Roach, Sex, and Gender-Bending Chemicals: The Feminization of Wild Fish in English Rivers

CHARLES R. TYLER AND SUSAN JOBLING

Feminization of the male roach, Rutilus rutilus*, a freshwater, group-spawning fish, is widespread in English rivers; among the causative agents are natural and synthetic steroidal estrogens and chemicals that mimic estrogens. In feminized male roach, concentrations of the egg-yolk protein vitellogenin are elevated, sex steroid hormone dynamics are altered, and gonad development is disrupted (most notably, a female reproductive duct or developing eggs [oocytes] are present in the testis). In some English rivers containing high levels of estrogens, all male roach sampled have been feminized to varying degrees. In the more severely affected males, individuals produce low-quality sperm with a reduced capability for fertilization. Laboratory studies have shown that the environmental estrogens responsible for inducing gonadal feminization in roach can also alter reproductive behavior, disrupting normal breeding dynamics (parentage) in the zebrafish, another group-spawning fish. Together these findings indicate that feminization of wild roach may result in adverse population-level effects, but this hypothesis has yet to be fully addressed.*

Keywords: roach, feminization, intersex, effluents, estrogen

The phenomenon of disruption of sexual development in roach (*Rutilus rutilus*), a common freshwater fish species belonging to the carp family, in English rivers was first reported more than 25 years ago. Sport fishermen noticed that a few wild roach, caught from a wastewater treatment works (WWTW) effluent settlement lagoon and at a site downstream of this discharge in the River Thames (London), were neither male nor female, but rather intersex—that is, the gonads contained both testicular and ovarian tissue (Sweeting 1981). Some fish undergo a sex change during normal development, becoming males first and then transforming into females, whereas others are females first, then males (Baroiller et al. 1999). Some fish even develop as hermaphrodites, with their gonads containing male and female parts at the same time. These adaptations, which allow fish to optimize reproductive output under varying environmental conditions, illustrate the plasticity, or ease of change, of sex in some fish species. The roach, however, is normally a single-sexed fish that develops as either a male or female, and individuals do not change sex during the course of their lives (Jafri and Ensor 1979, Schultz 1996); thus, the finding of intersex in roach was unusual.

Another chance observation, which occurred during our research with colleagues at the Centre for the Environment and Fisheries Advisory Services, established more evidence for gender disruption. Here, vitellogenin (VTG) was detected in male rainbow trout (*Oncorhynchus mykiss*) that had been exposed to the effluent of a WWTW. VTG is a precursor of yolk in developing eggs and is normally produced only in females under the stimulation of estrogen (Van Bohemen et al. 1982), suggesting that the WWTW effluents studied were estrogenic to fish (figure 1).

We set up an extensive survey to investigate this hypothesis. Rainbow trout and carp (*Cyprinus carpio*) were placed in cages in or close to the effluent discharges at 28 WWTW throughout England and Wales, and we measured blood VTG concentrations after a two- to three-week exposure period (Purdom et al. 1994). The results were astonishing. Almost all of the effluents studied were estrogenic and induced up to a millionfold increase in the amount of circulating VTG in males. Some concentrations of VTG measured in males were in excess of 50 milligrams per milliliter, representing more than half the blood protein content. These concentrations were higher than those found in fully mature females with thousands of large, yolky eggs in their ovaries. The phenomenon of estrogenic effluents has subsequently been established more widely across Europe—for example, in Germany (Hecker et al. 2002), Sweden (Larsson et al.

Charles R. Tyler (e-mail: c.r.tyler@ex.ac.uk) is with the Environmental and Molecular Roach Biology Group of the School of Biosciences, Hatherly Laboratories, at the University of Exeter in the United Kingdom. Susan Jobling (e-mail: susan.jobling@brunel.ac.uk) is with the Institute for the Environment at Brunel University in the United Kingdom. *© 2008 American Institute of Biological Sciences.*

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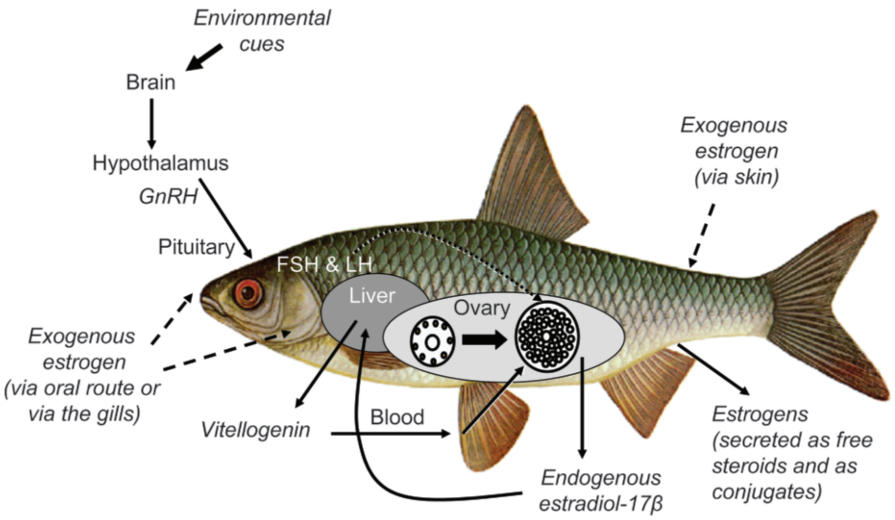

