BRIEF COMMUNICATION

Endocrine disruption in juvenile roach (*Rutilus rutilus*) from U.K. rivers: a preliminary study

Running title: Endocrine disruption in juvenile roach

N. Beresford*§, S. Jobling*, R. Williams† and J. P. Sumpter*

*Department of Biological Sciences, Brunel University, Uxbridge, Middlesex, UB8 3PH, U.K.

†Centre for Ecology and Hydrology, Crowmarsh Gifford, Wallingford, Oxfordshire, OX10 8BB, U.K.

§ Author to whom correspondence should be addressed. Tel: +44 1895 274000 ext. 2805; fax: +44 1895 274348; email: Nicola.Beresford@Brunel.ac.uk

Abstract:

Juvenile roach from seven rivers of varying water quality were examined for evidence of endocrine disruption. The majority of roach from five of these rivers had female-like reproductive ducts, but because the fish were sexually immature it is not known whether their germ cells would also be intersex. Nevertheless, the results suggest that juvenile, rather than adult, fish could be used in studies of endocrine disruption in wild fish populations.

Key words: endocrine disruption; reproductive ducts; juvenile fish; *Rutilus rutilus*; wild fish

A wide variety of chemicals released into the aquatic environment are believed to disrupt normal endocrine function in fish, thereby causing reproductive disorders and abnormalities (reviewed in Sumpter, 2002). Sewage treatment work (STW) effluents have been shown to be oestrogenic, inducing the synthesis of vitellogenin (a female specific yolk precursor) (Harries *et al*., 1997; Purdom *et al*., 1994) and inhibition of testicular growth (Harries *et al*., 1997) in male fish from U.K. rivers. A relationship has also been established between the widespread occurrence of intersexuality (gonads containing male and female tissue and/or female-like reproductive ducts) in roach and exposure to STW effluents (Jobling *et al*., 1998). In that study, the number of fish with normal testes was inversely proportional to the number of intersex fish, suggesting that this phenomenon was due to the feminisation of genetically male fish. Intersexuality in gudgeon (*Gobio gobio*) inhabiting U.K. rivers has also been reported (van Aerle *et al*., 2001), confirming that sexual disruption is not species specific. Additionally, exposure of juvenile roach to STW effluent induced a dose-dependent induction of vitellogenin (VTG) and formation of female-like reproductive ducts (ovarian cavities) in 'male' fish (Rodgers-Gray *et al*., 2001). Depuration resulted in a reduction in plasma VTG, but the feminised ducts were stably retained. No oocytes were seen in these feminised fish, suggesting that duct development is more sensitive to disruption than germ cell development. Intersexuality in fish, which appears to be associated with exposure to STW effluents, has also been reported in a number of other countries (for example, Harshbarger *et al*., 2000; Hashimoto *et al*., 2000).

To date, all field studies on endocrine disruption in fish have involved an assessment of the status of adult fish. In the most extensive study, this involved sampling well over 1000 fish (Jobling *et al*., 1998). However, the removal of mature fish is not desirable in most fisheries, especially if the species is one whose numbers are declining (e.g. trout and salmon: Burkhardt-Holm *et al*., 2002) and/or the species is one of high profile and significance (e.g. barbel, *Barbus barbus*). In such cases, although it would be extremely useful to know if the population was being adversely affected by endocrine disruption, this should not be demonstrated at the expense of a significant proportion of the adult fish. An alternative approach might be to sample young fish, which are much more plentiful. Few of these would survive to maturity and reproduce, and hence the removal of juveniles is less likely to affect the sustainability of the population (Matthews, 1971; Mills and Mann, 1985). Further, because much evidence indicates that many features of endocrine disruption, such as intersexuality and the 'feminisation' of the reproductive ducts, are induced at an early stage in development (Gimeno *et al*., 1997), the situation found in adults should be reflected in juveniles. Hence, in this study, juvenile roach from a range of rivers of varying water quality were investigated for evidence of endocrine disruption. The main criterion used for assessing the degree of oestrogenic activity was the formation of an ovarian cavity, which is typical for cyprinids but not necessarily for other families. This is a persistent feature (Rodgers-Gray, 2001) normally dependent on the presence of oestrogen (Piferrer, 2001).

