Causes and consequences of feminisation of male fish in English rivers

Science Report SC030285/SR

The Environment Agency is the leading public body protecting and improving the environment in England and Wales.

It's our job to make sure that air, land and water are looked after by everyone in today's society, so that tomorrow's generations inherit a cleaner, healthier world.

Our work includes tackling flooding and pollution incidents, reducing industry's impacts on the environment, cleaning up rivers, coastal waters and contaminated land, and improving wildlife habitats.

This report is the result of research commissioned and funded by the Environment Agency's Science Programme.

Published by:
Environment Agency, Rio House, Waterside Drive, Aztec West, Almondsbury, Bristol, BS32 4UD
Tel: 01454 624400 Fax: 01454 624409
www.environment-agency.gov.uk

ISBN: 1844322998

Further copies of this report are available from:
The Environment Agency's National Customer Contact Centre by emailing enquiries@environment-agency.gov.uk or by telephoning 08708 506506.

Authors:
Gross-Sorokin MY, Roast SD and Brighty GC

Dissemination Status:
Publicly available

Keywords:
endocrine disruption, fish, feminisation, steroid oestrogen, oestradiol, oestrone, ethinyloestradiol, risk assessment

Environment Agency's Project Manager:
Dr Geoff Brighty, Ecosystems and Human Health, Science Group, Wallingford, Oxon OX10 8BD

Science Project Number:
SC030285/SR

Science at the Environment Agency

Science underpins the work of the Environment Agency, by providing an up to date understanding of the world about us, and helping us to develop monitoring tools and techniques to manage our environment as efficiently as possible.

The work of the Science Group is a key ingredient in the partnership between research, policy and operations that enables the Agency to protect and restore our environment.

The Environment Agency's Science Group focuses on five main areas of activity:

- **Setting the agenda**: To identify the strategic science needs of the Agency to inform its advisory and regulatory roles.
- **Sponsoring science**: To fund people and projects in response to the needs identified by the agenda setting.
- **Managing science**: To ensure that each project we fund is fit for purpose and that it is executed according to international scientific standards.
- **Carrying out science**: To undertake the research itself, by those best placed to do it - either by in-house Agency scientists, or by contracting it out to universities, research institutes or consultancies.
- **Providing advice**: To ensure that the knowledge, tools and techniques generated by the science programme are taken up by relevant decision-makers, policy makers and operational staff.

Professor Mike Depledge Head of Science

Executive Summary

Background

Since the early 1980s there has been growing concern about the ability of substances discharged into the environment to disrupt the normal endocrine (hormone) function of wildlife. In particular, the apparent widespread feminisation of male fish in rivers has received particular attention. Following an assessment of the available data, the Environment Agency of England and Wales has concluded that the weight of evidence for the effects of endocrine disruption in fish is sufficient to develop a risk management strategy for biologically active effluents discharging to the aquatic environment. This paper provides the background information supporting the proposed England and Wales evaluation and demonstration programme of effluent technologies to reduce the endocrine disrupting potential of sewage effluent on fish in receiving waters. The Endocrine Disruption Demonstration Programme is intended to deliver a basis for future decision on investment in sewage treatment works (STW) infrastructure aimed at reducing the impacts to acceptable levels, if and where required.

Environmental Evidence

Scientific investigations in the UK, Europe, USA and Japan have demonstrated the occurrence of the intersex condition (developing eggs within the testis tissue and/or feminised reproductive ducts) and elevated concentrations of plasma vitellogenin (egg-yolk protein) in fish in freshwater and estuarine environments. The results confirm that this is a widespread issue. The UK and European epidemiological datasets have clearly demonstrated that the effects were associated with point source discharges, and incidence and severity directly related to the proportion of treated sewage effluent in rivers. Moreover, the apparent progressive nature of the intersex condition in male fish indicates that long-term exposure to such substances is an important factor in the development of the effects seen.

Causation

Studies from the UK, Europe and the United States have clearly identified natural (oestrone and 17β-oestradiol) and synthetic (17α-ethinyloestradiol) steroids as the main oestrogenically active substances present in domestic effluents. Although it is accepted that feminisation of fish can also occur through other mechanisms (e.g. through exposure to substances with anti-androgenic properties) and that weaker environmental oestrogens (e.g. alkylphenols) may also contribute to the overall effects observed, the role of steroid oestrogens (oestrone, 17β-oestradiol and 17α-ethinyloestradiol) in domestic effluents is implicit.

The use of an oestrogen-specific biomarker, the presence of vitellogenin in male, intersex and juvenile fish, has demonstrated that wild fish have been exposed to and have taken up oestrogenic substances discharged in effluents. Experimental exposures to effluents have also induced vitellogenin production and the development of ovarian cavities. Germ cell disruption (eggs in testis) could not be induced in one year exposures, but evidence suggests that this effect may to longer to be induced. Laboratory studies also clearly confirm a causal link between exposure to steroid oestrogens and endocrine disruption. Induction of plasma vitellogenin, disruption of mating behaviour, reduced number of eggs, reduced fertilisation effects, skewed sex ratios and morphological abnormalities have all been reported following laboratory exposure of fish to steroids.

Conclusion

The amount of experimental evidence available on causation of sexual disruption in wild fish, when taken with evidence from field studies, suggests strongly that natural steroids from STWs contribute significantly to oestrogenic endocrine disruption in fish. The widespread occurrence of the intersex condition in the UK, taken together with the evidence that moderate to severely intersex fish have reduced sperm quality, quantity and fertilisation success raises the concern that this phenomenon could potentially have chronic impacts on the sustainability of fish populations. The Agency believes that the weight of evidence is sufficient to develop risk management approaches for oestrogenically active sewage treatment work effluents.

Contents

1. Introduction

1.1 Background

Since the early 1980s there has been growing concern about the ability of substances discharged into the environment to disrupt the normal endocrine (hormone) function of wildlife. This concern and subsequent investigations have been prompted by field observations of changes in reproductive physiology and health of wildlife species from several taxonomic groups in many countries (e.g. Tyler *et al.*, 1998; Vos *et al.*, 2000). In particular, the apparent widespread feminisation of male fish in rivers, together with impacts on reproduction and survival of mollusc species, have received particular attention.

The level of concern over these effects led to a series of major national and international workshops, assessments and research programmes (e.g. Ankley *et al.*, 1998; deFur *et al.*, 1999; Taylor *et al.*, 1999; IPCS, 2002; Matthiessen *et al.*, 2002). These served to evaluate the effects from candidate contaminants, their pathways to the environment and the epidemiological impacts through risk assessments. Following an assessment of the available data, the Environment Agency of England and Wales (the Agency) has concluded that the weight of evidence for the effects of endocrine disruption in fish is sufficient to develop a risk management strategy for biologically active effluents discharging to the aquatic environment.

This paper provides the background information supporting the proposed England and Wales evaluation and demonstration programme of effluent treatment technologies to reduce the endocrine-disrupting potential of sewage effluent on fish in receiving waters. The Endocrine Disruption Demonstration Programme is intended to deliver a basis for future decisions on investment in sewage treatment works (STW) infrastructure aimed at reducing the impacts to acceptable levels, if and where required.

1.2 Scope of the review

This review concentrates on evidence of endocrine disruption-induced feminisation of fish and the causes and consequences of this phenomenon. Compared with the available literature on the endocrine systems of vertebrates, there is a paucity of information on impacts on invertebrate endocrine systems, and it is difficult, therefore, to make definitive statements about endocrine disruption in invertebrates. Perhaps the best-known example of endocrine disruption in invertebrates is the induction of imposex in the marine gastropod *Nucella lapillus* (dogwhelk) following exposure to anti-fouling paints containing tributyl-tin (TBT) (Matthiessen and Gibbs, 1998). In general, however, the effects of compounds known to cause comparatively distinct endocrine-disrupting effects in fish are variable in invertebrates, and often cannot be directly attributable to disruption of the hormonal system.

This review particularly focuses on evidence of oestrogenically active substances found in sewage effluents, and in particular that of steroidal hormones of human origin, for example the *natural hormones* oestrone and 17β-oestradiol, and the *synthetic hormone* 17α-ethinyloestradiol. A large number of other substances possessing oestrogenic activity have also been identified and are being taken through a risk assessment in the European Union presently (European Commission, 1999). However, the steroidal hormones represent the most potent group of substances *in vivo* as well as *in vitro*, compared with other oestrogenic substances under consideration. In addition, this review also considers *anti-androgenic* substances, as their overall effects may not be dissimilar to the effects of oestrogenic chemicals, in that they may suppress male hormone-dependent development and lead to feminising effects.

2. Environmental evidence

2.1 Biological effects in wild fish populations

A number of studies have reported the widespread occurrence of abnormal sexual development in freshwater and marine fish species (reviewed in sections 2.2–2.4). Surveys of wild fish in rivers and estuaries have found male fish with elevated concentrations of vitellogenin in their blood plasma and with a condition known as intersex (a condition in which the gonads contain developing eggs (oocytes) within the testis tissue and/or in which the reproductive ducts are feminised) (Jobling *et al.*, 1998; van Aerle *et al.,* 2001; Kirby *et al.*, 2004).

Vitellogenin is an egg-yolk protein produced in the liver of mature female fish in response to endogenous stimulation by the hormone oestrogen. The protein is transported via the blood to the ovary, where it is taken up by the developing oocytes and sequestered as egg yolk. Vitellogenin is not normally found in juvenile or male fish, due to the absence of endogenous oestrogenic stimulation. However, exposure to oestrogens can cause juvenile and male fish to produce vitellogenin, and the presence of this protein in male and juvenile fish is, therefore, used as a marker of exposure to oestrogenic substances (Purdom *et al.,* 1994). The implications of an abnormal induction of vitellogenin in fish are not well known, but high concentrations can lead to alterations in kidney development and disruptions of kidney function (Herman and Kincaid, 1988, 1991). The energy budget in early life stage fish is critically balanced and any inappropriate induction of vitellogenin as a consequence of oestrogen exposure is likely to impose an additional strain on survivorship capability.

Sexual differentiation in fish usually starts shortly after hatch and can occur over a period of a few weeks to many months (depending on the fish species and growth rates). It is a two-stage process, involving gonadogenesis (the development of the somatic tissues and the gonad duct) and gametogenesis (the differentiation of germ cells into sperm or eggs). Oestrogens play a central role in both gonadogenesis and gametogenesis in female fish, and the sexual development of males of many fish species can be reversed by the application of oestrogens during the period of sexual differentiation (see Rodgers-Gray *et al.*, 2001, and references therein). Combination of this evidence with the finding that the number of fish with normal testes was inversely proportional to the number of intersex fish (Jobling *et al.*, 1998) has led to the suggestion that the intersex phenomenon is due to the feminisation of male fish.

There is strong evidence demonstrating that most sewage effluents studied are oestrogenic (Purdom *et al.*, 1994; Harries *et al.*, 1996; Desbrow *et al.*, 1998), supporting the hypothesis that the feminisation observed in fish is due to an oestrogenic (feminising) effect.

There is a possibility that this “feminisation” may, in part, be due to anti-androgenic mechanisms (anti-masculinising) (see section 3.3). Whilst it is known that exposure to oestrogenic substances does lead to feminisation, exposure to substances that act antagonistically on the androgen systems/pathways may potentially result in similar feminising effects as oestrogens. Anti-androgens suppress the development of male characteristics, and may act in isolation or in combination with oestrogens found in effluent. Further research is necessary to investigate the specific modes of action of anti-androgens, to identify environmentally relevant contaminants causing such effects, and how they might act in concert with oestrogens. It has been demonstrated that ethinyloestradiol and some pesticides can also act in an anti-androgenic manner *in vivo* (Katsiadaki *et al.*, 2002; Defra, 2003). Nevertheless, the fact that many chemicals coexist in complex effluents indicates that many organic chemicals, not single ones, are probably responsible for endocrine-disrupting effects observed in wild fish. Notwithstanding this, for some environments, specific chemicals have been identified that are now known to be major contributor(s) to the effects seen. An example of this would be for steroid oestrogens and some of the feminising effects seen in wild fish living in UK rivers.