Figure 1. Vitellogenesis in roach. The solid line indicates the normal pathway of vitellogenin induction in female fish; endogenous estrogen is produced by the ovary under the stimulation of the pituitary hormones, follicle-stimulating hormone and luteinizing hormone; it passes into the circulation and induces vitellogenin synthesis in the liver. The vitellogenin then passes into the circulation and is sequestered by the developing oocytes and stored as yolk for the subsequent embryo. The dashed lines show the routes of exposure to exogenous estrogens in fish, these are oral and/or across the gill or skin surfaces. In male fish exposed to estrogen, the vitellogenin produced by the liver accumulates in the plasma.

1999), Denmark (Bjerregaard et al. 2006), Portugal (Diniz et al. 2005), Switzerland (Vermeirssen et al. 2005), and the Netherlands (Vethaak et al. 2005)—and in the United States (Folmar et al. 1996), Japan (Higashitani et al. 2003), and China (Ma et al. 2005).

Further studies using caged fish (rainbow trout, carp, and roach) and measuring VTG induction have demonstrated that WWTW effluent discharges in the United Kingdom vary widely in their estrogenic potency, depending on the influents received by the WWTW, the level and type of treatment that takes place in the WWTW (Kirk et al. 2002), and the level of influent and effluent dilution (Williams et al. 2003). Seasons also have been shown to affect the estrogenic potency of effluent, probably in connection with changes in the level of microbial activity (Harries et al. 1999). Studies on rivers receiving treated WWTW effluent have shown that the estrogenic activity can persist in the receiving rivers for many kilometers downstream of the point source of discharges and with considerable dilution of the effluent (Harries et al. 1995, 1997, Rodgers-Gray et al. 2000, 2001, Liney et al. 2005). Effluent concentrations as low as only 10% have been shown to induce a vitellogenic response in juvenile roach exposed for four months, and this effect concentration may be even lower for longer-term exposures (Rodgers-Gray et al. 2000). These findings have important implications for wild roach populations that spend much or all of their lives in effluent-contaminated rivers, where the river flow is often composed of 10% WWTW effluent (figure 2). In some rivers in the United Kingdom, during the summer months and periods of low water flow, half of the flow of the river can be composed of WWTW effluent, and in the most extreme cases, the complete flow of the river can be made up of treated WWTW effluent (Jobling et al. 1998). This situation in the United Kingdom is somewhat unusual compared with mainland Europe and the United States, where dilution rates in rivers are generally far higher. Indeed, this very likely explains why levels of VTG induction in wild male fish in the allied carp family in mainland European and US rivers are generally lower than those levels in wild roach in English rivers.

Sexual disruption in roach in UK rivers: A widespread phenomenon

To determine the extent of sexual disruption in wild roach populations in UK rivers, a national survey of those populations in 1995 analyzed for disruptions in gonad development (Jobling et al. 1998, Nolan et al. 2001). Roach were sampled at locations upstream and downstream of WWTW on eight rivers, and at five reference sites throughout England and Ireland. Microscopic analysis of the gonads collected revealed that a large proportion of the putative males were in fact intersex (figure 3).

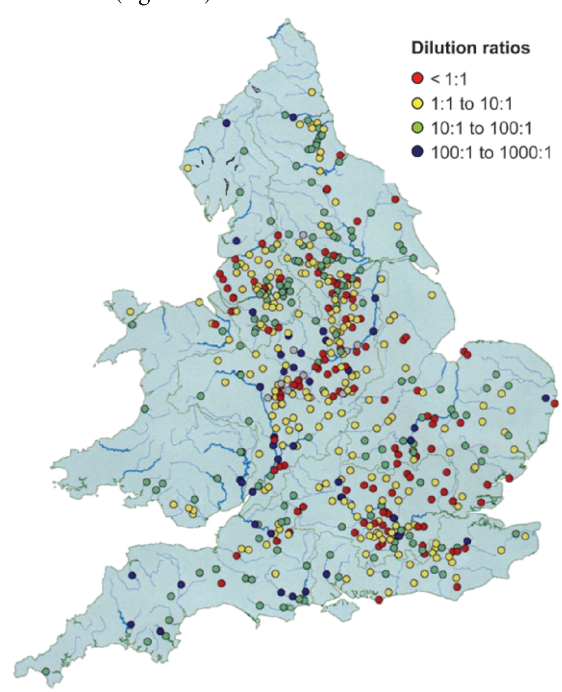

Figure 2. Dilution ratios of effluents discharged from major wastewater treatment works in rivers in England and Wales.

