# Endocrine disruption is reduced but still widespread in wild roach (*Rutilus rutilus*) living in English rivers

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SYNOPSIS

Assessment of wild fish in English rivers indicates endocrine disruption persists at most of the sites surveyed two decades ago

ABSTRACT

Endocrine disruption of wild fish, primarily resulting in the feminization of males, has been reported in English river sites for several decades. Estrogenic activity emanating from Wastewater Treatment Works (WwTW) has been conclusively demonstrated to be the main driver of these feminized phenotypes. Here we re-visit ten English river sites previously surveyed in the late 1990s and early 2000s to assess how the frequency and severity of feminization now compare with the historical surveys. In the contemporary assessment, 60% of the sites re-visited still showed endocrine disruption at the tissue organization level (oocytes present in otherwise male gonads; intersex) and 90% of sites had average male plasma vitellogenin concentrations (female-specific yolk protein; a sensitive biomarker of estrogen exposure) above natural baseline levels. In contrast to the historic surveys, none of the males sampled in the contemporary survey had ovarian cavities. At one of the larger WwTW, improvements to treatment technology may have driven a significant reduction in intersex induction, whereas, at several of the smaller WwTW sites, the frequencies of feminization did

not differ from those observed in the late 1990s. In conclusion, we show that although the severity of feminization is now reduced at many of the re-visited sites, endocrine-disrupting chemicals are still impacting wild fish living downstream of WwTW in England.

## INTRODUCTION

Endocrine disruption in fish arising from exposure to chemicals emanating from wastewater treatment works (WwTW) effluents has been under investigation since the 1990's. The original study in the UK identified elevated concentrations of a female egg yolk precursor, vitellogenin (VTG), in the plasma of caged male rainbow trout exposed to WwTW effluent[1] and studies that followed showed tissue level effects in fish living downstream of WwTW outfalls, or in fish experimentally exposed to WwTW effluents[2–6]. In those studies, normally gonochoristic fish species displayed intersex phenotypes whereby the gonadal tissue contains both developing eggs and sperm, and/or the fish had reproductive duct abnormalities i.e. oviducts present simultaneously with sperm ducts[4,7]. The intersex condition and elevated VTG plasma concentrations/gene expression in male fish have now been reported in wild fish populations globally[8–13]. Furthermore, fish with high levels of intersex have been shown to be adversely impacted, with altered gamete maturation and reduced reproductive success[5,14–16].

Estrogenic pharmaceuticals (e.g. the contraceptive hormone 17α-ethinylestradiol; EE2), industrial xenoestrogens (e.g. alkylphenols) and natural steroid estrogens (e.g. 17β-estradiol; E2, estrone; E1) identified within WwTW effluent are now well known to be the main drivers of intersex phenotypes in fish[17,18]. Furthermore, in the UK, the incidence and the severity of intersex in wild fish have been found to highly correlate with prediction models for natural and synthetic

estrogen river concentrations[19–22]. In England, there has been no large-scale assessment of endocrine disruption in fish (i.e. intersex and VTG induction) since the early 2000's.

In the last ten years, technological and operational improvements to WwTW at pilot and full scale have demonstrated reductions in estrogen concentrations and endocrine disrupting effects in fish[23,24]. Advanced water treatment technologies (e.g. ozonation, granular activated carbon) that can reduce micropollutants, such as estrogens, have not been employed at scale in the UK to treat wastewater. However, measures that have been implemented to reduce ammonia and nitrogen in WwTW effluents can also remove estrogenic steroids[25–27] and in turn this may have resulted in reduced estrogenic pollution in receiving waters.

Due to their toxicity to aquatic wildlife, the use of alkylphenols (e.g. nonylphenol (NP) and octylphenol (OP)) have been regulated under the European Commission's Water Framework Directive (WFD) since the early 2000s, and a reduction in NP has been associated with a reduced disruption of gonadal development in wild fish in selected rivers that were historically heavily contaminated with NP[28]. In the UK, the most current and far ranging data set on chemicals emanating from WwTW come from the Chemical Investigation Program(s) (CIP), implemented by the Environment Agency and the Water industry. The CIP(s) monitored a range of pollutants including steroid estrogens and xenoestrogens (e.g. NP) in WwTW effluents between 2010-2011 ("CIP1", 162 WwTW effluents from England, Scotland and Wales) and 2016-2019 ("CIP2", 605 WwTW across England and Wales)[29]. The data from the CIP1 and CIP2 indicate average concentrations of estrogenic steroids measured in WwTW effluents during these two monitoring periods were relatively similar, albeit with high variability for the earlier CIP (e.g. EE2 0.6 ng/L ± 2 ng/L in CIP1, 0.2 ng/L ± 0.16 ng/L in CIP2, mean average concentrations reported ± standard deviation; data from Tables 1 & 2 in Gardner *et al.*[29]) whereas NP was shown to

significantly reduce over the same period (NP 0.23 µg/L ± 0.16 µg/L in CIP1 to 0.14 µg/L ± 0.14 µg/L in CIP2)[29]. The CIP2 dataset is considerably larger than that for the first program, CIP1, which complicates making direct comparisons over time. Nevertheless, 75 sites were directly comparable between the two CIP datasets[29]. At these specific sites, average NP concentrations were significantly lower during the period for CIP2 compared with that for CIP1 (mean percentage change of 24%, Table 3 in Gardner *et al.*[29]). Unfortunately, not all chemicals were monitored at all sites at all times[29], including the steroid estrogens, so a more direct comparison of the concentrations of EE2 was not possible. Earlier available data, from a more limited number of sites (<10), indicate WwTW effluent estrogen levels of E1, E2 and EE2 were in the range of 1.4-76 ng/L, 2.7-48 ng/L and <0.2-7 ng/L respectively in the mid 1990's[17] and <0.4-12.2, <0.4-4.3, <0.5-3.4 ng/L in the early 2000's[20]. In the mid-1990's NP effluent concentrations were as high as 330 µg/L on the heavily polluted River Aire, and ranged from <0.2-6.7 µg/L elsewhere in England and Wales[30]. Therefore, data indicate an overall general reduction in estrogenic activity of WwTW effluents discharging into English rivers.

