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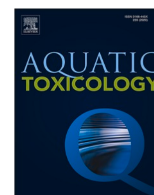

Feminizing effects of ethinylestradiol in roach (*Rutilus rutilus*) populations with different estrogenic pollution exposure histories

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ABSTRACT

Experimental exposures aimed at assessing the risks posed by estrogens in waste-water treatment work (WwTW) effluents to fish populations have rarely considered whether populations differ in their sensitivity to estrogenic compounds. This is despite evidence that selection at genes involved in the estrogen response has occurred in wild populations, and evidence that genotype can influence estrogen-response. In this study we compare the effects of a two-year exposure to a low measured concentration (1.3 ng/L) of ethinylestradiol (EE2) on the sexual development of roach (*Rutilus rutilus*) whose parental generation was sampled from two river stretches heavily contaminated with WwTW effluent and from two without any known WwTW effluent contamination. Exposure to EE2 significantly reduced the proportion of genetic males and induced a range of feminized phenotypes in males. Significantly, exposure also increased the proportion of genetic females with vitellogenic oocytes from 51 to 96%, raising the possibility that estrogen pollution could impact populations of annually spawning fish species through advancing female reproduction by at least a year. However, there was no evidence that river origin affected sensitivity to estrogens in either sex. Thus, we conclude that chronic exposure to low level EE2 has reproductive health outcomes for both male and female roach, but we find no evidence that the nature or magnitude of the response is affected by the population origin.

1. Introduction

Effluents from wastewater treatment works (WwTW) contain complex chemical mixtures including estrogens and estrogen mimics that are known to affect the reproductive health of wild fish (e.g. Kase et al., 2018; Kasonga et al., 2021). Estrogens play a major role in the sexual development of female fish, irrespective of the wide range of genetic and environmental mechanisms for initial sex determination that are displayed in different species of fish. As estrogen receptors are phylogenetically conserved across vertebrates and are also expressed in male fish, estrogenic contamination in the water can disrupt normal sexual development in male fish and induce feminization. Feminization of wild male fish exposed to WwTW effluents has been reported in Europe, America and Asia (e.g. Desbrow et al., 1998; Hinck et al., 2009) and has largely been attributed to the presence of natural and synthetic estrogens which have been detected in most effluents examined to date (Harries et al., 1997; Purdom et al., 1994; Ternes et al., 1999). Synthetic estrogens include ethinylestradiol (EE2), commonly used as the main active component of the female oral contraceptive pill. WwTW effluents have also been shown to have anti-androgenic activity (Katsiadaki et al., 2012) which may also contribute to feminization seen in wild fish (Jobling et al., 2009; Lange et al., 2015). Feminized phenotypes in males include the presence of the precursors of the egg yolk protein vitellogenin (VTG) in the blood (male fish do not normally produce this protein) (Purdom et al., 1994); feminized reproductive ducts (ovarian cavities), and the intersex condition - the presence of developing eggs in otherwise male gonads (Nolan et al., 2001). Experiments using wild roach (*Rutilus rutilus*) from rivers in England have shown that males with moderately-severely feminized gonads have a reduced fertility through *in-vitro* fertilization studies (Jobling et al., 2002b) and are less successful

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in siring offspring in group spawning scenarios (Harris et al., 2011).

Much of the information on the potential impact of estrogen pollution at a population level has been derived from controlled exposures over the period of sexual development. Results from some of these studies suggest that the estrogenic activity that results from the combination of estrogenic pollutants present in some polluted waters could impact on the sustainability of populations. Estradiol equivalents (E2Eq) are measures of predicted total estrogenic potency, calculated from the potencies and predicted concentrations of estradiol (E2), estrone (E1), and EE2 [that account for >80% of total estrogenic activity in domestic effluent (Harries et al., 1996; Nakada et al., 2004)]. E2Eq are estimated to exceed an average of 10 ng/L E2 equivalents in 1-3% of river stretches in the United Kingdom (Williams et al., 2009). Long-term exposures to 0.5-1 ng/L EE2, (equivalent to 5-10 ng/L E2Eq) have resulted in female-skewed sex ratios and decreased egg fertilization (e.g. Armstrong et al., 2015; Parrott and Blunt, 2005; Zha et al., 2008). Exposures to higher concentrations of EE2 (4-6 ng/L) than are typically found in rivers, but have occasionally been measured in some effluents (e.g. Desbrow et al., 1998; Larsson et al., 1999; Rodgers-Gray et al., 2000; Ternes et al., 1999), have resulted in male to female sex reversal and/or breeding failure (Kidd et al., 2007; Lange et al., 2001; Nash et al., 2004). Notably, the addition of 5–6 ng/L EE2 to a whole lake in Canada over three years resulted in the collapse of the fathead minnow population (Kidd et al., 2007). Complete sex reversal of all genetically male roach also been observed in an exposure to an undiluted WwTW effluent for 2 years (Lange et al., 2020b; Lange et al., 2011).

There are now several studies that have found that fish populations can differ in their sensitivity to pollution. This has been attributed to genetic adaptation that has resulted in increased tolerance of the harmful effects of contaminants such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyl (PCBs) (e.g. Di Giulio and Clark, 2015; Williams and Oleksiak, 2011; Wirgin et al., 2011); and metals (Paris et al., 2015). In contrast little is known of whether fish populations differ in their sensitivity to endocrine disrupting chemicals (EDCs). The only exception we could find in the literature to this was for adult killifish (*Fundulus heteroclitus*) from chemically impacted Newark Bay (USA) that were found to be desensitized when exposed to 17β-estradiol, compared to fish from a reference population, as assessed using responses of vitellogenin and choriogenin genes (Bugel et al., 2014). In that study, the mechanism of desensitization was not established, although later studies using genome wide comparisons identified evidence for selection at estrogen-responsive genes in killifish populations at other polluted sites (Reid et al., 2016). However, killifish is not closely related and is ecologically different (an estuarine fish) to the fish species in which the effects of estrogen pollution in contaminated rivers has been studied. Thus the extent to which populations of fish differ in their sensitivity to estrogens (or other EDCs) is unknown.

