Environmental Science & Technology

ARTICLE

pubs.acs.org/est

Implications of Persistent Exposure to Treated Wastewater Effluent for Breeding in Wild Roach (*Rutilus rutilus*) Populations

Anke Lange,[*,†] Gregory C. Paull,[†] Patrick B. Hamilton,[†] Taisen Iguchi,[‡] and Charles R. Tyler[*,†]

[†]University of Exeter, Biosciences, College of Life & Environmental Sciences, Exeter EX4 4PS, United Kingdom

[‡]Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, National Institutes of Natural Sciences, and Department of Basic Biology, School of Life Science, Graduate University for Advanced Studies, 5-1 Higashiyama, Myodaiji, Okazaki 444-8787, Japan

Supporting Information

ABSTRACT: Feminized responses are widespread in wild populations of roach, *Rutilus rutilus*, living in UK rivers, and some of these responses have been shown to arise as a consequence of exposure to wastewater treatment works (WwTW) effluent discharges and the endocrine disrupting chemicals (EDCs) they contain. The causation of the ovotestis condition in wild roach, however, has yet to be established. Furthermore, the impact of long-term exposure to WwTW effluents on the reproductive fitness of wild fish populations is not known, and this information is crucial for population level effect assessments. We undertook a chronic exposure of roach to a treated estrogenic wastewater effluent for up to 3.5 years to assess principally for effects on subsequent reproductive fitness, as determined through parentage analysis on offspring from a competitive breeding study. In generating the fish for the breeding study we found that exposure to full strength WwTW effluent until sexual maturity resulted in sex reversal in almost all males in the population; 98% of the exposed fish were phenotypic females, containing ovaries. Furthermore, fish exposed to a 50% dilution of WwTW effluent contained ovotestis (21% of the male roach) that was absent from the control population. In competitive breeding studies, and applying DNA microsatellites to assess parentage, we show that presumptive females exposed to sexual maturity to WwTW effluent bred normally, albeit in the absence of nonexposed females, but putative sex-reversed males breeding as females contributed poorly, if at all, in a breeding population, depending on the competition. These novel findings on sex reversal add a new dimension for impact assessments of exposure to WwTW effluents on fish populations.

INTRODUCTION

In wildlife, and to a lesser extent in humans, there is global evidence for adverse effects of exposure to endocrine disrupting chemicals (EDCs) (reviewed in ref 1). A wide range of EDCs have been identified, spanning steroid hormones, plasticizers, bisphenols, alkylphenols, pesticides, and many others,[2] and they act through a variety of mechanisms to alter hormone function. Most endocrine disrupting effects in wildlife have been established in populations living in or closely associated with the aquatic environment and effects in fish include delayed onset of sexual maturation, reduced gonadal growth, gonadal deformations, inhibition of spermiogenesis, lowered egg production and intersex, where gonads contain both male and female reproductive tissue (reviewed in ref 3). In the case of feminized wild roach (*Rutilus rutilus*) collected from UK rivers, they show reduced gamete quality[4] with the potential for adverse effects on breeding. Feminized phenotypes in wild male fish, including intersex (ovotestis), have been associated with point source wastewater effluent (WwTW) discharges, but only vitellogenin induction and feminized reproductive ducts have been induced experimentally in males exposed to these effluents.[5−7] Some of these feminized phenotypes in fish are also induced by steroidal estrogens at concentrations found in some WwTW effluent discharges.[8]

Roach are group spawners, a tactic most commonly adopted among freshwater cyprinids and although little work has been conducted on the intricacies of their specific mating behavior one study suggests they may adopt a lek-like mating system[9] where males gather on the spawning ground and establish dominance hierarchies. Sexual selection is strong in lek-like mating systems and choice of mates by females is not random.[10,11] Even though females choose males that hold a territory, multimale fertilizations are common.[9] Various EDCs have been shown to affect reproductive behaviors in fish[12,13] and in some cases to alter parentage outcomes in group spawning fish (e.g., zebrafish[14]).

Attempts have been made to predict effects of individual EDCs (through lab-based exposures) on fish populations,[15−18] and modeling approaches have been applied to investigate for the effect of compromised reproductive fitness, as a result of endocrine disruption, on fish populations.[19,20] Outcomes from these studies however, are extremely limited as none have taken into account the full spectrum of disruptions that might occur in exposed populations, including effects on reproductive behavior. This shortfall is, in part, due to the lack of required data to do so. The impacts of continuous, life-long exposure to EDCs or their mixtures on the reproductive fitness of wild fish populations are therefore not known. To start to address some of these key gaps in the data,

Received: September 22, 2010
Accepted: December 13, 2010
Revised: December 7, 2010
Published: January 5, 2011

we undertook a chronic exposure of roach (3.5 years in duration) to treated estrogenic wastewater effluent to assess the impacts on reproductive fitness as adults, assessed through parentage analysis on offspring resulting from a competitive breeding study.

MATERIAL AND METHODS

Long-Term Effluent Exposure. From 35 days posthatch (dph), roach were exposed to river water, 50% or 100% effluent for a period of up to three and a half years under ambient temperature and photoperiod and their gonadal development monitored at various intervals. The overall design of the exposure is shown in Figure S1 (Supporting Information). Detailed descriptions of the fish origin and husbandry, exposure, and the histological monitoring of gonadal development are also provided in the Supporting Information.

