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Widespread Sexual Disruption in Wild Fish

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A number of chemicals present in the environment have been shown to mimic or antagonize the actions of steroid hormones, an issue often described as "endocrine disruption/modulation". There is very little evidence, however, to support the hypothesis that exposure to endocrine-disrupting chemicals is a global environmental health problem. In this paper, we demonstrate a high incidence of intersexuality in wild populations of riverine fish (roach; *Rutilus rutilus*) throughout the United Kingdom. These reproductive disturbances are consistent with exposure to hormonally active substances and are associated with discharges from sewage treatment works that are known to contain estrogenic chemicals. This is the first documented example of a widespread sexual disruption in wild populations of any vertebrate and indicates that reproductive and developmental effects do result from exposure to ambient levels of chemicals present in typical British rivers.

Introduction

One of the most controversial issues in environmental science today concerns the potential risk to humans and wildlife posed by exposure to both natural and/or man-made chemicals that may interfere with reproduction and development (*1*−*3*). Considerable scientific evidence indicates that a multitude of environmental contaminants can modulate or mimic the actions of steroid hormones and, in some cases, produce biological responses qualitatively similar to those produced by endogenous hormones. Indeed, hormonal activity has become a widely recognized mechanism of toxicity, and a number of laboratory studies have shown that exposure to endocrine-modulating substances can impair reproductive function in adults of either sex, lead to irreversible abnormalities when administered during development, or cause cancer (see reviews in refs *4*−*7*). In addition, there are a few examples in wildlife and humans where high-level exposure (usually emanating from accidental chemical spills or from pre-existing residues of highly bioaccumulative substances) has resulted in well-documented, but geographically limited, population-level effects (*8*−*14*). Most of the chemicals that mimic hormones are many orders of magnitude less potent than their endogenous counterparts, and it therefore seems unlikely that low-level exposure will cause significant health problems. Notwithstanding this, it is entirely possible that exposures to mixtures of endocrine-modulating substances, at the concentrations present in the environment, may cause additive (*15*, *16*) or even synergistic effects. Furthermore, the presence of unknown chemicals that may be more potent than those known to date or whose actions do not follow a typical toxicological dose−response curve (*17*) cannot be ignored. The critical issue is whether sufficiently high levels of endocrine-modulating substances exist in the general environment to exert adverse reproductive effects on wildlife and/or humans. It is primarily this question that has formed the basis of the international concern about the possible environmental impact of endocrine-modulating substances. While this debate has been the subject of many international meetings and has received much publicity, the evidence to support the hypothesis that endocrine-modulating substances are a significant human and environmental health problem is lacking (for a recent review, see ref *18*). However, this conclusion seems to be due to the paucity of etiologic studies on this subject and not to definitive evidence in support of the converse hypothesis.

Rivers and estuaries throughout the world are repositories for enormous amounts of industrial and domestic waste containing thousands of chemicals, both natural and man-made. Almost all of the chemicals currently known to interact with the estrogen receptor, for example, some pesticides, alkylphenolic chemicals, phthalates, and bisphenol A are found in sewage treatment work effluents. The aquatic environment, therefore, presents an ideal medium in which to study the possibility of more widespread effects of endocrine-modulating substances on wildlife populations. Indeed, fish are one of the most thoroughly studied groups of wildlife in terms of the effects of chemicals on developmental and reproductive processes (*19*). In our own studies and those of other workers, exposure to estrogens and their mimics, particularly alkylphenolic chemicals, has been shown to cause the synthesis and secretion of vitellogenin, a female-specific protein, in male fish (*20*, *21*). Furthermore, the use of this biomarker of estrogen exposure has enabled us to demonstrate that effluents from sewage treatment works are estrogenic (*22*) and that these effects can persist in rivers several kilometers distant from the point of effluent entry (*23*, *24*). Other studies (*25*, *26*) have demonstrated the presence of higher than normal plasma vitellogenin concentrations in wild populations of carp in certain areas of the United States. Although vitellogenin is now a widely accepted biomarker of exposure to estrogenic substances (*27*), the ecological significance of elevated vitellogenin concentrations in the blood of male fish is unclear. In fish, as in all other vertebrates, estrogens play an important role in many reproductive and developmental processes, including sexual maturation (*28*) and sexual differentiation (*29*). Exposure to estrogens (reviewed in ref *30*) or estrogen mimics (*31*) during sexual differentiation has been shown to induce sex reversal and/or intersexuality, while exposure during sexual maturation can inhibit gonadal growth and development (*20*). The possibility exists that these effects may occur in wild populations of fish that are exposed to estrogenic substances in sewage effluents entering rivers. In this paper, we present strong evidence of adverse reproductive health effects in wild populations of a cyprinid fish, the roach (*Rutilus*

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FIGURE 1. Geographical locations of the rivers sampled. Simplified map of the British Isles showing the general geographical locations of the rivers, lakes, or canals from which fish were sampled during 1995 and 1996. Site A was the laboratory control, sites B−E received no sewage treatment work (STW) effluent, whereas rivers F−M received varying amounts of STW effluent from more than one STW.

rutilus), from a wide range of typical rivers throughout the British Isles. Our studies demonstrate that intersexuality is occurring on a large scale in U.K. rivers and is associated with exposure to effluents from sewage treatment works.

