The Identification of Oestrogenic Effects in Wild Fish – Phase II

R&D Technical Report W2-014/TR

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Key words
Sewage effluent, fish, roach, gudgeon, fathead minnow, rainbow trout, oestrogen, endocrine disruption, reproduction, steroid, alkylphenol, nonylphenol.

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FOREWORD

This report represents the concerted research undertaken over a 4-year period from 1997 to 2001 into the causes and consequences of oestrogenic effects in fish. It follows on from a field survey programme from 1995 to 1997 that identified the widespread nature of oestrogenic effects in wild fish in English rivers.

The research was a collaborative programme between Brunel University, Exeter University, Brixham Environmental Laboratory, Sheffield University, Essex and Suffolk Water and the Environment Agency. Chemical analyses of effluents were contracted to the Centre for Environment, Fisheries and Aquaculture Sciences

The primary investigators were Professor John Sumpter and Dr Susan Jobling (Brunel University) and Professor Charles Tyler (Exeter University, formerly from Brunel University). The research team comprised, Dr Karen Thorpe, Dr Ronny van Aerle, Dr Trevor Rogers-Gray, Suzy Coey, John Whitmore (Brunel University), Monique Nolan (Environment Agency), Dr David Kime, Dr Brian McAllister and Dr Kathryn van Look (Sheffield University and Carole Kelly (Centre for Environment, Fisheries and Aquaculture Sciences).

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The programme was assisted greatly by many others in the collaborating organisations including laboratory-based researchers at Brunel University, Sheffield University, Brixham Environmental Laboratory, Essex and Suffolk Water and the Environment Agency's National Fish Health Laboratory, Brampton, National Fish Farm, Calverton and Area fisheries teams across England. The authors wish to acknowledge with gratitude their invaluable support for this research programme.

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EXECUTIVE SUMMARY

Background

Previous research has established that certain natural and man-made chemicals can potentially interfere with the normal functioning of the endocrine systems of humans and animals. Such chemicals have been termed ***endocrine disrupters***. A number of adverse changes in humans and wildlife have been postulated as being related to exposure to such chemicals. The strongest evidence for such an association is the link between environmental exposure to certain chemicals (or mixtures of chemicals) and feminisation in a number of wildlife species. In particular, within the UK, evidence has accumulated that freshwater fish may be showing a range of effects associated with exposure to discharges from sewage treatment works.

The Environment Agency has a range of statutory duties that aim to protect the environment through effective control of pollution. As part of these duties, the Agency, in collaboration with other bodies, is undertaking an extensive research programme into the state of health of UK fish populations and the possible role played by treated sewage effluents.

Phase 1 Research Programme

The first phase of the research programme (1995-1998) established that a number of oestrogenic (feminising) effects were common in UK populations of the roach (*Rutilus rutilus*). These included a high incidence of intersex (the simultaneous appearance of male and female gonadal tissue in an individual) and, in males, production of the egg-protein precursor vitellogenin and reduction in the size of the gonads relative to body size (i.e. reduced gonadosomatic index). Additionally, it was found that the severity of intersex appeared to depend on the proportion or percentage of treated sewage effluent in the water body. Other studies in Europe and America have reported similar findings in related fish species.

The results from Phase I formed the basis of an Environment Agency Technical Report (Environment Agency, 1998), which informed the Agency's development of a strategy to reduce pollution of the aquatic environment by endocrine disrupting chemicals (Environment Agency, 2000). The technical report also identified a number of gaps in knowledge that are addressed by the second phase of the programme. Work undertaken in this second phase includes the detailed study of the pathology and occurrence of intersex in roach and gudgeon and assessment of the role played by sewage treatment effluent in its occurrence, and the development and use of an *in vivo* test system to assess the oestrogenic activity of individual and binary mixtures of substances. The findings to date from this second phase form the basis of this Technical Report.

Phase 2 Research Programme

Investigations in wild fish populations have confirmed the continued presence of intersex associated with treated sewage effluent exposure in roach populations within Great Britain, and established that the condition also exists in a second cyprinid species, the gudgeon. The geographical distribution of intersex severity appeared similar for these two species, although the proportion of a population showing intersex and the degree of effect were generally lower in the gudgeon than in the roach for any given sampling site (though the sampling regime for gudgeon was less extensive than for the roach). It was also noted that, as had previously been shown for roach, abnormal induction of vitellogenin occurred in some male and intersex gudgeon.

Further studies of roach showed that the intersex condition was associated with changes in the fish's expression of vitellogenin and also, with altered sex steroid hormone profiles. In addition, it was established that males and females showed heightened sensitivity to xenoestrogens at particular stages of their life cycle. This was shown to have consequences not only for the sexual development and differentiation of fish during their early life stages, but also in mature fish of this species with respect to the seasonal processes of sexual maturation and acquisition of secondary sexual characteristics during recrudescence. In particular, there was evidence of a delay in sexual maturation in male, but not female, roach taken from sites at which effluent exposure was known to occur.

Comparison of the gamete quality of wild-caught males and females from exposed and reference sites, including *in vitro* fertilisation assays, showed a reduction in sperm motility in males from exposed sites, and a general decrease in the overall quality of gametes (particularly sperm) in intersex fish from effluent-exposed sites. Studies following fertilisation and hatching success indicated that decreases in sperm quality (in milt volume and sperm motility in male fish) were related to the severity of the intersex condition in the individual fish assessed. Regressing (atretic) and vacuolated oocytes in females, were also noted in wild roach.

Groups of fish held in large aquaria at a water treatment works were exposed to sewage effluents for several months. Adult fish showed a transitory disturbance at the physiological level (e.g. vitellogenin induction), whereas even only relatively short exposure of larvae/juveniles resulted in non-reversible pathological changes (feminisation of reproductive ducts) as well as altered physiological parameters such as hormone and vitellogenin levels. The induction of eggs in testis was not induced experimentally.

Chemical analysis of effluent samples taken from Chelmsford STW identified the natural oestrogen oestradiol and its oxidative product oestrone, together with several anthropogenic alkylphenolic compounds (nonylphenol and octylphenol ethoxylates and their breakdown products, nonylphenol and octylphenol) as significant oestrogenic constituents. Concentrations of the synthetic oestrogen ethinyl estradiol (from oral contraceptive pills) were very low, in most cases lying below the detection limit. Seasonal variations in the oestrogenic composition and some physicochemical parameters of the effluent were observed.

A key question is whether the one form of the intersex condition (eggs in testis) is driven by exposure in the year of their birth or is a factor of continued (lifetime) exposure. Studies on wild populations of roach revealed that older fish were more severely affected with this form of intersex than younger individuals. This suggests that it is the continual exposure of fish to oestrogenic substances that drives the origins and severity of the eggs in testis condition, However, experimental evidence indicates that formation of gonad ducts (oviducts/sperm ducts) is effectively a 1-off process in early life. This information has implications for how hazards and risk are determined for fish populations.

Studies on the long-term exposure of adult roach to the effluent demonstrated a dosage- and time-dependent increase in vitellogenin production, although the apparent time dependency may have been influenced by temporal variations in the concentration of certain oestrogenic substances present in the effluent.

Laboratory-based studies on individual and binary mixtures of oestrogenic substances were performed using a short-term rainbow trout screening model (with vitellogenin as the principal end-point) that was developed as part of this programme. These studies demonstrated that the model was sensitive to oestrogenic stimulus and showed that, for the binary mixtures examined, the effects were additive. The finding of additivity suggests that, at least for some constituents of effluents, oestrogenic effects could be induced even if the individual substances were present at concentrations below their no effect level. This is of obvious importance when considering the environmental situation, where many constituents are present together at low levels.

It is possible that the various effects on reproductive development and performance in individual fish, as demonstrated by this research, may impact on population recruitment, particularly where the population age structure is skewed towards older fish or when dominated by relatively few year classes. This suggests that wild fish populations in the UK may be under stress as a result of exposure to oestrogenic treated sewage effluents, and other sources of such substances to the aquatic environment. Further research is required to clarify specific aspects and to determine the actual consequences for the sustainability of fish stocks in rivers receiving sewage effluents and recommendations for this and related work are made.

1. INTRODUCTION

1.1 Background to Endocrine Disruption

The endocrine system is composed of a number of glands whose function is to secrete into the blood or lymph a complex range of chemical messengers (hormones) which, through interaction with receptors in the cells of target tissues or organs, modify virtually all the vital processes of life, such as growth and development, metabolism, immunity and reproduction. Some form of endocrine system exists in most animals although, as a result of diverging evolutionary pathways, marked differences in the structure and function of the various glands and hormones exist between different groups of organisms. Nonetheless, particularly amongst the vertebrates, there is a high degree of conservation of function.

Over the past 40 to 50 years, there has been increasing recognition that the functions of the endocrine system are susceptible to specific interference by certain substances of natural or anthropogenic origin, either though mimicking or blocking hormonal action or by interference in some other way; substances showing such properties have become known as ***endocrine disrupters***. Several formal definitions have been developed, including one from a conference sponsored by the European Commission held in Weybridge, UK in 1996, which describes an endocrine disrupter as "an exogenous substance that causes adverse health effects in an intact organism, or its progeny, subsequent to changes in endocrine function" (EC 1997).

A number of authoritative reviews of the evidence for endocrine disruption in wildlife have been published in recent years (Kendall *et al*., 1998; Botham *et al*., 1999; IEH, 1999; Guillete & Crain, 2000). Overall, there is experimental and observational evidence suggestive of endocrine disruption in a number of wildlife species, including: the marked decline in populations of raptors and song birds in the late 1950s following exposure to organochlorine pesticides such as DDT; marked reductions in global populations of molluscs such as the dogwhelk, as a result of masculinisation (imposex) of females following exposure to marine organotin antifoulants (Matthiessen and Gibbs (1998); impaired immunocompetence in marine mammals exposed to organochlorine chemicals; and, in a number of European countries, reports of feminisation of fish exposed to sewage-treatment effluent. This latter finding is an aspect of particular concern to the Environment Agency. Changes induced by abnormal oestrogenic stimulation of fish include development of ovarian tissue in the gonads of apparently male fish (intersex), the production of an egg-yolk protein precursor, vitellogenin, by male and juvenile fish, and altered hormonal profiles.

1.2 The Environment Agency's Endocrine Disrupters Strategy

The Environment Agency has a range of duties to protect the environment and fisheries in the UK, and the technical report of the first phase of this programme (Environment Agency, 1998a), together with input from stakeholder responses to a consultative document 'Endocrine Disrupting Substances in the Environment: What should be done' (Environment Agency, 1998b), informed the further development of the Agency's strategy to reduce endocrine disrupting substances in the UK environment (Environment Agency, 2000). The strategy developed by the Agency has four main elements:

1 Cost-effective action to reduce the risk of potential endocrine disrupting substances causing harm to the environment
2 Monitoring targeted in high risk areas
3 Collaborative research to address areas of scientific uncertainty
4 Raising awareness of producers, users and the public.

As part of the Strategy's implementation, the Agency prepared a list of priority chemicals for which action would be considered in order to reduce their impact on the environment. Substances identified included organochlorine compounds, pesticides, natural and synthetic hormones, and breakdown products of certain surfactants. It was proposed that reductions in the environmental concentrations would be achieved by implementation of Environmental Quality Standards (EQS), and where such standards were not available, they would be derived. Reduction in environmental concentrations would involve: catchment management solutions; research into new waste treatment technologies; reduction in industrial and trade input; and review of licences for discharges and abstractions.

The focus for the Strategy in protecting the environment was those wildlife groups where risks of effects following exposure to oestrogens had been identified. In particular, fish were selected as a key group because of their sensitivity and apparent susceptibility to becoming intersex, and evidence for adverse health effects, in particular the intersex condition, arising from exposure to oestrogenically-active sewage effluents.

1.3 Oestrogenic Endocrine Disruption in Fish

1.3.1 Effects observed in fish

Native fish within the UK rivers are mostly single sex (gonochoristic) species. While male and female gonadal tissues are known to occur simultaneously in individuals of protandric or protogynous species, this is generally considered abnormal in such as the fresh water cyprinids, roach (*Rutilus rutilus*) and gudgeon (*Gobio gobio*) (van Aerle *et al*., 2001). However, limited evidence suggests that intersex may occur at very low levels in apparently unexposed populations of certain gonochoristic cyprinid species including the roach (Jobling *et al*., 1998; Jafri & Ensor 1979) and the bream (*Abramis brama*) (Slooff & Klootwjk, 1982).

Levels of intersex have been reported in wild populations of gonochoristic fish in the UK (Phase 1 of this programme, Jobling *et al.*,1998); Allen *et al.*,1999) Scandinavia (Christiansen *et al*, 2000), France (Flammarion *et al*., 2000; Minier *et al*., 2000), and Germany (Hecker *et al*., 2001) and the Netherlands (COMPREHEND, 2002), Species affected include the freshwater species grayling (*Thymallus thymallus*), roach, gudgeon, carp (*Cyprinus carpio*) and bream (*Abramis brama*), and estuarine species such as the flounder (*Platichthys flesus*).

Vitellogenin (vtg) synthesis - the production of egg protein by egg-laying females - occurs via oestrogen receptor pathways. As male fish do not naturally synthesise vtg, the measurement of this protein in male fish indicates recent exposure to oestrogenic substances and is therefore a valuable biomarker tool to indicate areas of the water environment contaminated with oestrogenic substances. This biomarker is transient in males, with blood vtg concentrations falling after several weeks following exposure to an oestrogen, however, the impact of abnormal induction of this protein on the overall functionality, survival and reproductive potential of individual male or juvenile fish is unknown (reviewed in Tyler *et al*., 1998), as are the potential consequences at the population level. High levels of vtg induction, however, may cause kidney failure (Herman and Kincaid, 1988). Endogenous steroid hormone concentrations in fish have also been shown to be susceptible to exposure to exogenous chemicals possessing hormonal activity although again the impacts on affected individuals are not well understood.

1.3.2 Feminisation of males, or masculination of females?

Although it is as yet not technically possible to establish the genotypic sex of fish that demonstrate the intersex condition, there are thought to be genetic males that have undergone abnormal phenotypic development (Tyler & Routledge 1998). Evidence that the intersex fish were derived from the feminisation of genetic males was derived from the fact that:

- the number of roach with normal testes in any population was inversely proportional to the number of intersex fish (Jobling *et al*., 1998);
- sewage effluent discharges in the UK are predominantly oestrogenic (Purdom *et al*., 1994; Harries *et al*., 1997; 1999; Rodgers-Gray *et al*., 2000, 2001); and
- the male and intersex fish contained VTG in the plasma (Jobling *et al*., 1998; van Aerle *et al*., 2001), which provided strong evidence that some of the populations of wild fish were being exposed, and responding to, oestrogenic (feminizing) contaminants.

1.3.3 Ecotoxicological studies - evidence for oestrogenic sustances causing effects

Experimental studies have shown a relationship between development of phenotypic sexual characteristic in gonochoristic fish and exposure to steroidal hormones. Exposure of fish to concentrations of 17 β-oestradiol of 100 ng/l and higher, even for relatively short periods of time (21 days), can inhibit testicular growth, cause deleterious effects on spermatogenesis, and even cause complete regression of the testes (Panter *et al*., 1998; Kinnberg *et al*., 2000; Halm *et al*., 2001). Induction of intersex, and even complete sex reversal can also be induced by exposure to high concentrations of natural steroid oestrogens (Nimrod and Benson, 1998; Imawatsu *et al*., 1999).

The potency of ethinylestradiol exceeds that of natural steroid oestrogens *in vivo* (reviewed in Environment Agency, 2002). Ethinylestradiol is between 20 and 50- times more potent than oestradiol-17β (Thorpe *et al*., 2001b) in inducing vtg. A 60% reduction in egg production was observed at concentrations of 5 ng l^{-1} in F_1 generation from multi-generation study with zebra fish (*Danio rerio*) (Nash and Kime, 2000) and a complete feminisation of fathead minnows (*Pimephales promelas*) was recorded at 4 ng l^{-1} (No Observable Effect Concentration- NOEC) of 1 ng l^{-1} (Länge *et al*., 2001). Similar effect thresholds based on intrinsic rate of population increase (λ) estimated from same study (Grist *et al*. 2002). Overall, it was concluded that the ethinyloestradiol is more potent by a factor of 10 compared with oestradiol.

Less is known about the effects of oestrone where the datasets are mostly of short-term (21 days) duration and study vtg production. A Lowest Observable Effect Concentration (LOEC) of 3.2 ng l^{-1} was reported for juvenile female rainbow trout by Thorpe *et al*. (2001b), although in adult male rainbow trout a higher LOEC of 44 ng l^{-1} has been reported (Routledge *et al*. 1998). Two studied the effect on gonad growth, with a LOEC of 318 ng l^{-1} being reported for adult male fathead minnow after 21 days of exposure.

Studies that have investigated the relative potency of the steroid oestrogens clearly suggest that oestrone is less potent than 17β-oestradiol. Of the studies in which test concentrations were confirmed by analysis, only two had adequate experimental details available (Routledge *et al*., 1998, Thorpe *et al*., 2001b). Both these studies investigated vtg induction as the end-point and suggest that oestrone is a factor of 3-5 times less potent than 17β-oestradiol.

Experimental studies have also shown a relationship between development of this phenotypic sexual characteristic in gonochoristic fish and exposure to mimics of steroidal hormones. Alkylphenolic chemicals have been studied most intensely. Octylphenol (OP), 4*tert*-nonylphenol (NP), di-ethoxylate and mono-carboxylate forms of nonylphenol (NP2EO and NP1EC) have all been shown to be oestrogenic to fish and have pronounced stimulatory effects on vitellogenin synthesis and can result in modifications in somatic growth rate and gonad growth (Jobling *et al*., 1996; Ashfield *et al*., 1998). 4*tert*-NP (in 3 week exposures) induces elevations in plasma VTG in fish at a threshold concentration of between 5.4µg/l and 10µg/ l(Thorpe *et al*., 2001a; Jobling *et al*., 1996; Hemmer *et al*., 2000; Harries *et al*., 2000). Exposure to 4*tert*-NP also causes disruptions in gonadal development, that include alterations in the testis structure (Kinnberg *et al*., 2000) and induction of ovo-testis. Studies on the biological activity of NP in fish has shown that exposure of juvenile Japanese medaka (*Oryzias latipes*) to 50 µg/l NP in water can result in the induction of hermaphroditism (ovo-testis), and exposure to a concentration of 100 µg p-NP/l results in complete feminisation (Gray & Metcalfe, 1997).

Furthermore, in fathead minnows, a 3 week exposure to 4 *tert*-NP at concentrations in the water between 0.65µg/l and 8.1µg/l resulted in a significant decrease in fecundity in females, and a reduction in the development of secondary sexual characteristics in males (Giesy *et al*.,2000; Harries *et al*., 2000). Octylphenol has been shown to induce elevations in plasma VTG in rainbow trout and male roach at threshold concentrations of 3µg/l (Jobling *et al*., 1996) and between 10µg/l and 100µg/l (Routledge *et al*., 1998) respectively, a potency of between 1/100th and 1/1000th of estradiol-17β (Routledge *et al*., 1998). Higher concentrations of OP have been shown to inhibit testis development in the summer flounder (*Paralichthys dentalis*; Mills *et al*., 2001) and inhibit spermatogenesis and induce oocytes in the testis of male Japanese medaka (Gronen *et al*., 1999). Some of the disruptive effects of 4 *tert*-octylphenol on reproduction in fish may occur as a consequence of altered expression of P450 aromatase (Navas & Segner, 2000).

1.3.4 Evidence for cause and effect from the field

In the UK, research has established that a number of natural and synthetic substances are present in treated sewage effluent at concentrations sufficient to elicit an oestrogenic response in fish exposed to such effluent. Identified substances include the natural steroid hormones oestrone and oestradiol, the synthetic hormone ethinyl estradiol (from oral contraceptive and hormone-replacement therapies), and synthetic industrial chemicals such as the alkylphenols, nonylphenol and their ethoxylates (Desbrow *et al*.,1998). Exposure-response studies subsequently confirmed that the amounts measured of these substances in effluents and receiving environments were sufficient to cause vitellogenin induction (as observed in wild fish and caged fish below effluent discharges) and, although this has not been demonstrated in the roach, instances of disruptions in gonadal development (altered sex cell development/intersex, as observed in laboratory controlled studies).

Although not conclusive, the information presented in section 1.3 suggests that these effluent-borne oestrogenic contaminants (either singly on in mixtures) were responsible, either partly or completely for the oestrogenic responses observed in wild and caged fish in rivers. However, a concerted research effort would be required to quantify the scale of the effects in wild fish populations and then to evaluate the causes and consequences to those populations of the adverse health impacts observed.

1.4 Collaborative Research Programme into Causes and Consequences of Oestrogenic Effects in Fish

1.4.1 Background

In 1995 the Environment Agency joined with the Natural Environment Research Council (NERC) to establish a major 5-year research programme at Brunel University. The first phase of this programme was designed to assess oestrogenic effects (in terms of incidence and severity) in fish in UK rivers receiving treated sewage effluent.

Subsequent to this first project, a further phase of research was developed to address the causes and consequences of the intersex condition in fish. This latter programme involved collaboration between Brunel University, Exeter University, Natural Environment Research Council, Environment Agency, Department of the Environment, Transport and the Regions (now Department of the Environment Food and Rural Affairs, Astrazeneca, Essex and Suffolk Water, and Sheffield University. Some investigations were conducted under contract to the Centre for Environment, Fisheries and Aquaculture Sciences.

1.4.2 Oestrogenic effects in wild fish – results of the first phase of the research

The first phase investigated the severity and extent of oestrogenic endocrine disruptive effects in fish in UK rivers and evaluated the link to effluents and water quality.

Between 1995 and 1996, roach were collected from 18 sites in the UK (8 rivers plus 5 reference sites). Study sites were identified on a number of rivers considered representative of the UK. Wherever possible, sites situated up and downstream of sewage treatment works (STWs) were selected for each river. Reference sites were chosen on the basis of apparent absence of exposure sources to effluents from sewage treatment works. Fish were caught by electro-fishing, and examined macroscopically and microscopically, with particular reference to gonadal structure. In addition, blood samples were taken, and plasma vitellogenin measured by radioimmunoassay.

An index of intersex was developed and applied to analyses of histopathological tissue sections of gonad material, in order to quantify the number of fish containing oocytes and oviducts, and the severity of the level of effect. This index relates to 0 (fully male) to 7 (fully female tissue)(Nolan *et al* 2001). Intersex fish were found at all sample and reference sites (Environment Agency, 1998). The incidence and severity at the reference sites were, however, generally lower (<15%) than for fish collected upstream (approx. 25%) or downstream (>50%) of STWs. Indeed, at two downstream sites all males sampled showed intersex. There was also an apparent increase in the severity of intersex with proximity to the STW, and with proportion of sewage effluent in the river. Similar patterns were observed for incidences of plasma vitellogenin induction and reduced gonadosomatic index (GSI) among male roach at the various sites. These results strongly suggested that abnormalities in the roach were widespread throughout the UK riverine environment, and were associated with the proportion of treated effluent present in the water body.

Concerns were raised that such effects, particularly the structural changes in the gonad, could have an adverse effect on the individual ability to reproduce and potentially impact at the population level. A particular concern was that the highest concentration of sewage effluent in rivers would be expected to occur at key stages of a fish's life cycle, namely spawning in spring and sexual differentiation in summer (Environment Agency, 1998).

This programme is an important element to understand hazards and risks to fish from oestrogenic endocrine disruption and propose appropriate risk management within the Agency's Strategic framework.

1.5 Phase II Research Programme

1.5.1 Objectives

Following the successful completion of the first phase of the research programme and having considered the identified gaps in knowledge and causes for concern, the Agency and other parties developed a second tranche of studies. The objectives set for this part of the programme were as follows:

- to investigate the occurrence of reproductive abnormalities in two freshwater fish species common in the UK, the roach and gudgeon. This would involve the assessment of the normal process of sexual development and the incidence and severity of intersex in roach, and the investigation of occurrence of intersex in the gudgeon;

- to investigate the effects of reproductive abnormalities on the reproductive health of wild roach, with particular reference to sexual maturation, gamete quality and quantity, endocrine function and gamete viability;

- to assess the cause and effect relationship between effluent concentration and oestrogenic response in roach using both adult and juvenile stages;

- to determine the relative importance of fish age, timing and duration of exposure to effluents; and

- to investigate interactions between mixtures of some environmentally-relevant oestrogenic compounds on juvenile fish.

This R&D Technical Report presents the research and findings generated within this collaborative programme, advancing considerably our understanding of the effects of effluent exposure in wild fish populations in the UK. By so doing, the Agency believes that future policy on environmental protection can be better formulated and future research needs in this area more precisely defined. The research results presented in this R&D Technical Report are mostly published in the peer-reviewed literature. Whereas this document provides a detailed synopsis of the research, full experimental details are presented in the cited literature.

The remainder of this section presents an overview of the experimental design of the work conducted under Phase II of the programme. Chapters 2 to 4 present the results of the individual components, and Chapter 5 discusses the significance of these findings. Conclusions and recommendations are presented in Chapters 6 and 7, respectively.

1.5.2 Programme components

The research programme was conducted as a number of contemporaneous components, as follows:

Status and effects in wild fish populations

This investigation was conducted as two separate studies. The first sought to further characterise the pathological basis of the intersex condition that had been found in the roach, and to establish if this also occurred in the gudgeon. (Choice of species is discussed further below). The incidence and severity of intersex in these species were also compared, to determine whether these fish species display similar sensitivities in relation to oestrogenic responses to treated sewage effluent. In a second study, wild populations of fish were studied to determine the impact on the quantity, quality and fertilisation success of gametes of environmental exposure to treated sewage effluent at river sites where intersex was known to occur in some individuals. In both studies, fish were collected from sites known to receive STW discharges, and from apparently unexposed 'reference' sites (see Section 1.4.3). Gonadal histopathology, vitellogenin expression and hormonal status were assessed in both studies, and gamete viability was assessed in the second study (see Section 2).

Assessment of cause and effect relationships for sewage effluents

This study had two components. The first consisted of field-controlled studies conducted at a sewage treatment works and investigated the impact of various dilutions of treated sewage effluent on roach at various stages of their lifecycle. The oestrogenic response in maturing (2-year old) fish was monitored following exposure to various dilutions of treated effluent.

In addition, juvenile roach were exposed, from 50 days post hatch, to effluent at graded dilutions under field-controlled conditions, for up to 150 days. Gonadal pathology and hormonal status were assessed. A group of fish exposed to 100% effluent was subsequently retained in clean water for a further 150 days, to investigate the reversibility of any oestrogenically-induced changes. In parallel with these investigations, samples of effluent were collected and their composition analysed in detail (see Section 3).

The second study was field-based and intended to examine the relationship between intersex, fish age and duration of exposure. To be able to do this with a high level of confidence, very significant numbers of fish are required. This aspect draws from the substantial dataset derived at the River Nene site over a 5-year period on intersex condition and age for individual fish. The assessment compares the age at capture versus degree of intersex, and also natal year against intersex. This study has yet to be fully reported but preliminary evidence is presented in this report.

Assessment of cause and effect relationships for single and binary mixtures of oestrogenic substances

STWs receiving domestic, industrial and/or agricultural waste, release a complex (and ill-defined) mixture of natural and synthetic chemicals into the aquatic environment following their partial or complete biodegradation or removal during the treatment process. Thus, identifying specific chemical(s) responsible for adverse effects observed in the field is difficult. Moreover, substances within these mixtures can interact to produce, potentially, a greater hazard to biota at concentrations below those at which the individual substances are considered to be harmful.

The final component of Phase II of the research programme was a mechanistic investigation of the oestrogenic responses elicited by individual substances and binary mixtures in a specially developed *in vivo* test system. The relative potency and additive effects of an alkylphenol, a pesticide and 3 steroidal estrogens were investigated using this developed test system. This work complemented the investigations detailed above on the effects of treated effluent and was intended to inform how mixture interactions could be evaluated and determine the implications for managing oestrogenic activity of effluents.
Investigations into the interactive effects of these oestrogenic substances were conducted at environmentally-relevant concentrations. Full details of this work are reported in Section 4.

