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Altered Sexual Maturation and Gamete Production in Wild Roach (*Rutilus rutilus*) Living in Rivers That Receive Treated Sewage Effluents[1]

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ABSTRACT

Disruption in gonadal development of wild roach living in U.K. rivers receiving large volumes of treated sewage effluent is manifest in a variety of ways, ranging from malformation of the germ cells and/or reproductive ducts to altered gamete production. Intersex fish were also found to have an altered endocrine status and an elevated concentration of plasma vitellogenin. Gonadal growth was inhibited only in severely intersex fish, whereas progression of spermatogenesis was delayed in a large proportion of all intersex and exposed male fish. In contrast to the effects observed in the intersex and exposed male fish, the maturation of ovaries in female fish inhabiting effluent-contaminated rivers appeared to be less obviously affected, although a higher incidence of oocyte atresia was found in the effluent-exposed fish compared with the reference fish. A positive correlation was found between the proportion of female tissue in the gonads of intersex fish and their plasma vitellogenin concentration, suggesting that vitellogenin can be an indicator for the level of gonadal disruption in intersex roach. The estradiol-17β concentration in intersex fish was intermediate between the concentration found in males and females, and the plasma testosterone was between 2- and 3-fold higher in intersex fish compared with male fish. These data suggest a link between altered endocrine status in intersex and female fish and gonadal disruption. Spermiation was also affected in roach living in effluent-impacted rivers: a lower proportion of fish were found releasing sperm, and in those intersex fish that were spermiating, a reduced milt volume and a reduced sperm density were found. All intersex fish had malformations of the reproductive duct(s), and in severely affected fish, the ducts were occluded, thus preventing release of gametes. In view of the widespread occurrence of intersexuality in wild fish populations in rivers throughout the United Kingdom, assessment of the reproductive capabilities of these intersex roach is clearly needed to understand the impact of this phenomenon on roach fertility.

environment, gametogenesis, seasonal reproduction, steroid hormones, toxicology

INTRODUCTION

In U.K. rivers, the occurrence of intersexuality (hermaphroditism) in wild roach (*Rutilus rutilus*; a cyprinid fish), characterized by the simultaneous presence of both male and female gonadal features in the same animal, is reported to be widespread [1]. In that study, most of the intersex roach were found to have both male and female reproductive ducts, and many also had female germ cells, or oocytes, within a predominantly male ''testis.'' The number, pattern, and developmental stage of oocytes within testicular tissue in intersex roach was found to vary greatly; the condition ranged from the presence of single, primary oocytes scattered randomly throughout testicular tissue in a mosaic fashion to large areas of ovarian tissue, clearly separated from testicular tissue, in severely feminized fish [2]. More recently, the intersex condition has been reported in other species of gonochoristic fish, including the gudgeon (*Gobio gobio*, U.K. [3]) and the flounder (*Platichthys flesus*, U.K. [4]; *Pleuronectes yokohamae*, Japan [5]), suggesting that sexual disruption is not a species-specific phenomenon and is not restricted to U.K. waters alone.

The exact causation of intersexuality is not known and currently under intense scrutiny. What is known, however, is that the populations of wild roach and gudgeon showing a high degree of intersexuality have been exposed to high concentrations of treated sewage effluents. Furthermore, exposure of early life-stage roach to treated sewage effluent has been shown to induce disruption (feminization) of the gonadal ducts in males [6]. Treated sewage effluents contain complex mixtures of endocrine-disrupting chemicals (EDCs), and these chemicals, both natural and synthetic, interfere with the normal hormonal processes that are crucial to the formation and maturation of the reproductive organs. Indeed, EDCs have now been linked to reproductive and developmental disturbances in several wildlife species either living in or closely associated with the aquatic environment [7].

Expression of the male or female phenotype can be altered, or reversed, by exposure of fertilized eggs, or embryos, to pharmacological concentrations of estrogens, androgens, or aromatase inhibitors (reviewed in [8]). Hence, the occurrence of intersexuality in wild fish populations has been associated with the widely reported presence of steroidal estrogens and their mimics in treated sewage effluents and their receiving waters [1, 9]. In wild roach in U.K. rivers, good evidence indicates that the intersex condition has resulted from exposure to environmental estrogens and, therefore, that the condition is a consequence of the partial feminization of genetic male roach rather than of masculin-

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ization of genetically female roach [1, 6]. The induction of vitellogenin (VTG; an estrogen-specific protein) in male roach exposed to treated sewage effluents [10] and the presence of VTG in male and intersex wild fish [1, 3, 5] has clearly established that these species are being subjected to an estrogenic challenge.

The widespread occurrence of hormonally active substances in surface waters has been an issue of concern for government regulators, scientists, and the public. To date, however, little is known regarding the consequences to wildlife of long-term exposure to these contaminants. Sex steroids play central roles in stimulating growth and development of the reproductive organs (ducts and the gonads) and in control of the reproductive cycle in all vertebrates, including fish. The roles of estrogens and androgens in gametogenesis are well characterized in fish, in which estrogens, principally estradiol-17β (E_2), are involved in control of the growth of the ovary whereas androgens (11-ketotestosterone [11-KT] and testosterone [T]) stimulate growth and development of the testes (reviewed in [11]). The plasma concentrations of each of these steroids, therefore, rise and fall in close synchrony with their seasonal roles in the development and maturation of the ovaries and testes. Altered patterns of synthesis, secretion, and/or function as a consequence of exposure to exogenous steroid hormones, or their mimics, could potentially alter their sexual cycling and/or their functioning. In some fish species [12] and in reptiles [13], the concentrations of E_2, T, and 11-KT, as well as the ratios between them, appear to be altered on exposure to EDCs, both in the laboratory and in the field, although the functional significance of these alterations has not yet been established.

In wild intersex roach, nothing is known regarding the plasma sex hormone profiles, the ability of ovarian and testicular parts of the gonad to mature, or what their reproductive capabilities are (e.g., whether they can produce functional gametes). Moreover, it is not known if intersex roach can produce sperm and/or eggs at the appropriate spawning time in their annual reproductive cycle [14]. Furthermore, our work to date has focused on the effects of treated sewage effluents on male fish; essentially, nothing is known regarding the effects of this exposure in female roach. If the consequences of the widespread exposure of fish to treated sewage effluents are to be understood, then the effects of the intersex condition in roach, at both the individual and the population level, need to be established. The aim of this study was to begin this process by comparing sexual maturation and gamete production in populations of wild roach (including intersex fish) living in water courses that receive no treated sewage effluent with those living in rivers that receive treated sewage effluents.