Materials and Methods

Sampling Regime. Wild populations of roach of mixed age and sex were sampled randomly, both upstream (where possible) and downstream of sewage treatment works, on each of eight rivers and from five reference sites throughout the British Isles (Figure 1) using electric fishing or netting methods. The rivers selected represented a range with regard to general water quality (from very good to poor). Other necessary criteria included (a) an abundance of roach, (b) the presence of both "upstream" and "downstream" sites, (c) the existence of data on the characteristics of sewage discharges that entered the rivers. Downstream sites were within 15 km of a sewage effluent outfall, while upstream sites were upstream of any significant effluent input. Upstream sites were usually several kilometers from corresponding downstream sites; the two sites being separated by one or more physical barriers. Fish sampled from these upstream sites were, in some cases, still subject to exposure to sewage effluents from smaller works located even further upstream. Descriptions of each capture site with regard to the characteristics of the sewage treatment works directly upstream of the capture point are given in Table 1. The impact of each sewage treatment works on each of the various capture sites was described using (a) the population equivalent (PE) of the sewage treatment works influent and (b) the amount of dilution that the effluent receives in the river at the point of capture. The population equivalent (PE) can be described as a measure of the "strength" of an influent or the "load" entering the sewage treatment works; polluting load

TABLE 1. Characteristics of the Fish Capture Sites

river	name	type of site	PE	dilution factor	effluent concn (adj PE)
F	Wreake/Eye	upstream	429	1 666	1
		downstream	51 950	8.6	6 037
G	Ouse	upstream	5 000	3 125	2
		downstream	198 546	69.9	2 829
H	Lea	upstream	NA[b]	NA	NA
		downstream	130 393	1.8	73 320
I	Arun	upstream	1 732	55.6	31
		downstream	107 250	3.8	28 636
J	Nene	upstream	22 143	9.8	2 270
		downstream	285 174	4.8	58 891
K	Trent	downstream[a]	982	3 333	1
L	Rea	downstream	2 000	17.1	117
M	Aire	downstream	674 717	7.1	94 939

[a] Unlike the other downstream sites, this site may have been impacted by effluents for many, diffuse sources rather than a single dominant point source. [b] NA, not applicable.

is usually defined as biochemical oxygen demand, and thus, 1 population equivalent is the amount of organic biodegradable load that has a 5-day biochemical oxygen demand (BOD5) of 60 g of oxygen per day. Population equivalent is therefore not an absolute measure of quality of the effluent, as it does not incorporate the differences in the level of treatment between various sewage treatment works. 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 average dilution factor of the 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 population equivalent) alone does not adequately explain the concentration of the effluent in the river at a particular point. Highly concentrated effluents from large sewage treatment works may have little impact if their dilution in the river is large. Conversely, sewage treatment works with small population equivalents may have 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. These figures are given in column 5 as adjusted population equivalents (to the nearest whole number).

It was impossible to find rivers in the U.K. that were inhabited by roach and that did not receive any effluent from sewage treatment works. The control sites were therefore composed of a selection of lakes and canals. A population of roach that were hatched in spring-fed water was included as a laboratory control. All control sites were selected only on the basis that they received no effluent from sewage treatment works, and hence the possibility of estrogenic contamination from diffuse sources (e.g., road or agricultural runoff) could not be excluded. Indeed, although it is well established that sewage treatment work effluents are particularly estrogenic when compared with effluent-free environments, the extent of general contamination of the environment by chemicals known to mimic estrogens is unknown presently.

Samples. A total of 60−100 adult roach was collected from each location, between September 20 and October 25, 1995 (river sites), and between the October 2 and October 23, 1996 (control sites). The laboratory control was collected on November 3, 1995. Blood was collected on site, via the caudal sinus, into 1-mL heparinized syringes containing aprotinin (2 TIU/mL). After centrifugation, the plasma was

frozen on dry ice for transportation and stored at −70 °C prior to vitellogenin analysis. Total length, total weight, and gonadal weight were determined for each fish. The gonadosomatic index was calculated using the following equation: gonadal weight/(total body weight − gonadal weight) and expressed as a percentage. Both gonads were removed and preserved in Bouins fixative for 6 h before removal to 70% alcohol in preparation for histological processing.

Histological Analysis. Gonads from each fish were divided into three equal portions. Representative transverse sections, 3−5 mm thick, were taken from the center of each portion to provide a total of 6 sections per fish; one section from each of the anterior, mid, and posterior regions of each gonad. The sections were then processed histologically, embedded in paraffin wax, and sectioned at 3μm. All sections were stained with Mayers Haematoxylin-eosin, mounted, and examined by light microscopy.

Quantification of Plasma Vitellogenin Concentrations. Quantification of vitellogenin in plasma samples was achieved using an established homologous carp radioimmunoassay (*32*) that has been validated for use with a wide variety of cyprinid fishes (*33*). Interassay variation was calculated using internal standards at three points on the standard curve and averaged at 22.7% (±5%, $n = 8$), while intra-assay variation was approximately 2% (±0.3%).

Statistical Analyses. The statistical analyses were carried out using STATVIEW and SUPERANOVA statistical programs (Abacus Concepts Inc., Berkeley, CA). Prior to analysis, data obtained were transformed where necessary to improve normality and homogenity of variance.

Plasma vitellogenin concentrations and gonadosomatic indices recorded from all samples were categorized according to the histological appearance of the gonads (male, intersex, or female). Two-way factorial analyses of variance (nested design) were then carried out to determine the effect of sampling site and category (upstream, downstream, or control) on these two variables.