In this study we investigated current levels of endocrine disruption (primarily the incidence and severity of the intersex condition, and levels plasma VTG) in roach (*Rutilus rutilus*), in selected English rivers. Roach are a European native gonochoristic freshwater fish species, common to lowland rivers in England, found in abundance in waters enriched by sewage discharges. In the UK and Europe, roach have been utilized for endocrine disruption assessments, both in the wild and under experimental conditions, for several decades[4,9,14,28,31]. We specifically designed the study to allow us to directly compare contemporary and historical levels of endocrine disruption in roach in rivers, by revisiting sites sampled in the late 1990's and early 2000's[4,19] where historically intersex was measured in roach (to varying degrees). With the advent of new

105 molecular methods to determine the genetic sex of roach[32], we also looked to confirm the
106 hypothesis that wild intersex roach in these English rivers are feminized males (rather than
107 masculinized females) and to assess the frequency of possible complete sex reversal in these
108 wild fish.

109 MATERIALS AND METHODS

110 **Historical survey data.** Historical roach data (including information on individual fish
111 morphometrics, age, phenotypic sex, intersex score, and plasma VTG) and site data (including
112 WwTW/site name, upstream and downstream national grid references, modelled river estrogen
113 concentrations (as per Johnson and Williams[33]), and WwTW population equivalent (PE)) were
114 obtained from files originally compiled and held by authors on this paper working on the topic
115 since the 1990s.

116 **Site selection for comparison of historical and contemporary levels of feminization.** Roach
117 data (e.g. fish morphometrics, age, intersex score, and plasma VTG) from a total of 73 sites (with
118 ordnance survey national grid reference number) derived from the previous two major surveys
119 (1995-98, 2000-2003; Figure S1 Supporting Information) were interrogated to determine site
120 suitability for reassessment. Criteria for inclusion included they were riverine (i.e. not lakes,
121 canals, fish farms) and that roach had previously been sampled and shown to exhibit feminized
122 phenotypes (e.g. intersex gonads). Ideally, samples also had been obtained from the sites visited
123 on more than one temporal occasion. Sites with data from both upstream and downstream of
124 WwTW effluent discharges were prioritized for inclusion. Sampling sites were also selected to
125 ensure representation across the full range of estrogenic potencies previously detected, as
126 indicated by the incidence and severity of gonadal feminization (i.e. low, moderate and high

intersex occurrence). Sites where data had been derived from only a few fish (less than 20 roach) were excluded to avoid statistical weakness. Cross-referencing with Environment Agency records, sites were excluded if they had recent (≤ 8 years) fish kills/restocking events, and where roach populations were not expected to be sufficient in number to sample. A total of ten sites were sampled in the autumn/winter of 2017 (Figure 1), all of which had at least one historical intersex dataset for comparison (see Table S1 Supporting Information).

**Ethics statement**. The use of animals for this study was approved by the institutional ethical review committee (Brunel University London Animal Welfare and Ethical Review Board). All procedures were conducted in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986 and conducted under the authority of the UK Home Office. 2017 Project Licence (PBB96C199).

**Field collection and transport.** Adult roach (≥10 cm in length) were collected from each river sampling site between 4th October 2017 and 6th December 2017 using electric-fishing methods by the Environment Agency (England) fisheries staff or licensed contractors. Roach were transported live back to Brunel University London's aquatics facility in a 250 L fiberglass fish transport tank filled with river water with constant aeration. The roach were then transferred to large glass tanks, with a dechlorinated tap water feed, in a temperature-controlled room (15 ˚C) prior to sampling. Roach were held in these tanks for a maximum of 48 hours prior to sampling.

**Tissue collection and analysis, morphometric measurement, age and genetic sex determination.** Roach were anaesthetized using neutrally buffered tricaine methanesulfonate (MS222, 500 mg/L). Blood samples were then taken from the caudal vein into heparinized syringes, and these samples were kept on ice until they were centrifuged at 7000 g for 5 minutes

149 (at 4 ˚C). Roach were then killed under anesthesia in accordance with UK Home Office
150 Regulations. Scales were collected from the flank of each fish. Fin clips were taken from the
151 caudal fin and placed directly into 100% molecular grade ethanol on ice. Fork-length (cm) and
152 wet weight (g) were recorded prior to dissection. The gonads were dissected out and weighed (g)
153 and each roach was sexed according to the macroscopic appearance of their gonads. Paired
154 gonads were preserved in Bouin's fixative for 24 hours, then rinsed and stored in 70% industrial
155 methylated sprits (IMS). The plasma samples were stored at -80 °C, fin clips were stored at 4 ˚C,
156 and scales were stored in paper envelopes at room temperature.

157 VTG analysis of plasma samples used a homologous carp vitellogenin enzyme-linked
158 immunosorbent assay (ELISA) kit (Biosense Laboratories), as per the manufacturer's
159 instructions. Fish scales were used for age estimation following the quality control methods
160 described in a previous study[34]. Fin clips were used for genetic sex identification, also as
161 previously described[32]. Portions of tissue representing the anterior, mid, and posterior regions of
162 each gonad were processed for histopathology as described by Nolan *et al.*[2]. Gonadal tissue
163 sections (3 µm thickness) were stained with haematoxylin and eosin. Six tissue sections per fish
164 were examined (blinded) under a light microscope. Microscopic sex, development stage
165 (spermatogenic/oogenic cell type) and intersex occurrence were recorded for each fish[2]. Intersex
166 indices were assigned to intersex fish according to Jobling *et al.*[19] (Table 1).

167 Table 1. Scaling of intersex severity according to Jobling *et al.*[19]

| Intersex severity score | Histological observation |
|---|---|
| **0** | Normal male testis |
| **1** | Multifocal ovotestis with 1-5 oocytes (usually singly) scattered among the testicular tissue |
| **2** | Multifocal ovotestis with 6-20 oocytes (often in small clusters) scattered among the testicular tissue |
| **3** | Multifocal ovotestis with 21-50 oocytes in clusters |

| | |
|---|---|
| **4** | >50 <100 oocytes. Section is usually multifocal and has the appearance of a mosaic of testicular and ovarian tissue |
| **5** | >100 oocyte, usually multifocal but could also be focal with clearly identifiable zones of ovarian and testicular tissue separated from the testicular tissue. |
| **6** | >50% of the gonad tissue on the section is ovarian and is clearly separated from the testicular tissue by epithelial cells and phagocytic tissues. |
| **7** | 100% of gonadal tissue on the section is ovarian. |