MATERIALS AND METHODS

Collection and Sampling of Wild Fish

In all, 206 adult wild roach were collected from two U.K. rivers that received treated sewage effluent, and a further 124 were collected from three reference sites. The river sites receiving treated sewage effluent were located on the Nene (Northamptonshire) and the Aire (Yorkshire). The nearest sewage treatment works (STWs) discharging into these rivers were located between 3 and 15 km upstream of the sampling sites. Details regarding the amount of treated effluent discharged at these sites have been given previously [1]. At both these river sites, populations of intersex roach were known to exist. The reference populations of roach were sampled from canals (reference 1: Royal Canal, Ireland, n = 43; reference 2: Grantham Canal, Leicestershire, n = 30) and from a spring-fed lake (reference 3: Lake Wartnaby, Leicestershire, n = 51). In these reference roach populations, the incidence of intersexuality varied between 1% and 4%. The fish collections were carried out at two time points during the annual reproductive cycle of the roach: one to assess and compare the progression of sexual maturation between the populations (autumn sampling: Aire, n = 51, 22 October 1995; Nene, n = 41, 23 October 1995; reference 1, n = 43, 25 October 1996; reference 2, n = 30, 26 October 1996), and one to coincide with the annual spawning (spring sampling: Aire, n = 44, 18 May 1998; Nene, n = 70, 22 May 1998; reference 3, n = 51, 19 May 1998). The reference site for the spring sampling (reference 3) was at a different location from that for the autumn sampling to facilitate the collection of spawning fish from all spring study sites over a 5-day period. Collection of the fish was by either electrofishing or netting methods, depending on weather conditions and the type of the body of water.

After capture, all fish were anesthetized individually using 0.1% (v/v) 2-phenoxyethanol, and blood was sampled via the caudal sinus. Blood was collected into 1-ml heparinized syringes, transferred directly into 1-ml microfuge tubes containing aprotinin (Sigma Chemical Co., Poole, Dorset, U.K.), and frozen at −20°C until required for quantification of VTG and/or sex steroids. All fish were then killed using standard procedures approved by the U.K. Home Office, and their lengths and weights were measured. Scales were taken for aging of the fish.

Analyses of Fish Sampled in the Autumn

Gonad analysis. For each fish collected, a ventrolateral incision was made in the abdominal cavity, and the gonads were photographed in situ. Detailed observations were made regarding their macroscopic appearance. The gonads were then removed, weighed to determine the gonadosomatic index (GSI; gonad weight/(body weight − gonad weight) × 100) and preserved in Bouin fixative (Sigma Chemical Co.) (~12 h) before transfer into 70% industrial methylated spirit (Sigma Chemical Co.) for storage before processing for histology. Paired gonads from each fish were divided into three equal portions: anterior, mid, and posterior. A central part (thickness, 3–5 mm) from each portion was taken and embedded in paraffin wax. Each of these gonad parts was then sectioned (thickness, 3 μm) to give a total of six transverse sections of tissue per fish (three from each gonad). All sections were mounted and stained with Mayer hematoxylin and eosin (Sigma Chemical Co.). Examination was by light microscopy using a range of magnifications (from 20× to 100×). For each section, the stage of development of the gonad (maturity), cell types present, intersex status, and any other abnormalities were recorded (including duct development). The sexual maturity of testicular tissue was assessed according to the method of Billard [15] for the roach. This method is based on determining the proportion of testicular cysts within each of five testicular lobules containing the different spermatogenetic cell types: spermatogonia A, spermatogonia B, and spermatocyte A. In the normal reproductive cycle of the roach, the proportion of cysts containing spermatogonia B increases during the summer, reaches a maximum in the autumn, and then decreases with the successive appearance of spermatocyte A (premeiotic cells [15]). The status of sexual development of the ovarian tissue was assessed by determining the proportional area of each of six sections occupied by oocytes of each developmental stage. Using the resulting data, each fish was then given a numerical score to denote its stage of maturity, based on the stage of development of the most dominant type of oocyte in the tissue, as follows: status 1, oogonia; status 2, primary oocytes; status 3, cortical alveolus oocytes; status 4, early to mid-vitellogenic oocytes; and status 5, mid- to late-vitellogenic oocytes. Oocytes were staged in both intersex and female fish using accepted criteria for classifying oocyte development in other oviparous fish (e.g., [16]). Measurements of the average size of oocytes in each stage, the thickness of the follicle, and zona radiata were also taken.

Plasma VTG and sex steroid analysis. Frozen plasma samples were thawed carefully on ice before assay for VTG and sex steroids. Plasma concentrations of VTG were measured using a carp VTG ELISA [17] that has been validated for use in the roach [18]. The interassay variation for the VTG ELISA in this study was 5%. Sex steroids were extracted from plasma samples using ethyl acetate. Then, E_2, T, and 11-KT were measured by specific radioimmunoassay. The detection limits and interassay variations (coefficients of variation) for the sex steroid assay were: E_2, 15 pg/ml and 19.1% (n = 5); T, 15.9 pg/ml and 17.3% (n = 4); and 11-KT, 366.7 pg/ml and 9.9% (n = 4).

Analyses of Fish Sampled in the Spring

After anesthesia, attempts were made to expel the gametes manually by applying gentle pressure to the abdominal cavity. If gametes were expelled, the volume of either milt (semen) or expelled eggs was then carefully measured, using a graduated test tube, to the nearest 0.1 ml, and the

sperm density of each fish was measured using a hemocytometer. In the spring sampling, many of the fish were spermiating or ovulating freely; therefore, particularly for the male fish, the methodologies used to assess the status of sexual maturity among the fish sampled in the autumn were not appropriate. (In spermiating males, it is often not possible to even discern the individual testicular lobules.) The status of sexual maturity in fish sampled in the spring was, therefore, ascribed simply to whether gametes could or could not be expelled from the fish. After expulsion of the gametes, the gonads were removed and weighed (to determine the GSI) and then fixed and processed for histology, as described above, and any morphological abnormalities were carefully documented. Gonadal analysis of any fish (male, female, or intersex) that had failed to ovulate or spermiate was carried out as described for the autumn sampling. It was also noted whether oocytes, eggs, and/or sperm were present in the reproductive ducts. Plasma sex steroids and VTG were measured as described above.

