

Evidence

Oestrogenic effects in wild fish:
comparing contemporary and historical
impacts

Project number SC170001

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Professor Doug Wilson
Director, Research, Analysis and Evaluation

Executive summary

Certain natural and man-made chemicals can interfere with the normal functioning of endocrine systems of both humans and animals. The strongest evidence for the effects of such endocrine disrupting chemicals in wildlife is the widespread feminisation of male fish reported in fish populations globally, linked to exposure to oestrogenic substances.

This report describes a study to investigate current levels of oestrogenic (feminisation) effects in wild roach populations in England and to compare these observations with those made in earlier investigations.

Pioneering studies previously carried out in the UK found that the feminisation of wild roach in rivers was widespread and was associated with exposure to Waste water Treatment Works (WwTW) effluents. The main chemicals associated with these effects are natural and synthetic oestrogens originating from human excretion, as well as oestrogen-mimicking alkylphenols that derive from the breakdown of industrial detergents.

An extensive research programme involving the sampling of 2,022 roach was undertaken between 1995 and 1998 to investigate the state of health of UK fish populations and the possible role played by treated sewage effluents. It showed that feminising effects linked to oestrogenic exposure were common in UK populations of the roach (*Rutilus rutilus*). These feminising effects included:

- a high incidence of intersex (the appearance of both male and female gonadal ducts and/or developing eggs and sperm in the gonads of individuals)
- in males, the presence of the egg-yolk protein precursor vitellogenin (VTG; a highly sensitive biomarker for oestrogen exposure) in blood plasma and reduction in the size of the gonads relative to body size (i.e. reduced gonadosomatic index).

The severity of feminisation (indicated by the number of eggs in an otherwise male testis) appeared to depend on the relative amount of WwTW effluent in the river, as well as the size and age of the fish.

Although moderately intersex fish have been shown to have reduced fertility, subsequent studies have found no evidence that the reduced fertility of individual males in effluent-contaminated stretches of a few selected English rivers results in impacts at the population level, as measured by genetic effective population size (N_e).

More recent studies have led to speculation that levels of intersex may now be lower within previously heavily polluted rivers (such as the River Aire), but that the situation may be worsening at formerly 'clean' sites. However, there have been no studies that have systematically compared current intersex levels to those reported previously.

To address this, a field survey was conducted in the autumn of 2017 to establish current oestrogenic effects in wild roach at sites which had previously contained feminised males. A total of 10 sites were successfully revisited in the autumn/winter of 2017 resulting in the sampling of 466 roach for comparative analysis.

The characteristics (endpoints) measured in the 2017 survey to assess the feminisation effects included:

1. Fish with both eggs and sperm in their gonad (ovotestis): these are with a binary yes/no response, providing a frequency of intersex within a population
2. Fish with ovotestis: each is given an intersex 'score', which reflects the severity or number of eggs in the testis
3. Fish with the feminised duct phenotype: recorded with a binary yes/no metric (referring to whether the ostensibly male fish has an oviduct or not)
4. Plasma VTG concentration measured as ng/ml

Each of the markers of feminisation is kept distinct in order to allow comparison of the 2017 data with that collected in historic surveys (in other words, 2017 intersex frequency is compared with historical intersex frequency, or 2017 intersex scores are compared with historical intersex scores).

In addition to the well-established feminised characteristics (e.g. intersex, feminised ducts, elevated male VTG), the genetic sex of the sampled fish was also determined to confirm whether intersex fish were feminised males or masculinised females, and to determine the possibility of fully sex-reversed fish. This novel endpoint was measured with the aid of a genetic sex probe, developed by the University of Exeter.

The results of this latest (2017) field survey illustrate that feminisation in wild male roach is still a widespread phenomenon, present at 60% of the sites sampled. For the majority (80%) of sites, the frequency of intersex has not significantly changed compared with historical surveys. Whilst a reduction in male plasma VTG was observed, levels remained elevated above the natural baseline for males at the majority of the sites sampled in 2017.

The results of the 2017 survey do however provide some indication of improvement compared with historical samples. No male fish were observed to have feminised ducts compared with historical analyses where 94% of sites were found to have males with feminised ducts. There was also a significant reduction in plasma VTG in males sampled at 7 of the 10 sites surveyed.

Collectively, the findings of this study are suggestive of a reduction in environmental exposure to oestrogenic substances but with continued impacts associated with chronic exposure, albeit at lower concentrations than those occurring historically.

A potential reduction in environmental oestrogenic concentrations may be associated with infrastructure upgrades at WwTw. Unfortunately an absence of chemical monitoring data prevented further exploration of this hypothesis.

Application of the genetic sex probe confirmed the hypothesis that the majority of intersex individuals are feminised genetic males (rather than masculinised females). The genetic sex probe also revealed the presence of wild sex-reversed fish, in which the gonad phenotype (assessed via histopathology) did not match with the genetic sex. Both male-to-female and female-to-male sex reversal was seen, but only at very low levels in the population, which may be a natural phenomenon in roach populations.

It should be emphasised that the 2017 survey was considerably smaller in scale (both in terms of the number of sites and the total numbers of fish sampled) than previous surveys, and therefore is not as wide ranging nor as representative of the whole of England as were previous surveys.

We recommend that any future monitoring programmes of UK rivers to assess changes in feminised responses in wild fish populations should operate over a longer collection period (perhaps two years). This would ensure the capture of adequate numbers of

fish for more robust statistical comparisons and would minimise the loss of important target sites from the survey due to unpredictable sampling challenges associated with weather conditions (e.g. high river flow due to heavy rainfall).

We also recommend that future analytical chemistry sampling campaigns consider monitoring sites with considerable biological data (that is, sites with historical and current intersex records), to facilitate an understanding of the links between specific chemicals and observations of biological effects.

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1 Introduction

1.1 Background

Endocrine disruption in fish, manifesting most notably as the feminisation of males, has been reported in fish populations globally (for example, Hecker et al 2002, Bjerregaard et al 2006, Tanna et al 2013). Much of this work stemmed from the original studies undertaken in the UK where caged male trout exposed to effluents from wastewater treatment works (WwTW) were found to have a precursor of egg yolk, vitellogenin (VTG), in the blood plasma (Purdom et al 1994). VTG is normally produced in the liver in females only under the stimulation of oestrogen (e.g. Purdom et al 1994, Tyler et al, 1996). In 1996, the Environment Agency commissioned a team of researchers at Brunel University to undertake a series of studies to investigate the feminisation (including levels of intersex – that is, the simultaneous appearance of male and female reproductive ducts and/or developing eggs and sperm in the gonadal tissue of an individual) in wild roach (*Rutilus rutilus*) populations. These studies demonstrated that intersex was largely attributed to discharges from WwTWs and further work identified that steroid oestrogens contained in these effluents were major contributors to these effects (Desbrow et al 1998, Tyler and Jobling, 2008).

The severity of feminisation (measured as the number of eggs seen in an otherwise male testis) has been linked to the proportion of WwTW effluent in the rivers in which the fish resided, as well as the size and age of the fish. This suggests that continual exposure of fish to oestrogenic substances drives the origin and severity of the ovotestis condition. However, experimental evidence indicated that formation of female gonad ducts (oviducts) alongside male sperm ducts can only be induced early in life when the gonad is forming (Liney et al 2005). There is also a dosage- and time-dependent increase in VTG production in male fish.

Subsequent studies showed reduced sperm quality in male fish that were feminised and that moderately intersex fish have a reduced capacity to compete in natural breeding scenarios (Jobling et al 2002, Harris et al 2011). However, a more recent assessment of the population genetics of English wild roach populations found no evidence for a reduced effective population size (N_e) in effluent-contaminated stretches of selected English rivers compared with less contaminated sites (Hamilton et al 2014).

Following the widespread detection of intersex in wild UK fish populations, risk assessment models were created to predict the location and severity of feminisation of wild roach/fish populations in river catchments and to identify areas where regulation of WwTW discharges to remove oestrogenic contaminants may be necessary. Steroid oestrogen exposure (categorized as either high, medium, or low “risk” on the basis of the assumed additive potency of equivalent doses of oestradiol) was modelled for the three steroid oestrogens at 45 sites on 39 rivers throughout the United Kingdom. Samples of roach were also obtained from these sites. Both the incidence and the severity of intersex in wild roach were significantly correlated with the predicted concentrations of the natural oestrogens (oestrone and oestradiol) and the synthetic oestrogen present in the contraceptive pill (ethinyloestradiol, EE2) (Jobling et al 2006). Predicted steroid oestrogen exposure was, however, less well correlated with the plasma VTG concentration measured in the same fish.

No correlation was found between any of the feminisation endpoints measured in the roach and the proportion of industrial effluents entering the rivers. A later study (Jobling, 2009), however, reported feminised endpoints associated with predicted steroid oestrogens, unidentified anti-androgens and the industrial chemical, nonylphenol (NP). The concentration of NP was also highly correlated with the

concentration of oestrone (Jobling et al 2009). Studies exposing roach to EE2 in the laboratory have shown that similar concentrations in some WwTW effluents induce the feminised responses (VTG induction and ovotestis) seen in wild roach populations (Lange et al 2009).

Due to their toxicity to aquatic wildlife, the alkylphenols 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 reduced feminisation of roach in selected rivers (Sheahan et al 2002). In 2013, due to concerns about the potential negative impacts on fish populations (in particular disruption of sexual development in fish), EE2, and the natural steroid, 17-beta-estradiol, were added to the European Commission's WFD priority substances' "watch" list. The aim of the "watch" list is to drive targeted EU-wide monitoring to support the prioritisation process in future reviews of the priority substances list.

The study of intersex in freshwater fish therefore remains of considerable interest in the UK and has been the subject of continued investigation by researchers from the University of Exeter and Brunel University, funded by the Natural Environmental Research Council (NERC) with support from both the Environment Agency and Defra. More recent studies have led to speculation that levels of intersex may now be lower in previously heavily polluted rivers (such as the River Aire), whereas the situation may be worsening at formerly 'clean' sites. However, many of these studies have looked at intersex opportunistically and there have been no studies that have systematically compared current intersex levels to those previously reported. Consequently, there are no data to robustly support any conclusions on the current levels and trends of intersex in wild freshwater fish in English rivers.

As a consequence, the Environment Agency wished to undertake a targeted investigation to investigate current levels of intersex (and other oestrogenic effects) in previously sampled wild roach populations and compare these levels with those observed in the previous studies undertaken in the 1990s. This project was undertaken by Brunel University and the University of Exeter, and this report details their findings.

1.2 Aims and objectives

The study addressed the following questions:

1. What are the current levels of intersex (and other oestrogenic effects, including full sex reversal) in wild roach at 10 previously sampled sites that have been shown in previous surveys to display a range of levels of intersex?
2. How do current levels of intersex compare with those observed from previous studies undertaken at the same sites in the late 1990's / early 2000's?
3. What are the possible explanations for any differences found between current and historically reported levels of intersex in roach populations?

1.3 Project approach and work flows

The project was awarded to the Brunel University and the University of Exeter with the lead provided by Brunel University. The field collection of wild roach was coordinated between the Environment Agency's project manager, Area teams and Brunel University. The roach sampling and tissue collection, fish ageing, blood plasma vitellogenin analysis and histopathology were conducted at Brunel University. Molecular sex-typing was conducted by the University of Exeter. Data on historical and

current WwTW population equivalent (PE) and treatment technologies used at target sites was collected by the Environment Agency. Analyses of the data and compilation of the report were carried out by Brunel University and the University of Exeter.

2 Methods

2.1 Historical data collection/compilation

Historical roach data (information on individual fish morphometrics, age, intersex score, and plasma VTG) and site data (including WwTW/site name, upstream and downstream national grid references, modelled river oestrogen concentrations, and WwTW PE) were held and compiled by members of the Brunel University and the University of Exeter teams.

Historical predicted river oestrogen concentrations from the Brunel/Exeter dataset were modelled as per Johnson and Williams (2004).

2.2 Site selection and field sampling

Brunel University and the University of Exeter research teams hold data sets from a total of 73 sites derived from the previous (1995-98) survey of feminisation in wild roach in English rivers and from a subsequent larger survey conducted in 2000-2003, carried out in partnership with the Environment Agency. An initial desk-based scoping exercise was undertaken to match suitable sampling sites with those assessed previously and thereby facilitate the main objective of this study: to compare the feminisation of roach in English rivers currently with that seen in previous surveys.

Riverine sites where roach had been studied previously on more than one occasion, and both upstream and downstream of WwTW effluent discharges, were prioritised for inclusion in the 2017 survey. Sampling sites were also selected to ensure representation across the full range of oestrogenic potencies previously detected, as indicated by the incidence and severity of feminisation (i.e. low, moderate and high intersex occurrence).

Lakes, canals and fish farms previously included as 'reference' sites in historical surveys were excluded from the list of possible sample sites. This is because the objective of the current study was to prioritise sites that had previous records of feminised roach. In addition, sites where data had been derived from only a few fish (less than 20 roach) were excluded to avoid statistical weakness. This first analysis produced a list of 55 sites for consideration.

This list was then interrogated to remove those sites with recent fish kills and restocking events (cross-referencing Environment Agency records), as this would confound the study by potentially including fish not originally from the sample location. Environment Agency area fisheries teams also informed site choice selection by providing information on the likelihood of sufficient numbers of roach for sampling based on recent fisheries survey data and the feasibility of sampling sites within the time limitations set for the project.

This site selection process resulted in twenty eligible study sites for possible inclusion in the survey (

Table 2.1, Figure 2.1). Three of these sites could not be included due to new information regarding recent restocking events and two further sites could not be included due to low numbers or the absence of roach as assessed through recent Environment Agency surveys. Of the remaining 15 possible sites, three rivers (the Don, Yorkshire Ouse and Ivel) were not sampled because they were in flood and/or too

turbid for electrofishing at the times of the proposed samplings. Further site sampling had to be cancelled; one due to a fish kill/pollution incident (River Mole) on the weekend preceding the proposed collection date, and another where water flows were too low to support the presence of any roach (River Lea upstream of East Hyde) (Table 2.1). In the final assessment, roach samplings were targeted for the remaining 10 sites (Table 2.2, Figure 2.1), all of which had also been sampled historically.

Table 2.1 Candidate and final (blue shaded) sampling locations for the 2017 survey.

Site name	River	Upstream NGR*	Downstream NGR*	Sampled/ not sampled
Upstream Melton Mowbray	R Eye	SK759188	SK735182	Sampled
Downstream Melton Mowbray	R Wreake	SK735182	SK705185	Not sampled – restocked frequently, last time in 2013
Upstream Naburn	R Ouse (Yorkshire)	SE412669	SE431660	Not sampled - no roach population now
Naburn	R Ouse (Yorkshire)	SE601472	SE580405	Cancelled - in flood
Upstream East Hyde	R Lea	TL121180	TL123178	Cancelled - not enough flow
Downstream East Hyde	R Lea	TL123178	TL128172	Sampled
Upstream Great Billing	R Nene	SP645596	SP668589	Sampled
Downstream Great Billing	R Nene	SP752597	SP834616	Sampled
Upstream Horsham	R Arun	TQ155302	TQ149297	Sampled
Downstream Horsham	R Arun	TQ149297	TQ119323	Sampled
Hampton Ferry	R Avon	SP030441	SP039436	Sampled
Clifton	R Ivel (Bedfordshire)	TL183414	TL182416	Cancelled - in flood
Horley	R Mole	TQ268436	TQ271451	Cancelled – recent fish kill
Pye Bridge	R Erewash	SK441526	SK446508	Not sampled – very low roach population
Lincoln	R Witham	SK999709	TF043714	Sampled
Ashford	Great Stour	TR025436	TR032430	Not sampled - heavily restocked in 2015 after fish kill
Wolseley Bridge, Rugeley	R Trent	SK020204	SK034202	Sampled
Blackburn Meadows	R Don	SK400914	SK405919	Cancelled - in flood
Chertsey	The Bourne	TQ015680	TQ035675	Sampled
Ilkeston (Hallam Fields)	R Erewash	SK483360	SK509336	Not sampled - restocked 2011

* National Grid Reference

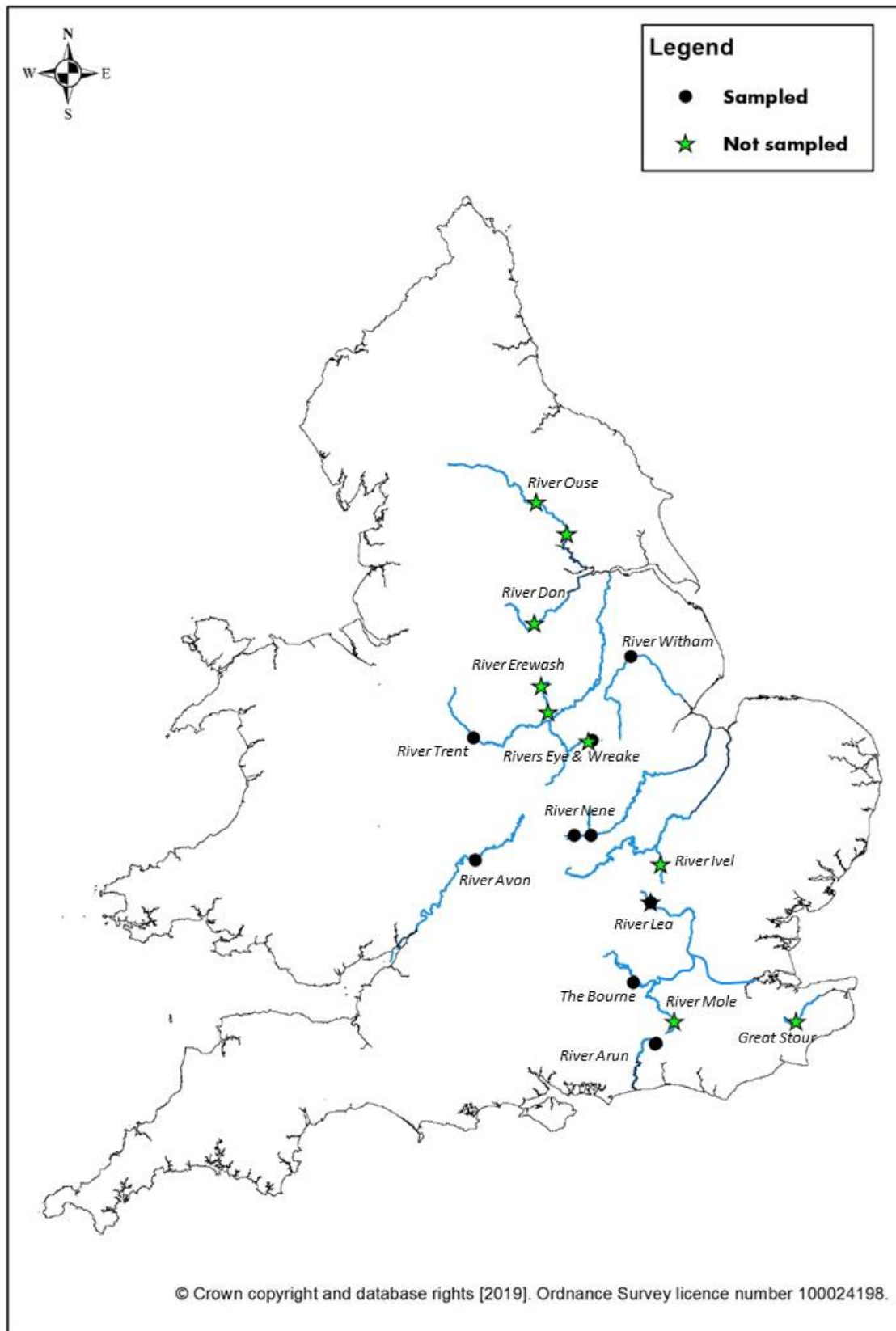


Figure 2.1 Map showing location of candidate (not sampled - star) and final (sampled – circle) sampling locations for the 2017 survey. Note, the close proximity of some upstream and downstream sites mean they display as a single point.

Table 2.2 Site specific information for the 10 sampling locations in the 2017 survey.

Site name	NGRs (maximum upstream and downstream points)	Historical PE	Historical Predicted river concentration ng/l (mean)				2017 PE	Sampling date(s) for historical samples and surveys	Sampling date 2017 survey
			E1	E2	EE2	E2 equ			
R Arun downstream of Horsham	TQ149297-TQ119323	63,048	7.3	0.45	0.12	4.11	70,068	Oct 1995, Apr 2000, Apr 2006, Apr 2008	3 Oct 2017
R Arun upstream of Horsham	TQ155302-TQ149297		0.27	0.02	0.01	0.18	1,201 + 1,032 ^{\$\$}	Oct 1995	11 Oct 2017
R Nene downstream of Great Billing	SP752597-SP834616	229,496	1.62	0.15	0.05	1.18	241,594	Oct 1995, Mar 1997, Apr 1998, Sept 1998, Apr 1999	23 Oct 2017
R Nene upstream of Great Billing	SP645596-SP668589		3.37	0.37	0.11	2.60	7,543 + 3,327 ^{\$}	Oct 1995	25 Oct 2017
The Bourne downstream of Chertsey	TQ015680-TQ035675	71,774	6.98	0.92	0.25	5.78	77,100	May 2002, Apr 2006	31 Oct 2017
R Avon at Hampton Ferry (Evesham)	SP030441-SP039436						24,643	Oct 1995	7 Nov 2017
R Eye upstream of Melton Mowbray	SK759188-SK735182	-*	-	-	-	-	-*	Sept 1995	13 Nov 2017
R Witham, Lincoln (Canwick)	SK999709-TF043714	97,348	3.87	0.45	0	1.74	109,813	July 2002	23 Nov 2017
R Lea downstream of East Hyde	TL123178-TL128172	104,558	14.15	1.85	0.51	11.63	166,000	Aug 1995, Apr 2000	28 Nov 2017
R Trent, Rugeley	SK020204-SK034202		4.93	0.51	0.15	3.70	23,066	Sept 1995	5 Dec 2017

Population equivalent (PE) of WwTW. National grid reference (NGR). * no WwTW facility upstream of River Eye fish capture site – not accounting for possible diffuse sources, \$Bugbrook and Weedon WwTW only no PE data for other upstream WwTW, e.g. Newnham, \$\$ PE for Warnham and Manning Heath WwTW.

2.2.1 Electric-fishing, transport and housing

Between 21 and 65 adult roach were collected from each sampling site location between 4th October 2017 and 6th December 2017 by Environment Agency teams or contractors using electric-fishing methods. Roach estimated to be ≤ 10 cm in length were returned to the river as these were considered likely to be sexually immature; that is gonadal development would be insufficient to determine the development of an intersex condition.

Roach were transported live back to Brunel University's aquatics facility in a 250l fibreglass fish transport tank with constant aeration. The roach were then transferred to large glass tanks, with a dechlorinated tap water feed, in a temperature-controlled room prior to sampling. Roach were held in these tanks for a maximum of 48 hours.

2.2.2 Roach tissue sample collection

Roach were anaesthetised using neutrally buffered tricaine methanesulfonate (MS222, 500mg/L). Blood samples were then taken from the caudal sinus into heparinised syringes, and samples were kept on ice until they were centrifuged at 7000 g for 5 minutes (at 4°C). The resulting plasma was stored at -80°C for later analysis of blood VTG (detailed below). Roach were then killed under anaesthesia and according to UK Home Office Regulations. Scales were collected from the flank of each fish and stored in paper envelopes at room temperature for subsequent age estimation. Fin clips were taken from the caudal fin and stored in 100% molecular grade ethanol at 4°C for genetic sex identification (Lange et al. in press). Fork-length (cm) and wet weight (g) were recorded prior to dissection. The gonads were isolated and weighed (g) and roach were sexed macroscopically according to the phenotypic appearance of their gonads. Gonads were preserved in Bouin's fixative for 24 hours, after which they were stored in 70% industrial methylated spirits (IMS) until histopathological processing and assessment.

The gonadosomatic index (GSI) was calculated as:

$$\text{GSI} = \text{gonad weight} / (\text{bodyweight} - \text{gonad weight}) \times 100$$

Vitellogenin was measured in the plasma using a homologous carp vitellogenin enzyme-linked immunosorbent assay (ELISA) kit (Biosense Laboratories), as per the manufacturer's instructions.

Determination of fish age

The age of each fish was estimated by counting the annuli (annual growth rings) on scales removed from the fishes' flank. Roach ageing was conducted by two people independently and a proportion (10%) of each of the ageing estimations were further assessed by another independent person to ensure accuracy.

Determination of genetic sex

For determining genetic sex, DNA was extracted from fin clips using the HotSHOT method (Truett et al., 2000) and then subject to polymerase chain reaction (PCR) using primers specific to a roach sex marker (Lange et al., in press). Given the presence-absence nature of the marker (present in males and absent in females), each sample was analysed using three different primer pair combinations to avoid possible erroneous sex determination due to failed PCR reactions. PCR reactions were carried out using GoTaq Flexi DNA Polymerase (Promega, UK), 1.5 mM MgCl₂, 0.2 mM dNTP mix, 0.2 μ M of each forward and reverse primer (and 2 μ l DNA in a total volume of 20 μ l. An initial denaturing step at 95 °C for 5 minutes was followed by 30 cycles of

denaturation (1 minute at 95 °C), annealing (30 s at 56° C) and extension (45 s at 72 °C), followed by a final extension of 5 minutes at 72 °C. Amplicons were resolved on 1.5 % agarose gels. As roach are known to hybridise with other coarse fish species (e.g. bream and rudd), all samples were genetically identified as pure-bred roach using the ITS1 nuclear ribosomal DNA region according to Wyatt et al. (2006), which in addition also served as positive control for successful DNA extraction.

Histopathological assessment of intersex

The gonads were cut into three sections (anterior, median and posterior), processed to impregnate the tissue with paraffin wax and mounted into paraffin blocks. Tissue sections (3µm) were stained with haematoxylin and eosin and examined under light microscope. Microscopic sex, development stage (spermatogenic/oogenic cell type) and intersex occurrence were recorded for each fish.

An intersex index score (Jobling et al. 2006) was then calculated for each male roach. This index describes the number of oocytes in the testes of intersex fish and, thus, the severity of this condition, using a numerical scale that ranged from 0 (all testis tissue) to 7 (all ovarian tissue) (Table 2.3). Testes sections are assigned to one of eight categories on the basis of the number of oocytes present. The arithmetic mean of the scores (measured on six sections per fish) for each intersex fish was used to derive an average intersex index (or severity score) for each sample.

Table 2.3 Scaling of intersex severity (after Jobling et al, 2006)

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.

2.3 Statistical methods

To facilitate comparability between the previous studies and the current survey we adopted the statistical methods employed by Jobling et al. (2006). Chi-squared or Fisher's exact test were used to analyse 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 results were compared separately for males and females. VTG data were first assessed for normality by D'Agostino & Person's test, and data sets found to be normally distributed were analysed using unpaired T-tests. Data sets found to not be normally distributed were Log₁₀ transformed and re-assessed for normality. If then normally distributed, the Log₁₀ transformed data were analysed using a T-test as above. However, if they were not normally distributed the non-transformed data were compared using Mann-Whitney U test.

ANOVA (parametric data) or Mann-Whitney (non-parametric data) were used to analyse for effects of location or time on intersex frequency/intensity. For intersex and VTG analyses α was set at 0.05, above which the null hypothesis - that there has been no effect of location or change in feminisation frequency/intensity of roach since the last national survey - was accepted. Analyses were conducted in GraphPad Prims 7.

Unless stated otherwise, data are presented as mean \pm standard deviation (SD).

2.4 Characterisation of Waste water Treatment Work (WwTW) effluent

As intersex and feminisation have been shown to be closely associated with the oestrogenic content of WwTW effluents and thus the oestrogenic activity of the receiving waters, details of the chemical composition, including steroid oestrogen and NP concentrations in the effluents upstream of the sites, are important for interpreting differences in the levels of feminisation compared with the historic surveys. Nationally, some WwTW have been part of large-scale monitoring programs (known as the Chemical Investigation Programmes (CIP 1 and 2)) in which chemical analysis of effluents has been conducted to monitor concentration oestrogenic compounds such as alkylphenols and steroid oestrogens. Data from the CIP were sought to provide information on current chemical composition of effluents.

The population served by a WwTW influences the oestrogenic load entering a WwTW and is used to estimate (along with effluent input and river flows/dilution factors) oestrogenic concentrations in effluent receiving waters. Historical population equivalents (PE) were collected from historic data sets from Brunel University and the University of Exeter, where available. Current PEs were supplied by the respective Water Companies via the Environment Agency.

The type of WwTW technology used can also have an impact on the oestrogenic content of WwTW effluents entering rivers. For example, activated sludge process (ASP) plants are known to be more effective than trickling filter plants for steroid oestrogen removal (e.g. Johnson et al. 2007). Additional treatment by tertiary (for example sand filters or reed beds) or advanced technologies (for example granular activated charcoal (GAC) or ozonation) has also been shown to reduce oestrogenic activity and steroid oestrogen concentrations of effluents (Baynes et al. 2012). Data on the on treatment technology used were obtained for 10 WwTW that release effluent at the river sites upstream of where the roach were sampled.

3 Results

This Section provides a summary of the results obtained. Additional information relating to fish age and related characteristics for the 2017 survey are presented in Appendix A. Detailed comparative data for individual river sampling sites (2017 survey results versus historical sampling) are included within Appendix B.

A total of 466 roach were caught across the 10 sampling sites revisited during the 2017 survey. The results presented in the main report focus specifically on the 3 research questions posed in Section 1.2 in relation to oestrogenic effects in male roach.

