

Assessment of Feminization of Male Fish in English Rivers by the Environment Agency of England and Wales

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In recent years there has been considerable concern over the ability of substances discharged into the environment to disrupt the normal endocrine function of wildlife. In particular, the apparent widespread feminization of male fish in rivers has received significant attention from regulators in the United Kingdom, the United States, Europe, and Japan. The U.K. and European epidemiological data sets have demonstrated that the occurrence of feminized fish is associated with effluent discharges and that the incidence and severity is positively correlated with the proportion of treated sewage effluent in receiving waters. Although weakly estrogenic substances may contribute to the overall effect, studies have concluded that steroid estrogens are the principal and most potent estrogenic components of domestic sewage. Extensive laboratory data sets confirm that steroid estrogens are capable of eliciting the effects observed in wild fish at concentrations that have been measured in effluents and in the environment. Based on evaluation of the available information, the Environment Agency (England and Wales) has concluded that the weight of evidence for endocrine disruption in fish is sufficient to develop a risk management strategy for estrogenically active effluents that discharge to the aquatic environment. *Key words:* endocrine disruption, ethinylestradiol, feminization, fish, estradiol, estrone, risk assessment, steroid estrogen. *Environ Health Perspect* 114(suppl 1):147–151 (2006). doi:10.1289/ehp.8068 available via <http://dx.doi.org/> [Online 21 October 2005]

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 the reproductive physiology and health of wildlife species from several taxonomic groups in many countries (Tyler et al. 1998; Vos et al. 2000). The apparent widespread feminization of male fish in rivers and the impacts on reproduction and survival of marine mollusc species have received particular attention.

Concern about these effects led to a series of major national and international workshops, assessments, and research programs (Ankley et al. 1998; International Programme on Chemical Safety 2002; Matthiessen et al. 2002; Taylor et al. 1999). These efforts evaluated the effects from candidate contaminants, their pathways to the environment, and their epidemiological impacts through risk assessments. The occurrence of widespread intersex in fish and the causes and consequences of this phenomenon have particular implications for the role of the Environment Agency of England and Wales in managing the aquatic environment and protecting the wildlife it supports. Following an assessment of the available data (Environment Agency 2004a), the Environment Agency 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 that discharge to the aquatic environment.

Our article brings together and critically assesses the relevant scientific information on biological effects observed in fish through eco-epidemiological studies, analyses of contaminant inputs from dominant point sources into receiving waters, laboratory evaluations of the effects of suspected causative chemicals on fish development and reproduction, and risk assessment. The Environment Agency has adopted a weight-of-evidence approach because there are many components to consider in demonstrating the linkages between effects seen in fish and their causes. The evidence and arguments presented in this article form the basis of the Environment Agency's rationale for developing and supporting the evaluation and demonstration program for effluent treatment technologies in England and Wales. The Endocrine Disruption Demonstration Programme, which is being developed in collaboration with government and the water industry, will deliver a basis for future decisions on investment in sewage treatment works (STW) infrastructure aimed at reducing the estrogenic activity in effluents if, and where, required.

The Environment Agency's Assessment of the Widespread Feminization of Fish

U.K. and European epidemiological data sets have clearly demonstrated the occurrence of intersex fish (fish with gonads containing male and female tissue, and/or feminized reproductive ducts) and its association with effluent discharges. These data sets have also demonstrated that incidence and severity of this inter-

sex condition are positively correlated with the proportion and estrogenic quality of treated sewage effluent in receiving waters [Center for Ecology and Hydrology (CEH) 2002; Jobling et al. 1998]. The induction of a female specific protein, vitellogenin, in male fish exposed to sewage effluents demonstrated that fish had been exposed to estrogenic substances in the receiving environment (Harries et al. 1997; Purdom et al. 1994) and supported the view that fish were being feminized. Reports of feminized fish have ranged from initial findings in roach (*Rutilus rutilus*) (Jobling et al. 1998; Thames Water 1981) to other wild freshwater species such as the gudgeon (*Gobio gobio*) (van Aerle et al. 2001), common bream (*Abramis brama*), common carp (*Cyprinus carpio*) (CEH 2002), and shovelnose sturgeon (*Scaphirhynchus platyrhynchus*) (Harshbarger et al. 2000). Feminization has also appeared in estuarine species such as the European and Japanese flounders (*Platichthys flesus* and *Pleuronectes yokohamae*, respectively) (Allen et al. 1999; Hashimoto et al. 2000; Kirby et al. 2004; Lye et al. 1997). These reports confirm that sexual disruption in fish is a general phenomenon and is neither species- nor habitat specific.

