that the reproductive cycle of a pulmonate snail responded to TSE in a similar way to my own snails. In my studies, increased reproductive output could potentially result in more snails in effluent (together with decreased mortalities late summer onwards). In the study of Watton and Hawkes (1984) more snails reproduced due to a decreased mortality in effluent (and delayed lifecycle in 50% effluent), and therefore, the underlying mechanisms are likely to be similar.

Several authors have suggested (see Introduction Chapter 4), that in general during increased (peak of reproduction under natural conditions) or periods of high reproduction in pulmonate snails there is an associated higher mortality rate, particularly under conditions of long day length (Bokhlen *et al.,* 1978; Bokhlen and Joosse, 1982; Watton and Hawkes, 1984). This "reproduction in the face of adversity" normally occurs at the expense of body growth and exhaustion of the snail's energy reserves that results in death (Bokhlen *et al.,* 1978). Interestingly however, in my own experiments, snails in effluent were bigger but not significantly so than those in river water apart from a period from late summer onwards (see Figure 5.3 and following

figures), and did not vary in their protein content from those snails in river water during Experiment 1 at any time despite reproducing more. This suggests that stress and impending death may not fully explain the reproductive response in effluent exposed snails.

The mortality rate in all treatments during the course of both summertime Experiments 1 and 3 was normal for pulmonates reproducing during summer months. Mortality of 28% and 60% has been reported for *P. corneus* in natural ponds and *L. peregra* in 25% effluent, respectively (Costil and Daguzan, 1995b; Watton and Hawkes, 1984), in one month during the peak of reproduction. Most likely and importantly in the study of Watton and Hawkes (1984), the replacement of adult *L. peregra* with juveniles occurred during mid summer with almost no overlap between generations; adults died at almost the exact same time juveniles appeared in the mesocosm. A distinctive feature of the lifecycle (adaptive plasticity) of *L. peregra* during the mesocosm experiment, the birth of their offspring was most likely precipitated high adult mortality at this time. Though the exact lifespan my own *P. corneus* is unknown, the total mortality seen in all treatments during the course of both experiments is likely, to be typical of this pulmonate species. Further, the presence of young snails did not seem to cause mortalities amongst my *P. corneus* snails. As with the snails of Watton and Hawkes, my own snails reproduced substantially less in river water than in effluent. However, since there is a close but complex relationship between reproduction and mortality, a causative comparison between mortality rates in river water and effluent treatments is difficult to assess. Increased reproductive rate could be a confounding factor affecting mortality rates; as the literature clearly demonstrates that increased reproduction can be the cause of increased mortality. Further stress (environmental) can increase mortality (via "R-related" strategy; increasing reproduction, and therefore, mortality), but importantly, as demonstrated by the study of Watton and Hawkes, lifecycle characteristics can also affect mortality during the peak of reproduction as it did in *L. peregra*, but appears not have with *P. corneus* in my own experiments. Therefore, a direct cause of the difference in mortality between snails in river water and effluent is also difficult to assess.

However, in my own experiments, exposure to effluent resulted in an overall increased mortality in *P. corneus* as compared to river water in the springtime experiment, which was likely due to increased reproductive rates, and therefore, stress during this springtime period. In contrast, decreased mortality rates in 25% effluent compared to river water was observed in the summertime experiment; a feature in common with the study of Watton and Hawkes (1984)(see Table 5.2), and was associated clearly with a decreased reproductive rate in these snails. However, high reproduction was associated with high mortality rates in Experiments 1 and 3, and visa-versa. In Experiment 2, with a much lower reproductive rate there was what might be considered an unusually high mortality rate, but this was likely due to the onset of winter conditions reduced feeding and lower water temperatures (and therefore, stress caused by abnormal reproduction at this time). Therefore, it appears that high reproduction was associated with high mortality rates (and visa-versa). Watton and Hawkes (1984) also demonstrated an association between high reproductive and mortality rates in *L. peregra* where snails in 25% effluent reproduced most and died earlier in the season. Similarly, fewer snails died in 50% effluent compared to 25% effluent, and they also reproduced less (per snail). Further, in 50% effluent where there was little overlap in the adults and young born that year, both age groups survived for an extended period of time.

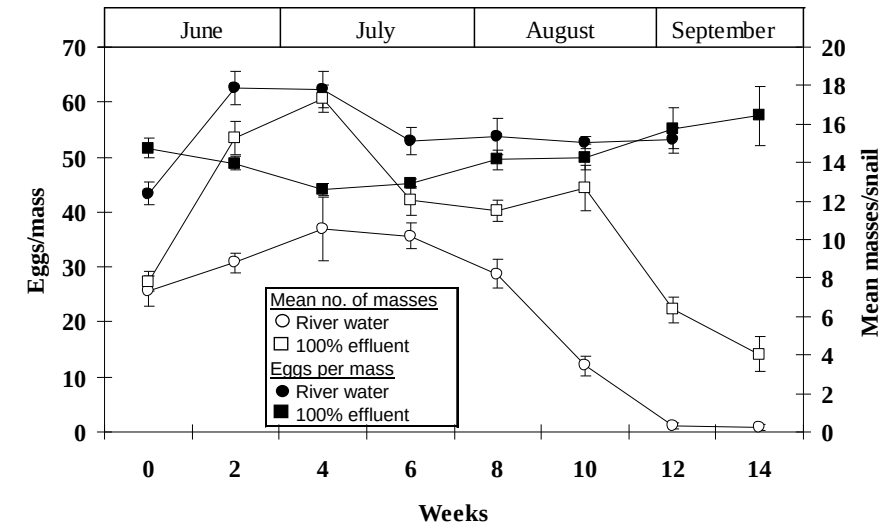
It is interesting to note that mortality of snails in 25% effluent was lower towards the end of my summer time experiment (experiment 3; weeks 8-12) when reproduction was less than in other effluent doses during this period (see Table 5.2); the lower mortality rate a feature common with *L. peregra* during summer in 50% effluent in the study of Watton and Hawkes, 1984. This may suggest that a delay occurred in the timing of mortality in the 25% effluent in experiment 3 also. Therefore, a mechanism may exist that prevents mortality from summer onwards when snails are decreasing their reproductive rate in effluent towards the end of their natural lifecycle. A possible underlying mechanism that might explain the amelioration of reproductive stress by effluent is unknown, though might suggest the involvement of oestrogens as a factor in this phenomenon.

Imposed upon the common background of physical environmental factors (day length and temperature) and their affects on reproduction, exposure to TSE resulted in

enhanced egg mass production without significant effects upon growth with the exception of a period late summer onwards (see Figure 5.6A; weeks 8 and 12, and Figure 5.6C). Most importantly, there was a failure of snails in all doses of effluent to undergo the characteristic autumnal decline in reproductive output that was so clearly seen in snails exposed to river water alone. The coincidental timing of the maximal effect of the effluent on the reproductive output of the snails during the summer of both experiments suggests that particular periods during the reproductive cycle of *P. corneus* snails may be more sensitive to the effects of TSE than others (see Figure 5.6). Two other studies have also demonstrated stimulatory effects of TSE on egg/embryo production in freshwater snails (Jobling *et al*., 2003, in *Potamopyrgus antipodarum*; Watton and Hawkes, 1984; in *L. peregra*).

Furthermore, in my experiments several reproductive parameters were affected during the maximal effect of the effluent (experiment 3, week 4). In 100% effluent, there was a reduction in both the number of eggs/mass and weight of individual masses in summer (see Figs. 5.8 and 5.9). Watton and Hawkes with *L. peregra* demonstrated a similar reduction in the number of eggs/mass in both 25 and 50% effluent compared to river water during the peak of reproduction. In my experiments with river water, a lower "normal" reproductive rate appeared to allow more eggs to be incorporated into each mass and increased, by comparison, the weight of individual masses. Casey (2005) reported a minimum production cost of an egg mass to snails. Indeed, anything below 43 eggs/mass represents energy inefficiency in egg mass production in *L. stagnalis* due to minimum quantities of packaging materials used to produce each mass. Since energy in our experiments was in excess, the effluent effects caused the snails to produce a greater number of smaller masses over time. In 100% effluent (Figure 5.28), the number of eggs per mass and weight of individual masses remained slightly enhanced instead of steadily declining as occurred in masses from snails in river water as autumn approached. Only Watton and Hawkes (1984) reported that 25 and 50% effluent caused snails to produce egg masses containing fewer eggs at the peak of reproduction, although there was no seasonal pattern to this effect.

A)



B)

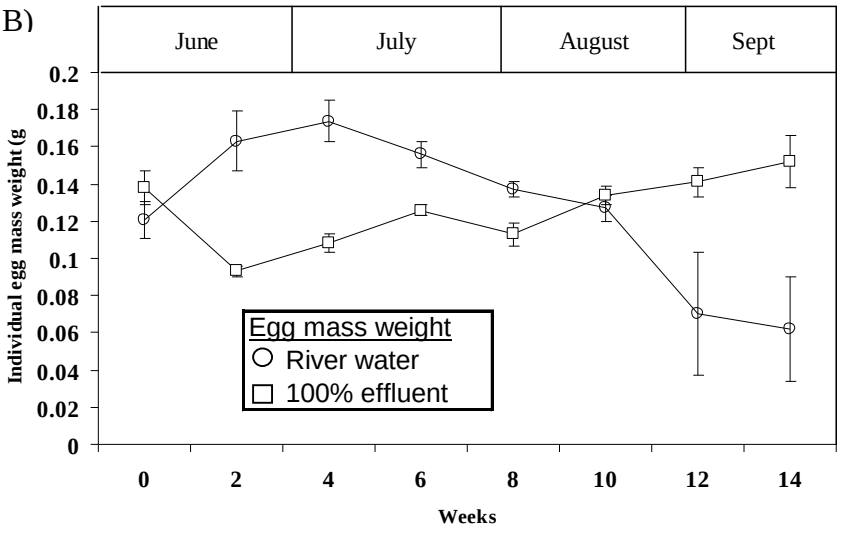


Figure 5.28 (A) Mean number of masses per snail (±SE) produced by groups (Experiment 3; 9 snails per group) of *P. corneus* exposed to river water, or 100% effluent, and eggs per mass from the same egg masses collected in river water or 100% effluent (Fig. 5.8C), and (B) Individual egg mass weight (±SE) of the same egg masses in river water or 100% effluent during experiment 3. Experiment 3, 25th May-13th September 2004.

At the midsummer peak of reproduction (weeks 2-4; 6th July) egg masses in river contained 62 eggs/mass, compared to 54 (25% effluent) and 53 (50% effluent), and 44 eggs/mass in 100% effluent. Therefore, during this part of the reproductive cycle it appears that at least CDCH production (and possibly DBH) was increased relative to river water in my experiments. Taken together, the results of these studies suggest that reproduction in *Planorbarius corneus* and also *L. peregra* are both sensitive to the effects of TSE and that these effects are likely to differ depending on the point in the reproductive cycle at which the exposure to TSE takes place.

There are many different characteristics of the treated sewage effluent that could have been responsible for the altered reproductive output of the effluent exposed snails in our experiments. The physico-chemical characteristics (e.g. pH, 02 concentration, turbidity, conductivity, nitrate concentrations, etc.) could have had an influence on reproduction (and development), but most of these parameters were not quantified as part of these studies. However, in the study of Watton and Hawkes (1984) where most of these parameters were measured, they were not found not to have an influence upon reproduction during any part of the reproductive season. However, one of the most obvious of these characteristics is treated sewage effluent as a food source, and the possibility that the effluent represented an additional food source for the snails relative to the river water alone leading to a possible enhanced reproductive output and growth in effluent exposed snails, relative to the reference snails. Moreover, a further possibility might be that the snails in the river water were starving relative to the effluent-exposed snails, and therefore, grew and reproduced less. In *P. corneus,* starved for approximately 2 months, for example, 95% of the polysaccharides, and 49% of the proteins were metabolised resulting in an overall 50.5% fall in dry weight (Emerson, 1967). In my experiments, however, there were no significant decreases in mantle protein, increases in polysaccharide hydrolyzing enzyme activity, or increases in weight loss; all snails grew well and gained in weight throughout all experiments.

In Experiment 1, the snails in river water grew to the same extent as those in effluent, whilst in Experiments 2 and 3, the snails growth in effluent was greater than those in river water, albeit only significantly ($p<0.05$) by the end of Experiment 3 in autumn and into winter. Perhaps importantly, most of the apparent differences in growth between river water and effluent dosed snails disappeared once growth rate, and

therefore, the initial size of the snails was taken into account. Interestingly, the study of Watton and Hawkes (1984) demonstrated that *L. peregra* snails in effluent grew at the same rate as those in river water despite additional food in the form of filamentous algae (*Cladophora sp.)*. The authors suggested that the presence of algae might have been the cause of increased reproduction in effluent exposed snails although they could not provide direct evidence to support this suggestion. Algae were also present in my own effluent tanks. However, there was no evidence in Experiment 1 that snails grew faster than in river water due to its presence, but in Experiment 3 snails growth was greater in effluent than in river water, but only during a relatively short period of time late summer. This suggests that this alga was not cause of the differences in growth seen. Further, the protein levels from snail mantles in 100% effluent were slightly lower (not significantly so) than those from river water during Experiment 1 indicating a similar nutritional status in both groups of snails. The activity of the polysaccharide (glycogen) hydrolyzing enzyme glycogen phosphorylase increased in all treatments equally as the reproductive season began in experiment 1 (Figure 5.14; by week 6). Joose and van Elk, 1986, fed *L. stagnalis* to considerable excess (Scheerboom, 1978) and demonstrated that the growth of both the body and albumen glands continued after egg laying began, glycogen levels in mantle tissues decreased during this time (which explains the increase in glycogen phosphorylase activity). Baturo *et al*., 1995 reported that the growth rate of food restricted *L. palustris* decreased when reproduction began, confirming the relationship between glycogen levels in mantle tissues and polysaccharide hydrolysing enzyme activity at this time. Together, this suggests that glycogen stores in mantle tissues of snails were similar during the peak of reproduction in all treatments during Experiment 1, and that the effluent did not have a significant positive nutritional effect on the snails compared to river water during the period of strong summer reproduction. Therefore, as food requirements and feeding play an important role in growth and reproduction in pulmonates (Baturo *et al.*, 1995), and each treatment received equal amounts of food, it seems likely that the effects on growth and reproduction were due to other properties of the effluent.

Chemicals present in the effluent could have had a direct toxicological effect (Jumel *et al.,* 2002) upon the snails, thus influencing their reproductive rate. However, the small differences in mortality rates between treatments and compared to river water

suggests only sub-lethal effects in any of the experiments described here. The possibility therefore exists that substances present in the effluent affected reproduction as a result of their action on the neuroendocrine system. In basomatophorans, such as *Planorbarius corneus*, both growth and reproduction are under the independent control of various types of neurosecretory cells located in the cerebrial region of the central nervous system (Baturo *et al.,* 1995). Female reproduction is under the control of the lateral lobes (LL), which respond to both external and endogenous stimuli (Baturo *et al.,* 1995; Bohlken *et al*., 1986, Ter Taat *et al*., 1983, Dogterom *et al*., 1985). Therefore, in my opinion, as "R strategists" (see Dillon, 2000), the chemical nature of the effluent could have stressed the snails to reproduce more, via a common neurological pathway which has already been suggested to have this effect (i.e. where excessive temperature fluctuations imitate conditions relating to the drying up of ponds or poor environmental conditions) resulting in more reproduction in snails. As yet, this phenomenon can only be explained as an external environmental influence (or signal) that can impact the central nervous system (affecting the lateral lobes) resulting in internal effects amongst snails (impacting the endocrine system), an increase of reproduction (Laufer and Downer, 1988). The action of the LLs results in production of both CDCH (caudodorsal cell hormone) and DBH (dorsal-body hormone) (increased egg and egg mass production) and inhibition of the light green cells (LGC) that produce a growth promoting substance (*viz*. GH). DBH specifically stimulates vitellogenesis and growth and synthesis in female accessory sex organs thereby modulating the number of eggs per mass (Jumel *et al*., 2002). CDCH, or egg-laying hormone, induces ovulation and oviposition, controlling the number of egg masses. At least one author has suggested that certain chemicals can affect the different forms of cytochrome P450 mono-oxygenases within snails` tissues affecting hormone balance (suppressing steroid synthesis) and leading to impaired reproductive performance (Baturo *et al.*, 1995), as demonstrated for polychlorobiphenyl exposure in *Asterias rubens* (Den Besten *et al*., 1991). Further, (CYP10) cytochrome P450 is specifically and abundantly expressed in the dorsal bodies that produce dorsal body hormone (DBH) and is involved in its synthesis, therefore reductions in activity of CYP10 could be responsible for reduced egg production (Teunissen *et al*., 1992). Bluzat and Seuge (1979) reported that pond snails (*L. stagnalis*) exposed to a low concentration of the pesticide Lindane produced fewer eggs than control animals and that at higher concentrations, the number of egg

masses was affected too (Jumel *et al.*, 2002). Similarly, in *L. stagnalis* exposed to Cadmium, the number of eggs per mass decreased at 200 µg/L, whereas egg mass production was totally inhibited at 400 µg/L (Gomot, 1998), probably the result of inhibition of the caudodorsal cells (Baturo *et al.*, 1995) by Cadmium. However, at 400 µg/L the effects of Cadmium were most likely due to toxicity as mortality in this treatment increased steeply after 2 weeks exposure to this metal ion.