2.2 Studies contributing to the present understanding of feminising effects of sewage effluent on fish in UK freshwaters

Initial findings of intersex roach (*Rutilus rutilus*) downstream of two STWs in the River Lea in the early 1980s (Thames Water, 1981) led to concerted investigations into the occurrence, causes and consequences of intersex fish in UK rivers. Caged male rainbow trout (*Oncorhynchus mykiss*), placed directly in or below sewage effluent outflows and at a range of distances downstream of STWs, produced the female-specific egg protein vitellogenin, demonstrating that fish had been exposed to oestrogenic substances (Purdom *et al.*, 1994; Harries *et al.*, 1996). For nine river stretches, an oestrogenic response was detectable only in undiluted sewage effluent or it was limited to the nearby downstream region. However, for three other stretches, effects were observed for considerable distances (1.5 and 5 km) downstream of the sewage outfalls (Harries *et al.*, 1996).

Studies progressed to investigations of effects in wild roach (*R. rutilus*) populations living below STWs. Wild roach were sampled from 18 sites on eight rivers and five reference locations (i.e. no direct large effluent inputs). A significant difference was observed between fish that were caught downstream from sewage discharge points and those that were caught from reference sites. Male fish caught from downstream sites exhibited a higher incidence of gonadal intersex (developing eggs in the testes and oviducts) and contained higher concentrations of plasma vitellogenin in their blood compared with those from reference sites. Moreover, the occurrence of intersex and plasma vitellogenin concentrations in fish at downstream sites was equal to or greater than observed in fish at upstream sites. The severity of the intersex condition varied from a few primary eggs within the testes to many hundreds of primary eggs and some secondary eggs. There were also differences in the severity of the effects on the ducts, from fish with both a sperm duct and (additionally) an oviduct to those which had only oviducts and lacked sperm ducts. The severity of the effect (measured by a numerical index – the intersex index) revealed that fish from the reference sites showed the lowest severity, fish from upstream sites showed a higher severity, and fish from downstream sites showed by far the greatest severity index (i.e. reference sites < upstream site < downstream site). A strong correlation was found between the approximate concentration of the sewage effluent in the river system (concentration of effluent) and the frequency and severity of effects (Jobling *et al.*, 1998).

Further sampling of roach from a selected number of sites together with a re-analysis of the new pooled field dataset from Jobling *et al.* (1998) indicated that the intersex condition was more pronounced in older fish than younger fish (Environment Agency, 2002a). The severity of the intersex condition (as measured by the intersex index) of wild fish of approximately six years of age was twice that of three-year-old fish. In fish below three years in age, intersex was a limited and rare condition, indicating that the expression of this condition is progressive and appears at the onset of puberty or after in roach (puberty occurs in wild roach in either their third or fourth year of life – age 2+ or 3+). The intersex condition was related more to the duration of exposure to effluent in the study rivers, than to the year of hatching and prevailing environmental conditions at that time.

Another survey, comprising two rivers and two lakes in the UK, established that the intersex condition was also present in another fish species, the gudgeon (*Gobio gobio*), confirming that the intersex condition is not limited to roach. Although the distribution pattern of intersexuality in gudgeon in the two rivers mirrored the pattern seen in roach, the occurrence and severity of intersex in gudgeon was generally lower than in roach for any given sampling site (van Aerle *et al.*, 2001). Reports of intersex occurrence in marine species (see section 2.3) confirm that sexual disruption is a general phenomenon in fish in the UK and is not species-specific.

One of the key questions arising from the reports of the widespread occurrence of intersex fish is whether the condition affects reproductive success. To begin to address this question, studies were undertaken to assess the sperm and egg (gamete) quality of wild-caught, intersex roach. Gametes from both male and female fish were removed artificially by application of gentle pressure to their abdomens. Using these methods, fewer of the moderately or severely feminised fish were found to release sperm (milt) compared with both “normal” male fish (from contaminated and reference sites), and less severely feminised intersex fish. Those feminised fish that were able to release milt (the majority) produced up to 50% less sperm (volume of sperm per gram of testis weight) than “normal” male fish. Sperm quality (i.e. motility, density and fertilisation success) was found to be negatively correlated with the degree of feminisation in intersex fish. In severely intersex fish, sperm motility was reduced by up to 50%, and fertilisation success was reduced by up to 75%, compared with less severely intersex, or unaffected, fish (Jobling *et al.*, 2002).

2.3 Studies contributing to the present understanding of feminising effects of endocrine disruption in UK estuarine and marine environments

Although there is less evidence of endocrine disruption in marine and estuarine fish by natural and synthetic steroids, evidence does exist that suggests that these hormones have the potential to cause adverse effects in marine and estuarine populations. Moreover, the effects of endocrine-disrupting compounds that have been widely reported for freshwater fish also occur in marine organisms (see Oberdörster and Cheek, 2001).

The EDMAR programme (Endocrine Disruption in the Marine Environment) was started in 1998 to investigate whether marine fish and invertebrates had impaired reproductive health due to endocrine disruption, and, if so, to identify possible causes and impacts on marine populations. Field evidence of endocrine disruption in marine and estuarine fish is summarised below (laboratory studies using marine and estuarine species are presented in section 3.4).

In wild populations of the sand goby (*Pomatoschistus minutus*), “morphologically intermediate papilla syndrome” (MIPS) was recorded from oestrogen-impacted sites. MIPS was most prevalent in the Mersey and Tees estuaries, and least prevalent at the reference sites (Kirby *et al.*, 2003). The significance of MIPS on reproductive success is not yet understood (Kirby *et al.*, 2003).

Very high levels of plasma vitellogenin in male flounder *Platichthys flesus* [i.e. greater than levels in females (Allen *et al.*, 1999)] and testes abnormalities have also been reported in >50% of male fish from estuaries where oestrogenic discharges were released (Lye *et al.*, 1997). Although evidence suggests that vitellogenin concentrations in flounder from some UK estuaries may be decreasing, the data do not support any conclusions regarding temporal variation or trends (Kirby *et al.*, 2004). Intersex has been recorded in wild flounder in some UK estuaries, with highest incidences in estuaries receiving high inputs of industrial and domestic effluents [the Mersey, Tyne and Clyde estuaries (Kirby *et al.*, 2004)]. The highest incidence of intersex in male flounder was 14%; the natural background occurrence of this condition is not known.

2.4 International evidence contributing to the present understanding of effects of sewage effluent on fish

2.4.1 Europe: COMPREHEND

The European Commission funded a project titled “Community Programme of Research on Endocrine Disrupters and Environmental Hormones (COMPREHEND)” in its Fifth Research Framework Programme. The overall objective of COMPREHEND was to assess the evidence for endocrine disruption in the aquatic environment in Europe, consequent to effluent discharge. The project focused on domestic and industrial effluents and aimed to determine their impacts on fish, and to identify the principal causative agents for any impacts observed. Collaborative partners included members from the Netherlands, France, Sweden, United Kingdom, Switzerland, Norway and Finland (CEH, 2002).

COMPREHEND demonstrated that oestrogenic effluents are widespread across Europe. Approximately one-third of the municipal STW effluents examined during the three-year programme were found to be strongly oestrogenic and capable of stimulating vitellogenin in juvenile or male fish. Samples of wild fish [common bream (*Abramis brama*), common carp (*Cyprinus carpio*), roach (*R. rutilus*) and gudgeon (*G. gobio*)], taken from the vicinity of municipal STWs known to discharge oestrogenic effluents, were found to have abnormally high concentrations of vitellogenin and increased incidence of intersex (CEH, 2002).

2.4.2 America and Japan

In a survey of a river known to be contaminated with chlordane and polychlorinated biphenyls (PCBs), Harshbarger *et al.* (2000) reported intersex gonads in shovelnose sturgeon (*Scaphirrhynchus platorynchus*). Various studies undertaken in America have reported elevated vitellogenin levels in male carp (*Cyprinus carpio*) and fathead minnow (*Pimephales promelas*) exposed to municipal sewage effluents (IPCS, 2002, and references therein). In Japan, the intersex condition has been reported in wild male flounder (*Pleuronectes yokohamae*) from Tokyo Bay and hypothesised to be attributable to environmental oestrogens (Hashimoto *et al.*, 2000). Furthermore, incidence of vitellogenin in male, Japanese common goby (*Acanthogobius flavimanus*) from several coastal bays in Japan correlates well with, amongst other things, oestradiol concentrations in those bays (Ohkubo *et al.*, 2003).

2.5 Conclusions

Investigations led by researchers in the UK, Europe, the USA and Japan have demonstrated the occurrence of the intersex condition and elevated concentrations of plasma vitellogenin in fish in freshwater and estuarine environments. The results confirm that this is a widespread issue. The UK and European epidemiological datasets have clearly demonstrated that the effects were associated with point source discharges, and that incidence and severity were directly related to the proportion of treated sewage effluent in rivers. Moreover, the apparent progressive nature of the intersex condition in male fish indicates that long-term exposure to such substances is an important factor in the development of the effects seen.

The use of an oestrogen-specific biomarker, the presence of vitellogenin in intersex, male and juvenile fish, has demonstrated that these fish have been exposed to, and have taken up, oestrogenic substances.

The widespread occurrence of the intersex condition in the UK, taken together with the evidence that moderate to severely intersex fish have reduced sperm quality, quantity and fertilisation success, raises the concern that this phenomenon could potentially have chronic impacts on the sustainability of fish populations.

3. Causation

3.1 Effluent exposure and feminisation

Whilst the field datasets indicated a causal link to effluents, direct exposure–effect studies were required to show response and concentration dependence, and life-stage-specific responses. To determine the effects of effluent exposure on sexual differentiation and development, juvenile roach (50 days post-hatch) were experimentally exposed to several “concentrations” of (primarily) domestic sewage effluent (dilutions of 0, 12.5, 25, 50 and 100%) for a further 150 days. Chemical analysis of 100% effluent confirmed the presence of oestradiol, oestrone and alkylphenols, whilst fish exposed to the effluent showed concentration-dependent vitellogenin induction, confirming that they had been exposed to oestrogenic substances. Exposure to the effluents resulted in feminisation of the reproductive ducts in a concentration-dependent manner, and all of the fish in 100% effluent had feminised reproductive ducts (oviducts). Although a 150 day depuration period in clean water led to a reduction in vitellogenin concentrations, there was no alteration of the feminised ducts. This indicates that feminised ducts observed in wild-caught roach are the result of exposure to effluents during early life stages and are a permanent effect. However, the eggs in testes condition was not induced in this study (Rodgers-Gray *et al.*, 2001).

Further studies were undertaken to assess the effects of effluent exposure on two sensitive life stages. Possible windows in the fish life-cycle during which fish may be sensitive to feminisation of the gonad include early life stage (encompassing the period from fertilisation of the egg to development of the reproductive organs) and the period of germ cell (developing egg and sperm cells) proliferation. The latter occurs every year around the time of spawning in annually breeding fish. Fertilised eggs (for early life stage assessment) and adult fish (for post-spawning assessment) were exposed to graded concentrations (from 0 to 100%) of two STW effluents. Industrial effluent constituted 6% at STW site A and 24% at STW site B. Induction of vitellogenin in exposed fish and chemical analysis of the effluents confirmed the presence of oestrogenic substances in the effluents. Vitellogenin induction was almost an order of magnitude higher at site A compared with site B, which reflected the higher concentrations of oestrogens measured in the effluent from site A. For the early life stage exposures there was a concentration-dependent increase in oviduct formation in developing young fish. However, there was no convincing evidence for the formation of eggs in testes in the early life stage or adult post-spawning fish exposures (Liney *et al.*, 2004; Environment Agency, 2004a).

The results of these experiments (Rodgers-Gray *et al.*, 2001; Liney *et al.*, 2004; Environment Agency, 2004a) confirm that exposure of fish to STW effluents for periods during certain life stages can induce some (oviducts), but not all (eggs in testes), of the characteristics of intersex seen in wild fish found below STWs. Further research continues to assess how this condition arises. So far long-term experimental studies have not been conducted, which may account for the failure to induce the ovotestis condition experimentally. Indeed, it is worth reiterating the fact that germ cell disruption in males (oocytes in the testis) is generally only seen in wild roach that are 2+ in age (see above), and all the exposures undertaken to date have been for less than one year.