3.1 Feminised roach in the 2017 survey

3.1.1 Vitellogenin induction

The majority of the male roach sampled in 2017 had plasma VTG concentrations above those reported as 'natural' or baseline concentrations for males of the species (≤ 50 ng/mL VTG). There were also significant differences in the concentration of plasma VTG in males between river sampling locations (Figure 3.1, Table 3.1). Plasma VTG levels in male roach from the River Witham ($32,199 \pm 47,326$ ng/mL VTG) were higher than those measured at all other sites sampled in 2017. On average the lowest male VTG levels were measured in roach from the River Nene upstream of Great Billing WwTW (27.86 ± 39.28 ng/mL VTG).

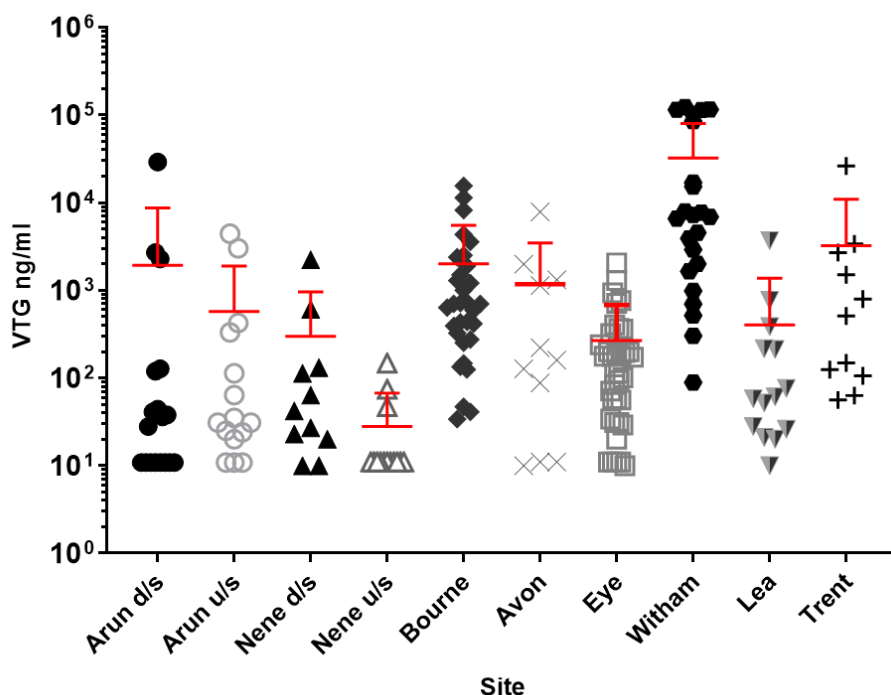


Figure 3.1 Plasma vitellogenin (VTG) concentration in male roach sampled at the different river/sites in 2017. Points (dots, triangles, crosses etc.) represent individual plasma concentrations; lower bar represents the mean and the upper error bar the standard deviation

Table 3.1. Results of oestrogenic effects analysis for male roach sampled in 2017

Site Name	No. fish (no. males)	Mean vtg (ng/ml)	% intersex males	Mean severity score
R Arun upstream Horsham WwTW	53 (15)	571.6 ± 1313	40	0.89 ± 0.71
R Arun downstream Horsham WwTW	56 (18)	1,919 ± 6816	17	0.44 ± 0.35
R Nene upstream Great Billing WwTW	42 (15)	27.86 ± 39.28	27	0.67 ± 0.49
R Nene downstream Great Billing WwTW	55 (11)	298 ± 663	0	0
Bourne downstream of Chertsey WwTW	54 (31)	2,000 ± 3522	35	0.56 ± 0.44
R Avon (Evesham)	33 (11)	1,172 ± 2308	0	0
R Eye	65 (37)	267.2 ± 413.3	22	0.56 ± 0.44
R Witham (Lincoln)	54 (23)	32,199 ± 47,326	0	0
R Lea downstream of East Hyde WwTW	34 (14)	402.3 ± 970.9	7	0.17 ± 0.00
R Trent (Rugeley)	21 (11)	3,233 ± 7698	0	0

3.1.2 Intersex frequency

Results from the gonad histopathology for the samples collected in 2017 revealed intersex roach (with ovotestis, i.e. both male and female reproductive tissues in their gonads) to be present at most of the survey sites (6 out of 10) (Table 3.1). Four sampling sites did not yield any intersex males. These were the River Nene downstream site, the River Avon (Severn), the River Witham and the River Trent. At most of these sites (3 out of 4), relatively small numbers of males were obtained for analysis. The site on the R Arun upstream of Horsham WwTW had the highest percentage, at 40% (6 out of 15 males).

3.1.3 Intersex severity

Mean intersex severity scores were observed to be below one at all sites (Figure 3.2, Table 3.1). The highest average intersex score was for intersex males from the River Arun site upstream of Horsham WwTW (0.89 ± 0.71, mean ± SD). The River Trent (n=11), River Avon (n=11), River Witham (n=23) and downstream River Nene (n=11) sites had no males with ovotestis, thus no intersex severity scores were assignable for these sites.

In the 2017 survey, none of the 185 phenotypic male roach analysed were found to contain ovarian cavities (female reproductive ducts).

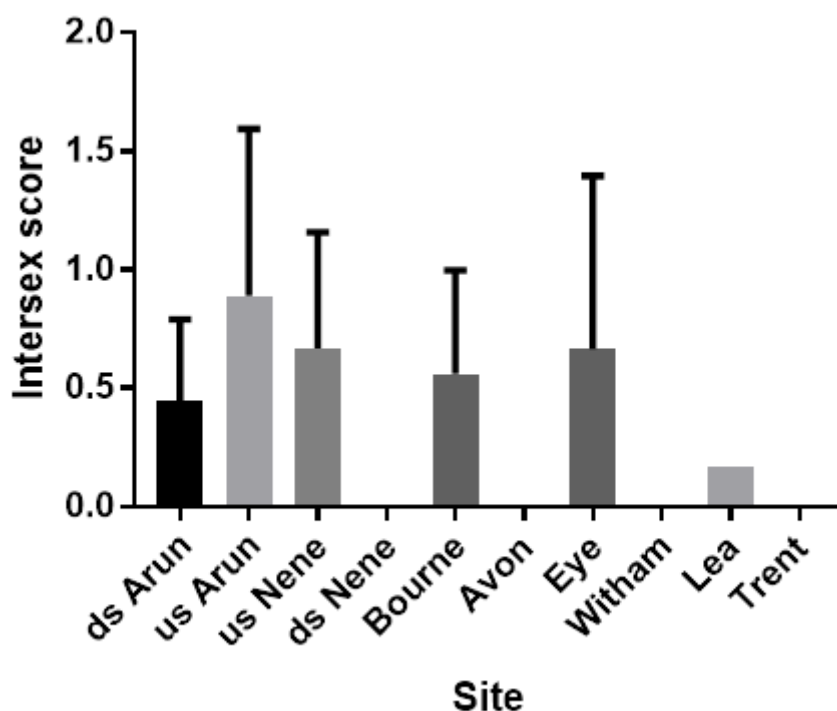


Figure 3.2 Average intersex (index) severity score of male roach sampled from 10 rivers in 2017. Error bars describe standard deviation

3.1.4 Phenotypic and genotypic sex ratio of roach sampled

Phenotypic sex (male or female) was determined from the gonad histopathology. At the majority of sites (7/10) there was a clear skew towards phenotypic females in the roach captured and sampled (Table 3.2). For two of the sites (Bourne and River Eye) sex ratios were skewed toward males and one site (River Trent) had a relatively even phenotypic sex ratio.

Using the genetic sex probe we found that the majority of the intersex fish (32 out of 33) were genetic males with feminised gonads (Figure 3.2). At the majority of sites (8/10) a small number of individuals (4.5% of the population) were found to have a mismatch between their genetic sex and the phenotypic sex determined via histopathology.

Six of the sites had sex reversed males (phenotypic females with male genotype); 4 at the upstream River Nene site, 3 at the River Arun downstream site, 2 at each of the River Avon and River Trent sites and 1 fish at each of the Bourne and River Witham. Five of the sites had roach that appeared to be sex reversed females (phenotypically male but with a female genotype); 3 of these fish occurred at the downstream River Arun site, 2 on both the Bourne and River Eye sites and 1 each at the upstream River Arun and the River Witham sites. None of the fish sampled at the downstream River Nene or River Lea sites were sex reversed.

Table 3.2 Number of phenotypic male and female roach sampled in the 2017 survey

Site name	Phenotypic females	Phenotypic males
R Arun upstream	38	15
R Arun downstream Horsham WwTW	38	18
R Nene upstream	27	15
R Nene downstream Great Billing WwTW	44	11
R Avon (Evesham)	22	11
R Witham (Lincoln)	31	23
R Lea downstream of East Hyde WwTW	20	14
Bourne downstream of Chertsey WwTW	23	31
R Eye	27	37
R Trent (Rugeley)	10	11
Total	280	186

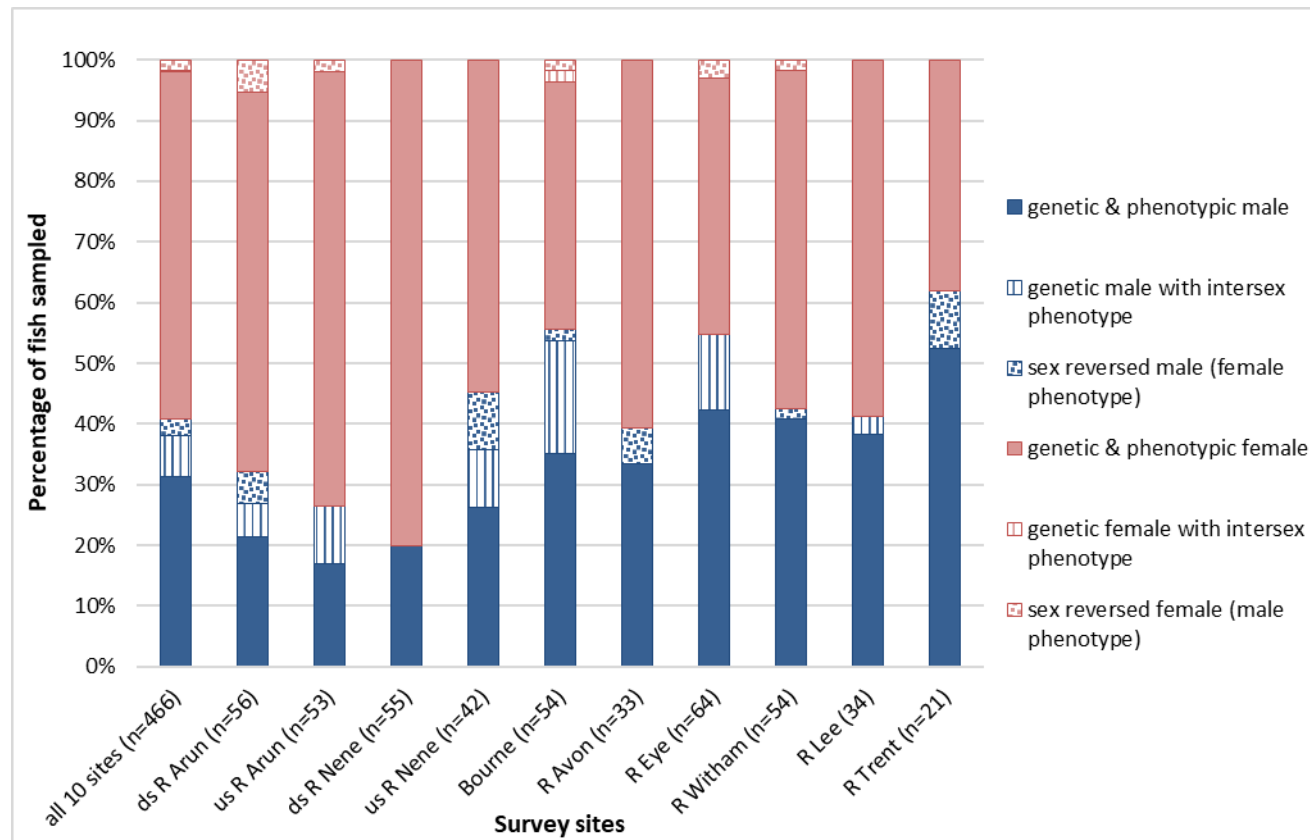


Figure 3.3 Phenotypic and genotypic sex types of roach sampled at 10 river sites in the 2017 survey.

3.2 Comparison of the 2017 survey findings with historical data sets

3.2.1 Sex ratio of samples

In both the historical and 2017 surveys, the phenotypic sex ratios of the sampled roach populations were generally skewed towards phenotypic females.

Comparison of genetic sex between historic and contemporary sites sampled was not possible as the genetic sex probe has only recently been developed and tissue samples for the historical samples were not available.

3.2.2 Intersex frequency and severity

Frequency

The frequency of intersex was lower at 9 out of the 10 sites (all except the River Arun upstream site) sampled in 2017 compared to the earliest previous survey occasion (see Table 3.3; Figure 3.4). However, a statistically significant difference was only observed at one site between the earliest sample (1995) and 2017: the River Nene downstream of Great Billing WwTW (see Appendix B for individual river comparisons; Figures B.4, B.5, B.6, B.10, B.11, B.12, B.15, B.16, B.19, B.20, B.23, B.24, B.27, B.28, B.31, B.32, B.35, and B.36). This site shows a clear downwards trend in intersex frequency over the six sampling occasions for which this site has been visited, with the highest intersex incidence (frequency) in 1995 and lowest in 2017 (see Figure B.11 Table 3.3; Figure 3.4).

The River Arun downstream of Horsham WwTW showed a significant difference in intersex occurrence between 2000 and 2017, but there was no clear downwards or upwards trend over the whole sampling timeframe (see Table 3.3; Figure 3.4).

A significant difference in intersex occurrence was also found for the River Lea between 1995 and 2000 (26% and 3% respectively) but the comparison was non-significant for 2017 despite remaining at low levels (7%).

Severity

The severity of intersex could not be compared between current and historical samples at 4 locations (River Nene downstream site, the River Avon (Severn), the River Witham and the River Trent) due to an absence of intersex males in the overall sample.

For the remaining sites where a comparison could be made, the severity of intersex condition was not statistically significantly different at the majority (4/5) of sites compared with the historical samples. A statistically significant reduction in intersex severity was only observed for the site on the Bourne downstream of Chertsey WwTW in both 2006 and 2017 when compared to the first survey conducted in 2002 (see Figure B.16; Table 3.3).

Table 3.3. Comparison of historical and current intersex frequency and severity at the 10 survey sites

Site Name	Sample date	% intersex males	Mean severity score
R Arun upstream Horsham WwTW	Oct 1995	31	0.27 ± 0.09
	Oct 2017	40	0.89 ± 0.71
R Arun downstream Horsham WwTW	Oct 1995	33	1.54 ± 2.26
	Apr 2000	61	2.47 ± 1.50
	Apr 2006	20* (2000)	2.42 ± 2.02
	Oct 2008	41	1.82 ± 1.69
	Apr 2017	17* (2000)	0.44 ± 0.35
R Nene upstream Great Billing WwTW	Oct 1995	33	0.70 ± 0.52
	Oct 2017	27	0.67 ± 0.49
R Nene downstream Great Billing WwTW	Oct 1995	65	2.42 ± 1.94
	Mar 1997	43	1.84 ± 1.49
	Apr 1998	45	2.68 ± 2.06
	Sept 1998	28* (1995)	1.89 ± 1.90
	Apr 1999	29* (1995)	0.26 ± 0.23* (1995-1999)
	Oct 2017	0* (1995/1997/1998)	0
Bourne downstream of Chertsey WwTW	May 2002	41	3.43 ± 1.77
	Apr 2006	39	1.02 ± 0.95* (2002)
	Nov 2017	35	0.56 ± 0.44* (2002)
R Avon (Evesham)	Oct 1995	12	0.56 ± 0.42
	Nov 2017	0	0
R Eye	Sept 1995	25	0.88 ± 0.74
	Oct 2017	22	0.67 ± 0.73
R Witham (Lincoln)	Jul 2002	22	1.33 ± 0
	Nov 2017	0	0
R Lea downstream of East Hyde WwTW	Aug 1995	26	1.96 ± 2.02
	Apr 2000	3* (1995)	0.33 ± 0.00
	Nov 2017	7	0.17 ± 0.00
R Trent (Rugeley)	Sept 1995	28	0.19 ± 0.06
	Dec 2017	0	0

* indicates a significant difference with the comparative year indicated in brackets.

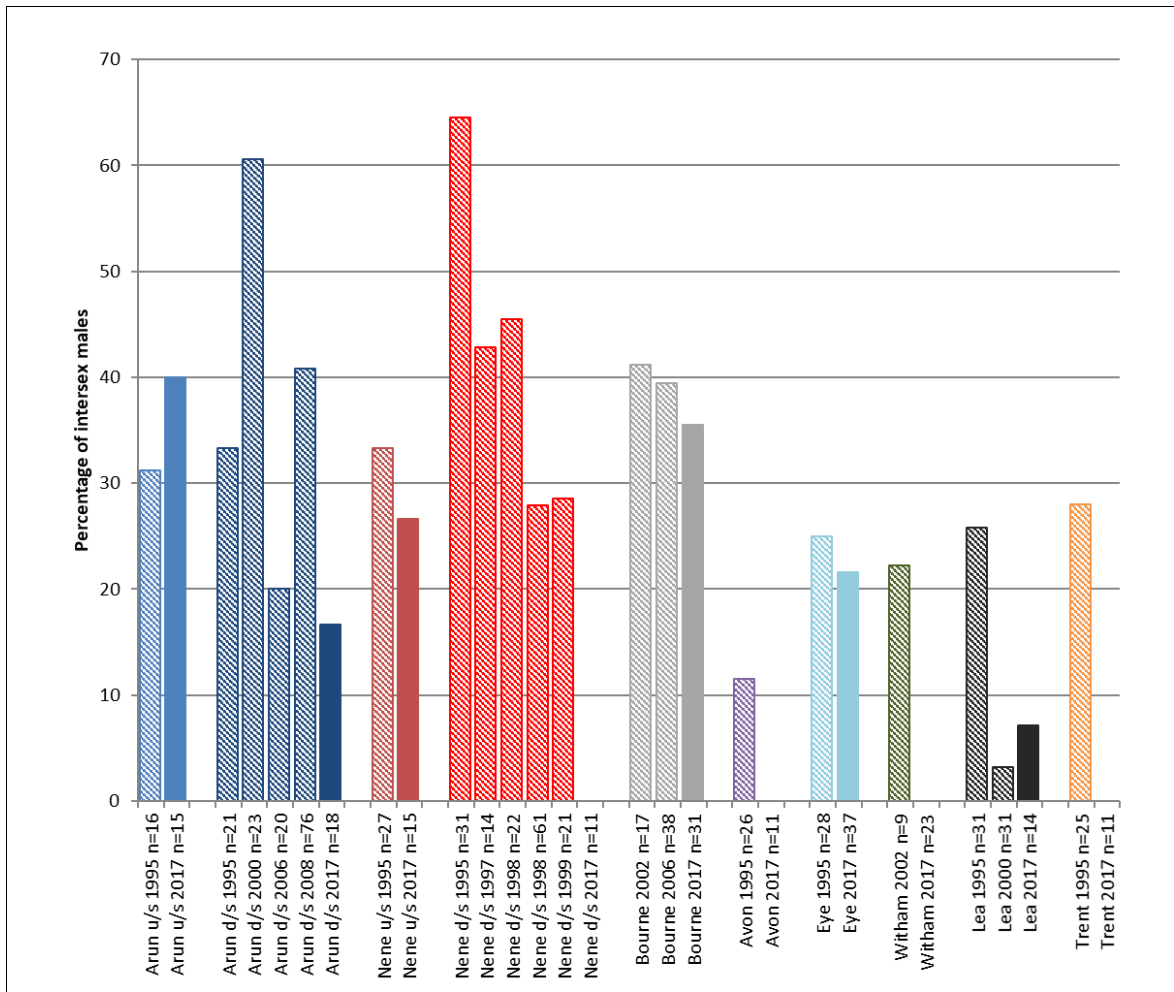


Figure 3.4 Percentage of phenotypic males with intersex gonads (eggs and sperm present) from surveys conducted since 1995 at ten river sites. Solid bars represent 2017 survey data and checked bars represent data derived from earlier surveys. Upstream site: u/s, downstream site: d/s, total number of males in sample: n=x.

3.2.3 Vitellogenin induction

When individual river survey sites were compared, 7 of the 10 revisited sites showed statistically significant decreases in plasma VTG concentrations (see Appendix B for individual river comparisons; Figures B.3, B.9, B.22, B.30 and B.34) in males (phenotypic males and intersex males) in the 2017 samples compared to the historical survey data (summarised in Figure 3.5). However at one site (the Bourne downstream of Chertsey WwTW) there was a statistically significantly elevated level of plasma VTG in males compared with those measured in the historical surveys (Figure B.14, Figure 3.5). The concentration of VTG in males sampled from the upstream River Nene site was within the range of natural baseline levels (<50 ng/ml).

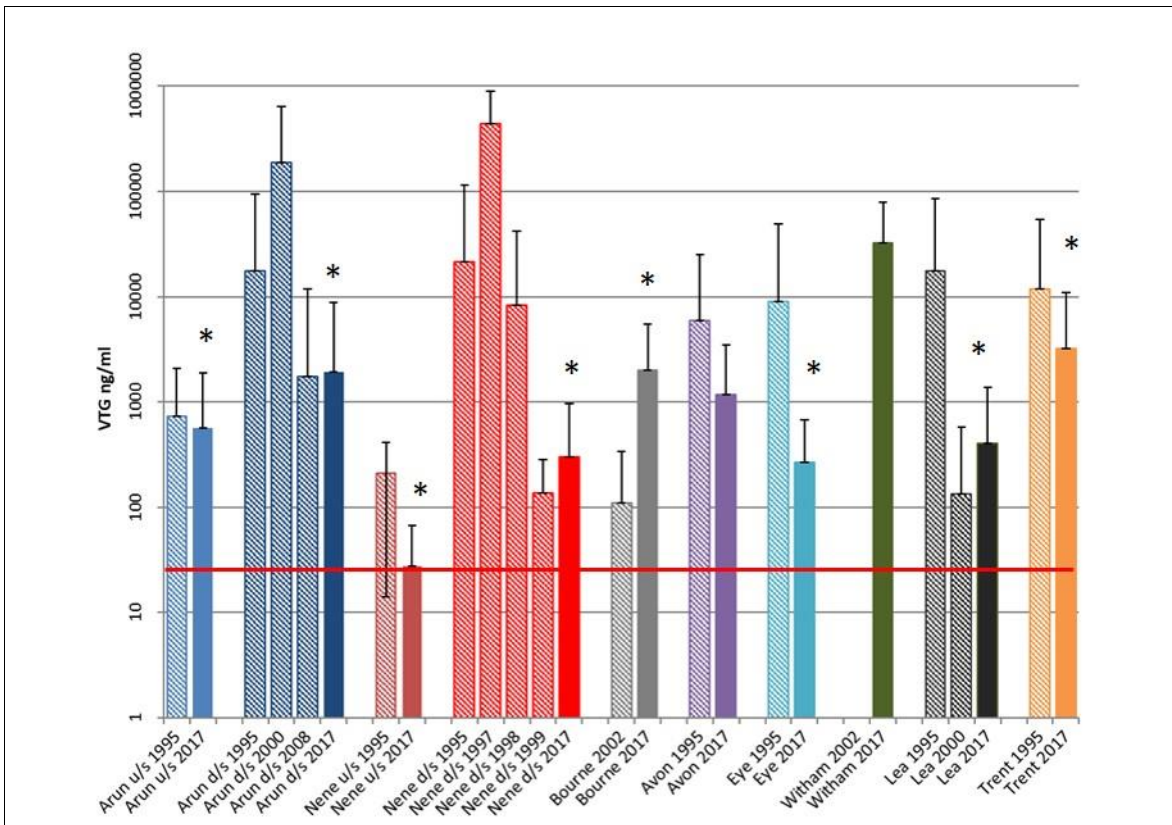


Figure 3.5 Male plasma vitellogenin (VTG) concentrations measured in surveys conducted since 1995 at ten river sites. Solid bars represent average 2017 VTG concentrations, checked bars are VTG concentration from earlier surveys. Error bars represent standard deviation. Stars indicate statistical significance between male VTG measured in 2017 and previous surveys ($P < 0.05$). Natural 'baseline' VTG concentrations in male roach are < 50 ng/ml represented by the horizontal red line.

3.3 WwTW effluent inputs at survey sites

3.3.1 Changes in population equivalents, WwTW technology improvements or changes to effluent quality

For all the sites where both historic and 2017 PE data were available, population growth, in terms of PE served by the WwTW, had increased in 2017 see (Table 2.2 and Appendix Table C.1). Great Billing WwTW on the River Nene had the lowest percentage PE increase (5%), whereas East Hyde WwTW on the River Lea had the highest percentage PE increase (59%) (Table 2.2).

Data on the on treatment technology for 10 WwTW that release effluent at the river sites upstream of where the roach are presented in Appendix Table C.1. One sample site, at the River Eye, did not have any known WwTW effluent inputs upstream, whereas some rivers had multiple WwTW upstream of the sampling sites.

Of the 10 WwTW, 8 used biological trickling filters as their main secondary treatment process. One used oxidation ditches (East Hyde) and one used activated sludge process (ASP) (Great Billing). Four WwTW also employed tertiary treatments such as sand filters (Horsham, Rugeley, Evesham) and Submerged Aerated Filters (Mannings Heath). Six of the ten WwTW sites had updated the treatment technology since 1998 (Great Billing, Horsham, Rugeley, Evesham, Mannings Heath, Warnham). The most common technology update was the addition of tertiary treatment. One site (Great Billing) had switched completely to an activated sludge process from a combination of a trickling filter and activated sludge system in 2001 (see Appendix Table C.1).

3.3.2 Changes in effluent chemical composition over time

Information about concentrations of steroid oestrogens and other known oestrogenic substances in discharges or the water column is required to inform the interpretation of differences in reproductive endpoints between sites, or between sampling occasions.

Unfortunately, data on steroid oestrogen concentrations from the CIP were not accessible within the timeframes of this project. It was found that the sites covered in the 2017 roach survey were not covered in the CIP Programme. However, the CIP data could still provide additional indirect information about possible changes in concentrations in steroidal, alkylphenolic and other oestrogenic chemicals measured in WwTW that have undergone similar upgrades to those upstream of the 2017 roach sampling sites.

4 Discussion

4.1 What are the current levels of intersex (and other oestrogenic effects) in wild roach?

4.1.1 Phenotypic sex and genetic sex

The majority of sites sampled in 2017 had a skewed sex ratio towards phenotypic females; this skew in sex ratio has previously been reported in wild roach at both 'reference' sites (sites receiving no known WwTW discharges) and at WwTW effluent-contaminated sites (see, for example, Environment Agency 1998, Bjerregaard et al. 2006), although others have reported an approximate 1:1 male: female ratio in wild populations (Geraudie et al, 2010).

Gonochoristic fish (such as roach) develop as either male or female, unlike many other fish species where sex changes occur as part of the natural process of sexual development. However, even gonochorists may retain some level of phenotypic sexual plasticity, including as adults, and they can change sex in response to various environmental influences. The development and use of a genetic sex marker for roach (with an accuracy of 99% for natural roach populations in the absence of intersex roach and 96% for wild populations with varying levels of intersex) has proven that wild intersex roach in UK rivers in this study arise as a consequence of the feminisation (demasculinisation) of males.

Sex-reversed males (i.e. phenotypic females that are genetically male) have been found to occur in fish chronically exposed to low oestrogen levels, including at the period of sexual differentiation (Kidd et al. 2007; Lange et al. 2009). The detection of some sex-reversed male roach in the 2017 study might suggest this could have arisen due to oestrogen exposure. However, the occurrence of sex-reversed females (i.e. phenotypic males that are genetic females) is more difficult to explain. It is possible that these individuals were genetic females, in which case sex reversal would imply complete masculinisation. This could result from exposure to androgens (masculinising chemicals), but in English rivers, androgen concentrations are not thought to be high enough to induce such a phenotypic change in roach. It is also possible that a low level of complete sex reversal might occur in wild roach populations, forming part of natural development processes that has not been identified previously due to the absence of the genetic sex marker. However, in other unpublished studies conducted at the University of Exeter, no sex reversal in roach raised in clean water laboratory conditions has been observed. Consequently, we are unable to explain the occurrence of sex-reversed females.

4.1.2 Vitellogenin induction and intersex frequency and severity

VTG induction in male fish is an extremely sensitive biomarker of exogenous oestrogen exposure for a range of fish species (Tyler et al, 1996, Lange et al, 2012). In male roach from all but one of the sites sampled (upstream of Great Billing WwTW on the River Nene), levels of plasma VTG were similar to those reported for juvenile females (Tyler et al, 1996), i.e. they were higher than levels found occurring naturally in males. Male roach from the site upstream of Great Billing WwTW were found to have plasma VTG similar to those reported in male roach from laboratory studies for un-exposed controls or 'reference' sites on river/lakes (Tyler et al, 1996, Geraudie et al, 2010), suggesting little or no oestrogenic exposure at this site.

Males in uncontaminated (reference) sites can exhibit seasonal increases in VTG, especially prior to spawning. However, generally these are at very low concentrations throughout the year, and the fluctuations are relatively small (Geraudie et al. 2010). This being the case, blood plasma levels of VTG in males between study sites, seasons and years of sampling are likely to be directly comparable.

Intersex frequency and severity generally appeared correlated with each other, with sites that had the highest frequency also having the highest severity. Interestingly, a higher frequency of intersex males and correspondingly higher intersex scores were found the upstream sites on the River Arun and River Nene compared with their downstream counterparts. This may reflect a higher standard of effluent treatment at larger urban WwTW resulting in greater oestrogen removal than small rural works. These larger works might also afford greater dilution downstream in the sampling sites. A more comprehensive analysis of upstream inputs would be required to verify such a hypothesis.