Statistical Analyses

Comparisons of hormonal concentrations and reproductive parameters were made with StatView (Cherwell Scientific, Oxford, U.K.). Failure to meet the assumptions of homoscedasticity and normality resulted in log transformation of the data before parametric analysis. Comparisons were made between individual sites and between sexes (male, female, and intersex; see Figs. 1–4) and also between males, females, and intersex fish from all sites (pooled data), regardless of their origin (see Figs. 1–4, insets). Differences between sites and/or sexes were assessed by ANOVA in all cases, and then pairwise comparisons were subsequently made with the Fisher test (all comparisons) and the Bonferroni test (for comparisons only with reference data). Comparisons with pooled reference data were also made (when stated), and these comparisons were statistically justified in each case. Pairwise correlation coefficients between hormone concentration and physiological indices were calculated by Pearson product-moment correlations for all data. With bivariate normal distributions, all nonnormal data were ranked, and the Spearman rho correlation test was applied. All data are reported as the mean ± SEM unless otherwise indicated.

RESULTS

Age at Maturity

All populations of roach captured from all sites (sites receiving treated sewage effluent: Nene, Aire, and reference sites [1, Royal Canal; 2, Grantham Canal; 3, Lake Wartnaby]) in the autumn and spring contained large proportions of maturing adult fish. The age structure of the population of Aire fish sampled in the autumn was bimodal, with predominant year classes of 3- and 6-yr-old fish, whereas the ages of the Nene and the reference fish varied between 2 and 6 yr and were normally distributed. In the spring, the age structure of the population of fish from the Aire was unimodal, with a predominant year class of 6 yr. In the spring, the Nene and Wartnaby roach populations also had unimodal age structures, with predominant year classes of 4 and 3 yr, respectively. No statistical differences were found between the proportions of male and female fish maturing in each year class at each site. Roach normally first mature when either 2 or 3 yr old. Females mature more slowly than males; hence, some females may not mature until they are 4 yr old [19]. With the exception of the Aire fish, all fish of 4 yr and older, sampled across all sites in this study, were sexually mature.

Gonadal Status and Sexual Development in Roach in the Autumn

Females. All female fish of 4 yr or older from both reference sites in the autumn were undergoing sexual maturation. The size of the ovaries and their stage of development in females from the reference sites were appropriate for roach ovaries midway through the seasonal reproductive cycle; hence, they contained oocytes in early to mid vitellogenesis [2]. Similarly, all the female fish sampled from the Nene, and most of the females from the Aire, were undergoing sexual maturation. No differences were found in the GSIs of the maturing females between both the reference sites (Royal Canal: 7.26 ± 0.30; Grantham Canal: 6.24 ± 0.49) and females from the Nene (6.7 ± 0.3) and Aire (5.9 ± 0.5) (Fig. 1a). Similarly, no differences were found in the status of gonadal development, as assessed by the presence of different stages of oocytes, between the reference sites and the Aire and the Nene (Fig. 1b). No differences were found in the sizes of oocytes of each stage or in the thickness of the follicle and zona radiata between the fish from the reference sites and the Aire and Nene (results not shown). The incidence of atresia (resorption of oocytes [16]), however, was higher in the ovaries of fish from the Nene and Aire compared with the females from either of the reference sites (Fig. 1c).

A small proportion (14.3%; 4 of 28 fish) of the fish from the Aire, aged between 4 and 7 yr, were sexually immature or indifferent. Two females from the Aire (ages, 3 and 7 yr) that were sexually immature had extremely small gonads (GSI, <1%) in which sexual differentiation appeared to be incomplete. Although the reproductive ducts in these fish were fully formed and appeared to be female, the gonads contained no primary or secondary oocytes and were composed largely of oogonia or undifferentiated germ cells. In a third female (age, 4 yr) from the Aire, the ovaries contained just a few primary oocytes interspersed with extensive amounts of proliferative connective tissue. In a fourth Aire female (age, 6 yr), the gonad contained primary and cortical alveolus-stage oocytes only. In these four fish, therefore, the gonadal status was abnormal and uncharacteristic with respect to the age of the fish and time of the reproductive season.

Males and intersex fish. At the reference sites, almost all phenotypically male fish appeared as histologically normal males. However, a small number of fish (reference 1: n = 4; reference 2: n = 3) were found with extremely low numbers of primary oocytes (one or two oocytes per gonad section on one or two sections) within otherwise normal testicular tissue. In these fish, the reproductive ducts were male and appeared to be normal. In accordance with their histological appearance, the gonads of both male and intersex fish from the reference sites were of a normal size for the age of the fish and the time in the reproductive season (Fig. 1a).

All phenotypically male fish from both the Aire and Nene were histologically intersex and contained female-like, aberrant reproductive ducts and, in some fish, oocytes at various stages of development within the testicular tissue. The GSI of these intersex fish was significantly lower (Nene: 2.2 ± 0.2; Aire: 2.5 ± 0.2) compared with the GSI of male or intersex fish from either reference site (Fig. 1a). In almost all the intersex fish (except for only one 3+ fish from the Aire), the testicular tissue within the gonads was undergoing sexual maturation, but spermatogenesis appeared to be delayed when compared with normal male fish (or with intersex fish) from either of the autumn reference sites. In the male fish from both reference sites, spermatogenesis was in the mid to late stages of mitosis and the early stages of meiosis; the most dominant cell type was spermatogonia B (occupying 96.2% ± 0.54% and 96.2% ± 0.72% of the cysts within each lobule for reference sites 1 and 2, respectively), together with smaller numbers of spermatocyte A (occupying 3.7% ± 0.6% and 3.0% ± 0.5% of the cysts within each lobule for reference sites 1 and 2, respectively). In contrast, in the gonads of the inter-