The incidence of intersexuality at each site was calculated by determination of the percentage of "perceived males" that were found to be intersex after histological examination. χ^2 analyses were then used to determine differences in the incidences of intersexuality between different sampling sites and between different categories of site (upstream, downstream, and control).

Differences in the severity of intersexuality (as defined by a numerical index) were initially analyzed using a two-way factorial ANOVA (nested design with "site category" and "river" as between factors and "section" as a within factor). This was followed by further analyses on individual sites, using one-way ANOVA followed by Fishers PLSD (to examine all pairwise comparisons) or Bonferroni/Dunn (control) test (to examine the differences between each sampling site and the field controls (pooled)).

In the intersex fish only, correlations were carried out to examine the possibility that relationships exist between GSI, plasma vitellogenin concentration, and/or intersex index; a significant correlation between any two of these variables would imply the presence of a common causal factor (for example, estrogen).

To investigate causality, the relationship between the concentration of sewage effluent in the river at each sampling site and intersexuality was examined using stepwise regression analyses on both the incidence and severity of intersexuality.

Results and Discussion

Incidence of Intersexuality. When examined macroscopically, all of the fish collected from these sites appeared to be either male or female. However, histological examination of the gonads revealed that a surprisingly large proportion of the males were in fact intersex, as defined by the simultaneous presence of both male and female gonadal characteristics. Intersex fish were found at all sites (Figure 2), although the incidence was much higher in those rivers that received sewage effluents than at the control sites (Figure 2, inset). The incidence of intersexuality in male fish ranged from 4%, in both the laboratory population and at one of the field control sites, to 100% in two populations of roach sampled from rivers J and M downstream of sewage treatment works. The proportion of intersex males at downstream sites ranged from 16% (river F) to 100% (rivers J and M); at upstream sites ranged from 11.7% (river H) to 44.4% (river J); and at the control sites ranged from 4% (sites A and B) to 18.1% (site E). A χ^2 analysis of these data showed that the differences in the incidence of intersexuality reached significance (>the mean incidence at control sites) in populations of fish downstream from sewage treatment works on rivers H, I, J, M ($p < 0.0001$), and L ($p = 0.0104$) and in both upstream ($p = 0.0003$) and downstream ($p < 0.0001$) populations of river J. The comparatively low incidence of intersexuality seen at many of the control sites was perhaps not surprising; while intersexuality (assessed macroscopically) is reported to be rare in roach (*34*, *35*) levels of up to 5% have been reported (at the microscopic level) in the carp (*36*) (*Cyprinus carpio*; a cyprinid fish related to the roach), and therefore, a low level of intersexuality could be considered "natural". Notwithstanding this, the results of our survey strongly suggest that the incidence of intersexuality in roach in U.K. rivers is, in many cases, considerably higher than expected and is associated with discharges from sewage treatment works (Figure 2, inset)

The Intersex Index. Gonadal sex can be manipulated in many teleost fish by exposure to pharmacological doses of sex steroids (either oestrogen's or androgens) or aromatase inhibitors (*37*); in broad terms, estrogens feminize and androgens masculinize. The labile period, when a fish is most susceptible to endocrine perturbation, is the time prior to morphological sex differentiation; specifically, just following hatching or at the juvenile stage. In our study, it was impossible to determine the genetic sex of the fish that we examined, as sex-specific probes for this species are not available; therefore, we were unable to establish whether the incidence of intersexuality was due to feminization or masculinization of genetic males or females, respectively. It was established, however, that the number of fish with normal testes in any population was inversely proportional to the number of intersex fish; this fact and the knowledge that sewage discharges contain estrogenic substances (*22*−*24*) strongly suggest that the incidence of intersexuality is due to feminization of genetically male fish rather than to androgenization of genetic females.

Sexual differentiation is a two-stage process involving gonadogenesis (formation of the structural and supporting elements of the gonad) and gametogenesis (the proliferation and differentiation of the germ cells; e.g., ref *38*). Clearly, high doses of steroid hormones are capable of redirecting development so that either the reproductive ducts (ovarian cavity terminating in an oviduct or sperm duct) and/or the primordial germ cells differentiate in a manner opposite to that of the genotypic sex of the individual (reviewed in ref *30*). Similarly, exposure to high doses of chemicals that mimic estrogens has also been shown to cause feminization of the ducts and/or the germ cells if exposure occurs during early life (*31*, *39*). In addition, it seems that redifferentiation of the germ cells (but not the ducts) is possible if exposure occurs during adulthood (*40*, *41*). In view of this information, both the presence of developing eggs (oocytes) and/or an ovarian cavity were used as diagnostic features (Figure 3) to characterize intersexuality in the fish captured during this study. In some intersex fish, the degree of intersexuality was

