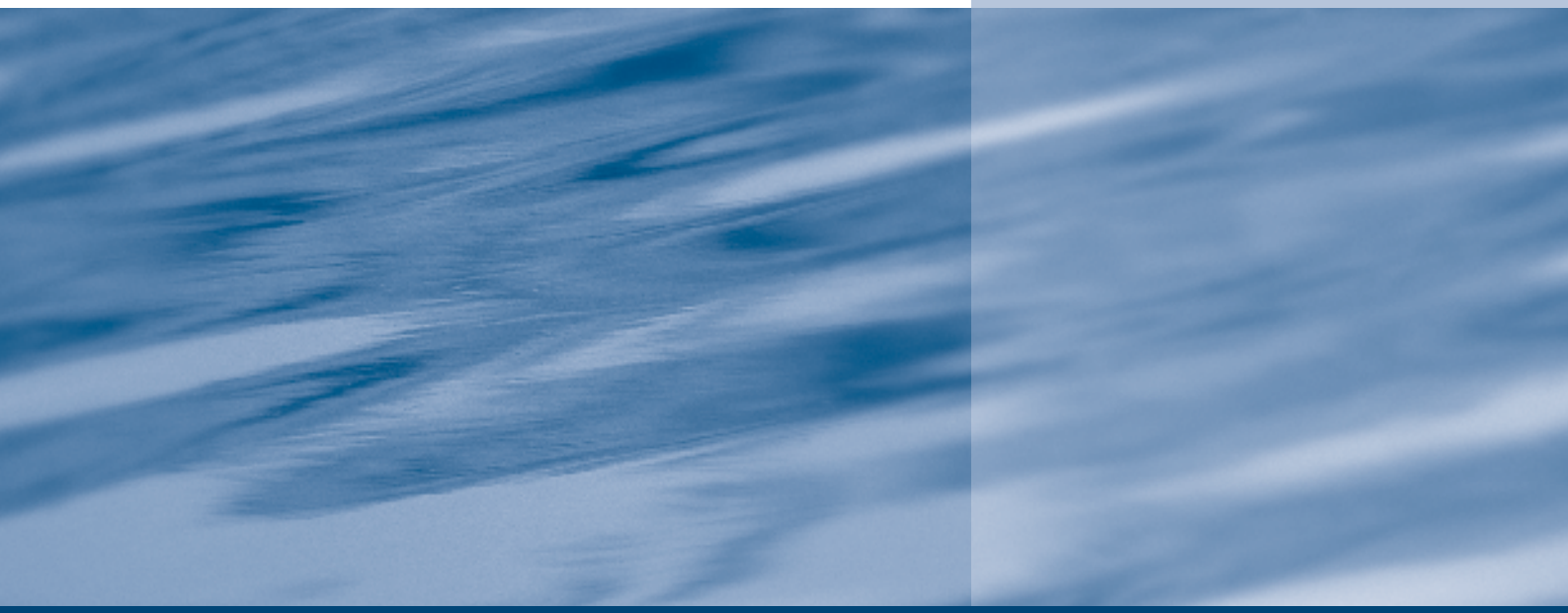

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The Endocrine Modulating Effects of Wastewater Treatment Works Effluents

'Sensitive windows' for inducing germ-cell intersex in the Roach (Rutilus rutilus)

Science Report SC990020/SR

UK WATER INDUSTRY RESEARCH LIMITED
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Acknowledgements from the Researchers

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Executive Summary

A U.K. survey published by Jobling et al (1998) has established that there is a widespread occurrence of feminised male fish living downstream from wastewater treatment works (WwTW). These feminised males had developing eggs (oocytes) in their testes and/or oviducts (feminised reproductive ducts) as well as sperm ducts. The condition where both male and female features occur in the same gonad is referred to as intersex. The feminised males also had abnormal concentrations of reproductive hormones (oestrogen and androgen) in their blood and contained a blood protein, called vitellogenin, which is normally only produced by female fish in response to oestrogens. Moreover, in the most severely intersex fish sperm quality and quantity was reduced and the ability of the fish to breed was impaired. Controlled experiments by Rodgers -Gray et al (2001) have clearly shown that exposure of adults (2-5 years old) or juvenile fish (50 days old) to WwTW effluents can induce both the production of the female yolk protein, vitellogenin (in both adults and juveniles), and the formation of oviducts (in young male fish). It has not, however been shown that exposure to WwTW effluents under controlled conditions induces the formation of oocytes in the testes, which is the most severe form of intersex.

Possible windows in the life cycle during which fish may be sensitive to feminisation of the gonad include the period encompassing fertilisation of the egg and the development of the reproductive organs (ovaries and testes and associated reproductive ducts) and during the proliferation of the germ cells (developing eggs and sperm) which occurs every year around the time of spawning in annually breeding fish.

The objective of this project, therefore, was to assess the effects of exposure of fish to WwTW effluents from two different sewage treatment works during the two life periods believed to be the most vulnerable to disruption of sexual development.

The percentage of trade influent entering the two treatment works studied was 6% at site A and 24% at site B. The population equivalents also differed between the sites and were 137,000 at site A and 312,700 at site B. Fertilised eggs or adult fish were exposed to a graded concentration of the effluents (from 0% effluent to 100% effluent) diluted with river water or tap water until 300 days after hatching (in the early life stage experiments) or for a period of 2 or 4 months in the two adult experiments. There was a difference in the source of fish between the two adult trials; the first trial (for 2 months only) used fish that had not previously been exposed to oestrogen whilst the second trial used wild fish that had previously been exposed to oestrogen.

Biological samples were taken at 50, 100, 200 and 300 days after hatching (in the juvenile fish study) and at the end of each trial (in the adult trials). In addition, in the early life stage experiment, a sample of fish from each treatment was transferred to clean dechlorinated tap water after only 60 days exposure and reared until they were 300 days old.

In the early life stage experiments, vitellogenin induction occurred in effluent exposed fish at both of the study sites, thus confirming that the effluents were oestrogenic. This oestrogenic response was almost an order of magnitude higher at site A compared to site B, reflecting the higher concentrations of oestrogens measured in the effluent at site A. There was also an increase in the proportion of developing young fish with oviducts with increased concentration of effluent. All of the fish in the highest effluent concentrations had an oviduct. This effect appeared to be irreversible as the oviduct was retained in the fish that were reared in clean water after an initial effluent exposure. There was, however, no evidence of developing eggs in the testes of the male fish in any of the treatments at any of the time points sampled.

In the adult roach experiments, for fish not previously exposed to oestrogen, there was an increased production of vitellogenin by the male fish in response to increased concentration of effluent. The development of the testis was also suppressed by effluent exposure, particularly at site B (which may not necessarily be as a consequence of exposure to oestrogen). The trial with fish previously exposed to oestrogen, however, (where fish had elevated concentrations of vitellogenin at the outset of the study), revealed a gradual clearance of vitellogenin from the blood, except for fish exposed to the highest dose of effluent. There was no evidence that either of the two effluents induced the development of intersex in either of the adult fish trials. Some of the fish that had previously been exposed to oestrogen before the experiment began had oocytes in their testes, and the number of eggs in their testes appeared to increase during the trial. This occurred in both the exposed and control fish, however, and was not due to exposure to the effluents.

When taken together, the results of these three experiments suggest that exposure of fish to WwTW effluents for relatively long periods during certain parts of the life cycle can induce some (vitellogenin and oviducts), but not all (eggs in testes), of the characteristics of intersex seen in wild fish that reside in rivers downstream from effluent discharge points.

The general health effects of exposure of young fish to treated sewage effluents were also examined and the utility of vitellogenin as a biomarker for these general health effects was examined. Vitellogenin was detected in body tissues including the liver (where it is synthesised), the kidneys and in the gonads (ovaries and testes) of the exposed fish and was most apparent in the fish exposed to the effluent at site A. Indeed, more detailed examination of the kidneys showed that there was an increase in the diameter of the kidney tubules and in the number of glomeruli in the kidneys with increased dose of effluent in fish at both of the study sites. Moreover, exposure to the highest concentration of effluent at site B, induced development of new nephrons by the kidneys of the exposed fish, reflecting the increased demand for filtration and removal of inappropriately produced vitellogenin and of pollutants. In some cases, these effects were retained when fish were transferred to clean water for 240 days following exposure, indicating a long-term effect of the effluent on the kidney development. The significance of the changes in the kidney is not known and they may reflect an acclimation to the effluent. There were no signs of overt kidney damage as a consequence of the effluent exposures.

Technical Executive Summary

There have been a number of studies documenting a widespread occurrence of sexual disruption in various freshwater and marine fish species. It has been established that effluents from wastewater treatment works (WwTW) are oestrogenic to fish, and exposures under controlled conditions have been shown to induce vitellogenin (VTG – an oestrogen dependent yolk precursor) synthesis and the formation of female gonadal ducts in male fish when the exposure occurs during early life. A published UK survey has shown a high incidence of intersexuality (gonads that have altered gonad ducts and/or altered sex cell development – developing eggs [oocytes] in the testis) in roach (*Rutilus rutilus*) populations living downstream of WwTW effluent outlets and this was correlated with concentration of effluent from WwTW at these sites. In controlled exposures, however, WwTW effluents have not been proven to induce alterations in sex cell development (oocytes in testis in male fish). Possible windows of sensitivity for induction of altered sex cell development in the roach are during very early life, the period encompassing fertilisation and embryogenesis to the onset of morphological sexual differentiation, and during germ cell proliferation, occurring around spawning in adult fish. In this project roach were exposed to effluents from two different WwTW for periods during early life (embryos up to 300dph) and as post-spawning adults (when germ cell proliferation occurs) and effects of these effluents assessed on gonad development (gonad ducts and germ cells- via histology) and vitellogenin induction (as a biomarker of oestrogen exposure). In this work a histological analysis of the kidney and immunolocalisation of VTG in a selection of tissues were also undertaken in fish exposed to WwTW effluent during early life to investigate for potential wider health implications of exposure to oestrogenic effluents. Assessments were also made to establish if alterations in somatic tissue and gonad development correlated with VTG induction.

The study effluents

Roach were exposed in field tank systems at two UK WwTW. Trade influent to the WwTW made up approximately 6% (WwTW A) and 24% (WwTW B) of the total influent. The population equivalents of the works influent were approximately 137,000 (WwTW A) and 312,700 (WwTW B). Measured effluent exposure concentrations were close to nominal throughout all trials. Fish losses occurred in every trial, and to varying degrees, and there were differences in growth of the fish between treatments, but they did not correlate with the effluent concentration.

Oestrogen content of the effluents

Seven day composite effluent samples were collected from both study sites and analysed for steroid oestrogens (17β-oestradiol, oestrone and 17α-ethinylestradiol). alkyphenolic compounds (octylphenol, nonylphenol and nonylphenol mono- and diethoxylates), and bisphenol A using gas chromatography-mass spectrometry. Steroid oestrogen concentrations in both

effluents varied. For WwTW A, oestrone concentrations ranged between 37 and 70 ng/L and 17β-oestradiol between 0.7 to 10 ng/L. At WwTW B concentrations of oestrone were between 2.3 and 7.8 ng/L and 17β-oestradiol between not detectable and 3.6 ng/L. The synthetic oestrogen 17α-ethinylestradiol was detected intermittently and only in the WwTW A effluent (up to 1.5ng/L, limit of detection =0.5ng/L). Measured concentration ranges of alkylphenolic compounds at the two study sites were between 0.17 and 11 μg/L at WwTW A and between 0.03 and 2.98 μg/L at WwTW B). Bisphenol-A was detected at concentrations between 0.02 and 0.23 μg/L (WwTW A) and between 0.03 and 0.4 μg/L (WwTW B).

Effects of exposure to WwTW effluents during early life on sexual development

For exposures to effluent from WwTW during early life, fertilised roach eggs were hatched and exposed to graded concentrations of effluent from fertilisation until 300dph to allow the completion of germ (sex) cell differentiation. Biological sampling was carried out at 50dph, 100dph, 200dph and 300dph. At 60dph, 60 fish from each treatment were transferred to clean water to depurate in order to compare a short term exposure to treated WwTW effluent in early life (fertilisation to 60dph) with a longer term exposure from fertilisation through to the completion of sexual differentiation (300dph).

Vitellogenin induction occurred in early life stage roach at both study sites, confirming the oestrogenic nature of the effluents. The vitellogenic response in fish at WwTW A was almost an order of magnitude higher than in fish exposed to the effluent at WwTW B, reflecting the higher concentrations of steroid oestrogens at WwTW A. At 300dph there was a concentration-related vitellogenic response in the fish at WwTW B. Fish held in clean water from 60 to 300dph contained very little body VTG showing a clear capacity for the clearance of VTG.

At 200dph and 300dph not all fish had completed sexual differentiation. Gonadal phenotypes of fish included males and females, and fish that had not completed sexual differentiation. There was a concentration-related response in the number of fish with male germ cells and ovarian cavities and all fish with male germ cells had feminized reproductive ducts in the highest effluent concentrations (100% - WwTW A, 80% -WwTW B). There were no discernable differences in the status of the ovaries in control fish compared with effluent exposed females. Depurated male fish that had been exposed previously to effluent at both study sites from fertilization to 60dph then held in clean water to 300dph retained the female duct, indicating that this effect may be permanent. There was no evidence of germ cell disruption in any of the fish examined for any of the treatments.

In summary, exposure to the study WwTW during early life induced VTG synthesis, the formation of a female reproductive duct (ovarian cavity) in testes of male fish, but did not disrupt germ cell development (induce oocytes in testis).

Effects of exposure to WwTW effluents on sexual development in adult fish

Two experiments were carried out to investigate the effects of exposure to WwTW effluent on germ cell development during the post-spawning period in adult male roach. One trial investigated the effects of the test effluents in 'clean' fish (fish that had not had prior exposure to oestrogen; 2 month exposure at WwTW A only) and the other (by chance) used fish that had been exposed to oestrogen prior to the effluent exposures (both study sites, WwTW A - 4 months exposure, WwTW B - 2 months exposure). The gonadosomatic index (GSI) decreased during the trial across all the treatments in both trials in line with normal seasonal patterns for sexual development. There was a strong indication of a suppressive effect of effluent exposure on gonad development/recovery at WwTW B. For the 'clean' fish a vitellogenic response occurred at concentrations of effluent at and above 50% (WwTW A). In males previously exposed to oestrogen and containing VTG there was a gradual clearance of VTG during the trial (at the end of the exposure only fish in the WwTW A 100% effluent had VTG titres above the control fish).

There was no evidence that exposure of post-spawning adult male roach to either of the two test WwTW effluents induced alterations in germ cell development (oocytes in the testis). This was the case for male fish both with and without previous exposure to oestrogen. Some fish that had received exposure to oestrogen prior to the exposure to the WwTW effluent were intersex and the severity of the intersex condition increased over the trial, but this was not due to the effluents. A possible explanation for this finding is that some of the germ cells in the males exposed to oestrogen prior to the study were programmed to develop into oocytes and did so during the period of germ cell proliferation that follows spermiation and spawning.

In summary, exposure of adult post-spawning males to the study WwTW induced VTG synthesis, but had no effect on the gonad duct or germ cell formation in male fish.

Effects of exposure to WwTW effluents during early life on fish health

Histological analysis of the kidneys derived from fish exposed during early life to the test effluents showed there were no observable differences in renal blood vessel structure between control and effluent-exposed fish. Mean tubule diameter of roach nephrons, however, was affected (increased) and in an effluent concentration-dependent manner at both sites. A concentration-dependent enhancement of nephron tubule diameter was also observed in fish exposed to effluent at both sites from fertilization to 60dph and then depurated for 240 days. There was a concentration-dependent increase in the number of glomeruli in the roach at WwTW B (200dph) and this effect was present in the depurated fish derived from the exposures at WwTW A. There was an effect of effluent exposure on nephrogenesis (basophilic clusters and developing nephrons) in the highest effluent exposure group (80%) at WwTW

B at 200 and 300dph. For depurated fish long term effects of effluent exposures on kidney development occurred for the roach exposed to full strength effluent at WwTW A, and for the 80% effluent at WwTW B.

In summary, exposure of roach during early life to WwTW effluents induced effects on kidney structure and some of these effects persisted even after depuration in clean water. The effects seen were consistent with the requirement to accommodate a higher filtration rate of the blood (due to the presence of induced vitellogenin/contaminants). The changes in the kidney may reflect an acclimation to the effluent and there were no signs of overt kidney damage.

In effluent-exposed fish at both sites, VTG was detected by immunostaining in the liver, its site of synthesis, in the kidneys, specifically around the tubules and in the gonad (ovary of non vitellogenic females and in the testis). This was most apparent in fish exposed to the effluent at WwTW A. In fish exposed to the WwTW, increased VTG induction was associated with increased numbers of glomeruli, (WwTW B) increases in size of kidney tubules, and higher numbers of basophilic clusters and developing nephrons (reflecting the increased demand for filtration of VTG for its removal from the circulation). Together these data show that abnormal VTG induction places an increased load on kidney function and is able to permeate into many of the body's tissues. The significance of this (if any) is not known. For effluent concentrations inducing an ovarian cavity in male fish there was an associated elevation in VTG in these exposure groups.

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1 Glossary

Basophilic cluster – A cluster of cells in the kidney that stain strongly with basic dyes. A basophilic cluster is the first stage of *de novo* nephron neogenesis, the process of renal repair in fish.

Depuration – The process of depurating or freeing from foreign or impure matter.

Eosinophilic – Readily staining with eosin.

Germ cell – A reproductive cell; gamete or cell giving rise to a gamete.

Glomerulus – The knot of blood capillaries in the kidney that are surrounded by the dilated end (Bowman's capsule) of a kidney tubule.

Gonochorists – Single sexed animals.

Haemopoetic tissue – blood forming tissue

Intersex – Condition where both male and female features occur in the same gonad.

Lumen – Internal space of any tubular or sac-like organ

Mesogonium – Primordial reproductive duct.

Nephrogenesis – development of the kidney or kidney tissue.

Nephron – The individual structural and functional unit of the vertebrate kidney, comprises a glomerulus, Bowman's capsule and a convoluted tubule.

Oocyte – Female ovarian cell in which meiosis occurs to form the egg. Cells undergoing first meiotic division are often termed primary oocytes, after which they become secondary oocytes which undergo the second meiotic division to become mature eggs.

Oviduct, ovarian cavity – female reproductive duct, carries eggs from the ovary to the exterior.

Spermatogonium – Primordial male germ cell, gives rise to spermatocytes.

Undifferentiated – Fish that have not undergone to the process of translation of genetic into phenotypic sex.

Vitellogenin (VTG) – A female specific yolk protein produced in the liver in response to oestrogens.

2 Project Aims

The principle aim of this research project was to investigate possible periods or "windows" in the life history of the roach that may be sensitive to oestrogenic effects of effluents from wastewater treatment works and to assess if these exposures resulted in intersex (the simultaneous presence of both male and female structures in a single gonad).

The original aims of the project were:

- **Determine the effects on sexual differentiation and development of exposure of fish embryos to Site A and Site B WwTW effluents.**
- **Determine the effects of exposure to Site A and Site B WwTW effluents on sex cell development in post-spawning fish.**
- **Investigate the utility of vitellogenin induction as a biomarker for adverse health effects in native UK fish.**

Under these three main aims there were five objectives, and they were to:

1) Determine the effects on sexual differentiation and development of exposure of fertilised roach eggs/embryos to Site A WwTW effluent.

2) Determine the effects on sexual differentiation and development of exposure of fertilised roach eggs/embryos to Site B WwTW effluent.

3) Determine the effects of exposure to the Site A WwTW effluent on sex cell development in adult post-spawning roach.

4) Determine the effects of exposure to the Site B WwTW effluent on sex cell development in adult post-spawning roach.

5) Investigate the utility of vitellogenin induction as a biomarker for general health effects in native UK riverine fish.

3 Introduction

There have been a number of studies documenting a widespread occurrence of sexual disruption in various freshwater and marine fish species. It has been established for some time that effluents from wastewater treatment works (WwTW) are oestrogenic to fish, resulting in the induction of vitellogenin (VTG – an oestrogen dependent yolk precursor) in male fish (Purdom et al. 1994). In 1998 the results of a UK survey were published showing that there was a high incidence of intersexuality in roach (*Rutilus rutilus*) populations living downstream WwTW effluent outlets (Jobling et al. 1998) and this was correlated with concentration of effluent from WwTW at these sites. Intersex is the term given to a range of effects on gonad development, including gonadal duct disruption where the male duct is feminised to form a female-like ovarian cavity, and/or the presence of both male and female germ cells within the same gonad (Nolan et al. 2001). This was deemed as unusual, given the knowledge that roach are gonochorists (single-sexed animals) and hermaphroditism is not thought to be a normal phenomenon in this species. More recent studies have shown that intersex fish have both a reduced milt volume and sperm density (Jobling et al. 2002) and reduced fertility (Jobling et al. 2002). Thus, there is the potential for the intersex condition to lead to population-level effects. Sexual disruption in wild fish inhabiting UK rivers is not restricted to the roach and intersex has been identified in other gonochoristic fish, such as the gudgeon (van Aerle et al. 2001).

WwTW effluents are a complex mixture of chemicals and they have been shown to contain many oestrogenic chemicals, including; natural steroid oestrogens -17b-oestradiol and oestrone, synthetic oestrogens-ethinylestradiol derived from the contraceptive pill, alkylphenolic chemicals – nonylphenol and octylphenol and their ethoxylates, and bisphenols (Desbrow et al. 1998). All of these chemicals have been shown to be oestrogenic to fish causing the induction of VTG (Routledge et al. 1998; Tyler and Routledge 1998; Thompson et al. 2000).

Many controlled exposures to WwTW effluents conducted in the UK and more widely in Europe have now shown that treated effluents induce VTG synthesis in a range of fish species, and the potency of the effluents depend on the treatment process and the level of effluent dilution (Harries et al. 1997). Furthermore, all life stages studied appear to be responsive for this oestrogenic effect (Rodgers-Gray et al. 2001).

It has not been established which life stages in fish are more sensitive to the oestrogenic effects of WwTW effluents. Early life stages of fish, however, may be particularly sensitive to exogenous hormonal signals during the period when gonadogenesis occurs; in some species of fish it is possible to influence or reverse the phenotypic sex by exposing them to high doses of exogenous steroids during early life (Patino 1997; Baroiller and D'Cotta 2001). Laboratory studies have shown that it is possible to induce gonadal duct disruption and germ cell disruption in fish by exposing them to some of the oestrogenic chemicals found in WwTW effluent, but at concentrations higher than that found in the environment (Gray et al. 1999; Koger et al. 2000). WwTW

effluents however, are complex mixtures of many oestrogenic compounds and these chemicals have been shown to be interactive (additive) in their effects (Thorpe et al. 2001; Silva et al. 2002; Thorpe et al. 2003). There are also many other components present in WwTW effluent that may have effects on the endocrine system, for example chemicals that can act as androgens, anti-oestrogens or anti-androgens (Jalabert et al. 2000). Thus the component chemicals in WwTW effluents have to be considered together to assess their potential effects for inducing sexual disruption in fish.

A possible window of sensitivity for sexual disruption in the roach is during very early life, the period encompassing fertilisation and embryogenesis to the onset of morphological sexual differentiation. A previous study has shown that exposing early life stage roach (50 to 150dph) induces gonadal duct disruption (feminisation) but in that study the simultaneous presence of male and female germ cells in the same gonad did not occur (Rodgers-Gray et al. 2001). The precise timing of the genetic programming for sex differentiation in the roach is not known, and it may begin before 50dph. The aim of the first part of this project (objectives 1 and 2) was to investigate the effects of exposure to WwTW effluent on sexual development in roach, from fertilisation through to the completion of the period of gonadogenesis.

Sexual development and differentiation in fish has been demonstrated to be very plastic and can be influenced by a range of environmental factors in early life, including temperature and exposure to xenobiotics (Patino 1997; Jalabert et al. 2000). Less is known about the potential plasticity of differentiated germ cells in gonochoristic fish. Yamamoto demonstrated that application of steroids could induce sex reversal in juvenile medaka (*Oryzias latipes*) that had undergone sexual differentiation, both male to female, and female to male (Yamamoto 1953; Yamamoto 1958). Egami (1955) documented that it was possible to induce ova-testis in sexually mature male medaka by several methods, namely high temperature, irradiation by x-rays, starvation, and by exposure to oestradiol. Concentrations of oestradiol were not given for that study. Egami concluded the treatments described had caused damage to the testes and testis-ova originated from spermatogonia during recovery and spermatogenesis (Egami 1955a; Egami 1955b; Egami 1955c; Egami 1955d; Egami 1955e). Shibata and Hamaguchi (1988) exposed sexually mature male medaka to 160 μg/L oestradiol for periods of between 6 to 30 days and showed that the induction of ova-testis was apparent in the early stages of exposure (0-6 days) but further exposure resulted in both the inhibition of spermatogenesis, and no further development of testis-ova. When fish were depurated (removed to clean water), spermatogenesis was restored and testis-ova developed in some of the regenerating testes. After quantifying the different sex cells present in testicular cyst structures, they concluded that testis-ova originated from spermatogonia B (Shibata and Hamaguchi 1988). They also concluded that testis-ova that developed in the depuration period after the termination of the oestradiol exposure originated from spermatogonia B which had been transformed during the exposure period. Exposure of male common carp to 1 μg/L oestradiol for 3 months has been reported to inhibit spermatogenesis and induce intersex (Gimeno et al. 1998).

The roach is an annually reproducing fish that spawns in UK rivers during early May. During the post-spawning there is a short interval of gonadal regression (lasting 3-6 weeks). Renewed gonadal growth and germ cell proliferation subsequently occurs throughout the summer and autumn and then arrests during the winter. The final stages of gonadal growth and maturation occur during early spring prior to the annual spawning period. It is possible that roach are sensitive to endocrine disrupting effects of WwTW effluents during this period of gametogenesis, resulting in altered germ cell development. Recent research has indicated that the severity of intersex in wild roach increases with age (S. Jobling, pers. comm.). This may be due either to longevity of exposure and/or repeated exposure during the sensitive period of germ cell proliferation proceeding spawning.

In the second phase of this project (Objectives 3 and 4), the adult, post-spawning period of sexual development was investigated as a possible window for disruption of sex cell development.

Vitellogenin induction is now used widely as a biomarker for exposure to oestrogenic and chemicals and their mixtures (including effluents). VTG and vitelline envelope proteins are among the most sensitive biomarkers for oestrogenic chemicals in fish (Thomas Jones et al., 2004**).** Extremely high concentrations of VTG have been shown to cause kidney failure (Herman and Kincaid 1988) and statistical associations have been shown between intersex in wild roach and plasma titres of VTG (Jobling et al. 1998). The value of VTG induction as a biological effect measure for the wider health implications of exposure to oestrogenic chemicals has not been established. To develop such an understanding there is the need to investigate the impact of VTG induction on somatic tissues, notably tissues that are involved with the metabolism and excretion of VTG - the kidney and liver.

VTG and other proteins are eliminated from the body via the kidney, where they can accumulate. In fish exposed to oestrogenic chemicals, eosinophilic material (including VTG) localises in the tubules and Bowman's capsule [oestradiol - (Folmar et al. 2001; Zaroogian et al. 2001), ethinylestradiol (Schwaiger et al. 2000; Weber et al. 2003) and lindane (Wester et al. 1985)]. The presence of VTG in the kidney in oestrogen-stimulated fish has been shown through immunohistology (Folmar et al. 2001). Ethinylestradiol administered at high doses (500μg/kg body weight by injection; Schwaiger et al. 2000) has been reported to cause severe haemorrhage within the kidney tubule lumen. Laboratory exposures of flounder to high doses of the oestrogenic alkylphenolic compounds, octylphenol and isomers of DDT, however, were not found to induce the kidney pathology seen in fish injected with oestradiol in the same study (Zaroogian et al. 2001). A study on carp exposed to nonylphenol via the water at environmentally relevant concentrations did not result in any observable renal disruption (Weber et al. 2003).

Field studies have reported renal disruption in fish sampled from rivers (Schrank et al. 1997; Schmidt-Posthaus et al. 2001), lakes (Koponen et al. 2001) and estuaries (Simpson et al. 2000; Stentiford et al. 2003) known to be contaminated with a variety of endocrine disrupting chemicals. The fish

collected from the field in these studies, however, had been exposed to a mixture of many aquatic pollutants and pathology observed in these cases may not be due only to the effects of EDCs but also to other chemical toxicants.

In the final part of this project, Objective 5, a histological analysis of the kidney and immunolocalisation of VTG in a selection of tissues were undertaken in fish exposed to WwTW effluent during early life (the most sensitive period for disruption in tissue development –see later) to investigate for potential wider health implications of exposure to oestrogenic WwTW effluents. A depuration study further investigated if any of the changes seen were transient or if there were longer-term health consequences. Assessments were also made to establish if alterations in somatic tissue and gonad development correlated with VTG induction.

The study species: Roach (*Rutilus rutilus*)

The roach was the chosen species for study for the following reasons:

- It is native to UK Rivers,
- It is a member of a very large family of fish living in UK freshwaters (Cyprinidae- the carp family)
- It one of the most common cyprinid fish living in UK rivers and comprises up to 50% of the fish biomass in lowland rivers.
- We have a good knowledge of the reproductive biology of the roach and have developed the required analytical tools to measure for oestrogenic effects in this species (VTG induction, histological markers for gonadal feminisation etc.).
- The roach has been our sentinel species for studies on the impact of effluents from WwTW for almost 10 years and thus we have a greater understanding of the intersex condition in the roach compared with any other fish species.

In the roach we have established the following:

- Intersex is widespread in roach populations living in effluent contaminated rivers.
- A basic knowledge on the ontogeny of sexual differentiation (germ cell and duct development).
- Early life is a critical window of sensitivity for oestrogen-induced disruption of gonadal duct formation.

4 Methodology

4.1 The Experimental Set-up for Effluent Exposure Studies

To undertake this project, it was necessary to hold fish in effluent for extensive periods of time. To do this field tank systems (mesocosms) were established at two UK WwTW, both equipped to supply effluent and diluent water to the tank systems. Both mesocosms were situated in the open air, and thus the fish were subjected to ambient seasonal environmental fluctuations (e.g. photoperiod, temperature). The mesocosm system enabled us to expose roach to real effluent discharges, with the natural fluctuations that occur in effluent composition. The composition of treated WwTW effluent is known to vary and depends on the composition of the influent to the works, the amount of dilution of the influent and the effluent of the WwTW, and the efficacy of treatment processes. There may be daily and seasonal differences in the effluent from a particular WwTW (Rodgers-Gray et al. 2000). The mesocosm facilities were used to investigate the effects of exposure to treated WwTW effluent on gonadal development and VTG induction in early life stage roach and in post-spawning adult roach. During the studies both effluents were sampled and analyzed for the presence of key oestrogenic chemicals (steroid oestrogens, alkylphenolic chemicals and Bisphenol A) in parallel with the biological analyses on the exposed fish. Prior to the start of experiments each mesocosm system was left to run for 1-2 weeks to ensure the pumps, flow meters and other associated equipment were performing consistently.

4.2 The Exposure Effluents

Effluent quality is usually assessed by its biological oxygen demand (BOD), a measure of the quantity of oxygen utilised by aquatic organisms over a 5 day period, expressed as mg/L/d. Higher quality effluents have a BOD lower than 10, poorer quality effluents have a BOD higher than 10. The ‘strength’ of the influent to a WwTW is quantified by the population equivalent (p.e.). One p.e. is the organic biodegradable load with a five-day biochemical oxygen demand (BOD5) of 60 g of oxygen per day. Influents to WwTW consist of a complex mixture of chemicals of domestic or industrial origin. Domestic influents can consist of urine, faeces, soap, paper, and synthetic detergents. Trade influents can contain more varied chemical components. It has been demonstrated that some trade influents can contain high concentrations of chemicals with known oestrogenic activity (Rudel et al. 1998).

Treatment of wastewater in WwTW generally consists of two or three treatment stages, namely primary, secondary and in some cases tertiary. Influents to WwTW usually pass through a preliminary grill filter prior to any treatment to remove any large material. Primary treatment usually consists of a sedimentation tank to settle out primary sludge. Secondary treatment involves biological treatment of the sewage; either through the use of trickle filter systems or activated sludge. These processes aim to remove much of

the suspended particulate matter and reduce the BOD and aid the oxidation of ammonia to nitrites and nitrates to nitrates by microbial oxidation. Tertiary treatment can include a variety of processes and often the tertiary treatment employed depends on the specific characteristics of the influent at a particular WwTW. Sand filters can be used to further remove suspended solids and reduce BOD. Other techniques are sometimes used to remove nitrogen, phosphorus and ammonia. Certain advanced forms of tertiary treatment can remove some metals, industrial chemicals and other types of specific contaminants.

There are other factors that can affect the quality of effluent from a WwTW. Ambient temperature can affect microbiological populations and therefore the efficiacy of biological treatments. High rainfall can result in dilution of influent to the WwTW, and also in a reduced retention time through the works. Dilution factor of the effluent in the receiving water may affect any subsequent impact on that water body.

It has been demonstrated that most UK sewage effluents studied are oestrogenic, albeit to varying degrees, and that this oestrogenicity can persist for considerable distances downstream from the effluent discharge (Purdom et al. 1994; Harries et al. 1997). It has been documented that WwTW effluents are oestrogenic in other European countries (Wahli et al. 1998; Hecker et al. 2002); and in Japan (Hashimoto et al. 2000) and the USA (Folmar et al. 2001). A link has also been shown between population equivalent and number and severity of intersex roach found in the river downstream from the works (Jobling et al. 1998).

In this project the effects of effluents on sexual development in roach were carried out at two UK WwTW with different population equivalent values, different influents and different treatment processes.

WwTW A

WwTW A supplied the treated effluent to the mesocosm system at Site A. Industrial influent to this treatment works makes up approximately 6% of the total influent and the population equivalent of the works influent is approximately 137,000. Preliminary treatment of influent to the works consists of screens and grit removal. Subsequent to this the influent is divided to pass along one of three paths for treatment, and these are to either the North works (20% of flow), South works (30%) and East works (50%). Each works has its own primary sedimentation tank. Secondary treatment at the North and South works comprise of trickling filters and re-circulation whilst the East works employs bubble diffused air activated sludge treatment. The effluent is not subject to tertiary treatment at this site and effluents from the North, South and East works are combined to produce the final effluent that is discharged into the brackish stretch of a local river. Extensive studies on the effluent from this WwTW have shown it to be oestrogenic to fish (Harries et al. 1999; Rodgers-Gray et al. 2001).

WwTW B

WwTW B has a higher industrial component to the population equivalent, making up approximately 24% of the total influent to the works. The population equivalent is approximately 312,700, over twice the population equivalent of the influent to WwTW A. Preliminary treatment of influent to the works consists of screens and primary settlement. Subsequent to this the influent is divided 2 ways. A plant comprising of bubble diffused air activated sludge treatment takes 60% of the flow and the further 40% is treated by means of biological phosphorus removal activated sludge plant. Both plants discharge into final settlement tanks. After settlement, the effluent from both tanks is combined and discharged into a local river. Direct studies on the oestrogenicity of the effluent from WwTW B had not been previously undertaken. There are, however, reports of widespread intersex in populations of wild roach living in the river downstream of this treatment works discharge.

4.3 The Mesocosm Systems

4.3.1 Site A Mesocosm

The experimental facility at Site A has been used for previous exposures of fish to assess the oestrogenicity of the treated WwTW A effluent (including those described by (Routledge et al. 1998; Harries et al. 1999; Rodgers-Gray et al. 2001). The mesocosm at this site was comprised of six, 600L tanks. See Fig. 1 and Plate 1a for a schematic and photograph of the mesocosm at site A, respectively. The system was designed to enable each tank to receive different concentrations of effluent, allowing concentration-dependant effects of effluent exposure to be investigated.

Final effluent was supplied to the mesocosm system by a submersible pump placed in the effluent channel. The effluent first passed through a 100 μm filter (cleaned daily) before passing through flow meters to each tank's supply pipe. Flow meters allowed the rate of flow into each tank to be adjusted and recorded daily. This allowed the maintenance of desired effluent concentrations. The diluent water at this site was river water from a local river upstream of the WwTW discharge. River water underwent UV disinfection to reduce any possible disease risk for the exposed fish. UV treatment of water is effective at eliminating many viral, bacterial and fungal fish pathogens. UV treatment, however, is less effective at destroying infective stages of pathogens such as the protozoan *Ichthyophthirius multifliis* which causes white spot disease in fish (Andrews 1988). An absolute control of tap water (run through a header tank and an activated charcoal filter to remove chlorine was also employed (see Plate 1.d; fish fry in particular are sensitive to chlorine). All diluent water supply pipes were fitted with flow meters. Flows were recorded daily and adjusted accordingly to maintain the nominal effluent concentrations assigned to each tank. Wastewater left the tanks through a standpipe that was guarded by mesh to prevent fish escaping. Each tank was fitted with aerators to support the fish and tanks were covered with mesh to prevent predation of fish by piscivorous birds and animals. These covers also prevented fish from jumping out of the tanks.

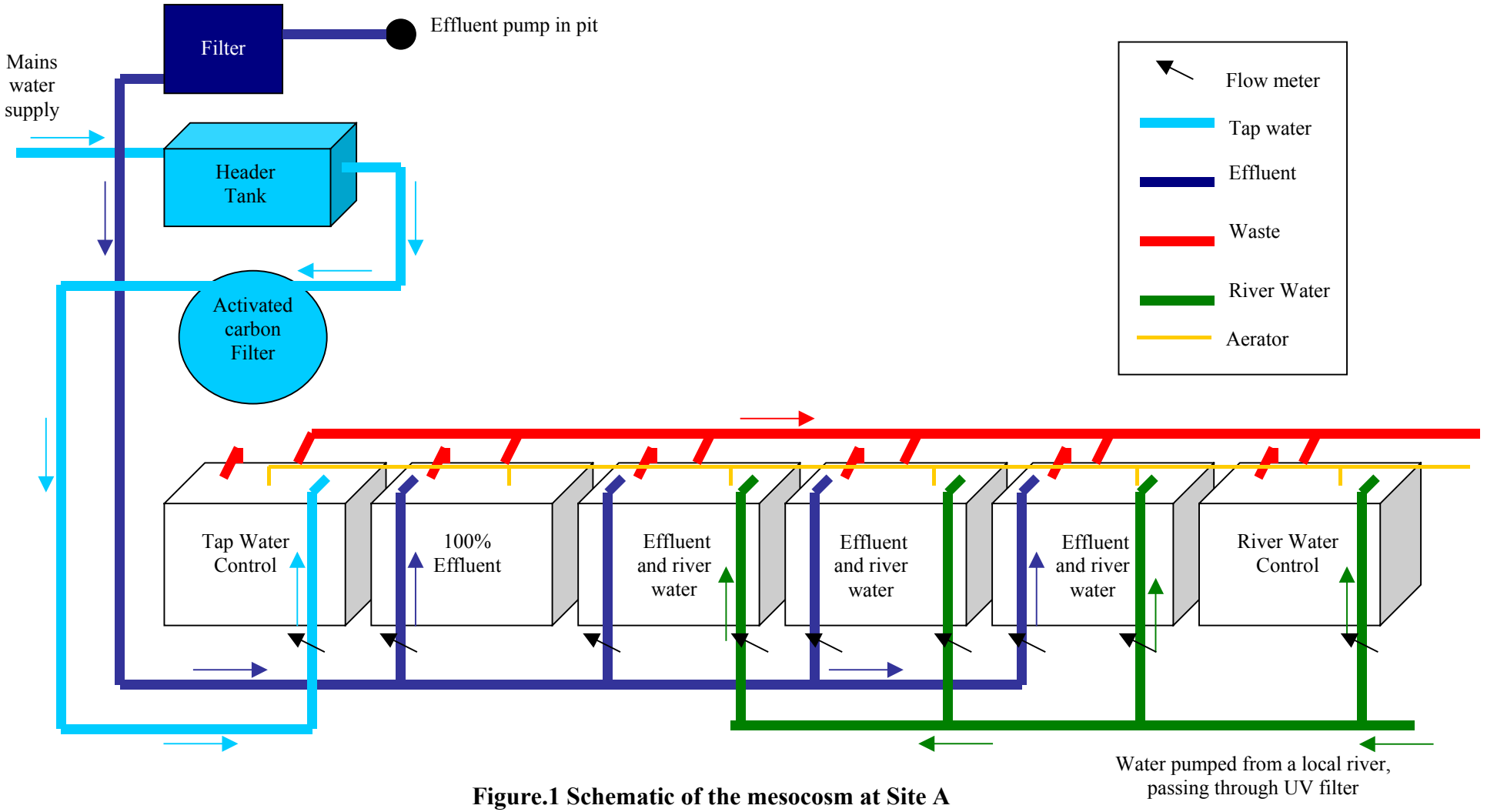


Figure.1 Schematic of the mesocosm at Site A

4.3.2 Site B Mesocosm

The mesocosm at site B was constructed during the initial part of the project in April 2001. The system was comprised of 6, 700L tanks. Fig. 2. and Plate 1b-d show a schematic and photographs of the mesocosm set up at Site B, respectively. Final treated effluent was pumped via a submersible pump placed in the final effluent channel of the WwTW (Plate 1c) and pushed through a 100 μm Spin-clear filter. This filter was self-cleaning through an automated backwash that occurred approximately once every hour (thus reducing the manual workload at this site). River water was not available at this site, and therefore the diluent used was tap water. Tap water from the mains supply at the WwTW was passed though a header tank and activated charcoal filter before reaching the tanks to remove chlorine. All water supplies were controlled by a system of flow meters, which allowed the rate of flow to be monitored and adjusted as required. All tanks were fitted with aerators to support the fish and tank covers to prevent predation and escape of fish.