3.2 Effluent toxicity identification and evaluation: UK/COMPREHEND/USA

Since the production of vitellogenin is stimulated via oestrogen/hormone–receptor interactions, it was hypothesised that the range of feminising effects observed in caged and wild fish was due to oestrogenic substances present in the effluent. The oestrogenic substances were suspected to be either natural oestrogens or synthetic substances capable of mimicking oestrogen. A wide variety of structurally diverse synthetic chemicals are known to mimic oestrogens, and many have been detected in the environment. These include alkylphenols (which are breakdown products of industrial surfactants), bisphenol A, phthalates and a variety of pesticides and herbicides.

A study was undertaken by Desbrow *et al.* (1998), which aimed to identify the oestrogenic substances in seven domestic STW effluents. The effluents were fractionated into chemically similar components, and each fraction was tested for oestrogenic activity in an oestrogen-specific bioassay (the yeast oestrogen screen; Routledge and Sumpter, 1996). The study found that two natural steroid hormones (oestrone and 17β-oestradiol), and one synthetic steroid hormone (17α-ethinyloestradiol, used in contraceptive hormone therapies), were the most significant oestrogenically active substances in the effluents (Desbrow *et al.*, 1998). The natural hormones were consistently present at high levels (oestrone at 1–80 ng L^{-1}, and 17β-oestradiol at 1–50 ng L^{-1}), whereas 17α-ethinyloestradiol was generally below the detection limit of approximately 1 ng L^{-1} (except at one site). In subsequent concentration–response experiments, it was demonstrated that there were sufficient hormones in the effluents to induce the vitellogenic responses in fish that characterised previous field research (Routledge *et al.*, 1998). Although these results do not rule out other weakly oestrogenic compounds from contributing to the overall effect, the authors concluded that oestrone, 17β-oestradiol and 17α-ethinyloestradiol were likely to be the major causes of the oestrogenic activity present in effluents arising from "domestic" sources (Desbrow *et al.*, 1998).

A similar fractionation study was performed to determine the relative contribution of alkylphenol polyethoxylates to the oestrogenic activity of an effluent from an STW known to receive particularly high concentrations (compared with other STWs in the UK) of alkylphenol polyethoxylates, mainly from the textile trade. The study suggested that alkylphenol polyethoxylates may contribute the majority of the oestrogenic activity in the effluent from this STW. However, the authors concluded that this assessment was based on a number of uncertainties, and conclusions should be considered tentative (Sheahan *et al.*, 2002). It is of interest to note that oestrogenic activity and biological response were present in effluents during a period when the majority of the local textile traders were not discharging, and this was attributed to the natural oestrogens oestradiol and oestrone (Sheahan *et al.*, 2002).

In the COMPREHEND programme (see section 2.4) similar studies to identify the main causative agents in effluents identified steroid oestrogens as the principal oestrogen components of domestic sewage. Oestrone and 17β-oestradiol concentrations were generally in the 0–10 ng L^{-1} range, whilst 17α-ethinyloestradiol was often below or at the limit of detection (1 ng L^{-1}). Bisphenol A was detectable in municipal effluents, but at concentrations less than 5 µg L^{-1}, and nonylphenol was generally below 2 µg L^{-1}. Given the relative potencies of the various oestrogenic compounds measured in municipal STW effluents, COMPREHEND concluded that natural and synthetic steroids, of human origin, were by far the most important oestrogenic components and are responsible for most of the oestrogenic effects observed in fish. COMPREHEND concluded that "strongly oestrogenic municipal effluents will be reasonably commonplace across mainland Europe, as they are in the UK, and, therefore, there is the potential for oestrogenic endocrine disruption of aquatic wildlife in all countries (the situation with industrial effluents will depend very much upon the nature of the industrial processes)" (CEH, 2002).

Bioassay-directed fractionation of three municipal STWs and several environmental samples in America were undertaken to identify and quantify oestrogenic substances present. Oestradiol and 17α-ethinyloestradiol were found to represent 88 to 99.5% of the total oestrogen equivalents in the samples analysed (Snyder *et al.*, 2001). Although alkylphenols were detected, in general they were found to contribute less than 0.5% of the total oestrogen equivalents in the samples (Snyder *et al.*, 2001).

In a recent survey commissioned by the Agency, final effluents from 43 STWs in England and Wales were sampled on two occasions in 2003 for their oestrogenic quality (Environment Agency, 2004b; Gross-Sorokin *et al.*, 2004). One, two or all three of the steroid oestrogens oestrone, 17β-oestradiol and 17α-ethinyloestradiol were detected at virtually all sites. Reported concentrations were in the ranges of <0.5–100 ng L^{-1} for oestrone, <1–22 ng L^{-1} for 17β-oestradiol, and 1.0–3.2 ng L^{-1} for 17α-ethinyloestradiol. Nonylphenol concentrations in the final effluents were generally low, with the majority of concentrations in the range of <1–3 µg L^{-1} (of 43 sites, only eight had concentrations above 3 µg L^{-1} and none higher than 7.7 µg L^{-1}). The oestrogenic potency of nonylphenol (see section 3.5) suggests that nonylphenol (at least in isolation) is not likely to elicit an oestrogenic response in fish downstream, once dilution is taken into consideration. It is likely that there is a contribution from this chemical (and of other industrial chemicals that are known to have weak oestrogenic activity), in combination with steroid oestrogens in complex mixtures such as effluents, in causing intersex and vitellogenin induction in wild fish populations, but present evidence suggests that this is not the major contribution to oestrogenic activity in mainly domestic effluents. Notwithstanding this, the long-term effects of low concentrations of nonylphenol on vitellogenin induction and gonadal disruption in roach are unknown.

3.3 Anti-androgenic activity in effluents

The recent national survey of 43 UK effluents commissioned by the Agency confirmed the presence of oestrogenic activity *in vitro*, but also revealed the widespread occurrence of *in vitro* anti-androgenic activity (Environment Agency, 2004b; Gross-Sorokin *et al.*, 2004). The widespread nature of the anti-androgenic activity measured in the treated final effluents in this study suggests that it is of domestic origin, but this has yet to be determined.

The detection of anti-androgenic effects at this stage does not detract from the focus of removal of steroid oestrogens from effluents in the UK demonstration programme, but suggests that anti-androgenic biological effects should also be taken into consideration when monitoring and evaluating the success of treatment options during the demonstration programme.

3.4 Laboratory studies on the feminising and reproductive effects of steroid oestrogen exposure

Results from laboratory studies have provided substantial datasets to show that exposure to natural and synthetic steroids found in STW effluents induce the range of feminising effects seen in wild fish, and in some cases at environmentally relevant concentrations.

3.4.1 Vitellogenin induction

17β-Oestradiol

Exposure of early life stages of fathead minnow (*Pimephales promelas*) to comparatively low concentrations of 17β-oestradiol (25–100 ng L^{-1}, nominal) led to vitellogenin induction (Tyler *et al.*, 1999). Panter *et al.* (1998) also reported an increase in plasma vitellogenin in fathead minnow (*P. promelas*) following exposure to 17β-oestradiol (>100 ng L^{-1}), and the response was concentration-dependent. In an attempt to link this work to likely environmental exposures of fish to 17β-oestradiol, further experiments were conducted exposing male *P. promelas* to intermittent exposure of 17β-oestradiol. Significant induction of vitellogenin was reported following pulses of 3 day exposure every 6 days to 17β-oestradiol at 120 ng L^{-1} (Panter *et al.*, 2000). Furthermore, the extent of vitellogenin induction in fish exposed intermittently was similar to that in fish exposed continuously to the same concentration of 17β-oestradiol, indicating that metabolism of vitellogenin is slow. The results suggest that fish in the vicinity of STW effluent discharges are susceptible to endocrine-disrupting effluents even under intermittent exposure, and irrespective of flow regimes.

Studies in Japanese medaka (*Oryzias latipes*) have also demonstrated increased vitellogenin induction following exposure to 17β-oestradiol concentrations as low as 5 ng L^{-1} (Tabata *et al.*, 2001). Vitellogenin induction has also been reported in early life stage zebrafish (*Danio rerio*) following exposure to 17β-oestradiol at 100 ng L^{-1}, and in adult fish at 25 ng L^{-1} (males) or 100 ng L^{-1} (females) 17β-oestradiol (Brion *et al.*, 2001). In rainbow trout (*Oncorhynchus mykiss*), following exposure to up to 45 ng L^{-1} 17β-oestradiol, vitellogenin concentrations increased significantly, with a predicted threshold concentration of <10 ng L^{-1} (Routledge *et al.*, 1998). Similarly, Thorpe *et al.* (2000) also report a concentration-dependent increase of plasma vitellogenin in female *O. mykiss* following exposure to <5–247 ng L^{-1} 17β-oestradiol. In addition, significant vitellogenin induction has also been reported in the roach *(Rutilus rutilus)* following exposure to 17β-oestradiol [although only at higher concentrations, e.g. 100 ng L^{-1} (Routledge *et al.*, 1998)].

Oestrone

Male fathead minnows *(Pimephales promelas)* exposed to nominal concentrations of 31.8 ng L^{-1} oestrone developed significantly increased concentrations of plasma vitellogenin (Panter *et al.*, 1998). Furthermore, significant induction of vitellogenin in rainbow trout (*Oncorhynchus mykiss*) following exposure to 44 ng L^{-1} oestrone was reported by Routledge *et al.* (1998), who suggested that the threshold concentration for vitellogenin induction by oestrone was between 25 and 50 ng L^{-1}. Thorpe *et al.* (2001) reported a similar increase in plasma vitellogenin following exposure to similar concentrations of oestrone, suggesting a vitellogenin LOEC of 3.2 ng L^{-1} and EC_{50} of 70 ng L^{-1}.

17α-Ethinyloestradiol

In fathead minnows (*Pimephales promelas*) exposed to 10 ng L^{-1} 17α-ethinyloestradiol (nominal concentration), the degree of vitellogenin induction showed a significant, positive correlation with exposure duration (van Aerle *et al.*, 2002). Fenske *et al.* (2001) also reported a clear concentration-dependent response for 17α-ethinyloestradiol and vitellogenin induction, and a LOEC of <1.67 ng L^{-1}. In zebrafish (*Danio rerio*) exposed to 20 ng L^{-1}, plasma vitellogenin levels were more than 30,000 times higher than in control fish (Fenske *et al.*, 2001). Wenzel *et al.* (2001) also reported vitellogenin induction in male *D. rerio* exposed to 10 ng L^{-1} from hatching, and that levels of this protein remained high even following a 100 day depuration period in clean water. Plasma vitellogenin induction also occurred in rainbow trout (*Oncorhynchus mykiss*) following exposure to 17α-ethinyloestradiol at >0.1 ng L^{-1} (Purdom *et al.*, 1994). Similar results were reported by Sheahan *et al.* (1993), with vitellogenin induction occurring following a 28 week exposure to 1 ng L^{-1}; and Jobling *et al.* (1996) reported vitellogenin induction in *O. mykiss* following a 21 day exposure to 1.79 ng L^{-1}. Thorpe *et al.* (2001) have estimated vitellogenin LOEC and EC_{50} values of 1 ng L^{-1} 17α-ethinyloestradiol for *O. mykiss.* Similar results have been reported for marine species; for example, prolonged exposure to 6 ng L^{-1} 17α-ethinyloestradiol induced vitellogenin mRNA expression of male sand gobies (*Pomatoschistus minutus*) (Robinson *et al.*, 2003).

3.4.2 Reduced male sexual characteristics and altered behaviour

17β-Oestradiol

In their studies in fathead minnow (*Pimephales promelas*), Panter *et al.* (1998) reported that inhibition of testicular growth (i.e. reduction of gonadosomatic index) accompanied the increase in vitellogenin levels; however, this was only at comparatively high concentrations of 17β-oestradiol [>320 ng L^{-1} (Panter *et al.*, 1998)]. Exposure to (comparatively high concentrations of) 17β-oestradiol has also been reported to cause morphological changes in the secondary sexual characteristics of *P. promelas* (Miles-Richardson *et al.*, 1999). Male fish demonstrated reductions in size of breeding tubercles following exposure to 272 ng L^{-1}, but fatpads were only affected at much higher concentrations (27,240 ng L^{-1} 17β-oestradiol) (Miles-Richardson *et al.*, 1999). Although reductions in these secondary sexual characteristics lasted for several months, the relevance of their reduction to reproductive success has not been demonstrated (Miles-Richardson *et al.*, 1999). In contrast with other fish species tested, no significant changes in GSI were recorded in guppy (*Poecelia reticulata*) following exposure to up to 1000 ng L^{-1} 17β-oestradiol (Toft and Baatrup, 2001).