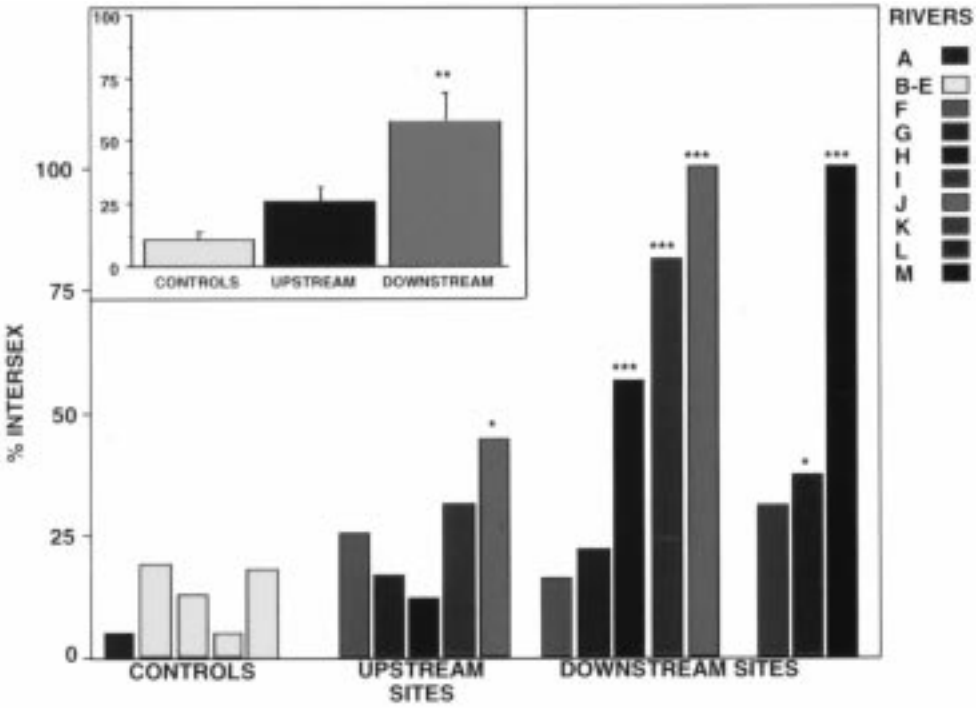

FIGURE 2. Incidence of intersexuality in samples of male roach from various rivers. The proportion of intersex roach (containing oocytes in their testes and/or with female reproductive ducts) in rivers (F−M), lakes or canals (B−E) in England and southern Ireland and in a laboratory control population (A). Sites B−E received no sewage treatment work (STW) effluent, whereas rivers F−M received varying amounts of STW effluent from more than one STW. Rivers F−J were sampled both upstream and downstream of major STWs (the two sites on these rivers were several kilometers apart and separated by one or more physical barriers). The inset diagram illustrates the general trends in the data when results from control, upstream, and downstream sites were pooled. The asterisks denote significance from the field control sites (B−E) at the following significance levels: *, $p = 0.05$; **, $p = 0.01$; ***, $p = 0.001$.

"slight", while in other examples, more than 50% of the gonadal tissue was ovarian. In these individuals, the sperm duct was absent and was replaced by an ovarian cavity. This large variation in the degree to which individual fish were feminized was observed even within populations of fish that were collected from the same site. Consequently, a numerical index, the intersex index, which ranged from 0 to 7, was devised to describe the degree of feminization in each fish (Figure 3). Comparisons between sections from the same fish revealed no significant differences in the intersex index (ANOVA; $p = 0.1$). In addition, oocytes observed within intersex individuals were similar in size and appearance to oocytes of a similar stage observed in female fish in the same population. Primary oocytes were most commonly observed, although secondary oocytes were noted in some of the fish (intersex index = 5−7) collected from downstream sites at which the mean intersex index was high.

Comparisons between the different sampling sites indicated that the mean intersex index varied considerably (Figure 4***); it ranged from 0.33 (at some control sites) to 2.3 (on river M; downstream). A two-way factorial analysis of variance (nested design) with "site category"(control, upstream, downstream) and "river"(nested) as factors showed that the intersex index was significantly higher at almost all river sites than at the control sites (ANOVA; $F = 33.206$, $p < 0.0001$). In addition, an examination of the overall trend in the data (Figure 4, inset) and of comparisons between sites on the same river (rivers H−J; Figure 4) revealed that the intersex index was higher at sites downstream from sewage treatment works as compared to those that were upstream (ANOVA; $F = 47.123$, $p = 0.0001$). It is noteworthy, however, that differences in the intersex index between upstream and downstream populations of fish were not apparent on all of the rivers sampled. On two rivers (F and G), the upstream and downstream populations did not differ with regard to the intersex index (Fishers PLSD; $p > 0.05$), despite the fact that the fish caught from the downstream site on river G were collected less than 100 m from the entry point of the effluent.

Biomarkers of Estrogen Exposure: (a) Vitellogenin. An analysis of the plasma vitellogenin concentrations provided strong evidence that some populations of fish were being exposed to estrogenic contaminant(s) (Figure 5). In general, the vitellogenin concentrations measured in intersex fish were intermediate between the concentrations found in males and those found in females, regardless of the type of site from which the fish were collected (ANOVA for overall significance; $F = 858.851$; $p = 0.0001$). Concentrations found in females were, as expected, at least 50-fold higher than those found in either male or intersex fish. Vitellogenin concentrations in male fish collected from sites downstream of sewage treatment works were significantly higher ($F = 17.526$; $p = 0.0001$) than in males from either upstream sites or from control sites, suggesting exposure to estrogen. In the intersex fish, differences in the vitellogenin concentrations were also dependent on the origin of the sample ($F = 4.560$, $p = 0.0044$), although in this case, the upstream samples were not significantly different from the reference samples ($p > 0.05$). The considerable intrasite variability in the plasma vitellogenin concentrations was reminiscent of the variability in the intersex index. In addition, when all intersex fish were considered together, there was a weak ($r = 0.404$), although highly significant ($p < 0.0001$), positive correlation between the intersex index and plasma vitellogenin concentrations; thus, elevated vitellogenin concentrations in wild fish populations could provide some indication of the likely histological state of the gonad.