A number of studies have described the reduction in numbers of eggs per mass and in egg masses as a result of exposure to chemicals (Canton and Sloof, 1977; Seuge and Bluzat, 1982; Woin and Bronmark, 1992; Gomot, 1998; Jumel *et al*., 2002). Unlike my findings, the majority of these studies report decreases in reproductive output with increasing dose of chemical. However, few studies have examined the relationship between reproduction, growth (energy balance), and mortality. One exception is a study in which *L. stagnalis* was exposed to formesan (diphenyl ether herbicide), where food restricted (or starved) snails demonstrated glycogen mobilization (from mantle tissue) with subsequent decreased growth but without increased reproduction (Jumel *et al,* 2002). Similarly in terms of effects upon growth**,** Baturo *et al.*, (1995) exposed *L. peregra* to the chlorinated fungicide HCB (hexachlorobenzene) and reported increased egg mass production with an associated decrease in growth rate. Both authors suggested that energy for reproduction was diverted away from growth, and that stimulation of LL resulted in increased DBH and CDCH production and inhibited LGC function in exposed snails compared to snails in control water. In contrast, snails in my experiments were supplied with an excessive amount of food (i.e. lettuce), resulting in energy available for both growth and reproduction. Berrie *et al.*, 1966 described the rapid growth of both the body and albumen glands in a natural population of *Lymnaea stagnalis* in spring. At the peak of reproduction, albumen glands showed a considerable reduction in size and it is likely that these snails were food restricted. As snails bodies and albumen glands continued to grow throughout Experiment 1, it is possible that we avoided a switch of energy away from growth to reproduction, due to either an excess of available energy for both processes, or because of other effects of the effluent.

It is theoretically possible that hormonally active (estrogenic) chemicals in the effluent were responsible for the increase in reproduction observed in effluent exposed snails. This type of effect has been well documented in fish (Purdom *et al.*, 1994, Harries *et al.*, 1996, Harries *et al.*, 1997), though the endocrinological route via which the effect could occur in snails is largely unknown. Further, laboratory experiments have demonstrated an increase in reproduction of *P. corneus* exposed to the synthetic-steroid oestrogen (EE2) or xeno-oestrogen BPA (Rachel Benstead pers. comm.). Oehlmann *et al.*, 2000, and Schulte-Oehlmann *et al.*, 2004 reported stimulatory effects of the synthetic-steroid oestrogen (EE2), alkylphenolic chemicals (4-NP, and 4-tert OP) and bisphenol-A on reproduction in the tropical snail *Marisa cornuarietis* and in the marine prosobranch *Nucella lapillus* (EE2 not tested). Duft *et al.*, 2003b reported similar stimulatory effects of OP, NP, and BPA on the production of new embryos in the brood pouch of the freshwater mudsnail *Potamopyrgus antipodarum*. All of these chemicals were measured in the effluent during my experiments described during this chapter (Tables 5.1A-C).

These results of the chemical analyses of water in treatment tanks and consequent reproductive output of snails may indicate that *P. corneus* snails were more responsive to the effects of alkylphenolic chemicals than estrogenic chemicals in the TSE. Due to varying organic and bacterial content in individual treatment tanks, the "oestrogenic" concentration within tank each is not a straightforward function of the dilution of full strength effluent with river water. Further, whether due to these effects, or others, such as a natural variation in the chemistry of TSE, the concentration of alkylphenolic chemicals and oestrogens varied widely between time points sampled (see Tables 5.1A-C). However, even allowing for these variations, the difference in the chemical content between effluent treatments and river water could help explain the differences in mortality and reproduction observed both between and within experiments. By week 10 of Experiment 3, there were particularly high concentrations of alkylphenolic chemicals (NP2EO, NP, OP) and BPA, but not steroidal estrogens (comparatively low compared to week 14). While snails in river water at this time (week 10) closely followed the seasonal decline in egg mass production, snails in 50% and 100% effluent had atypically high seasonal reproductive output (that had increased over the previous 2-week period) (Fig. 5.6C). At the end of the experiment (week 14), reproductive output had decreased

dramatically in river water, and the same was cseen in 50% and 100% effluent after week 10. By this time, alkylphenolic content had dropped drastically, and steroidal oestrogen content risen dramatically compared to week 10, indicating that levels of steroidal estrogens failed to maintain reproduction in 50% and 100% TSE. Therefore, it could be that comparatively high alkylphenolic content had a much greater effect on reproductive output of snails than high estrogen content.

The way in which effluent affects reproduction in *P. corneus* snails could be many. Nevertheless, overall reproduction in *P. corneus* was increased as occurs when vertebrates, such as fish species (Purdom *et al*., 1994, Harries *et al*., 1996, Harries *et al.*, 1997), are exposed to the mixture of estrogenic compounds found in TSE. However, whether or not this effect can be classed as an "oestrogenic effect" is presently unknown. There is evidence that a seasonal increase of reproduction in *P. corneus* in effluent may be associated with a small increase in mortality. Exposure to effluent at the peak of reproduction caused snails to produce smaller (lighter and less fecund) egg masses although a higher rate of reproduction. These snails also failed to undergo the normal seasonal decrease in both egg mass production and individual egg mass size. Associated with this effect, was a decreased mortality rate amongst snails in 25% effluent, an effect more dramatically illustrated by the study of Watton and Hawkes, 1984 and increased growth in effluent compared to snails in river water.

5.5 Conclusion

Whatever the cause, the results of my experiments provide considerable evidence that exposure of adult *Planorbarius corneus* to a STW effluent disrupts the seasonal reproductive cycle with possible small concurrent increase in mortality early summer, with decreased mortality and increased growth late season (autumn). Further, in our studies TSE had affects on other parameters of reproduction during the seasonal peak, in 100% effluent the number of eggs/mass and egg mass weight was reduced compared to egg masses from snails in river water control. Importantly snails in effluent failed to undergo the normal seasonal decline in egg mass production in autumn. The mechanisms underlying these effects need considerable further elucidation, and the effects at the population level (e.g. hatchability, survivability, and

reproductive performance of F1`s etc.) are presently unknown. However, my preliminary data strongly suggests that effluent does not affect the hatchability of baby snails from eggs. Still though, *P. corneus* has proved to sensitive to effects of TSE and therefore is a good candidate species upon which to study the effects of xenobiotics in a temperate aquatic environment.

5.6 Discussion

5.6.1 *Viviparus viviparus*

In this chapter, the effects of a graded treated sewage effluent on the seasonal response of the prosobranch gastropod mollusc *Viviparus viviparus* are described. My results indicate that *V. viviparus* adopted a reproductive strategy that was tightly influenced by daylight (Ultimate factor). Water temperature appeared to have comparatively less influence upon this species of freshwater snail.

Despite this, my results indicate that the various doses of treated sewage effluent produced a different seasonal reproductive response compared to *V. viviparus* held in river water. Although not clearly dose dependant, snails exposed exposed to 100% effluent experienced a second large increase in their reproductive rate during late summer (August-September) with considerably more mature embryos released relative to river water.

This second peak of reproduction was accompanied by a dose dependant decrease in the number of youngest unshelled embryos in the brood pouches of female snails, perhaps suggesting an increased rate of maturation of immature embryos into mature shelled embryos prior to to partuition. These phenomena where clearly observed in my experiments (Summer to Autumn) where in 100% effluent the possible early maturation of young unshelled embryos into mature embryos was followed by the subsequent release of a significantly increased number of mature embryos. These phenomena may have population level effects as females that fail to cease reproducing towards autumn and increase their body weight prior to winter may suffer

higher mortality rates, and further, consequent effects upon the increased number of embryos released is unknown. This extra peak of reproduction had no significant effects on female body growth or mortality compared to river water snails. Snails in all other effluent doses also experienced an additional increase in their reproduction, although this effect was variable between doses and over time.

Furthermore, mature embryos released in spring from snails exposed to effluent weighed significantly less than those in river water suggesting they were premature. Interestingly, in what must have been a separate phenomenon, there was a failure of mature embryos born in effluent (significantly in 100% effluent), to undergo the seasonal decline of weight observed in baby snails in river water towards Autumn.

By mid-Summer, snail fecundity (new embryo production) appeared to be inversely related to the reproductive output. Indeed, a dose-related decrease in the number of newly recruited (unshelled) embryos with inceasing effluent dose, as well as an increase in the number of mature embryos released in effluent (100% effluent only consistently significant) were observed.

Although mature shelled embryos in the brood pouches of female snails during early summer (Figure 5.27; week 6) did not weigh significantly more or less than those from the brood pouches of snails in river water, approximately one month later during July (Figure 5.27B; weeks 0-2, and 2-4) babies born in effluent weighed significantly less than those in river water. Although the underlying mechanism(s) are unknown, the loss of weight post partuition might suggest these babies were premature (due to effects of the effluent on embryos in pallial oviducts), and exhibited a failure to thrive after being born in effluent. Alternatively, a negative effect of the effluent (endocrine disruption or toxicity) upon the health of these baby snails could have occurred post partuition. As the decrease in weight post partuition upon recently born snails occurred early summer only, this might suggest a particular seasonal effect at this time.

My results also suggest that phenotypic plasticity may have occurred allowing a second reproductive peak late summer in response to TSE when only a single peak occurred in river water during spring. There is to my knowledge only a single report

describing the seasonal reproductive profile of a viviparid with a similar lifecycle (Ribi and Gebhardt, 1986; *Viviparus. ater*), that could produce two reproductive peaks under favourable conditions; (one in Spring and another in late Summer), and one under less suitable conditions, suggesting that our results may not be unusual for these viviparid snails.

Interestingly, increased rate of new embryo production was previously reported in *P. antipodarum* exposed to the same TSE used in our own experiments (Jobling *et al*., 2003). With this prosobranch, there was a dose related increase in new embryo production within 14 days when exposed to TSE. In my own experiment 1 (Figure 5.24A), the opposite effect occurred; dose dependant decreases in new embryo production after 12 weeks. However, the rate of maturation of unshelled embryos into mature embryos is unknown, and it maybe that in our own experiments these new embryos matured more quickly than those in river water resulting in the increased reproduction observed in effluent late summer. In the experiment by Jobling *et al*., 2003, reproduction or birth rate was not measured, and therefore, the relationship between reproduction and fecundity was unknown. Due to differences in experimental design, it is not possible to compare seasonal effects of TSE between these two species of prosobranch mollusc.

During spring there were comparatively equal small mortality rates in all treatments (Table 5.5). This was associated with a decrease in reproduction (with a coincidental increase in fecundity) due to increases in day length (Fretter and Graham, 1962). However, in autumn, there were particularly large increases in mortality rates that also occurred in equally in all treatments (Table 5.5), a period where there were large seasonal seasonal decreases in day length (but not water temperatures). This suggests that day length rather than effects of the TSE, may have been responsible for their demise. It is possible, therefore, that this comparatively long lived viviparid (commonly 6 years, sometimes 11 years) snail adopts a life cycle that includes a late summer die off (senescence) amongst the largest adults (oldest adults, and late born juveniles) of the population (Fretter and Graham, 1965). However, true senescence in a viviparid has been cited once before by where *V. ater* adults were observed to die

off gradually (low death rate over time) during their last autumn/winter (Van Cleave and Lederer, 1932). Further, growth data indicates that snails that reproduced more (Fig. 5.27A) were not significantly larger or smaller (weight without shells and embryos) than those in river water (Fig. 5.20).

Shells of dead snails indicated no seasonal change in the preponderance for larger or smaller snails to die (Fig. 5.22), nor where there any between treatment effects upon the size of snails dieing during any time period. This suggests that mortality occurred equally across snails of all size classes, and that day length and not reproductive effort (or size, therefore age) was the cause. Therefore, since the largest (therefore oldest) snails were not responsible for increased mortality rates in autumn, and the death rate was far in excess of what would be observed if senescence was the cause, this data together suggests a mechanism other than senescence was responsible, and that TSE also, was not the cause.

The mortality rate of snails varied between 8.3% and 71.2% and was higher in Experiment 3 than Experiment 1. During the course of both experiments, the mortality rate in all treatments was higher than might be expected under ideal experimental conditions. Viviparids held in predator secure cages from March to October (see Chapter 4 Introduction), had relatively lower mortality rates (Ribi and Gebhardt, 1986). Metal ion toxicity, which was implicated in the mortality of *P. antipodarum* snails in effluent (Watton and Hawkes, 1984), is unlikely to be the cause of mortality in effluent in my experiments as snails died equally in both river water and TSE. As part of their lifecycle, *V. viviparus* produce a few embryos per season over several seasons, and therefore, die at the end of their natural lives, and not due to the reproductive effort of that season (like pulmonates). During the final 6 weeks of experiment 3, reproductive rate was highest in 100% effluent, compared to the reproductive rate of snails in river water, yet their mortality rates were very similar. Taken together, this suggests that TSE did not influence mortality of snails due to a secondary effect of increased reproduction. Rather, a negative effect of the conditions within the experimental system itself on the snails may be responsible for the increased mortality observed in all treatments towards autumn.

As discussed with a pulmonate snail *P. corneus* (see section 5.6.1.2; detailed in the Discussion), the chemical nature of the effluent and/or excessive temperature fluctuations may have caused the snails to adapt a form of semelparity in which a single large reproductive event precedes mortality (Dillon, 2000, Calow, 1978). Alternatively, long days as well as rises in water temperature can cause an increase in reproductive activity as 16L-8D days can increase eggs numbers by as much as x10-x20 in pulmonates such as *L. stagnalis* (Bohlken, 1978). This increased reproduction can be at the expense of body growth, and exhaustion of the snail's energy reserves (resulting in death) could explain the mortality observed in my experiments. There may have been a high cost (in energy terms) to the possibly greater fecundity (new embryo production) and reproduction in 100% effluent during early Summer (June onwards) as indicated by the high variability in the mortality data by week 6 of Experiment 1 (see Table 5.5). However, snails in effluent survived at least as well, if not better than snails in river water in autumn, suggesting that increased reproduction in effluent at this time did not negatively affect their health. Taken together, this data suggests that the increased reproduction in effluent from mid-summer onwards was not the cause of mortalities, at least not immediately.

In late spring/early summer (to week 6 of Experiment 1), there was a suggestion of increased growth in effluent with little effect upon mortality (Figures. 5.17A and Table 5.5). In late summer (week 12 of Experiment 3), high reproductive rates in 100% effluent had no effect upon growth and only slightly increased mortality rates (Experiment 3) compared to other treatments (Figures 5.17 and Table 5.5). This suggests only minor energy costs of increased reproduction of snails in effluent. Furthermore, snails in river water and effluent doses up to 50% grew less in early summer (Experiment 1; weeks 0-6), and grew more late summer (Experiment 1; weeks 6-12) without increased reproduction, and survived better than snails in 100% effluent**.** When taken together, this evidence suggests only a slight negative effect of 100% effluent overall on snail survival (if at all).

The seasonal change in the number of new embryos in the brood pouch of females and mature embryo numbers released during both experiments suggests the effects of effluent occurred over some considerable time period, possibly months. Indeed, the effects of effluent would have to occur throughout the comparatively long gestation

time of viviparid embryos (Van Cleave and Lederer, 1932; approximate 8 month period) in order to produce the combined pattern of new and mature embryos (and embryos released) seen over a season. This suggests that *V. viviparus* were sensitive to the effects of TSE, if not throughout the entire gestation period of embryos, then over a considerable part of it. As mentioned (Chapter 5, Introduction), one other study has also demonstrated stimulatory effects of TSE on embryo production in a freshwater prosobranch snail (Jobling *et al*., 2003, in *Potamopyrgus antipodarum*). In this study, only a single parameter of reproduction was demonstrated to be affected; the production of new embryos. In my experiments, only the reproductive rate (release of mature embryos), was demonstrably increased with increasing TSE concentration. However, the numbers of embryos in the brood pouch has provided the first evidence that mature embryos (when born) were replaced by new embryos at this time, thereby suggesting both an increased rate of new embryo recruitment or fecundity, and increased reproductive rate. When the total numbers of embryos in the brood pouch are added to numbers of mature embryos released, the data suggests that snails in effluent were not depleted of embryos compared to snails in other treatment groups. The data also suggests that the overall increase in reproduction in 100% effluent was not at the expense of growth of adults, or was the cause of mortalities. There was also evidence that reproduction in 100% effluent during early summer produced young that were released prematurely, as both the weight of shelled embryos in the brood pouch, and the weight of mature embryos released in 100% effluent from late June to mid July were less than the weight of embryos in river water. This would suggest a further period of time over which the TSE had effects on developing embryos, whether this occurred in the pallial oviducts of female snails and/or post partuition.