4.2 How do current levels of feminisation compare with those observed in previous studies?

Roach with ovotestis were present at the majority of sites surveyed in 2017 as was observed in the historical surveys. Furthermore, the frequency of the intersex condition was found not to be significantly different in 2017 compared with historical samples for 80% of the study sites. Whilst a reduction in male plasma VTG was observed, levels remained elevated above the natural baseline for males at the majority of the sites sampled in 2017. Collectively, these results indicate that exposure to oestrogenic substances in the environment is still occurring with measurable effects on male roach reproductive health.

However, the results of the 2017 survey indicate some improvement compared with historical samples. The frequency of intersex occurrence was lower at 9 out of the 10 sites. The frequency of roach with feminised reproductive ducts or male ovarian cavities had declined markedly in 2017 with an absence of this characteristic across all study sites in 2017 compared with a presence at 94% of sites surveyed historically (ranging from one or two individuals to all of the individuals sampled at the different sites in historical samples). Previous research has found that feminised ducts in males is the result of concentration dependent exposure to oestrogens during critical early life stages and is a permanent effect (Liney et al., 2005).

One possible explanation for the results observed in this study is that oestrogen concentrations have fallen below levels required to elicit the feminised duct response. This hypothesis is supported by the observation of a significant reduction in plasma VTG in males sampled at the majority (7/10) of sites surveyed. The continued presence of ovotestis, a progressive condition linked to the age and size of fish (Jobling et al., 2006), suggests the observed impacts are associated with chronic exposure to environmental oestrogens, albeit at lower concentrations than those occurring historically.

The findings of the 2017 study in English rivers are similar to the results of a survey of roach in Denmark (Christiansen et al., 2002). Relatively low levels of intersex (7 – 27% of males sampled) were observed and female ovarian cavity was not found in their intersex roach.

Whilst the severity of intersex was not significantly different at the 5 sites where a comparison could be made, the intersex condition was not detected in male roach at 4 locations during the 2017 survey. These 'zero' responses prevent statistical comparison at these sites. When considered in relation to the entire 73 historical data

sets held by Brunel University and the University of Exeter, an absence of intersex males has only been observed at 6 sites previously, which includes 'control/reference' sites. The majority of these sites (8/10) are, however, associated with a small number of male roach (< 12) in the overall sample which results in low statistical power. Consequently, this introduces uncertainty regarding the interpretation of these findings.

4.3 Possible explanations for the differences observed?

4.3.1 Effect of age class and/or body size of sampled roach

Age has been shown to be a significant factor in intersex severity (Tyler and Jobling 2008) and growth rate can affect sexual development in roach (Paull et al. 2008). Therefore, both age and size (length/weight) are important factors to consider when comparing intersex frequency and severity between surveys. For example, intersex severity has been shown to increase with the age of the fish sampled (Tyler and Jobling 2008), therefore comparing a mixed age sample with a later sample dominated by younger fish could result in an underestimation of intersex severity (or *vice versa*).

Most sites yielded younger fish in 2017 compared to earlier surveys (see Appendix B): there was only one survey site where no significant differences were found between the age and size of roach at the different survey times (River Witham, Table B8). Three sites (Bourne – Table B5; River Eye – Table B7; and River Trent- B10) had, on average, older (and larger) fish in the historical samples (1995 and 2002) compared with those collected in 2017. At four of the sites (River Arun both upstream and downstream, River Nene upstream and River Lea) fish collected in the 1995 samples were younger but larger than those sampled in 2017, and at one site (River Avon) the fish were not significantly different in age but were again significantly larger in the historical sample. These comparisons could indicate differences in the growth rates of the fish during different sampling periods. Growth rates can vary due to a range of factors such as water quality, food availability and stocking density.

It is not possible to definitively account for the effects of fish size or growth across the study years on the impacts of the feminised responses seen in the roach, but it is the scientific opinion of the authors that this would have little bearing on the overall interpretation of the data nor the conclusions drawn relating to the observed feminised effects or differences occurring in the roach over the study years.

4.3.2 Changes in chemical composition of effluents discharged upstream of sample sites

Although VTG was still elevated in male roach above natural baseline levels at the majority of sites sampled in 2017, compared with historical surveys it was significantly reduced at several of the study sites, suggesting that oestrogenic contamination may have declined at those sites.

The data and information obtained from the Water Companies suggest that since 1998 more than half of the WwTW upstream from the fish sampling sites, for which data have been provided, have updated their treatment technology (namely Great Billing, Horsham, Rugeley, Evesham, Mannings Health and Warnham), often with the addition of tertiary treatment technology and in one case switching from trickling filter beds to activated sludge process. In addition to the technology upgrades, legislative changes resulting in reduced alkyphenol use and disposal may have reduced the overall oestrogenic load entering the survey sites. These additions and/or improvements are

likely to have reduced the concentrations of oestrogenic substances in the final effluents, and in the river water downstream, but at present we are not able to confirm this due to lack of analytical data for the key causative oestrogenic chemicals known to induce the feminised effects in wild roach populations (including steroidal oestrogens and alkylphenolic chemicals).

In the 1990s, some UK WwTW effluents and rivers were found to have high concentrations of alkylphenol polyethoxylates, with maximum effluent and river concentrations of NP (a breakdown product from alkylphenol polyethoxylates) reaching 330 µg/l and 180 µg/l, respectively, on the heavily impacted River Aire (Blackburn & Waldock 1995, Blackburn et al. 1999). Other UK effluents and rivers had lower levels (<0.2-12 µg/L) (Blackburn & Waldock 1995). Alkylphenols are now regulated under the WFD. This class of surfactants was voluntarily phased out in the UK and their use banned from the early 2000s. Recent WwTW effluent monitoring suggests alkylphenol (including NP) levels have reduced, but still persist (e.g. NP < 0.1-0.64 µg/L – Severn Trent monitoring data 2015-2016). However, without site-specific data it is not possible to conclude if concentrations of alkylphenols at the sample sites visited in this survey have declined.

The lack of CIP or other chemical monitoring data means that the causative reasons for any differences observed cannot be fully explored. Whilst CIP2 monitoring sites were unfortunately found not to be co-located with the fish sampling sites, some useful insight might still be gained from exploring these data (when available) for general trends in the concentrations of these chemicals for effluents where similar WwTW upgrades have occurred.

4.3.3 Survey breadth and sample size

In comparison with previous intersex surveys undertaken by the Environment Agency, Brunel University and the University of Exeter, the survey in 2017 was relatively small and geographically limited. Nevertheless, revisiting exactly the same locations as previous surveys has clearly illustrated that feminisation is still occurring in roach, with both frequency and severity (for intersex gonads in male roach) at comparable levels to those seen in historical surveys and with plasma VTG levels, whilst reduced, remaining above what is considered 'natural' baseline concentrations.

For three out of the four sites where no intersex males were recorded the total numbers of male roach sampled were low (n=11, Figure 3.4), which is less than half of the targeted numbers required for a strong comparable analysis. Given this limitation, in future surveys, multiple sampling trips might usefully be incorporated into survey plans, ensuring the collection of sufficient numbers of males to reduce uncertainty in the intersex assessments.

4.4 Key messages and recommendations

The results of this 2017 field survey, in which 466 roach were sampled across 10 previously surveyed sites, showed feminisation in male roach is still a widespread phenomenon. Intersex males were detected at 60% of sites revisited. For the majority of sites (80%), the frequency of intersex has not significantly changed compared with historical surveys.

However, with a level of caution, the evidence suggests that there may be an improving picture with respect to overall oestrogenic exposure and resulting feminisation effects in roach at the survey sites studied. The complete absence of ovarian ducts in 2017, which is in marked contrast with observations in historical surveys (1995-2008), suggests oestrogenic concentrations are below the levels required to elicit this

response. This finding is supported by the reduction in blood plasma VTG levels in males at most sites compared with historical sampling.

Nevertheless, the continued presence of intersex males indicates that chronic exposure to oestrogenic substances at biologically active levels, as indicated by elevated male blood plasma VTG above 'natural' baseline levels, continues to impact the reproductive health of male fish.

The absence of chemical monitoring data prevented further exploration of current environmental oestrogenic concentrations and therefore of the effectiveness of work carried out to upgrade WwTw infrastructure.

Application of a newly developed genetic sex marker for roach supports the hypothesis that most intersex individuals in the UK are feminised genetic males (rather than masculinised females). Use of the genetic probe has also revealed the presence in wild roach populations of sex-reversed fish, albeit at a low incidence; the significance of this is unknown. It may be a natural phenomenon given that the reversal occurs both ways (males to females, and females to males).

The analyses of intersex incidence for some of the study sites in this survey were limited by the small number of males captured. In future, surveys should consider multiple samplings (perhaps conducted over a period of 2 years) to best ensure the collection of greater numbers of males (minimally 20). This would assist in improving statistical confidence in results and reducing uncertainty in the assessment of the effectiveness of interventions intended to reduce chemical concentrations in the environment.

As highlighted above, the 2017 survey was proportionally much smaller in scale (both in terms of site numbers and total numbers of fish sampled) than previous surveys, and therefore may not be as representative of the whole of England as were previous surveys. We therefore recommend that in future surveys, a greater geographically range of sites are re-visited to provide a more comprehensive, up-to-date picture of the incidence of intersex in roach populations nationally.

We also recommend that, in future, national sampling campaigns for the biological assessment of feminisation of fish include targeted chemical analyses, at the very least for steroidal oestrogens and alkylphenolic chemicals, to enable a more comprehensive understanding of the factors causing changes in biological effects. Ideally this should be co-ordinated with fish sampling to allow the biological metrics to be directly linked to chemical exposure.

References

- BAYNES, A., GREEN, C., NICOL, E., BERESFORD, N., KANDA, R., HENSHAW, A.J., CHURCHLEY, J.H. AND JOBLING, S. (2012) Additional Treatment of Waste Water Reduces Endocrine Disruption in Wild Fish – a Comparative Study of Tertiary and Advanced Treatments. *Environmental Science & Technology*, 46(10), 5565- 5573.
- BJERREGAARD, L.B., KORSGAARD, B. AND BJERREGAARD, P. (2006) Intersex in wild roach (*Rutilus rutilus*) from Danish sewage effluent-receiving streams. *Ecotoxicology and Environmental Safety*, 64(3), 321-328.
- BLACKBURN, M.A. AND WALDOCK, M.J. (1995) Concentrations of alkylphenols in rivers and estuaries in England and Wales. *Water Research*. 29(7),1623-1629.
- BLACKBURN, M.A., KIRBY, S.J. AND WALDOCK, M.J. (1999) Concentrations of alkylphenol polyethoxylates entering UK estuaries. *Marine Pollution Bulletin*. 38(2),109-118.
- CHRISTIANSEN, L.B., WINTHER_NIELSEN, M. and HELWEG, C. (2002) Feminisation of fish. The effect of estrogenic compounds and their fate in sewage treatment plants and nature, Environmental Project, No. 729.
- DESBROW, C., ROUTLEDGE, E.J., BRIGHTY, G.C., SUMPTER, J.P. AND WALDOCK, M. (1998) Identification of estrogenic chemicals in STW effluent, 1: Chemical fractionation and in vitro biological screening. *Environmental Science and Technology*. 32, 1549-1558.
- ENVIRONMENT AGENCY (1998) *The identification of oestrogenic effects in wild fish*. R&D Technical Report W119. Bristol: Environment Agency.
- ENVIRONMENT AGENCY (2002) *The Identification of Oestrogenic Effects in Wild Fish – Phase II*. R&D Technical Report W2-014/TR. Bristol: Environment Agency.
- ENVIRONMENT AGENCY (2004) *Causes and consequences of feminisation of male fish in English rivers*. Science Report SC030285/SR. Bristol: Environment Agency.
- ENVIRONMENT AGENCY (2008) *Catchment Risk Assessment of Steroid Oestrogens from Sewage Treatment Works*. Science Report SC030275/SR3. Bristol: Environment Agency.
- GERAUDIE, P., GERBRON, M., HILL, E. AND MINIER, C. (2010) Roach (*Rutilus rutilus*) reproductive cycle: a study of biochemical and histological parameters in a low contaminated site. *Fish Physiology and Biochemistry*. 36, 767–777.
- HAMILTON, P.B., NICOL, E., DE-BASTOS, E.S.R., WILLIAMS, R.J., SUMPTER, J.P., JOBLING, S., STEVENS, J.R. AND TYLER, C.R. (2014) Populations of a cyprinid fish are self-sustaining despite widespread feminization of males. *BMC Biology*. 12(1), p1.
- HARRIS, C., HAMILTON, P.B., RUNNALLS, T.J., VINCIOTTI, V., HENSHAW, A., HODGSON, D., COE, T., JOBLING, J., TYLER, C.R. AND SUMPTER, J.P. (2011) The

Consequences of Feminization in Breeding Groups of Wild Fish. *Environmental Health Perspectives*. 119(3), 306-311.

HECKER, M., TYLER, C.R., HOFFMANN, M., MADDIX, S. AND KARBE, L. (2002) Plasma bio-markers in fish provide evidence for endocrine modulation in the Elbe River, Germany. *Environmental Science and Technology*. 36, 2311-2321.

HICKS, K.A., FUZZEN, M.L.M., MCCANN, E.K., ARLOS, M.J., BRAGG, L.M., KLEYWEGT, S., TETREAULT, G.R., MCMASTER, M.E., AND SERVOS, M.R. (2017) Reduction of Intersex in a Wild Fish Population in Response to Major Municipal Wastewater Treatment Plant Upgrades. *Environmental Science and Technology*. 51(3), 1811-1819.

JOBLING, S., NOLAN, M., TYLER, C.R., BRIGHTY, G. AND SUMPTER, J.P. (1998) Widespread sexual disruption in wild fish. *Environmental Science and Technology*. 32, 2498-2506.

JOBLING, S., COEY, S., WHITMORE, J.G., KIME, D.E., VAN LOOK, K.J.W., MCALLISTER, B.G., BERESFORD, N., HENSHAW, A.C., BRIGHTY, G., TYLER, C.R. AND SUMPTER, J.P. (2002) Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biology of Reproduction*. 67(2), 515-524.

JOBLING, S., WILLIAMS, R., JOHNSON, A., TAYLOR, A., GROSS-SOROKIN, M., NOLAN, M., TYLER, C.R., VAN AERLE, R., SANTOS, E. AND BRIGHTY, G. (2006) Predicted Exposures to Steroid Estrogens in U.K. Rivers Correlate with Widespread Sexual Disruption in Wild Fish Populations. *Environmental Health Perspectives*. 114 (Suppl 1), 32–39.

JOBLING, S., BURN, R.W., THORPE, K., WILLIAMS, R. AND TYLER, C. (2009) Statistical Modeling Suggests that Antiandrogens in Effluents from Wastewater Treatment Works Contribute to Widespread Sexual Disruption in Fish Living in English Rivers. *Environmental Health Perspectives*. 117(5), 797-802.

JOHNSON, A. AND WILLIAMS R., (2004) A model to estimate influent and effluent concentrations of estradiol, estrone, and ethinylestradiol at sewage treatment works. *Environmental Science and Technology*. 38(13), 3649-3658.

JOHNSON, A., WILLIAMS, R., SIMPSON, P. AND KANDA, R. (2007) What difference might sewage treatment performance make to endocrine disruption in rivers? *Environmental Pollution*. 147(1), 194-202.

KIDD, K.A., BLANCHFIELD, P.J., MILLS, K.H., PALACE, V.P., EVANS, R.E., LAZORCHAK, J.M. AND FLICK, R.W. (2007) Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Science U.S.A.* 04(21), 8897-901.

LANGE, A., PAULL, G.C., COE, T.S., KATSU, Y., URUSHITAN, H., IGUCHI, T. AND TYLER, C.R. (2009) Sexual Reprogramming and Estrogenic Sensitization in Wild Fish Exposed to Ethinylestradiol. *Environmental Science and Technology*. 43(4), 1219-1225.

- LANGE, A., KATSU, Y., MIYAGAWA, S., OGINO, Y., URUSHITANI, H., KOBAYASHI, T., HIRAI, T., SHEARS, J.A., NAGAE, M., YAMAMOTO, J., OHNISHI, Y., OKA, T., TATARAZAKO, N., OHTA, Y., TYLER, C.R. AND IGUCHI, T. (2012) Comparative responsiveness to natural and synthetic estrogens of fish species commonly used in the laboratory and field monitoring. *Aquatic Toxicology*. 109, 250-258.
- LINEY, K.E., JOBLING, S., SHEARS, J.A., SIMPSON, P. AND TYLER, C.R. (2005) Assessing the sensitivity of different life stages for sexual disruption in roach (*Rutilus rutilus*) exposed to effluents from wastewater treatment works. *Environmental Health Perspectives*. 133(10), 1299-1307.
- MORTHORST, J.E., MATHIESEN, K.K., HOLBECH, H., PEDERSON, K.L. AND BJERREGAARD, P. (2018) Vitellogenin concentrations in feral Danish brown trout have decreased: An effect of improved sewage treatment in rural areas? *Environmental Toxicology and Chemistry*. 37(3), 839-845.
- NOLAN, M., JOBLING, S., BRIGHTY, G., SUMPTER, J.P. AND TYLER, C.R. (2001) A histological description of intersexuality in the roach. *Journal of Fish Biology*. 58, 160-176.
- PAULL, G.C., LANGE, A., HENSHAW, A. AND TYLER, C.R. (2008) Ontogeny of sexual development in the roach (*Rutilus rutilus*) and its interrelationships with growth and age. *Journal of Morphology*. 269(7), 884-895.
- PURDOM, C.E., HARDIMAN, P.A., BYE, V. J., ENO, N. C., TYLER, C.R. AND SUMPTER, J.P. (1994) Estrogenic effects of effluents from sewage treatment works. *Journal of Chemical Ecology*. 8, 275-285.
- SCOTT, A.P., HILL, T., BERESFORD, N., RUNNALLS, T.J., SUMPTER, J.P. AND ELLIS, T. (2013) Out-of-season production of 17,20 β -dihydroxypregn-4-en-3-one in the roach *Rutilus rutilus*. *Journal of Fish Biology*. 83, 233–249.
- SHEAHAN, D.A., BRIGHTY, G.C., DANIEL, M., JOBLING, S., HARRIES, J.E., HURST, M.R., KENNEDY, J., KIRBY, S.J., MORRIS, S., ROUTLEDGE, E.J., SUMPTER, J.P. AND WALDOCK, M.J. (2002) *Environmental Toxicology and Chemistry*. 21(3), 515-519.
- TANNA, R.N., TETREAU, G.R., BENNETT, C.J., SMITH B.M., BRAGG, L.M., OAKES, K.D., MCMASTER, M.E. AND SERVOS, M.R. (2013) Occurrence and degree of intersex (testis-ova) in darters (*Etheostoma* spp.) across an urban gradient in the Grand River, Ontario, Canada. *Environmental Toxicology and Chemistry*. 32(9), 1981-1991.
- TRUETT, G.E., HEEGER, P., MYNATT, R.L., TRUETT, A.A., WALKER, J.A. AND WARMAN, M.L. (2000) Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques*. 29(1), 52-54.
- TYLER, C.R., VANDER EERDEN, B., JOBLING, S., PANTER, G. AND SUMPTER, J.P. (1996) Measurement of vitellogenin, a biomarker for exposure to oestrogenic chemicals, in a wide variety of cyprinid fish. *Journal of Comparative Physiology B Biochemical, Systems, and Environmental Physiology*. 166(7), 418-426.

TYLER, C.R. AND JOBLING, S. (2008). Roach, Sex, and Gender-Bending Chemicals: The Feminization of Wild Fish in English Rivers. *BioScience*. 58(11), 1051–1059.

WYATT, P.M.W., PITTS, C.S. AND BUTLIN, R.K. (2006) A molecular approach to detect hybridization between bream *Abramis brama*, roach *Rutilus rutilus* and rudd *Scardinius erythrophthalmus*. *Journal of Fish Biology*. 69, 52-71.

List of abbreviations

CIP	Chemical Investigation Programme
DNA	Deoxyribonucleic acid
E1	estrone
E2	oestradiol
E2 Equ	oestradiol equivalent
EE2	ethinylestradiol
ELISA	enzyme-linked immunosorbent assay
GSI	gonadal somatic index
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
♀	female
♂	male

Appendix A: Detailed results for 2017 roach survey

Roach length, weight and age

Roach from the River Nene downstream of Great Billing WwTW were, on average, the largest sampled in size, with fork-length of 17.05 ± 1.63 cm and total weight of 79.44 ± 29.66 g. Roach sampled from the River Trent site were, on average, the smallest in size (11.59 ± 2.34 cm, 24.42 ± 26.1 g, Figure A.1).

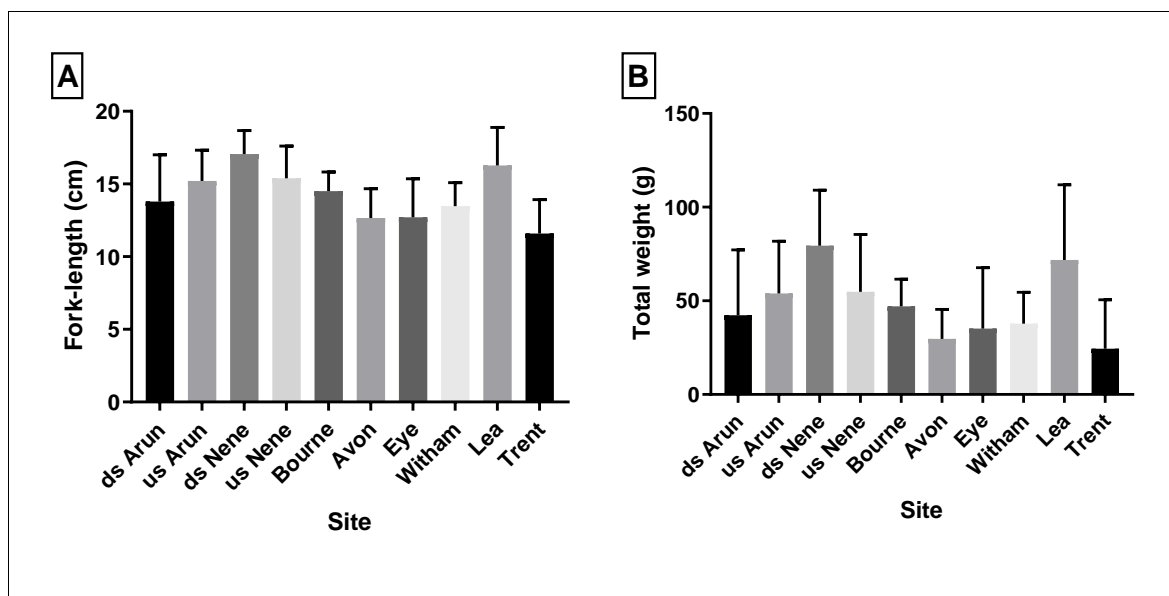


Figure A.1 Comparison of roach sizes in terms of (A) fork lengths and (B) total weights from the 10 river sites sampled in 2017. Columns indicate mean length (cm) or weight (g), bars describe standard deviation

In the 2017 sampling, the minimum age of roach was two years (sampled from the Bourne and River Trent) and the maximum age was nine years (sampled from River Arun, upstream and downstream sites, and the River Eye). From the River Nene downstream of Great Billing and River Arun upstream of Horsham, six year old fish were the most frequent (mode) sampled; five year old fish were most frequently sampled from the River Witham, River Avon, upstream River Nene and downstream River Arun, in the Bourne four year old fish were most frequent, three year olds were most frequently sampled from the River Lea, and in the River Trent sample those most frequently sampled were two years old. Figure A.2 illustrates the range (minimum, median and maximum) of ages of roach from the 10 sites sampled in 2017.

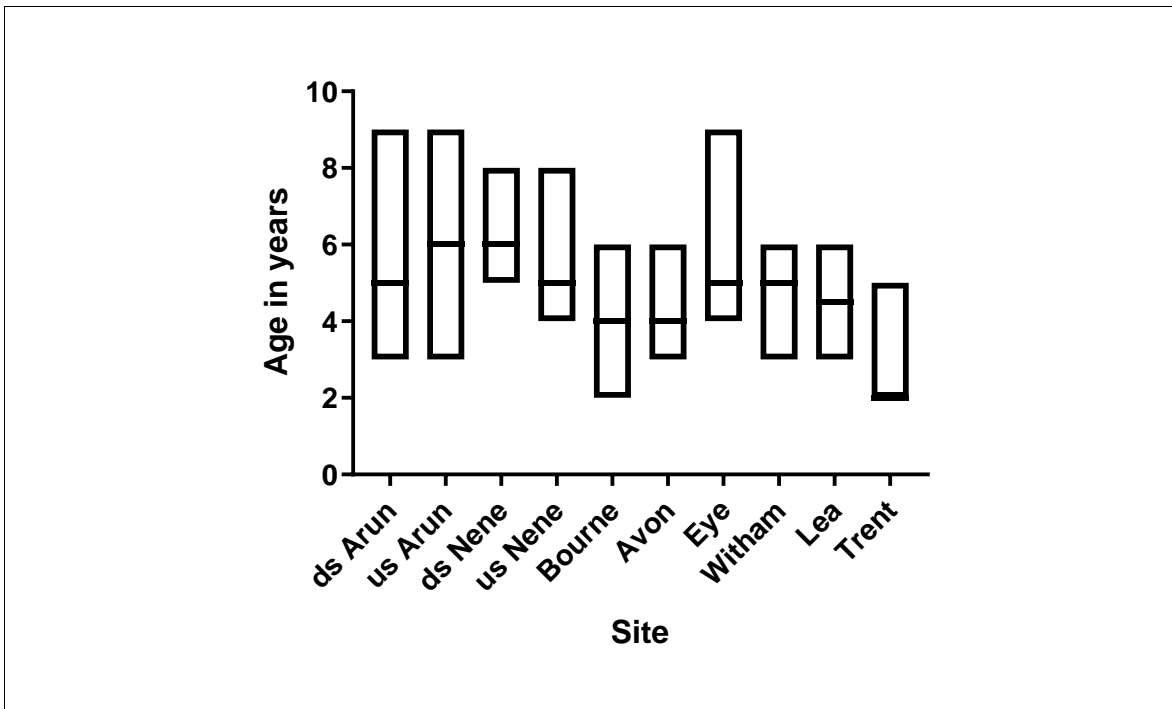


Figure A.2 Floating bar plot of the ages of roach sampled from the 10 river sites in 2017. Boxes represent minimum and maximum ages, line represents the median age

Gonadal somatic index (GSI)

Data for the gonadal somatic index (GSI) were grouped by phenotypic sex. In males, on average the GSI was largest in males from the Bourne (mean \pm SD, 5.6 ± 3.28) and smallest in fish from the River Arun downstream of Horsham WwTW (2.87 ± 0.48). Generally, males sampled in November (Bourne, River Avon, River Eye, River Witham and River Lea) had a higher GSI compared with those sampled in October (downstream River Arun, upstream River Arun, downstream River Nene and upstream River Nene, Figure A.3). Statistically, the males from the River Eye and the River Witham had larger testes (higher GSIs) compared with those sampled at the downstream River Arun, upstream River Arun and downstream River Nene. The GSI in males at the downstream River Arun site was also significantly lower than males sampled from the Bourne, River Avon and the River Lea.

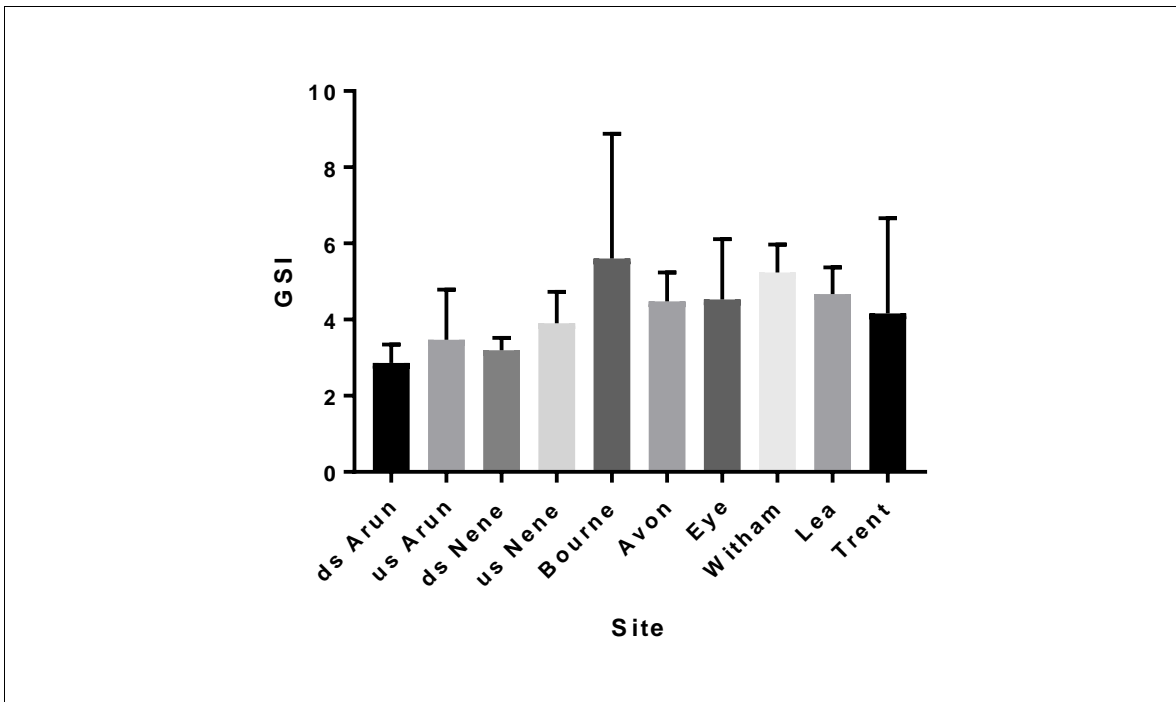


Figure A.3 Gonadal somatic index (GSI) in male roach sampled from 10 river sites in the 2017 survey. Columns indicate mean GSI, bars describe standard deviation

Females from the R Trent had on average the lowest GSI (3.01 ± 4.49) and those from the River Lea and Bourne sites had the highest GSI (10.23 ± 4.52 , 10.19 ± 4.69 , respectively, Figure A.4).