It is of interest that the vitellogenin concentrations in female fish collected from the downstream sites were at least 2-fold lower than in females from either the control sites or the upstream sites ($p = 0.0001$), while there were no significant differences between the upstream and downstream sites (p

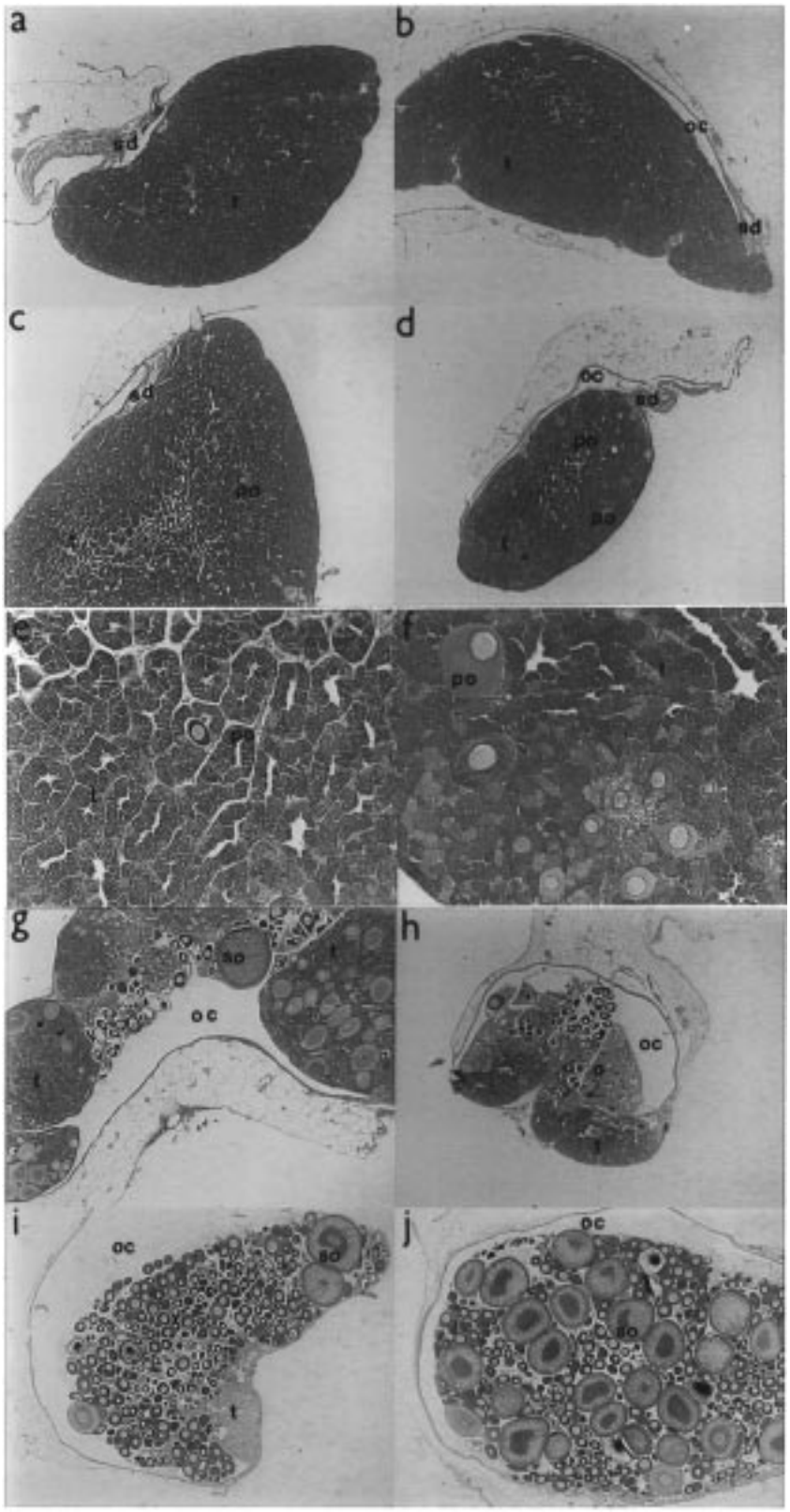

FIGURE 3. Intersex index. Intersexuality was diagnosed in six gonadal sections from each fish using both the appearance of the germ cells and the reproductive ducts. Intersexuality was characterized by the appearance of female characteristics in a typically male tissue and the progressive disappearance of male characteristics. The degree of intersexuality in each section from intersex specimens was assessed using a numerical scale, the intersex index, which ranged from 0 to 7. 0: Panel a (Index = 0) illustrates a completely male gonadal section, with sperm duct (sd) and testicular germ cells (t). Panels b and c (Index = 2) are testicular sections in which both a sperm duct and an ovarian cavity (oc) are present (panel b) or in which the duct is normal but the germ cells are composed of both testicular (t) germ cells and primary oocytes (p.o). The occurrence of primary oocytes in these sections was low (panel c). Indices of 2 or 3 described sections that were intersex in terms of both the germ cells and the reproductive ducts (panel d). 2 = sections in which both male (sd) and female (oc) ducts are present; oocytes (p.o) occurred occasionally. 3 = as score 2 except that primary oocytes occurred frequently