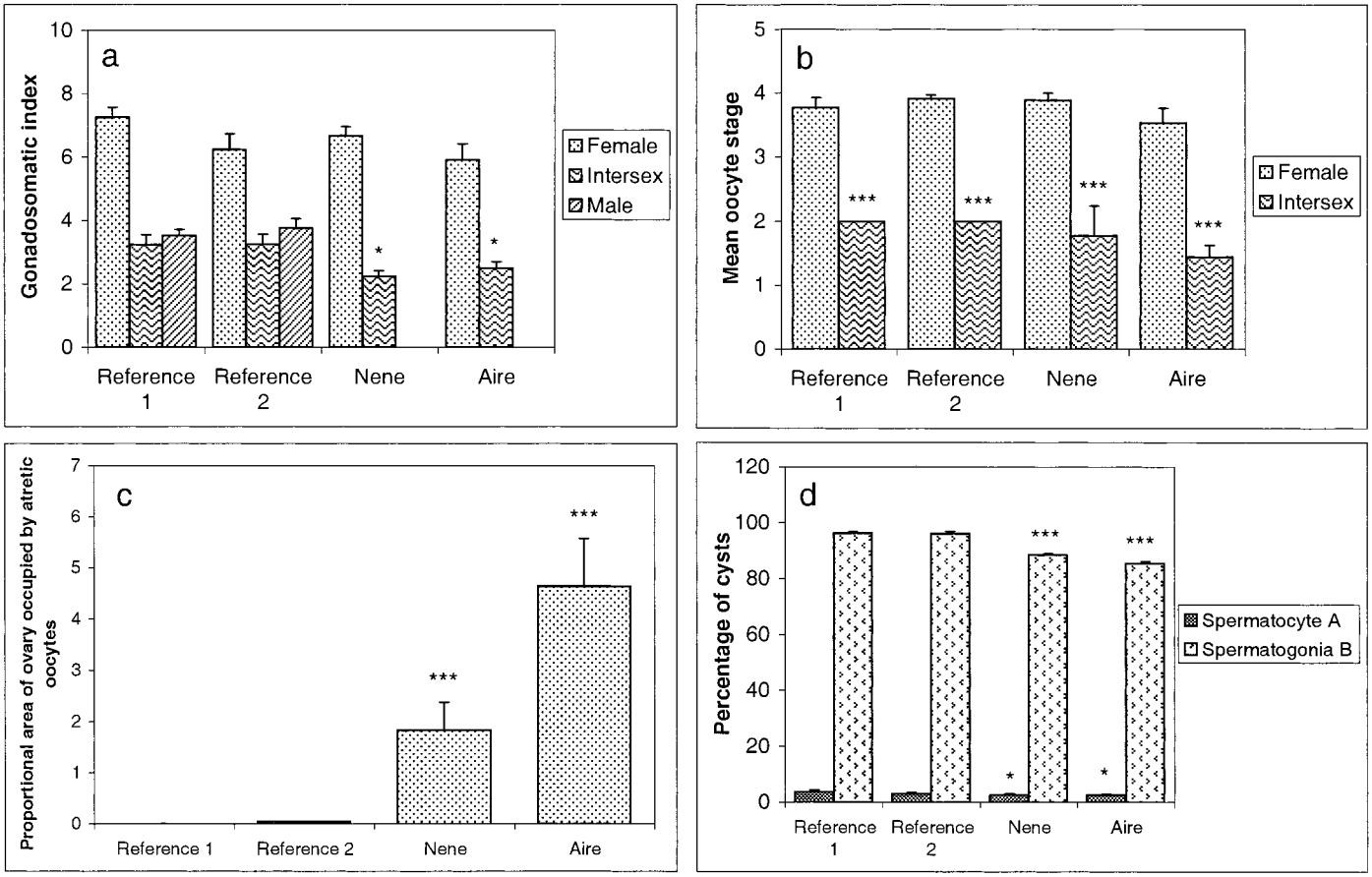

FIG. 1. Status of the gonads of roach sampled from reference sites and sites receiving STW effluent. **a**) GSIs in male, female, and intersex roach from the reference and river (Nene and Aire) sites. Asterisks denote significant differences between intersex fish and pooled reference male fish. **b**) Maturity of the ovarian tissue in the ovaries or ovotestes of female (reference 1 and 2) or intersex (Nene and Aire) roach sampled in October. Asterisks denote significantly lower values than in reference females. **c**) Preovulatory atresia in female roach sampled in October. Asterisks denote significant differences compared to the references. **d**) Maturity of the testicular tissue or ovotestes in male (reference 1 and 2) and intersex (Nene and Aire) roach sampled in October. Asterisks denote differences that were significantly lower than reference males. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

sex fish from both the Nene and Aire, both fewer spermatogonia B (88.5% ± 0.45% in the Nene fish and 85.3% ± 0.68% in the Aire fish) and fewer spermatocyte A (2.6% ± 0.5% in the Nene fish and 2.6% ± 0.4% in the Aire fish) were found. All these differences between the proportions of the different male cell types in the Aire and Nene fish were statistically different from those of fish from both reference sites (pooled data) (Fig. 1d).

In contrast with the testicular tissue in the intersex fish, the ovarian tissue in these animals, in most cases, did not appear to be maturing (Fig. 1b). In general, intersex gonads were characterized by variable numbers of oogonia (status 1) and primary oocytes (status 2). In some intersex fish, cortical alveolus-stage (status 3) and early vitellogenic oocytes (status 4) were also present, albeit in much lower numbers compared with oogonia and primary oocytes. No significant differences were found in the ages of the intersex and male fish between any of the sites. However, an indication was observed that the degree of feminization (in intersex fish) increased with age; hence, older fish appeared to be more feminized. This observation was not, however, statistically significant.

Plasma VTG Concentrations

The concentrations of plasma VTG in males from the Royal Canal and Grantham Canal (the reference sites) were 100 ± 40 and 15 ± 9 ng/ml, respectively (Fig. 2a). No significant differences were found in the VTG concentrations between male and intersex fish within both reference sites. The VTG concentrations in female fish collected from the reference sites were between 20 000- and 30 000-fold higher than those in the males and were as expected for the time in their reproductive cycle. The VTG concentrations in female fish obtained from the Nene and the Aire were similar to that in the reference females. Intersex fish obtained from both the Nene and Aire had much higher plasma VTG concentrations ($P < 0.001$) than those of the male and intersex fish from the reference sites. Indeed, the plasma VTG concentrations of the intersex fish from the Nene closely approached that of the female fish at this and the other sites (Fig. 2a). When data from all the sampling sites were pooled and then categorized into male, intersex, and female fish, the VTG concentrations in the intersex fish were intermediate between the concentrations found in male and female fish (Fig. 2a, inset). A significant, positive correlation ($r = 0.419$, $P = 0.0054$) was found between the proportion of tissue in the gonads of intersex fish that was female and their VTG concentrations. (As a corollary, a negative correlation [$r = -2.81$] was found between the proportion of male tissue in the intersex gonad and the VTG concentration, although this relationship was not statistically significant.)