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In populations of roach living in rivers downstream of WWTW outfalls, the proportion of intersex males in population samples of 50 to 100 fish ranged between 16% and 100%. In contrast, the incidence of intersex in roach at the upstream sites (also downstream from more distant WWTW) was generally much lower (11% to 44%; Jobling et al. 1998). In 2002–2003, a more extensive survey of wild roach at 51 UK river locations receiving WWTW effluent discharges found intersex roach at 86% of these sites, firmly establishing the widespread nature of sexual disruption in roach in English rivers (figure 4; Jobling et al. 2006).

The gonad types most commonly found in the wild "feminized" male roach surveyed included those with single oocytes, or with small nests of oogonia, interspersed throughout an otherwise normal testis (figure 3; Nolan et al. 2001). In the most extreme cases, however, half the testis was composed of ovarian tissue. In some individuals, the sperm duct that enables the sperm to be released was absent, replaced by an ovarian cavity (figure 3; Jobling et al. 1998, Nolan et al. 2001, van Aerle et al. 2001). The degree of sexual feminization was greater in roach living immediately downstream of the WWTW discharges. An analysis of the extensive data on intersex in wild populations of roach has shown that both the incidence of intersex in the population and the degree of intersex within those individuals are highly positively correlated with age (figure 5; Jobling et al. 2006), with oocytes appearing commonly in the testis only when the roach are two or more years old.

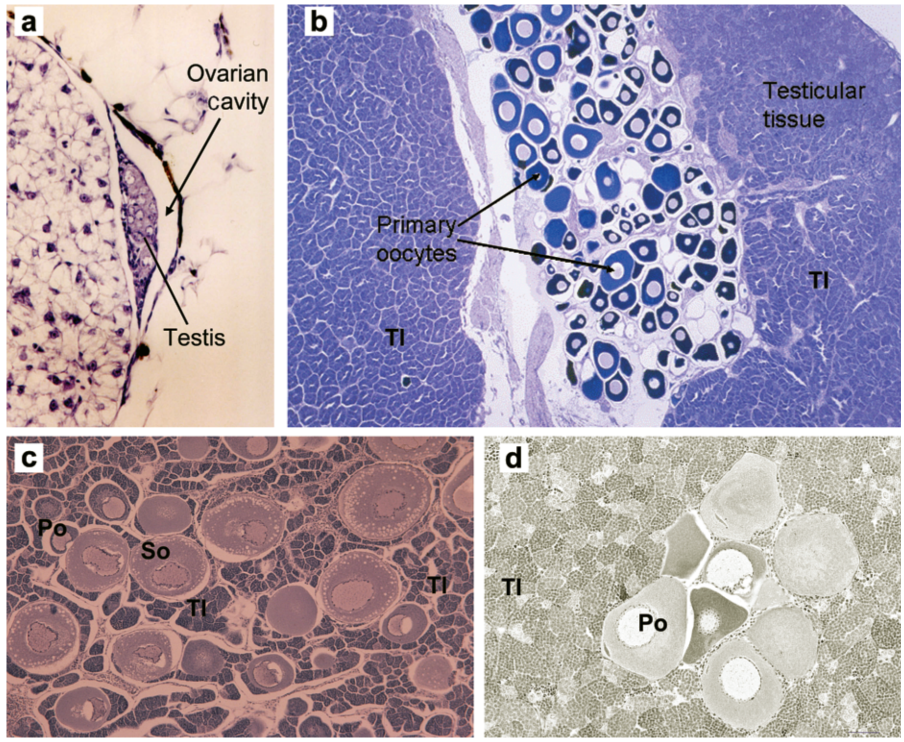

*Figure 3. Histological sections showing intersex phenotypes in roach (*Rutilus rutilus*). (a) Presence of a femalelike ovarian cavity in an otherwise normal testis, (b) severely intersex gonad showing a testis containing a large number of primary oocytes at a single focus, (c) severely intersex gonad with large numbers of both primary and secondary oocytes dispersed throughout the testis, and (d) mildly intersex gonad with a small number of primary oocytes found at focal points throughout the testis (the more common condition in roach living in English rivers). Abbreviations: Tl, testis lobule; Po, primary oocyte; So, secondary oocyte. The scale bar represents 100 micrometers.*

Sexual disruption in fish in UK rivers occurs in species other than the roach, including the gudgeon (*Gobio gobio*; van Aerle et al. 2001). Like roach, the gudgeon is a member of the carp family, but its lifestyle is different from that of the roach; the gudgeon lives on the bottom of the river, close to the river sediments, whereas the roach lives midwater in the river. Altered sexual development in other species of wild fish belonging to the carp family (e.g., bream, *Abramis brama*) has also been reported in other European countries (Hecker et al. 2002). In contrast with the findings for roach and gudgeon, however, a study of perch (*Perca fluvialitis*) and pike (*Esox lucius*), which are top predatory fish living in the same English rivers as roach and gudgeon, did not find severe sexual disruption; this suggests that these species may have less susceptibility or lower sensitivity to the chemical disruption of sex (Vine et al. 2004).

Are intersex roach in English rivers feminized males or androgenized females?