In the sand goby (*Pomatoschistus minutus*), "morphologically intermediate papilla syndrome" (MIPS) was induced following exposure to oestrogens, but the condition was not very "plastic" and almost certainly life-stage-specific, whereby chronic exposure at early life stages to 10 ng L^{-1} oestradiol led to the full development of MIPS in adult fish (Kirby *et al.*, 2003). The significance of MIPS on reproductive success has not been demonstrated (Kirby *et al.*, 2003).

Oestrone

Although oestrone exposure has been reported to affect gonad development (expressed as GSI) in fathead minnow (*P. promelas*), significant effects occurred only at the comparatively high concentration of 318 ng L^{-1} [and, surprisingly, not at 993 ng L^{-1} (Panter *et al.*, 1998)].

17α-Ethinyloestradiol

Jobling *et al.* (1996) reported inhibited testicular growth (GSI) in rainbow trout (*Oncorhynchus mykiss*) following a 21 day exposure to 1.79 ng L^{-1}; whilst exposure to >10 ng L^{-1} led to reduced testes somatic index in male zebrafish (*Danio rerio*) (Van den Belt *et al.*, 2001). Furthermore, it has also been suggested that pre-exposure to 17α-ethinyloestradiol disrupts sexual/mating behaviour of *D. rerio*, since spawning success of unexposed females when paired with pre-exposed males was reduced (Van den Belt *et al.*, 2001). In addition, mating behaviour in zebrafish ceased completely in mature males exposed to 10 ng L^{-1} (resulting in no spawning), and although mating behaviour and spawning returned following depuration for 100 days in clean water, fertilisation success was only 4% of control values (Wenzel *et al.*, 2001). In addition, exposure to 6 ng L^{-1} 17α-ethinyloestradiol affected maturation (including testis size, seminal vesicle size and nuptial colouration) of male sand gobies (*Pomatoschistus minutus*) (Robinson *et al.*, 2003).

3.4.3 Sex ratios

17β-Oestradiol
The ratio of males to females in Japanese medaka (*Oryzias latipes*), following continuous post-hatch exposure to 17β-oestradiol, was unaffected at 100 ng L^{-1} (7:11, male:female); however, a completely female population was produced at 1000 ng L^{-1} [0:19, male:female (Tabata *et al.*, 2001)]. Skewed sex ratios in *O. latipes* have also been reported by other authors (e.g. Nimrod and Benson, 1998; Metcalfe *et al.*, 2001). All fish exposed to 10 ng L^{-1} 17β-oestradiol from 5 day larval stage to 83 days developed as females, obviously providing no further reproductive output within the treated population (Nimrod and Benson, 1998). Similar results were reported by Metcalfe *et al.* (2001), although skewed sex ratio was only found in fish exposed to 100 ng L^{-1} 17β-oestradiol. Early life stages of zebrafish (*Danio rerio*) exposed to 100 ng L^{-1} 17β-oestradiol tended to develop as females, producing a significantly skewed population (Brion *et al.*, 2001).

Oestrone
Exposure to high concentrations of oestrone can significantly affect sex ratios in fish. For example, when exposed to >1000 ng L^{-1} oestrone from 1 day post-hatch until 15 mm in length (ca. 100 days post-hatch), almost all test Japanese medaka (*O. latipes*) developed into females, producing sex ratios that differed significantly from controls (Metcalfe *et al.*, 2001).

17α-Ethinyloestradiol
A 56 day post-hatch exposure to 4 ng L^{-1} 17α-ethinyloestradiol resulted in a significantly skewed sex ratio of 85:5 (female:male) (Länge *et al.*, 2001). After 172 day post-hatch exposure to >16 ng L^{-1}, no males were recorded at all (Länge *et al.*, 2001). Skewed sex ratios have also been reported in Japanese medaka (*O. latipes*), with 91% of fish developing as females following exposure to 100 ng L^{-1} 17α-ethinyloestradiol (Metcalfe *et al.*, 2001).

3.4.4 Intersex

17β-Oestradiol
Intersex has been recorded in Japanese medaka (*O. latipes*) exposed to 100 ng L^{-1} 17β-oestradiol (Tabata *et al.*, 2001). Other authors have also reported intersex in medaka; for example, Metcalfe *et al.* (2001) reported ovotestes in fish exposed to 10 ng L^{-1}. In the zebrafish (*D. rerio*) all early life stage fish exposed to 100 ng L^{-1} developed female-like reproductive ducts, with 75% showing this condition at lower concentrations, e.g. 25 ng L^{-1} (Brion *et al.*, 2001).

Oestrone
In their study with Japanese medaka (*O. latipes*), Metcalfe *et al.* (2001) reported severe intersex in fish exposed to >10 ng L^{-1} oestrone from 1 day post-hatch until 15 mm in length (ca. 100 days post-hatch). At 1000 ng L^{-1}, most fish developed into females; however, those fish that did develop into males exhibited well-developed ovotestes, a condition that was also apparent at lower exposure concentrations [10–100 ng L^{-1} (Metcalfe *et al.*, 2001)].

17α-Ethinyloestradiol
In fathead minnows (*Pimephales promelas*), 60% of males exposed to 10 ng L^{-1} 17α-ethinyloestradiol developed intersex (van Aerle *et al.*, 2002). However, although male *P. promelas* developed feminised ducts, no oocytes were found (van Aerle *et al.*, 2002). Also, a 56 day post-hatch exposure to 4 ng L^{-1} resulted in 11% of males having ovotestes (Länge *et al.*, 2001). Following a two month post-hatch exposure to 100 ng L^{-1} 17α-ethinyloestradiol, all male medaka (*Oryzias latipes*) were sex-reversed,

having developed ovaries (Scholz and Gutzeit, 2000). Metcalfe *et al.* (2001) also reported intersex in medaka, with ovotestes being present in males at all concentrations >10 ng L^{-1}. Based on their studies, Metcalfe *et al.* (2001) estimated an LOEC of between 10 and 100 ng L^{-1} for 17α-ethinyloestradiol effects on medaka. Similarly, Seki *et al.* (2002) reported the induction of ovotestis in *O. latipes* following exposure to 63.9 ng L^{-1} 17α-ethinyloestradiol.

3.4.5 Egg production/reproductive output effects

17β-Oestradiol
Exposure to a nominal concentration of 120 ng L^{-1} 17β-oestradiol inhibits egg production of female fathead minnows (*Pimephales promelas*), and this response is concentration-dependent (Kramer *et al.*, 1998). Similarly, when female medaka (*Oryzias latipes*) previously exposed to 17β-oestradiol [e.g. 1660 ng L^{-1} (Nimrod and Benson, 1998); 272 ng L^{-1} (Shioda and Wakabayashi, 2000)] were mated with untreated males, they produced significantly fewer eggs compared with untreated females. In zebrafish (*Danio rerio*), age of first egg laying was delayed following exposure to 100 ng L^{-1}, as well as the concentration-dependent inhibition of egg production (Brion *et al.*, 2001). Ohn *et al.* (2000) – cited in Van den Belt *et al.* (2001) – also reported reduced egg production in female zebrafish exposed from the embryo stage. In guppy (*Poecelia reticulata*), reproductive success was reduced due to 17β-oestradiol exposure, whereby matings between exposed males and virgin females produced 70% less offspring than in control fish (Toft and Baatrup, 2001).

17α-Ethinyloestradiol
Following exposure to 10 ng L^{-1} 17α-ethinyloestradiol, 60% of male fathead minnows (*P. promelas*) showed reduced sperm production (although likely effects of this on reproductive success are not clear) (van Aerle *et al.*, 2002). Given that reduced egg production in female fathead minnows also occurred at 10 ng L^{-1} (Schweinfurth *et al.*, 1996), however, it is likely that reproductive output of *P. promelas* is compromised following exposure to 10 ng L^{-1} 17α-ethinyloestradiol. Female medaka (*O. latipes*) showed significant reduction in ovary weight and reduction in egg production (i.e. 80% reduction) following exposure to 10 ng L^{-1} (Scholz and Gutzeit, 2000). Exposure to 17α-ethinyloestradiol has also been reported to affect reproductive success of *D. rerio* (Van den Belt *et al.*, 2001). A concentration-dependent reduction in number of females able to spawn was reported at nominal concentrations ≥ 10 ng L^{-1} (measured concentrations confirmed at 75% of nominal). Non-spawning females from the 10 and 25 ng L^{-1} exposures had significantly regressed ovaries, with reduced size and lack of mature oocytes compared with control fish. Similarly, male fish were affected at concentrations ≥ 10 ng L^{-1} (Van den Belt *et al.*, 2001). Fertilisation success was reduced to below 70% at 10 ng L^{-1}, and reduced to only 6% at 25 ng L^{-1} (Van den Belt *et al.*, 2001).

Wenzel *et al.* (2001) reported a complete life-cycle test examining the effects of 17α-ethinyloestradiol on zebrafish (*D. rerio*) through to the early life stage of the F_2 generation. A concentration-dependent impact on fertilisation was found at ≥ 1.1 ng L^{-1} (Wenzel *et al.*, 2001). Further to this, Segner *et al.* (2002), using the same concentrations as those used by Wenzel *et al.* (2001), identified that the life stage during sexual differentiation was particularly susceptible (including delayed and reduced spawning, and lowered fertilisation rate) to 17α-ethinyloestradiol exposure (≥ 3 ng L^{-1}).

Similar effects have been reported for marine species. For example, following exposure to 17α-ethinyloestradiol, fertile egg production of female sand gobies (*Pomatoschistus minutus*) was reduced by >90% due to reduced fecundity and failure to spawn (Robinson *et al.*, 2003).

3.4.6 Growth and potential population effects

17β-Oestradiol
In the Atlantic salmon (*Salmo salar*), exposure to 100 ng L^{-1} 17β-oestradiol also significantly affected growth and a plasma growth factor [plasma insulin-like growth factor I (IGF-I)], the effects being seasonally dependent (Arsenault *et al.*, 2004). Furthermore, exposure to 100 ng L^{-1} 17β-oestradiol disrupted the transformation from parr to smolt (smoltification – a physiological transformation to enable migration to saline waters) in *S. salar* (Arsenault *et al.*, 2004).

17α-Ethinyloestradiol
A general lack of population level effect studies has been identified and highlighted as a research gap in the literature (Taylor *et al.*, 1999). This lack may be due in part to the difficulty in designing and undertaking such studies. In an attempt to estimate population level impacts of oestrogen exposure, Grist *et al.* (2003) applied modelling techniques to a fish life-cycle study by Länge *et al.* (2001). Following a 56 day post-hatch exposure to 4 ng L^{-1} 17α-ethinyloestradiol, juvenile growth was reduced (Länge *et al.*, 2001). Grist *et al.* (2003) reanalysed the data of Länge *et al.* (2001) to estimate effects on population growth. Using a linear model, the critical concentration of 17α-ethinyloestradiol estimated to reduce the intrinsic growth rate to zero (i.e. no population growth) was 3.11 ng L^{-1} 17α-ethinyloestradiol (Grist *et al.*, 2003). Importantly, the reduction in population growth was due to reduced fertility and not to direct mortality due to 17α-ethinyloestradiol exposure (Grist *et al.*, 2003).

3.5 Laboratory studies on the feminising and reproductive effects of nonylphenol exposure

Laboratory studies have provided datasets, which show that exposure of fish to nonylphenol can induce a range of feminising effects seen in wild fish. The results of a recent survey of final effluents in England and Wales (see section 3.2) indicated that concentrations of nonylphenol at the majority of sites were at or below the thresholds for induction of vitellogenin and other somatic endpoints, once dilution is taken into account (Environment Agency, 2004b; Gross-Sorokin *et al.*, 2004).

3.5.1 Vitellogenin induction

Significant elevations of vitellogenin levels were demonstrated in 3-week laboratory exposures of rainbow trout (*Oncorhynchus mykiss*) to a nonylphenol (NP) concentration of 20 μg L^{-1} (Jobling et al., 1996). Thorpe *et al*. (2001) reported lowest observed effect concentrations (LOEC) of 6.1 – 6.4 μg L^{-1} for vitellogenin induction following two week exposures of the same species. In medaka (*Oryzias latipes*) exposed from fertilised eggs to 60d post-hatch, Seki et al. (2003) reported a LOEC of 11.4 μg L^{-1}. Intermittent exposures over a longer time period (ten days per month for four months) resulted in significant vitellogenin induction in adult male rainbow trout at 1 and 10 μg L^{-1} (Schwaiger *et al*., 2002). In the offspring of exposed fish, only the females revealed elevated vitellogenin levels (Schwaiger *et al*., 2002).

