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Models and Murkiness: Evaluating Fish Endocrine Disruption in the Laboratory and the Field.

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ABSTRACT

Much analysis of endocrine disruption in fishes has focused on measuring vitellogenin as a biomarker of estrogenic disruption. Vitellogenin (VTG) is a specific and convenient marker of estrogen exposure, but it does not provide information about effects on sexual development, reproductive success, or other subtle changes in reproductive physiology. Using medaka as a model system, we conducted two laboratory studies evaluating multiple endpoints of endocrine disruption. In the first, we examined the effects of anthracene as a potential estrogen antagonist and in the second, we examined the effects of DDT as an estrogen agonist. For both compounds, developing medaka were exposed for either two or eight weeks post-hatch. Subsamples were taken after 2, 4, and 8 weeks for quantification of vitellogenin (both compounds) and histological examination of the gonads (DDT exposed fish). After fish reached sexual maturity, mating pairs established. Percentage of fertilized eggs and percentage of embryos surviving 16 days post-hatch were quantified. Two week exposure to anthracene had no effect on mating success, but reduced the proportion of estrogen-induced sex reversal in co-exposed fish. Eight week exposure to anthracene reduced the percentage of fertilized eggs and co-exposure to estradiol prevented the anthracene effect. We concluded that anthracene disrupts estrogen action, but not via the estrogen receptor as no changes in VTG production occurred. In the second laboratory study, we found that DDT feminized developing fish, producing a female-skewed sex ratio in adults after both 2 and 8 week exposures. Fertility and hatching success were significantly reduced in a time and dose dependent manner. DDT had no effect on VTG expression after a 2 week exposure, but did induce VTG production after 8 weeks. However, fertility and hatching success were more sensitive to estrogenic disruption than were gonad differentiation and VTG expression. These laboratory studies clearly showed multiple effects of endocrine disruption by xenobiotics. Field studies tend to be murkier. How prevalent is endocrine disruption in wild fish and what is its impact? Our field work has focused on longear sunfish (Lepomis megalotis) exposed to unbleached kraft mill effluent. Fish were sampled twice monthly during the spring and summer reproductive season and once monthly during the fall and winter of the past two years. Males and females were captured and bled for analysis of VTG, estradiol, testosterone (T), and 11-ketotestosterone (11KT) levels. Males upstream and downstream of the effluent outfall have similarly low levels of VTG, indicating that they are not exposed to an estrogenic substance. Males downstream of the outfall have slightly lower T levels and slightly higher estradiol levels than males upstream and the ratio of T to estradiol is significantly lower in downstream males, indicating that exposure to mill effluent has a slightly demasculinizing effect on males. VTG and hormone levels do not differ in females upstream and downstream of the effluent. We conclude that there is a subtle effect of kraft mill effluent on reproductive physiology of adult male sunfish, but no morphological effects are evident. Endocrine disruption is occurring in wild populations, but we still can't fully measure the impacts.

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Measuring Biomarkers of Endocrine Disruption

Evaluating the occurrence and consequences of endocrine disruption in aquatic animals is an important component of evaluating ecosystem health. The occurrence of endocrine disruption is assessed by measuring biomarkers that reflect hormonal status or indicate the level of hormonal function. The consequences of hormonal dysfunction are less often measured and more often extrapolated from the observed changes in morphology or physiology. Biomarkers to be measured should include markers of exposure and markers of effect. For instance, much analysis of endocrine disruption in fishes has focused on measuring vitellogenin (VTG) as a biomarker of estrogenic disruption. Production of VTG by an oviparous vertebrate specifically indicates exposure to estrogen, whether endogenous or exogenous. However, VTG production does not necessarily indicate any specific morphological or physiological effects of that exposure. Similarly, measurement of circulating steroid hormone levels can indicate whether there is a deviation from the control or "normal" levels, but does not indicate what the consequences may be. In contrast, measurement of morphological or functional variables that respond to hormones can provide some assessment of effect, rather than simply exposure. For example, to evaluate reproductive disruption, gonad morphology can be examined, as can traditional measures of reproductive success such as fertility and hatching success. The difficulty in examining markers of effect is that a variable such as hatching success integrates many inputs, not just hormonal ones. When possible, both biomarkers of exposure and biomarkers of effect should be measured in the same individuals.