Selection of fish species

Over the course of this phase of the research programme, a number of different species representative of different families of fish were employed, and both wild populations and captive-bred fish examined.

The roach was selected for the studies on the status and effects of wild populations and on cause and effect relationships for sewage effluents as earlier work, including Phase I of this programme, had clearly established it as a species that was susceptible to effluent exposure. This had raised important questions regarding the potential consequences for UK roach and other cyprinid populations. Where required for the experimental studies, captive-bred fish from Calverton, Nottinghamshire were used.

Another cyprinid, the gudgeon was selected as the second species for studying effects in wild populations since it is found within the same water bodies as the roach but has a different reproductive strategy and inhabits a different ecological niche. Unlike the roach, gudgeon are asynchronous fish, spawning several times during their reproductive season. In addition, gudgeon are benthic-dwellers and hence live in close contact with sediments, compared to the water column habitat of the roach. Thus, a comparison of effects between the species would provide information in the possible consequences for different fish species inhabiting different ecological niches.

Captive-bred rainbow trout (*Oncorhynchus mykiss*) were used for studies on effects of effluent dilution and of single and binary mixtures of specific substances, since it is known to be susceptible to oestrogenic substances (Harries *et al.*, 1996), has a long history of use in regulatory toxicity, and is well suited for both laboratory studies and *in situ* monitoring. Additionally, sensitive and quantitative assays for rainbow trout vitellogenin already exist. Further, this species is representative of a different fish family, the salmonids, and inhabits a different ecological niche from the above-mentioned cyprinids.

Selection of study sites

The sample and reference sites used for the studies on wild fish populations are detailed in Table 1. These sites were selected using the following criteria:

- presence of wild populations of the selected fish species (roach and gudgeon) in water-bodies that were known to receive sewage effluent discharges or, for reference sites, that had no definable exposure to sewage effluent;

- for the sample sites, the sources of raw effluent feeding into the sewage treatment works were representative of the typical UK situation of receiving predominantly domestic but also some industrial influent;

- sample sites were known to have indigenous roach populations showing a significant incidence of intersex; and

- sites were chosen from among those that had previously undergone investigation, so as to extend the existing knowledge base.

During Phase I it had proved difficult to identify truly pristine control sites (Environment Agency, 1998), and for this series of studies reference sites were selected on the basis of having no known effluent input and a low incidence of intersex, together with the requirement for a control site that could be classified as ‘running water’. In Phase I, the Royal Canal, Eire was identified as satisfying these criteria and was therefore used as a control site for comparisons with effluent-receiving rivers in the Phase II investigation.

For the work on cause and effect relationships with sewage effluents, Chelmsford STW was chosen because it was considered representative of STWs in the UK, with the majority of the raw effluent it receives arising from domestic sources and a lesser input (14%) from industrial sources. It was also the site of previous studies and, hence, existing information was available on the oestrogenic activity and chemical composition of the effluent.

Table 1: Sampling sites for roach and gudgeon

Site	Site description	Date of roach Sampling (Phase 1 / Phase 2)	Date of gudgeon sampling
River Aire, West Yorkshire	Effluent from domestic and industrial sources	October 1995 May 1998 September 1998 May 2000	September 1995 May 1998 August 1998
River Lea, Essex	Effluent from mostly domestic sources	September 1995 May 2000	September 1995 October 1998
Lakeside Fisheries The Midlands	Raw sewage from domestic cesspits and possibly pesticide run-off	N/A	November 1997
Longton Park Lake, Stoke-on-Trent	Receives no treated effluent; contamination from other sources cannot be discounted	N/A	February 1998 March 1998
River Nene, Northamptonshire	Receives effluent from mostly domestic sources	October 1995 May 1998 September 1998 May 1999	N/A
Lake Wartnaby, Leicestershire	Spring-fed lake, free from treated effluent; other sources of contamination cannot be discounted	May 1998 May 1999	N/A
Grantham Canal, Leicestershire	Receives no treated effluent; contamination from other sources cannot be discounted	October 1996	N/A
Royal Canal, Co Kildare, Eire	Receives no treated effluent; contamination from other sources cannot be discounted	October 1996	N/A
River Calder, Yorkshire	Effluent from industrial and domestic sources	May 2000	N/A
River Arun, Sussex	Effluent from domestic and industrial sources	October 1995 May 2000	N/A
River Blackwater, Suffolk	Effluent from mostly domestic sources	May 2000	N/A

2. STATUS AND EFFECTS IN WILD FISH POPULATIONS

Phase I of the research programme demonstrated a high incidence of intersex in wild populations of roach in UK rivers that appeared to be associated with exposure to treated sewage effluents. In this chapter, studies focusing on the characterisation of intersex in two fish species, roach and gudgeon, and the implications for the reproductive health of UK fish populations are reported.

2.1 Investigation of Reproductive Abnormalities in Roach and Gudgeon

2.1.1 Experimental design

A detailed study of the histopathology of intersex in the roach was undertaken, and a sampling programme on wild populations was performed to establish whether intersex occurred in a second species, the gudgeon. A comparison of intersex incidence and severity was undertaken for these species to investigate whether they showed similar responses to the effluents. In addition, a sampling campaign for roach was undertaken over two consecutive spawning seasons in 1999 and 2000. Adult roach were collected from the River Nene in 1999 and from the rivers Aire, Lea, Arun, Calder and Blackwater in 2000 (see Table 1). All sampling sites were situated 3 – 15 km downstream from point source STW effluent discharges.

Briefly, fish were sampled from sites where intersex in roach had been established as prevalent during Phase I, and were examined for oestrogenic effects on vitellogenin induction, hormonal status and histopathology of the reproductive tissues. Findings were compared with fish sampled from the selected reference sites. Fish were anaesthetised using 0.1% 2-phenoxyethanol, and blood samples taken from the caudal sinus into 1 ml heparinised syringes. After centrifugation, plasma was stored at –80 ^{o}C before analysis as detailed below. The fish were sacrificed under anaesthesia, and the gonads examined macroscopically to determine the phenotypic sex, weighed, and then fixed in Bouins solution before storage in 70% alcohol pending histological processing (see below).

Details of the individual methodological procedures undertaken during this study are described below:

Meristic measurement

After sacrifice, the age of each fish was estimated by counting the annuli on scales removed from the flank, and body length and bodyweight were measured. The gonads were isolated and weighed and fish were sexed according to the phenotypic appearance of their gonads. In spawning fish, gonads were stripped prior to weighing. The gonadosomatic index (GSI) was calculated as:

$$GSI = \text{Gonad weight}/(\text{Bodyweight} – \text{Gonad weight}) \times 100$$

Histopathological examination of gonads

The gonads were cut into three sections (anterior, median and posterior), mounted in paraffin blocks and six sections cut from each block**.** Sections were stained with haematoxylin and eosin, and examined under light microscopy.

The incidence and severity of intersex was recorded for roach using the categorisation system the Intersex Index (see section 1.4.2) that was developed for Phase I of the programme (Environment Agency, 1998). This was used to score numerically the degree of intersex observed, with a score of 0 representing a normal male and 7 representing a normal female. These data were also recorded for gudgeon although, because of differences in the manifestation of intersex in this species, a different scoring system was adopted:

Category A: < 5 primary oocytes per tissue section
Category B: > 5 primary oocytes per tissue section
Category C: presence of primary and secondary oocytes in a discrete area/region of the testes clearly separated from testicular tissue.

Vitellogenin analysis

Vitellogenin was measured in the plasma using a homologous carp vitellogenin radioimmunoassay or enzyme-linked immunosorbent assay (ELISA), both of which are validated for use with both roach and gudgeon (Tyler *et al*., 1996, 1999).

2.1.2 Results

Meristic measurement

When the influence of effluent exposure on fish growth was considered, no discernible effect on body length or bodyweight at a given age was noted for gudgeon or roach. In gudgeon, GSI was not clearly affected by exposure to oestrogenic effluents, although interpretation was difficult as the fish at the various sites were sampled at different stages within their reproductive cycle. Although fish were initially sexed on the basis of gonadal morphology, further histological examination found that fish classified as intersex possessed similar size gonads to those classified as male; this provides further support for the hypothesis that these intersex fish are a result of the feminisation of male fish.

Gonadal pathology

Examination of the gonads of adult male and female roach showed that reproductive development in this species was that of normal synchronous fish, where gametogenesis is a continuous process throughout the year. In gudgeon, gonadal development and function has been described elsewhere (Lahnsteiner *et al*., 1994; Kestemont, 1987) and therefore a full characterisation was not necessary in this study.

Determination of Intersex

Work in Phase I had demonstrated the presence of several grades of intersex in roach and resulted in the development of the Intersex Index. Histological examination of the gonads, however, revealed that all of the phenotypic male fish from the river Nene were intersex, whilst in all of the Wartnaby (control) males, the testes appeared normal (no feminization was evident). In all intersex (Nene) fish, both male and female reproductive ducts were present, although only a few individuals had testes that contained low numbers of developing oocytes within primarily testicular tissue.

There were no significant differences in either the length, weight or age of the fish collected from the Rivers Lea, Arun, or Aire, whilst in contrast, the fish collected from the Nene, Blackwater and the Calder were significantly younger than the fish from any of the other rivers ($p<0.001$; data not shown). In most of the fish collected, the gonads appeared to be of the expected size and appearance for sexually mature male or female roach, although 3 fish from the River Arun were macroscopically intersex. Histological examination of the gonads of the macroscopically male fish from all of the rivers combined revealed that 46% were intersex.

Subsequent scoring of roach caught in several rivers in 1999 and 2000 established that compared with ranges of 0 to 2.7 in the River Aire, less than 1 in the River Lea (only two fish were intersex), 0 to 7 in the River Arun, 0.8 to 3.8 in the River Calder and 0 to 1 in the River Blackwater.

In all, the proportions of male roach which were intersex from each site were: 100% (Nene); 96% (Arun); 93% (Aire); 92% (Calder); 31% (Blackwater), and 9% (Lea). The Intersex Indices for these fish ranged from 0 to 7 (Arun), 0 to 2.7 (Aire), 0 to 1 (Blackwater), and 0.8 to 3.8 (Calder). Only two fish from the River Lea were intersex (indices 0.7 and 0.3). For fish from the Nene an average of 0.95 was recorded (range not reported).

Histological analyses

Histological examination of gonadal tissue from roach from various rivers, lakes and streams established two basic patterns of intersex, as revealed by the distribution of ovarian tissue within the testis, namely focal and the more common, multifocal form. In multi-focal intersex, oocytes at various stages of development occurred either singly or in clusters in a mosaic-like pattern throughout the testicular tissue, with some oocytes actively undergoing meiosis. The relatively low incidence of female tissue within the testes appeared to cause minimum disruption to the overall testis structure (see Figure 1).

Figure 1: The histology of multifocal ovo-testis in wild roach

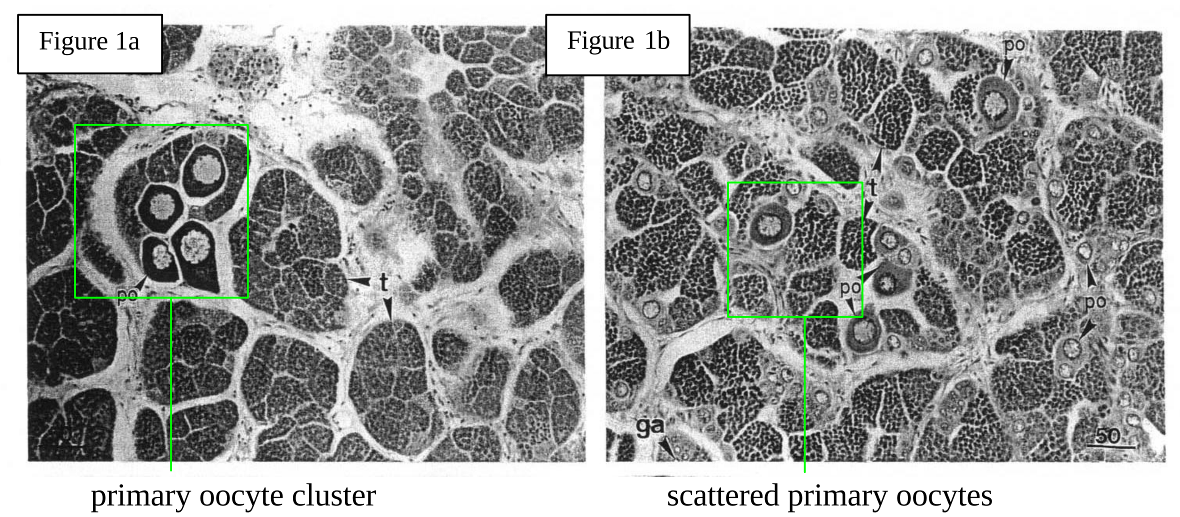


Figure 1a) Mosaic type intersex gonad with a cluster of primary oocytes (po) surrounded by testicular tissue (t). **Figure 1b)** Mosaic type intersex with numerous primary oocytes (po) scattered throughout the testicular tissue. **Reprinted with permission from 'Nolan *et al*., (2001). A histological description of intersexuality in the roach. *J Fish Biol*, 58, 160-176. Copyright 2001, Academic Press Ltd.**

Focal intersex was characterised by the presence of oocytes in a discrete area or 'central cavity' in the testes, clearly separated from testicular tissue. The cavities differed in size and shape and in the number of oocytes present. In advanced stages, considerable disruption to the normal testicular architecture was apparent with, in some cases, associated retarded spermatogenesis (Figure 2).

In both these types of intersex in the roach, the majority of male and female germ cells appeared to be developing normally within the same gonad, with only a few oocyte clusters appearing degenerative. In female and intersex roach, there was increased cytoplasmic vacuolation in primary oocytes in the fish exposed to effluents (see Figure 3) and this was thought to be associated with chronic oestrogenic exposure.

Figure 2: The histology of focal ovo-testis in wild roach

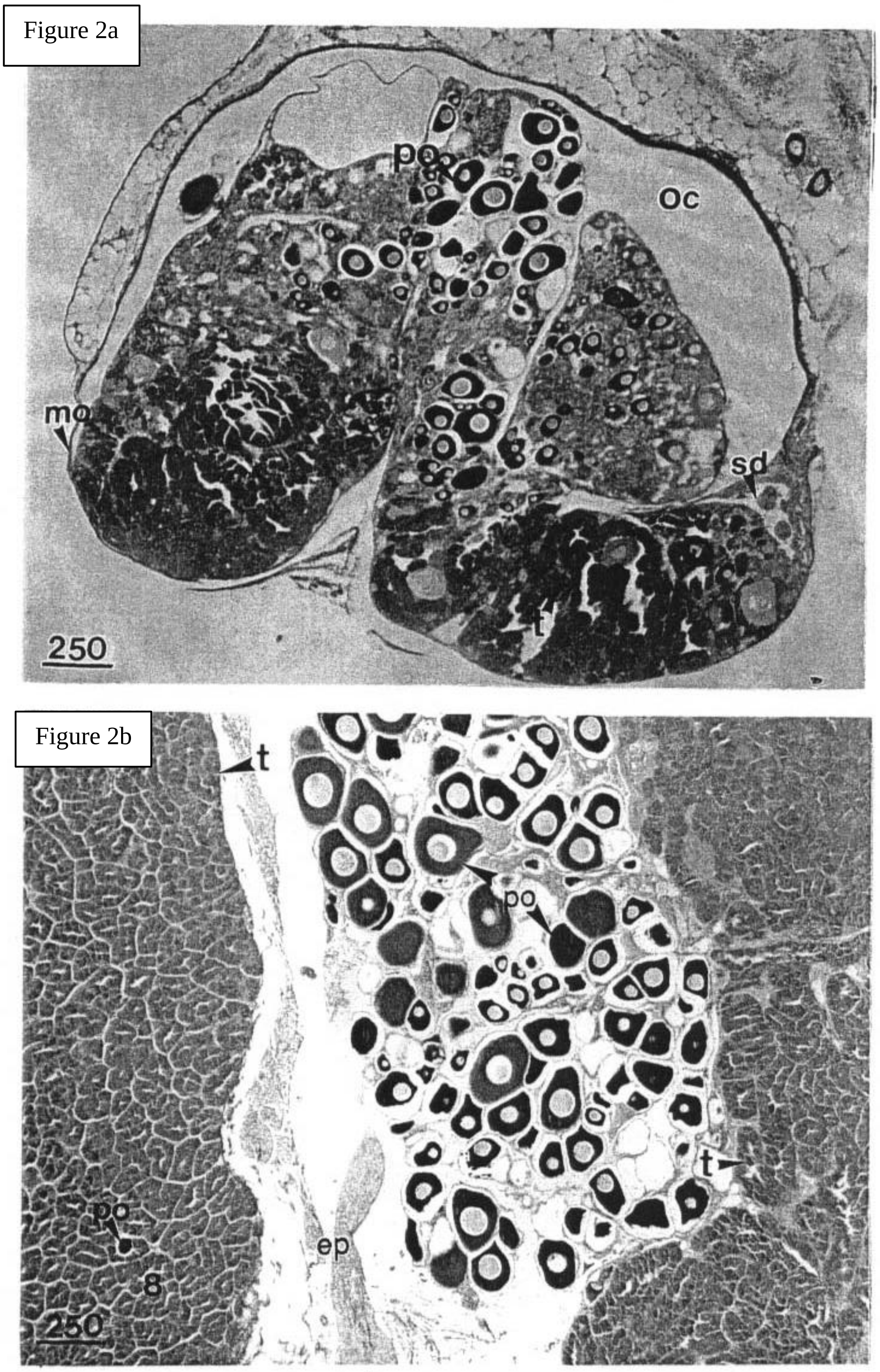
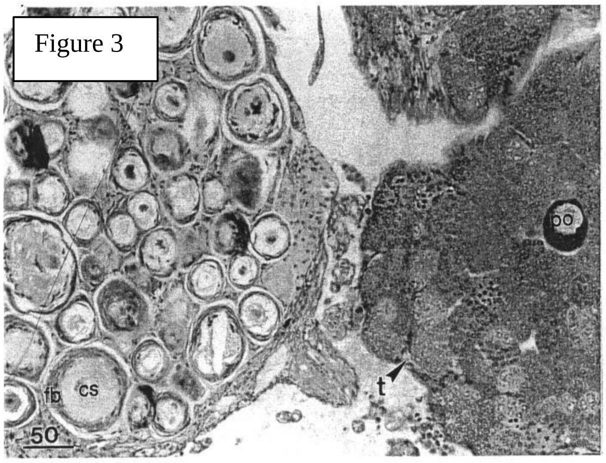


Figure 2a) Transverse section through a severely intersex 'zoned' gonad, with a clearly identifiable ovarian cavity (oc), sperm duct (sd) and numerous primary oocytes (po). Testicular tissue (t) can be seen in the ventral portion of the gonad. **Figure 2b)** Gonadal tissue in a zoned intersex gonad showing primary oocytes (po) within a defined area, separated from the testicular tissue (t) be epithelial cells (ep) and phagocytic tissues

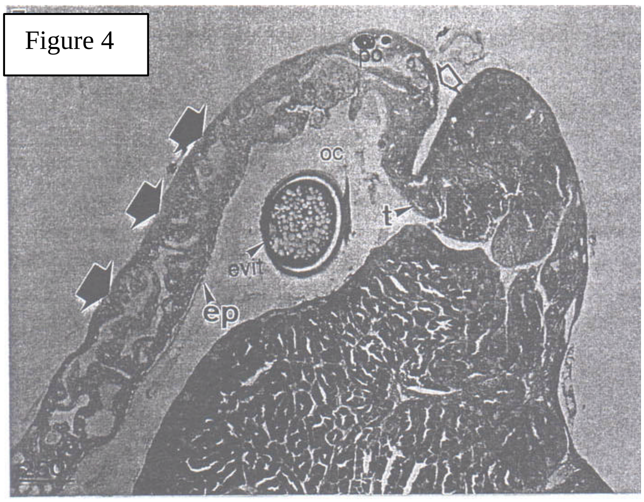
Figure 3: The histology of degenerating oocytes in wild roach



Numerous cyst-like structures (cs; presumptive degenerate oocytes) enclosed in concentric layers of fibroblast-like cells (fb) adjacent to testicular tissue (t) from an intersex gonad. Primary oocyte (po).
Reprinted with permission from 'Nolan *et al.*, (2001). A histological description of intersexuality in the roach. *J Fish Biol*, 58, 160-176. Copyright 2001, Academic Press Ltd.

Abnormalities of the reproductive ducts (gonoducts) were also observed in roach showing focal or multifocal intersex. The most common malformation was the simultaneous occurrence of male reproductive ducts and ovarian tissue or 'feminised' ducts, and was considered another manifestation of intersex. In severe cases of feminisation, ducts were malformed, appearing blind-ended, blocked or shrunken; in a few cases they were absent, being replaced by a fully formed ovarian cavity. Owing to the natural variability of duct size in roach, it was not possible to confirm an association between abnormalities of the duct and the severity of intersex (see Figure 4).

Figure 4: The histology of malformed reproductive ducts in wild roach



Severely intersex gonadal tissue showing the ovarian cavity (oc) and vestiges of the sperm duct (large solid arrows) within the wall of the ovarian cavity. The position where the true sperm duct would be is marked by an open arrow. **Reprinted with permission from 'Nolan *et al.*, (2001). A histological description of intersexuality in the roach. *J Fish Biol*, 58, 160-176. Copyright 2001, Academic Press Ltd.**

The sampling regime for gudgeon was less extensive than that undertaken for roach, with fewer fish, of a narrower age distribution, being caught at various times of the year. However, gudgeon have a shorter lifespan than roach so this would be seen as being broadly representative of the reproducing population. Two basic patterns of intersex were discernible in gudgeon, a mild form and a severe form. The mild form (Categories A and B) presented as a normal male testis containing isolated primary oocytes at the margin, or clusters of oocytes in the centre. The severe form (Category C) comprised a higher proportion of primary and secondary oocytes interspersed across the testis, with the gonad being oddly shaped and containing degenerating tissue. In light of the variation attributable to seasonal differences, abnormalities in the gonoducts were not investigated further. The majority of gudgeon showing intersex had immature oocytes, and the gonads were only slightly affected, whereas severe intersex was associated with degenerative gonadal tissue (see Figure 5).

Figure 5: The histology of normal and intersex gonadal tissue found in wild gudgeon

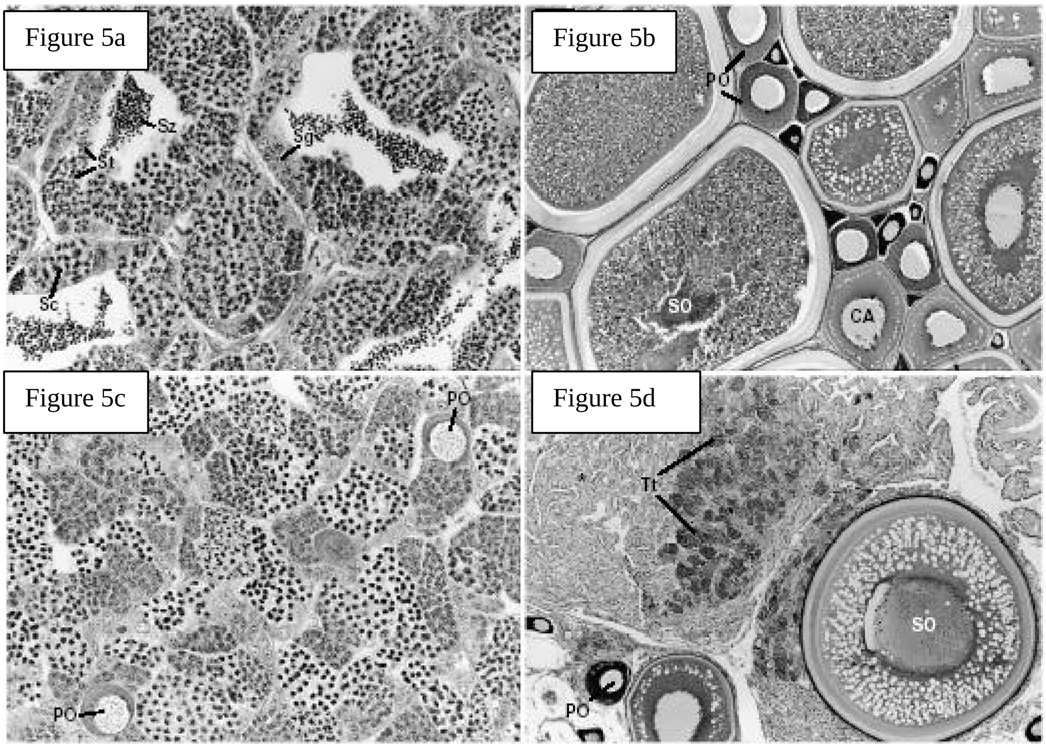


Figure 5a) Normal male testis, displaying spermatogonia (Sg), spermatocytes (Sc), St – spermatids (St) and spermatozoa (Sz).
Figure 5b) Normal female ovary in vitellogenesis, displaying primary oocytes (po), secondary oocytes (so) and CA – cortical alveolus stage oocytes.
Figure 5c) An intersex gonad containing primary oocytes (po) only (category A).
Figure 5d) A grossly intersex gonad containing both primary oocytes (po) and secondary oocytes (so), adjacent to testicular tissue (Tt), (Category C).

Inter-site comparisons of intersex incidence and severity

Examination of captured gudgeon demonstrated incidences of intersex at all sites except for Crossflats on the River Aire, West Yorkshire. The greatest percentage of fish with intersex occurred at a reference site, Lakeside Fisheries, while the severity of intersex was greatest in gudgeon from Thwaite Weir on the River Aire, West Yorkshire (downstream of Esholt STW). An intersex incidence of 6% was noted at the reference site Longton Park Lake, near Stoke-on-Trent (see Figure 6).

Figure 6: The geographical occurrence and magnitude of severity of intersexuality in gudgeon

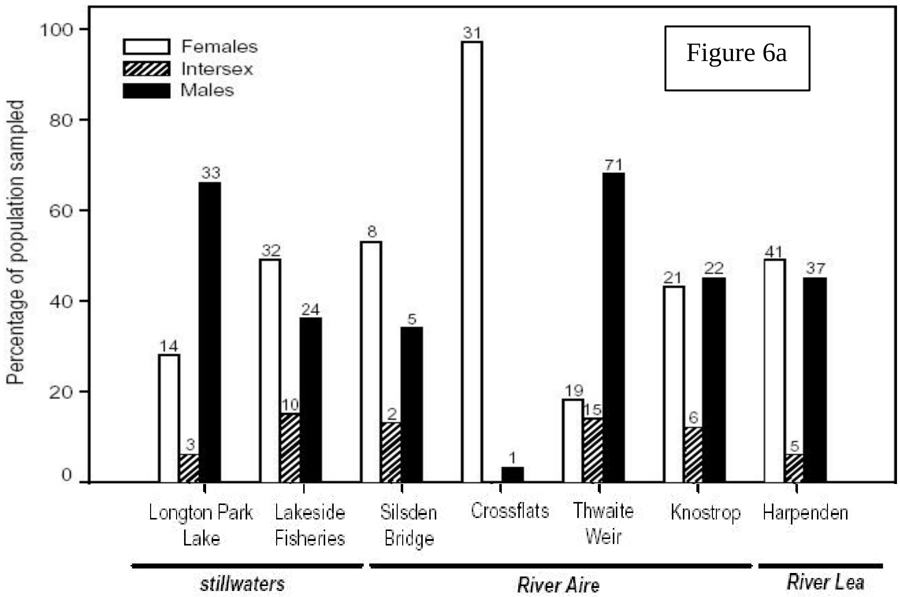


Figure 6a) Percentage of male, female and intersex gudgeon captured at the different sampling sites. Numbers above bars indicate the total numbers of fish in each group.