3.5.2 Intersex, sex ratio and reproductive effects

Yokota *et al.* (2001) studied the chronic effects of NP exposure on the reproduction of medaka over two generations. Continuous exposures of the parental generation (F_0) from 24 hrs post fertilisation to NP concentrations of 17.7 and 51.5 μg L^{-1} resulted in the development of the intersex condition in four of 20 fish and eight of 20 fish, respectively. Mean fertility in the 17.7μg L^{-1} treatment group was decreased to 76% of that in the controls, although due to the small sample size the statistical significance could not be determined. In the F_1 generation, the sex ratio of males to females was 1:2 in the 17.7 μg L^{-1} treatment as opposed to the approximate 1:1 ratio in the control or lower concentration treatments. In addition, two of 20 and five of 20 fish were found to be intersex in the 8.2 and 17.7 μg L^{-1} exposures, respectively (Yokota *et al.*, 2002). These data are consistent with a LOEC of 11.6 μg L^{-1} reported by Seki *et al.* (2003) for the induction of intersex in medaka exposed from fertilised eggs to 60d post-hatch.

To simulate NP exposures that fish are likely to encounter in the environment more realistically, Schwaiger *et al.* (2002) exposed adult rainbow trout intermittently for a four month period. Exposure to 10 μg L^{-1} NP resulted in a reduction in hatching success of the offspring due to higher mortalities occurring early in development. The sex ratio of the offspring was not found to be different from the controls, however, in single cases intersex occurred in both males and females. Analysis of sex steroid levels revealed a 2-fold increase of oestradiol in male and 13-fold elevation of testosterone in female progeny. The authors concluded that NP exposures lead to hormonal imbalances in both male and female offspring, but it remains unclear whether the low percentage of intersex is a consequence or just a normal feature within the frame of the physiological variability (Schwaiger *et al.*, 2002).

3.6 Response of fish following exposure to combinations of oestrogens

Although data from single chemical exposure–response testing are predominant in the literature, effluent fractionation assessments have confirmed that effluents are complex mixtures of contaminants, including oestrogenic substances. Exposure–response experiments performed by Thorpe *et al.* (2001) have confirmed the additive nature of dissimilar oestrogens (nonylphenol and oestradiol) in mediating an oestrogenic biological response. 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.

Further studies on three steroidal oestrogens (17β-oestradiol, oestrone and ethinyloestradiol) in rainbow trout (*Oncorhynchus mykiss)* similarly demonstrated relative potencies of these steroids for vitellogenin induction (ethinyloestradiol was approximately 11 to 27 times more potent than 17β-oestradiol, while 17β-oestradiol was 2.3 to 3.2 times more potent than oestrone). In addition, concentration additivity was demonstrated for ethinyloestradiol and 17β-oestradiol at environmentally relevant concentrations (Thorpe *et al.*, 2003).

These studies with mixtures of steroidal oestrogens and/or industrial chemicals demonstrated oestrogenic responses in fish even though concentrations of the individual substances in the mixture were lower than previously assessed “No Observable Effect Concentration” (NOEC) values for the individual substances. This indicates that assessments of environmental hazards and risks for single substances may not be adequately protective when other oestrogenic substances are also present and act via the same toxic mechanism(s).

3.7 Fate of natural oestrogens in sewage works and the environment

Natural steroid oestrogens are excreted in the urine of humans, but they are primarily in conjugated forms (i.e. glucuronides and sulphates), which are considered to be biologically inactive. However, studies of the fate and behaviour of steroid oestrogens during sewage treatment have provided evidence that these biologically inactive steroid conjugates can be deconjugated by micro-organisms to the free, biologically active forms. In aerobic batch experiments, two glucuronide conjugates of 17β-oestradiol (which were added to activated sludge) were shown to be readily cleaved, forming free steroids (Ternes *et al.*, 1999, cited in Environment Agency, 2002b). In another study, exposure of male fathead minnow (*Pimephales promelas*) to the glucuronide conjugate of 17β-oestradiol did not result in an oestrogenic response (as measured by induction of vitellogenin). However, an oestrogenic response was observed in the fish when they were exposed to effluent generated in laboratory simulations of sewage treatment processes, to which the conjugated oestradiol had been added (Panter *et al.*, 1999a). It is also possible that oestrogen conjugates may cleave to some extent prior to entering STWs because of bacterial activity in sewers (Baronti *et al.*, 2000; D'Ascenzo *et al.*, 2003).

Several studies have examined the rates of removal of steroid oestrogens from effluents entering domestic STWs. These usually involve the measurement of oestrogen concentrations in the sewage influent and effluent, from which percentage removal rates are calculated. Reported removal abilities of STWs have been found typically to be in the ranges 64–99% for 17β-oestradiol, 61–83% for oestrone, and 64–85% for 17α-ethinyloestradiol (Environment Agency, 2002b, and references therein). Degradation half-lives of 0.2–8.7 and 0.1–10.9 days for 17β-oestradiol and oestrone, respectively, were found in aerobic degradation studies using surface waters from three UK rivers. However, 17α-ethinyloestradiol was more persistent in river water, with a half-life approximately 10 times that of 17β-oestradiol (Williams *et al.*, 2001). In the same study, it was found that steroid oestrogens will bind preferentially to suspended and bed sediment, but in modelled scenarios, there would be insufficient particulate matter to adsorb all steroid present. Taken together, these data indicate that, although degradable, steroid oestrogens would persist in the water column for several days, leading to exposures to aquatic life over distances up to 10 km downstream (variations depending on ambient flow and weather conditions).

Vertebrate steroidal oestrogens and androgens are also present in estuarine waters (Thomas *et al.*, 2001, 2004). Most oestrogenic and androgenic activities in estuaries are in the sediment phase (adsorbed to sediment particles), although pore-waters from sediments in the Tyne estuary show oestrogenic activity (Thomas *et al.*, 2001, 2004). However, estuaries are often highly industrialised areas, so estuarine waters also contain oestrogenic industrial discharges as well as domestic effluents. Nevertheless, data suggest that 17β-oestradiol derived from domestic STW effluent plays a significant role (up to 90%) in the *in vitro* oestrogenic activity of concentrates from estuarine surface waters (Thomas *et al.*, 2001).

3.8 Conclusions

Studies from the UK, Europe and the United States have all examined the major constituents present in domestic STW effluents. These studies have clearly identified the natural (oestrone and 17β-oestradiol) and synthetic (17α-ethinyloestradiol) steroids as the main oestrogenically active substances present. Although it is accepted that feminisation of fish can also partially be attributable to substances with anti-androgenic properties and that weaker environmental oestrogens may also contribute to the overall effects observed, the role of oestrogens (and, therefore, oestrone, 17β-oestradiol and 17α-ethinyloestradiol) is implicit. Laboratory studies clearly confirm a causal link between exposure to these compounds and endocrine disruption. Induction of plasma vitellogenin, disruption of mating behaviour, reduced number of eggs, reduced fertilisation effects, skewed sex ratios and morphological abnormalities have all been reported following laboratory exposure of fish to these steroids.

Although some effects appear to be reversible following depuration in clean water (e.g. plasma vitellogenin concentrations), others are not readily reversible (e.g. morphological abnormalities). Pulsed exposure (perhaps a more environmentally realistic exposure regime) appears to be as equally disruptive as continuous exposure.

It is recognised that steroids are typically excreted (and, therefore, enter STW) in conjugated forms that are biologically inactive (i.e. are not in a form that can affect biota). However, experiments have proven that sewage treatment processes reactivate these compounds, so that effluents are again oestrogenically active when they exit the STW and enter rivers.

Other oestrogenically active substances have also been detected in domestic effluents. These include alkylphenols (APs) and alkylphenol polyethoxylates (APEs). In most cases, these exist at concentrations at or below the known threshold for induction of vitellogenin and other somatic endpoints. However, from studies evaluating additivity between oestrogenic substances, the demonstration that chemically dissimilar as well as similar substances can act in an additive manner on oestrogenic endpoints indicates that the oestrogenic hazard should consider the total oestrogenic burden within an effluent.

From the weight of evidence, it can be concluded that:

(a) oestrogenically active STW effluents cause feminisation of fish;
(b) the main oestrogenically active compounds present in most domestic STW effluents include the steroid oestrogens oestrone, 17β-oestradiol and 17α-ethinyloestradiol;
(c) these compounds have been measured reliably at or above concentrations that can cause a range of effects with the potential to reduce the reproductive outputs of fish;
(d) these steroids can act with each other and other weaker oestrogens in an additive manner to induce some oestrogenic effects;
(e) STW processes actively increase oestrogen activity in their effluents through the degradation of conjugated forms; and
(f) when released into river systems, these substances will persist within river reaches sufficiently to lead to exposure to aquatic wildlife.

The amount of experimental evidence available on causation of sexual disruption in wild fish, when taken with evidence from field studies, suggests strongly that natural steroids from STWs contribute significantly to oestrogenic endocrine disruption in fish.

4. UK spatial survey and risk assessment

Following an evaluation of the studies on the effects of effluents on freshwater fish, the Agency committed itself to evaluating the full geographical extent of endocrine-disrupting effects in English and Welsh fisheries, and its relationship to the quality and quantity of sewage effluent discharged into the receiving waters. The initial UK research programmes between 1994 and 2001 had focused only on a limited number of sites, some of which were considered to be so-called "hot-spots" where the concentration of treated sewage effluents was high or where the discharge of particular industrial oestrogenic chemicals was extensive. It was important, therefore, to broaden the spatial extent of the assessments so that a balanced view of prevalence of endocrine-disrupting effects in the fish populations could be obtained.

A further component within the assessment was to develop and use a model to predict the likely degree and spatial extent of steroid oestrogen contamination of rivers throughout the UK, in order to indicate fisheries at risk of developing oestrogenic effects. The model incorporated information on the loads of steroids entering STWs, removal rates within the works and the dilution on entry into the aquatic environment. Each river stretch below a particular STW was examined and placed into a high-, medium- or low-risk category based on the concentrations of steroid oestrogens predicted to be present and their known effects measured in various species of fish in laboratory studies.

The model identified 142 high-risk sites, 192 medium-risk sites and 132 low-risk sites from a database of STWs with population equivalents greater than 10,000. Wild roach (*Rutilus rutilus*) were sampled from selected sites within each of 21 high-, 30 medium- and six low-risk categories (57 total) and examined in order to identify any endocrine-disrupting effects. Overall 1615 roach were sampled, 467 from predicted high-risk sites, 782 from predicted medium-risk sites and 280 from predicted low-risk sites (Taylor *et al*. 2004; Environment Agency, 2004c). Data were gathered on the length, weight and age of all fish captured. Analyses for plasma vitellogenin concentrations and intersex (incidence and severity) were carried out and correlated with the predicted high-, medium- and low-risk predictions from the model.

The severity of feminisation was quantified using a numerical index describing the incidence of ovotestis (developing eggs within the testes), the severity (number of eggs within the testes) and feminised reproductive ducts.

Two-hundred and eighteen intersex roach (a third of all "males") were intersex, as defined by the simultaneous presence of both testicular and ovarian tissue within the gonads. Intersex roach were found at 44 (86%) of the 51 sites within all 5 Regions sampled. Of the intersex fish, 117 had abnormal reproductive ducts (most of these had a single sperm duct together with an oviduct in one or both of the gonads). In 23 fish one or both gonads contained an additional sperm duct, as well as an oviduct. Both abnormal reproductive ducts and ovotestis were seen in 39 fish with a further 40 fish with just eggs in testis. The prevalence of intersex varied between sites (from 100% to 0%) and there was a large variation in the severity of the condition at these sites.

This spatial survey confirmed a number of findings from previous investigations:

- Prevalence of duct disruption did not vary with age of the fish.

- Both the proportion of “male” fish with ovotestis and the severity of the condition increased with age.
- Prevalence and severity of intersex was higher where the concentration of effluent in the river was higher.