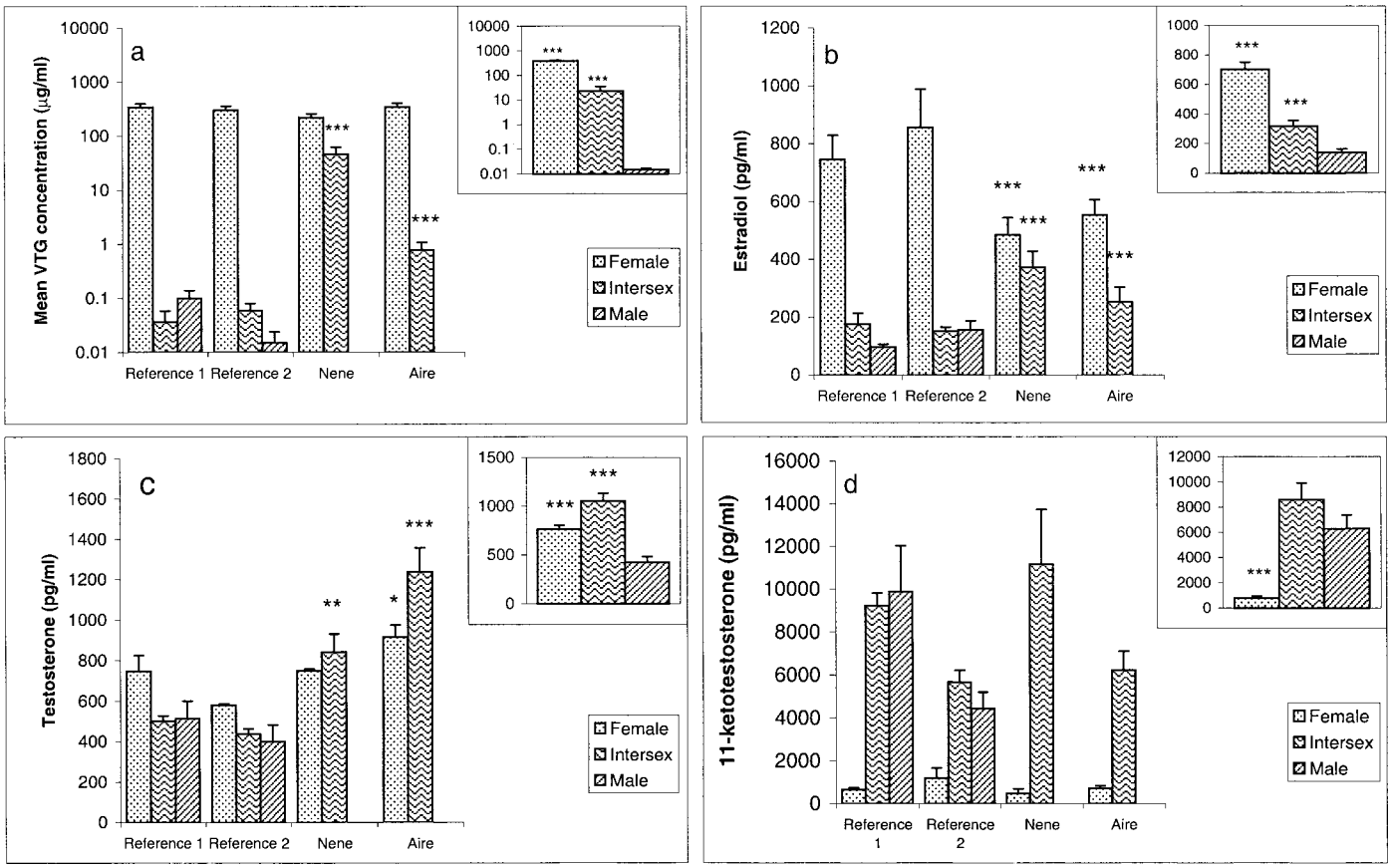

FIG. 2. Sex steroid hormone and VTG concentrations in male, female, and intersex wild roach sampled in October. **a**) VTG. **b**) E_2. **c**) T. **d**) 11-KT. The main figures present data from the individual sites; inset figures present pooled data from all four sites. Asterisks on the main figures represent significant differences in concentrations between intersex fish or exposed females and pooled reference males or females, respectively. Asterisks on the inset figures refer to significant differences between intersex fish or females and males. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Plasma Concentrations of Sex Steroids

Sex steroid concentrations in the plasma of fish across all sites varied greatly, depending on the sex of the fish and its state of sexual maturity. Sex steroid concentrations, however, did not vary with the order of sampling (e.g., from fish 1 to 30), thus indicating minimal effects of confinement stress on steroid hormone synthesis.

Estradiol-17β. The concentrations of plasma E_2 measured in male, female, and intersex fish from all sites varied both between sites and between sex groupings within the same site (Fig. 2b). In the reference fish, plasma E_2 concentrations were higher ($P < 0.001$) in females compared with males and intersex fish. No differences were found in the E_2 titers for the respective groups of male, female, or intersex fish between the two reference sites. Furthermore, for each reference site, no statistically significant differences were found in the plasma E_2 concentrations between the male and intersex fish from the same location. The E_2 concentrations in the female fish sampled from the Nene and Aire, however, were approximately half those measured in female fish from either of the reference sites. In addition, the intersex fish sampled from the Nene or the Aire had plasma E_2 concentrations approximately 2-fold higher than those in the normal males from either of the reference sites ($P < 0.001$). When all data for male, intersex, and female fish were pooled across all the sampling sites, the E_2 concentrations in the intersex fish were intermediate between the concentrations found in male and female fish (Fig. 2b, inset).

Testosterone. In general, the concentrations of plasma T varied between male, female, and intersex fish derived from the same site (Fig. 2c). Comparing the two reference sites, however, no differences were found in the plasma T concentrations between similar groups (male, female, or intersex). Plasma T concentrations were highest in the intersex fish from the Nene (842 ± 89 pg/ml) and Aire (1238 ± 119 pg/ml). For the intersex fish in the Aire, this T concentration was significantly higher than that in the intersex fish at both of the reference sites, while in the intersex Nene fish, the T concentration was significantly higher than that in the intersex fish in reference 2 only. The plasma T concentrations in the intersex fish were up to 3-fold higher relative to those in the males at the reference sites. When all data for male, intersex, and female fish were pooled across all the sampling sites, the T concentrations in the intersex fish were significantly higher than those in either males or females sampled at that time (Fig. 2c, inset).

11-Ketotestosterone. At all four sites, female fish had much lower 11-KT concentrations (below the reliable detection limit of the assay) than either male or intersex fish (Fig. 2d). No significant differences were found in the 11-KT concentrations between the male and intersex fish or between the four groups of intersex fish.