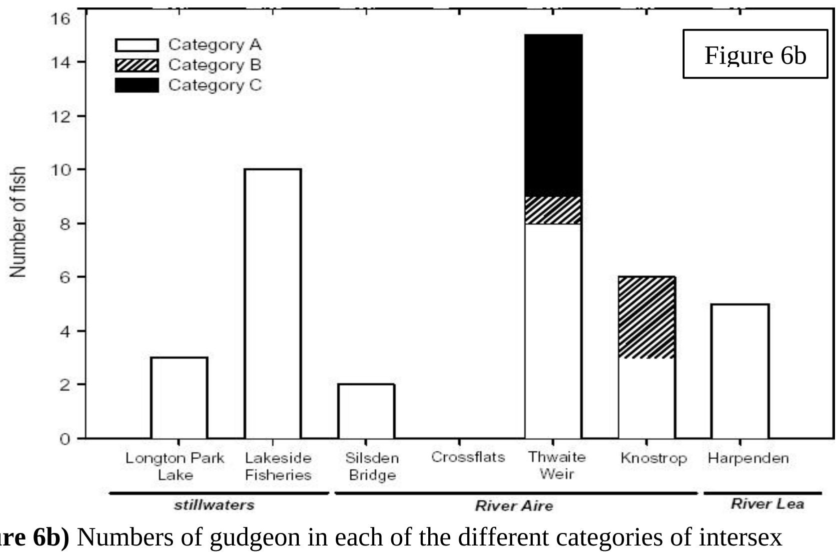


Figure 6b) Numbers of gudgeon in each of the different categories of intersex
Reprinted with permission from van Aerle *et al.*, (2001). Sexual disruption in a second species of wild cyprinid fish (the gudgeon, *Gobio gobio*) in UK freshwaters. *Environ Toxicol Chem*, 20 2841-7. Copyright 2001, SETAC

Although the gudgeon sampling regime was less extensive than that for roach, the gudgeon work was nevertheless considered of importance in determining the extent and type of intersex in a second species of cyprinid. The low incidence of intersex gudgeon found at Harpenden on the River Lea mirrors the situation observed in roach sampled from this site (Jobling *et al.,* 1998). Comparisons of intersex incidence and severity in gudgeon and roach caught at the same sampling sites on the River Aire, West Yorkshire, demonstrated that differences in the oestrogenic response to effluent exposure exist in these two species. In gudgeon, there was only a low incidence of intersex and an apparent small intersex severity gradient. However, similarities in the geographical distribution of severity were evident.

Whereas all phenotypic male roach from the River Aire showed intersex, less than 50% of phenotypic male gudgeon displayed intersex, and only three categories (two severity patterns) of intersex were defined in the gudgeon, compared to eight in the roach (Environment Agency, 1998). The apparent differences in response seen in these species could potentially arise from a variety of factors, such as different lifestyles (feeding and reproductive strategies, etc) affecting exposure level, and differing species sensitivities to oestrogenic stimulus, or could be a result of sampling at different stages of the reproductive cycle of the two species. Nonetheless, it is important to note that the disruption of germ cell development was evident in roach and gudgeon and distribution of severity of intersex in fish in the River Aire was similar for each species, with the most severely affected fish being those sampled from Thwaites Weir, Leeds. The differences in response between these two species may also be a reflection of the shorter lifespan of gudgeon and hence shorter time of exposure to effluent, and also to predominant year of birth of the gudgeon as opposed to roach collected from the same site.

Vitellogenin concentrations in gudgeon

It was not possible definitively to assess the influence of treated sewage effluent exposure on vitellogenin induction in the different gudgeon populations because sampling was undertaken at different times at the various sites. Vitellogenin titres are known to be transient, but nonetheless, levels of plasma vitellogenin in gudgeon from effluent-exposed sites were higher than in fish from the reference sites, indicating that these fish were exposed to oestrogenic substances (see Figure 7).

The equally high vitellogenin concentrations in fish caught from Longton Fisheries suggest that these were also exposed to oestrogenic compounds, although the source is unknown.

Figure 7: The plasma vitellogenin concentrations found in wild gudgeon

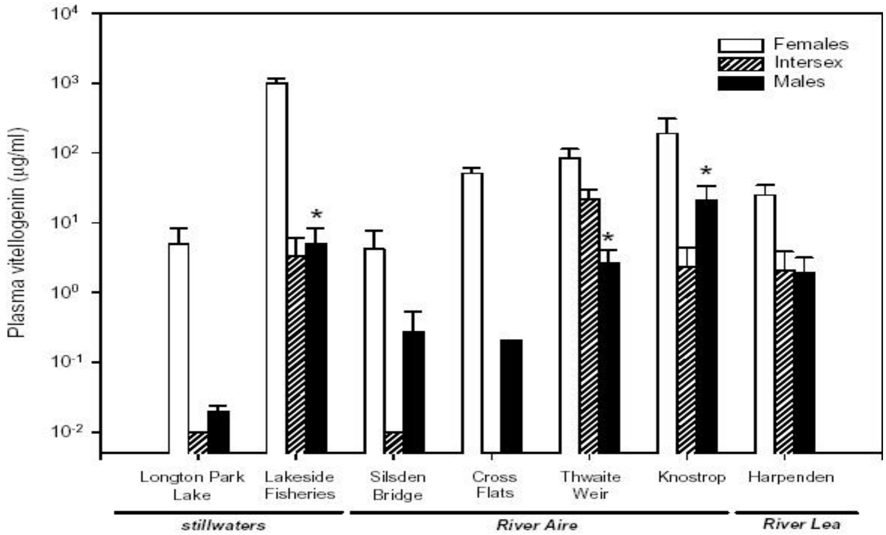


Figure 7) Plasma vitellogenin concentrations in female, male and intersex gudgeon collected from the different sampling sites. Numbers are given as means + S.E.M. (* - significant difference).
Reprinted with permission from van Aerle *et al.*, (2002). Sexual disruption in a second species of wild cyprinid fish (the gudgeon, *Gobio gobio*) in UK freshwaters. *Environ Toxicol Chem*, 20 2841-7. Copyright 2001, SETAC.

2.2 Assessment of Reproductive Health of Wild Roach

This study was undertaken to investigate whether the levels and severities of intersex recorded in Phase I (Environment Agency, 1998) were sufficient to affect the reproductive health of the roach, in particular their sexual maturation and *in vitro* fertilisation success.

2.2.1 Effects on sexual maturation of wild roach

Experimental design

In this investigation, fish from sites with an established incidence of intersex and from other 'reference' sites, were collected during the periods of sexual maturation (autumn) or spawning (spring). Fish were collected from the Rivers Nene and Aire (test sites), and the Royal Canal, Co. Kildare, Eire, Grantham Canal and Lake Wartnaby ('reference' sites; see Table 1), between October 1995 and May 1998. Fish collected in the autumn were examined for effects of effluent exposure on vitellogenin induction, hormonal status and gonadal histopathology. Fish collected in the spring were hand-stripped and the gamete quantity assessed. Gonadal histopathology, level of vitellogenin induction and hormonal status were also determined.

Blood steroid and vitellogenin analyses

Samples of plasma were taken from the fish and stored at –20°C. The steroid hormones 17β-oestradiol, testosterone and 11-ketotestosterone were extracted from plasma using ethyl acetate. After centrifugation, the solvent was evaporated and the dried extract re-suspended in buffer before quantification using specific radioimmunoassays (RIA; Carragher *et al.*, 1989).

Samples were not analysed for all steroids on each occasion. Vitellogenin was quantified following the methods of Tyler *et al*., (1996), (1999).

Histopathological examination of gonads - Sexual maturation

For female roach, the stage of sexual maturation was determined by scoring the proportion of prominent oocytes present in tissue sections, as follows:

1 Oogonia
2 Primary oocytes
3 Cortical alveolus stage oocytes
4 Early to mid vitellogenic oocytes
5 Mid to late vitellogenic oocytes

Oocytes were staged using accepted classification criteria for oviparous fish (Tyler & Sumpter, 1996). Assessment of sexual maturation in male roach was based on the method of Billard (1986), in which the proportions of testicular lobules to testicular cysts containing spermatogonia/ spermatocytes are assessed at different stages of development. In addition, for males, the degree of intersex development was determined on the basis of the stage of sexual development, cell types present, and extent of intersex apparent. Other abnormalities were also recorded. The developing male and female germ cells were characterised by measuring cell and nuclear diameters.

The gonads of wild roach caught in spring were assessed for histopathological change to the gonad and reproductive ducts. For each roach, the stage of sexual development, gonadal cell types, intersex status and the presence/absence of other abnormalities were recorded.

Gamete production

The gametes from wild roach were obtained by hand-stripping and assessed under field conditions. The volume of gametes produced was measured using a graduated test tube, while sperm density was determined for the milt using a haemocytometer. Adult fish were subsequently examined *post mortem* as per Section 2.1. In addition, relative gonad milt volume was determined as:

Relative gonad milt volume = Volume of milt/ weight of stripped gonad

Relative female fecundity rate was determined as:

Relative female fecundity rate = Number of eggs/ body weight

2.2.2 Results

Meristic measurement

For roach sampled during the autumn period, the majority caught from the River Aire were aged 3+ or 6+ years old, while those collected from the River Nene or reference sites showed a normal distribution of ages between 2+ to 6+ years. In autumn of 1997, the GSI of effluent-exposed male roach was less than that for reference site fish (p<0.001). However, no such difference was noted for females.

At the spring 1998 sampling, roach from the River Nene and Lake Wartnaby were predominantly 4+ and 3+ years, respectively, but those from the River Aire were predominantly 6+ years of age. A significant decrease in GSI was noted in non-spermiating roach.

Vitellogenin concentrations

Autumn (1997) sampling of roach showed no site-related differences in plasma vitellogenin level in females (see Figure 8). However, fish showing intersex from effluent-exposed sites had higher concentrations than either male or intersex fish from reference sites (p< 0.001). Pooling of the samples from the effluent-exposed intersex roach showed a vitellogenin concentration that was intermediate between that for pooled samples of males or females from the reference sites, indicating males were producing vitellogenin for eggs developing within their testes. In intersex fish from effluent-exposed sites, there was a positive association between vitellogenin concentration and the proportion of female tissue present in the gonad (r = 0.419, p = 0.0054).

Figure 8: Plasma vitellogenin concentrations in wild roach sampled in the autumn of 1997

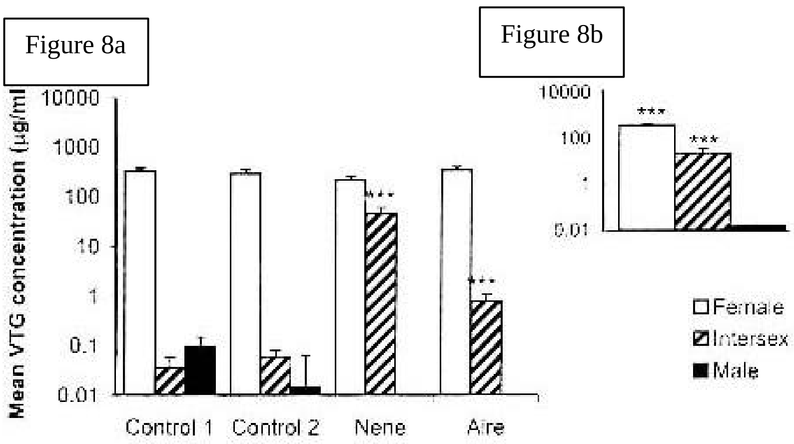


Figure 8a) Plasma vitellogenin concentrations in male, female and intersex wild roach.
Figure 8b) Pooled plasma vitellogenin concentrations of male, female and intersex wild roach. (Asterisks represent significant differences with control males only).
Asterisks (* p<0.05, ** p<0.01, *** p<0.001).
Reprinted with permission from Jobling *et al*., (2002a). Altered sexual maturation and gamete production in wild roach living in rivers that receive treated sewage effluent. *Biol Reprod*, 66 272-281. Copyright 2002, Society for the Study of Reproduction.

Sex steroid concentrations

Oestradiol concentrations in female roach from effluent-exposed sites were approximately 50% lower than those for females from the reference sites in autumn 1997 (p<0.001), whereas concentrations in effluent-exposed intersex fish were two-fold higher than in male or intersex roach from reference sites ($p<0.001$). When data for effluent-exposed intersex fish and male and female reference fish were compared, the oestradiol concentration of effluent-exposed intersex fish was intermediate between those for males and females from the reference sites (see Figure 9a, b). For fish from the reference sites, concentrations in male and intersex fish were lower than in females.

Differences were also noted in testosterone concentrations in autumn. Females from the River Aire had significantly higher concentrations than those from reference sites, whereas intersex fish from exposed sites showed significantly higher testosterone concentrations than reference male or reference intersex fish (see Figure 9c,d).

Figure 9: Plasma steroid concentrations in wild roach sampled in autumn 1997

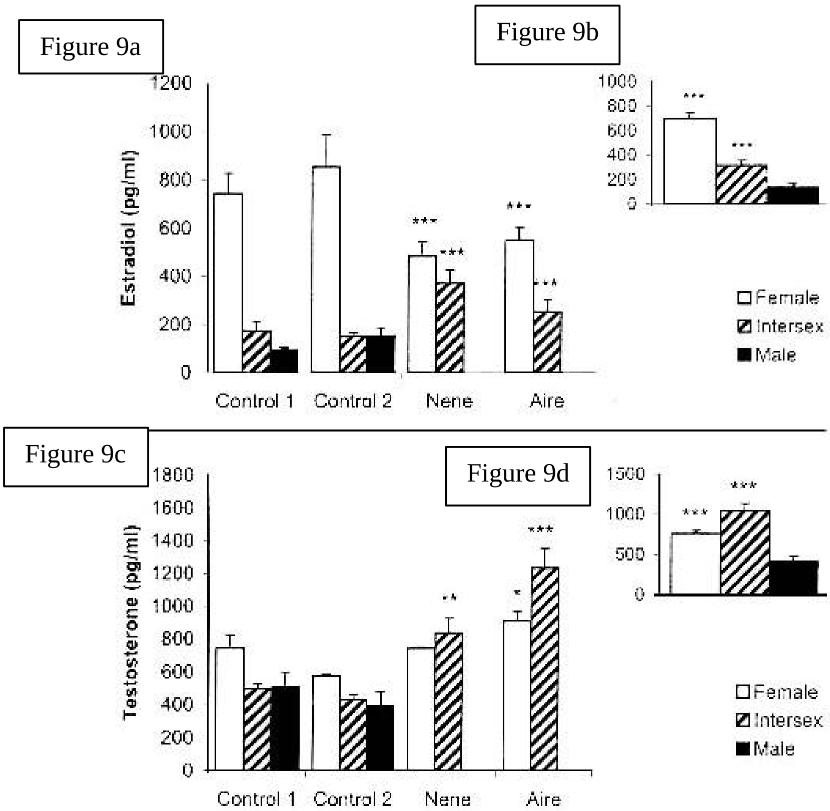


Figure 9a) Estradiol concentrations in male, female and intersex wild roach. **Figure 9b)** Pooled estradiol concentrations in male, female and intersex wild roach. (Asterisks represent significant differences with control males only).
Figure 9c) Testosterone concentrations in male, female and intersex wild roach. **Figure 9d)** Pooled testosterone concentrations in male, female and intersex wild roach. (Asterisks represent significant differences with control males only).
Asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

However, no similar effect was observed for 11-ketotestosterone, with concentrations being higher in male and intersex fish than effluent-exposed or reference site females. Concentrations of these hormones were noted as being extremely low in male and intersex fish in spring, however, the data are not reported owing to the difficulties in interpreting the rapid seasonal changes in hormonal profiles of fish prior to spawning.

Gonad histology

Among roach caught during autumn, all phenotypically male roach from effluent-exposed sites were found to have intersex, with low numbers of oocytes and also feminisation of the reproductive duct. In contrast, among phenotypic males from reference sites only 4% displayed intersex.

In spring 1998, no intersex fish were found at the reference sites while 15% of the phenotypic male roach from the River Nene and 38% from the River Aire had abnormally-shaped testes and/or absent, blind-ended or blocked reproductive ducts. Histopathology confirmed intersex in 85 and 100% of phenotypic males from the rivers Nene and Aire, respectively. Among females, no significant differences in gonadal histopathology were noted.

Sexual maturation and gamete production in male fish

In autumn 1997, an increase in the incidence of atretic oocytes was noted in effluent-exposed females compared with fish from Lake Wartnaby (Figure 10a). A few females (aged 4+ to 7+ years) from the River Aire were also sexually less mature than females aged 4+ years from the River Nene or control sites. The gonads of male roach from effluent-exposed sites appeared to be maturing at a slower rate than in male or intersex fish from reference sites ($p<0.001$) (see Figure 10b).

Figure 10: The effects of effluent exposure on the gonadal maturation of wild roach caught in autumn 1997

Figure 10a

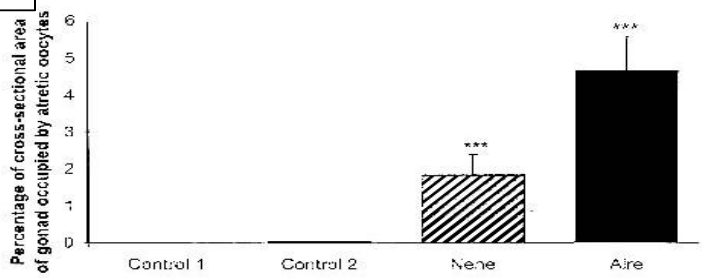


Figure 10b

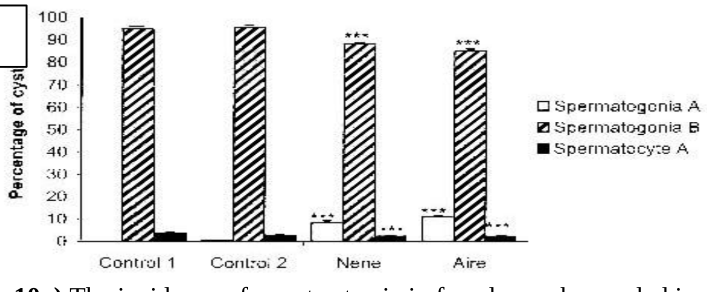


Figure 10a) The incidence of oocyte atresia in female roach sampled in autumn.
Figure10b) Maturity of the testes or ovo-testes in male (control 1 and control 2) and in intersex (Nene and Aire) roach sampled in October (* denotes significant reduction).

At the spring 1998 sampling, the majority of intersex fish from the effluent-exposed sites showed an early stage (Stage 1 or 2) of oogenesis, with synchronous development of male and female gametes in a few individuals. Intersex fish from effluent-exposed sites also showed a delay in sexual maturity compared with those from the reference location. The percentage of males producing sperm at the reference site was 100%, compared with only 49% and 57.1% for intersex fish from the Rivers Nene or Aire, respectively (see Figure 11).

In contrast, ovulation in female roach was in progress at each site, and had been completed in 80% of females from the reference sites, 87% from the River Nene and 91% from the River Aire, confirming that the state of sexual maturity in females was similar at all locations and, overall, their readiness for spawning. It is of interest that three true hermaphrodite fish (i.e. producing ova and milt simultaneously when hand stripped) were caught in the River Aire

Figure 11: The effects of effluent exposure on sperm production of wild roach collected in spring 1998

Figure 11a

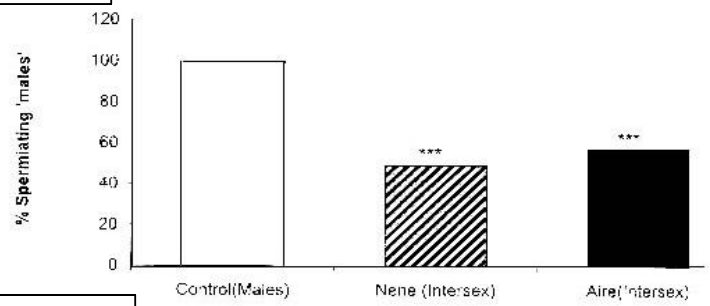


Figure 11b

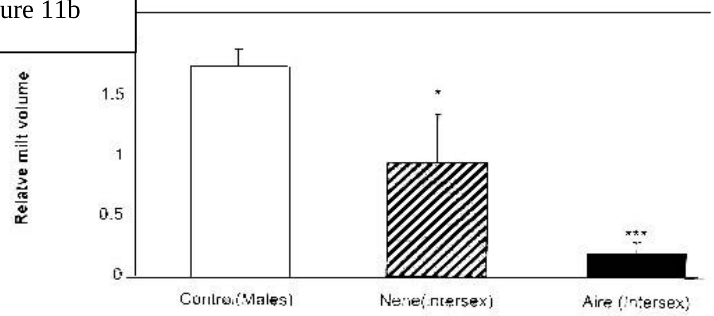


Figure 11a) Proportions of spermiating fish in populations of wild roach.
Figure 11b) Relative milt volume in spermiating male and intersex roach from rivers receiving STW effluents compared with males from a control lake.

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Gamete production

In male roach assessed in the field in the spring of 1999, milt volume and sperm densities were lower in effluent-exposed roach (see Figure 12). The majority of intersex roach that were able to expel gametes produced sperm. When stripped, all female roach produced only small numbers of ova. Owing to uncertainty as to the stage of spawning at the time of capture, the results from these fish could not, however, be clearly interpreted. These fish were subsequently taken for spawning experiments to assess gamete viability..

Figure 12: Gamete production in control and effluent-exposed roach collected during spring 1999

Figure 12a

Figure 12b

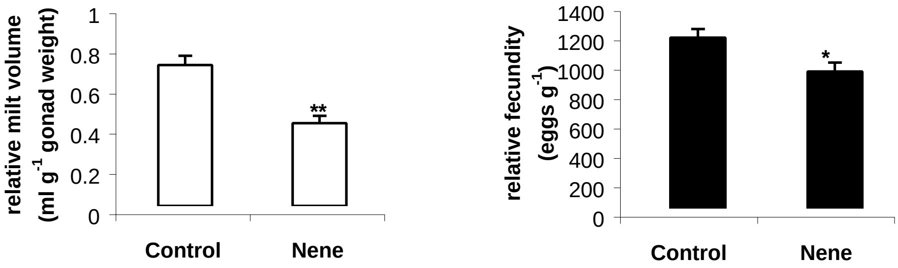


Figure12a) Relative milt volumes of the male/intersex roach in control and effluent-exposed (Nene) groups. **Figure 12b)** Relative fecundity of the female roach from control and effluent-exposed (Nene) groups. Error bars represent SEM and asterisks denote significant differences from control groups at significance levels *p< 0.05; **p< 0.01.

2.2.2 Assessment of gamete quality and the relationship between intersex severity and *in vitro* fertilisation success in wild roach

Experimental design

In these investigations, mature adult male, female and intersex roach were collected from effluent–exposed sites with an established incidence of intersex and also from a ‘reference’ site during May (1999 and 2000), the usual period of spawning.

Fish were grouped according to phenotypic sex (males and females only at this stage), and transported to the Environment Agency’s fish research laboratory at Calverton, Nottinghamshire. Here they were housed in glass fibre tanks in a recirculating system, at an ambient water temperature of 13.2 +/- 1.5 ^{o}C.

These fish were induced to spawn through injection of pituitary hormone, and the resulting gametes assessed for quantity, quality and *in vitro* fertilisation success.

Collection of gametes

Following acclimatisation, fish were hormonally induced to spawn by a series of intraperitoneal injections of carp pituitary extract (CPE) under anaesthesia, following normal fish farm procedure. Milt and eggs were collected by manual stripping. The fish were sacrificed and meristic measurements were taken.

Gamete quality and quantity

The volume of collected milt was measured and the relative milt volume calculated. The milt was retained in extender solution at $4^{o}C$ to preserve its viability for the *in vitro* fertilisation assays (see below) and assessment of motility using Computer Assisted Sperm Analysis (CASA: Hobson Vision Ltd, Baslow, UK). For CASA analysis, sperm were activated in distilled water and videotaped under light microscopy until motility ceased. Motility was assessed over 15 second intervals. Sperm density was determined by stopping the tapes 2, 5 and 10 seconds into each recording, counting all the sperm and averaging the results.

In addition to the male analyses, a further sample of female roach was hormonally induced to spawn and the numbers of eggs counted manually. The relative fecundity of each female was then calculated.

Assessment of fertilisation success

The following experimental cross-fertilisation studies were used to assess *in vitro* fertilisation success of wild roach:

<u>1999 reproduction season:</u>

1. Control milt x Control eggs: to assess the ability of gametes from normal male roach to fertilise ova from normal female fish.

2. Milt from effluent exposed fish x Control eggs: to compare male gamete quality between effluent exposed and unexposed male roach.

3. Control milt x Eggs from effluent exposed fish: to determine whether gamete quality is reduced in effluent-exposed female fish.

<u>2000 reproduction season</u>

4. Milt from intersex fish of varying severity x Control eggs: to examine the relationship between the intersex severity and fertilisation success.

For studies 1 to 3, approximately 300 eggs from each female roach from the reference site or the River Nene were exposed to milt from each of 22, 16 and 18 phenotypic male roach respectively from either the reference site or the River Nene; making 59, 40 and 50 crosses in total. Semen was diluted 1:1000 in distilled water. Gamete viability was determined by assessment of embryo viability 24 (+/- 2) hours post-fertilisation (hpf). For each *in vitro* fertility study, the numbers of viable embryos in three randomly selected fields of view were photographed, and the proportion of fertilized or viable eggs/embryos was calculated.

For the fourth study, approximately 300 eggs from one of three female control roach were exposed to milt from either a single male or intersex roach, of the 94/105 spermiating fish collected from the five rivers in the May 2000 sampling campaign.

Embryo viability was examined 24 (+/- 2) hpf, and at the eyed stage (when the eyes of the developing embryos are clearly visible to the naked eye). To calculate the number of viable embryos, all of the embryos in each batch were photographed in a single field of view and the proportion of viable embryos was expressed as a percentage of the total. Hatching rate was calculated by counting the number of hatched larvae at the "swim-up" stage and expressing this as a percentage of the number of eggs initially fertilised.

Histopathological analysis of gonads

The gonads of male and female roach were processed using the same histological methodology as described in Section 2.1.

Groups of intersex roach with similar manifestations of the condition were placed into the following categories of gonadal feminisation:

1. Ovarian cavity only – feminisation of the reproductive ducts only, no oocytes present in the testes.
2. Some oocytes – oocytes (mainly primary) present in the testes, ranging from one oocyte in one section to oocytes comprising up to 33% of the observed gonadal tissue.
3. Severely feminised – oocytes comprise a large proportion of the observed tissues (>33%) with both primary and secondary oocytes often being present.

Results

Meristic measurements

There were no significant differences in length, weight or age of the roach caught in the 1999 reproductive season or from the rivers Lea, Arun or Aire, during the 2000 reproductive season. However, roach caught from the rivers' Blackwater and Calder during the 2000 reproductive season were significantly younger compared with the other fish caught during this period ($p<0.001$). There was an apparent decrease in the number of River Nene intersex fish that had tubercles on the surface of their skin (an indicator of male spawning condition) compared with those of male fish from the reference site although this was not significant. However, of the roach caught during the 2000 reproductive season, there were no significant differences in the proportions of fish bearing tubercles (after induction by CPE) between either the groups of fish obtained from different rivers or intersex and male fish.

Histopathological analysis of gonads

Macroscopic examination of the gonads from roach sampled in 1999 suggested that those from the River Nene and Lake Wartnaby consisted of normal mature male and female fish; the gonads appearing to be of the expected size and appearance for sexually mature roach. However, microscopic examination of the gonads revealed that 100% of the phenotypic male fish from the River Nene were intersex (with an average Intersex Index of 0.95) whilst in all of the Wartnaby (control) males the testes appeared normal (no feminisation was evident). In all intersex (Nene) fish, both male and female reproductive ducts were present, although only a few individuals had testes that contained low numbers of developing oocytes within primarily testicular tissue.