Plasma vitellogenin concentrations (in females) varied depending on their sexual status at the time of sampling (vitellogenin concentration is known to vary seasonally by 3-4 orders of magnitude). There was a strong suggestion that this also occurred in the “male” fish, with lower concentrations of vitellogenin immediately following spawning compared with those sampled in the autumn. Inter-site differences in vitellogenin concentrations in intersex fish sampled concurrently were correlated with the concentrations of effluent to which the fish were exposed. When taken together, the results of this study would strongly support the hypothesis that the elevated concentrations of vitellogenin seen in wild intersex fish are as a result of exposure to environmental oestrogens, and some of this induction is accounted for by elevated concentrations of endogenous oestradiol, that occurs as a result of disruption of steroidogenesis and ovarian tissue development within the testis.

Further analysis of the fish data using pooled datasets for fish taken from high, medium and low risk category sites are summarised here in graphical form. The data demonstrate clearly that both the incidence (Figure 1) and the severity (Figure 2) of intersex in wild roach collected from 57 different sites were strongly correlated with their predicted exposure to steroid oestrogens derived by use of the hydrological model. The finding of vitellogenin in the blood of these fish confirms that they had recently experienced an oestrogenic stimulus.

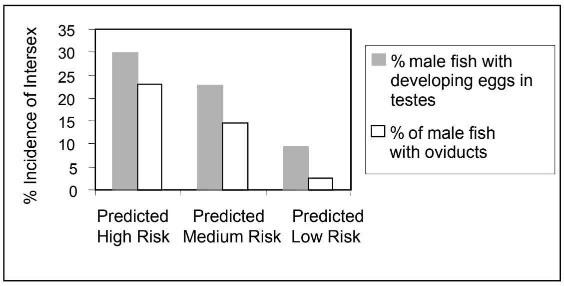

Figure 1. Incidence of the intersex condition in male roach taken from 57 sites in the national spatial fisheries survey (2002/2003) predicted to be at high, medium and low risk of oestrogenic effects.

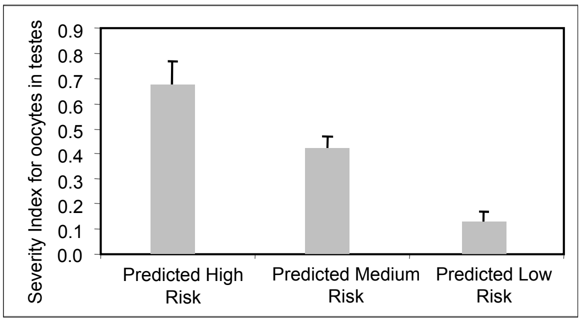

Figure 2. Severity of the intersex condition in male roach taken from 57 sites in the national spatial fisheries survey (2002/2003) predicted to be at high, medium and low risk of oestrogenic effects.

There was a clear relationship between the age of the intersex fish and the incidence and degree of feminisation found. Within each risk category, the most severely feminised fish were found in the oldest year classes. The relationship between age and the incidence and severity of intersex for predicted high-risk (r^2 = 0.9412), medium-risk (r^2 = 0.9336) and low-risk (r^2 = 0.7624) categories could be described by the three mathematical equations shown in Figure 3. For any given fish in any of these risk categories, providing the age of the fish is known, the intersex index could be predicted.

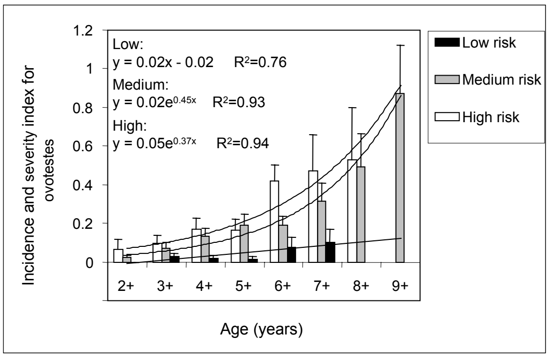

Figure 3. The relationship between the age of the intersex fish and the combined incidence and severity of feminisation found, within predicted risk categories

When taken together, these data strongly suggest that intersexuality in wild fish is caused by exposure to sewage discharges and that the condition is a progressive condition that further develops as the fish grow older.

Overall, the survey confirmed that over one third of the male population of roach sampled from rivers across England were feminised, even where the impact of sewage discharges was lower than the previously sampled so-called “hot spots”. These effects were positively correlated with the predicted steroid oestrogen load in the receiving water below the STW discharge. This clearly establishes that sexual disruption in roach is a widespread phenomenon in the ambient environment in English and Welsh rivers and is strongly associated with oestrogenic sewage effluent discharges.

5. Discussion

This review brings together relevant scientific information on biological effects observed in fish through eco-epidemiological studies, analysis of contaminant inputs from dominant point sources into receiving waters, laboratory evaluations of suspected causative chemicals, and their effects on fish development and reproduction. In addition, it draws together, through risk assessment, the relationships between inputs and responses in wild fish. This is a weight-of-evidence approach, since there are many components to consider in demonstrating the linkages between effects seen in fish and what is causing them.

What are we defining as "endocrine disruption", "feminisation" and "oestrogenic" versus "anti-androgenic" effects?

Endocrine disruption has been taken as a "collective noun" for a range of mechanisms that are hypothesised to lead to effects mediated through a disruption of the endocrine system, following chemical exposure. The mechanisms of action might be through: receptor binding (leading to agonist or antagonistic consequences); interference with hormone synthesis (enzymatic processes); hormone balance, transport and binding; receptor synthesis and turnover; or any combination of these. Identifying a substance as an "oestrogen" implies exclusivity to a mode of action of that substance, and is simplistic, whereas the reality is likely to be much more complex.

It is known that *oestrogenic* substances are also capable of binding to other receptors (Routledge and Sumpter, 1996) or inducing other endocrine-disrupting responses, although often at much reduced potencies (viz. 17α-ethinyloestradiol acting as an anti-androgen) (Katsiadaki *et al.*, 2002). Without the specific knowledge of every potential site of action of a chemical in an organism, as might be possible through studying the whole genome of an organism, evidence for exposure–effect comes from *functional* assays, which by their nature often focus on a single biological response. Therefore, in developing conclusions, the focus is on chemicals that can lead to the part-feminisation of male fish in rivers, mediated by chemicals for which there is a plausible linkage to mechanisms of disruption in fish. Although substances acting through anti-androgenic mechanisms may be a contributory factor, the current evidence base is strongest for chemicals that act *oestrogenically.*

Are the effects seen in fish inducible through exposure to oestrogens?

There is a substantial body of evidence from the laboratory that chemicals acting as oestrogens do induce a range of biological effects, which have been reported in fish in English rivers (vitellogenin, oviduct malformations, ovotestis). The substances are shown to be oestrogens because they are capable of inducing oestrogen-specific responses *in vivo* (for example, vitellogenin), and binding to oestrogen response elements in screening assays *in vitro.* In addition to effects observed in the field, laboratory studies have also demonstrated a number of further oestrogenic effects, including altered behaviour, reduced growth and lessened reproductive output.

Are the effects seen in fish inducible through exposure to oestrogenically active effluents?

Direct exposure to effluents has induced a number of "feminising" effects, including vitellogenin and duct disruption. Other relevant endpoints have not been demonstrated yet; this remains a key science investigation area. In particular, the genesis of female germ cells in male testis tissue has not been induced experimentally following effluent exposure. This might be because:

- the effluents studied do not contain the substances that induce germ cell disruption;
- the effluents studied do not contain sufficient amounts of the required substances to induce germ cell disruption; and/or
- the experimental fish have not been exposed for long enough or at the appropriate life stage to induce germ cell disruption.

In essence, it is more straightforward to continue with scientific investigations to mimic, as closely as possible, the conditions usually experienced by wild fish exposed to effluents, in order to examine this issue thoroughly. To date, roach (*Rutilus rutilus*), the wild sentinel fish species, has not been maintained beyond two years post-hatch in controlled effluent exposure conditions. One current hypothesis is that the development of the eggs in testis condition is a result of longer-term exposure. This premise is supported by analysis of roach datasets gathered between 1995 and 2000 (n = 229) for three rivers. Germ cell alteration originates in the third year of life, following the cycle of gametogenesis and gonad maturation and leading up to the fishes' first spawning, and its severity then increases with increasing age of the fish (Environment Agency, 2002a). Whereas sperm/oviduct formation is known to occur in the first year of life, and was disrupted experimentally in exposures up to one year, so far it has not been possible to maintain natural environmental conditions over long-term experimental studies. This might account for the failure to induce the ovotestis condition experimentally in roach with effluents.

Are there oestrogenic substances in effluents? And are they at sufficient concentrations that are likely to induce the effects in wild fish?

Toxicity investigation and evaluation (TIE) studies on many effluents indicate that steroidal oestrogens are the most potent known oestrogens to be found in effluents. Exposure–response studies with many fish species have confirmed a range of potencies and relevant feminising effects that they can mediate. Moreover, those studies have indicated "Predicted No-Effect Concentrations" (PNECs) for steroid oestrogens of 0.1 ng L^{-1} (17α-ethinyloestradiol), 1 ng L^{-1} (17β-oestradiol) and 3 ng L^{-1} (oestrone) (Environment Agency, 2002b). Steroid concentrations measured in studies of STW effluents, in studies in experimental systems using effluent dilutions, and in limited studies of receiving waters have demonstrated that such concentrations exceed these PNECs. In addition, modelling studies have predicted concentrations higher than these PNECs for certain river stretches below STWs. Furthermore, these steroids have been shown to be additive when mixed together in an environmentally relevant mixture, and also with other oestrogenic (but structurally dissimilar) substances. This additivity is reflected in a combined PNEC for steroid oestrogens of 1 ng L^{-1}, which takes into account the relative potency of each steroid and their additive effects (Environment Agency, 2002b).

What is the correlation between the effects observed, and both predicted and measured exposure to effluent, in the environment?

Even as far back as 1981, when the occurrence of intersex fish was first linked to effluent quality, the evidence for effects drawn from the eco-epidemiological studies was evaluated against the proportion, and oestrogenic (or feminising) quality, of effluents in receiving waters.

Evidence gathered from the eco-epidemiological studies indicated that fish downstream of effluent point sources were more likely to be intersex, and at a greater severity, than those fish upstream or in unimpacted waters. Both incidence and severity were positively correlated to the proportion of effluent to which those fish were exposed. Moreover, the data used were for fish of mixed age, and remarkably, given that intersex severity can differ across age groups, statistically significant correlations were still found.

Other widespread surveys have also revealed the prevalence of the intersex condition in roach (*Rutilus rutilus*) (and in less extensive surveys, the gudgeon, *Gobio gobio*) in English fisheries. By apportioning fishery sites to categories of high, medium and low exposure to steroids arising from effluents, the pooled datasets on severity and incidence of intersex indicate a strong positive correlation between incidence, severity and the predicted exposure to effluents.

The influence of age on the manifestation of the intersex condition remains an interesting phenomenon. Evidence presented in this review indicates that severity and incidence of intersex increase with age across all predicted exposures (high, medium and low), suggesting that this is a progressive condition. It also indicates that the greatest response occurred in sites predicted to be high-exposure sites, followed by medium-, and then low-exposure sites. These pooled datasets have clearly shown a strong positive correlation to exposure to effluents.

Does the intersex condition in male fish affect reproductive health?

Induced spawning of wild male roach, shown (retrospectively) to be moderate to severely intersex, demonstrated that these fish were less able to produce and release viable gametes. The viability of subsequent *in vitro* egg fertilisations was also significantly reduced. Although it has not been possible to follow a natural spawning cycle in roach, similar experiments with other fish species indicate that other endpoints might also be compromised, particularly final maturation of gametes and spawning behaviour. Whilst the results at face value indicate that the fertility of intersex fish is reduced over that of normal males, the *in vitro* reproduction experiments may have indicated a more positive reproductive outcome than is seen in the wild.

What are the main uncertainties?

The crux of the argument within this review is the hypothesis of “feminisation” of male fish. Without markers for the genetic sex, it cannot be proven unequivocally. However, if it can be assumed that sex ratios are broadly 50:50 in proportion, at least in the young fish within the population, then the field data indicate that the proportion of intersex males was inversely proportional to the number of normal males. Moreover, the datasets from Rodgers-Gray *et al.* (2001) indicate that females differentiated first in all treatments (with clear oviducts) and that undifferentiated fish (that subsequently suffered duct disruption) were likely to be males. The application of genomic and molecular tools is now under way to elucidate this information.

