The identification of oestrogenic effects in wild fish

Brunel University
Environment Agency National
Fish Health Laboratory

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The Identification of Oestrogenic Effects in Wild Fish

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This output is the first Technical Report from Project W2/i636. It covers the period September 1995 to September 1997 and presents the progress towards the Agency's project's objectives within the overall research programme funded by the Natural Environment Research Council at Brunel University. It is to be used by Regional fisheries and water quality staff for information as to the state of oestrogenic impacts in fish and the association with sewage effluents. The report is also to be used by Agency staff involved with the project to target further investigations of fish reproductive health and impacts on fisheries.

Research Collaboration
This document was produced under R&D Project i636 in collaboration with Brunel University, the Natural Environment Research Council and the project team for the Environment Agency.

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EXECUTIVE SUMMARY

In the early 1980s, routine biological assessments of roach *Rutilus rutilus* from the River Lea, Hertfordshire, revealed abnormalities to reproductive organs. In particular, male roach were found to be *hermaphrodite,* (better termed, intersex) with eggs within the testis, indicating that the males had been feminised. Subsequent research identified sewage effluents as inducing oestrogenic (feminising) impact on fish. Although the oestrogenic nature of sewage effluents had been established, the extent of impacts on wild fish in UK rivers was not clear.

The Environment Agency has a range of statutory duties that aim to protect the environment and contribute to sustainable development through the effective control of pollution. It also has specific duties with regard to the maintenance, improvement and development of fisheries. The emerging knowledge was of concern and in 1995, the Agency developed a five-year collaborative research project with Brunel University, supported jointly with the Natural Environment Research Council, to assess the effects of oestrogenic effluents on fish populations. The aims of the initial research phase were to assess the extent and severity of oestrogenic effects in fish in rivers that receive sewage effluent and to evaluate links to water quality and effluent inputs. The first phase has now been completed and the report focuses on the findings of this part of the programme.

Sampling sites that held a resident population of roach, (including some that were designated under the EC Fisheries Directive) and were receiving effluent from sewage treatment works (STW) were selected. Some rivers were sampled up and downstream of effluents where there was an impassable barrier between sites. Agency fisheries staff caught up to 100 roach from 18 sites from a range of 8 rivers and 5 reference (no effluent inputs) locations. Fish were dissected to remove gonads and blood samples were subsequently analysed for plasma egg proteins (vitellogenin). Gonads were taken for histological analysis of the intersex condition (presence of eggs in testis tissue) and any further abnormalities. A numerical index of the intersex condition was developed, based on presence of numbers of eggs in testis tissue and other abnormalities such as the presence of an oviduct (scale from 0 all male tissue, to 7 all female tissue).

Fish were identified as male or female from microscopic examination, but microscopic analysis was required to measure the intersex condition. Although the intersex condition was observed in at least some male roach from all sites, the proportion of males with the condition varied, with reference sites having a mean value of less than 10 %, compared to two sites downstream of STW effluent discharges where all males were intersex. Most riverine sites were in the range of 25 to 60 % of males showing the condition. Reference, up and downstream site comparisons showed that the incidence in downstream fish was equal to or greater than the condition in the upstream fish. Reference sites had the lowest incidence, i.e. reference $<$ upstream $\geq$ downstream. A strong correlation ($p< 0.0002$, R^2 0.68) was found between the numbers of fish with the intersex condition and the adjusted population equivalent (population equivalent of the treatment works / dilution factor) for the relevant river reach. Similarly, fish downstream of sewage works discharges had a lower gonadosomatic index (gonad weight as a proportion of total body weight) and elevated plasma vitellogenin levels compared with reference sites. Tissue abnormalities ranged from a few primary eggs within the testis to many hundreds of secondary eggs and oviducts instead of sperm ducts. Severity of the effect (measured by intersex index) revealed that reference sites showed the lowest impact, with fish from upstream sites showing a higher severity, and the

downstream sites an even greater impact. i.e. reference < upstream < downstream. A correlation was found ($P<0.0002$, R^2 0.31) between the severity of impact in male fish and the adjusted population for the relevant river reach.

The intersex condition is widespread in fish in rivers but the severity varies, peaking where effluents receive little dilution. The correlation of the intersex condition, plasma vitellogenin, and gonadosomatic index to the proportion of sewage effluent in the river confirms the role of effluents influencing these effects in wild fish. However, strict cause and effect relationships between particular substances and biological responses remain elusive.

Fish from reference sites also showed some degree of intersex, indicating that this might arise spontaneously within a population. However, the frequency and severity of effect was low in reference fish compared with the river fish, indicating that river fish had been exposed to oestrogenic substances within sewage effluents. The intersex condition appears to be a permanent effect, whereas plasma vitellogenin and GSI are likely to vary with the degree of exposure and the time of year. Supportive work on gudgeon (and a limited extent on dace, chub and bream) shows the same effect as for roach, indicating effects in many fish species of ecological relevance.

The project's interim findings have established the priorities for the remaining two years of work, particularly to investigate the reproductive health of affected fish, to evaluate the intersex condition in another fish species and to investigate the association of such abnormalities to sewage effluent (and oestrogenic substances within effluent).

