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Effects of Exposure to WwTW Effluents over Two Generations on Sexual Development and Breeding in Roach *Rutilus rutilus*

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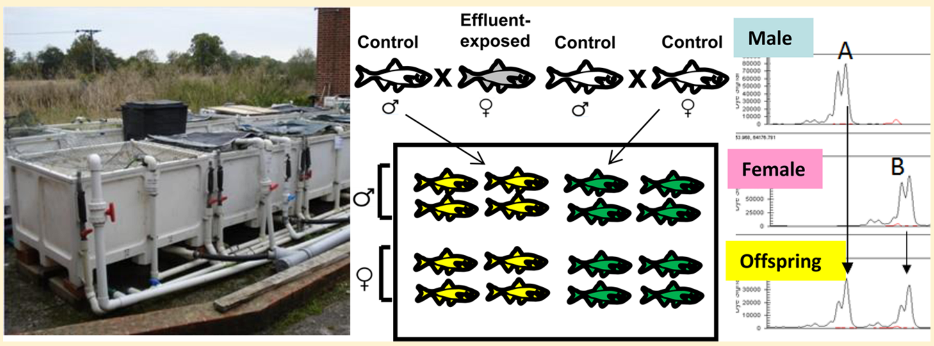
Supporting Information



ABSTRACT: Exposure to environmental estrogens in wastewater treatment works (WwTW) effluents induces feminized responses in male fish, including the development of eggs in male testes. However, the impacts on the offspring of exposed fish are not well understood. In this study, we examined whether roach (*Rutilus rutilus*) from mothers that had been exposed to an undiluted WwTW effluent from early life to sexual maturity had altered susceptibility to gonadal feminization and an impaired capacity to reproduce. For males from both WwTW effluent exposed mothers and dilution water exposed mothers, effluent exposure for up to 3 years and 9 months induced feminized male gonads, although the intersex condition was relatively mild. There was no difference in the severity of gonadal feminization in roach derived from either WwTW effluent exposed or dilution water exposed mothers. Furthermore, a breeding study revealed that roach with effluent-exposed mothers reproduced with an equal success as roach with mothers exposed to clean water. Roach exposed to the effluent for 3 years in this study were able to reproduce successfully. Our findings provide no evidence for impacts of WwTW effluent exposure on reproduction or gonadal disruption in roach down the female germ line and add to existing evidence that male roach with a mild intersex condition are able to breed competitively.

INTRODUCTION

Wastewater treatment work (WwTW) effluents contain tens of thousands of chemicals, including natural and pharmaceutical steroid estrogens. There is substantial evidence that exposure of male fish to WwTW effluents causes feminization, and that severely feminized male gonads impair breeding success of those individuals.[1−3] Feminized male phenotypes include the production of the female yolk protein precursor vitellogenin,[4] feminized reproductive ducts and the presence of both male and female germ cells in the male gonad.[5]

The estrogenic activity of WwTW effluents predominantly results from the presence of steroid estrogens emanating from human excretion. These include estradiol (E2), its breakdown product, estrone (E1) and the pharmaceutical estrogen 17α-ethinylestradiol (EE2), a component of the female contraceptive pill.[6] Other substances detected in effluents shown to be estrogenic include the pharmaceutical metformin,[7] alkylphenoxy polyethoxylates (APEOs) and their breakdown products,[8] and plasticizers (e.g., bisphenol A[9]). These chemicals may also contribute to the feminization of male fish in some rivers receiving high level industry discharges. Natural plant estrogens occur widely in effluent discharges,[10] but they are relatively weak in potency compared with steroidal estrogens.[11] There is also some evidence supporting the involvement of chemicals that can act as antiandrogens contribute to the feminization of fish in some rivers.[6,12−18]

In the United Kingdom, concern regarding the impacts of estrogenic effluents on fish health and fish populations led to a £40 M investment to evaluate the ability of various secondary and tertiary treatment processes to remove estrogens from effluents[19,20] and more recently a £100 M chemical investigation program. From this work, it has been established that although some tertiary processes, such as activated carbon,

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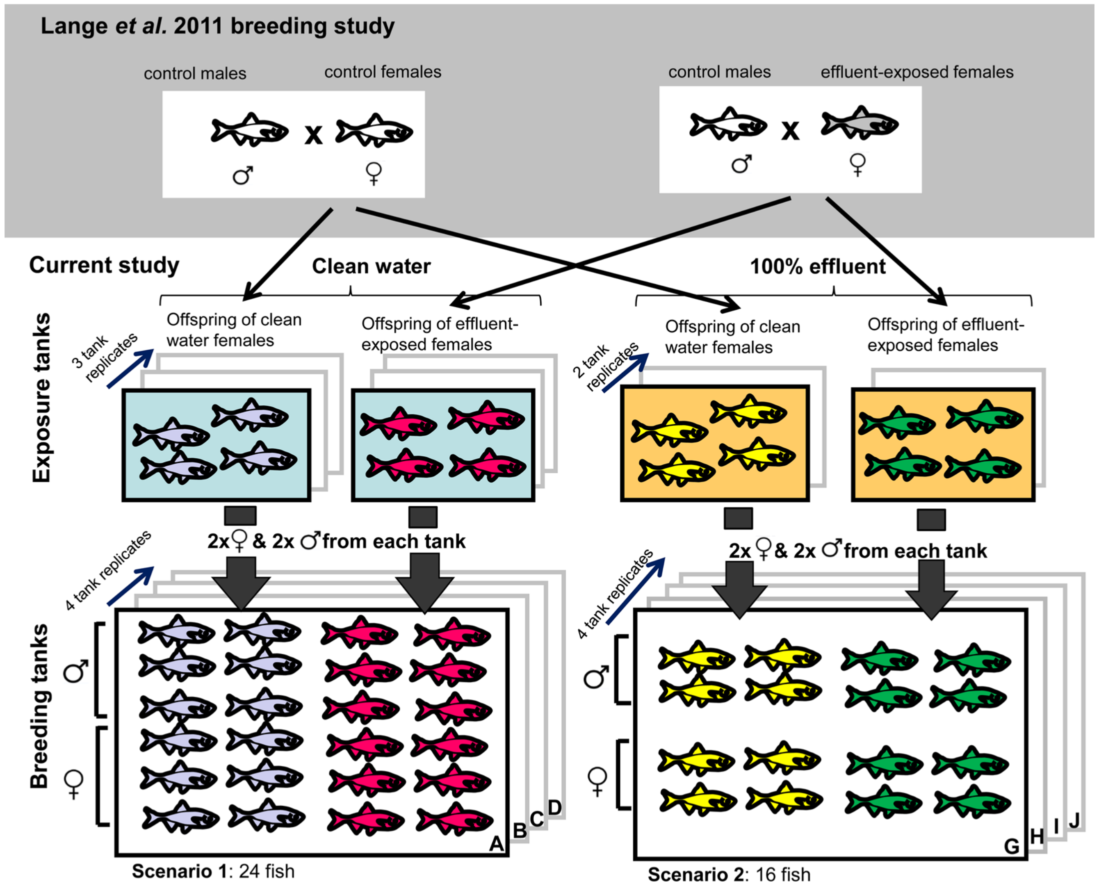