Adult wild fish were used in Bugel et al.'s study (2014). Therefore, the influence of genetic and epigenetic effects (e.g. from previous exposure to pollutants) on the estrogen response would have been difficult to establish. Contrasting with Bugel et al.'s (2014) work, laboratory studies on zebrafish and on roach have indicated the potential for estrogen exposure to sensitize them to further estrogen exposures (Green et al., 2018; Lange et al., 2009; Nash et al., 2004). Therefore, in order to understand differences in sensitivity between wild populations, it is important to control for early life exposure.

The aim of the current study was to compare how established roach populations with different estrogen exposure histories respond to a chronic exposure of estrogen at a potency predicted to induce phenotypes observed in wild roach living in effluent polluted rivers. We used the offspring of roach taken from the wild in order to control for the potential confounding effect of sensitisation from early-life exposure.

2. Materials and methods

2.1. Choice of rivers for parental roach

Roach (*Rutilus rutilus*), a cyprinid fish, is native and widely distributed in English rivers and most of Europe. It feeds mostly on algae and small invertebrates including aquatic insect larvae and molluscs (Mann, 1973). It spawns annually in the spring with each female producing between 800-50,000 eggs depending on body size (Mann, 1973; Papageorgiou, 1979). Individuals up to 19 years old have been reported (Vollestad and Labeelund, 1990), but the oldest individuals generally found in UK rivers are ~8-9 years in age (Jobling et al., 2006). In the wild females generally reach sexual maturity at 3 or 4 years whereas males reach sexual maturity at 1-2 years (Vollestad and Labeelund, 1990).

The parental roach used in this study were derived from four populations - two from the Thames Catchment and two from the Humber Catchment (Fig. 1). In each catchment one population came from a stretch of river heavily contaminated with WwTW effluent and the other came from a stretch with no known upstream WwTW discharges. The contaminated river stretches were in the River Mole (Thames Catchment) and the River Wreake (Humber Catchment). These river stretches have predicted total estrogen potencies of 5.3 ng/L and 7.2 ng/L estradiol equivalents (E2eq) respectively, and are within the top 5% for of river stretches in England modeled for risk of estrogen exposure (Williams et al., 2009).

E2Eq are estimated from using geographical information systems-based model (LF2000-WQX), which predicts the concentrations of E1, E2, and EE2 in each WwTW effluent from the size of the population served by and per-capita excretion values. E2Eq also account for the types of treatment of each upstream WwTW and the dilution by river water (Williams et al., 2009). E2eq are then calculated using the

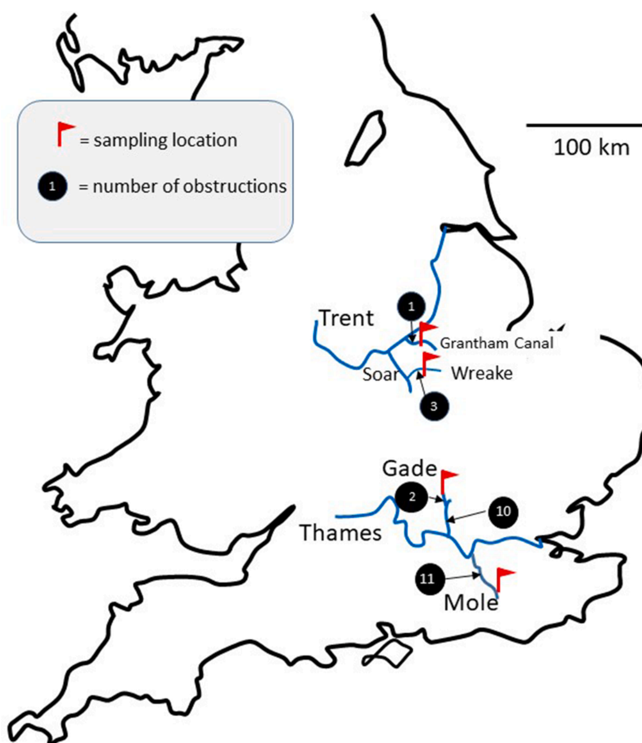

Fig. 1. Simplified map of the England and Wales showing the general locations of the origins of the parents of the laboratory bred fish; these offspring were used in the experimental exposure to ethinylestradiol. Numbers in black circles indicate the number of obstructions that restrict movement of fish (either locks or weirs) from the sampling location and the confluence with the next river.

following formula E2eq=¼[E1]+[E2] +10x[EE2] where the concentrations of E1 and E2 are multiplied or divided according to their predicted estrogenic potencies (Williams et al., 2009). E2Eq correlate with the actual incidence and severity of intersex in fish found downstream of WwTWs (Jobling et al., 2006). Previous surveys found that 39% of male roach from the River Mole had moderate numbers of oocytes in their testes (Hamilton et al., 2020). This compared to 17-29% from the River Wreake, 11-18% from the Grantham Canal, and 18% from the River Gade; for these rivers the males generally had low numbers of oocytes in the gonads (Hamilton et al., 2020; Jobling et al., 2002a; Jobling et al., 1998; Jobling et al., 2006).

In population genetic analyses, roach populations in the Humber Catchment grouped together and separately from populations in Thames Catchment; roach populations in these catchments may have been largely separated since the last ice age (Hamilton et al., 2020). Within the Humber Catchment, roach from the Grantham Canal and the River Wreake grouped with populations from the River Trent. The River Wreake flows into the River Trent (via the River Soar) and obstructions either side of the sampling location restrict movement (Hamilton et al., 2020) – Fig. 1. The stretch of the Grantham Canal has had no connection to the River Trent for 50 years (Hamilton et al., 2020) and the roach population from this section of the canal appears genetically isolated from nearby populations in the River Trent (Hamilton et al., 2020). Population genetic analysis has demonstrated that while the populations from the River Mole and River Gade are genetically similar to each other, migration is restricted between these populations and those in the main River Thames. This is consistent with the presence of weirs downstream of the sampling locations on these rivers (Hamilton et al., 2020; Hamilton et al., 2014) – Fig. 1.

2.2. Two Year Developmental Exposure to EE2

We used the offspring of wild fish from the study sites for the exposure to EE2. This ensured that fish used in the exposure had no direct prior exposure to estrogen between hatching and the start of the exposure. Pre-spawning sexually mature adult roach were captured by electrofishing by the Environment Agency (England) fisheries teams and were induced artificially to spawn using established procedures with carp pituitary extract (CPE), as previously described (Jobling et al., 2002b).