In fish, as in all other vertebrates, estrogens (female sex hormones) and androgens (male sex hormones) play important roles in determining the sex of an individual (Yeoh et al. 1996), and environmental exposure to estrogen, androgens, or other chemicals that interfere with the balance of natural sex hormones can cause altered sexual development and even complete sex reversal. In theory, therefore, intersex wild roach could arise as a consequence of the males' exposure to estrogens or the females' exposure to androgens. Unlike the case for mammals and some other animal species, there are no genetic sex probes available for roach to provide a definitive answer to this question. Nonetheless, the evidence supporting the hypothesis that intersex roach in English rivers arise from the feminization of genetic males is substantive: (a) the number of roach with normal testes in wild populations that have been studied is inversely proportional to the number of intersex roach (Jobling et al. 1998, 2006); (b) WWTW effluent discharges into UK rivers are estrogenic (Purdom et al. 1994, Harries et al. 1997, 1999, Rodgers-Gray et al. 2000, 2001, Jobling et al. 2003) or antiandrogenic, which would

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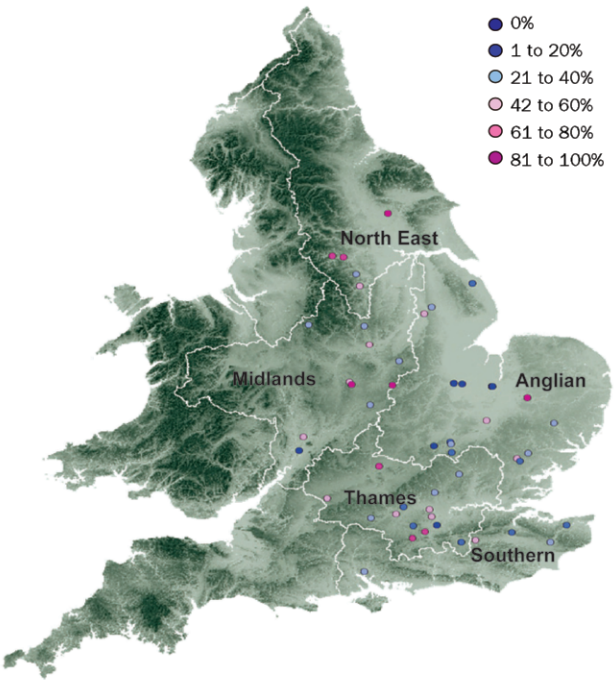

Figure 4. Extent of sexual disruption in roach in English rivers. Intersex was present at 44 (86%) of 51 sites surveyed, and there was an overall incidence of intersex in 23% of the males sampled. Colored symbols indicate the incidence of intersex at the different river sites surveyed.

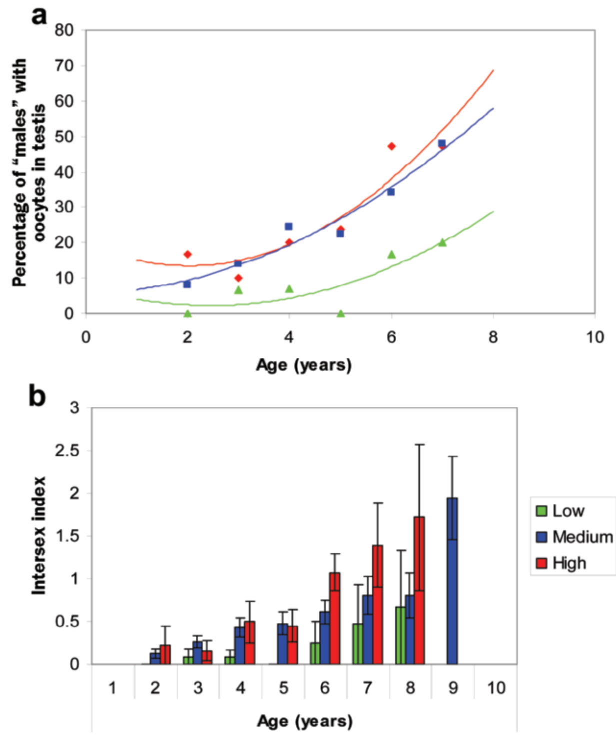

Figure 5. Relationship between the age of wild roach and (a) the proportion of intersex roach sampled from wild populations, and (b) the degree of sexual disruption at low (green), medium (blue), and high (red) estrogen-exposed sites. The intersex index, a measure of the degree of feminization of the testis, runs from 0 to 7, with 0 a normal testis, 1 to 6 a testis with increasing levels of female tissue, and 7 a normal ovary (see Jobling et al. 1998 for the derivation of the intersex index; data derived from a total of 604 fish). Source: Reproduced with permission from Jobling and colleagues (2006).

further enhance any feminization of males, but rarely androgenic (Environment Agency 2007); (c) wild male and intersex roach contain VTG in the plasma (figure 6; Jobling et al. 1998, 2002a); and (d) the plasma levels of 11-ketotestosterone, the main male sex hormone in roach, and estradiol-17b in wild intersex roach are more similar to those in normal males than to those in normal females (figure 6). Thus the evidence strongly suggests that some wild roach populations were exposed and responded to estrogenic contaminants.