Figure 1. Experimental setup for the breeding study conducted after maintaining the fish for 3 years in either clean water conditions, or exposed to 100% WwTW effluent. The mothers and fathers of the fish colored purple and yellow were kept in clean water conditions, whereas the mothers of fish in red and green were exposed to an undiluted effluent for approximately three years, as described in Lange et al.[26] The actual sex ratios differed from the intended ratio (shown here) in some cases (Table S1).

are highly effective, they are expensive and incur a greater carbon footprint than more widely adopted secondary treatment processes.[19,21]

Much of the current knowledge on the impacts of treated estrogenic effluents is derived from studies on the cyprinid fish roach (*Rutilus rutilus*), which commonly occurs in sewage-contaminated rivers in the United Kingdom. Endocrine disruption in fish was first reported when a low (5%) incidence of intersex was found in wild roach populations living just downstream of a WwTW effluent discharge into the River Lee in the UK.[22] Later surveys found roach with feminized gonads at 86% of UK river locations surveyed.[23,24] All feminized phenotypes seen in wild roach have been induced through controlled exposures to WwTW discharges[19,25,26] and to EE2,[27,28] with induction of female germ cells in an otherwise male gonad requiring persistent exposure during the period of gonadal differentiation.

A major concern is whether WwTW effluents impact fish populations by affecting reproductive output. Several experimental exposures to EE2, at concentrations that have been measured occasionally in WwTW effluents and encompassing the period of sexual development, have found that exposure can result in complete feminization and/or reproductive failure.[28−32] Notably, exposure of an entire lake in Canada for 3 years to 4−6 ng EE2/L resulted in the collapse of the fathead minnow (*Pimephales promelas*) population residing in this lake.[31] These exposures, however, exceed typical river concentrations of estrogens.[33] Nevertheless, EE2 exposures at concentrations <1 ng/L, equivalent to the combined estrogenic potency of all estrogens found at heavily polluted river stretches, have found impacts on reproductive output that could potentially impact populations.[32,34,35] A competitive breeding study found the majority of wild intersex roach caught from two WwTW impacted UK rivers were able to breed successfully, although gonadal feminization impaired breeding success of male fish by up to 76% dependent on the severity of the feminization.[2] Impacts on roach at a population level in the wild have also been examined using population-genetic analysis. An analysis of population-genetic structures of roach in southern England found high levels of genetic diversity in polluted river stretches, and also identified some populations confined to polluted river stretches that are not reliant on immigration from unpolluted river stretches. However, in that analysis a substantial impact on effective population sizes, which relates to the number of breeding fish, could not be excluded.[36]

There is also concern that exposure to WwTW effluent could harm the offspring of exposed fish. Schwindt et al.[37] found that the offspring of female fathead minnows exposed to EE2 (3.2 ng/L) had reduced survival, even though the embryos had never been exposed to waterborne EE2. The mechanism was unknown, but it was suggested that changes in DNA methylation patterns could be responsible.[37] There is good evidence that exposures to a range of chemical contaminants present in effluents, including estrogens[38,39] alter the DNA

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methylation patterns of adult fish.[31,32] Additionally, studies using zebrafish (*Danio rerio*) have shown DNA methylation patterns are retained to a much greater extent than in mammals, where the majority of these patterns are "wiped clean" during early embryonic development.[40] In roach, exposure to EE2 (4.0 ng/L) for 120 days caused sensitization to this estrogen when subsequently exposed 398 days after the original exposure, as measured by induction of estrogen receptor 1 (*esr1*), estrogen receptor 2b (*esr2b*), and gonadal aromatase (*cyp19a1a*) transcript levels.[28] Again, it is not known whether this resulted from altered DNA methylation patterns. Thus, at present there is no information on whether WwTW effluent exposure impacts on gonadal development or reproductive success of the offspring of exposed fish.

In a previous study conducted in our research group, roach were exposed for over 3 years to a treated WwTW effluent that contained sufficient estrogenic activity to induce sex reversal of genetic males.[26] It will have also contained a cocktail of thousands of other chemicals including metals[41] and pharmaceutical products[42] found in WwTW effluents. At 50% dilution, this induced gonadal feminization (intersex and ovarian cavities) in male fish whereas exposure to the undiluted effluent resulted in an all-female population. In breeding scenarios that employed roach derived only from the exposure to 100% effluent, there was reproductive failure due to the absence of males. However, females exposed to 100% effluent reproduced successfully with males grown in clean water. In the present study, the offspring of these WwTW exposed females and clean water males were exposed to effluent from the same WwTW for up to 3 years and 9 months. An assessment of the reproductive ability of fish derived from effluent exposed mothers against fish derived from clean water mothers was subsequently assessed using competitive breeding trials and parentage analysis with DNA microsatellites. This was undertaken after maintaining these fish in effluent or clean water for 3 years. In this study, we thus aimed to investigate for effects of exposure to WwTW effluent over two generations in the roach and assess whether maternal exposure to WwTW effluent alone causes sexual disruption and/or affects reproductive health of the offspring.