Sexual Maturation and Gamete Production in Roach Sampled in the Spring

Gamete quantity. Roach are group spawners, and they spawn in a single event that takes place in the spring over 1 or 2 days. Most of the females from all the sampling sites had already ovulated (references: 80%, n = 15; Aire: 87%,

n = 21; Nene: 91%, n = 27), and eggs could be expressed from most or all of the remaining females from sites both exposed and not exposed to effluent. It was not possible to get reliable fecundity estimates, however, even in the fish that were ovulating, because it could not be established whether they were starting to ovulate or had already ovulated some of their eggs. All the males from the reference site were spermiating (Fig. 3a). The mean milt volume and sperm density of these fish were 1.74 ml/g (testis weight) and 4 200 000 sperm/μl, respectively (Fig. 3, b and c). The production of sperm by intersex and male fish from either the Nene or the Aire (the rivers receiving STW effluent) was highly variable compared with that of the reference males. Only 50% (n = 12) and 57% (n = 12) of the macroscopically male fish from the Nene and Aire, respectively, produced milt (compared with 100% of the reference males) (Fig. 3a). Milt volume was reduced in intersex and male fish from the Nene and Aire compared with that of the reference males (Fig. 3b), and sperm density was also reduced in intersex and male fish from the Aire compared with reference males (Fig. 3c). Three fish from the Aire produced both mature eggs and sperm simultaneously and, thus, were true hermaphrodites. Two of these individuals produced very few eggs (10 and 12); thus, their fecundity was much lower than that of normal female roach, which produce between 5000 and 50 000 eggs per spawning. The third fish produced large quantities of eggs and little milt. Sperm density in these simultaneous hermaphrodites was also much reduced compared with that of normal male fish.

Gonadal status. Macroscopic observations of the gonads indicated that, in 8.3% (n = 2) of ''male'' fish from the Nene and 38% (n = 8) from the Aire, the gonads were of an abnormal shape. In some cases, development of the gonad in an anterior direction and/or a posterior direction (from its point of origin on the mesonephros) was reduced. In other cases, the reproductive ducts were absent or terminated before the opening of the genital pore. In two cases, the reproductive ducts appeared to be blocked, even though the testes were fully mature (gentle abdominal pressure resulted in rupture of the gonads and expulsion of the milt into the abdominal cavity). The GSI in spermiating intersex fish was similar to that in spermiating reference male fish (2.69 ± 0.5 vs. 2.94 ± 0.16, respectively), whereas in nonspermiating intersex fish, the GSI was significantly lower (1.28 ± 0.13) than that in normal male (spermiating) fish or spermiating intersex fish ($P < 0.001$) (data not shown). As with the autumn sampling, the ages of the intersex and male fish did not differ significantly between sites.

Histological examination of the gonads of all fish confirmed that 22 of the 24 ''males'' from the Nene (two fish were normal males) and all of the ''males'' from the Aire were, in fact, intersex. In contrast, at the reference site, only normal males and females were found.

The histological appearance of the intersex fish varied greatly. The major types of disruption are given in Table 1. The gonads of each fish were categorized according to whether or not milt and/or eggs could be expelled and according to the sexuality of the reproductive ducts and the germ cells.

Histopathological examination confirmed the following points: First, in intersex fish that were able to expel gametes, the gametes were found to be present in the male and/or the female reproductive ducts. These gametes were usually sperm. Second, when female germ cells were present in the gonads of intersex fish, they were usually in the early

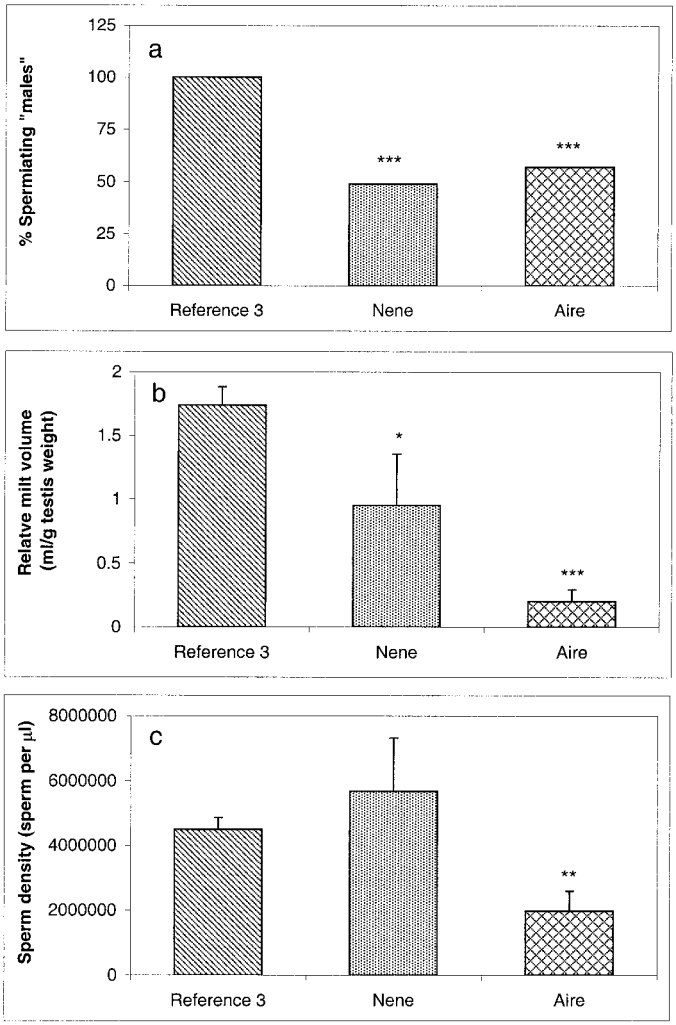

FIG. 3. Sperm production in populations of wild roach. **a**) Proportions of ''male'' fish producing milt. **b**) Relative milt volume of spermiating male (reference) and intersex (Nene and Aire) roach. **c**) Sperm density of spermiating male and intersex roach. Asterisks denote significant differences from reference data. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

stages of development. In a small number of fish, developing oocytes and sperm cells appeared to have matured simultaneously and in synchrony with that for normal development for gametes in males (sperm) and females (eggs). Third, the gonads in several of the nonspermiating intersex fish were small and, histologically, appeared to be immature, even though the fish were of reproductive age, thus suggesting a delay in sexual development. Fourth, some nonspermiating intersex fish were probably unable to release mature sperm due to blockage (occlusion) of the reproductive ducts. Fifth, some nonspermiating intersex fish had mature testicular tissue but did not release sperm (despite an unobstructed sperm duct), thus suggesting a delay in spermiation.