Identity of the chemicals that cause sexual disruption in roach in English rivers

A positive correlation has been shown between the proportion of wild intersex roach in the populations studied and the concentration of the effluent at the different sampling sites (Jobling et al. 1998). Furthermore, a link has been shown between high effluent discharges into a river and an abnormally high proportion of juvenile roach with a feminized reproductive duct (Beresford et al. 2004). In addition to these indications is definitive evidence that effluents from WWTW induce sexual disruption: in a series of roach exposures to effluents undertaken under controlled conditions, all of the feminine characters seen in wild roach could be experimentally induced (Rodgers Gray et al. 2000, 2001, Gibson et al. 2005, Liney et al. 2005, 2006, Tyler et al. 2005).

The question of which chemicals in WWTW effluents are responsible for the feminization of roach in English rivers has now largely been answered. Effluents from WWTW contain a wide range of chemicals with estrogenic activity, albeit with widely varying potencies. Fractionating the effluents and screening those fractions with genetically engineered yeast cells that respond to estrogens, combined with high-quality analytical chemistry, determined that approximately 80% (or more) of the estrogenic activity is contributed by the natural steroidal estrogens estradiol-17ß (E_2) and estrone (E_1) (Desbrow et al. 1998, Rodgers-Gray et al. 2000, 2001), together with the synthetic estrogen ethinylestradiol (EE_2), a component of the contraceptive pill (figure 7). This fractionation procedure shows that in some WWTW effluents, horse estrogens used in hormone-replacement therapy (Gibson et al. 2005) and alkylphenolic chemicals (derived from the breakdown of industrial surfactants used in cleaning agents, paints, etc.) also contribute to the estrogenic activity. Alkylphenolic chemicals have been shown to be especially prevalent in WWTW that receive significant inputs from the wool-scouring industries.

Roach exposures in the lab to steroidal estrogens and alkylphenolic chemicals have induced VTG synthesis, gonadduct disruption, and oocytes in the testis (albeit for the last

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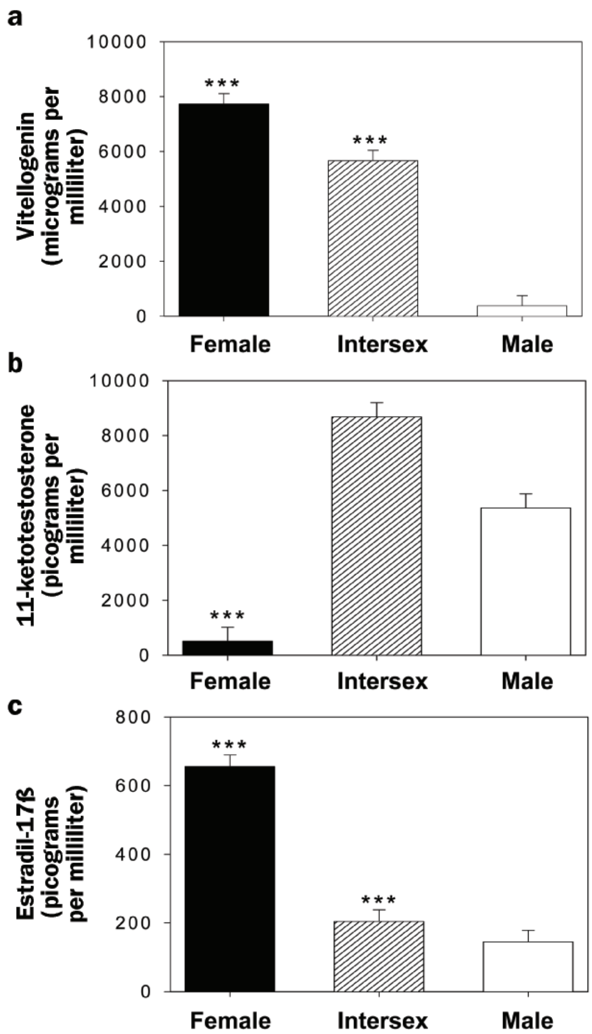

Figure 6. Endocrine status of roach (**Rutilus rutilus*****) sampled from wild populations in the United Kingdom. Fish from effluent-contaminated waters were collected from the Rivers Nene (Northhamptonshire) and Aire (Yorkshire). Roach from sites uncontaminated with treated sewage effluent were sampled from the Royal Canal, Ireland, Grantham Canal, Leicestershire, and from a spring-fed lake at Wartnarby, Leicestershire. Fish were collected in October. Plasma vitellogenin (a), 11 ketotestosterone (b), and estradiol-17ß (c) in male, female, and intersex fish. Asterisks represent significant (*****$p < 0.001$*****) differences of intersex males or exposed females from pooled control males or females, respectively. Source: Adapted from Jobling and colleagues (2002a).***