■ MATERIALS AND METHODS

Experimental Design. The roach used in this work originated from two of the breeding scenarios of a study conducted in 2008[26] (Figure 1): (1) female fish that had been exposed to an undiluted (100%) effluent from 35 days post hatch (dph) to three years in age and that reproduced with males kept in clean water (control) conditions for 2 years or (2) both male and female fish that had been kept in clean water conditions for 2 years. Thus, both scenarios had clean water (control) fathers but one had WwTW effluent exposed mothers and the other had clean water exposed mothers. Fertilized roach eggs from the two tank replicates of each breeding scenario were separately maintained in clean water as described previously and hatched between the 5th and 22nd May 2008.[26] When fry were sufficiently robust to be transferred to the field site, fry from the two replicate tanks for each scenario were then (7/16/2008) combined and transferred into four 1 m^3 tanks ("treatment tanks") that were supplied with dechlorinated tap water [filtered through granulated active charcoal (GAC) (clean water)] or an undiluted (100%) WwTW effluent. During the exposure, roach were fed with γ-irradiated bloodworm (*Chironomus* sp.), dry coldwater flake food (TetraMin), and Cyprico Crumble EX dry food (Coppens International bv, Helmond, The Netherlands). The fish were kept under ambient temperature and photoperiod and each tank was aerated. Flow rates were maintained at 5 L/min. In April 2010 (at 2 years of age), the fish from each clean water tank were subdivided into three "treatment" tanks and fish from each effluent tank were subdivided into two "treatment tanks" in order to reduce densities and encourage growth. The WwTW effluent originated from a treatment works the employs two types of treatment technologies: biological filters (BF) and activated sludge (AS) that run in parallel. Approximately 50% of the flow is treated in two BF streams (30% to one, 20% to the other) and the rest is treated in the AS plant (ASP). The effluent is blended before being discharged. Two AS lanes ran in parallel on the ASP stream until November 2009 when a third lane was installed that was 3465 m^3 in volume (the same as the original 2), therefore increasing the ASP stream capacity by ~50%.

The employed level of exposure (100% effluent) is higher than occurs in rivers, although treated effluents can make up a large proportion of the flow in some rivers, particularly during dry weather conditions. Using data from Jobling et al.[24] for 44 lowland river stretches, Lange et al.[26] calculated the average proportion of river flow comprising effluent was 27%, with the most polluted of these having an average of 50%. However, the proportion and quality of effluent in receiving river waters changes with annual and seasonal variations in rainfall. For instance, in the River Lee downstream from Harpenden and East Hyde WwTWs, the average proportion is 70%, increasing to 90% in dry weather conditions.[36] Higher rainfall and low temperatures lead to poorer effluent quality due to low retention time and low bacterial activity, respectively. Moreover, effluents treated via trickling filters alone are known to be of poorer quality, and contain more estrogenic substances than those treated through the activated sludge process.[43]

Breeding Study. A competitive breeding experiment was carried out in 2011 after the fish had been held for approximately 3 years in either full strength WwTW effluent or in clean water. Roach breed once a year, generally between April and May, depending on temperature and photoperiod. In April (4/6/2011), roach were assorted into eight "breeding tanks", each supplied with dechlorinated water for the period of the breeding experiment. The breeding study was conducted in clean water rather than effluent, to enable us to monitor the breeding process and facilitate collection of the embryos after spawning. These tanks were the same design as those used for the exposure, with the exception of the inclusion of a layer of Enkamat (a three-dimensional synthetic mat consisting of randomly placed nylon filaments) placed on the bed of each breeding tank as a spawning substrate.[26] The aim was to have an equal number of males and females in each breeding tank; sex was determined based on secondary sex characteristics: roughness of skin caused by breeding tubercles and milt production in males. While setting up the breeding tanks, fin clips were taken from each fish and stored in 100% ethanol, so that the specific tank history of each fish could be traced after sampling by matching DNA microsatellite genotypes.

Two breeding scenarios were employed (Figure 1), each with an equal number of roach with clean water and effluent-exposed mothers from the Lange et al. study.[26] Each of the four breeding tanks of the first scenario (Tanks A−D) held 24 roach from clean water tanks, of which 12 were from tanks with effluent-exposed mothers and the other 12 were from clean

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water mothers. Fish judged to be the most sexually mature (based on production of milt and roughness of the skin for males and body shape for females) were placed in Tank A, then B etc., so that Tank D contained those fish judged to be least mature, which were also generally the smallest in body size. This was done in order to match fish of similar size and maturity so to minimize differences in reproductive success due to these factors within each breeding tank; larger male roach have previously been shown to be more successful in completive breeding scenarios.[2] Each of the four tanks of the second scenario (Tanks G–J) contained 16 roach from the effluent exposed tanks (fewer fish were available than for the clean water fish), of which 8 were from exposed mothers and 8 from clean water mothers. These were also organized such that tank G contained the fish that appeared to be the most mature and Tank J the least mature. Three adults from the clean water fish died during the experiment, but there were no deaths in the effluent-exposed fish. After sampling, sex ratios differed slightly from the expected ratios (Table S1), as sex determiantion using external characteristics can be difficult, particulary for smaller fish and those that have been exposed to effluent; WwTW exposure has been shown to suppress the development of male secondary sex features, as shown for male fathead minnow.[44]

Eggs were removed from tanks on the 15th and 20th of April 2011 by cutting out a section of the spawning substrate and no spawning occurred after this. Fertilized eggs were hatched at the University of Exeter and 100, 5 dph fry were terminated by lethal anesthesia using benzocaine (ethyl-*p*-aminobenzoate) and placed in 100% ethanol for parentage analysis. 2 weeks after the eggs were removed from the breeding tanks, parent fish were terminated on the 5th of May by lethal anesthesia using benzocaine (ethyl-*p*-aminobenzoate), and according to UK Home Office procedures. Fork length and wet weight were recorded and a fin clip was taken and stored in 100% ethanol for subsequent DNA microsatellite analysis. The remainder of the body was preserved in Bouin's solution for histological analysis to assess gonadal development.

During the breeding study, 36 fish from the effluent exposed tanks were kept in clean water then returned to be exposed to 100% effluent until they were sampled in April 2012 (4/24/2012), after 3 years and 9 months of exposure, for histological analysis. Gonads from these fish were removed from the body cavity prior to preservation in Bouin's solution, so the presence of feminized ducts could not be determined. After fixation, all samples were processed for histological analyses and an assessment was made of alterations in germ cell development and/or to the structural organization of the gonad due to the exposure.

Parentage Assignment and Genetic Sex Testing. DNA was extracted from fin tissue of all adults and from the fry using the HotSHOT[45] extraction method. Each adult and 50 offspring from each tank (except tank J, where only 46 fry were available) were genotyped using 19 DNA microsatellites described previously.[36,46] The origin of each fish was determined by matching the microsatellite genotypes from fins taken at sampling to those from fins taken during the setting up of the breeding study using GenAlEx 6.5.[47] The programs Colony v2.0.5.0[48] and Cervus 3.0.7.[49,50] were used for parentage analysis. For the Colony analysis, we did not assign sexes to the fish, to allow for the possibility of intersex fish reproducing as either males or females, although in a previous breeding study wild intersex fish reproduced only as males.[2] In Colony we used full likelihood with long runs. A PCR-based genetic sex test for roach (unpublished) was applied to determine the genetic sex of all adult fish.