5 Effects of exposure to effluent from WwTW during early life on sexual differentiation in roach (objectives 1 and 2).

5.1 Methodology

5.1.1 Experimental design

Experiments were set up to investigate the effects of treated sewage effluent on sexual differentiation in roach. See Fig.3 for a diagram of the experimental design. Fertilised roach eggs were exposed and allowed to hatch in effluent in both of the purpose built mesocosms at the Site A and Site B sites. The exact timing of sexual differentiation in the roach is not known, although a previous study has shown that female germ cell differentiation is complete at 200dph but male germ cell differentiation was not (Rodgers-Gray et al. 2001). In this study, fish were exposed to graded concentrations of effluent from fertilisation until 300dph to allow the completion of germ cell differentiation. Biological sampling was carried out (where numbers of surviving fish were sufficient) at 50dph, 100dph, 200dph and 300dph. At 60dph, 60 fish from each treatment were transferred to clean water to depurate in order to compare a short term exposure to treated WwTW effluent in early life (fertilisation to 60dph) with a chronic exposure from fertilisation through to the completion of sexual differentiation (300dph).

At both experimental sites six tanks were supplied with graded concentrations of treated sewage effluents. For exposure experiments involving roach eggs and fry the large volume of the mesocosm tanks was inappropriate, so a smaller glass reinforced plastic tank with a capacity of approximately 50 litres was placed inside each of the larger tanks, and raised on bricks to allow easy viewing (Plate 2). The overflow from this smaller tank was filtered through a cylinder of fine stainless steel mesh to prevent loss of larvae to the waste pipe. Fish were later transferred to the larger mesocosm tanks at approximately 70 days post hatch.

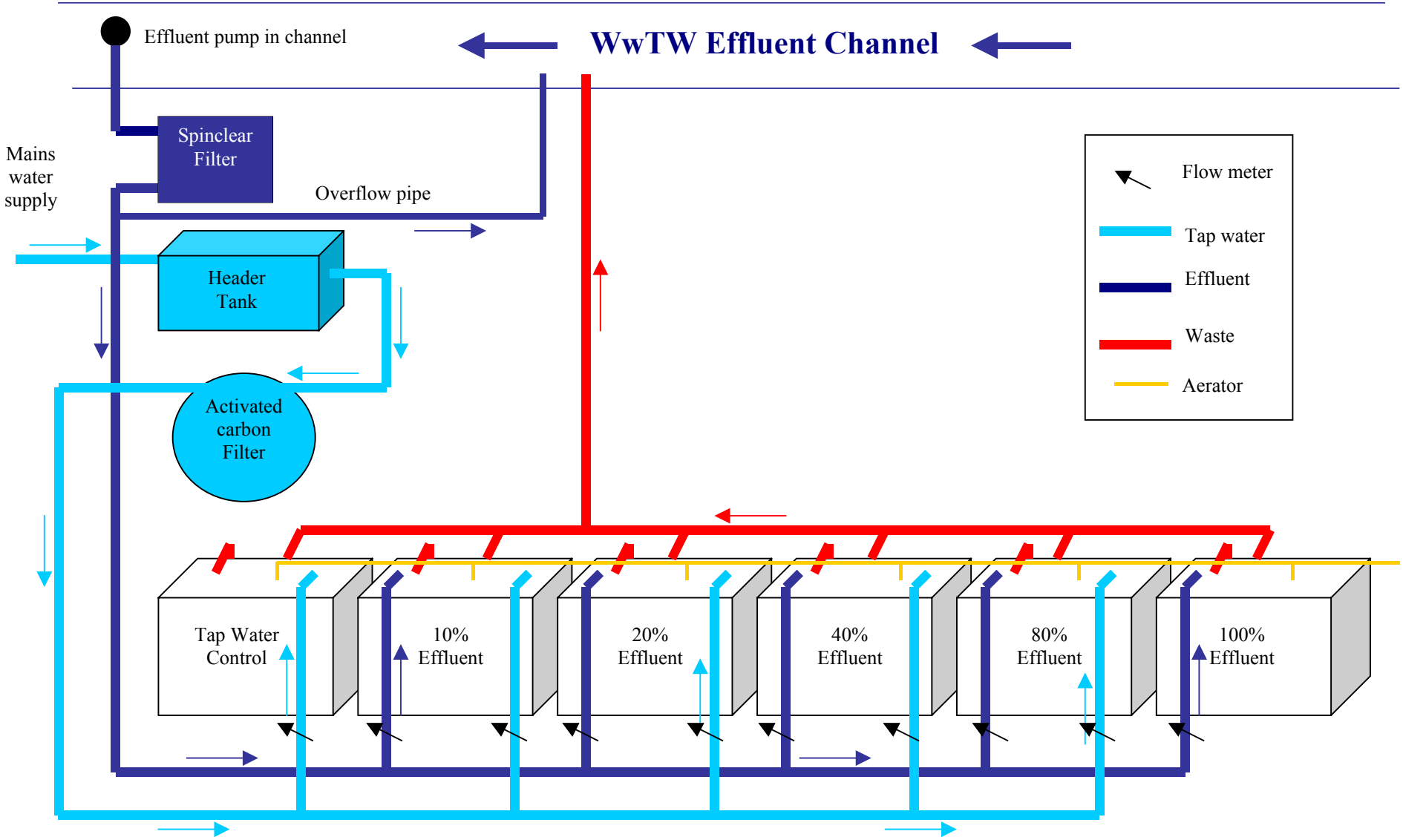


Figure.2 Schematic of the mesocosm at Site B

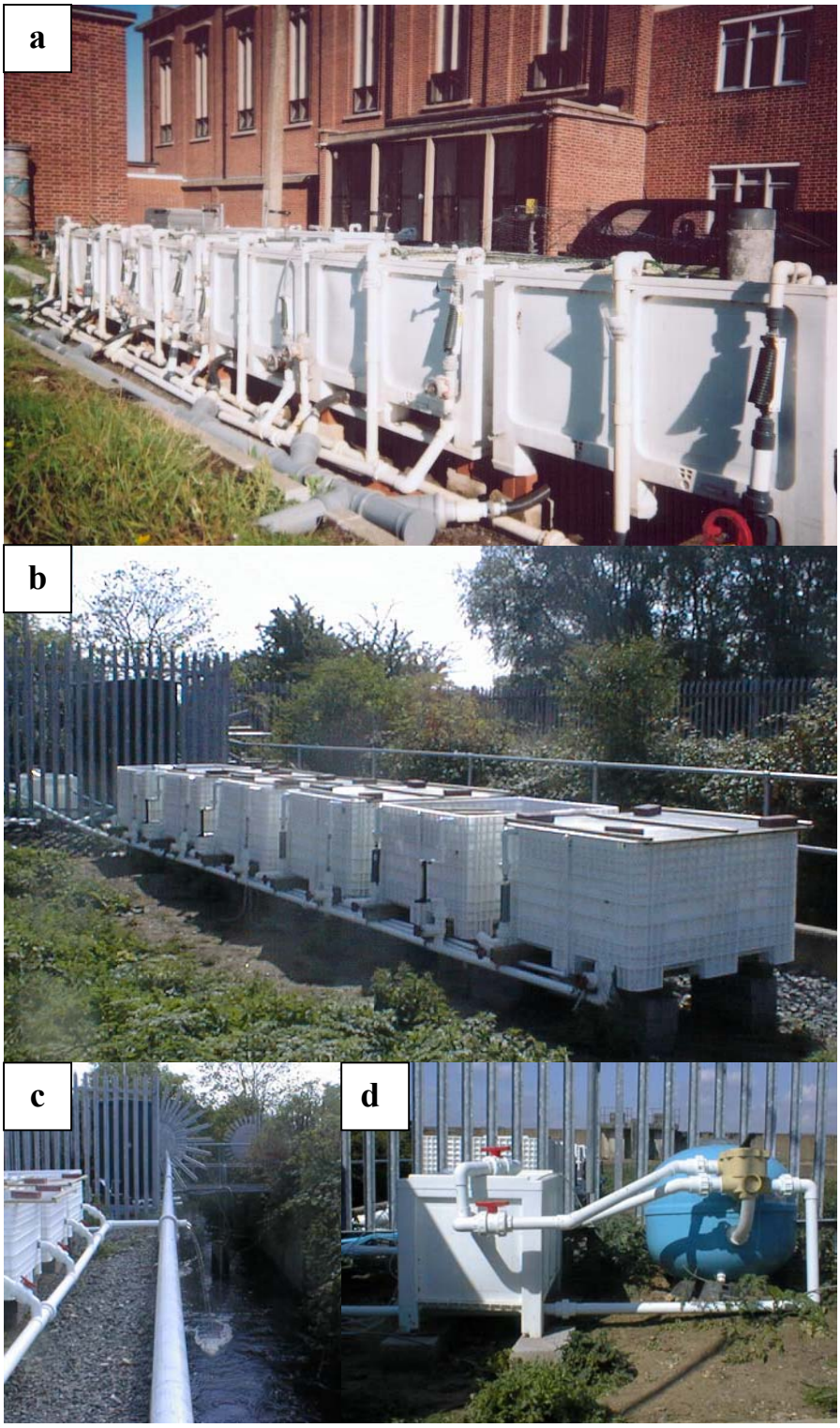


Plate 1a – The mesocosm system at Site A, showing the six tanks that housed the fish and the system of flow meters that controlled the concentration of effluent and diluent water entering the tanks. **Plate 1b** – The mesocosm system at Site B. **Plate 1c** – The final effluent channel at Site B where the submersible pump was situated. **Plate 1d** – The header tank and activated charcoal filter at Site B. The filter removed chlorine from the diluent tap water. A similar system was in place at Site A.

Nominal effluent concentrations at Site A were 100%, 40%, 20%, 10%, with river water and dechlorinated tap water controls. At Site B WwTW nominal effluent concentrations were 100%, 80%, 40%, 20%, 10% and the control tank received dechlorinated tap water. The flow rate through each of the tanks totalled 5 L/min and flow rate and water temperature were monitored daily. The tanks were aerated to ensure sufficient oxygen supply.

Adult broodstock fish were collected by Environment Agency staff from the River Trent, Nottingham in May 2001 and induced to spawn using intra-peritoneal injections of carp pituitary extract according to established protocols. Gametes were pooled (4 females and 6 males for Site A and 5 females and 6 males for Site B) and then fertilized eggs were placed on raised mesh hatching trays in each exposure tanks. The embryos were cleaned of sediment twice daily by gently pushing water towards the embryos with a Pasteur pipette. From hatching, fry were fed newly hatched artemia until approximately 60dph, when commercial cyprinid food pellets were introduced (Calverton Fish Farm, UK) and artemia feeds gradually phased out. For the remainder of the trial, fish were fed food pellets once or twice daily. Sixty roach were sampled from each treatment at each of the 50, 100, 200 and 300 dph sampling points and used to measure whole body VTG in individuals and assess their gonadal status via histopathology. Due to the relatively low numbers of surviving fish at Site A, the experiment at this site was sampled and terminated at 200 dph. At 60 dph, 60 fish from each surviving treatment were transferred to clean water at The University of Exeter and maintained for a further 240 days. These fish were sampled at 300 dph to coincide with biological sampling of fish at Site B STW.

5.1.2 Measurement of oestrogenic compounds in the two study effluents

Seven day composite effluent samples were collected from both study sites at days 0-7 days, days 27-33 and days 53-60. Samples of effluent were analysed for steroid oestrogens; 17β-oestradiol, oestrone and 17α-ethinylestradiol, and alkyphenolic compounds; octylphenol, nonylphenol and nonylphenol mono- and diethoxylates. Analysis methods used solid phase extraction to isolate the compounds of interest followed by analysis by gas chromatography-mass spectrometry (Blackburn and Waldock 1995). All chemical analyses were carried out by CEFAS, Burnham on Crouch, UK.

5.1.3 Biological Sampling

At each sampling point 60 fish from each treatment were sacrificed with a lethal dose of anaesthetic (MS-222 or Benzocaine, according to Home Office recommendations). Thirty fish were weighed (mg) and their lengths measured (mm), for growth analyses, and then placed into cryovials, frozen on dry ice and then transferred for long term storage at –20°C for subsequent analysis of VTG. Thirty fish were fixed for 24 hours in Bouin's solution and stored in 70% industrial methylated spirits prior to processing for histological analysis.

5.1.4 Measurement of Vitellogenin

Homogenisation and subsequent quantification of whole body VTG (using a carp VTG ELISA, that has been validated for use in the roach) was carried out according to Tyler et al. (1999).

5.1.5 Gonadal Histology

Transverse tissue blocks for gonad sectioning were prepared by cutting the fish trunk either side of the dorsal fin. Samples were then embedded in paraffin wax, sectioned at 5 μm, mounted and stained with haemotoxylin and eosin. Slides were then analysed by light microscopy. The gonadal status of the fish was determined (Nolan et al. 2001), and any abnormalities in development assessed (abnormal sex ducts or altered germ cell development).

5.1.6 Statistical Analyses

All statistical analyses were carried out using Sigmastat v2.0 (Jandel Scientific). Statistical significance was accepted at $p<0.05$ for all comparisons. Inter-group differences were assessed using one-way ANOVA (parametric, for normalised data) or Kruskal-Wallis test (non-parametric). Multiple comparisons tests were performed using post-hoc analyses for parametric or non-parametric data.

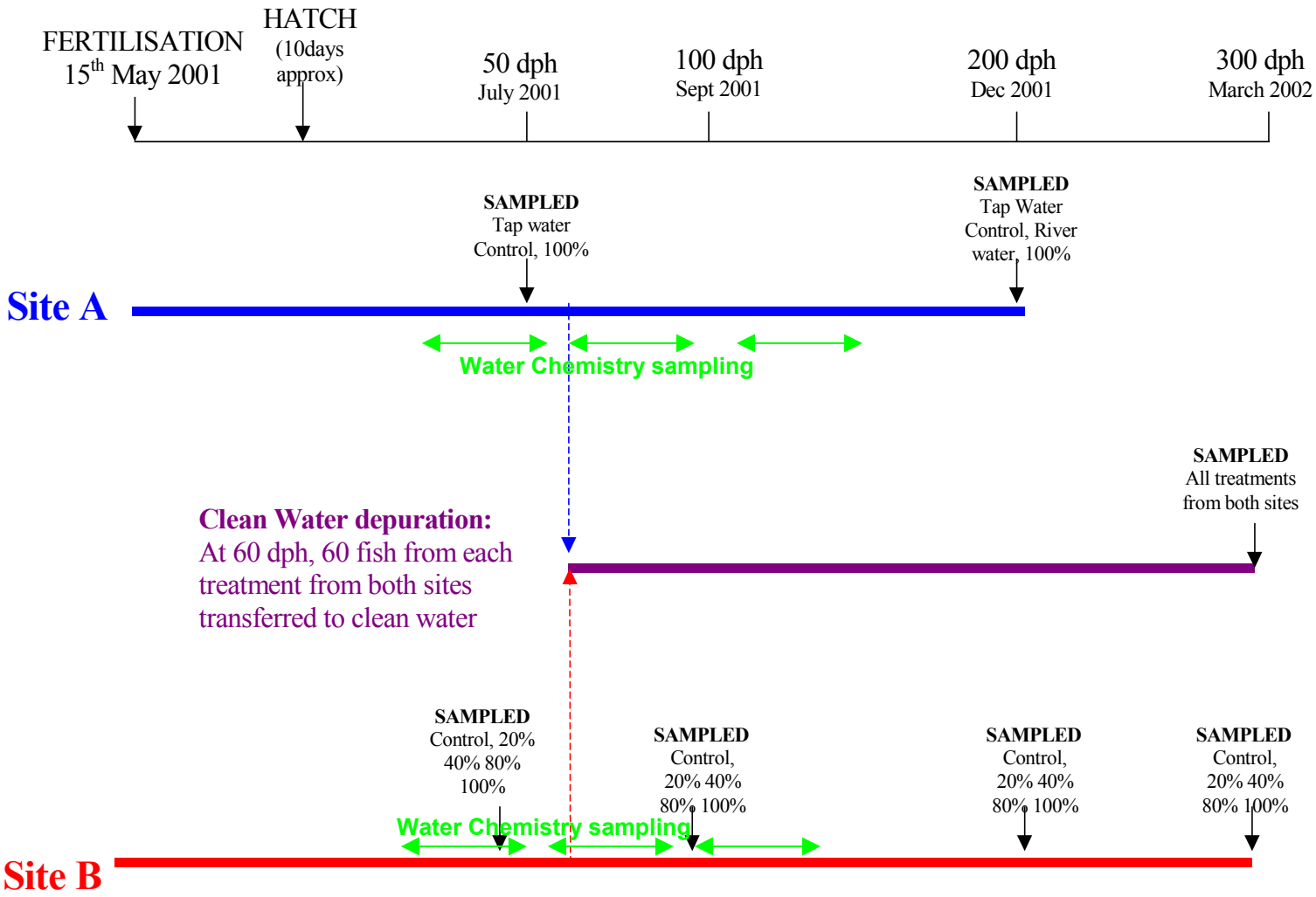


Figure.3 Exposure and Sampling Protocol for Early Life Stage Roach Studies – Fertilised roach eggs were deployed at both sites in May 2001. Hatching occurred approximately 10 days after fertilisation. Fish were reared and sampled for biochemical and histopathological analyses at 50, 100, 200 and 300 dph, where surviving numbers of fish would allow. At 60 dph, 60 fish from each treatment were transferred to clean water for depuration. At 200 dph the effluent exposure at Site A was terminated due to low surviving numbers of fish. Seven day composite effluent samples were collected from both sites at 0-7days, 27-33days and 53-60days and analysed for key oestrogenic chemicals. Details of the sampling protocols are given in the main text.

Plate 2. Hatching tanks for roach. To allow monitoring of the developing roach embryos, smaller glass reinforced plastic tanks were fitted into the large mesocosm system tanks. Outflow from these tanks was filtered through fine stainless steel mesh cylinders to prevent loss of fry. Fertilized eggs were placed into mesh hatching trays which were raised above the bottom of the tank to prevent settlement of sediment on the eggs.

5.2 Results Measured concentrations of the treated sewage effluent

Site A – The measured concentrations of WwTW effluent to which the roach were exposed between fertilization and 50 dph were 0% (Tap water control), 0% (River water control), 15.0+/-1.3%, 24.9+/-1.4%, 42.6+/-2.2% and 100% (mean concentration +/- standard error of the mean; Fig 4a). From 50 dph to 200 dph in the surviving treatment groups at this site the measured exposures were 0% (Tap water control), 0% (River water control) and 100% WwTW effluent.

Site B - The measured concentrations of WwTW effluent to which the roach were exposed between fertilization and 50 dph were; 0%, 8.0+/-0.7%, 17.8+/-0.9%, 36.6+/-1.6%, 78.3+/-1.0% and 100%. From 50 dph to 100 dph the measured exposure regimes were; 0%, 17.0+/-1.0%, 33.6+/-1.3%, 77.8+/-0.8% and 100%, for the period between 100 dph and 200dph; 0%, 12.4+/-0.8%, 33.9+/-1.3% and 81.0+/-2.5%, and for the period between 200dph and 300dph, the exposure regimes were; 0%, 15.7+/-0.6%, 35.1+/-0.6% and 77.0+/-0.4% (mean concentration +/- standard error of the mean; Fig 4b).

5.2.2 Concentrations of steroid oestrogens and alkyphenolic chemicals in the test effluents.

Chemical analysis of the effluents showed that concentrations of steroid oestrogens were higher at Site A effluent compared with Site B (Fig 5a). At Site A concentrations of oestrone in each 7 day composite sample were in the range 37 to 63 ng/L and 17β-oestradiol 0.7 to 3.6 ng/L, whereas at Site B concentrations of oestrone were between 3.3 and 7.8 ng/L and 17β-oestradiol between 0.7 and 3.6 ng/L. The synthetic oestrogen 17α-ethinylestradiol was not detected in either effluent at any of the sampling points (limit of detection of 0.5 ng/L). Concentrations of alkylphenolic compounds were slightly higher in the Site B effluent (Fig 5b). At Site A nonylphenol was detected between 0.17 and 0.3 μg/l with Site B ranging between 0.62 and 0.92 μg/L. Concentrations of nonylphenol mono- and di-ethoxylates were between 0.43 and 0.82 μg/L at Site A and 0.79 and 2.7 μg/L at Site B. Concentrations of octylphenol were between 0.06 and 0.19 μg/L at Site A and 0.1 and 0.41 μg/L at Site B.

5.2.3 Measured temperatures in the exposure tanks

The temperature of the effluent/water fluctuated with the ambient temperature in all exposure tanks at both exposure sites. There were no significant differences in water temperature between the various treatments at any one time point at either site (Fig 6).

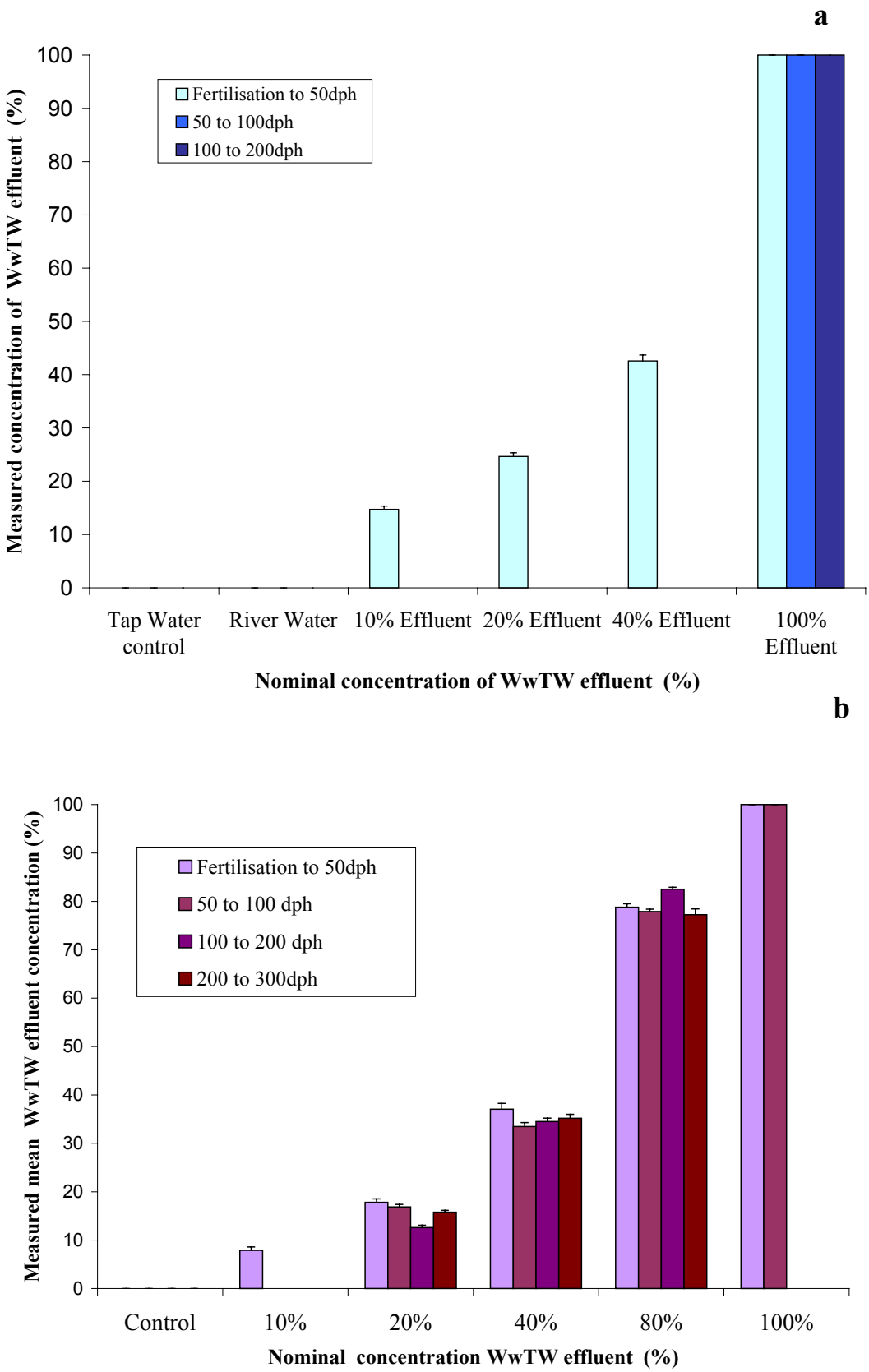


Figure 4. Measured concentrations of effluent during the early life stage fish study at **(a)** Site A and **(b)** Site B. The graphs show mean percentage concentration +/- standard error of the mean. Missing data points are due to termination of that exposure group due to low surviving numbers of fish.

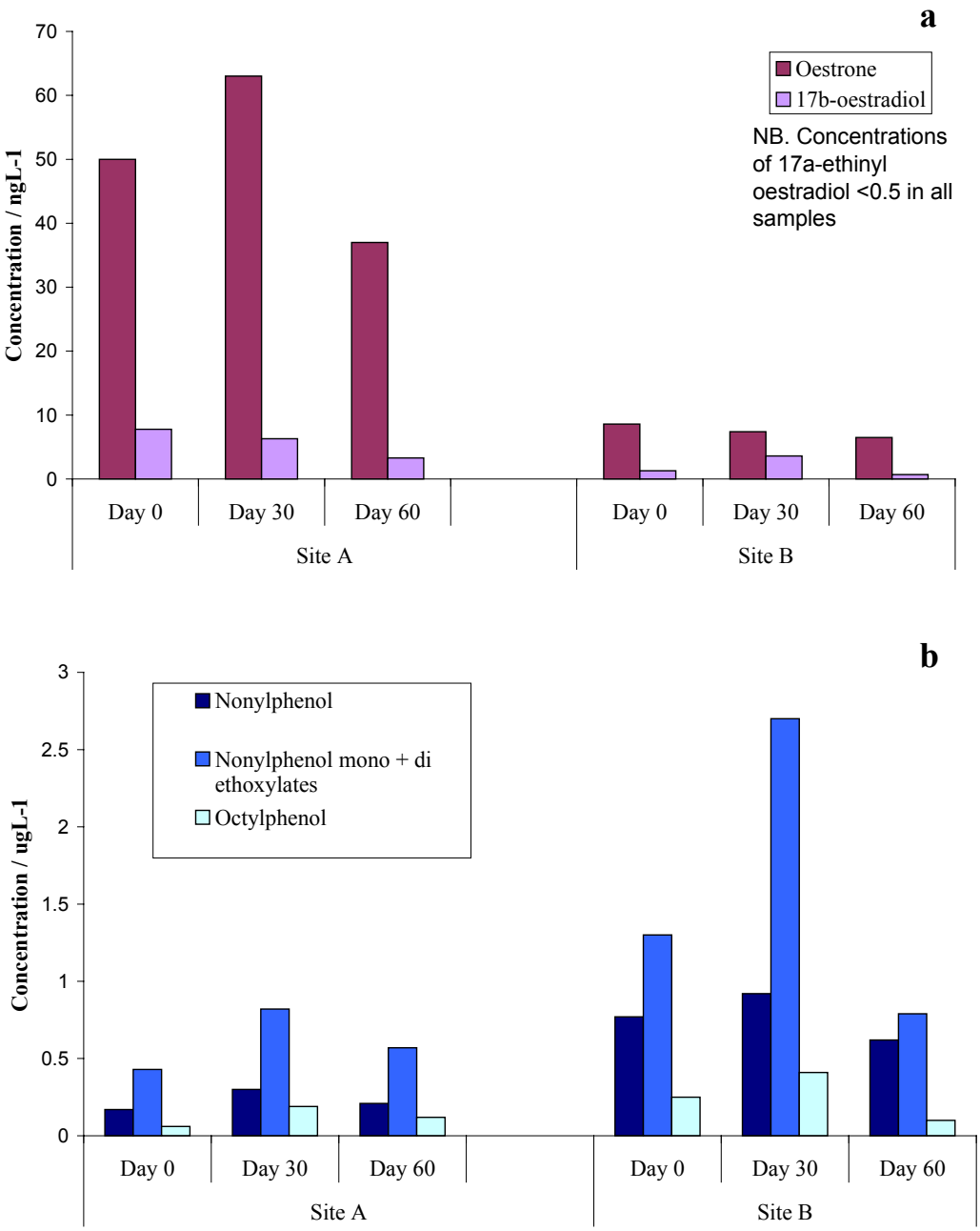


Figure 5. Concentrations of known oestrogenic compounds in the two test effluents during the first period the of the fish exposures (day 0 to day 60), **(a)** steroids and **(b)** alkylphenolic compounds

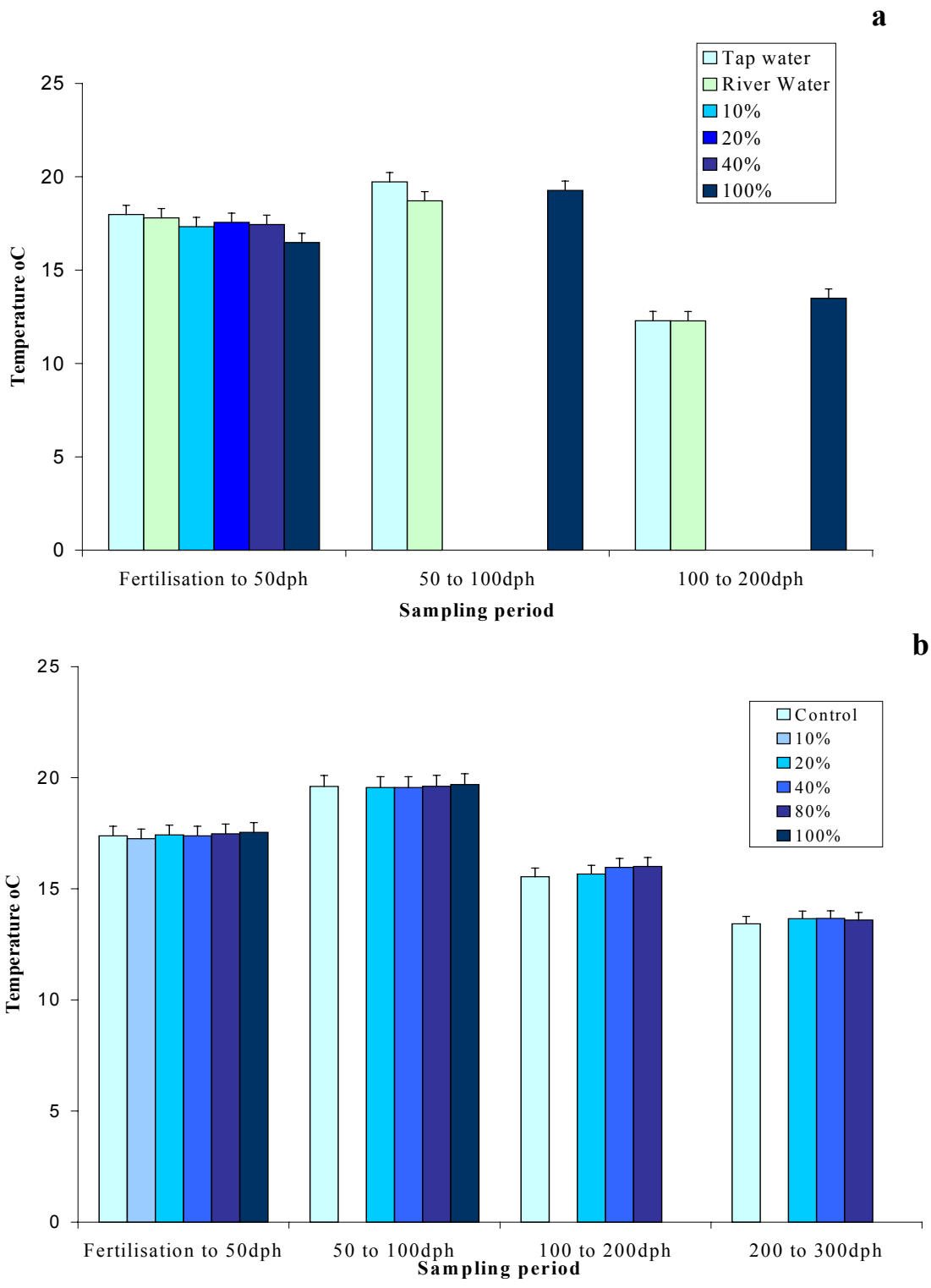


Figure 6. Temperature of the water/effluent in each of the exposure tanks covering the periods between the sampling points **(a)** Site A, May 2001 to December 2001 **(b)** Site B, May 2001 to March 2002. Water temperature fluctuated with ambient temperature. There were no significant differences between the treatment tanks for any one time point.

5.2.4 Survival of Roach

The survival of fish in the different treatment regimes is described below relative to the sampling intervals.

10dph – At 10dph there were no surviving fish in the 100% effluent tank at Site A. At this time approximately 200 fry from the tap water control tank were transferred to the 100% effluent treatment. For the remaining report these fish are referred to as the 100% effluent exposure group, but it should be noted that they were exposed to the 100% Site A WwTW effluent at Site A from 10dph, and not from fertilization.

60dph to 200dph – At the time of the transfer of fish from the effluent exposures to clean water for depuration, survivorship in all treatments was high. Subsequent to this, however, there were significant mortalities in the 10% and 40% effluent groups at Site A and in the 10% effluent exposure group at Site B. Furthermore, there was an outbreak of white spot in the fish held in the 100% effluent tank at Site B at around 100dph. The high level of mortalities in all of these tanks meant that there were insufficient numbers of fish to continue these experimental groups and they were terminated prior to the 200dph sampling period.

200dph to 300dph – Surviving treatment groups at Site A (tap water, river water and 100% effluent) were sampled at 200dph. There were insufficient numbers of fish at Site A to continue the study and thus the experiment was terminated at this at site at 200dph. The exposures at Site B continued to 300dph at which point fish were sampled and the experiment was terminated. At each sampling point 30 fish were sampled for VTG analysis and 30 for histopathology from each treatment. A summary of the treatment groups sampled at 200 and 300dph at both sites are listed in Table 1.

Sampling point	Site	Treatment groups sampled (% effluent)
200dph	**Site A**	Control Tap water
		River water
		100%
	Site B	Control Tap water
		20%
		40%
		80%
300dph	**Site B**	Control Tap water
		20%
		40%
		80%

Table 1. Treatment groups sampled at each time point at both of the effluent exposure sites. Sample sizes were 30 fish for histopathology and 30 fish for VTG analysis from each treatment group.

Depurated fish – 60 to 300dph – At 60dph, 60 fish from each initial treatment from both sites were transferred to clean water for depuration. However, not all these fish survived to sampling at 300dph. Numbers of fish sampled for VTG analysis and histopathology at 300dph are listed in Table 2.

Site	Treatment	Number of fish sampled for VTG analysis	Number of fish sampled for histopathology
A	**Tap water**	0	0
	River	20	29
	10%	20	26
	20%	30	31
	40%	15	17
	100%	20	20
B	**Control**	0	23
	10%	20	24
	20%	19	28
	40%	14	22
	80%	29	30
	100%	20	18

Table 2. Numbers of depurated fish in each treatment group sampled at 300dph

5.2.5 Effects of effluent exposure on fish growth.

There were differences in growth of fish between treatments at each sampling point. At 50 dph at Site A, fish in the 100% effluent treatment group were significantly bigger than fish from the tap water control group ($p<0.05$). At 200 dph fish in both the river water control and the 100% effluent treatment groups were significantly larger than fish in the tap water control group ($p<0.05$). Differences in growth were also observed at Site B. The differences in growth were not found to be related to concentration of the effluent (Fig 7) or the health effects seen.

5.2.6 Whole body vitellogenin concentrations

Whole body VTG analysis of surviving fish at 200dph from both sites (Fig 8a) showed that fish exposed to high concentrations of Site A WwTW effluent had much higher VTG concentrations (almost an order of magnitude higher) than fish exposed to WwTW effluent at Site B. The whole body concentration of fish exposed to 100% effluent at Site A was 5684+/-546 ng/ml VTG compared with 771+/-122 ng/ml VTG in fish exposed to 80% effluent at Site B. This biological effect in the Site A fish mirrored the much higher steroid

concentrations contained in the Site A effluent. Whole body VTG concentrations from surviving fish at 300dph (Fig 8b) showed a concentration-related response with higher VTG concentrations associated with higher effluent concentrations at Site B (significantly different from controls at 40% and 80% effluent). The maximum concentration of VTG in fish exposed to 80% Site B effluent at 300dph was 1709+/-211 ng/ml (compared with 33+/-9 ng/mL in the controls). Fish held in clean water from 60 to 300dph contained very little body VTG and ranged between 29+/-8 ng/ml and 108+/-42 ng/ml VTG. There were no significant differences in whole body VTG concentrations between treatment groups of depurated fish.

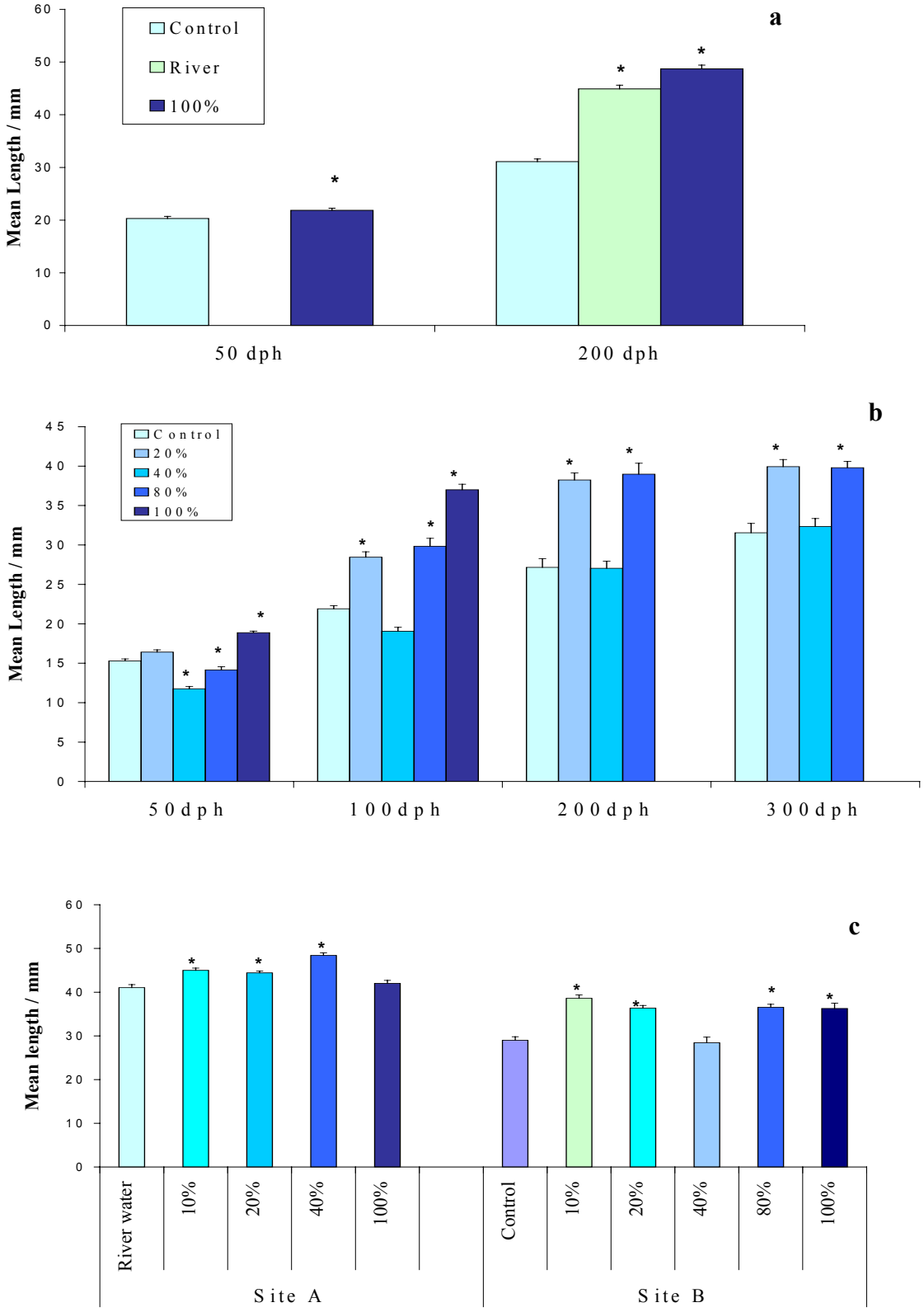


Figure 7. Size of fish at the sampling points **(a)** Site A **(b)** Site B and **(c)** depurated fish at 300dph. Asterisks denote significance from control within a sampling point *, $p<0.05$.