Equal volumes of milt from 10 males were mixed with approximately equal quantities of eggs from females from the same river. Five females were used for both the Grantham Canal and River Gade, 3 from the River Mole and 2 from the River Wreake, dictated by availability. Fertilized eggs were kept in triplicate 'holding' tanks from each river where they were allowed to hatch. For the exposures, 33-41 days after hatching the fry were deployed into 40 L glass tanks under flow-through conditions with water. Each tank contained 52 fry; 15 from each from the Mole, Gade and Grantham and 7 from the Wreake (as fewer fry were available).

Eight of these tanks were dosed at final nominal concentration of 1.75 ng/l EE2 with a flow rate of 100 ml/min, from 6-7 weeks post hatching and the remaining 8 tanks were maintained as controls (dilution water only). The EE2 exposure concentration was chosen based on published data for inducing feminized characteristics in males within 2 years, but it was unlikely to cause complete sex reversal of genetic males (Lange et al., 2009). For the dosing, stock EE2 (Sigma-Aldrich, Poole, U. K) was diluted to a concentration of 500 mg EE2/L in ethanol. Twice a week this stock was used to make a 25 μg EE2/L 'dosing stock' in a 2L bottle by diluting with water. This 'dosing stock' was then fed continually (by a peristaltic pump) into a header tank together with dilution water, and mixed continually with a magnetic stirrer. This made a final nominal concentration of 1.75 ng/L EE2 which was then distributed to all 8 EE2 treatment tanks. The final nominal concentration of ethanol was 0.00000035% v/v. Control roach were similarly maintained in flow-through dilution water tanks; no ethanol was added. After 3 months of exposure fry were moved into 135 L tanks with water flow rates into each tank of 640 ml/minute, while maintaining the same exposures and the groupings of fish. Again all 8 exposure tanks received EE2-dosed water at a final concentation from a single header tank. Further details of the maintenance protocol of the fish are given in the Supplementary Information.

Overall, the mortality rate over the 2 years was 6%. Fish were monitored twice daily for health assessments (normal swimming and feeding behaviors) and any fish showing signs of poor health, including from fin nipping imposed by other tank members were removed and euthanized to adherence to good fish welfare practice. Over the study this equated to 2% of fish in the control group and 12% of fish in the EE2 exposed group. Approximately once a month fish were sampled to reduce densities and to maintain similar densities between the different tanks.

After 1 year in the exposure study, 151 control and 106 exposed roach were sampled to reduce fish densities in tanks, and again this included removing those with nipped fins. At the end of the 2 year exposure a total of 365 fish were sampled (184 from the water control and 181 from the EE2 treatment); only fish sampled at this final time point were subsequently processed for analysis of sexual disruption. All animal work was carried out in accordance with the EU Directive for the protection of animals used for scientific purposes (2010/63/EU) and UK Animals Scientific Procedures Act (ASPA) 1986. Experimental procedures were carried out under personal and project licenses granted by the UK Home Office under ASPA, and ethically approved by the Animal Welfare and Ethical Review Body at the University of Exeter.

2.3. Confirmation of Estrogenic (EE2) Exposure

Water samples were taken from each tank (EE2 and controls) at 2 month intervals throughout the 2-year exposure (16 sets of samples in total) and were treated as per Beresford *et al.* (2016). Estrogenicity was quantified using the yeast estrogen screen (YES) and EE2 concentrations were confirmed by LCMS/MS (25% of samples) using standard protocols (Beresford et al., 2016). Results presented here for YES use EE2Eq, the response equivalent to the reference concentration of EE2. See Supplementary Information for further details.

2.4. Roach Sampling and Analyses

The juvenile laboratory-grown roach were sampled after one and two years of exposure, but only those after two years were assessed for the effects of estrogen exposure. Fish were terminally anaesthetized with buffered 250 mg/L MS-222 (Sigma-Aldrich) as approved by the U.K. Home Office (Animals (Scientific Procedures) Act 1986). For vitellogenin (VTG) analysis, blood was collected using a heparinized haematocrit tube, the blood was centrifuged at 7,000 g for 5 min at 4°C and the plasma collected and stored at -80°C until analysis. Fish standard length (cm) and wet weight (g) were recorded for each fish. Fulton's condition factor (K) was calculated from the following formula: (K) = (weight/length^3)*100. Tail fin tissue was collected and stored in 100% ethanol for molecular parental and genetic sex assignment. For histopathology, fish were excised ventrally and their whole bodies were preserved in Bouin's fixative (Sigma-Aldrich) for 24 hrs, after which tissues were stored in 70% industrial methylated spirits (IMS).

2.5. Histopathology

To determine the effects of the laboratory exposure of EE2 on sexual development, the gonads of roach at the end of the 2-year exposure were processed, sectioned and stained for histopathological assessment and their developmental stages were assessed using standard histopathology methods and protocols (Beresford et al., 2016; Jobling et al., 2006; Nolan et al., 2001). This was done 'blind'; the researchers undertaking the analysis of histology did not know the treatement, genetic sex or

parentage until all the histopathology was complete. Due to the small size (young age) of the roach, whole bodies (containing the gonads) were sectioned, rather than dissected gonads. For histological processing, four sections were taken spanning the posterior, middle and anterior of gonads. Sections were cut at 3 μm in thickness and stained with hematoxylin and eosin. Sections observed for each portion of the gonads were serial. Each male fish was given a whole number 'intersex score' (of between 1-7; those with a score of '7' were initially identified as female, but were given a score of 7 after genetic sex testing) to classify the level of male gonadal disruption based on the number of oocytes present in the testes (as defined by Jobling *et al.* (2006)), with the highest observed score in any of the four tissue sections recorded (G-L in Fig. 2). Phenotypic sex (male or female) was recorded for each fish; we refer to both normal male and intersex fish as 'males'. Ovarian cavities in males can occur as a consequence of exposure to estrogen during early life and therefore presence of female-like reproductive ducts (ovarian cavities) (Nolan et al., 2001) in males was also recorded.