Statistical Analysis. To deduce whether reproductive success significantly differed between fish with exposed mothers and clean water mothers, influences on reproductive performance were assessed by fitting linear mixed-effect (LME) models using the proportion of offspring sired per fish as the response variable. A random (breeding) tank effect was included in each model, as reproductive performance of each fish depends on the other fish in the same breeding tank. Fixed factors included in the full models suspected of influencing reproductive performance were size (length), treatment tank (i.e., tank the fish was in prior to the breeding study) and maternal exposure. For male fish exposed to effluent, the presence of oocytes was also included as an additional fixed factor in the analysis. Minimum adequate models (MAM) were obtained by sequentially removing fixed factors with the highest p values from the full model, until all factors in the model had a p value <0.05. MAM, with p values <0.05 validated using permutation tests. To do this, the proportion of offspring for each fish was shuffled randomly within each tank 1000 times. p values for the permutation tests were calculated as the proportion of permutation slopes that were equal or greater to the observed slope.[2] Throughout the paper, variation is given as standard error of the mean (SEM). All statistical analyses were conducted in 'R'.[51]

Analysis of Effluent Quality (Ammonia) and Estrogen Content. The WwTW serves a population equivalent of ~138 000. The steroid estrogen content for this effluent has been measured previously, most recently in 2005, where average concentrations of E1 were 42.1 ng/L, 17α-E2 was 0.17 ng/L and 17β-E2 was 2.49 ng/L, and EE2 was 0.57 ng/L. Equine estrogens (used in hormone replacement therapy) 17ß-dihydroequilenin (17β-Eqn) and equilenin (Eqn) were measured at 0.10 and 0.43 ng/L, respectively.[52] For this study, ammonia content was used as a proxy for effluent quality; over a daily cycle, ammonia content correlates closely with steroid estrogen content.[53] Ammonia, nitrates and nitrites and total oxidized nitrogen (TON) were quantified using discrete automated colorimetric analysis with an Aquakem 600 Photometric Analyzer at Northumbrian Water Scientific Services. Nitrate concentrations were calculated from TON and nitrite concentrations. Additionally, a spot sample was taken for measurement of steroid estrogen content during exposure of the fish in this study in June 2012 for chemical analysis (Severn Trent Services). Estrogens were quantified using LC-MS/MS, using an Agilent system for LC and AB SCIEX 5000 for MS/MS, and nonylphenols were quantified using GC–MS.

RESULTS AND DISCUSSION

Gonadal Feminization. Exposure of roach to the undiluted WwTW for 3 three years resulted in feminization of male gonads, consistent with other published experimental exposures of roach to WwTW effluents[19,26,54] and studies of wild fish.[23] We found no evidence for disruption of sexual development in males kept in clean water conditions irrespective of maternal exposure to WwTW effluent (Figure 2, Figure S1). In contrast, all male roach sampled after 3 years of exposure to 100% effluent had female-like ducts. Feminized ducts form in the first few months of development[26,55] and in the previous study[26] ducts were formed in fish by 67 dph. However, in this study the ducts must have formed at an older age as some of the

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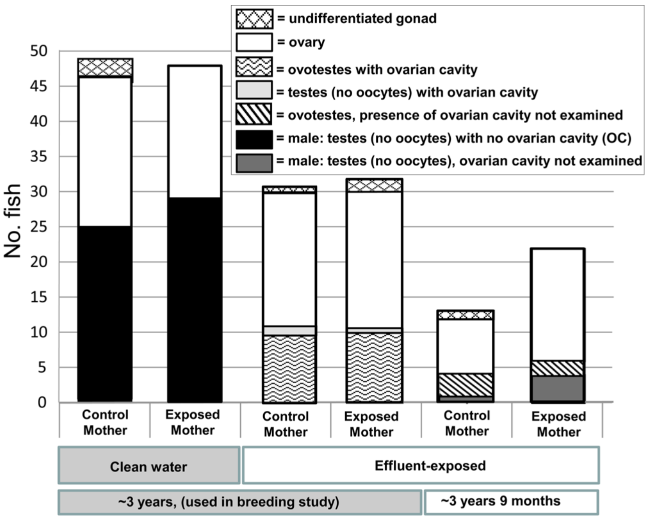

Figure 2. Sex ratios determined by gonadal histology of roach exposed to either clean water conditions or 100% WwTW effluent for 3 years and 3 years and 9 months, showing comparisons between fish with WwTW exposed mothers and "control" mothers (kept in clean water conditions). For roach with undifferentiated gonads, application of a genetic sex test revealed that for 3 year clean water fish, two were genetic males and two were genetic females; for effluent exposed fish sampled at 3 years, three were males and two were females and the one undifferentiated effluent exposed fish sampled at 3 years and 9 months was a genetic female.

experimental fish were 70 dph when they were first transferred to effluent. Low numbers of primary oocytes were found scattered throughout the testicular tissue in the gonads of most (85%) of the males exposed to the WwTW effluent (Figure 2). No oocytes were observed in four of the effluent exposed males. The development of eggs in male gonads requires persistent exposure and gonads tend to become increasingly feminized throughout the exposure.[26,28] Average lengths and weights for clean water roach were 8.5 ± 0.8 cm, 8.0 ± 2.9 g compared to 8.9 cm ±1.2 cm and 9.0 g ± 2.8 cm for exposed roach (Figure S2). For clean water fish, those with exposed mothers were, on average, significantly longer (ANOVA, F = 10.8, p = 0.0015) but lengths did not significantly differ between males and females (p = 0.42). For the exposed fish, females were significantly longer than males (ANOVA, F = 8.5, p = 0.0051), but lengths did not significantly differ between fish with effluent-exposed mothers compared to those with clean water mothers (p = 0.69).

After an additional 9 months of exposure, four male fish had moderate numbers of oocytes in each gonadal section and no oocytes were observed in the gonads of five male fish (Figure 2, Figure S1). Average length of these fish was 12.2 ± 1.0 cm and average weight was 27.4 ± 7.6 g. At both sampling points, there were similar levels of disruption in the gonads of fish with exposed mothers compared to fish where the mothers were exposed to clean water (Figure 2). Application of a genetic sex test suggested that sex-reversal of genetic males due to effluent exposure had not occurred. For roach kept in clean water, 80 of 87 fish for which sex could be clearly determined histologically had matching of genetic and histological sex. All 7 fish in which genotype and phenotype did not match were genetic females with histological male gonads. Failure of the genetic test to identify males may potentially result from genetic polymorphisms in the primer binding sites, or recombination between this region and the "true" sex determination gene. For roach kept in effluent (combined for both sampling times), genetic sexes matched histological sex for 86 of 90 fish where

