Male-Biased Sex Ratios of Fish Embryos near a Pulp Mill: Temporary Recovery after a Short-Term Shutdown

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In a previous study we showed that broods from the viviparous eelpout (Zoarces viviparus) were significantly male biased in 1998 in the vicinity of a large kraft pulp mill on the Swedish Baltic coast. One suggested hypothesis was that masculinizing compounds in the effluent were affecting gonadal differentiation of the embryos, resulting in skewed sex ratios. In this article, we present further evidence for a causal relationship between the exposure to the effluent and the male-biased sex ratios. Analyses of historical samples showed that the eelpout produced male-biased broods close to the mill in 1997 in addition to 1998. During 1999, the mill was shut down for 17 days, coinciding with the period when the gonads of the eelpout embryos differentiate. Subsequently, in the fall of 1999, the sex ratios were no longer male biased; however, the following year (2000), a significant male bias reappeared. Investigations at 13 sites for up to 4 years showed a relatively stable sex ratio around 50/50, with the exceptions by the mill and with few observations of deviating ratios at other sites. Several reports document endocrine disturbances in fish near pulp and paper mills, including the expression of male secondary sex characters in female fish. The repeatedly identified male bias at the investigated mill, the normalization after mill shutdown, and the reappearance the following year indicate that pulp mill effluents also can affect sex ratios of nearby fish. Key words: androgenic, endocrine disruptors, gonadal differentiation, masculinization, ovary, testis. Environ Health Perspect 110:739–742 (2002). [Online 11 June 2002] Figure 1 was corrected on 29 July 2002.

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Pulp mills release large amounts of complex effluents more or less continuously into our aquatic environment. In general, the toxicity of these effluents has decreased substantially during the past decades through more closed systems, introduction of nonchlorine bleaching, and more efficient secondary treatment of the effluents (1). Despite these improvements, there is ample evidence for disturbed benthic algal and invertebrate communities in the recipients (1–3). Effects on the hepatic cytochrome P450 system of fish are also regularly observed, as is interference with their reproductive system (4–11), including depressions of circulating steroid hormones, delayed maturation, smaller gonad size, and altered expression of secondary sex characters. In the United States and New Zealand, female mosquito- light (Gambusia sp.) exposed to pulp and paper mill effluent, both in the field and in the laboratory, develop an elongated anal fin resembling the male gonopodium (12–15). Overexpression of male secondary sex characters has also been found in eels (Anguilla rostrata) (16) and white suckers (Catostomus commersonii) (17) exposed to paper/pulp mill effluents. Because androgens are known to induce male secondary sex characters in many fishes, the hypothesis was generated that the effluents contain androgenic compounds (12). In addition to the effects on secondary sex characters, androgens (and estrogens) can direct gonadal sex toward male (or female) development in many fish species if applied at a critical time at or just before the onset of gonadal differentiation (18). Exposure experiments with eggs from fathead minnows (Pimephales promelas) and a diluted Canadian bleaching Kraft mill effluent produced not only a greater proportion of adult fish expressing male secondary sex characters, but also a greater proportion of males as judged by the presence of testis (19). These findings led us to investigate the sex ratios of embryos of the viviparous eelpout (Zoarces viviparus) in the vicinity of a large kraft pulp mill on the Swedish Baltic coast (20). The eelpout undergo gonadal differentiation as embryos and are simple to sex before birth (20). Thus, in contrast to most oviparous species, accurate sex ratios from individual broods are easy to obtain from the field. The sex ratios of embryos sampled in 1998 were significantly male biased (42% females) close to the mill, whereas the normal sex ratio appeared to be around 50% females at all studied reference sites (20). We therefore hypothesized that masculinizing components in the effluent were causing the male-biased broods. Our aim in the present study was to investigate further the link between pulp mill effluent exposure and male-biased sex ratios of the eelpout. We therefore investigated the consistency of the response over 4 years at the mill and at several reference sites. We also included sites close to other industrial activities to investigate the specificity of the cause. We discuss the data in context of the classic criteria used in ecotoxicologic research (21).