To assess for developmental effects of EE2 exposure on spermatogenesis, the presence of extensive multiple developmental phases of spermogenic cells (asynchronous development) was also scored as present/absent (Johnson et al., 2009) and males were also given a score of between 1 and 10 (a modified Johnsen Score (Johnsen, 1970)) with 1 being the least developed testes (absence of both germ cells and Sertoli cells) and 10 being the most mature where the majority of tubules contained spermatozoa (see Supplementary information Table S1).

Genetic females were scored between 1 and 7 based on the developmental stages of oocytes, with 7 being the most developed i.e. 'mostly vitellogenic stage oocytes' and 6 also possessing vitellogenic oocytes (see Supplementary Information Table S2).

2.6. VTG ELISA

VTG in blood plasma was measured using a commercial VTG ELISA kit (Biosense Laboratories) as described previously (Tyler et al., 1996).

2.7. Parentage assignment and genetic sex testing

DNA was extracted from the fin tissue of all adults and fry, and a suite of DNA microsatellite markers (Hamilton et al., 2014) was used to conduct parentage analysis using Colony v2.0.5.0. (Jones and Wang, 2010). A PCR-based test was used to identify genetic sex; PCR amplification of the ITS1 nuclear ribosomal DNA region was included in this test to verify successful isolation of DNA (Lange et al., 2020b). See detailed methods in Supplementary Information.

2.8. Statistical analysis

All statistical analyses were conducted in 'R' (R Core Team, 2012) using the data from fish sampled at the final sampling point, ~2 years post hatching. We first examined the impacts of 'tank' and on fish length using data from both genetic sexes. Normality and variance of length data were assessed using histograms. An analysis of variance (ANOVA) was used to determine whether average length differed between tanks. The mean lengths, weights and condition factors of the fish in each tank were determined and these values were used to conduct T-tests to test for differences between the exposed and control tanks. Due to differences in the size of fish, all subsequent statistical models included 'tank' as a random factor to control for inter-tank correlations.

Using data from both genetic sexes, we examined interactions between 'exposure' and 'river' (i.e. river origin of the parents), and between exposure and genetic sex, on length of the fish. Then the influences of exposure and 'river' on genetic sex ratio were assessed, again including both genetic sexes. All subsequent analyses were conducted on either only genetic males (gonadal feminization, ovarian cavities, spermatogenesis score, asynchrony, VTG) or genetic females (oocyte progression, VTG), and included fish length as a fixed effect, as size can influence sexual maturity (Paull et al., 2008).

For each response variable the effect of 'river' was first examined by a comparing model with an interaction term between river and exposure (river*exposure+length) to one that excluded this interaction (river+exposure+length); a significant interaction would indicate that both river and exposure influenced the response variable. If no significant interaction was identified, the influence of each explanatory variable was assessed by comparing a 'full' model including all the explanatory variables (e.g. river+exposure+length) to a model excluding this term (e.g. 'exposure+length' to assess the influence of river).

Analyses of categorical responses were conducted using generalized linear mixed models (GLMM, binomial family, using the logit as a link-function), using the package nlme (Pinheiro et al., 2020) and p-values presented in this study were derived using models comparisons assessed using chi-square tests on the log-likelihood values. All other analyses were conducted by fitting linear mixed-effect (LME) models using the package lme4 (Bates et al., 2015) and p-values are based on model maximum-likelihood versions of the mixed models. For analysis of gonadal feminization, males with oocytes in testes or sex reversed males (intersex index = 7) were coded as '1' and those with no gonadal feminization were coded as '0'. Both histological spermatogenesis score and oogenesis score were arcsin transformed whereas plasma VTG concentrations log 10 transformed to improve model fit.

3. Results

3.1. Captive Breeding Exposure Experiment

After approximately two years of exposure, 365 fish were examined by histology. Parentage analysis revealed that there were only eight offspring of fish from the River Wreake, so these fish were excluded from all analyses. This left a total of 357 fish from the contaminated River Mole and the two reference sites (River Gade and Grantham Canal). Of these there were 179 exposed to EE2 and 178 were in control conditions. Genetic sex testing revealed that 185 were genetic males and 172 were genetic females.

In the control tanks, the average concentration of EE2 was 0.08 ng/L (± 0.08 ng/L) as measured by LCMS/MS or 0.07 ng/L (± 0.13 ng/L) EE2Eq as measured by YES. Limits of detection were 0.05 ng/L for LCMS/MS and 0.02 ng/L for YES. In the EE2 dosed tanks the average concentration was 1.29 ng/L (± 0.33 ng/L) as measured by LCMS/MS or 1.63 ng/L (± 0.61 ng/L) EE2Eq as measured by YES. There were small differences in the concentrations at different sampling points and between tanks (Supplementary Information Figure S1). This type of 'tank' effect had been anticipated. To account for this potentially confounding effect, our experimental design included placing the offspring of fish from each of the four rivers into each tank and 'tank' was included as a random factor in our statistical analyses of the effects of river origin.

3.2. Fish survival

At the final sampling point, there were significantly fewer surviving offspring of the fish from the River Mole (11%) compared to offspring of fish from the River Gade (49%) and the Grantham Canal (39%) despite the experiment starting with equal numbers of fish from each location (e.g. T-test for Mole and Grantham ($p<0.0000001$). Survival of the offspring of roach from the River Wreake was also lower, although fewer were included in the experiment and these were excluded from subsequent analyses. On average mortality was low (6 %), and 7% of the fish that were showing signs of poor health were removed from the tanks and euthanized for adherence with good animal welfare practice.