In my experiments, the second reproductive peak observed in effluent exposed snails (experiment 3 weeks 6 to 8 onwards), produced baby snails that failed to undergo the "normal" seasonal decline in weight seen in mature embryos in river water. This was most likely a post partuition effect since their weights in the pallial oviduct before partuition were equivalent to those in river water. However, an effect during gestation in the pallial oviduct that was expressed post partuition cannot be ruled out. Whether the baby snails in river water or effluent were an unhealthy or abnormally developing snail group is also presently unknown (though weight loss in river water intuitively

seems unusual). To my knowledge no other author has studied these parameters in prosobranchs before, let alone in TSE exposed viviparids, and therefore, the underlying mechanisms can only be speculated upon. However, during the reproductive cycle it appears that both CDCH (or equivalent) and DBH production were increased in effluent relative to river water in my experiments mid-summer onwards. Taken together, the results of my studies strongly suggest that *Viviparus viviparus* is sensitive to the effects of TSE, and that these effects are likely to differ depending on the point in the reproductive cycle at which the exposure to TSE takes place.

There are many different characteristics of the treated sewage effluent that could have been responsible for the enhanced reproductive output and possibly increased fecundity of the effluent exposed snails in my experiments. The most obvious of these would appear to be the possibility that the effluent represented an additional food source for the snails relative to river water alone leading to enhanced reproductive output and growth in effluent exposed snails, especially as viviparids can be suspension feeders and TSE is heavily laden with organic material. A further possibility is that the snails in the river water were underfed relative to the effluent-exposed snails, and therefore, grew and reproduced less.

In experiment 1, the snails in river water grew significantly less than those in 50% effluent (25% effluent almost significant) over the first six weeks of the experiment, though this pattern was reversed over weeks 6 to 12 with snails in 100% effluent growing significantly the least (Figure 5.19B). During experiment 3, all snails underwent negative growth, and it may be that mortality amongst snails masked growth effects. However, this hypothesis is difficult to confirm from the data obtained largely due to the high mortality rates in experiment 3 compared to experiment 1. Though there were apparent seasonal differences in the growth and reproductive rate between river water and effluent groups, overall, the general small differences in growth suggests that snails were not starved in river water, nor was effluent a significant food supply. Moreover, these growth effects may be smaller than thought when the weight of shells (Figure 5.20) and embryos (Figure 5.21) are taken into account, though these analyses were only undertaken for experiment 3. As no analysis of viviparid snail tissues was undertaken, the nutritional status of the snail groups in

each of the treatments is unknown. Food requirements and feeding play an important role in growth and reproduction in pulmonates (Baturo *et al.*, 1995), however, these prosobranchs both grow more slowly and reproduce considerably less in each season than their distantly related cousins the pulmonates. Despite this, had effluent been a significant additional food source to the snails I believe growth effects would have been apparent in younger snails. Further, snails in river water where not starving compared to snails in TSE as good growth during summer in river water suggested (Figure 5.19 and Chapter 4; discussion of effects of river water on *V. viviparus*; section 4.8.1). Therefore, taken together this data suggests that the effluent (including subsequent algal growth) did not have a significant positive nutritional effect on the snails compared to river water. As equal amounts of food were available in each treatment, and any differential nutritional affects on growth were not significant, the effects of TSE upon reproduction are likely to be due other properties of the effluent.

Chemicals present in the effluent could have had a direct toxicological effect upon the snails (Jumel *et al.*, 2002), thus influencing their reproductive rate. However, the changing seasonal differences in mortality rates between treatments compared to river water suggests only sub-lethal effects in either of the experiments described here. This leaves the possibility that substances present in the effluent affected reproduction as a result of their action on the neuroendocrine system. In basomatophorans, such as *Planorbarius corneus*, the endocrine control of both growth and reproduction are well described (see Chapter 4 Introduction). In contrast, in prosobranchs only a group of cells both positionally and functionally similar to the paired DBs found in pulmonates have been identified. Further, though a hormone thought to be functionally similar to DBH has been identified, no further details regarding the endocrine control of reproduction (and growth) are available for prosobranchs. Therefore, any hypothesis that relies on knowledge of the endocrine system of prosobranchs is largely hypothetical. Nevertheless, some commonality of reproductive response to xenobiotics has been established with both marine prosobranch molluscs and other invertebrates in both laboratory and field experiments. At least one author has suggested xenobiotics can affect the different forms of cytochrome P450 mono-oxygenases within snails` tissues affecting hormone balance (suppressing steroid synthesis) and can lead to impaired reproductive performance (Baturo *et al.*, 1995), as demonstrated for polychlorobiphenyl exposure in *Asterias rubens* (Den Besten *et al.*,

1991). Further, (CYP10) cytochrome P450 is specifically and abundantly expressed in the dorsal bodies that produce dorsal body hormone (BDH) and is involved in its synthesis, and could therefore be responsible for reduced egg production (Teunissen *et al.*, 1992). Bluzat and Seuge (1979) reported that pond snails (*L. stagnalis*) exposed to a low concentration of the pesticide Lindane produced fewer eggs than control animals and that at higher concentrations, the number of egg masses was affected too (Jumel *et al.*, 2002). Similarly, in *L. stagnalis* exposed to Cadmium, the number of eggs per mass decreased at 200 µg/L, whereas egg mass production was totally inhibited at 400 µg/L, possibly as a result of inhibition of the caudodorsal cells (Baturo *et al.*, 1995). Though there are many accounts of the effects of xenobiotics on the reproductive performance of freshwater basomatophorans (see discussion Chapter 4), there are a few reports of their effects upon freshwater prosobranchs.

The effects of hormonally active (oestrogenic) chemicals have been well documented in fish (Purdom *et al.*, 1994, Harries *et al.*, 1996, Harries *et al.*, 1997). However, the endocrinological route via which reproductive effects of effluent could occur in snails is largely unknown. Oehlmann *et al.*, 2000, 2004 reported stimulatory effects of the synthetic-steroid oestrogen (EE2), alkylphenolic chemicals (4-NP, and 4-tert OP) and bisphenol-A on reproduction in the tropical snail *Marisa cornuarietis* and in the marine prosobranch *Nucella lapillus* (EE2 not tested). Duft *et al.*, 2003 reported similar stimulatory effects of OP, NP, and BPA on the production of new embryos in the brood pouch of the freshwater mudsnail *Potamopyrgus antipodarum*. All of these chemicals were measured in the effluent during my experiments described during this chapter (Table 5.1). Moreover, due to varying organic and bacterial content, the "estrogen" concentration within each treatment is thought not to be a straightforward function of the dilution of full strength effluent with river water, and the concentration of oestrogens varied widely between time points sampled. The difference in the chemical content between effluent treatments and river water could explain the differences reproduction both between and within experiments.

During experiment 3, there were particularly high concentrations alkylphenolic chemicals (NP2EO, NP, OP) and BPA, but not estrogens, at the end of week 10 (the converse was true at the end of the experiment) (Figure 5.1C). However, by the end of the experiment, though water temperature decreased in all treatments**,** while snails in

river water at this time through weeks 8 to 12 closely followed a seasonal decline in both the numbers of live born snails released and their mean weight (Figs. 5.26A and B), snails in 50 and 100% did neither, and continued to reproduce without these babies decreasing weight thereafter (seasonally atypical). These results of the chemical analyses of water and consequent reproductive output of snails may indicate that like *P. corneus* snails, *Viviparus vivipraus* were more responsive to the effects of alkylphenolic chemicals than estrogenic chemicals in the TSE. This may be important as in particular one of the major effect of the effluent observed, the second (only in effluent) reproductive peak was observed late summer 2004 when concentrations of alkylphenolic compounds were found to be particularly high.

Though the actions of effluent upon reproduction in *V. viviparus* snails could be many, overall reproduction was increased in a similar way to when vertebrate fish species (Purdom *et al*., 1994, Harries *et al*., 1996, Harries *et al.*, 1997) are exposed to compounds found in TSE. However, whether or not an "estrogenic effect" occurred here, is unclear. There is some evidence that in this invertebrate species a seasonal increase of reproduction in effluent occurred, which was not associated with any significant increase in mortality by the end of either experiment as indicated by both of my experiments.

5.73 Conclusion

In conclusion, these results provide considerable evidence that exposure of adult *Viviparus viviparus* to a STW effluent disrupts their seasonal reproductive cycle without concurrent increases in mortality rate or significant effects upon growth.

Reproductive disruption upon exposure to TSE has also been demonstrated in other freshwater pulmonate species, both in my own experiments with *Planorbarius corneus* (chapter 5), and with *Lymnaea peregra* (Watton and Hawkes, 1984), as well as in a prosobranch species *Potamopyrgus antipodarum* (Jobling *et al.*, 2003). In my own experiments reproductive rate was considerably increased in autumn resulting in a second reproductive peak (only observed in 100% effluent) and to a lesser degree in

25% and 50% effluent. Further, TSE had effects on other parameters of reproduction during the autumnal reproductive peak, in the 100% effluent dose mature embryos released failed to undergo the seasonal decline in their weight seen in snails in all other treatments (including 25% and 50% effluent). The failure of baby snails to loose weight in autumn in 100% effluent might positively affect their chances of survival. However, whether short-term gain in survival rate would remain in the longer term is unknown.

The mechanisms underlying these effects upon both adults and their offspring need considerable further elucidation, and the effects at the population level (e.g. survivability, and reproductive performance of F1`s etc.) are presently unknown. However, *V. viviparus* (both adults and offspring) has proved to be sensitive to effects of TSE over, possibly, if not through the entire spring thorough autumn time period then over a considerable part of it, and therefore is a good candidate species upon which to study the effects of xenobiotics in a temperate aquatic environment, given some species specific ecological constraints relating to the experimental setup (flow rate, food source).

Chapter Six: A Preliminary Study looking for Evidence of Developmental Disruption

6.1 Introduction

Two orders of the gastropods, the opisthobranchs, and the pulmonates, consist of hermaphrodite species. But even in the prosobranchs, considered to be gonochoristic, the phenomenon of sex reversal occurs in many species (e.g. *Patella vulgata*, *Crepidulata fornicata*). Therefore, the endocrinology of reproduction in gastropods is almost entirely limited to the study of the regulation of hermaphroditism or sex reversal (Joose, 1972). As research into the endocrinology of reproduction amongst gastropods has progressed little since the mid 1980`s (see Introduction chapter 4), very little detailed reseach has documented the histology (or morphology) of the full reproductive system of these molluscs. Therefore, certain aspects of the biology of pulmonate snails has received extensive study (Berrie, 1966); including the processes of insemination and ovulation that give a detailed account of the histology of the gonad, and of the sequence of events involved in spermatogenesis and oogenesis. However, more recently renewed interest in the gonadal and reproductive development of molluscs has followed studies into the effects of EDCs on mollusc reproduction, as reproduction is a sensitive endpoint in other taxa.

Sexual disruption has been studied to a far greater degree in other animal species. An important part of the study of sexual disruption is the determination of the sex of the individual animal. Mechanisms controlling sex determination differ between vertebrate groups. In mammals and birds, the process of sex determination is under genetic control, while in some reptiles though genetics play a role in sex determination (snakes, and lizards), sex determination is controlled by environmental factors (Johnston *et al*., 1995). In mammals, genetic sex is not determined by exposure to "hormones" whatever time that exposure occurs; however, the morphology of the reproductive system and the sexual behaviour of the resulting offspring can be affected (Vom Saal, 1981). In many reptiles, temperature-activated genes encode steroidogenic enzymes and hormone receptors, which, in turn may alter

the subsequent sex of the offspring. In this class of vertebrates, both gonadal and genetic sex depends on the temperature at which the egg is incubated (Wibbels, *et al.*, 1993, Wibbels, *et al.*, 1994). Few studies have been conducted on sex determination in amphibians and fish, however, examples of both male and female heterogamety, and temperature dependency, occur. Although many teleost species are functionally hermaphroditic, the majority of them have separate sexes. It is generally accepted that a pair of sex chromosomes determines sex; the heterogametic sex can be the male or female, depending on the species. In some species of fish temperature markedly influences sex ratio (Conover and Heins, 1987).

Sexual differentiation is the physical translation of the genetic sex. As with sex determination, the mechanisms controlling sex differentiation differ between vertebrate groups. Although the genotypic sex is entirely determined by the compliment of genes, in many animals, hormonal and/or environmental factors can all play a role in gonadal differentiation. Sexual differentiation in all groups of fish, birds, and in some amphibians is labile and can be influenced by steroids and enzymes controlling their synthesis (Piferrer, *et al.*, 1994). In the case of molluscs and echinoderms, the involvement of steroids in sex determination is far from clear (Lafont, 1991); as is the basis of genetic sex determination

Further, other developing animals have been reported to very sensitive to oestrogens (the feminizers in effluent). In male rats, exposure to synthetic oestrogens causes morphological damage to the testes (Mclachan and Dixon, 1976). Exposure of pregnant female rats has been shown to have deleterious effects on fertility of both male and female offspring due to reproductive tract developmental abnormalities (Newbold and McLachan, 1985). As in mammals, oestrogenic chemicals can be detrimental to fish; high doses of 17ß-oestradiol administered to juvenile rainbow trout caused kidney failure and death, probably as a result of excess vittellogenin accumulation (Herman and Kincaid, 1988). Similarly, the ostrogenic pesticide ß-hexachlorocyclohexane can cause induction of vitellogenesis and hermaphroditism in juvenile guppies and medaka, respectively (Wester and Canton, 1986; Wester *et al.*, 1985). When taken together, the physiological effects of oeestrogenic chemicals on animals appear to be manifested primarily in the gonads.

In the past few years, there have been a number of studies documenting the widespread occurrence of sexual disruption in freshwater fish species (Jobling *et al*., 1998, van Aerle *et al*., 1999). An extensive study of wild roach (*Rutilus rutilus*) found "males" that also had female germ cells, "female-like" reproductive tracts (ovarian cavities) and elevated concentrations of plasma vitellogenin (VTG), a female-specific protein (Sumpter & Jobling, 1995). Furthermore, feminization of males in a second wild fish species, the gudgeon (*Gobio gobio*), has been reported in the same UK Rivers (van Aerle, *et al*., 1999). Some exposed wild fish populations display a range of reproductive problems, including retarded or disrupted testicular development (Lye *et al*., 1998), masculinisation of females (Howell *et al*., 1980), and anomalous reproductive behaviour (Jones and Reynolds, 1997). In freshwater and estuarine species a widespread incidence of intersex has been reported throughout the U.K. and in some other parts of Europe. Intersex fish are characterized by the simultaneous presence of both male and female gonadal characteristics in the same animal (Nolan *et al*., 2001). With an ever-growing data set associating these intersex fish with oestrogenic treated sewage effluent discharges, these histopathological changes have been suggested to result from the feminisation of males, though direct evidence of this is lacking (Rodgers-Gray *et al*., 2001). Comparable results in molluscs are rare and hitherto described only in laboratory experiments with gonochoristic snails exposed to Bisphenol A and OP. In *Marisa*, these xeno-oestrogens induced a complex syndrome of alterations in female *Marisa* referred to as "superfeminization" at the lowest concentrations. Affected snails were characterized by the formation of additional female organs, an enlargement of the accessory pallial sex glands, as well as gross malformations of the pallial oviduct resulting in an increased female mortality, and a massive stimulation of oocyte and spawning mass production (Oehlmann *et al*., 2000). The histopathological analyses of the gonads of both sexes during the *Marisa* parental generation test gave no indication that spermatogenesis or oogenesis were affected by either BPA or OP in the applied concentration range.

6.2 Morphology of the gonads and associated structures of pulmonate snails

In pulmomate and prosobranch snail species, only the structure of the gonads and their associated sexual structures have been well described in the literature. Very few studies have documented the effects of EDCs on the sexual determination/ development of freshwater gastropod molluscs. The following sections describe in detail the morphology of the gonads and related structures of pulmonates (and the lesser studied prosobranchs), and include where possible, further morphological details on the species of snail used in my studies; results from which are presented in this chapter.

6.2.1 Morphology of the Genital Tract

The gonad of pulmonates consists of a large number number of sac like acini (Joose, 1968)(see Figure 6.1). In each acinus male and female sex cells develop simultaneously. These acini are connected with the spermoviduct by the vasa efferentia (Joose *et al.*, 1968). The spermoviduct bifurcates at its distal end, and from here the female cells are transported into the oviduct and the male cells into the vas deferens. The oviduct has two distinct accessory glands glands; the albumen gland and the nidamnental gland.