Materials and Methods
Sex ratio analyses. The embryos of the viviparous eelpout initiate gonadal differentiation in late September or early October while still in the mother’s ovary (20). The sex of the embryos can be distinguished easily and accurately before birth by morphologic differences between the embryonic testes and ovary (20). We performed sex ratio analyses in situ on Bouin’s-fixed embryos sampled from late October to mid November as described previously (20). In total, we sexed 15,895 embryos from 425 adult females. We treated all animals in accordance with the requirements of the ethical committee in Gothenburg, Sweden. Sampling sites. The investigated mill is a kraft pulp mill located on the Swedish Baltic coast. The mill is using total chlorine-free bleaching technology (bleaching process Q/OP/ZQ/PO), and it alternates between softwood and hardwood pulp production. Activated sludge treatment was introduced during the summer of 1998, replacing aerated lagoons for effluent treatment. The effluent earlier entered the Baltic Sea via a 5-km-long tube equipped with a 1.5-km-long diffuser. The extension of the effluent plume has been described elsewhere (22). In 1997, the lid covering the end of the diffuser was open, probably resulting in a somewhat different plume, and in 1999, a 250-m-long diffuser permanently replaced the old one (23). From 20 September to 6 October

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1999, the mill instituted a planned complete shutdown of its pulp production to prepare for an increased production capacity from 550,000 to 800,000 metric tons of pulp per year. Over a few days before and after this stop, production gradually decreased and increased, respectively. This period comprised the time just before and during the expected onset of morphologic differentiation of the embryonic gonads of the eelpout (20). There have been shutdowns during other years as well, but long before or after the onset of gonadal differentiation of the eelpout. During a shutdown, organic material usually leaks continuously from the mill, but the character of the effluent is probably much different from that of normal production (23).

A river with a long history of organochlorine and heavy metal pollution (24) enters the Baltic Sea approximately 10 km north of the pulp mill outfall. In 1999 and 2000, the investigation was expanded to include an additional site 10 km north of the mill outfall, to evaluate if water from this river could have caused the previously observed male bias. To investigate whether other types of stressors could affect the sex ratios, we sampled eelpout in 1999 on the Swedish West coast in the vicinity (1–2 km) of an oil refinery in Borjudden, close to the effluent outfall from Stenungsund petrochemical industries (< 1 km) and two sites in the Göta River Estuary. The latter two receive a mixture of sewage and industrial effluents from the Gothenburg region and are located 1 and 3 km downstream from the outfall of the local sewage treatment plant. The reference sites have previously been used in regional environmental monitoring programs (physiologic studies of eelpout and perch (Perca fluviatilis)) (25–31).

Statistical analyses. For each year, we pooled sex ratios from the preselected reference sites and compared them with sex ratios of each of the other sites using Student’s t-tests (no Bonferroni correction). When results from residual analyses did not meet the assumptions of parametric tests, we performed randomization analyses without replacement according to Manly (32).

Results
In 1997 the embryonic sex ratios were significantly male biased (45.5% females). A. A. north of the mill compared with the pooled reference sites ($p = 0.0015$), whereas sex ratios at the site 1.2 km south of the mill showed a similar trend but were not significantly different from the reference sites ($p = 0.057$; Figure 1). Further south in the discharge gradient the sex ratios approached reference levels. This pattern was very similar to that previously reported for 1998 when the two closest sites were significantly male biased (42.2% and 46.1%) compared with the four control sites ($p = 0.0005$ and $p = 0.042$), and a similar gradual normalization was apparent as moving south from the mill (Figure 1) (20).

In 1999, the year when the mill had a planned shutdown period (see “Materials and Methods”), we found no significant differences between the two pooled preselected reference sites and any other site. We found no trends suggesting a male bias at the site closest to the river mouth (10 km north) or closer to the mill (Figure 1). On the other hand, the northernmost reference site showed a significantly lower sex ratio compared with 1998 ($p = 0.046$).

In 2000, the sex ratios at the site closest north of the mill was again male biased (38.7%) compared with the pooled references ($p = 0.0004$; Figure 1). Also, two of the three sites south of the outfall were male biased (41.3%, 41.7%; $p = 0.020$, 0.014). The site closest south of the outfall (47.0%) and the site closest to the river mouth (48.0%) did not differ significantly from the pooled references ($p = 0.94$ and $p = 0.71$, respectively).

In 1999, we studied the specificity of the sex ratio deviations by sampling fish from a reference site in Slagertak (same as in 1998), an oil refinery, a petrochemical industry, and two sites in the Göta River Estuary, all on the Swedish west coast. The sex ratios were close to 50/50 at all sites and no site differed significantly from the control site (Figure 2).