3.3. Fish size

At the end of the two year exposure average fish length was 8.2 cm (+- stdev 0.87 cm), average fish weight was 9.0 g +-3.0 g and average

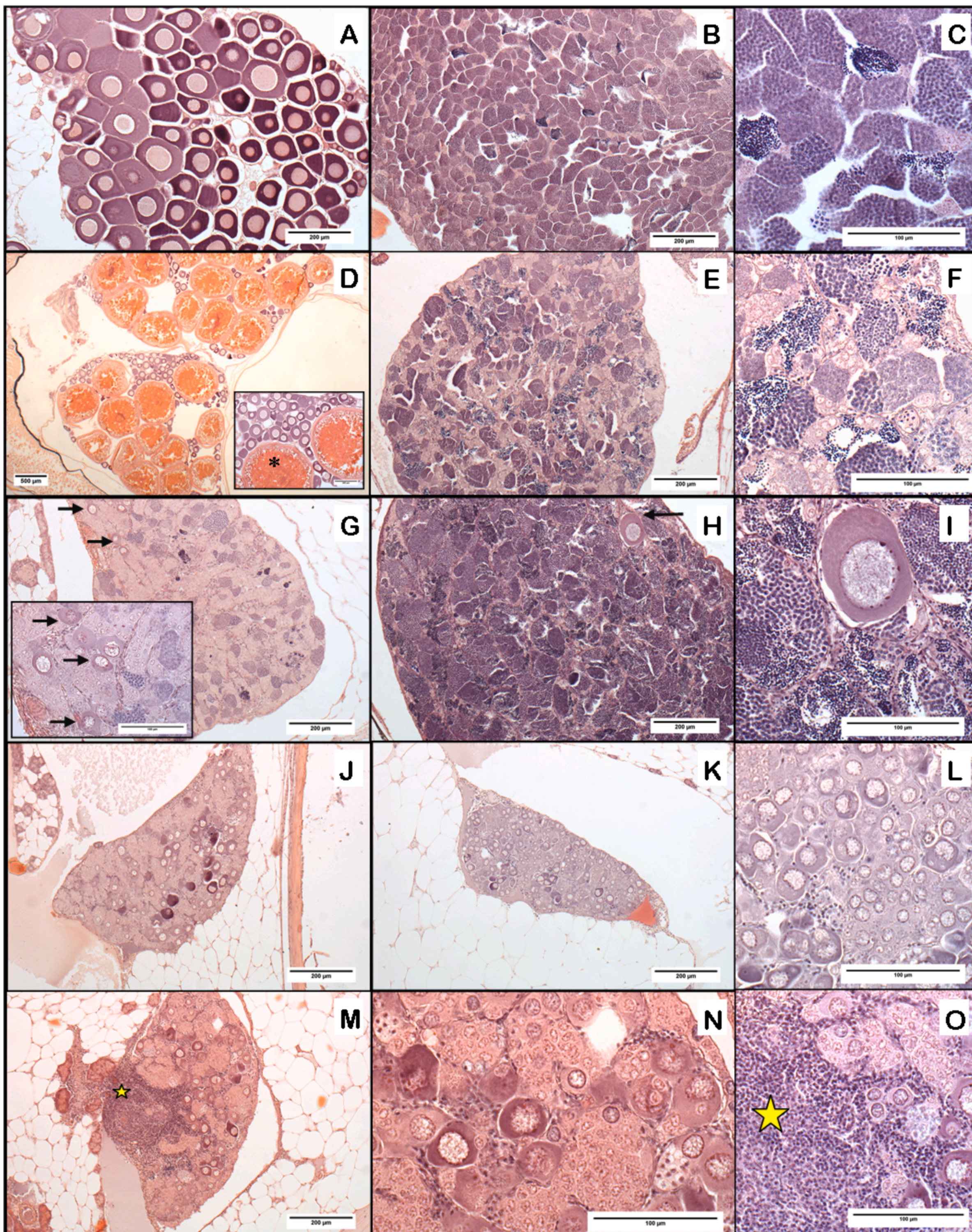

Fig. 2. Histological micrographs of gonads from roach exposed to a measured concentration of 1.3 ng/L ethinylestradiol (EE2) for 2-years. The top two panels show examples of genetic females and genetic males with the development score we saw most frequently in the dilution water (A-C) and EE2 (D-F) exposures i.e. in dilution water females had a mode score of 4 (A), whereas in the EE2 they had a mode score of 6 (D, * is an example of a vitellogenic oocyte). For the males in both the dilution water (B-C) and the EE2 (E-F) exposures the mode testicular development score was 8, however, the males had extensive asynchronous development. The next panel (G-I) shows genetic males with mild intersex alongside either early spermatogenesis (G) or advanced (full) spermatogenesis (H-I). Black arrows; testicular oocytes. J-L are images of genetic males that are seemingly "sex reversed" and M-O is also a "sex reversed" genetic male but with lymphocyte (yellow star) infiltration which somewhat resembles sperm in appearance.

Fulton's condition factor was 1.59 (Supplementary Information, Figures S2, S3). There were no differences in the average length (T-test, p = 0.38), weight (p = 0.31), or condition factor (p = 0.91) of the fish in the control tanks compared to those in the exposure tanks – Figure S2. There were, however, significant differences in the length of fish between tanks (ANOVA, DF = 15, F = 2.6, p = 0.0017).

Fish length was not influenced by river origin (likelihood ratio test, (LRT) = 0.96, p = 0.62)). However there was a significant interaction between genetic sex and exposure on the length of fish (LRT = 4.46, p = 0.034); genetic males were smaller than genetic females in control tanks, but there was no difference in length between the sexes in the exposed tanks (Supplementary Information, Figure S2). See Supplementary Information 1, Figures S3, for length, weight and condition factor broken down by river, genetic sex and exposure, and Table S3 for full results of statistical analysis.

3.4. Genetic sex ratio

The proportion of genetic males in the EE2 exposed tanks was 45%, compared to 61% in the control tanks (Fig. 3); this difference was statistically significant ($\chi^2(1) = 5.57$, p = 0.018). Genetic sex ratio was unaffected by the river origin of the parental fish (p = 0.78) - see Supplementary Information, Table S3 for the full results for the of statistical analyses.