KEYWORDS

sewage effluent, fish, roach (Rutilus rutilus), gudgeon (Gobio gobio), oestrogen, endocrine disruption, reproduction

1. INTRODUCTION

1.1 Background

The endocrine system is a complex web of chemical messengers (hormones) and their receptors that control the vital processes of growth, metabolism and reproduction. Over the past 15 years, there has been increasing knowledge regarding the ability of a wide range of substances to interact with the endocrine (hormonal) system of organisms. Such substances, termed *Endocrine Disruptors*, have been shown to bind with hormonal receptors to stimulate or block biological processes, mimicking or interfering with the naturally-produced (endogenous) hormones and their actions. The potential for adverse impacts on wildlife and humans has led to investigations of the causes and consequences of exposure to such substances.

The components of the endocrine system that have received the most attention with respect to endocrine disruption are those associated with reproduction, particularly oestrogens (female) and androgens (male) (reviewed in Tyler, 1998). Examples of impacts on the reproduction in a range of organisms have been documented and attributed to specific substances. Some of the earliest data on the reproductive risks posed by endocrine disrupting substances was produced in the 1960's. In the Great Lakes Area of North America, the eggs from some gull colonies were found to be heavily contaminated with DDT, a toxic and persistent organochlorine pesticide (reviewed in Fry, 1995). Successful reproduction in these birds declined precipitously, whilst many of the female gulls were found to be sharing nests with other females (Fry *et al*, 1987). More recently, male alligators born in Florida's Lake Apopka (which received a large pesticide spill in 1980) have been reported to have phalli one half to one third the normal size (Guillette *et al*, 1996). In addition, the follicles in the exposed females possessed not only abnormal oocytes (eggs cells) but also a greater number of oocytes than normal (Guillette *et al*, 1994).

Abnormal occurrences of intersexuality have also been described in wildlife populations, for example the dog whelk *Nucella lapillus* ("imposex") and the mosquito fish, both of which were found to be related to environmental pollution. In the case of the dog whelk, the presence of male sexual organs in female whelks was found to be directly correlated with pollution of the marine environment by tributyltin (TBT) (Gibbs *et al*,1988; reviewed in Matthiessen and Gibbs, 1998). TBT has recently been shown to block aromatase enzyme activity thereby inhibiting the production of oestradiol and leading to elevated androgen concentrations. The induction of male secondary sexual characteristics in female mosquito fish was thought to be caused by effluents from paper mills; the precise nature of the causative agent is unknown (Bortone *et al*, 1989; Bortone, 1994).

In freshwaters, roach (*Rutilus rutilus*) from sewage treatment works lagoons, and downstream from sewage effluents discharged to the River Lea, were found to be "hermaphrodite". This early study in 1981 (Table 1.1) detailed a 5% incidence of hermaphroditism in samples of 100 mature roach at 2 separate sites (Thames Water, 1981). One or both of the gonads in these fish consisted of both ovarian and testicular tissue. In addition, the gonads of apparently normal specimens of either sex contained more ovarian tissue and less testicular tissue, determined by gonadosomatic index (gonad weight as a proportion of total body weight) than was found in other populations of roach at other sites. This indicated that the fish had been exposed to substances that induced a feminising, response.

Table 1.1 Incidence of hermaphroditism in two groups of roach captured at two separate sites on the River Lea.

SITE	Male	Female	Hermaphrodite	Parasitised
Rye Meads	16	55	3	26
East Hyde	72	18	6	0

The East Hyde group of fish were captured at a location on the river Lea, downstream of East Hyde sewage treatment works. Approximately 30% of these fish were infested with *Ligula intestinalis* (Parasitised), a parasite which prevents the development of the gonads. Rye Meads represents a sample fish capture from the final settlement tanks at Rye Meads sewage works.

1.2 Research Investigations

The findings in the River Lea catchment led to a concerted investigation to determine what factors might cause such responses in fish. The chemical influence on fish in the River Lea was hypothesised to be derived from sewage effluent discharges. Test bioassays were developed to assess whether the effluents contained causative agents that were oestrogen-like. Studies published by Purdom *et al* (1994) revealed that male fish placed directly in the sewage effluent showed physiological responses usually associated with high circulating concentrations of oestrogens, that is, they produced very large amounts of the female-specific blood egg-protein vitellogenin. This nation-wide survey showed that this effect was common to most sewage effluents, although it varied in its magnitude between individual sewage works discharges.

The research by Purdom *et al* (1994) showed that the presence of vitellogenin in blood represents a valuable biomarker for the exposure of fish to oestrogens in the aquatic environment. It was clear that sewage effluents were capable of causing oestrogenic effects in fish, but at that time the main oestrogenic contaminant(s) had not been identified. Similarly, whether the oestrogenic contaminants could cause the hermaphrodite condition was not known.

Further research designed to assess the oestrogenic impact of effluents in rivers demonstrated significant effects in caged fish as far as 5km downstream from the point of discharge (Harries *et al*, 1996). This response ranged from a slight elevation of blood levels of vitellogenin to a large rise, enlarged livers and suppressed testis growth. The enlargement of liver tissue was seen as a direct response to the excessive vitellogenin production from liver hepatocyte cells. Similar effects were observed directly after exposure in the laboratory to the oestrogenic chemicals nonylphenol (Jobling *et al*,1996), and the human contraceptive hormone ethinyloestradiol (Purdom *et al*,1994).

In addition to the biological responses and effects in fish, there was a need to identify which substances in sewage effluents were capable of causing the observed responses. The Environment Agency published results of a detailed investigation which assessed seven sewage effluents for

oestrogenic activity (Environment Agency, 1998). The effluents were fractionated into chemically-similar components, and each fraction was tested for oestrogenic activity in an oestrogen-specific bioassay. The causative substances were subsequently identified and quantified. The project concluded that, within effluents that were mainly domestic in origin, the most significant oestrogenically-active substances were the natural steroid hormones 17β-oestradiol and oestrone and the synthetic hormone ethinyloestradiol. In subsequent dose-response experiments (steroid dose vs induction of vitellogenin synthesis), it was shown that there was sufficient hormone in effluents to induce the vitellogenic responses which characterised previous field research. In addition, there was some evidence of additivity between the hormones, indicating that it was the total steroid concentration that determines the oestrogenic activity of the effluent.