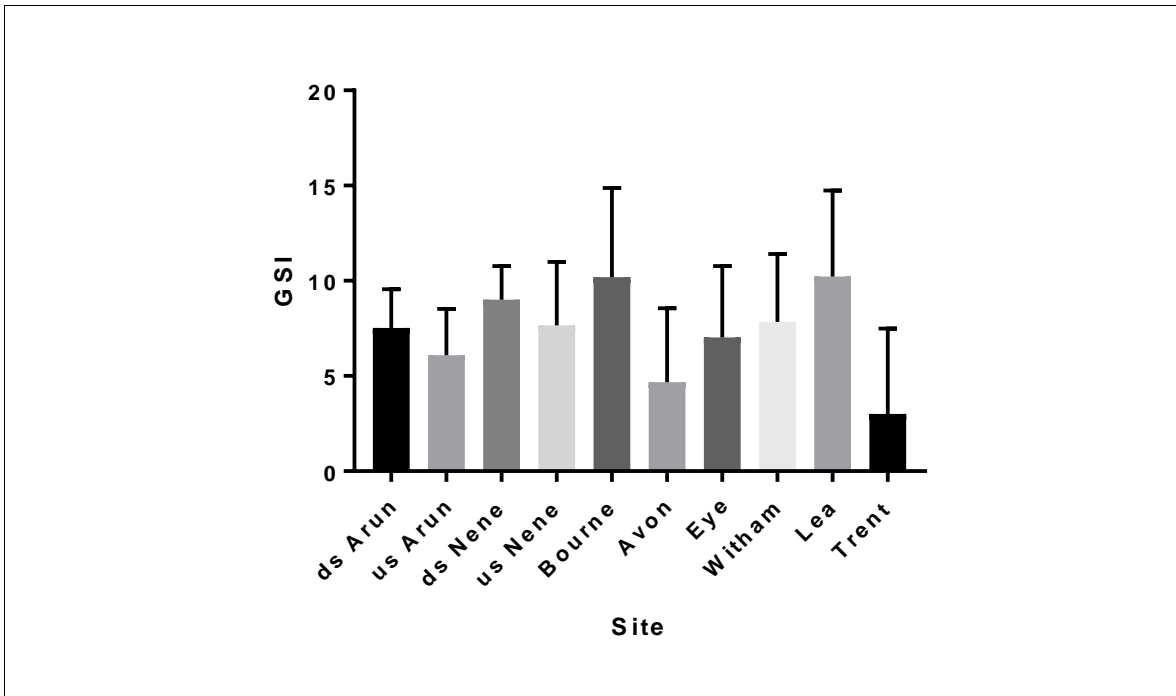


Figure A.4 Gonadal somatic index (GSI) in female roach sampled from 10 river sites in the 2017 survey. Columns indicate mean GSI, bars describe standard deviation

Other pathologies and conditions

Abnormalities and obvious parasite infections were noted during sampling and tissue analysis (by dissection of the whole fish and microscopic assessment of the gonads). The most frequent parasite noted was black spot, which was particularly common in roach from the River Eye and River Lea (Table A.1). Other parasites such as leeches, and internally, nematode worms, were also found occasionally. The cestode *Ligula intestinalis* was identified in one roach from the Bourne (Table A.1). During dissection, a number of both male and female roach had noticeable spots, lumps or cysts on the surface of their gonads. Upon microscopic inspection (histology) these were found to be myxosporidia cysts in the males. Females were found to contain atretic oocytes or parasite infections (for example, encysted parasites). When parasites were observed inflammatory or proliferative immune responses (for example, granulomatous inflammation, fibrosis) were also seen. In some individuals, similar immune responses were also observed (especially fibrosis) without obvious parasite infection. Atretic oocytes were observed in females from all sites, however they were especially frequent (>80%) in female roach from the River Arun, from both up and downstream locations. In males, testicular dysgenesis was observed in some individuals ($\leq 20\%$). Both these conditions (atretic oocytes and testicular dysgenesis) have been previously reported in association with exposure to oestrogenic wastewaters, environmental oestrogens and other pollutants. However, although some sites had higher levels of atretic oocytes or testicular dysgenesis than others, no specific trend, i.e. lower levels upstream of a WwTW compared to downstream, were observed in this survey.

Table A.1 Abnormalities, parasite infections and pathologies observed in roach sampled in the 2017 survey

River sampling site	No. of roach with parasite infections	No. of roach with immune cells/ pathology	Number of females with atretic oocytes	Number of males with testicular dysgenesis
River Arun downstream of Horsham	myxosporidia cysts (2♂), unidentified parasite in gonad (1♀)	lymphocyte infiltration (3♀), fibrosis (10♀)	32/38 (84%)	2/18 (11%)
River Arun upstream of Horsham	Blackspot (3♂, 1♀), Unidentified parasite in the gonad (1♀),	granuloma (1♂), fibrosis (1♂, 14♀), lymphocyte infiltration (7♀)	33/38 (87%)	3/15 (20%)
River Nene downstream of Great Billing	Nematode worms (2♂, 6♀) Blackspot (2♀), Leach (1♀), Unidentified parasite in gonad (1♀, 1♂)	lymphocyte infiltration (1♀), fibrosis (1♀)	21/44 (47%)	0
River Nene upstream of Great Billing	Blackspot (2♂)	lymphocyte infiltration (2♀), fibrosis (5♀), one ♂ with 3 white tumours on swimbladder	13/27 (48%)	0
Bourne downstream of Chertsey	Blackspot (1♂), <i>Ligula intestinalis</i> (1♂),	lymphocyte infiltration (2♂), fibrosis (4♂, 2♀)	6/31 (19%)	1/23 (4%)

	Unidentified encysted parasite(s) in gonad (5♂)			
River Avon Hampton Ferry	Nematode worms (1♂, 4♀)	fibrosis (2♀)	2/22 (9%)	2/11 (18%)
River Eye upstream of Melton Mowbray	Blackspot (13♂, 6♀)	fibrosis (1♂, 4♀)	5/27 (19%)	4/37 (11%) One ♂ with only one gonad
River Lea downstream of East Hyde	Blackspot (2♂, 10♀), myxosporidia cysts (1♂)	fibrosis (2♀)	10/20 (50%)	1/14 (7%)
River Witham (Lincoln)	Blackspot (2♂, 1♀) Leach (1♂)	fibrosis (2♂, 5♀) lymphocyte infiltration (1♂, 1♀) Exophthalmia (1♂)	1/31 (3%)	1/23 (4%)
River Trent (Rugeley)	Blackspot (3♂, 1♀)	fibrosis (1♀)	1/10 (10%)	0

Appendix B: Detailed river by river comparison of historical and 2017 roach surveys

River Arun upstream and downstream of Horsham

Roach sampled in 1995 from the upstream site on the River Arun ranged in age between 2 to 6 years (median 5 years). In 2017, roach sampled were between 3 and 9 years old (median 6 years) (Figure B.1). Roach in the 2017 samples were significantly older than those sampled in 1995 ($P < 0.0001$). In the 1995 sampling, fish length (22.1 ± 4.3 cm) and weight (145.9 ± 83.3 g) were significantly greater in length and heavier than those sampled in 2017 (15.21 ± 2.13 cm, 53.89 ± 27.97 g respectively, $P < 0.0001$) (Table B.1).

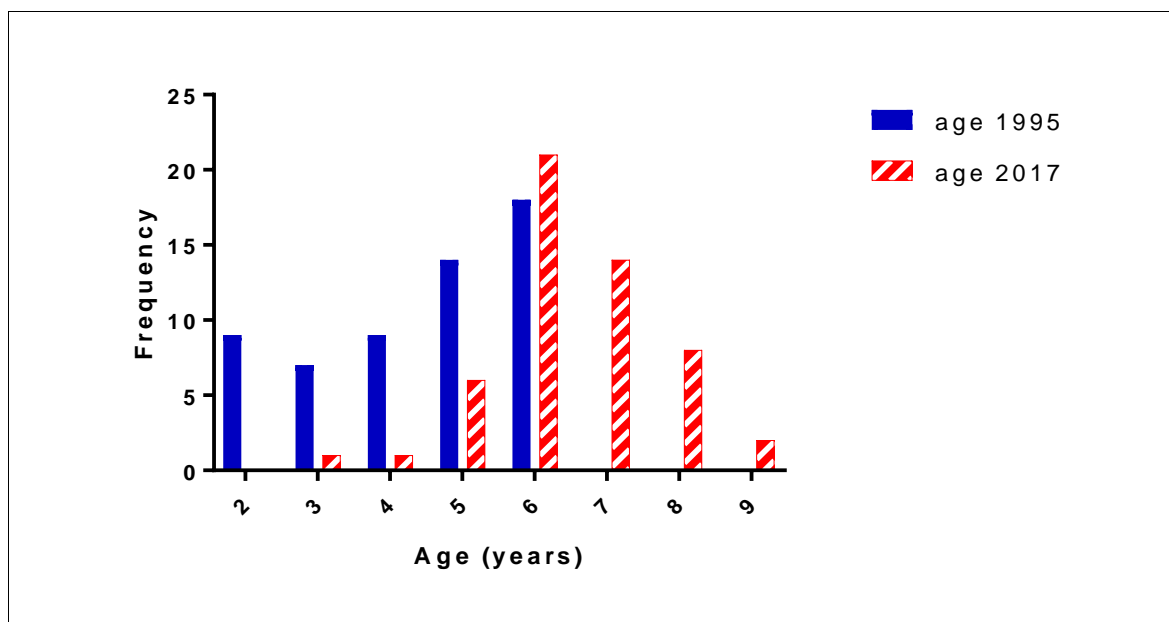


Figure B.1 Age distribution frequency of roach sampled from the upstream site on the River Arun in 1995 and 2017

In the downstream River Arun sampling site, in 1995 and 2000 roach ranged in age between 2 to 7 years (median 3 and 4, respectively); in 2006 and 2008 they ranged between 3 to 7 years old (median 5 years for both) and in 2017 between 3 to 9 years old (median 5 years) (Figure B.2). There were significant differences in age between the roach sampled across the different dates ($P < 0.0001$), with significantly younger fish sampled in 1995 compared with those sampled at all other sampling dates, and with the 2017 sample having a higher average age roach than those sampled at the other dates. The ages of roach in the intervening years (2000, 2006 and 2008) were similar to one another.

There were significant differences in length and weight of the roach sampled over the five time points ($P < 0.0001$ for both). The roach sampled in 1995 (17.29 ± 2.14 cm) were significantly greater in length than in 2000 (15.44 ± 2.76 cm, $P = 0.003$), 2006 (15.45 ± 1.26 cm, $P = 0.005$) and 2017 (13.79 ± 3.22 cm, $P < 0.0001$) but not 2008 (16.75 ± 2.67 cm). In terms of weight, roach sampled in 2017 (42.35 ± 34.79 g) were

significantly lighter compared with those sampled in 1995 (65.5 ± 31.77 g, $P = 0.0002$), 2006 (64.45 ± 20.25 g, $P = 0.002$) and 2008 (78.65 ± 31.94 g, $P < 0.0001$), but not 2000 (63.94 ± 41.74 g).

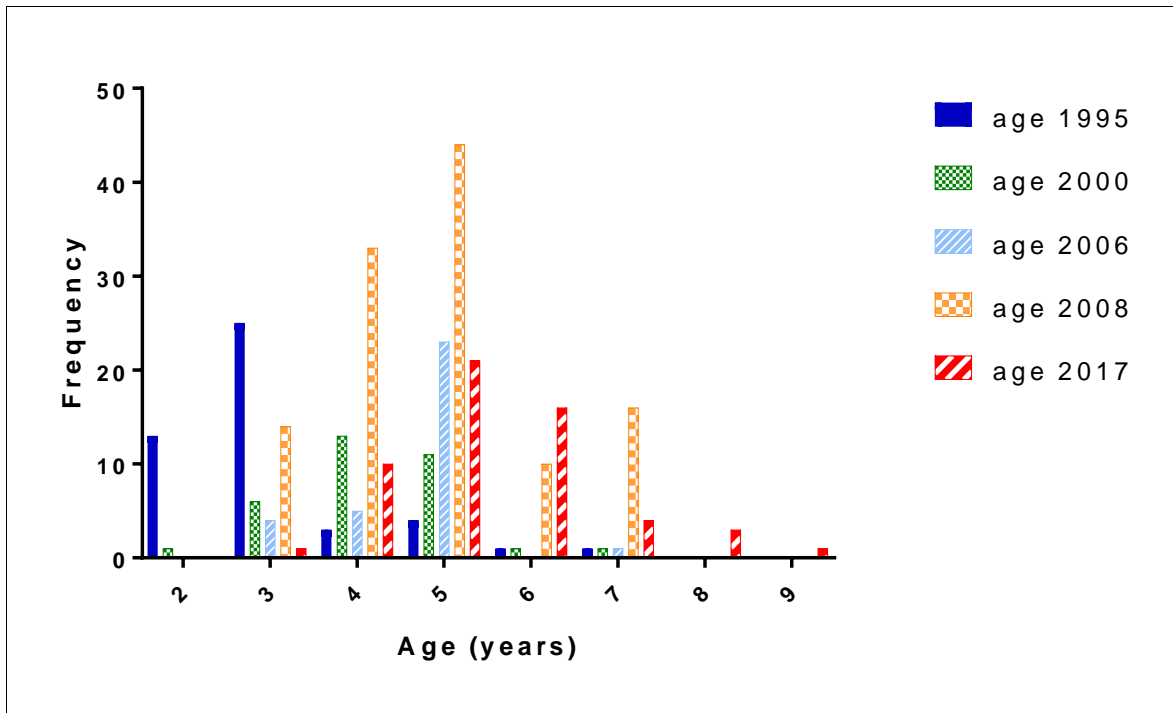


Figure B.2 Age distribution frequency of roach sampled from the site downstream of Horsham WwTW on the River Arun in 1995, 2000, 2006, 2008 and 2017

For the River Arun the phenotypic sex ratio was skewed towards females in both the 1995 (upstream 75%, downstream 61%) and 2017 (upstream 72%, downstream 68%) sampling occasions (Table B.2). There was no significant difference in sex ratio between these two sampling occasions at the different sites.

For the site upstream on the River Arun, no significant difference was found in the male GSI between 1995 and 2017 (3.60 ± 0.51 and 3.47 ± 1.32 , respectively). A significant difference was found in the GSI in females, with those from 1995 having a larger ovary (GSI) compared with those sampled in 2017 (9.71 ± 1.38 and 6.1 ± 2.42 , $P < 0.0001$, T-test). The same pattern was found at the downstream site on the River Arun with no significant difference between the GSI in males 1995 or 2017 (1995: 2.98 ± 0.84 compared with 2017: 2.87 ± 0.48). Again, significant differences were found between the GSI in females with 1995 females having a relatively larger ovary than the fish sampled in 2017 (10.17 ± 1.70 vs. 7.52 ± 2.04 , $P < 0.0001$).

In both 1995 and 2017 the average downstream VTG plasma concentrations in males were higher than those from the upstream site in the corresponding year. In 2017, VTG concentrations in males at the downstream site of the River Arun (1919 ± 6816 ng/ml (mean \pm SD)) were lower than those measured in 1995 ($17,689 \pm 76,381$ ng/ml, $P < 0.0001$) at the same site. Compared with the downstream River Arun site, the differences between plasma VTG in males at the upstream River Arun site were less pronounced between 2017 and 1995 (2017: 571.6 ± 1313 ng/mL, 1995: 733.6 ± 1351 ng/mL), yet VTG concentrations were still significantly reduced ($P = 0.0148$) in the 2017 sampling occasion compared with 1995 (Figure B.3).

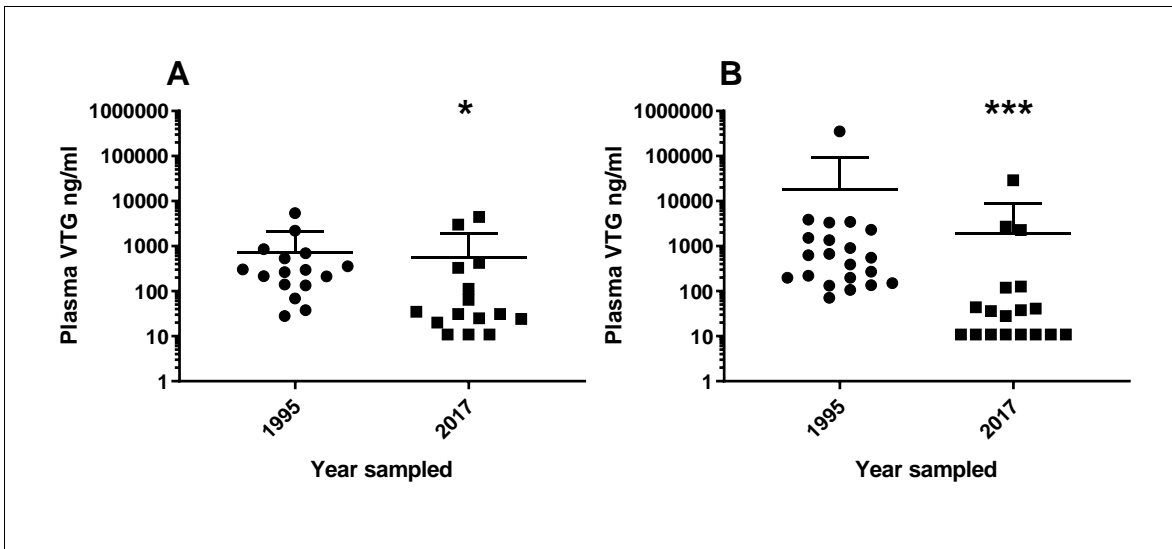


Figure B.3 Plasma vitellogenin (VTG) concentrations in male roach sampled from the River Arun sites (A) upstream and (B) downstream of Horsham WwTW in 1995 and 2017. Points (dots, squares) on graph represent individuals, long horizontal line indicates mean, short horizontal line indicates standard deviation. Star * indicates statistical significant decrease in VTG between sampling dates (A* P = 0.0148, B * P < 0.0001)**

For the River Arun, the plasma VTG concentrations for females (at both the upstream and downstream sites) were higher in the 2017 sampling, compared with 1995 (Table B.12).

In the upstream River Arun site, 31% (5/16) of males had oocytes in their testis in 1995 compared with 40% (6/15) in 2017 (Figure B.4); this apparent difference was not statistically significant.

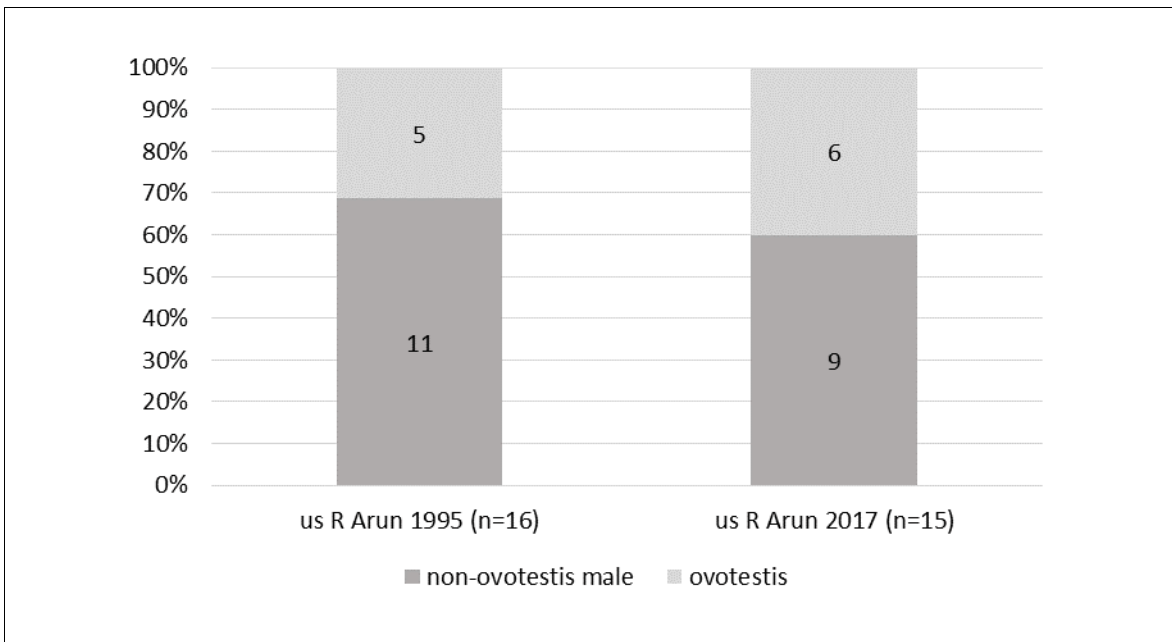


Figure B.4 Percentage of phenotypic males sampled from the upstream Arun site with normal testes (non-ovotestis, dark grey bar) or intersex (ovotestis, light grey). The total number of phenotypic males is indicated by n=x in parentheses for each sampling date, and number of individuals for each type of male is indicated by the number in each column

At the downstream River Arun site, males with ovotestis accounted for 33% (7/21) of all the males in 1995, 61% (20/33) of males in 2000, 20% (4/20) in 2006, 41% (31/76) in 2008 and 17% (3/18) in 2017 (Figure B.5). Due to multiple historical sampling at the sample site downstream of Horsham WwTW, a test for statistical difference was first conducted for all the dates collectively. Using this method there was a significant difference in the frequency of males with ovotestis at the different time points ($P = 0.0083$). To determine which years significantly differed from each other, further pairwise comparisons were made. From these analyses it was found that in 2000 the frequency of males with oocytes in their testis were significantly higher than in 2006 ($P = 0.005$) and 2017 ($P = 0.033$). However, none of the other pairwise comparisons were significant, and there was no apparent general trend in frequency of ovotestis over time.

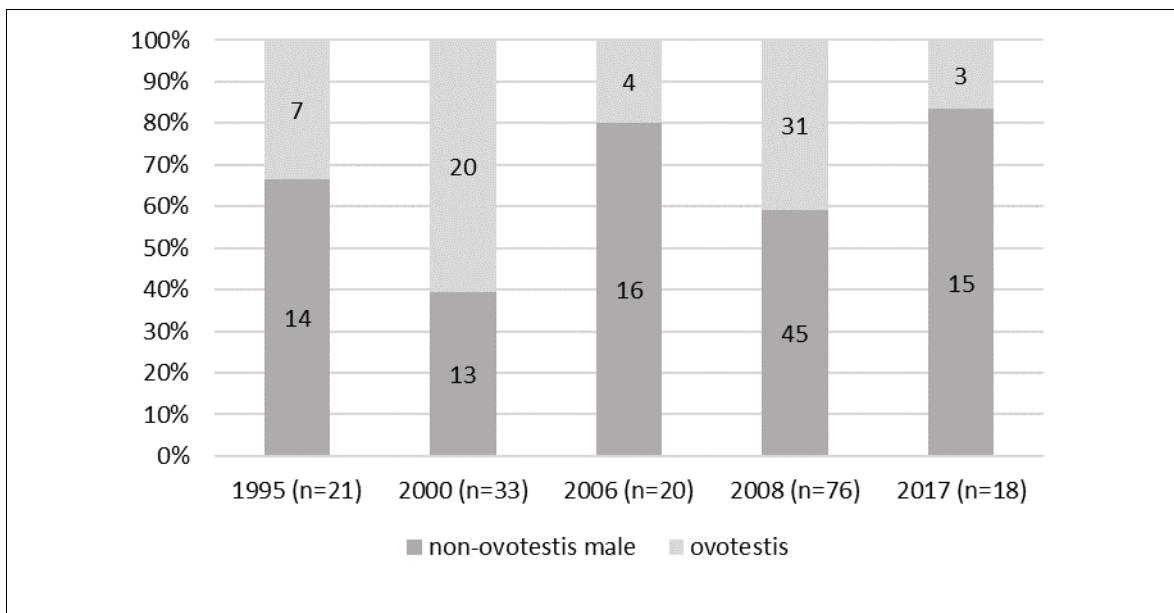


Figure B.5 Percentage of phenotypic males from the downstream River Arun site with, normal testes (non-ovotestis, dark grey bar) or intersex (ovotestis, light grey). The total number of phenotypic males is indicated by n=x in parentheses for each sampling date, and the number of individuals for each type of male is indicated by the number in each column.

For the River Arun site upstream of Horsham WwTW, the mean intersex index score in 1995 was 0.27 ± 0.09 , and 0.89 ± 0.71 in 2017, with no significant differences between the intersex scores for the two sampling dates (Figure B.6).

For the River Arun site downstream of Horsham WwTW, the mean intersex index score was 1.54 ± 2.26 in 1995, 2.47 ± 1.50 in 2000, 2.42 ± 2.02 in 2006 and 1.82 ± 1.69 in 2008. In 2017 the mean intersex index score was 0.44 ± 0.35 . No significant difference was found between the intersex index scores over the different sampling periods ($P < 0.05$) (Figure B.6).

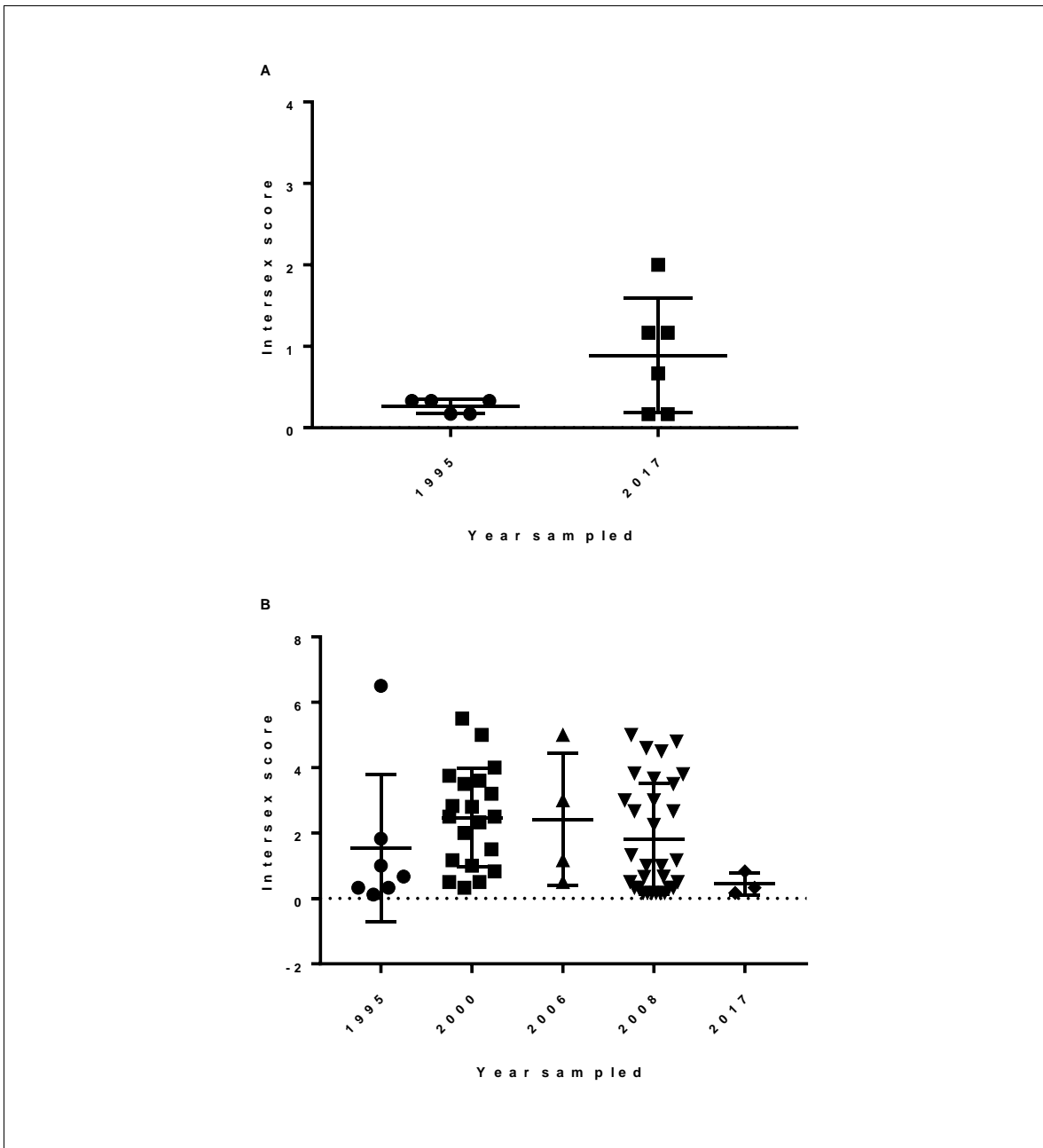


Figure B.6 Intersex index scores for roach sampled from the River Arun (A) upstream and (B) downstream of Horsham WwTW. Points (dots, triangles, etc.) represent individual intersex scores. Horizontal bars indicate the mean and standard deviation in each case.