3.5. Impacts on genetic males

A range of gonadal phenotypes were observed in genetic male fish exposed to EE2 (Figures 2 & 3). We found no evidence that the river origin of the parental fish influenced any of the gonadal characteristics measured in genetic males, or plasma VTG levels (p > 0.05 - see Supplementary Information, Table S3 for full details of statistical analyses). Feminized gonads (intersex condition or female-like gonads) occurred in 30% (23 of 76) of genetic males exposed to EE2, compared to 1 of 105 in the control group ($\chi^2(1) = 14.7$, p = 0.00013) – Fig. 3. For feminized genetic males within the exposure group the gonads of 12 had low numbers of oocytes in otherwise male gonads (intersex index 1 and 2) and the gonads of 11 resembled those of immature females (stages 3 and 4, peri-nucleolar oocytes) and were scored an intersex index of 7 – Fig. 2). Ovarian cavities occurred in 56% of genetic males in the exposure group and were absent in the control group (Fig. 3, $\chi^2(1) = 28.5$, p $= 9.3 \times 10^{-08}$) - see Additional file 1, Table S3 for full results of statistical analysis).

Assessed via histological sections, EE2 exposure inhibited spermatogenesis (LRT = 10.6, p = 0.0011) (See Supplementary Information, Figure S4). Exposure also resulted in a higher proportion of genetic males with extensive asynchronous gonadal development (See E and F in Fig. 2 and Supplementary Information Figure S4) from 3.7% in the control group to 37.7% in the exposed group ($\chi^2(1) = 14.4$, p = 0.00015). Blood plasma VTG was significantly elevated in genetic males exposed to EE2 (LRT = 60.4, p < 0.0001) (See Supplementary Information, Figure S5, Table S3).

3.6. Impacts on genetic females

Ninety six percent of the exposed females possessed vitellogenic oocytes (categories 6 and 7 on the oogenesis development scale, Table S2), compared to 51% (37/73) of the control group (Fig. 4) – (LRT = 16.0, p < 0.0001), see Supplementary Information 1, Table S3). There was no influence by river origin of the parental fish on the oogenesis development scale (p = 0.67). VTG concentrations in unexposed genetic females varied widely whereas all exposed genetic females had high blood plasma VTG levels. There was a significant interaction between exposure and river (LRT = 11.5, p = 0.0032) indicating that river origin affected the magnitude of VTG response to EE2. However, this appears to result from differences between unexposed genetic females; all exposed genetic females had similar VTG levels but the unexposed female offspring of fish from the River Mole had lower VTG levels than the female offspring of fish from the other rivers, particularly the River Gade (Supplementary Information 1, Figure S5).

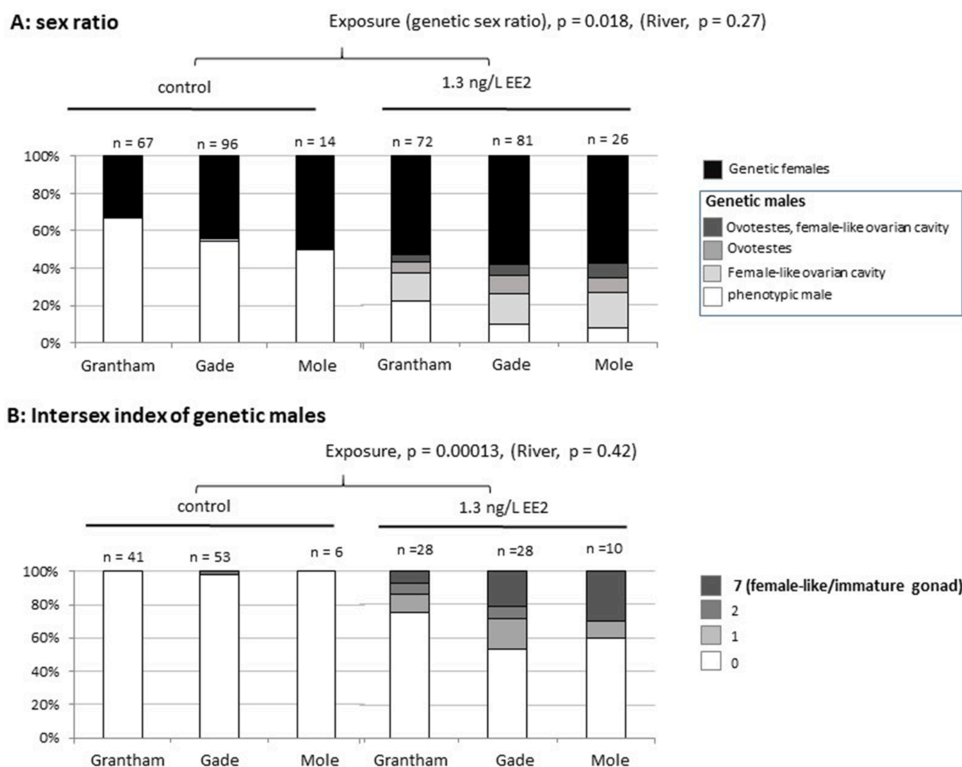

Fig. 3. Effects of exposure to a measured concentration of 1.3 ng/L ethinylestradiol (EE2) and river of origin of the parental fish on the sexual development of genetic male roach. A: Sex ratio. B: Intersex index of genetic males as defined by Jobling et al. (Jobling et al., 2006). This scale ranges from 0 (not intersex, no oocytes in detected in the testes), <0-2 are mildly intersex (low numbers of oocytes in the testes), 2-4 have moderate numbers of oocytes, 5-6 are severely intersex and 7 are histologically female.

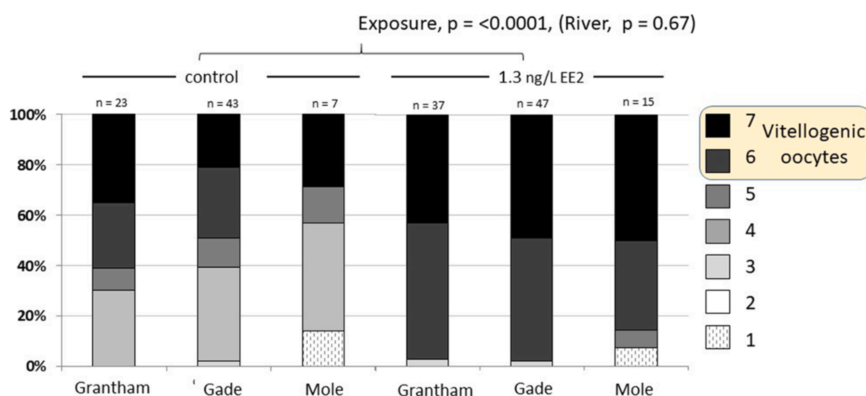

Fig. 4. Stages of gonadal development in genetic female roach after 2 years in either control or a measured concentration of 1.3 ng/L ethinylestradiol (EE2) exposure conditions. Numbers within bars represent the developmental stages of oocytes, with 7 being the most mature. 1 = no germ cells present, 2 = oogonia/primary oocytes only, 3 = some peri-nucleolar oocytes, 4 = predominantly peri-nucleolar and Balbiani stage oocytes, 5 = some cortical alveolus stage oocytes, 6 = some vitellogenic stage oocytes but less than 50%, and 7 = predominantly vitellogenic stage oocytes.