The gonads of almost all the female fish were spent (all mature oocytes had been ovulated). Large areas of the gonadal tissue contained postovulatory follicles and developing oogonia/primary oocytes undergoing the initial phases of growth. Histopathological comparisons between the gonads in female fish from the Nene, the Aire, and the reference site confirmed that the females were at a similar stage in the annual reproductive cycle. A small number of mature female fish (Aire: n = 3; Nene: n = 1) had not

TABLE 1. Types of histopathological sexual disruption in intersex fish collected from Nene and Aire rivers at spawning time.

Type of disruption[a]	No. of fish		
	Spermiating	Non-spermiating	Both ovulating and spermiating
Disruption of the reproductive ducts			
Sperm duct (open)	3		
Ovarian cavity (open)	3	3	2
Sperm duct and ovarian cavity (open)	11	9	
Two sperm ducts and one ovarian cavity (open)	6	1	
Occluded sperm duct, ovarian cavity, or both	1	4	
Germ cells present			
Only male germ cells (sperm present)	12		
Only male germ cells (sperm absent)		5	
Only female germ cells			1[b]
Both male and female germ cells (sperm present)	11	6	
Both male and female germ cells (sperm absent)	1	5	
Both male and female germ cells (both sperm and fully mature oocytes present)		1	1

[a] Disruption of the reproductive ducts and the germ cells is described separately for all fish (n = 43). Reproductive ducts were classified as open in cases where the duct extends to the genital pore and occluded where one or both ducts terminated anterior to the genital pore and were closed at the posterior end. The presence of male, female, or intersex (both male and female) germ cells was assessed for each gonad. The presence or absence of mature male (spermatozoa) or female (oocytes) germ cells was also noted. The patterns of disruption seen in the ducts, germ cells, or both were further categorized under three groupings, depending on whether the fish was spermiating, nonspermiating, or releasing both eggs and sperm.
[b] Although sperm were found in the milt released, the histological appearance of the spent gonad was female.

ovulated, and their gonads contained large numbers (as much as 50% of the gonad) of large, atretic oocytes. The ducts, however, were normal in all cases.

Plasma Concentrations of VTG and Sex Steroids in Roach During the Spawning Period

The plasma concentration of VTG in males at the reference site (reference 3: 65.7 ± 5.4 ng/ml) was similar to that measured in the males during the autumn at the other reference sites. The plasma concentration of VTG in the female fish at the different sites was lower than that in reference females from the previous sampling, but no differences were found between females obtained from the different sites in the spring sampling (Aire: 552 ± 76 μg/ml; Nene: 431 ± 134 μg/ml; Wartnaby: 381 ± 77 μg/ml). In the intersex roach, VTG concentrations were also low (34.5 ± 15.2 ng/ml).

Concentrations of sex steroids (E_2, T, and 11-KT) were extremely low both in intersex fish and in males during the spawning period (results not shown).

DISCUSSION

The results of this study confirmed that almost all the ''male'' roach inhabiting the Nene and Aire were intersex, as defined by the simultaneous presence of both male and female reproductive tissue [1]. Intersexuality in these fish was manifest in a wide variety of ways, including malformation of the gonads and/or reproductive ducts and altered gamete production. Intersex fish (sampled in the autumn) also had an altered endocrine status and elevated concentrations of plasma VTG.

During the annual reproductive cycle of the roach, an intense period of gonadal growth occurs in the summer [20] such that, during the autumn, the gonads of males and females would be expected to constitute approximately 6%–7% and 3%–4% of the total body weight, respectively [21]. The GSIs of both males and females sampled in this study were of the expected size. Data from the reference fish show that intersex gonads can be of a normal size when the condition is mild, but alternatively, they can be smaller than expected in more severely affected fish. These findings were not unexpected, because published studies have reported an inhibition of gonadal growth in intersex fish [1]. Because the GSI of intersex fish depends, at least in part, on the severity of the condition, it is not possible to distinguish intersex fish in a population based on their relative GSI alone.

Spermatogenesis was clearly inhibited in many of the intersex fish from the Aire and Nene, but the cause of this delay is not known. Delays in spermatogenesis and concomitant effects on gonadal growth have, however, been well documented in laboratory studies investigating the effects of exposure of male fish to either natural or environmental estrogens during sexual maturation (e.g., [22–24]) and at concentrations known to be present in treated effluents from STWs [9].

In contrast with the growth and development of the testes, the maturation of ovaries of female fish inhabiting effluent-contaminated rivers appeared to be less obviously affected. During the autumn, the majority of adult female fish appeared to be undergoing vitellogenesis at comparable rates, regardless of where they were living. No significant differences were found across all the sample sites in oocyte size, thickness of the zona radiata, or stage of oocyte development. However, a higher incidence of oocyte atresia was found in the effluent-exposed fish compared with the reference females. In addition, the incidence of atresia in effluent-exposed females appeared to be higher in the spring than in the autumn; in some individuals in the spring, 50% of the cross-sectional area of the gonad was composed of atretic oocytes. These results would suggest that ovulating fish might have a lowered fecundity relative to females from the reference sites, in which a very low incidence of atresia was found. Atresia is thought to be an uncommon event in healthy females, and it has been linked to poor nutrition, environmental stress [25], and starvation [26]. No differences were found in the condition factor between the groups of fish; thus, little evidence of starvation was seen. It is possible, therefore, that the increased incidence of atresia in the females from contaminated waters is due to prolonged exposure to environmental stressors. Exposure to EDCs (including the synthetic estrogen ethinylestradiol [27] and the antiandrogen vinclozolin [28]) is known to

induce atresia in fish. Taken together, these field and laboratory findings raise the possibility that atresia in wild female roach could occur as a result of exposure to hormonally active contaminants.