Effluents are known to be complex mixtures of organic and inorganic substances. Identification of major hazardous contaminants in effluents, and ascribing a significance to each (or each group of) contaminant(s) is challenging, particularly when the potency of the other coexisting substances might be low. Steroid oestrogens are the most potent oestrogenic substances identified to date in sewage effluent, but whether they act solely or in combination with other substances to induce the observed effects is not known, but that potential exists when acting by the same mechanism. It is likely that understanding of such combination effects will remain elusive, until effluents are tested through further TIE studies with a wider range of biological effect measures, particularly *in vivo* assays.

Mechanisms of action are now being pursued with the advent of genomic tools and technologies. It is important to consider that substances might act by different mechanisms and that the science of endocrine disruption, which has “loosely” attributed an activity to a substance, will need to revisit this nomenclature as specific gene responses are discovered. In the specific issue of intersex fish and causal substances, oestrogens in *in vivo* laboratory assessments have been shown to induce these effects. Further work needs to be performed assessing anti-androgenic mechanisms of action. In any event, with both activities prevalent in sewage effluent, untangling the relative importance of these two mechanisms of action on similar endpoints when faced with complex mixtures may prove intractable. However, the discovery of anti-androgenic activity does not undermine the weight of evidence for the hazards and risks arising from exposures to oestrogens – indeed, it adds to the concern that effluents contain substances with wider biological activity, which is directly linked to the effects seen in wild fish.

6. Conclusions

Based on the weight of scientific evidence presented, the following conclusions can be drawn:

Fish in English rivers have been and are being exposed to oestrogenic substances discharged to those rivers in sewage effluents.

Exposure to oestrogenic effluents can lead to oestrogenic responses in male fish, including production of vitellogenin and disruption of formation of sperm ducts. Ovotestis has not been induced experimentally in effluents. The effects on testis tissue “development” observed in wild and experimental situations would appear to be permanent and progressive.

There is a prevailing incidence of ovotestis and duct disruption in wild fish populations across more than 50 riverine sites in England. At some sites, the levels of effect within the “male” fish sampled indicate that there may be reproductive consequences in local roach populations.

The incidence and severity of these responses in wild fish have been positively correlated to the proportion of effluent within the river system. Further, the recent survey data for incidence and severity of intersex, aligned to predicted high, medium and low exposure to oestrogenic substances from effluents, show positive, statistically significant, relationships to the levels of effect observed in the fish.

The consequence of the intersex condition is that moderate to severely affected male fish have significantly poorer reproductive capacity. Whereas minor levels of severity do not compromise reproductive health, the apparent progressive nature of the effects within males indicates that individual male fish will become affected later in life.

Laboratory studies have confirmed the potency and effects of exposure of fish to steroidal oestrogens. These studies have identified a wide range of biological effects, from induction of vitellogenin to population-level effects on reproduction. When exposed in combination, these substances act together both with other steroidal substances and with other oestrogenic chemicals, indicating that the overall oestrogenic “burden” of a complex effluent should be measured (by bioassay), and not just single chemicals (by direct chemistry).

Measured concentrations of steroidal oestrogens in effluents are at or higher than predicted no-effect concentrations determined from laboratory ecotoxicological studies. Both modelled scenarios and the limited ambient monitoring for steroids indicate that sufficient steroid concentrations exist at some sites, leading to an oestrogenic response.

The overall conclusion is that there is sufficient weight of evidence to show that sewage effluent discharges are leading to the feminisation of male fish in English rivers. The effect on reproductive health has been shown in moderate to severe levels of effects, and the progressive nature of the condition indicates that males’ functionality will decline as they get older. Oestrogenic activity of effluent is playing a significant role in causing some of those effects, although it is possible that other substances within the complex nature of effluents may also lead to general health, and specifically reproductive health, outcomes. The implication of these effects is potential impact on the sustainability of some fish populations. However, the effects appear to be permanent, progressive and can be concluded as *harm* to individual male fish.

7. References

Allen Y, Scott AP, Matthiessen P, Haworth S, Thain JE, Feist S (1999) Survey of estrogenic activity in United Kingdom estuarine and coastal waters and its effects on gonadal development of the flounder Platichthys flesus. Environmental Toxicology and Chemistry 18: 1791-1800

Ankley G, Mihiach E, Stahl R, Tillett D, Colburn T, McMaster S, Miller R, Bantle J, Campbell PM, Dickerson R, Fry M, Giesy J, Gray LE, Guiney P, Hutchinson T, Kennedy S, Kramer V, LeBlanc G, Mayes M, Nimrod A, Patino R, Peterson R, Purdy R, Ringer R, Thomas P, van der Kraak G, Zacharewski T (1998) Overview of a workshop on screening methods for detecting potential (anti-) oestrogenic/androgenic chemicals in wildlife. Environmental Toxicology and Chemistry 17: 68–87.

Arsenault JTM, Fairchild WL, MacLatchy DL, Burtidge L, Haya K, Brown SB (2004) Effects of water-borne 4-nonylphenol and 17 beta-estradiol exposures during parr–smolt transformation on growth and plasma IGF-I of Atlantic salmon (*Salmo salar* L.). Aquatic Toxicology 66: 255–265.

Baronti C, Curini R, D'Ascenzo G, Di Corcia A, Gentili A, Samperi R (2000) Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. Environmental Science and Technology 34: 5059–5066.

Brion F, Triffault G, Palazzi X, Garric J, Laillet B, Porcher J, Tybaud E, Tyler CR, Flammarion P (2001) Biological effects of exposure of various life stages of zebrafish to environmental concentrations of 17β-estradiol. Platform presentation at the 11th Annual Meeting of SETAC Europe, 6–10 May 2001, Madrid, Spain.

CEH (2002) Community Programme of Research on Environmental Hormones and Endocrine Disrupters (COMPREHEND). Final Report (January 2002). Centre for Ecology and Hydrology.

D'Ascenzo G, Di Corcia A, Gentili A, Mancini R, Mastropaqua R, Nazzari M, Samperi R (2003) Fate of natural estrogens conjugates in municipal sewage transport and treatment facilities. Science of the Total Environment 32: 199–209.

Defra (2003) Evaluation of the anti-androgenic activity of selected pesticides. Defra project code A1150.

deFur PL, Crane M, Tattersfield LJ, Ingersoll CG, Stahl RG, Matthiessen P, LeBlanc G (1999) In: Workshop on Endocrine Disruption in Invertebrates: Endocrinology, Testing, and Assessment (EDIETA), Eds. deFur PL, Crane M, Ingersoll C, Tattersfield LJ. SETAC Press, pp. 1–6.

Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M (1998) Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and *in vitro* biological screening. Environmental Science and Technology 34: 1548–1558.

Environment Agency (2002a) The identification of oestrogenic effects in wild fish – Phase II. R&D Technical Report W2–014/TR.

Environment Agency (2002b) Proposed Predicted-No-Effect-Concentrations (PNECs) for natural and synthetic steroid oestrogens in surface waters. R&D Technical Report P2-T04/1.

Environment Agency (2004a, in press). Endocrine Modulating Effects of Wastewater Treatment Effluents. Science Report P2-214/TR

Environment Agency (2004b, in press) Assessment of the (anti-)oestrogenic and (anti-) androgenic activity of sewage treatment works final effluents. R&D Technical Report P6-021/TR

Environment Agency (2004c, in press) Spatial survey of the extent of sexual disruption in wild roach in English rivers. R&D Technical Report P6-018/TR

European Commission (1999) Community Strategy for Endocrine Disrupters – a range of substances suspected of interfering with the hormone systems of humans and wildlife. Commission Communications Com 706.

Fenske M, van Aerle R, Brack S, Tyler CR, Segner H (2001) Development and validation of a homologous zebrafish (Danio rerio Hamilton-Buchanan) vitellogenin enzyme-linked immunosorbent assay (ELISA) and its application for studies on estrogenic chemicals. Comparative Biochemistry and Physiology C – Toxicology & Pharmacology 129: 217-232

Grist EPM, Wells NC, Whitehouse P, Brighty G, Crane M (2003) Estimating the effects of 17 alpha-ethinylestradiol on populations of the fathead minnow *Pimephales promelas*: Are conventional toxicological endpoints adequate? Environmental Science and Technology 37: 1609–1616.

Gross-Sorokin M, Williams RJ, Tyler CR, Thorpe K, Johnson I, Brighty G (2004) Risk assessment of the impact pf steroid oestrogens discharged from selected sewage treatment wrks. Poster presentation at the 14th Annual Meeting of SETAC Europe, 18-22 April 2004, Prague, Czech Republic

Harries JE, Sheahan DA, Jobling S, Matthiessen P, Neall P, Routledge EJ, Ryecroft R, Sumpter JP, Tylor T (1996) A survey of estrogenic activity in United Kingdom inland waters. Environmental Toxicology and Chemistry 15: 1993–2002.

Harshbarger JC, Coffey MJ, Young MY (2000) Intersexes in Mississippi River shovelnose sturgeon sampled below Saint Louis, Missouri, USA. Marine Environmental Research 50: 247–250.

Hashimoto S, Bessho H, Hara A, Nakamura M, Iguchi T, Fujita K (2000) Elevated serum vitellogenin levels and gonadal abnormalities in wild male flounder

(*Pleuronectes yokohamae*) from Tokyo Bay, Japan. Marine Environmental Research 49: 37–53.

Herman RL, Kincaid HL (1988) Pathological effects of orally-administered estradiol to rainbow trout. Aquaculture 72: 165–172.

Herman RL, Kincaid HL (1991) Effects of orally-administered steroids on lake trout and Atlantic salmon. Progressive Fish-Culturist 53: 157–161.

IPCS (2002) Global Assessment of the State-of-the-Science of Endocrine Disruptors. Eds. Damstra T, Barlow S, Bergman A, Kavlock R, van der Kraak G. WHO/IPCS/EDC/02.2 <http://ehp.niehs.nih.gov/who/>.

Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP (1996) Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. Environmental Toxicology and Chemistry 15: 194-202.

Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP (1998) Widespread sexual disruption in wild fish. Environmental Science and Technology 32: 2498–2506.

Jobling S, Coey S, Whitmore JG, Kime DE, van Look KJW, McAllister BG, Beresford N, Henshaw AC, Brighty G, Tyler CR, Sumpter JP (2002) Wild intersex roach (*Rutilus rutilus*) have reduced fertility. Biology of Reproduction 67: 515–524.

Katsiadaki I, Scott AP, Hurst, MR, Matthiessen P, Mayer I (2002) Detection of environmental androgens: A novel method based on enzyme-linked immunosorbent assay of spiggin, the stickleback (*Gasterosteus aculeatus*) glue protein. Environmental Toxicology and Chemistry 21: 1946–1954.

Kirby MF, Bignell J, Brown E, Craft JA, Davies I, Dyer RA, Feist SW, Jones G, Matthiessen P, Megginson C, Robertson FE, Robinson C (2003) The presence of morphologically intermediate papilla syndrome in United Kingdom populations of sand goby (*Pomatoschistus* spp): Endocrine disruption? Environmental Toxicology and Chemistry 22: 239–251.

Kirby MF, Allen YT, Dyer RA, Feist SW, Katsiadaki I, Matthiessen P, Scott AP, Smith A, Stentiford GD, Thain JE, Thomas KV, Tolhurst L, Waldock MJ (2004) Surveys of plasma vitellogenin and intersex in male flounder (*Platichthys flesus*) as measures of endocrine disruption by estrogenic contamination in United Kingdom estuaries: Temporal trends, 1996 to 2001. Environmental Toxicology and Chemistry 23: 748–758.

Kramer VJ, Miles-Richardson S, Pieron, SL, Giesy JP (1998) Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (*Pimephales promelas*) exposed to waterborne 17β-estradiol. Aquatic Toxicology 40: 335–360.

Länge R, Hutchinson TH, Croudace CP, Siegmund F, Schweinfurth H, Hampe P, Panter GH, Sumpter JP (2001) Effects of the synthetic estrogen 17 alpha-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). Environmental Toxicology and Chemistry 20: 1216–1227.

