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Masculinization of Female Mosquitofish in Kraft Mill Effluent-Contaminated Fenholloway River Water Is Associated with Androgen Receptor Agonist Activity

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Female mosquitofish *(Gambusia affinis holbrooki)* downstream from Kraft paper mills in Florida display masculinization of the anal fin, an androgen-dependent trait. The current investigation was designed to determine if water contaminated with pulp-mill effluent (PME) from the Fenholloway River in Florida displayed androgenic activity *in vitro* and to relate this activity to the reproductive status of female mosquitofish taken from this river. We tested water samples for androgenic activity from a reference site upstream of a Kraft pulp and paper mill on the Fenholloway River, from 3 sites downstream from the mill, and from another reference site on the Econfina River, also in Florida, where there is no paper mill. We also examined anal fin ray morphology in mosquitofish from these rivers for evidence of masculinization. Eighty percent of the female mosquitofish from the Fenholloway River were partially masculinized while another 10% were completely masculinized, based upon the numbers of segments in the longest anal fin ray (18.0 ± 0.4 vs. 28.1 ± 0.9 [$p < 0.001$]) in the Econfina River vs. the Fenholloway River, respectively). In a COS whole cell-binding assay, all 3 PME samples displayed affinity for human androgen receptor (hAR) ($p < 0.001$). In addition, PME induced androgen-dependent gene expression in CV-1 cells (co-transfected with pCMV hAR and MMTV luciferase reporter), which was inhibited by about 50% by coadministration of hydroxyflutamide (1 μM), an AR antagonist. Water samples collected upstream of the Kraft mill or from the Econfina River did not bind hAR or induce luciferase expression. When CV-1 cells were transfected with human glucocorticoid receptor (hGR) rather than hAR, PME failed to significantly induce MMTV-luciferase expression. Further evidence of the androgenicity was observed using a COS cell AR nuclear-translocalization assay. PME bound hAR and induced translocalization of AR into the nucleus. In contrast, AR remained perinuclear when treated with water from the control sites (indicating the absence of an AR ligand). Interestingly, PME also displayed "testosterone-like" immunoreactivity in a testosterone radioimmunoassay, whereas water from the reference sites did not. In summary, water collected downstream of the Kraft mill on the Fenholloway River contains unidentified androgenic substances whose presence is associated with masculinization of female mosquitofish.

Key Words: **environmental androgens; Kraft pulp and paper mill effluent; masculinized female mosquitofish anal fin; gonopodium; androgen receptor; glucocorticoid receptor; AR binding; AR gene expression; *in vitro*; Fenholloway River, Florida.**

Exposure to anthropogenic compounds that interfere with normal function of the endocrine system is a major concern, due to the potential effects on both human and wildlife reproduction and development (Colborn and Clement, 1992). Although, environmental contaminants with estrogenic and antiandrogenic properties have well-documented effects in both mammalian laboratory models and in wildlife (Gray *et al*., 1994; Guillette *et al*., 1994; 1996; Harries, 1996; Kelce *et al*., 1997; McLachlan *et al*., 1984; Mylchreest and Foster, 1998; Shelby *et al*., 1996; Sonnenschein and Soto, 1998; Sumpter and Jobling, 1993; Tyler *et al*., 1998; White *et al*., 1994) toxicants with androgenic activity have not been identified. Although antiandrogens exert their effects primarily in the fetal male, androgen agonists are more likely to alter female reproduction and development. In this regard, several field and laboratory studies have hypothesized the existence of environmental androgens based on the presence of masculinized female fish from bodies of water in North America and Europe (Bortone and Cody, 1999; Cody and Bortone, 1997; Drysdale and Bortone, 1989; Hegrenes, 1999; Howell and Angus, 1999; Wells *et al*., 1999). Female mosquitofish living downstream from the Buckeye Kraft mill (KM) on the Fenholloway River in Florida display an anal fin that is masculinized, having a greater number of segments and/or being elongated, resembling in part a male-like gonopodium (Davis and Bortone,

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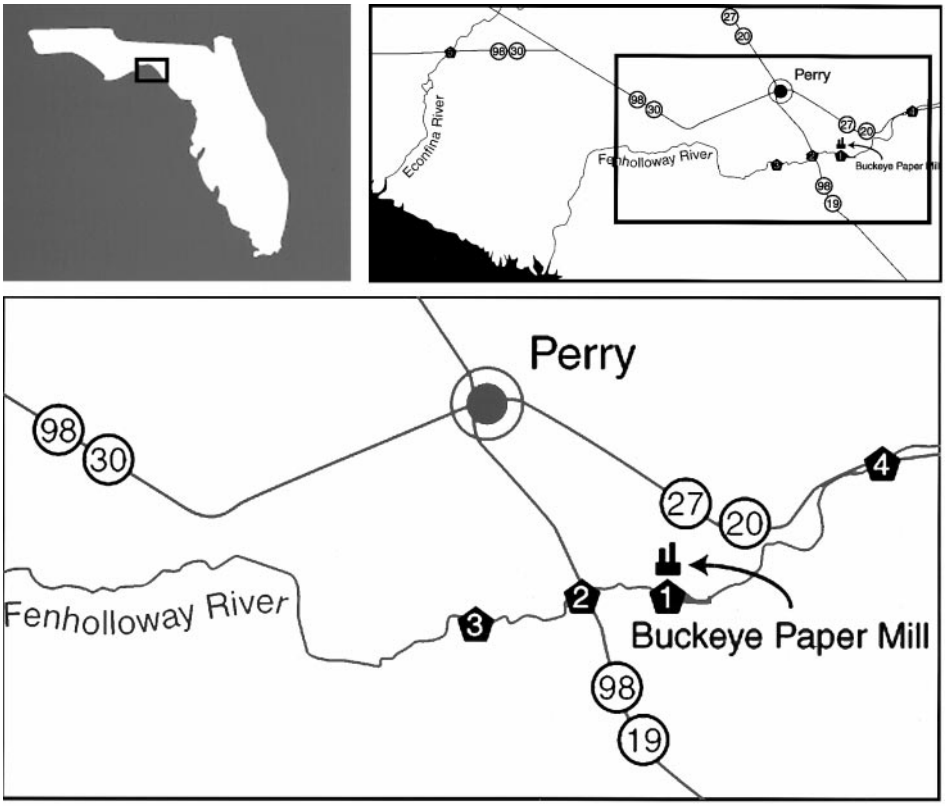