4. Discussion

In this study comparing the responses of roach derived from three different populations, we show that long-term exposure to estrogen feminized genetic males and affected female reproductive development. The average measured exposure concentration (1.3 ng/L EE2) is within the range reported in WwTW effluents (e.g. Rodgers-Gray et al., 2001; Ternes et al., 1999; Thorpe et al., 2006) and is equivalent to the estrogenic potency predicted for the 1-3% most polluted of river reaches in England (Williams et al., 2009). The slightly higher reported values in the exposure tanks as measured by the YES bioassay (1.6 EE2eq ng/L) may result from capturing endogenous estrogens excreted by the fish themselves. In the present study the intersex prevalence and severity were similar to that observed for wild roach of this age from river stretches heavily contaminated with WwTW effluents (Jobling et al., 2006). However, VTG concentrations measured in the present study in male fish were higher than reported from wild male fish in such contaminated river stretches, including the locations from where the parental fish were captured (Jobling et al., 1998), indicating that these roach had experienced a higher estrogen exposure than wild fish. Other effects of exposure included a reduced proportion of genetic males and induction of a “sex reversal” phenotype in 15% of genetic males. Significantly, in females this persistent low-level estrogen exposure induced premature sexual development, with an increase in the proportion of fish with vitellogenic oocytes from 51 to 96%. The significant differences between the VTG levels of females derived from the different rivers in the control tanks is difficult to explain; a genetic influence is possible given that the fish from the River Mole came from only three females, or an effect exposure to contaminants due to maternal transfer.

The main objective of this study was to compare responses to estrogen exposure between roach derived from the different populations. We found no differences in response in either sex, despite differences in the exposure histories of these populations and genetic differences between the Grantham population and the two Thames populations (Hamilton et al., 2020). To our knowledge the only other study comparing sensitivity of fish populations to estrogens has been conducted on killifish where fish taken from a polluted site were desensitized to estrogen exposure compared to exposed fish from a reference location (Bugel et al., 2014), which differs from our findings for responses in the roach. A possible explanation for these differences is our use of offspring of fish caught from the wild, rather than using the adult fish taken directly from the wild as in the killifish study. In our study on the roach the offspring from the different rivers in each tank experienced identical exposures during development. This reduced the potential influence of sensitization to estrogen, where early life exposure can increase sensitivity to estrogen exposure as an adult (Green et al., 2018; Lange et al., 2009; Nash et al., 2004). Developing oocytes can accumulate contaminants such as polychlorinated biphenyls (PCBs) and legacy pesticides through maternal transfer (Birceanu et al., 2015; Ostrach et al., 2008), so we cannot exclude the possibility that developing offspring were exposed these contaminants prior to hatching. Our previous work, however, found that that lifelong experimental exposure of female roach to an undiluted estrogenic WwTW effluent had no impact on the sexual development of their offspring when they were reared in clean water, or the responses of these offspring to an estrogenic effluent, when compared to the offspring of parentally unexposed fish (Hamilton et al., 2015). Together with the results of the present study, for roach there is no evidence to date that estrogen exposure of adults affects the sexual development of their offspring, or their sensitivity to estrogen, for example through transgenerational epigenetic inheritance.

For killifish widespread genetic adaptation that has led increased tolerance to various hydrocarbon contaminants at polluted sites. Variants of estrogen receptor 2b and estrogen receptor–regulated genes are enriched within outlier gene sets for pollution tolerant populations, indicating that they are involved genetic adaptation (Reid et al., 2016). By contrast, our previous population genetic analyses (using 217 SNPs) found no evidence of selection at estrogen receptors, and no enrichment of variants of estrogen responsive genes in comparisons of populations from polluted and ‘clean’ rivers (Hamilton et al., 2020). The results of the present study are consistent with these results.

Nevertheless, in our previous study there were large differences in the allele frequencies in the genes for androgen receptor and *Cyp1A* in comparisons of roach populations from the Trent Catchment and the Thames Catchment (Hamilton et al., 2020). These allele frequency differences provided evidence that genetic selection involving these genes had occurred in populations from the Thames Catchment and/or populations from the Humber Catchment since the separation of these groups of populations, potentially at the end of the last ice age, ~10,000 years ago. The results of the current study suggest that this selection was unrelated to sensitivity to estrogenic pollutants. Further work comparing the sensitivity of populations to androgenic and anti-androgenic contaminants would help to indicate whether selection at the androgen receptor was driven by pollution by EDCs, particularly as anti-androgens may contribute to feminization of male fish (Jobling et al., 2009; Lange et al., 2015).

Despite finding no differences in sensitivity to estrogen between the three populations of fish, the study provides new information into the reproductive health effects of exposure to a lower (and more environmentally relevant) concentration of estrogen than used in most long-term studies. For instance, we show that 30% of genetic males developed feminized gonads: either ovotestes (the intersex condition) or a ‘sex reversal’ phenotype. Previously induction of the ovotestes in roach has been reported at an exposure concentration of 4 ng EE2/L for a shorter period (120 days) during early life (Lange et al., 2009), whereas there was no gonadal feminization observed in 17 of 18 male roach exposed to an even lower dose (0.3 ng/L) for two years (Lange et al., 2009). A similar ‘sex reversal’ phenotype to that observed here, characterized by small ovaries with incomplete sexual differentiation and with some

possessing primary oocytes (Jobling et al., 2002a), was previously observed in wild roach from the River Aire, heavily polluted with estrogenic pollutants at the time (Jobling et al., 1998). This contrasts with the phenotype seen in most males that had undergone complete feminization in persistent exposures to higher estrogen concentrations, which is similar to that of genetic females (Lange et al., 2009; Lange et al., 2011). Nevertheless, recent application of a genetic sex test to wild roach populations from impacted sites has revealed that while complete sex reversal may occur in the wild it is rare (less than 5%), even at impacted sites (Baynes et al., 2019; Lange et al., 2020a).