Evaluating endocrine disruption in laboratory models

Laboratory studies of model species provide controlled conditions under which to deliver known doses of compounds and allow close adherence to sampling schedules and balanced experimental designs. Following individuals is also much easier than in field sampling protocols. We chose to use laboratory studies of medaka (*Oryzias latipes*) to conduct an integrated analysis of biomarkers of exposure (VTG production) and effect (sex differentiation, reproductive success). The objective was to determine the sensitivity of each marker to contaminant exposure and to examine the possibility that markers of exposure might predict effects.

Anti-estrogen

In the first study, we exposed medaka to a potential environmental anti-estrogen, anthracene (Cheek et al., 2001a). Anthracene is a polyaromatic hydrocarbon (PAH), a class of compounds that are known to suppress reproduction in fish. Field surveys of benthic fish, including English sole (*Pleuronectes vetulus*), rock sole (*Pleuronectes bilineatus*), and starry flounder (*Platichthys stellatus*) exposed to hydrocarbon-contaminated sediments showed that females had depressed levels of estradiol (E2) and reduced fecundity when spawning was induced in the laboratory (Johnson et al., 1988; Johnson et al., 1998; Spies and Rice, 1988). Laboratory exposure studies of domestic fathead minnows (*Pimephales promelas*) and wild-caught English sole have also shown that PAH-exposed animals have decreased reproductive success (Collier et al., 1986; Diamond et al., 1995; Hall and Oris, 1991). Effects varied from complete inhibition of spawning in English sole captured at a highly contaminated site (Collier et al., 1986) to reduced egg production and hatching success in fathead minnows exposed to anthracene and fluoranthene (Diamond et al., 1995; Hall and Oris, 1991).

Interestingly, in studies of wild-caught English and rock sole, plasma estradiol (E2) levels were reduced in fish from highly contaminated sites, but plasma vitellogenin levels were not (Johnson et al., 1988; Johnson et al., 1998). Still, soles from the most contaminated sites were less likely to spawn after gonadotropin releasing hormone stimulation and had the lowest percentage of fertilized eggs and the lowest numbers of normal larvae after hatch (Johnson et al., 1988; Johnson et al., 1998).

In order to evaluate the apparently anti-estrogenic activity of PAHs, we exposed developing medaka to anthracene (ANT) alone and in combination with E2. If anthracene is an anti-estrogen, we would predict that it could inhibit estrogen-induced processes in fish, including VTG production and feminization of fry. In addition, we predicted that ANT exposure would suppress reproductive success. To identify the critical window for ANT and E2 exposure, we exposed medaka fry during sex differentiation only (for 2 weeks

post-hatch) or throughout sex differentiation and puberty (for 8 weeks post-hatch). Treatments included a water control (no solvent), a solvent control (triethylene glycol, TEG), 0.27 (g/L E2, 12 (g/L ANT, 20 (g/L ANT, 12 (g/L ANT + 0.27 (g/L E2, and 20 (g/L ANT + 0.27 (g/L E2. ANT concentrations were selected based on laboratory studies with fathead minnows showing that nominal doses of 12 and 20 (g/L (ppb) reduced reproductive success (Hall and Oris, 1991). These low dissolved concentrations provide conservative estimates of the potential impact of PAH exposure on field populations, given that sediment concentrations as high as 17 ppt (parts per thousand) have been measured in contaminated waterways (G. Flowers, personal communication). Fry were subsampled for analysis of whole body VTG concentration at 2, 4, and 8 weeks post-hatch, then transferred to clean water for grow-out until 3 months of age. At 3 months, adults were sorted into mating pairs in order to assess the impact of parental exposure on production and survival of offspring. Three categories of mating pairs were created: both parents treated, only the female parent treated, or only the male parent treated. Details of the experimental set up and methods are provided in (Cheek et al., 2001a).

VTG production

ANT did not induce VTG production in fish of any age, regardless of exposure duration (Fig. 1). As expected, E2 did induce vitellogenesis, even in fish as young as 2 weeks post-hatch. ANT did not inhibit E2-induced vitellogenesis, suggesting that ANT does not act as an estrogen receptor antagonist.