COLLECTION SITES AND WATER-QUALITY PARAMETERS

Site	Water (C)	pH	Salinity (ppt)	Water Clarity (Secchi disk - cm)
1	27.0	7.63	1.0	7
2	27.0	7.69	1.0	14
3	25.0	7.28	1.0	14
4	20.5	7.53	0.0	45
5	22.0	7.92	0.0	80

FIG. 1. Map of the water (Sites 1–5) and fish (Sites 3 and 5) collection sites from May 1999 and water quality parameters measured concurrently at these sites. Water was collected on the Fenholloway River and the Econfina River in the panhandle region of Florida. Water collected adjacent to the Kraft mill plant (Buckeye plant) is labeled Site 1 (located at the point of effluent discharge), while Sites 2 and 3 are located approximately one-half mile and 2 miles, respectively, downstream from Site 1. The water collection site upstream of the plant is labeled Site 4, and the site on the Econfina River, on which there is no paper mill, is labeled Site 5.

1992; Howell *et al*., 1980). In the mosquitofish (*Gambusia affinis holbrooki)*, the anal fin of the male serves as a gonopodium, an intromittent organ for internal fertilization in this live-bearing fish. Masculinization has also been reported for female mosquitofish from other bodies of water in Florida (Davis and Bortone, 1992) and other fish species in the Great Lakes as a consequence of exposure to pulp mill effluent (PME; Wells *et al*., 1999).

In sexually dimorphic vertebrate species, males and females possess species-specific, androgen-dependent, and estrogen-dependent secondary sex characteristics. Although androgen-exposure induces the formation of a male-like anal fin in the female mosquitofish, the presence of masculinized female fish does not necessarily indicate that an endocrine-disrupting chemical (EDC) acts as an AR agonist, as other mechanisms of action could also produce these effects. For example, it has been hypothesized that inhibition of aromatase activity, the enzyme that converts testosterone to estradiol, could masculinize fish by causing an accumulation of testosterone (Orlando *et al.*, 1999).

In the present study, male and female mosquitofish were collected simultaneously with some of the water samples from the Fenholloway and Econfina Rivers (Fig. 1). The Econfina River is an uncontaminated “reference” site that arises from the same headwaters as the Fenholloway River. We hypothesized that since mosquitofish are masculinized downstream from the

discharge of the Buckeye-Kraft mill this water would display androgenic activity. In the current study we used *in vitro* mammalian cell-based assays containing the human androgen (hAR) or glucocorticoid (GR) receptors to determine if either androgenic- or glucocorticoid-like activity was present in the river water downstream from the plant. GR-induced gene expression was examined in PME samples, because it has been suggested that corticosteroids might be present and induce masculinization in the female fish, and secondly, because both GR and AR will induce luciferase gene expression via the MMTV promoter *in vitro*. Similar gene-expression assays with AR from fish have not yet been developed.

Another objective of our investigation was to relate this endocrine activity in the water sample to the reproductive status of female and male mosquitofish. We wanted to confirm that female mosquitofish in the Fenholloway River were indeed masculinized and to provide an expanded description of the reproductive systems of male and female fish, using a large sample size throughout the year to precisely and accurately describe the condition of the mosquitofish in this river. In our study, body length and weight, anal-fin length, and the numbers of segments in the longest ray of the anal fin were measured in over 500 male and female mosquitofish. Further assessments on these fish (including gonad and liver weights, ovarian histology, with counts of the numbers of follicles and embryos, and ovarian aromatase activity; Orlando *et al.,* in preparation) will be presented elsewhere. This study provides *in vitro* data demonstrating the presence of androgenic activity in PME and data confirming masculinization of the anal fin of the female mosquitofish.

MATERIALS AND METHODS

Collection of water samples. Water samples were collected from 5 sites in the Florida panhandle in May of 1999 in pre-cleaned, EPA-approved amber bottles (Fisher, cat # 05–719–77). Site 1 was at the point of effluent discharge from the Buckeye mill. Sites 2 and 3 were approximately one-half mile and 2 miles downstream from Site 1, respectively (Fig. 1). Site-4 water was collected on the Fenholloway River above the mill, and water was collected from another "control/reference" site (Site 5) on the Econfina River near the bridge on route 98. The Econfina River was selected as a reference site because it arises from the same headwaters as the Fenholloway River (Fig. 1). The temperature (at 6-in. depth), salinity, pH, and clarity (using a Secchi disk) was measured when water samples were collected. Bottles remained sealed until they were submerged below the water; the caps were then removed and filled. Bottles were capped under water, placed on ice, and returned to the University of Florida. They were refrigerated until shipping on the following morning via overnight express to the U.S. EPA, Research Triangle Park, NC. Upon receipt, the samples were stored at 4°C. Mosquitofish were collected from Sites 3 (PME) and 5 (Econfina River).

Collection and analysis of male and female mosquitofish. Several hundred male and female mosquitofish were collected at 3 different times of the year from the Fenholloway (contaminated Site 3, near the Carlson Bridge on SR 361A) and Econfina (near the bridge on SR 98) Rivers. The data included herein are from female mosquitofish collected in May 1999 ($n = 50$ and $n = 49$ from the Econfina and Fenholloway Rivers, respectively) when water samples were taken for *in vitro* analysis of androgenic activity. Fish were transported in coolers and placed in aquaria with fresh water from their respective rivers. The following day, fish were anesthetized and the following morphometric data were collected: total body weight and length, anal fin length, number of segments in the longest ray of the anal fin, and liver, gonad, and brain weights. These data, the number of segments in the anal fin being the most robust, sexually dimorphic trait in this species, are presented here to confirm the abnormal, male-like sexual phenotype of the female fish in the Fenholloway River. Additional morphometric, endocrine (ovarian aromatase activity, body testosterone concentration, and ovarian histological data are presented in a companion paper, Orlando *et al*., in preparation).