168

169 **Historical roach sex determination**. Methods to determine the genetic sex of roach have only
170 recently been developed[32], so the published historical datasets have not included this metric.
171 However, genetic sex can be determined from archived tissue samples. The sex-specific marker
172 used in this assessment is present in males and absent in females[32]. DNA was extracted from
173 individual fin clips using the HotSHOT method[35]. Briefly, a small section of fin tissue was
174 incubated in 75 µl alkaline lysis reagent (25 mM NaOH, 0.2 mM $Na_2EDTA$) at 95 °C for 45 min.
175 The samples were placed on ice for 5 minutes before the adding 75 µl neutralizing reagent (40
176 mM Tris-HCl, pH 5.0). The extracted DNA served as template in three separate PCR reactions
177 using three different primer combinations of two sense and three antisense primers (Table S3
178 Supporting Information). If amplification was successful for at least two out of three PCRs
179 reactions, the individual was assigned as a genetic male. PCR reactions were carried out using
180 GoTaq Flexi DNA Polymerase (Promega, UK), 1.5 mM $MgCl_2$, 0.2 mM dNTP mix (Thermo
181 Scientific, UK), 0.2 µM of each forward and reverse primer (Eurofins Genomics, Germany) and
182 2 µl DNA in a total volume of 20 µl. An initial denaturing step at 95° C for 5 min was followed
183 by 30 cycles of denaturation (1 min at 95 °C), annealing (30 s at 56° C) and extension (45 s at 72
184 °C), followed by a final extension of 5 min at 72 °C. Amplicons were resolved on 1.5% agarose
185 gels. PCR bands were scored blindly of the donor fish and only after scoring was completed
186 were the phenotypic and genetic sex results combined and compared[32]. All individuals were
187 verified as genetically pure-bred roach using species-specific forward primers and a universal

cyprinid reverse primer designed to the ITS1 nuclear ribosomal DNA region according to Wyatt *et al.*[36]. In addition, this amplification also served as positive control for successful DNA extraction.

**Statistical analysis.** To facilitate comparability between the previous studies and the current survey we adopted the statistical methods employed by Jobling *et al*.[19]. Although current practice might treat intersex scores as categorical data rather than continuous data, Jobling *et al*.[19] recorded individual intersex scores as an average of the intersex scores (Table 1) observed in each tissue section. The database we have for the historical samples only holds these averaged intersex scores for each fish. Therefore, the same methods were employed here to aid direct comparisons of the historic and 2017 intersex data. ANOVA (parametric data) or Mann-Whitney (non-parametric data) were used to analyze for effects of location or time on intersex intensity.

Chi-squared or Fisher's exact test were used to analyze possible differences in sex ratio (male vs. female) and male intersex frequency ('normal' male vs. intersex male) at different sampling time points. For rivers with multiple sampling occasions Chi-squared was also used for trend analysis for frequency of intersex males (both Fisher's exact and Chi-squared analyses were two-sided; statistical significance was considered at $\alpha \leq 0.05$).

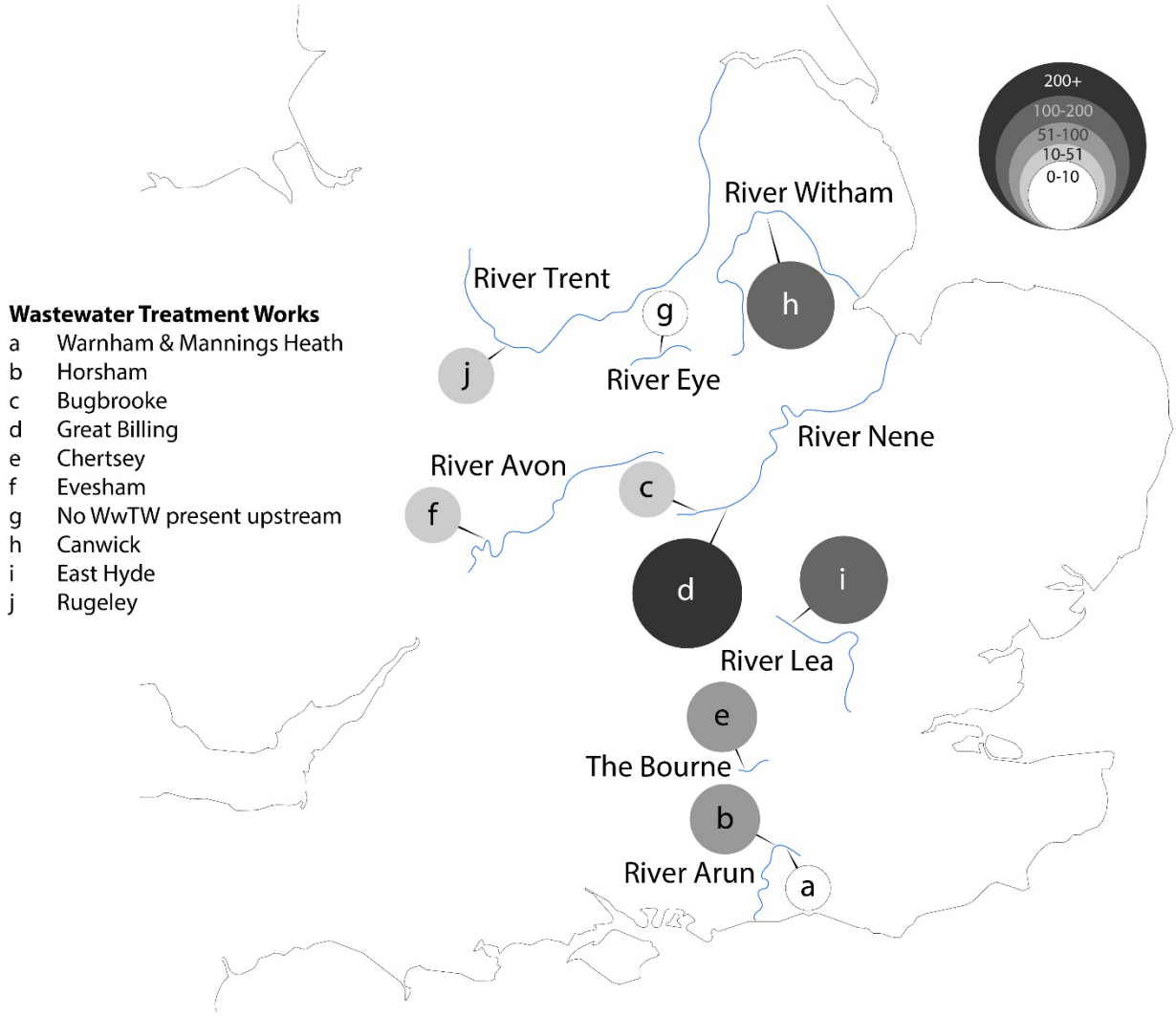
Results for plasma VTG were compared separately between males and between females (here "male" represents both phenotypic intersex males and non-intersex males combined). VTG data were first assessed for normality by D'Agostino & Person's omnibus K2 test[37] (GraphPad Prism 7), and data sets found to be normally distributed were analyzed using unpaired T-tests. Data sets not normally distributed were Log10 transformed and re-assessed for normality. If then normally distributed, the Log10 transformed data were analyzed using a T-test as above. However, if they

were not normally distributed the non-transformed data were compared using Mann-Whitney U test.