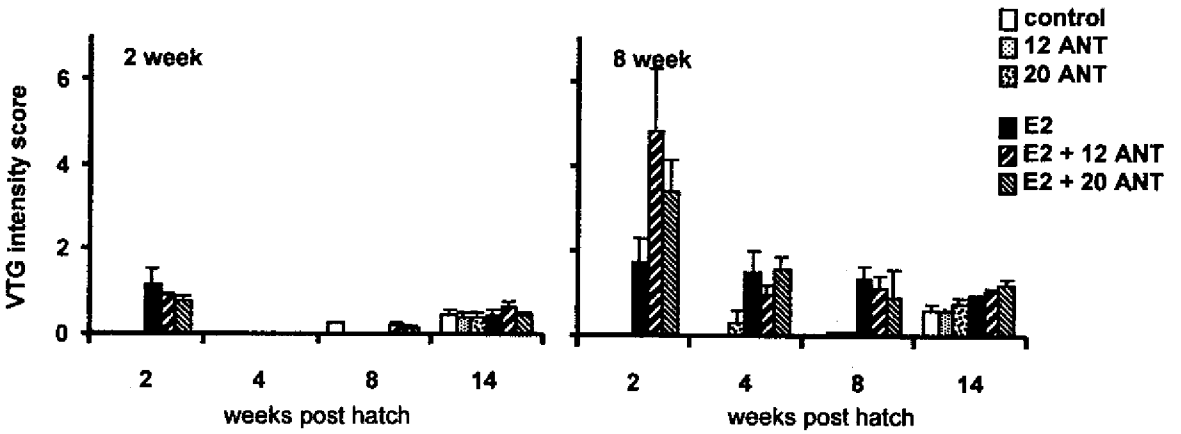

Figure 1. ANT does not induce or inhibit VTG synthesis.

Sex ratio

We quantified sex ratio in adults based on external morphology. Males have a longer, rectangular anal fin and a notched dorsal fin, while females have a shorter, triangular anal fin and an un-notched dorsal fin. ANT alone did not alter sex ratio regardless of dose or exposure duration, indicating that it does not inhibit the action of endogenous estrogens present during sex differentiation. E2 feminized fish, producing 83% females and 8% feminized males after 2 weeks (Table 1) and 100% females after 8 weeks. Feminized males had ambiguous fin morphology but spawned as males. Both doses of ANT prevented E2-induced feminization during a 2 week exposure, but rather than increasing the number of normal males, the higher dose doubled the number of feminized males (Table 1). ANT could not block E2-induced feminization during an 8 week exposure. Taken together, these results suggest that the efficacy of ANT as an anti-estrogen is both time and estrogen-dose dependent.

Table 1. Sex ratio of adults after 2 week post-hatch exposure to E2 alone (0.27 (g/L) or in combination with ANT (12 or 20 (g/L).

Treatment	# males	# feminized males	# females
E2	2	2	20
E2 + 12 ANT	6	3	15
E2 + 20 ANT	5	7	12

Reproductive Success

For both exposure periods, reproductive success was quantified as (1) the number of fertilized eggs per treated individual, (2) the percentage of fertilized eggs per treated individual, and (3) the percentage of a treated individual's embryos surviving for 72 hours post hatch.

After a 2 week developmental exposure, ANT alone had no effect on male or female reproductive success, but ANT + E2 treatment of males significantly reduced reproductive success. Because some, but not all males in the ANT + E2 mating pairs were feminized, we compared fertilization and hatching success between feminized males and normal males at the same doses of E2 and ANT. Feminization reduced the number of fertilized eggs to less than 4% of those fertilized by normal males (Fig. 2). This suggests that E2, not ANT reduced sperm production in feminized males. None of the eggs fertilized by feminized males hatched, while 40 – 60% of those fertilized by normal males did, indicating that sperm quality was affected as well as sperm quantity.