Preparation of water samples by solid-phase extraction. For some assays, site water was concentrated using solid-phase extraction (SPE) C-18 columns (J.T. Baker, #7020–06, 6-ml Bakerbond Octadecyl Extraction Columns). Columns were attached to a vacuum manifold and primed with 4 ml of methanol (Fisher Scientific), followed by 2 ml of distilled water. Columns remained wet during the entire priming process. After conditioning, 40 ml of site water was passed over a column. The column was then dried under vacuum for 1 h. The columns were eluted with 2 3-ml aliquots of methanol. In the CV-1 assay, the methanol was evaporated under vacuum and the sample was resuspended in 300 μl of phosphate-buffered saline (100 mM) plus 1% gelatin for the testosterone RIA; 500 microliters of serum-free DMEM (Gibco Brl.) for the COS assay; or 2 ml of DMEM plus 5% dextran charcoal-stripped serum (Hyclone).

Preparation of water samples: Dosing media made with site water. For some assays, powdered DMEM (Gibco) with 3.7 g $NaHCO_3$ (ICN Biochemicals) was reconstituted with 1 liter of site water from each site and adjusted to a pH of 7.4. Media were sterile filtered (0.2-micron, Nalgene bottle-top filters), supplemented with 5% dextran charcoal serum (HyClone) and antibiotics (Gibco), and stored at 4°C wrapped in aluminum foil in the dark until use in the CV-1 transcriptional-activation assay.

COS whole cell human androgen receptor (hAR) binding assay. The COS whole cell-binding assay was used to evaluate the ability of the SPE concentrated water samples from 40 ml of each (dried down and resuspended in 500 μl of medium) from 5 sites to compete with [^{3}H] R1881 (a synthetic androgen) for binding to the hAR. In 3 blocks, 2 replicates per block, COS cells (SV-40 transformed monkey kidney line ATCC # CRL-1650) were transiently transfected with the hAR expression vector pCMVhAR as described by Wong *et al*. (1995). COS cells were plated at 200,000 cells/well in 12 plates and transfected with 1 μg of pCMVhAR (from Dr. Elizabeth Wilson, UNC at Chapel Hill). After a 3-h transfection period, cells were washed with DPBS and incubated overnight in 2 ml 10% FBS DMEM. Twenty-four hours later, medium was aspirated and replaced with 200 μl of serum-free/phenol red-free DMEM with R1881 plus 200 microliters of medium made from SPE-extracted site water (400-μl incubation volume, with a concentration factor of 40×). Cells were incubated for 2 h with 5 nM [^{3}H] R1881 at 37°C under an atmosphere of 5% CO_2. Nonspecific binding (NSB) was determined by adding 100-fold molar excess of unlabeled R1881 to NSB tubes. Cells were washed in phosphate-buffered saline and lysed in 200 μl ZAP (0.13 M ethylhexadecyldimethylammonium bromide with 3% glacial acetic acid). The lysate was added to 5 ml OPTI-fluor scintillation cocktail (Packard Bioscience, The Netherlands) and radioactivity was counted using a Beckman LS 5000 TD counter (Beckman, Irvine, CA).

CV-1 AR and GR 40-dependent transcriptional activation assays. Three experiments, each with several replicates, were conducted to determine if PME induced AR- or GR-dependent gene expression in CV-1 cells (monkey kidney line, ATCC # CCL-70). In these experiments, 200,000 CV-1 cells were plated in a 60-mm dish and then transiently cotransfected with 1 μg pCMVhAR and 5 μg MMTV-luciferase reporter using 5 μl Fugene reagent in 95 μl serum-free medium as per the manufacturer's protocol (Boehringer Mannheim, Germany). Twenty-four h after transfection, medium was aspirated and replaced with 2 ml of DMEM plus 5% DCC (1) reconstituted extracted sample (20-fold concentration factor) or (2) with 2 ml medium prepared with water from each of the 5 sites (without any concentration). Cells were then incubated at 37°C under 5% carbon dioxide. After 5 h of exposure, medium was removed, and cells were washed once with phosphate-buffered saline and harvested with 500 μl

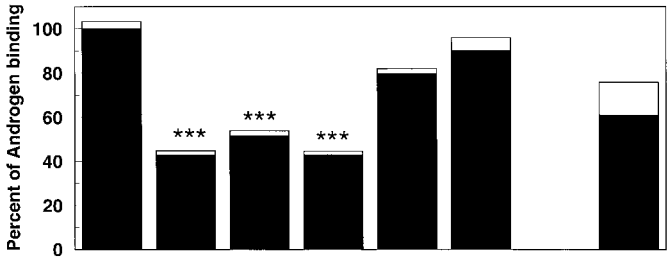
lysis buffer (Promega). Relative light units of 0.05-ml aliquots of lysate were determined using a Monolight 2010 luminometer (Analytical Luminesce).

Experiments 1 and 2 examined AR agonist activity using extracted and unextracted site water. In the first experiment, cell media were made with site water (4 replicates), while in a second experiment, 20 ml SPE extracted site water (prepared as described above from 40 ml divided into 2 duplicates, 3 replicates) was used to determine if the androgenic activity eluted with the more lipophilic fraction. In the first experiment, 1 μM hydroxyflutamide was added to half of the samples with PME to see if this potent antiandrogen would block the PME-induced luciferase activity. Cells also were exposed to 1 nM DHT as a positive control. A third experiment was conducted to determine if PME would induce glucocorticoid-dependent transcriptional expression. CV-1 cells were transfected as above with 1 μg of pCMVhGR instead of pCMVhAR. CV-1 cells containing the glucocorticoid receptor (GR) were then dosed with media made with site water (2 ml, concentration factor 0.5 $\times$) from all 5 sites, incubated, and luciferase activity measured as above for CV-1 cells transfected with the hAR. In addition, CV-1 cells were incubated with 1 nM dexamethasone, a potent GR agonist, which induces MMTV-luc gene expression as a positive control (3 replicates).