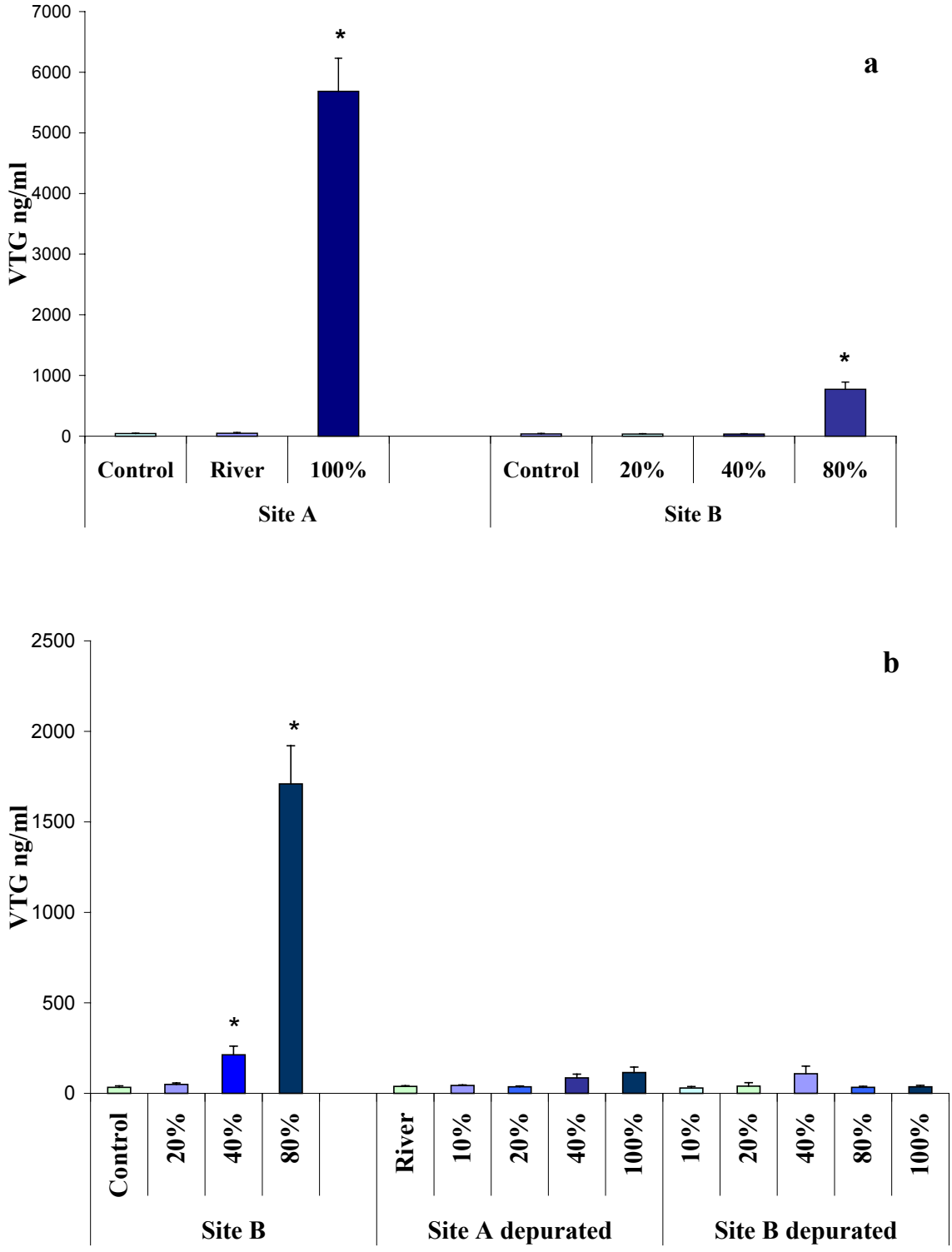


Figure 8. Whole body concentrations of vitellogenin in roach **(a)** exposed from fertilization to 200dph to both effluents and **(b)** 300dph roach, exposed continuously from fertilization to 300dph to effluent from Site B WwTW, and fish exposed from fertilization to 60dph (both sites) and then transferred to clean water to depurate to 300dph. * indicates significance from control $p<0.05$

5.2.7 Gonadal Histopathology

Histological processing and analysis was carried out on all samples collected at 200 and 300 dph.

200dph Roach

At 200dph not all fish had completed sexual differentiation. Gonadal phenotypes of fish at 200 dph included males and females, and fish that had not completed sexual differentiation. The frequency of each of these phenotypes in each treatment group is shown in Figure 9a. The primordial gonad of an undifferentiated fish contained several primordial germ cells (PGCs) surrounded by stromal cells. The gonad was attached to the body wall by a single point of attachment, the mesogonium (Plates 3 and 4). Definitive female fish (Plates 5) contained ovaries with the gonad attached to the mesentery by two points of attachment forming the ovarian cavity (female reproductive duct). Two types of germ cells were observed in phenotypic female fish. Some individuals had ovaries that contained only oogonia (Plate 6). Females at a more advanced stage of development had larger ovaries with both oogonia and primary oocytes in the perinucleolar stage (Plates 7 and 8). The lamellar structure of the ovary could often be discerned (Plate 7). There were no obvious differences in the development of ovaries in females in the controls compared with the effluent exposed females. In the control presumptive males (Plate 9) the testes had a single point of attachment to the mesentery, forming the sperm duct (Plates 10 and 11). Germ cells (presumptive spermatogonia A) were developing in clusters or cyst-like structures. This was more apparent in more advanced fish (Plate 11). Some male fish that had been exposed to WwTW effluent at both study sites had feminized reproductive ducts. These testes contained male germ cells (spermatogonia A, and both spermatogonia A and B in more advanced fish) but were connected to the body wall by two distinct points of attachment forming a ‘female-like’ duct or ovarian cavity (Plates 12, 13 and 14). There was no evidence of germ cell disruption in any of the fish examined in any of the treatments.

300dph Roach

Gonad histopathology of roach at 300dph revealed that not all fish had completed sexual differentiation. The frequency of each gonad phenotype in each treatment group is shown in Figure 9b. Undifferentiated fish were histologically very similar to undifferentiated fish examined at 200dph (Plate 15). Ovaries of phenotypic female fish contained primary oocytes (Plate 16). There were no discernable differences in the status of the ovaries in control fish compared with effluent exposed females.

The testes in phenotypic males in the controls contained spermatogonia A, or both spermatogonia A and B, as in 200dph roach. Some males were more developed and the testes were comprised of well-defined cysts of spermatogonia A and B (Plate 17). Several individuals had very advanced testes containing spermatogonia A and B, spermatocytes and spermatids within the cyst structures (Plates 18 and 19). As observed in the 200dph

effluent exposed fish, some males that had been exposed to effluent at Site B had retained a feminized reproductive duct (Plate 20). Feminized ducts occurred even in the more advanced testes that contained spermatocytes and spermatids (Plate 21). Depurated male fish that had been exposed previously to effluent at both study sites from fertilization to 60dph then held in clean water to 300dph retained the female duct, indicating that this effect may be permanent. There was no evidence of germ cell disruption in any of the fish examined.

Incidence of duct feminisation

The frequency of ovarian cavity or 'female-like' reproductive ducts in sexually differentiated fish (male and female) is shown in Figure 11. At 200dph all sexually differentiated fish in the 100% effluent exposure tank at Site A and the 80% effluent exposure tank at Site B had an ovarian cavity. This was a significant deviation from the controls ($p<0.001$) where the incidence of ovarian cavities was 45% and 64% at Site A and Site B, respectively – Fig. 11a). Thus in the higher effluent concentrations at both sites (80% and 100%) all fish with male germ cells had feminized reproductive ducts (Fig. 12a), This phenomenon also occurred in all the fish held in 100% effluent at Site B at 300 dph (all sexually differentiated individuals had ovarian cavities). The incidence of ovarian cavities in the control fish sampled at 300dph was 31%. At 300dph there was also a higher incidence of ovarian cavities in the 40% effluent exposure group at Site B (55%) compared with the control ($p<0.05$).

There was a concentration-related response in the number of fish with male germ cells and ovarian cavities, 0% in the control group and 5%, 25% and 100% incidence in the 20%, 40% and 100% effluent groups respectively. However, the only treatment group that differed statistically from the control was the 100% effluent exposure group ($p<0.001$).

In the depuration treatment groups at 300dph, even after 240 days in clean water, all fish initially exposed to 40% and 100% effluent at Site A had feminized reproductive ducts. Ninety seven percent and all fish exposed to the 80% effluent and full strength effluent at Site B, respectively, possessed 'female-like' reproductive ducts, regardless of germ cell sex (Fig. 12, significantly different from control $p<0.001$). When fish with male germ cells are considered there was a concentration-related response (Fig. 3.9b). None of the males derived from control tap water at Site B contained an ovarian cavity. There was a very low incidence of feminized ducts in males derived from the depurated river water and 20% effluent groups but they did not differ significantly from the control. No males with ovarian cavities were observed in the control tap water group or depurated 10% effluent exposure treatment. The incidence of feminized ducts in the 20%, 40%, 80% and 100% exposure groups were 9%, 33%, 88% and 100% respectively.

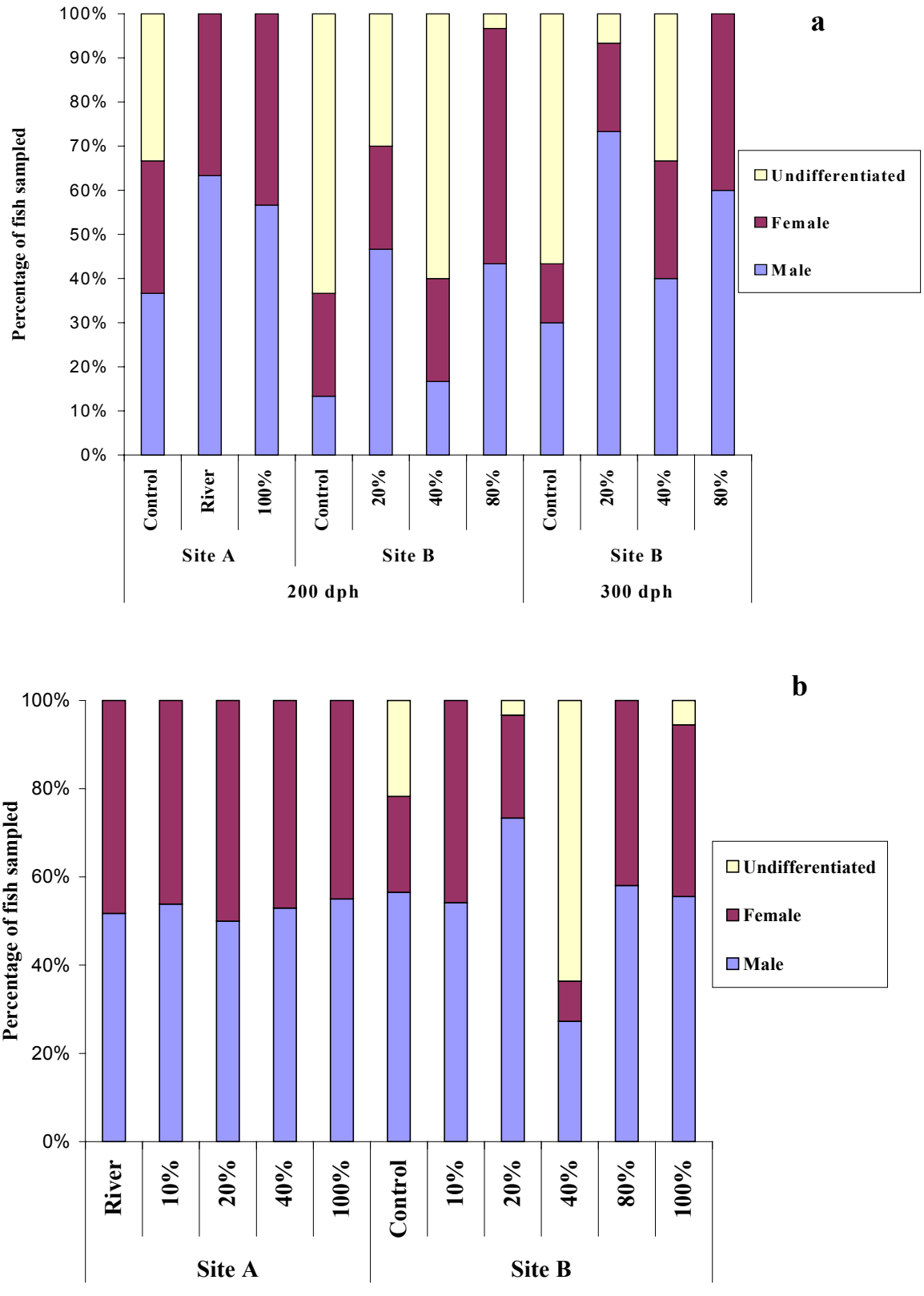


Figure 9. Percentage of phenotypic male, female and sexually undifferentiated fish at each sampling point. **(a)** Fish exposed continually from fertilization to 200dph (sites A and B) and 300dph (site B) **(b)** Fish exposed from fertilization to 60dph and depurated to 300dph.

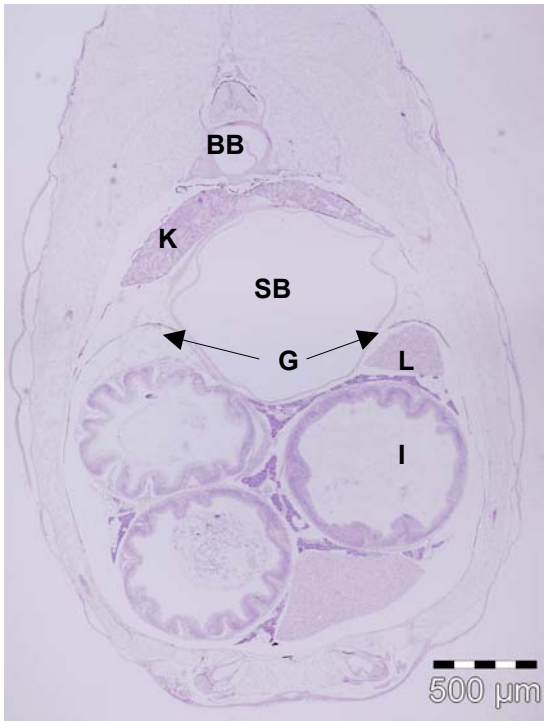


Plate. 3 Photomicrograph of a transverse section through the mid-portion of a 200dph roach that has not completed sexual differentiation. The plate shows the backbone (BB), kidney (K), swim bladder (SB), liver (L), intestine (I), and position of the undifferentiated gonads (G).

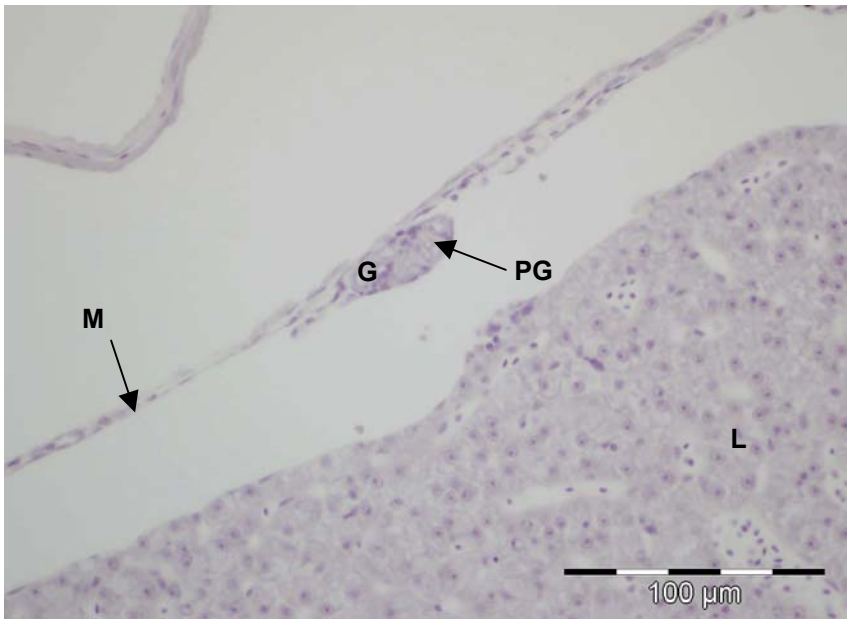


Plate. 4. Photomicrograph of an undifferentiated gonad (G) of a 200dph roach. The undifferentiated gonad contains several primordial germ cells (PGC) surrounded by stromal cells. The plate shows the liver (L) and mesentery (M).

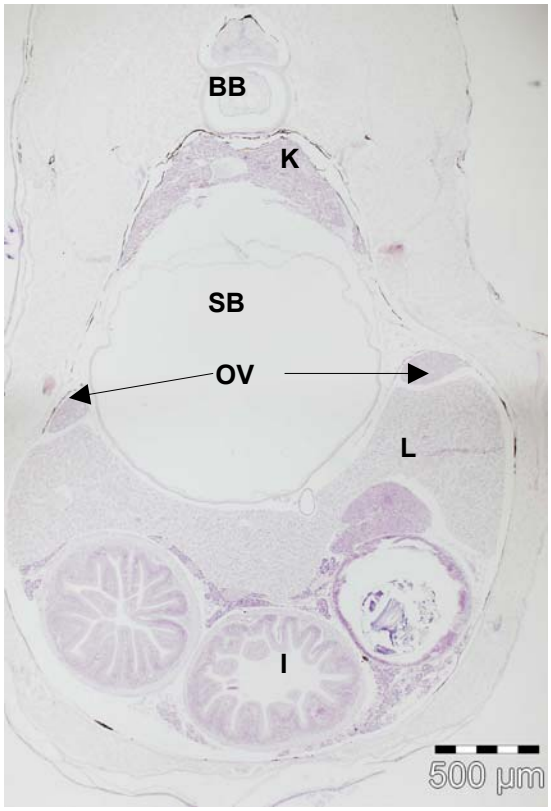


Plate. 5. Photomicrograph of a transverse section through the mid-portion of a 200dph female roach reared in tap water. The plate shows the backbone (BB), kidney (K), swim bladder (SB), liver (L), intestine (I), and ovaries (OV).

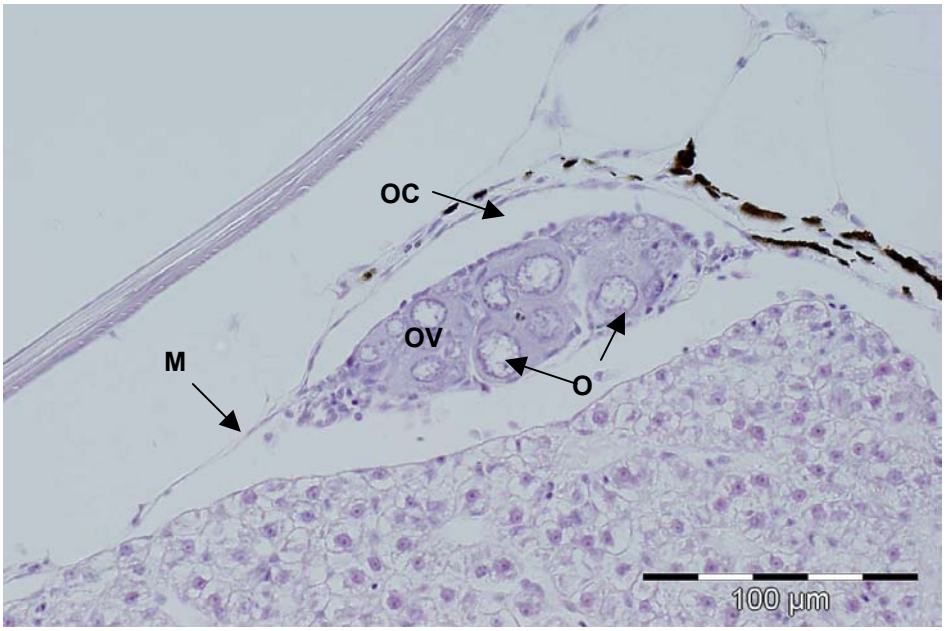


Plate. 6. Photomicrograph of an ovary (OV) of a 200dph roach reared in tap water. The ovary contains oogonia (O) but no primary oocytes. The ovary is connected to the mesentery (M) by two points of attachment forming the ovarian cavity (OC).

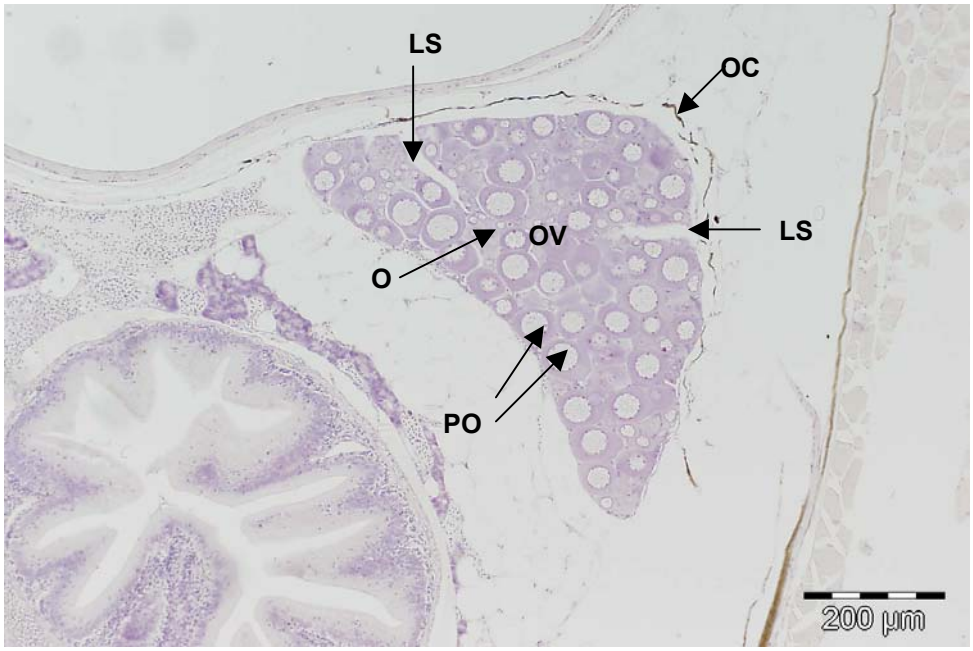


Plate. 7. Photomicrograph of an ovary (OV) of a 200dph roach reared in tap water. The ovary contains oogonia (O) and primary oocytes (PO). The plate also shows the ovarian cavity (OC) and lamellae structure (LS).

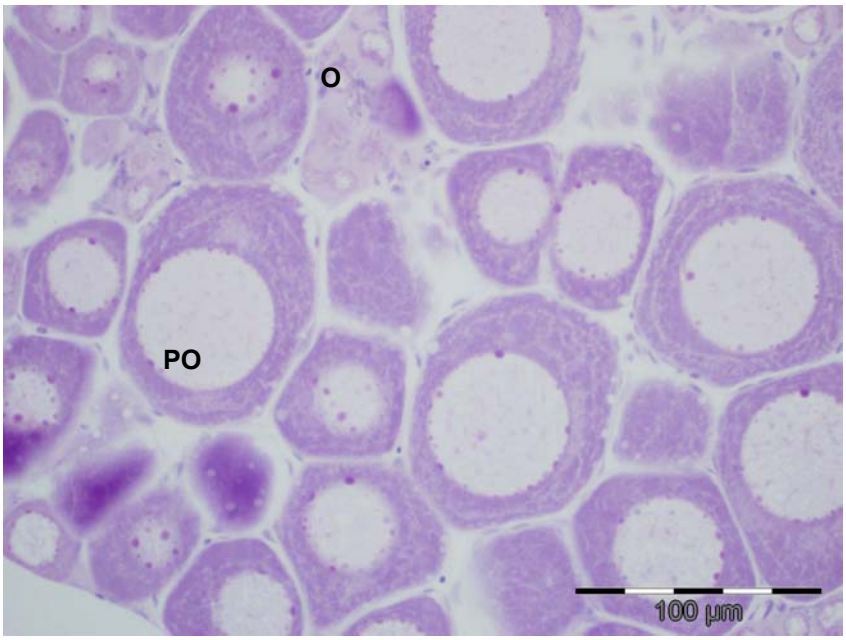


Plate. 8. Photomicrograph of an ovary of a 300dph roach under high power. The ovary contains primary oocytes (PO) at the perinucleolar stage and oogonia (O) in the interstices between the oocytes.

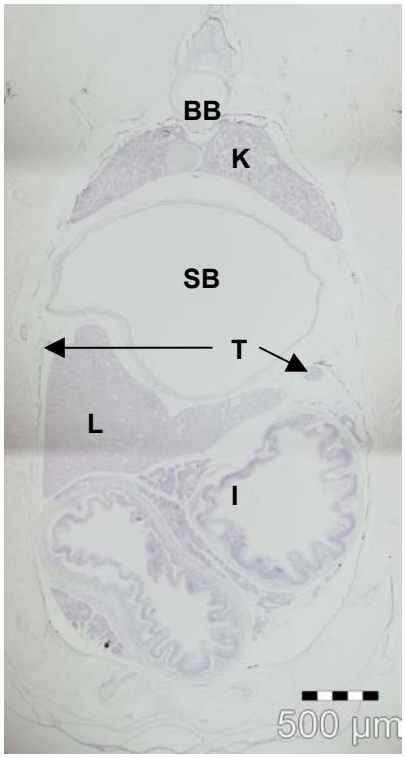


Plate. 9 Photomicrograph of a transverse section through the mid-portion of a 200dph male roach reared in tap water. The plate shows the backbone (BB), kidney (K), swim bladder (SB), liver (L), intestine (I) and testes (T).

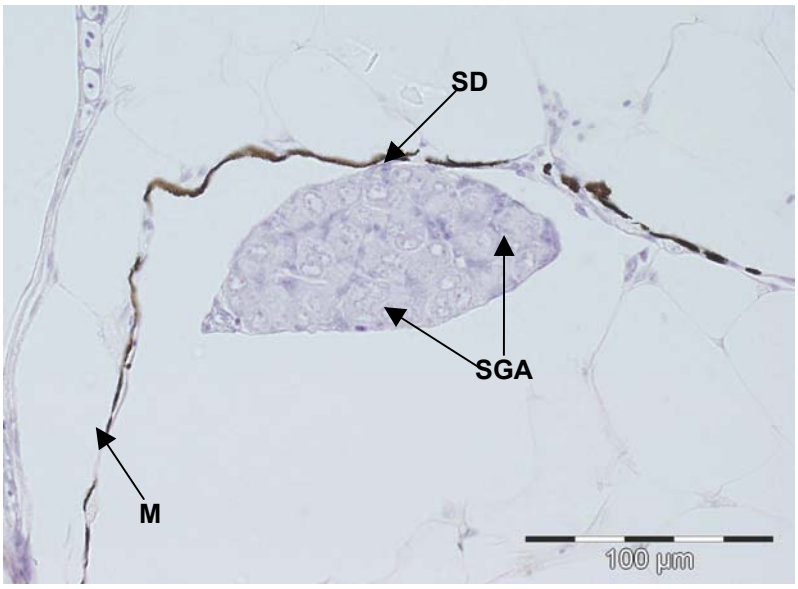


Plate. 10. Photomicrograph of a testis of a 200dph roach reared in tap water. The testis contains spermatogonia A (SGA) and is connected to the mesentery (M) by a single point of attachment; the sperm duct (SD).

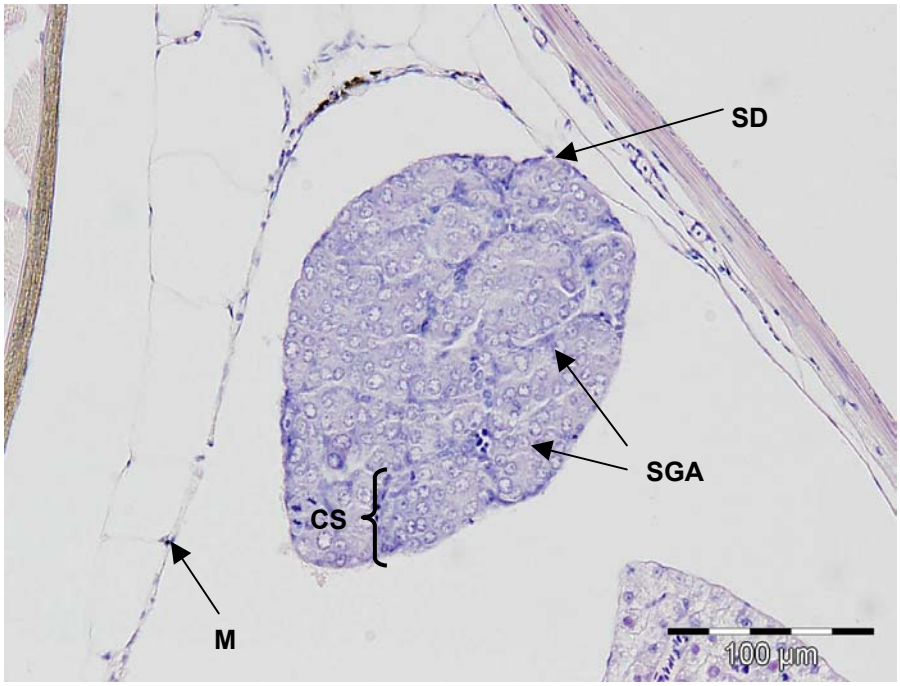


Plate. 11. Photomicrograph of a testis of a more developed 200dph roach reared in tap water. The testis contains spermatogonia A (SGA) and is connected to the mesentery (M) by a single point of attachment; the sperm duct (SD). Germ cells are developing in cyst structures (CS).

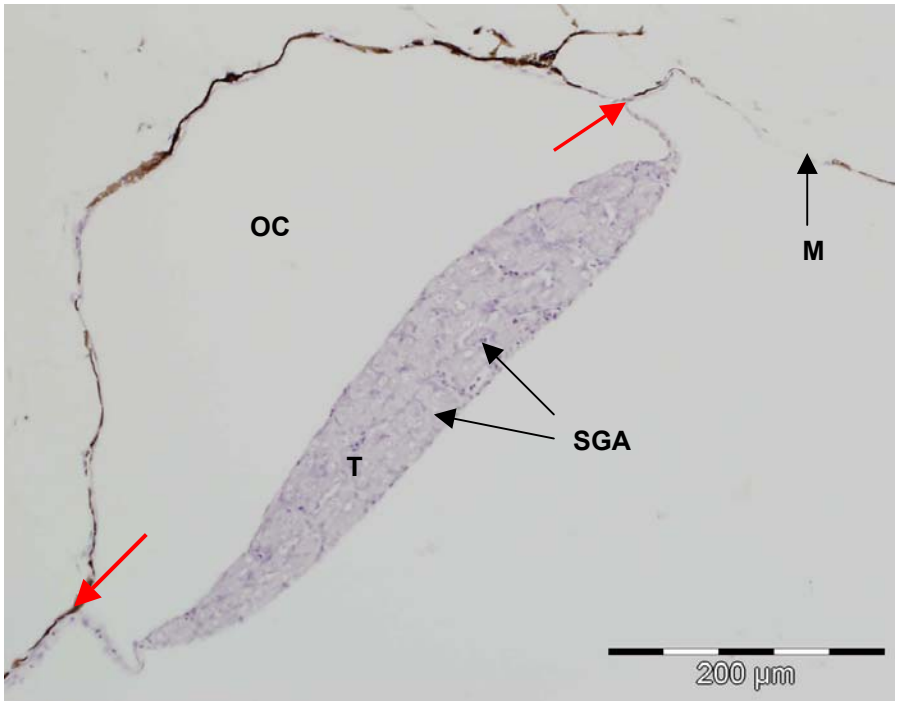


Plate. 12. Photomicrograph of a testis (T) of a 200dph roach reared 100% effluent at site B. The testis contains spermatogonia A (SGA) and is connected to the mesentery (M) by two points of attachment (marked by red arrows), forming a 'female-like' ovarian cavity (OC).

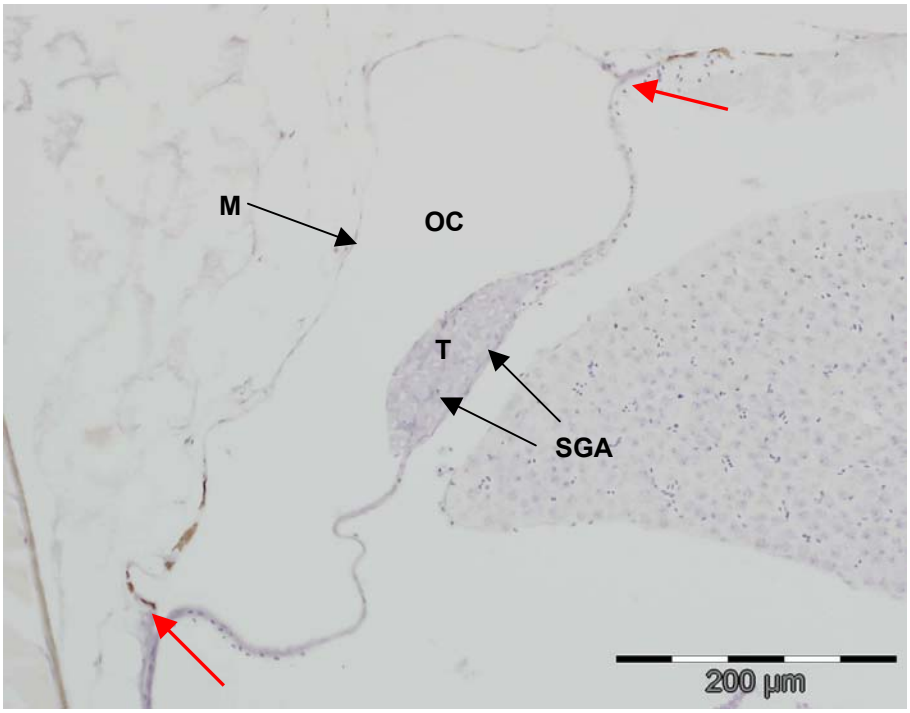


Plate. 13. Photomicrograph of a testis (T) of a 200dph roach reared 100% effluent at site A. The testis contains spermatogonia A (SGA) and is connected to the mesentery (M) by two points of attachment (marked by red arrows), forming a 'female-like' ovarian cavity (OC).

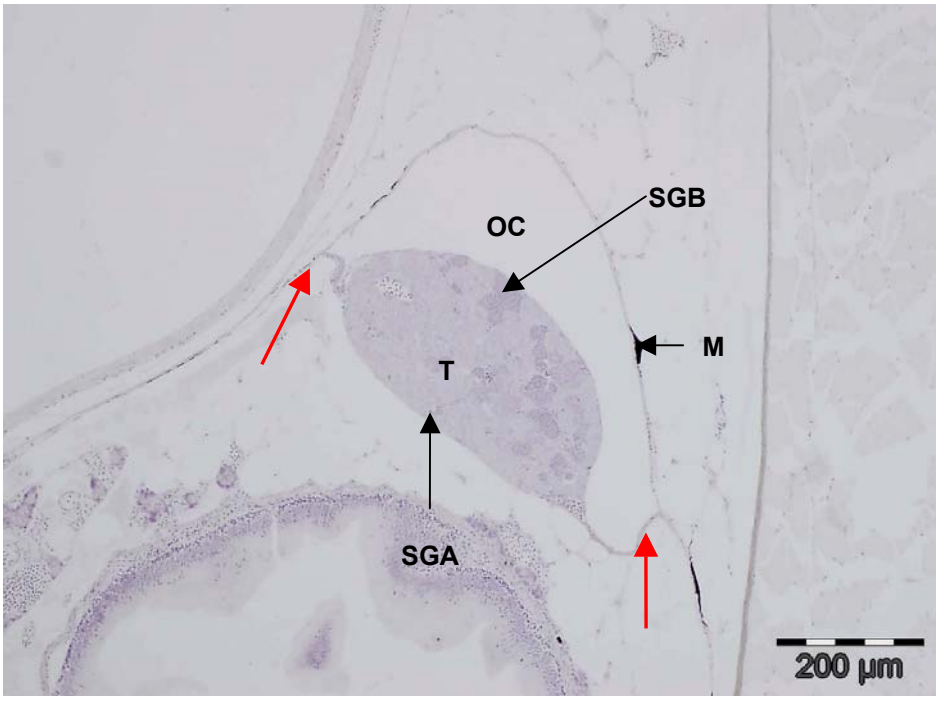


Plate. 14. Photomicrograph of a testis (T) in a more developed 200dph roach reared in 100% effluent at Site A. The testis contains spermatogonia A (SGA) and spermatogonia B (SGB) developing in cysts and is connected to the mesentery (M) by two points of attachment (marked by red arrows), forming a 'female-like' ovarian cavity (OC).

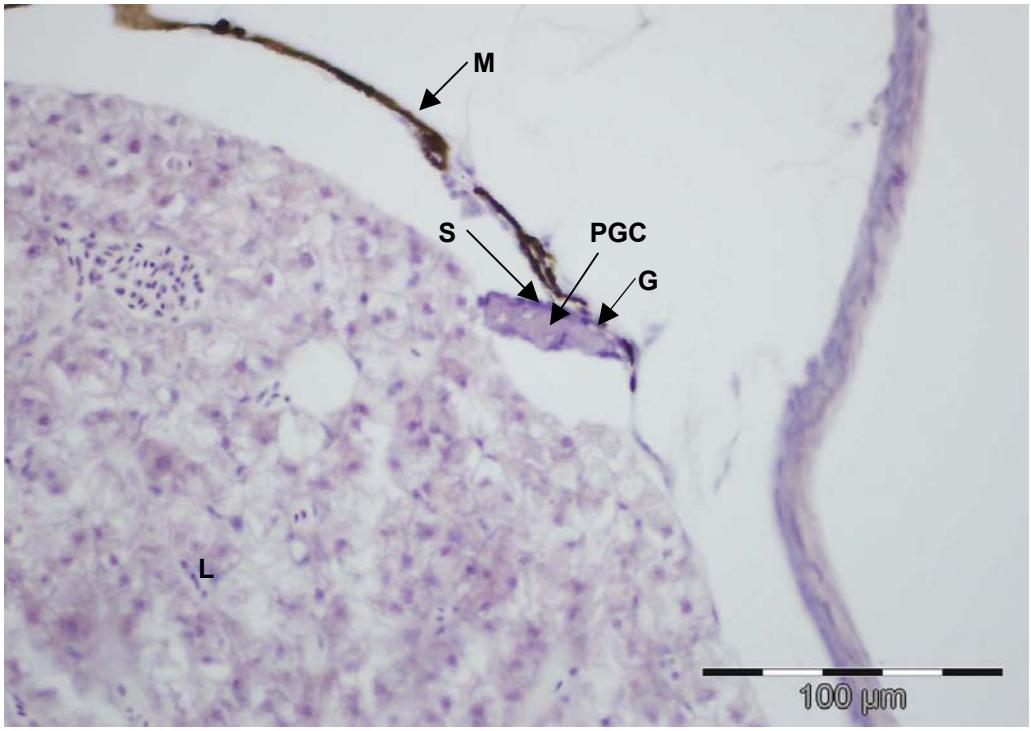


Plate. 15. Photomicrograph of an undifferentiated gonad (G) in a 300dph roach. The undifferentiated gonad contains several primordial germ cells (PGC) surrounded by stromal cells (S). Liver (L) mesentery (M).