Table B.1 Summary data for roach from the River Arun upstream of Horsham WwTW sampled in 1995 and 2017

Year sampled		1995	2017
Month sampled		Oct	Oct
Total roach n		63	53
Phenotypic Male no.		16	15
Phenotypic Female no.		47	38
Age (years)	Minimum	2	3
	Mode	6	6
	Maximum	6	9
Length (cm)	Minimum	13.5	11.6
	Mean \pm SD	22.1 \pm 4.3	15.21 \pm 2.13
	Maximum	29.0	21.9
Weight (g)	Minimum	26.1	21.8
	Mean \pm SD	145.9 \pm 83.3	53.89 \pm 27.97
	Maximum	349.8	177.5
GSI – ♂ mean \pm SD (n)		3.60 \pm 0.51 (11)	3.47 \pm 1.32 (15)
GSI - ♀ mean \pm SD (n)		9.71 \pm 1.38 (32)	6.1 \pm 2.42 (37)

Table B.2 Summary data for roach from the River Arun downstream of Horsham WwTW sampled in 1995, 2000, 2006, 2008 and 2017

Year sampled		1995	2000	2006	2008	2017
Month sampled		Oct	Apr	Apr	Apr	Oct
Total roach n		54	34	37	117	56
Phenotypic Male no.		21	33	20	76	18
Phenotypic Female no.		33	1	17	41	38
Age (years)	Minimum	2	2	3	3	3
	Mode	3	4	5	5	5
	Maximum	7	7	7	7	9
Length (cm)	Minimum	13.5	11.7	13.2	11.8	8.0
	Mean \pm SD	17.29 \pm 2.14	15.44 \pm 2.76	15.45 \pm 1.26	16.75 \pm 2.67	13.79 \pm 3.22
	Maximum	23.5	22.9	17.7	26.0	23.0
Weight (g)	Minimum	27.9	23	35.2	23.4	6.9
	Mean \pm SD	65.5 \pm 31.77	63.94 \pm 41.74	64.45 \pm 20.25	78.65 \pm 31.94	42.35 \pm 34.79
	Maximum	177	204	118.6	170.3	137
GSI – ♂ mean \pm SD (n)		2.98 \pm 0.84 (21)	3.51 \pm 1.93 (32) (nc)	8.11 \pm 8.01 (19) (nc)	4.29 \pm 2.12 (76) (nc)	2.87 \pm 0.48 (18)
GSI - ♀ mean \pm SD (n)		10.17 \pm 1.70 (33)	35.94 (1) (nc)	22.29 \pm 10.6 (17) (nc)	12.52 \pm 10.95 (41) (nc)	7.52 \pm 2.04 (38)

nc indicates data non-comparable as sampled during different season/month to 2017 survey.

River Nene upstream and downstream of Great Billing WwTW

In 1995, roach sampled from the River Nene site upstream of Great Billing WwTW ranged in age between 3 and 7 years (median 4 years). In 2017 they ranged between 4 and 8 years old (median 5 years) (Figure B.7). The roach sampled in 2017 were significantly older than those sampled in 1995 ($P = 0.0001$). Roach from the 1995 sample were significantly greater in length (19.33 ± 2.13 cm, $P < 0.0001$) and heavier (89.3 ± 33.24 g, $P < 0.0001$) than those sampled in 2017 (15.39 ± 2.22 cm, 54.76 ± 30.72 g) (Table B.3).

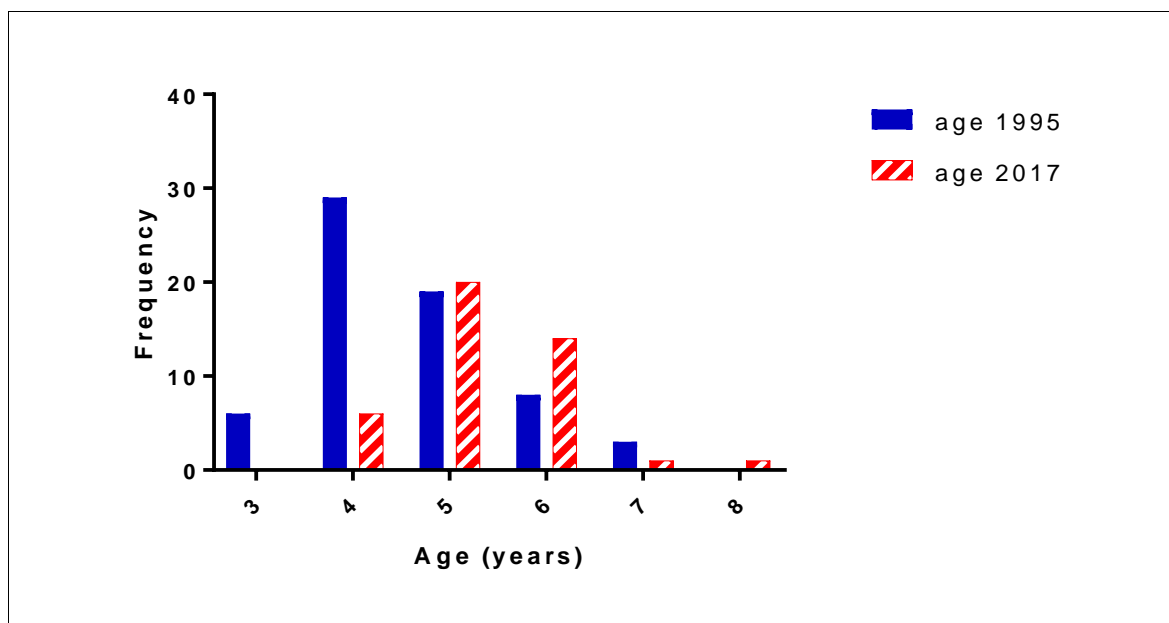


Figure B.7 Age distribution frequency of roach sampled from the River Nene site upstream of Great Billing WwTW in 1995 and 2017

For the River Nene site downstream of Great Billing WwTW, roach sampled in 1995 ranged in age between 2 and 6 years (median 4 years), in 1997 between 3 to 8 years (median 4 years), in April 1998 between 3 to 7 years (median 4 years), in September 1998 between 1 to 6 years (median 3 yrs), in 1999 between 2 to 7 years (median 4 years) and in the 2017 sample they ranged between 5 and 8 years (median 6 years) (Figure B.8). The roach in the 2017 cohort were significantly older than those sampled in other years ($P < 0.0001 - 0.0002$). Roach from the September 1998 sample had the youngest cohort ($P < 0.0001 - 0.0001$). On average, roach sampled in 2017 were also greater in length (17.05 ± 1.63 cm) and heavier (79.44 ± 29.66 g) than those sampled in other years. The roach sampled in 2017 were significantly longer ($P < 0.0001$) than those sampled in 1997 (14.47 ± 2.26 cm), April 1998 (14.37 ± 2.33 cm), September 1998 (13.39 ± 2.65 cm) and 1999 (13.87 ± 2.44 cm) but not than those sampled in 1995 (16.5 ± 1.21 cm). The roach sampled in 1995 were also significantly longer than those sampled in 1997 ($P = 0.0016$), 1998 (April and Sept.) and 1999 ($P < 0.0001$, for all three). The roach sampled in 2017 were also significantly ($P < 0.0001$) heavier than those sampled in 1995 (52.68 ± 13.71 g). As weight changes seasonally in roach (especially in females), comparisons have been limited to fish collected in 1995 and 2017 as they were sampled in the same season. Weight data had also not been collected for the September 1998 sampling (Table B.4).

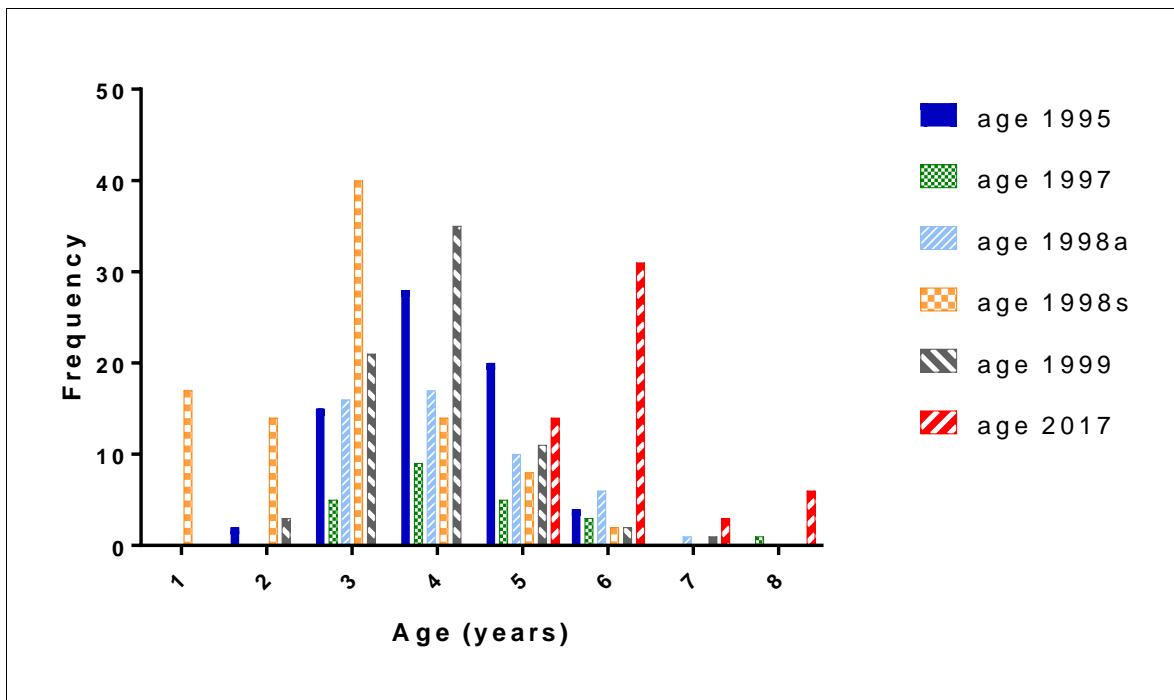


Figure B.8 Age distribution frequency of roach sampled from the site on the River Nene downstream of Billing WwTW in 1995, 1997, April 1998 (1998a), September 1998 (1998s), 1999 and 2017

There were higher numbers of female roach from the River Nene site upstream of Great Billing WwTW from both the 1995 (58%) and the 2017 (63%) surveys, though there was no significant difference in sex ratio between these two time points. In the 1995 sample taken from the River Nene site downstream of Great Billing WwTW the sex ratio was only slightly skewed towards females (54%), whereas in the 2017 sample phenotypic females dominated (80%). There was a significant difference in the sex ratios between these two time points ($P = 0.0038$).

For the River Nene site upstream of Great Billing WwTW, no significant difference was found in the male GSI between 1995 and 2017 (1995: 3.59 ± 1.09 , 2017: 3.91 ± 0.82) or females (1995: 8.28 ± 2.88 , 2017: 7.67 ± 3.33). In comparison, at the site downstream of Great Billing WwTW there was a significant difference in male GSI between the two sampling dates; males sampled in 2017 had significantly larger GSIs (3.20 ± 0.33) than those sampled in 1995 (2.23 ± 0.92 , $P = 0.005$). At the same site, GSI in female roach from the 2017 sample was also significantly higher (9.00 ± 1.78 , $P < 0.0001$) than in females from the 1995 survey (6.38 ± 1.34).

For males, from both 1995 and 2017 sampling occasions, average plasma VTG concentrations were higher from roach sampled at the downstream site (1995: $21,373 \pm 92,214$ ng/ml, 2017: 298 ± 663 ng/ml) than from those at the upstream site (1995: 211.5 ± 197.3 ng/ml, 2017: 27.86 ± 39.28 ng/ml) in the same year. However, in 2017 both upstream and downstream VTG concentrations in males were significantly lower than those in the 1995 survey ($P < 0.0001$, $P < 0.0001$, Figure B.9). Whereas plasma VTG in females was higher in the 2017 sampling at both upstream and downstream sites (Table B.12).

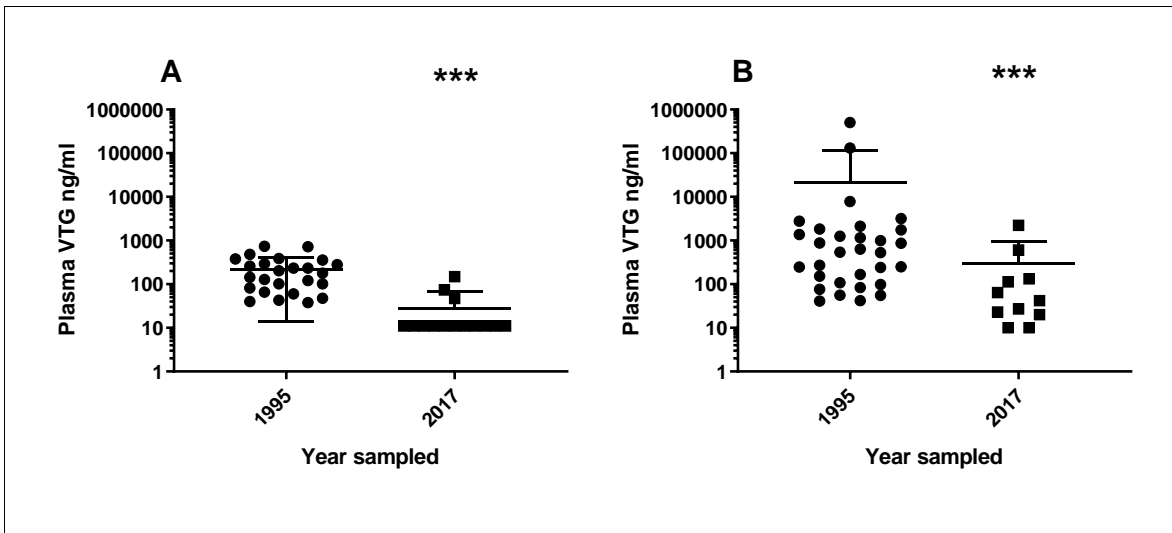


Figure B.9 Plasma vitellogenin (VTG) concentrations from male roach sampled from the River Nene (A) upstream and (B) downstream of Great Billing WwTW in 1995 and 2017. Points (dots, squares) represent individuals. Horizontal lines describe mean and standard deviation. Stars* indicates statistical decrease in VTG between sampling dates (*) P<0.0001)**

From the River Nene site upstream of Great Billing WwTW, 33% (9/27) of phenotypic males were found to have ovotestis in the 1995 sample, compared with 27% (4/15) in 2017. This difference was not statistically significant (Figure B.10).

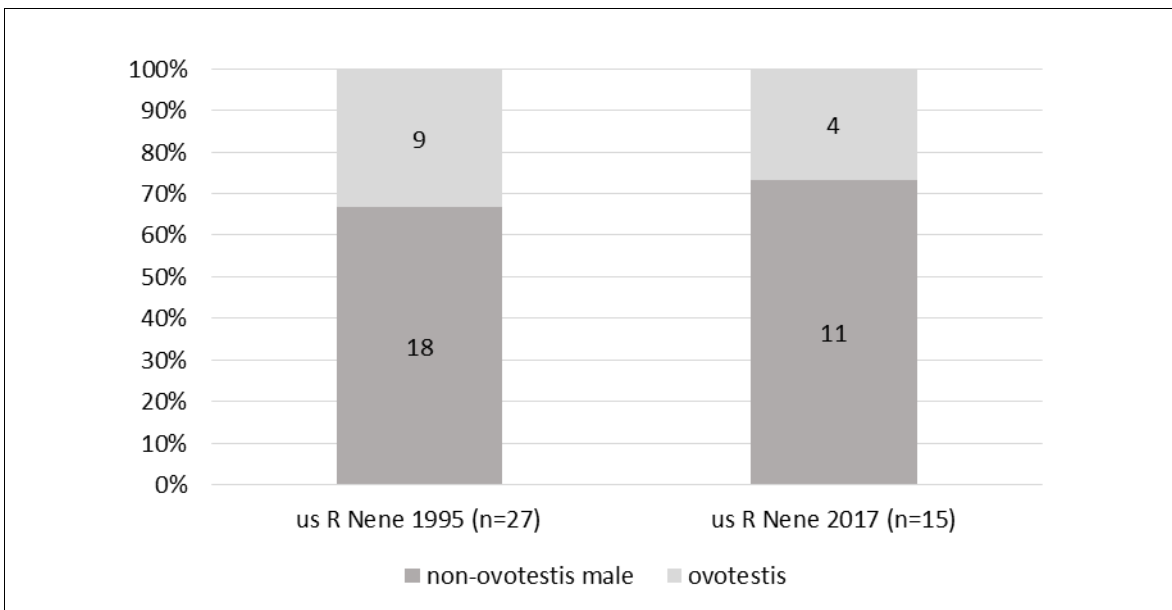


Figure B.10 Percentage of phenotypic males from the upstream River Nene site with normal testes (non-ovotestis, dark grey bar) or intersex (ovotestis, light grey). The total number of phenotypic males is indicated by n=x in parentheses for each sampling date, and the number of individuals for each type of male is indicated by the number in each column

From the River Nene site downstream of Great Billing WwTW, males with ovotestis accounted for 65% of phenotypic males (20/31) in 1995, 43% (6/14) in 1997, 45% (10/22) in April 1998, 28% (17/61) in September 1998, 29% (6/21) in 1999 and 0% (0/11) in 2017 (Figure B.11). Overall there was a significant difference in the frequency of males with ovotestis sampled at the different time points (P = 0.0011) when all sampling data were considered collectively. To determine which years significantly

differed from each other, further pairwise comparisons were made. These analyses found that the frequency of males with ovotestis were significantly higher in the 1995 ($P = 0.002$), 1997 ($P = 0.0196$) and April 1998 ($P = 0.0129$) samples than in the 2017 sample. The roach sampled in 1995 were also found to have significantly higher ovotestis frequency than those sampled in September 1998 and in the 1999 survey ($P = 0.0014$ and 0.0277 , respectively) (Figure B.11).

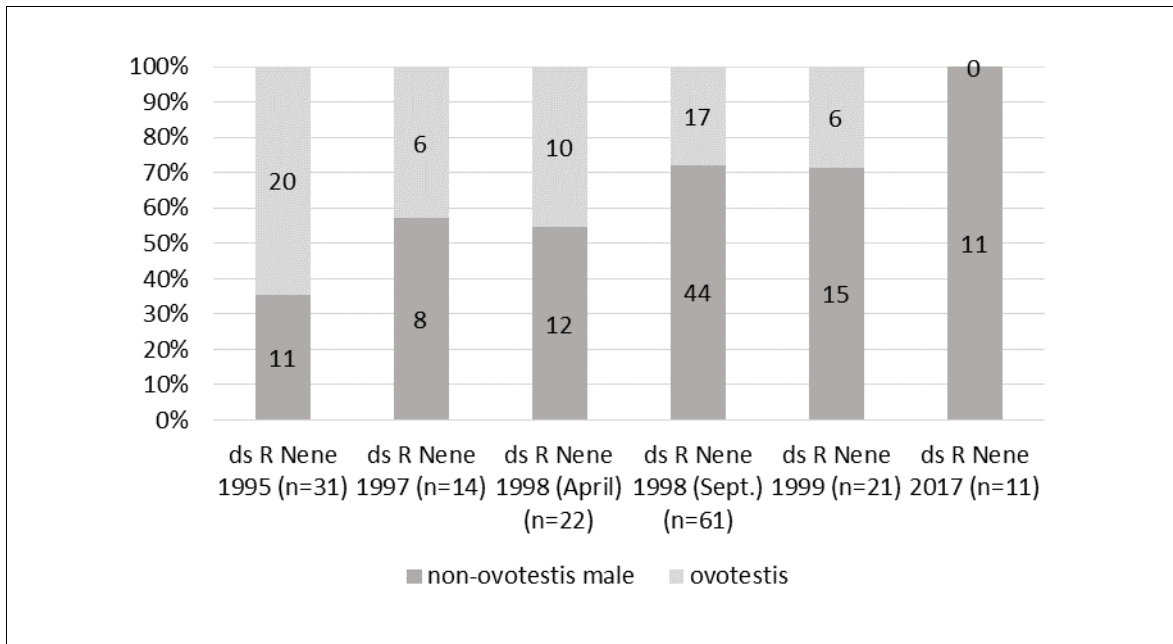


Figure B.11 Percentage of phenotypic males sampled from the River Nene site downstream of Great Billing WwTW with normal testes (non-ovotestis, dark grey bar) or intersex (ovotestis, light grey). The total number of phenotypic males is indicated by n=x in parentheses for each sampling date, and the number of individuals for each type of male is indicated by the number in each column.

From the River Nene site upstream of Great Billing, the mean intersex index score in 1995 was 0.70 ± 0.52 compared with 0.67 ± 0.49 in 2017. There was no statistically significant difference in intersex index scores between roach sampled on these two dates (Figure B.12).

From the River Nene site downstream of Great Billing WwTW the mean intersex index score was 2.42 ± 1.94 in 1995, 1.84 ± 1.49 in 1997, 2.68 ± 2.06 in the April 1998 group and 1.89 ± 1.90 in the September 1998 group. In the 1999 sample the mean score was 0.26 ± 0.23 and in the 2017 survey no intersex individuals were found. Therefore, 2017 could not be included in the statistical analysis (Figure B.12). However, a significant difference in the intersex index score was found between the other survey dates (1995-1999) overall ($P = 0.0115$), with the intersex scores in 1999 being significantly lower than those recorded in the 1995 survey and the April 1998 survey ($P = 0.0077$ and 0.0169 , respectively).

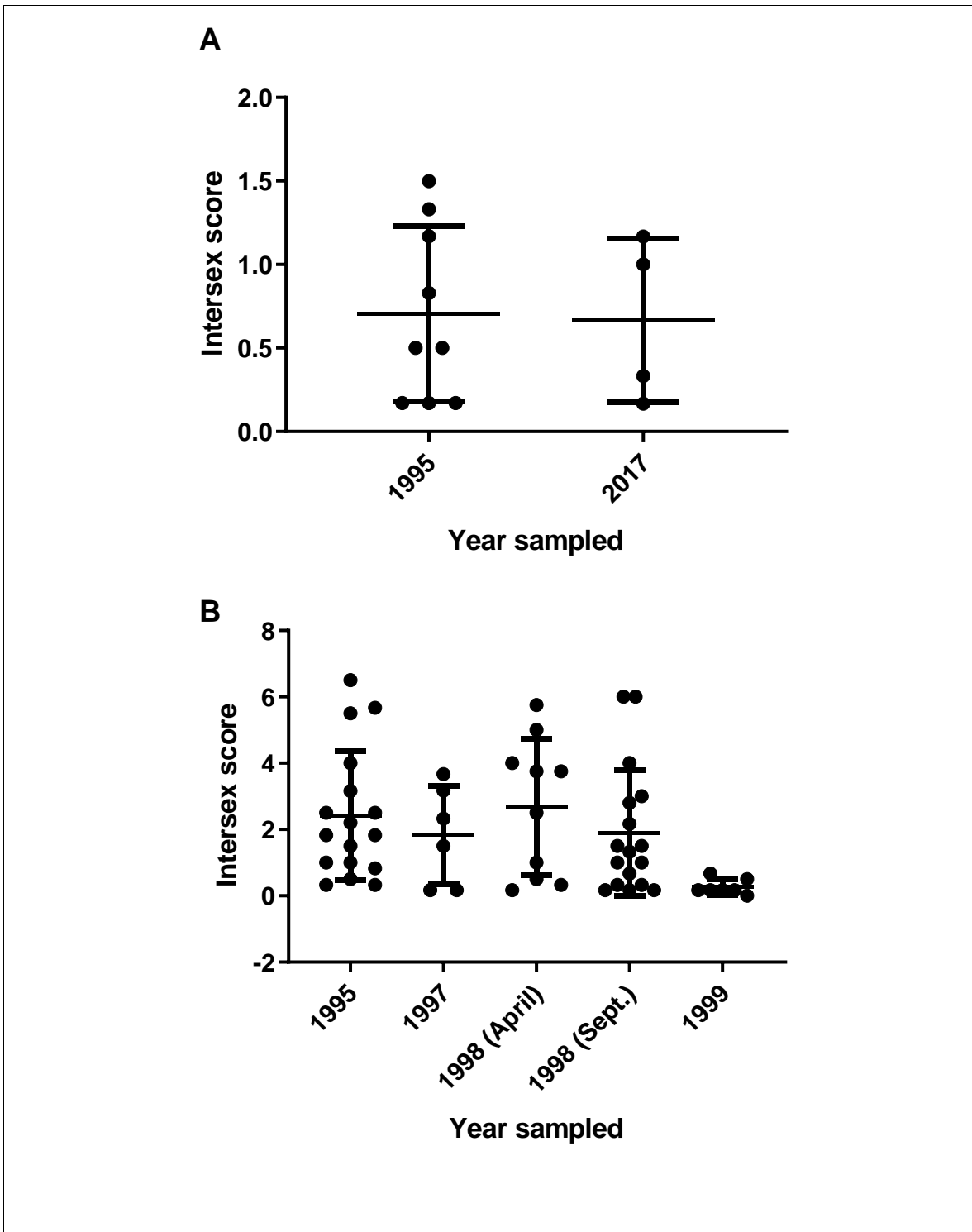


Figure B.12 Intersex index scores for roach from the River Nene (A) upstream and (B) downstream of Great Billing WwTW. Points (dots) represent individual intersex index scores. Horizontal bars indicate the mean and standard deviation

Table B.3 Summary data for roach from the River Nene upstream of Billing WwTW

Year sampled		1995	2017
Month sampled		Oct	Oct
Total roach n		64	42
Phenotypic Male no.		27	15
Phenotypic Female no.		37	27
Age (years)	Minimum	3	4
	Mode	4	5
	Maximum	7	8
Length (cm)	Minimum	12.5	12.6
	Mean \pm SD	19.33 \pm 2.13	15.39 \pm 2.22
	Maximum	26.3	22.5
Weight (g)	Minimum	18.0	23.3
	Mean \pm SD	89.3 \pm 33.24	54.76 \pm 30.72
	Maximum	219.2	164.4
GSI - ♂ mean \pm SD (n)		3.59 \pm 1.09 (26)	3.91 \pm 0.82 (15)
GSI - ♀ mean \pm SD (n)		8.28 \pm 2.88 (38)	7.67 \pm 3.33 (27)

Table B.4 Summary data for roach from the River Nene downstream of Billing WwTW

Year sampled		1995	1997	1998	1998	1999	2017
Month sampled		Oct	Mar	Apr	Sept	Apr	Oct
Total roach n		70	25	69	97	74	55
Phenotypic Male no.		31	14	22	61	21	11
Phenotypic Female no.		37	11	46	33	53	44
Age (years)	Minimum	2	3	3	1	2	5
	Mode	4	4	4	3	4	6
	Maximum	6	8	7	6	7	8
Length (cm)	Minimum	13.5	10.0	10.5	8.5	9.5	14.3
	Mean \pm SD	16.5 \pm 1.21	14.47 \pm 2.26	14.37 \pm 2.33	13.39 \pm 2.65	13.87 \pm 2.44	17.05 \pm 1.63
	Maximum	19.5	18.5	20.0	21.5	21.0	22.3
Weight (g)	Minimum	25.3	16.0	16.5	No record	14.2	43.4
	Mean \pm SD	52.68 \pm 13.71	51.53 \pm 24.35	47.29 \pm 24.34	No record	47.9 \pm 32.74	79.44 \pm 29.66
	Maximum	90.4	103.4	122.2	No record	190.9	197.5
GSI - ♂ mean \pm SD (n)		2.23 \pm 0.92 (31)	8.63 \pm 3.79 (14) (nc)	1.31 \pm 0.86 (22) (nc)	No record	4.36 \pm 1.37 (21) (nc)	3.20 \pm 0.33 (11)
GSI - ♀ mean \pm SD (n)		6.38 \pm 1.34 (37)	11.53 \pm 3.62 (11) (nc)	2.64 \pm 1.51 (27) (nc)	No record	14.31 \pm 9.52 (46) (nc)	9.00 \pm 1.78 (44)

No indicated data not comparable as sampled during different season/month. Weight not recorded in September 1998.

The Bourne downstream of Chertsey

The ages of the roach sampled from the Bourne site downstream of Chertsey WwTW ranged between 3 to 7 years (median 6 years) in 2002, 2 to 6 years (median 3 years) in the 2006 survey, and between 2 to 6 years (median 4 years) in 2017 (Figure B.13). The roach sampled in 2002 were significantly older than those sampled in 2006 and 2017 ($P < 0.0001$). There was a significant difference between the samples taken at those three times in terms of fork-length of the roach sampled ($P < 0.0001$) with those sampled in 2002 having significantly greater length (18.75 ± 3.09 cm, $P < 0.0001$) than those sampled in the 2006 (14.27 ± 1.57 cm) or 2017 (14.5 ± 1.33 cm). The roach sampled in 2002 were also much heavier (121.6 ± 64.85 g) than those sampled in 2006 (42.01 ± 16.49 g) and 2017 (47.11 ± 14.46 g) (Table B.5). However, direct statistical comparisons have not been made due to the impact of seasonality on weight and the different months in which the roach were sampled.