COS cell immunocytochemistry. The following experiment was conducted to visualize, by immunofluorescence, the ligand-induced nuclear translocation of hAR in COS cells. Two chamber slides (Nunc) were seeded with 100,000 cells/chamber in 2 ml DMEM (Gibco) supplemented with 10% FBS (HyClone) after which cells were transfected with 0.5 μg pCMVhAR (as per Wong *et al*., 1995). Following transfection, 2 ml DMEM plus 5% DCC medium was prepared from site water (without concentration), added to slides, and incubated for 24 h at 37°C under 5% CO_2. The next day, medium was removed, cells washed once with DPBS (Dulbecco's phosphate buffered saline), allowed to dry for 45 min at room temperature, fixed for 10 min with 95% ethanol (−20°C), blocked with 5% BSA (Sigma, in DPBS), and incubated overnight with primary AR antibody (Affinity Bioreagents, 1:1000) at 4°C. The following day, cells were washed once with DPBS and incubated with fluorescently labeled secondary antibody (Molecular Probes) for 30 min at room temperature. To visualize the nuclei, cells were counter-stained with DAPI (Sigma), a DNA stain, mounted with fluoromount (Electron Microscopy Sciences), and the slides examined using a Nikon Optiphot-2 Microscope at 200 $\times$ magnification. The localization of the AR was classified as either perinuclear or nuclear in a blinded fashion from 10 randomly selected fields from a slide for each site. In another experiment, cells were exposed to 1 nM DHT, as a positive control.

Testosterone (T) radioimmunoassay (RIA). A testosterone RIA was conducted as previously described (Kelce *et al*., 1997 modifed from Cochran *et al*., 1981; Ewing *et al*., 1984; and Schanbacher and Ewing, 1975) with the water samples from the 5 sites (3 replicates per sample). Forty ml of water was extracted using an SPE protocol described above (100% methanol elution). After evaporation of the methanol, samples were resuspended in 300 μl PBS-G (100mM), vortexed 30 s and placed in a 45°C water bath. These samples have been concentrated 133-fold (from 40 ml to 0.3 ml). After 10 min, samples were removed from the water bath, at which time [^{3}H] testosterone (10,000 DPM in 100 μl, 1 mCi/ml, DuPontNEN), T-antibody ((1:10,000) ICN in 100 μl) and 400 μl PBS-G were added to each sample. Samples were then vortexed and incubated overnight at 4°C. On the following day, a charcoal mixture (1 ml) was added to each sample for 20 min, and then each sample was vortexed (30 s) and centrifuged (10 min at 2000 $\times$ *g*). After centifugation, the supernatant was combined with 15 ml of OPTI-fluor scintillation cocktail (Packard Bioscience, The Netherlands) in a 20-ml plastic scintillation vial and the level of radioactivity in the samples was quantified by scintillation counting for 2 min (Beckman LS 5000 TD, Irvine CA).

Statistical analyses. Binding data were expressed as percent bound minus nonspecific binding, whereas the gene expression data were expressed as fold induction above the medium value, calculated for each replicate. When warranted, fold induction data were log-transformed to correct for heterogeneity of variance, typical for this sort of biological response. In addition, the CV-1 AR and GR assay data were recalculated as percent of the DHT (for AR) or dexamethasone (for GR) positive controls so the effects of PME could be compared to these potent inducers of gene expression. *In vitro* data and the number of segments in the anal fin were analyzed by ANOVA using PROC GLM available with SAS version 6.08 on the U.S. EPA's IBM mainframe. Statistically significant effects ($p < 0.05$, F statistic) were examined using the LSMEANS procedure (*t*-test) to compare the sites with the media control group and river water from the 2 reference/control sites. Site-related differences in the distribution of AR (nuclear versus perinuclear), detected using immunohistochemistry, were analyzed using χ^2 and Fisher's exact tests.



	Con	Site 1	Site 2	Site 3	Site 4	Site 5		FLUT
■ Mean	100.0	43.0	51.6	43.0	79.8	90.2		61.0
□ SE	3.3	1.8	2.2	1.5	2.2	5.8		14.7

FIG. 2. COS whole-cell androgen receptor (AR)-binding assay using water (concentrated from 40 ml, with a final concentration factor of 40 $\times$) from all 5 sites. Water samples from 5 sites were examined for AR binding, including a reference site upstream (Site 4) and 3 downstream (Sites 1–3) from the Kraft mill paper plant on the Fenholloway River and one from the Econfina River (Site 5), on which there is no paper mill. In this assay, 40 ml of water from each site was concentrated through a C-18 column (6 replicates) for Sites 1–5 (8 replicates for control medium and 5 for hydroxyflutamide). Only PME from Sites 1–3 and hydroxyflutamide (1 nM) significantly inhibited binding of [^{3}H] R1881 (a synthetic androgen) to the AR as compared to medium control ($p < 0.001$), upstream reference, Site 4 ($p < 0.01$), and the Econfina River reference, Site 5 ($p < 0.001$).

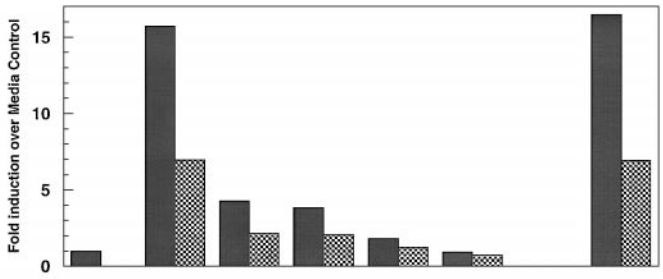
RESULTS

COS Cell AR Binding Assay

PME (concentrated from 40 ml) from Sites 1–3 downstream from the Buckeye plant significantly inhibited R1881 AR binding by approximately 50–60% (compared to media control, $p < 0.001$; upstream site, $p < 0.01$; and Econfina River site, $p < 0.001$). In contrast, water from the 2 reference sites did not significantly inhibit R1881 binding to the AR as compared to media control (Fig. 2). The binding activities of the concentrated water samples from three PME sites downstream from the KM were comparable in potency to 1 μM hydroxyflutamide (Fig. 2).