6.2.2 Gametogenesis

In the freshwater pulmomate snails, the acini are compartmentalised; the female cells develop abluminally (Laufer and Downer, 1985). The female compartment is separated from the male cells by a continuous layer of Sertoli cells, which has its limits at the germinal epithelial ring. From this ring all cell types originate. Thus, the male compartment is the acinar lumen. These observations indicate that male and female cells develop in compartments that are physiologically different and this may also have consequences for the accessibility of hormones (de Jong-Brink *et al*., 1983). A further consequence of this morphology of the ovotestis is that ovulation of mature oocytes includes not only their liberation from the follicles but also the passage through the Sertoli cell barrier. The female cells enter the premeiotic phase and as a rule do not multiply mitotically after their differentiation. They continually remain in contact with the acinar wall. The oocytes migrate away from the acini opening in the

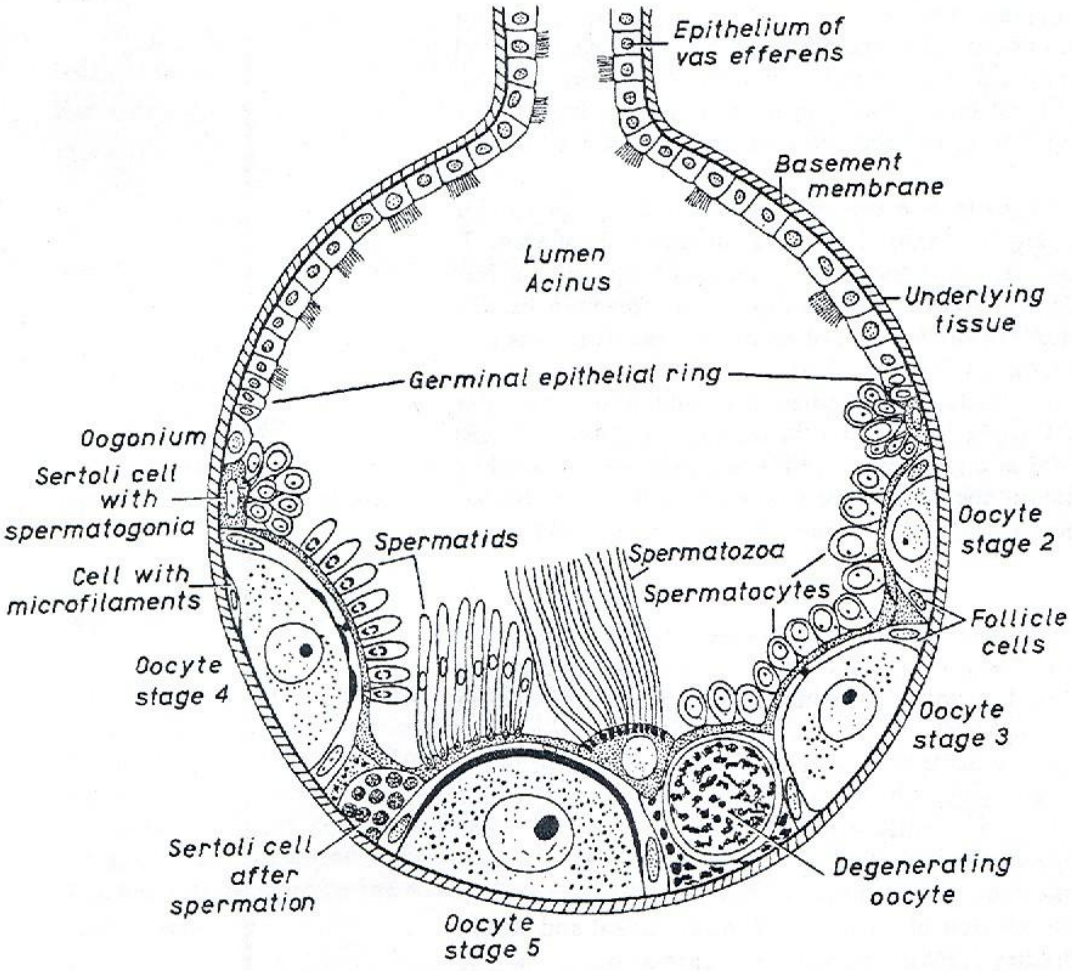


Figure 6.1 Schematic representation of a longitudinal section through an acinus of the ovotestis of *L. stagnalis*. The continuous layer of Sertoli cells separates the male from female cells. Picture taken from Laufer and Downer (1988).

previtellogenetic stage (by amoeboid movement; Bretschneider and Raven, 1951). In the vitellogenetic stage they are surrounded by a number of follicle cells (*viz* nurse cells) and remain sessile (Joose, 1972). The spermatogonia are displaced in the same direction by their Sertoli cells.

In *Lymnaea stagnalis*, the migration of the oocytes is necessary for them to reach the vitellogenetic areas. The male cells show mitotic activity after their differentiation. They are grouped upon Sertoli cells. During spermatogenesis the Sertoli cells move along the acinar wall and over the previtellogenetic oocytes to the rim of the vitellogentic area where spermiation occurs (Joose and Reitz, 1969).

The ovotestis of *Planorbarius corneus* contains the female gametes (oogonia and oocytes) as well as the male gametes (spermatogonia, spermatocytes, spermatids, and spermatozoa) (Watermann *et al*., 2007). According to the observations of Merton (1930) in *Planorbis* the spermatogonia and oogonia differentiate from the germinal epithelium near the ciliated vas eferens of the acinus. The oogonia migrate to a retro-epithelial position and become sessile when suurounded by nurse cells during the phase of vitellogenesis. The maturation of the gametes is sustained and processed by specialized agametic epithelial cells (*viz* nurse cells) of the acini. As a result of these processes the ripe gametes are found on the floor of the bottle shaped acini (Boer and Cornelisse, 1968). Oocytes are fertilised in the hermaphrodite duct, where this duct gives rise to a female and male duct. A detailed description of normal oogenesis and spermatogenesis in *P. corneus* is given in Merton (1930) and Starke (1971).

In protandrous species the juvenile gonad is bisexual, but when the animals reach sexual maturity, the male line first develops further. In *Patella vulgata* the sex reversal takes place at a variable period of the life cycle, but always during a period of post spermatogenetic rest (Chroquet, 1971). Simultaneous hermaphrodites show a short period of protandry (e.g. *Helix aspersa*, Guyard 1969).

6.3 Methods

Baby snails that been exposed to Treated Sewage Effluent (see section 2.2.1) were analysed for possible developmental effects. Adult viviparid snails were introduced into the experimental system at the beginning of June (04`), and Experiment 3 was terminated on the 13th September. Therefore, their babies could have been a maximum of 5 months old when they were collected two weeks after the end of the experiment.

The gonads from the adult parental (F0) generation of *P. corneus* collected at the end of Experiment 3 were also analysed for histopathologiccal effects. The gonads were fixed by the same methodology as other snail tissues (including F1 viviparid babies); see section 2.5.5.1 onwards. The fixed gonads of these adult planorbid snails, along with the fixed bodies of the F1 generation of baby *P. corneus* (collected with F1 viviparids) were sent to Burkard Waterman (LimnoMar, Hamburg, Germany), for the practical reasons described in section 2.5.5.

A number of baby *V. viviparus* snails collected in the summer of 2004 from the Grand Union Canal and were used to compare the sex ratio of males to females, and to look for any developmental abnormalities in the young snails amongst this population.

6.3.1 Collection of baby Viviparid (F1) snails

At the end of Experiment 3, all treatments (river water, 25%, 50%, and 100% effluent) were continued in each of the experimental tanks.

Baby snails born during the experiment remained free in treatment water until collected for further analyses two weeks after the end of this experiment, after which time tanks were temporarily drained and baby viviparid snails (and *P. corneus*) were removed. The baby snails were collected into labelled 500-ml polypropylene flasks and were immediately transferred back to the laboratory. In the laboratory, snails were kept alive in glass tanks (maximum of 24-hours) in river water until they could be euthanised using 1.5% $MgCl_2$. Once euthanised, snails were divided into three groups

nominated “small”, “medium”, and “large” according to size, as an aid to analyses. The approximate sizing of baby snails was performed visually before they were weighed and the length of their shells measured. The shell of each snail was then cracked with pliers and all shell removed; the soft parts remaining were weighed again, and finally placed in labelled 1.5-ml eppendorfs containing bouins solution (Sigma) for fixation (see section 2.5.5.2).

Baby *P. corneus* snails born in the experimental system were collected and treated in the same manner as baby viviparid snails. Both species of snails were fixed for morphological and histopathological analyses as described in sections 2.5.5.1 onwards.

6.3.2 Collection of juvenile *V. viviparus* from the Grand Union canal for Morphological and Histopathological analyses

Juvenile *V. viviparus* snails were collected from the same site on the canal as the adults used in both Experiments 1 and 3, and were therefore from the same population, see section 2.1. Juveniles were immediately transported back to the laboratory and kept in glass tanks containing canal water. Morphological observations of the tentacles of live juvenile snails were made to enable the splitting of these snails into two groups (juvenile males and juvenile females) before they were counted, weighed, measured and euthanised. Males clearly had “stubby” right tentacles that were easily identified (even in the smallest snails), whilst females had two slim tentacles.

Due to the observation of unusual tentacle morphology in some snails, where the right tentacle was not as slim as those of female snails, but not as thick or as “stubby” as those of males, a third group was designated “unknown” and analysed for their sexual characteristics by my morphological and histological methods. These snails were fixed in the same manner as juvenile F1 *V. viviparus* snails.

6.3.3 Morphological and Histopathological methods used to analyse baby *V. viviparus* snails

Whole bodies of fixed baby viviparid snails were analysed by both morphological and histopathological methods, as described in sections 2.5.5 to 2.5.5.5. The fixed gonads of the parental (F0) generation of *V. viviparus* were analysed by the same histopathological methods.

6.4 Results

6.4.1 Sex ratio and incidence of intersex in baby F1 *V.viviparus* snails

The numbers, length and weight (with shells), and sex ratios of the baby snails collected from each of the treatment tanks in Experiment 3 derived from my morphological and histopathological analyses are presented in Table 6.1. The same analyses for the juvenile *V. viviparus* snails collected from the Grand Union Canal O4` are presented in Table 6.2. In both analyses, tables contain the number of "undetermined" snails where my morphological and histopathological analyses were unable to confirm the sexual characteristics, and therefore sex of these snails. For example, in Table 6.2 there were 43 "undetermined" snails in the group of snails classified as female based on morphology of tentacles. Here the qualitative information gained from the analysis of their tentacles by histology was poor. Therefore, the sex of these snails could not be confirmed. In each of the snails that remained "undetermined", part (or parts) of my analyses was incomplete, and the sex of the snail could not be determined definitely.

Amongst snails collected from my mesocosm, the percentage of determined snails ranged from 36.4 % to 60.0 % in river water and from 5.9 % to 75.0 % in 25% effluent (Table 6.1). Amongst snails collected from the canal, 81.1% of proposed males collected had their sex successfully determined, compared to only 23.3% of proposed females, and 46.1% of proposed intersex snails.

The overall (combined) sex ratio (M:F) of juvenile snails was 1:4.0 in river water, and 1:2.1 in juvenile snails from 25% effluent. Amongst the different sized snails collected, the male to female sex ratio varied widely between different sizes of snails from river water, and also amongst the different sized snails from 25% effluent. Amongst river water snails, the male to female sex ratios were 1:1 and 1:7, and amongst snails from 25 % effluent the sex ratio varied between 1:1 and 1:4.5.

Overall, the proportion of intersex snails found in snails from river water was higher than in those snails from 25% effluent (28.5% in river water compared to 7.4% in

25% effluent). Intersex snails were identified in all sizes of snails collected from river water, including the "small" sized snails that averaged 8.8 mm in length. Intersex snails were not found amongst the smallest sized snails collected from 25% effluent, however, the sex of only a single snail of this size could be identified. Intersex snails were identified in both the larger sizes of snails collected from river water and 25% effluent.

Overall, from observation of the external morphology of the left and right tentacles of juvenile viviparids collected from the Grand Union canal the sex ratio of males to females was 1:1.1 (excluding presumptive "intersex" snails) (Table 6.2). However, analysis by my own morphological and histological methods failed to confirm this ratio as many of the presumptive females could not be analysed (43 females remained "undetermined"). Amongst the juvenile snails with unusual external right tentacle morphology (designated initially as "unknown" when alive) the sex ratio of males to females (based on 6 snails) was 1:1.5. Of the 6 snails analysed, one snail (0.8% of the total number of canal snails in which the sex could be determined).

None of the baby F1 viviparid snails found to be intersex relied upon my morphological observations alone. All intersex snails were confirmed by histopathogical methods. A total of seven baby snails were found to be intersex (4 snails from the river water tank, 2 from the 25% effluent tank, and 1 snail from the Grand Union canal), and my histological analysis was able to show a male right tentacle and 3 orifices in the mantle tissue (including a female opening). In addition, in a single intersex snail from the river water tank, the histological analysis of gonadal tissue (by Burkhard Waterman, Limnomar (see section 2.5.5)) revealed a gonad in active spermatogenesis. In addition, morphological analyses of these 7 snails showed 3 appeared to have male right tentacles, and in a single specimen a female opening was noted on the mantle tissue. In the remaining 3 specimens, either the material was unsuitable for observation of the sexual characteristics of interest (too badly damaged), or my observations could not confirm the nature of the sexual characteristic of interest (not definitely male nor female).

Table 6.1 Details of the baby viviparid snails studied. Baby snails were collected at the end of experiment 3. Values of lengths and weights are means (+/-SE) in all cases.

Treatment	Size of snail	Number of snails	(m:f:I)	Intersex percentage	Length (mm)	Weight (g)
	small	11	1:1:2 (7 undetermined)**	40%	8.8+/-0.17	0.24+/-0.01
River water	large	15	1:7:2 (6 undertmined)**	20%	11.3+/-0.22	0.47+/-0.02
	TOTAL	**26**	**2:8:4 (13 undetermined)****	**28.5%**		
	small	17	0:1:0 (16 undetermined)**	0%	9.3+/-0.28	0.28+/-0.01
25% effluent	large	40	8:16:2 (10 undetermined)**	7.7%	11.7+/-0.19	0.55+/-0.03
	TOTAL	**57**	**8:17:2 (26 undetermined)****	**7.4%**		

** Undetermined; snails could not be sexed

Table 6.2 Details of the baby viviparid snails studied. Baby snails were collected from the Grand Union canal (04`) near Uxbridge, Middlesex. Values of lengths and weights (+/-SE) are means in all cases.

Treatment	Size of snail	Number of snails	(m:f:I)	Intersex percentage	Length (mm)	Weight (g)
"Males"	Juveniles	53	43:0:0 (10 undetermined)**	0%	16.6+/-0.23	1.22+/-0.05
"Females"	Juveniles	56	0:13:0 (43 undetermined)**	0%	16.9+/-0.33	1.39+/-0.07
"Unknown"	Juveniles	13	2:3:1 (7 undetermined)**	14.3%	21.5+/-4.56	3.02+/-1.20
	TOTAL	**122**	***	**1.6%**		

** Undetermined; snails could not be sexed

*** Due to large number of undetermined snails, a reliable sex ratio cannot be calculated

6.4.2 Typical Morphology of F1 baby snails

Plates 6.1 and 6.2 show typical light microscope pictures of the inner surface of the mantle tissue from baby viviparid snails. The first two plates show the typical sexual openings of a baby female snail with a ureter, anus, and female opening (*viz* vagina). The second plate shows the typical sexual openings of a male snail with a ureter, and anus only. All pictures displayed are of snails where the sex was confirmed using all analytical methods. Of the intersex snails identified, none were sufficiently advanced that the female opening could be seen by my morphological analysis; hence no picture is available.

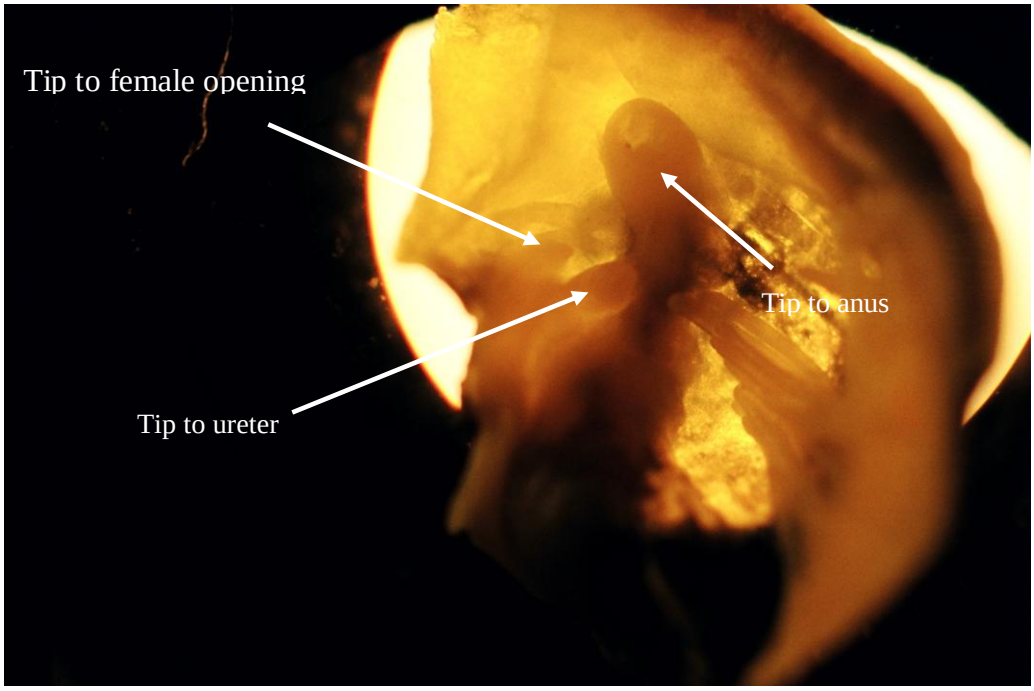


Plate 6.1 F1 female *V. viviparus*. The orifices can clearly be seen in this "female" snail.