Breeding Experiment and Parentage Analyses. The competitive breeding experiment comprised of four different scenarios (Figure S1, Supporting Information), each of which was run in duplicate. The male:female sex ratio adopted was conducted to ensure competition for the females between the males. Fish were allowed to breed naturally and spawning occurred in six out of the eight tanks. Within six days of spawning, all fertilized eggs were removed and transported to the laboratory. Hatched fry were randomly sampled for parentage analysis, gonads of parental fish were sampled for histopathology, and fin clips were collected from all fish to subsequently identify individual reproductive success in the parentage assessments. A detailed description of the breeding experiment, subsequent sampling, and details on the parentage analyses is provided in the Supporting Information.

RESULTS

Feminized Gonadal Development in Roach Exposed to Effluent. The principal objective of this study was to assess the impacts of long-term exposure to a WwTW effluent on reproductive fitness in roach. In generating these fish, however, we monitored their sexual development in the different treatment regimes, and here we briefly report on those findings. During early life (until 161 dph, corresponding to 126 days of exposure) all males exposed to 100% effluent showed feminized responses (Figures 1and S2−S5). Distinction between presumptive sex at the earlier life stages (67 and 91 dph) was based on the shape of the gonad, the number of points of attachment of the gonad to the peritoneal wall, and the distribution of somatic cells relative to primordial germ cells (for details see ref 21). All fish exposed to 100% effluent ($n = 59$ fish) had female-like duct characteristics, in contrast to those kept in river water ($n = 53$ fish) where 40% could be assigned as males and 53% as females (the sex of remaining fish were not clearly identifiable at this time). In the 50% WwTW exposure regime ($n = 58$ fish), 21% of the fish analyzed were males, 29% females and the remaining 50% of the fish could not be assigned to either gender (based on the sex cells), but they all had a female-like duct. In the males exposed to 50% effluent, 20% showed morphological characteristics of males (sex cells) and females (ovarian-like cavity). Fish in the 50% and 100% effluent exposure groups showed both time- and concentration-related trends for the induction of the estrogen dependent yolk precursor vitellogenin (Figure S6, Supporting Information).

Male fish exposed to 50% effluent for periods beyond 161 dph contained ovotestes. Indeed, more than half of the males exposed to 50% effluent, sampled at 390 dph and 1061 dph, had ovotestes,