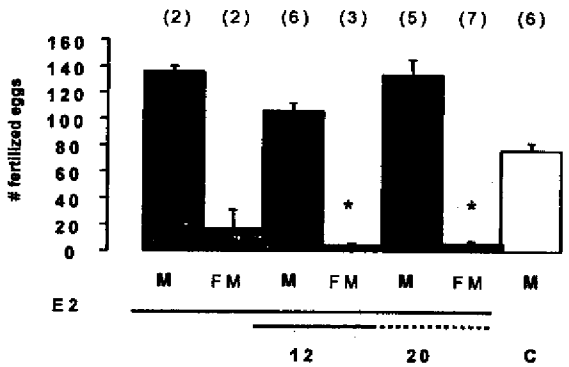

Figure 2. Number of fertilized eggs in normal (M) and feminized males (FM) treated with E2 or ANT + E2 for 2 weeks post-hatch. Numbers in parentheses are numbers of individuals. C, control (solvent). * indicates means significantly different ($p < 0.05$) between males and feminized males within the same treatment.

After an 8 week developmental exposure, females treated with 20 (g/L ANT had noticeably fewer fertilized eggs than controls (Fig. 3). Females treated with E2 + ANT had the same number of fertilized eggs as controls, suggesting that E2 prevented the ANT-induced reduction. When both partners were treated, 20 µg/L ANT significantly decreased the percentage of fertilized eggs (Fig. 4). Males treated with ANT showed no changes in reproductive success, suggesting that ANT preferentially affected eggs (Figs 3 and 4). Neither ANT nor E2 affected hatching success of embryos from treated males or females. The apparently female-specific effect of ANT may be due to transfer of lipophilic contaminants from the female to her eggs. Parental transfer of lipophilic contaminants into gametes (eggs or sperm) can be a significant route of contaminant elimination in fishes (Guiney et al., 1979; Mac and Edsall, 1991).

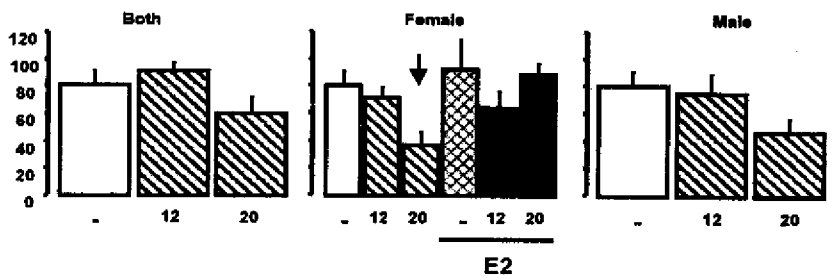

Figure 3. Number of fertilized eggs produced by adults exposed for 8 weeks during development.

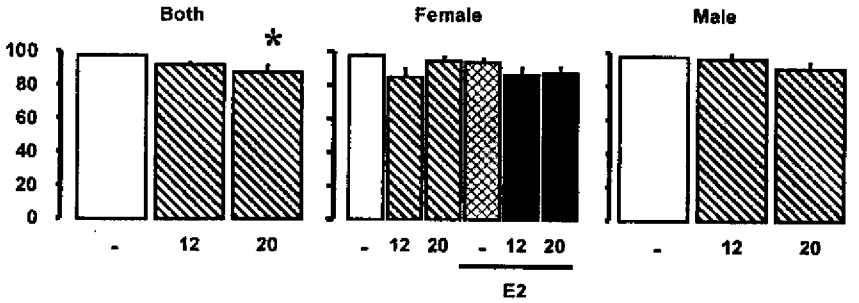

Figure 4. Percentage of fertilized eggs produced by adults exposed for 8 weeks during development.

Environmental Estrogen

This study addressed two issues: 1) the effects of developmental estrogen exposure on individual fitness (number of offspring) and 2) the utility of VTG as a predictive biomarker for altered sexual development and impaired reproduction (Cheek et al., 2001b). We predicted that developmental exposure to environmental estrogens would have an organizational or permanent effect (Arnold and Breedlove, 1985) on gonad morphology and reproductive function, but would have an activational or transitory effect on VTG production, i.e. exposed animals will produce VTG only while the stimulus is present. If this is the case, VTG serves as an excellent biomarker of current estrogenic exposure, but may not indicate organizational effects such as altered sex differentiation and impaired reproductive function.