CV-1 AR-dependent Transcriptional Activation Assay for Androgen Agonist Activity

PME from the sites downstream from the Buckeye plant induced androgen-dependent transcriptional activation in CV-1 cells that were transiently cotransfected with the hAR



	Con	Site1	Site2	Site3	Site4	Site5		DHT
■ No OHF	1.0	15.7	4.3	3.8	1.8	0.9		16.5
▨ OHF		7.0	2.1	2.0	1.2	0.7		6.9

OHF; 1 micromolar hydroxyflutamide added to media, an AR antagonist

FIG. 3. CV-1 assay for androgenic activity, using unextracted site water (without concentration). In this assay, luciferase expression is induced by hAR agonists. CV-1 cells were transiently cotransfected with hAR and MMTV-luc promoter-reporter plasmids (see Materials and Methods for details). Medium was made with water from the 5 collection sites (4 replicates), including one upstream (Site 4), 3 downstream (Sites 1–3) from the Kraft mill paper plant on the Fenholloway River), and one (Site 5) on the Econfina River, on which there is no paper mill. PME from Sites 1–3 significantly induced luciferase activity as compared to medium control ($p < 0.01$) and the Econfina reference, Site 5 ($p < 0.01$, analyzed using log-transformed data to correct for heterogeneity of variance). These results demonstrate that the androgenic activity is seen in water contaminated with PME on the Fenholloway River. In addition, the induction of luciferase activity by DHT or PME was significantly reduced by co-administration of the potent antiandrogen hydroxyflutamide (1 μM; $p < 0.01$; data not shown). Both DHT-induced luciferase activities were inhibited by about 50% by coadministration of this AR antagonist. This indicates that the PME-induced luciferase expression is mediated via the AR.

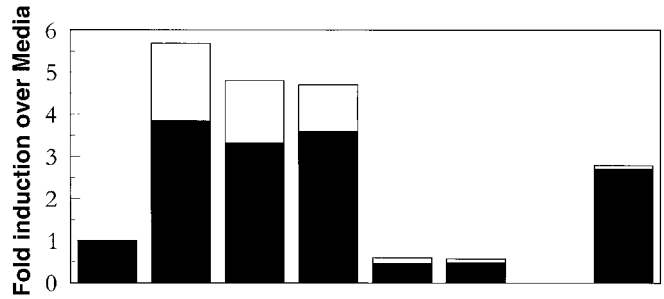
and the MMTV-luciferase reporter. Unextracted water samples from PME Sites 1–3 induced luciferase activity by 15.7-, 4.3-, and 3.8-fold over media ($p < 0.01$ using log10-transformed data) and the Econfina reference-site water ($p < 0.01$; Fig. 3). Site-1 water (unextracted) also elevated luciferase expression significantly, when compared to water from the reference site upstream of the Kraft mill ($p < 0.001$). Further, the induction of luciferase activity by DHT or unextracted PME from the 3 sites downstream of the plant was significantly attenuated from 8- to 3.7-fold by 53% ($p < 0.01$ for the main effect of hydroxyflutamide treatment in a 2-way ANOVA) by co-administration of 1 μM hydroxyflutamide potent antiandrogen.

PME from Sites 1–3 (extracted from 20 ml) also significantly induced luciferase activity by 3.9-, 3.3-, and 3.6-fold, respectively, as compared to water samples from the upstream (0.46-fold) and Econfina (0.49-fold) sites and media control (Fig. 4). Extracted and non-extracted PME displayed androgenic activity eqivalent to the positive control androgen, DHT (1 nM), which represents a maximal response in this assay.

CV-1 GR-dependent Transcriptional Activation Assay for Glucocorticoid Agonist Activity

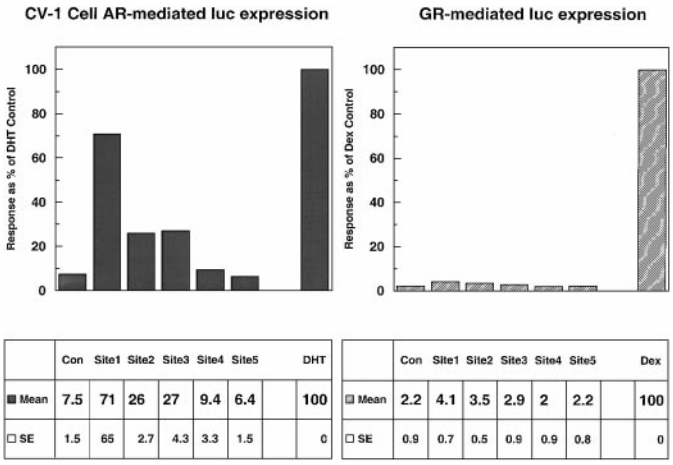
To determine if glucocorticoid-like activity was displayed by PME, CV-1 cells were cotransfected with the GR and the MMTV luciferase reporter and then treated with medium made with PME. In contrast to the induction in luciferase seen above with the hAR, PME did not significantly induce the MMTV-luciferase promoter-reporter via the GR, whereas the positive control, 1 nM dexamethasone, induced GR-dependent MMTV-luciferase expression by nearly 85-fold above medium values. The F-value for the ANOVA model for fold induction, which compared the 5 sites to the medium control, was not significant ($F = 0.76$ (5,12 df), $p > 0.8$).

When the data from the CV-1 AR- and GR-dependent assays are displayed as percent response of the appropriate positive control (DHT for AR and dexamethasone for GR), it is evident that PME has considerable AR agonist activity, but induces little or no gene expression via the GR (Fig. 5). The slight simulation of GR at Site 1, the highest value in the water samples, did not differ significantly from the medium control value (4.04 ± 2.83 for Site 1 versus 1.0 for medium control, $p > 0.15$).



	Con	Site1	Site2	Site3	Site4	Site5		DHT
■ Mean	1.00	3.85	3.33	3.60	0.46	0.49		2.71
□ SE		1.83	1.47	1.10	0.14	0.08		0.08

FIG. 4. CV-1 assay for androgenic activity using methanol-extracted (20 ml per individual sample, final concentration factor of 20 ×) site water samples. In this assay, luciferase expression is induced by hAR agonists. CV-1 cells were transiently cotransfected with hAR and MMTV-luc promoter-reporter plasmids (see Materials and Methods for details). Water samples from 5 sites were examined, including one upstream and 3 downstream (Sites 1–3) from the Kraft mill paper plant on the Fenholloway River and one from the Econfina River (Econ), on which there is no paper mill (3 replicates). Water samples from all 3 downstream sites contaminated with PME and the positive control, DHT, all significantly ($p < 0.05$, as analyzed by log-transformed data to correct for heterogeneity of variance) induced luciferase activity as compared to medium control, and both of the uncontaminated reference sites (the upstream Sites 4 and 5 on the Econfina River). The induction of androgen-dependent luciferase expression in the CV-1 cell by PME from the downstream sites (Sites 1–3) was similar to that of the positive control DHT (1 nM).