Juvenile roach (0+) were sampled randomly by netting methods between 19 to 21 September 2000, from seven rivers in the U.K.. The impact of sewage treatment works on water quality at the various capture sites is described in Table I, based on the population equivalent (PE) of the sewage treatment works influent and the amount of dilution the effluent receives in the river at the point of fish capture (Jobling *et al*., 1998). The fish were sacrificed using a lethal dose of MS222 anaesthetic prior to fixation *in toto* in 10% neutral buffered formaldehyde. After measuring total fish lengths, mid-body transverse sections (3-5 mm) were embedded in paraffin wax and histologically sectioned at 5 m. Sections were mounted, stained with Harris' haematoxylin and eosin, and examined by light microscopy for the type of germ cells and reproductive ducts present.

Statistical analyses were carried out using the statistical program StatView (Abacus Concepts Inc., Berkeley, CA). The lengths of fish from different sites were compared by a Kruskal-Wallis test (nonparametric, as the data could not be normalised) and multiple comparisons were performed using Scheffé's post hoc test. The presence or absence of an ovarian cavity was compared with the expected ratio and between the different sites by ^{2} analysis or by Fisher's Exact Test (when the frequency in one or more cells was less than 5). Regression analysis was used to determine if a relationship existed between effluent contribution and either the total fish length or the percentage of fish with female-like reproductive ducts. Statistical significance for all tests was accepted at $p<0.05$.

Two distinct gonad types were clearly visible: either club-shaped, with one point of attachment to the mesentery (Figure 1a), or elongated, with two points of attachment forming an ovarian cavity (probable female; Figure 1b). The ducts were not studied posterior to the gonad, and the condition of the ducts beyond the point of attachment to the gonad is therefore not known. Very few of the female-like gonads contained cells which had developed into primary oocytes; only 25% of those from the River Aire and 10.5% from the River Dearne.

The majority of fish from the Rivers Aire (95%), Dearne (95%), Don (85%), Rother (86%) and Tees (95%) had an ovarian cavity, and were found to have significantly different ratios of 'male' to 'female' (based on the appearance of the ducts) from the expected ratio of 1:1 (Figure 2a). There were significant differences between the 'male' to 'female' ratios at the various sites. Since the lengths of the fish (Figure 2b) were significantly different between sites, it is possible that some of the different ratios were due to differences in maturity. However, there were no significant differences between the lengths of the fish from the River Aire and River Swale, River Dearne and River Ouse, River Don and River Ouse, River Ouse and River Rother, River Rother and River Swale, River Swale and River Tees, yet the 'male' to 'female' ratios of these pairs of sites were significantly different. Additionally, whilst the River Dearne and the River Don had significantly more roach with ovarian cavities than those from the River Swale, the roach from both the River Dearne and River Don were significantly shorter than those from the River Swale, indicating that they were likely to be younger.

There was no significant relationship between the effluent contribution and either the total fish length or the percentage of fish with female-like reproductive ducts. When the effluent contribution data were log transformed (to improve normality of distribution) and compared with the percentage fish with female-like reproductive ducts, linear regression analysis was still not significant ($p=0.074$), but non-linear power regression analysis showed that the relationship was weakly significant ($p=0.037$).

Sexual development is a two-stage process, involving gonadogenesis (development of reproductive ducts) and gametogenesis (germ cells development). In roach, gonadogenesis mainly takes place between 50 and 150 days post hatch (dph). Gametogenesis occurs later; female germ cells (primary oocytes) appearing between 100 and 200 dph and male germ cells (spermatogonia) between 200 and 350 dph (Rodgers-Gray *et al*., 2001). Roach spawn in May, therefore the juvenile fish used in this study were approximately 130 dph on the date of capture. Since reproductive ducts are not fully formed until 150 dph, some of the 'male' fish may still be undifferentiated and may still develop ovarian cavities, thus further increasing the percentage of juvenile fish with female-like reproductive ducts. Whether they would then have gone on to develop gonads containing both male and female tissue is not known. Nolan *et al*. (2001) reported that all intersex individuals had an ovarian cavity, and that the most common malformation was the presence of individuals with both sperm duct and an ovarian cavity. In this study, sperm ducts were not visible in individuals with ovarian cavities. However, as the size of the sperm duct is generally reflective of the stage of reproductive cycle (Nolan *et al*., 2001), being bigger in the mature fish, the juvenile fish with ovarian cavities may have developed a functional sperm duct at a later stage.