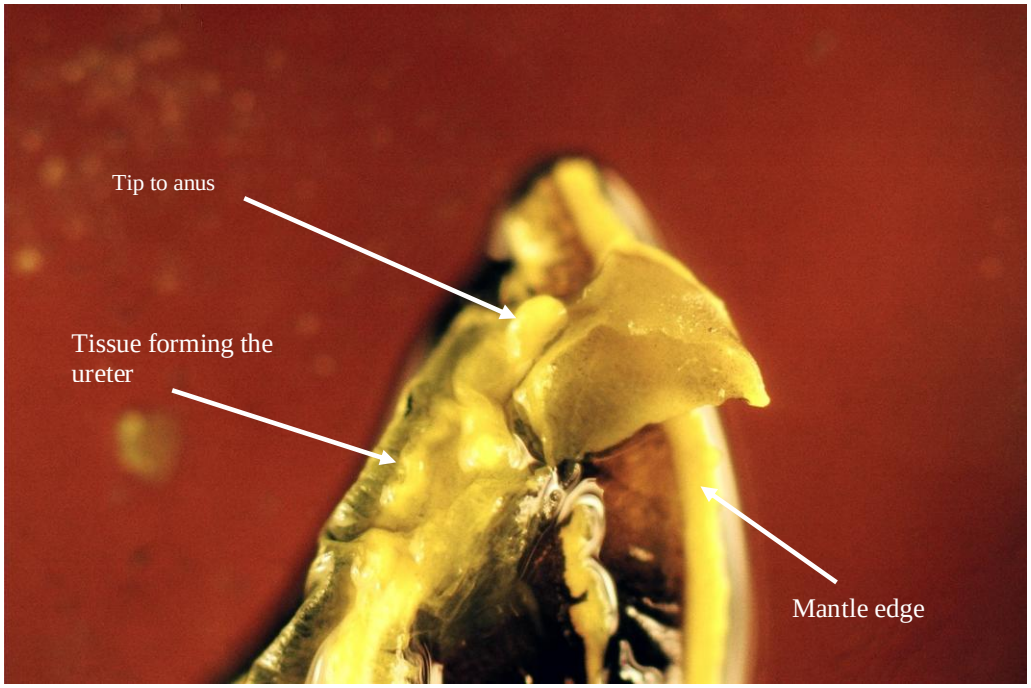


Plate 6.2 F1 Male *V. viviparus*. No female opening is present, and therefore, this mantle tissue appears to have "male" sexual orifices only.

Plates 6.3, 6.4, and 6.5 show typical light microscope pictures of the left and right tentacles (with “nose” in between) from baby viviparid snails. Plate 6.3 is a picture of a typical snail where the tentacles look female in form, that is, no hook with a pore was visible. Plate 6.4 is a picture of a typical snail where the right tentacle appears to be male in form; the right tentacle was more bulbus with a hook, sometimes with a visible pore. Plate 6.5 is a picture of a snail where the right tentacle was thought to be male in form. However, the snail was found to be “intersex” upon determination of all the sexual characteristics studied (i.e three openings were found in the mantle tissue).

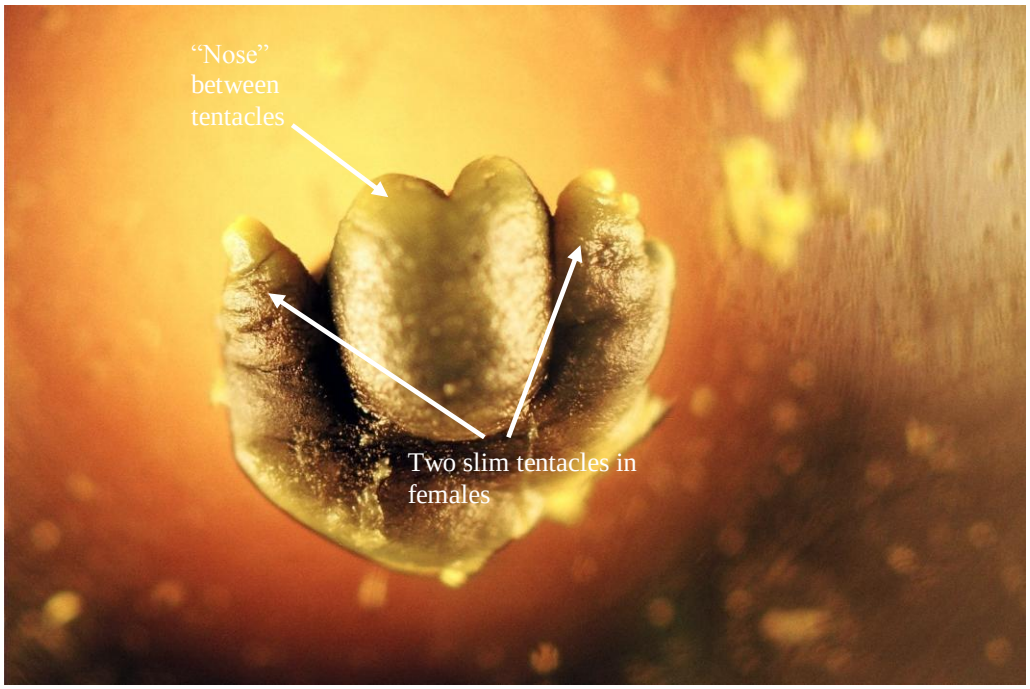


Plate 6.3 Morphological characteristics of a female *V. viviparus* showing two identical slim tentacles.

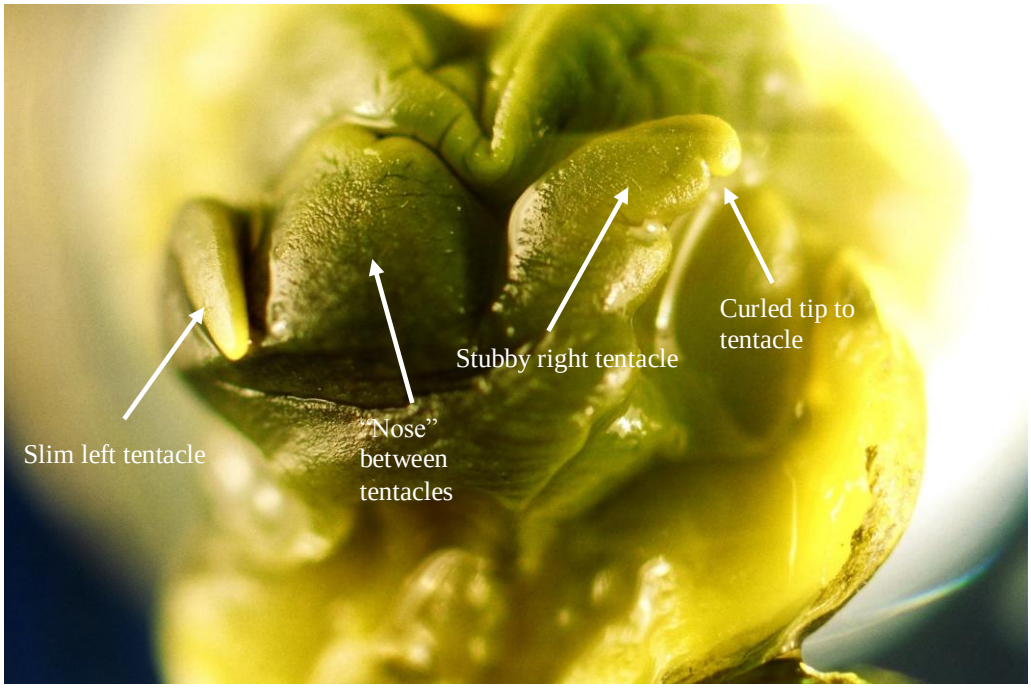


Plate 6.4 Morphological characteristics of a male *V. viviparus* showing the characteristic stubby right tentacle.

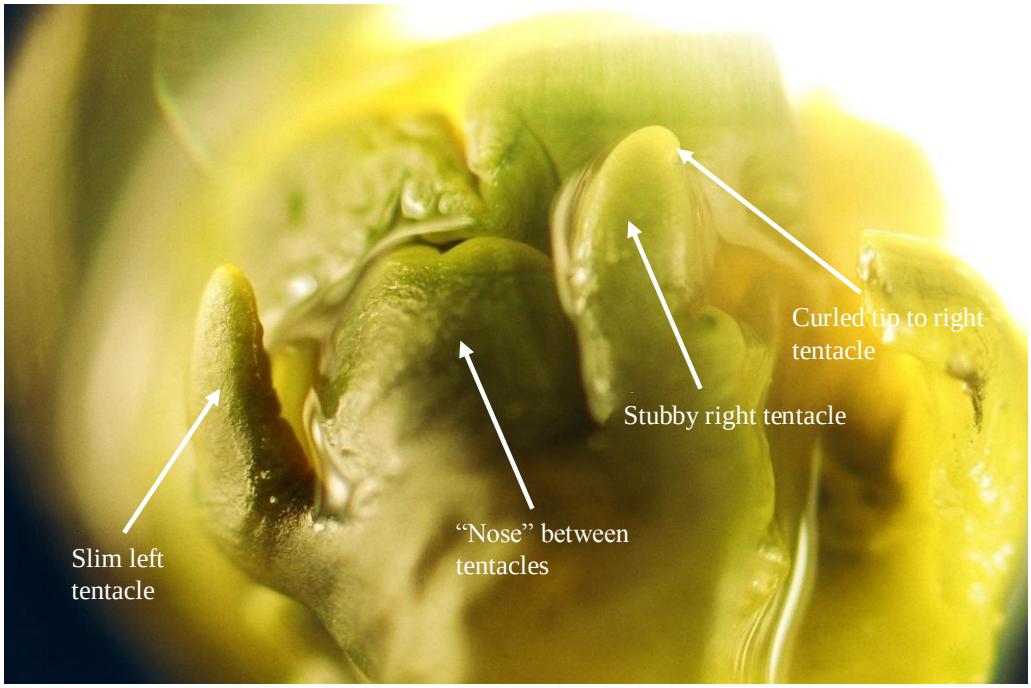


Plate 6.5 An "intersex" *V. viviparus* with a male right tentacle morphology.

6.4.3 Typical Histology of baby F1 snail tissues

Plates 6.6-6.8 show typical light microscope pictures of transverse sections through mantle tissue from baby viviparid snails. Plate 6.6 is a picture of mantle tissue from a typical female snail that has 3 sexual openings (ureter, anus, and female opening (*viz* vagina)). Plate 6.7 is a picture of a typical male snail that has 2 sexual openings (ureter and anus). Though all "intersex" snails I identified were found to have three sexual openings in the mantle tissue (like female snails) no suitable pictures of the 7 snails identified to be "intersex" were taken. By chance, either none of the pictures taken by camera were suitable (inferior quality) for display here, or no picture was taken.

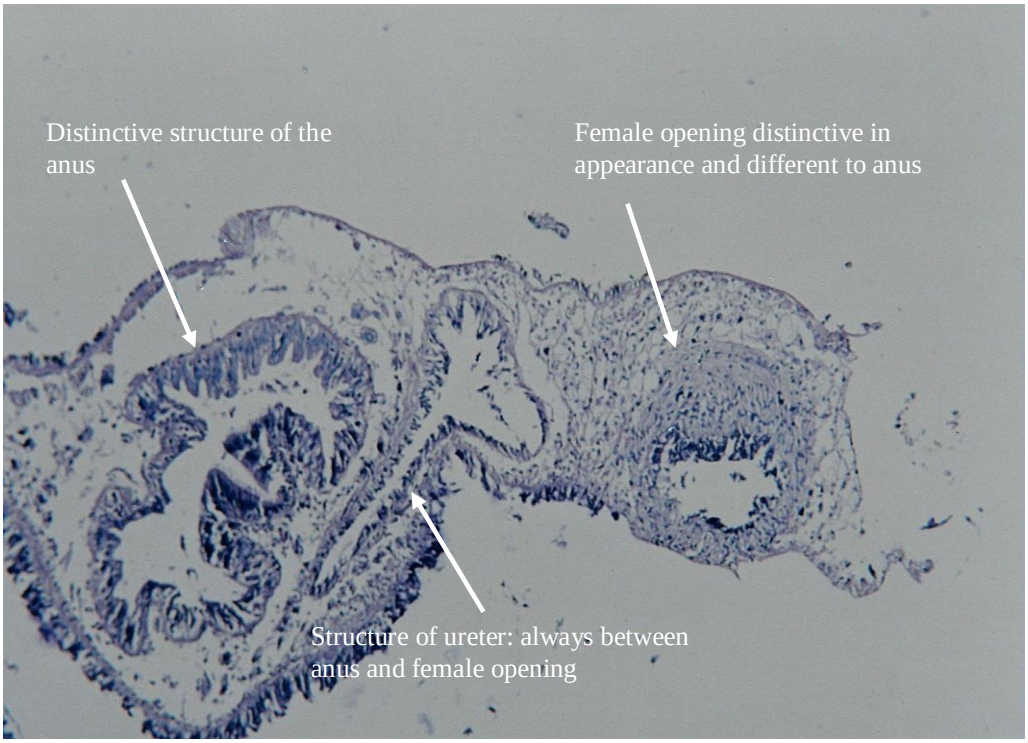


Plate 6.6 shows the 3 sexual openings, anus, ureter, and female opening and their relative position to each other in the mantle tissue from a typical female.

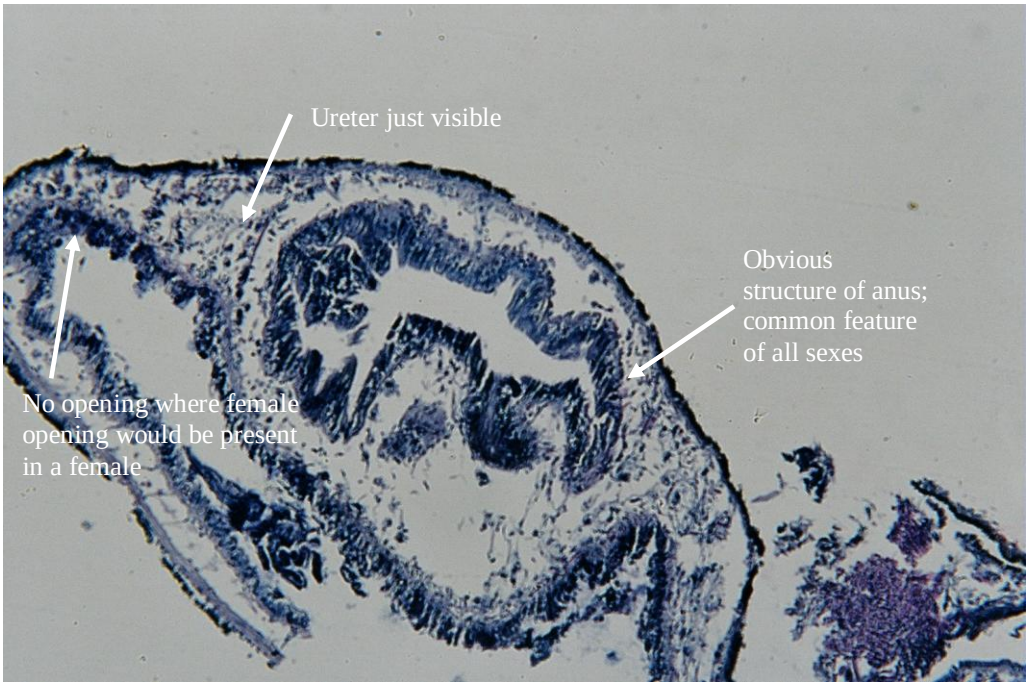
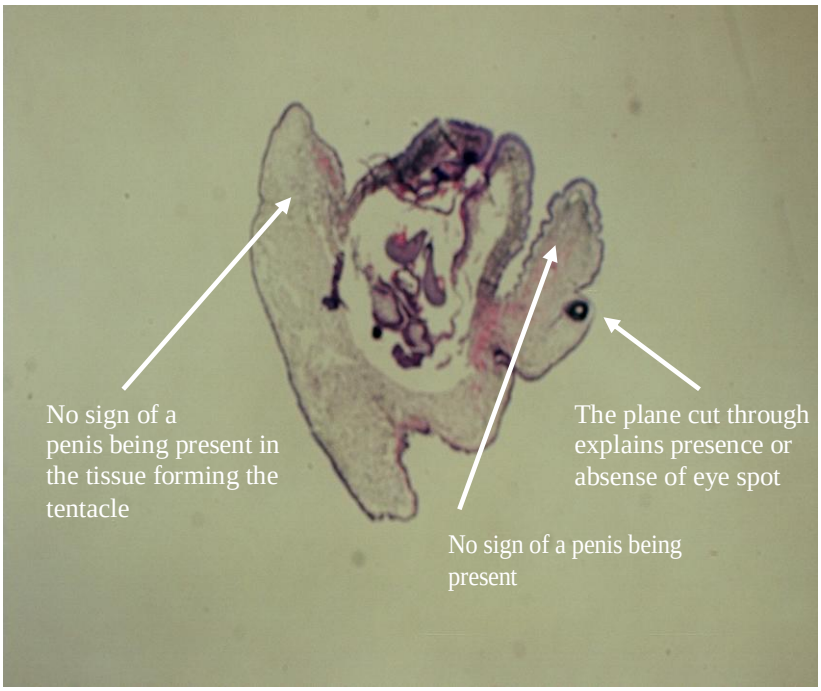


Plate 6.7 shows the 2 sexual openings anus and ureter, and their relative position to each other in the mantle tissue from a typical male.