FIGURE 3 (CONTINUED). and were often in clusters. Panels e and f illustrate the difference between the infrequent (Indices 1 and 2) and frequent (Index 3) occurrence of oocytes [oocyte frequency was assessed in 3−4 random fields of view per section (magnification: ×100): oocytes occurring in less than 2 fields of view = infrequent, clusters or groups of oocytes occurring in more than 2 fields of view = frequent]. More severe cases of intersexuality (Indices 4−7, panels g−j) were characterized by the absence of a sperm duct and the presence of an obvious ovarian cavity. 4 (panel g) = sections in which oocytes (which may be primary and/or secondary, so) were frequent, although still interspersed with testicular tissue (t). 5 (panel h) = large, continuous areas of the section that were testicular while less than 50% of the gonadal tissue were ovarian; oocytes may be primary and/or secondary. 6 (panel i) = as score 5 except that 50% or more of the gonadal tissue were ovarian, and oocytes may be primary and/or secondary. 7 (panel j) = 100% of the gonadal tissue is ovarian; oocytes may be primary and/or secondary. Magnification: panels a−d and g−j are ×20 and e−f are ×100.

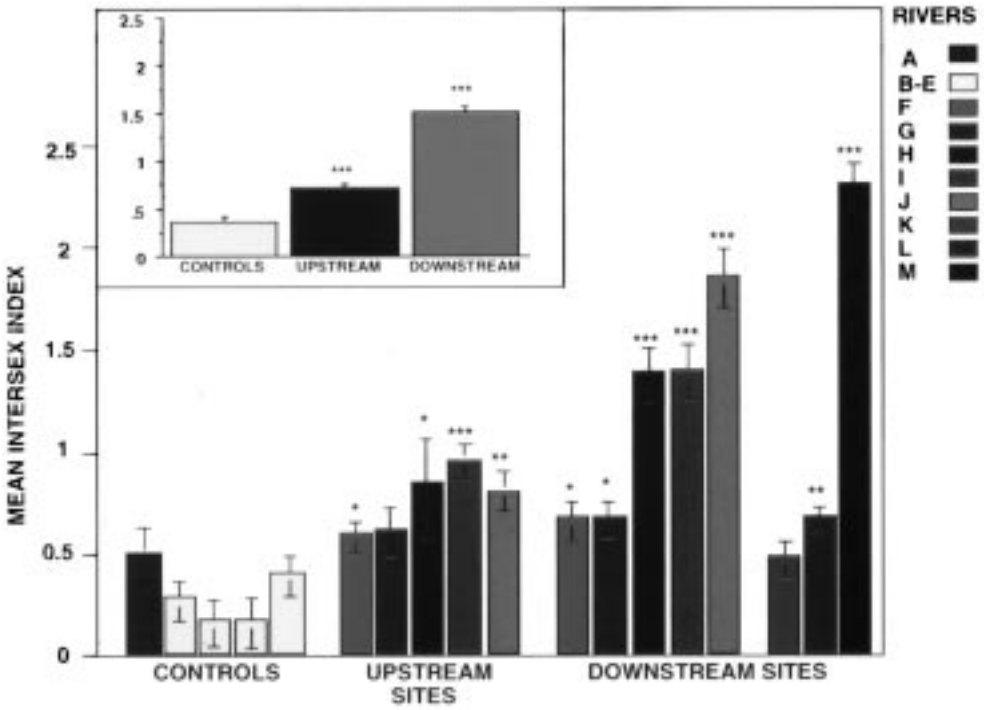

FIGURE 4. Intersex index in samples of roach from various rivers. The degree of intersexuality in populations of roach in rivers (F−M), lakes, and canals (B−E) in England and southern Ireland and in a laboratory control population (A). Sites B−E received no sewage treatment work (STW) effluent, whereas rivers F−M received varying amounts of STW effluent from more than one STW. Rivers F−J were sampled both upstream and downstream of major STWs (the two sites on these rivers were several kilometers apart and separated by one or more physical barriers). Intersexuality was assessed using the intersex index, which ranged from 0 (histological male) to 7 (histological female). The arithmetic mean of the scores (6 sections per fish) for all intersex fish was used to derive an average intersex index for each site or group of sites (inset). The asterisks denote significance (as analyzed by ANOVA on log-transformed values) from the field control sites (B−E) at the following significance levels: *, $p = 0.05$; **, $p = 0.01$; ***, $p = 0.001$.

< 0.05). The reason(s) for these differences is not known and can only be speculated upon presently.

(b) Gonadal Weight. Although the presence of vitellogenin in the blood of male fish is the most widely accepted biomarker of estrogen exposure (*27*), there are other physiological and biochemical measurements that can be viewed as general indicators of endocrine modulation. In particular, inhibition of testes growth in male fish has been reported in connection with exposure to oestrogens (*20*, *42*). However, the size of the gonad relative to body weight, expressed as the gonadosomatic index (GSI), is not an unequivocal indicator of estrogen exposure since GSI decreases have also been reported in male fish in response to exposure to other contaminants that are not estrogenic. Notwithstanding this, an inverse correlation between plasma vitellogenin concentrations and GSI in adult male fish exposed to estrogens or xenoestrogens in water has been demonstrated (*20*). Similar observations were made here in our study of wild fish populations; the gonadosomatic indices were on average higher in males and females sampled at the control sites than at either the upstream (males, $p = 0.051$; females, $p = 0.001$) or downstream sites (males, $p = 0.0027$; females, $p = 0.0001$), while in the intersex fish, small, although very significant, differences in the gonadosomatic index were observed at all types of site (control > upstream > downstream; $p = 0.0001$).