Primordial germ cells are thought to be bipotential, remaining undifferentiated until exposed to hormonal and other influences that transform them into developing spermatogonia or oogonia (Shibata and Hamaguchi, 1988). Steroids play a critical role in the differentiation of the gonads into the two sexual types, and probably also for the maintenance of these conditions (Devlin and Nagahama, 2002). The major oestrogenic compounds in STW effluents include natural oestrogens (17 β-oestradiol and oestrone) and the synthetic oestrogen 17 α-ethynylestradiol (Desbrow *et al*., 1998), with concentrations sufficiently high to induce vitellogenin synthesis in roach (Routledge *et al*., 1998). STW effluents frequently contribute 50% flow to rivers, and as much as 90% in periods of low rainfall in some rivers in the U.K. Additionally, it has been demonstrated that the oestrogenic effects of STW effluents persist for several kilometres downstream from the point of effluent entry (Harries *et al*., 1997). Thus, fish are often exposed to high concentrations of STW effluent. Only a weak statistical relationship was seen between the percentage of fish with female-like reproductive ducts and effluent contribution. However, the precise chemical(s) responsible for the formation of female-like reproductive ducts are not known, and the effluent contribution only gives an indication of the quantity of effluent and not the actual concentrations of the causative chemicals. In addition, no information is available on the endocrine activity of the effluents or the receiving waters, or how that changes downstream at the site of capture (see Johnson and Sumpter, 2001, for a discussion of some of these issues). In this study, fish from only two of the rivers (Ouse and Swale) had 'normal' sex-ratios, and effluent contributions at both of these sites were very low. In contrast, the five rivers having the highest effluent contributions (albeit these covered a considerable range) all had an unexpected high proportion of fish with ovarian cavities, suggesting 'feminisation' of the males. Therefore, this weak link between effluent quality and 'feminised' ducts in wild juvenile fish populations, suggests that young fish could be used to monitor endocrine disruption in the aquatic environment.

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Table I. Characteristics of the fish capture sites.

River	Location of Capture Site		PE of STW effluent	Dilution factor of effluent in river	Effluent contribution to river at site of sampling (adjusted for PE)
	Latitude	Longitude			
Aire	53° 43’ 47” N	1° 7’ 26” W	75032	170.6	440
Dearne	53° 30’ 1” N	1° 15’ 2” W	186452	5.3	35180
Don	53° 30’ 23” N	1° 11’ 25” W	704352	7.9	89158
Ouse	54° 0’ 41” N	1° 11’ 28” W	33000	584.4	56
Rother	53° 21’ 58” N	1° 20’ 57” W	25829	20.4	1266
Swale	54° 8’ 8” N	1° 20’ 19” W	12500	815.0	15
Tees	54° 28’ 57” N	1° 33’ 8” W	105000	76.4	1374

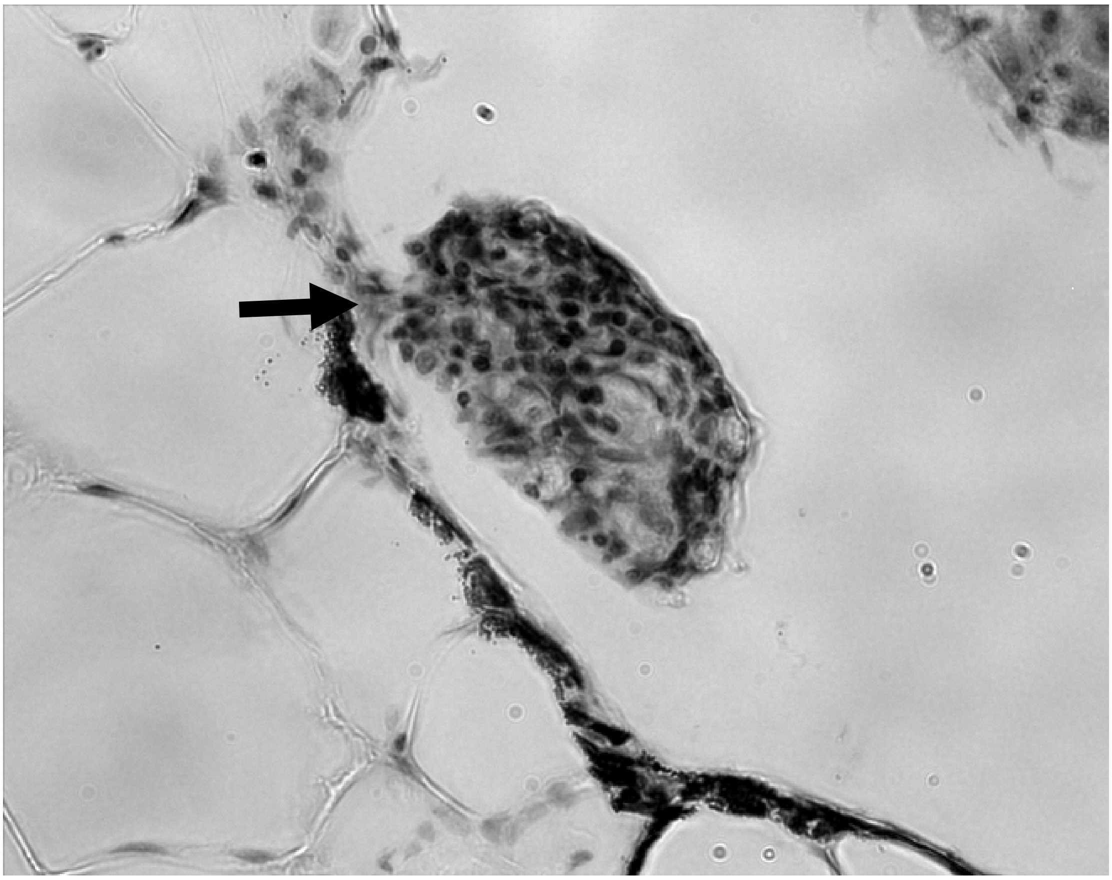
PE = Population equivalent, the unit of measure used to describe the ‘strength’ of an effluent. This measure of the biological oxygen demand of an effluent incorporates both domestic and industrial components.