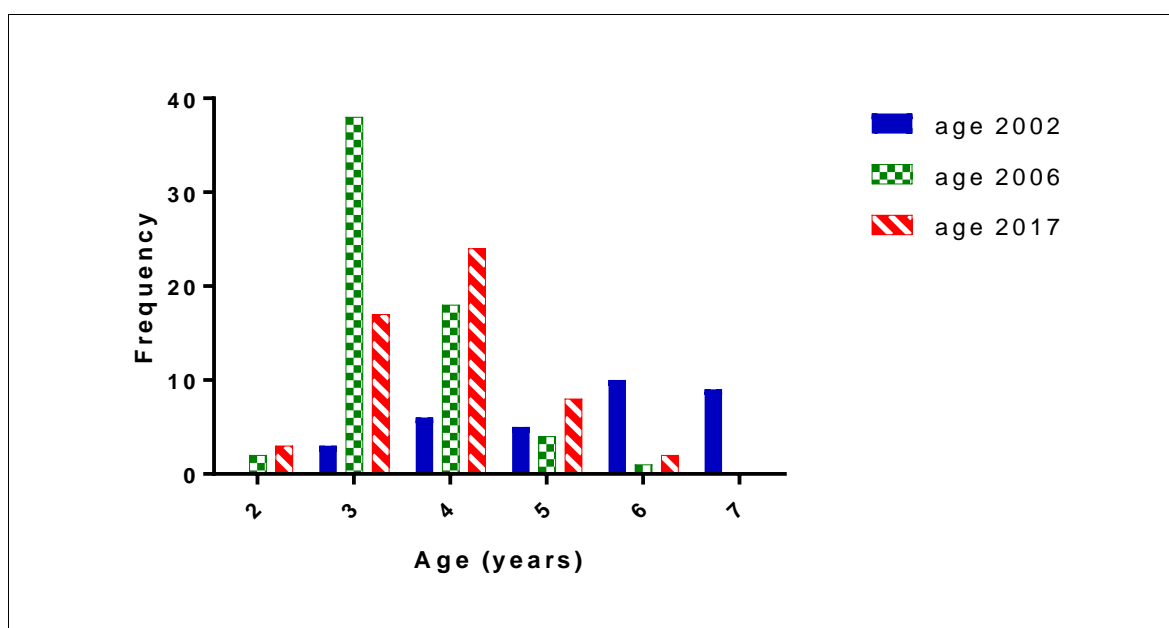


Figure B.13 Age distribution frequency of roach sampled from the site on the Bourne downstream of Chertsey WwTW, sampled in 2002, 2006 and 2017

From the Bourne site, phenotypic females were less prevalent on all three sampling occasions (2002: 47%, 2006: 40% and 2017: 43% female). There were no significant differences in sex ratio between these sampling periods from the Bourne site.

GSI was not compared between the 2002 (May), 2006 (April) and 2017 (November) surveys due to the different seasons/months the samples were taken and the strong influence of seasonality for this index.

The first sampling occasion for the Bourne site was in May 2002 and the average plasma VTG concentration for males then was 109.1 ± 226.4 ng/ml, compared with 2000 ± 3522 ng/ml from the November 2017 survey. Male plasma VTG was significantly higher in 2017 than in 2002 ($P < 0.0001$) (Figure B.14 and Table B.11).

For females sampled in the May 2002 survey, the average plasma VTG concentration was $29,548 \pm 88,347$ ng/ml, compared with $5,001,361 \pm 3,038,389$ ng/ml from the November 2017 sample (Table B.12). However, statistical comparison was not made for the females due to the different sampling periods (time of year/reproductive stage).

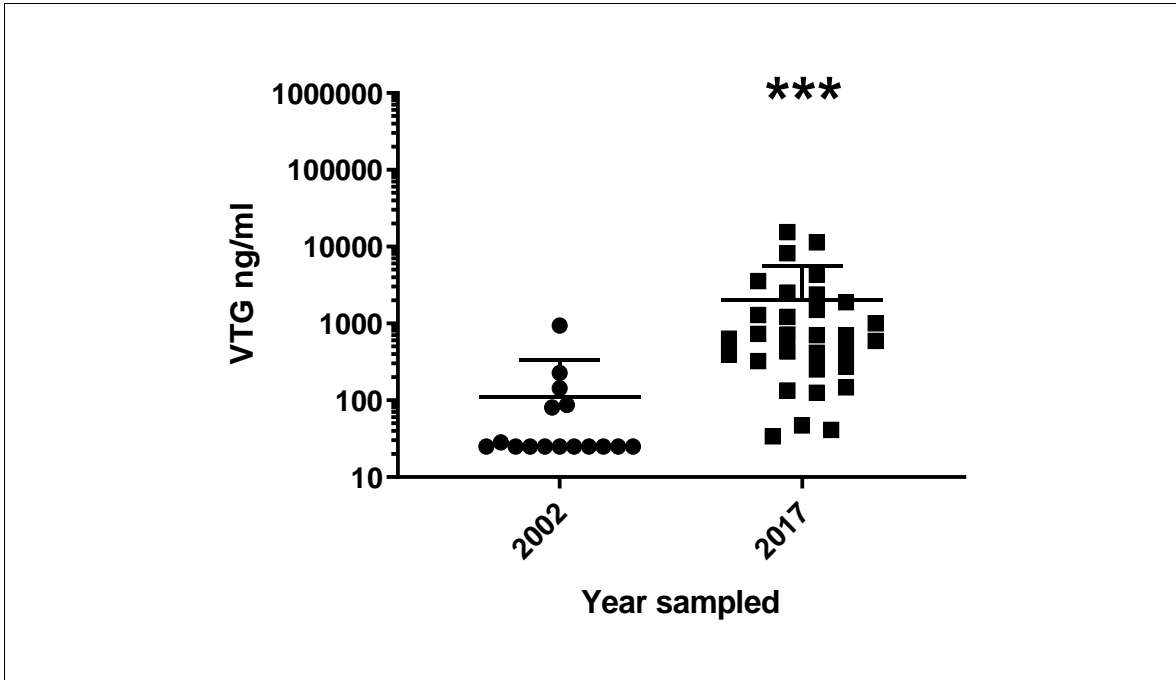


Figure B.14 Plasma vitellogenin (VTG) concentrations for male roach sampled from the Bourne site downstream of Chertsey WwTW in 2002 and 2017. Points (dots, squares) represent individuals. Horizontal lines describe mean and standard deviation. Star * indicates statistical increase in VTG between sampling dates (*) $P < 0.0001$**

From the Bourne site downstream of Chertsey WwTW, 41% (7/17) of the phenotypic males had ovotestis in the 2002 survey, 39% (15/38) in the 2006 survey and 35% (11/31) in the 2017 survey. No statistical difference was found in the frequency of ovotestis between these sample dates (Figure B.15).

The mean intersex index score for the Bourne site was 3.43 ± 1.77 in 2002, 1.02 ± 0.95 in the 2006 survey and 0.56 ± 0.44 in the 2017 survey (Figure B.16). A significant difference was found in intersex severity between these sampling occasions ($P = 0.0013$), with the roach sampled in 2002 having significantly higher intersex index scores than those sampled in 2006 and 2017 ($P = 0.0095$ and 0.0012 , respectively). No significant difference was found between the intersex index scores from the 2006 and 2017 surveys.

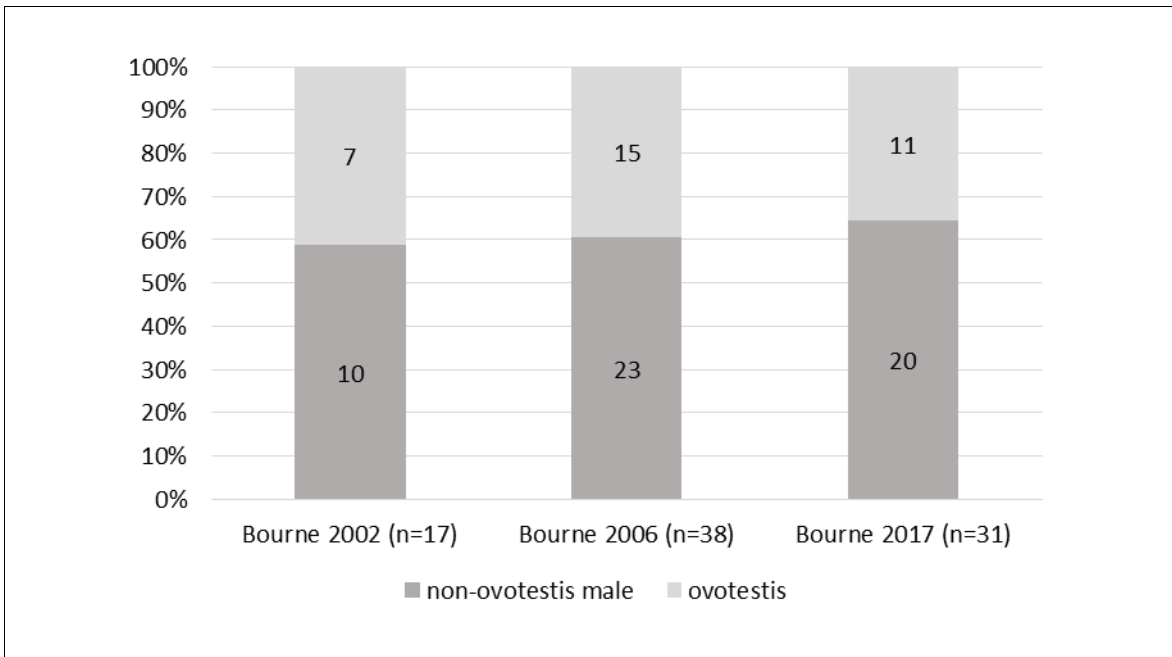


Figure B.15 Percentage of phenotypic males from the Bourne site downstream from Chertsey WwTW, with normal testes (non-ovotestis, dark grey bar) or intersex (ovotestis, light grey). The total number of phenotypic males is indicated by n=x in parentheses for each sampling date, and number of individuals for each type of male is indicated by the number in each column

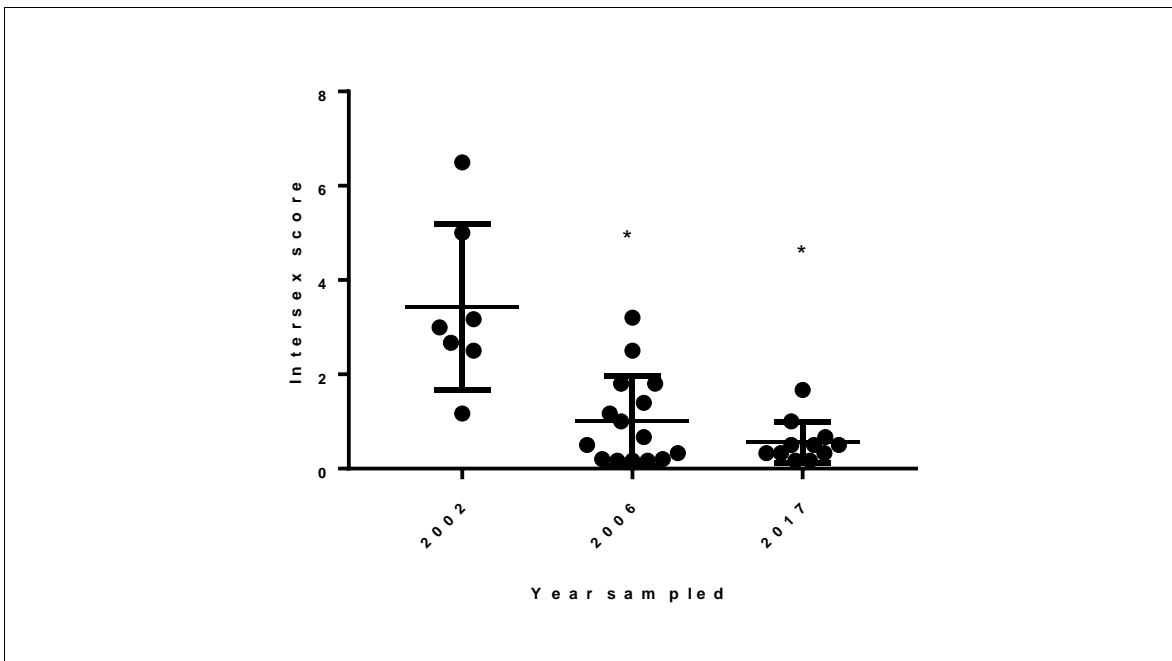


Figure B.16 Intersex index scores for roach from the Bourne site downstream of Chertsey WwTW sampled in 2002, 2006 and 2017. Points (dots) represent individual intersex scores. Horizontal bars indicate the mean and standard deviation. Stars indicate statistically significant difference from the 2002 sample

Table B.5 Summary data for roach from the Bourne downstream of Chertsey WwTW

Year sampled		2002	2006	2017
Month sampled		May	Apr	Nov
Total n		33	63	54
Phenotypic Male no.		17	38	31
Phenotypic Female no.		15	25	23
Age (years)	Minimum	3	2	2
	Mode	6	3	4
	Maximum	7	6	6
Length (cm)	Minimum	14	11.2	11.9
	Mean \pm SD	18.75 \pm 3.09	14.27 \pm 1.57	14.5 \pm 1.33
	Maximum	25.5	18.9	18.0
Weight (g)	Minimum	43.6	17.6	24.4
	Mean \pm SD	121.6 \pm 64.85	42.01 \pm 16.49	47.11 \pm 14.46
	Maximum	309.8	94.0	82.0
GSI - ♂ mean \pm SD (n)		1.26 \pm 0.32 (17) (nc)	3.85 \pm 1.15 (38) (nc)	5.6 \pm 3.28 (30)
GSI - ♀ mean \pm SD (n)		2.27 \pm 0.53 (15) (nc)	3.89 \pm 2.69 (24) (nc)	10.19 \pm 4.69 (23)

Data not comparable (nc) as sampled during different season/month.

River Avon at Hampton Ferry

From the River Avon site at Hampton ferry, the roach ranged in age between 2 and 6 years (median 4 years) in the 1995 sample and 3 and 6 years old (median 4 years) in 2017 (Figure B.17). There was no significant difference in age between the 1995 and 2017 samples from the R Avon. Roach from the 1995 survey were significantly greater in length (17.21 ± 2.22 cm, $P < 0.0001$) and heavier (63.69 ± 34.19 g, $P < 0.0001$) than those sampled in 2017 (12.66 ± 2.01 cm and 29.74 ± 15.68 g) (Table B.6).

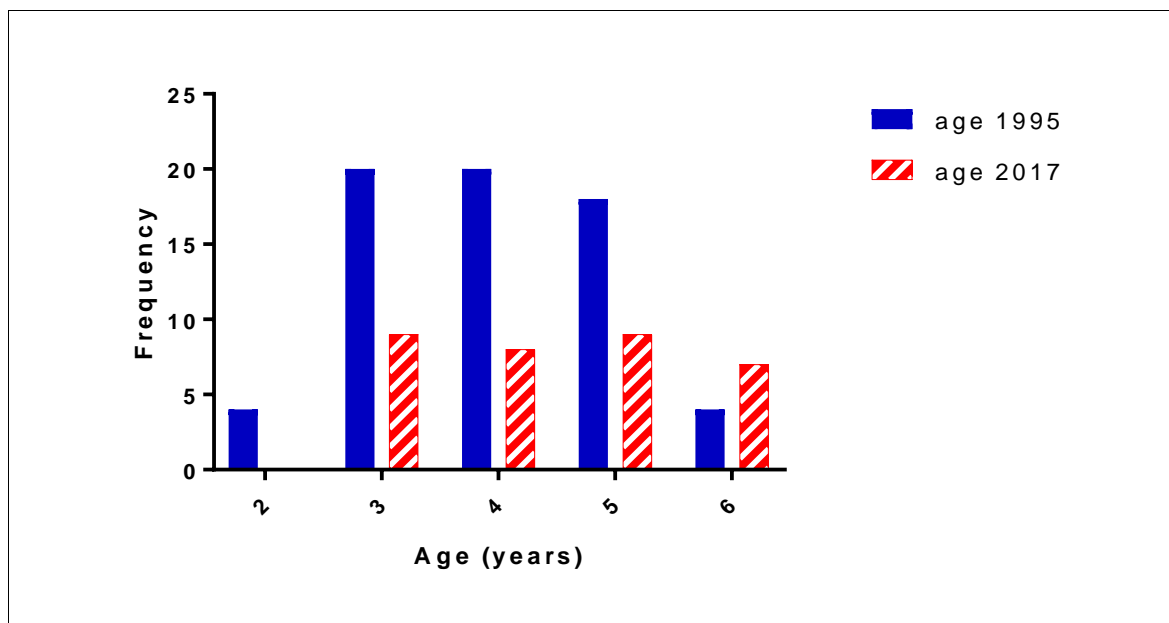


Figure B.17 Age distribution frequency of roach sampled from the River Avon in 1995 and 2017

From the River Avon site at Hampton Ferry, phenotypic females predominated in samples in both 1995 (64%) and 2017 (67%) however no significant differences between sex ratio at these two sampling times were found.

From the River Avon, the GSI was significantly lower in male roach sampled in 1995 compared with males from the 2017 survey (3.22 ± 0.72 and 4.48 ± 0.75 respectively, $P < 0.0001$). In females from the River Avon site, GSI was not significantly different between the two sampling occasions (1995: 5.76 ± 3.10 and 2017: 4.67 ± 3.89).

Male plasma VTG concentrations from the River Avon were on average 1172 ± 2308 ng/ml in 2017 compared with 5961 ± 18925 ng/ml in 1995. Male plasma VTG was not significantly different between the two sampling occasions at the R Avon (Figure B.18 and Table B.11).

The plasma VTG concentrations in females from the R Avon site were on average $3,031,534 \pm 4,158,873$ ng/mL in 2017 compared with $138,603 \pm 224,735$ ng/ml in 1995 (Table B.12).

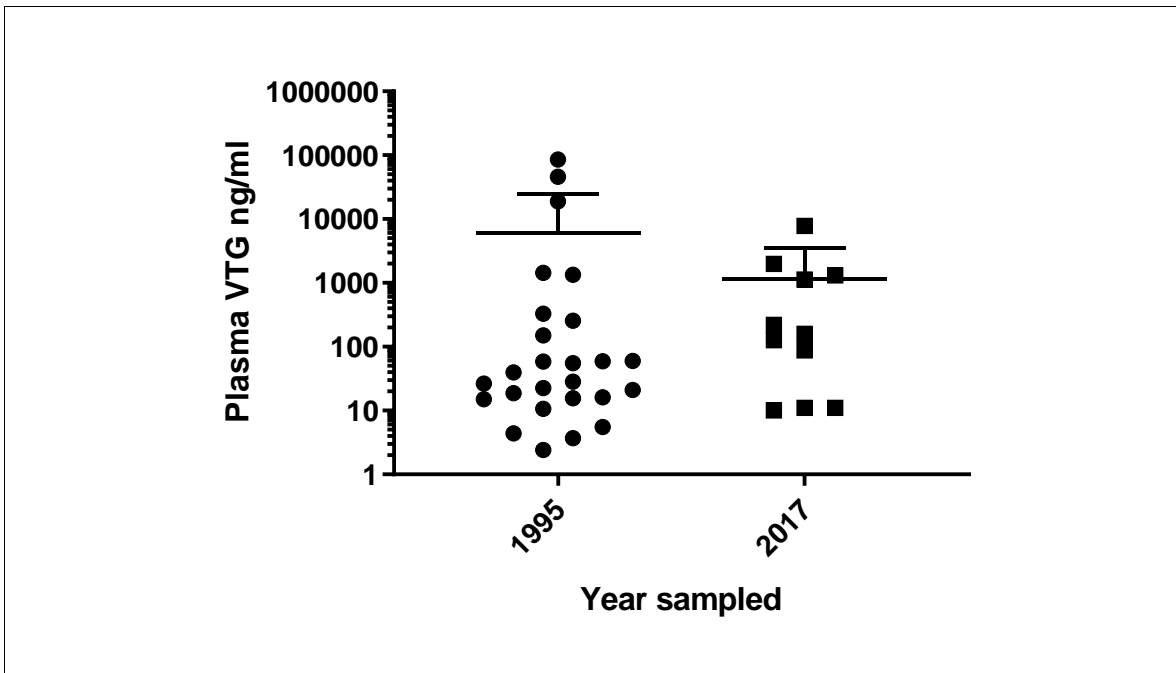


Figure B.18 Plasma vitellogenin (VTG) concentrations from male roach sampled from the River Avon at Hampton Ferry in 1995 and 2017. Points (dots, squares) represent individuals. Horizontal lines indicate the mean and standard deviation

From the River Avon site 12% (3/26) of phenotypic males had ovotestis in the 1995 survey, and none (0/11) had ovotestis in 2017. However, there was no statistically significant difference (according to Fisher's exact test, $P > 0.05$) between the two sampling occasions (due to lower male numbers in the 2017 survey) (Figure B.19).



Figure B.19 Percentage of phenotypic males from the River Avon survey site with normal testes (non-ovotestis, dark grey bar) or intersex (ovotestis, light grey). The total number of phenotypic males is indicated by n=x in parentheses for each sampling date, and the number of individuals for each type of male is indicated by the number in each column

The mean intersex index score of the roach surveyed at the River Avon site in 1995 was 0.56 ± 0.42 (Figure B.20). In the 2017 survey none of the roach from the River Avon site had the intersex condition (Figure B.20), so statistical analysis could not be conducted for this endpoint.

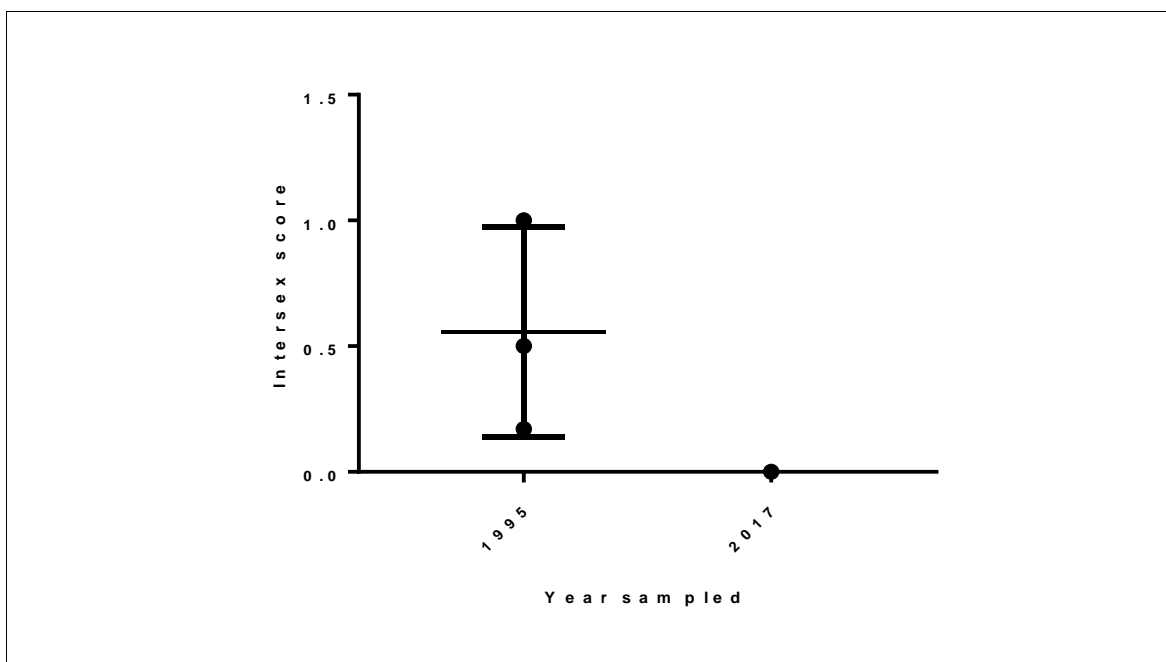


Figure B.20 Intersex index scores for roach sampled from the River Avon in the 1995 and 2017 surveys. Points (dots, triangles, etc.) represent individual intersex scores. Horizontal bars indicate the mean and standard deviation

Table B.6 Summary data for roach from the River Avon at Hampton Ferry

Year sampled		1995	2017
Month sampled		Oct	Nov
Total roach n		72	33
Phenotypic Male no.		26	11
Phenotypic Female no.		46	22
Age (years)	Minimum	2	3
	Mode	3	5
	Maximum	6	6
Length (cm)	Minimum	13.5	10.0
	Mean \pm SD	17.21 ± 2.22	12.66 ± 2.01
	Maximum	26.3	16.3
Weight (g)	Minimum	24.3	11.8
	Mean \pm SD	63.69 ± 34.19	29.74 ± 15.68
	Maximum	255.4	57.9
GSI - ♂ mean \pm SD (n)		3.22 ± 0.72 (26)	4.48 ± 0.75 (11)
GSI - ♀ mean \pm SD (n)		5.76 ± 3.10 (46)	4.67 ± 3.89 (22)

River Eye upstream of Melton Mowbray WwTW

From the site on the River Eye upstream of Melton Mowbray WwTW, the ages of the roach ranged between 2 and 11 years (median 5 years) in the 1995 survey and between 4 and 9 years (median 5 years) in the 2017 survey (Figure B.21). The roach from the 1995 sample were significantly older than those from the 2017 sample ($P=0.0033$). For both fork-length and total weight, the roach sampled in 1995 were also significantly larger (length: 19.05 ± 5.07 cm vs. 12.7 ± 2.66 cm, and weight: 112.6 ± 109.8 g vs. 35.2 ± 32.47 g, $P < 0.0001$) than those from the 2017 sample (Table B.7).

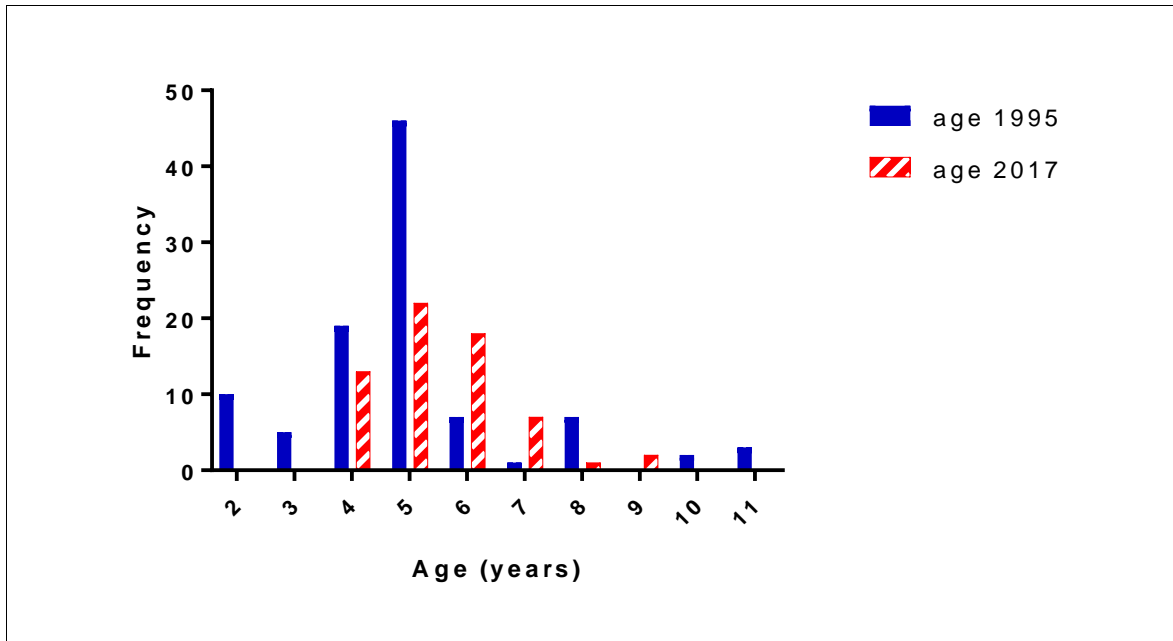


Figure B.21 Age distribution frequency of roach sampled from site on the River Eye in 1995 and 2017

From the River Eye site upstream of Melton Mowbray, the sex ratio was skewed towards phenotypic females in the 1995 (72%) survey, while the opposite was found in the 2017, with only 42% of the 2017 sample being female. There was a significant difference in the sex ratios between these two time points ($P = 0.0002$).

The GSI of male roach sampled from the River Eye site in 1995 was 2.37 ± 0.85 and in 2017 it was 4.53 ± 1.58 . For females the GSIs for this site was 6.02 ± 2.33 in 1995 and 7.02 ± 3.76 in 2017. However, GSI (gonad size) increases seasonally and the 1995 roach were collected in September, as compared to November for the 2017. It is conceivable that the gonads could be growing during this time period, prior to full maturation in the spring, so the data may not be directly comparable.

In the 2017 survey, males from River Eye site had significantly ($P = 0.0214$) lower plasma VTG concentrations (267.2 ± 413.3 ng/ml) than those sampled in the 1995 survey (8934 ± 39874 ng/ml) (Figure B.22 and Table B.11).

The females from the River Eye site had mean VTG concentrations of $5,482,257 \pm 4,780,142$ ng/ml in 2017 compared with $187,005 \pm 123,598$ ng/ml in 1995 (Table B.12). However, as with GSI, female plasma VTG increases with season and therefore blood samples taken in November would be expected to be have higher levels of VTG than those taken in September.

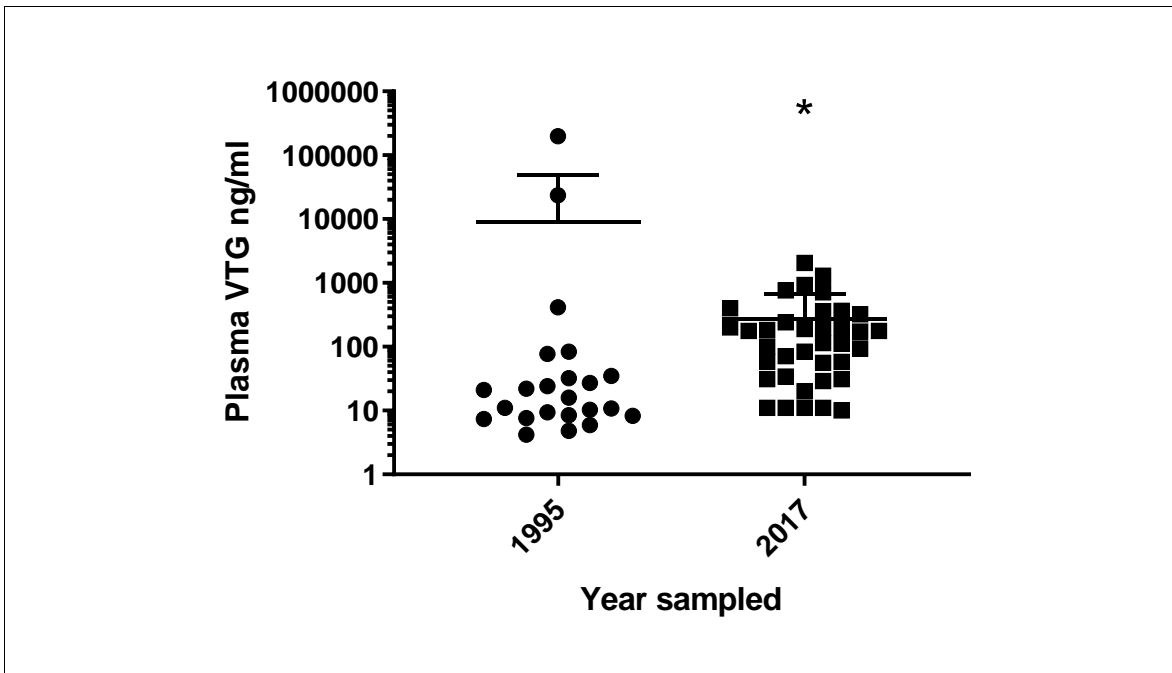


Figure B.22 Plasma vitellogenin (VTG) concentrations from male roach sampled from the River Eye site. Points (dots, squares) represent individuals. Horizontal lines indicate the mean and standard deviation. Star * indicates statistical decrease in VTG between 1995 and 2017 samples

From the River Eye, in the 1995 sample 25% (7/28) of phenotypic males were found to have ovotestis, and in the 2017 survey 22% (8/37) were similarly affected. No statistically significant difference was found in intersex frequency between these two dates (Figure B.23).