1.3 Establishing Causes and Consequences of Endocrine Disruption

Several highly-toxic substances, including the insecticide DDT, the antifoulant paint active ingredient tributyltin and the combustion bi-products polychlorinated-*p*-dibenzodioxins, have now been shown to act upon the endocrine system. These substances are already subject to regulatory control in Europe due to their toxicity and persistence. However, concern about endocrine disrupting substances has become more acute recently because many substances that interact with the endocrine system are less toxic (and not subject to regulation), including certain alkylphenolic chemicals, natural and synthetic steroids, some phthalates, as well as a number of pesticides (Tyler, 1998). Most of these substances are present in the environment and hence wildlife populations (and humans) could be exposed to them via a number of routes. These research presented in Sections 1.1 and 1.2 are just a few of many studies linking reproductive changes with exposures to endocrine disrupting substances. However, developing strict causal relationships between substances and effects in the environment remains elusive due to the complex nature of inputs to the environment and interactions between substances.

Several recent reports have also identified declines in semen quality in humans over the past four decades and increases in cancers such as breast, prostate and testicular in which oestrogens play a role (reviewed in Toppari *et al*, 1995). However, it should be emphasised that some scientists have questioned many of the reported adverse effects, and some studies have not been repeatable. It is presently very unclear what effects are produced by exposure to hormone-mimicking chemicals, and what doses/concentrations are required to induce these effects.

1.4 Quantifying Impacts on Natural Populations

Since 1993, there have been many scientific workshops in the United States and Europe (for example European Commission, 1996) to address the issue of endocrine disruption. These workshops concluded that the findings of the scientists around the globe were of sufficient concern to warrant concerted research on the issue. About 50 synthetic (and some natural) chemicals have been found to interfere with the workings of the hormonal or endocrine system. Although the scientific community agrees that exposure to large doses of these chemicals can cause serious damage to the reproductive system, it is uncertain whether similar, but more subtle, effects are occurring in the general population.

In the UK, the fact that sewage effluents are oestrogenic leads to the hypothesis that exposure to such effluents can influence the natural indigenous fish populations downstream of the discharge point. Reproductive effects have consequences for successful reproduction, recruitment and population dynamics. It is clear, therefore, that some evaluation of the impact of oestrogenic pollution on natural populations of animals is needed.

1.5 Collaborative Research Programme

In April 1995, the Natural Environment Research Council (NERC) funded Brunel University to investigate the effects of environmental endocrine disrupters (primarily oestrogenic substances) on wild fish.

The objectives of the NERC-funded programme are:

- *to determine whether the oestrogenic chemicals in sewage treatment work effluent are affecting native fish;*

- *to determine the severity of these effects (if they occur), and assess whether the reproductive potential of the fish is impaired.*

These objectives directly relate to the Environment Agency's statutory duties for the maintenance, improvement and development of fisheries and the control of pollution in the aquatic environment. The programme of work was developed with the collaborative support of the Environment Agency under the auspices of the Agency's Concordat with NERC which ensures that fundamental research is applicable to relevant end-users.

An important prerequisite for detailed controlled laboratory and field trails on endocrine disruption is to assess the natural status of the native, wild populations of fish. The information generated determines whether there is an oestrogenic impact on wild fish and leads on to sound hypothesis development and experimental design. A collaborative study on the reproductive health of wild fish was developed as the initial phase of the NERC programme, with the overall objective:

to identify and assess oestrogenic effects in riverine roach *Rutilus rutilus* and gudgeon *Gobio gobio* in British rivers of varying water quality.

The specific objectives were:

- to collate and assess information on the known impact of oestrogenic substances on fish (blood; gonad; health);

- to determine measurable parameters which indicate an oestrogenic response in fish;

- to develop criteria for the selection of river sites and overall experimental design, to include: availability of fish; access; ease of sampling/manpower requirements; co-ordination and liaison with sites sampled for National R&D Project 490, and DoE Projects; statistical requirements; inter and intra-river controls;

- to identify appropriate rivers and sites suitable for collection and sampling of roach and gudgeon;
- to undertake the sampling programme;
- to perform histological analysis on gonadal material and analysis of blood-plasma parameters;
- to collate the results and assess in the context of oestrogenic effects and correlation where possible with water quality data;
- to produce a report presenting the assessment of oestrogenic responses in native wild fish.

The Agency, through its routine fisheries sampling and investigation programme in England and Wales, has undertaken the capture of fish from a range of sites. The Agency's National Fish Health Laboratory, which undertakes specialist fish health and histological analysis, has processed the samples and jointly evaluated and interpreted the histological sections.

2. RESEARCH STRATEGY

2.1 Experimental Design

The objective of the project was to identify the level of intersex in fish populations living in rivers of varying quality and to determine whether reproductive abnormalities were linked with point source effluent input. Wild fish samples were taken from rivers of varying quality (using criteria based on the Environment Agency's General Quality Assessment Scheme) particularly above and below point sources of effluents where possible. It was proposed to employ a wide range of analytical techniques on the sampled fish, in order to provide as clear a picture of their reproductive condition as is possible.