effect, concentrations in the lab were generally higher than those found in effluents and receiving rivers) (Blackburn and Waldock 1995, Tyler and Routledge 1998, Metcalf et al. 2001, Yokota et al. 2001, van Aerle et al. 2002, Hill and Janz 2003), strongly supporting the involvement of those estrogens and chemicals in the feminization of wild roach. Researchers recently found that exposing roach to 4 nanograms of EE_2 per liter, a concentration found in some of the more heavily contaminated effluent discharges, induced complete sex reversal, resulting in an all-female population. Adding further to the hypothesis that steroidal estrogens play a major role in causing intersex in wild roach, a recent study on wild roach at 45 sites on 39 UK rivers found that both the incidence and severity of intersex were significantly correlated with the predicted concentrations of the E_1, E_2, and EE_2 present (Jobling et al. 2006). Steroidal estrogens and alkylphenolic chemicals also bioconcentrate up to 40,000-fold in roach (Larsson et al. 1999, Gibson et al. 2005), enhancing their feminizing effects.

It should be recognized that some estrogenic chemicals other than steroid estrogens and alkylphenolic chemicals may play a role, although a more minor one, in the feminization of roach. WWTW effluents contain a very wide range of chemicals—including plasticizers such as phthalates and bisphenols, and various pesticides and herbicides—that have estrogenic activity in roach. Individually, these chemicals are unlikely to play a significant role in the disruption of sex in wild roach, given their relatively lower estrogenic potency compared with steroidal estrogens; as part of a mixture, however, they may contribute to that disruption. Indeed, *in vivo* studies in the roach and other fish species have shown that estrogenic chemicals in combination have additive feminizing effects (Thorpe et al. 2001, 2006).

A further dimension, and complication, in establishing the chemical causation of sexual disruption in the roach, many WWTW effluents in the United Kingdom have been shown to be strongly antiandrogenic (Environment Agency 2007), and this activity will further increase the feminizing effects of these effluents. Efforts to identify these antiandrogens are now under way, using a targeted fractionation process similar to the one used to identify the estrogenic contaminants but employing a recombinant yeast containing the androgen (rather than the estrogen) receptor. Clearly, to fully appreciate the relative importance of the individual endocrine-disrupting chemicals and the potential impact of effluents on sexual development in roach and other aquatic wildlife, it is essential to consider the concentrations of all the different estrogens and antiandrogens in the effluent, their uptake capability, and their interactive effects.

It is also worth noting that molecular approaches to studying changes in gene expression have shown that the feminizing effects of environmental estrogens and antiandrogens share both common and distinct pathways of effect; this work is starting to provide valuable insights into how some of the chemicals of primary concern cause sex-disrupting effects (Filby et al. 2007a, 2007b, Tyler et al. 2008).

The biological significance of feminized responses in roach

Feminized roach, which can often be found several kilometers downstream from any point of WWTW effluent input, are widespread throughout English rivers. The ecological importance of these feminized responses in roach in UK rivers, however, will depend on whether the reproductive competence of these roach is compromised, and if so, how

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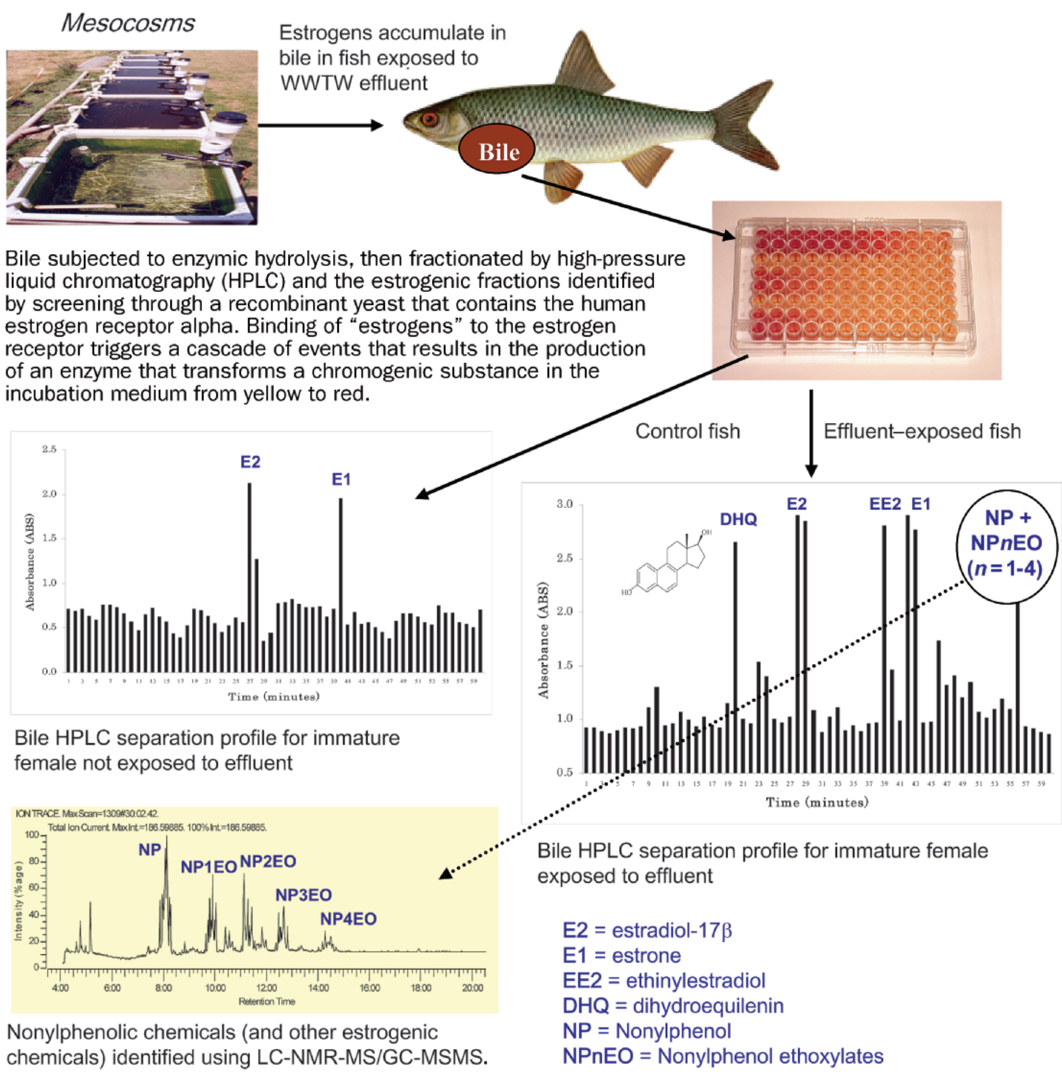