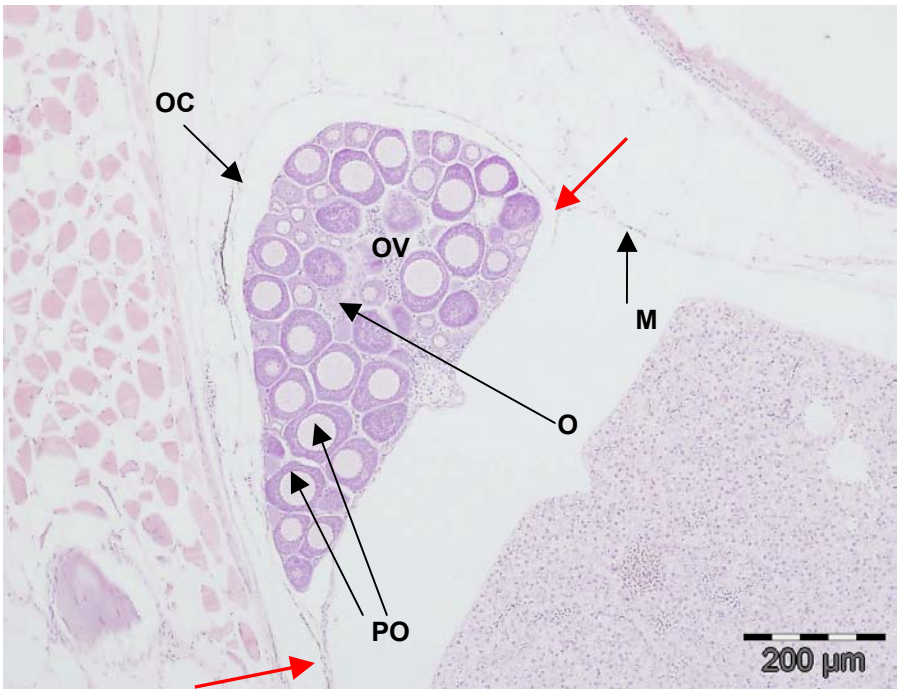


Plate. 16. Photomicrograph of an ovary (OV) of a 300dph roach reared in tap water. The ovary contains oogonia (O) and primary oocytes (PO). The plate also shows the two points of attachment to the mesentery (M) (marked by red arrows) forming the ovarian cavity (OC).

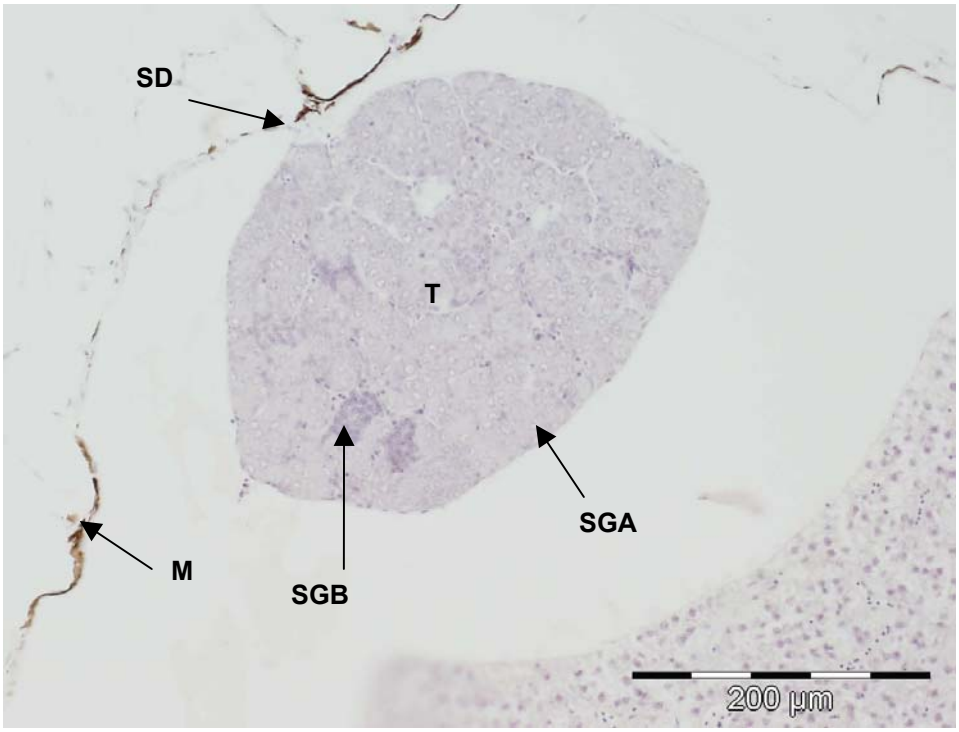


Plate 17. Photomicrograph of a testis in a 300dph roach reared in tap water. The testis contains spermatogonia A (SGA) and spermatogonia B (SGB) developing in cysts and is connected to the mesentery (M) by one point of attachment forming the sperm duct (SD).

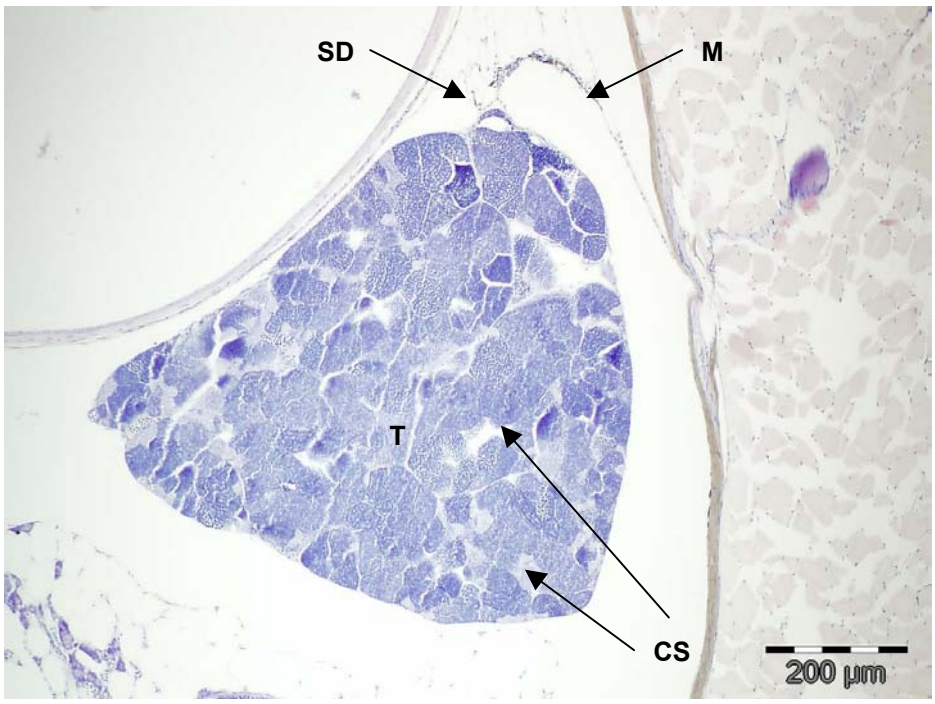


Plate 18. Photomicrograph of a testis in a more developed roach at 300dph reared in tap water. The testis contains germ cells developing in cyst structures (CS) and is connected to the mesentery (M) by one point of attachment forming the sperm duct (SD).

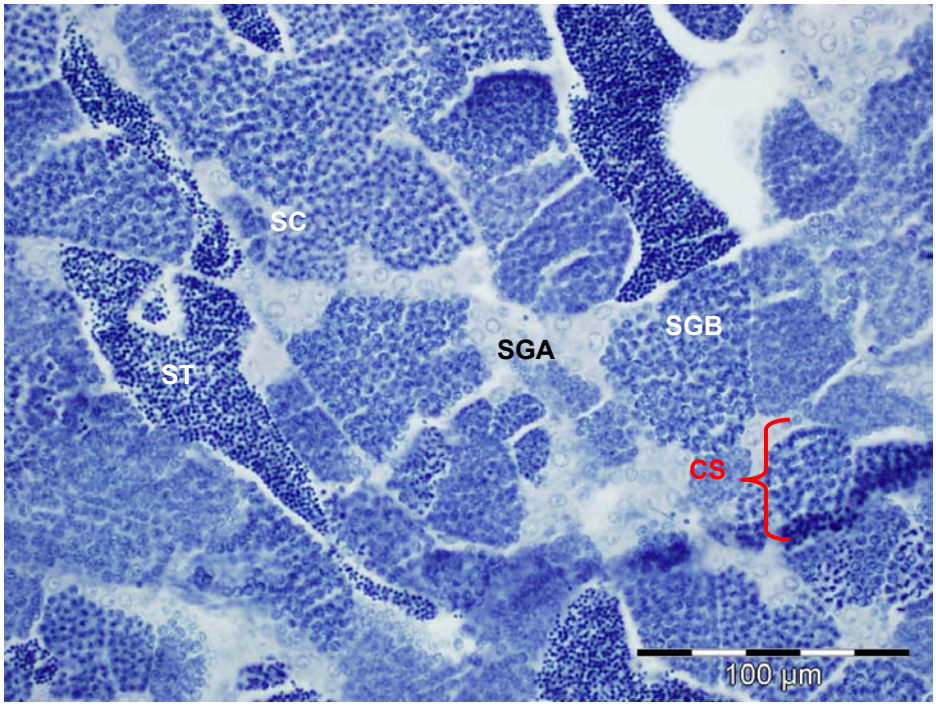


Plate. 19. Photomicrograph of a testis in a roach at 300dph, viewed under high power. Spermatogonia A (SGA), spermatogonia B (SGB), spermatocytes (SC) and spermatids (ST) are all present and developing synchronously in cyst structures (CS).

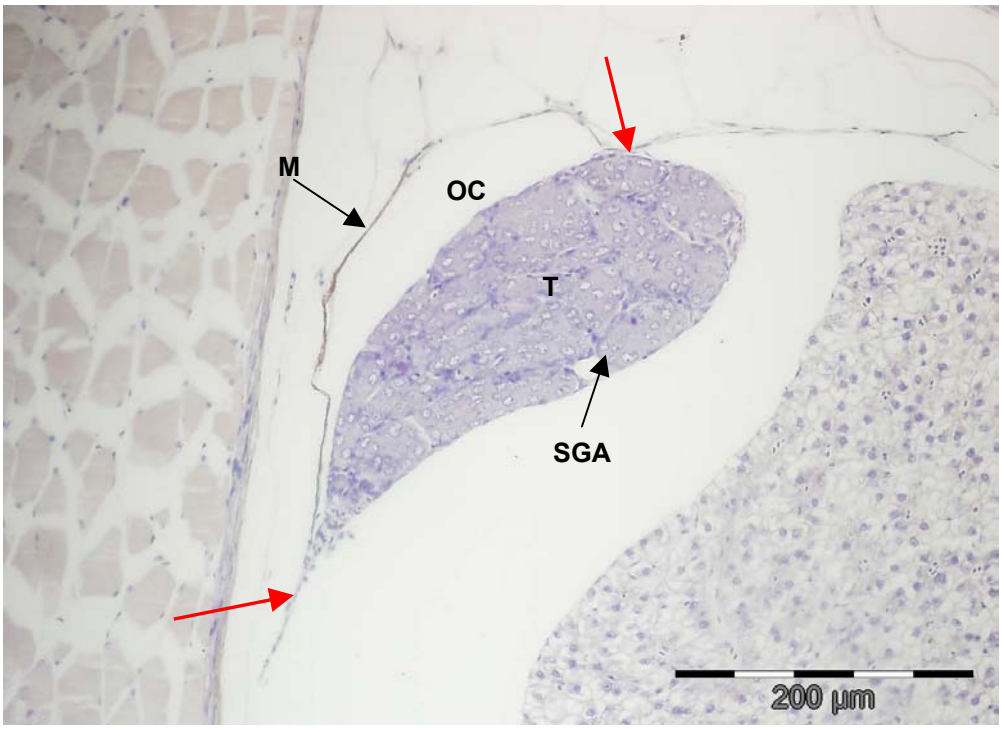


Plate. 20. Photomicrograph of a testis (T) of a 300dph roach reared in 100% effluent at Site B. The testis contains spermatogonia A (SGA) and is connected to the mesentery (M) by two points of attachment (marked by red arrows), forming a 'female-like' ovarian cavity (OC).

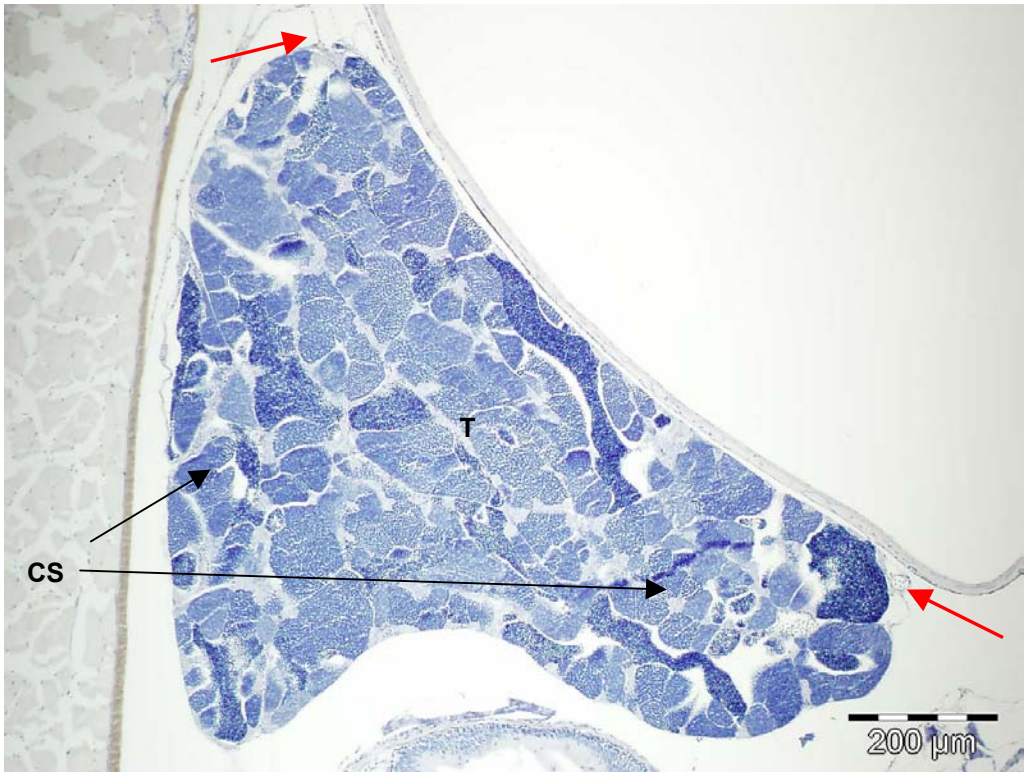


Plate 21. Photomicrograph of a testis in more developed 300dph roach reared in 100% effluent at Site B. The testis contains germ cells developing in cyst structures (CS) and is connected to the mesentery by two points of attachment (marked by red arrows) forming a 'female-like' ovarian cavity.

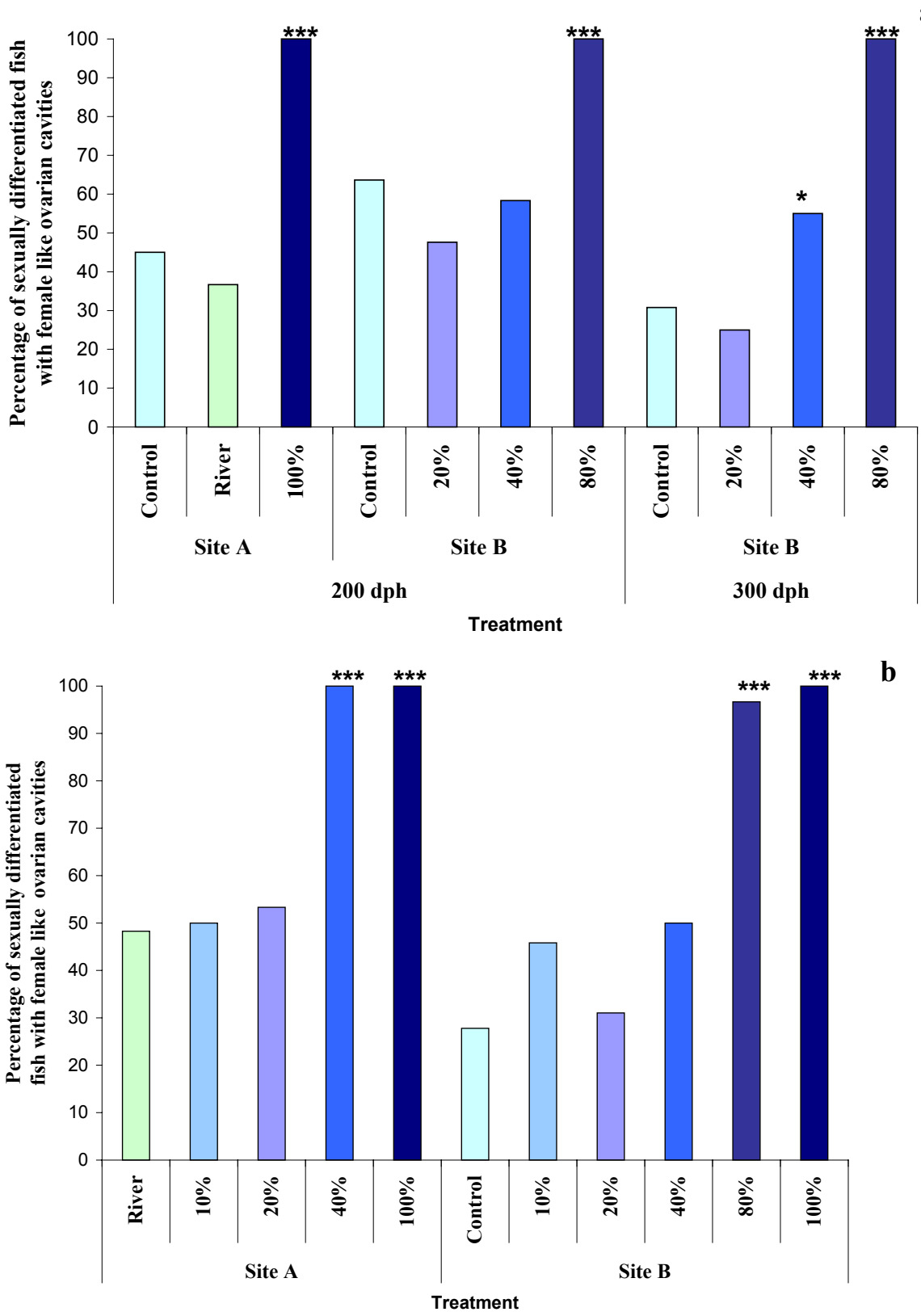


Figure 11. Percentage of sexually differentiated roach (phenotypic male and female) that had female-like reproductive ducts (ovarian cavities) at each sampling point. **(a)** Fish exposed continually from fertilization to 200dph (Site A and B) and 300dph (Site B) **(b)** Fish exposed from fertilization to 60dph and depurated to 300dph. Asterisks denote significance from control within a sampling point; * $p<0.05$, ***$p<0.001$

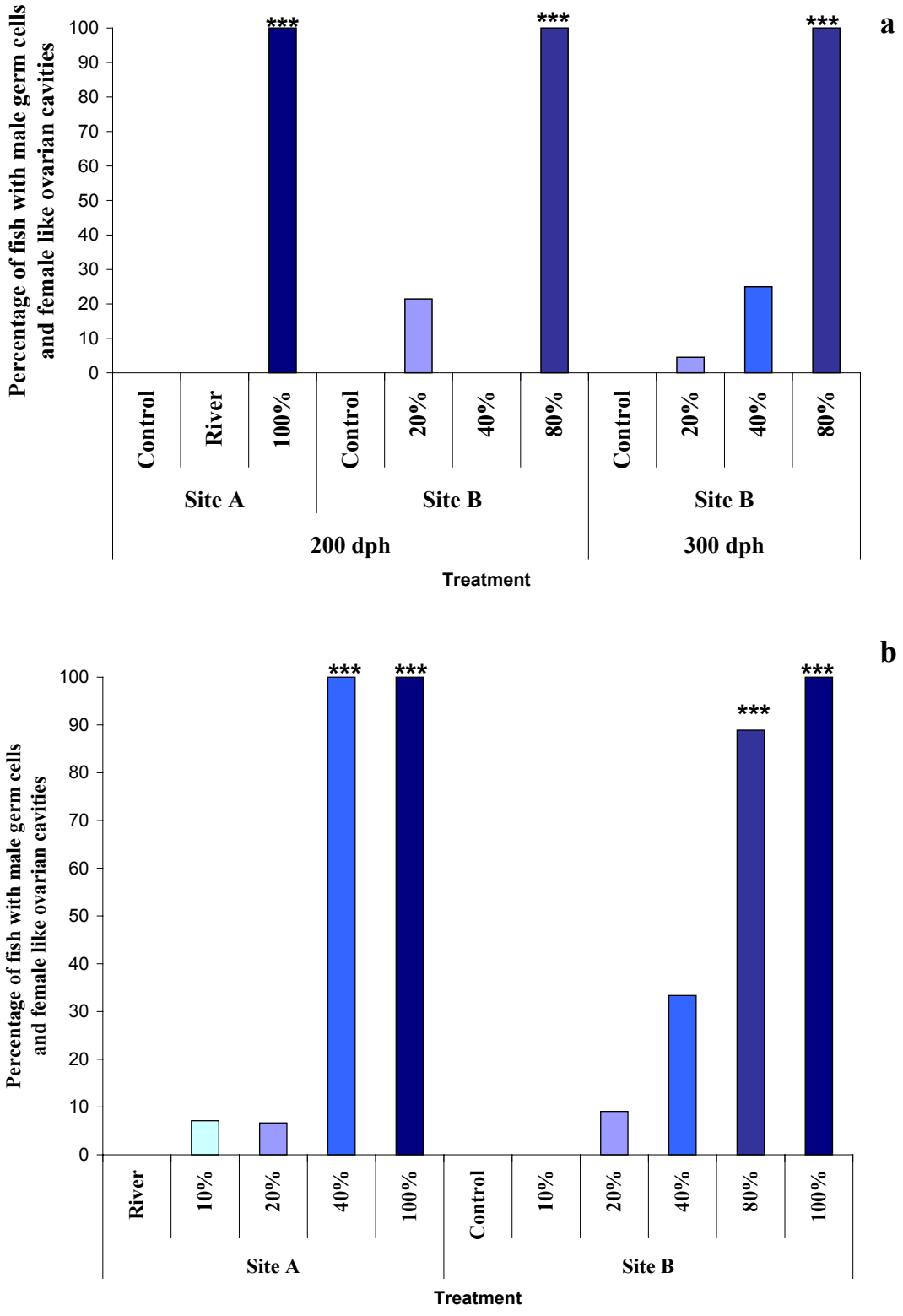


Figure 12. Percentage of phenotypic male fish (with male germ cells) with 'female-like' reproductive ducts (ovarian cavities) **(a)** Fish exposed continually from fertilization to 200dph (Site A and Site B) and 300dph (Site B) **(b)** Fish exposed from fertilization to 60dph and depurated to 300dph. Asterisks denote significance from control within a sampling point; ***p<0.001.

5.3 Discussion

Concentrations of known oestrogenic chemicals differed between the two sites. Site A WwTW effluent contained higher concentrations of steroid oestrogens ($p<0.05$) compared with Site B WwTW effluent. In contrast, the effluent derived from Site B contained higher concentrations of alkylphenolic compounds compared with Site A. This perhaps reflects the different influents to the works and different treatment processes employed (See: Exposure Effluents). Both effluents were oestrogenic to fish inducing a vitellogenic response, however at 200dph the response to full strength (100%) Site A effluent was an order of magnitude higher than the response to 80% effluent at Site B, mirroring the higher concentrations of steroid oestrogens at Site A (steroid oestrogens, such as oestradiol are around 1000-fold more potent at inducing a vitellogenic response in fish than nonylphenol – the more potent of the alkylphenolics (Metcalfe et al. 2001). The magnitude of the vitellogenic response in roach was similar to that established in previous studies in this species for this effluent (Rodgers-Gray et al. 2001). Further exposure of roach to 300dph in the Site B 80% effluent resulted in a 2-fold increase in the VTG titre. This increase may be the result of higher oestrogenic chemical concentrations in the period 200-300dph, as it is now well established that the oestrogenic activity of effluents can vary on a seasonal basis. However, no analytical chemistry was carried out at this time to be able to confirm this. Alternatively, the higher concentration of VTG in fish at Site B after 300 days of exposure (versus 200 days), may simply be as a consequence of some of the oestrogenic chemicals building up in the bodies of the fish (bio-concentration), which has been well established for both steroid oestrogens and alkylphenolic chemicals (Larsson et al. 1999). The transient nature of the VTG induction was demonstrated in the fish undergoing depuration, where there was a concomitant clearance of VTG from the circulation. It should be realised however, that some fish in UK rivers live in environments that are constantly supplied with mixtures of oestrogenic chemicals sufficient to induce VTG. In these cases there is more likely to be a health consequence for such an exposure.

There were differences in growth of fish between each of the treatments. The growth rate of fish is known to be influenced by a number of factors, including the diet (feeding ration), the population density and the water temperature (although there were no significant differences in temperature between the treatments in this study). It was not possible to estimate the numbers of fish in each of the tanks accurately throughout the early part of the trial, and hence it was difficult to provision a single ration level relative to fish biomass throughout. This problem was further exacerbated by the fact that variable amounts of natural food were obviously also present in each of the tanks. Growth rate may also be affected by exposure to xeno-oestrogens present in the effluents. Laboratory exposure of rainbow trout to environmentally relevant concentrations of alkylphenolic chemicals (including the alkylphenolic chemicals identified in the exposure effluents in this study) has been shown to increase somatic growth (Ashfield et al. 1998). This difference in growth may be important when considering timing of sexual differentiation and sex ratios (Devlin and Nagahama 2002). Not all fish had completed sexual differentiation

in this study even at 300dph and therefore it is not meaningful to investigate if there were significant differences in sex ratio between the treatments. The length of the exposure was initially based on previous study that indicated all roach complete sexual differentiation by 350dph (Rodgers-Gray 2001). The roach in that study, however, were depurated from 200 to 350dph (December to March) in an indoor constant temperature aquarium, and therefore the fish grew more quickly. In turn, there is the possibility of a faster rate of sexual development in those fish, compared with the fish that were kept at ambient and naturally fluctuating temperatures in this study.

Exposure of juvenile roach to high concentrations of treated WwTW effluent from fertilisation to 200dph and 300dph resulted in the feminisation of the male reproductive duct. At 200dph all males in the higher effluent concentrations at both sites were found to have feminised ducts. At 200dph at Site B this effect was not found to be concentration-related, but a concentration-related response was observed at 300dph at this site. This is probably due to the fact that more fish had reached sexual differentiation at 300dph. Fish that were exposed to effluent from both sites and then transferred to clean water at 60dph to depurate also exhibited this duct disruption, indicating that the effect on the duct was permanent. Exposure to a 40% concentration of effluent at Site A resulted in all fish with phenotypic male germ cells containing feminised ducts. Site B depurated fish also demonstrated a concentration-response for ovarian cavity induction, however this was only significant from the control in the 80% and 100% effluent groups. A third of the males in the Site B 40% effluent exposed group had feminised ducts, but this may not have been significant due to the relatively low number of fish that had completed sexual differentiation in this group at 300dph. These data, together with previous studies on the roach (Rodgers-Gray et al. 2001), indicate that the window for disruption of duct development includes the period between fertilisation and 60dph.

The causative agent(s) of duct disruption is (are) unknown. Feminisation of the reproductive duct has been induced in male fish during laboratory exposure to oestrogenic chemicals commonly found in WwTW effluents, including oestradiol (Gimeno et al. 1996), ethinylestradiol (van Aerle et al. 2002) and an alkylphenolic compound (Gimeno et al. 1997; Gimeno et al. 1998). The concentrations of these oestrogenic chemicals required to do this, however, were higher than in the effluents analysed in this study. Furthermore, the fact that duct disruption occurred at Site B shows that relatively low concentrations of steroid oestrogens and/or alkylphenolic chemicals alone or in combination can induce duct disruption in roach.

The functional significance of duct feminisation in males has yet to be established fully. It has been shown that grossly feminised roach with severely disrupted ducts have a decreased fertility (Jobling et al. 2002). In extreme cases in intersex fish, the duct is blocked and thus they are unable to release any gametes. However, it is not known if the fertility of fish would be impacted where the gonad is less severely affected with a feminised duct.

There was no evidence of the presence of both male and female sex cells present in the same gonad in any of the fish examined, showing that the two

test effluents did not induce germ cell disruption in roach for the life period of exposure studied. It is possible that the WwTW effluents studied were not sufficiently oestrogenic to induce intersex in roach (this would contrast with the evidence from field studies on roach). It may also be possible that germ cell disruption is a consequence of a longer term exposure to treated sewage effluent, or arises as a consequence of exposure at a different life period (e.g. post-spawning- see later). In the final analysis, however, based on the data presented, effluents from the WwTW studied did not cause sex cell disruption for exposures during early life.

6 Effects of exposure to WwTW effluent on sex cell development in adult post-spawning roach (Objectives 3 and 4)

6.1 Overall Experimental design

Two experiments were carried out to investigate the effects of exposure to WwTW effluent on germ cell development during the post-spawning period in adult male roach. The original intention was to conduct only a single experiment, however, subsequent to completing the first trial in 2002 it was discovered that the roach used for the study had contained VTG in their plasma and the testes of some males contained oocytes (they were in fact intersex) prior to the effluent exposure study, indicating they had been exposed previously to oestrogen(s). The site from which these roach were derived was believed originally to be oestrogen-free, but there had been no analytical chemistry conducted to confirm this. It was therefore, necessary to undertake a further trial to investigate if the post-spawning period of germ cell proliferation was a sensitive window for the induction of intersex, and for this trial it was possible to obtain roach reared throughout their lives on borehole water (to ensure no previous exposure to oestrogen(s). Male roach used in the first study were of a mixed age classes. The roach selected for the second trial were 3+ years in age and in their first spawning season. The need to repeat the post-spawning fish exposure study put a considerable additional workload on to the project, but was vital to address the fundamental questions set out in this part of the proposal. Furthermore, the fact that the roach for the first trial had been exposed to a low-level of oestrogen contamination prior to the study meant that we were able to investigate the effects of a pre-exposure to oestrogenic chemicals on the subsequent impact of exposure to WwTW effluent during the period of germ cell proliferation in adult fish.

Experiments were set up to investigate the effects of treated sewage effluent on adult male roach exposed in the period of germ cell proliferation that occurs after (annual) spawning. See Figure 13 for a diagram of the experimental design.

6.1.1 Effects of Exposure to WwTW Effluent on Adult Post-Spawning Roach – Site A (two month exposure)

This experiment describes the effects of exposure to the Site A WwTW effluent on male roach in the post-spawning period. The fish employed in this study had been maintained on borehole water and this not subject to previous

exposure to exogenous oestrogen. For the trial, 4 mesocosm tanks at Site A were supplied with a series of graded concentrations of effluent and river water (see Fig 1 for details of the mesocosm system). Nominal effluent concentrations were 100%, 50%, 25% and control river water (tap water used as an additional control in the early life stage experiment was not available at this time). The flow rate through each of the tanks totalled 6 L/min and was monitored daily. The tanks were aerated to ensure sufficient oxygen supply. Roach were exposed for a 2 month period from July to September 2003*. 112 sexually mature and spermiating 3+ male roach were supplied by Calverton Fish Farm, Environment Agency, Calverton, UK. On day 0, 12 fish were sampled for biological analyses (pre-exposure sample) and the remaining fish were deployed into the mesocosm tanks (25 fish per treatment). Fish were fed commercial cyprinid pellets daily. After 2 months exposure, the experiment was terminated and the fish sampled for biological analysis.

** This trial was originally set up in May 2002, immediately after the normal spawning date of roach, but almost all of the fish died shortly after their deployment into the mesocosm tanks. We were not able to ascertain the reason for the demise of the fish, but were able to re-start this study with a further population of roach derived from the same source in early July 2002, as described above and this trial proceeded without significant fish mortalities. During the trial in May 2002 at WwTW A, we further exposed a small population of 1+ roach that had been previously exposed to that effluent (from fertilization to 60dph) to investigate the possible effects of re-exposure to this WwTW effluent on sexual development. All of these fish also died shortly after their deployment and we were unable to repeat this work.*

6.1.2 Effects of Exposure to WwTW Effluent in Adult Post-Spawning Roach that have previously been exposed to an exogenous source of oestrogen – Site A and Site B (four month exposure)

In this study the effects of exposure to oestrogenic WwTW effluents was examined on germ cell development during the post spawning period in male fish that had been exposed previously to environmental oestrogen(s).

Five mesocosm tanks at Site A were supplied with graded concentrations of effluent and river water (see Figure 1 for details of the mesocosm system). Nominal effluent concentrations were 100%, 50%, 25%, river water and tap water as an absolute control. At Site B four mesocosm tanks were supplied with graded concentrations of effluent and tap water (see Figure 2 for details of the mesocosm system). Nominal effluent concentrations were 100%, 50%, 25% and a control tap water. The flow rate through each of the tanks at both sites totalled 6 L/min and was monitored daily. The tanks were aerated to ensure sufficient oxygen supply. 200 sexually mature and spermiating wild male roach of mixed ages were obtained from a commercial supplier in May 2002. On day 0, 20 fish were sampled for biological analyses (pre-exposure sample) and 20 fish deployed into each mesocosm tank at both sites. Fish were fed daily with commercial cyprinid pellets. At Site A, roach were exposed

for a 4-month period from May to September 2002 and the fish sampled in July (2 month exposure) and September (4 months exposure). The experiment at Site B was sampled and terminated in July, after a 2 month exposure.

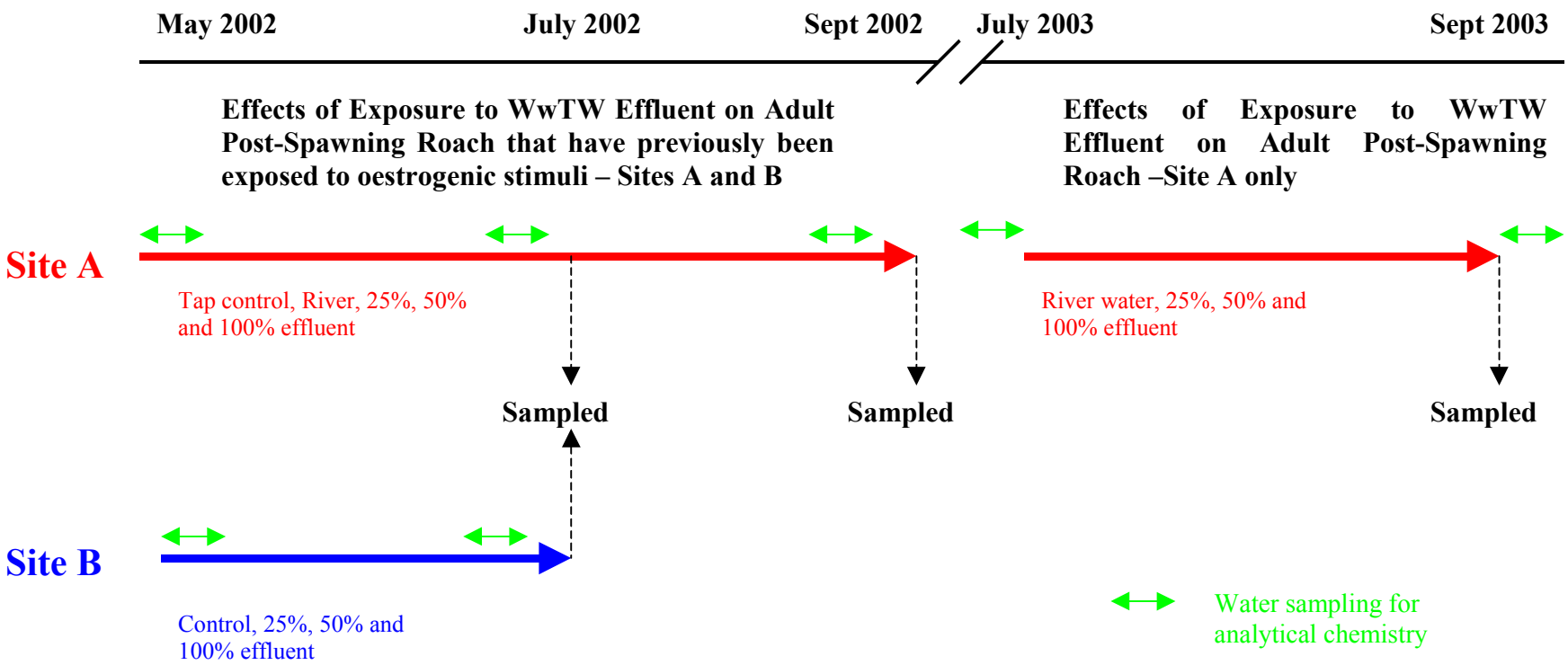


Figure 13. Exposure and Sampling Protocol for Post-spawning Adult Roach Trials – At the start of each experiment adult roach were obtained and a pre-exposure samples taken. ***2002 experiment***: Adult roach that had previously been exposed to an oestrogenic stimulus were deployed into mesocosms at both sites in May 2002. The Site B trial was terminated in July 2002 due to an outbreak of white spot disease causing mortality of fish at this site. Fish at site A were sampled at this time. The trial at Site A was terminated in September 2002. ***2003 experiment***: Adult roach raised in bore-hole water were deployed into the mesocosm at Site A in July 2003. This exposure was terminated and the fish sampled in September 2003. Seven day composite effluent samples were collected from both sites during the exposure periods for steroid and alkylphenolic chemical analysis.

6.1.3 Measurement of Oestrogenic Compounds in the Exposure Effluents

Seven day composite effluent samples were collected from both sites at the start and end of each exposure (see Figure 13 for details of timing of chemistry sampling). Composite samples were analysed for steroid oestrogens; 17β-oestradiol, oestrone and 17α-ethinylestradiol, and alkylphenolic compounds; octylphenol, nonylphenol and nonylphenol mono- and di-ethoxylates. Daily samples (2.5L) of full strength effluent were collected and refrigerated until processing. The methods used to measure these chemicals are described above.

6.1.4 Biological Sampling

At each sampling point roach were anaesthetized (MS-222 or Benzocaine, according to Home Office procedures) and blood samples collected, spun at 15000 rpm for 3 minutes and the plasma collected and stored on dry ice until storage at –20°C. After blood sampling the fish were sacrificed and their fork-length (mm) and weight (g) recorded. Several scales were collected from each roach from the area just anterior to the dorsal fin for aging of the fish. The gonads were then removed and fixed in Bouin's solution for 6 hours before transfer into 70% IMS until histological processing and analysis.

6.1.5 Condition factor

Condition factor (K) was calculated for each individual fish using the formula:

$$K = \frac{\text{weight (g)}}{\text{length (cm)}^3} \times 100$$

6.1.6 Age of roach

Roach scales were cleaned in a dilute detergent solution to remove surface debris. Scales were then mounted between two glass microscope slides and the number of annual checks (zones of closely spaced circuli) counted to determine age. Age analysis was carried out by the Environment Agency, National Fisheries Laboratory, Brampton, UK.

6.1.7 Gonadosomatic Index

Gonadal status (state of sexual maturity) was assessed using the gonadosomatic index (GSI), and was calculated for each individual fish using the formula:

$$GSI = \frac{\text{gonad weight (g)}}{\text{total body weight (g)} - \text{gonad weight (g)}} \times 100$$

6.1.8 Measurement of Vitellogenin

Quantification of plasma VTG was carried out using the carp VTG ELISA according to Tyler et al. (1999).

6.1.9 Gonadal Histopathology

Three transverse sections were cut from each fixed gonad, one each from the anterior, middle and posterior regions. The resulting sections were then dehydrated and embedded in paraffin wax and sectioned at 5 μm. Sections were stained with Haematoxylin and Eosin, mounted and examined by light microscopy. Gonad sections were examined for presence of oocytes in the testes and ovarian cavities, and any other gross abnormalities.

6.1.10 Statistical Analyses

All statistical analyses were carried out using Sigmastat v2.0 (Jandel Scientific). Statistical significance was accepted at $p<0.05$ for all comparisons. Inter-group differences were assessed using one-way ANOVA (parametric, for normalised data) or Kruskal-Wallis test (non-parametric). Multiple comparisons tests were performed using post-hoc analyses for parametric or non-parametric data.

6.2 Results - Effects of exposure to a WwTW effluent on adult post-spawning roach – Site A (two month exposure)

6.2.1 Measured Concentrations of WwTW Effluent

The actual concentrations of treated sewage effluent to which the roach were exposed were 0% (River water control), 26.5+/-0.9%, 48.7+/-1.0% and 100% (mean concentration +/- standard error of the mean; Figure 14).