2.1.1 Selection of test fish species

The roach was chosen as the indicator species for several reasons:

- it is the most common coarse fish in British lowland rivers, often comprising over 50 % of the biomass of the coarse fishery;
- intersex has been reported in wild roach at a very limited number of sites in British rivers;
- the "biomarker" for oestrogenic contamination, the egg-protein vitellogenin, can be detected and accurately measured in blood from this species using an established assay.

The gudgeon was chosen as a second study species, due to its benthic habit, which may lead to it being exposed to contaminants that were associated with the sediment rather than the water column. Where possible, 100 roach were collected from each site. The relatively high sample number was chosen to ensure sufficient numbers of both males and females, and also sufficient numbers of intersex animals for analysis. The sample size was calculated to ensure that subsequent statistical analysis would be soundly based. Although it was initially proposed to collect similar numbers of gudgeon from all sites, this proved to be impractical due to both fishing and sampling time constraints. However, gudgeon were obtained from some of the sites where numbers of roach were low.

2.1.2 Selection of sampling sites

The rivers were selected based on the following criteria:

- presence of the selected species (roach and gudgeon);
- inputs of sewage effluent to river;
- support of Agency Fisheries staff to collect fish;
- whether the site had undergone previous investigations (in order to build on the existing knowledge base);
- overall list of sampling sites to represent a range of water qualities.

Wherever possible, 2 sites per river were sampled with one site in a reach downstream from a sewage treatment works discharge point and the other upstream. An important prerequisite was that the upstream and downstream sites should be separated by a physical barrier, such as a weir or lock. This barrier was expected to prevent the downstream fish migrating upstream, but would not necessarily prevent downstream movement of fish.

2.1.3 Sampling programme

The initial sampling programme was undertaken in September and October 1995 and was completed by the end of October 1996. It was hoped that all of the necessary samples would be collected in 1995 over a 6-8 week period. However, because the abnormalities were found to be much more prevalent than was expected, even in the better quality rivers studied, a second sampling programme was undertaken in 1996 at the same time of the year in order to provide fish from the necessary control rivers/still waters .

Finding control sites proved difficult, since it was not possible to find rivers in the UK that were both inhabited by roach, and did not receive sewage discharges. A selection of canals and still-waters were located in England and Southern Ireland. Roach were sampled and the material was taken for analysis. These sites were then visited in October 1996, together with four additional "control" sites. Table 2.1 provides a summary of the 22 sites which were sampled and gives the numbers of fish obtained at each site.

Roach maintained at the National Coarse Fish Farm at Calverton were used as a control. These fish were maintained in ponds and over-wintered in bore-hole water, under normal fish culture conditions. Samples of roach aged 2+ years were maintained at Calverton Fish Farm and sampled throughout 1995 and 1996 in order to obtain a seasonal profile of the parameters under study. Careful documentation of gonadal development in both male and female roach was not available from the literature and this information was essential for the interpretation of the field data.

2.2 Analysis of Fish Samples

2.2.1 Field measurements

The fish were electrofished and samples of gonads, blood, and scales (for age determination) were removed from the fish on site. Meristic measurements of length and weight were also taken. Analysis of fish age, histological sectioning and preliminary analysis of the gonads was undertaken at the Agency's National Fish Health Laboratory. Analysis of the histological sections for gonadal abnormalities and plasma for vitellogenin was conducted at Brunel University.

2.2.2 Endocrine Parameters

Blood plasma samples were assayed for vitellogenin by radioimmunoassay developed for carp (*Cyprinus carpio*). Measurement of plasma vitellogenin concentrations (in males) provides a very good index of overall oestrogen exposure (Tyler *et al*, 1996).

Table 2.1 Sampling programme undertaken for wild roach and gudgeon

Region	River / stillwater	Site	Date	Fish Caught
Midlands	Wreake	D/S Melton Mowbray STW and weir	September 1995	62 male roach 38 female roach
Midlands	Eye	U/S Melton Mowbray STW and weir	September 1995	32 male roach 68 female roach
Midlands	Trent	Wolsey Bridge	September 1995	38 male roach 43 female roach 3 male gudgeon
North East	Aire	U/S Silsden Bridge	September 1995	7 male gudgeon
North East	Aire	Crossflats, Leeds just D/S Marley STW	September 1995	33 gudgeon 4 roach 2 brown trout
North East	Aire	Thwaite Weir, 7.5 km D/S Marley STW, U/S Nostrop STW	September 1995	36 male roach 36 female roach 10 male gudgeon 13 female gudgeon
North East	Aire	Swillington Bridge D/S Nostrop STW	September 1995	2 male roach 16 gudgeon
Thames	Lea	U/S East Hyde STW above weir		22 male roach 16 female roach
Thames	Lea	D/S East Hyde STW		32 male roach 38 female roach 23 male gudgeon 18 female gudgeon

Table 2.1 (Cont/d...) Sampling programme undertaken for wild roach and gudgeon

Region	River / stillwater	Site	Date	Fish Caught
Anglian	Nene	D/S Billing STW	October 1995	31 male roach 39 female roach
Anglian	Nene	U/S Billing STW, Upper Heyford bridge, above Northampton	October 1995	29 male roach 36 female roach
Midlands	Avon	Evesham	October 1995	38 male roach 34 female roach
Southern	Arun	D/S Horsham STW, D/S weir	October 1995	21 male roach 33 female roach
Southern	Arun	U/S Horsham STW	October 1995	16 male roach 45 females
Midlands	Rea	trout fishery	October 1995	16 male roach 45 female roach
North East	Yorkshire Ouse	U/S Naburn STW, above York	October 1995	28 roach
North East	Yorkshire Ouse	D/S Naburn STW	October 1995	81 roach
Midlands	Grantham Canal	Wartnaby	April 1996	17 male roach 36 female roach
Midlands	Wartnaby Lake	Wartnaby	July 1996	41 male roach 14 female roach