In summary, when both the plasma vitellogenin and GSI measurements are viewed together with the observations on intersexuality, they provide very compelling evidence that populations of wild fish inhabiting many rivers are being exposed to estrogenic contaminants and that these contaminants are, in most cases, present at higher concentrations on river stretches directly downstream from large sewage treatment works. The incidence and severity of intersexuality as described here is both alarming and intriguing since almost all of even the downstream sites were several kilometers away from any point of sewage discharge, and hence the samples collected were truly representative of wildlife populations in typical river ecosystems. In addition, observations on a secondary species, the gudgeon (*Gobio gobio*), from several rivers were very similar to those made on the roach, indicating that these effects are unlikely to be species specific (results not shown).

Causality. This study was designed to investigate a range of typical rivers of varying water quality throughout the U.K. in an attempt to determine whether evidence for endocrine disruption could be detected in the resident populations of fish. Discharges from sewage treatment works located on

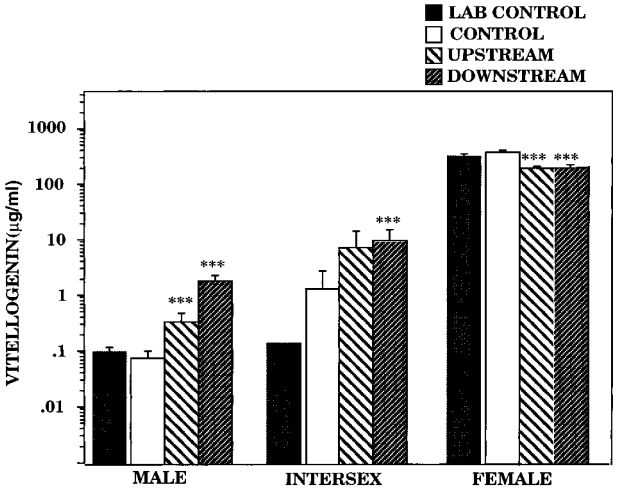

FIGURE 5. Plasma vitellogenin concentrations in samples of roach from various rivers. Concentrations of plasma vitellogenin in populations of roach of mixed sex in rivers, lakes, and canals in England and southern Ireland and in a laboratory control population (A). The "field controls" represent data from four lakes or canals (B−E) that received no sewage treatment work (STW) effluent, whereas "upstream" and "downstream" samples represent data from five (F−J) or eight (F−M) rivers, respectively; all of which received varying amounts of STW effluent. Downstream samples of fish were taken within 15 km of a sewage effluent outfall, while upstream sites were upstream of any significant effluent input. These sites were usually several kilometers from the corresponding downstream site; the two sites being separated by one or more physical barriers. Asterisks denote significance (assessed by ANOVA on log-transformed values) from the field control sites (B−E) at the following significance levels: *, $p = 0.05$; **, $p = 0.01$; ***, $p = 0.001$.

these rivers could then be examined to determine whether their characteristics could be associated with any of the physiological responses evident in these fish. The most obvious indicator of endocrine disruption appeared to be the presence of a high proportion of intersex fish at many sites; the number of males being inversely proportional to the number of intersex fish. When each site was categorized according to both the population equivalents of the nearest sewage works and the average annual dilution factor of the effluent in the river (Table 1), a highly significant relationship ($r^2 = 0.683$, $F = 28.997$, $p = 0.0002$) between the proportion of intersex fish and the concentration of the effluent (expressed as adjusted population equivalents) in the river was apparent. Thus, for the range of sites studied, the proportion of intersex fish in any sample of macroscopically male fish could perhaps be predicted, using a linear equation, from the average concentration of effluent constituents in the river; high concentrations of effluent were predictive of high incidence of intersexuality, while low concentrations of effluent predicted a low incidence of intersexuality. The average annual concentration of effluent was also regressed against the intersex index using the data obtained from all sampling sites. A regression plot of the data (Figure 6) showed that a positive relationship between the two variables existed ($r^2 = 0.312$, $p < 0.0001$, $n = 150$). Thus, the average intersex index in a population of roach from a particular site could be estimated, using the average concentration of sewage effluent in the river as a predictor. The scatter of the points suggests that the intersex index, and hence the degree of exposure to estrogen(s), may vary tremendously, even within populations of fish that were sampled from the same site. This is not surprising when differences in the time/timing of exposure, fish movement, and migration are all taken into account. Although, on many of the rivers, 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.

In conclusion, these results strongly suggest that the concentration of sewage effluent in a river is a major causal factor in the evolution of intersexuality in fish. Furthermore, the association between the degree of intersexuality and the plasma vitellogenin concentration suggests that the two effects have a common cause and, therefore, that the estrogenic constituents of sewage effluents are responsible