Discussion
We have shown that sex ratios of eelpout broods in the vicinity of a pulp mill were male biased in 1997, 1998, and 2000. In 1999, the mill shut down at a time when the embryos normally undergo gonadal differentiation (20). When we sampled the eelpout later in the fall, the sex ratios were normalized; however, the following year the eelpout by the mill again produced male-biased broods. We hypothesize that the effluent from the mill is causing the male bias, and a reduced exposure to the effluent during the period of gonadal differentiation in 1999 allowed the eelpout to produce broods with normal sex ratios.

Fox (21) has defined a set of ecologic criteria to be used to evaluate whether a causal relationship is likely to be present between an environmental stressor and an observed response. These include probability, time order, strength, specificity and consistency of association, predictive performance, and coherence (theoretical, factual, biologic, and dose–response). Below, we have tried to apply these criteria to the male-biased sex ratios by the Swedish mill.

The probability of a causal relationship refers to statistical significance. Indeed, over 3 years, a statistically significant male bias developed at one or both of the two sites closest to the mill compared with the preselected reference sites. A statistically significant correlation may imply causality, but it is a strong argument only when it can be backed up by other information. The time order is such a criterion, which ideally should include not only appearance of the effect after the stressor is introduced, but also disappearance of the effect when the stressor is removed and reoccurrence when the stressor is introduced again. The investigated mill has been releasing effluent for several decades, although exposure may have varied with different processes, production, and alternating currents in the area (22). No historical samples of eelpout embryos are available to investigate sex ratios before the mill was built. However, the temporary shutdown in 1999 of the mill during the onset of the gonadal differentiation of the eelpout opened up an unusual opportunity to demonstrate the disappearance of the effect after a presumably reduced exposure. A previous study at a large pulp mill in Jackfish Bay, Lake Superior, Canada, showed that circulating 11-ketotestosterone levels of male white suckers recovered within 2 weeks after a maintenance shutdown (6). This suggested that the fish were able to approach a normal physiology in a relatively short time, implying that at least some of the endocrine

\[ \text{Distance from mill} \]

\[ \text{Figure 1. Embryonic sex ratios of eelpout broods (mean ± SEM) sampled at reference sites (ref) or at different distances (north (N) or south (S)) from a pulp mill on the Swedish Baltic coast from 1997 to 2000.} \]

\[ ^* p < 0.05, ^{**} p < 0.01, ^{***} p < 0.001: \text{significant difference from the pooled reference sites within the same year.} \]

Previously published data from 1998 (20) have been included with permission from the Society of Environmental Toxicology and Chemistry.
disruptors were relatively rapidly metabolized and/or excreted by the fish. The recovery to normal sex ratios of the eelpout after the 17-day pulp mill shutdown and the reappearance of the male bias the following year agree with the observations at Jackfish Bay.

The strength of association refers to the relative occurrence of the effect when the stressor is present compared with when it is not. Over the 4 years of the investigations, we had one observation of significantly male-biased sex ratios at a presumably clean site (100 km north, 1999) and one observation at a site where the exposure levels are expected to be very low or zero (26 km south, 2000), revealing that eelpout sex ratios may show some degree of natural, or at least unexplained, variation. However, at the two sites closest to the mill, four of six possible observations (excluding the year of the shutdown) were significantly male biased, and one observation in 1997 was very close to significance \( (p = 0.057) \). This represents a much higher relative occurrence.

We addressed the specificity of the potential cause by sampling eelpout exposed to other potential stressors—that is, near an oil refinery; near a petrochemical industry; near a river with organochlorine and heavy metal pollution; and at two sites with a mixed exposure situation. At none of these sites were the sex ratios significantly skewed. The disappearance of the effect by the mill in 1999 also gives some information about the specificity, suggesting no causal relationship between any permanent conditions at the two sites closest to the mill and the skewed sex ratios. Such conditions, present throughout the investigation, include alterations of the benthic fauna and flora in the close recipient. The most obvious alteration is a strong reduction in Fucus vesiculosus, the dominating large brown algae in the Baltic, hosting a whole ecosystem of small animals \( (29,33,34) \).

Regarding the consistency of association upon replication, we demonstrated the male bias over 3 years. We performed the actual sex ratio analyses with different personnel each year, showing independence of the examiner. A lab study exists \( (19) \), but to our knowledge no field study other than at this mill \( (20\), present study\), has yet shown a change in sex ratio in fish associated with pulp mill effluent exposure. This warrants further investigations at this and other mills, ideally with more species. However, the possibly related effects of pulp and paper mills on male secondary sex characters in different fish species have been identified repeatedly at several times and locations \( (12,14–17) \).