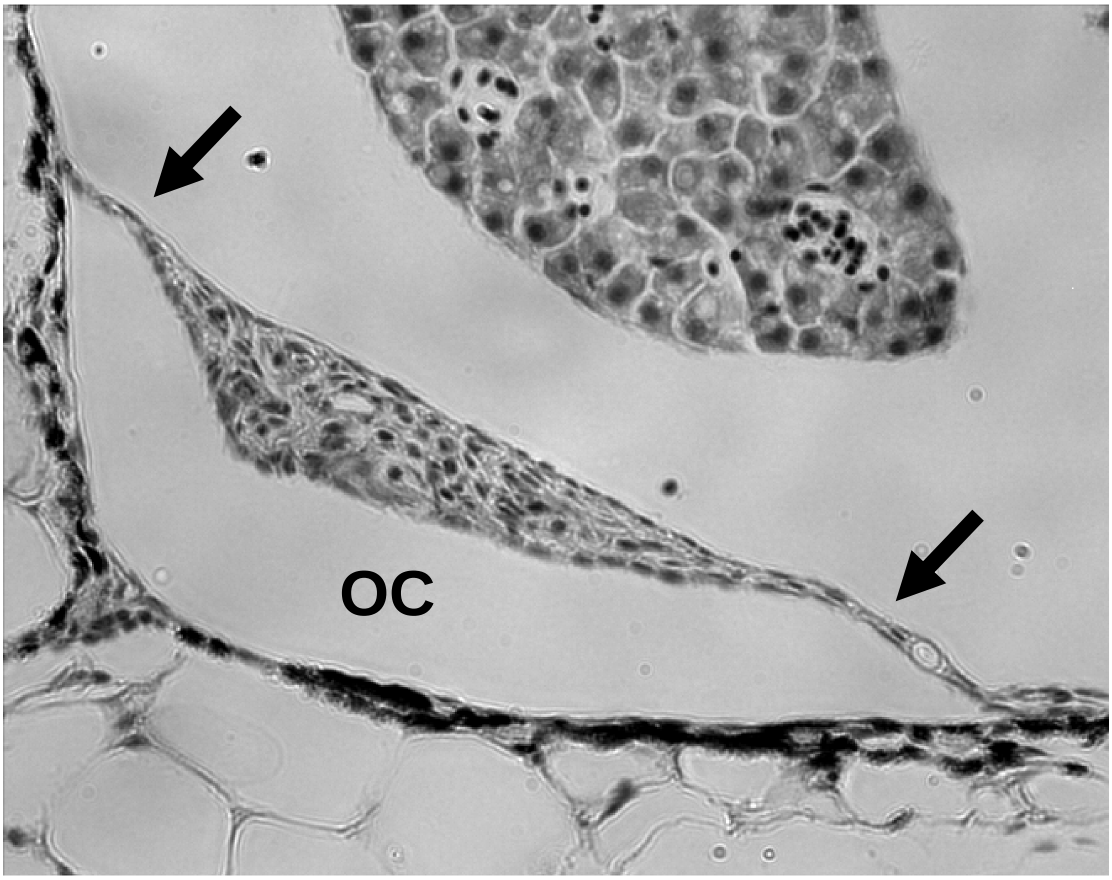
Figure Legends:

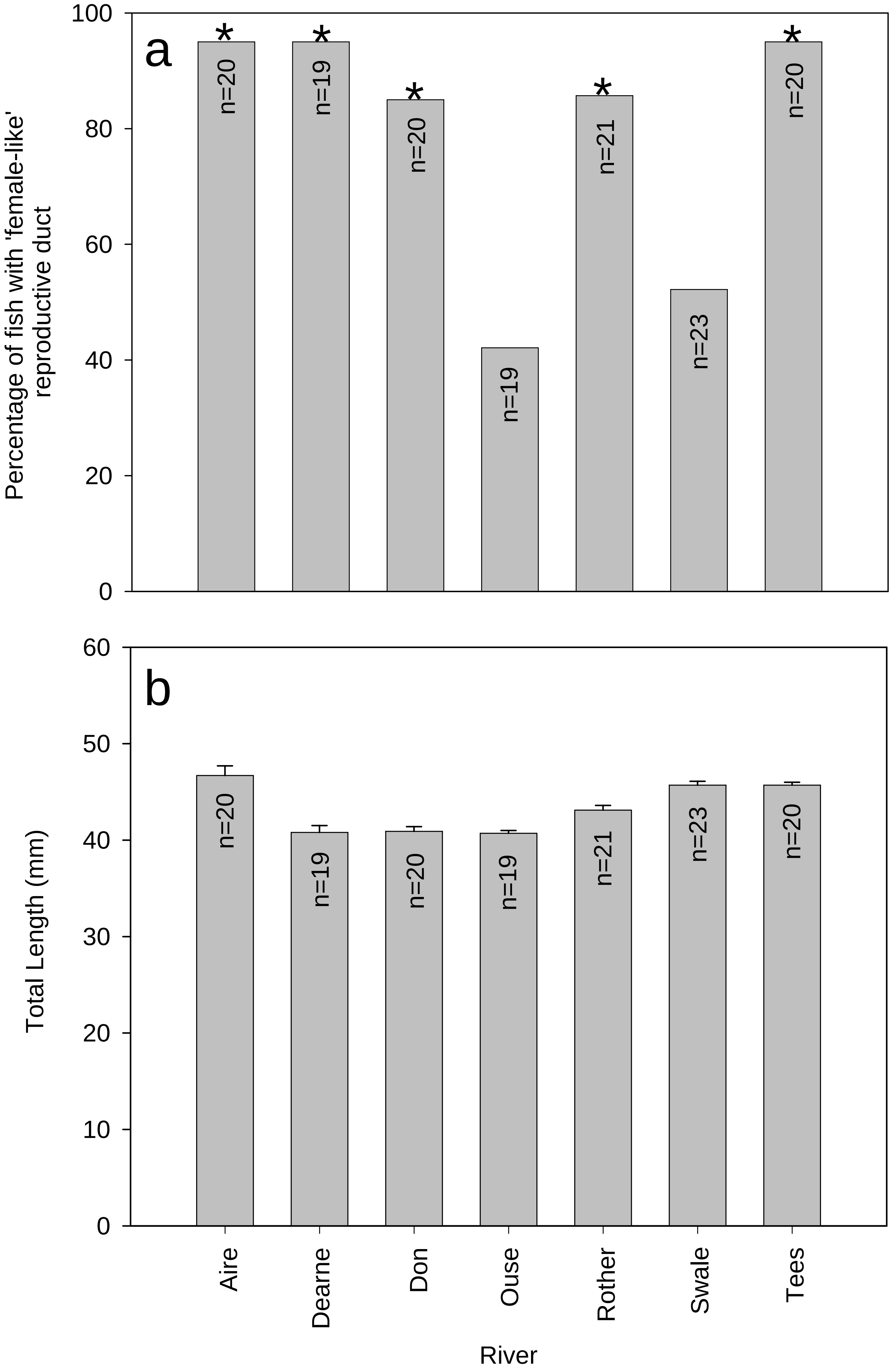
Figure 1. Gonadal histopathology of (a) a gonad with one point of attachment, and (b) a gonad with two points of attachment, forming an ovarian cavity (OC) [400x]. Arrows mark point(s) of attachment of the gonad to the body wall.

Figure 2. Duct morphology and size of juvenile roach collected from seven rivers. (a) Percentage of fish with a female-like reproductive duct, and (b) total fish lengths.

- Significantly different from the expected ratio of 1:1.




OC


a
b
Percentage of fish with 'female-like' reproductive duct
Total Length (mm)
River
100
80
60
40
20
0
60
50
40
30
20
10
0
n=20
n=19
n=20
n=19
n=21
n=23
n=20
*
Aire
Dearne
Don
Ouse
Rother
Swale
Tees