The presence of low, but higher than basal, concentrations of plasma VTG in males from one of the reference sites (Royal Canal) are consistent with observations from some previous studies of wild [1] and laboratory-maintained male fish [17]. It is not known, however, whether these plasma VTG concentrations result from exposure to low levels of estrogens via the diet and/or water or are a consequence of endogenous estrogen. There could also be interpopulation variability in the basal concentrations of plasma VTG between the different reference sites. The elevated concentrations of plasma VTG in the intersex fish from the Nene and Aire in the autumn indicate that these fish had been exposed to significantly higher levels of estrogen, or estrogen mimics, than fish at the reference sites. A positive correlation between the proportion of tissue in the gonads of intersex fish that was female and their plasma VTG concentration indicated that VTG in intersex fish (measured in the autumn) is an indication of the level of gonadal disruption in the roach. This confirms previous work that demonstrated a weak, positive correlation between plasma VTG titer and the intersex index (a numerical score used to describe the degree of feminization of the gonad [1]), and it emphasizes the utility of VTG as a biomarker for endocrine disruption by estrogenic compounds. Laboratory studies have shown a negative correlation between plasma VTG and testicular growth and development in males exposed to estrogens or estrogen mimics (e.g., [22, 24]). The concentrations of EDCs in each of the rivers were not measured in this study; hence, we were unable to assess whether the level of exposure to these chemicals was sufficient to induce the effects seen in the fish. Both effluent discharges directly upstream of the points of capture are, however, known to contain steroidal estrogens and/or estrogen mimics, and in the Aire, these contaminants have also been measured at concentrations high enough to explain the increased VTG concentrations in the wild roach captured during this study [9, 29]. It is unclear, however, whether the increased VTG production is due entirely to exposure to exogenous estrogens, because intersex fish also have elevated endogenous estrogen concentrations due to disruptions in steroidogenesis.

Plasma sex steroid hormone concentrations and the ratios of estrogens to androgens have been used as indicators of exposure to suspected EDCs in both fish [12, 30] and reptiles [13, 31, 32]. To date, however, the validity of using these parameters to predict real reproductive dysfunction (e.g., intersexuality, reduced or retarded testicular growth and development) has not been proven. Indeed, an important consideration often overlooked in studies of this nature has been that the concentrations of sex steroid hormones will vary in maturing adult fish, depending on the stage of their reproductive cycle [33]. Furthermore, steroid levels can also be affected by handling or confinement stress [34, 35]. For all species studied (and for which documented evidence exists in the literature), information regarding sex steroid concentrations in different populations of animals from different localities (e.g., contaminated and noncontaminated) is either very limited or nonexistent. Care has to be taken, therefore, when using plasma titers of sex steroids and ratios of these steroids to assess possible alterations in reproductive function. In the present study, however, intersex fish from the Nene and Aire did appear to have sex steroid profiles differing from those of the male or the intersex fish from the reference sites (collected at the same time of the year), with significantly higher titers of both E_2 and T in the effluent-exposed Nene and Aire fish. The E_2 concentrations in the intersex fish in the autumn were intermediate between those found in males and females and strongly support the hypothesis of an alteration/disruption of steroidogenesis in intersex fish. Female roach from the Nene and Aire sampled in the autumn had low plasma E_2 concentrations compared with those of the reference females. Many chemicals, especially those in the effluent of pulp and paper mills, disrupt steroid synthesis by the ovaries [36], and chemicals of this nature probably are also present in the effluent-contaminated waters of the Nene and Aire. Reduced plasma E_2 concentrations have been shown to have a negative influence on egg quality [37], and it is interesting to note that the prevalence of atresia was also higher in the Nene and Aire females compared with that in the reference females. Fecundity was not measured in this study; therefore, it is not yet known if prolonged exposure to estrogenic effluents has detrimental effects on egg production, as has been documented with steroidal estrogens in laboratory studies [27].

Spermiation in the roach normally begins in early to mid May and continues for a period of approximately 3 wk [20]. During this period, the quantity of milt produced per single stripping will vary from 0.1% to 2% of body weight (unpublished results). In this study, the collection of milt was carried out during the spawning time, when many of the females were spontaneously ovulating. All the males from the reference sites were spermiating, whereas only 50% and 57% of the intersex fish from the Nene and Aire, respectively, produced sperm. It is possible that the differences in milt production between the intersex fish and the normal males (from the reference site) were due to differences in the sampling times (with respect to the cycle of milt production). Having said this, there did not appear to be any differences between the sites with respect to the timing of ovulation, and in seasonally spawning fish, the reproductive cycles of the male and female are usually in synchrony. Furthermore, the timing of spawning in seasonally spawning cyprinids is closely linked to the water temperature, and because the water temperature varied by only 0.2°C (on the day of capture) between the sites, it seems unlikely that differences in water temperature would explain the differences in milt production observed between the intersex and male fish.

A separate, but nevertheless related, point concerns the size of the gonads of spermiating male fish compared to those of spermiating intersex fish. Our results suggest intersex fish that release sperm naturally have gonads of a similar size to those of spermiating male fish. This, therefore, would indicate that the gonads of intersex fish have the capacity to reach maximal size, at least in some individuals. Notwithstanding this, the spermiating intersex fish had a reduced milt production (and sperm density in the Aire fish) compared with the spermiating male fish; hence, a deleterious effect of intersexuality on milt production, regardless of gonad size, was observed. The reasons for this are unclear. In almost all the spermiating intersex fish, in histological sections, milt could be seen both in the ovarian cavity (the female reproductive duct) and in the sperm duct(s); therefore, it was assumed that both ducts could transport sperm to the exterior. The quality of the sperm was not determined in this study; hence, the functionality of the feminized ducts could not be assessed. That a small

number of roach from each of the two impacted river sites produced both male and female gametes demonstrated that simultaneous hermaphrodites exist in the wild. The milt obtained from these fish, however, was extremely dilute, and the eggs produced were few in number in two of the fish. In the third fish, predominantly female gametes were produced. The ability of these fish to self-fertilize was not tested and is, in part, a focus of our future research.

The reason for the presence of sexually immature or indifferent roach of a reproductive age in the Aire, but not in any other site, is not known. Sexually immature or indifferent gonads are characteristically found in roach infested with the cestode parasite *Ligula intestinalis*, which exerts its effects by inhibition of gonadotrophin-releasing hormone, the hypothalamic hormone that ultimately controls the growth and development of the gonad [38]. No signs of parasitic infestation were observed in any of the roach, however, from any of the sites; therefore, it seems reasonable to assume that the effects seen are due to exposure of the fish to gonadal toxicants, genotoxins, or EDCs.

In conclusion, the results of this study suggest that the reproductive success of roach living in rivers that receive treated sewage effluents is very likely to be compromised. These detrimental effects are not restricted to the males; they also occurred in females. Other field studies have demonstrated a decreased reproductive success in fish as a consequence of exposure to xenobiotics, including known endocrine disruptors, in polluted habitats such as industrialized estuaries and coastal waters [39–42]. In most cases, including in the current study, the ecological significance of these phenomena on reproductive output at the individual and population levels is unclear. In view of the widespread occurrence of intersexuality in wild fish populations in rivers throughout the U.K., assessment of the reproductive capabilities of these intersex roach is clearly needed to understand the impact of this phenomenon on roach fertility.

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