For intersex and VTG analyses $\alpha$ was set at 0.05, above which the null hypothesis - that there has been no effect of location or change in feminization frequency/intensity of roach since the first/original survey point - was accepted. For the Rivers Arun and Nene (both up and downstream), Avon, Eye, Lea and Trent the first survey point was in 1995, for the Bourne and River Witham the first survey point was in 2002. Analyses were conducted in GraphPad Prism 7. Unless stated otherwise, data are presented as mean ± standard deviation (SD).

## RESULTS AND DISCUSSION

A total of 466 roach (186 males and 280 females) were caught in the 2017 survey across the revisited 10 (historical) sampling sites (see Table S1 Supporting Information for site information). Eight out of the ten sites surveyed in 2017 were first sampled in 1995 and three of these sites (River Lea, River Arun downstream Horsham WwTW and River Nene downstream Great Billing WwTW) had been sampled on multiple earlier occasions (two, four and five times, respectively) prior to the 2017 survey. Two of the 2017 study sites, had first been surveyed in 2002, with one site sampled twice prior to 2017 (River Bourne downstream of Chertsey WwTW) (Table S1 Supporting Information).




228

229 **Figure 1.** Map of sites sampled in 2017, circles size and shade represent the WwTW 2017
230 Population equivalent (PE) for the upstream WwTW listed on the left hands side of the map (a-j)
231 PE is a means of quantifying the organic strength of wastewater discharges and reflects the net
232 waste of the human population and industrial facilities discharged into the WwTW. An accurate
233 PE is vital for the prediction of steroid estrogen concentrations (also see Table S1 Supporting
234 Information for more details).

235 **Assessment of endocrine disruption markers in wild roach over time.** In male fish, blood
236 plasma VTG is a sensitive biomarker of estrogenic exposure. In 2017, the majority of male (both

phenotypically intersex and non-intersex males combined) roach sampled from the ten locations had plasma VTG concentrations above those considered "natural" for males from non-polluted references sites (≤ 50 ng/ml VTG)[38,39]. There was considerable variation between individual VTG concentrations in males at any one sampling location (Figure 2A), as has been reported in wild fish in other studies (e.g.[40]), however, on average, male roach from the River Witham had the highest plasma VTG concentrations and males from River Nene upstream of Great Billing WwTW, the lowest (32,199 ± 47,326 ng/ml VTG and 27.86 ± 39.28 ng/ml VTG, respectively) (Figure 2A).

When individual river sites were compared across time, average male plasma VTG concentrations were significantly higher at one site only (River Bourne, 2002, 109.4 ± 227.5 ng/ml vs. 2017 2000 ± 3522 ng/ml $p < 0.0001$) and at significantly lower level for seven of the sites revisited in 2017 compared with the earliest historical samplings for each site (1995 or 2002, Table S2 Supporting Information) (Figure 2B). For one site, River Witham, VTG concentrations were recorded for two male fish only (at 256 and 25 ng/ml VTG respectively) in the historic dataset. Due to the low sample number in 2002, no statistical analysis could be effectively conducted to assess for possible changes in plasma VTG for this site. In the 2017 survey, plasma VTG was found to be above "natural" or baseline concentrations for most sites and most time points; the exception to this was the upstream River Nene site where they were within the range of natural baseline levels found in male roach from non-polluted reference sites (<50 ng/ml VTG, as per[38,39]) in 2017 (Figure 2B).

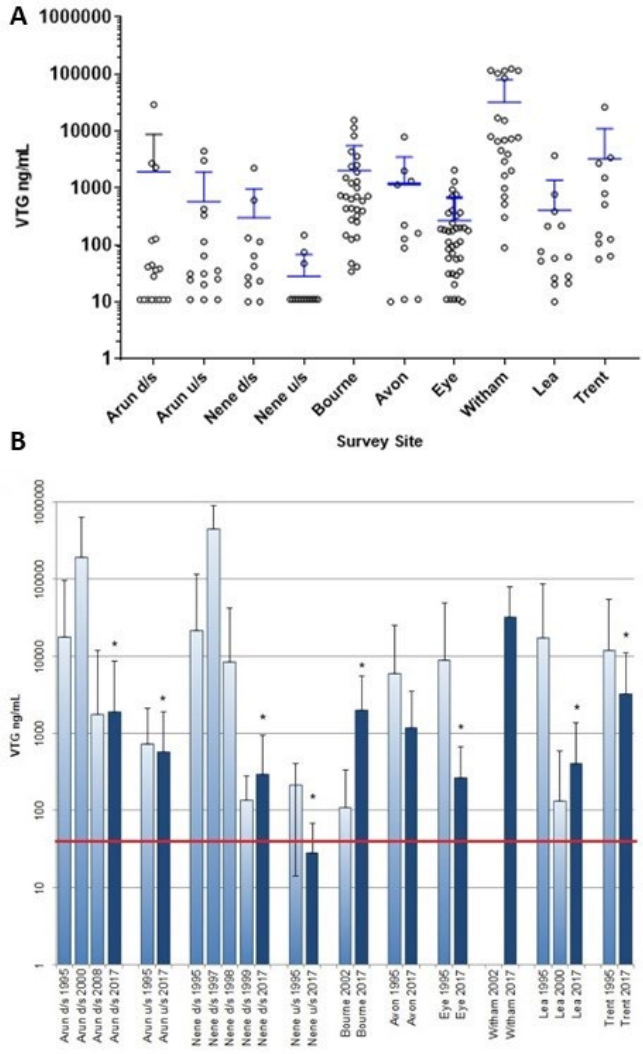



**Figure 2.** Mean plasma vitellogenin (VTG) concentration (ng/ml) in blood plasma of male roach from ten English rivers. A) VTG measured in male roach from the 2017 assessment; circles represent individual plasma VTG concentrations, lower horizonal bar represents the mean and the upper error bar the standard deviation for each sample site. B) VTG measured in male roach surveys conducted since 1995 at ten river sites. Error bars represent standard deviation. Stars indicate statistical significance between VTG measured in 2017 and the earliest previous survey (i.e. either 1995 or 2002 depending on site) ($p<0.05$). Natural 'baseline' VTG concentrations in