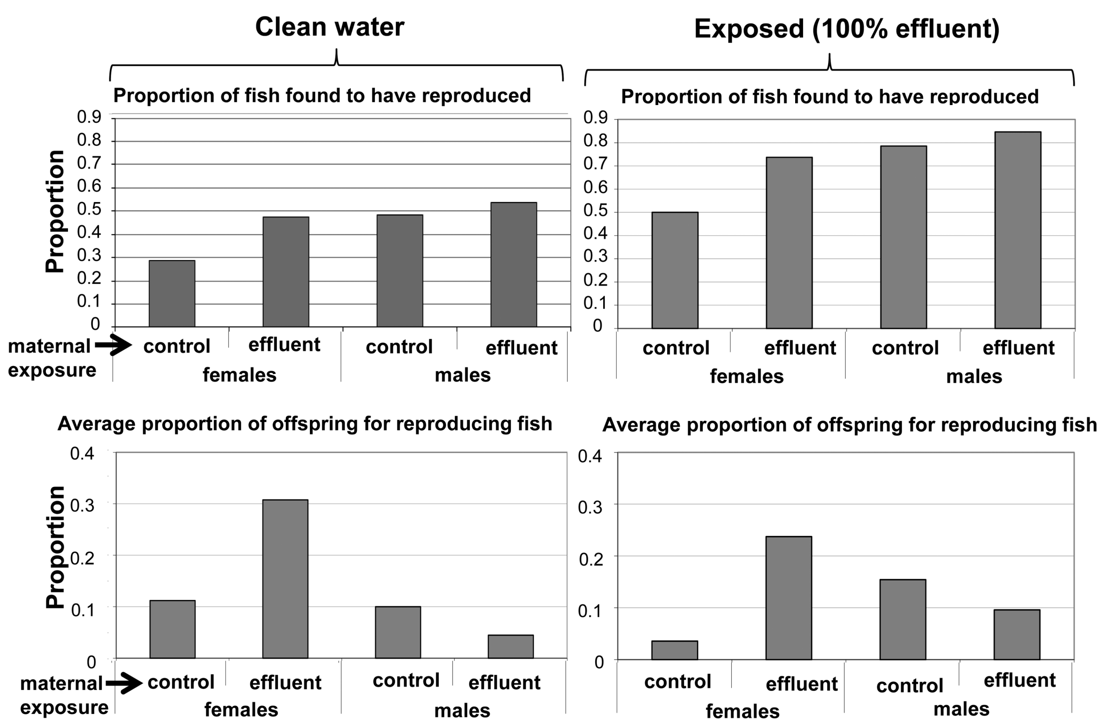

Figure 3. Reproductive success of male and female roach in two breeding scenarios. The results summarize reproductive output of four breeding tanks, each with 24 fish for the clean water tanks or four tanks each with 16 fish for the effluent-exposed tanks. Each tank contained a similar number of males and females and an equal number of fish with mothers that were exposed to an effluent for three years in[26] and fish with "control" mothers that were kept in clean water conditions in the same study.

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sex could clearly be determined histologically. Only one histological female gave a male genetic genotype, but this is within the error rate for this test so it does not constitute strong evidence for sex reversal. Overall, these results do not suggest that maternal exposure influenced sexual development of the fish in these offspring.

Breeding Success. Spawning occurred in all (eight) breeding tanks. Parentage analysis revealed a higher proportion of fish kept in effluent (81% of males and 62% of females) reproduced, compared to those kept in clean water conditions (50% of males and 37% of females), Figure 3. The difference is likely a result of the larger size of the fish kept in effluent (Figure S2), which may result from additional nutrients and food obtained from the effluent. Wild male roach reach sexual maturity after 2−3 years, compared with 3−4 years for females,[56] and the rate of sexual development is dependent on growth.[55] It is therefore likely that a higher proportion of the roach kept in effluent were sexually mature. We cannot, however, exclude a direct effect of exposure to estrogen or other substances in effluent advancing puberty, but this has never been tested experimentally. These proportions of fish reaching sexual maturity at 3 years are lower than those from the Lange et al. study,[26] a likely consequence of the larger fish in that study, where body lengths were 11.0−13.2 cm for those in clean water and 12.2−14.5 cm for effluent-exposed fish. The ability of exposed females to breed confirms the results of the previous exposure to this effluent,[26] and is also consistent with the finding that the majority of wild females sampled from WwTW contaminated rivers breed successfully.[2] All intersex fish that reproduced did so as males, consistent with the results of a previous breeding study using wild roach.[2]

Roach with effluent-exposed mothers and roach with clean water (control) mothers both reproduced in the breeding trial (Figure 3). For nonexposed females, no factors included in the analysis correlated with reproductive success ($p \geq 0.12$). For nonexposed males, there was no significant relationship between maternal exposure and the proportion of offspring sired (full linear mixed effects (LME) model, $p = 0.81$). However, males of greater length were significantly more successful at siring offspring (linear mixed effects (LME) model coefficient = 0.052, $p = 0.0005$, permutation $p < 0.002$), a relationship that has been found previously for roach in a group spawning scenario.[2] For tanks with exposed fish, females with exposed mothers (LME model coefficient = −0.13, $p = 0.0069$, permutation $p = 0.009$) and those of greater length (LME model coefficient = 2.17, $p = 0.038$, permutation test $p = 0.011$) produced significantly more offspring. The greater success of females with exposed mothers may be a consequence of the greater average weight of these fish, despite the fish being of similar length (Figure S2). No factors measured (treatment tank, parental exposure, presence of oocytes in testes) significantly correlated with male reproductive success (full LME model, $p \geq 0.08$). Roach with ovarian cavities and those with low numbers of oocytes in their testes reproduced successfully, as found previously.[2]

Overall, our finding that roach exposed to a WwTW effluent, and those whose mothers were exposed were able to breed successfully, is consistent with some of the data that are available for wild roach populations living in effluent-polluted rivers: breeding studies have found that the majority of roach from several effluent-exposed rivers were capable of reproduction.[1,2] Furthermore, evidence from a population genetic analysis suggests that populations of roach exist in effluent-polluted river stretches that receive minimal immigration from unpolluted river stretches.[36] However, fish species vary in their sensitivity to estrogen exposure,[57,58] so these results for roach do not allow us to draw definitive conclusions on the treat of WwTW effluent exposure on other fish species. For instance, a study on the Chinese rare minnow (*Gobiocypris rarus*) found complete reproductive failure for an exposure to 0.2 ng EE2/L, equivalent to 2 ng/L E2eq, a concentration that has not been known to affect reproduction in other fish species[35] and is at the low end for the estrogenic content in most WwTW effluents tested.