We chose to use o,p'DDT as a model environmental estrogen for several reasons. First, although DDT use was banned in the United States in 1973, DDT isomers and metabolites are extremely stable and are globally distributed at concentrations ranging from 0 to 10 ppm (Simonich and Hites, 1995). Second, o,p'DDT is known to be a relatively potent environmental estrogen in several vertebrates (Fry and Toone, 1981; Palmer et al., 1998; Palmer and Palmer, 1995; Soto et al., 1994). Finally, DDT feminizes developing male medaka (Edmunds et al., 2000; Metcalfe et al., 2000).

We exposed developing medaka to o,p'DDT for two or eight weeks post hatch and examined the effects of exposure on VTG synthesis, sex differentiation, and reproductive success. Fish were exposed to 0, 0.5, 1.0, 2.5, and 7.5 ppb o, p' DDT, then sampled 2, 4, and 8 weeks after hatch to examine VTG expression and gonad development. After exposure, fish were transferred to clean water, grown to sexual maturity, and placed in mating pairs. Eggs were collected for 7 days and scored for fecundity (number of eggs), fertility (% fertilization), and hatching success (% hatching). Details of methods are found in (Cheek et al., 2001b).

Vitellogenin synthesis

In fry exposed to DDT for 2 weeks post-hatch, VTG production was not induced in juveniles (up to 8 weeks old) or altered in adult fish (17 weeks post-hatch)(Fig. 5). VTG expression did increase over time, as expected with the onset of sexual maturity. When fry were continuously exposed to DDT for 8 weeks post-hatch, VTG production was induced in a dose and time dependent manner (Fig. 5). Once exposure ended at eight weeks post-hatch, VTG production was attenuated. These data indicate that DDT had transitory, activational effects on VTG synthesis.

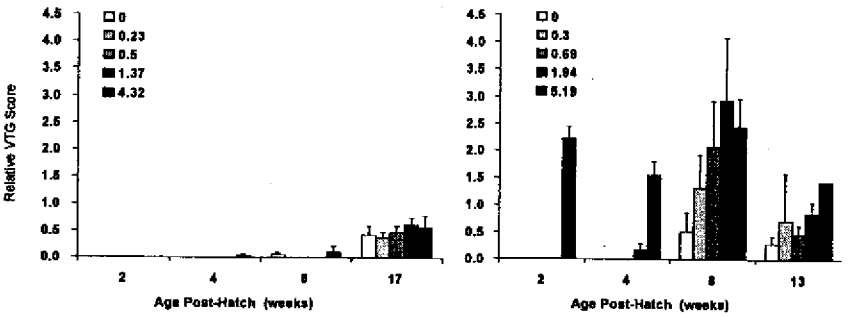

Figure 5. VTG synthesis in response to 2 (left panel) or 8 week (right panel) exposure to DDT.

Sex differentiation

Developmental exposure to DDT caused a female-skewed sex ratio in adults (Fig. 6). When exposure occurred during the first two weeks post-hatch, only the highest dose caused feminization, but when fry were exposed for eight weeks post-hatch, both the highest doses caused a significantly female-skewed sex ratio (Fig. 6). Cross-sectional sampling of exposed fry (n = 6 individuals per dose per sampling time) suggested that feminization was a progressive process. In fry exposed for two weeks post-hatch, a disproportionate number of ovaries were observed in four and eight-week old fry exposed to the highest doses of DDT. In some fry exposed for eight weeks post-hatch, ovotestes appeared after two and four weeks exposure to the highest doses of DDT, while after eight weeks only ovaries were observed. However, two males exposed to 1.94 (g/L DDT and sampled at sexual maturity (13 weeks) had ovotestes, suggesting that incomplete feminization occurred in these fish. These observations strongly suggest that o,p' DDT-induced feminization is due to gonadal reorganization and not to differential mortality of DDT exposed male fish.

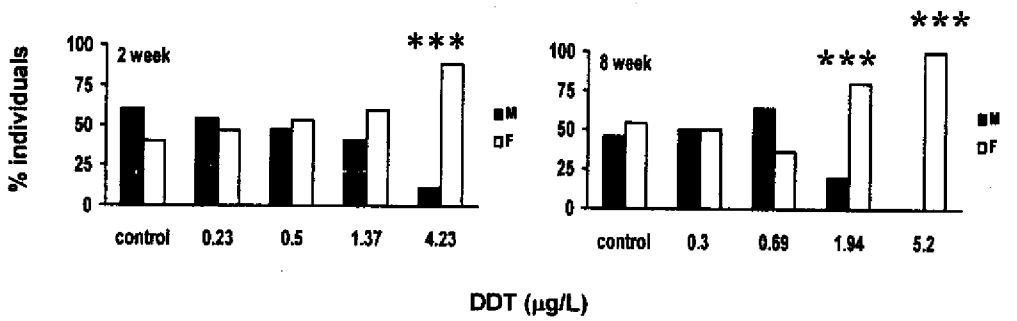

Figure 6. Adult sex ratio of medaka exposed to DDT for 2 or 8 weeks during development.