	Con	Site1	Site2	Site3	Site4	Site5		DHT
■ Mean	7.5	71	26	27	9.4	6.4		100
□ SE	1.5	65	2.7	4.3	3.3	1.5		0

	Con	Site1	Site2	Site3	Site4	Site5		Dex
▨ Mean	2.2	4.1	3.5	2.9	2	2.2		100
□ SE	0.9	0.7	0.5	0.9	0.9	0.8		0

FIG. 5. CV-1 assay for glucocorticoid and androgen receptor-mediated activity with medium made from site water (without concentration). In this assay, luciferase expression is induced by hGR agonists like dexamethasone. CV-1 cells were transiently cotransfected with hGR and MMTV-luc promoter-reporter plasmids (see Materials and Methods for details) and treated with medium made with site water. The GR agonist activity of water samples from all 5 sites was examined (3 replicates), including one upstream (uncontaminated) and 3 sites downstream (PME Sites 1–3) from the Kraft mill paper plant on the Fenholloway River and one from the Econfina River (Site 5-Econ), FL, on which there is no paper mill. Neither PME-contaminated nor reference site water induced GR-dependent luciferase activity. In contrast, the potent glucocorticoid receptor agonist dexamethasone (DEX, 1 nM) induced luciferase expression by about 85-fold over medium. PME significantly induces AR-dependent gene expression (expressed as a percentage of DHT, the positive control) whereas GR-dependent gene expression is not significantly affected (as a percentage of 1 nM dexamethasone, the positive control).

Testosterone Radioimmunoassay (RIA) of Reference Site Water and PME

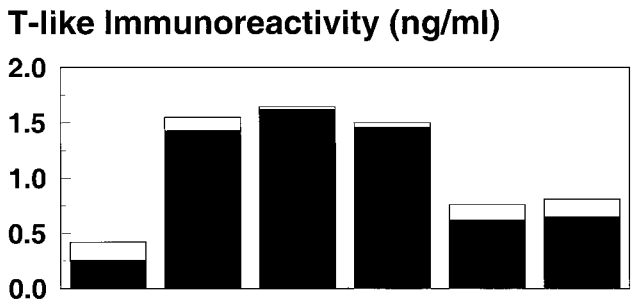
PME samples from Sites 1–3 (SPE extracted from 40 ml site water) displayed elevated T-like radio-immunoreactivity as compared to the reference sites (Fig. 6). "Testosterone-equivalent" values for 40 ml of extracted water from Sites 1, 2, and 3 were 1.43 ± 0.12, 1.62 ± 0.02, and 1.46 ± 0.04 ng/ml, respectively ($p < 0.001$ greater than medium or values for Sites 4 and 5). The values for Sites 4 and 5 were 0.62 ± 0.14 and 0.65 ± 0.16 ng/ml, respectively. Although these results are consistent with the hypothesis that PME contains T as a result of microbial metabolism of phytosterols in PME, they are not at all definitive, in that high concentrations of other chemicals in PME also may cross-react with the antibody for T and give false-positive results. However, we find it interesting that this activity continues to track with the androgenic fraction of PME, and we intend to continue to measure T-RIA immunoreactivity in conjunction with further fractionization and identification of the androgenic substance(s) in PME.

AR Distribution Determined by Immunocytochemistry

In COS cells, the location of the AR is perinuclear in the unliganded state, but when a chemical binds AR, this receptor-ligand complex undergoes a conformational change, which then binds with another liganded AR, forming a homodimer. These homodimers are then actively translocated from their perinuclear position into the nucleus where they bind hormone-response elements on androgen-dependent genes, regulating transcription. This process was visualized using immunolabeling of the AR with a fluorescent tag. COS cells exposed to PME from the 3 sites downstream of the Kraft mill displayed fluorescence throughout the nucleus. This indicates that androgenic ligand(s) present in PME bound to the AR and were translocated into the nucleus of the cell (Figs. 7A and 7C). However, after exposure to medium alone or medium prepared with the reference water samples (water from Site 4, upstream of the Kraft Mill, and Site 5, from the Econfina River), the AR remained perinuclear (Figs. 7B and 7D; medium: 1/10 nuclear; Site 1: 7/10 nuclear; Site 2: 7/10 nuclear; Site 3: 8 or 9/10 nuclear; Site 4: 2/10 nuclear; Site 5: 0/10 nuclear; overall $\chi^2 = 25.9$, $p < 0.0001$; Fishers exact probability for medium versus Sites 1 and 2, $p < 0.03$, for medium versus Site 3, $p < 0.01$; Site 4, upstream, versus Sites 1–3 pooled, $p < 0.001$).

Fish and Water Samples

Water quality parameters are presented in Figure 1. Sites 1, 2, and 3 (PME) river water temperature and salinity were increased and clarity was decreased in the Fenholloway River as compared to the Econfina River and the site upstream from



	Media	Site 1	Site 2	Site 3	Site 4	Site 5
■ Mean	0.26	1.43	1.62	1.46	0.62	0.65
□ SE	0.16	0.12	0.02	0.04	0.14	0.16

FIG. 6. Radioimmunoassay (RIA) of testosterone-like radioimmunoreactivity in 40 ml of methanol-extracted water samples (final concentration factor of 133 ×). Water samples from 5 sites were examined (3 replicates), including one uncontaminated upstream site (Site 4) and 3 PME-contaminated sites downstream (Sites 1–3) from the Kraft mill paper plant on the Fenholloway River and one uncontaminated site from the Econfina River (Econ), on which there is no paper mill (Site 5). Only PME contaminated Sites 1–3 cross-reacted with testosterone antibody to produce a significant testosterone-equivalent reading ($p < 0.001$) as compared to either reference sites. Values are presented as ng/ml testosterone-equivalent derived from the testosterone standard curve generated in the RIA.