Comparison of Effluent Composition Between the Two Studies. The mild gonadal feminization of males observed in this study contrasts with the results of the previous 3 year exposure study to effluent from the same WwTW that resulted in an all-female population.[26] This difference could be explained by (1) the slower growth rate of the roach in this study, as the rate of sexual development is dependent on size in roach;[55] (2) a later start of exposure (between 55 and 70 dph), compared to 35 dph in the first study; and/or (3) lower estrogenic activity of the effluent in this study. The increase in the capacity of the ASP in November 2009 would have increased hydraulic retention times (HRT). Increasing HRT has previously been shown to reduce both ammonia concentrations and natural estrogens.[59] Estrogen concentrations were not measured in either study. However, exposure concentrations of ammonia, nitrate, and nitrite differed between the two studies (Figure S3) and a study found ammonia and estrone concentrations to correlate strongly over a 24 h period within a WwTW.[53] Conversely, there was no consistent relationship between the two when comparing between different WwTWs.[43] Thus, effluent quality differed between the two studies, which may be associated with different estrogen concentrations. The absence of any intensive chemical testing for estrogens, or other chemicals that may contribute to estrogenic activity,[16] does not allow us to draw any definitive conclusion on the nature of the differences in chemical content in the study effluent over time.

Further evidence that estrogen concentrations may have differed between the studies comes from a spot sample of effluent taken in June 2012, ~2 months after the exposure period in the present study. Measured concentrations of steroidal estrogens were 19 ng/L E1, 2.1 ng/L E2, and no EE2 was detected (detection limit = 0.1 ng/L). This equates to an estrogenic potency of 8.4 E2 equiv (E2eq), from the concentrations and potency of all the steroid estrogens.[33] Of the nonylphenols tested, only NP1EC was detected at a concentration of 409 ng/L whereas nonylphenol monoethoxylate (NP1EO), nonylphenol diethoxylate (NP2EO), and long chain nonylphenol polyethoxylates (NP3-12EO) were below the limits of detection at 50 ng/L. This is substantially lower than measurements taken between September and November of 2005 during the Lange et al.[26] exposure study, which gave an estrogen potency equivalent to 22 E2eq[52] with individual estrogens measured at 42 ng/L E1, 2.7 ng/L E2, and 0.57 ng/L EE2. A modest reduction in estrogenic activity may be all that is required to explain the differences in feminization. For instance, male roach exposed to 50% effluent for almost 3 years in the previous study had ovotestes, but these had a few oocytes per gonadal section, whereas those exposed to 100% effluent in the same study were completely feminized.[26] In the present study, a 50% reduction in estrogen concentration, compared that the

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Lange et al. study[26] may be sufficient to explain the difference between the levels of feminization observed in the two studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b03777.

Figure S1, examples of gonadal histology of exposed fish; Figure S2, lengths and weights of fish in the breeding study; Figure S3, ammonia, nitrate and nitrite concentrations measured in the effluent in this study and in Lange et al.;[26] Table S1, length and weight of fish and sex ratios in the breeding tanks (PDF).

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Author Contributions

The paper was written through contributions of all authors. P.H., E.N., A.L., S.J., and C.T. participated in the design of the research. P.H., E.N., A.L., E.B., and L.B. participated in data collection and analysis. All authors have given approval to the final version of the paper.

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Notes

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■ ABBREVIATIONS

ANOVA	analysis of variance
APEOs	alkylphenoxy polyethoxylates
AS	activated sludge
ASP	activated sludge plant
cyp19a1a	gonadal aromatase
E2	estradiol
EE2	ethinylestradiol
esr1	estrogen receptor 1
esr2b	estrogen receptor 2b
HRT	hydraulic retention time
MAM	minimum adequate models
NP1EC	linear mixed-effect LME
NP3-12EO	long chain nonylphenol polyethoxylates
NP1EO	nonylphenol monoethoxylate
NP2EO	nonylphenol diethoxylate
NP1EC	nonylphenolic acetic acid
PCR	polymerase chain reaction
TON	total oxidized nitrogen
WwTW	wastewater treatment works

■ REFERENCES

(1) 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* (2), 515−524.

(2) Harris, C. A.; Hamilton, P. B.; Runnalls, T. J.; Vinciotti, V.; Henshaw, A.; Hodgson, D.; Coe, T. S.; Jobling, S.; Tyler, C. R.; Sumpter, J. P. The consequences of feminization in breeding groups of wild fish. *Environ. Health Persp.* **2010**, *119* (3), 306−311.

(3) Fuzzen, M. L. M.; Bennett, C. J.; Tetreault, G. R.; McMaster, M. E.; Servos, M. R. Severe intersex is predictive of poor fertilization success in populations of rainbow darter (*Etheostoma caeruleum*). *Aquat. Toxicol.* **2015**, *160*, 106−116.

(4) Purdom, C. E.; Hardiman, P. A.; Bye, V. V. J.; Eno, N. C.; Tyler, C. R.; Sumpter, J. P. Estrogenic effects of effluents from sewage treatment works. *Chem. Ecol.* **1994**, *8* (4), 275−285.

(5) Nolan, M.; Jobling, S.; Brighty, G.; Sumpter, J. P.; Tyler, C. R. A histological description of intersexuality in the roach. *J. Fish Biol.* **2001**, *58* (1), 160−176.

(6) Routledge, E. J.; Sheahan, D.; Desbrow, C.; Brighty, G. C.; Waldock, M.; Sumpter, J. P. Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. *Environ. Sci. Technol.* **1998**, *32* (11), 1559−1565.

(7) Niemuth, N. J.; Jordan, R.; Crago, J.; Blanksma, C.; Johnson, R.; Klaper, R. D. Metformin exposure at environmentally relevant concentrations causes potential endocrine disruption in adult male fish. *Environ. Toxicol. Chem.* **2015**, *34* (2), 291−296.

(8) Jobling, S.; Sheahan, D.; Osborne, J. A.; Matthiessen, P.; Sumpter, J. P. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environ. Toxicol. Chem.* **1996**, *15* (2), 194−202.

(9) Blackburn, M. A.; Kirby, S. J.; Waldock, M. J. Concentrations of alkyphenol polyethoxylates entering UK estuaries. *Mar. Pollut. Bull.* **1999**, *38* (2), 109−118.

(10) Lundgren, M. S.; Novak, P. J. Quantification of phytoestrogens in industrial waste streams. *Environ. Toxicol. Chem.* **2009**, *28* (11), 2318−2323.

(11) Gutendorf, B.; Westendorf, J. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. *Toxicology* **2001**, *166* (1−2), 79−89.