Reproductive Success

Developmental exposure to DDT had a relatively small impact on female fecundity (egg number). After a two week developmental exposure, the highest doses of DDT reduced fecundity of exposed females, whether they were mated with control males or exposed males. These results indicate that DDT directly affects ovaries. After an eight week developmental exposure, so few individuals (n = 2 females) survived the highest DDT dose that no mating pairs could be tested. Even with longer exposure, lower doses did not alter female fecundity (Table 2).

Table 2. Effect of DDT exposure of females and males on female fecundity. * indicates treatments that differ significantly from the control.

Exposure Duration	DDT (µg/L)	Exposed female	Exposed Male	Both Exposed
2 weeks	0	59.0 ± 6.3	59.0 ± 6.3	59.0 ± 6.3
	0.23	52.8 ± 5.5	81.7 ± 11.5	34.2 ± 5.6
	0.50	41.3 ± 5.2	44.8 ± 8.7	59.5 ± 10.9
	1.37	34.0 ± 9.3*	46.7 ± 5.6	45.7 ± 10.3
	4.32	35.8 ± 4.7*	-	9.0 ± 6.0*
8 weeks	0	33.3 ± 4.6	33.3 ± 4.6	33.3 ± 4.6
	0.30	52.7 ± 5.4	32.3 ± 4.7	45.3 ± 6.2
	0.69	58.0 ± 7.8*	32.0 ± 4.0	34.3 ± 6.7
	1.94	47.6 ± 8.1	16.5 ± 8.5	21.5 ± 2.8

Female and male fertility (% fertilization) were significantly reduced by developmental exposure to DDT. After two or eight week developmental exposure, female fertility was significantly reduced by all doses of DDT. Male fertility was significantly decreased by doses ≥ 0.5 µg/L after a two week developmental exposure and by doses ≥ 0.3 µg/L after an eight week developmental exposure.

Female and male fitness (% hatching) were drastically reduced by developmental exposure to DDT. After a two week developmental exposure, all doses except the highest significantly reduced hatching of embryos from exposed females. Hatching was also reduced for males exposed to doses (0.5 µg/L and mated with control females. When both parents were exposed, hatching was significantly reduced at all doses except 0.5 µg/L. After an eight week developmental exposure to any dose of DDT, embryo hatching was catastrophically reduced, regardless of whether males or females were treated. In most cases, less than 20% of embryos hatched.

In summary, short term developmental exposure caused feminization at the highest dose, and reduced fertility and hatching success at much lower doses, but did not alter VTG synthesis in juveniles or in adults. Long term developmental exposure feminized males at the two highest doses and drastically reduced fertility and hatching success at all doses. Long term exposure did induce VTG synthesis in juveniles and adults at the lowest dose, but VTG production was attenuated once the estrogenic stimulus was removed. Both experiments indicate that DDT had permanent, organizational effects on sexual development and reproductive function, but transitory, activational effects on VTG production. Organizational responses were more sensitive to disruption than were activational responses; relative sensitivities were: hatching success > fertility >> gonad differentiation >> VTG production.

Conclusion from laboratory studies

Together, these studies indicate that VTG is an accurate marker of recent exposure to an environmental estrogen, but it is not a predictor of physiological effects such as changes in gonad development and reproductive function. Because this marker of exposure is less sensitive to disruption than markers of effect, such as gonad morphology, the abnormal presence of VTG may be taken as an indicator that fry and juveniles developing in the same environment may be at risk for reproductive impairment.