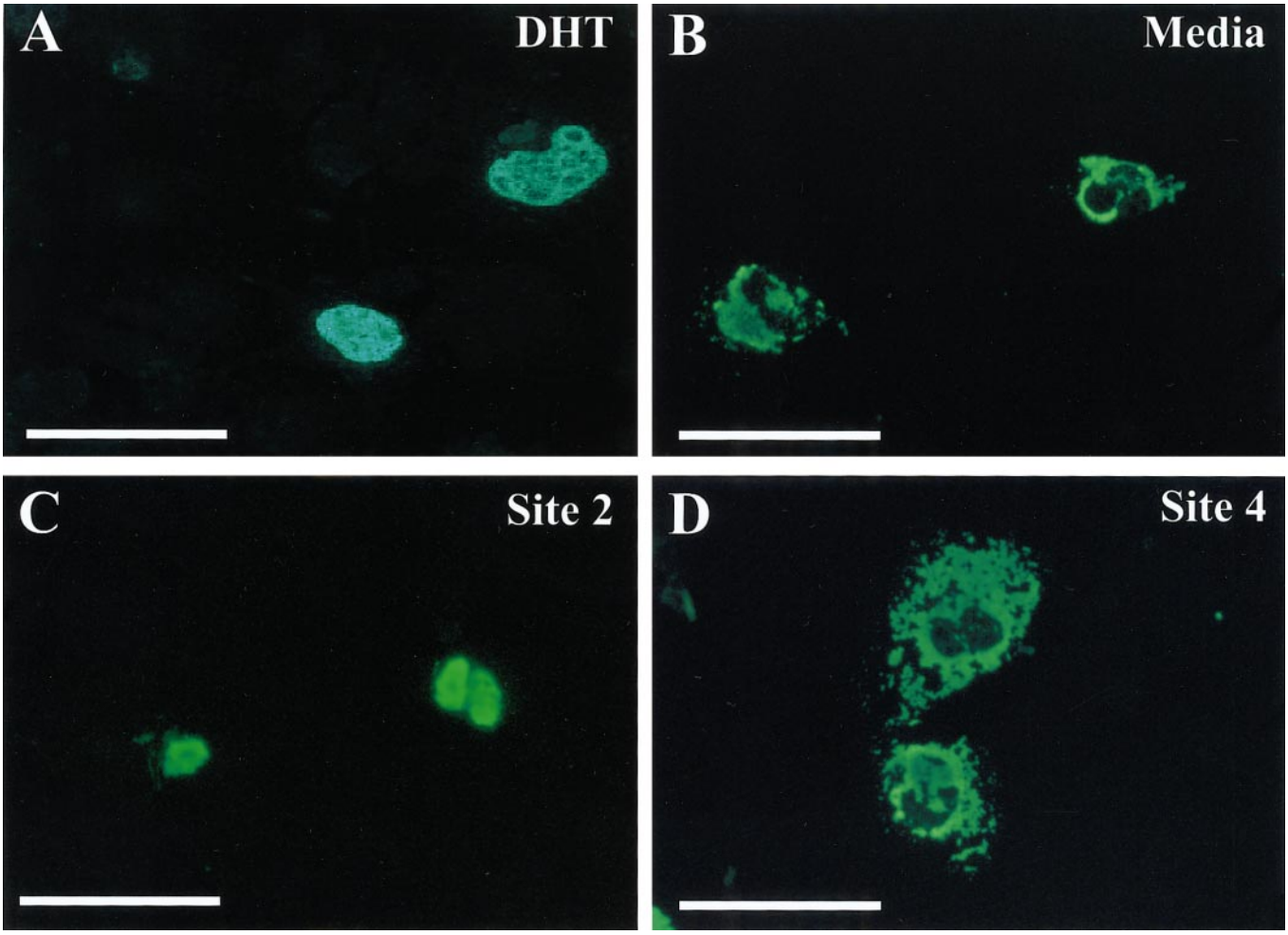


FIG. 7. Immunocytochemical localization of fluorescently tagged androgen receptor (AR) using medium made with site water (without concentration). Translocalization of AR from perinuclear to the nuclear region of COS cells takes place after ligand binding. COS cells exposed to medium made from PME from the 3 sites downstream of the Kraft mill or the positive control, DHT, displayed fluorescence throughout the nucleus. This result indicates that androgenic ligand(s) present in PME bound to the AR and were translocated into the nucleus of the cell. However, after exposure to medium alone or medium prepared with the reference water samples (water from Site 4, upstream of the Kraft Mill, and Site 5, from the Econfina River), the AR remained perinuclear (see Results for details, overall $\chi^2 = 25.9$, $p < 0.0001$). The potent endogenous androgen, dihydrotestosterone (DHT, 1 nM), is partially effective in inducing AR translocation (A). Medium prepared from PME Site 2 (C) contained chemicals that bound AR that was then translocated into the nucleus (as identified by the display of fluorescence throughout the nucleus in 7/10 cases). The medium control sample (B, 9/10 perinuclear) and medium made from water from the Fenholloway River upstream reference, Site 4 (D, 8/10 perinuclear) did not significantly induce nuclear translocalization of hAR. In these samples, the fluorescence remained perinuclear, as AR ligands were absent from these samples (10 fields were evaluated in a blinded fashion for each site). Scale bars represent 1.5 nm. The DHT panel was generated in a previous study, and the scale bar is approximately the same as in B, C, and D.

the Buckeye plant. Female fish from Site 3 on the Fenholloway River displayed several indices of masculinization as compared to Econfina River female fish, including an increased number of segments in the longest ray of the anal fin (Fig. 8) and anal fin length (AFL), adjusted for body size (S), also was increased in female fish (AFL/S $= 2.06 \pm 0.05$, Fenholloway River, versus 1.80 ± 0.02, Econfina River). The numbers of segments in the longest ray of the anal fin were increased as compared to females from the Econfina river (from 18.0 ± 0.4 [$n = 50$] to 28.1 ± 0.9 [$n = 49$]; $p < 0.001$, Econfina versus Fenholloway River, respectively). Based upon the distributions of the number of segments in the anal fin ray, 80% of the female mosquitofish from the Fenholloway River were partially masculinized. In about 10% of these, the numbers of segments in the longest ray of the anal fin fell within the frequency distribution displayed by male fish from the Econfina River, being completely masculinized for this endpoint. Hence, about 90% of the female mosquitofish on the Fenholloway River from Site 3 are either partially or completely masculinized, and only 10% are unaffected, based on segment numbers in the ray of the anal fin. Overall body size was reduced in PME-exposed females as was unadjusted anal fin length (data not shown).