In this study we found that estrogen exposure delayed spermatogenesis and increased the proportion of males showing extensive asynchronous development (i.e. with several stages of spermatogenesis observed within the same testes). Inhibition of spermatogenesis by estrogen has been attributed to reduced testosterone levels in somatic cells in the testes as the effect can be reversed by additional androgen treatment (Luiz Henrique de Castro et al., 2018). Asynchronous development is a relatively common phenotype in wild male roach downstream of WwTW discharges (Jobling et al., 2002a). These results demonstrate that estrogen pollution alone is sufficient to explain the occurrence phenotypes in polluted rivers although the reproductive consequences of these phenotypes are unclear.

Through the application of the genetic sex test in the present study we also show that EE2 exposure significantly reduced the proportion of genetic males from 61% to 45%. Studies in fathead minnow have found that exposure to 0.32 ng/L EE2 significantly altered the sex ratio towards females, but were unable to confirm genetic sex (Parrott and Blunt, 2005). For roach, reduced proportions of genetic males have previously been observed in individual tanks dosed with 0.3 ng/L EE2 for 250 dph (Lange et al., 2009) and WwTW effluent for 3 years (Hamilton et al., 2015). In the present study, however, the altered genetic sex ratio can specifically be linked to estrogen exposure as our statistical analysis used tank-level replication thus is robust to potential 'tank' effects. This may have resulted from sampling of males earlier due to ill health; 12 % of the exposed fish were removed due to poor health compared to 2% of the control. A possible explanation for reduced survival could be the energetic costs of VTG production in males, or the health impacts of egg yolk precursors such as VTG on the liver and kidneys, as has been suggested previously (Folmar et al., 2001). However, we did not test the genetic sex of fish removed before the final sampling point. The application of a genetic sex test (Lange et al., 2020a) has also revealed biases towards genetic females in roach populations from both polluted and clean river stretches. However, assessing the true sex ratio in the wild is complicated by potential differences in behavior between the sexes; determining the impact of pollution over and above this variability is challenging (if not impossible). Thus, in the present study we show that estrogen pollution could potentially alter the sex ratio in wild populations of roach through mortality resulting from sex-specific stress, without the complete feminization of genetic males.

In the wild, female roach generally mature at 3-4 years in age and they spawn annually. We found exposure to EE2 increased the proportion of females with vitellogenic oocytes from 51% in the controls to 96%, so some of these estrogen stimulated females would likely have spawned one year earlier than is normal. Our previous studies have found that exposure to undiluted WwTW effluent increased the proportion of female roach that reproduced after 3 years, compared to those kept in clean water conditions (Hamilton et al., 2015). Previously we considered elevated food sources in the WwTW effluent exposures may have advanced growth and therefore also advanced female sexual development (Hamilton et al., 2015). The present study suggests, however, that estrogen exposure alone is sufficient to induce earlier maturation, as food sources were identical and there were no significant differences in size between the females in exposed and control conditions. The mechanism is unknown, though may relate to earlier onset of puberty. Indeed, Le Page *et al.* (2011) speculated that because xenoestrogens affect GnRH neuron development, and in zebrafish the expression of kiss2 is upregulated by E2 (Servili et al., 2011), estrogenic EDCs have the potential to disrupt the onset of puberty.

Arguably, adverse impacts of a pollutant on the sexual development of females have greater potential to affect population sizes than adverse effects on males. Although premature sexual development of female roach could enhance population growth rates, the timing of first reproduction has been shown to be under natural selection to maximize lifetime reproductive output in other fish species (Barot et al., 2004). Therefore, females that reproduce a year early could be disadvantaged, for example by diverting resources from growth towards egg production, potentially increasing mortality or impairing reproductive output in subsequent years. Our study found no significant difference in the average size of females in exposed and control tanks (Figure S2). However, given that gonads comprise approximately 7% of the body weight in small prespawning female roach, which decreases to about 2% after spawning (Mann, 1973; Papageorgiou, 1979), it is therefore possible that early maturation affected somatic growth. Thus, we illustrate the potential for impacts of persistent estrogen exposure on populations through effects on females, at concentrations that induce no, or mild gonadal feminization in most males after two years of exposure.

A limitation of this study is that we were only able to compare responses from three locations, so it is still possible that population differences exist. In addition survival of the offspring of fish from the Mole and Wreake was lower than for the Gade and Grantham. It is not clear why this was the case, although the offspring from these sites were from comparatively few females, so maternal effects may have impacted on survival. As this was the case for both those exposed to estrogen and those in control tanks, it is unlikely to have altered our conclusions regarding effects of estrogen exposure. We highlight also the lack of an ethanol control in our experiemental design. However, in the EE2 treatment tanks the final concentration of ethanol was extremly low at 0.00000035% v/v ethanol and in previous long term exposures (including for roach over a period of 2 years) we found no differences in the gonadal status of roach between controls (no ethanol) and those exposed to ethanol concentrations 30,000 times higher than we used in this study (Lange et al., 2009; Yokota et al., 2001).

We conclude that chronic exposure to low level EE2 has reproductive health outcomes for both male and female roach. However, we find no evidence that the magnitude of the response was influenced by differences in parental and/or historical exposure to estrogen pollution, differences in allele frequencies at the androgen receptor and *Cyp1A*, and genetic separation of the roach populations from the Humber and Thames catchments for potentially 10,000 years.

Author's contributions

The manuscript was written through contributions of all authors. PH, SJ and CT obtained the funding for this research. PH, AB, EN, SJ and CT participated in the design of the research. PH, TUW, EN, AB participated in data collection. PH, CRT, AB wrote the paper. NB, MS, GH conducted analysis of water samples. TUW conducted analysis of DNA microsatellites. All authors have given approval to the final version of the manuscript. We are grateful to the two anonymous reviewers and the editor for their insightful comments.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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