The initial U.K. surveys focused on a limited number of sites and included some considered to be so-called hot-spots, where concentrations of sewage effluents were high (Environment Agency 2002a; Harries et al. 1997; Jobling et al. 1998). The Environment Agency next committed itself to evaluating the full spatial extent and risk of endocrine disruption in English fisheries. Modeling of steroid concentrations in effluents (Johnson and Williams 2004), using a database of STWs with population equivalents greater than 10,000, placed 142 river stretches into a high-risk category, 192 into medium-risk, and 132 into

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low-risk. Of the wild roach (*R. rutilus*) collected from selected sites within each of these categories, one-third of all “male” fish were intersex, with intersex fish found at 44 (86%) of the 51 sites sampled (Environment Agency 2004a). Further analysis of pooled data sets from within the high-, medium-, and low-risk categories clearly demonstrated that both the incidence and severity of intersex fish were correlated with their predicted exposure to steroid estrogens. Furthermore, within each risk category, the most severely feminized fish were found in the older year classes (Jobling et al. 2006), thereby confirming findings of previous investigations (Environment Agency 2002a). In fish younger than 3 years of age, intersex was found to be a rare condition, thus indicating that the expression of this condition is progressive and appears at, or after, the onset of puberty in roach. Incidence and severity of the intersex condition were related more to the duration of exposure to effluent than to the year of hatching or to the prevailing environmental conditions in the study rivers (Environment Agency 2002a).

While these field data sets indicated a correlation between intersex and effluent exposure, direct exposure–effect studies were required to demonstrate concentration-dependent and life stage–specific responses. Developmental stages during which fish may be sensitive to feminization of the gonads include early life-stage (encompassing the period from fertilization of the egg to development of the reproductive organs) and the period of germ cell (developing egg and sperm cells) proliferation. Direct-exposure studies with early life-stage roach (from fertilized egg and juveniles) confirm that effluents induce a number of feminizing effects, including vitellogenin induction and duct disruption, in a concentration-dependent manner. Although depuration in clean water led to a reduction in vitellogenin concentrations, there was no correction of the feminized ducts. These studies indicate that feminized ducts in wild-caught roach are the result of exposure to effluents during early life stages and are a permanent effect. Other relevant end points, particularly the genesis of female germ cells in male testes following experimental exposures to effluents, have not yet been demonstrated (Environment Agency 2004b; Rodgers-Gray et al. 2001); this phenomenon remains a key area for investigation. One current hypothesis is that the development of the eggs-in-testis condition is a result of longer-term exposure, a premise supported by the apparent progressive nature of the intersex condition revealed by analysis of roach data sets gathered between 1995 and 2000 (Environment Agency 2002a). Germ cell disruption in male fish is generally only seen from the third year of life, 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 has been disrupted in experimental exposures up to 1 year (Environment Agency 2004b), to date it has not been possible to maintain natural environmental conditions over longer-term experimental studies. This constraint might account for the failure to induce the eggs-in-testis condition experimentally in roach with effluents.

The Environment Agency’s focus on endocrine disruption currently centers around the feminization of male fish. Without markers for genetic sex, this hypothesis cannot be proved unequivocally; however, the application of genomic and molecular tools is now under way to test this theory. Regardless, the effects of estrogens on sexual differentiation in fish, the presence of estrogenically active substances in effluents, and the inversely proportional relationship between number of fish with normal testes and number of intersex fish (Jobling et al. 1998) strongly support the male fish feminization hypothesis. Moreover, the data sets from Rodgers-Gray et al. (2001) indicate that females differentiate first in all treatments (with clear oviducts) and that undifferentiated fish (that subsequently suffered duct disruption) are likely to be males.