Table 2.1 (Cont/d...) Sampling programme undertaken for wild roach and gudgeon

Region	River / stillwater	Site	Date	Fish Caught
Midlands	Calverton Fish Farm		ongoing, monthly from November 1995	
Midlands	Grantham Canal	Grantham Canal	October 1996	18 male roach 25 female roach
Midlands	Wartnaby Lake	Wartnaby	October 1996	25 male roach 26 female roach
Anglian	Lincoln Counter Drain	Counter Drain	October 1996	23 male roach 31 female roach
Irish Fish Board	River Dee Southern Ireland	Tom's Bridge	October 1996	20 male roach 34 female roach
Irish Fish Board	Royal Canal Southern Ireland	Kilcock	October 1996	22 male roach 29 female roach
Irish Fish Board	River Finn Southern Ireland	Ann's Bridge	October 1996	50 roach

2.2.3 Gonadal Growth and Histology

The gonadosomatic index (GSI) was determined for all fish. Gonadal tissue was fixed in Bouins fixative for six hours and then stored in 70% alcohol until taken and prepared for histological sectioning. Ovaries and testes were then cut into three (anterior, median and posterior) portions and all three were then mounted in the same wax block. Sections were cut on a sledge microtome at 3 : m, floated on a water bath and mounted on slides with *Vectabond* adhesive. A total of six sections were prepared per fish; 1 section from each of the anterior, mid and posterior regions of each gonad. Slides were stained with Mayer's Haematoxylin and Eosin, sealed with DPX mountant and examined under light microscopy.

Before assessing the nature of the oestrogenic effects, it was necessary to consider what biomarkers of effect might be encountered. Gonadal sex can be manipulated in many teleost fish by exposure to very high doses of sex steroids (either oestrogen's or androgens) or aromatase (the enzyme responsible for the conversion of androgen to oestrogen) inhibitors (Piferrer *et al*, 1994). The labile period, when fish are most susceptible to sex reversal, is the time prior to morphological sex differentiation, specifically, just following hatching or at the juvenile stage. In this study, it was impossible to determine the genetic sex of the fish that were examined, as sex-specific probes for this species are not available. Therefore, whether the incidence of the intersex condition was due to feminisation of genetic males or masculinisation of genetic females, respectively, could not be established. However, the normal external appearance of male testes that subsequently were found to have microscopic abnormalities the strongly suggests that the incidence of the intersex condition was due to feminisation of genetically male fish, rather than to masculinisation of genetic females.

Sexual differentiation is a two stage process, involving gonadogenesis (the formation of the structural and supporting elements of the gonad - the female ovarian cavity and oviduct, or male sperm duct) and gametogenesis (the proliferation and differentiation of the germ cells which will eventually form the eggs and sperm in females and males), respectively. High doses of steroid hormones (such as oestrogens and androgens) are capable of redirecting development so that either the reproductive ducts and/or the undifferentiated germ cells differentiate in a manner opposite to that of the genetic sex of the individual (reviewed in Hunter and Donaldson, 1983). Similarly, exposure to high doses of chemicals that mimic oestrogens has also been shown to cause feminisation of the ducts and/or the germ cells if exposure occurs during early life (Gimeno *et al*, 1996; Gray and Metcalfe, 1997).

In view of this information, both the presence of developing eggs (oocytes) and/or an ovarian cavity were used as diagnostic features to characterise and quantify the intersex condition in the fish captured during this study.

Table 3.2 Characteristics of the Fish Capture Sites

RIVER	NAME	TYPE OF SITE	POPULATION EQUIVALENT	DILUTION FACTOR	EFFLUENT CONCENTRATION
F	WREAKE/EYE	UPSTREAM	429	1666	1
		DOWNSTREAM	51950	8.6	6037
G	OUSE	UPSTREAM	5000	3125	2
		DOWNSTREAM	198546	69.9	2829
H	LEA	UPSTREAM	N/A	N/A	N/A
		DOWNSTREAM	130393	1.8	73320
I	ARUN	UPSTREAM	1732	55.6	31
		DOWNSTEAM	107250	3.8	28636
J	NENE	UPSTEAM	22143	9.8	2270
		DOWNSTREAM	285174	4.8	58891
K	TRENT	DOWNSTREAM*	982	3333	1
L	REA	DOWNSTREAM	2000	17.1	117
M	AIRE	DOWNSTREAM	674717	7.1	94939

*Unlike other "downstream" sites, this site may have been impacted by effluents from many diffuse sources, rather than a single dominant point source.