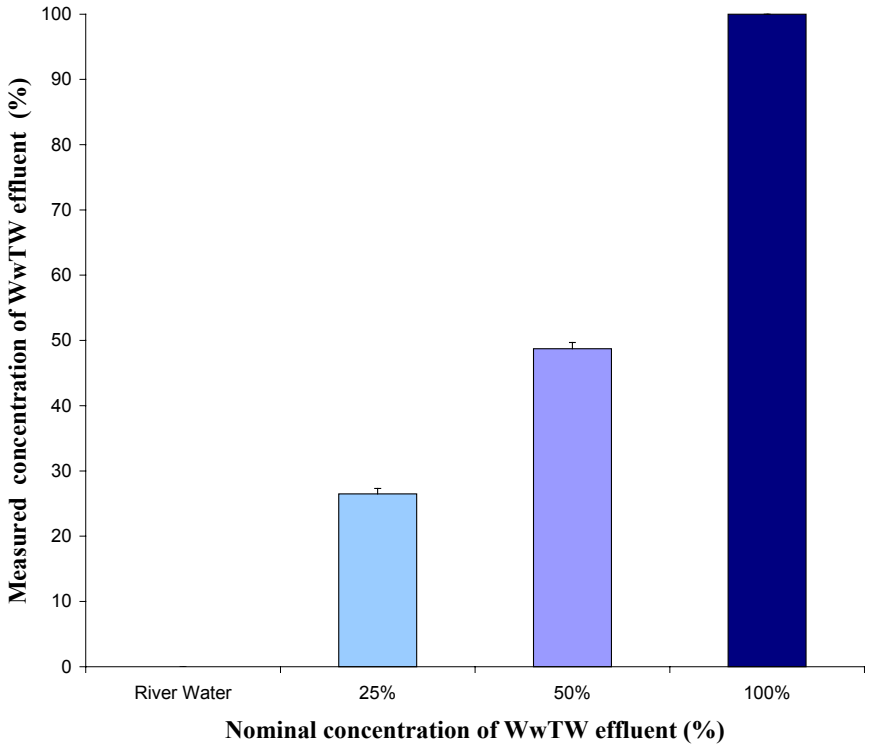


Figure 14. Measured concentrations of WwTW effluent that roach were exposed to during the study. The graph shows mean percentage concentration +/- standard error of the mean.

6.2.2 Concentrations of Oestrogenic Compounds in the Exposure Effluent

Concentrations of oestrone in the 7 day composite effluent samples ranged between 60 to 70 ng/L and between 2.7 and 10 ng/L for 17β-oestradiol. The synthetic oestrogen 17α-ethinylestradiol was not detected in the first composite sample in June (limit of detection of 0.5 ng/L), but was measured at a concentration of 1.5 ng/L in the September composite sample (Fig.15). Nonylphenol was detected at concentrations between 0.17 and 0.3 μg/L in June and between 0.92 and 1.1 μg/L in September. Concentrations of nonylphenol mono- and diethoxylates were between 4.1 and 11μg/L. Concentrations of octylphenol were between 0.01 to 0.03 μg/L. Bisphenol A was also detected at concentrations between 0.13 and 0.23 μg/L. See Figure 15.

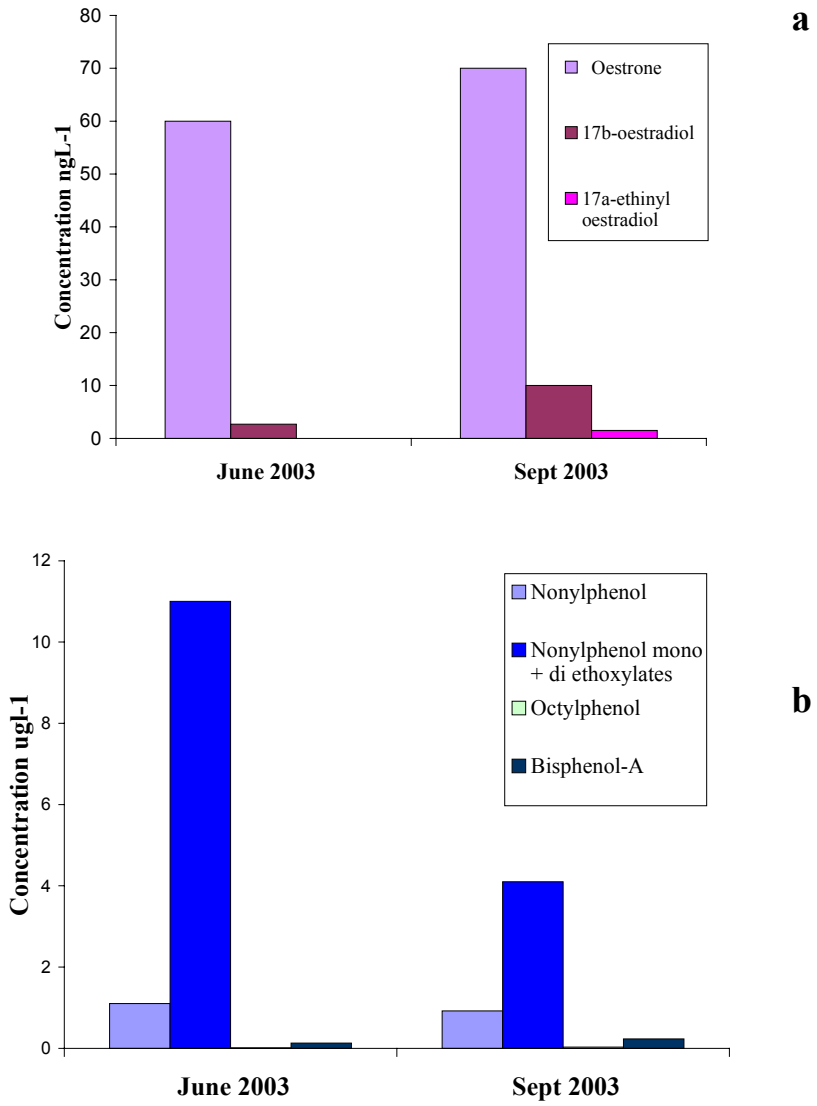


Figure 15. Concentrations of known oestrogenic compounds in effluents during the exposure to Site A WwTW effluent at Site A, **(a)** steroids and **(b)** alkylphenolic compounds and bisphenol A.

6.2.3 Survival of Roach

At the start of the exposure 25 male roach were deployed into each mesocosm tank. There were differences in differential survival between the treatments (due to disease and some fish escaping). Numbers of surviving (and sampled) fish at the end of the trial are shown in Table 3.

Treatment (Nominal effluent concentration)	Number of surviving fish
Control river water	18
25%	23
50%	25
100%	24

Table 3 – Numbers of surviving fish at the end of the exposure period.

6.2.4 Condition Factor

The condition factor of the fish increased during the trial across all the treatments from a pre-exposure sample mean of 1.06+/-0.04 (Figure 16). Condition factor in control river water fish at the end of the exposure was 1.26+/-0.01. The condition factor in the 25%, 50% and 100% effluent exposed fish were 1.30+/-0.01, 1.34+/-0.01 and 1.32+/-0.01, respectively. There was a significant difference in condition factor between control and 50% effluent exposed fish only (enhanced condition in the 50% effluent fish, $p<0.05$).

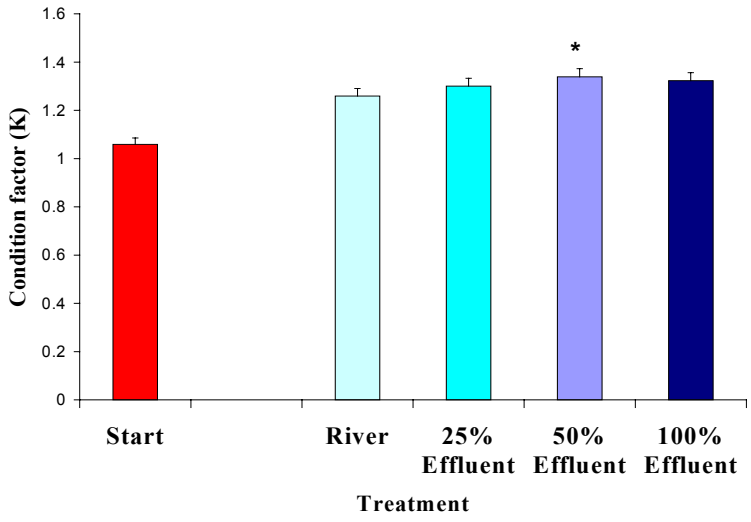


Figure 16. Condition factor (K) (+/- standard error of the mean) of roach sampled before and after the exposure period. * Indicates significance from control $p<0.05$

6.2.5 Gonadosomatic Index

The GSI of the fish immediately prior to the exposures was 6.04+/-0.03. The GSI decreased during the trial across all the treatments, in line with normal seasonal patterns for sexual development (the fish were producing sperm; Figure 17). In the river water fish, the GSI at the trial completion was 2.25+/-0.31. All the effluent exposure groups had significantly lower GSIs than the river water control ($p<0.05$); 25% effluent treatment = 1.71+/-0.12, 50% effluent =1.72 +/-0.11, 100% effluent =1.70+/-0.10.

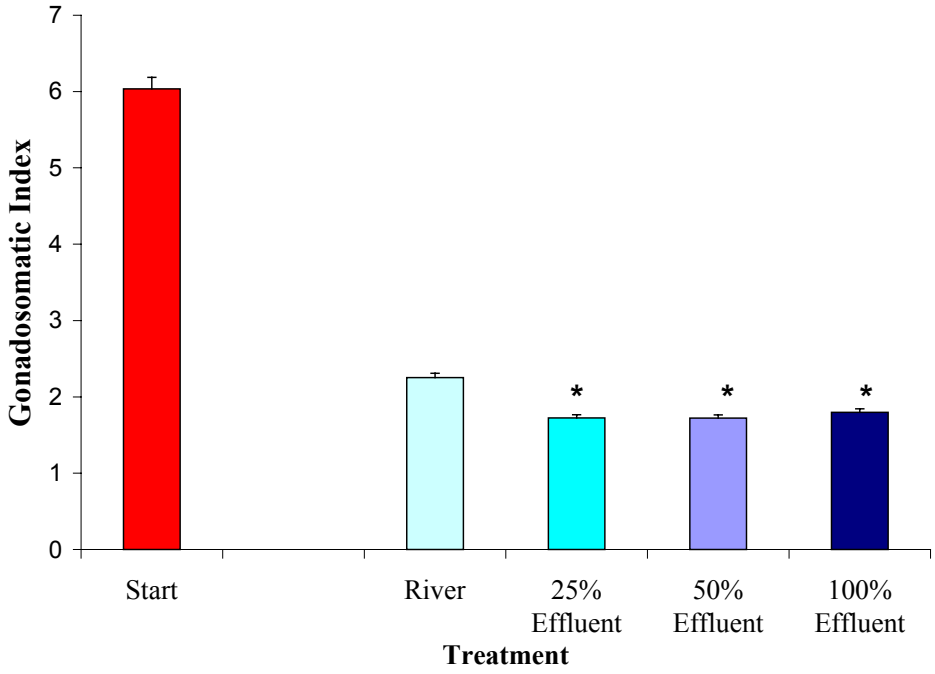


Figure 17. Gonadosomatic Index (GSI) (+/- standard error of the mean) of roach sampled before and after 2 months exposure to the Site A effluent. * Indicates significance from control $p<0.05$

6.2.6 Plasma Vitellogenin Concentrations

The plasma VTG concentration in the male fish prior to the effluent exposures in July was 226+/-67 ng/mL. In September the river water fish had a mean plasma VTG concentration of 91+/-22 ng/mL. Plasma VTG in the 25% effluent exposed fish was 38+/-15 ng/mL (no induction). Fish exposed to 50% and 100% effluent had plasma VTG concentrations of 2332+/-329 ng/mL and 2223+/-561 ng/mL respectively (both significantly higher than in the controls, $p<0.05$, Figure 18), confirming the effluent was oestrogenic to adult fish. The magnitude of the vitellogenic responses in the 50% and 100% effluent exposed fish were not significantly different from each other.

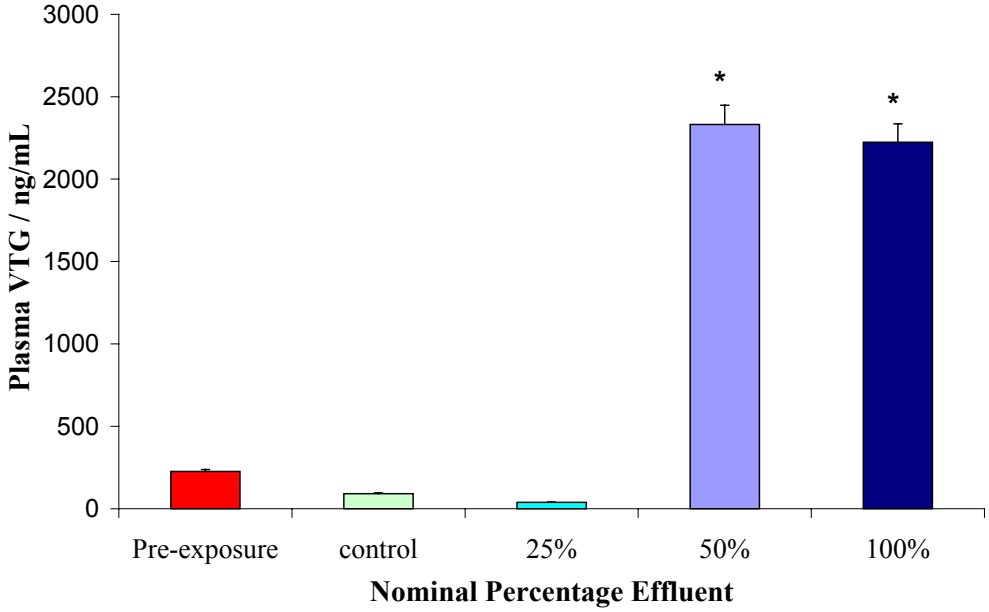


Figure 18. Plasma vitellogenin concentrations (+/- standard error of the mean) in roach sampled before and after exposure to Site A treated sewage effluent. * Indicates significance from control $p<0.05$.

6.2.7 Gonadal Histopathology

Pre-exposure roach (July)

Twelve spermiating male fish were sampled in July and 3 sections prepared from each testis. All sections showed the presence of large amounts of spermatozoa in the testis lobules. A section though a typical testis of a 3+ spermiating male roach is shown in Plate 22. A single fish was identified as intersex with a small number of primary oocytes in the perinucleolar stage contained within the otherwise normal testicular tissue (Plate 23).

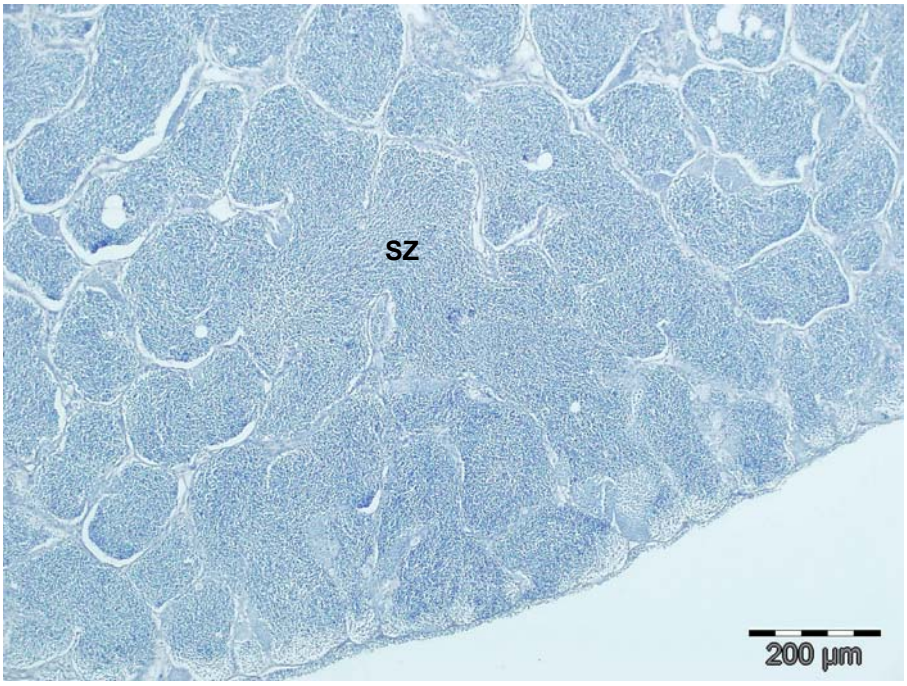


Plate 22. Photomicrograph of a typical testis of a normal 3+ spermiating male roach sampled in July. The testis is filled with spermatozoa (SZ).

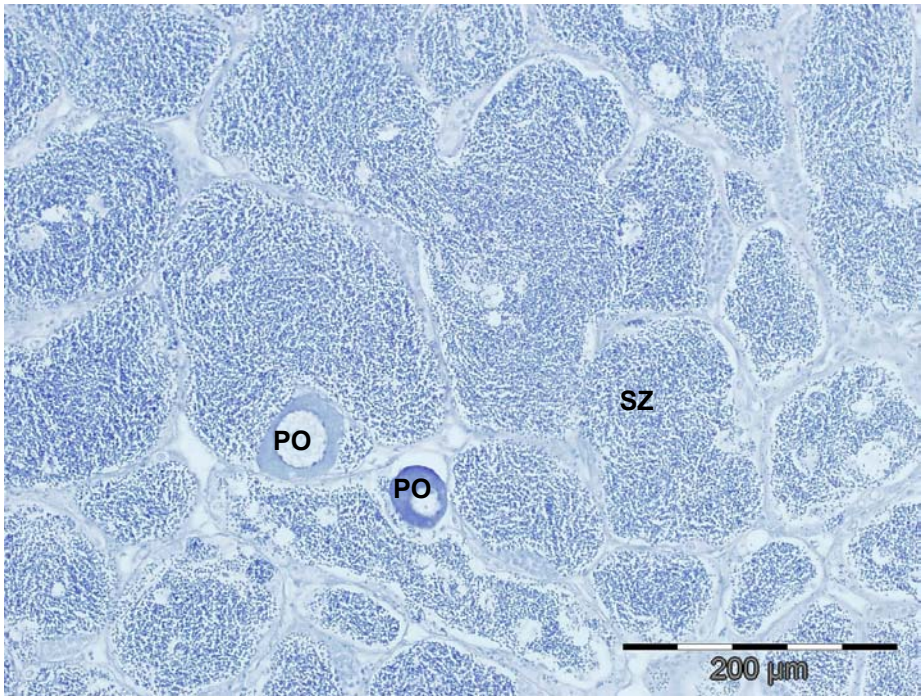


Plate 23. Photomicrograph of the single intersex 3+ spermiating male roach in the population sampled in July. The testis is filled with spermatozoa (SZ) but also contained a few primary oocytes (PO).

Post-effluent exposure (September)

Male fish sampled in September had ceased spermiating. Analysis of gonad sections demonstrated that the testes contained spermatogonia A, spermatogonia B and spermatocytes but no spermatozoa were observed in any of the sections (Plates 24 and 25). One fish sampled from the control river water exposure group was identified as intersex and contained a small number of primary oocytes in the perinucleolar stage (Plate 26). There were no obvious differences between the testes of fish sampled from the effluent exposure groups and the river water control group.

Photomicrographs of an ovary in a typical female 3+ roach sampled in September are provided for comparison (Plates 27 and 28). The ovary contained primary oocytes and vitellogenic oocytes.

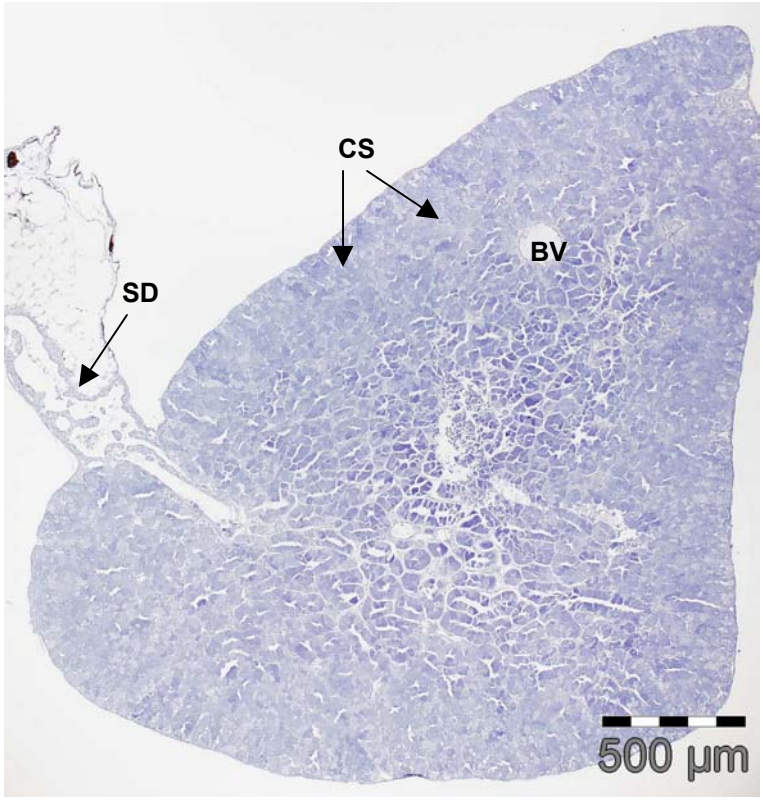


Plate 24. Photomicrograph of a typical testis of a normal 3+ male roach sampled in September. The plate shows the sperm duct (SD), a blood vessel (BV) and germ cells developing in cyst structures (CS).

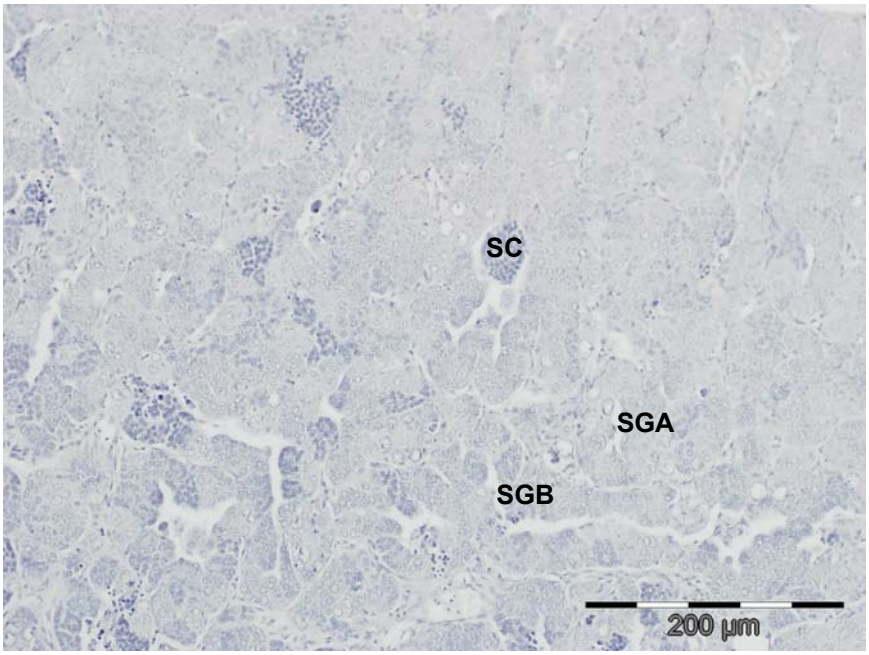


Plate 25. Photomicrograph of a typical testis of a normal 3+ male roach in September. The plate shows the spermatogonia A (SGA), spermatogonia B (SGB) and spermatocytes (SC).

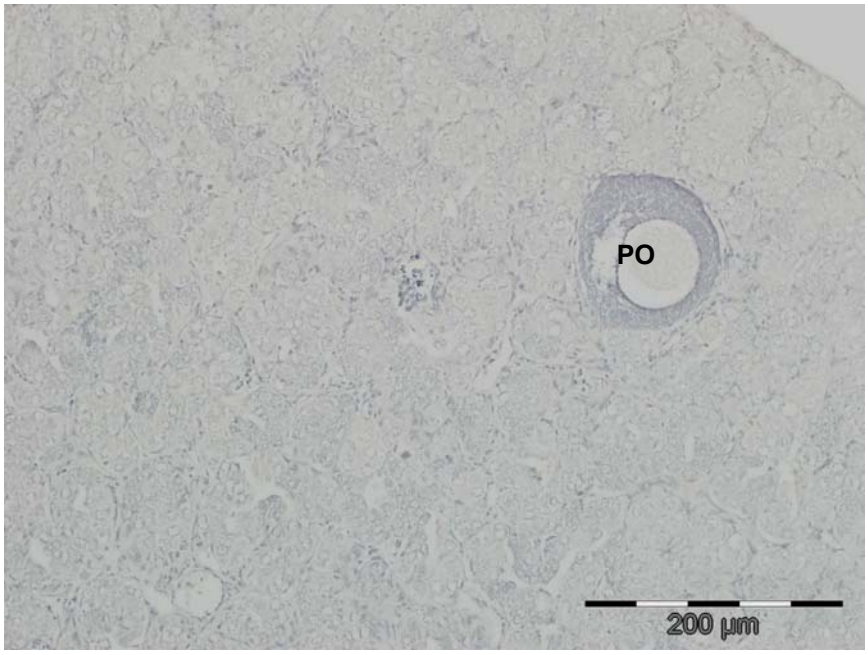


Plate 26. Photomicrograph of an intersex 3+ spermiating roach sampled in July. The plate shows a primary oocyte (PO) amongst the male germ cells.

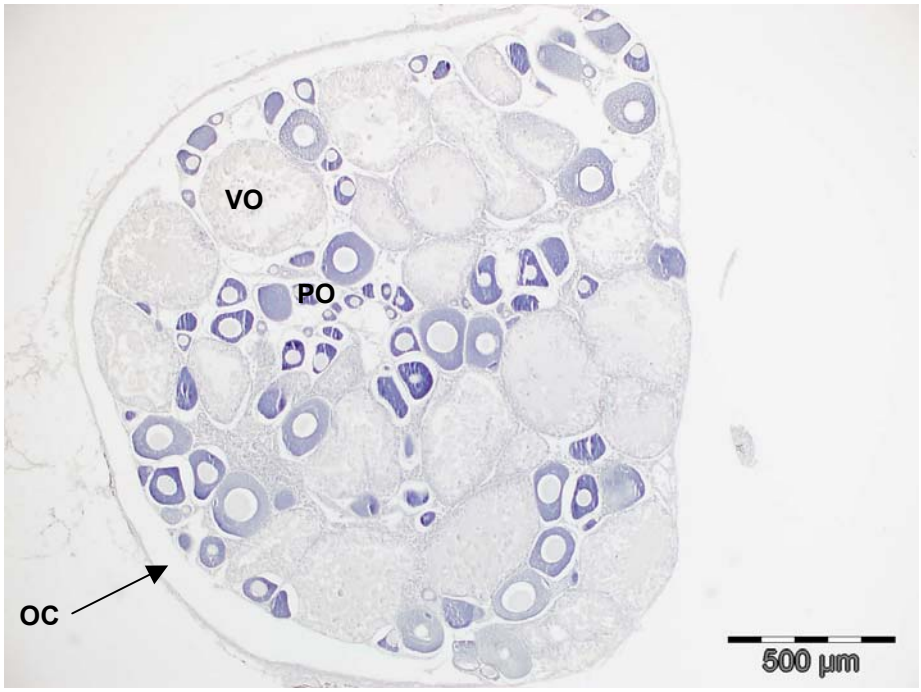


Plate 27. Photomicrograph of a typical ovary of a 3+ female roach showing primary oocytes (PO), vitellogenic oocytes (VO) and the ovarian cavity (OC).

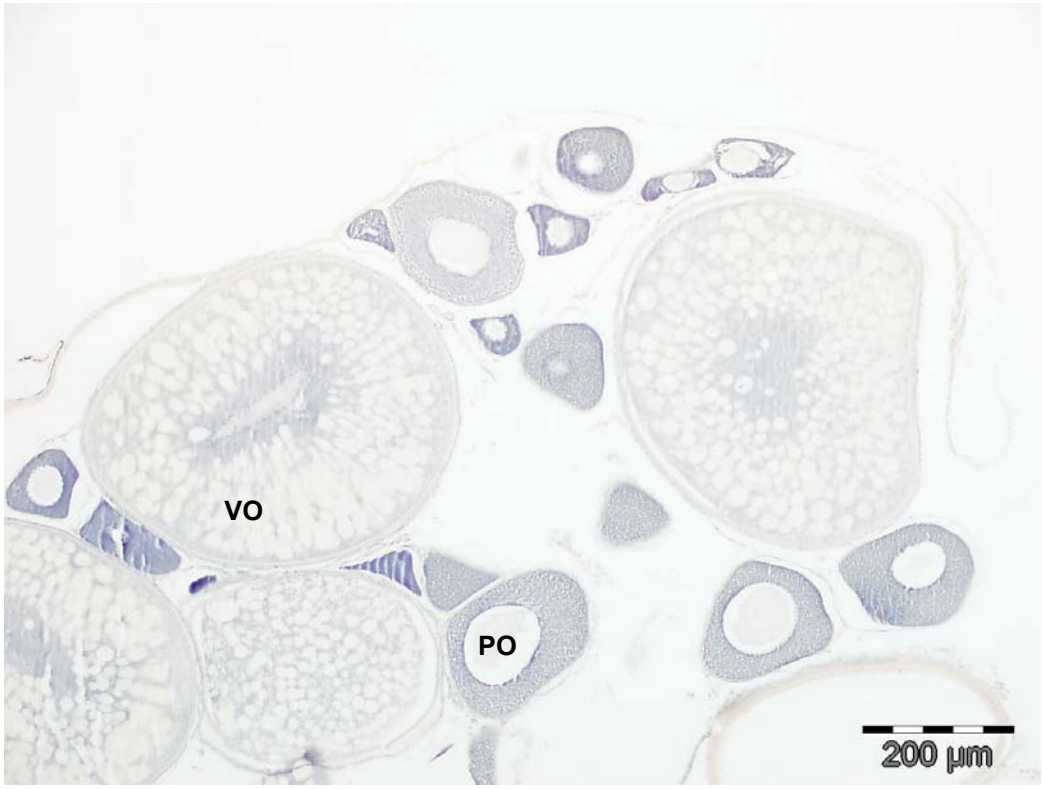


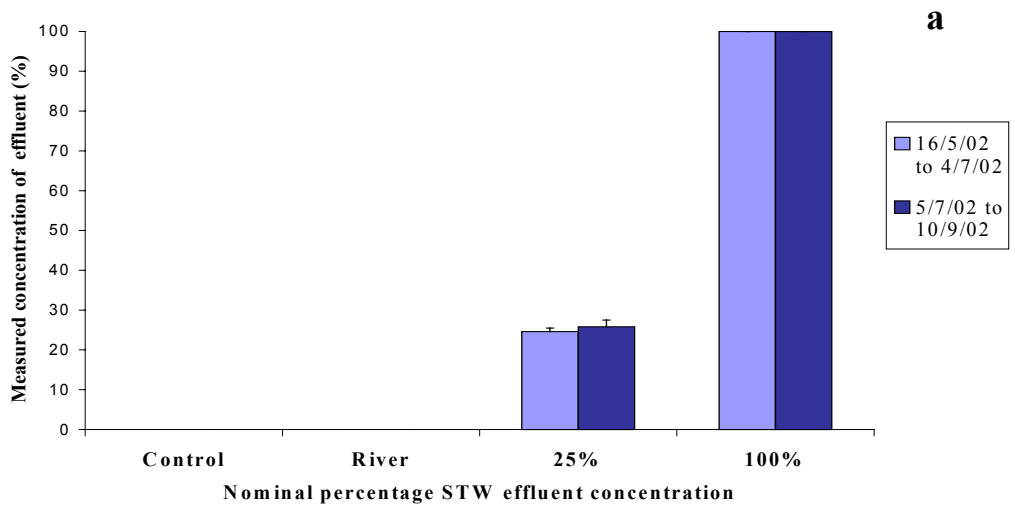
Plate 28. Photomicrograph of a typical ovary in a 3+ female roach showing the presence of primary oocytes (PO) and vitellogenic oocytes (VO).

6.3 Results - Effects of exposure to a WwTW effluent on adult post-spawning roach that had received prior exposure to oestrogen(s) – Site A and Site B (four months exposure)

6.3.1 Measured Concentrations of Sewage Treatment Works Effluent

Site A – The measured concentrations of Site A WwTW effluent to which the roach were exposed between May when the exposure started and July were 0% (Tap water control), 0% (River water control), 24.4+/-0.9% (25% effluent) and 100% (full strength effluent; mean concentration +/- standard error of the mean; Fig 19a). Between July and September the measured concentrations of effluent were 0% (Tap water control), 0% (River water control), 27.5+/-1.7% (25% effluent) and 100% (full strength effluent; mean concentration +/- standard error of the mean; Fig 19a).

Site B - The measured concentrations of treated sewage effluent to which the roach were exposed between May, when the exposure started and July when the exposure at this site was terminated were 0% (Tap water control), 25.1+/-1.3% (25% effluent), 46.6+/-1.5% (50% effluent) and 100% (full strength effluent; mean concentration +/- standard error of the mean; Fig 19b).



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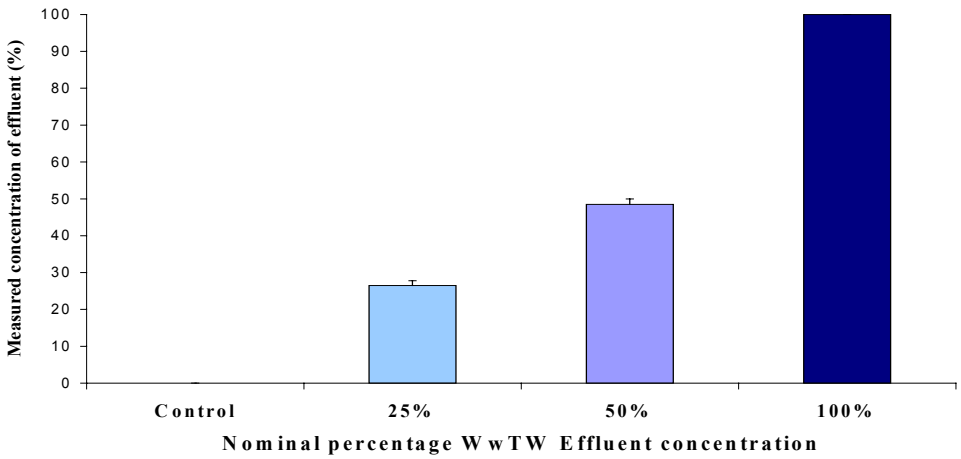

Figure 19. Measured concentrations of effluent that roach were exposed **(a)** Site A, May to July –to the first sampling, and July to termination of the experiment in September **(b)** Site B, May to July – the only sampling for this site. The graphs show mean percentage concentration +/- standard error of the mean

6.3.2 Concentrations of Oestrogenic Compounds in the Exposure Effluents

Chemical analysis of the effluents showed that concentrations of steroid oestrogens were higher in the Site A WwTW effluent compared with the effluent from Site B WwTW, as found in the previous trials (Fig. 20a). At Site A, concentrations of oestrone in each 7 day composite sample were between 20 and 57 ng/L and 17β-oestradiol between 2.3 and 6.4 ng/L, compared with ranges between 2.3 and 3.4 ng/L for oestrone at Site B. 17β-oestradiol was detected in one of the site B composite samples at a concentration of 1.1ng/L and was below the limit of detection (1ng/L) in the second. The synthetic oestrogen 17α-ethinylestradiol was detected (0.5ng/L) only in the second 7 day composite sample at Site A. Measured concentrations of alkylphenolic compounds at the two study sites were similar (Fig 20b.). At Site A, nonylphenol was detected in the range between 1.38 and 1.90 μg/L and in the range between 0.89 and 2.98 μg/L at Site B. Concentrations of nonylphenol mono- and diethoxylates were not detected in samples from either site (detection limit of 0.5 μg/L). Concentrations of octylphenol were between 0.03 and 0.11 μg/L at Site A and between 0.5 and 0.14 μg/L at Site B. Bisphenol-A was detected in two of the three composite samples collected from Site A at concentrations between 0.02 and 0.05 μg/L and in both samples collected from Site B (0.03 and 0.4 μg/L).

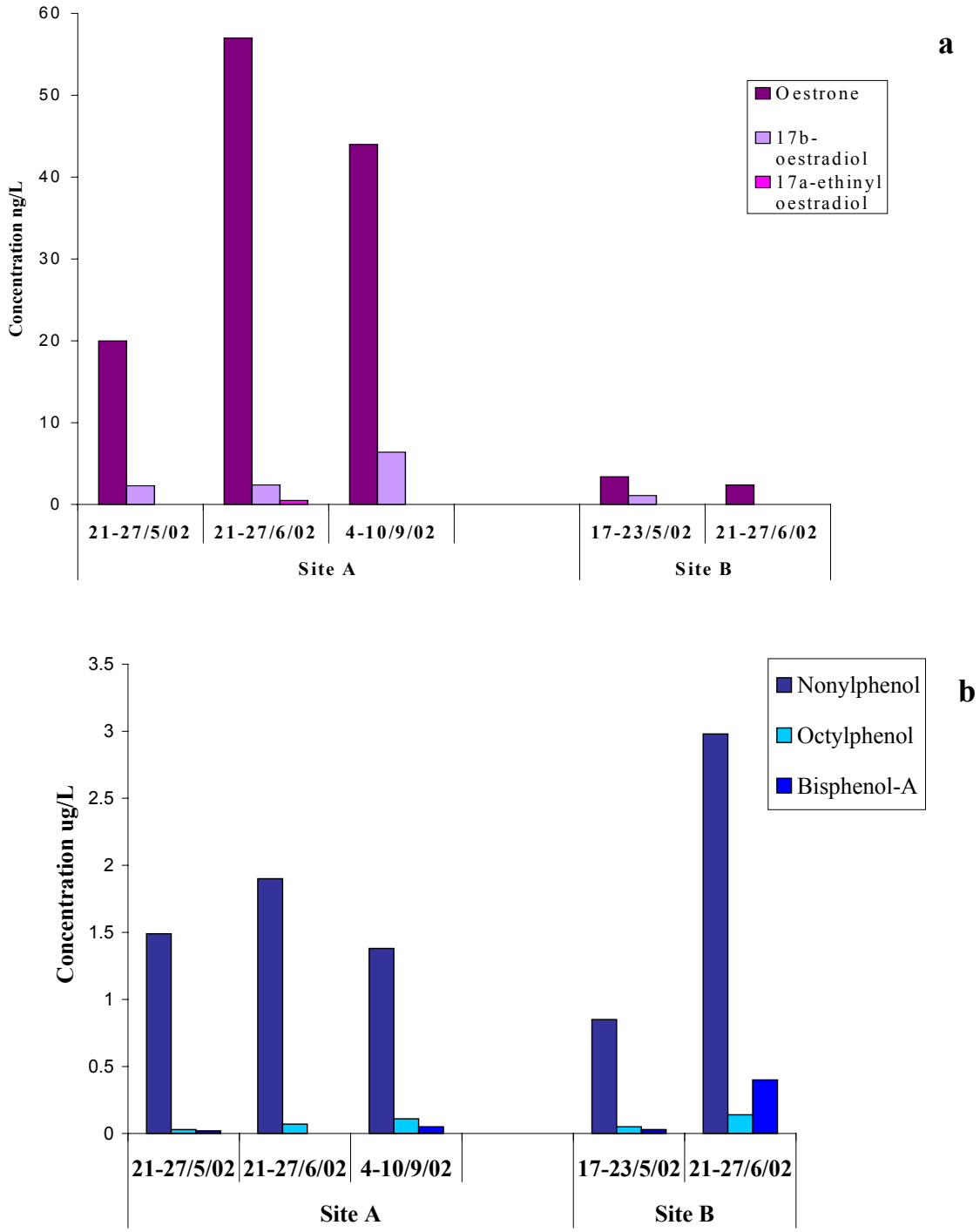

Figure 20. Concentrations of known oestrogenic compounds in effluents during the exposures at Site A and Site B, **(a)** steroids and **(b)** alkylphenolic compounds and bisphenol A

6.3.3 Survival of Roach

There were differences in survival of roach at Site B due to an outbreak of white spot disease in July 2002, which prompted the termination of the exposure at this site at this time. There were no surviving fish in the 50% effluent treatment at Site A due to a disease outbreak and loss of fish (which we could not account

for, but may have been due to predation by herons). Numbers of fish analysed for each sampling point are shown in Table 4.