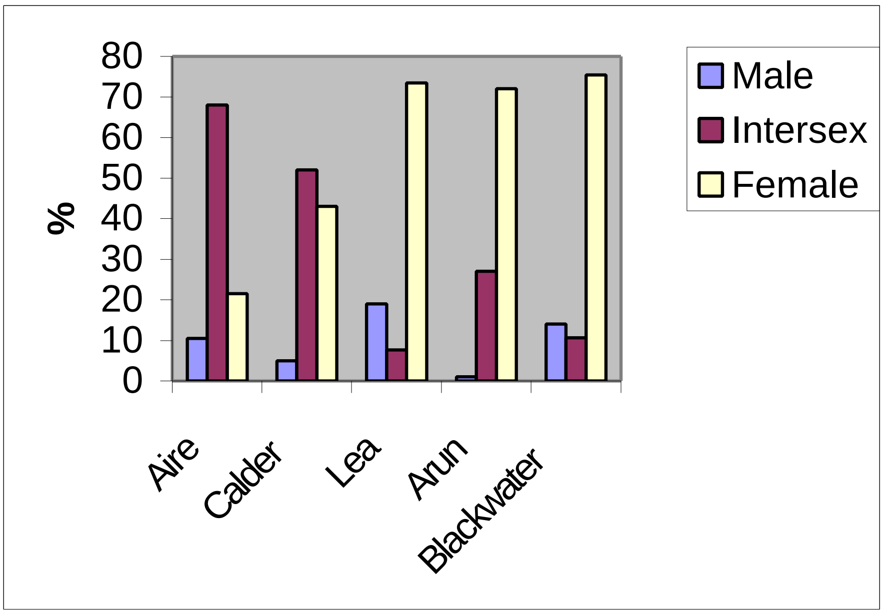
Macroscopic examination of roach sampled in the year 2000 indicated that the majority of the gonads were the expected size and shape for adult male and female roach, with the exception of three fish from the River Arun, that exhibited gross intersex malformations. However, microscopic examination revealed that 46% of all roach collected in the 2000 sampling campaign were intersex. When separated into groups representative of their river of origin, it was found that > 90 % of 'male' roach from the rivers Aire, Arun and Calder were intersex, whereas the incidences of intersex in fish from the rivers Blackwater and Lea were 31% and 9 %, respectively.

Of the histologically normal "mature" males identified in the sampling from the Blackwater and Lea, 12 and 16 %, respectively, were found to have immature gonads that were exhibiting inhibition of spermatogenesis and gonadal growth. Of the 105 roach collected in this sampling campaign, 41 were histologically normal males, 31 had an ovarian cavity only, 19 had some oocytes in testicular tissue, and four were severely feminised males.

Sex Ratio

Interestingly, the proportions of male, female and intersex fish varied widely, from 1% male, 72% female and 27% intersex (on the R. Arun) to 19% male. 73% female and 8% intersex (on the River Lea). The River Aire sample contained the greatest proportion of intersex fish (68%), whilst the River Blackwater sample contained the greatest proportion of females (see figure 13).

Figure 13: Sex ratios of roach taken for spawning experiments in 2000



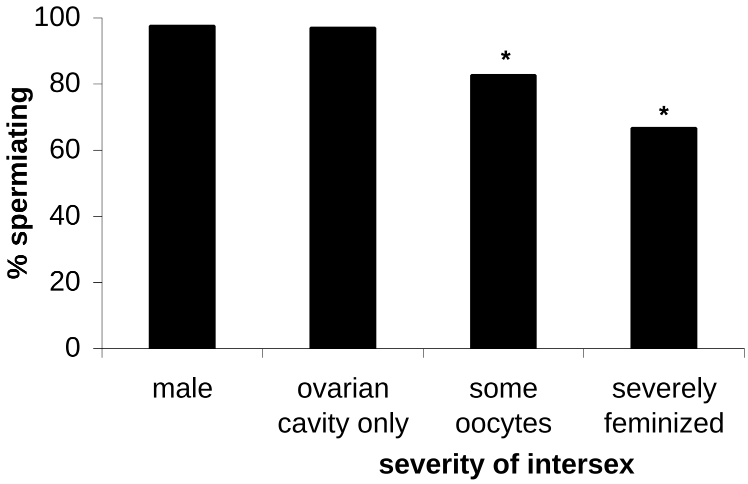
Gamete quantity

Of the roach sampled during 1999, 97.7% of the male roach from the reference sites and 85.8% from the River Nene released sperm when hormonally induced to spawn. The milt volume produced by effluent exposed fish was significantly reduced ($p<0.01$). The proportions of ovulating female fish in each group were 81.0% for the River Nene and 71.0% for the controls. No significant difference was noted in the number of roach releasing gametes (sperm or eggs) from effluent-exposed sites compared with the reference site, although calculation of the relative fecundity rates in females from the River Nene showed a significantly lower value ($p<0.05$).

Induction of spawning of the roach collected during the year 2000 sampling campaign, resulted in 97.6% spermiation of normal males, compared with 97.0% of males with ovarian cavities, 82.6% of males with ovarian cavities and oocytes and 66.7% of severely feminised males. A significantly higher proportion of the fish in the latter two groups (17.4% and 33.3% respectively) did not spermiate ($p<0.05$) (see Figure 14).

The majority of non-spermiating fish had testes that were immature despite being 3 to 5 years of age. Male roach would be expected to mature at least by their fourth year of life. The remainder had lobules of testicular tissue containing sperm although none was released after induction of spermiation. Induction of spawning in female fish resulted in 100 % ovulation. The volume of milt released by the intersex fish collected during the 2000 spawning period was not significantly decreased compared to controls and there was no relationship between milt volume and intersex index ($p>0.05$).

Figure 14: Effect of intersexuality on milt production in roach collected in the 2000 reproductive season



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Sperm analyses

CASA analysis of milt from roach sampled from the River Nene during the 1999 reproductive season demonstrated a significantly lower percentage of motile sperm between 20 and 50 seconds post-activation and a slower velocity between 5 to 35 seconds post-activation ($p<0.05$), compared to controls, although sperm density was not a determining factor (see Figure 15).

In roach caught in the 2000 reproductive season, no significant effects on relative milt volume were observed between the fish as grouped by severities of the intersex condition.. Decreased sperm density with increasing intersex severity was apparent, but was only statistically significant in severely feminised roach ($p<0.05$; see Figure 16a). No significant correlation was observed between sperm density and the Intersex Index ($p>0.05$; see section 2.1.1).

There were also subtle differences in sperm motility (both in the percentage of motile sperm and their swimming velocity) between the intersex and male fish. Significant differences were found to occur in the initial 5-20 seconds post activation and in the severely feminised group (Figure 16b). Compared with histologically normal male fish, the percentage of motile sperm was unaffected in fish with ovarian cavities, but was significantly reduced ($p<0.01$) during the initial 5 to 20 seconds post-activation in fish with some oocytes (Figure 16c). In severely feminised fish, the mean proportion of motile sperm was reduced by about 30%, but this was not statistically significant, owing to the small sample size and high variability of motility in this group of severely feminised fish. Overall, the mean percentage of motile sperm appeared to decrease with increasing severity of intersex, although there was no statistically significant relationship between the two.

Figure 15: Sperm characteristics of control and effluent-exposed (River Nene) roach from the 1999 reproductive season

Figure 15a

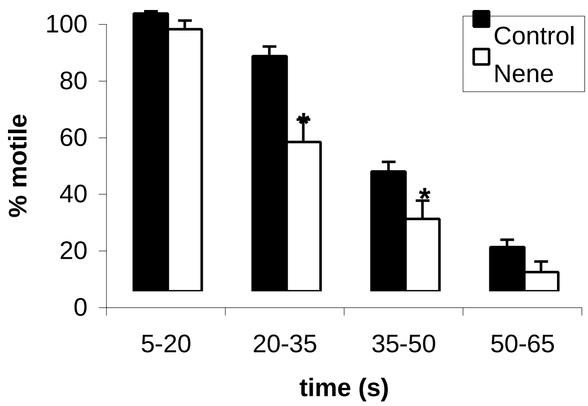

Figure 15b

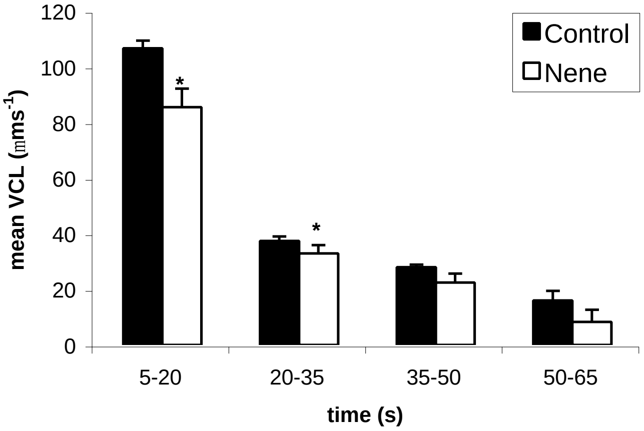

Figure 15a) Percentage of sperm motile in control and effluent-exposed (Nene) roach at various times after activation. **Figure 15b)** Mean curvilinear velocity (VCL) of motile sperm from control and effluent-exposed (Nene) roach at various times after activation. Error bars represent SEM and asterisks denote significant differences from control at significance level *p< 0.05.

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Figure 16: Sperm characteristics of male and intersex roach collected during the 2000 reproductive season

Figure 16a

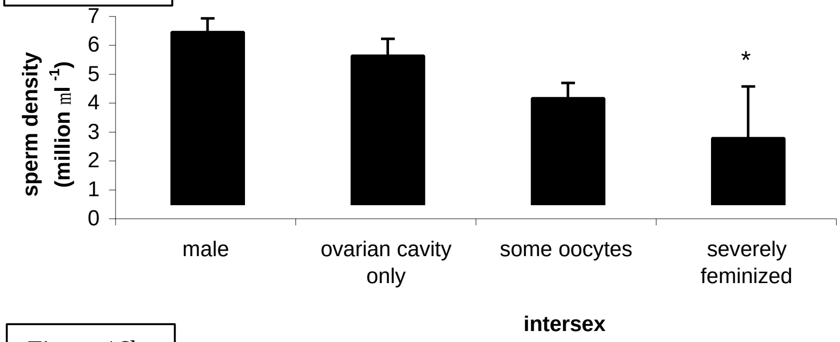

Figure 16b

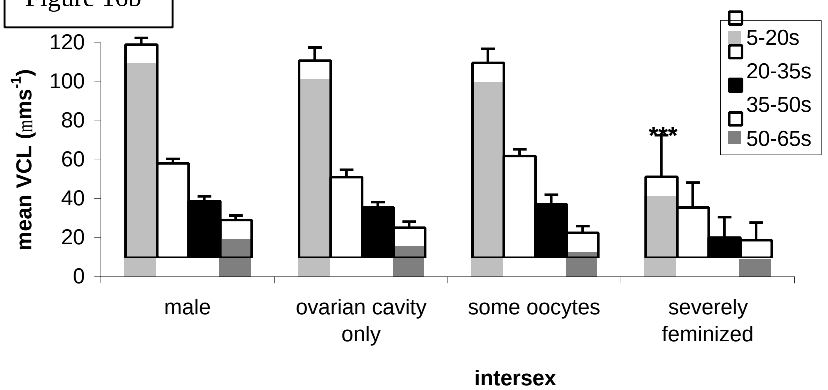

Figure 16c

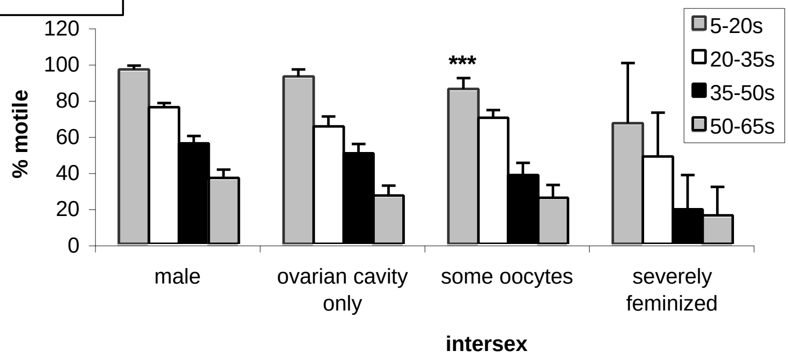

Figure16a) Mean sperm density of male roach and roach with different degrees of intersexuality.
Figure 16b) Mean curvilinear velocity (VCL) of motile sperm from male and intersex roach at various time after activation.
Figure 16c) Percentage of sperm motile in male and intersex roach at various times after activation.
Error bars represent SEM and asterisks denote significant differences from males at significance level *p<0.05; ***p<0.001.
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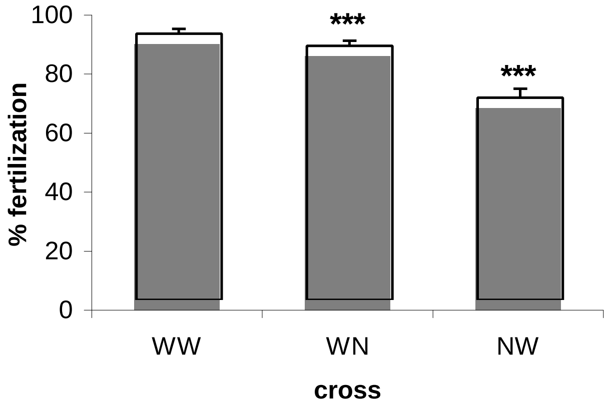
Differences in sperm velocity between the intersex and male fish were confined to the initial 5-20 seconds post activation, with a marked decrease (up to 50%) in swimming velocity in sperm from Category 3 fish compared with sperm from Category 1 and 2 fish ($p<0.01$) or from male fish ($p<0.001$; see Figure 16b).

***In vitro* fertilisation success**

Cross-fertilisation studies on sperm from roach caught in 1999 showed fertilisation success for effluent-exposed roach to be significantly lower for both eggs from River Nene fertilised by milt from the reference sites (89%, $p<0.001$) and for reference eggs and milt from the River Nene (68%, $p<0.001$; see Figure 17), compared with a reference site success rate of 93%. Although fertility was reduced for both studies involving gametes from exposed sites, the effect of effluent exposure appears markedly greater in male than in female roach.

Figure 17: The reproductive success of control and effluent-exposed roach in the 1999 reproductive season

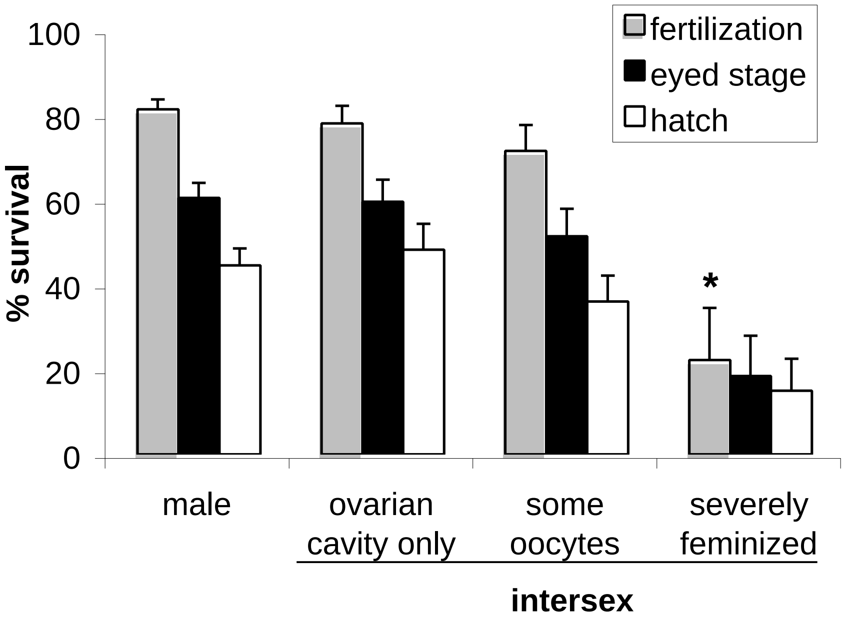


Milt and eggs were collected from control (Lake Wartnaby, W) and effluent-exposed (River Nene, N) roach and used to establish 3 crosses: WW (control males x control females), WN (control males x exposed females); NW (exposed males x control females). The percentage of fertilised eggs was determined after 24 hours. Error bars represent SEM and asterisks denote significant differences from the control cross (WW) at significance level ***$p<0.001$.

Roach caught in spring, 2000 displayed inter- and intra-river variability in fertilisation success and no significant differences were observed in the fertilisation success of the males and individual intersex fish ($p>0.05$). However, when the intersex fish were grouped according to the Category of gonadal feminisation, *in vitro* fertilisation success was significantly decreased in Severely feminised fish ($p<0.05$; see Figure 18).

There was also a significant negative relationship between the Intersex Index of affected roach and their fertilisation success ($r = -0.603$; $p<0.001$). In all groups, the proportions of surviving embryos fell at each developmental stage assessed. This effect was magnified in eggs fertilised with sperm from intersex fish and the numbers of hatching embryos decreased in line with an increase in paternal intersex severity.

Figure 18: Reproductive success of male and intersex roach collected during the 2000 reproductive season



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2.3 Conclusions

Incidence of oestrogenic effects in native fish populations

- Wild roach populations in English rivers receiving treated effluent are showing a range of reproductive health effects including ovo-testis, feminisation of the reproductive ducts in males, disruption and asynchrony of sexual maturity, and occurrence of atretic oocytes.

- In UK wild gudgeon populations, intersex was also evident, although at a lower incidence and severity than in roach. Such differences could be due to different life histories. However, geographical distributions of intersex severity were similar for the two species.

Consequences of the oestrogenic effects observed

- In late-spring, male roach taken from effluent-exposed sites and assessed for spawning readiness showed delayed spermatogenesis compared with males from control sites, whereas females were not at a dissimilar stage. Spawning readiness was concluded to be asynchronous in some wild roach populations.

- Examination of gamete viability in wild roach at spawning showed reduced milt volume and sperm motility in males, and atretic and vacuolated oocytes in females. Furthermore, *in vitro* fertilisation studies established significant reductions in the viability of gametes from male and female roach exposed to effluents, particularly for sperm.

- There was a significant negative relationship between the Intersex Index of affected roach and their fertilisation success ($r = -0.603$; $p<0.001$). In all groups, the proportions of surviving embryos fell at each developmental stage assessed, but this effect was magnified (and much more variable) in embryos produced by severely intersex fish.

- Factors that influenced a reduction in male fertility in fish from effluent-exposed sites included reduced sperm density, lower proportion of sperm that were motile and reduced duration of motility. Severely-feminised male fish showed the greatest variability in the sperm characteristics measured.

3. ASSESSMENT OF CAUSE AND EFFECT RELATIONSHIPS TO SEWAGE EFFLUENTS

3.1 Background

These studies were designed to examine the apparent influence of exposure to treated sewage effluent on the reproductive health of freshwater fish in the UK as noted during the first phase of the research programme. Previous studies (Harries *et al*., 1999) at Harpenden STW, in 1994, had revealed, after 3 weeks of exposure to graded dilutions (0, 25, 50, 75 or 100%) of STW effluents elevated plasma vitellogenin levels in males exposed to 50 or 100% effluent compared with controls ($p<0.001$). Evaluations using effluent from Chelmsford STW in 1996 and 1997 revealed a similar dosage-related induction of vitellogenin at all the dilutions tested.

These results confirmed the oestrogenic nature of the Harpenden and Chelmsford STW effluents as revealed by the induction of the oestrogenic biomarker, vitellogenin, over a 3-week exposure period. No somatic or organisational effects were discerned, but such a short-term exposure experiment does not enable the study of development effects that are likely to arise following chronic (or even lifetime) exposure, or at specific times during the lifecycle when development is occurring. Therefore, to assess the cause effect relationship for the native cyprinid fish populations showing adverse health effects, particularly on their reproductive development, longer-term experiments were required, and at different developmental stages in a cyprinid fish lifecycle.

3.2 Long-term Temporal Changes in the Oestrogenic Composition of Treated Sewage Effluent and Its Biological Effects on Fish – *Experiment A*

3.2.1 Experimental design

Studies were conducted to assess oestrogenic effects on adult roach following exposure to treated sewage effluent.

Male and female roach, housed in large Perspex tanks at Chelmsford STW for four months from November 1997 to March 1998, were exposed to flowing treated effluent at various concentrations (0, 12.5, 25, 50 or 100%) diluted with river water. Before exposure and after 1, 2 or 4 months, subgroups were anaesthetised for blood sampling and then killed. Length and weight of the fish were recorded, gonads weighed and the GSI calculated. The gonads were then subject to histopathological examination., and the blood plasma vitellogenin level determined using a carp enzyme-linked immuno-sorbent assay (ELISA).

Chemical analysis

In addition to these investigations, detailed chemical analyses were performed on samples of effluent taken from Chelmsford STW during November 1997 to March 1998 (Experiment A). Effluent samples were also taken during July to December 1998 (Experiment B), and again were subjected to detailed analysis.

Composite samples of effluent were obtained by taking duplicate 400 ml samples at approximately the same time, daily for five days. The samples were subsequently combined before analysis. Samples were taken at commencement of the study (time zero) and after 1, 2 or 4 months. Physicochemical parameters were measured and compared with fluctuations in the chemical composition (see Table 2).

Assessment of oestrogenic activity and identification of oestrogenic compounds used a toxicity identification and evaluation (TIE) approach (Mount & Anderson-Carnahan, 1988), in which the effluent was fractionated and tested using an oestrogen-responsive recombinant yeast screen (YES) assay (Routledge & Sumpter, 1996)**.** Oestrogenic substances (that is the steroid hormones identified during TIE and alkylphenolic ethoxylates detected in preliminary studies; Desbrow *et al*., 1998) were extracted from the effluent fractions using a C18 silica bonded solid-phase extraction (SPE) cartridge, eluted with ethyl acetate followed by dichloromethane (DCM) for alkylphenolic ethoxylates, or twice with DCM for the steroids, and identified by GC-MS.

3.2.2 Results

Meristic measurement

No effects of effluent exposure were noted on growth, survival or condition of the fish. GSI showed increases with time in both sexes across all treatments, in line with normal seasonal development.

Vitellogenin concentrations

Plasma vitellogenin concentrations in adult females were unaffected by any of the effluent dilutions tested. In males exposed to river water alone, vitellogenin concentrations remained unchanged throughout the 4 month exposure period.

Among males exposed to effluent, a consistent dosage-related increase in vitellogenin was noted throughout the study period. At 100% effluent, there was a significant induction of vitellogenin at all time points ($p<0.01$).

Statistical significance was also attained after 1 month’s exposure at an effluent concentration of 37.9 ± 2.3%, and at 4 months at an effluent level of 9.4 ± 0.9% and 17.53% ± 1.19. A strong correlation between time and vitellogenin was determined, indicating a temporal lowering of the threshold for vitellogenic response ($p<0.001$, $r = 0.839$; see Figure 19) .

Gonadal pathology

No treatment-related changes in gonadal pathology were observed in either adult male or female roach.

Chemical analysis of effluents in Exposure

The natural oestrogens 17β-oestradiol and oestrone were identified by TIE as major contributors to ‘in vitro’ oestrogenic activity of the Chelmsford STW effluent, on the occasions investigated (see table 2). The ratio between the two compounds showed marked variation during Experiment A, but was relatively constant in Experiment B when oestrone was present at a much higher concentration (oestrone concentrations three to 13 times higher than for oestradiol).

Figure 19: The effects of treated effluent exposure on plasma vitellogenin concentrations in male roach

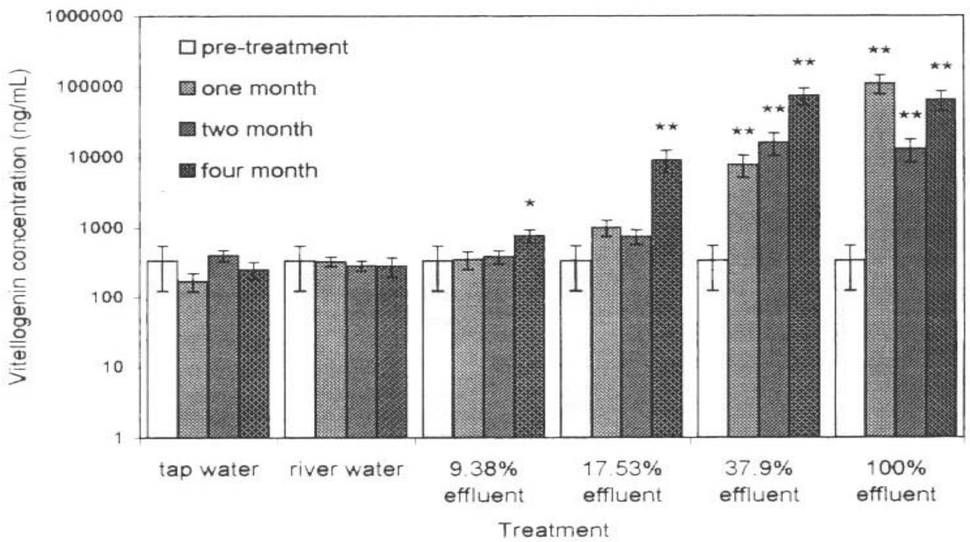

Figure 19) Mean vitellogenin concentration (ng/ml) ± SEM, in adult male roach exposed to various concentrations of treated sewage effluent (* p<0.05, ** p<0.01).

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Overall, concentrations of these substances were three-fold higher in Experiment A than Experiment B (see Table 2). The synthetic steroid hormone 17α-ethinyl estradiol was also detected intermittently outside, but not during, the experimental periods. The alkylphenolic compound, nonylphenol, and its ethoxylates (mono- and di-ethoxylates) were found at higher concentrations than the steroidal oestrogens in both trials. Alkylphenol concentrations varied markedly, however, being much greater in Experiment B than Experiment A. Nonylphenol was below the level of detection in effluents analysed in Experiment A.

Linear regression analyses did not identify any apparent relationships between the measured concentrations of steroids or nonylphenolic compounds in the treated sewage effluent and the physicochemical parameters of the treated sewage effluent.

Table 2: Concentrations of oestrogenic substances and physicochemical properties of the final effluent at Chelmsford STW over the two trial periods

	Trial A				Trial B			
Oestrogenic substances (ng/l)	**November**	**December**	**January**	**March**	**July**	**August**	**October**	**December**
Oestradiol	7	51	71	88	4	8.8	4.4	6.3
Oestrone	104	15	59	220	31.5	27	56	34
Ethinyl estradiol	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Nonylphenol	ND	ND	ND	ND	2700	1200	2400	1600
Nonylphenol Ethoxylates	<200	1000	2000	< 200	350	8900	8300	6500
Octylphenol	NA	NA	NA	NA	190	30	80	50
Physicochemical properties	Mean ± SEM				Mean ± SEM			
Temperature (^{o}C)	12.3 ± 0.38				17.2 ± 0.65			
Rainfall (mm: mean per day)	2.4 ± 1.15				0.5 ± 0.32			
pH	7.59 ± 0.06				7.43 ± 0.06			
Suspended solids (mg/l)	15.8 ± 1.58				16.3 ± 1.04			
Ammonia (mg/l) as N	1.34 ± 0.26				1.61 ± 0.19			
Nitrite (mg/l) as N	0.81 ± 0.09				0.71 ± 0.12			
Nitrate (mg/l) as N	67.9 ± 2.81				93.9 ± 3.82			
BOD[a]	7.43 ± 0.66				6.2 ± 0.48			
Dissolved oxygen (mg/l)	7.51 ± 0.12				7.2 ± 0.13			
Total bacteria counts[b] (cfu)	63 624				123 503			

a biochemical oxygen demand   b bacterial counts include faecal coliforms, streptococci and E. coli

ND not detected SEM standard error of mean

NA not available

3.3 Effect of Treated Sewage Effluent Exposure on Reproductive Duct Development in Juvenile Roach – *Experiment B*

3.3.1 Experimental design

A preliminary study was undertaken to identify the critical time periods of sexual differentiation in male and female roach. Juvenile roach were reared in dechlorinated tap water and gonadal pathology examined (as per Section 2.1) at 50, 100, 150 or 200 days post-hatch (dph). The findings from this study were used to define the sensitive periods of sexual differentiation (duct formation and germ cell formation) in roach and ensure that the experimental effluent exposure durations in the main study coincided with these developmental periods.