Plates 6.8-6.10 show typical light microscope pictures of longitudinal sections through the left and right tentacles (with “nose” in between) of baby viviparid snails, respectively. Plate 6.8 is a picture of typical “female” tentacles, none of these snails studied showed signs of any penal tissue developing. Plates 6.9A-C are pictures of typical “male” tentacles in which the right tentacles contained developing penal tissues. Plates 6.10A and B are pictures from typical “intersex” tentacles; like males, these snails typically had right tentacles that were male in nature with developing penal tissue.



Plates 6.8 both tentacles are female in their nature, with no male characteristics seen.

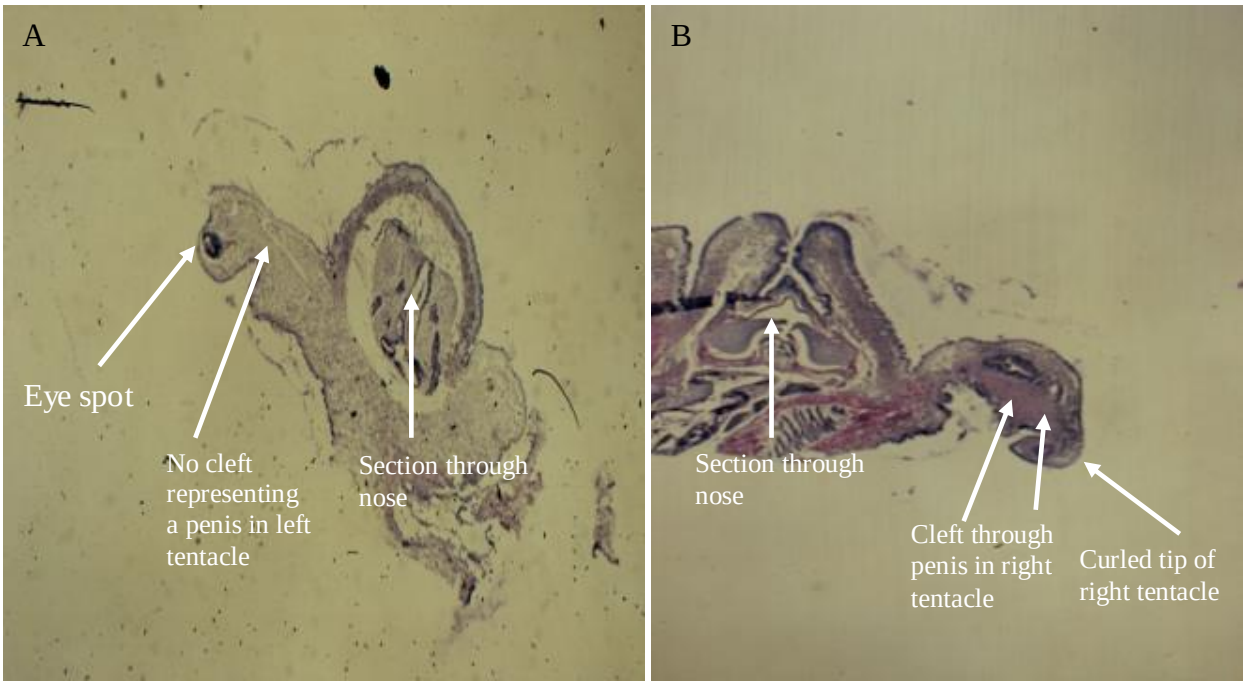


Plate 6.9 shows a left tentacle, and 6.9B a right tentacle with male tissue forming within the male right tentacle tissues.

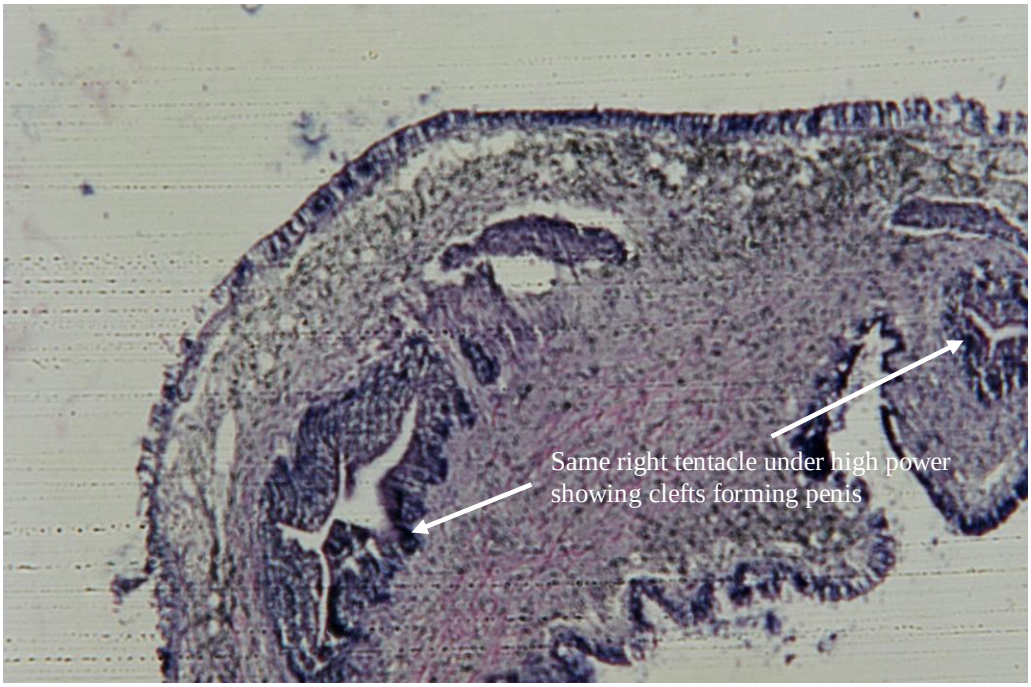


Plate 6.9C shows the same right tentacle with male tissue clearly forming.

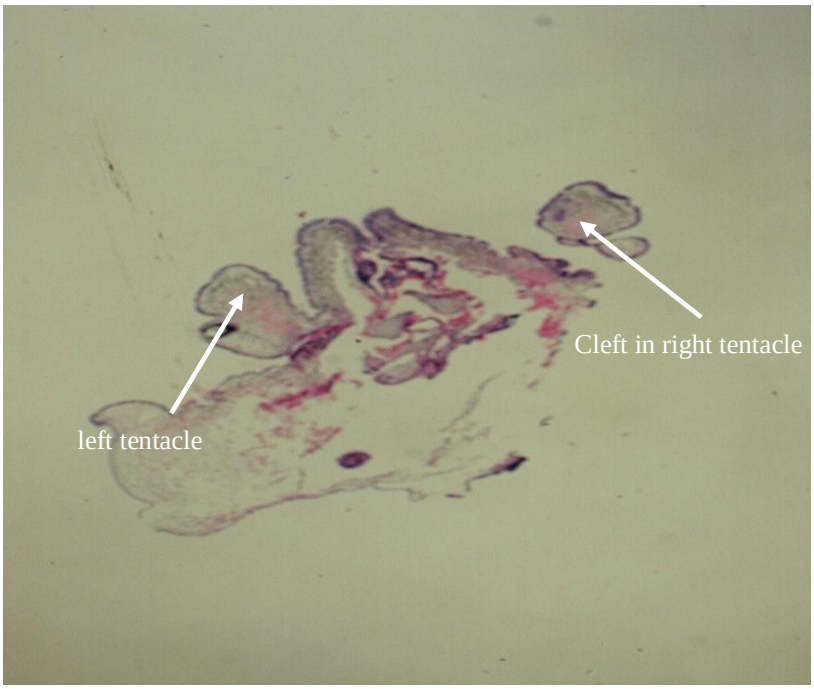


Plate 6.10 shows that typically in “intersex” snails the right tentacle is male in form.

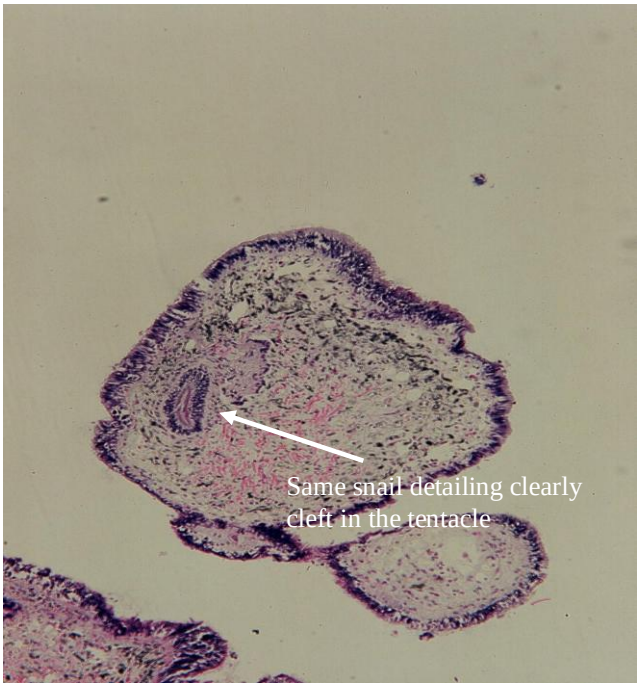


Plate 6.10B shows the same right tentacle, with clearly male tissue forming.

Plates 6.11-6.13 show typical light microscope pictures of transverse sections through the gonads of baby viviparid snails. Plate 6.11 is a picture of a female gonad, with oogenesis taking place. There were no signs of any male gonadal tissue developing. Plate 6.12 is a picture of a male gonad, with spermatogenesis in various stages and no signs of oogenesis (as expected). Plate 6.13 is a picture of a gonad displaying the characteristics of a male gonad. Spermatogenesis was taking place in the gonad of this snail; however, the presence of three openings on the mantle of this snail demonstrated it to be an “intersex” snail.

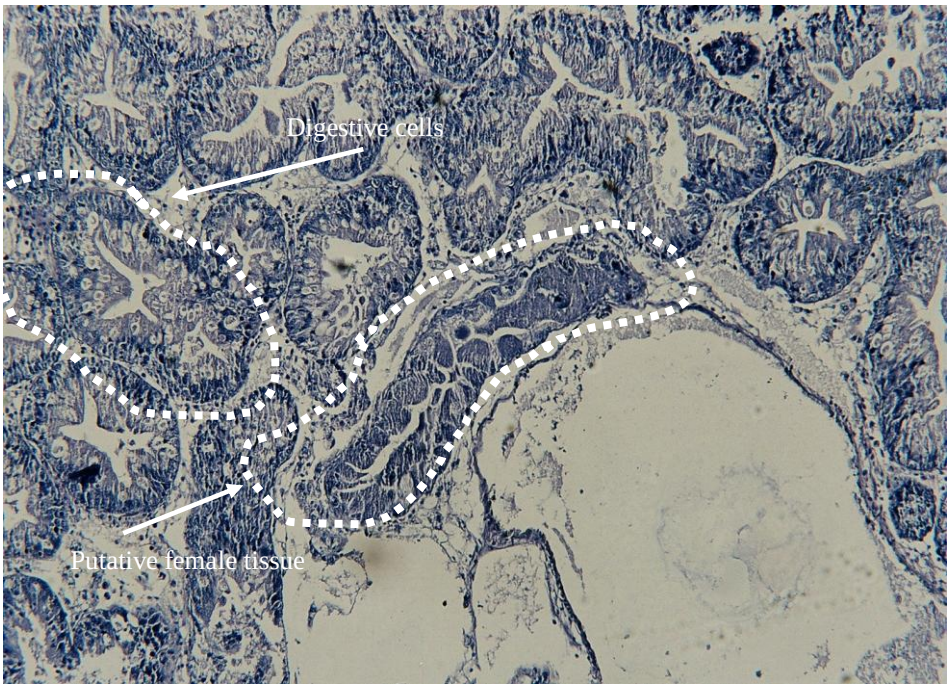


Plate 6.11 shows female tissue and where oogenesis takes place.

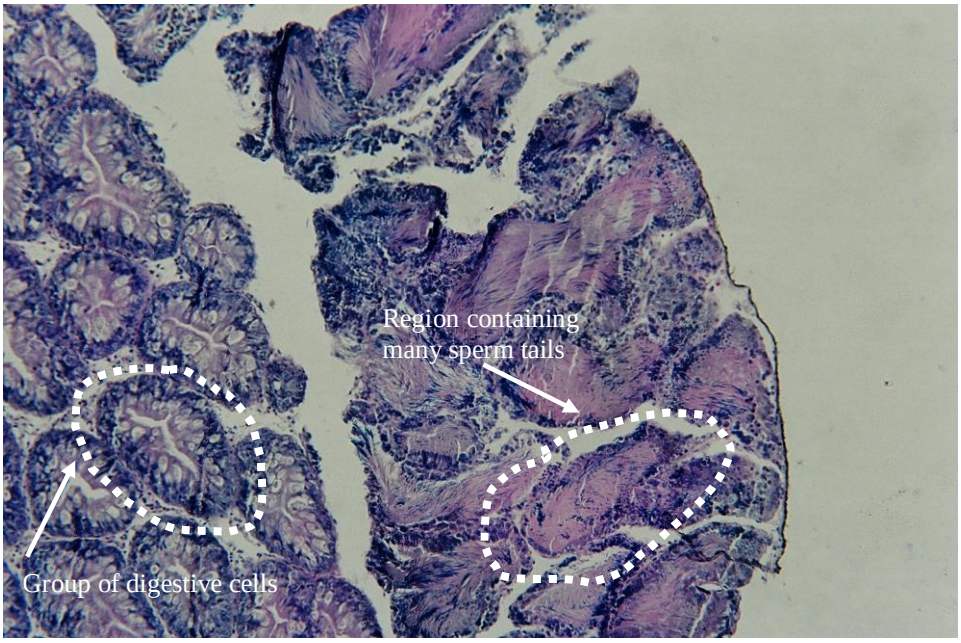


Plate 6.12 shows male tissue with spermatogenesis taking place.

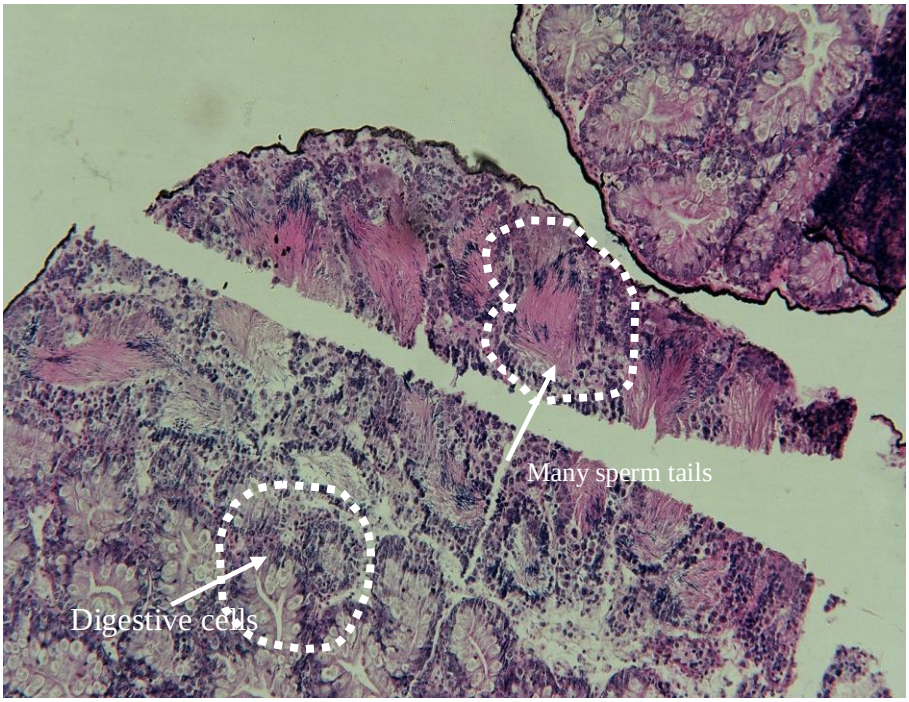


Plate 6.13 shows spermatogenesis within the gonad of an “intersex” snail (male right tentacle plus three openings on mantle).

6.4.4 Typical Morphology of Grand Union Canal snails

Plates 6.14 shows typical light microscope pictures of the inner surface of the mantle tissue from a baby viviparid snail. This plate shows the typical sexual openings of a proposed male snail with a ureter, and anus only. Unfortunately, no pictures are available of a female snail; though females were identified by my methodologies, none provided a suitable picture to present here. Of the intersex snails identified in the canal, none were advanced enough in their development for the female opening to be seen morphologically.