Date	Site	Treatment (Nominal effluent concentration)	Number of fish sampled
May	-	Initial sample	20
July	A	River water control	8
		25%	8
		50%	0
		100%	8
	B	Tap water control	20
		25%	14
		50%	12
		100%	12
September	A	Tap water control	13
		River water control	12
		25%	10
		50%	0
		100%	11

Table 4 – Numbers of fish sampled from each treatment at each of the sampling points in July and September 2002.

6.3.4 Condition Factor

The condition factor (K) of the fish was higher in the control and all the treatments groups at the termination of the experiments compared with the fish sampled at the start of the study (1.29+/-0.02; Figure 21).

Site A - In July there were no significant differences in condition factor between control river water and effluent exposed fish. Condition factor of control river water fish in July was 1.51+/-0.05, in the 25% effluent exposed fish, 1.52+/-0.04, and in the fish exposed to full strength effluent, 1.53+/-0.03. Similarly in September there were no differences in condition factor between the controls and all effluent treatment groups: controls- 1.52+/-0.05; river water fish -1.44+/-0.03, 25% effluent exposed fish - 1.46+/-0.02, fish exposed to 100% effluent - 1.49+/-0.03 (mean concentration +/- standard error of the mean; Fig 21).

Site B – The condition factor of control fish at the end of the exposure in July was 1.32+/-0.03, in the 25% effluent exposed fish, 1.36+/-0.02, in the 50% effluent exposed fish, 1.34+/-0.02, and in fish exposed to 100% effluent, 1.44+/-0.03 (mean concentration +/- standard error of the mean; Fig 21). The only significant difference in condition factor occurred between the controls and 100% effluent exposed fish (an enhanced condition in the effluent exposed fish; $p<0.05$).

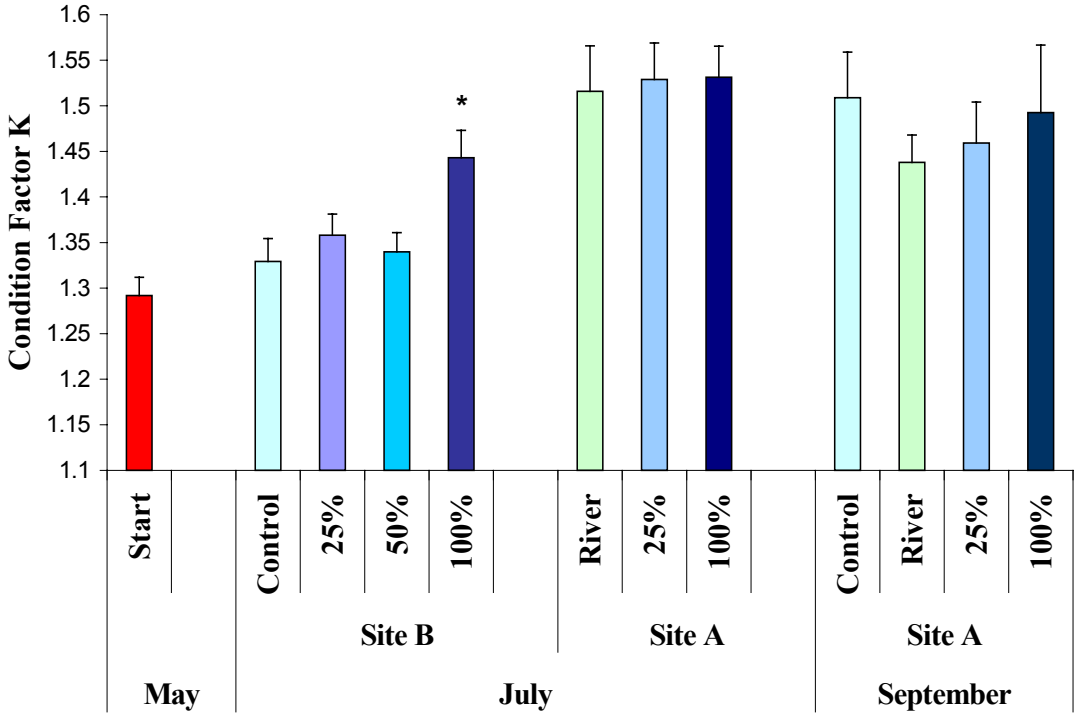

Figure 21. Condition factor (K) (+/- standard error of the mean) of roach sampled before and after exposure to Site A and Site B WwTW effluent. * Indicates significance from control $p<0.05$.

Age of Fish

The roach used in this trial were of mixed age, ranging between 3+ and 8+ years, with most in the 5+ year class (see Fig.22).

Figure 22. Age distribution of male roach in used in this trial

6.3.5 Gonadosomatic Index

The GSI of the roach prior to the effluent exposures was 3.93+/-0.26. As in the first adult post-spawning trial, the GSI decreased during the experiment across all the treatments in line with normal seasonal patterns for sexual development (Fig. 23).

Site A – In July the control river water fish had a GSI of 1.37+/-0.06. The GSIs in the 25% and 100% effluent exposed fish were 1.26+/-0.06 and 1.75+/-0.12, respectively. The GSI in the 100% effluent fish was significantly higher than in the river water controls ($p<0.05$). There were no differences in GSI between the effluent exposed fish and the control fish sampled in September; tap water fish = 1.69+/-0.09, river water fish = 2.19+/-0.21, 25% effluent =1.93+/-0.10 and 100% effluent exposed fish =1.90+/-0.12 (mean concentration +/- standard error of the mean; Fig 23).

Site B – In July control fish (tap water) had a GSI of 2.48+/-0.23. GSIs in the effluent exposed fish were; 25% =1.91+/-0.20, 50% effluent =1.62+/-0.26, 100% effluent =1.64+/-0.13 (mean concentration +/- standard error of the mean; Fig 23). The GSI was significantly lower in the 50% and 100% effluent exposed fish compared with the controls, indicating suppression in gonad development/recovery after spermiation.

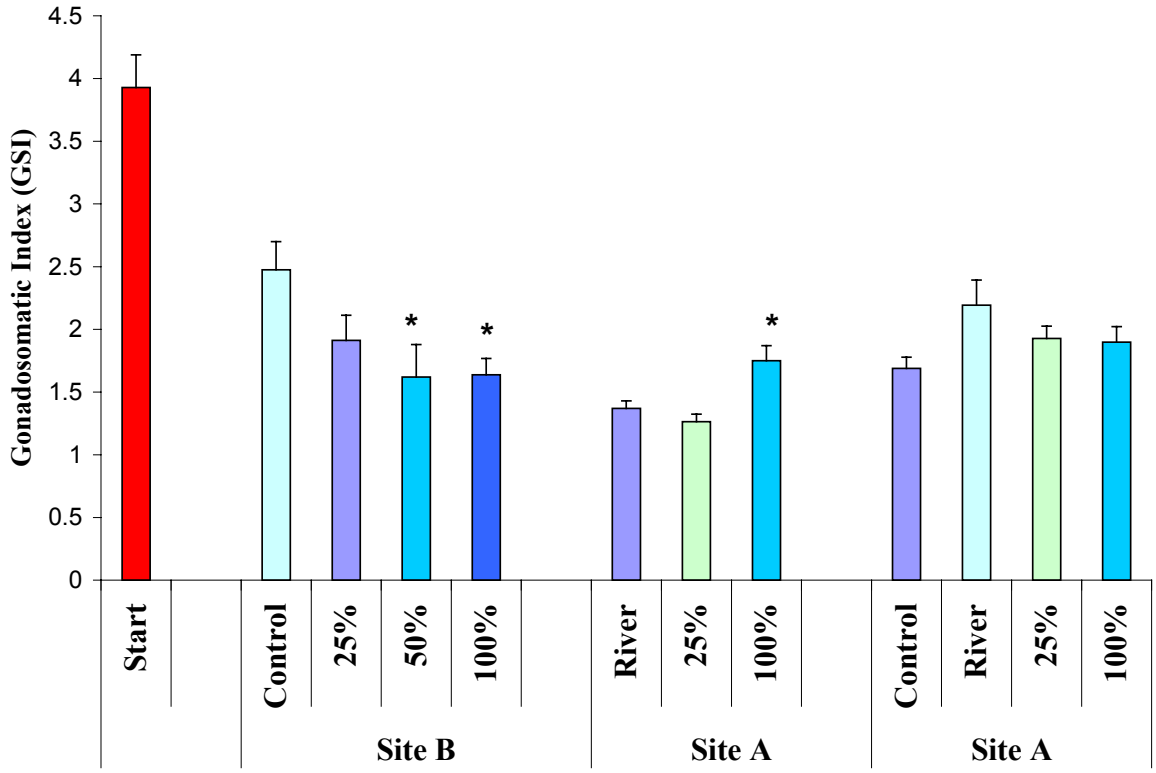

Figure 23. Gonadosomatic Index (GSI) (+/- standard error of the mean) of roach sampled before and after exposure Site A and Site B WwTW effluent. * Indicates significance from control $p<0.05$.

6.3.6 Plasma Vitellogenin Concentrations

The mean plasma VTG concentration of the fish at the start of the study was 1531+/-426 ng/mL, indicating that the fish had previously been exposed to an oestrogenic stimulus or stimuli (Figure 24).

Site A – In July, 2 months after the start of the experiment, the control river water fish had mean plasma VTG concentration of 62+/-9 ng/mL. In these fish therefore, a clearance of the VTG present in these fish at the outset of the trial had occurred. Fish in the 25% effluent showed a similar pattern of VTG clearance (94+/-27 ng VTG /mL). Fish exposed to 100% effluent had a significantly elevated concentration of plasma VTG concentration (4017+/-1013 ng/mL; $p<0.05$). The titre of plasma VTG in the 100% effluent exposed fish was approximately 3-fold higher than in the pre-exposure fish.

A very similar picture was seen for the plasma VTG in these fish in September; controls; 53+/-21 ng/mL, river water fish; 56+/-7 ng/mL, 25% effluent, 64+/-11 ng/mL and 100% , 4768+/-563 ng/mL (significantly higher than in the controls; mean concentration +/- standard error of the mean; Fig. 24).

Site B – Control tap water fish sampled at Site B in July had mean plasma VTG concentration of 191+/-42 ng/mL. The 25% and 50% effluent exposed fish had plasma VTG concentrations of 101+/-27 ng/mL and 61+/-25 ng/mL respectively (no significant differences from controls). Male fish in the 100% effluent

exposure contained 525+/-136 ng/mL (significantly different from controls; p<0.05; mean concentration +/- standard error of the mean; Fig 24).

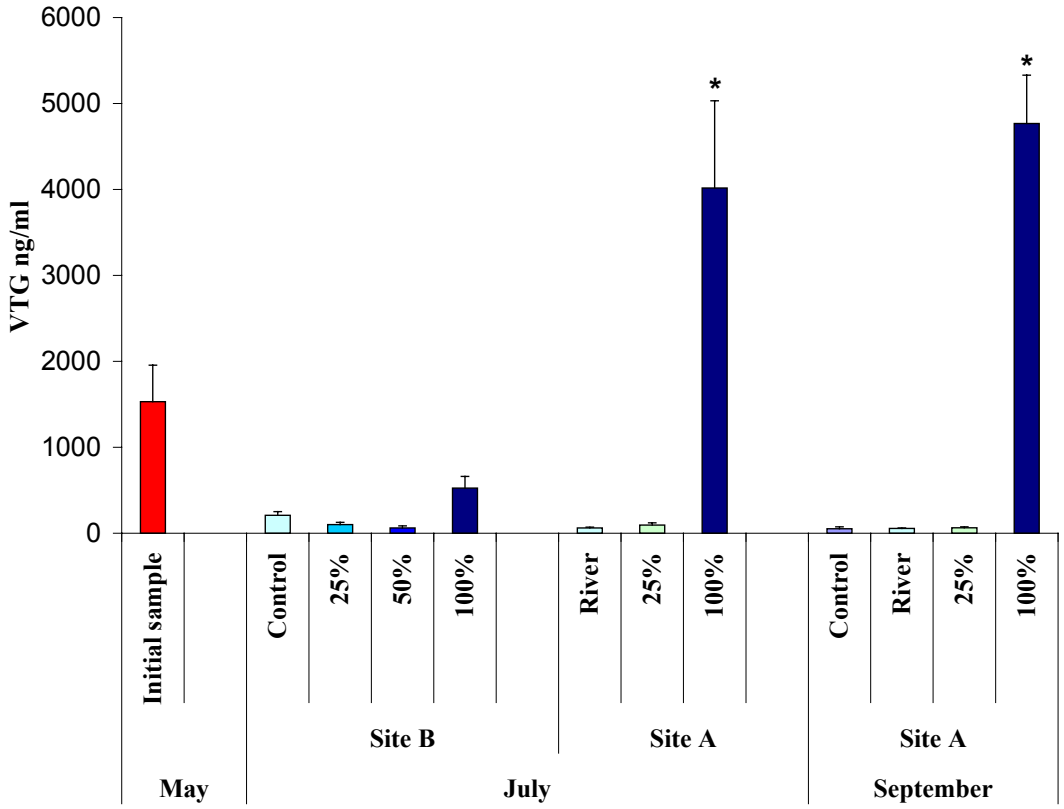

Figure 24. Plasma vitellogenin concentrations (+/- standard error of the mean) in roach sampled before and after exposure to WwTW effluents at Site A and Site B. * Indicates significance from control p<0.05.

6.3.7 Gonadal Histopathology

Intersex fish were found at every sampling point in every treatment. Testes sections were analyzed and fish assigned to one of three categories; 0= normal (no oocytes), 1=a few oocytes (10 or less per section, and principally comprised of primary oocytes) and, 2= many oocytes (more that 10 oocytes per testes section, many of which were often in the cortical alveolus stage of development). The percentages of fish in each category for each exposure group are shown in Figure 25. There were no significant differences between treatments and sampling points and numbers and severity of the intersex condition (Figure 26).

Pre-exposure fish (May)

All fish sampled in May were spermiating. All sections through the testes were found to contain spermatozoa in lobules. A section though a typical testis of a normal spermiating male roach for this study group is shown in Plate 29. Testes also contained dispersed cysts of spermatogonia A and spermatogonia B (Plate 30). Intersex fish were identified and classified as having few oocytes (Plate 31) or many oocytes (Plate 32). All intersex fish had spermatozoa contained in lobules.

July Sampling– Site A and Site B

Normal and intersex fish were found in every effluent exposure group and control groups at both sites. Normal male roach had ceased spermiating and testes contained spermatogonia A, spermatogonia B and spermatocytes developing synchronously within cyst structures (Plate 33). Spermatids were not seen. The intersex condition included clusters of primary oocytes nested in an otherwise normal testicular tissue (Plate 34), and, in the more severe condition with secondary oocytes (Plate 35).

September sampling – Site A

Normal males and intersex fish were found in every effluent exposure group and in the river water control. Testes in normal males were histologically very similar to those analyzed in July. Testes contained spermatogonia A, spermatogonia B and spermatocytes developing synchronously within cyst structures (Plate 36). Intersex fish were similar to that observed the fish sampled in July, where some fish contained a few primary oocytes (Plate 37) and others were more severely intersex and contained secondary stage oocytes (Plate 38).

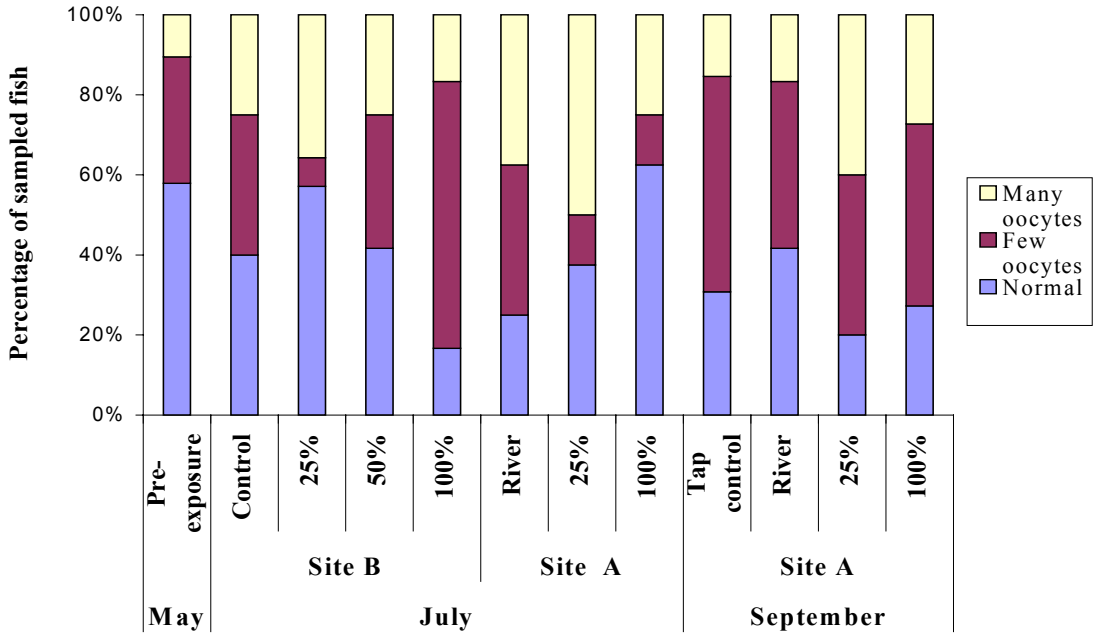

Figure 25. Percentage of intersex and normal fish in roach prior to and subsequent to exposure to the Site A and Site B WwTW effluents.

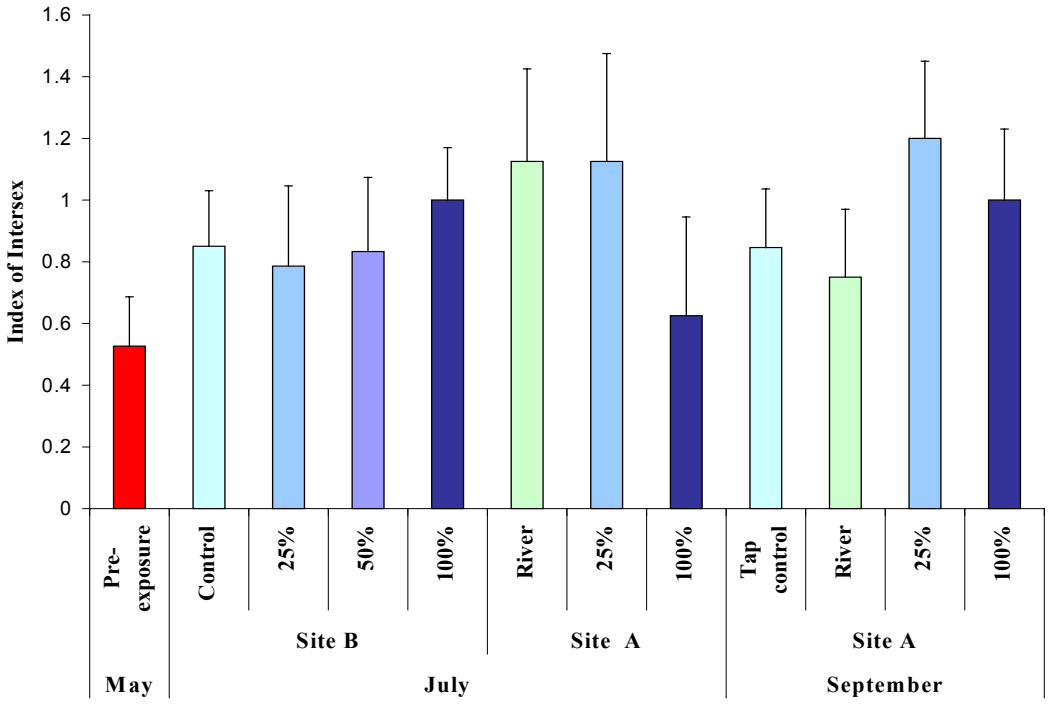

Figure 26. Mean index of intersex in roach before and after exposure to the Site A and Site B WwTW effluents.

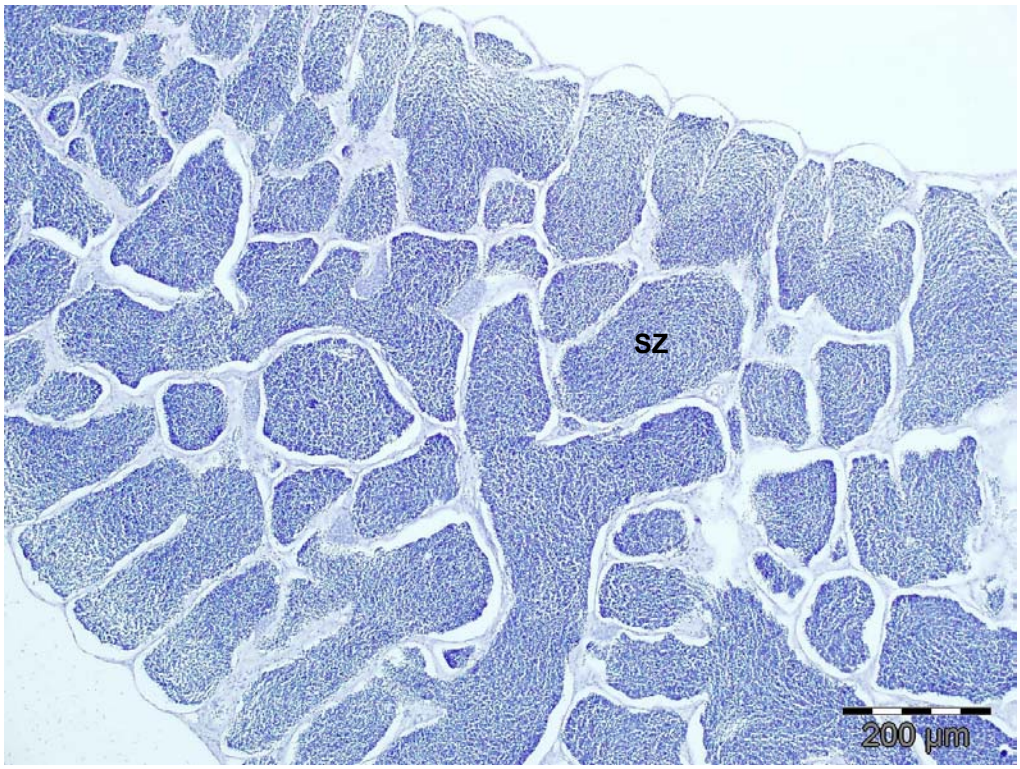

Plate 29. Photomicrograph of a typical testis in a normal spermiating male roach in May. The testis is filled with spermatozoa (SZ).

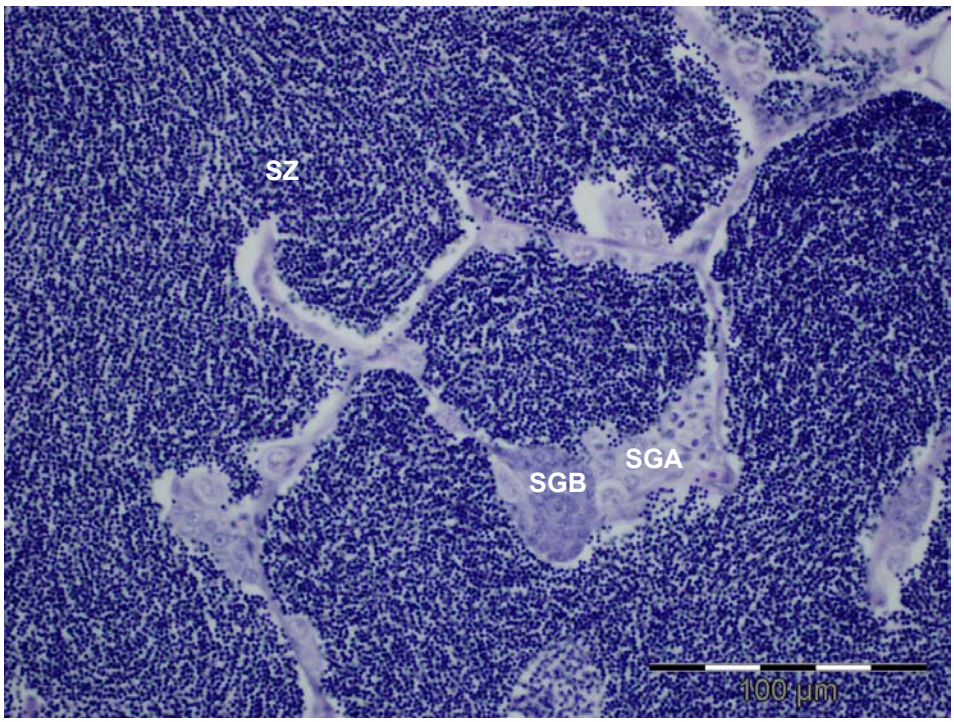

Plate 30. Photomicrograph of a testis in a normal spermiating male roach in May (high power magnification). The testis is filled with spermatozoa (SZ). Also visible are cysts of spermatogonia A (SGA) and spermatogonia B (SGB).

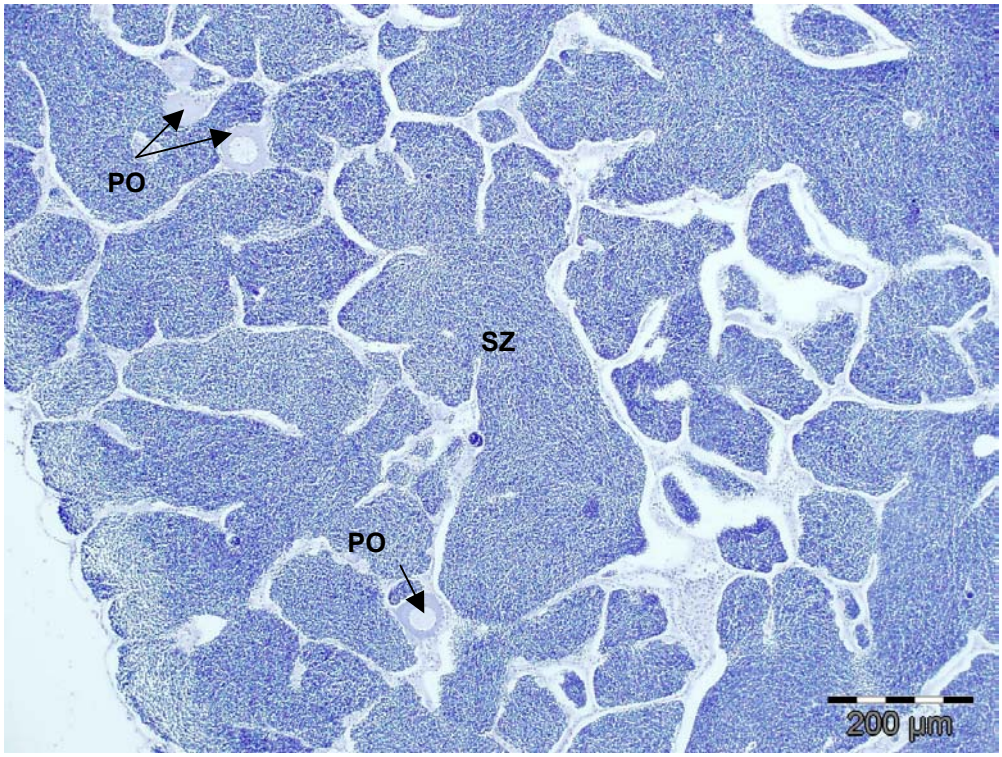

Plate 31. Photomicrograph of an intersex spermiating roach in May. The testis is filled with spermatozoa (SZ) and also present are primary oocytes (PO).

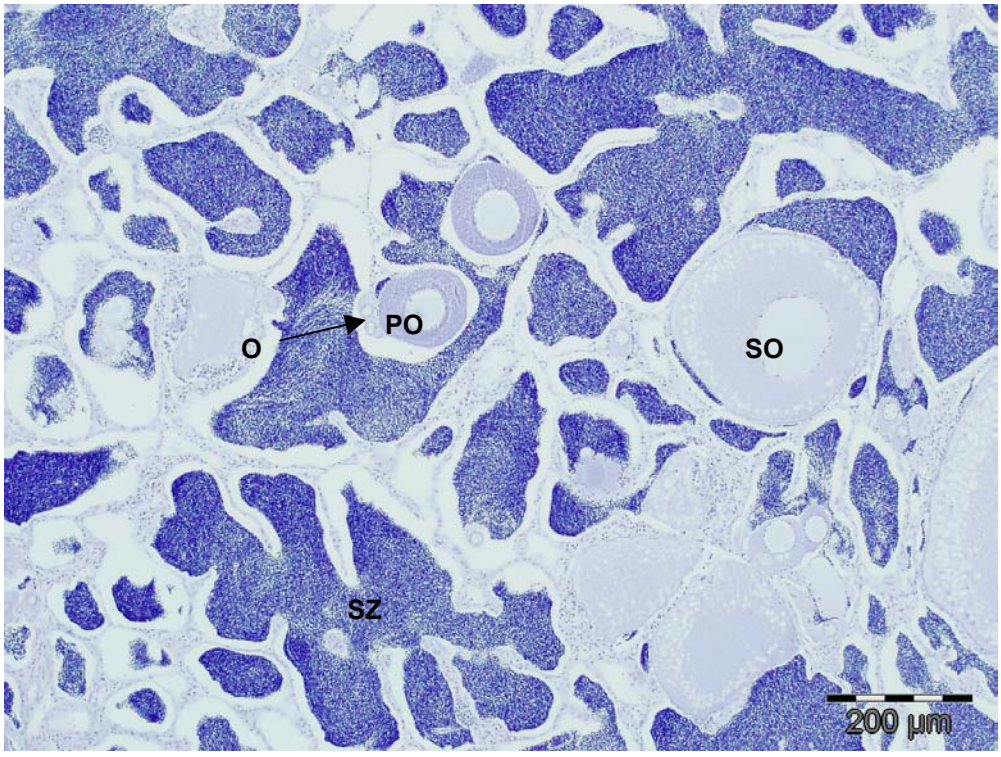

Plate 32. Photomicrograph of a more severely intersex and spermiating roach sampled in May. The testis contains spermatozoa (SZ), oogonia (O), primary oocytes (PO) and larger secondary oocytes (SO).

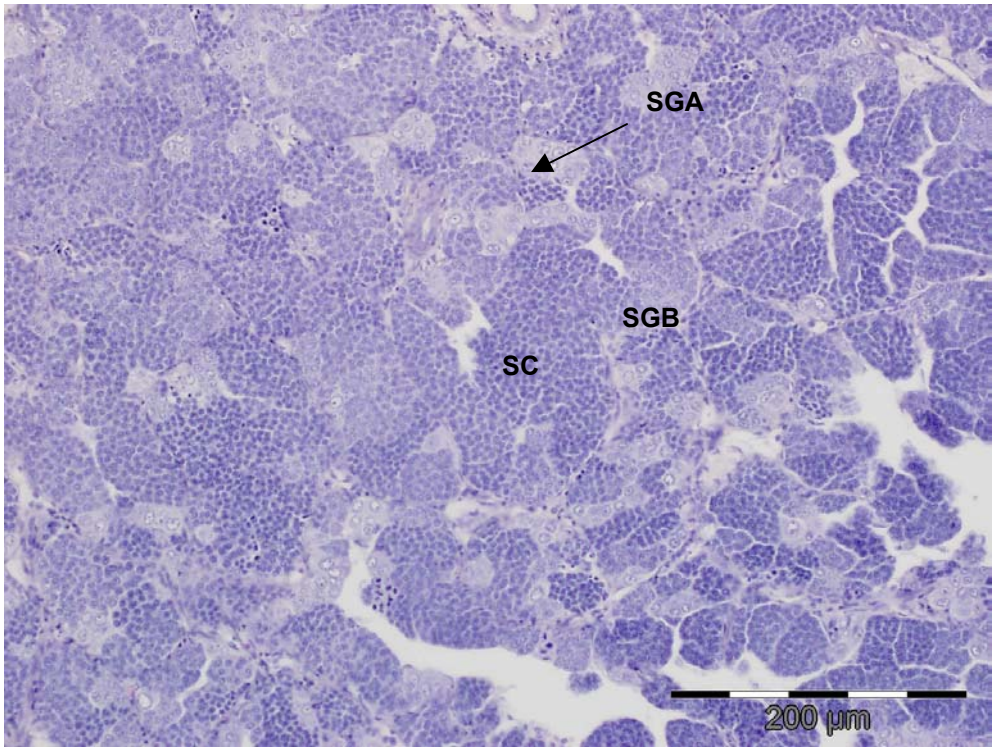

Plate 33. Photomicrograph of a typical testis in a normal male roach sampled in July. The testis contains cysts of spermatogonia A (SGA), spermatogonia B (SGB) and spermatocytes (SC).

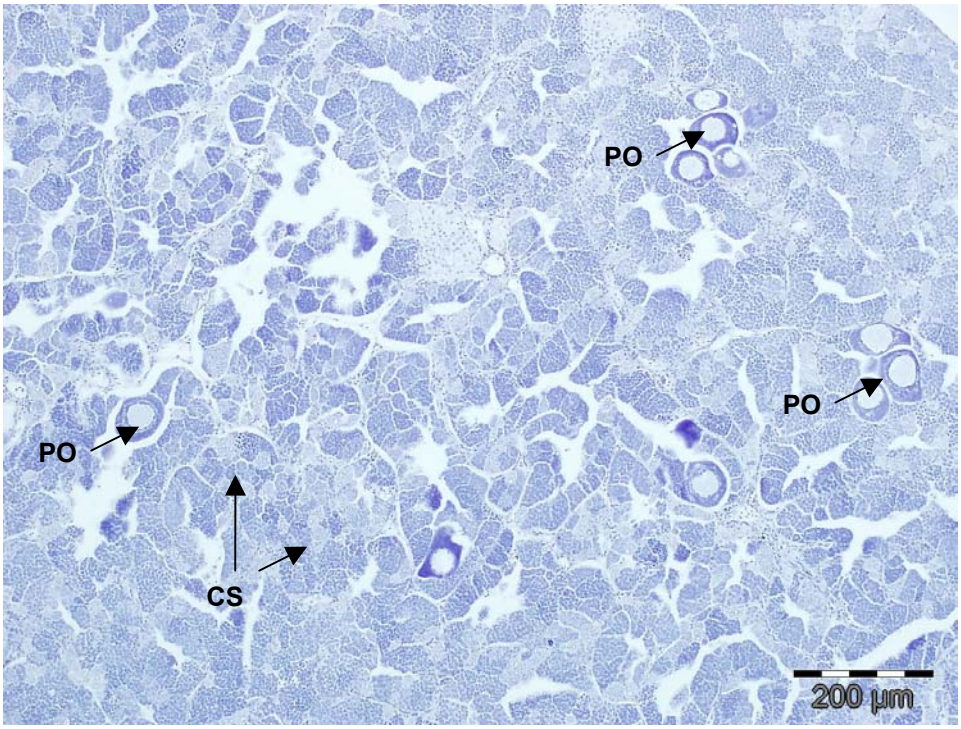

Plate 34. Photomicrograph of an intersex roach sampled in July. The gonad contains male germ cells developing in cyst structures (CS) and primary oocytes (PO).

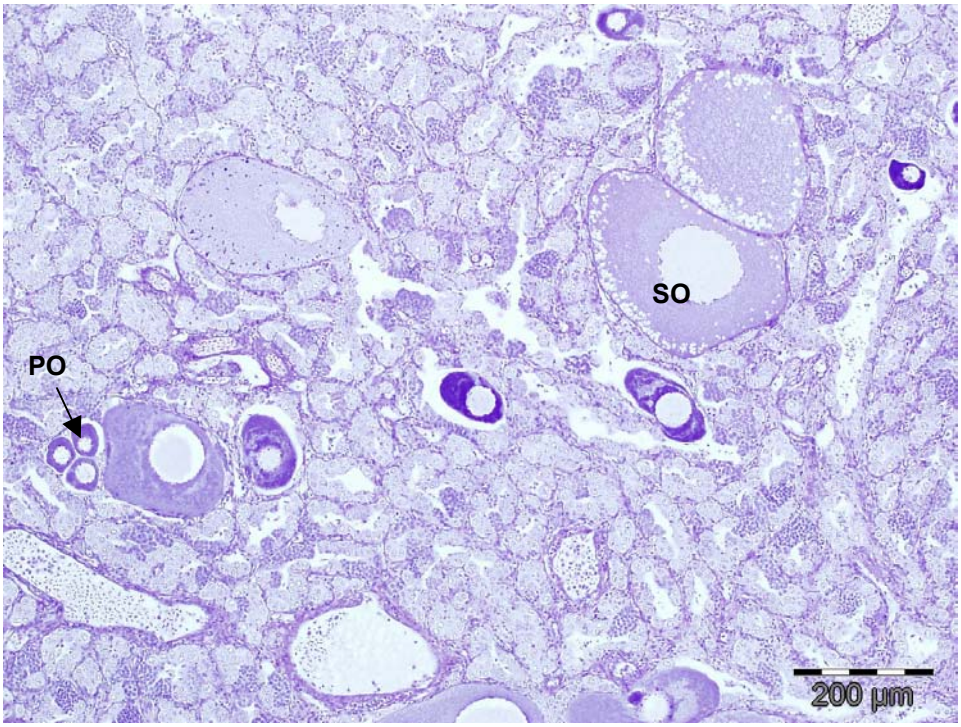

Plate 35. Photomicrograph of the gonad in a more severely affected intersex roach in July. The testis has male germ cells and also primary oocytes (PO) and larger secondary oocytes (SO).

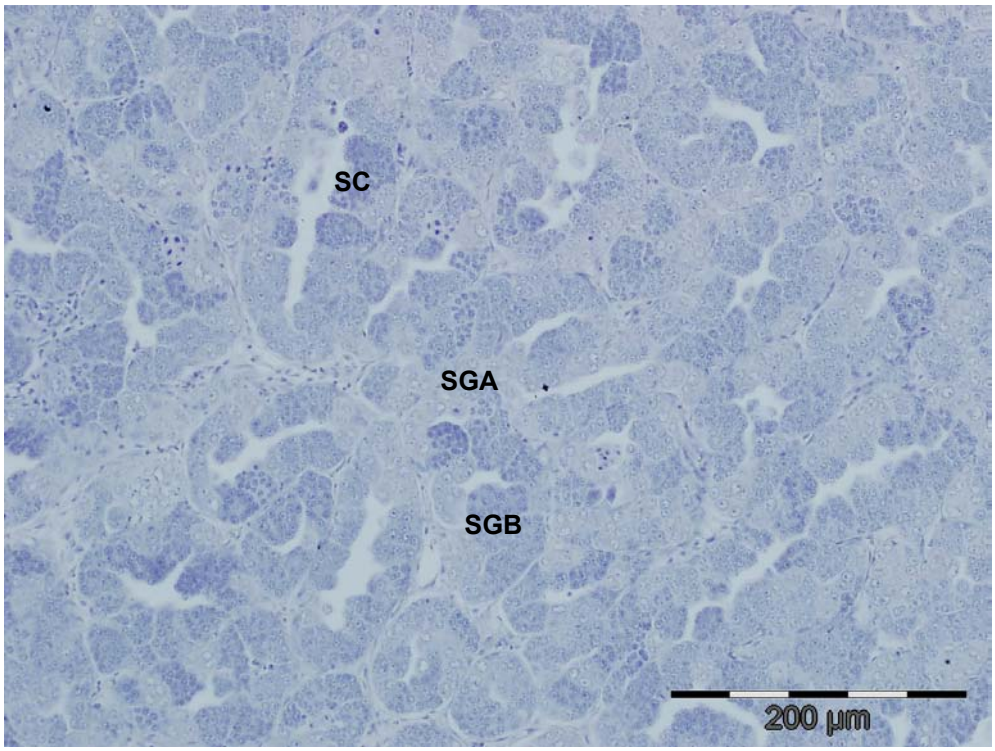

Plate 36. Photomicrograph of a typical testis in a normal male roach in September. The testis contains cysts of spermatogonia A (SGA), spermatogonia B (SGB) and spermatocytes (SC).