Liney KE, Jobling S, Simpson P, Tyler CR (2004) Investigating windows of sensitivity of exposure to treated sewage effluent during sexual development in roach (*Rutilus rutilus*). Poster presentation at the CREDO Cluster Workshop on the Ecological Relevance of Chemically-Induced Endocrine Disruption in Wildlife, University of Exeter, United Kingdom, July 5-7, 2004.

Lye CM, Frid CLJ, Gill ME, McCormick D (1997) Abnormalities in the reproductive health of flounder *Platichthys flesus* exposed to effluent from a sewage treatment works. Marine Pollution Bulletin 34: 34–41.

Matthiessen P, Gibbs PE (1998) Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. Environmental Toxicology and Chemistry 17: 37-43

Matthiessen P, Allen Y, Bamber S, Craft J, Hurst M, Hutchinson T, Feist S, Katsiadaki I, Kirby M, Robinson C, Scott S, Thain J, Thomas K (2002) The impact of oestrogenic and androgenic contamination on marine organisms in the United Kingdom – summary of the EDMAR programme. Marine Environmental Research 54 (3–5): 645–649.

Metcalfe CD, Metcalfe TL, Kiparissis Y, Koenig BG, Khan C, Hughes RJ, Croley TR, March RE, Potter T (2001) Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by *in vivo* assays with Japanese medaka (*Oryzias latipes*). Environmental Toxicology and Chemistry 20: 297–308.

Miles-Richardson SR, Kramer VJ, Fitzgerald SD, Render JA, Yamini B, Barbee SJ, Giesy JP (1999) Effects of waterborne exposure of 17 beta-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). Aquatic Toxicology 47: 129–145.

Nimrod AC, Benson WH (1998) Reproduction and development of Japanese medaka following an early life stage exposure to xenoestrogens. Aquatic Toxicology 44: 141–156.

Oberdörster E, Cheek AO (2001) Gender benders at the beach: Endocrine disruption in marine and estuarine organisms. Environmental Toxicology and Chemistry 20: 23–36.

Ohkubo N, Mochida K, Adachi S, Hara A, Hotta K, Nakamura Y, Matsubura T (2003) Estrogenic activity in coastal areas around Japan evaluated by measuring male serum vitellogenin in Japanese common goby (*Acanthogobius flavimanus*). Fisheries Science 69: 1133–1143.

Ohn S, Gessbo A, Steinholz A, Norrgren L (2000) Zebrafish (*Danio rerio*) – a candidate to evaluated endocrine disrupting chemicals. In: Zebrafish for Testing Endocrine Disrupting Chemicals. TemaNord 2000, 555. Nordic Council of Ministers, Copenhagen, Denmark, 65pp. Cited in Van den Belt *et al.* (2001).

Panter GH, Thompson RS, Sumpter JP (1998) Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. Aquatic Toxicology 42: 243–253.

Panter GH, Thompson RS, Beresford N, Sumpter JP (1999a) Transformation of a non-oestrogenic steroid metabolite to an oestrogenically active substance by minimal bacterial activity. Chemosphere 38: 3579–3596.

Panter GH, Thomspon RS, Sumpter JP (2000) Intermittent exposure of fish to estradiol. Environmental Science and Technology 34: 2756–2760.

Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Sumpter JP (1994) Estrogenic effects of effluents from sewage treatment works. Chemistry and Ecology 8: 275–285.

Robinson CD, Brown E, Craft JA, Davies IM, Moffat CF, Pirie D, Robertson F, Stagg RM, Struthers S (2003) Effects of sewage effluent and ethynyl oestradiol upon molecular markers of oestrogenic exposure, maturation and reproductive success in the sand goby (*Pomatoschistus minutus* Pallas). Aquatic Toxicology 62*:* 119–134.

Rodgers-Gray TP, Jobling S, Kelly C, Morris S, Brighty G, Waldock MJ, Sumpter JP, Tyler CR (2001) Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. Environmental Science and Technology 35: 426–470.

Routledge EJ, Sumpter JP (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environmental Toxicology and Chemistry 15: 241–249.

Routledge EJ, Sheahan D, Desbrow C, Brighty GC, Waldock M, Sumpter JP (1998) Identification of estrogenic chemicals in STW effluent. 2. *In vivo* responses in trout and roach. Environmental Science and Technology 32: 1559–1565.

Scholz S, Gutzeit HO (2000) 17-α-ethinylestradiol affects reproduction, sexual differentiation and aromatase gene expression of the medaka (*Oryzias latipes*). Aquatic Toxicology 50: 363–373.

Schwaiger J, Mallow, U, Ferling, H, Knoerr S, Braunbeck T, Kalbfus W, Negele RD (2002) How estrogenic is nonylphenol? A transgenerational study using rainbow trout (*Oncorhynchus mykiss*) as a test organism. Aquatic Toxicology 59: 177-189.

Schweinfurth H, Länge R, Gunzel P (1996) Environmental fate and ecological effects of steroidal estrogens. IBC Conference Proceedings of Oestrogenic Chemicals in the Environment, London, 9–10 May 1996.

Segner H, Carol K, Fenske M, Jansen CR, Maack G, Pasco D, Schafers C, Bandenbergh GF, Watts M, Wenzel A (2002) Identification of endocrine disrupted effects in aquatic invertebrates and invertebrates: report from the

European IDEA project. Ecotoxicology and Environmental Safety 54: 302–314.

Seki M, Yokota H, Maeda M, Tadokoro H, Kobayashi K (2003) Effects of 4-nonylphenol and 4-*tert*-octylphenol on sex differentiation and vitellogenin induction in medaka (*Oryzias latipes*). Environmental Toxicology and Chemistry 22: 1507 – 1516.

Seki M, Yokota H, Matsubura H, Tsuruda Y, Maeda M, Tadokoro H, Kobayashi K (2002) Effect of ethinylestradiol on the reproduction and induction of vitellogenin and testis–ova in medaka (*Oryzias latipes*). Environmental Toxicology and Chemistry 21: 1692–1698.

Sheahan DA, Bucke D, Matthiessen P, Sumpter JP, Kirby, MF, Neall P, Waldock M (1993) The effects of low level 17α-ethinylestradiol upon plasma vitellogenin levels in male and female rainbow trout (*Oncorhynchus mykiss*) held at two acclimation temperatures. In: *Sublethal and chronic effects of pollutants on freshwater fish*. (Ed R Muller and R Lloyd), pp. 99 –112. Fishing News Books.

Sheahan DA, Brighty GC, Daniel M, Kirby SJ, Hurst MR, Kennedy J, Morris S, Routledge EJ, Sumpter JP, Waldock MJ (2002). Estrogenic activity measured in a sewage treatment works treating industrial inputs containing high concentrations of alkylphenolic compounds – A case study. Environmental Toxicology and Chemistry 21: 507–514.

Shioda T, Wakabayashi M (2000) Evaluation of reproductivity of medaka (*Oryzias latipes*) exposed to chemicals using a 2 week reproduction test. Water Science and Technology 42: 53–60.

Snyder SA, Villeneuve DL, Snyder EM, Giesy JP (2001) Identification and quantification of estrogen receptor agonists in wastewater effluents. Environmental Science and Technology 35: 3620–3625.

Tabata A, Kashiwada S, Ohnishi Y, Ishikawa H, Miyamoto N, Itoh M, Magara Y (2001) Estrogenic influences of estradiol-17 beta, p-nonylphenol and bis-phenol-A on Japanese Medaka (*Oryzias latipes*) at detected environmental concentrations. Water Science and Technology 43: 109–116.

Taylor AAL, Nolan M, van Aerle R, Santos EM, Tyler CR, Brighty G and Jobling S (2004) Spatial Survey of the extent of sexual disruption in wild roach living in rivers in England and Wales. Poster presentation at the CREDO Cluster Workshop on the Ecological Relevance of Chemically-Induced Endocrine Disruption in Wildlife, University of Exeter, United Kingdom, July 5-7, 2004.

Taylor MR, Holmes P, Duarte-Davidson R, Humfrey CDN, Harrison PTC (1999) A research strategy for investigating the ecological significance of endocrine disruption: report of a UK workshop. Science of the Total Environment 233: 181–191.

Ternes TA, Kreckel P, Mueller J (1999) Behaviour and occurrence of estrogens in municipal sewage treatment plants – II. Aerobic batch experiments with activated sludge. Science of the Total Environment 223: 91–99.

Thames Water (1981) Hermaphrodite roach in the River Lee, London, UK. Thames Water.

Thomas KV, Hurst MR, Matthiessen P, Waldock MJ (2001) Characterization of estrogenic compounds in water samples collected from United Kingdom estuaries. Environmental Toxicology and Chemistry 20: 2165–2170.

Thomas KV, Balaam J, Hurst M, Nedyalkova Z, Mekenyan O (2004) Potency and characterization of estrogen-receptor agonists in United Kingdom estuarine sediments. Environmental Toxicology and Chemistry 23: 471–479.

Thorpe KL, Hutchinson TH, Hetheridge MJ, Sumpter JP, Tyler CR (2000) Development of an *in vivo* screening assay for estrogenic chemicals using juvenile rainbow trout (*Oncorhynchus mykiss*). Environmental Toxicology and Chemistry 19: 2812–2820.

Thorpe KL, Hutchinson TH, Hetheridge MJ, Scholze M, Sumpter JP, Tyler CR (2001) Assessing the biological potency of binary mixtures of environmental estrogens using vitellogenin induction in juvenile rainbow trout (*Oncorhynchus mykiss*). Environmental Science and Technology 35: 2476–2481.

Thorpe KL, Cummings RI, Hutchinson TH, Scholze M, Brighty G, Sumpter JP, Tyler CR (2003) Relative potencies and combination effects of steroidal estrogens in fish. Environmental Science and Technology 37 (6): 1142–1149.

Toft G, Baatrup E (2001) Sexual characteristics are altered by 4-tert-octylphenol and 17 beta-estradiol in the adult male guppy (*Poecilia reticulata*). Ecotoxicology and Environmental Safety 48: 76–84.

Tyler CR, Jobling S, Sumpter JP (1998) Endocrine disruption in wildlife: A critical review of the evidence. Critical Reviews in Toxicology 28: 319–361.

Tyler CR, van Aerle R, Hutchinson TH, Maddix S, Trip H (1999) An *in vivo* testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. Environmental Toxicology and Chemistry 18: 337–347.

van Aerle R, Nolan M, Jobling S, Christiansen LB, Sumpter JP, Tyler CR (2001) Sexual disruption in a second species of wild cyprinid fish (the gudgeon, *Gobio gobio*) in United Kingdom freshwaters. Environmental Toxicology and Chemistry 20: 2841–2847.

van Aerle R, Pounds N, Hutchinson TH, Maddix S, Tyler CR (2002) Window of sensitivity for the estrogenic effects of ethinylestradiol in early life-stages of fathead minnow, *Pimephales promelas*. Ecotoxicology 11: 423–434.

Van den Belt K, Verheyen R, Witters H (2001) Reproductive effects of ethynylestradiol and 4-*t*-octylphenol on the zebrafish (*Danio rerio*). Archives of Environmental Toxicology and Contamination 41: 458–467.

Vos JG, Dybing E, Greim HA, Ladefoged O, Lambre C, Tarazona JV, Brandt I, Vethaak AD (2000) Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. Critical Reviews in Toxicology 30: 71–133.

Wenzel A, Schafers C, Vollmer C, Michna H, Diel P (2001) Research efforts towards the development and validation of a test method for the identification of endocrine disrupting chemicals. Final Report Contract B6–7920/98/000015.

Williams RJ, Johnson AC, Smith JL, Jurgens MD, Holtaus K (2001) Fate and behaviour of steroid oestrogens in aquatic systems. Environment Agency R&D Technical Report P2–162/TR.

Yokota H, Seki M, Maeda M, Oshima Y, Tadokoro H, Honjo T, Kobayashi K (2001) Life cycle toxicity of 4-nonylphenol to medaka (*Oryzias latipes*). Environmental Toxicology and Chemistry 20: 2552-2560.

We welcome views from our users, stakeholders and the public, including comments about the content and presentation of this report. If you are happy with our service, please tell us about it. It helps us to identify good practice and rewards our staff. If you are unhappy with our service, please let us know how we can improve it.

Publication Code	SCHO0704BIBD-E-P