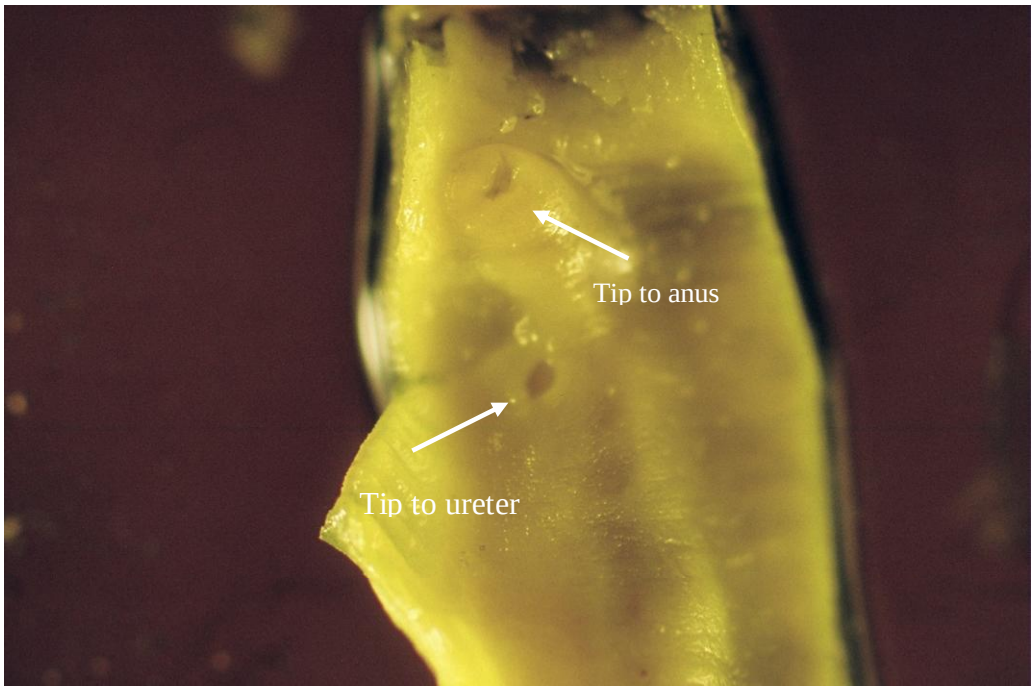


Plate 6.14 Sexual openings of a male snail on the inner surface of the mantle tissue; ureter and anus only.

6.4.5 Typical Histology of Grand Union Canal snails

Due to time constraints no pictures of the histological analyses of canal snails were undertaken, however, slides were analysed by the same method as F1 baby viviparid snails.

6.4.6 Histological analyses of adult (F0) and baby (F1) *P. corneus* gonads

During the baseline period, 45% of the adult (F0) snails contained a gonad in a ripening stage with evidence of spermatozoa and oocytes present in the acini. The remaining snails did not have clearly defined gonadal tissue at this time. By the end of the exposure period, all adult snails had gonadal tissue, and there were no differences in acini size between *P. corneus* in any of the treatment groups (data not shown)**.** Arrests in spermatogenesis in the male part of the gonadal acini together with the occurrence of immature gametes in the vas deferens were particularly prevalent in the effluent exposed snails. Moreover, in 100% effluent, unbound spermatids were found in the acini of the snails, indicating Sertoli cell dysfunction. In contrast, oogenesis in the female gonadal acinus of the F0 snails appeared to be largely similar between the river water and effluent-exposed groups.

In the developmentally-exposed (F1) animals, histological evaluation of the gonad in some snails showed that both the control and effluent-exposed snails had a well developed gonad in active oogenesis and spermatogenesis (Plate 6.15). The degree of maturation of the germ cells was most advanced in the river water exposed snails (where no evidence for an arrest in spermatogenesis was seen), and a disturbance (up to an arrest) of spermatogenesis occurred in a increasing proportion of the individuals with increasing concentrations of effluent. As with the F0 snails, the underlying mechanism appeared to be insufficient maturation of the spermatids due to progressive degeneration of Sertoli cells. In addition, in some specimens exposed to 100% effluent, spermatogenesis was arrested from the level of spermatocytes up to spermatozoa (Plate 6.16). The perimeter of the F1 acini increased steadily with

increasing concentration of the effluent. A significant difference from the control was noted with exposure to 25%, 50% and 100% effluent (Figure 6.2). A visual inspection of Figure 6.2 would suggest that these differences were found to be statistically significant slightly surprising, however, although the author was not contacted personally, the standing of the laboratory in question would strongly support the authenticity of these results. Arrests in spermatogenesis coincided with an increase in resorption rate in the 50% and 100% effluent exposure groups (data not shown). Microliths (calcifications associated with resorption of cells) were found in the gonads of some of the F1 snails. An evaluation of the microlith-index as a ratio between normal oocytes and microliths showed a reduction in the 25% effluent group, and a significant increase in the 50% effluent group relative to the river water control (data not shown).

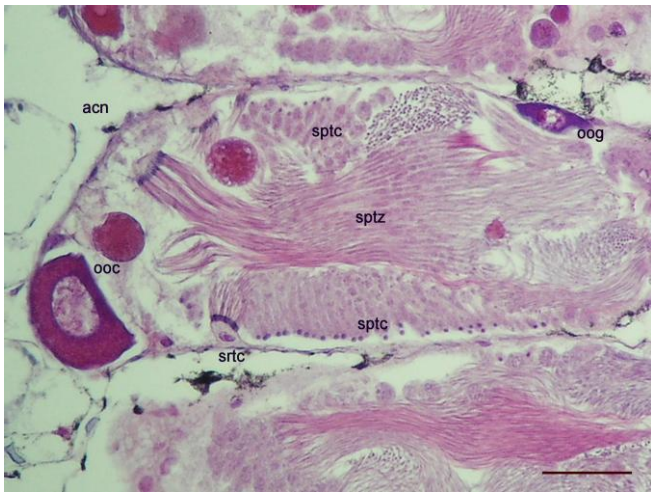


Plate 6.15 Normal gametogenesis in acinus of F1 specimen of river water. ooc = oocyte, sptc = spermatocytes, srtc = Sertoli cells (nutritive phagocytes), sptz = spermatozoa, acn = acinus wall, bar = 50µm

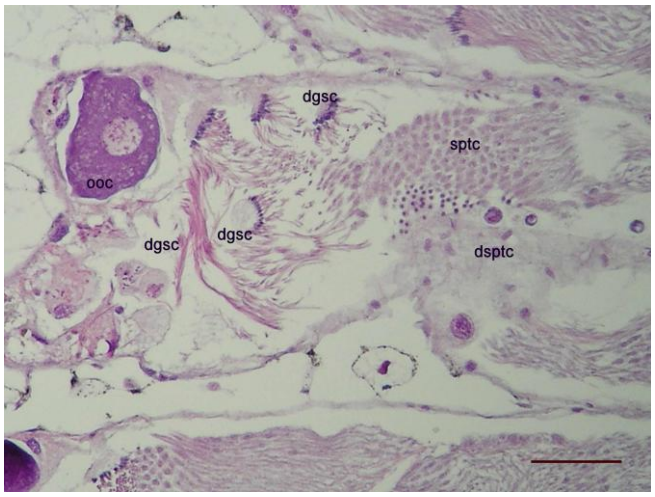


Plate 6.16 Gametogenesis in acinus of F1 specimen exposed to100% effluent with arrest in spermatogenesis. sptc = spermatocytes, dsptc = degenerated spermatocytes, dgsc = degenerated Sertoli cells (nutritive phagocytes), bar = 50 µm

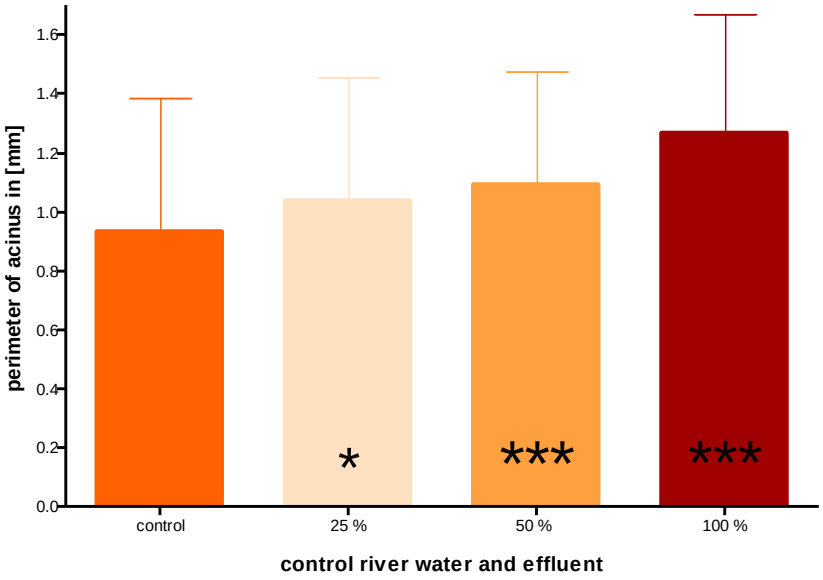


Figure : *Planorbarius corneus,* perimeter of acini in mm, mean & standard deviation, 16 to 20 animals per group, 10 measurements per animal, statistics: Anova with Newman-Keuls Multiple Comparison Test, significant differences: T6 vs T4 P<0.05, T6 vs T5+T7 P<0.001

Figure 6.2 Acini radius of (F1) effluent exposed *P. corneus*, taken from Waterman *et al*, unpublished data; whole bodies of F1 snail samples were sent away for expert histological analysis, for the reasons stated in section 2.5.5.

6.5 Discussion

6.5.1 Sex ratio of baby (F1) viviparid snails

Of the baby viviparid snails that developed in my experimental system for up to 5 months, and were analysed for their sexual characteristics, around 50% in either treatment (river water or 25% effluent) had their sex successfully determined (Table 6.2). However, of the juvenile snails collected from the canal, a far higher percentage of the males (91.1%) had their sex successfully determined, than females (32.2%) (intersex sex determination success rate was 46.2%). This data suggests it was apparently easier to determine the sexual characteristics of one sex, the males, than the females; this could in theory have biased the sex ratio obtained (and the number of intersex snails found). The data in table 6.2 suggests that the "female" tentacles where hard to pick up by my histological methodology; since the failure to section the tentacles of this group of snails was responsible for the high rate of "undetermined" sexual characterstics (and therefore sex) of this group of snails.

As the practical work proceeded I became aware of the limitations of my methodologies. These limitations could be allowed for as I proceeded with the analyses of tissue samples. To illustrate, it soon became obvious that if female openings were present their presence was obvious in the mantle tissue; their presence was evident whilst cutting on the microtome. For right tentacles, I set a minimum of 70-80% coverage to determine whether a cleft was present in a tentacle or not: 100% coverage was the total cross sectional area of the widest width of a tentacle whilst cutting on the microtome; by continuing to cut on the microtome the best samples were obtained for staining and analysis.

Therefore, with the combined values of experience and method improvements**,** of the snails that I did manage to sex, I am confident that I did correctly determine the sex of each snail. However, the affect upon the sex ratio of the number of undetermined snails amongst the baby F1 viviparid analysed snails is unknown, and it cannot be ruled out entirely that this could have created a biased sex ratio.

Therefore, for use a guide to the sex ratio of the canal snails, it would seem reasonable to use the morphological charactors of live snails to determine their sex in this case. 53 “male” snails and 56 “female” snails were collected giving a male to female sex ratio of 1:1. Further of the “male” and “female” snails collected from the canal that I did manage to determine the sex of by my morphological and histological methods, in each case, my analyses were in agreement with the sex of snails determined morphological analysis of live snail tentacles (also suggesting this may be a fairly reliable way to determine the sex of snails, though snails with abnormal tentacle morphology have first to be removed from the group). This suggests encouragingly, that my methods did correctly determine the sex of juvenile snails. This data perhaps confirms a 1:1 sex ratio amongst newborn vivparid snails from the canal. This further implies that intersex amongst this group was approximately 1 in 62 snails or 1.8% (Table 6.2: 1 snail in every 7 “unknown” snails being intersex).

However, as stated (chapter 4), the actual sex ratio of viviparids in the wild is unknown. It is likely that at birth the male/female sex ratio maybe approximately 1:1, but as the lifespan of males is considerably less than that of females; this ratio changes in favour of females with the increasing age of snail populations. This might be expected given that a male could easily mate with several females in early summer.

6.5.2 Intersex in F1 viviparids

In order to gauge whether the level of intersexuality found amongst my F1 viviparids was “normal” we first have to establish what “normal” is. Amongst vertebrate species (that have also been exposed to EDs in experiments), fish species have been, by comparison, extensively researched. In the field, a single hermaphrodite Artic charr (*Salvenius alpinus*) was caught in Loch Rannoch, Scotland (Fraser, 1997), and this occurrence was attributed to the fish having possibly hatched in the vicinity of an effluent plant (indicating the existence of this single intersex fish was of significance). However, male flatfish from the Mersey and Tyne estuaries contained elevated oestradiol levels, and up to 18% of males from these estuarine locations are intersex individuals exhibiting ovotestis (Scott *et al*., 1999). However, in general the

occurrence of intersexuality in gonochorist fish is thought to be low (e.g. no more than 5% in carp/roach).

Other than imposex/intersex in TBT-exposed gastropod molluscs, relatively few field cases of environmental ED have been described in marine invertebrates. However, higher incidences of intersex, and female-biased sex ratios, where observed amongst populations of the marine/estuarine amphipod *Echinogammarus marinus* at polluted compared with reference sites in east Scotland (Ford *et al*., 2004). Total incidence of intersex varied between approximately 5-8% at reference sites and approximately 14-15% at polluted sites. A high prevelance of intersex has previously been observed in harpactioid copepods from the Firth of Forth, though no causal agent was identified, however, these animals were collected in the vicinity of sewage discharges (Moore & Stevenson, 1994). The development of ovotestes in male lobsters was observed following exposure to sewage effluent (Sangalang & Jones, 1993), and also an increased incidence of embryonic malformations was reported in the amphipod *Monoporeia affinis* originating from sites receiving industrial effluents (Sundelin & Eriksson, 1998). In laboratory experiments, indications of hermaphroditism, reduced gnathopod size and females biased sex ratios were reported in the amphipod, *Hyallela azteca* exposed to EE2 (Vandenburg *et al*., 2003).

Although the rates of intersex in crustaceans decribed above may serve as a general guide for invertebrare species, it is clear that little is known about molluscan species. Indeed, there are no reports in the literature of the "natural" or increased rate of intersex in a molluscan species. For my own exposure experiments, therefore, the best and only guide to "natural" rates of intersex in *V. viviparus* can only come from specimens collected from the local canal (where the adults used in my experiments originated). From the specimens collected from the canal, intersex was identified at a rate of 1.6 % (1 snail in 62 specimens); from river water snails, intersex was found at a rate of 28%, and at a rate of 7.4% in snails from 25% effluent. Clearly then the rate of intersex in both these treatments appears to be considerably higher than in the snails collected from the canal (and what could be considered to be the parental generation), and comparable with what figures are available for invertebrates exposed to pollution in the wild.

6.5.3 Histological analyses of adult (F0) and bay (F1) P. corneus gonads

In the developmentally-exposed (F1) animals, the observed enlargement of the acini may be due to an increased number of oocytes indicating an oestrogenic effect of the effluent. Comparable results in other molluscs are rare and hitherto described only in laboratory experiments with gonochoristic snails exposed to bisphenol A and 4-tert-octylphenol (Oehlmann *et al*., 2000; Oehlmann *et al*., 2006).

The resorption index, reflecting an increasing number of resorbed oocytes and or Sertoli cells per acinus, was elevated at concentrations of 50 and 100% effluent. In the normal ovotestis a low number of deficient oocytes and Sertoli cells after spermiation undergo apoptosis and are resorbed by the nutritive phagocytes. This rate is well known and described for *P. corneus* and other pulmonate species like *B. glabrata* (De Jong-Brink *et al.*, 1996; Jong-Brink *et al*., 1976). The increase in resorption may indicate a disturbance in vitellin synthesis in oocytes and/or elevated numbers of defective Sertoli cells.

The occurrence of microliths in the gonad, especially in the testis has been reported in both mammals and fish (Vegni-Talluri *et al*., 1980; Renshaw, 1998). They may arise from degenerating male gametes surrounded by glycoproteins serving as centres for the deposition of calcareous compounds. Their significance is still unclear, but the original cell damage may be followed by a variety of pathological disorders including neoplasia (Zastrow *et al*., 2005). The distribution of the microliths in the apex of the acinus of the snail gonads suggests that they may represent a late stage of resorbed oocytes or spermatozygnema with glycoproteins or ceroids serving as a core for the calcification. Thus, the microliths index may reflect a disturbance in spermatogenesis and/or maturation of the oocytes.

Therefore, dose-dependant effects of the effluent on gonadal abnormalities (microliths and atresia) in the offspring were apparent. The mechanisms underlying these effects and their consequences to the sustainability of mollusc populations are presently unknown and need considerable further elucidation.