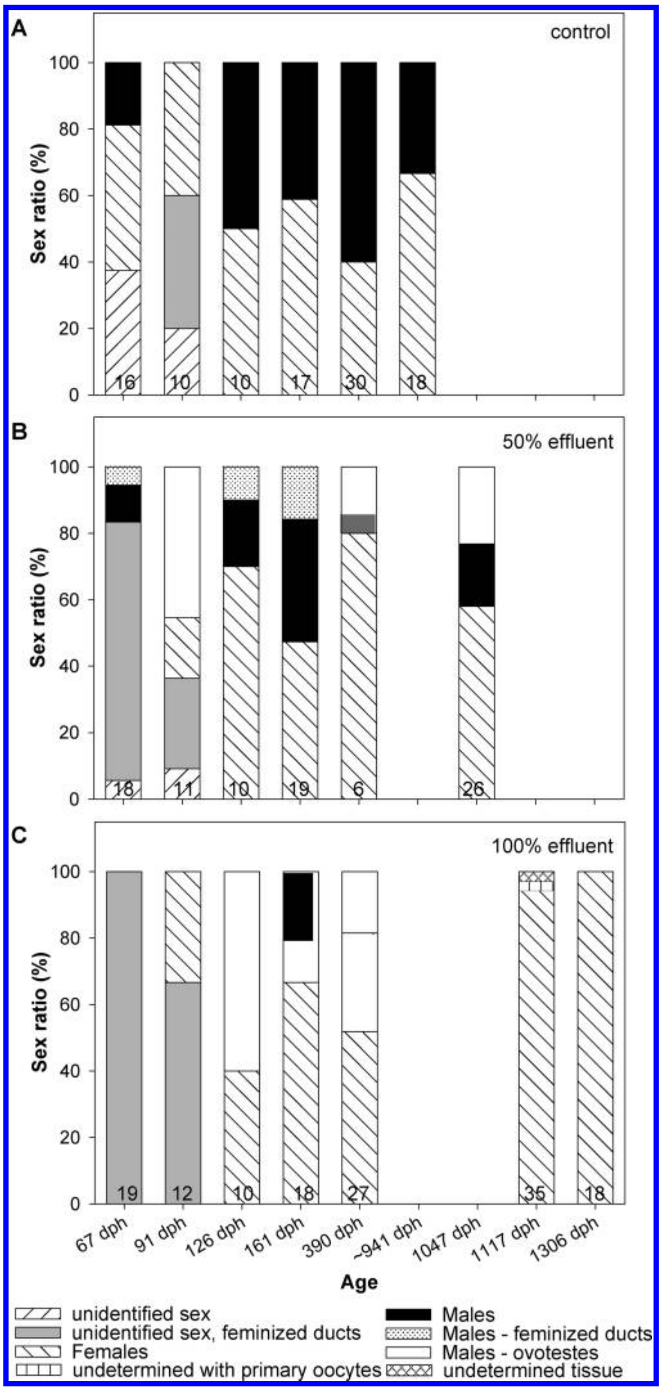

Figure 1. Observations on the effects of long-term exposure to WwTW effluent on gonadal sex in roach: (A) nonexposed fish, (B) fish exposed to 50% effluent, and (C) fish exposed to 100% effluent. Exposure to 50% effluent was conducted up to 1047 dph (approx 3 years) and fish exposed to 100% effluent until 1306 dph (approx 3.5 years). From 390 dph onward, sex was determined based on the sex cells present in gonads. Numbers in each bar represent the number of fish analyzed from each treatment at that time point. Due to the loss of the original nonexposed fish caused by a white spot infection during the course of the experiment, these were replaced with roach derived from parent fish from the same river as the original experimental fish.

albeit they were mildly intersex (a few oocytes per gonadal section). Analyses at these life stages were conducted on dissected gonads, and thus it was not possible to score duct phenotypes. Nevertheless, previous work has shown that induced feminization of ducts during early life in roach is irreversible[22,23]). At 390 dph, we found that roach exposed for almost one year to 100% WwTW effluent still maintained a near 1:1 sex ratio of females to males (52% females and 48% males). However, all of these males had feminized reproductive ducts and 31% had ovotestes. After a further two and a half years of exposure to 100% WwTW effluent (1306 dph), and corresponding to the end of the effluent exposure period, none of the roach examined (18 fish in total) contained any discernible testicular tissue suggesting an all-female population (Figures 1 and 2). In a further 35 fish that were exposed to 100% effluent and subsequently used for the breeding study, only 2 fish contained gonadal tissue that was not well-defined, but may have been testicular in nature (see Figure S12, Supporting Information).

Impacts of Long-Term Effluent Exposure on Breeding Success. During the setting up of the breeding experiment, the sex of the control fish was readily distinguishable externally based on body shape and secondary sex characteristics (urogenital papilla and skin roughness - presence of breeding tubercles). Effluent-exposed fish were sexed based on their body morphology only, because there was no obvious development of breeding tubercles in males and there were no obvious differences in the urogenital papilla between the sexes.

Gonadal histopathology conducted on fish from the breeding experiment showed that gonads of most nonexposed fish (35 out of 36) were likely to have spawned, based on the stages of the germ cell present. Testes contained cysts of spermatogonia (predominantly spermatogonia A, but spermatogonia B were also present) and ovaries contained predominantly primary oocytes and postovulatory follicles. No abnormal developmental features were observed (Figures S7, S9−S10, S13−S16, Supporting Information). For effluent-exposed fish, histological analyses revealed that 33 out of 35 were phenotypic females. The remaining two fish contained gonadal material that was testicular-like in nature but was nevertheless atypical (see Figure S12, Supporting Information). For the 33 `females' with an ovarian phenotype from the effluent exposure group, there were essentially two histological gonadal phenotypes: (1) gonads characterized by the predominance of primary oocytes, but also containing postovulatory follicles and, (2) gonads characterized by the presence of large numbers of atretic secondary oocytes, indicating that these fish may not have spawned (Figures S8, S11−S16, Supporting Information). Primary oocytes were the predominant cell type in the gonads of presumptive males (defined on their prespawning body shape).

Spawning occurred in both replicate tanks for three of the four breeding scenarios (Figure 3); fish in the tanks that contained all effluent-exposed fish failed to reproduce, explained by the all-female gonadal phenotypes seen for this scenario. Parentage analysis revealed multimale (and multifemale) fertilization for most of the other tanks. All of the nonexposed females and effluent-exposed presumptive females reproduced, and 17 of the 24 (71%) nonexposed males sired offspring.

In both tanks that contained nonexposed fish only (scenario 1), five of the six males sired offspring. The individual reproductive success (proportion of offspring sired) for these fish ranged between 2 and 39% (Figure 3A,B). The smallest male in each control tank failed to sire any offspring, although the success of all males did not depend on body size, as shown using a linear mixed effects model using tank as a random factor (Likelihood Ratio Test $p = 0.46$). However, the size variation of these males was limited in these two tanks, with body lengths ranging from 11.0 to 12.9 cm and from 11.7 to 13.2 cm, respectively. In the breeding groups consisting of presumptive effluent-exposed females and a combination of nonexposed and effluent-exposed presumptive males (scenario 4), nonexposed males sired all of the offspring sampled. All presumptive effluent-exposed females spawned, and their contributions to the breeding outcome ranged between 25 and 39% in replicate 1 and 24−47% in replicate 2. In one replicate, one nonexposed male sired all of the resulting fry, whereas in the second replicate, all three nonexposed males sired between 2 and 55% of the offspring (Figure 3E,F). In tanks containing nonexposed females and a combination of nonexposed and effluent-exposed presumptive males (scenario 3), nonexposed males again sired 100% of the offspring and individual success of these nonexposed males in both replicate tanks ranged between 29 and 40% and 0−73%, respectively. Interestingly, fish that were considered to be effluent-exposed presumptive males reproduced as females, albeit at a much lower level compared with the control females in these tanks (0−2% compared with 4−48% in replicate 1, and 4−17% compared to 2−40% in replicate 2; Figure 3C,D)).