Assessment of Endocrine Disruption in Wild Fish

Several investigators have demonstrated endocrine disruption in fishes living in rivers or estuaries receiving industrial and sewage effluents (Folmar et al., 1996; Jobling et al., 1998; McMaster et al., 1996; Munkittrick et al., 1998), yet relative to laboratory analyses, very little work has focused on demonstrating that endocrine disruption is occurring in wild populations of fish. The purpose of this study was to determine whether endocrine disruption is occurring in a wild population of longear sunfish (*Lepomis megalotis*) in a river receiving unbleached kraft mill effluent. Our objectives were (1) to examine individual site fidelity in order to quantify the likelihood that fish receive long term exposure to effluent

and (2) to measure sex hormone and VTG levels in male and female longear upstream and downstream of the mill. We sampled fish once monthly during the non-reproductive season (Nov 2000 to April 2001 and Sept 2001 to April 2002) and twice monthly during the reproductive season (May – Aug 2001 and 2002). Fish were collected from two sites upstream and two sites downstream of the effluent outfall. Individuals were marked by elastomer injection, bled, palpitated for gamete expression, and released.

Site Fidelity

Over a two year period, 767 longear were marked. Of these, 17 (2.5%) were recaptured. Nearly all recaptures occurred at the site of initial capture (82%), indicating strong site fidelity of adults. This is important for interpretation of our reproductive data because we observed only one fish that migrated from a downstream to an upstream location and no fish that migrated from upstream to downstream of the effluent. We can reasonably assume that fish downstream of the paper mill effluent have remained in that location, at least as adults.

Biomarkers of Exposure

VTG levels were low in male fish throughout the year, and did not vary between fish captured at upstream and downstream locations. This suggests that the paper mill effluent is very unlikely to contain estrogen mimicking compounds. In contrast, VTG levels in females were highest from May to August in both years. During the first reproductive season, female VTG levels did not vary between upstream and downstream locations until August and September when females downstream of the paper mill had lower VTG levels. During the second reproductive season, females captured downstream of the paper mill had lower VTG levels at every sampling date. River flow volume differed between the two years, with lower water levels during the second sampled season.

E2 levels were low in male fish throughout the year, but did vary between fish captured at upstream and downstream locations in some months. In females, E2 levels were high from April to August and low during the fall and winter. E2 levels did not vary between fish captured at upstream and downstream locations. T levels in females did vary between locations, however. During the first reproductive season, T levels were similar in females from both locations, except at the end of season in September when fish captured downstream of the paper mill had lower T levels. During the second reproductive season, T levels were consistently lower in females captured downstream of the paper mill. T levels in males increased in February and remained elevated until late August. Fish downstream of the paper mill had significantly lower T levels in February, suggesting a slight delay in preparation for spring and summer spawning.

11-ketotestosterone (the major androgen in male fish) in male longear was high throughout both reproductive seasons. However, at the end of both seasons, 11KT levels were significantly lower in males collected downstream of the paper mill, suggesting that spermatogenesis may end earlier in effluent-exposed fish than in unexposed fish.

Conclusions from Field Study

Hormone and VTG levels are not the same in male and female longear sunfish captured upstream and downstream of an unbleached kraft mill effluent outfall. Water quality, including temperature and dissolved oxygen, is the same upstream and downstream. Conductivity is higher downstream of the paper mill, reflecting the influence of the effluent. The most likely cause of the difference in hormone levels is the presence of the effluent. We are fairly confident that these biomarkers of exposure indicate that longear are experiencing endocrine disruption. We did not invest in measuring biomarkers of effect until we could show that disruption was occurring.

CONCLUSIONS

Quantifying endocrine disruption and its effects on development and reproductive success is much simpler in laboratory experiments than in the field. Laboratory experiments allow us to explore the relationships between biomarkers of exposure, such as VTG and hormone levels, and the more integrative biomarkers of

effect, such as gonadal development and reproductive success. These laboratory experiments have shown that VTG is a very effective marker of estrogen exposure, but it does not directly predict effects on gonad development and reproduction. In assessing endocrine disruption in wild populations, we can begin by measuring the simplest and most economical markers of exposure before trying to quantify the effects of endocrine disruption. However, high levels of individual variation and temporal changes in rainfall and effluent discharge and dilution rates can strongly influence interpretation of results obtained in wild fish. Regardless of these difficulties, the importance of the question about whether wild populations are affected should drive further study.

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