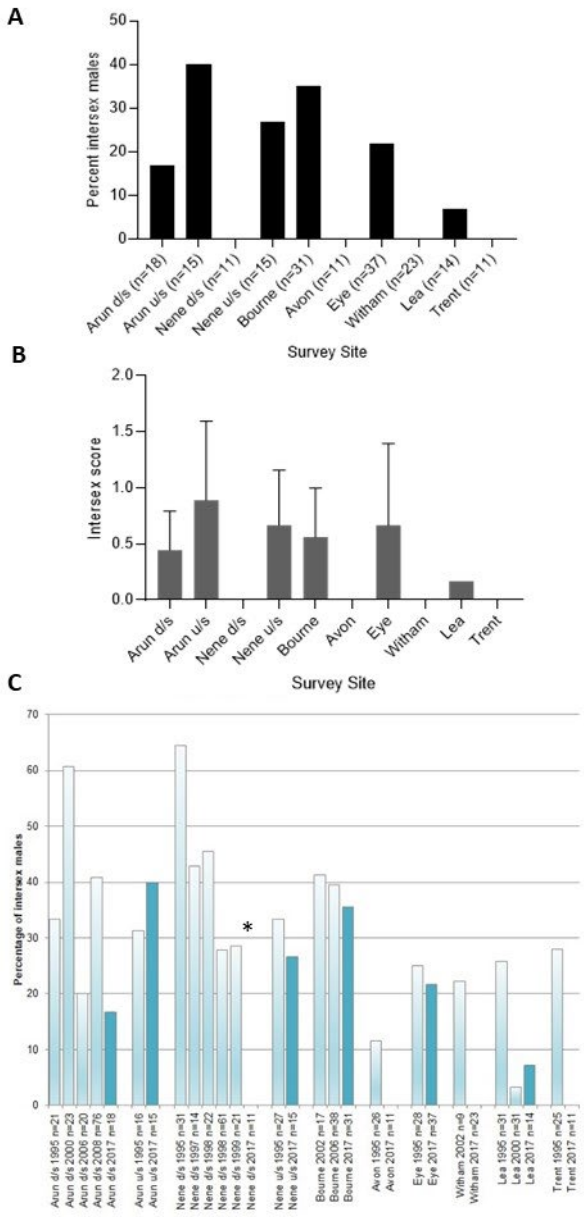
male roach from non-polluted reference sites are <50 ng/ml, represented by the horizontal red line.

Histopathological analysis of the gonad tissues revealed intersex roach (i.e. with both male and female reproductive tissues in their gonads; ovotestis) at 6 out of the 10 sites sampled in 2017 (Figure 3A). In the 2017 survey, on average 14.8 ± 15.6% of males were intersex, compared with 31.6 ± 14.0 % intersex males from the earliest surveys at each site (1995 or 2002, depending on site, see Supporting Information Table S4) (2017 range 0-40% intersex, 1995/2002 range 12-65% intersex). In 2017, the River Arun upstream of Horsham WwTW had the highest percentage of intersex males (40%, 6 out of 15 males). This site also had the highest average intersex score (0.89 ± 0.71), indicating higher levels of intersex severity in this location (Figure 3B). Of the four sampling sites where no intersex was found in 2017, three of these sites had relatively small numbers of males sampled (i.e. 11 males each from the River Nene downstream site and the Rivers Avon and Trent).

Intersex frequency appeared to be higher (but not statistically significantly) at one site in 2017 (River Arun upstream Horsham WwTW), and was at very similar frequencies over the sampling periods for three sites (Rivers Nene upstream of Great Billing, Bourne and Eye) (Figure 3C and Table S4 Supporting Information). At the River Nene downstream of Great Billing, intersex occurred at a lower frequency in 2017 compared with in 1995 (65% versus 0%, respectively, $p = 0.0002$), with a downwards trend in intersex frequency evident across the six sampling occasions between 1995 and 2017 (where no intersex individuals were found in 2017) (Figure 3C, Table S4 Supporting Information). In the River Arun downstream of Horsham and River Lea sites, the temporal data were less clear. At the downstream site on the River Arun, intersex occurrence was significantly lower in 2017 compared with 2000 (17% versus 61%, respectively, $p = 0.0033$), but

with no obvious trend, with the frequency of intersex fluctuating up and down across the sampling time points between these dates and no statistically significant difference between the earliest sampling point in 1995 (33% intersex) and 2017 (17% intersex) (Figure 3C and Table S4 Supporting Information). For the River Lea, in 2000 there was a significantly lower incidence of intersex compared with 1995 (3% versus 26%, respectively, $p = 0.0261$), but there was no statistical difference in intersex frequency over the period between 1995 to 2017 (in 2017, the intersex incidence was 7%; Figure 3C, Table S4 Supporting Information). At the remaining three study sites (Rivers Avon, Witham and Trent) no intersex was recorded in 2017, but the intersex incidence was also low in earlier years at these sites and there were no statistical differences over the sampling occasions (Fisher's exact test $p = 0.5399$, 0.0726, 0.0756, respectively, Table S4 Supporting Information).

In 2017, no intersex males occurred at the Rivers Nene (downstream site), Avon, Witham and Trent, and thus no statistical comparisons were possible for intersex severity with the historical data for these four locations. A significantly lower intersex severity was observed for the site on the Bourne only. Here intersex severity was lower in both 2006 and 2017 when compared to the first survey conducted in 2002 ($p = 0.0095$ and 0.0012, respectively, see Table S5 Supporting Information). For the remaining sites, the severity of intersex condition did not differ statistically compared with the historical samples (Table S5 Supporting Information).