■ DISCUSSION

In this experiment, we showed that exposure of roach to a treated WwTW effluent from early life to sexual maturity altered sexual (germ cell) development in males, and the degree of effect appeared to be related to the concentration of the effluent exposure and the longevity of exposure. Extensive studies on this effluent over a period of ten years have shown it to be estrogenic to fish, with total estrogenicity (as assessed in the recombinant yeast screen[24]) ranging between 11.1 ng and 38.2 ng E2EQ/L (with individual steroid estrogen concentrations of 15−195 ng/L for estrone; 4−88 ng/L estradiol and 1.5−7.9 ng/L ethinylestradiol).[22,23,25,26] We further show that exposure to a full strength WwTW effluent resulted in essentially an all-female population. The apparent cumulative feminizing effect of WwTW effluent on males supports studies in wild roach populations where the incidence of intersex and the degree of ovotestis (in English rivers) are highly positively correlated with age.[27,28] Furthermore, some of the phenotypic females in the long term exposure to full strength WwTW effluent showed inappropriate timing of gamete maturation that would consequently result in their reproductive failure in the wild; roach populations normally breed once annually at a well-defined seasonal time point (in late April to early May). Some of the presumptive effluent-exposed females also appeared to show a greater level of ovarian atresia, but contrasting with the impacts in males, they were able to breed, albeit this was assessed for females in the absence of any competition with nonexposed females.

Observations on WwTW Effluent Exposure Effects on Testicular Development. After three years of exposure to 50% WwTW effluent, more than half of the exposed males were intersex, albeit the condition was relatively mild (individual primary oocytes were scattered throughout otherwise `normal' testicular tissue). Previous studies on intersex roach have shown that gametes produced in fish with moderate to severe intersex condition are of lower quality but appear relatively normal for mildly intersex fish.[29] In the wild, however, roach can live more than 10 years[30] and, given the findings for the exposure of males to full strength treated effluent, longer exposures to a 50% effluent concentration is likely to induce more severe effects progressively with age. Furthermore, it is not known whether exposure to effluent affects behaviors which could potentially affect their breeding capability in natural populations.

In the population exposed to full strength WwTW effluent for three years almost all males had undergone a complete phenotypic