A key question arising from the reports of widespread occurrence of intersex and elevated vitellogenin levels in fish is whether these conditions affect reproductive success. 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). In addition, the inappropriate induction of vitellogenin as a consequence of estrogen exposure may result in the diversion of vital proteins or lipids and a loss of calcium from the scales (Carragher and Sumpter 1991), which may make fish more susceptible to disease. Studies assessing the gamete quality of wild-caught intersex roach demonstrated that moderately to severely feminized fish had reduced fertility and were less able to release milt (sperm) compared with both “normal” male fish and less severely feminized intersex fish (Jobling et al. 2002). Those able to release milt produced up to 50% less (volume of sperm per gram of testis weight) than normal male fish. Sperm quality (i.e., motility, density, and fertilization success) was also negatively correlated with the degree of feminization in intersex fish. In severely intersex fish, sperm motility was reduced by up to 50%, and fertilization success was reduced by up to 75%, compared with less severely intersex, or unaffected, fish (Jobling et al. 2002).

The ecological significance of endocrine disruption in fish is a further question frequently raised by researchers in this field because the implications of the intersex

condition and reduced fertility, which are observed at the individual level, have not yet been demonstrated for wild populations. A general lack of population-level effect studies has been identified and highlighted as a research gap (Taylor et al. 1999), possibly caused, in part, by the difficulty in designing and undertaking such studies. To estimate population-level impacts of estrogen exposure, modeling techniques have been applied to ecotoxicological data for steroids. For example, Grist et al. (2003) applied a linear model to the fish life-cycle data of Länge et al. (2001) and estimated that environmentally relevant concentrations of ethinylestradiol reduced the intrinsic growth rate to zero (i.e., no population growth).

Field data sets from many years of fish surveys have identified environmental variables such as temperature as key drivers of population success (Environment Agency 2003). To date, population models of wild coarse fish have not taken into account endocrine-disrupting effects. It is now possible to factor the effects observed during *in vitro* fertilization experiments into the available fish life-table models, and this development has been identified as a key science need. However, the widespread and high occurrence of the intersex condition in the United Kingdom, along with currently available evidence that moderately to severely intersex fish have reduced sperm quality, quantity, and fertilization success, raises the concern that this phenomenon may have chronic impacts on the sustainability of fish populations. The Environment Agency and the U.K. government have taken the view that the observed effects can be considered harmful and, together with the reasonable likelihood of population level effects, that this damage is unacceptable for the long term.

Identification of Causative Substances

Identifying the main contaminants responsible in effluents, which are complex mixtures of organic and inorganic substances, and ascribing a significance to each (or each group of) contaminant(s) is challenging, particularly when the potency of co-existing substances might be low. The Environment Agency’s focus is on chemicals for which there is a plausible linkage to mechanisms of feminization in fish. A wide variety of structurally diverse, synthetic chemicals are known to mimic estrogens, and many have been detected in the environment. These include alkylphenols (breakdown products of industrial surfactants), bisphenol A, phthalates, and a variety of pesticides and herbicides. Modes of action are currently being investigated with newly developed genomic tools. It is important to consider that substances might act by different

or multiple mechanisms (e.g., estrogenic and/or antiandrogenic) and that the terminology of endocrine disruption, which has attributed an activity to particular substances, may need to be revisited as specific gene responses are discovered. However, the evidence to date from the United Kingdom, Europe, and the United States has clearly identified steroid estrogens as the most potent estrogenically active substances present in domestic effluents (CEH 2002; Desbrow et al. 1998; Snyder et al. 2001).

Using an estrogen-specific bioassay (the yeast estrogen screen; Routledge and Sumpter 1996) to determine estrogenic activity in fractionated effluents, Desbrow et al. (1998) found estrone, 17 β -estradiol and 17 α -ethinylestradiol to be the most significant estrogenically active substances in seven domestic effluents. The natural hormones were consistently present at high levels (estrone at 1–80 ng/L, and 17 β -estradiol at 1–50 ng/L), whereas the synthetic hormone 17 α -ethinylestradiol was generally below the detection limit (approximately 1 ng/L). Subsequent concentration–response experiments demonstrated that there were sufficient hormones in the effluents to induce the vitellogenin production in fish that characterized previous field research (Routledge et al. 1998). Studies conducted in the United Kingdom (Desbrow et al. 1998), across Europe in the COMPREHEND program (CEH 2002), and in the United States (Snyder et al. 2001) also identified estrone, 17 β -estradiol, and 17 α -ethinylestradiol as the major causes of the estrogenic activity present in effluents arising from domestic sources. However, these studies did not rule out other weakly estrogenic compounds (e.g., nonylphenol and bisphenol A) from contributing to the overall effect.