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311 **Figure 3.** Comparison of the frequency of intersex in male roach, from surveys conducted at ten
312 English river sites. A) Percentage of phenotypic males with intersex gonads (eggs and sperm
313 present) measured in roach from the 2017 assessment, B) Average intersex index scores in intersex
314 roach from the 2017 assessment, as per Jobling *et al.*[19], error bar represent standard deviation. C)
315 Percentage of phenotypic males with intersex gonads in roach surveys 1995 to 2017. Dark bars

316 represent 2017 survey data and graduated bars represent data derived from earlier surveys. Stars
317 indicate statistical significance between intersex frequency measured in 2017 and the earliest
318 previous survey (i.e. either 1995 or 2002 depending on site) ($p<0.05$). Upstream site: u/s,
319 downstream site: d/s, total number of males sampled from each river site: n=x.

320 Contrasting with previous surveys in the UK[4,41], in 2017 none of the 186 phenotypic males
321 sampled contained ovarian cavities (female reproductive ducts) as determined histologically. In
322 the data from the 10 historical sites, only on one occasion were there no males sampled with an
323 oviduct phenotype (R Eye in 1995, see Table S6 Supporting Information) whereas in the
324 remaining surveys between 1995 and 2008, between 3% and 100% of the male fish exhibited the
325 oviduct phenotype (Table S6 Supporting Information). Experimental investigations have shown
326 that feminized ducts in male fish are the result of estrogenic exposure during a critical window of
327 development in early life (pre-60 days post hatch for roach[42]) and the feature is permanent once
328 induced[18]. This suggests fish sampled in 2017 had not experienced high enough concentrations
329 of estrogenic chemicals during this critical window to induce this phenotype in males. The roach
330 in the 2017 survey were between 2-9 years old (Table S7 Supporting Information), suggesting
331 estrogenic concentrations were not high enough to induce male ovarian cavities in late
332 spring/early summer of 2008-2015 at any of the revisited sites (Figure S2 Supporting
333 Information).

334 In 2017, at some sites surveyed there was no positive correlation between plasma VTG content
335 in males and percentage of males in the population with intersex gonads. For example, in the
336 River Witham, that had the highest male plasma VTG concentrations, no intersex males were
337 identified (0 out of 23 males), and in the River Nene upstream of Great Billing WwTW male
338 roach had the lowest male plasma VTG concentrations but 27% of the males exhibited intersex

339 gonads (n=15). Timing of exposure and sampling is likely to be a key factor explaining these
340 observations. Adult male roach can develop oocytes in their testes (testis-ova; intersex) if they
341 are exposed to WwTW effluent during the period of gonad regrowth[24]; there is an annual
342 "sensitive window" for the induction of the testis-ova intersex phenotype in this annually
343 spawning species. In contrast, plasma VTG concentrations in fish reflect a more recent exposure
344 history to estrogens and is furthermore transient[43,44]. Thus, levels of plasma VTG provide an
345 indication of the estrogenic exposure at a time proximate to when the fish were captured (days-
346 weeks), rather than months or even years earlier (in the case of testis-ova or male oviduct).

347 In the two rivers where samples were collected from both "upstream" and "downstream" sites in
348 2017 (Nene and Arun), there was the unexpected observation that the upstream sites had higher
349 intersex frequencies and severities compared with those downstream of larger WwTW effluent
350 discharges, which contrasts with previous survey findings more generally (e.g.[4]). However, these
351 two "upstream" sites are not completely free of WwTW inputs; there are two small trickling
352 filter WwTW above the "upstream" River Arun site and one small trickling filter WwTW above
353 the "upstream" River Nene site (Figure 1, Table S1 & S8 Supporting Information). The
354 frequency of intersex in males was not significantly different in the upstream sites (Arun or
355 Nene) between the 1995 and 2017 samples. Therefore, the change in intersex frequency pattern
356 is driven by the lower intersex frequencies downstream of the larger WwTW. Previous studies in
357 the UK have used WwTW PE, effluent flow and river flow to predict river estrogen
358 concentrations[19,33]. Here we also used the historic predicted estrogen concentrations to describe
359 the possible estrogen exposure scenarios of our survey sites (Table S1 Supporting Information).
360 Jobling *et al*.[19], used predicted estrogen concentrations to determine possible risk of inducing
361 intersex and VTG using no effect concentrations (NOEC) and low effect concentrations (LOEC)

362 for these biological responses to set risk boundaries. With low risk sites having < 1 ng/L
363 predicted E2 equivalent, medium risk sites 1-10 ng/L E2 equivalent and high-risk sites > 10 ng/L
364 E2 equivalent. Using this method, Jobling *et al.*[19] found a clear trend with intersex frequency and
365 severity with increasing "risk", but surprisingly not with VTG ("male", "intersex" and "female"
366 were assessed separately). Similar analysis with our smaller number of sites provides a slightly
367 different picture. VTG in males (both non-intersex and intersex combined) showed a general
368 pattern of higher average VTG concentration with increased risk category in the historic samples
369 (Figure S2 Supporting Information), whereas in the 2017 survey the highest average male VTG
370 concentrations were seen in the medium risk category (Figure S2 Supporting Information). For
371 intersex frequency, the pattern also differed compared with that seen in the larger survey by
372 Jobling *et al.*[19], where in the historic sample the medium risk category had the highest percentage
373 of intersex males, and in the 2017 survey the pattern indicates increasing intersex frequency at
374 lower risk sites (Figure S3 Supporting Information). Between the periods of the earliest surveys
375 in 1995/2002 to 2017, the WwTW PE has generally increased (Table 1 Supporting Information),
376 and it is likely dilution factor (driven by effluent flow and river flow) has not changed.
377 Therefore, both the observation of the "downstream" sites having lower levels of endocrine
378 disruption (feminization) compared with the "upstream" sites, and the mis-match with historic
379 predicted estrogen concentrations, could be driven by changes to WwTW technology at some
380 formally heavily polluted sites, resulting in improvements in the removal of EDCs in the
381 discharged wastewaters.