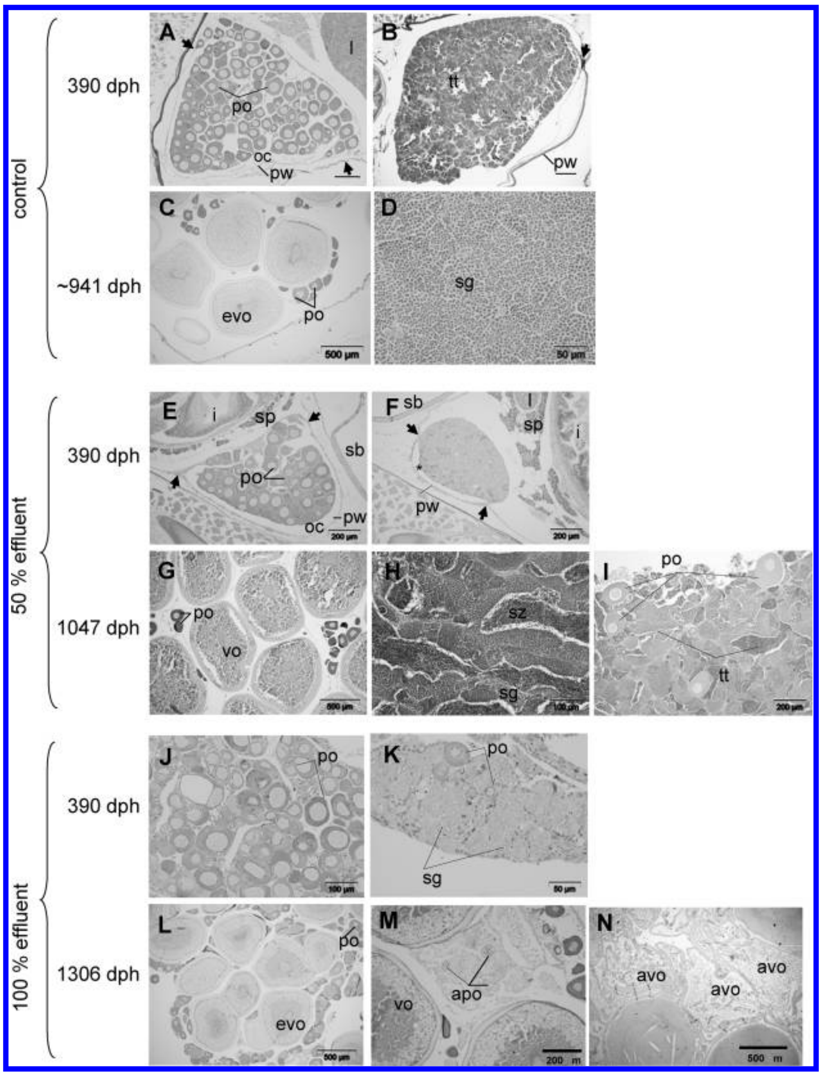

Figure 2. Representative gonad sections of fish exposed to dilution water, 50% and 100% effluent from 390 dph until the termination of individual exposure treatments. A-D: gonads of control females (left) and males (right) at 390 and ~955 dph. E-I: gonads of fish exposed to 50% effluent (E,F — ovary and testes, respectively at 390 dph; G — ovary at 1047 dph; H,I — normal testis and ovotestis, respectively at 1047 dph); J-N: gonads of fish exposed to 100% effluent (J — ovary and K — ovotestis at 390 dph; L-M — ovaries at 1306 dph). apo/avo, atretic primary/vitellogenic oocytes; I, intestine; oc, ovarian cavity; po, primary oocyte; pw, peritoneal wall; sb, swim bladder; so, secondary oocyte; sp, spleen; tt, testicular tissue; vo, vitellogenic oocyte; sg, spermatogonia; sz, spermatozoa; *, ovarian-like cavity.

sex reversal. In a laboratory study exposing roach to 17α-ethinylestradiol at 4 ng/L (a concentration found in some of the more polluted WwTW effluents) from fertilization to 720 dph all the resulting fish also contained ovaries only. In that study there were two gonadal cohorts in terms of the developmental status of the germ cells, one identical with nonexposed females, and one, which was presumed to be sex-reversed males, at a significantly less advanced stage,[8] but there was no such clear distinction in the presumptive sex-reversed males compared with females in the effluent-exposed fish in this study. Interestingly, studies on roach populations in UK rivers heavily polluted with estrogenic WwTW effluents have reported populations with a sex ratio skewed toward females.[31]

Observations on WwTW Effluent Exposure Effects on Ovarian Development. Follicular atresia is a common degenerative process in the ovary of fish and can be observed at all stages of the reproductive cycle, but it normally occurs most frequently during the postspawning period.[32] Despite the clear pattern of female development, gonads of several fish exposed continuously to 100% effluent for 3.5 years showed severe disruption and contained degenerative primary and secondary oocytes. Furthermore, the presence of oocytes close to full maturity in some of the effluent-exposed fish sampled in late autumn suggested either a retarded maturation rate (oocytes should have been ovulated in the previous spring) or enhanced maturation rate (oocytes to mature in the following spring), and any such effects in wild populations could have major breeding ramifications for the individual affected. As there is no genetic sex probe available that we could apply for sexing the roach, we are unable to say whether fish most affected for these conditions were sex-reversed males. The reasons for the altered timing in oocyte development in some of the effluent-exposed fish may be because of abnormal endocrine signaling and/or due to the lack of phenotypic males in the population to drive the synchronization of the final maturation and spawning process. A higher prevalence of oocyte atresia has been reported previously in female roach living in effluent-contaminated rivers, and these fish also had altered sex steroid hormone profiles compared to females from reference sites.[29]