(12) Jobling, S.; Burn, R. W.; Thorpe, K.; Williams, R.; Tyler, C. Statistical modeling suggests that antiandrogens in effluents from wastewater treatment works contribute to widespread sexual disruption in fish living in English rivers. *Environ. Health Persp.* **2009**, *117* (5), 797−802.

(13) Bhatia, H.; Kumar, A.; Du, J.; Chapman, J. C.; McLaughlin, M. J. Co-treatment with the non-steroidal anti-androgen drug, flutamide and the natural estrogen, 17*β*-estradiol does not lead to additive reproductive impairment in juvenile Murray rainbowfish (*Melanotaenia fluviatilis*). *J. Appl. Toxicol.* **2015**, *35* (11), 1241−1253.

(14) Green, C.; Brian, J.; Kanda, R.; Scholze, M.; Williams, R.; Jobling, S. Environmental concentrations of anti-androgenic pharmaceuticals do not impact sexual disruption in fish alone or in combination with steroid oestrogens. *Aquat. Toxicol.* **2015**, *160*, 117−127.

(15) Lange, A.; Sebire, M.; Rostkowski, P.; Mizutani, T.; Miyagawa, S.; Iguchi, T.; Hill, E. M.; Tyler, C. R. Environmental chemicals active as human antiandrogens do not activate a stickleback androgen receptor but enhance a feminising effect of oestrogen in roach. *Aquat. Toxicol.* **2015**, *168*, 48.

(16) Niemuth, N. J.; Klaper, R. D. Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish. *Chemosphere* **2015**, *135*, 38−45.

(17) Sohoni, P.; Tyler, C. R.; Hurd, K.; Caunter, J.; Hetheridge, M.; Williams, T.; Woods, C.; Evans, M.; Toy, R.; Gargas, M.; Sumpter, J. P. Reproductive effects of long-term exposure to bisphenol a in the fathead minnow (*Pimephales promelas*). *Environ. Sci. Technol.* **2001**, *35* (14), 2917−2925.

DOI: 10.1021/acs.est.5b03777
Environ. Sci. Technol. 2015, 49, 12994−13002

(18) Furuichi, T.; Kannan, K.; Giesy, J. P.; Masunaga, S. Contribution of known endocrine disrupting substances to the estrogenic activity in Tama River water samples from Japan using instrumental analysis and in vitro reporter gene assay. *Water Res.* **2004**, *38* (20), 4491−4501.

(19) Baynes, A.; Green, C.; Nicol, E.; Beresford, N.; Kanda, R.; Henshaw, A.; Churchley, J.; Jobling, S. Additional treatment of wastewater reduces endocrine disruption in wild fish-a comparative study of tertiary and advanced treatments. *Environ. Sci. Technol.* **2012**, *46* (10), 5565−5573.

(20) Filby, A. L.; Shears, J. A.; Drage, B. E.; Churchley, J. H.; Tyler, C. R. Effects of advanced treatments of wastewater effluents on estrogenic and reproductive health impacts in fish. *Environ. Sci. Technol.* **2010**, *44* (11), 4348−4354.

(21) Owen, R.; Jobling, S. Environmental science: The hidden costs of flexible fertility. *Nature* **2012**, *485* (7399), 441.

(22) Sweeting, R. A. *Hermaphrodite roach in the River Lee, Internal report*; Thames Water, Lea Division: Herts, U. K., 1981.

(23) Jobling, S.; Nolan, M.; Tyler, C. R.; Brighty, G.; Sumpter, J. P. Widespread sexual disruption in wild fish. *Environ. Sci. Technol.* **1998**, *32* (17), 2498−2506.

(24) 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 UK rivers correlate with widespread sexual disruption in wild fish populations. *Environ. Health Persp.* **2005**, *114*, 32−39.

(25) 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 Persp.* **2005**, *113* (10), 1299−1307.

(26) Lange, A.; Paull, G. C.; Hamilton, P. B.; Iguchi, T.; Tyler, C. R. Implications of persistent exposure to treated wastewater effluent for breeding in wild roach (*Rutilus rutilus*) populations. *Environ. Sci. Technol.* **2011**, *45* (4), 1673−1679.

(27) Lange, A.; Katsu, Y.; Ichikawa, R.; Paull, G. C.; Chidgey, L. L.; Coe, T. S.; Iguchi, T.; Tyler, C. R. Altered sexual development in roach (*Rutilus rutilus*) exposed to environmental concentrations of the pharmaceutical 17 alpha-ethinylestradiol and associated expression dynamics of aromatases and estrogen receptors. *Toxicol. Sci.* **2008**, *106* (1), 113−123.

(28) 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* (4), 1219−1225.

(29) Nash, J. P.; Kime, D. E.; Van der Ven, L. T. M.; Wester, P. W.; Brion, F.; Maack, G.; Stahlschmidt-Allner, P.; Tyler, C. R. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environ. Health Persp.* **2004**, *112* (17), 1725−1733.

(30) Lange, R.; Hutchinson, T. H.; Croudace, C. P.; Siegmund, F.; Schweinfurth, H.; Hampe, P.; Panter, G. H.; Sumpter, J. P. Effects of the synthetic estrogen 17 alpha-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* **2001**, *20* (6), 1216−1227.

(31) 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* (21), 8897−8901.

(32) Parrott, J. L.; Blunt, B. R. Life-cycle exposure of fathead minnows (*Pimephales promelas*) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. *Environ. Toxicol.* **2005**, *20* (2), 131−141.

(33) Williams, R. J.; Keller, V. D. J.; Johnson, A. C.; Young, A. R.; Holmes, M. G. R.; Wells, C.; Gross-Sorokin, M.; Benstead, R. A national risk assessment for intersex in fish arising from steroid estrogens. *Environ. Toxicol. Chem.* **2009**, *28* (1), 220−230.

(34) Armstrong, B. M.; Lazorchak, J. M.; Murphy, C. A.; Haring, H. J.; Jensen, K. M.; Smith, M. E. Determining the effects of a mixture of an endocrine disrupting compound, 17 alpha-ethinylestradiol, and ammonia on fathead minnow (*Pimephales promelas*) reproduction. *Chemosphere* **2015**, *120*, 108−114.

(35) Zha, J.; Sun, L.; Zhou, Y.; Spear, P. A.; Ma, M.; Wang, Z. Assessment of 17 alpha-ethinylestradiol effects and underlying mechanisms in a continuous, multigeneration exposure of the Chinese rare minnow (*Gobiocypris rarus*). *Toxicol. Appl. Pharmacol.* **2008**, *226* (3), 298−308.