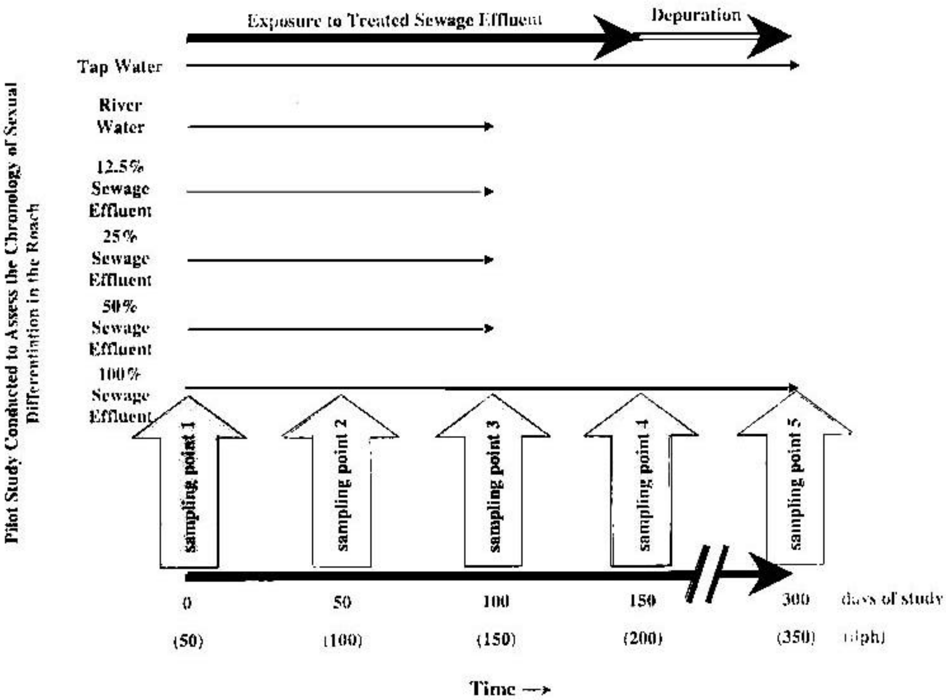
In the main study, male and female juvenile roach were housed in tanks under through-flow conditions and exposed to treated sewage effluent from Chelmsford STW at nominal concentrations of 0 (river water only), 12.5, 25, 50 or 100% effluent (see Figure 20). An additional control group was held in dechlorinated tap-water. The groups exposed received actual mean concentrations of 0%, 12.0 ± 0.9%, 23.6 ± 0.8%, 47.4 ± 1.2% and 100%, respectively. Subgroups were taken for investigation at specific time points in the study:

- Sampling Point 1 (50 dph) - prior to exposure.
- Sampling Point 2 (100 dph) - after 50 days exposure to effluent.
- Sampling Point 3 (150 dph) - after 100 days exposure to effluent.
- Sampling Point 4 (200 dph) - for fish exposed to 100% effluent and the tap-water controls only; assessed for effects on germ cell differentiation.
- Sampling Point 5 (350 dph) - fish exposed to 100% effluent from 50 to 200 dph, concentrations followed by a recovery period of 150 days in clean water, before examination (to determine if any exposure-induced effects were reversible and to further assess male sexual development).

On each occasion, the length and weight of the fish in the relevant subgroup were measured, and histopathology of the gonad and reproductive duct assessed. Fish were also sampled for either vitellogenin or 17β-oestradiol level; whole body homogenates were analysed for vitellogenin (see Section 4.2). At Sample Points 1-3, 17β-oestradiol level was determined for pooled body homogenates of five fish per group, using a competitive-binding radioimmunoassay (Carragher *et al.*, 1989).

In addition, samples of treated sewage effluent, collected contemporaneously with each fish sampling time point, were analysed for natural and synthetic steroid hormones and alkylphenolic compounds, as detailed previously (Section 3.2.1).

Figure 20: Flow diagram of the experimental design whereby juvenile roach were exposed to various levels of treated sewage effluent at various stages of sexual development



Reprinted with permission from Rodgers-Gray *et al.*, (2001). Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. *Environ Sci Technol*, 35, 462-470. Copyright 2001, American Chemical Society.

3.3.2 Results

Meristic measurement

Between 50 and 100 dph, fish exposed to 100% effluent showed a increase in bodyweight compared with other treatments ($p<0.05$). However, the magnitude of this effect was unaffected by an additional 50 days exposure to this level of effluent, and there were no significant changes at the other effluent concentrations examined at any occasion. After 150 days depuration in clean water, intersex fish (‘feminised males’; see below) weighed significantly more compared with the tap water control male fish ($p<0.05$).

Vitellogenin analysis

Exposure of juvenile fish from 50 dph to graded dilutions of effluent for 50 days resulted in a dosage-related increase in whole body vitellogenin level, compared with controls ($p<0.001$; Sample point 2). Exposure to 100% effluent from Sample point 2 for 50 days until 100 dph resulted in an 11-fold increase in whole-body vitellogenin level, while levels in control fish only increased 5-fold over this period**.** However, following 100 days exposure at 150 dph, whole body vitellogenin levels no longer showed any relationship to effluent exposure (Sample point 3).

Among roach exposed to 100% effluent, there was evidence of a time-dependent increase in vitellogenin. Sampling at 150 and 200 dph showed a significant increase in the levels of whole-body VTG in fish of this treated group after 100 or 150 days exposure. When fish that had been exposed to 100% effluent from 50 to 200 dph were allowed a recovery period of 150 days in tap water (to 350 dph; Sample point 5), whole body vitellogenin levels fell between 200 and 350 dph, although they remained higher than the unexposed controls on both occasions ($p<0.001$). In addition, among fish that had been allowed a recovery period after exposure to 100% effluent, the vitellogenin level for those showing intersex (i.e. 'feminised males') was comparable with that of females in the exposed group at 350 dph.

Sex steroid concentrations

The investigation of hormone concentrations levels in this study relates to the exposure of juvenile roach from 50 dph to graded concentrations of effluent for 100 days. Exposure for 50 days (Sample point 2) resulted in no treatment related change in whole body 17β-oestradiol levels. However, when exposure was extended to 150 dph (i.e. exposed for 100 days), a dosage-related elevation in whole body oestradiol was apparent ($p<0.05$).

Gonadal pathology

Pathological examination of the gonads of the juvenile roach showed that the gonads were undifferentiated at 50 dph, and that the development of the reproductive duct in both males and females occurred between 50 and 150 dph. However, development of the gametes was found to be discernable only at a later stage of the life cycle. Female germ cell differentiation occurred between 100 and 200 dph, while male germ cell differentiation was not apparent until after 200 dph.

Dosage- and time- dependent occurrence of female-like reproductive ducts was evident at 100 and 150 dph after exposure to graded concentrations of treated effluent from 50 dph. Indeed, all fish held in 100% effluent showed female-like reproductive ducts at 100 dph, following 50 days exposure (Figure 21a).

Fish held in tap water for 150 days following initial exposure to 100% effluent were found still to display female-like ducts regardless of their germ cell sex (Figure 21b). (Note: Based on findings from other treatment regimens, all fish in this group can be assumed to have developed a female-type reproductive duct during the exposure period).

With regard to germ cell differentiation, juvenile roach exposed to graded concentrations of treated effluent from day 50 post hatch showed no discernible changes by 100 dph (Sample point 2). However, after 100 days exposure to 100% effluent (150 dph) there was a significant increase in number of roach bearing primary oocytes ($p<0.001$).

No increase in the number of oocytes per fish were noted in those given lower effluent exposures, all accompanied by the presence of a female-type reproductive duct. After an additional 150 days exposure (300 dph; Sample point 5), the numbers of fish with primary oocytes at 100% effluent was significantly higher than for the controls (Figure 22). At that time point, male germ cells were evident.

Figure 21a: Development of oviducts in juvenile roach following exposure to graded concentrations of sewage effluents

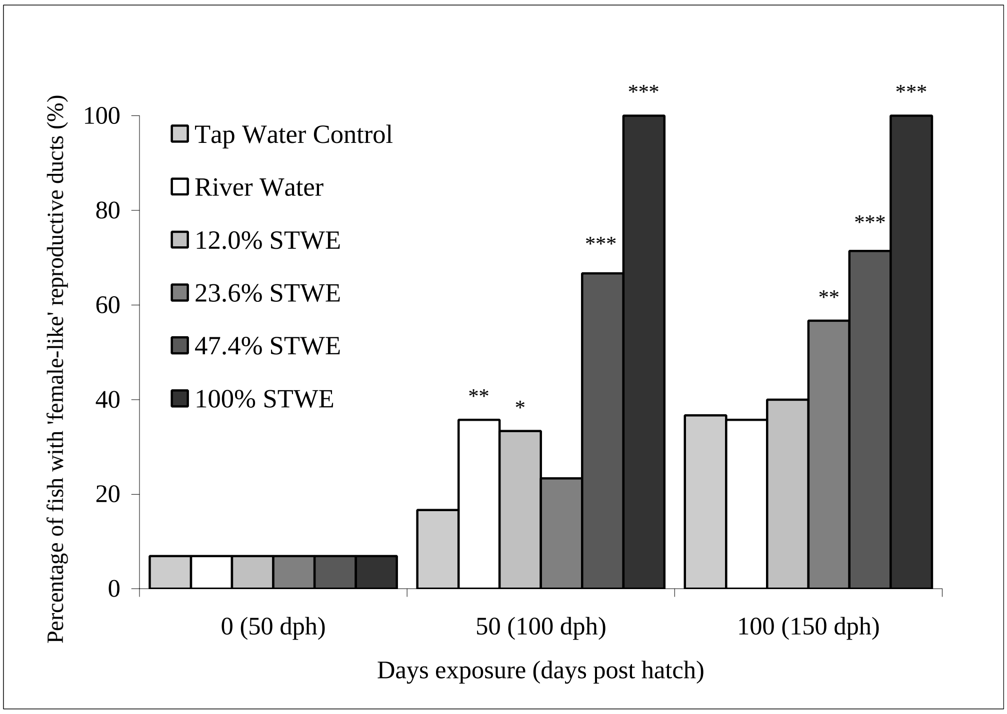


Figure 21b: Development of primary oocytes in juvenile roach following exposure to graded concentrations of sewage effluents

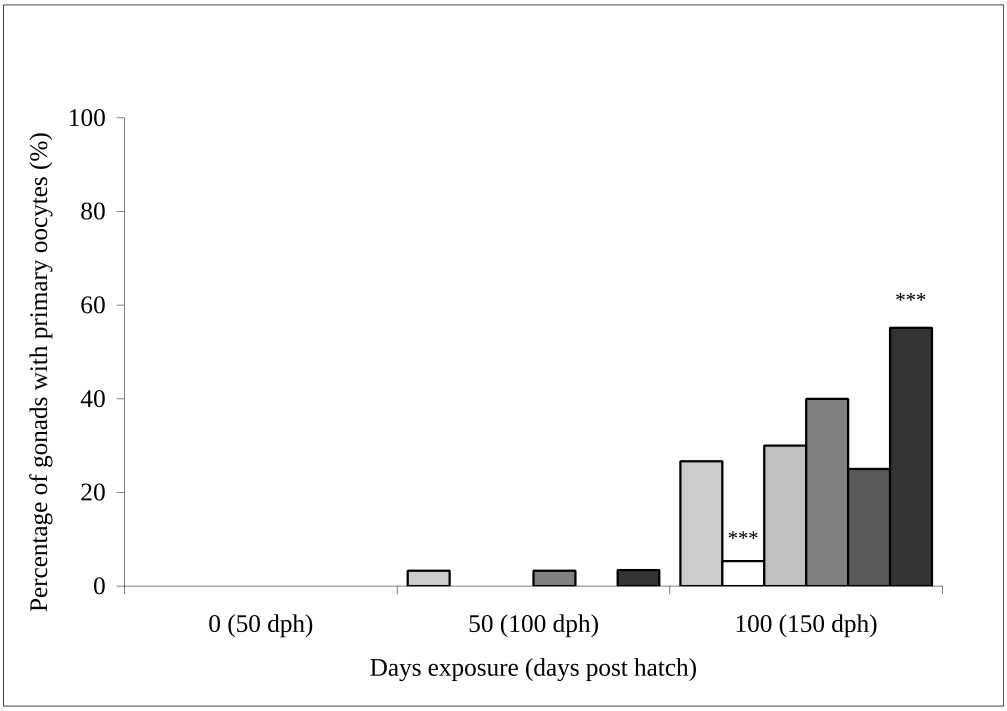
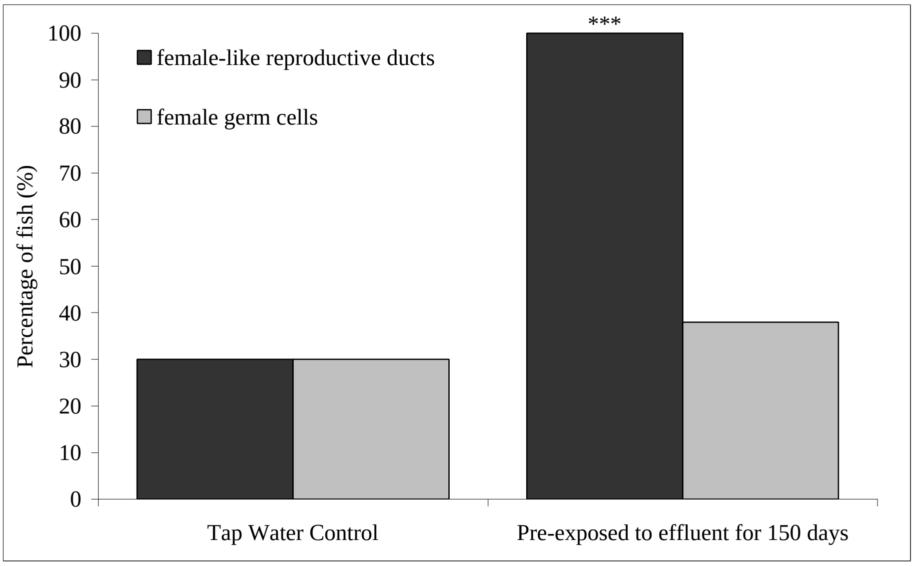


Figure 22: Change in development of female reproductive tracts and germ cells in juvenile roach pre-exposed to effluent and tapwater



Note: both groups "grown on" in tapwater for 150 days following earlier 150 days exposure to effluent and tapwater (control)

3.4 Cause and Effect Relationship in Wild Populations

3.4.1 Experimental Design

This study comprised the second component of the assessment of the causes and effects of intersexuality in wild roach. It was a field based study intended to assess the prevalence (proportion of fish affected) and severity (Intersex Index) of intersex in wild roach in relation to the age of the fish (at capture) or to the year in which they were born. The hypotheses to be tested were that:

- intersexuality is manifest during early life and therefore, both the prevalence of intersexuality and the severity of feminisation is higher in fish born during drought years (when the proportion of effluent in the rivers is higher) than in those born during wetter years.
- Intersexuality is manifest in early life, but is progressive the longer the fish is exposed to effluents

Biological Measurements

Briefly, histopathological analysis of intersex was carried out on "male" roach gonadal samples collected from a variety of sites (from those listed in Table 1) both upstream and downstream of sewage treatment works between 1995 and 2000 (n=530). The number of fish at each site with altered reproductive ducts and/or ovotestes was counted and the degree of feminisation of individual fish was recorded using the Intersex Index.

The age of each fish was estimated by counting the annuli on scales removed from the flank and the year of birth was calculated. The body weight and length and the plasma vitellogenin concentration concentrations were measured in each fish as described in the section 2.1.1.

Analysis of River and Effluent Flow

Data on the annual and monthly flow rates of both effluent and river water in sampled rivers was collated from the Environment Agency, water companies and from the Centre fo Ecology and Hydrology (CEH) at Wallingford. These data (either modelled or measured) was used to approximate the proportional monthly flow of effluent in selected rivers at the point of fish capture. Using these data, annual and monthly estimates of the mean proportion of effluent in selected river at the point of capture could be calculated.

Statistical Analyses

For all sampling sites, the proportion of “males” with altered (feminised) reproductive ducts and/or with ovotestes was plotted against the age at capture, or against the year in which the fish were born. Differences in the occurrence of these histopathological effects between different cohorts of fish were analysed using Chi-squared Analysis. Differences in the degree of feminisation between different cohorts of fish, or between fish of different ages, were analysed using one-way ANOVA, followed by Fishers PLSD test for multiple comparisons.

It is important to note that the degree of feminisation (intersex index) is known to vary greatly with the variations in exposure to effluents between different rivers or on different sites on individual rivers both above and below discharges. For this reason, meaningful analyses of differences between cohorts of the intersex index can only be carried out by comparing cohorts of fish from a single site (e.g. the River Nene downstream of Billing STW), or by pooling the data obtained from several downstream sites that are similarly exposed to effluents. In this study, therefore, data from the downstream sites on the rivers Aire, Arun, and Nene (n=229) were analysed both separately, and in combination (pooled data).

The hydrological data were assessed together with the biological data to attempt to elucidate any links between perceived exposure to effluent and the incidence and severity of intersexuality in each year class of fish at each site.

3.4.2 Results

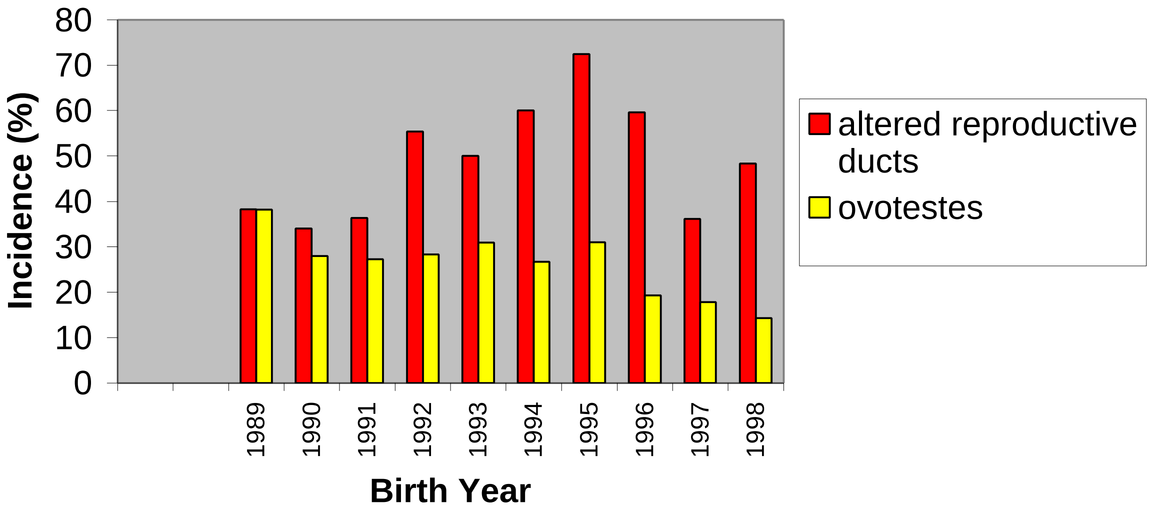
Pathological examination of the gonads of the fish showed that, of the 530 male or intersex fish examined, 366 fish between 1 and 9 years old at the time of capture (born between 1986 and 1998) were intersex.

Hypothesis 1: Intersex is driven by exposure in the year of birth

Prevalence of Intersex Fish

The proportion of male fish with female reproductive ducts varied by more than 38% in the fish born between 1989 and 1998; 34% of the "males" in the 1990 year class were intersex compared with 72.41% of the 1995 year class (Figure 23). During the same period, however, the variation in the proportion of male fish with ovotestes differed by only 3% from 28% in the 1990 year class, to 31% in year class 1995. In summary, there were significant inter-year class differences in the proportion of male fish with altered (feminine) reproductive ducts, although there were no significant inter-year class differences in the proportion of "male" fish with ovotestes (oocytes in their testes).

Figure 23. Variation in incidence of intersex characteristics in pooled fish from rivers Nene, Aire and Arun



The Degree of feminisation (0=male; 7=female)

Intersex indices and years of birth for intersex and male fish collected on the rivers Aire (captured in 1995, 1997, and 1998; n=82), Nene (captured in 1995, 1997 and 1998 and 2000; n=94), and Arun (captured in 1995 and 2000; n= 49) were considered.

River Arun (below Horsham STW): The mean intersex index varied from 0.871 +/-0.155 in the fish born in 1997 to 2.100 +/- 0.505 in the 1995 year class (figure 24a). There were no overall significant differences in the intersex index between the various birth cohorts (p=0.6733).

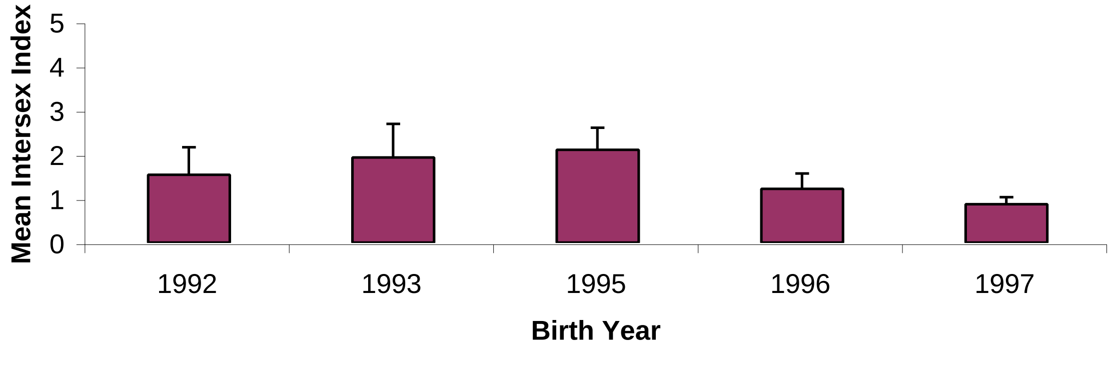


Figure 24a. Variation in mean intersex index in male roach from the river Arun

River Aire (at Thwaite Weir): The mean intersex index varied from 1.060+/-0.133(n=5) in fish born in 1997 to 3.887+/-0.802(n=3) in fish born in 1989.(figure 24b) Overall, there appeared to be a gradual decrease in the mean intersex index with time and hence, fish born in earlier years appeared to be more severely feminised than those born later. The differences in the degree of feminisation between the various year classes were statistically significant (p=0.0073). Those fish born during 1991 were significantly more feminised than those born in 1995, 1996 and 1997, whilst those born in 1992 were significantly more feminised than those born in 1996

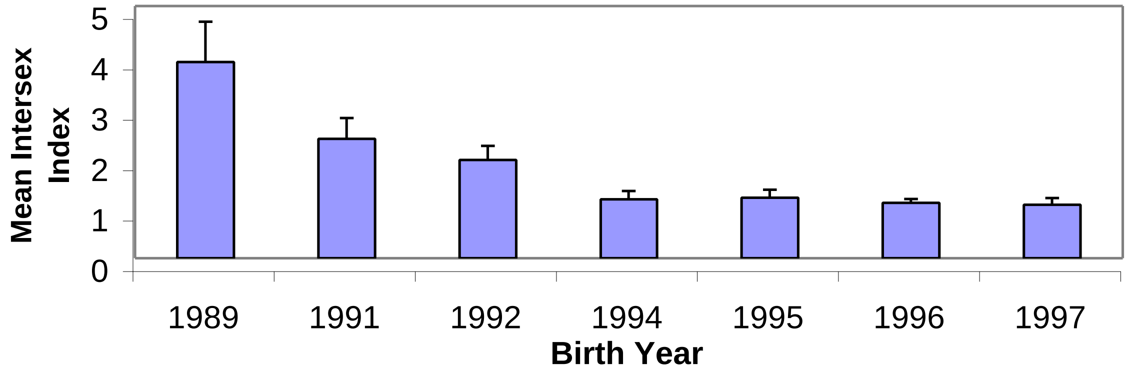
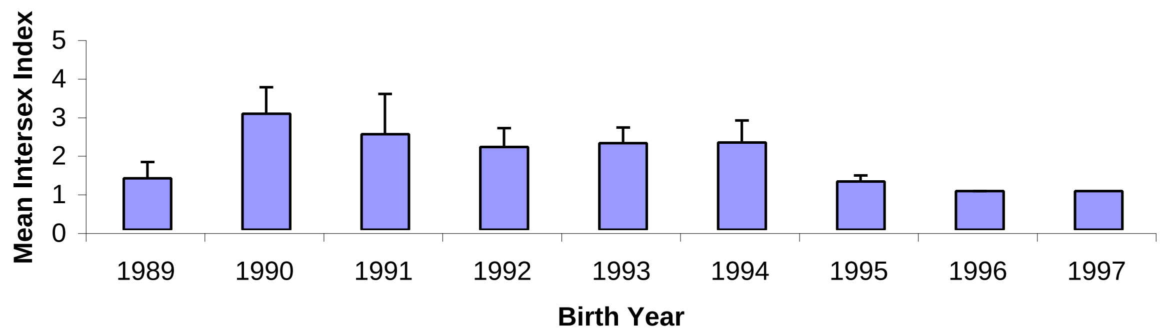


Figure 24b. Variation in mean intersex index in male roach from the river Aire

River Nene (Below Billing STW): The river Nene at Earls Barton was the most intensively studied of all of the sites where 4 separate samples, totalling 94 intersex or male fish were taken during the years 1995, 1997 and 1998. The mean intersex index varied from 1.0 (+/- 0) in fish born in 1997 (n=9) to 3.003+/- 0.420(n=9) in the 1990 year class. In general, 1996 < 1995 <1989 <1992 <1993 <1994 <1991< 1990.

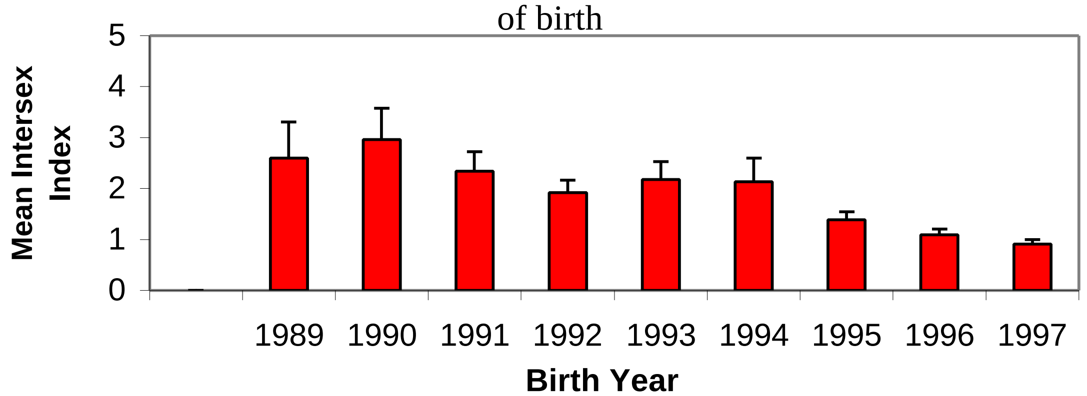
There were no overall significant differences in the mean intersex indices between any two of the cohorts (p=0.0938). (Figure 24c)

Figure 24c. Variation in mean intersex index in male roach from the river Nene



Pooled Data:When data from the Nene, Aire and Arun were considered together, the mean intersex index varied from 0.906+/- 0.093 in year class 1997, to 2.788+/- 0.7 (in the 1990 year class). There were no significant differences in the mean intersex index between any year classes(p=0.1148). (Figure 24d**)** **,**

Figure 24d Variation in mean intersex index in pooled sample of male roach from the Rivers Aire, Nene and Arun, versus year of birth



Relationships between effluent exposure and intersexuality

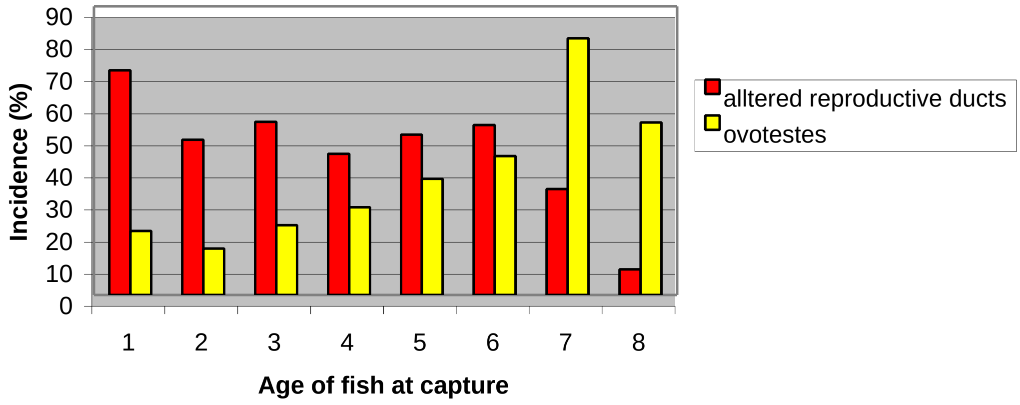
The proportion of effluent in each river at the point of capture varied greatly, both between rivers and between different months or years at the same point. Two rivers (the Aire and Nene) were chosen for further analysis as the largest samples of fish were collected from these two sites. Data for the flows on the other rivers was not calculated, due to insufficient raw data with which to perform the analyses.