In our previously published study, we presented the first data on the male bias close to the mill \( (20) \). In this follow-up study, we had the opportunity to test the predictive performance of our hypothesis. At all times, the observed sex ratios by the mill have been similar to the predictions from the hypothesis.

There is clear coherence between skewed sex ratios and pulp mill effluent exposure. Two research groups recently identified the presence of androgen receptor ligands in the Fenholloway River, which is heavily contaminated by pulp mill effluent \( (35,36) \). Androstenedione, a moderately strong steroidal androgen and also a precursor for more potent androgens, was found in a concentration of 0.14 nM \( (35) \). Female mosquitofish in the Fenholloway River display male secondary sex characters \( (35,36) \). Thus, androstenedione is a good candidate for causing the observed masculinizations of mosquitofish; but the relative contribution of other steroid receptor ligands remains to be evaluated. Hewitt et al. \( (37) \) showed that fish exposed to kraft pulp mill effluent accumulate androgen and estrogen receptor ligands in their livers. Taking the findings of induced male secondary sex characters into consideration, we have extensive evidence for the presence of masculinizing agents in pulp and paper mill effluents. Also, a physiological mechanism for androgen exposure leading to male-biased sex ratios has been established \( (18) \). For all 3 years when we observed an effect in the recipients of the effluent, the north site closest to the mill showed the lowest male-to-female sex ratio. Also, we found the strongest male bias after the increase in production capacity in 2000, and two sites farther south in the gradient were also male biased. These observations could represent a dose–response relationship, albeit the data are not entirely clear. For example, the site north of the mill (1.7 km) consistently displayed a stronger male bias compared with the site immediately south of the mill, which is closer to the outfall (1.2 km), and in 2000 the sex ratio was not male biased at the closest site south of the outfall. Because the gonadal differentiation of fish, in general, is sensitive to hormones only during a short time window, the direction of the effluent plume at a particular time point may be of importance. The normalization of the sex ratios by the mill after the 17-day shutdown in 1999 supports the hypothesis that the male bias is not caused by chemicals accumulated over long periods of time.

As an alternative hypothesis, we have earlier discussed exposure to potentially increased temperatures near the mill \( (20) \). As is true for exogenous hormonal exposure, altered environmental temperatures have the potential to affect gonadal differentiation in many fish species \( (38) \). At present, we have little support for a significant temperature gradient around the mill outfall \( (29) \), but ample data suggest the presence of endocrine-disrupting compounds in pulp mill effluents. To address further the temperature hypothesis, we would like to gather more detailed temperature data in the proximity of the outfall and at reference sites. Another alternative hypothesis is that the sex ratios are skewed near the mill because of parental “choice.” One of the most important factors determining the fitness of an individual is the sex ratio of its offspring. Hence, an ability to adjust offspring sex ratios to certain conditions has developed in many animal species \( (39) \). Some common cues related to offspring sex ratios in different species include size of mother, number of embryos, and the size of the offspring. These, however, are not important predictors of offspring sex ratios in the eelpout \( (20) \). Nor is sex-selective early embryonic mortality a mechanism by which the observed male bias is brought about \( (20) \). A sex ratio of approximately 50% female embryos appears to be the norm for eelpout at a variety of sites in the Baltic, Kattegat, and Skagerrak. We observed skewed sex ratios only twice in the present study, except at the three sites closest to the mill. Under some natural environmental conditions eelpout may produce broods with skewed sex ratios. Although it would be very coincidental, it cannot be ruled out that such conditions were present by the mill in 1997, 1998, and 2000 but not in 1999.

Field data can rarely supply finite evidence for a causal relationship; however, by applying the above criteria, the probability of causality can be judged. Our interpretation is that a substantial amount of data must be ignored to accept the null hypothesis, that no causal relationship exists between exposure to the effluent from the mill and the male bias of the sex ratios. Although skewed sex ratios were found in fathead minnows exposed to another kraft pulp mill effluent in a laboratory experiment \( (19) \), well-performed dose–response exposures with effluent from the presently investigated mill would provide additional information.

We have had some success with spawning eelpout in captivity, overcoming some of the difficulties in successfully catching, transporting, and holding...
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