Chapter Seven: Discussion

Comparative Effects upon Development and Reproduction of TSE on a Pulmonate (*P. corneus*) and Prosobranch (*V. viviparus*) species

7.1 Mortality

In the springtime effluent exposure with *P. corneus* (Eperiment 1) mortality in effluent from mid-June into July was higher (though not significantly so) than in river water. This coincided with increased reproduction in effluent compared to river water, though these phenomenons were not repeated the following year. Experiment 1, water temperature was very variable, and reproduction also increased dramatically from springtime onwards. This suggests that the observed mortality in *P. corneus* in effluent may have been due to the secondary effects of reproductive stress. The highly changeable water temperatures are likely to have caused stress to these snails at this time of year, and the unusually high reproductive rate (by summer) due to the favourable environmental conditions in my experimental setup (notably food quantity at this time of year) would have further increased this stress. However, it is also possible that my *P. corneus* snails could have perceived the increasing temperatures in spring as a pond drying up event; causing "reproduction in the face of perceived adversity" and increased mortality rates. In the only comparable study of a pulmonate snail available by Watton and Hawkes (1984), numbers of *L. peregra* also increased dramatically from spring into early summer in effluent compared to river water (presumably due to reproduction), but no increased mortality was apparent. However, only dead shells that could be recovered were counted, therefore actual mortality was unknown. It may have been that the density of adult snails was lower at times (density release increasing reproduction per snail) or reproductive rate was lower in some treatments (when density was higher) at times explaining this increased density of adult snails without apparent increased mortality during the reproductive season. In *V.*

viviparus, there were no apparent effects of effluent upon mortality of adult snails until mid-July into August of Experiment 3. The mortality rates at this time were higher in 100% effluent, and these mortalities coincided with the second reproductive peak in this treatment. Therefore, my experiments have demonstrated that increased reproduction can cause increased mortality (or visa-versa with *P. corneus*) in my experimental mesocosm system, in both these species. Therefore, to my knowledge, my report maybe the first to demonstrate this link between reproduction and mortality not only in a pulmonate species of snail, but in a prososbranch species also. In the study by Jobling *et al.*, 2004, which used the same experimental system, the prosobranch *P. antipodarum* (different lifecycle characteristics) had higher mortality and fecundity rates (new embryo production) in 100% effluent. However, this mortality was likely due heavy metal toxicity. In our prosobranch mollusc (*V. viviparus*), effluent also appears to have increased fecundity (perhaps without effects upon mortality had the effluent not been toxic). As increased mortality in 100% effluent in Experiment 3 coincided with increased reproduction during this period of time only, it seems unlikely that toxicity of the effluent could explain these results. In what might be a separate phenomenon, the young snails born to my adult *V. viviparus* in early summer lost weight (compared to river water snails) at a time when they would be expected be growing fast and putting on weight in preparation for winter. Therefore, a second (negative) effect upon health of young viviparids (that could lead to their mortality) was apparent in effluent during Experiment 3.

An explanation for these results in both species could include that the oestrogenic properties of the effluent where stimulatory to reproduction, but the effluent was also toxic (e.g. heavy metals) to very young viviparid snails, as the early life stages of snails are known to be most vulnerable to chemical toxicants (see section 1.4.3). Although the effects of effluent on newborn *P. corneus* are unknown, my hatching experiments suggested no negative effects of effluent on the hatching of embryos. Therefore, it is seems unlikely that the health of baby *P. corneus* were affected, and negative health effects of effluent during summer on baby viviparids may be a taxa specific effect, and may not be due to toxicity. However, between species (and taxa) differences in their susceptibility to toxicity in effluent may help explain these results.

Towards Autumn, in what might be a separate phenomenon amongst adult *P.corneus*, there was a decreased mortality rate in the 25% effluent treatment only. However, this might simply be explained by a lower reproductive rate in this treatment only; a lower reproductive rate that persisted from the baseline period (when river water only was running in the tank) to at least the middle of summer. Although the lower reproductive rate could be a tank effect, this phenomenon demonstrates again the relationship between reproduction and mortality in my experiments. In contrary to this, however, an association between lower reproductive output and mortality rates in *V. viviparus* was not demonstrated in my experiments.

In conclusion, the results of my experiments with a pulmonate species confirm the data produced by *in vitro* experiments where increased reproduction is associated with increased mortality. Further, my results demonstrated that the effluent was responsible for increased mortality rates, a secondary effect of increased reproduction in both these species, though the evidence for this was far greater with *P. corneus* than with *V. viviparus*. In addition, my results with *P. corneus* suggest an opposite effect; a lower reproductive rate was associated with a lower mortality rate, the first time such a phenomenon has been reported.

7.2 Growth

In both species of snail, the effects of effluent upon growth were strongly mediated by season. In both species, any effects of effluent upon growth were transitory, and influenced by seasonal factors; such as reproductive rate (higher or lower) that changed throughout the reproductive cycle. In *P. corneus* there was a slight indication that growth in effluent exceeded that in river water during spring when the growth of snails (including ASOs) is known to be maximal, and reproductive rate comparative low (compared to summertime). No further indications of growth differences were noted until the peak of reproduction had passed, and day length shortened into autumn and towards winter. At this time the growth of snails in effluent exceeded that in river water. Possible explanations for this phenomenon (and perhaps springtime growth in effluent) may include the anabolic effects of chemicals contained within the effluent. Therefore, these effects of effluent were only apparent when reproduction was sub-

maximal, and more energy could be diverted towards growth. However, this suggestion is highly speculative as there is no real evidence to back up this hypothesis. An alternative hypothesis is that increased availability of organic material occurred in the effluent tanks towards winter that the snails consumed in preference to lettuce.

Unlike *P. corneus* increases in fecundity (new embryo production) of *V. viviparus* in effluent compared to river water during spring coincided with increases in growth. This phenomenon most probably can be explained by the way in which energy is devoted to reproduction (lifecycle characteristics) in both species. In the slow growing and comparatively slow reproducing prosobranch there was enough energy available even during the peak of reproduction for growth to be increased by effluent, but not in the faster growing and reproducing pulmonate where a higher proportion of energy available was most likely devoted to reproduction.

By mid summer, growth in *V. viviparus* in effluent was probably lower in effluent than in river water, with no growth differences between effluent and river water from mid summer onwards. However, the health of viviparids in my experimental system was compromised at this time (mid summer onwards), and therefore, comparison of their growth rate with *P. corneus* during this period is not possible.

Overall, in both species whilst in apparent good health, the effects of effluent upon growth were small. They were particularly small compared to the effects of either day length or reproductive rate upon growth during the reproductive cycle for both these parameters. Though day length and reproductive rate were very closely linked, the effect of either parameter upon growth during my experiments was seen over the reproductive season. Both factors strongly influenced growth in these species. In contrast, effluent had comparatively little effect upon growth; a result that is perhaps is surprising and somewhat counter intuitive. However, though to my knowledge, my study is the first research of its type in a semi-natural system (or *in vitro*). Furthermore, in conclusion, my results for both species show that any possible nutrative effect of the effluent did not to significantly effect growth.

7.3 Reproduction and Growth of F1`s

Reproductive effort as defined by the number of eggs produced by a female (or carbon content of the offspring) (Dillon, 2000) was increased in *P. corneus* exposed to effluent. Most definitely the number of egg masses (increased CDCH) produced per snail was increased (during the peak of reproduction), as was the total weight of egg masses produced per snail. Though the eggs in most egg masses were counted (only a few were not at the very peak of reproduction) it also appears that more eggs per snail were produced in effluent. This indicates a direct effect on DBH and as well as CDCH production, and therefore on the ASO`s, and implies a direct effect of effluent on the gonads, as more egg cells would need to be matured to facilitate increased reproduction.

Reproduction was also increased in 100% effluent as indicated by a second reproductive peak in *V. viviparus,* and similar effects upon their reproductive endocrinological pathways is therefore implied in this prosobranch mollusc. Therefore, most likely the equivalent to DBH was also increased in this prosobranch mollusc. There was also an implied increased rate of new embryo production (increased fecundity) in *V. viviparus* in effluent during springtime (equivalent to egg cell production in a pulmonate) suggesting similar reproductive endpoints have been affected in both taxa. However, as stated very little is known about the endocrinological control of reproduction in prosobranchs apart from a role of the equivalent to DBH found and identified in pulmonates. Due to the high degree of variation in the endocrinological control of reproduction seen in various molluscan taxa, the endocrinological pathways that were affected in my species were not necessarily or likely to be exactly the same in both species. Therefore, very few further comparative comments regarding the hormonal control of reproduction in both these species can be made.

Throughout my experiments I have established the reproductive effects of effluent upon a pulmonate species of mollusc, namely *P. corneus*. Importantly, these effects were demonstrated to be repeatable effects; reproductive rate was increased in effluent in this snail species in all three of my experiments. Further, though the data was less convincing, this same effluent also increased reproduction in the prosobranch

V. viviparus, producing a second reproductive peak in 100% effluent only. Therefore, in conclusion it appears that effluent increased reproduction in both these species of freshwater gastropod mollusc.

In *P. corneus*, the stimulatory effects of effluent occurred during the peak of reproduction onwards, from early summer into winter in this pulmonate species for reasons that are unknown. In *V. viviparus*, during the first peak of reproduction (typically in spring for viviparids) there also may have been a stimulatory effect on reproduction resulting in the birth of premature young (and increased fecundity) as indicted by the low weight of recently born baby snails in effluent. This stimulatory effect resulting in the birth of low weight baby snails may have been slight because the maternal viviparid snails had only been in my experimental setup for a relatively short period of time, comparatively little time for effluent to have any affect, as gestation of viviparid embryos is comparatively long. Alternatively the effects of effluent could have been post partuition (i.e. toxic effects of effluent upon newborn snails) in which case other properties of the effluent may have been responsible for the reproductive effects on adult viviparids in spring. Further, the second peak of reproduction of *V. viviparus* in 100% effluent from mid summer towards Autumn was the result of a stimulation in reproduction, this after these adult snails had been in my setup a considerably longer period of time (2.5 months in effluent).

Thus, in both taxa, there was a failure to undergo a seasonal decline of particular reproductive parameter(s) clearly seen in snails in river water. In *P. corneus* the number of egg masses produced and weight (number of eggs per mass) of egg masses was affected towards the end of the breeding season where there was a failure of these parameters to decline. These are parameters where maternal influence could be the decisive factor, as egg mass production is internal in pulmonates, within their bodies. Similarly, there was an end of season affect with *V. viviparus*, where recently born baby snails failed to undergo a seasonal weight loss seen in baby snails born in river water. However, this effect was in baby snails and, once born, they were not under maternal influence. It is possible that the growth effect of effluent in F1 viviparids occurred before the mature embryos were born, and was expressed post partuition. Alternatively, the effect of effluent could have been upon the baby snails after their

birth, an effect separate to, or in addition to, affects that occurred upon maturing embryos before their birth.

The stimulatory effects of effluent on egg mass production in adult *P. corneus* snails during the late season might be expected to have caused weight loss in these adult snails, and a failure to increase energy stores before winter. However, no weight loss occurred during my experiments, possibly because they were well fed. The lower weight of baby *V. viviparus* born from effluent exposed snails in the spring may have been caused by a shorter gestation period due to increased embryo production. The second reproductive peak of viviparids in effluent late summer could also be viewed as a failure of a reproductive parameter to decline; this also could have caused weight loss (or a failure to increase body weight) in adult viviparid snails with winter approaching. These hypotheses to explain the results I observed in both species are not mutually exclusive, how and if these phenomena observed in my experiments are linked (e.g. chemical/s in effluent, and their MOA/s) is unclear at present.

Common to the end of the reproductive season for both species were high concentrations of alkylphenolic compounds identied in TSE, but these chemicals could only explain effects upon reproduction observed at this time. This implies other chemicals (and/or alkylphenolic chemicals) contained in TSE may have had affects earlier (or during) the reproductive season. In conclusion, this study demonstrated reproductive effects of effluent upon both a pulmonate and prosobranch species of freshwater gastropod mollusc over a period of months, and not only during the normal peak of reproduction in both species. Further, developmental effects in the offspring and a failure to gain weight before winter could affect the ability of these snails to survive winter. These effects (reproductive and developmental) could indicate stimulatory effects of TSE, and therefore, may have serious lifecycle and population levels effects upon these species in the wild. How these effects of TSE might affect wild populations of snails is unclear.

7.4 Further work and future perspectives

7.4.1 Reproductive effects of TSE upon freshwater snails

Further work in my opinion could investigate further the phenomena of increased reproduction, a failure to undergo a seasonal decline in reproductive parameters, and the developmental effects seen in F1 baby snails I have observed. Many papers indicate from a nomenclature point of view the use of the terms "parental generation" (FO), and then the "first generation" (F1) of snails` born to the parental generation. Often used for laboratory investigations often into the effects of EDCs, these terms can be found in many papers. However, I feel this nomenclature is misleading in some circumstances. My results have demonstrated the importance of a high degree of plasticity of response (growth, mortality, and reproduction pattern etc.) of freshwater molluscan species especially when placed into a new environment. Further, at the beginning of my experiments little was known about the "parental generation" of snails that were used in my experiments, specifically their "home" environments before being moved; water temperature profile, food availability, and the stability of that environment (dessication events, risk of predation, and severe weather events storms etc.). Although the canal water (from which the viviparids were collected) was demonstrated not to be oestrogenic in the yeast assay screen, few other details are known about this environment. In the case of *P. corneus* little is known about their physical environment (rifes by definition maybe small and possibly temporary bodies of water), or chemical environment, though a regular effluent input is highly unlikely.

Therefore, it is difficult to compare their new environment to their previous. We might infer that for *P. corneus* the mesocosm appeared very favourable (due to high reproductive rate), but the same assurity cannot be made for *V. viviparus* due to their poor health in autumn. I would contend, therefore, that although technically my snails were the F0 generation once in my experimental system, in fact the process of adaptation to new or changing environmental conditions, is an ongoing process through the many generations of snails that have been exposed to the effects man has had on freshwater bodies (since our dramatic increase in population numbers). Their previous environment may have programmed them in a certain way, for example,

sensitised or desensitised them to chemicals in the water (as well as programming them to important ecological parameters such as temperature and food supply). Therefore, the source of snails may well influence the results of experiments once these snails have been moved to a new environment. Further, the movement of snails to a new environment (physical and chemical) would result in physiological changes during adaptation that might be complex and confounding and take several generations to settle down.

This theoretical argument explains my suggestions for further work needed; for *P. corneus*, to test their reproductive and developmental responses to effluent in a multi-generation test system. If my adult "F0" generation of *P. corneus* were placed into the mesocosm in graded concentrations of TSE (with a river water, or perhaps better, tap water control) then reproduction could be assessed, and a generation of F1 baby snails could be allowed to mature to adulthood before winter. The number of F1 adults snails in each treatment could be counted (before winter), and a subsample of snails from each treatment taken for histological assessment of snails` tissues. The live F1 snails, once counted, could then be replaced into treatment tanks and allowed to overwinter in their own treatment water. These adult F1 snails may or may not survive, but where they did survive the next generation could be assessed for the same endpoints as the parental FO generation. Population numbers in each treatment tank in springtime would be an endpoint measured. Other endpoints would be an estimate of reproduction, and histological analysis of snail tissues, for each generation of snails. However, since a multigeneration test relies on the survival of F1 snails overwinter, and the subsequent survival and reproduction of F1 snails, and neither is guaranteed but is part of the experimental design; how many generations of snails that might form a multigeneration test would be determined by factors beyond our control.

Evidence from my experiments of effects of effluent on both F1 development and adult reproduction in viviparids should be repeated, with some alterations and improvements to the experimental design. If possible, the entire early season peak of reproduction should be observed. The adult viviparids seemed very robust early season; moving them to the sewage treatment plant neither caused mortalities nor seemed to interfere with reproduction. This may be somewhat surprising given that they were moved during a peak of reproduction. However, in order to start an

experiment earlier, the snails would need to be collected earlier, and this may prove difficult. Whether these snails would still be found in shallow water at this time is unknown. However, it is a desirable goal to confirm that effluent had no (or little) effects upon reproduction during this early season (and main reproductive) peak; as snails born in spring are largely carried over from the previous year this part of the reproductive cycle may not tell us much about the effects of TSE at this time. This change in experimental design would also improve the observation of growth effects early season also. Though it may be difficult to achieve in a single experiment, the observation of fecundity (unshelled, shelled, and total embryos in the pallial oviduct) and reproduction from spring through to autumn, and a possible second reproductive peak would help clarify the relationship between fecundity and reproduction over the season. As this relationship is unclear at present, more detail is required to understand it more thoroughly, however, at present, only a destructive method is available to count embryos in the brood pouch. To my knowledge, no x-ray machine capable of taking pictures of embryos in brood pouches of live snails is available. However, the experimental design needed to cover this entire period would, by comparison to own experiments, be very long. My own experiments were long and time consuming, and an even longer experiment may simply not be feasible (perhaps counting newborn snails monthly could help). Nevertheless, the aim would be to test the repeatability of results; thereby to clarifying the relationship between fecundity and reproduction, and for the first time to if increasing the time these snails were in effluent affects their response to effluent during the first reproductive peak and onwards.

The comments I have already made regarding multi-generation testing for the pulmonate species *P. corneus* also hold true for this prosobranch species. The between taxa differences in their response to effluent over generations would also be worthy of scientific investigation. By my own estimations (based on the minimum time to first reproduction observed) from my own morphological and histological observations, three consecutive generations ("F0", "F1", and "F2") all reproducing could be produced in a two-year experiment (e.g. January 09` to January 11`). Although ideally more generations would be better, such an experiment involving just three generations would exceed the length of time any similar experiment has been run in the experimental set-up.

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