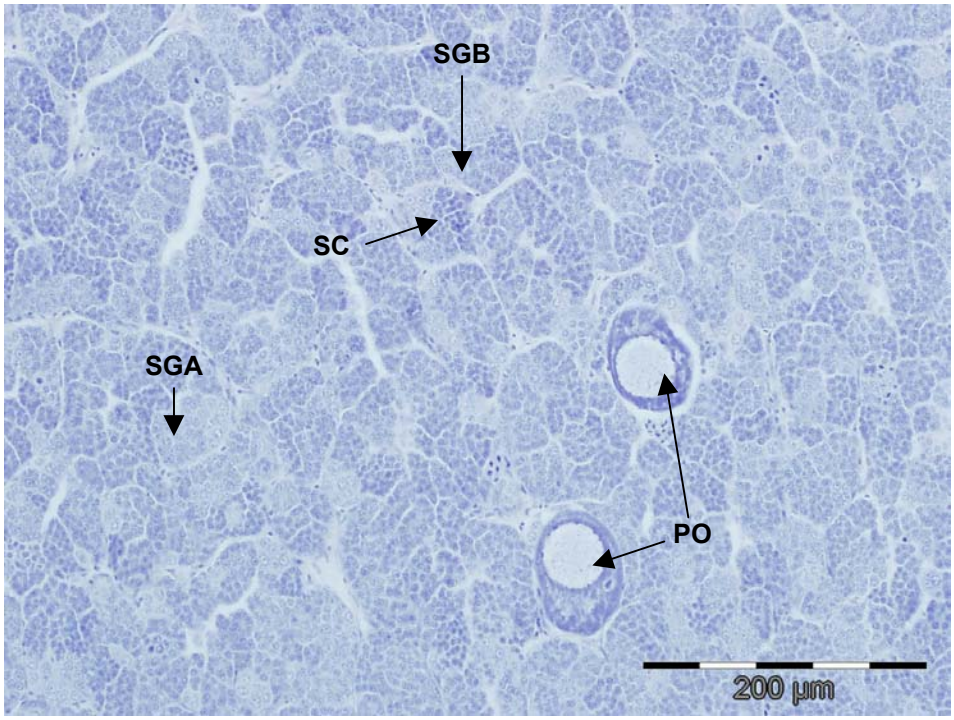

Plate 37. Photomicrograph of a typical testis of an intersex roach sampled in September. The testis contains cysts of spermatogonia A (SGA), spermatogonia B (SGB) and spermatocytes (SC). Also present are primary oocytes (PO).

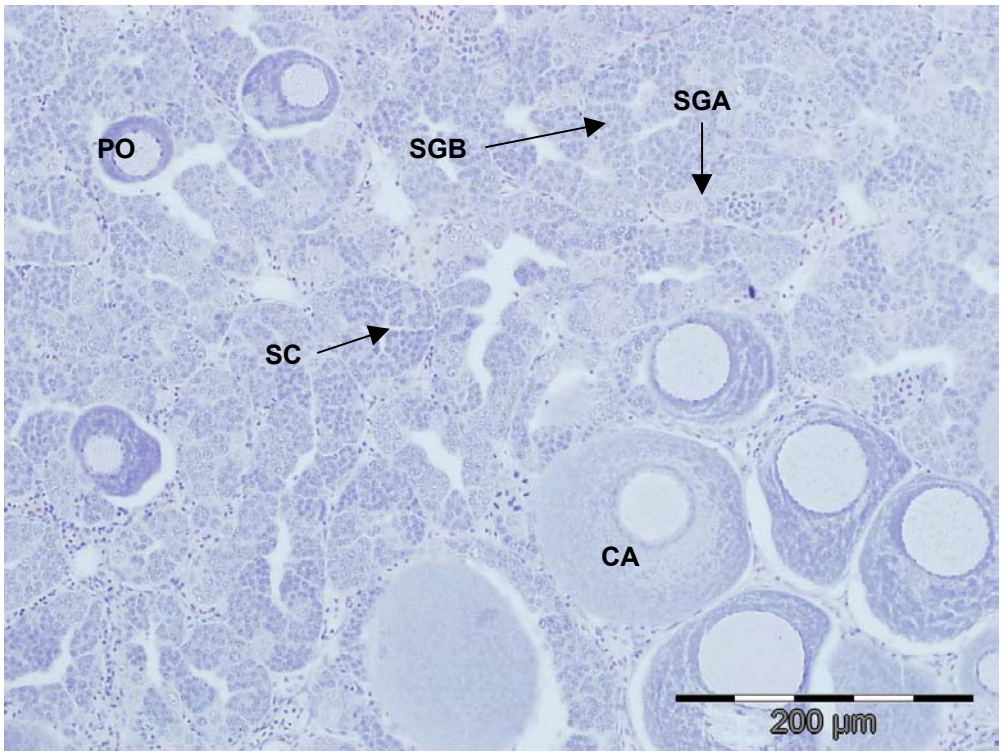

Plate 38. Photomicrograph of a typical testis of a severely intersex roach in September. The testis contains cysts of spermatogonia A (SGA), spermatogonia B (SGB) and spermatocytes (SC). Also present are primary oocytes (PO) and larger oocytes in the cortical alveolus stage (CA).

6.4 Discussion

The measured concentrations of effluent flowing through the mesocosm tanks at both sites were close to nominal throughout all trials showing a good operating reliability for both systems. The adult fish trials confirmed the effluent derived from the WwTW at Site A had a consistently higher concentration of steroid oestrogens compared with Site B effluent. Furthermore, the concentrations of oestrone and oestradiol at Site A were consistent across the different sampling periods. The synthetic oestrogen, ethinylestradiol, derived from the contraceptive pill was detected (intermittently) in the Site A effluent only. The highest concentration of ethinylestradiol detected in the Site A effluent was sufficient to induce VTG induction in fish (Purdom et al., 1994). Alkylphenolic chemicals in the WwTW effluent at Site B were again generally higher than at Site A, and this probably reflects the greater trade influent to the works at Site B. The higher concentrations of nonylphenol measured in the Site B WwTW effluent are sufficient to induce both VTG induction in fish, and intersex in the medaka (*Latipes oryzias*), one of the most sensitive species for chemical induction alteration of sexual development (Gray et al. 1999; Knorr and Braunbeck 2002). Concentrations of alkylphenolic chemicals in the effluent were highly variable, which may reflect differences in the influents received, works efficiency and/or levels of dilution in the treatment works (Rodgers-Gray et al. 2000). Concentrations of Bisphenol A (up to 0.4 μg/L) alone would be insufficient to induce oestrogenic effects in fish (Sohoni et al. 2001). It is now well established that mixtures of oestrogenic chemicals can be additive in their effects in vivo (Thorpe et al. 2001; Thorpe et al. 2003) and the totality of the oestrogenic chemicals measured in both effluents is sufficient to induce VTG induction in fish (which it indeed does). The effective concentrations of natural steroid oestrogens, synthetic steroid oestrogens and alkylphenolic chemicals for inducing feminising effects in fish are described in detail in (Tyler and Routledge 1998).

Survival of the fish in the adult exposure trials was highly variable. In the trial with adult males that had not previously experience oestrogen exposure, overall survival was almost 90%, however for the trial where fish were derived from a wild population (and had been exposed to oestrogen prior to the study), the overall survivorship was approximately 50%. Disease, escapes and predation all contributed to these losses. There was no effluent concentration-related survivorship, indicating that the effluents were not toxic to the fish (indeed the condition of the fish in the higher effluents improved with time).

The increased condition of the fish with time across the treatments was as expected. During gonadal maturation a large amount of energy is drawn from somatic growth and condition to fuel gamete development. After the spawning season fish gain condition as energy is no longer required to support rapid gonad growth and is directed to somatic growth. The adult post spawning male fish followed this pattern. The higher concentrations of effluent appeared to enhance condition recovery. This may be as a consequence of a greater availability of natural food, or indeed because less energy was being partitioned to gonad recovery (an associated inhibitory effect on testis recrudescence).

The reduced gonad mass (GSI) in the adult post-spawning trials was in line with normal seasonal patterns for sexual development (the fish were producing sperm). There was no consistent pattern in the effect of effluent exposure on post-spawning gonad recrudescence; in some case there appeared to be an inhibitory effect

The vitellogenic responses in males exposed to the effluents confirmed the findings of the early life stage studies showing that the effluent WwTW at Site A was the more potent and this was likely as a consequence of the associated higher titres of steroid oestrogens in this effluent. Both effluents at full strength induced a vitellogenic response in male fish that had received prior exposure to oestrogen. At Site B, however, the concentration of plasma VTG in the 100% effluent exposed fish was lower (approx 33%) of that in the pre-exposure fish, suggesting that the environment in which these fish were derived was more strongly oestrogenic than the Site B effluent.

There was no evidence that exposure of post-spawning adult male roach to either of the two test WwTW effluents (for both naive, or male previously exposed to oestrogen) induced alterations in germ cell development (oocytes in the testis). In fish that had received exposure to oestrogen prior to the exposure to the WwTW effluent, there was an increase in the severity of the intersex condition, but this was the case for all treatments and controls. Thus, this effect was not deemed to be a function of the constituents in the WwTW effluents. A possible explanation for this finding is that some of the germ cells in the males exposed to oestrogen prior to the study had been programmed to develop into oocytes and did so during the period of germ cell proliferation that follows spermiation and spawning.

Thus, we have not identified a life stage window in the roach that is susceptible to germ cell disruption by oestrogenic effluents from WwTW effluents; neither early life, or the post-spawning period are sensitive periods for germ cell re-programming for the oestrogenic mixtures found in WwTW effluents. These data, together with data showing an age-related relationship between the severity of intersex (degree of germ cell disruption) and fish age, suggest that it is most likely that oocytes in the testis of male roach arise as a consequence of long term exposure to oestrogenic chemicals.

7 The utility of vitellogenin induction as a biomarker for general health effects in native UK riverine fish (Objective 5)

It is well established that VTG induction can be used as a biomarker of oestrogen exposure in fish (Hansen et al. 1998; Jones et al. 2000). Male fish exposed to oestrogenic stimuli can produce concentrations of circulating VTG similar to those found in mature females (Maitre et al. 1985; Leguellec et al. 1988). Very high levels of VTG synthesis have been shown to induce adverse health effects, including kidney failure (Herman and Kincaid 1988). In this study the health effects of lower levels of VTG induction were investigated.

The kidney is involved in the homeostatic control of fluid and salt balance in fish. The kidney is also the route of elimination of VTG and other protein metabolites from the body and VTG and its products of degradation can accumulate in the tubules and Bowman's capsule. The teleost kidney is an elongate dark red organ located ventral to the spine and above the swim bladder. The anterior or head kidney of fish has a haematopoietic role, similar to that of the bone marrow in higher vertebrates. Endocrine and lymphoid tissue is also present in the head kidney. The posterior or trunk kidney is principally involved in filtering the blood plasma for the excretion of metabolic waste products and the control of osmotic balance (Powell 2000). The trunk kidney is composed of nephrons surrounded by haematopoietic and lymphoid tissue. The nephron is the individual structural and functional unit of the vertebrate kidney The nephron generally has a highly vascularised renal corpuscle or glomerulus, a ciliated neck segment, two proximal segments and a distal segment leading to a collecting duct system (Reimschuessel 2001). The glomerulus is the principal site of ultra-filtration and urine formation (Yokota et al. 1985). The glomerulus is enveloped by the Bowman's capsule which is connected to the neck of the tubule. The proximal tubule is the principal site of re-absorption of filtrate. Several sugars, low molecular weight proteins and most amino acids are reabsorbed in this section of the tubule. Re-absorption and secretion of ions into the lumen occurs in both the proximal and distal tubule sections (See Figure 27 for a simplified diagram of the fish nephron).

In fish, injured nephrons have the capacity for repair following toxicant induced damage. Injured epithelial cells may be replaced provided the basement membrane of the nephron remains intact (Reimschuessel 2001). The process of renal regeneration in the fish nephron follows an initial phase of cell death and denuding of the basement membrane. A flattened basophilic epithelium repopulates the denuded membrane (Reimschuessel et al. 1990). Fish also have also been demonstrated to have the ability to develop new nephrons after renal damage; *de novo* nephron neogenesis (Reimschuessel et al. 1990;

Reimschuessel et al. 1993) and that this process can occur after repeated renal damage (Salice et al. 2001). During the process of *de novo* nephron neogenesis, a cluster of basophilic cells (Basophilic cluster; BC) forms and develops a cavity to produce the renal vesicle. This grows and becomes first C-shaped and then S-shaped. One end of the S shape indents further and forms the glomerulus and Bowman's capsule, whilst the other end grows out further and fuses with the archinephric (collecting) duct (Figure 28). The tubules elongate, coil and intertwine as the nephron develops (Reimschuessel 2001).

After nephrotoxic damage the number of developing nephrons (DN) has been shown to increase. This effect has now been reported many fish species at various life stages [(rainbow trout (Reimschuessel et al. 1993), goldfish (Reimschuessel et al. 1990), tilapia (Augusto et al. 1996) and the zebrafish (Reimschuessel 2001)]. Exposure to a variety of renal toxicants have been demonstrated to induce this response, including hexachlorobutadine (Reimschuessel et al. 1990), gentamicin (Augusto et al. 1996; Salice et al. 2001) and tetrachloroethylene (Reimschuessel et al. 1993). Field evidence indicates that wild fish sampled from sites of known contamination have higher numbers of BCs and DNs than fish of the same species taken from reference sites (Cormier et al. 1995). It has been proposed that BCs and DNs may be useful biomarkers for assessing nephrotoxicity (Cormier et al. 1995).

Early life stages of organisms are generally considered most sensitive to disruption by toxicants, notably during the period of organogenesis. The aims of this phase of the study were to identify any changes in the kidneys of fish exposed to effluent during early life and to establish if this disruption correlated with VTG induction. Some fish exposed to the WwTW effluent were then subject to depuration to investigate if any of the induced changes were fixed. Parameters of the kidney investigated included changes in gross morphology of the kidney, tubule diameter, occurrence of; glomeruli, basophilic clusters and developing nephrons.

Immunohistochemistry was further employed to examine for the presence of VTG accumulation in both somatic (kidney, liver, intestine etc.) and in gonadal tissues. Assessments were also made to establish if alterations in gonad development correlated with VTG induction.

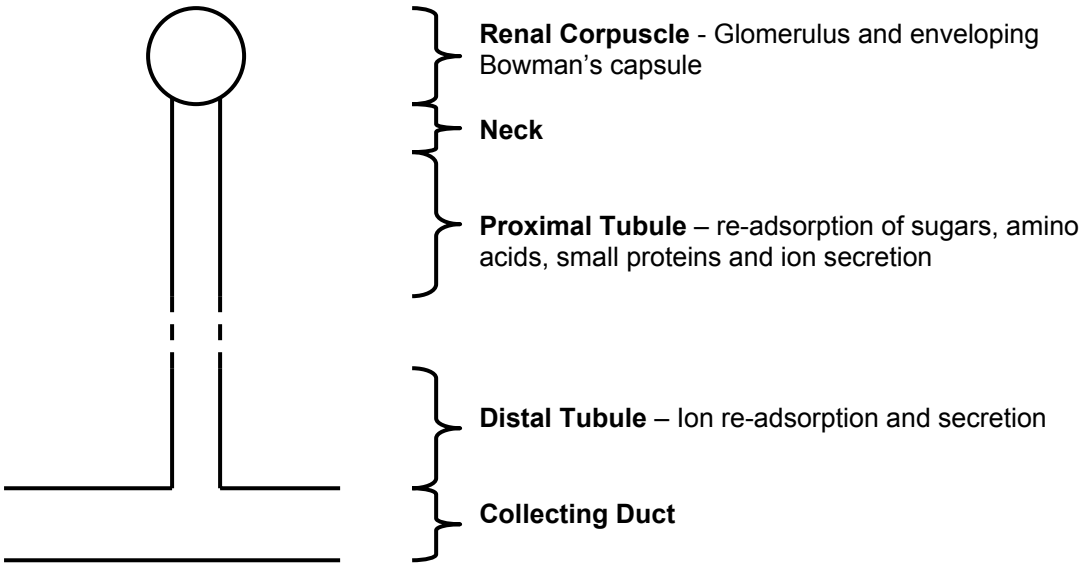

Figure 27. Simplified diagram of a typical freshwater fish nephron (adapted from (Elgar 2000)

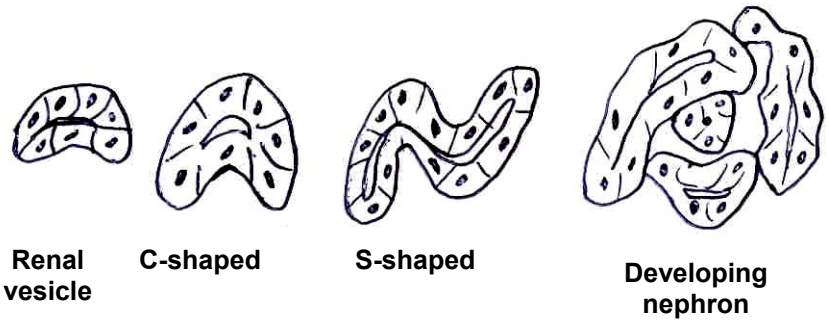

Figure 28. Development of the renal tubule. A cavity develops within a solid mass of basophilic cells forming the renal vesicle. This grows and first becomes C-shaped in form and then S-shaped. One end of the S-shaped structure indents further and forms the glomerulus and Bowman's capsule, whilst the distal end extends and fuses with the archinephric (collecting) duct. The tubules elongate, coil and inter twine as the nephron develops (Reimschuessel 2001).

7.1 Overall Experimental design

Histological samples were obtained from roach from the early life stage exposure to WwTW effluent detailed under the work in objectives 1 and 2. In brief, fertilised roach eggs were deployed into graded concentrations in mesocosm systems at the two study WwTW and at 60dph, 60 fish from each exposure group were transferred to clean water for depuration. Fish were sampled and processed for histological analysis at 200dph (both WwTW) and 300dph (Site B WwTW, and depurated fish from both sites).

7.1.1 Kidney histomorphology:

Whole body transverse tissue blocks were prepared by cutting the fish trunk either side of the dorsal fin in order to obtain a section through the trunk kidney. Samples were then embedded in paraffin wax, sectioned at 5 μm, mounted and stained with haematoxylin and eosin. Two sections of each sample were prepared for analysis by light microscopy. Each section was assessed for any abnormalities and the numbers of glomeruli, developing nephrons and basophilic clusters counted over the whole kidney cross-section. Kidney tubule diameter was measured for 10 nephrons in each kidney section. Measurements were taken using image analysis software (analySIS® v3.2, Soft Imaging System, GmHB).

7.1.2 Vitellogenin Immunohistochemistry:

Immunohistochemistry allows the identification of cell bound antigens *in situ* by means of a specific antigen-antibody reaction tagged with a visible label. Immunohistochemistry was used to detect the presence of VTG in whole body section of juvenile roach using a carp-VTG polyclonal antibody (which has been validated for use on the roach (Tyler et al. 1996). Tissue was incubated with the 1st (unlabelled) antibody (anti carp-VTG, raised in rabbit) and a labeled 2nd antibody applied (Goat anti-rabbit IgG, conjugated with peroxidase). The horseradish peroxidase label splits hydrogen peroxide and the oxygen released converts a colorless chromagen (in this case 3,3'-Diaminobenzidine (DAB) into a coloured end product (in this case a brown precipitate). The location of the brown staining gives the location of the VTG antigens in the tissue.

For immunohistochemistry, glass microscope slides were treated with Vectabond (Vector Laboratories, UK) to aid attachment of paraffin sections to the slide and prevent detachment during processing. Whole body sections of juvenile roach were cut at 5 μm, applied to treated slides at 3 sections per slide and heated at 55°C for 30 minutes. The protocol for VTG immunohistochemical staining is given in Table 5. Sections were then re-hydrated thorough a series of graded alcohols, water and washing buffer (0.01M Sodium phosphate buffer containing 0.9% NaCl (9 g/L), pH 7.4). Blocking buffer (washing buffer containing 1% bovine serum albumin) was then applied to sections. A solution of rabbit anti-carp VTG antibody (1:1000 in blocking buffer) and 0.03% hydrogen peroxide was applied to 2 sections on the slide at 20 μL/section. The hydrogen peroxide acts to block endogenous peroxidase activity in the tissue. A solution of blocking buffer and 0.03% hydrogen peroxide was applied to the third section on each slide to demonstrate any non-specific binding of antibody. Slides were incubated for 2 hours at 37°C. Slides were then washed twice in washing buffer to remove unbound primary antibody. Goat anti-rabbit IgG peroxidase conjugated second antibody (Sigma, 1:100 in blocking buffer) was then applied to all sections at 20 μL/section and the slides incubated for 1 hour at 37°C. Slides were then washed twice in washing buffer to remove unbound 2nd antibody. DAB and hydrogen peroxide solution was then applied (Sigma Fast 3,3'-Diaminobenzidine (DAB) kit (Sigma, UK) was made just prior to application - 1 DAB and 1 substrate tablet were added to 1 mL distilled water. Dissolved tablets gave a solution comprised of 0.06M Tris buffer, 1.6 mg/mL

urea hydrogen peroxidase and 0.7 mg/mL DAB). After approximately 5 minutes, slides were placed in water to stop the colour reaction. Sections were then counterstained with Haematoxylin, dehydrated and mounted with DPX mountant.

Immunohistochemistry was carried out on 5 whole body sections from 5 male and 5 female fish sampled from control and 100% effluent treatments at Site A and control and 80% effluent treatments at Site B (the highest effluent exposure concentration at this site). Thus, in total 200 sections were analyzed using immunohistochemistry.

7.1.3 Statistical Analyses

All statistical analyses were carried out using Sigmastat v2.0 (Jandel Scientific). Statistical significance was accepted at $p<0.05$ for all comparisons. Inter-group differences were assessed using one-way ANOVA (parametric, for normalised data) or Kruskal-Wallis test (non-parametric). Multiple comparison tests were performed using post-hoc analyses for parametric or non-parametric data.

Solution	Temperature	Immersion Time	Function
Histoclear	RT	15 minutes	Rehydration of tissue
100% IMS		2 minutes	
90% IMS		2 minutes	
70% IMS		2 minutes	
Water		2 minutes	
Washing buffer		5 minutes	Stabilize pH to allow antibody to function
Blocking buffer		10 minutes	Block endogenous antibodies in the tissue
1st Antibody; anti-cVTG (1:1000) and 0.03% H_2O_2	37°C	2 hours	Antibody binds to VTG. H_2O_2 blocks endogenous peroxidases
Washing Buffer	RT	5 minutes	Remove unbound 1st antibody
Washing Buffer		5 minutes	
2nd Antibody; anti-rabbit IgG (1:2000)	37°C	1 hour	Binds to 1st antibody. Labeled with peroxidase
Washing Buffer	RT	5 minutes	Remove unbound 2nd antibody
Washing Buffer		5 minutes	
DAB and H_2O_2		5 minutes	Colour reaction
Water		5 minutes	Stop reaction
Haematoxylin		3 minutes	Counterstain
Water		10 minutes	Wash
1% Hydrochloric Acid in IMS		40 seconds	Resolves stain
Water		20 seconds	Wash
Saturated Li_2CO_3 solution		10 seconds	Bluing agent – Raises pH to achieve purple colour
Water		20 seconds	Wash
70% IMS		2 minutes	Dehydration of tissue
90% IMS		2 minutes	
100% IMS		5 minutes	
Histoclear		5 minutes	

Table 5. Immunohistochemistry Protocol

7.2 Results

7.2.1 Kidney histopathology

Plate 39 shows a typical cross-section of a 200dph roach. The trunk kidney is saddle shaped and positioned above the swim bladder. Kidney tubules are surrounded by haematopoietic tissue (Plate 40a). In controls, kidney tubules and glomeruli in all samples appeared normal in structure (Plate 40b and 41a). There was no evidence of degeneration, haemorrhage or accumulation of eosinophilic material in the tubule lumen. There were no observable differences in renal blood vessel structure between control and effluent exposed fish.

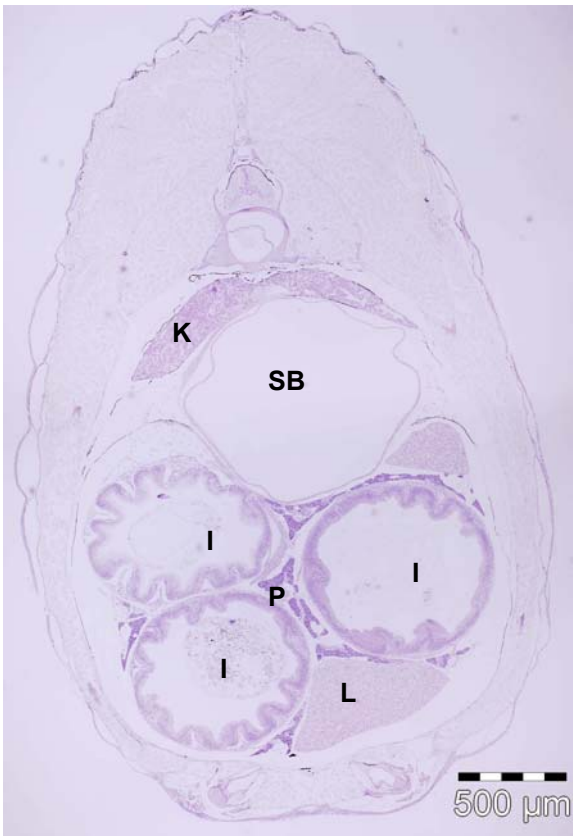

Plate 39. Photomicrograph of a transverse section through the mid-portion of a 200dph roach. The plate shows the position of the kidney (K), swim bladder (SB), liver (L), intestine (I) and pancreatic tissue (P).

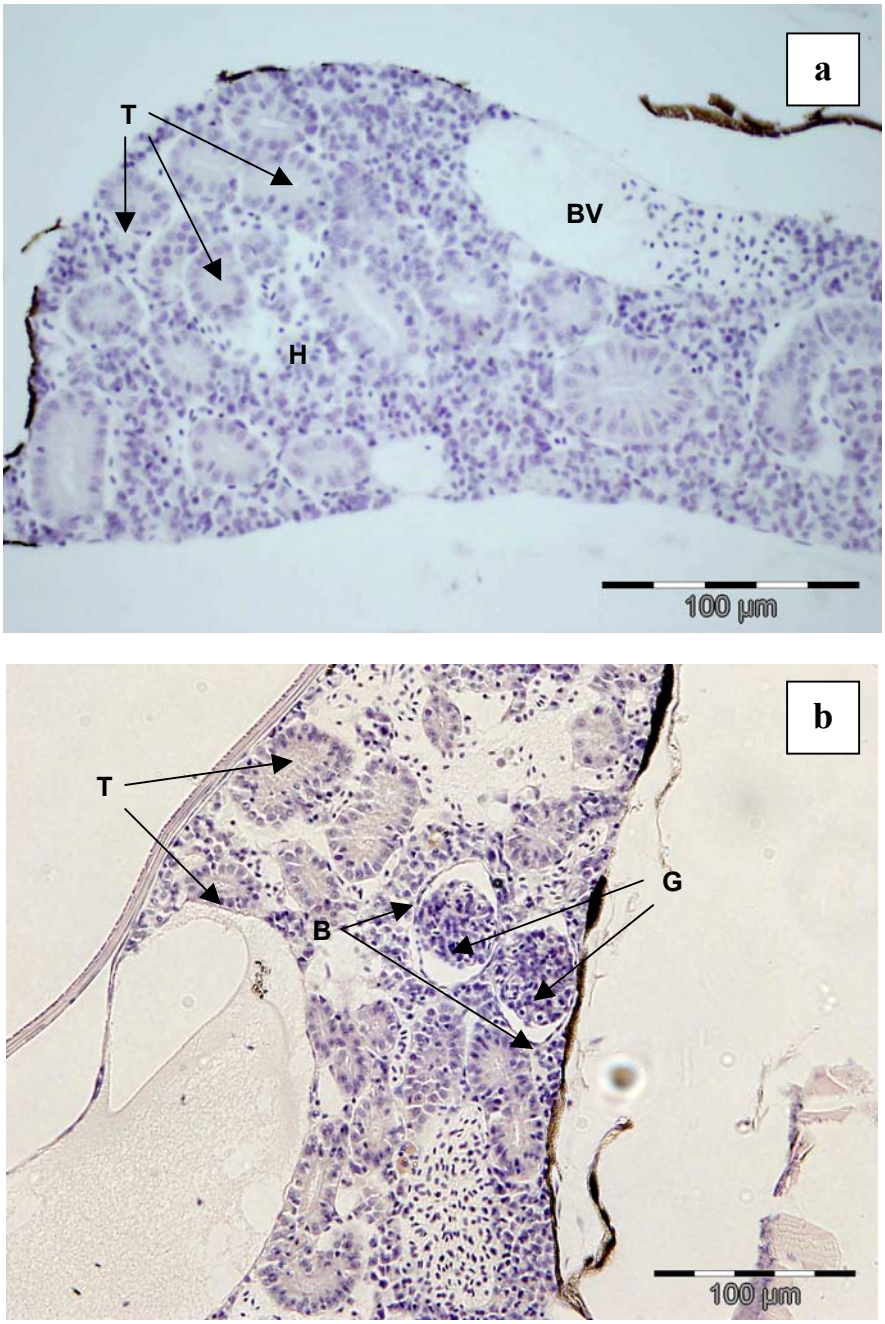

Plate 40. Photomicrographs of transverse sections through the trunk kidney of **(a)** 200dph roach, showing transverse renal tubules (T) embedded in haematopoietic tissue (H). a blood vessel is also visible (BV) and **(b)** 300dph roach showing transverse renal tubules (T), glomeruli and associated Bowman's capsules (B).

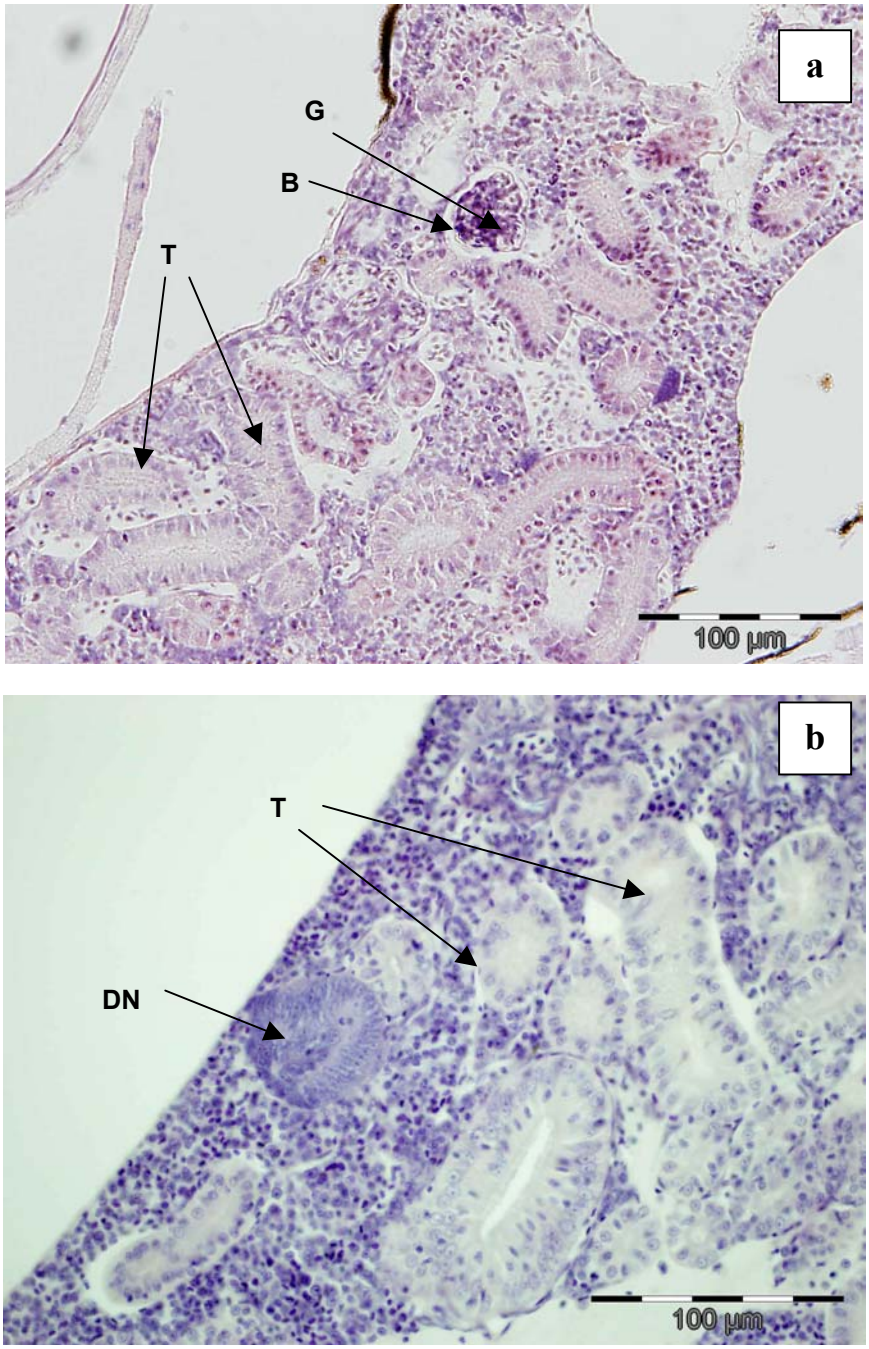

Plate 41. Photomicrographs of transverse sections through the trunk kidney of **(a)** 200dph roach, showing transverse renal tubules and longitudinal tubules (T), and a glomerulus (G) and Bowman's capsule (B), **(b)** 300dph roach showing a developing nephron (DN) in the C-shaped stage of development, lying amongst normal mature tubules (T).

Tubule diameter: At 200dph mean tubule diameter of roach nephrons had increased in a concentration-dependant manner with increasing effluent concentrations at both sites. Tubule diameter was larger in every effluent concentration (at both sites) compared with controls ($p<0.001$). At Site A, fish in the river water also had a wider tubule diameter compared with the control fish at this site ($p<0.05$, see Figure 29). Within each treatment group and in controls, there were no gender specific or size related differences. At 300dph at Site B tubule diameter of nephrons had increased in a concentration-dependant manner with increasing effluent concentration. Tubule diameter in all effluent

concentrations was greater than in the control ($p<0.001$, see Figure 29). A concentration-dependent enhancement of nephron tubule diameter was also observed in fish exposed to effluent at both sites from fertilization to 60dph and then depurated for 240 days. Fish exposed to effluent concentrations of 20% and higher had significantly larger tubule diameters than in the control fish at both sites ($p<0.05$). Within each treatment group and in the controls there were no gender specific or size related differences.

Kidney Glomeruli: Glomeruli and associated Bowman's capsules were observed in many of the trunk regions of the kidney cross-sections. In controls there was no evidence of any disruption, haemorrhage, or accumulation of eosinophilic material in the Bowman's capsule. All glomeruli observed were considered to be normal and healthy. There were no gender specific or size related differences in the occurrence/appearance of glomeruli. At 200dph, there was no significance difference in occurrence of kidney glomeruli between control and effluent exposed fish at Site A. There was a concentration-dependant increase in the number of glomeruli in the roach at Site B. However, this was only significant from the controls for the 100% effluent exposure treatment group ($p<0.05$, Figure 30). At 300dph, at Site B there appeared to be a concentration-dependant increase in glomeruli, but this was not statistically significant (Figure 30). Depurated fish derived from the exposures at Site A appeared to exhibit a concentration dependant increase in the number of glomeruli, however this was only significant for the 100% effluent treatment group. There was no significant difference between the control and treatment groups of depurated fish from Site B (Figure 30).

Basophilic Clusters and Developing Nephrons: At 200dph at Site A, there were no differences in occurrence of BCs or DNs between control and effluent exposed fish. In 200dph roach at Site B there were no differences in occurrence of DNs between the different treatment groups and controls. In the roach at Site B, however, there was a higher occurrence of BCs in the 80% effluent exposed group compared with the control (Figure 31). At 300dph for the roach at Site B there was a higher occurrence of BCs in the 40% effluent group (Plate 42, $p<0.05$), but not in the 80% effluent exposed fish. There was a higher occurrence of DNs in the 80% effluent exposed fish ($p<0.05$) but not in lower effluent concentration treatment groups (Figure 31). There were no differences in the occurrence of BCs or DNs between effluent exposed and controls in the depurated fish (See Figure 31). There were no gender specific or size related differences in the occurrence of BCs and DNs.

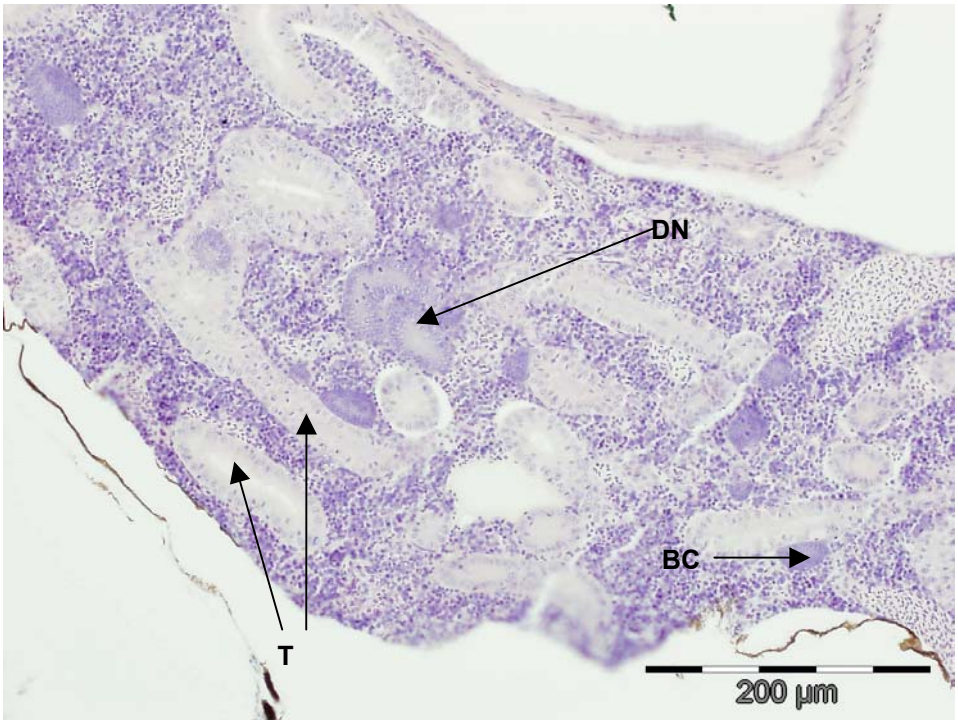

Plate 42. Photomicrograph of a transverse section through the trunk kidney of a 300dph roach exposed to effluent for 300 days at Site B. Several basophilic clusters (BC) and developing nephrons (DN) were present at various stages of development, amongst mature tubules (T).