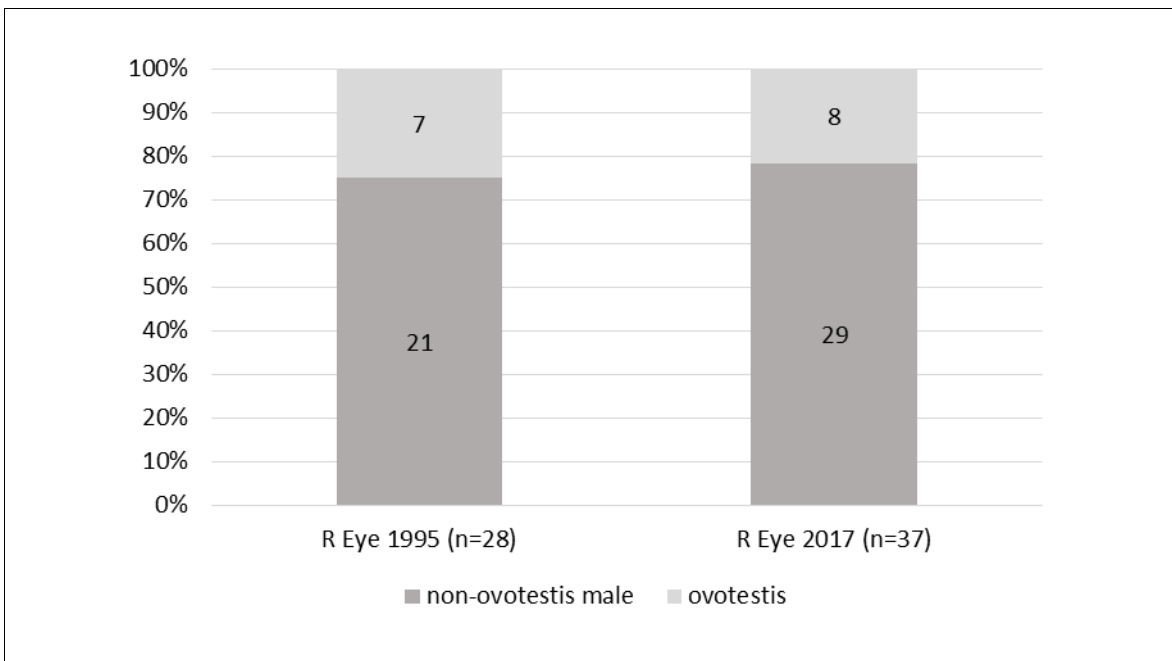


Figure B.23 Percentage of phenotypic males from the River Eye survey site with normal testes (non-ovotestis, dark grey bar) or intersex (ovotestis, light grey). The total number of phenotypic males is indicated by n=x in parentheses for

each sampling date, and the number of individuals for each type of male is indicated by the number in each column

From the River Eye site, the mean intersex index score in the 1995 survey was 0.88 ± 0.74 compared with 0.67 ± 0.73 in 2017. No statistically significant differences in intersex index score were found between the two sampling dates at the River Eye site (Figure B.24).

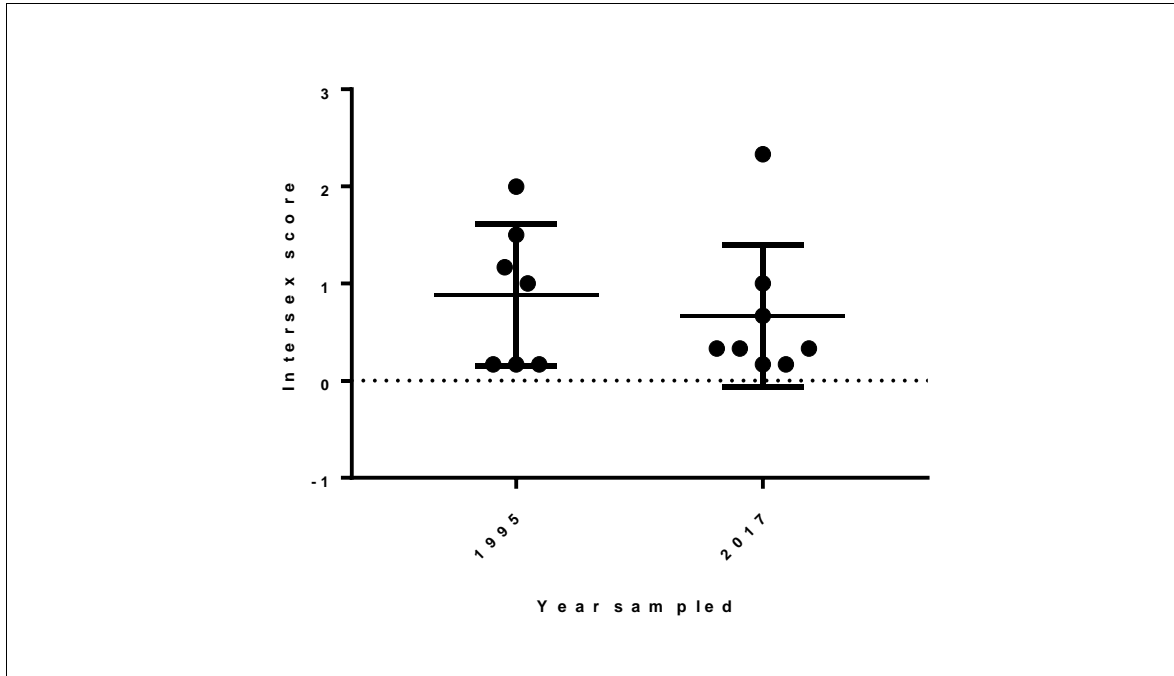


Figure B.24 Intersex index scores for roach from the River Eye in the 1995 and 2017 surveys. Points (dots, triangles, etc.) represent individual intersex scores. Horizontal bars indicate the mean and standard deviation

Table B.7 Summary data for roach from the River Eye upstream of Melton Mowbray

Year sampled		1995	2017
Month sampled		Sept.	Nov.
Total roach n		100	65
Phenotypic Male no.		28	37
Phenotypic Female no.		72	27
Age (years)	Minimum	2	4
	Mode	5	5
	Maximum	11	9
Length (cm)	Minimum	9.5	9.4
	Mean \pm SD	19.05 ± 5.07	12.7 ± 2.66
	Maximum	32	22.6
Weight (g)	Minimum	9.6	10.4
	Mean \pm SD	112.6 ± 109.8	35.2 ± 32.47
	Maximum	461.5	178
GSI - ♂ mean \pm SD (n)		2.37 ± 0.85 (28)	4.53 ± 1.58 (37)

River Witham downstream of Lincoln WwTW

The roach from the River Witham site ranged in age between 3 and 11 years (median 5 years) in the 2002, and between 3 and 6 years (median 5 years) in 2017 (Figure B.25). There were no statistically significant differences in age between the two dates at the River Witham site. There were also no significant differences between the fork-lengths in the 2002 and the 2017 surveys (14.28 ± 3.36 cm compared with 13.48 ± 1.61 cm, respectively). On average the weight of the roach sampled in 2002 (52.22 ± 42.18 g) was higher than of those from the 2017 survey (37.87 ± 16.72 g). However, weight may change seasonally (especially in females) and therefore these were not compared statistically (Table B.8).

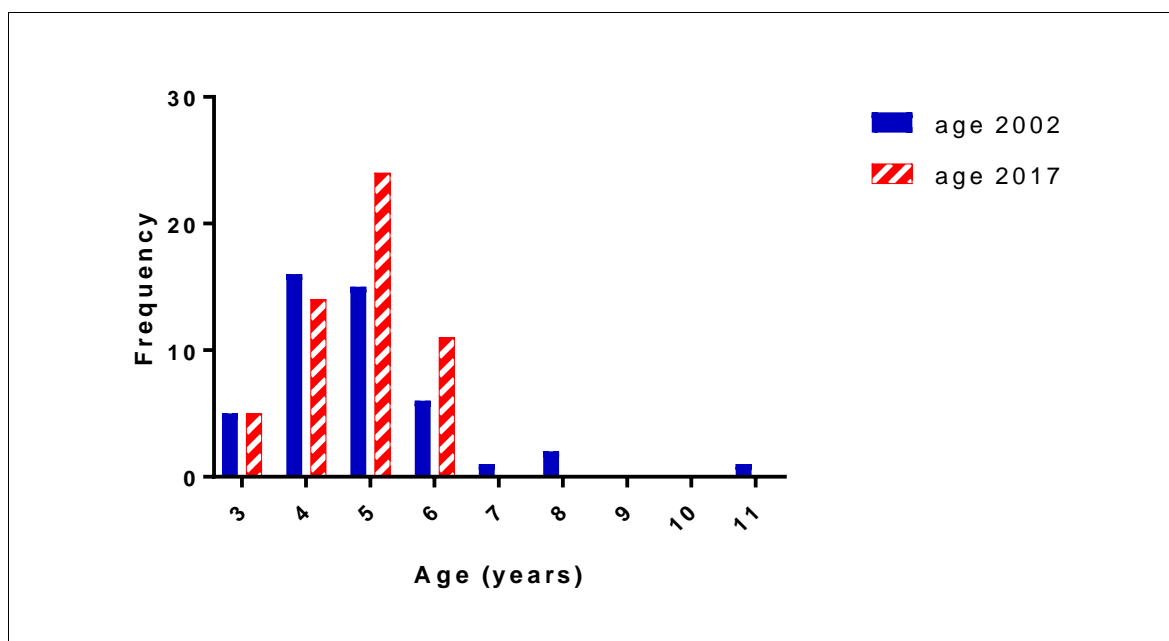


Figure B.25 Age distribution frequency of roach sampled from the River Witham in the 2002 and 2017 surveys

From the samples taken at the River Witham site the sex ratio was skewed towards phenotypic females, although this was more extreme in the 2002 survey (81%) than in the 2017 survey (57%). A significant difference ($P = 0.0110$) was found between these two time points for sex ratio.

Due to the different seasons/months the roach were sampled, GSI was not compared between the 2002 (July) and the 2017 (November) surveys.

For the River Witham 2002 survey, there was only plasma VTG data for two of the male fish (256 and 25 ng/ml VTG respectively). Whereas in the 2017 survey (November), 23 males had plasma VTG measured, with concentrations ranging from 89 ng/ml to 123,270 ng/ml (mean was $32,199 \pm 47,326$ ng/mL). Due to the low sample number ($n=2$) in 2002, no statistical analysis was conducted for changes in VTG concentration (Figure B.26 and Table B.11).

For females from the River Witham site, more comparable numbers were analysed at each time point (28 and 31, respectively). In July 2002, average female plasma VTG was 476 ± 339.8 ng/ml compared with $6,328,311 \pm 4,683,086$ ng/ml from females in the November 2017 survey. (Table B.12). However, direct statistical comparisons were not conducted due to the different sampling periods (time of year/reproductive stage).

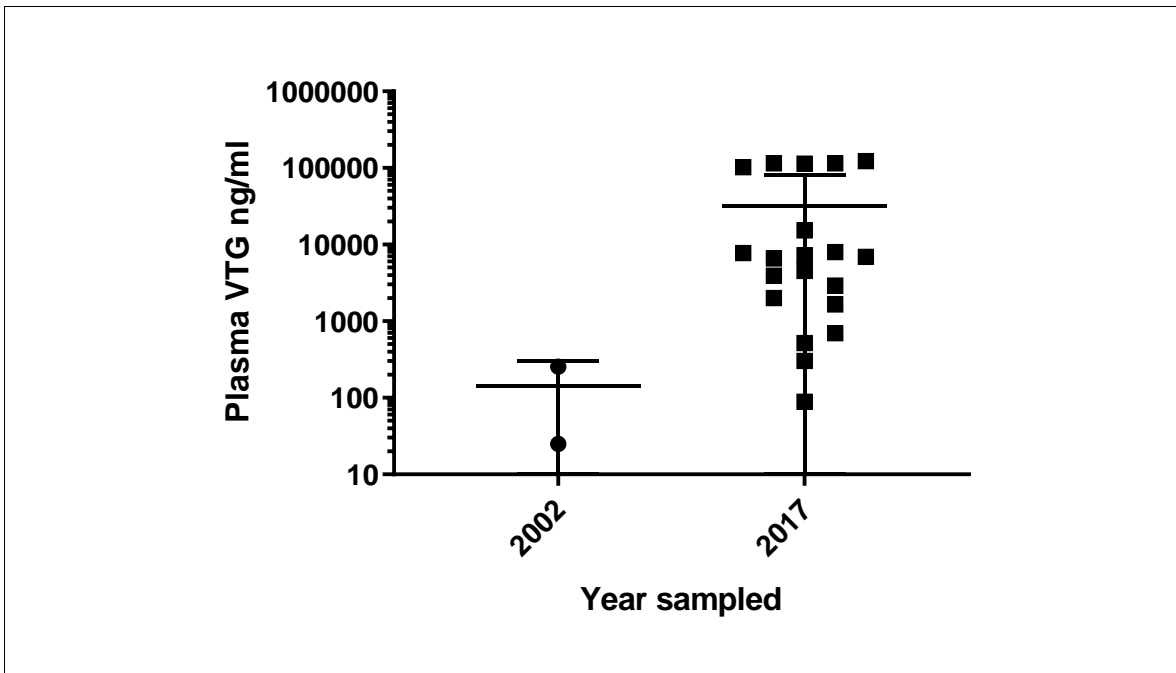


Figure B.26 Plasma vitellogenin (VTG) concentrations from male roach sampled from the River Witham in the 2002 and the 2017 surveys. Points (dots, squares) represent individuals. Horizontal line indicate the mean and standard deviation

From River Witham site, 22% (2/9) of the males were found to have ovotestis in the 1995 survey. In the 2017 survey none of the males (0/22) had ovotestis. However, no statistically significant differences (according to Fisher's exact test, $P > 0.05$) were found between the two sampling dates (Figure B.27).

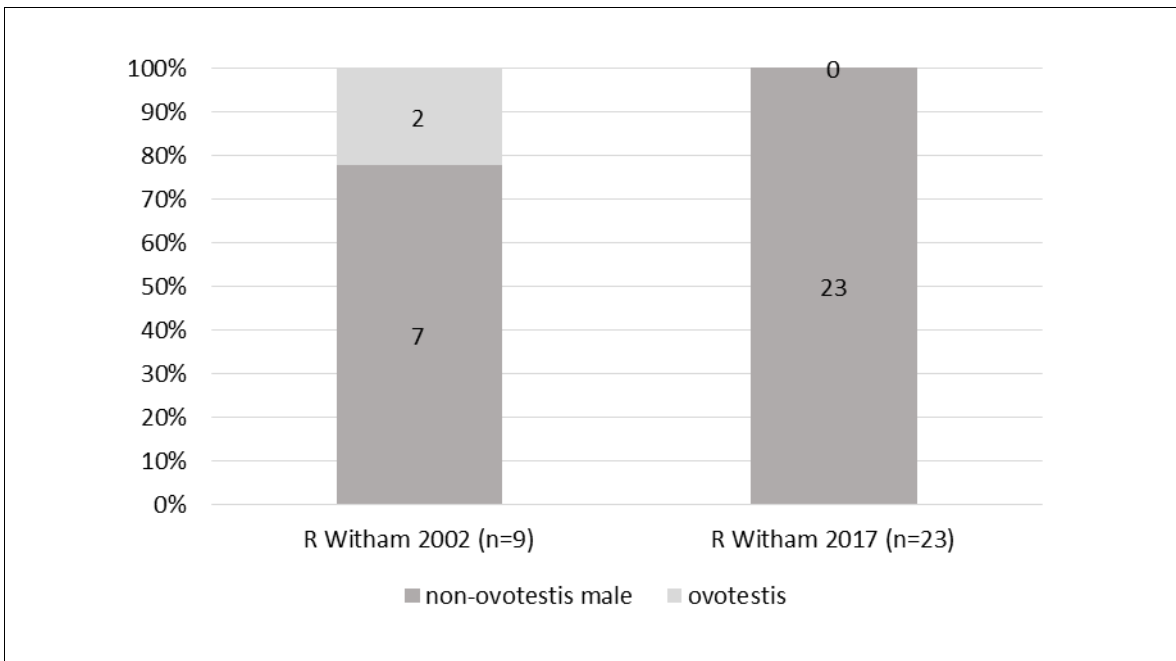


Figure B.27 Percentage of phenotypic males from the River Witham site with normal testes (non-ovotestis, dark grey bar) or intersex (ovotestis, light grey). The total number of phenotypic males is indicated by n=x in parentheses for each sampling date, and the number of individuals for each type of male is indicated by the number in each column

From the River Witham site, the mean intersex index score for the 2002 survey was 1.33 ± 0 . In the 2017 survey there were no intersex individuals so no statistical comparison could be made for this endpoint (Figure B.28).

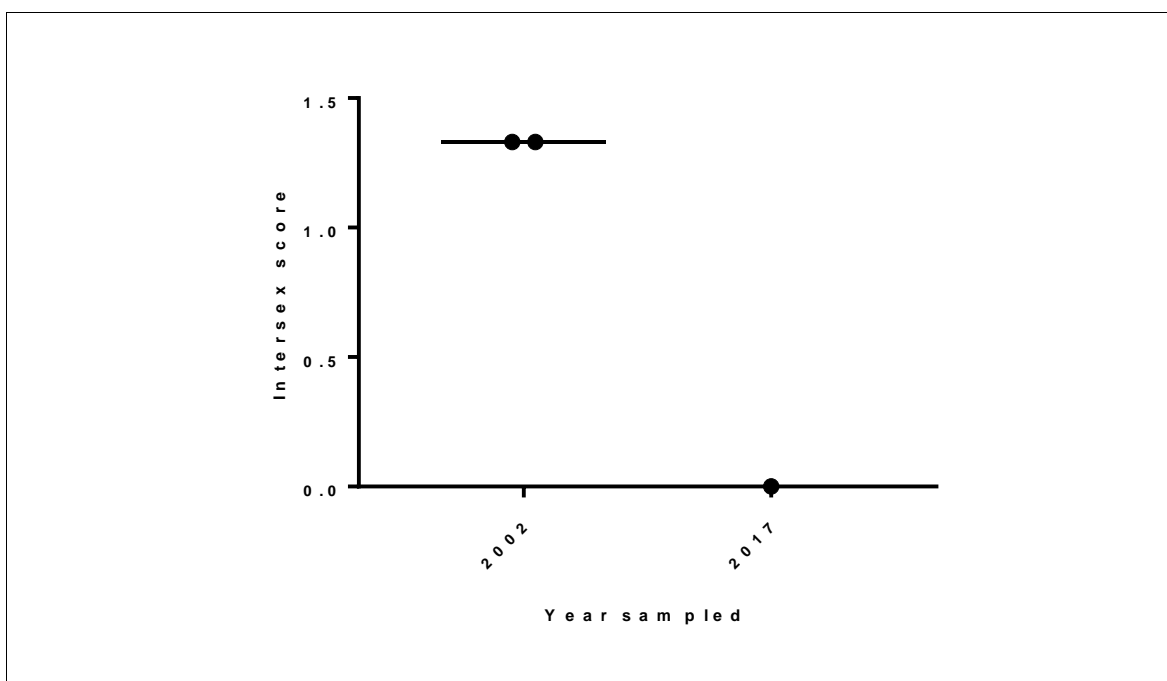


Figure B.28 Intersex index scores of roach from the River Witham in the 2002 and 2017 surveys. Points (dots) represent individual intersex scores, long horizontal bar represents the mean

Table B.8 Summary data for roach from the River Witham Lincoln

Year sampled		2002	2017
Month sampled		July	Nov.
Total roach n		47	54
Phenotypic Male no.		9	23
Phenotypic Female no.		38	31
Age (years)	Minimum	3	3
	Mode	4	5
	Maximum	11	6
Length (cm)	Minimum	6.4	11.5
	Mean \pm SD	14.28 \pm 3.36	13.48 \pm 1.61
	Maximum	23.8	17.4
Weight (g)	Minimum	3.2 (nc)	20.4
	Mean \pm SD	52.22 \pm 42.18 (nc)	37.87 \pm 16.72
	Maximum	214.4 (nc)	85.3
GSI - ♂ mean \pm SD (n)		1.72 \pm 1.19 (8) (nc)	5.24 \pm 0.73 (23)
GSI - ♀ mean \pm SD (n)		1.98 \pm 0.88 (38) (nc)	7.85 \pm 3.56 (31)

nc indicates data not comparable as sampled during different season/month

River Lea downstream of East Hyde WwTW

At the site on the River Lea downstream of East Hyde WwTW, the roach sampled in the 1995 were between 1 and 6 years old (median 3 years). In 2000 they aged were between 3 and 7 years (median 4 years) and in the 2017 sample they were between 3 and 6 years (median 3 years) (Figure B.29). There was a significant difference between the ages of the roach sampled in 1995, 2000 and 2017 at the R Lea site, with those from the 1995 survey being significantly younger than those from the 2000 ($P < 0.0001$) or 2017 ($P = 0.0432$) surveys. The roach sampled in the 1995 survey had significantly greater fork-length (21.75 ± 3.49 cm, $P < 0.0001$) than those from the 2000 (14.87 ± 2.06 cm) and 2017 (6.28 ± 2.60 cm) surveys. The roach from the 1995 survey were also heavier (138.7 ± 68.12 g) than those sampled in the 2000 (48.35 ± 25.72) and 2017 (71.86 ± 40.14 g) surveys. However, due to the different times of year (August, April and November) it is not appropriate to statically compare this.

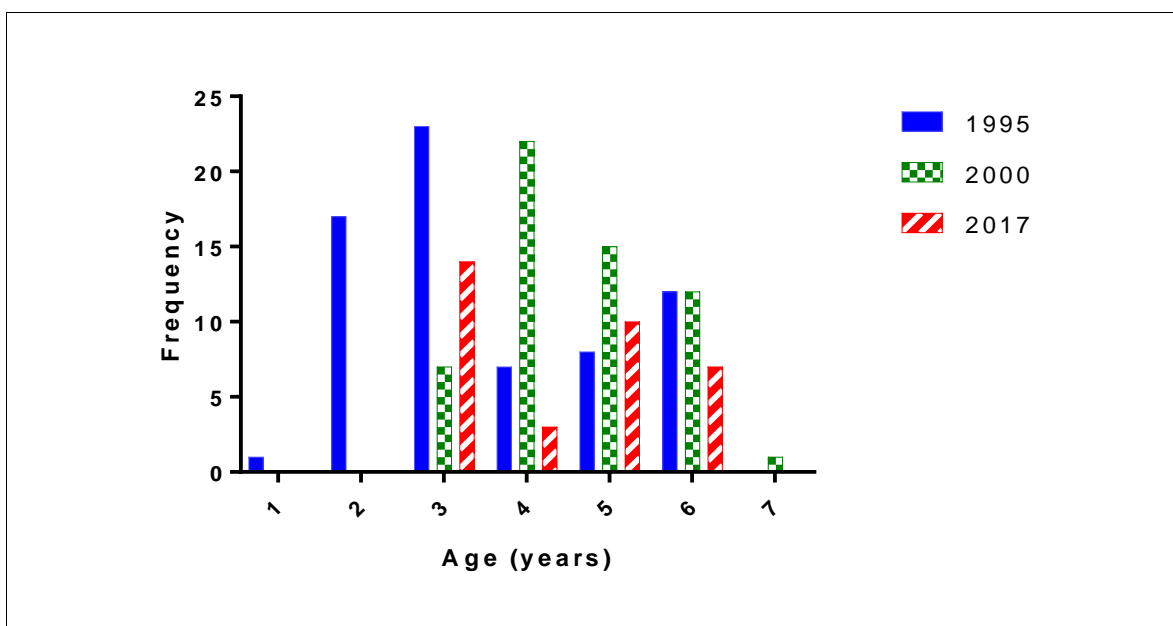


Figure B.29 Age distribution frequency of roach sampled from the River Lea downstream of East Hyde WwTW in 1995 and 2017

From the site on the River Lea downstream of East Hyde WwTW, the sex ratios were slightly skewed towards phenotypic females on both sampling occasions (1995: 56%, 2017: 59%), however, there was no significant difference in sex ratio between the two sampling dates.

Due to the different sampling months/seasons, GSI was not compared between the 1995 (August), 2000 (April) and 2017 (November) surveys.

For the first sampling occasion on the River Lea downstream of East Hyde in August 1995, the average male plasma VTG concentration was $17,536 \pm 68,355$ ng/ml, compared with 132.9 ± 449 ng/mL measured in roach sampled in April 2000 and 402.3 ± 970.9 ng/ml in those sampled in November 2017. There was a significant difference in male VTG when the data were analysed collectively ($P = 0.0037$), with a significantly higher VTG concentration in 1995 than 2017 ($P = 0.0028$) (Figure B.30 and Table B.11).

For female roach sampled from the River Lea site, the average plasma VTG concentration was $222,387 \pm 185,759$ ng/ml in the 1995 (August) survey compared with $81,024 \pm 120,966$ ng/ml in the 2000 (April) survey and $4,470,060 \pm 3,000,496$

ng/ml in the 2017 (November) survey. (Table B.12). As plasma VTG fluctuates significantly with season in female roach, no statistical comparisons were made for females from this site.

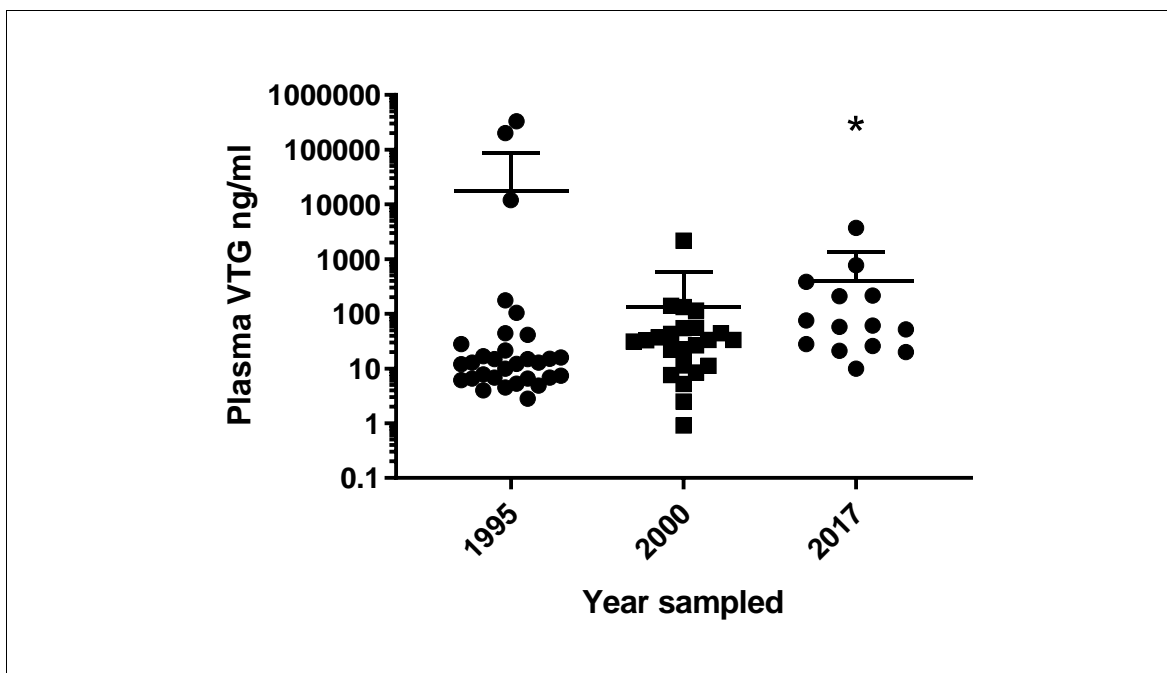


Figure B.30 Plasma vitellogenin (VTG) concentrations from male roach sampled from the River Lea downstream of East Hyde WwTW in 1995, 2000 and 2017. Points (dots, squares) represent individuals. Horizontal lines indicate mean and standard deviation. Star * indicates statistically significant decrease in VTG between the 1995 and 2017 samples

From the River Lea site downstream of East Hyde WwTW, 26% (8/31) of the phenotypic males had ovotestis in the 1995 survey, 3% (1/31) in the 2000 survey and 7% (1/14) in 2017. Significant differences were found between the three sampling dates ($P = 0.028$) when compared overall. Male ovotestis frequency was found to be significantly higher in the 1995 survey than in the 2000 survey ($P = 0.0261$). However, there was no statistically significant difference between the 1995 and 2017 surveys, or between the 2000 and the 2017 surveys (Figure B.31).