The Environment Agency recently commissioned a survey of 43 STWs in England and Wales to provide national information on the estrogenic activity of priority STW effluents (Environment Agency, in press (b)). Twenty-five of these sites were previously predicted to be high- or medium risk, and those sites were the ones where the greatest level and severity of effects were found in the spatial survey of intersex in roach [Environment Agency, in press (a)]. The results supported the previous findings of Desbrow et al. (1998). One, two, or all three of the steroid estrogens were detected at virtually all sites, with reported concentrations in the ranges of < 1–100 ng/L for estrone, < 1–22 ng/L for 17 β -estradiol, and < 1.0–3.2 ng/L for 17 α -ethinylestradiol. Nonylphenol (an alkylphenol) concentrations in the final effluents were generally low, with the majority in the range of < 1–3 μ g/L; of 43 sites, only 8 had concentrations > 3 μ g/L, and none was higher than 7.7 μ g/L [Environment Agency, in press (b)]. The estrogenic potency of

nonylphenol suggests that, in isolation, it is not likely to elicit an estrogenic response in fish downstream of discharges once dilution is taken into consideration. However, the long-term effects of low concentrations of nonylphenol on vitellogenin induction and gonadal disruption in roach are unknown.

The role of estrogens in vitellogenin induction and the development of the intersex condition is implicit. Substantial data sets from laboratory studies have clearly confirmed that exposure to steroids at environmentally relevant concentrations can induce the effects observed in wild fish. Effect concentrations for vitellogenin induction have been reported as low as 5 ng/L for estradiol (Tabata et al. 2001), 3.2 ng/L for estrone (Thorpe et al. 2001), and around 1 ng/L for ethinylestradiol (Fenske et al. 2001; Thorpe et al. 2001). The intersex condition has been recorded in various fish species following exposures to concentrations as low as 10 ng/L each for estradiol and estrone, (Metcalf et al. 2001; Tabata et al. 2001) and 4 ng/L for ethinylestradiol (Länge et al. 2001). Other estrogenic effects observed included altered mating behavior (Wenzel et al. 2001), skewed sex ratios (Länge et al. 2001), reduced testicular growth (Jobling et al. 1996), and reduced reproductive output (van den Belt et al. 2001; Wenzel et al. 2001).

Data from single-chemical exposure studies dominate the literature, but effluent fractionation assessments have confirmed that estrogenic substances occur in mixtures. Studies on binary mixtures performed by Thorpe et al. (2001) confirmed the additive nature of structurally dissimilar estrogenic compounds (nonylphenol and estradiol), at environmentally relevant levels, in eliciting an estrogenic biological response (vitellogenin induction in rainbow trout). Ethinylestradiol and estradiol are also additive in mixtures at environmentally relevant concentrations (Thorpe et al. 2003). Importantly, estrogenic responses in fish were induced even though concentrations of the individual substances in the mixtures were lower than previously assessed no observable effect concentrations (NOECs) for the individual substances (Thorpe et al. 2001, 2003). Assessments of environmental hazards and risks for mixtures of substances that act via the same toxic mechanism(s) may not be adequately protective if they are made using data derived for individual substances. Therefore, assessment of the estrogenic hazard of a complex effluent should consider the total estrogenic burden within that effluent.