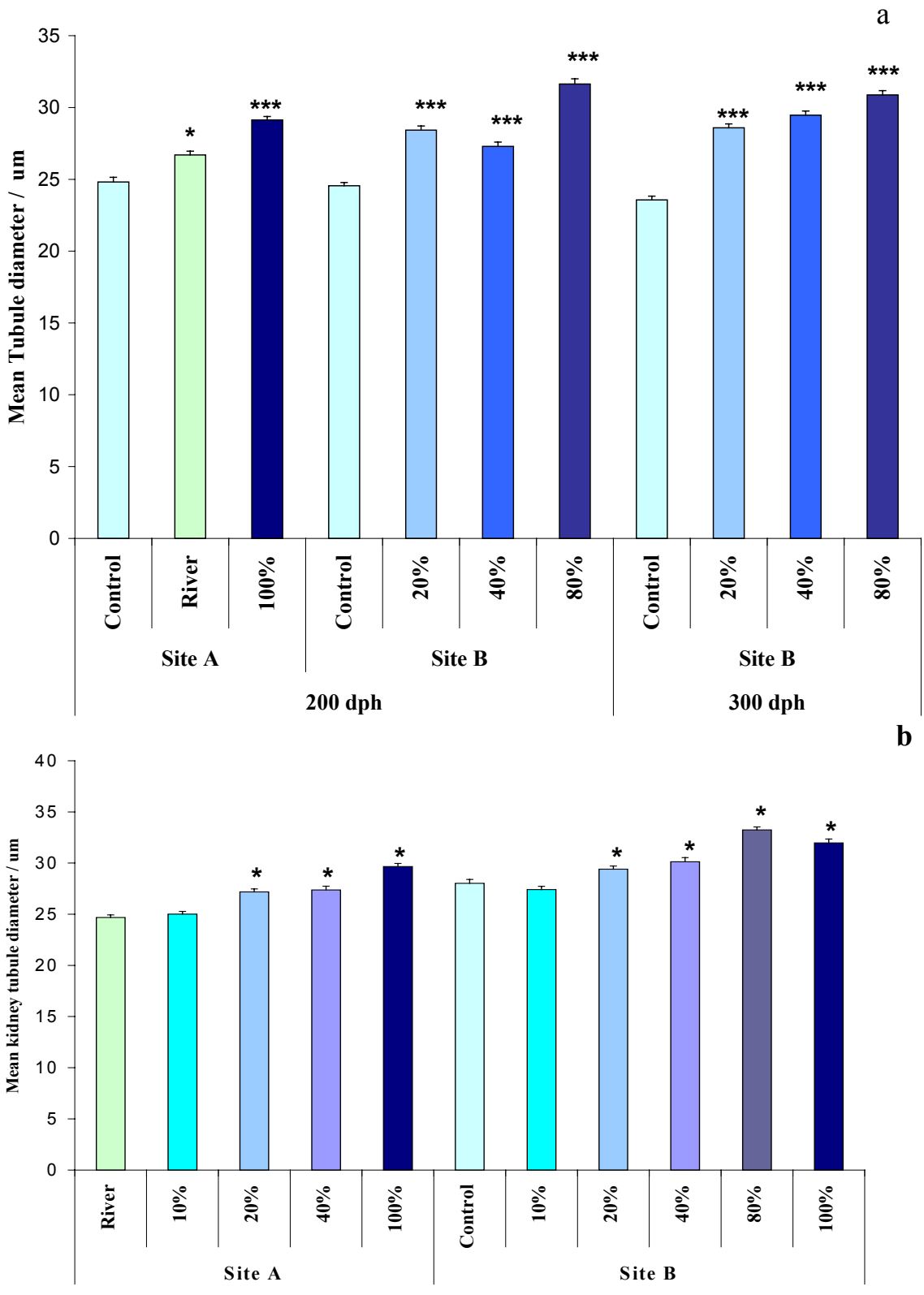

Figure 29. Kidney tubule diameter of early life stage roach (+ standard error of the mean) **(a)** Roach exposed from fertilization to 200dph (Sites A and B) and 300dph (Site B) **(b)** 300dph roach exposed from fertilization to 60dph to WwTWs effluent at both study sites and depurated in clean water for 240 days. Asterisks denote significance from control * $p<0.05$, *** $p<0.001$

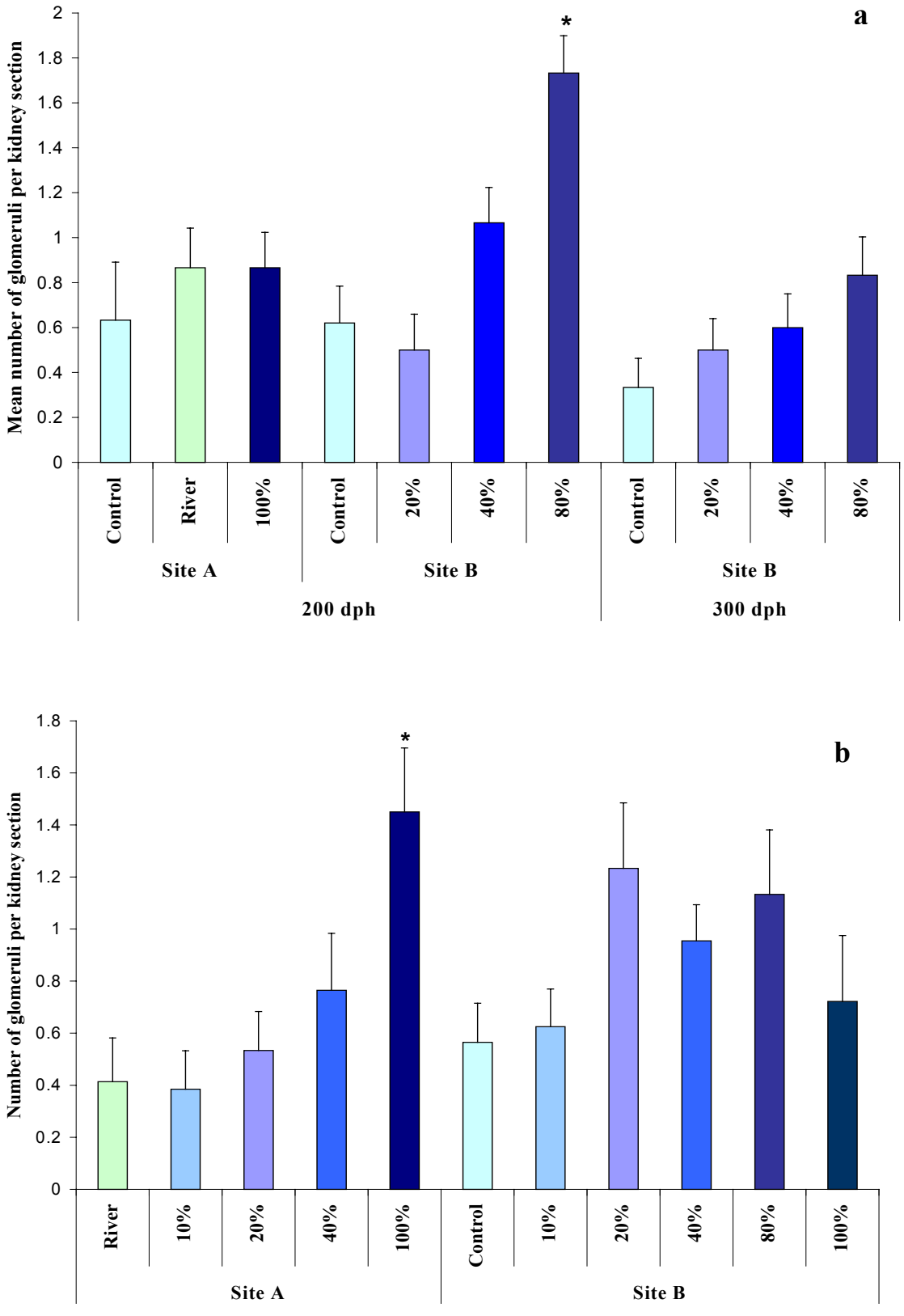

Figure 30. Number of glomeruli per kidney section in early life stage roach (+standard error of the mean). **(a)** Roach exposed to effluent from fertilization to 200dph (Site A and Site B) and to 300dph (Site B) **(b)** 300dph fish, exposed from fertilization to 60dph to WwTW effluent at both sites and depurated in clean water for 240 days. Asterisks denote significance from control * $p<0.05$, ***$p<0.001$

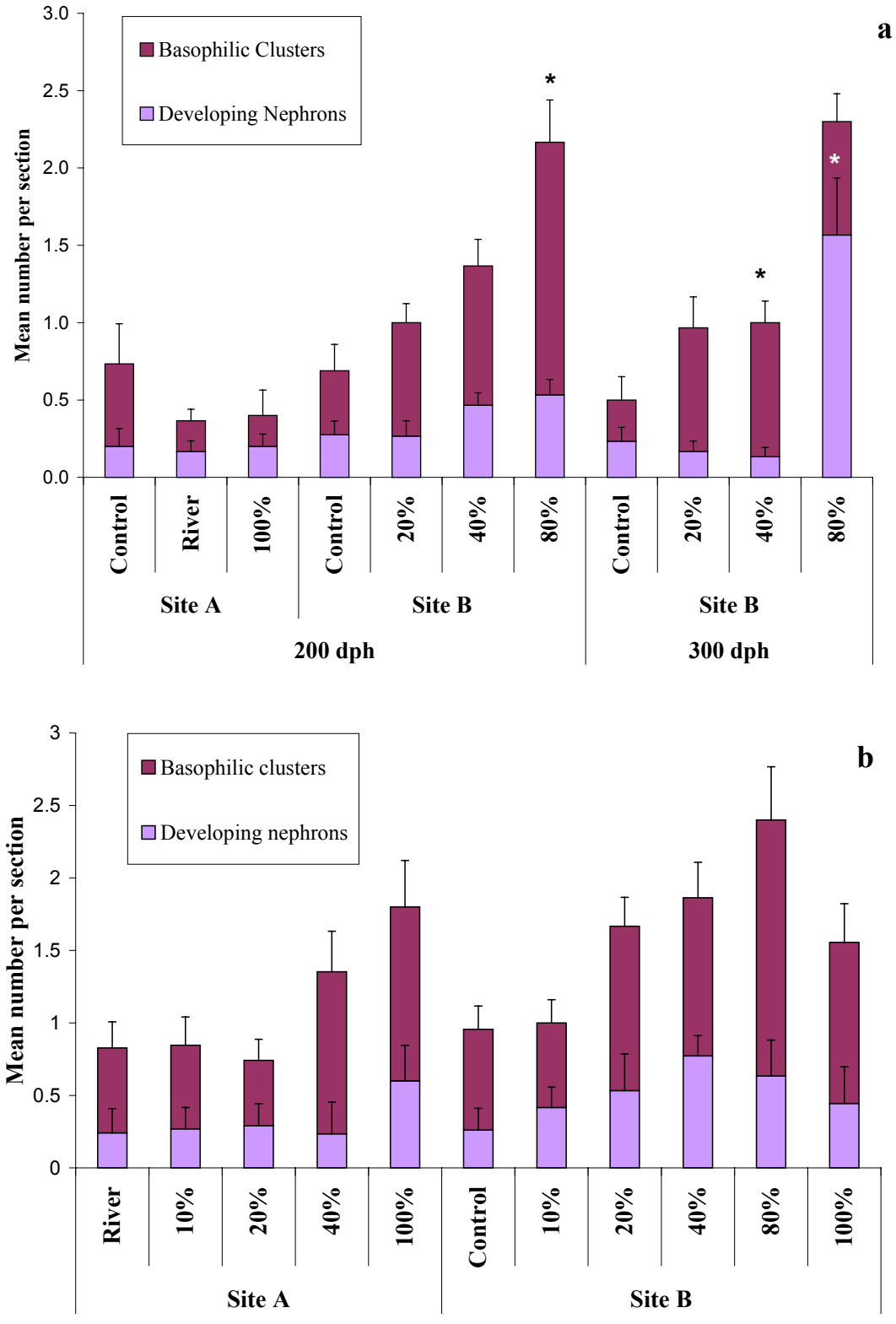

Figure 31. Number of basophilic clusters and developing nephrons per kidney section in roach exposed to the study WwTW effluents (+standard error of the mean). **(a)** Roach exposed from fertilization to 200dph (Site A and Site B) and to 300dph (Site B) **(b)** 300dph roach exposed from fertilization to 60dph to WwTW effluent at both study sites and depurated in clean water for 240 days. Asterisks denote significance from control * $p<0.05$.

In summary, there was an effect of effluent exposure on nephrogenesis (BCs and/or DNs) in the highest effluent exposure group (80%) at Site B at 200 and 300dph. For depurated fish long term effects of effluent exposures on kidney development occurred for the roach exposed to full strength effluent at Site A, and for the 80% effluent at Site B.

7.2.2 Localisation of Vitellogenin in body tissues of WwTW effluent exposed roach

The concentrations of VTG in whole body homogenates of 30 fish from each of the immunohistochemistry treatment groups is shown in Table 6. In brief, control fish had very low VTG concentrations. Fish exposed to the WwTW effluent at Site A effluent had a measured VTG concentration approximately three times higher than the VTG concentration in fish exposed at Site B.

Site	Age	Exposure group	Mean VTG ng/ml (+/- std error)
Site A	200dph	Control	42 +/- 8
		100% Effluent	5684 +/-545
Site B	300dph	Control	33 +/- 8
		80% Effluent	1709+/-211

Table 6 – Measured VTG concentrations of whole body homogenates of ELS fish

In controls, VTG was not detected on any of the sections in any tissue in either male or female fish (the detection capability for the immunolocalisation technique employed is approximately 100ng). This corresponded with VTG measurements for whole body homogenates and the status of sexual development in females (pre-vitellogenesis).

In effluent exposed fish from both sites VTG was detected in all sections. DAB staining on the sections in roach derived from the Site A was stronger than in fish derived from Site B, reflecting the higher VTG levels in fish sampled at this site. There were no apparent differences in level or location of VTG between effluent exposed male and female fish.

In effluent exposed fish at both sites VTG was detected in the liver, its site of synthesis, and in particular in hepatic blood vessels (Plate 43). VTG was observed to accumulate in the kidneys specifically around the tubules. This was most apparent in fish exposed to the effluent at Site A. There was no evidence of accumulation of VTG within the kidney tubule lumen or Bowman's capsule as has been reported in previous studies (Folmar et al. 2001). There was little VTG accumulation in the kidney of fish exposed to effluent at Site B (Plate 44).

In the ovary and testis of effluent exposed fish VTG was detected in the blood vessels and between the germ cells (oocytes in the ovary; Plate 45, and spermatogonia in the testis; Plate 46). VTG was not more concentrated in the gonads compared with the somatic tissues studied.

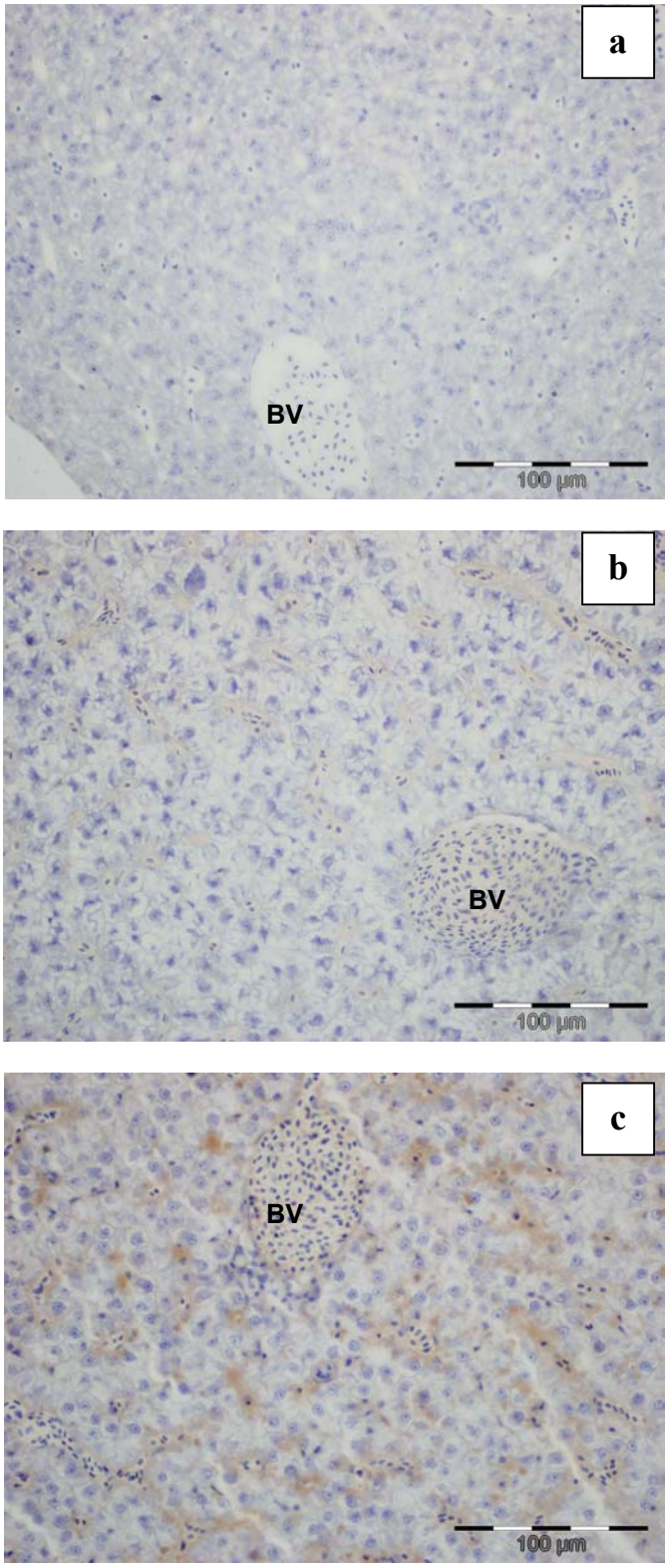

Plate 43. Photomicrographs of liver sections immunostained with VTG antibody; **(a)** Control fish at 200dph, **(b)** Fish exposed to 80% effluent at Site B for 300 days **(c)** Fish exposed to 100% effluent at Site A for 200 days. Blood vessels (BV) are shown on each of the plates. Location of VTG is indicated by the brown staining (Haematoxylin counterstain).

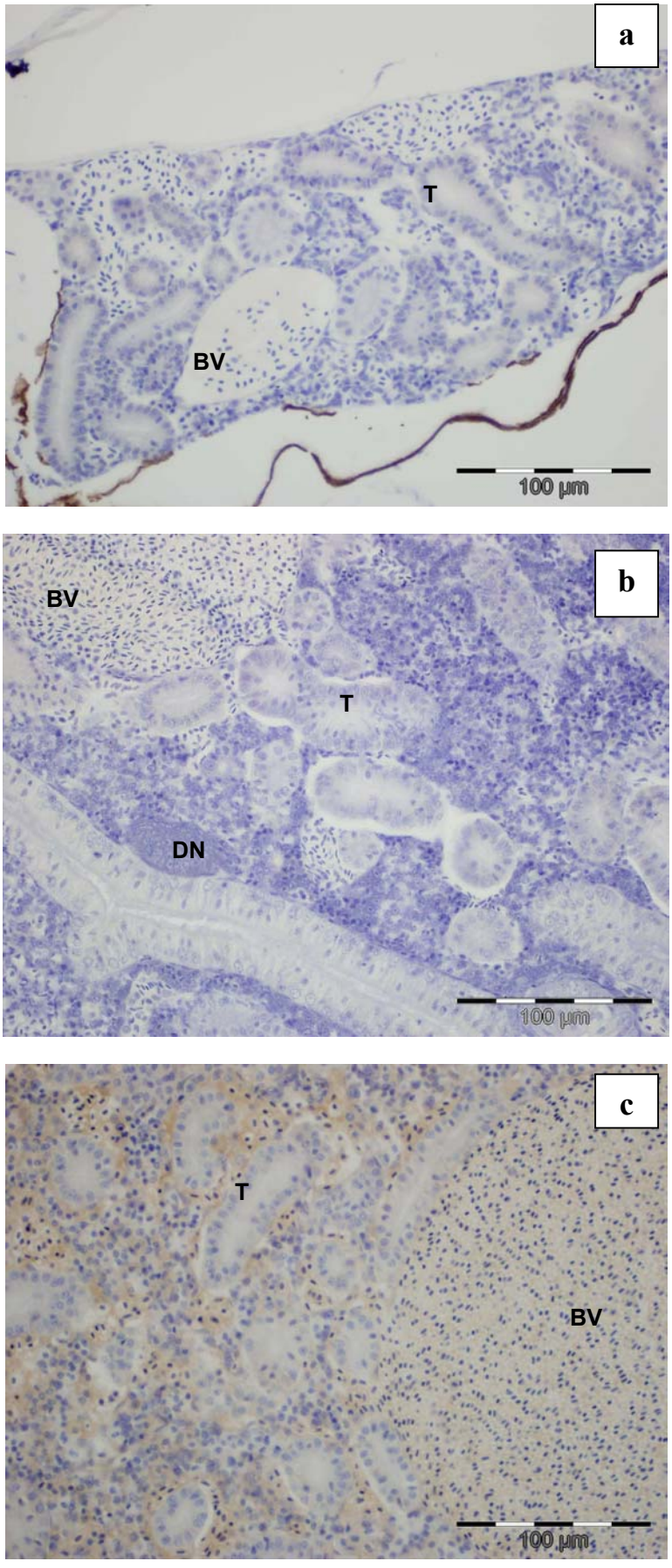

Plate 44. Photomicrographs of kidney sections immuno-stained with VTG antibody; **(a)** Control fish at 200dph, **(b)** Fish exposed to 80% effluent at Site B for 300days **(c)** Fish exposed to 100% effluent at Site A for 200days. Blood vessels (BV) and kidney tubules (T) are indicated on each of the plates. A developing nephron (DN) is present in section (b). Location of VTG is indicated by the brown staining (Haematoxylin counter stain).

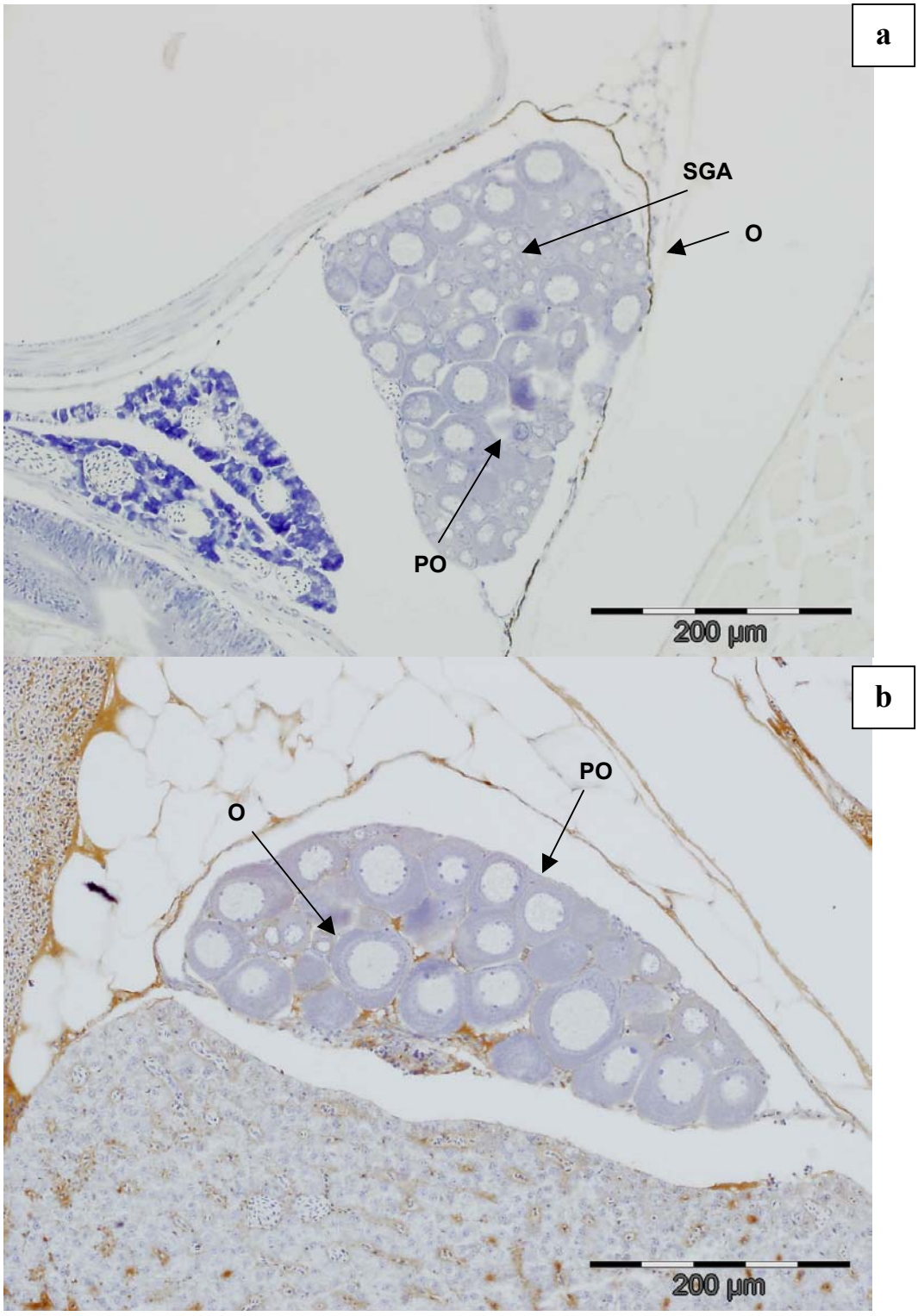

Plate 45. Photomicrographs of ovary sections immunostained with VTG antibody; **(a)** Control female, **(b)** Female exposed to 100% effluent at Site A. Both ovaries contain primary oocytes (PO) and oogonia (O). Location of VTG is indicated by the brown staining (Haematoxylin counterstain).

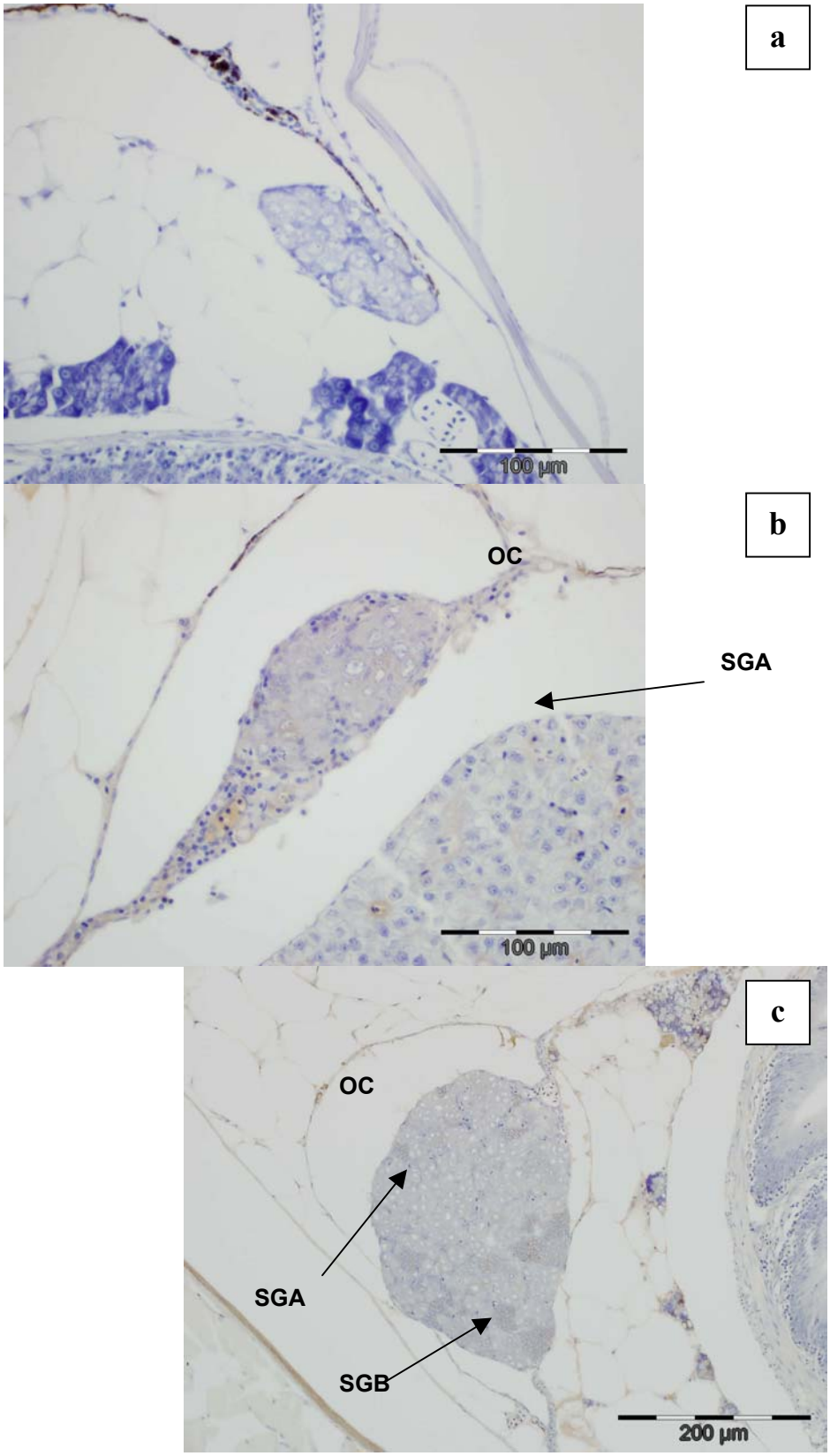

Plate 46. Photomicrographs of testis sections at 200dph immunostained with VTG antibody; **(a)** Control male, testis contains presumptive spermatogonia A (SGA), **(b)** Male exposed to 100% effluent from Site A, SGA present, **(c)** a more advanced male exposed to 100% effluent at Site A. The testis contains SGA and spermatogonia B (SGB). Both effluent exposed males have feminised reproductive ducts (ovarian cavity; OC). Location of VTG is indicated by the brown staining (Haematoxylin counterstain).

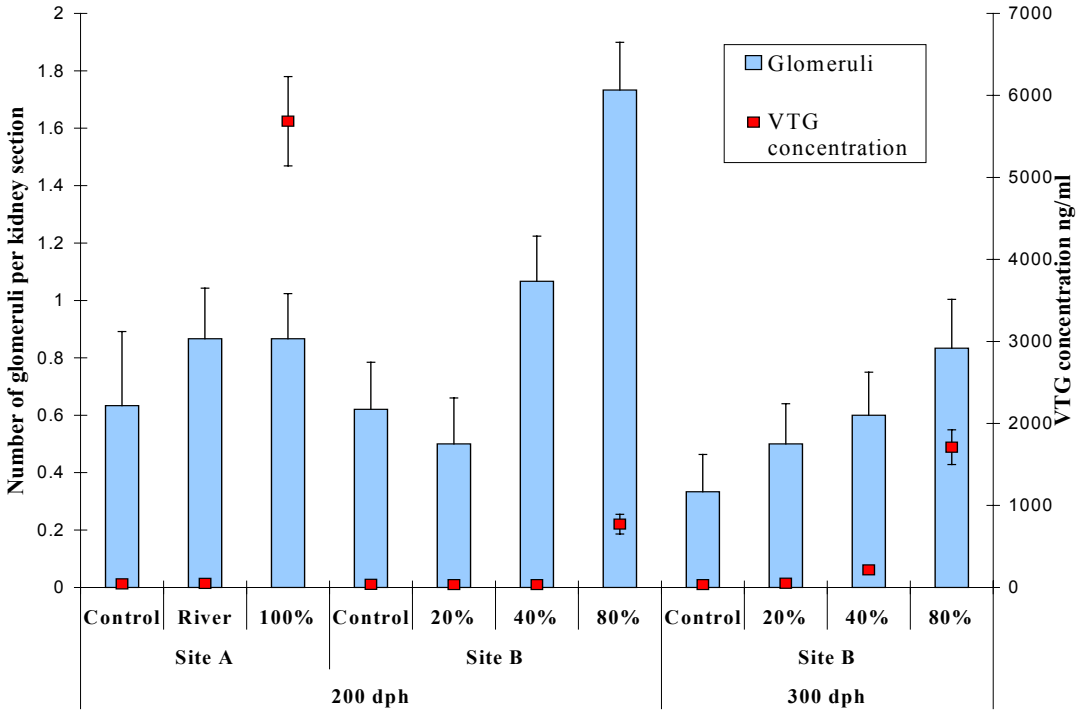

Figure 32. Vitellogenin concentrations in whole body homogenates and numbers of glomeruli per kidney section in roach exposed to WwTW effluents for 200days (Site A and Site B) and 300 days (Site B).

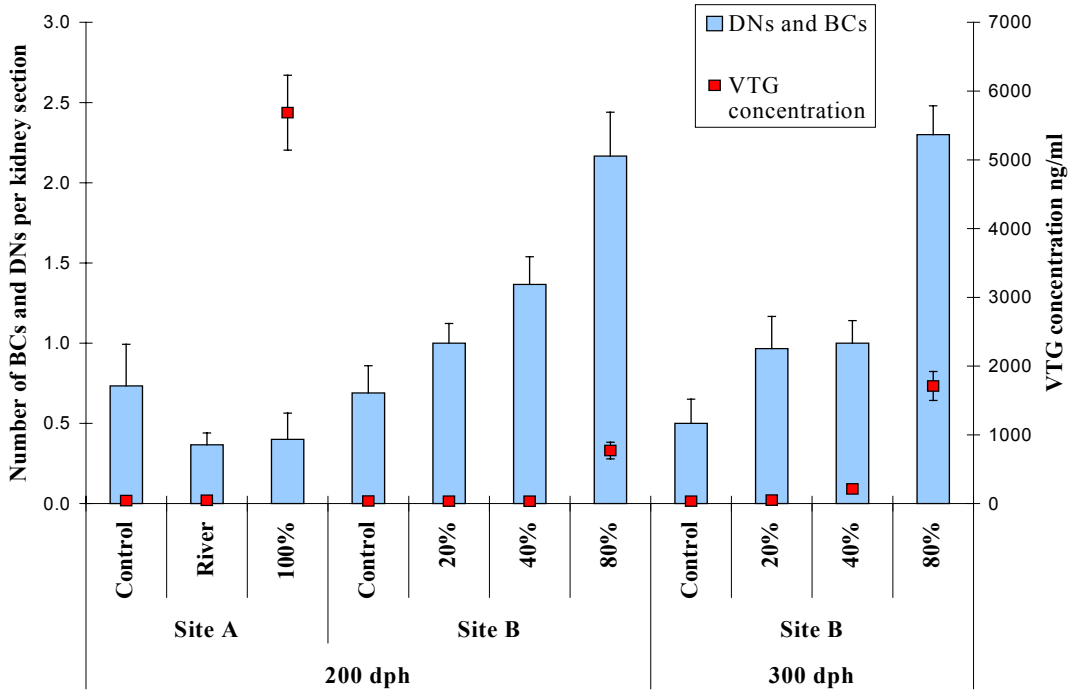

Figure 33. Vitellogenin concentrations in whole body homogenates and numbers of basophilic clusters and developing nephrons per kidney section in roach exposed to WwTW effluents for 200days (Site A and Site B) and 300 days (Site B).

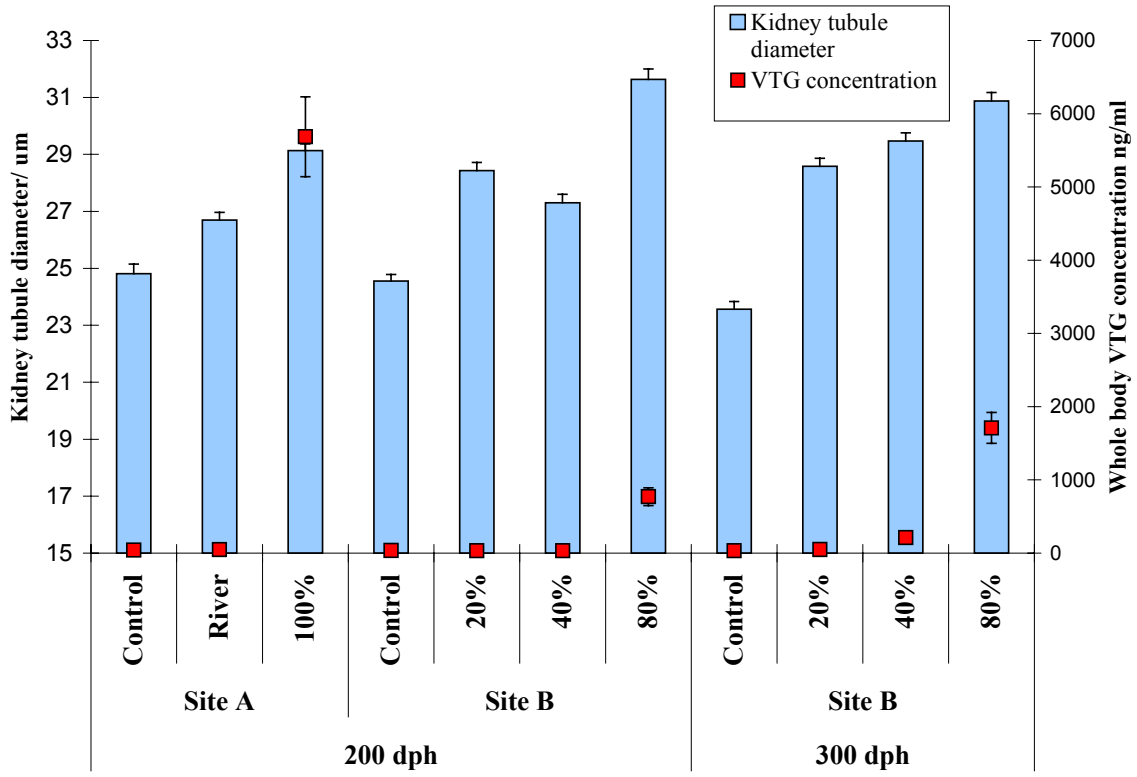

Figure 34. Vitellogenin concentrations in whole body homogenates and kidney tubule diameter in roach exposed to WwTW effluents for 200days (Site A and Site B) and 300 days (Site B).

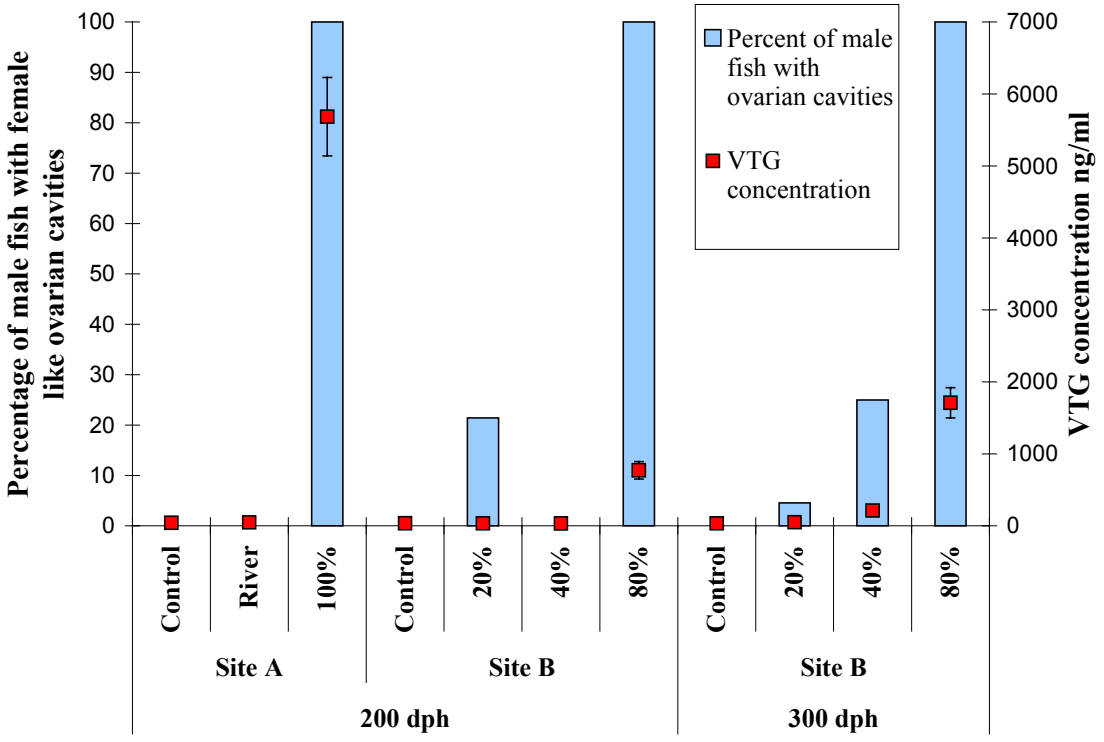

Figure 35. Vitellogenin concentrations in whole body homogenates and percentage of male fish with an ovarian cavity in roach exposed to WwTW effluents for 200days (Site A and Site B) and 300 days (Site B).

Vitellogenin induction compared with body tissue effects

In fish exposed to the WwTW, increased VTG induction was associated with increased numbers of glomeruli, (Site B) increases in size of kidney tubules, and higher numbers of BCs and DNs. For effluent concentrations inducing an ovarian cavity in male fish there was an associated elevation in VTG in these exposure groups.

7.3 Discussion

There were clear effects of effluent exposure on kidney development and links between some for the effects seen and VTG induction. Tubule diameter was greater in fish exposed to effluent compared with controls and this was linked with higher concentrations of VTG. The fish exposed to effluent at Site A however, had a mean VTG concentration three times higher than the fish at Site B, but their kidney tubules were narrower in dimension. This implies that VTG and other oestrogen inducible proteins are not necessarily the only contributing factor in kidney tubule size; WwTW effluents are complex mixture of chemicals, a number of which may impact on the kidney. Vitellogenin was observed by immunohistochemistry to accumulate in the kidneys of fish exposed to both WwTW effluents. However, of the renal health parameters investigated, fish at Site B (where the level of VTG induction was lower compared with Site A) were observed to have higher incidence of the renal repair and de novo nephrogenesis. This would further suggest that this renal damage is not a function of VTG induction, but as a consequence of other factors contained within the effluent. The higher incidence of glomeruli in the kidney of fish exposed to the higher effluent concentrations is likely to be as a consequence for the greater blood filtration rates required to clear VTG from the circulation. Immunohistochemistry demonstrated that VTG induced by effluent exposure permeated both somatic and gonadal tissues in both males and females. The females studied were immature and the ovaries had not entered vitellogenesis and thus the developing oocytes were not at a stage of development capable of sequestering VTG. We do not know what the consequences of VTG accumulation in non-target organs other than the kidney would be, but it is likely to induce disruptions in osmotic balance and thus exchange in electrolytes between the blood and tissues. These studies on the kidney provide no evidence of severe renal damage in any of the fish exposed to effluent from either site. Furthermore, a clear capacity for renal repair was shown on removal of roach from the oestrogenic effluents. This would suggest that short-term exposures to the effluents are unlikely to have major health consequences at the level of the kidney.

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