Figure 7. Toxicity identification and evaluation process adopted to identify the mixture of estrogens taken up into fish exposed to effluents from wastewater treatment works and contributing to feminized responses. Data derived from Gibson and colleagues (2005).

this affects the size, structure, and genetic integrity of the population.

VTG induction in wild roach does not necessarily imply an adverse effect on reproductive health, because VTG can be cleared from the blood following removal of the estrogenic stimulus (Liney et al. 2005, Lange et al. 2008). Induction of VTG at very high levels, however, has been associated with the cessation of breeding in some fish species (Thorpe et al. 2007); it has also been shown to induce kidney damage or failure (Herman and Kincaid 1988). Inappropriate VTG induction in roach at an early life stage could result in energetic costs, and this imbalance could potentially affect roach survivorship in the wild.

The ability of intersex roach to produce gametes has been shown to be highly variable, and is dependent on the degree of disruption in the reproductive ducts and altered germ-cell development. Small numbers of wild roach have been found that cannot produce any gametes at all because of the presence of severely disrupted gonadal ducts. In the majority of intersex roach found, viable male gametes are produced, but they are of poorer quality than those from normal males in aquatic environments that do not receive WWTW effluent (Jobling et al. 2002b). Fertilization and hatchability studies have shown that intersex roach even with a low level of gonadal disruption are compromised in their reproductive capacity and produce fewer offspring than roach from uncontaminated sites under laboratory conditions (Jobling et al. 2002b). Jobling and colleagues (2002b) found an inverse correlation between reproductive performance and severity of gonadal intersex (figure 8).

Researchers have also found that male roach living in effluent-contaminated rivers in England have altered sex steroid hormone profiles, altered spawning time, and reduced sperm production (Jobling et al. 2002a), all of which are likely to affect the reproductive capability and success of those individuals. Appropriate timing of gamete synthesis and

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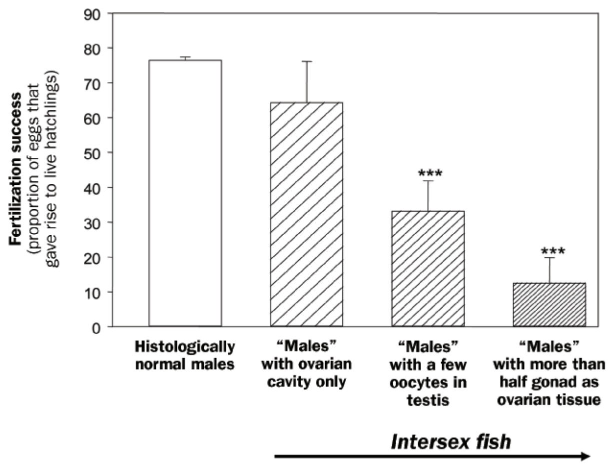

Figure 8. Relationship between the degree of sexual disruption and reproductive performance, defined by the proportion of eggs that gave rise to live hatchlings, in wild roach (*Rutilus rutilus*) sampled from the River Arun. Intersex fish were grouped into three classes according to the degree of severity of the intersex condition: (a) feminized ducts only, (b) gonads containing some oocytes, (c) 50% or more of the gonad is female tissue. Asterisks represent significant (*p < 0.001) differences between intersex fish and males. Adapted from Jobling and colleagues (2002b).

preparation for their release is vital for reproductive success in seasonally breeding roach. Furthermore, researchers have also found that female roach living in rivers heavily contaminated with treated effluent have a higher incidence of ovarian atresia, potentially reducing the number of eggs produced (Jobling et al. 2002b).