Descriptions of each capture site are given with regard to the characteristics of the sewage treatment works directly upstream of the capture point. The population equivalent (P.E) can be described as a measure of the "strength" of the influent of a works: 1 P.E is the amount of organic biodegradable load which has a biochemical oxygen demand (BOD5)of 60g of oxygen per day. The average dilution factor of the effluent in the river at the capture site was calculated using hydrometric data on monthly river flows, together with actual sewage flows, in order to provide an estimate of the dilution factor of effluent in the river over a period of several years (encompassing the life spans of the captured fish wherever possible). The use of either of these variables (the dilution factor or the P.E) alone does not adequately explain the concentration of the effluent in the river at a particular point. For example, highly concentrated effluents from large sewage treatment works have little impact if their dilution in the river is large. Conversely, sewage treatment works with smaller population equivalents may cause a larger impact if the dilution factor is low. The absolute concentration of effluent at each site could thus be approximated by adjusting the population equivalent to allow for the degree of dilution of the effluent in the river. For the purposes of this comparison, equivalent treatment at the STWs is assumed. These figures are given in column 6 as adjusted population equivalents (to the nearest whole number).

intersex fish in the sample and the concentration of the effluent (expressed as adjusted population equivalents), presented in Figure 3.4.

Using data obtained from all sampling sites, a positive *relationship* was found between the concentration of effluent when regressed against the intersex index (r^2=0.312, p<0.0001, n=150) (Figure 3.5).

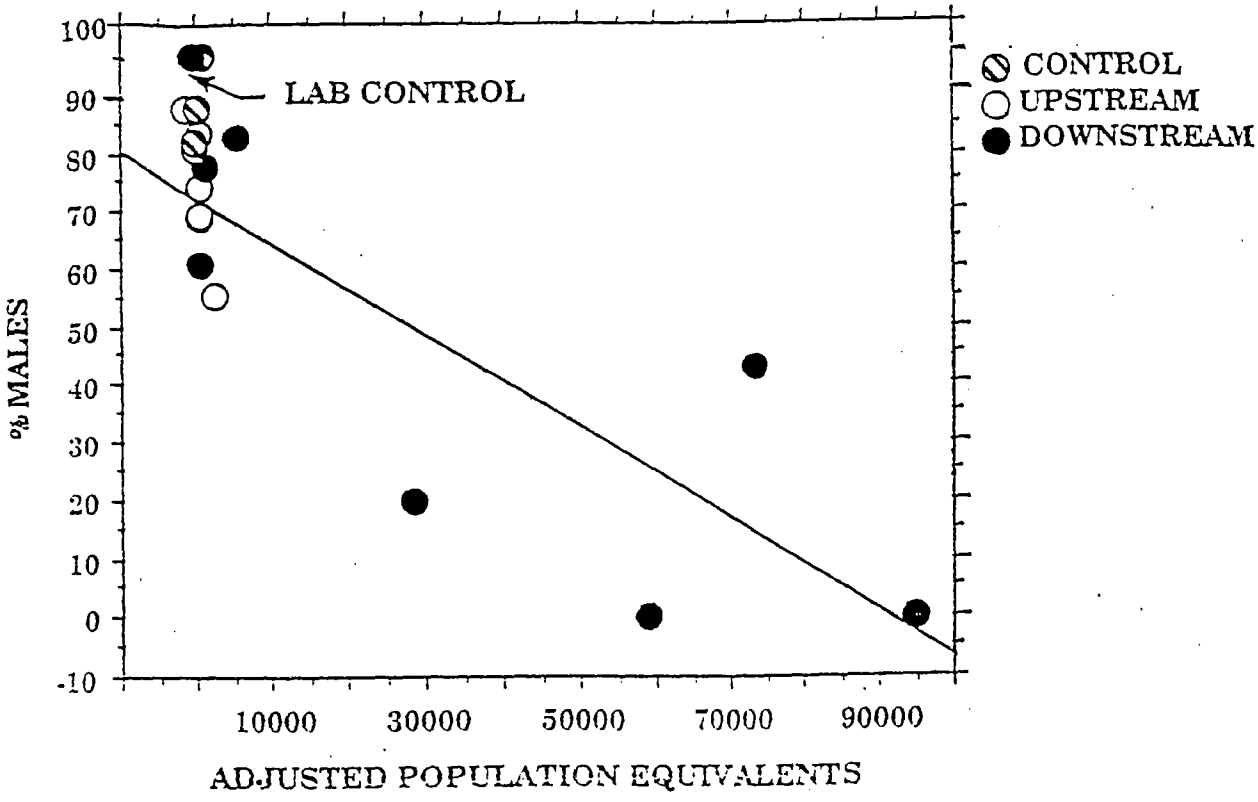

Figure 3.4 Correlation between the concentration of sewage effluent in river water and the incidence of the intersex condition in roach

Refer to Table 3.2 for derivation of adjusted population equivalent.
Each point represents the mean intersex index of individual fish. Where this overlaps, the point is slightly displaced.

4 DISCUSSION

4.1 Oestrogenic Responses in Wild Fish

The investigation has quantified the incidence of oestrogenic impacts on a species of wild fish in English rivers. The comparatively low incidence of the intersex condition seen at many of the reference sites concurred with the literature, as being a rare phenomenon, although historically the intersex condition has been assessed macroscopically (Jafri *et al*, 1979; Schulz, 1996). Incidences of intersex of up to 5% have been reported at the microscopic level in the carp (*Cyprinus carpio*, a cyprinid fish related to the roach; Komen *et al*, 1989). It is impossible to determine whether a low incidence of the intersex condition is "natural", arising spontaneously within a population or due to diffuse sources of oestrogenic substances in the environment, such as those present in run-off from agricultural land, or from groundwater seepage from septic tanks or farm waste. Overall, the results from this study indicate that in English rivers the intersex condition can arise, and the incidence in male roach is, many cases, considerably higher than the apparently normal baseline.