From the River Lea site downstream of East Hyde WwTW, the mean intersex index score was 1.96 ± 2.02 in the 1995 survey, 0.33 ± 0.00 ($n = 1$) in the 2000 survey and 0.17 ± 0.00 ($n = 1$) in the 2017 survey (Figure B.32). Due to low sample size statistical analysis was not conducted for this metric (Figure B.32).

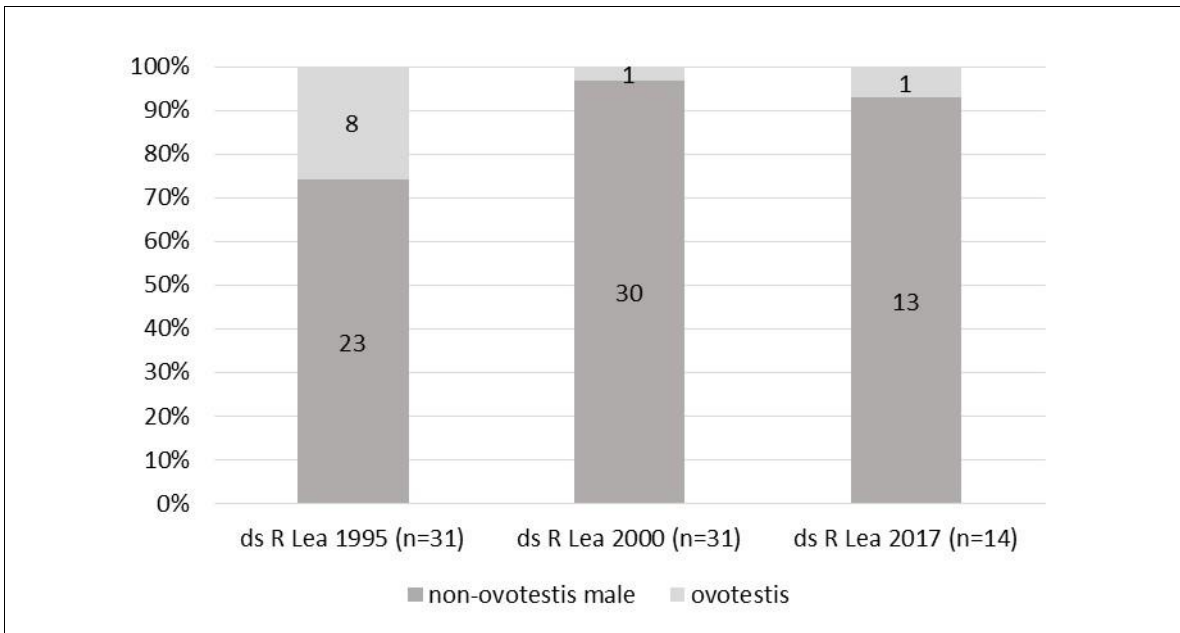


Figure B.31 Percentage of phenotypic males from the River Lea downstream of East Hyde WwTW with normal testes (non-ovotestis, dark grey bar) or intersex (ovotestis, light grey). The total number of phenotypic males is indicated by n=x in parentheses for each sampling date, and the number of individuals for each type of male is indicated by the number in each column.

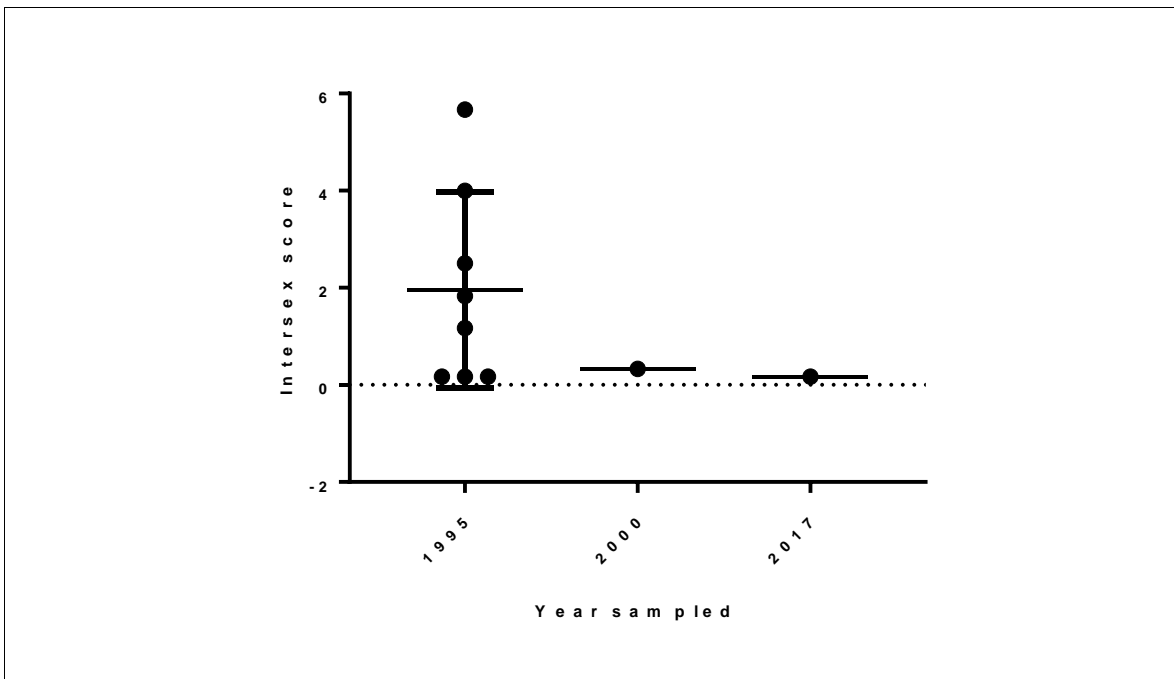


Figure B.32 The intersex index scores of roach from the River Lea site downstream of East Hyde WwTW in the 1995, 2000 and 2017 surveys. Points (dots) represent individual intersex index scores. Horizontal bars represent the mean and standard deviation

Table B.9 Summary data for roach data the River Lea downstream of East Hyde WwTW

Year sampled		1995	2000	2017
Month sampled		Aug.	Apr.	Nov.
Total roach n		69	57	34
Phenotypic Male no.		31	31	14
Phenotypic Female no.		38	25	20
Age (years)	Minimum	1	3	3
	Mode	3	4	3
	Maximum	6	7	6
Length (cm)	Minimum	15.5	10.2	12.6
	Mean \pm SD	21.75 \pm 3.49	14.87 \pm 2.06	16.28 \pm 2.60
	Maximum	29.5	21.6	21.2
Weight (g)	Minimum	35	2	27
	Mean \pm SD	138.7 \pm 68.12	48.35 \pm 25.72	71.86 \pm 40.14
	Maximum	302.1	166	159.3
GSI - ♂ mean \pm SD (n)		2.44 \pm 1.14 (31) (nc)	2.75 \pm 3.20 (31) (nc)	4.67 \pm 0.70 (14)
GSI - ♀ mean \pm SD (n)		6.00 \pm 2.06 (39) (nc)	2.95 \pm 2.86 (25) (nc)	10.23 \pm 4.52 (20)

nc indicated data not comparable as sampled during different season/month

River Trent

From the site on the R Trent at Hampton Ferry, the roach ranged in age between 1 and 9 years (median 3 years) in the 1995 survey, and between 2 and 5 years (median 2) in the 2017 survey (Figure B.33). There was a significant difference in age between the two sampling dates ($P < 0.0001$). The roach sampled from the R Trent in 1995 were significantly ($P < 0.0001$) greater in length (16.6 ± 2.15 cm) than roach sampled in 2017 (11.59 ± 2.34 cm) and were heavier (59.92 ± 31.94 g in 1995 compared with 24.42 ± 26.1 g in 2017) (Table B.10).

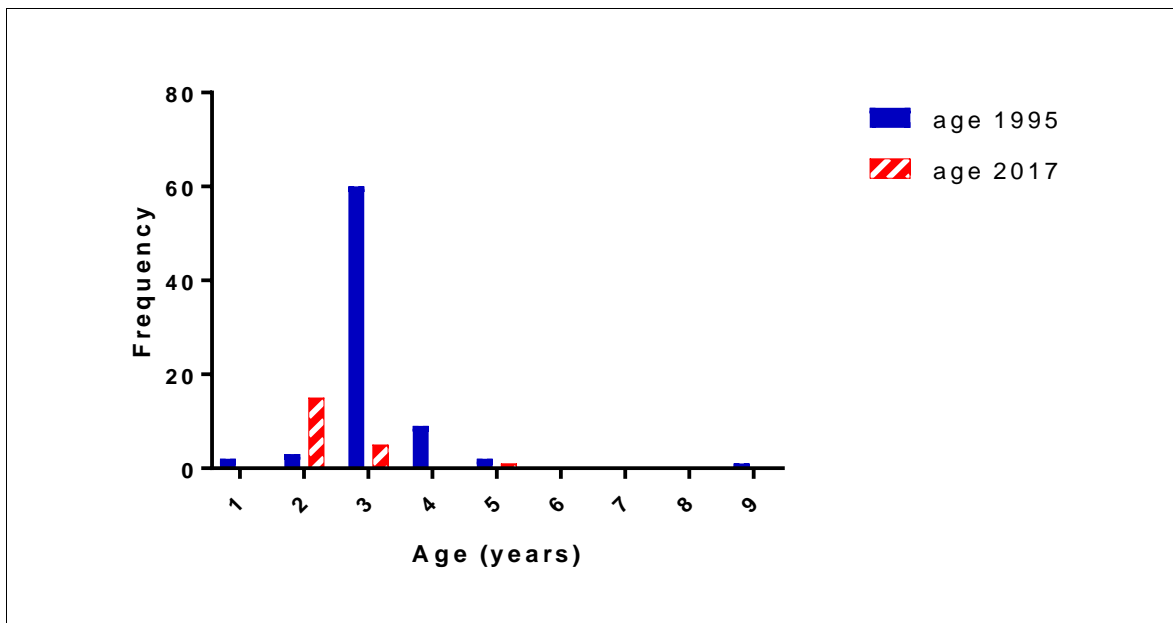


Figure B.33 Age distribution frequency of roach sampled from the site on the River Trent in 1995 and 2017

At the River Trent sample site, sex ratio was skewed towards phenotypic females in the 1995 survey (63%), whereas in the 2017 survey it was skewed toward phenotypic males (52%). However, there was no statistically significant difference found between the two sampling occasions for sex ratio ($P = 0.3092$).

Male GSI was 2.61 ± 1.29 in 1995 and 4.17 ± 2.49 in 2017. For females, GSI was 5.49 ± 2.36 in the 1995 sample and 3.01 ± 4.49 in 2017. However, due to the different seasons/month of the sample collection (September 1995 and December 2017) statistical analysis was not conducted for differences in GSI.

For the River Trent site, male plasma VTG concentrations were significantly ($P = 0.0005$) lower in the 2017 survey (3233 ± 7698 ng/ml) than those from the 1995 survey ($11,807 \pm 41941$ ng/ml) (Figure B.34 and Table B.11).

For females at the River Trent site, plasma VTG concentrations were $87,927 \pm 85745$ ng/ml in 1995 and $69,235 \pm 217,186$ ng/ml in 2017) (Table B.12). However, direct statistical comparisons were not made between females at this site due to the different time of year and reproductive stage of the fish at the difference sampling periods and the influence this can have on female plasma VTG concentrations.

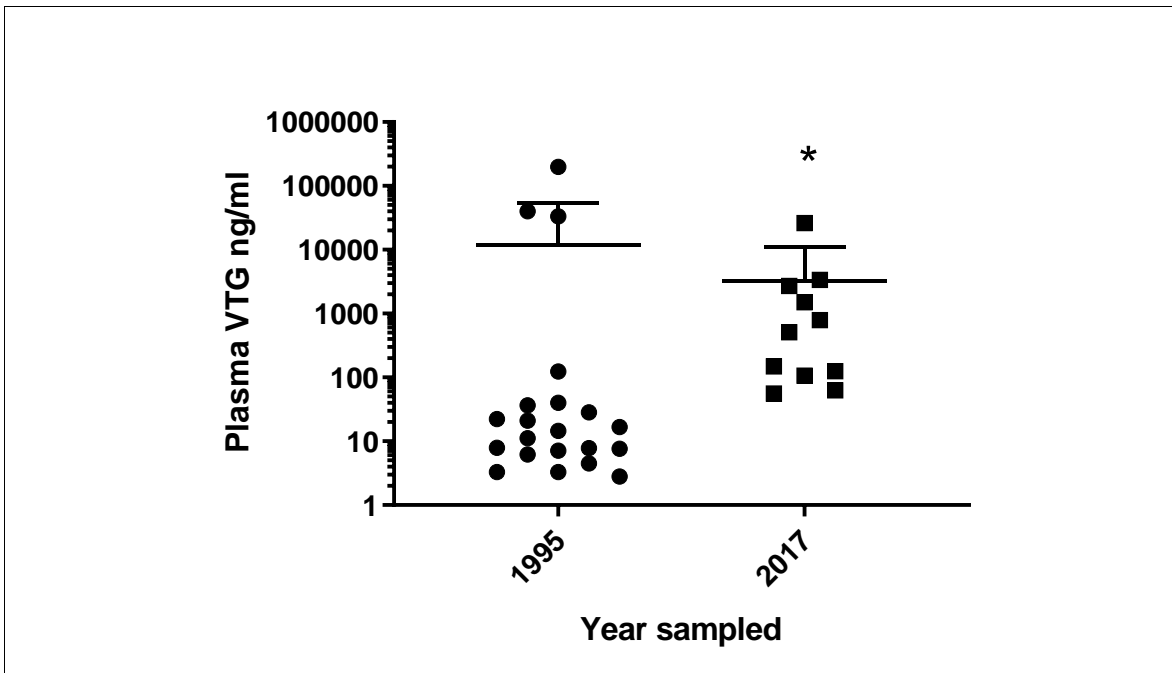


Figure B.34 Plasma vitellogenin (VTG) concentrations from male roach sampled from the River Trent in the 1995 and 2017 surveys. Points (dots, squares) represent individuals. Horizontal lines indicate mean and standard deviation. Star * indicates statistical decrease in VTG between 1995 and 2017

In the samples from the River Trent site, 28% (7/25) of the phenotypic males had ovotestis in the 1995 survey, and none of the males had ovotestis (0/11) in 2017. However, there was no statistically significant difference between the ovotestis frequencies of the two samples from the River Trent (Figure B.35).

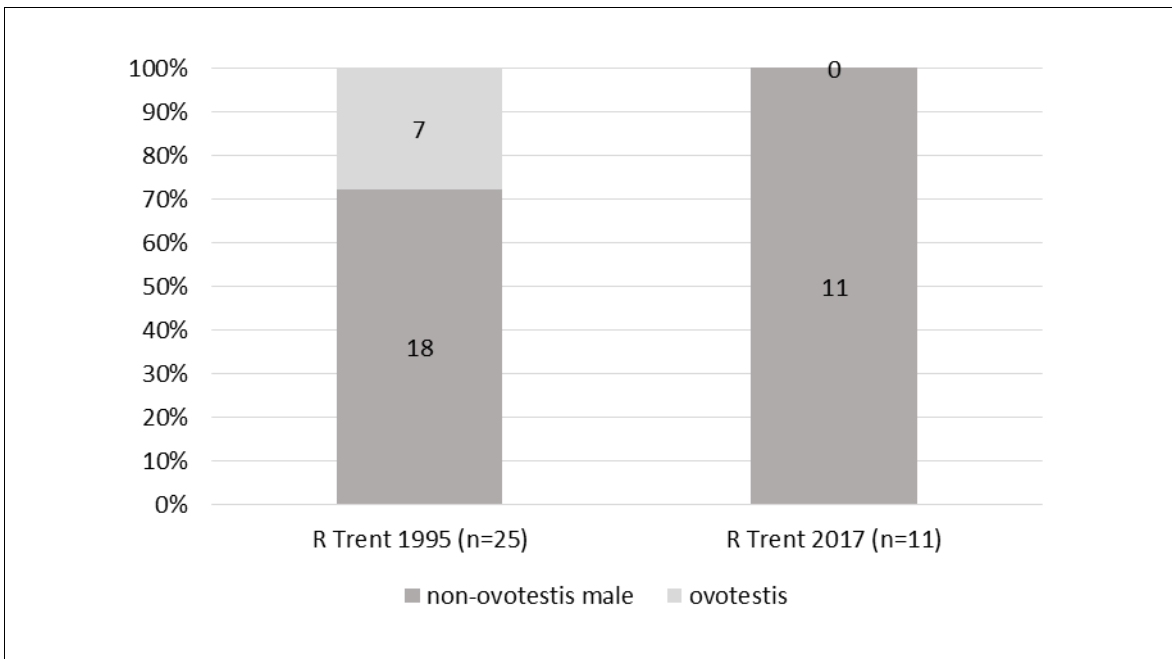


Figure B.35 Percentage of phenotypic males from the River Trent with normal testes (non-ovotestis, dark grey bar) or intersex (ovotestis, light grey). The total number of phenotypic males is indicated by n=x in parentheses for each sampling date, and the number of individuals for each type of male is indicated by the number in each column

For the R Trent site, the mean intersex index score from the 1995 survey was 0.19 ± 0.06 . In the 2017 survey there were no intersex individuals and therefore no statistical comparison could be made for intersex severity (Figure B.36).

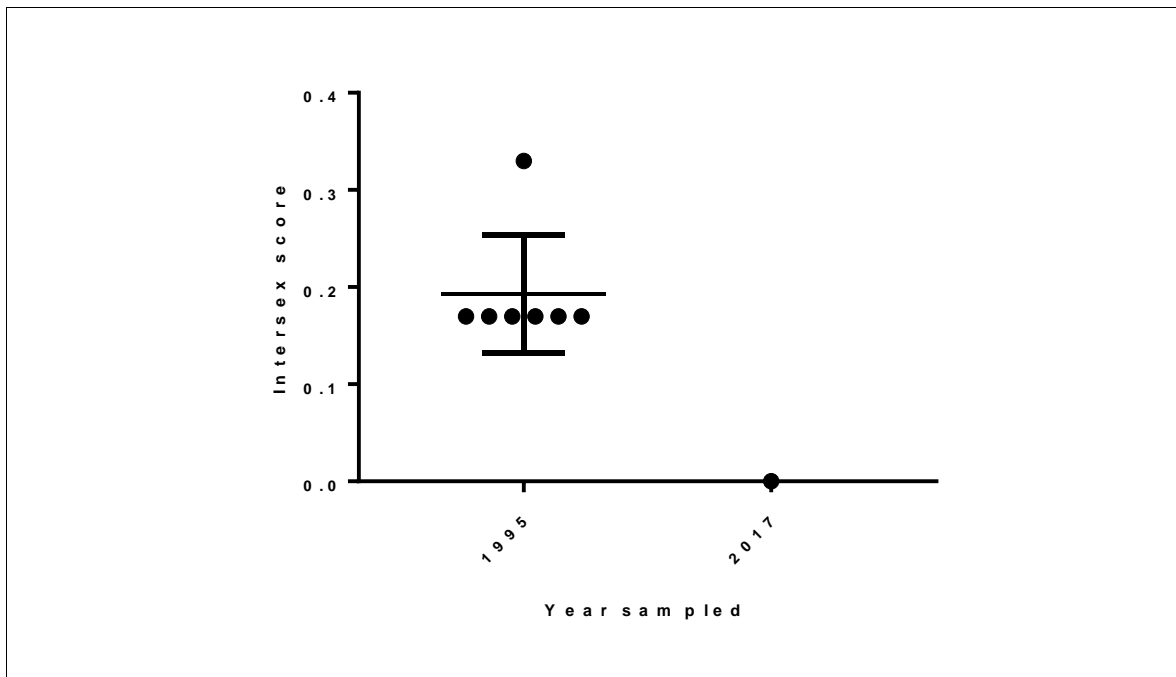


Figure B.36 Intersex index scores for roach from the River Trent site in the 1995 and 2017 surveys. Points (dots) represent individual intersex scores. Horizontal bars indicate the mean and standard deviation

Table B.10 Summary data for roach from the River Trent

Year sampled		1995	2017
Month sampled		Sept	Dec.
Total roach n		80	21
Phenotypic Male no.		25	11
Phenotypic Female no.		42	10
Age (years)	Minimum	1	2
	Mode	3	2
	Maximum	9	5
Length (cm)	Minimum	9	9.6
	Mean \pm SD	16.6 \pm 2.15	11.59 \pm 2.34
	Maximum	27	19.8
Weight (g)	Minimum	6.3	10.5
	Mean \pm SD	59.92 \pm 31.94	24.42 \pm 26.1
	Maximum	280	127.1
GSI - σ mean \pm SD (n)		2.61 \pm 1.29 (25)	4.17 \pm 2.49 (10)
GSI - σ mean \pm SD (n)		5.49 \pm 2.36 (42)	3.01 \pm 4.49 (10)

Table B.11 Vitellogenin plasma concentrations measured in male roach sampled in historic surveys (1995 or 2002) and in this survey (2017)

Site	d/s Arun		u/s Arun		d/s Nene		u/s Nene		Bourne		Avon		Eye		Witham		Lea		Trent	
Month	Oct.	Oct.	Oct.	Oct.	Oct.	Oct.	Oct.	Oct.	May	Nov.	Oct.	Nov.	Sept.	Nov.	Jul.	Nov.	Aug.	Nov.	Sept.	Dec
Year	1995	2017	1995	2017	1995	2017	1995	2017	2002	2017	1995	2017	1995	2017	2002	2017	1995	2017	1995	2017
N =	21	18	16	15	31	11	27	14	16	31	26	11	25	37	2	23	31	14	23	11
VTG ng/mL																				
Mean	17689	1919	733.6	571.6	21373	298	211.5	27.86	109.4	2000	5961	1172	8934	267.2	140.5	32199	17536	402.3	11807	3233
SD	76381	6816	1351	1313	92214	663	197.3	39.28	227.5	3522	18925	2308	39874	413.3	163.3	47326	68355	970.9	41941	7698
SEM	16668	1607	337.9	339	16562	199.9	37.98	10.5	56.87	632.5	3711	695.9	7975	67.94	115.5	9868	12277	259.5	8745	2321
VTG ng/mL																				
Min	71	10.85	28	10.85	41	10	0	11	25	34	2.4	10	0.91	10	25	89	2.8	10	0	56
Med	553	32	281.5	31	542	42	144	11	25	702	33.85	160	11	114	140.5	6912	12.8	59.5	11.1	510
Max	351000	29044	5400	4434	502000	2228	735	148	935.2	15532	86000	7831	199000	2054	256	123270	331000	3697	198000	26184

N = number of males in the analysis. Mean = arithmetic mean; SD = standard deviation of the mean; SEM = standard error of the mean; Min = minimum value; Med = median value; Max = maximum value.

Table B.12 Vitellogenin plasma concentrations measured in female roach sampled in historic surveys (1995, 2002) and in this survey (2017)

Site	d/s Arun		u/s Arun		d/s Nene		u/s Nene		Bourne		Avon		Eye		Witham		Lea		Trent	
Month	Oct.	Oct.	Oct.	Oct.	Oct.	Oct.	Oct.	Oct.	May	Nov.	Oct.	Nov.	Sept.	Nov.	Jul.	Nov.	Aug.	Nov.	Sept.	Dec.
Year	1995	2017	1995	2017	1995	2017	1995	2017	2002	2017	1995	2017	1995	2017	2002	2017	1995	2017	1995	2017
N =	33	35	47	38	37	44	37	27	15	23	46	22	70	27	28	31	39	20	41	10
VTG ng/mL																				
Mean	21962 6	283516 8	29952 1	33693 01	23655 5	523276 7	27883 8	30434 98	29548	500136 1	13860 3	303153 4	18705 5	548225 7	476	6328 311	22538 7	447006 0	87927	69235
SD	11173 6	278745 0	14824 4	24248 36	14509 9	238667 2	31560 4	21475 45	88347	303838 9	22473 5	415887 3	12359 8	478014 2	339.8	4683 086	18575 9	300049 6	85745	21718 6
SEM	19451	471165	21624	39336 0	23854	359804	51885	41329 5	22811	633548	33135	886675	14773	919939	64.22	8411 07	29745	670931	13391	68680
VTG ng/mL																				
Min	159	43	133	11	2740	45	128	11	25	61	4.4	10	3.7	10	25	72	4.4	60	4.8	10
Med	20400 0	170476 5	30700 0	36428 39	20400 0	552556 0	19200 0	30552 65	1505	518885 5	44500	123	18000 0	575259 3	461.5	6141 957	20700 0	434240 8	77000	332.5
Max	52700 0	105055 99	59700 0	90967 02	59500 0	140797 06	16900 00	64216 64	33937 2	102323 85	12000 00	121024 99	41500 0	187495 41	1407	1603 9305	65600 0	118831 78	25000 0	68735 5

N = number of females in the analysis. Mean = arithmetic mean; SD= standard deviation of the mean; SEM = standard error of the mean; Min = minimum value; Med = median value; Max = maximum value.

Appendix C: Wastewater technologies and inputs

Table C.1 Wastewater treatment work technology at survey sites and changes between historic and 2017 surveys

WwTW name	Inputs to river	Wastewater treatment technology on site	Estimated retention time	Chemical dosing	PE	Industrial / Trade PE	Updates to technology since 1998
Horsham	R Arun	Secondary trickling filters, deep bed sand filters	-	No, ferric chloride added for phosphate removal	2008 - 77,512 2009 - 76,881 2010 - 76,832 2011 - 76,487 2012 - 77,994 2013 - 79,207 2014 - 74,860 2015 - 77,717 2016 - 72,075 2017 - 70,068	6% industrial (of which 39% tip leachate, 2% metal finishing, 23% swimming pools, 9% commercial laundry, 2% vehicle washing, 23% recycling centre, 2% misc)	01/02/08 New nitrifying and carbonaceous plastic media trickling filters, new deep bed sand filter and ferric chloride dose for phosphate removal 02/02/14 - additional carbonaceous plastic media trickling filter and recirculation added July 2015 - Installation of tertiary Mecana cloth pile filter to assist deep bed sand filter. Feb 2017 - Installation of sodium hydroxide dosing to trickling filter feed to increase pH and optimise nitrification
Warnham	R Arun	Secondary trickling filters, no tertiary	-	No, ferric chloride added for phosphate removal	2008 - 1,215 2009 - 1,308 2010 - 1,337 2011 - 1,344 2012 - 1,367 2013 - 1,377 2014 - 1,180 2015 - 1,192 2016 - 1,197 2017 - 1,201	0.1% industrial (vehicle wash)	Nov 2003: 2 new PSTs, 2 new plastic media trickling filters in series, 2 new humus tanks
Mannings Heath	R Arun	Secondary trickling filters, tertiary Submerged Aerated Filters (SAF)	-	No phosphate removal required	2008 - 1,008 2009 - 980 2010 - 994 2011 - 994 2012 - 1,004 2013 - 1,006	No consented trade discharges in catchment	2000 - one new Primary settlement Tank (PST), one new trickling filter, 2 new humus tanks May 2005 - new 6mm 2D screen 20/12/05: Tertiary nitrifying SAF and final settlement tank installed

					2014 - 1,001 2015 - 1,008 2016 - 1,025 2017 - 1,032		
Rugeley	R Trent	Biological trickling filter + tertiary sand filter	8-12 hours	Yes PolyAluminium chloride	2007 – 23571 2017 - 23066	TE Load PE 2007 – 233 TE Load PE 2017 - 82	2014 - Sand filters + Chemical dosing added
Evesham	R Avon	Biological trickling filter + tertiary sand filter	8-12 hours	Yes - Ferric sulphate	2007 – 23031 2017 - 24643	TE Load PE 2007 – 775 TE Load PE 2017 - 877	2006 - Sand filters + Chemical dosing added
Canwick	R Witham	Double filtration filter works	-	-	2007 – 99892 2008 - 99645 2009 - 100980 2010 - 100935 2011 - 105296 2012 - 107951 2013 - 108969 2014 - 110018 2015 - 110230 2016 - 109713 2017 - 109813	2007 - 6137 2008 - 7611.17 2009 - 5603 2010 - 2355.07 2011 - 2929.79 2012 - 3279 2013 - 3993 2014 - 4019 2015 - 4997.19 2016 - 3860 2017 - 4069.6	No
Great Billing	R Nene	Activated sludge process	-	-	2007 - 222063 2008 - 218436 2009 - 221280 2010 - 225210 2011 - 222015 2012 - 219028 2013 - 236599 2014 - 239031 2015 - 237975 2016 - 241594 2017 - 241478	2007 - 73147 2008 - 66827.75 2009 - 67171 2010 - 58718.18 2011 - 69633.25 2012 - 96669 2013 - 93247 2014 - 80632 2015 - 89307.21 2016 - 826021.7 2017 - 69575.3	Change from being a blend of filter works and activated sludge to all activated sludge about 2001

Bugbrooke	R Nene	Trickling filter	-	-	2007 - 7105 2008 - 8121 2009 - 6400 2010 - 7002 2011 - 6914 2012 - 6775 2013 - 7000 2014 - 6389 2015 - 7149 2016 - 7545 2017 - 7543	none	No
Chertsey	Bourne	Primary settlement Tank (PSTs), biological filters, no tertiary	45-60 mins hydraulic retention	Ferric sulphate dosed	Current PE: 77,100	Trade permits in catchment for Cr, Cu, Pb, Ni, Zn	No significant changes
East Hyde	R Lea	PSTs, oxidation ditches, final settlement, no tertiary	30 mins hydraulic retention	Ferrous chloride is dosed	Current PE: 166,000	Trade permits in catchment for Cd, Cr, Cu, Fe, Pb, Hg, Ni, Zn	No significant changes

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