(36) Hamilton, P.; Nicol, E.; De-Bastos, E.; Williams, R.; Sumpter, J.; Jobling, S.; Stevens, J.; Tyler, C. Populations of a cyprinid fish are self-sustaining despite widespread feminization of males. *BMC Biol.* **2014**, *12* (1), 1.

(37) Schwindt, A. R.; Winkelman, D. L.; Keteles, K.; Murphy, M.; Vajda, A. M. An environmental oestrogen disrupts fish population dynamics through direct and transgenerational effects on survival and fecundity. *J. Appl. Ecol.* **2014**, *51* (3), 582−591.

(38) Liu, Y.; Chen, S.; Liu, S. Z.; Zhang, Y. Y.; Yuan, C.; Wang, Z. Z. DNA methylation in the 5 ′ flanking region of cytochrome P450 17 in adult rare minnow *Gobiocypris rarus* - Tissue difference and effects of 17 alpha-ethinylestradiol and 17 alpha-methyltestoterone exposures. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* **2014**, *162*, 16−22.

(39) Aniagu, S. O.; Williams, T. D.; Allen, Y.; Katsiadaki, I.; Chipman, J. K. Global genomic methylation levels in the liver and gonads of the three-spine stickleback (*Gasterosteus aculeatus*) after exposure to hexabromocyclododecane and 17-beta oestradiol. *Environ. Int.* **2008**, *34* (3), 310−317.

(40) Macleod, D.; Clark, V. H.; Bird, A. Absence of genome-wide changes in DNA methylation during development of the zebrafish. *Nat. Genet.* **1999**, *23* (2), 139−140.

(41) Karvelas, M.; Katsoyiannis, A.; Samara, C. Occurrence and fate of heavy metals in the wastewater treatment process. *Chemosphere* **2003**, *53* (10), 1201−1210.

(42) Gagné, F.; Blaise, C.; André, C. Occurrence of pharmaceutical products in a municipal effluent and toxicity to rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Ecotoxicol. Environ. Saf.* **2006**, *64* (3), 329−336.

(43) Johnson, A. C.; Williams, R. J.; Simpson, P.; Kanda, R. What difference might sewage treatment performance make to endocrine disruption in rivers? *Environ. Pollut.* **2007**, *147* (1), 194−202.

(44) Minarik, T. A.; Vick, J. A.; Schultz, M. M.; Bartell, S. E.; Martinovic-Weigelt, D.; Rearick, D. C.; Schoenfuss, H. L. On-site exposure to treated wastewater effluent has subtle effects on male fathead minnows and pronounced effects on carp. *J. Am. Water Resour. Assoc.* **2014**, *50* (2), 358−375.

(45) Truett, G. E.; Heeger, P.; Mynatt, R. L.; Truett, A. A.; Walker, J. A.; Warman, M. L. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques* **2000**, *29* (1), 52−54.

(46) Hamilton, P. B.; Tyler, C. R. Identification of microsatellite loci for parentage analysis in roach *Rutilus rutilus* and 8 other cyprinid fish by cross-species amplification, and a novel test for detecting hybrids between roach and other cyprinids. *Mol. Ecol. Resour.* **2008**, *8* (2), 462−465.

(47) Peakall, R.; Smouse, P. E. GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes* **2006**, *6* (1), 288−295.

(48) Jones, O. R.; Wang, J. COLONY: a program for parentage and sibship inference from multilocus genotype data. *Mol. Ecol. Resour.* **2010**, *10* (3), 551−555.

(49) Marshall, T. C.; Slate, J.; Kruuk, L. E. B.; Pemberton, J. M. Statistical confidence for likelihood-based paternity inference in natural populations. *Mol. Ecol.* **1998**, *7* (5), 639−655.

(50) Kalinowski, S. T.; Taper, M. L.; Marshall, T. C. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol. Ecol.* **2007**, *16* (5), 1099−1106.

(51) R Core Team *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2012.

DOI: 10.1021/acs.est.5b03777
Environ. Sci. Technol. 2015, 49, 12994−13002

(52) Tyler, C. R.; Filby, A. L.; Bickley, L. K.; Cumming, R. I.; Gibson, R.; Labadie, P.; Katsu, Y.; Liney, K. E.; Shears, J. A.; Silva-Castro, V.; Urushitani, H.; Lange, A.; Winter, M. J.; Iguchi, T.; Hill, E. M. Environmental health impacts of equine estrogens derived from hormone replacement therapy. *Environ. Sci. Technol.* **2009**, *43* (10), 3897−3904.

(53) Kanda, R.; Churchley, J. Removal of endocrine disrupting compounds during conventional wastewater treatment. *Environ. Technol.* **2008**, *29* (3), 315−323.

(54) Liney, K. E.; Hagger, J. A.; Tyler, C. R.; Depledge, M. H.; Galloway, T. S.; Jobling, S. Health effects in fish of long-term exposure to effluents from wastewater treatment works. *Environ. Health Persp.* **2005**, *114*, 81−89.

(55) 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* (7), 884−895.

(56) Davies, C. E.; Shelley, J.; Harding, P. T.; McLean, I. F. G.; Gardiner, R.; Peirson, G. *Freshwater fishes in Britain: The species and their distribution*; Harley Books: Colchester, U. K., 2004.

(57) Tyler, C. R.; Spary, C.; Gibson, R.; Santos, E. M.; Shears, J.; Hill, E. M. Accounting for differences in estrogenic responses in rainbow trout (*Oncorhynchus mykiss*: Salmonidae) and roach (*Rutilus rutilus*: Cyprinidae) exposed to effluents from wastewater treatment works. *Environ. Sci. Technol.* **2005**, *39* (8), 2599−2607.

(58) Miyagawa, S.; Lange, A.; Hirakawa, I.; Tohyama, S.; Ogino, Y.; Mizutani, T.; Kagami, Y.; Kusano, T.; Ihara, M.; Tanaka, H.; Tatarazako, N.; Ohta, Y.; Katsu, Y.; Tyler, C. R.; Iguchi, T. Differing species responsiveness of estrogenic contaminants in fish is conferred by the ligand binding domain of the estrogen receptor. *Environ. Sci. Technol.* **2014**, *48* (9), 5254−5263.

(59) Petrie, B.; McAdam, E. J.; Lester, J. N.; Cartmell, E. Assessing potential modifications to the activated sludge process to improve simultaneous removal of a diverse range of micropollutants. *Water Res.* **2014**, *62*, 180−192.

DOI: 10.1021/acs.est.5b03777
Environ. Sci. Technol. 2015, 49, 12994−13002