On the river Nene at Earls Barton (below Billing STW), the driest years appeared to be 1990 and 1995 whilst the wettest periods occurred in 1992, and 1993 and 1998. On the river Aire, the driest periods were around 1995 and 1996. Furthermore, when both the hydrological and biological data were considered together, there did not appear to be any consistent relationship between the intersex index and exposure to effluent during the first year of life. Notwithstanding this, the prevalence of fish with feminised reproductive ducts could be loosely associated with exposure to effluent during early life as the incidence of this phenomenon appeared higher in 1995 and 1996 than in 1992 and 1998. It is important to note here that these comparisons are observations, only, and were not found to be statistically significant.

Hypothesis 2: Intersex is driven by the number of years fish are exposed to effluents

Prevalence of Intersexuality in fish from different age groups:
The proportions of fish with ovotestes increased with increased age at capture from 14% (n=63) in fish aged 2+, to 80% in the fish aged 7+ (n=13; figure 25). Conversely, however, the variation in the proportion of male fish with feminised reproductive ducts varied less, differing by only 21% from 33% in 7+ fish to 54% and 48% in 3+ and 2+fish respectively (n=165).

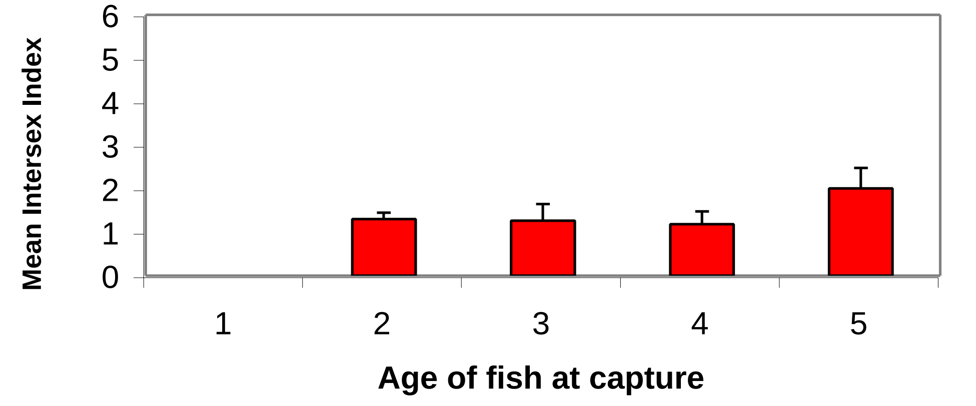
Figure 25 Prevalence of intersexuality in roach from the Rivers Nene, Aire and Arun



The Degree of feminisation (0=male; 7=female) in fish from different age groups:
Intersex indices and age at the time of capture were assessed on groups of fish collected from the rivers Aire, Nene, and Arun (n=229).

River Arun (below Horsham STW)
The mean intersex index in fish aged 5+ (n=12) was higher (2.008+/- 0.470) than in those aged 2+ (1.3+/-0.150), 3+(1.27+/- 0.38) or 4+(1.185+/- 1.060; figure 26a). However, there were no statistically significant differences in the Intersex Index between the different cohorts of fish (p=0.1515)

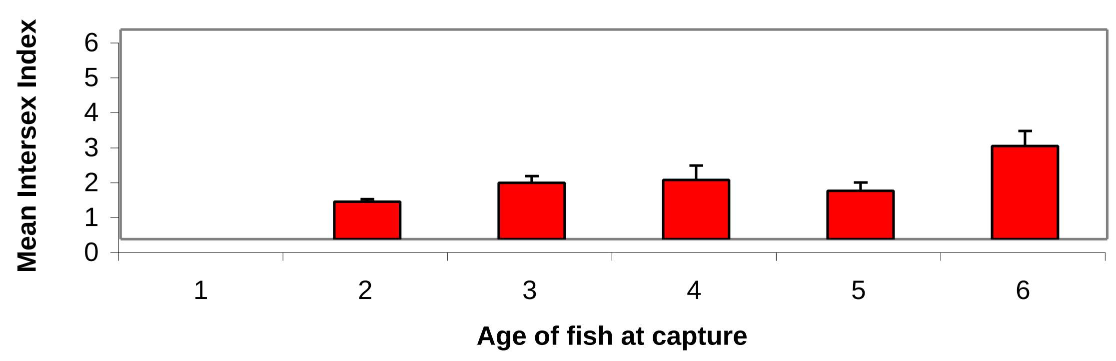
Figure 26a Prevalence of intersex in roach from the River Arun



River Aire (at Thwaite Weir, below Knostrop STW).

Figure 26b summarises the relationship between age at capture and the intersex index in wild roach collected from Thwaite weir on the River Aire. The mean intersex index increased gradually from 1.071+/- 0.75 (n=16) in fish aged 2+, to 2.669+/-0.429 (n=17) in fish aged 6+. In general, there were statistically significant differences in the intersex index between the fish of different ages (p=0.0030). Furthermore, multiple comparisons tests revealed that fish aged 6+ were statistically more feminised than those aged 2,3 or 5+.

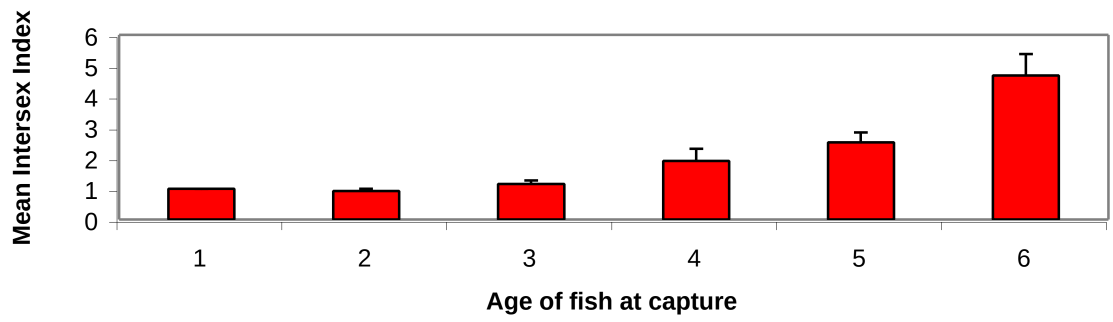
Figure 26b Prevalence of intersex in roach from the River Aire



River Nene (below Billing STW)

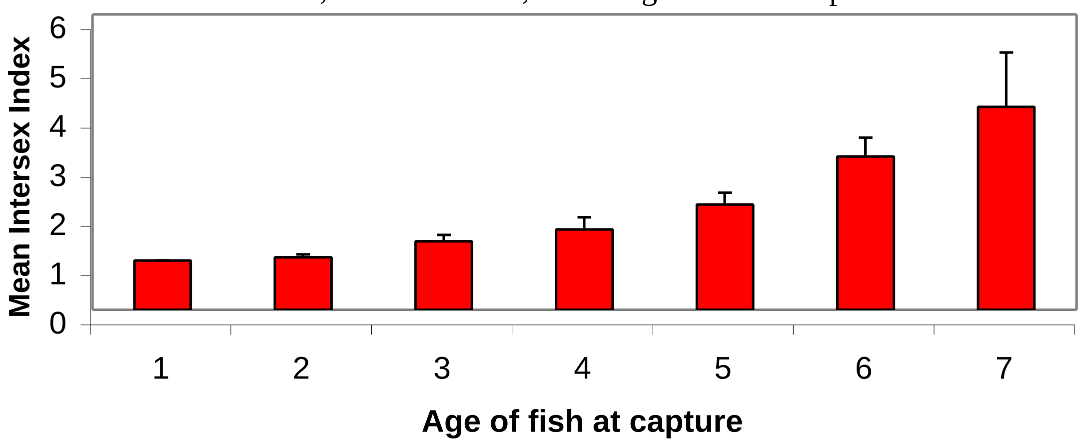
Figure 26c summarises the relationship between age at capture and the intersex index in wild roach collected from this site. As the graph clearly illustrates, there was a gradual, but nonetheless statistically significant increase in the mean intersex index with increased age at capture(p <0.0001). The intersex index increased from 0.929(+/- 0.189, n=7) in 2+ fish, to 4.6+/-0.678 in 6+ fish (n=5). Furthermore, multiple comparisons tests revealed that fish aged 6+ were significantly more feminised than fish aged 5+, 4+, 3+ 2+ or 1+, whilst those aged 5+ were significantly more feminised than 3+, 2+ or 1+ fish. All other differences in the intersex index between fish of different ages were not significant at the 95% level.

Figure 26c Prevalence of intersex in roach from the River Nene



Pooled Data:
When data from all of these sites was pooled, the mean intersex index varied from 1.00+/- 0.0 (fish aged 1+; n=4) to 4.122+/-1.107 (for fish aged 7+; n=3). There was a gradual, although very significant increase in the degree of feminisation with increased age at capture (1+<2+<3+<4+<5+<6+<7+; p<0.0001, Figure 26d). Fish aged 7+ were significantly more feminised than those aged 5+, 4+, 3+ 2+ or 1+, whilst those aged 6+ were significantly more feminised than those aged 4+, 3+, 2+ or 1+ and those aged 5+ were significantly more feminised than those aged 3+ or 2+. There were no significant differences in the intersex index between fish aged 4+ (n=45) and 3+ (n=71), 4+ and 5+ (n=43), 1+(n=4) and 2+(n=27) or between 2+ and 3+.

Figure 26d Prevalence of intersex in pooled sample of male roach from the Rivers Arun, Aire and Nene, versus age of fish at capture



Plasma vitellogenin concentrations

The vitellogenin measurements for all fish samples throughout the 5 year period have not been fully analysed. Preliminary results, however, suggest that plasma vitellogenin concentrations in the intersex fish aged 5 and 6+ are significantly higher than in those aged 2, 3 or 4+.

3.5 Conclusions

Induction of oestrogenic effects by experimental exposure to sewage effluents

- Studies exposing fish to sewage effluents for up to 4 months established that feminised reproductive ducts were induced at an effluent exposure of 25% during sexual development and differentiation in male juvenile (0+) roach. No disruption of ducts was observed in 1+ and 2+ fish exposed to effluents, indicating a lesser degree of, or no, sensitivity to this effect in fish that have already completed their reproductive duct development.

- Preliminary evidence for germ cell disruption was found in one individual but this needs to be further investigated following a longer growing-on period for these exposed fish.

- Vitellogenin was also induced in roach of all ages (0+, 1+ and 3+), and at exposures as low as 9% effluent (in tapwater). Induction by exposure to sewage effluent showed dose-dependence and indicated that sensitivity of fish to oestrogenic substances may increase over time.

Development of oestrogenic effects in wild populations with continual exposure to sewage effluents

- Assessment of long-term datasets on intersex condition (eggs in testis, and feminisation of sperm ducts) of wild roach from rivers Aire, Nene and Arun, comparing age at capture (evaluating the duration of lifetime exposure) and year of birth (indicating local exposures at the early life stage) indicate that duration of lifetime exposure is a major influence on the severity of the intersex condition

- No relationship was found between intersex (eggs in testis) and year of birth

- No relationship was determined for the severity of intersex (measured as presence of oviducts), for either the duration of lifetime exposure, or low flows conditions in the native river.

- These studies indicate that germ-cell-related feminisation of male adult roach may occur throughout life, and therefore that fish may become more feminised as a result of continual exposure to oestrogen as adults.

- Taken together with experimental data, these data indicate that feminisation of the reproductive ducts and the germ cells, respectively, may be quite separate phenomena, induced at different times during the fishes' life cycle.

Identification of oestrogenic substances in sewage effluents

- Determination of the chemical composition of samples of treated sewage effluent to which fish were exposed confirmed the presence of specific oestrogenic compounds, but that some of these varied markedly, with a probable seasonal influence and treatment efficiency influence.

- Oestrogenic components identified in treated effluents included the natural steroids oestradiol and oestrone, alkylphenol ethoxylates and their degradation products nonylphenol and octylphenol. Levels of the synthetic steroid ethinyl estradiol were below the limit of detection in the majority of samples analysed. Owing to substantial temporal variations, and the mixture of oestrogenic substances present, attributing activity to an individual substance is not possible.

4. ASSESSMENT OF CAUSE AND EFFECT RELATIONSHIPS TO SINGLE AND BINARY MIXTURES OF OESTROGENIC SUBSTANCES

4.1 Background

This part of the Phase II research programme comprised the study of the effects in fish of exposure to specific representative oestrogenic substances and mixtures.

Initial activities involved the development of a suitable enzyme-linked immunosorbent assay (ELISA) assay and an *in vivo* fish test model. In general, although *in vitro* tests are extremely useful in screening for specific toxic properties, *in vivo* tests have several advantages. In particular, they take into account the influence of bioaccumulation and metabolism of the chemical under test and permit investigation of a wider range of endpoints. They are therefore likely to identify a wider range of toxic effects than an *in vitro* model. For example, an *in vivo* test may allow the identification of toxicity operating by mechanisms that do not involve oestrogen receptor mediated processes. Development of the *in vivo* fish model was followed by investigations of responses of various endpoints to individual substances and binary mixtures.

4.2 Development of an *In Vivo* Screening Assay for Oestrogenic Substances using Juvenile Rainbow Trout

The basic approach to developing the model was to adapt a fish early life-stage design, such as OECD Test Guideline 204 (prolonged toxicity assay *in vivo*). The OECD test involves exposing juvenile fish of one of a number of standard test species to particular substances under flow-through conditions for 21 days. For the current model development the rainbow trout was employed (see Section 1.4.2), and females were used because of logistical difficulties in sourcing mixed sex supplies, given the yearly breeding cycle.

4.2.1 Experimental design

In the test, female juvenile trout were acclimatized for 10 days to flow-through tanks, under standard conditions for this species. Fish were then exposed to a test material for 21 days. Control of exposure concentrations was achieved by peristaltic pumping of stock solutions into the tanks at a constant rate (achieved levels were analysed weekly).

Subgroups of fish were destructively sampled prior to treatment and on exposure days 7, 14 and 21. The endpoints measured were: bodyweight, body length, gonadosomatic (GSI) and hepatosomatic (HSI) indices (calculated as for GSI; Section 2.1.1), and plasma vitellogenin by an established homologous radioimmunoassay (Sumpter, 1985).

Initial investigations focused on the impact of feeding rates on the endpoints studied. This was to establish how different feeding regimes affected the concentration of plasma VTG, body growth etc. over the period of the screening assay (14-21 days). This was to minimise any possible variability in our chosen endpoints over the test period.

Fish were fed proprietary trout pellets at 1, 2 or 4% bodyweight/day. Full methodological details are given in Thorpe *et al*., 2000.

4.2.2 Results

Feeding at 2 or 4% per day had a marked effect on bodyweight in both male and female fish. Therefore a feeding rate of 1% bodyweight/day was subsequently defined as a suitable standard rate (Thorpe *et al*., 2000) as on this ration, there was no change in the chosen endpoints over the test period. Although males and females were used in these initial trials to establish suitable baseline conditions, subsequent investigations employed only female trout, because of difficulties experienced in obtaining supplies of mixed sex fish.

Exposure of the juvenile female trout to oestrogenic test substances was shown to produce concentration-dependent induction of plasma vitellogenin that was optimal after 14 days, and this study duration was therefore selected for subsequent studies.

4.3 Assessment of *In Vivo* Activity of Individual Substances and Binary Mixtures Using the Juvenile Trout *In Vivo* Screening Assay

4.3.1 Experimental design

Test system validation

A number of reference substances known to have oestrogenic activity (see Section 1.4.1) were assessed using the developed *in vivo* screening method (Section 4.2), thereby providing information of value in validating the methodology. The treatment concentrations are listed in Table 3.

Table 3: Concentrations of test compounds used for the validation study

Reference chemical	Nominal exposure level
17β-oestradiol	1.0, 3.2, 10, 32, 100, 320 ng/l
4-*t*-nonylphenol	0.32, 1.0, 3.2, 10, 32, 100 μg/l
Methoxychlor	0.056, 0.18, 0.56, 1.8, 5.6, 18.0 μg/l

Dilution water (dechlorinated tap water) and vehicle (solvent) controls were included in the design for each test.

Water samples were taken from the holding tanks at weekly intervals. Levels of 17β-oestradiol were determined using solid-phase extracted, derived and subject to gas chromatography-mass spectrometry (GC-MS). Nonylphenol was also solid-phase extracted and analysed using high performance liquid chromatography (HPLC), while methoxychlor was liquid-liquid extracted and analysed by gas chromatography-electron capture detection (GC-ECD). The results (Section 4.3.2) were interpreted on the basis of time-weighted means of the actual concentrations.

4.3.2 Comparison of individual and binary mixtures of oestrogenic substances

In addition to the validation exercise, the test system was employed in the investigation of responses to 17β-oestradiol, 4-*tert*-nonylphenol and methoxychlor, individually and as binary mixtures in three sets of experiments.

The 14-day juvenile trout assay (see Section 4.2) was used, focusing on vitellogenin induction as the end-point of interest. Binary mixtures were achieved by passing the test solutions from each primary mixing vessel into a second mixing vessel, before passing to the fish holding tank. For each set of experiments, a 'fixed ratio' design was used to keep a constant ratio of substances, but varying the total quantities of the mixture introduced to the fish.

Nominal and achieved concentrations for the single and binary mixture exposures over 14 days are detailed below (Tables 4, 5 and 6). Dilution water and solvent controls were included in each set. Juvenile females were exposed to five levels of individual substances and three of each binary mixture in each of the following sets of experiments:

Table 4: Experiment 1 – Individual substances and fixed ratio binary mixtures of 17b-oestradiol and 4-t-nonyl phenol

Oestradiol (ng/l)			Nonylphenol (µg/l)		
Nominal	Single*	Mixture*	Nominal	Single*	Mixture*
2.4	2.3 ± 0.0	NA	2.4	1.8 ± 0.4	NA
4.2	4.7 ± 0.2	4.9 ±0.3	4.2	3.6 ± 0.3	3.3 ± 0.4
7.5	7.2 ± 0.2	7.4 ± 0.2	7.5	6.1 ± 0.7	6.1 ± 1.1
13.5	13.3 ± 0.4	12.6 ± 0.2	13.5	10.2 ± 0.6	10.2 ± 1.1
24.0	21.3 ± 0.3	NA	24.0	12.2 ± 3.8	NA

NA not applicable * measured concentrations

Table 5: Experiment 2 – Individual substances and fixed ratio binary mixtures of 17b-oestradiol + methoxychlor

Oestradiol (ng/l)			Methoxychlor (µg/l)		
Nominal	Single*	Mixture*	Nominal	Single*	Mixture*
2.4	4.6 ± 1.2	NA	2.4	1.4 ± 0.1	NA
4.2	4.2 ± 0.0	4.3 ± 0.0	4.2	4.4 ± 0.3	3.8 ± 0.2
7.5	7.9 ± 0.4	7.2 ± 0.1	7.5	4.6 ± 0.3	5.7 ± 0.4
13.5	12.6 ± 0.1	12.8 ± 0.0	13.5	13.0 ± 0.3	13.2 ± 0.8
24.0	23.0 ± 0.2	NA	NA	NA	NA

NA not applicable * measured concentrations

Table 6: Experiment 3 – Individual substances and fixed ratio binary mixtures of 4-*t*-nonylphenol + methoxychlor

Nonylphenol (µg/l)			Methoxychlor (µg/l)		
Nominal	Single*	Mixture*	Nominal	Single*	Mixture*
2.4	1.8 ± 0.1	NA	2.4	2.0 ± 0.1	NA
4.2	2.9 ± 0.2	3.0 ± 0.3	4.2	4.3 ± 0.1	3.5 ± 0.2
7.5	6.4 ± 0.4	6.3 ± 0.6	7.5	6.5 ± 0.2	6.8 ± 0.5
13.5	11.0 ± 0.7	11.9 ± 0.7	13.5	14.3 ± 2.1	11.4 ± 1.2
24.0	16.3 ± 2.0	NA	24.0	20.2 ± 0.7	NA

NA not applicable * measured concentrations

Treatment of results

There are two main statistical models available for analysis of potentially additive effects of mixtures. In the model of concentration addition (CA) it is assumed that compounds act via the same mechanism (Loewe & Muischnek, 1926). In the model of response addition it is assumed that they act via independent pathways (Bliss, 1936). Available evidence suggests that xenoestrogens mimic 17β-oestradiol by binding to oestrogen receptors and thereby eliciting an agonist and/or antagonist response, thus the CA model was used to investigate the nature of chemical interactions in these studies.

In the CA model, sigmoidal curves are fitted to describe the relationship between the plasma vitellogenin level and the chemical concentration. To assess whether the effects of a binary mixture are additive, one curve is fitted to the total concentration of the two substances in a mixture and a second curve is used to describe the response predicted by the model of concentration addition. Overlap of the two curves indicates that the two compounds act in an additive manner. A 'confidence level' bootstrap is fitted around each curve to help assess the significance of any overlap.

The CA model is considered to be the most scientifically-acceptable approach to both the design and assessment of substance interactions (antagonism, additivity or synergy) in dose response experiments.

4.3.3 Results

Testing of reference substances

For each of the test substances, measured concentrations were lower than nominal, probably due to absorption to the apparatus (as all the compounds are hydrophobic) although biodegradation or bioaccumulation may also be factors.

With 17β-oestradiol or 4-*t*-nonylphenol, dosage-related induction of plasma vitellogenin was found; lowest observed effect concentrations (LOEC) were 8.9 ng/l and 16 µg/l, respectively, emphasising the markedly higher potency of the natural ligand. The higher concentrations of methoxychlor tested were toxic although lower concentrations induced vitellogenin, with a lowest observed effect concentration (LOEC) of 9.8 µg/l (see Figure 27).

Figure 27: The effects of exposure to oestrogenic substances on plasma vitellogenin concentrations in female juvenile trout

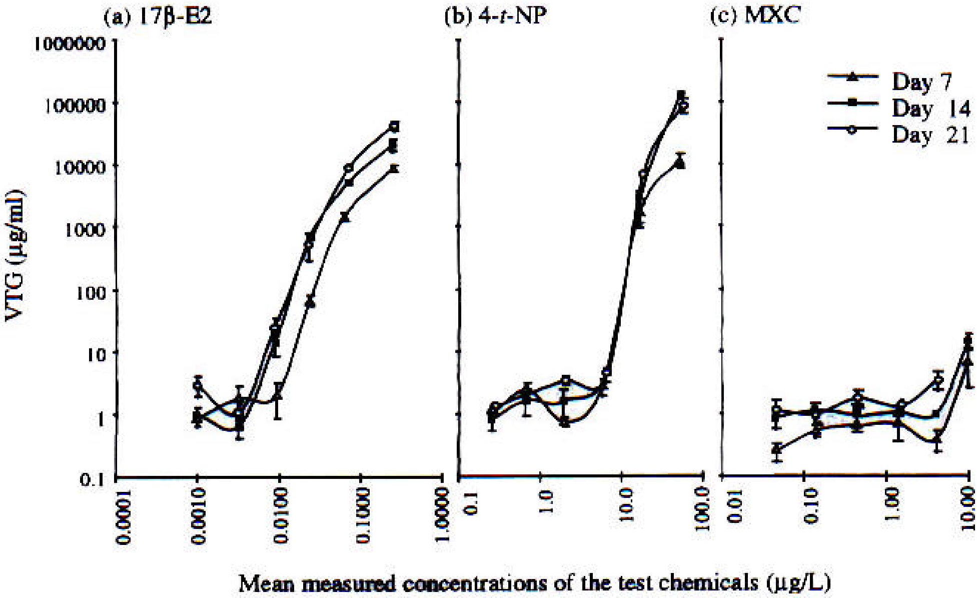


Figure 27) Plasma vitellogenin concentrations in female juvenile trout exposed to a) 17-ß oestradiol, b) 4-tert-nonylphenol and c) methoxychlor for 7, 14 and 21 d. data shown as the mean ± SEM.
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Individual and binary mixtures of oestrogenic substances

Plasma vitellogenin concentrations on day 0 were 500±80, 310±50 and 540±50 ng/ml for the three sets of experiments, respectively, and there was no detectable increase in level in the distilled water or solvent controls in each case. Findings in the treated fish were as follows:

Experiment 1 – There were no mortalities in fish exposed to oestradiol or nonylphenol or the binary mixture. Dosage-related plasma vitellogenin induction was noted with each chemical and for the mixture. LOECs and corresponding plasma vitellogenin levels are presented in Table 7. Comparison of the data for the mixtures with those predicted by the CA model showed that the effect was one of additivity (see Figure 28a).

Figure 28: The effects of exposure to single substances and binary mixtures on vitellogenin concentrations in female juvenile trout

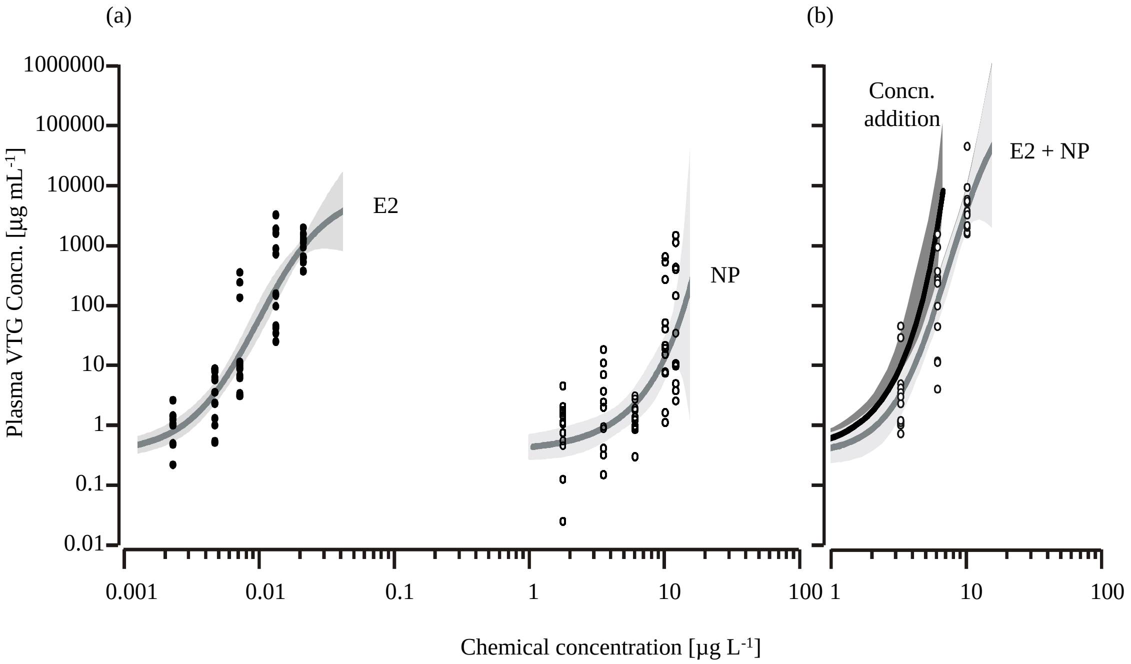

Figure 28a) Plasma vitellogenin concentrations in female juvenile rainbow trout exposed to 17ß-oestradiol (E2) or 4-tert-nonylphenol (NP) and **Figure 28b)**, serial dilutions of binary mixtures of both E2 + NP at 1:1000 concentration ratio.