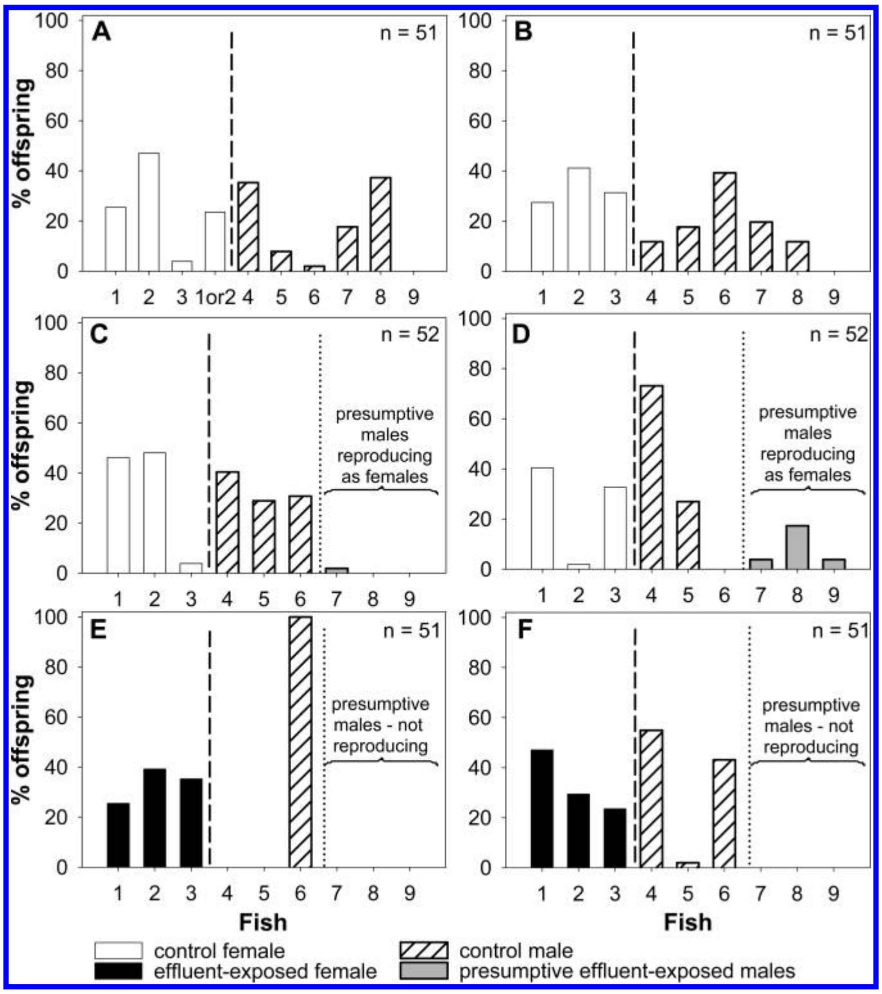

Figure 3. Reproductive success of females and males in the different breeding scenarios. The experiment consisted of four different breeding scenarios each containing 3 females and 6 `males' with different combinations of nonexposed and effluent-exposed fish. Fish were allowed to breed naturally and resulting fry were sampled at 4 dph for DNA microsatellite analysis and parentage assignment. Results are shown for scenario 1 (all nonexposed fish; A,B); scenario 3 (nonexposed females x nonexposed + presumptive effluent-exposed males; C,D); and scenario 4 (presumptive effluent-exposed females x nonexposed + presumptive effluent-exposed males; E,F). No breeding occurred in scenario 2 (all effluent-exposed fish). For each tank, fish originating from the different exposures are ranked by size with the first fish being the largest and the last the smallest, left to right. In (A) 12 fry could not be assigned clearly to one of the three females. Dashed lines on the figures separate females from males and dotted lines separate control from effluent-exposed presumptive males.

Effects of WwTW Effluent Exposure on Breeding Success. In the competitive breeding studies, all females and the majority of males bred successfully in the control tanks, albeit that the relative contribution of individuals varied widely. It is known that multimale and indeed multifemale fertilizations occur in roach;[9] however, the variability in reproductive success is not known for this species. In mating systems with elaborate male—male competition, the winners usually get most mates and sire most of the offspring, but female choice may also play a role.[33]

The finding that females exposed to full strength effluent throughout their lives were able to reproduce successfully (with control males) is important given the degree to which female breeding success and life-history traits drive population demographics. Some fish derived from the full strength effluent exposure designated as `effluent-exposed presumptive males' reproduced as females, but they did so at much lower success rates compared with nonexposed females in those tanks; the relative proportion of fry produced by the effluent-exposed presumptive males combined was 2 and 25% in the two replicate tanks compared to 98 and 75% for nonexposed females. In the breeding scenarios with presumptive sex-reversed males (phenotypical females), with presumptive effluent-exposed females and nonexposed males, none of the presumptive sex-reversed males contributed to the offspring produced in either of the replicate tanks, indicating a lack of breeding compatibility and/or capability under competition. A reduced reproductive success in the presumptive sex-reversed males aligns with studies in other fish species where sex reversal has been induced experimentally via exposure to estrogenic chemicals.[34,35]

Implications for Roach Populations. The ramifications of our findings for the sustainability of wild roach populations are far from clear. Recent modeling approaches suggest that environmental feminization could potentially result in moderate population increases due to female-biased sex ratios (increased reproductive potential).[20] We know nothing, however, about the ability of the offspring derived from sex-reversed males to reproduce. More severe environmental feminization could reduce the number of males (and/or their ability to reproduce) to a point where this factor limits population growth and could even lead to population declines.[20] Whether/when this becomes a deciding factor for roach populations will depend on how many males normally contribute

dx.doi.org/10.1021/es103232q |Environ. Sci. Technol. 2011, 45, 1673–1679

in the breeding process, proportional success of individuals, and other factors. An important issue that has not been considered in all of the work reported on this nature to date is the potential loss of genetic variability in populations containing feminized fish due to a decrease in the effective population size that may occur. Genetic diversity is fundamentally important for the sustainability of healthy populations and to allow for adaptation to contaminated environments or other changes to their habitats.[36,37]

In conclusion, we have shown that life-long exposure of roach to an estrogenic WwTW effluent appears to induce complete sex reversal of males, resulting in an all-female population, but that putative sex-reversed individuals breeding as females contribute poorly, if at all, in a breeding population, depending on the competition. Histological assessment of the effluent-exposed roach has also provided strong support that WwTW effluent can induce all of the feminized phenotypes, including the formation of ovotestis that occur in males in wild populations living in UK rivers. Our findings likely represent what may occur for wild roach exposed to WwTW effluents under some of the worst case scenarios, but they nevertheless present new considerations for assessments on the impacts of WwTW effluent discharges on fish populations.

■ ASSOCIATED CONTENT

S Supporting Information. Histopathology of gonadal development (nonexposed and exposed fish) and histological appearance of pre- and postspawning gonads from control fish used for breeding. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +44.1392.269298 (A.L.); +44.1392.264450 (C.R.T.); Fax: +44.1392.263700; E-mail: A.Lange@exeter.ac.uk (A.L.); C.R.Tyler@exeter.ac.uk (C.R.T.).