382 Different WwTW technologies/processes are known to the influence estrogenic activity of final
383 effluents, with some processes removing estrogenic chemicals more effectively than others, for
384 example, activated sludge process is considered more effective than trickling filters, and the

addition of tertiary treatments, like sand filters, have been shown to reduce estrogenic activity of final effluents[24,45]. Although there is no comparable data for effluent estrogen concentrations at any of the sites and time points, treatment technology upgrades had been reported at some of the sites. For example, Great Billing WwTW on the River Nene, which had significantly lower frequency of intersex and concentrations of plasma VTG in male roach, switched from trickling filter to activated sludge process circa. 2001 (Table S8 Supporting Information) which may have reduced estrogenic activity of the WwTW effluent entering the river. Horsham WwTW on the River Arun, which had significantly reduced male VTG, but not intersex frequency, had a deep bed sand filter added circa. 2008 (Table S8 Supporting Information). No WwTW technology upgrades were reported for the small WwTW upstream of the "upstream" Nene sample site (Table S8 Supporting Information), where intersex frequency was similar at the two survey points. Average male plasma VTG was relatively low in the 1995 survey at the upstream Nene site but significantly lower in the 2017 survey. In contrast, WwTW upgrades had been made at the two upstream WwTW on the River Arun in the early 2000's (e.g. new media for trickling filters, new humus tanks, nitrifying submerged aerated filters, see Table S8 Supporting Information), where male roach had a more moderately lower plasma VTG in the 2017 survey, and slightly higher intersex frequency than in 1995, suggesting effluent quality may not have improved, even with these treatment works upgrades. However, more recent data (circa. 2021) on the frequency of combined sewer overflows (cso) at these River Arun sites (Manning Heath & Warnham) indicates relatively high spilling rates of longer duration than observed at sites with lower intersex frequencies (See Table S11, Supporting Information)[46]. To our knowledge, the impact of cso releases on estrogenic activity and subsequent endocrine disruption of wild fish has not been investigated in the UK. However a study from a WwTW cso in Vermont, USA,

408 measured E2 concentrations of 1-10 ng/L in the cso outflow, compared to <0.5 ng/L in same
409 WwTW treated effluent[47] and although cso releases were infrequent Phillips *et al.*[47] estimated
410 the cso accounted for around 30% of the annual load for E2 at the Vermont site's receiving
411 water[47]. Therefore, release of untreated sewage, via cso, could be an additional endocrine
412 stressor at sites where they occur frequently, especially if they occur during sensitive windows of
413 disruption.

414 The pattern of some WwTW being upgraded but others not, found in this study, is likely to
415 reflect the UK picture more generally. Data collected by Statistical Office of the European Union
416 (Eurostat) for the European Environment Agency, on Member State's urban wastewater
417 collection and treatment indicate that in 1995 20% of the UK population's wastewater was either
418 not treated, or only underwent primary wastewater treatment, 64% underwent secondary
419 treatment and 14% underwent tertiary treatment[48]. By 2005, this had improved considerably with
420 56% of the populations' wastewater undergoing secondary treatment, and 43% undergoing
421 tertiary treatment. However, the Eurostat data shows this progress has slowed; in 2017 57% of
422 the UK population's wastewater underwent tertiary treatment, in comparison to more than 90%
423 for the populations of Germany, Denmark, Austria and the Netherlands[48]. The 2020 EU-27
424 average was 68.9% of the populations wastewater undergoing tertiary treatment[48].

425 For the River Eye there is no-known upstream municipal WwTW above the site sampled, but
426 there was an intersex incidence of 22% and the males had elevated levels of plasma VTG
427 concentrations (Figure 1, Figure 2, Table S1 Supporting Information). It is difficult to reconcile
428 these observations, but we cannot exclude other potential sources of upstream estrogenic
429 contamination and our findings here are not unprecedented. For example, studies in rural
430 Denmark suggested environmental estrogens derive from domestic sewage treatment facilities,

such as septic tanks, after observing elevated plasma VTG in caged brown trout (*Salmo trutta*)[40,49]. Furthermore, high density livestock farming and the use of animal manures and sewage sludge on farmland can also be a diffuse source of estrogens entering surface waters (e.g.[50,51]).

Our findings suggest there has been a reduction in environmental estrogenic exposure between the mid 1990's and the contemporary assessment. These improvements may be driven by WwTW upgrades and improved environmental regulations. However, the pattern of improvements is not universal, and some sites had a static or worsening picture. An absence of comprehensive chemical monitoring data at these specific sites prevented further investigation of drivers for the observed changes (e.g. WwTW upgrades, NP regulations). The possibility that cso could be contributing to estrogenic load and endocrine disruption in fish in UK rivers is also an area which requires further investigation given the paucity of data currently available.

**Assessment of roach sex ratio, phenotypic and genetic sex.** The "normal" or "natural" sex ratio in wild roach populations is not known. Wild fish behaviors may affect analyses that derive from "spot location" samples, because fish may aggregate in sex-specific groups outside of breeding season. Female biases in sex ratios have been documented in WwTW effluent-contaminated rivers as well as those without known WwTW discharges[9], whereas others have reported a more equally balanced sex ratio[39]. In our study, the phenotypic sex ratios of the sampled roach populations were generally skewed towards phenotypic females in both the historical and 2017 surveys (Table S9 Supporting Information).

Via gonadal histopathology we found there was a skew towards phenotypically female roach at seven out of the ten sites in the 2017 survey. The opposite was the case for the Rivers Eye and

Bourne and the sex ratio was equal for the River Trent. Applying the genetic sex probe, we were able to compare the genetic sex of individual fish with their gonadal phenotype. For the intersex fish (those with both male and female gametes in the gonad), more than 96% (32 out of 33) were found to be genetic males (represented by diamond pattern bars in Figure 4). The majority of roach had matching genetic and phenotypic sex (represented by solid pink or blue bars in Figure 4). However, both sex-reversed males (genetically male, with a female phenotype) and sex-reversed females (genetically female, with a male phenotype) were identified in the 2017 survey (represented by stripped bars in Figure 4). Eight out of the ten sites had some individuals with a phenotype/genotype mis-match. The downstream site on the River Arun had the highest frequency of sex-reversed individuals (10.7%), whereas none of the fish sampled at the River Lea or the downstream River Nene site were found to be sex-reversed (Figure 4).