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Experiment 2 – 58% mortality was noted with methoxychlor at 13.0 µg/l MXC, but was only 25% in fish exposed to the mixture of methoxychlor at 13.2 µg/l with oestradiol at 12.8 ng/l. Individually, these substances showed a dosage-related response, as did the mixture (Figure 29). LOECs and plasma vitellogenin levels are presented in Table 7. It was noted that the effect of this mixture did not correspond to the CA model predictions, showing a less than additive effect (see Figure 29b).

Figure 29: The effects of exposure to single substances and binary mixtures on vitellogenin concentrations in female juvenile trout

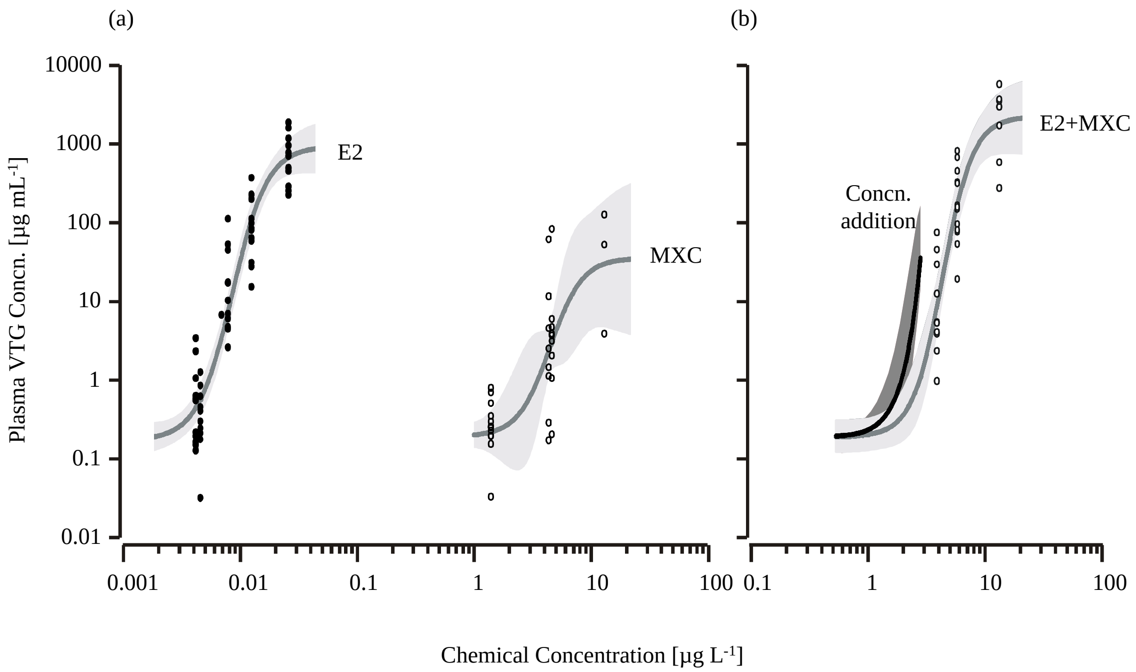

Figure 29a) Plasma vitellogenin concentrations in female juvenile rainbow trout exposed to 17ß-oestradiol (E2) or methoxychlor (MXC) and **Figure 29b)**, serial dilutions of a binary mixtures of both (E2 + MXC) at 1:1000 concentration ratio.
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Experiment 3 – With methoxychlor, 100% mortality occurred by 6 days at 20.2 μg/l and 50% mortality by 14 days at 14.3 μg/l. Fish exposed to the mixture (11.4 μg/l methoxychlor + 11.9 μg/l nonylphenol) showed 58% mortality overall. Both chemicals and the mixture again demonstrated dosage-related induction of vitellogenin; for LOECs and vitellogenin levels see Table 7. It was not possible to assess whether this mixture acted in an additive manner because of the impact of fish mortality on derivation of the dose-response curves (see Figure 30).

Figure 30: The effects of exposure to single substances and binary mixtures on vitellogenin concentrations in female juvenile trout

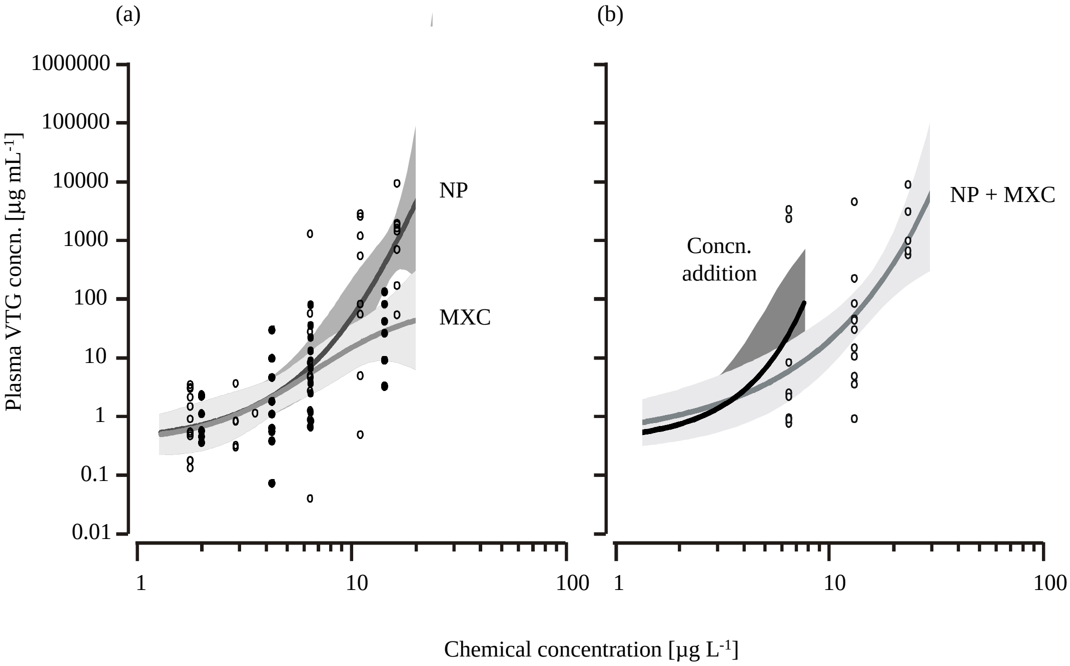

Figure 30a) Plasma vitellogenin concentrations in female juvenile rainbow trout exposed to methoxychlor (MXC) or 4-tert-nonylphenol (NP), and **Figure 30b)**, serial dilutions of binary mixtures of both MXC + NP at 1:1concentration ratio.

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Table 7: Results of the concentration additivity study for nonylphenol, methoxychlor and 17b -oestradiol

	Chemical/Mixture	LOEC (mg/l)*	Plasma vitellogenin (ng/ml)
Experiment 1	E2	4.7*	4100 ± 940
	NP	6.1	1450 ± 230
	E2 + NP (1:1000)	4.9* + 3.1	8720 ± 4370
Experiment 2	E2	4.4*	9340 ± 6590
	MXC	7.9	22460 ± 8660
	E2 + MXC (1:1000)	4.3* + 3.8	18340 ± 7680
Experiment 3	NP	6.4	128760 ± 117540
	MXC	6.5	16390 ± 7160
	NP + MXC (1:1)	3.0 + 3.5	723055 ± 481814

E2 17β-oestradiol NP 4-t-nonylphenol MXC methoxychlor

*E2 concentrations are in ng/l.

4.4 Relative Potencies and Additive Effects of Steroidal Oestrogens in Fish

Using the juvenile rainbow trout assay, developed and validated as described in sections 4.2 and 4.3 respectively, the relative potencies and additive effects of steroidal estrogens on vitellogenin induction in female juvenile rainbow trout were assessed.

4.4.1 Experimental design

Relative potencies of steroidal oestrogens in fish

To assess the relative potencies of the steroidal oestrogens, groups of 12 female juvenile rainbow trout were exposed for a period of 14 days to either oestradiol or oestrone at 0, 1.0, 3.2, 10, 32.0, 100, and 320 ng/l or ethinylestradiol at 0, 0.1, 0.32, 1.0, 3.2, 10 and 32 ng/l. Solvent and dilution water controls were also included. Fish were sacrificed and blood samples were taken on either day 0 or day 14; the blood samples were assayed for vitellogenin titre (see section 4.1).

The additive effects of steroidal oestrogens in fish

To assess the additive effects of two steroidal oestrogens, groups of 12 female juvenile rainbow trout were exposed for a period of 14 days to either oestradiol at 0, 1.0, 10.0, 25.0, 175 and 750 ng/l, ethinyl estradiol at 0, 0.04, 0.4, 1.0, 7.0, and 30.0 ng/l, or to a binary mixture of oestradiol and ethinylestradiol at respective concentrations of 5.0 and 0.2 ng/l, 12.5 and 0.5 ng/l or 87.5 and 3.5 ng/l. Solvent and dilution water controls were also included. Fish were sacrificed and blood samples were taken on either day 0 or day 14; the blood samples were assayed for vitellogenin titre (see section 4.1).

Treatment of results

Results were analysed using the same methods as described in section 4.3.2.

4.4.2 Results

There were no fish mortalities in either the relative potency or the additivity studies. The vitellogenin titre of the fish held in the solvent control and the dilution water control tanks did not noticeably alter over the test period. The mean measured concentrations of the test substances were used to calculate the relative potencies and additivity of the test compounds (data not reported).

Relative potencies of steroidal oestrogens in fish

The Effect Concentrations for 50% of the fish population (EC_{50}), the lowest observable effect concentrations (LOECs) and the no observed effect concentrations (NOECs) of the three steroidal oestrogens are reported in Table 8. The results for oestradiol and ethinyl estradiol are similar to the results from previous studies of similar duration of exposure and species.

The results for oestrone showed a lower LOEC and NOEC than that of oestradiol, which is not as expected, given the higher EC50 value for oestrone. This reflects both the experimental dosing regime used, but particularly the shallow slope of the oestrone exposure-response curve compared to that of oestradiol which is much more steep.

Table 8: Results of the relative potency study for oestrene, ethinyl oestradiol and 17B-oestradiol

Oestrogen	LOEC (ng/l)	NOEC (ng/l)	EC_{50} (ng/l)
E1	3.3	0.74	80
E2	14	4.8	28
EE2	1	0.21	1.1

The relative potencies of the steroidal oestrogens were determined from their respective EC_{50}'s (Figure 31). In female juvenile rainbow trout, ethinyl estradiol was found to be 25 times more potent than oestradiol and 70 times more potent than oestrone; oestradiol was 2.8 times more potent than oestrone.

Figure 31: Plasma vitellogenin response in juvenile rainbow trout following exposure to ethinyloestradiol, 17B oestradiol or oestrone

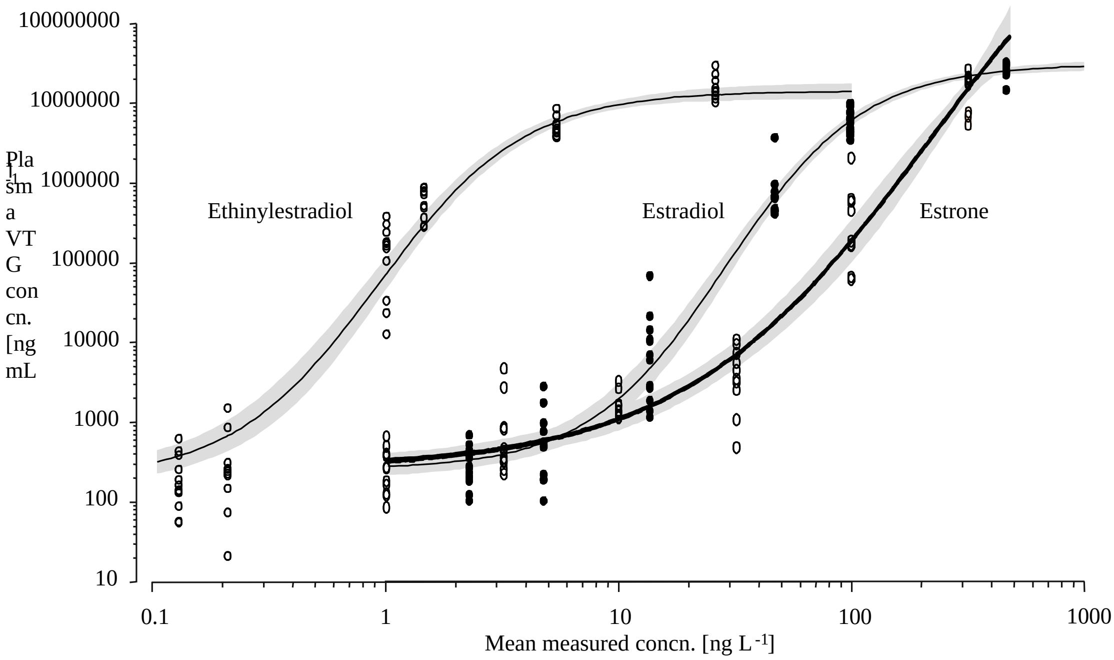

Figure 31) Plasma vitellogenin (VTG) concentrations in female juvenile rainbow trout exposed to estradiol-17β (central curve; closed circles), ethynylestradiol-17α (left hand curve; open circles) and estrone (right hand curve; open circles). In some cases the VTG concentrations were very similar between fish within a treatment, therefore not all data points are visible. For each of these exposures the 95% confidence belts (light grey shaded regions) of the fitted concentration-response relationships (black lines) are shown.

The additive effects of steroidal oestrogens in fish

The LOECs and NOECs of the individual and mixtures of E2 and ethinyl estradiol concentration additivity study are reported in Table 9. A NOEC was not detectable for the binary mixture, as all mixtures tested induced vitellogenin.

Table 9: Results of the concentration additivity study for ethinyl oestradiol and 17b - oestradiol

Chemical/Mixture	LOEC (ng/l)	NOEC (ng/l)
E2	22	9.6
EE2	7.6	1.13
E2 + EE2 (1:25)	5.0 + 0.2	ND

ND not detected

The exposure of juvenile trout to individual test compounds resulted in NOECs of 9.6 ng/l and 1.13 ng/l for oestradiol and ethinyl estradiol respectively. The exposure of juvenile trout to binary mixtures of oestradiol and ethinyl estradiol, resulted in a dose-dependent increase in plasma vitellogenin that was statistically significant at all the concentrations tested in this study (see figure 31). A comparison of the observed effects on vitellogenin titre with those predicted from the model of concentration addition (see section 4.3.2), showed that the binary mixture acted in a concentration additive manner, supporting the hypothesis that both these compounds act via the same mechanism (i.e., the estrogen receptor). More importantly, the concentrations at which the test mixtures induced vitellogenic response in juvenile fish were lower than the observed NOECs for the individual test compounds.

These findings indicate that individual oestrogenic substances, when combined with other oestrogenic substances in STW effluents, are biologically active at concentrations at which they were previously thought to be inactive.

Figure 32: Assessment of additivity of ethinyloestradiol and oestradiol on vitellogenin synthesis in juvenile rainbow trout

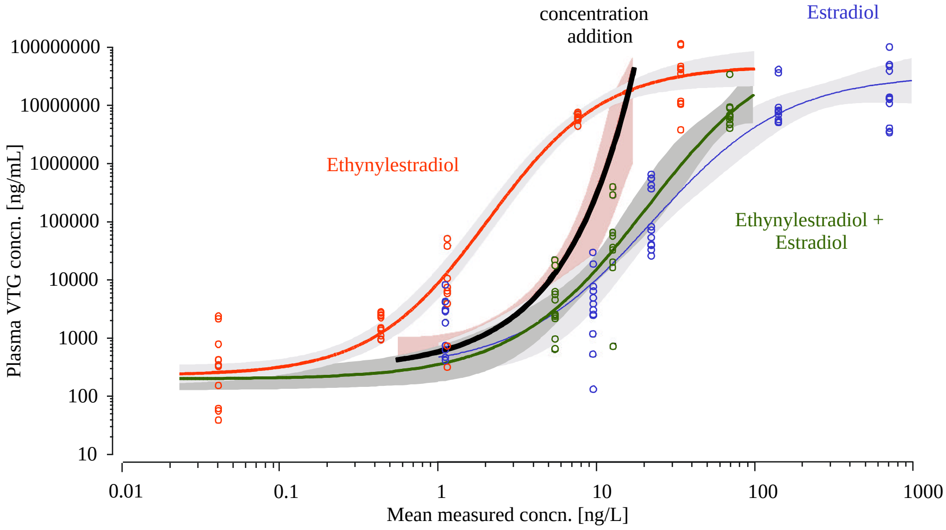

Figure 32): Plasma vitellogenin (VTG) concentrations in female juvenile rainbow trout exposed to estradiol-17β, ethynylestradiol-17α and fixed ratio binary mixtures (25:1) of estradiol-17β and ethynylestradiol-17α. In some cases the VTG concentrations were very similar between fish within a treatment, therefore not all data points are visible. For each of these exposures the 95% confidence belts (shaded regions) of the fitted concentration-response relationships are shown. The expected VTG response for the binary mixture, calculated using the model of concentration addition, is shown as a bold line with a 95% confidence bootstrap belt (shaded region). (Thorpe *et al* 2001b)

4.5 Conclusions

Interactions between oestrogenic substances

- Studies on binary mixtures of selected reference substances established that their oestrogenic activity (at least in terms of vitellogenin induction in rainbow trout) was additive, and demonstrated that a mixture of chemically–dissimilar oestrogens, 17β-oestradiol and 4-*t*-nonylphenol, could elicit effects at environmentally-relevant levels of the individual substances.

- Repeated studies on 3 steroidal oestrogens similarly established exposure response measures to vitellogenin and their relative potencies in this reliable assay system. Similarly, concentration additivity was demonstrated for ethinyloestradiol and 17β-oestradiol at environmentally-relevant concentrations.

- Concentration additivity studies with mixtures of steroidal estrogens and/or industrial chemicals showed that the concentrations of the mixture components that induced oestrogenic activity, were lower than the previously assessed No Observable Effect Concentration (NOEC) values for the induction of activity by individual substances. The implications of these findings is that environmental standards for single substances may not be adequately protective if other oestrogenic substances are also present and act via the same toxic mechanism(s).

5. DISCUSSION

This chapter draws together key issues arising from the research programme taken as a whole. The results emerging from this current programme are discussed in relation to the conclusions made in the earlier R&D Technical Report. The conclusions from the earlier work are presented as section headings, whilst the text that follows discusses the new findings.

5.1 Oestrogenic Responses in Wild Fish

The Phase 1 programme concluded: *Wild male roach in rivers that receive sewage effluent exhibited a range of gonadal and physiological abnormalities consistent with exposure to oestrogenic substances.*

5.1.1 Oestrogenic responses observed in roach

The research has assessed the various oestrogenic effects considered to be "adverse". Vitellogenin has been consistently shown to arise in both male and female fish in response to exposure to oestrogens via the water column. This biomarker response is both rapid (within 10 days to significant elevation) and transient and was found in fish of all ages tested (fry, juveniles and adults). Vitellogenin was also produced in young fish after several months exposure to a 1:11diluted effluent. It is likely that all fish exposed to oestrogens via sewage treatment works effluents at such dilutions will produce vitellogenin.

As was observed in the field work conducted in 1995 to 1997, the intersex condition in male fish was identified in all fish populations sampled in these recent studies where wild fish were taken for assessments at spawning. Severities of the intersex condition were also variable, with fish from rivers with the larger proportion of effluent being the most severely affected. The intersex condition was again characterised by the presence of oocytes in the testis and oviducts in addition to, or instead of, sperm ducts.

A striking finding in this further study has been the proportion of intersex, male and female roach in the populations sampled at spawning. Whereas a broadly equal proportion of male and female may have been expected, sex ratios of the these groups being taken for spawning were skewed towards female or intersex fish. The high ratio of female gudgeon in the River Aire (97%) is a cause for concern and warrants further investigation. Nagler *et al*. (2001) recently reported high incidences of the male 'Y' chromosome in chinook salmon from the Pacific coast of North America that were phenotypically female (possessed ovaries). Whereas the genetic sex of the fish inn this study cannot be identified, there is a possibility that some of the females may have been completely sex-reversed males.

5.1.2 Oestrogenic effects in a second species

One of the major objectives for this study was to assess the physiology of a related species that would be found with roach in UK rivers. The investigations confirmed that some gudgeon populations included intersex fish. Whilst the gudgeon sampling programme was less extensive, the spatial distribution of affected fish was similar to the roach, showing a greater severity and incidence in rivers that had higher proportions of sewage effluent. In particular, the most severely affected fish of both species were collected from Thwaites Weir, Leeds on the River Aire. The elevation of vitellogenin level seen in both intersex and male roach or gudgeon in the River Aire confirmed that oestrogenic exposure was occurring at the time of sampling, and a common cause seems likely. Also, although the incidence and severity of intersex in gudgeon was generally less than in roach, the geographical pattern of severity was similar

The high incidence of low severity intersex and increase in vitellogenin in male and intersex gudgeon from a reference site, Lakeside Fisheries, suggest that oestrogenic substances from non-STW sources may enter this water body. However, the occurrence of intersex at other, apparently unexposed, reference sites poses the possibility that intersex may occur naturally in wild populations; the finding of low vitellogenin levels in intersex fish from these reference populations supports this hypothesis. The incidence of intersex at reference sites was generally below 10%, and it may be that populations can withstand such a degree of intersex without significant consequence.

This evidence for effects in another fish species suggests that, generally, any cyprinid fish species that is exposed to effluents in these environments would be expected to be at risk of becoming intersex. However, the implications for any particular species will depend on a range of factors, including life history (longevity, reproductive strategy) route of uptake of contaminants (food source, ambient environment), sensitivity to effects (timing in lifecycle) and pattern of exposure (location of fish within environment, duration and periodicity).

5.1.3 Relationship between age, year of birth, prevalence and severity of intersex

The research results strongly suggest that wild intersex roach become more feminized with increased age/duration of exposure. Furthermore, the data indicate that feminisation of the reproductive ducts and the germ cells, respectively, may be quite separate phenomena induced, and therefore potentially disrupted, at different times during a fish life cycle.

Intersexuality (of both the ducts and the germ cells) has been historically associated with early life stage (ELS) exposure of fish to oestrogens, androgens or aromatase inhibitors and not, as seen here, with longevity of exposure. Indeed, the emerging literature suggests that sexual differentiation that occurs in ELS fish is under the control (at least partially) of steroidogenic enzymes such as aromatase and c17,20, lyase. Formation of the female gonad appears to be stimulated by aromatase, whilst the male germ cells differentiate after a rise in the expression of c17,20, lyase. Such events will need to be evaluated separately in order to understand how endocrine disrupting substances cause their effects (see also section 5.2).

No-one has yet identified a window of sensitivity to feminisation during adult life and hence the results presented here are not immediately intuitive. It is known, however, that new recruitment of the primordial germ cells (PGCs) in seasonally spawning fish occurs each spawning season (and therefore, annually in the roach) and that there there is an increase in the total number of germ cells with increased age (Billard, 1986). If the PGCs are bipotential, then feminisation of the newly recruited germ cells could occur annually, leading to increases in the number of oocytes in the testis with time.

In some of the fish collected (those from the River Arun), the increased degree of feminization with age was perhaps not as pronounced as seen in the fish collected from the rivers Nene and Aire. The reason for this is not known, although it could be due to

- a reflection of greater exposure or bioaccumulation of a feminising substance in the germ cells of Nene fish compared with the Arun fish, or
- exposure of the two groups of fish to different chemicals with different oestrogenic potencies, and/or with different mechanisms of action.

Notwithstanding this, when the data for the Arun, Aire and Nene fish were pooled, the results provide very convincing evidence for a relationship between longevity of exposure and increased feminisation.

In addition to the degree of feminisation, the prevalence of fish with ovotestes also increased with increased age at capture. It is possible that this phenomenon occurs because normal male fish die younger than intersex fish, and hence intersex fish contribute progressively more to the proportion of fish of any particular age group as the years go by. The fact that intersex fish are reproductively compromised, for example, may enhance their life expectancy, as very little effort is put into reproduction.

Whilst this is a plausible explanation for the increased prevalence of intersex fish with increased age in several populations studied, it cannot be used to explain the increase in the severity of effect (as referenced against the Intersex Index) with increased age at capture. This phenomenon must occur as a result of long-term exposure of the fish to the effluents throughout life and/or to repeated exposure during a sensitive window during the annual reproductive cycle. The existence of such a window is now being tested experimentally, but is part of on-going research.

There is some evidence, from the River Aire, that the the degree of feminisation may have decreased over time, and hence, the fish of age 3+ caught in 1995 were more feminised (intersex index = 2.26+/- 0.37 ;n=13) than those of the same age caught during 1998 (Intersex Index = 1.03 +/- 0.056; n=9) and in 2000 (n= 0.883 +/- 0.207 ; n=6). The reason for this is not known, although it is likely to reflect a lower exposure to oestrogenic substances during 1995 to 1998 and 1997 to 2000 than during 1992 to 1995. This may have occurred due to the significant decreased discharge of alkylphenolic surfactants into the River Aire that has occurred during this period. Due to the large month by month variation in the proportional effluent flow in each river, however, and with insufficient knowledge of the periods of sensitivity (timing and length), it is difficult to directly relate the changes in effluent exposure or the changes in the discharge of a specific chemical (such as nonylphenol) to the incidence and severity of intersexuality. This apparent improvement to oestrogenic effects in these fish will need to be followed as further improvements to water quality are achieved in this catchment.

Feminisation of the ducts appeared to vary, with year of birth being the most prevalent in the year classes 1994 to 1996. The prevalence of ovotestes in the same year classes, however, remained relatively low and did not vary much between the different year classes. Feminisation of the reproductive ducts can occur in roach as a result of exposure to effluents during the period of sexual differentiation (which occurs through the summer and autumn) and it seems likely that the increased prevalence of female ducts found in the fish born 1995 and 1996 reflects an increased exposure to treated sewage effluents during this period. An alternative explanation for the data is that both the ducts and the germ cells of sexually differentiating fish are sensitive to the feminising effects of effluents, although the former are much more sensitive than the latter.

Whatever the explanation for the results presented, the implications of this increase in intersexuality with increased age should be carefully considered, as they could include an effect on population stability if the older fish (on which the fishery is dependent) are less successful in breeding.

5.1.4 Reproductive development towards spawning

Preliminary evidence for asynchrony of gamete maturation at spawning between the sexes was found in wild adult roach that were exposed to effluent. Sampling in autumn showed that effluent exposed males and some females had delayed gonad development compared with roach from reference sites. Fish from the rivers Aire and Nene collected in late-spring showed delayed spermatogenesis, at a time when the majority of females had begun spawning. In those male roach that were spermiating, reduced milt volume and sperm motility were also noted. These observations in male fish are considered to be unusual, as cyprinid males would be expected to spermiate in advance of the final egg maturation in females, and the spawning period.

Moreover, male roach from the River Nene showed a reduction in numbers of tubercles, a male secondary sexual characteristic, and one that is associated with elevated blood androgen concentrations. This response similarly reflects delayed sexual maturation. Such an effect has been shown experimentally in fathead minnows exposed to nonylphenol (Harries *et al*., 2000); although this observation was not apparent in similar studies using effluent-exposed fish from other rivers.