■ ACKNOWLEDGMENT

We thank the following for their support of this work: members of the Environmental and Molecular Fish Biology group at the University of Exeter, Alan Henshaw and his team at Calverton Fish Farm (UK Environment Agency), and Afsaneh Janbakhsh and David Walker at Essex and Suffolk Water. This work was funded by the UK Natural Environmental Research Council (NE/D002818/1 and within the Environmental Genomics Programme (NER/T/S/2002/00182)), DEFRA and the UK Environment Agency to CRT, and a grant in support of the United Kingdom and Japan Cooperation Research from the Ministry of the Environment, Japan to TI.

■ REFERENCES

(1) Hotchkiss, A. K.; Rider, C. V.; Blystone, C. R.; Wilson, V. S.; Hartig, P. C.; Ankley, G. T.; Foster, P. M.; Gray, C. L.; Gray, L. E. Fifteen years after "Wingspread" - Environmental endocrine disrupters and human and wildlife health: Where we are today and where we need to go. *Toxicol. Sci.* **2008,** *105*, 235–259.

(2) Jobling, S.; Nolan, M.; Tyler, C. R.; Brighty, G.; Sumpter, J. P. Widespread sexual disruption in wild fish. *Environ. Sci. Technol.* **1998,** *32*, 2498–2506.

(3) Scholz, S.; Kluver, N. Effects of endocrine disrupters on sexual, gonadal development in fish. *Sex. Dev.* **2009,** *3*, 136–151.

(4) Jobling, S.; Coey, S.; Whitmore, J. G.; Kime, D. E.; Van Look, K. J. W.; McAllister, B. G.; Beresford, N.; Henshaw, A. C.; Brighty, G.; Tyler, C. R.; Sumpter, J. P. Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biol. Reprod.* **2002,** *67*, 515–524.

(5) Diniz, M. S.; Peres, I.; Magalhaes-Antoine, I.; Falla, J.; Pihan, J. C. Estrogenic effects in crucian carp (*Carassius carassius*) exposed to treated sewage effluent. *Ecotox. Environ. Safe* **2005,** *62*, 427–435.

(6) Diniz, M. S.; Peres, I.; Pihan, J. C. Comparative study of the estrogenic responses of mirror carp (*Cyprinus carpio*) exposed to treated municipal sewage effluent (Lisbon) during two periods in different seasons. *Sci. Total Environ.* **2005,** *349*, 129–139.

(7) Rodgers-Gray, T. P.; Jobling, S.; Kelly, C.; Morris, S.; Brighty, G.; Waldock, M. J.; Sumpter, J. P.; Tyler, C. R. Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. *Environ. Sci. Technol.* **2001,** *35*, 462–470.

(8) Lange, A.; Paull, G. C.; Coe, T. S.; Katsu, Y.; Urushitani, H.; Iguchi, T.; Tyler, C. R. Sexual reprogramming and estrogenic sensitization in wild fish exposed to ethinylestradiol. *Environ. Sci. Technol.* **2009,** *43*, 1219–1225.

(9) Wedekind, C. Lek-like spawning behaviour and different female mate preferences in roach (*Rutilus rutilus*). *Behaviour* **1996,** *133*, 681–695.

(10) Jacob, A.; Evanno, G.; Renai, E.; Sermier, R.; Wedekind, C. Male body size and breeding tubercles are both linked to intrasexual dominance and reproductive success in the minnow. *Anim. Behav.* **2009,** *77*, 823–829.

(11) Kortet, R.; Vainikka, A.; Rantala, M. J.; Jokinen, I.; Taskinen, J. Sexual ornamentation, androgens and papillomatosis in male roach (*Rutilus rutilus*). *Evol. Ecol. Res.* **2003,** *5*, 411–419.

(12) Larsen, M. G.; Hansen, K. B.; Henriksen, P. G.; Baatrup, E. Male zebrafish (*Danio rerio*) courtship behaviour resists the feminising effects of 17α-ethinyloestradiol - morphological sexual characteristics do not. *Aquat. Toxicol.* **2008,** *87*, 234–244.

(13) Martinovic, D.; Hogarth, W. T.; Jones, R. E.; Sorensen, P. W. Environmental estrogens suppress hormones, behavior, and reproductive fitness in male fathead minnows. *Environ. Toxicol. Chem.* **2007,** *26*, 271–278.

(14) Coe, T. S.; Hamilton, P. B.; Hodgson, D.; Paull, G. C.; Tyler, C. R. Parentage outcomes in response to estrogen exposure are modified by social grouping in zebrafish. *Environ. Sci. Technol.* **2009,** *43*, 8400–8405.

(15) Grist, E. P. M.; Wells, N. C.; Whitehouse, P.; Brighty, G.; Crane, M. Estimating the effects of 17α-ethinylestradiol on populations of the fathead minnow *Pimephales promelas*: Are conventional toxicological endpoints adequate? *Environ. Sci. Technol.* **2003,** *37*, 1609–1616.

(16) Meng, Y. B.; Lin, B. L.; Tominaga, M.; Nakanishi, J. Simulation of the population-level effects of 4-nonylphenol on wild Japanese medaka (Oryzias latipes). *Ecol. Modell.* **2006,** *197*, 350–360.