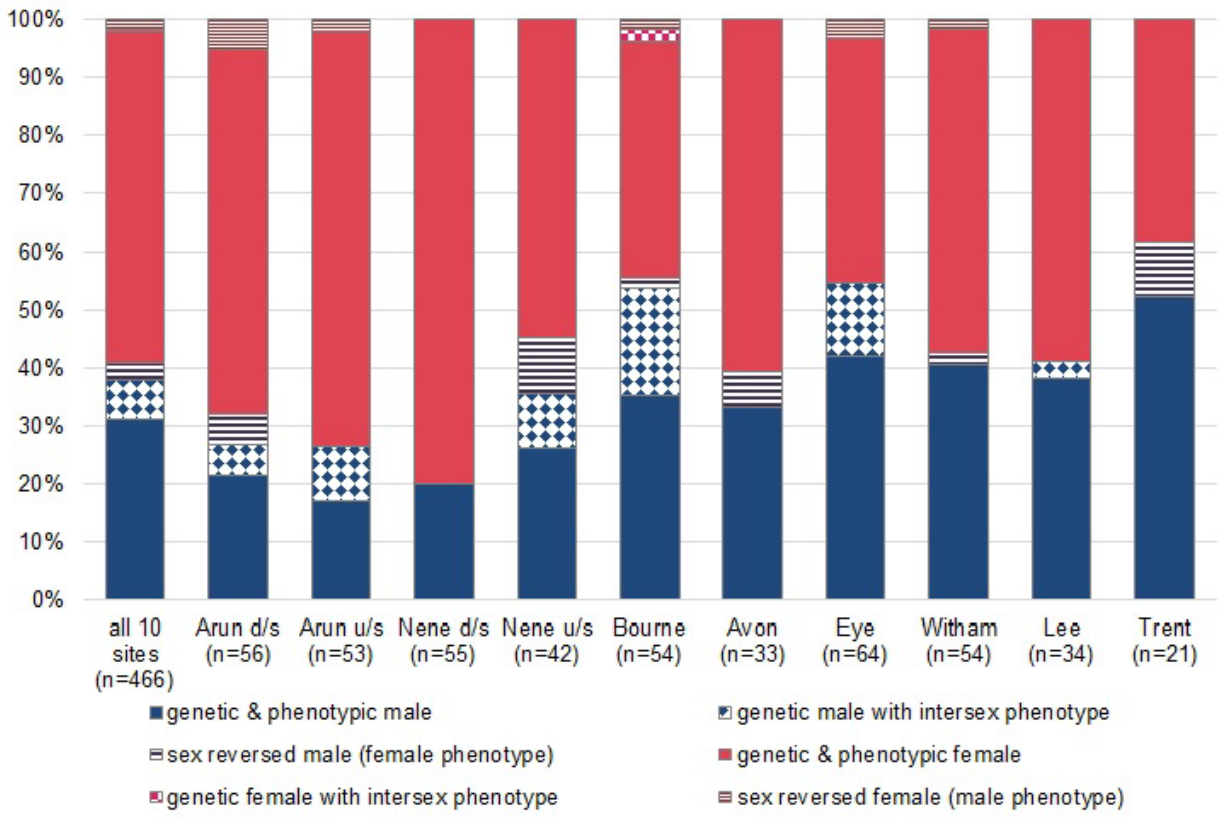

**Figure 4.** Percentage of different phenotypic and genotypic sex types of roach sampled at 10 river sites in the 2017 assessment. Phenotypic sex identified via gonad histopathology (microscopically). Genetic sex identified via molecular marker[32]. Total number (n=x) of roach analyzed (all sexes) from the site in 2017.

A small cohort of historic samples from the same river sites investigated throughout this study were available to carry out genetic sex assessments (three sites: Arun, Nene and Bourne). These samples were collected during an investigation into roach breeding and parental success, which used male dominated breeding groups (6 males to 3 females)[14,52]; the sex ratio is artificially skewed. Nevertheless, these historic samples also demonstrate low levels of sex reversal in both genetic male and genetic female roach. In this historic cohort, two genetic females with a male phenotype were identified from the River Arun, downstream of Horsham WwTW, and one genetic male with a female phenotype was identified from the river Bourne sample[32]. As with the 2017 cohort, none of the roach from the site downstream of Great Billing on the River Nene had mis-matched genetic/histological gonad phenotypes (Table S10 Supporting Information). Therefore, we confirm both that the majority of intersex roach in the 2017 survey were feminized males, and that low frequencies of sex reversal, both male-to-female and female-to-male, were common at the majority of sites visited in 2017 and also in the smaller selection of sites from this historic sampling. These findings support work by Lange *et al*.[32], who investigated the genetic sex of roach from a wide range of field and lab based studies. It is currently unknown if this phenomenon is natural in roach populations, or if it is driven by some external factor. Similar to Lange *et al*.[32], in our study, sex-reversed phenotypes in wild roach were not only associated with estrogenic/WwTW effluent polluted sites but also at sites without known WwTW effluent inputs.

## ASSOCIATED CONTENT

**Supporting Information**. The following files are available free of charge.

The Supporting information file (PDF) includes additional experimental details, including further information about survey sites, survey data and statistical analysis.

## AUTHOR INFORMATION


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### Author Contributions

AB, AL, CRT and SJ obtained the funding. AB, AL, CRT, SJ and KW participated in the design of the research. AB, EB and NB participated in data collection, generation and analysis of 2017 field samples: scale ageing, vitellogenin and histopathological data. SJ provided historical roach datasets. AB conducted the analysis of historical data in comparison to data collected in 2017. AL conducted the data generation and analysis of molecular samples. AB, AL, CRT and SJ wrote the paper. All authors read and approved the final manuscript. All authors have given approval to the final version of the manuscript.


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## COMPETING INTERESTS

The authors declare that they have no competing interests.

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## ABBREVIATIONS

CIP, Chemical Investigation Program; CSO, combined sewer overflows, E1, estrone; E2, estradiol; E2 Equ, estradiol equivalent; EE2, ethinylestradiol; ELISA, enzyme-linked immunosorbent assay; IMS, industrial methylated sprits; MS222, tricaine methanesulfonate; NGR, national grid reference; NP, nonylphenol; OP octylphenol; PCR, Polymerase chain reaction; PE, population equivalent; VTG, vitellogenin; WFD, Water Framework Directive; WwTW, wastewater treatment works.

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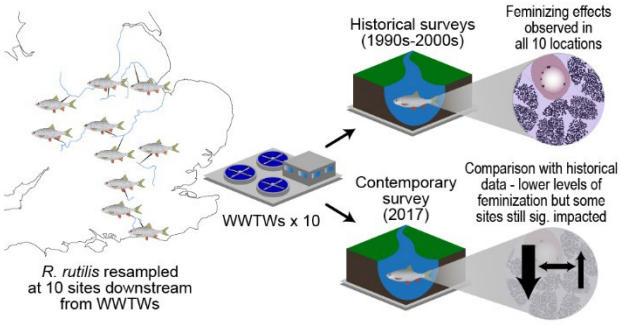
719 GRAPHICAL ABSTRACT

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