Based on available data and its strategy on endocrine disruption (Environment Agency 2000), the Environment Agency has derived the following predicted no effect concentrations (PNECs) for steroid estrogens: 0.1 ng/L

for 17 α -ethinylestradiol, 1 ng/L for 17 β -estradiol), and 3 ng/L for estrone. Recognizing that steroids are unlikely to occur in isolation, a combined PNEC for total steroid estrogens of 1 ng/L estradiol equivalents was derived, which takes into account the relative potency of each steroid and their additive effects (Environment Agency 2002b). Measurements of steroid estrogens in the Environment Agency's national survey of STW effluents have demonstrated that steroids are present in some effluents at concentrations at or higher than the PNEC derived for steroids (Environment Agency 2002b). In addition, modeling studies undertaken as part of the Environment Agency's risk assessment of effluents have predicted concentrations higher than these PNECs in the downstream receiving environment, leading to unacceptable exposures of aquatic life that inhabit certain river stretches below STWs.

The Environment Agency's Risk Management Strategy

The Environment Agency is responsible for the protection of the environment through the control of pollution and has specific duties for the protection of fish. Following an assessment of the available data (Environment Agency 2004a), the Environment Agency concluded that the weight of evidence for the causes and consequences of endocrine disruption in fish reinforces the need to look at cost-effective ways of minimizing exposure to these endocrine-disrupting substances.

There are a number of regulatory mechanisms available to manage industrial chemicals and reduce the inputs of certain endocrine-disrupting chemicals to the environment. Emissions can be controlled through discharge consents for point source discharges. However, the control of diffuse sources is more difficult, although substances are on the European Market, control at the source through restrictions on the marketing and use of a substance is one option. We undertook a European Union risk assessment on nonylphenol, a recognized endocrine-disrupting chemical. Nonylphenol has a wide variety of applications, is commonly present in sewage effluent, and could lead to risks to the environment. As a result, restrictions on the use of nonylphenol for such applications have now been imposed across Europe.

Voluntary initiatives can also be important. For example, following recommendations of the U.K. Chemicals Stakeholder Forum, the U.K. government has negotiated a voluntary agreement with the suppliers of nonylphenols, octylphenols, and their respective ethoxylates in which suppliers have agreed not to promote octylphenol as a substitute for nonylphenol. The U.K. voluntary initiative is therefore particularly important in

preventing the development of octylphenol, another endocrine disruptor that has been found in sewage effluent, as a substitute for nonylphenol. Signatories to the voluntary agreement have agreed not to manufacture or import new formulations or products containing these substances and to reformulate existing products to remove these substances as a matter of urgency.

However, a different approach is required for the risk management of natural and synthetic steroid estrogens released into rivers via sewage effluent. Further investigation is required to assess the effectiveness of existing or enhanced sewage treatment processes in removing or reducing these substances and the associated costs in order to inform future decisions on how to control this type of pollution.

The Environment Agency, in collaboration with government and the water industry, has developed a demonstration program to conduct this sewage treatment assessment as part of the water industry's environmental program from 2005 to 2010. The program will assess the effectiveness and associated costs of current sewage treatment technologies for removing steroid hormones from effluent and will also conduct full-scale demonstration trials of the most promising advanced treatment technologies, such as granular activated carbon. The program involves an effluent-testing program comprising a combination of chemical determination and biological activity monitoring (i.e., *in vitro* and *in vivo* assays). The program will evaluate the ability of treatment methods to remove steroid estrogens, to reduce estrogenic activity of final effluent, and consequently, to reduce risks of intersex in the fishery. In addition, environmental monitoring (e.g., histology and population parameters) and population modeling will be undertaken in supplementary research programs to evaluate the effectiveness of advanced treatment technologies by providing important information on changes to environmental quality. This goal will be achieved by using chemical and biological monitoring tools developed through recent scientific programs.

The demonstration program is required to provide all stakeholders with the confidence that any potentially substantial investment in treatment technology will effectively reduce steroid estrogens in final STW effluents and minimize the risks of harm to fish downstream of STWs associated with their endocrine-disrupting effects. While there is little doubt that effluents significantly influence the development of coarse fish, the degree of risk from discharging individual or multiple effluents into a river system will vary and therefore so will the risk management options. It is an appropriate response, given the number of remaining uncertainties regarding the long-term significance of intersex in fish populations, the need

for, and means of, risk reduction, and how endocrine disruption-related hazards are best regulated to protect fish and the health of aquatic ecosystems.

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