(17) Miller, D. H.; Ankley, G. T. Modeling impacts on populations: fathead minnow (*Pimephales promelas*) exposure to the endocrine disruptor 17β-trenbolone as a case study. *Ecotox. Environ. Safe* **2004,** *59*, 1–9.

(18) Raimondo, S.; Hemmer, B. L.; Goodman, L. R.; Cripe, G. M. Multigenerational exposure of the estuarine sheepshead minnow (*Cyprinodon variegatus*) to 17β-estradiol. II. Population-level effects through two life cycles. *Environ. Toxicol. Chem.* **2009,** *28*, 2409–2415.

(19) An, W.; Hu, J. Y.; Giesy, J. P.; Yang, M. Extinction risk of exploited wild roach (*Rutilus rutilus*) populations due to chemical feminization. *Environ. Sci. Technol.* **2009,** *43*, 7895–7901.

(20) Cotton, S.; Wedekind, C. Population consequences of environmental sex reversal. *Conserv. Biol.* **2009,** *23*, 196–206.

(21) Paull, G. C.; Lange, A.; Henshaw, A. C.; Tyler, C. R. Ontogeny of sexual development in the roach (*Rutilus rutilus*) and its interrelationships with growth and age. *J. Morphol.* **2008,** *269*, 884–895.

(22) Liney, K. E.; Jobling, S.; Shears, J. A.; Simpson, P.; Tyler, C. R. Assessing the sensitivity of different life stages for sexual disruption in roach (*Rutilus rutilus*) exposed to effluents from wastewater treatment works. *Environ. Health Perspect.* **2005,** *113*, 1299–1307.

(23) Rodgers-Gray, T. P.; Jobling, S.; Morris, S.; Kelly, C.; Kirby, S.; Janbakhsh, A.; Harries, J. E.; Waldock, M. J.; Sumpter, J. P.; Tyler, C. R. Long-term temporal changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. *Environ. Sci. Technol.* **2000,** *34*, 1521–1528.

(24) Routledge, E. J.; Sumpter, J. P. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ. Toxicol. Chem.* **1996,** *15*, 241–248.

(25) Environment Agency. *Biological effect measures in fish- application to treated sewage effluent*; Bristol, UK, 2007; p 36.

(26) Thorpe, K. L.; Maack, G.; Benstead, R.; Tyler, C. R. Estrogenic wastewater treatment works effluents reduce egg production in fish. *Environ. Sci. Technol.* **2009,** *43*, 2976–2982.

(27) Jobling, S.; Williams, R.; Johnson, A.; Taylor, A.; Gross-Sorokin, M.; Nolan, M.; Tyler, C. R.; van Aerle, R.; Santos, E.; Brighty, G. Predicted exposures to steroid estrogens in U.K. rivers correlate with widespread sexual disruption in wild fish populations. *Environ. Health Perspect.* **2006,** *114*, 32–39.

(28) Tyler, C. R.; Jobling, S. Roach, sex, and gender-bending chemicals: The feminization of wild fish in English rivers. *Bioscience* **2008,** *58*, 1051–1059.

(29) Jobling, S.; Beresford, N.; Nolan, M.; Rodgers-Gray, T.; Brighty, G. C.; Sumpter, J. P.; Tyler, C. R. Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biol. Reprod.* **2002,** *66*, 272–281.

(30) Vøllestad, L. A.; L'Abée-Lund, J. H. Reproductive biology of stream-spawning roach, *Rutilus rutilus*. *Environ. Biol. Fishes* **1987,** *18*, 219–227.

(31) Environment Agency. *The identification of oestrogenic effects in wild fish—Phase II. R&D Technical Report W2-014/TR*; Bristol, UK, 2002; p 102.

(32) Miranda, A. C. L.; Bazzoli, N.; Rizzo, E.; Sato, Y. Ovarian follicular atresia in two teleost species: a histological and ultrastructural study. *Tissue Cell* **1999,** *31*, 480–488.

(33) Jacob, A.; Nussle, S.; Britschgi, A.; Evanno, G.; Muller, R.; Wedekind, C. Male dominance linked to size and age, but not to 'good genes' in brown trout (*Salmo trutta*). *BMC Evol. Biol.* **2007,** 7.

(34) Kidd, K. A.; Blanchfield, P. J.; Mills, K. H.; Palace, V. P.; Evans, R. E.; Lazorchak, J. M.; Flick, R. W. Collapse of a fish population after exposure to a synthetic estrogen. *Proc. Natl. Acad. Sci. U.S.A.* **2007,** *104*, 8897–8901.

(35) Vos, J. G.; Dybing, E.; Greim, H. A.; Ladefoged, O.; Lambre, C.; Tarazona, J. V.; Brandt, I.; Vethaak, A. D. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. *Crit. Rev. Toxicol.* **2000,** *30*, 71–133.

(36) Bickham, J. W.; Sandhu, S.; Hebert, P. D. N.; Chikhi, L.; Athwal, R. Effects of chemical contaminants an genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. *Mutat. Res.-Rev. Mutat. Res.* **2000,** *463*, 33–51.

(37) Vaha, J. P.; Erkinaro, J.; Niemela, E. Primmer, C.R. Life-history and habitat features influence the within-river genetic structure of Atlantic salmon. *Mol. Ecol.* **2007,** *16*, 2638–2654.