The cause of the delay in spermiation (or non spermiation) and the absence of secondary sexual characteristics in males is uncertain but could possibly relate to deformities of the reproductive duct, immaturity of the gonads or other factors such as depressed blood androgens. Nevertheless, whatever the causes, asynchrony would have important consequences for spawning success and population sustainability.

The research findings strongly suggest that exposure to treated effluents can impact on developmental and reproductive processes in the roach throughout the reproductive cycle. Moreover, increased duration of exposure lowers thresholds for effluents to cause impacts on vitellogenin. Temperate cyprinid species, such as roach and gudgeon, have continuous yearly reproductive cycles, maturing throughout autumn/winter months and spawning in spring (Nolan *et al*., 2001). The roach has been shown to be sensitive to effluent not only during early life development and differentiation (when morphological effects such as intersex may arise) but also during adult life, when production of viable gametes, fertility and, eventually, population recruitment may be at risk.

5.1.5 Reproductive health

The Phase 1 programme concluded: “*Although the reproductive and general health effects of the measured abnormalities are not clear, it is possible that reproduction of individual fish may be impaired*”.

This is clearly one of the major questions that needs to be answered if we are to understand the full implications of these effects to fish populations. The research has made significant progress towards understanding the consequences of male fish being intersex. Undertaking this work poses a number of difficulties, including the artificial nature of any experiment.

It would be desirable to mimic natural spawning to determine whether intersex led to effects on reproductive health, and to determine what those effects were influenced by. However, the ability to determine meaningful results (via controlled experiments) with wild fish (as opposed to laboratory maintained breeding populations) is generally not possible.

The main approach taken was to assess gamete quality in as controlled an environment as possible so that the factors determining fertility could be assessed. Therefore an *in vitro* approach was used in order that not only gamete quality could be assessed, but that other variables, such as the process of fertilisation, could be closely controlled. Using this approach, a first evaluation of the implications of the intersex condition for male roach was made.

In vitro fertilisation experiments established a relationship between intersex severity and impaired fertility, as measured by a number of physiological parameters – sperm density, sperm motility, fertilisation of ova and survival of larvae to early-life stage. In addition, wild male fish from effluent–rich rivers also suffered reduced fertility, but without showing any gross morphological changes to their reproductive system that would be indicative of intersex. Observations of reduced or absent secondary-sexual characteristics could be evidence for altered circulating blood androgen concentrations, implying that other sexual development processes may be being affected by exposure to environmental oestrogens. From this work, fish living in similar effluent-receiving waters may well have compromised fertility, whether displaying intersexuality or not.

As stated above, undertaking *in vitro* reproduction experiments does not reflect the true environmental situation. Factors such as the synchrony of maturation of both sexes, competition between males in pursuit swimming to fertilise the eggs, sperm competition, availability of substrate, predation and abiotic effects (such as water quality) have all been excluded from these assessments. The data presented should be considered to be the best possible reproductive outcome as gametes were manipulated optimally for egg fertilisation. This being the case, it is possible that environmental factors may act disproportionately on population reproductive success, such that intersex fish of even low severities show a greater reduction in reproductive performance compared to unaffected fish in the natural situation.

Whereas male roach displaying high intersex severities are physiologically impaired, understanding the true performance of moderate (and even low) intersex condition fish, is the important consideration ecologically. This is because it is likely to be the most widespread form of the condition in fish populations in English fisheries.

5.2 Causality and Correlation with Oestrogenic Substances and Sewage Effluents

The Phase 1 programme concluded:

- *A strong positive significant correlation was found between the incidence of the intersex condition and the proportion of sewage effluent in the river.*
- *A positive significant correlation was found between the severity of the intersex condition and the proportion of sewage effluent in the river.*

5.2.1 The link between sewage effluent and oestrogenic responses in fish

The earlier research indicated a causal “associative” link of the intersex condition to sewage effluents, both extent and severity, in groups of male fish. As is the case for the reproductive experiments, understanding the causal relationship requires controlled experiments. The programme established a series of exposure-response experiments where responses were observed in roach of different ages exposed to graded dilutions of a well-characterised effluent, over a period of several months. These studies have provided valuable information on the time course of some of the oestrogenic responses seen in wild fish. Whereas most of the literature on exposure-response to effluents in the field is of short-term duration (2-6 weeks), these experiments were significantly longer and for the larvae, covered critical developmental periods. The work reflects a more relevant time period, although falls short of whole life exposure, as experienced by the wild fish.

The research established that treated sewage effluents induce dosage-related oestrogenic responses – vitellogenin (all fish tested), and oviducts (> 25% effluent, in larval roach). In addition to the expected vitellogenin induction, juvenile roach exposed to Chelmsford STW effluent during gonadogenesis showed dosage- and time-dependent feminisation of reproductive ducts when exposed from 50 days post hatch for 100 or 150 days. When exposed fish were transferred to clean water, vitellogenin induction was partially reversed but the feminisation of duct architecture in ‘male’ fish remained, showing the permanent nature of this morpholigical change, but the temporal nature of vitellogenic response.

At the time point of 150 days, the juvenile male and intersex (by virtue of presence of an oviduct) roach had not developed female-like primordial germ cells. Roach, in common with other temperate cyprinid (and many other species) fish, undergo two stages in reproductive development – the development of the reproductive tract (usually in early life stages – *gonadogenesis* – a single developmental process that is irreversible) and the generation and release of gametes (follows gonadogenesis – gametogenesis – a cyclical process of gamete development, release at spawning). Whereas development of the reproductive tract occurs within the gonadogenesis phase, gametogenesis is likely to occur after the gonad architecture is formed. These distinct phases of development in our test species will need to be understood if the exposure-response relationship is to be fully elucidated.

There are potentially several reasons therefore why the disruption of the gametogenic processes was not induced:

- fish were exposed from a later stage in their development than is necessary to induce / initiate this effect

- The effluent did not contain the substances, or sufficient quantities of substances, necessary to induce this effect
- The fish need lifetime or subsequent exposures to induce this effect
- The effect may not be apparent until the fish are older
- The effect may be induced at a later developmental stage, e.g. post-spawning gametogenesis

Finding the causes of oocytes in testis remains an important objective and further investigations are underway.

5.2.2 Assessing implications of lifetime environmental exposure to oestrogens

The exposure-response experiments are not a direct comparison with the wild situation as the fish were not exposed from the egg stage, and fish were exposed for discrete (albeit long) periods. It is now possible to expose fish for many months, however, exposing a fish species such as roach from egg stage to maturity (a 3-year period) in a controlled manner is practically impossible, so it is likely that such information is best generated by moving to surrogate related species with shorter, but similar, life histories.

Research is still continuing to determine whether gametogenesis in fish is disrupted at developmental stages before and after the exposure periods tested here, and also in the adult stage. However, some preliminary clues as to what might be important factors have been determined from assessments of wild populations (see section 5.3). In addition, juvenile roach exposed to 100% effluent have now been grown-on to beyond their third birthday, a point where they would be expected to be sexually mature. These fish will be assessed to see the current state of their gonads.

The study has also suggested a temporal element exists, where over extended periods, effects (vitellogenin) can be induced at lower effluent concentrations. Sampling of fish at different time intervals during the experiment enabled exposure duration to be assessed, yielding clear evidence for lowering the threshold for response as duration of exposure increased. This indicates that previous assessments of vitellogenin response may underestimate the response in wild fish exposed over the longer term. This implies that effluents previously considered to have low oestrogenic activity should be re-assessed over longer time periods, and more generally, a re-evaluation of how effluents are assessed for their biological effects will be needed.

Winter exposure of adult male roach to Chelmsford STW effluent also induced dosage- and time-related vitellogenin production. There were no effects on vitellogenin levels in females over this period, nor were there changes in male or female roach gonad pathology or GSI in males or females. In the context of the life history of a fish and its protection from adverse health effects, it is important to assess what the fish is exposed to and its timing, and how sensitivity of the fish will vary during its gonadogenesis developments, and thereafter in the gametogenic cycles. Further research is required to confirm whether and how sensitivity to oestrogenic substances increases over time, but these results show that juvenile fish (50-150 dph) are more sensitive to exposure to oestrogenic effluents than adults with respect to altered duct formation, whereas elevations of blood vitellogenin concentrations are similarly affected.

5.2.3 Variation in effluent quality, and oestrogenic activity

Several studies have shown temporal variations in the levels of the oestrogenic substances in treated effluent, and this variation has been suggested to relate to changes in the physical properties and chemical composition of the effluent (such as biochemical oxygen demand and dissolved oxygen level). Also, factors such as microbial activity, rainfall or temperature (Ternes, 1998; CES, 1993; Rodgers-Gray *et al*., 2000, Johnson and Sumpter, 2001) could impact on sewage treatment through, for example, effects on microbial breakdown. If a relatively constant influent composition is assumed, such factors could affect the oestrogenic potency of the treated effluent.

Temporal variability in the composition of Chelmsford STW effluent was observed (from Table 2): during the winter period (November 1997 to March1998), concentrations of alkylphenol ethoxylates were low and nonylphenol was absent. During summer, concentrations of these compounds were 4- to 5-fold higher. Concentrations of oestradiol and oestrone were highest in the winter period and concentrations of both showed a clear correlation with BOD (an indicator of treatment efficiency). The elevated concentrations of the steroidal oestrogens detected here in the winter effluents would occur at the time of sexual maturation of adult roach. The highest concentrations of alkylphenolic compounds detected in this study occurred during the summer, a time of spawning and early embryo development. Although not a significant component of this study, understanding what oestrogenic substances fish and other aquatic life are exposed to is essential in assessing risks of exposure, effect and to their sustainability.

The concentrations of the natural steroids, oestrone and oestradiol, measured at Chelmsford STW were consistent with measurements made in the UK (Desbrow *et al*., 1998) and Israel (Shore *et al*., 1993), and were present at generally 1000-fold lower concentrations than alkylphenolic and alkylphenolic ethoxylate compounds in the same effluent (Rodgers-Gray *et al*., 2000; 2001). Although concentrations of steroid oestrogens were much lower, their (e.g. oestradiol) potency in vitellogenin induction has been shown to be 1000 fold greater compared with nonlyphenol (Thorpe *et al.,* 2001a). The concentrations present exceeded that observed to induce vitellogenin previously (dosed as single substances) in laboratory assessments (Routledge *et al.*, 1998; Thorpe *et al.*, 2000; 2001a). Taking these considerations into account, the oestrogenic activity in the Chelmsford sewage effluent cannot be ascribed to a single chemical component of STW effluent and it is likely that it is as a result of a combination of the substances measured, with the possibility of other substances presently unknown.

5.2.4 Assessing environmental mixtures

The traditional view has been that hazardous properties of a substance are assessed on an individual substance basis, and that single-substance-based standards are derived, sufficient to protect the environment against adverse health effects. It is known that environmental discharges contain many substances, only some of which have had environmental standards derived. Moreover, these substances may interact with each other in an additive, synergistic or antagonistic manner – these considerations are not generally taken into account. The possibility exists that substances acting via the same mechanism may be at or below environmental standard concentrations, yet the total amount of the different substance present may still lead to an adverse health effect.

How substances interact has been a contentious scientific issue, and in particular, how such interactions can be reliably assessed. Analytical chemistry assessments of effluents referenced above have shown that environmental discharges contain mixtures of oestrogenically-active substances, and in some cases, data are reported showing diurnal and seasonal variations in concentrations. This is the environmental reality – fish are exposed to mixtures of chemicals throughout their lives. What is important to understand in assessing risks from particular environmental discharge and setting relevant environmental targets is whether substances present in these mixtures can act via the same mechanism to induce an effect, or multiple effects.

Since endocrine disruption became a well-studied scientific area, consideration of how endocrinally-active substances could interact to cause effects in wildlife has received increased attention. This work therefore complemented the direct effluent research where "real world" effluent exposures to fish were performed.

5.2.5 Cause and effect for single and binary mixtures of oestrogenic substances

Given the lack of mechanistic information on other endpoints, demonstrating interactions on the oestrogen system was taken forward using the vitellogenin synthesis, a receptor–mediated response. This enabled the development and validation of a relatively short-term test methodology. The OECD Test Guideline 204, used to assess the effects on fish of flow-through exposure to chemicals, was successfully adapted into a screening test for oestrogenic activity for a 14-day exposure period and has received favourable scientific peer review (Thorpe *et al.*, 2000; 2001a, b) and at the OECD. This method can now be used to screen for receptor-mediated oestrogenic activity and has been shown to be adequately responsive to be conducted over a 10-day period, and with high reproducibility.

Studies on individual exposures to oestradiol and 4-*t*-nonylphenol in the *in vivo* test model gave dosage-dependent responses on vitellogenin production. Findings for methoxychlor suggest that this chemical is only weakly oestrogenic, even at concentrations that are acutely lethal. Studies investigating the relative potencies of steroidal oestrogens in this test system have shown that ethinyloestradiol is 70 times more potent than oestrone and 25 times more potent than oestradiol. The vitellogenic responses determined for ethinyloestradiol and oestradiol using this test system compare favourably with those found in similar studies using steroidal estrogens and rainbow trout (Routledge *et al.*, 1998; Thorpe *et al.*, 2000), although ethinyloestradiol appears to be even more potent than suggested by these previous authors.

Of particular interest are the effects of binary mixtures using this test system. Whereas managing single exposure:response systems is straightforward, and even dosing of several substances simultaneously is practicable, the problem is consideration of how the exposure regime is determined and then responses analysed subsequently. The only scientifically acceptable way to assess whether interactions are additive, synergistic or antagonistic is using the model of concentration addition (Loewe and Muischnek, 1926) The oestrogenic response data generated in this programme represent the first such data from well-conducted experiments – other authors have reported various interactions but these must be considered to be of lower quality since the exposure response was not based on equipotent dosing.

These binary mixture exposures established that 17β-oestradiol and 4-*t*-nonylphenol act in a concentration additive manner, that is they are acting via the same mechanism and elicit the same response in the fish, in a predictable manner. In contrast, the binary 17β-oestradiol/ methoxychlor mixture showed a less than additive effect indicating that methoxychlor may elicit a vitellogenic response through a different mechanism to 17β-oestradiol. Methoxychlor is metabolised in the liver of rainbow trout to HPTE, which has been postulated to act as a mixed agonist/antagonist in the presence of 17β-oestradiol (Ghosh *et al*., 1999, Gaido *et al*., 1999). Unfortunately, the high level of mortality obtained with the binary 4-t-nonylphenol/methoxychlor mix precluded a detailed analysis of the nature of any interaction in this experiment. Investigation of the concentration additivity of ethinylestradiol and oestradiol revealed that these two substances also act in an additive manner and that the concentrations of the compounds in the mixture that induced oestrogenic activity were lower than the NOEC values for the individual compounds.

The first significant finding of the binary mixture experiments is t structurally (and chemically-unrelated) substances (oestradiol and nonylphenol) can act together to elicit an additive oestrogenic response. The second finding is that structurally-related substances (ethinyl oestradiol and oestradiol) also act additively, which may be expected, but that the degree of additivity was at concentrations (of each substance) lower than that previously considered to be the no-effect concentration for that substance. These findings indicate that substance interactions are likely to be very important in assessing the risk to fish and other wildlife from oestrogenically-active discharges. In order to protect wildlife in receiving environments from oestrogenic effects, permits based solely on single-substance standards will not provide adequate protection from oestrogenic effects. Alternative approaches that integrate the activity of all oestrogenically-active substances present in an environmental discharge will need to be developed.

This emphasises the need to consider, during any risk assessment programme addressing endocrine disrupting effects, the possibility of interactions among individual components of the complex mixture that constitutes treated sewage effluent.

5.3 Implications for Fish Populations

The Phase I programme concluded*: The significance of the impacts on the male fish at the population level is not known but requires investigation.*

The results of Phase II of the research programme pose a number of important questions regarding the impact of treated sewage effluent on UK fish populations.

Studies have demonstrated that despite having differing ecological niches and reproductive strategies, UK populations of roach and gudgeon are showing changes (including intersex and vitellogenin induction) indicative of exposure to environmental oestrogens. There is, however, some evidence to suggest that a low level of intersex may exist naturally in these species, although the degree of feminisation (numbers of oocytes in testes) found in fish at control sites was very low (only one or two oocytes per gonad sample) compared with those found downstream of sewage treatment works (in which the oocytes might constitute more than 50% of the surface area of the gonad sections sampled). The exact cause of these disruptions in sexuality is undetermined, although it is clear that effluents from sewage treatment works containing oestrogenic contaminants are a major causal factor.

The changes caused by exposure to treated effluent are now known to extend beyond intersex and vitellogenin induction (the latter at least being apparently partially reversible), to include significant effects on early life development, sex differentiation, adult sexual maturation and fertility. There is also some preliminary evidence to suggest a skewing of the sex ratio in favour of females in some rivers, and it seems therefore possible that full sex reversal, resulting in a gradual skewing of the sex ratio, *may* be occurring. These most recent studies, of wild fish populations from the rivers Nene and Aire, indicate that feminisation of adult roach may occur throughout life, and therefore that fish may become more feminised as a result of continual exposure to oestrogen as adults. Furthermore, the data also indicate that feminisation of the reproductive ducts and the germ cells, respectively, may be quite separate phenomena, induced at different times during the fish's life cycle.

Perhaps the most significant results are those that provide evidence (based on *in vitro* fertility assessments) of reduced fertility of both males and females, and asynchronous sexual maturation. The hatching success of eggs fertilised with sperm from male fish with mild to severe intersex, showed that offspring viability was decreased with an increase in intersex severity. These findings provide strong evidence to indicate a relationship between the level of the effect (intersex) and the degree of reduction in the ability to produce good quality gametes.

How these effects relate to the population level consequences cannot be assigned at this time, however, as there are many other uncertain factors that might affect the ability of intersex fish to breed in the wild. These include the ability to compete with other males to fertilise the eggs from females (both behavioural competition and sperm competition). In addition, the numbers and ages of the fish in a population that contribute their genes to the next population represent a further unknown factor.

The reproductive health of severely intersex fish (such as was found in the rivers Nene and Aire) is significantly compromised, such that their ability to reproduce at all is questionable. What remains a more important issue is the degree to which moderately-affected fish, comprising more of the population in at least the rivers Nene and Arun, are actually able to produce viable gametes. Notwithstanding all of this, to determine the effects of intersexuality at the population level, or rather, how the widespread incidence of intersexuality affects the population dynamics of the roach is a highly complex question that is not easily addressed.

The evidence presented suggests that the widespread nature of intersexuality provides reasons for concern for fish population sustainability, particularly in urban areas where the proportion of effluent reuse is high, and therefore a high degree of exposure to sewage effluents containing mixtures of oestrogenic substances, is likely.

6. CONCLUSIONS

The main findings of the Phase II research programme are:

6.1 Incidence of Oestrogenic Effects in Native Fish Populations

- In UK wild roach populations, exposure to treated effluent is associated with ovo-testis, feminisation of the reproductive ducts in males, disruption of sexual maturity, occurrence of atretic oocytes, and spawning asynchrony.

- In UK wild gudgeon populations, intersex was evident in effluent-exposed fish, although at a lower incidence and severity than in roach. However, geographical distributions of intersex severity were similar for the two species.

6.2 Consequences of the Oestrogenic Effects Observed

- In late-spring, male roach taken from effluent-exposed sites and assessed for spawning readiness showed delayed spermatogenesis compared with males from control sites, whereas females were not at a dissimilar stage. Spawning readiness was concluded to be asynchronous in some wild roach populations.

- Examination of gamete viability in wild roach at spawning showed reduced milt volume and sperm motility in males, and atretic and vacuolated oocytes in females. Furthermore, *in vitro* fertilisation studies established significant reductions in the viability of gametes from male and female roach exposed to effluents, particularly for sperm.

- Factors that influenced reduced male fertility in fish from effluent-exposed sites included reduced sperm density, lower proportion of sperm that were motile and reduced duration of motility. Severely-feminised male fish showed the greatest variability in the sperm characteristics measured.

6.3 Induction of Oestrogenic Effects by Experimental Exposure to Sewage Effluents

- Studies exposing fish to sewage effluents for up to 4 months established that feminised reproductive ducts were induced at an effluent exposure of 25% during sexual development and differentiation in male juvenile (0+) roach. No disruption of ducts was observed in 1+ and 2+ fish exposed to effluents, indicating a lesser degree of, or no, sensitivity to this effect in fish that have already completed their reproductive duct development.

- Preliminary evidence for germ cell disruption was found in one individual but this needs to be further investigated following a longer growing-on period for these exposed fish.

- Vitellogenin was also induced in roach of all ages (0+, 1+ and 3+), and at exposures as low as 9% effluent (in tapwater). Induction by exposure to sewage effluent showed dose-dependence and indicated that sensitivity of fish to oestrogenic substances may increase over time.

6.4 Development of Oestrogenic Effects in Wild Populations with Continual Exposure to Sewage Effluents

- Assessment of long-term datasets on intersex condition (eggs in testis, and feminisation of sperm ducts) of wild roach from the rivers Aire, Nene and Arun, comparing age at capture (evaluating the duration of lifetime exposure) and year of birth (indicating local exposures at the early life stage) indicate that duration of lifetime exposure is a major influence on the severity of the intersex condition

- No relationship was found between intersex (eggs in testis) and year of birth

- No relationship was determined for the severity of intersex (measured as presence of oviducts), for either the duration of lifetime exposure, or low flows conditions in the native river.

- These studies indicate that feminisation of adult roach may occur throughout life, and therefore that fish may become more feminised as a result of continual exposure to oestrogen as adults. Furthermore, the data also indicate that feminisation of the reproductive ducts and the germ cells, respectively, may be quite separate phenomena, induced at different times during the fish's life cycle.

6.5 Identification of Oestrogenic Substances in Sewage Effluents

- Determination of the chemical composition of samples of treated sewage effluent to which fish were exposed confirmed the presence of specific oestrogenic compounds, but that some of these varied markedly, with a probable seasonal influence and treatment efficiency influence.

- Oestrogenic components identified in treated effluents included the natural steroids oestradiol and oestrone, alkylphenol ethoxylates and their degradation products nonylphenol and octylphenol. Levels of the synthetic steroid ethinyl estradiol were below the limit of detection in the majority of samples analysed. Owing to substantial temporal variations, and the mixture of oestrogenic substances present, attributing activity to an individual substance is not possible.

6.6 Interactions between Oestrogenic Substances

- Studies on binary mixtures of selected reference substances established that their oestrogenic activity (at least in terms of vitellogenin induction in rainbow trout) was additive, and demonstrated that a mixture of chemically–dissimilar oestrogens, 17β-oestradiol and 4-*t*-nonylphenol, could elicit effects at environmentally-relevant levels of the individual substances.

- Repeated studies on three steroidal oestrogens similarly established exposure response measures to vitellogenin and their relative potencies in this reliable assay system. Similarly, concentration additivity was demonstrated for ethinyloestradiol and 17β-oestradiol at environmentally-relevant concentrations.

- Concentration additivity studies with mixtures of steroidal estrogens and/or industrial chemicals showed that the concentrations of the mixture components that induced oestrogenic activity, were lower than the previously assessed No Observable Effect Concentration (NOEC) values for the induction of activity by individual substances. The implications of these findings is that environmental standards for single substances may not be adequately protective if other oestrogenic substances are also present and act via the same toxic mechanism(s).

6.7 Implications for Fish Populations

- The findings from this programme indicate that population recruitment in UK fish stocks exposed to treated sewage effluent could be impaired, due to lower male fertility, and that several stages within the life- and reproductive (gonad and gamete development) cycles are vulnerable to oestrogenic endocrine disruption.

- The evidence presented suggests that the widespread nature of intersexuality provides reasons for concern for fish population sustainability, particularly in urban areas where the proportion of effluent reuse is high, and therefore a high degree of exposure to sewage effluents containing mixtures of oestrogenic substances, is likely.

7. RECOMMENDATIONS FOR FUTURE RESEARCH

7.1 An Approach to Assess Population-Level Effects

There has been a consistent view at many international meetings that endocrine disruption should be assessed "at the population level". To date, there is only one clear example of the effects of endocrine disruption where population-level (and likely community-level) effects have been seen, that of tributyl-tin on some bivalve and gastropod molluscs species. In that case, agreement on the need to control TBT came long after populations had been lost in many locations across the world, but before mechanisms of action of TBT had been attributed to interference with endocrine-related processes. Finding the cause of population loss was the imperative, but how that arose following exposure to the causal substance(s) was also important but nonetheless a secondary question.

In the example of oestrogenic effects in wild fish, the situation is somewhat different. In this example, data have been gathered indicating impairment of reproductive health, that can (particularly in extreme cases) reduce or cease reproductive function. This indicates that population sustainability may be at risk and in principle (if exposure to effluents is the driving factor), fish in those catchments can be ascribed as at high, medium or low risk depending on quantities and qualities of effluent discharged to them. The information available is indicative of risk, but how does that compare to the performance of populations in these catchments – their sustainability?

Determining the sustainability of fish populations, and any impacts on reproductive health in particular, is very complex. Monitoring of wild fish is possible for fish above a certain size (say 10cm) even in the largest of rivers within the UK, but assessing success of recruitment, particularly for cyprinid fish, is less straightforward for very small fish. Reliance is placed on enumerating fish that are at least 2 to 3 years old, therefore reflecting reproductive health 2 to 3 years previously of the population. Given the potential number of pressures that can affect recruitment, determining the importance of any one factor is not likely to be possible unless all other relevant factors are assessed also. The baseline information available for fish in the wild is not currently sufficient to deliver this relative assessment of pressures.

However difficult it is to derive assessments of sustainability from wild populations, this could be determined through laboratory assessments and extrapolation through modelling to a wild situation. If this was also to evaluate risks from particular environmental situations, this would need to incorporate:

- Exposure:effect relationships;
- Effect:consequence for ontogeny of development;
- Reproductive performance both controlled (assess gamete quality) and natural spawning (assess role of behaviour);
- Larval/juvenile fish development; and,
- Modelling of density dependence-related species (where other factors are also characterised).

Such an exercise is only completely practicable in a laboratory species, such as the fathead minnow (*Pimephales promelis*) or zebrafish (*Brachydanio rerio).* For a UK native species, only certain aspects of this would be possible, which would be valuable as a cross reference to the laboratory fish, where data would be more concerted.

Unless the information base on the dynamics of wild populations is significantly improved, deriving a sound understanding of how intersex affects population sustainability will remain elusive. Therefore, the best available science suggests that further work in wild fisheries should focus on using data at the level of *the individual* and using laboratory model species to infer linkages and risks to wild populations. Such model species, such as the cyprinid fish the fathead minnow, should also be used as tools to establish the level of effect against a range of endocrine-related endpoints and then applied in testing for environmental discharges and chemical risk assessment.

7.2 Other Key Future Activities

7.2.1 To establish the effects of exposure on sensitive early development periods, thereby identifying the periods when fish are most susceptible to the induction of intersex effects, and therefore require the greatest level of protection

7.2.2 To assess the development and reproductive health of mature fish previously exposed (as larvae) to effluents in earlier experiments.

7.2.3 To understand the health consequences (reproductive or otherwise) of vitellogenin induction in male and juvenile fish and establish linkages between this exposure biomarker to other (permanent) health effects (e.g. induction of intersex, sex ratios)

7.2.4 To establish integrative test strategies for use in hazard assessment of endocrine disrupting substances, in particular with regard to the implications of time-dependent aspects.

7.2.5 To develop a genetic probe that can determine the genotype of intersex and female fish in roach and gudgeon populations and for an ecotoxicologically-relevant test species. This should be applied both to archived material already gathered from wild populations to confirm true sex ratios within the population, future river surveys of cyprinid fish and to fish exposed *in experimentia* in the development of fish early-life stage and chronic testing protocols.

7.2.6 To develop a risk-based approach to identify and prioritise sites where fisheries may be at high risk of induction of oestrogenic effects. This should include further assessments of wild fish populations to determine the spatial extent of the intersex condition and how that relates to known water quality and quantity datasets.

7.2.7 To take forward a risk-management framework that can demonstrate both the need for, and the benefits of, remedial measures. This would include an assessment of current impact, causal oestrogenic substances and sewage treatment processes that can reduce or eliminate them.

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