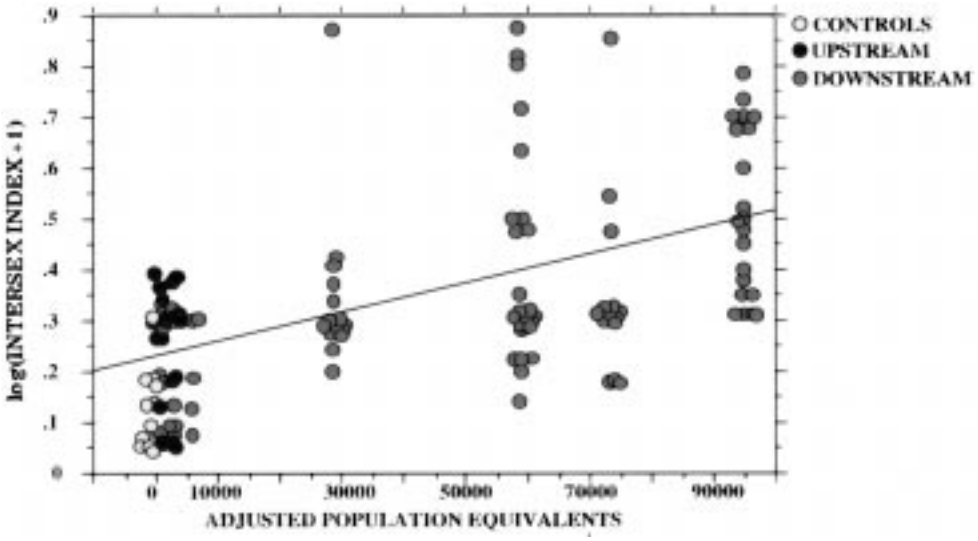

FIGURE 6. Relationship between the concentration of sewage effluent in river water and the degree of intersexuality in wild populations of roach throughout the British Isles. The effect of sewage effluent concentration (measured as adjusted population equivalents) on the average intersex index in populations of roach from a range of rivers, lakes, and canals (controls) in England and southern Ireland and in a laboratory (control) population. Each point represents the mean intersex index in individual fish; where overlap occurs, the points are shown slightly displaced. The characteristics of the influent from the nearest and largest sewage treatment works relative to each capture site are given in Table 1. The relationship between the average concentration of effluent (adjusted population equivalents) at each site and the severity of intersexuality can be explained by the linear regression equation $\log(Y + 1) = 0.203 + 2.88^{-6}(X)$, $r^2 = 0.312$ (ANOVA $p < 0.0001$, $n = 150$).

for the occurrence of intersexuality in wild fish populations. While a "typical" effluent does not exist, attempts to identify the main estrogenic chemicals using a toxicity-based fractionation approach have revealed that natural (oestradiol and oestrone) and synthetic (ethinylestradiol, from birth control pills) estrogens, presumably excreted by humans, are present in effluents, including some of the specific effluents that impact the sampling sites in this study. Furthermore, although the concentrations of these substances in the effluents were extremely low (in the tens of nanograms per liter range; (*43*) when replicated in laboratory experiments, they were high enough to induce vitellogenin synthesis in male fish (*44*). Other authors (*45* have reported similar concentrations of natural and synthetic ostrogens in effluents and river water in another European country, suggesting that the presence of these extremely biologically active chemicals in effluents from sewage treatment works is general. It should be noted, however, that estrogenic xenobiotics, such as the alkylphenolic compounds, are also present in many sewage effluents (*46*, *47*); in particular, they are major constituents of some industrial effluents. These discharges have been shown to cause estrogenic effects along large stretches of some rivers (*24*), and thus the role of these chemicals in the overall cause of the biological effects seen in wild fish populations should not be underestimated. Furthermore, estrogenic chemicals that have not yet been tested in aquatic organisms in vivo, such as the phthalates, bisphenol A, and many pesticides, are also present.

Fisheries Implications. The ecological implications of these findings are dependent on the reproductive competence of the roach that are intersex. Both the quality and quantity of gametes produced by these fish must be assessed to determine the physiological significance of intersexuality and, therefore, its impact on wild fisheries in the U.K. Parallels can perhaps be drawn between the findings reported here and those reported in the 1980s on mollusk populations that were heavily affected by the aquatic contaminant tributyl tin (TBT). This anti-fouling agent caused a condition termed imposex, in which female mollusks developed male sexual organs (penis and sperm duct); in the most severe cases this developmental abnormality caused sterility and thus led to the extinction of certain species of mollusks in some areas (*48*). Similarly, the absence of a sperm duct in a male fish (which we have observed in cases where the intersex index exceeds 4) would certainly prevent the release of viable sperm, while the ability of these individuals to produce viable eggs is questionable. It is well-known that the survival of any fish population is largely determined by the relationship between the size of the spawning stock and the annual number of offspring (recruits) produced together with the subsequent survival of these recruits on entering the fishery. It is probable, therefore, that populations of roach in at least some locales are adversely affected.

Effluent characteristics can change drastically with annual and seasonal variations in rainfall; for example, the average annual flow rate of river J at a particular gauging station was almost 4 times higher in 1993 than in 1991, while during the summer months, river flows can fall to zero in some regions. The importance of these fluctuations in river flow and hence effluent concentration is realized when one considers that the roach (like many U.K. cyprinid fish) spawns in spring, and therefore sexual differentiation in the juveniles occurs during the summer when effluent concentrations are at their highest, in some cases reaching 100% of the flow of the river annually. Furthermore, periods of drought, such as experienced in the U.K. during 1995 and 1996, would be expected to have a pronounced deleterious impact on both the numbers of roach that are intersex and on the intersex index. It is important to note that these conditions are far from unusual; water quality in the U.K. is generally thought to be good and improving, particularly when compared with many other European countries where sewage is often discharged into rivers and canals after little or no treatment. Perhaps the most disturbing fact is that discharges from sewage treatment works are an inevitable consequence of human existence, and hence estrogenic contaminants could have a global impact on all populations of riverine fish exposed to sewage discharges. Indeed, if global warming is confirmed and continues, water use continues to increase, and water reuse schemes continue to be implemented, the impact of sewage discharges on riverine populations of fish would be expected to increase unless the efficiency of sewage treatment works, and hence effluent quality, were to improve substantially.

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