Laboratory studies on a variety of other fish species have further shown that exposure to environmental estrogens, antiandrogens, and estrogenic effluents can alter normal breeding behavior, and this too can affect the reproductive success of individuals. Data generated from studies in the zebrafish (Nash et al. 2004) have also implied that reproductively compromised males can disrupt the normal interactions of healthy males with females, thereby impairing their ability to breed successfully in breeding colonies.

To gain a more complete understanding of the impact of sexual disruption on the reproductive fitness of individuals, we recently initiated parentage studies using DNA microsatellites in fish populations that include either intersex roach or species that have been treated with environmental estrogens to disturb the normal pathway of sexual development (zebrafish). Through these breeding experiments, we hope to find out how well affected males compete with one another to breed with females, and to establish how the genetics of the population may be affected.

The question of whether wild populations of roach are adversely affected by endocrine disruption has yet to be resolved. However, given that a large proportion of the males are intersex in many populations in English rivers, and that estrogenic chemicals can affect reproductive behavior, there is a strong likelihood of population-level effects. Individual-level consequences of toxicant exposure can be weak predictors of population-level consequences (Forbes and Calow 1999), and extrapolating the effects of sexual disruption from the individual to the population is the most challenging—but arguably the most needed—direction for studies of endocrine disruption. Modeling approaches have been proposed to support investigations into the potential for population-level effects of sexual disruption in roach. Extensive life-table data sets are available for the roach from some of the studies reported above and in data banks held by the UK Environment Agency as part of UK monitoring programs. Attempts are now under way to gather all of these data and to build appropriate models, but these data sets are far from complete, and the challenges to produce effective models with strong predictive powers for intersex effects on wild populations are still considerable.

Even in the absence of data on population-level effects, the likelihood for harm to the fishery in English rivers is sufficient for the UK Environment Agency to act on the precautionary principle to remediate the effects of endocrine-disrupting chemicals (EDCs) discharged through WWTW. The UK Environment Agency and UK water companies are now jointly investigating the effectiveness of available treatment technologies for EDC removal in WWTW. At selected WWTW sites, they are also implementing enhanced treatment processes. Biological and chemical monitoring programs on the effluents subsequently discharged, and the responses in wild roach populations downstream of these WWTW, will soon tell us how successful the remediation process has been for the fishery.

Concluding thoughts

Although significant advances have been made in developing our understanding of many aspects of the feminization process in roach in English rivers, even after 25 years of study we are not able to say with any degree of certainty that there are substantive population-level changes in abundance or composition in wild populations as a result of EDC exposure. This does not mean that they have not occurred, but simply that we do not have data that are sufficiently comprehensive to make that judgment. Evidence from the field suggests that in some regions of the United Kingdom, recruitment of roach into the fishery has declined, but these observations may be due to changes in the nutrient composition of the river, habitat alterations, or many other factors. Indeed, even in the rivers most heavily affected by estrogenic WWTW effluents in England—rivers in which all of the male roach have been feminized to a greater or lesser extent—recruitment of roach is still observed. This finding, however, is complicated by the fact that restocking with roach is a common occurrence in these rivers, most notably after pollution incidents.

Enhancing our understanding of the impacts of EDCs on roach populations requires more comprehensive information on the species' normal population dynamics, ideally over ex-

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tensive time periods, as roach are reasonably long-lived (10 years or more). The monitoring programs the UK Environment Agency has operated for roach do not provide data that are sufficiently comprehensive for interpretations of the population-level effects of pollutants. Furthermore, most of these fish monitoring programs are no longer operating for English rivers.

In addition to knowledge of the population dynamics of wild roach, we need more complete knowledge of roach breeding biology to understand the implications of EDC exposure on roach populations. For example, to assess the level (and degree) of intersex that can be sustained by a roach population, we need to know how many individuals normally contribute to the breeding process. If, perhaps, a relatively low percentage of the males contribute their genes to the next generation, as is quite likely in comparison with other animal species, then it might be assumed that a reasonably high level of intersex could be tolerated in the population. Yet this is a simplistic view, and abundance is not the only consideration in the long-term maintenance of a viable and healthy population. Laboratory findings indicating that environmental estrogens can alter the breeding dynamics of fish, for example, potentially have major implications for dominance and the normal sexual selection process, if it occurs in wild roach populations.

In conclusion, our research into the feminization of roach in English rivers has significantly advanced understanding of the effects of estrogenic chemicals on the sexual development and function of fish, and, more widely, it has heightened significantly our awareness of the more subtle effects of environmental pollutants discharged into the environment. Nevertheless, the key question, What does it all means for the population?—a question that could be applied equally to most potentially harmful or potentially harmful chemicals discharged into the environment—has yet to be answered. This is a research challenge that we are now actively seeking to meet in our work on roach.

Other fascinating questions that arise from our work on feminized roach in English rivers include whether roach are adapting to the environmental estrogen exposure over time (and, if so, how their endocrine physiology is changed to accommodate this adaptation), and whether estrogen exposure is affecting the sexual selection process. To address these questions fully will most likely take at least 25 more years of research.

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