The intersex condition is widespread in male roach fish in rivers but the severity varied. Some fish were found to have few primary oocytes within apparently normal testis tissue. In extreme cases, ovarian tissue dominated, with the characteristic lobular appearance of the testis tissue being reorganised into small groups of spermatocyte cells. Even in these severely affected fish, the external appearance of the testis did not appear abnormal. Therefore, microscopic examination is required to reveal the nature of the abnormalities and suggests that previous assessments of riverine populations (see Table 1.1) underestimated the actual extent and severity of the intersex condition.

The presence in males of oviducts, as opposed to sperm ducts, is further evidence for exposure oestrogenic substances. Gimeno *et al* (1997) identified female cells in juvenile male carp testes after exposure to oestrogenic substance tertiary pentylphenol. The presence of an oviduct in many male roach suggests that this response may be as sensitive as that of the formation oocytes in testes. Furthermore, as gonadogenesis in cyprinids takes place at the juvenile stage, the change to such cell types suggests that the males were exposed, and therefore responded, to oestrogenic substances at the juvenile stage.

Other parameters assessed in the study, plasma vitellogenin and gonadosomatic index (GSI), provided strong supporting evidence that the river fish were being exposed to oestrogenic substances. In male and intersex fish below sewage effluent discharges, vitellogenin concentrations were significantly elevated compared to their respective fish at reference sites. Vitellogenin was significantly elevated in male and intersex fish taken from upstream sites, suggesting that other upstream oestrogenic inputs, possibly some distance away, were also affecting the fish.

Although the presence of vitellogenin in the blood of male fish is widely accepted as a biomarker of oestrogen exposure (Sumpter and Jobling, 1995), inhibition of testes growth in male fish has also been reported as a consequence of exposure to oestrogens (Billard *et al*, 1981; Jobling *et al*, 1996). In this study the gonadosomatic index (GSI) was suppressed in both males and females fish from riverine sites compared to reference sites. Taken together, the suppression of gonad

growth in females could be associated with the lower vitellogenin concentrations measured.

The intersex condition appears to be a permanent effect, whereas plasma vitellogenin and GSI are likely to vary with the degree of exposure and the time of year. Supportive work on gudgeon (*Gobio gobio*) (and a limited extent on dace, *Leuciscus leuciscus*, *chub Leuciscus cephalus* and bream *Abramis brama*) shows the same effect as for roach, indicating effects in many fish species of ecological relevance. In the preliminary observations in gudgeon, the similar findings to that seen in the roach indicates that the effects seen are not likely to be species specific.

Taken together the plasma vitellogenin and GSI data, with the observations on the intersex condition provide compelling evidence that populations of wild fish inhabiting many rivers are being exposed (and affected by exposure) to oestrogenic contaminants.

4.2 Causality and Correlation with Sewage Effluents

Both the incidence and severity of the intersex condition were positively correlated with proportion of sewage effluent at those river sites. This information strongly indicates that the intersex condition was caused by exposure to STW effluent. The data also confirm previous research which found that fish caged within and below sewage outfalls showed oestrogenic effects in response to exposure to substances within the effluent (Purdom *et al*, 1994; Harries *et al*, 1996). In these studies, the responses observed were elevated blood vitellogenin concentrations and in extreme situations, suppressed GSI.

Severity of the intersex condition appeared to peak where effluents received little dilution in the river, and not necessarily at sites receiving the "largest" effluents (determined in terms of population equivalent). The correlation of the intersex condition, plasma vitellogenin, and gonadosomatic index to the proportion of sewage effluent in the river also confirms the role of effluents influencing these effects in wild fish.

The data indicated that the mean intersex index in a population of roach could be estimated using the average concentration of sewage effluent in that river, although the correlation was less strong than for the incidence. The scatter of the data points suggested that the intersex index (and hence, the degree of exposure to oestrogen) may vary widely, even within a population of fish sampled from the same site. This may relate to differences in the timing and duration of exposure to oestrogen due to differences in fish movement and migration in that watercourse. Although on many of the rivers studied, physical barriers between upstream and downstream populations of fish may prevent the upstream migration of fish, the downstream movement, and therefore, mixing of upstream and downstream populations, could not be ruled out.

Whilst a "typical" effluent does not exist, attempts to identify the main oestrogenic chemicals using a toxicity-based fractionation approach has demonstrated that both natural (oestradiol and oestrone) and synthetic oestrogens (ethinylestradiol, from birth control pills), presumably excreted by humans and alkylphenolic compounds are found in STW effluents (Environment Agency, 1998). Furthermore, the concentrations of natural and synthetic oestrogens in effluents (natural oestrogens in the tens of nanograms per litre range) are high enough to induce vitellogenin synthesis in male fish maintained in the laboratory. Other authors (Stumpf *et al*, 1996) have reported similar concentrations of natural and synthetic oestrogens in effluents and river water in

other European countries. Alkylphenolic compounds are major constituents of some industrial effluents (Ahel and Giger, 1985; Blackburn and Waldock, 1995) and STW discharges containing high concentrations of alkylphenolic compounds have been shown to induce oestrogenic effects in fish for long stretches in the river below the discharges (Harries *et al*, 1996, 1997). Strict cause and effect relationships between particular substances and biological responses observed in this study remain elusive.

The results presented in the report strongly suggest that the concentration of sewage effluent in a river is a major causal factor in the evolution of the intersex condition in wild fish. The association between the degree of intersexuality and the plasma vitellogenin concentration suggests that the two biological effects have a common cause and that the oestrogenic constituents of sewage effluents are responsible for the occurrence of intersexuality in wild fish populations.

4.3 Implications for Fish populations

The presence of oocytes and oviducts in male testis, reduced GSI and elevated vitellogenin could clearly have adverse effects on the reproductive capacity and general health of the fish, although the ecological implications have yet to be determined. It is possible that the testis can function in intersex fish in the normal way, producing viable sperm at spawning. However, it is also possible that abnormal development of the sperm duct, as a consequence of exposure to oestrogenic substances, will prevent the release of the sperm. Both the quality and quantity of gametes produced in these fish needs to be assessed to determine the physiological significance of intersexuality to the individual fish. Any effect on gamete production and / or quality to the individual may impair the contribution of that fish to the population.

The survival of a fish population is determined by the size of the spawning stock and the annual number of offspring (recruits) produced, together with the subsequent survival of these recruits. From on the observations in this study, it is probable that populations of roach in at least some locales are adversely affected. The effect on the population from this impact will need to be assessed in comparison with other pressures on fish populations, for example, habitat, general water quality and food in order to determine its significance.

Effluent characteristics may show seasonal variations, due, at least in part, to differences in rainfall. The average annual flow rate in the River Nene at one gauging station was almost 4 times higher in 1993 than in 1991. In the summer months, river flow fell to zero at some sites. The significance of these fluctuations in river flow (and effluent concentration) is realised when the roach (like many UK cyprinid fish) spawn in the spring, and sexual differentiation in the juveniles occurs during the summer months, when effluent concentrations are at their highest. In some cases sewage effluent comprises 100% of the river flow. Periods of drought, such as those experienced in the UK during 1995 and 1996, would be expected to have a pronounced effect on the incidence of intersex. Further work is required to investigate the incidence and severity of the intersex condition in fish of known age to establish whether they are correlated with the year of hatch and sexual differentiation.

5. CONCLUSIONS

The main project findings are:

- Wild male roach in rivers that receive sewage effluent exhibited a range of gonadal and physiological abnormalities consistent with exposure to oestrogenic substances;
- Abnormalities in male roach ranged from elevated plasma vitellogenin and suppressed testis growth to the presence of large numbers of oocytes in the testis tissue and a substitution of sperm ducts by oviducts;
- The extent of abnormalities varied between reference sites and river sites upstream and downstream of sewage effluent discharges;
- A strong positive significant correlation was found between the incidence of the intersex condition and the proportion of sewage effluent in the river;
- A significant positive correlation was found between the severity of the intersex condition and the proportion of sewage effluent in the river;
- The intersex condition was evident at reference sites but at significantly lower proportions than river sites;
- Although the reproductive and general health effects of the measured abnormalities are not clear, it is possible that reproduction of individual fish may be impaired;
- The significance of the impacts on the male fish at the population level requires further investigation.

6. FUTURE WORK WITHIN EXISTING RESEARCH PROGRAMME

6.1 Relationship between the Intersex Condition and Fish Age

It is currently unclear from the data whether the differences in the intersex condition in fish varies with age and sewage concentration due to the length of the exposure period to sewage effluent (e.g. 4 years versus 7 years), and/or to the timing (in the fish's life cycle) of the exposure to effluent. If early life stages of fish are the more susceptible to endocrine disruption than sub adult/adult fish, as has been suggested in the literature, then it is likely that the exposure to effluent encountered during the year of hatch will be a key factor in determining the degree of intersexuality. This cause-effect relationship is suggested for the 1995 and 1996 year classes but more fish samples are required to generate sufficient data to test this hypothesis.

6.2 Investigation of Reproductive Abnormalities in other Fish Species

Further assessments will be made in the second chosen fish species, the gudgeon, to determine whether intersex is a phenomenon .common to fish in UK rivers exposed to STW effluents.

6.3 Assessment of Cause and Effect Relationships to Sewage Effluents

Current work is assessing whether exposure of juvenile, 1+ and 2+ roach to sewage effluents causes the reproductive abnormalities documented in roach from rivers. These fish are being exposed to a range of dilutions of oestrogenic sewage effluent in an experimental tank system over extended periods (3-6 months). The effluent is also being analysed for oestrogenic contaminants, including oestrogenic steroids and alkylphenols. The data will provide further information on the quantity of sewage effluent (and concentration of oestrogenic substances) required to induce the intersex condition. Fish of ages 35 days to 2 + years will be exposed to provide evidence for sensitivity of the different life stages to oestrogenic substances, particularly at sexual differentiation.

6.4 Assessment of Cause and Effect Relationships to Oestrogenic Substances

In support of the sewage effluent exposure experiments, a programme of work is underway to assess the response of fish to individual, and mixtures, of oestrogenic substances present in STW effluents at environmentally-relevant concentrations.

6.5 Assessment of Reproductive Health of Wild Roach

Future work will assess the reproductive competence of intersex roach. This is pivotal in determining the potential impact of widespread intersexuality in wild roach populations. This work will involve collecting mature wild roach from spawning grounds in selected rivers where populations are heavily impacted by endocrine disrupters and comparing gamete production and quality with control populations from reference sites and captive fish stocks held at Calverton Fish Farm.

In these studies the aim will be to determine whether intersex fish can produce functional gametes, and if so whether they can be released (whether the gonadal duct is functional). The quality of gametes that are released (both eggs and sperm) will be assessed through breeding experiments and supported by histological assessments of the spawned fish.

6.6 Assessment of Impacts at the Population Level

Following on from the work presented in sections 6.1 to 6.5, population data for fish at the impacted sites will be collated. This will be to determine whether the presence and absence of fish (whether intersex or "normal") in those populations can be correlated to the oestrogenic impacts of sewage effluents.

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