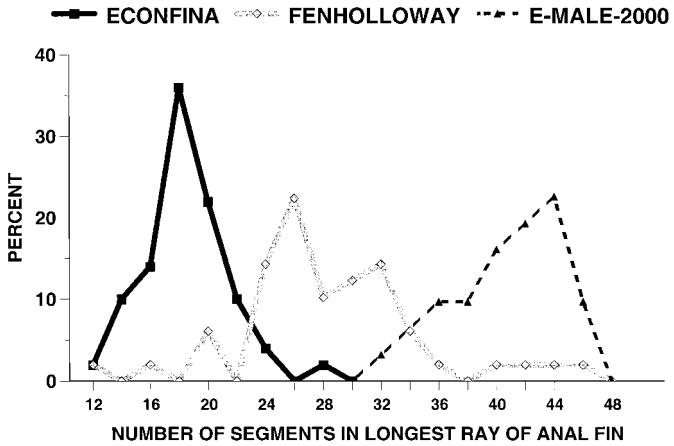


FIG. 8. Distributions of the numbers of segments in the longest ray of the anal fin of male (Econfina River reference) and female (Fenholloway River PME site) mosquitofish. Numbers of segments in the longest anal fin were measured for fish collected from Site 3 (downstream of the Kraft mill, "PME-exposed site") and from the Econfina River (reference site, Site 5). Female fish from the Fenholloway River displayed several indices of masculinization as compared to the Econfina, including an increased number of anal fin segments, a more male-like phenotype. The numbers of segments in the longest ray of the anal fin were increased as compared to females from the Econfina river (from 18.0 ± 0.4 to 28.1 ± 0.9 ($p < 0.001$), Econfina versus Fenholloway Rivers, respectively). Based upon the distributions of the number of segments in the anal fin ray (shown here) 80% of the female mosquitofish from the Fenholloway River were partially masculinized. In about 10%, the numbers of segments in the longest ray of the anal fin fell within the frequency distribution displayed by male fish from the Econfina River, being completely masculinized for this endpoint. Frequency distributions for female mosquitofish from the Econfina and Fenholloway Rivers (1999 sample) are compared to male mosquitofish (E-MALE-2000) sampled from the Econfina River in 2000. No male fish were sampled in 1999 so the 2000 data are displayed to show the degree of masculinization seen in female fish; 2000 values in females are nearly identical to the 1999 values (Orlando *et al*., in preparation).

DISCUSSION

In this study, we found that water contaminated with PME from the Buckeye Kraft pulp and paper mill on the Fenholloway River contains androgenic activity that appears to be of sufficient potency to account for the masculinization of female mosquitofish (*Gambusia affinis holbrooki*) collected from Site 3 downstream from the mill. PME from all 3 contaminated sites contains a chemical, or mixture of chemicals, that bind hAR and induce androgen-dependent gene expression *in vitro*. In contrast to the effects of water contaminated with PME, water samples collected upstream of the plant or from the nearby Econfina River, on which no paper mill is located, do not bind AR or display androgenicity. The role of hAR in this response is confirmed by the observations that (1) the PME-induced gene expression in CV-1 cells cotransfected with hAR and the MMTV-luciferase reporter can be attenuated by coadministration of the potent antiandrogen hydroxyflutamide, (2) exposure of COS cells to PME induces nuclear transport of AR (which occurs only in the liganded state), and (3) the MMTV-luciferase promoter-reporter is not induced by PME when cotransfected with hGR, and hence, PME-induced luciferase expression is not due to corticosteroid-like activity.

Consistent with our observations about the androgencity of PME, another research group also has presented data indicating that androgenic activity is displayed by Fenholloway River water (Howell and Angus, 1999). Jenkins *et al*. (2000, 2001) identified 2 fractions of Fenholloway River samples with androgenic activity. One of these peaks contained the steroidal androgen androstenedione at a concentration of 0.14 nM (also described 15 ng androstenedione per liter by Raloff, 2001). In female mosquitofish, it is possible that androstenedione would be converted in tissues to more androgenic steroidal androgens such as testostereone and dihydrotestosterone as in mammals (Labrie *et al*., 1989). However, it is uncertain if 0.14 nM androstenedione alone would be of sufficient potency to produce the responses that we observed *in vitro* or if it would directly masculinize female mosquitofish in the field. There may be other androgens along with androstenedione present in the PME. In our study, testosterone was measured by RIA in the water samples from the 5 sites (3 replicates per sample), because it had been hypothesized that PME contains androgens, possibly including testosterone, produced from microbial metabolism of phytosterols (Conner *et al*., 1976).

The data presented herein are derived from river water samples collected concurrently with mosquitofish, so the *in vitro* data could be compared with the data on the reproductive physiology and morphology of the male and female mosquitofish from Sites 3 (Fenholloway) and 5 (Econfina; Orlando *et al*., in preparation). Masculinization of the female mosquitofish was confirmed by measuring the number of segments in the anal fin rays, which is an androgen-dependent secondary sex characteristic. Male mosquitofish from the Econfina or Fenholloway Rivers display about twice as many segments in the longest ray on the anal fin as a normal, unexposed female (40 versus 18, Econfina male versus female, respectively) while the anal fin is only about 25% longer in the male than in the normal female. Female mosquitofish from Site 3 on the Fenholloway River are masculinized, as indicated by an overall 55% increase in the numbers of segments in the longest ray of the anal fin as compared to females from the Econfina River ($p < 0.001$). Masculinization has been achieved in female mosquitofish and killifish in the laboratory with exposures to either PME or metabolites of microbial degradation of chemicals present in the PME by such microbacterium as *Mycobacterium smegmata* (Conner *et al*., 1978; Denton *et al*., 1985). These observations led to the hypothesis, confirmed in the current study, that PME contained androgenic substances because masculinization of the ray segments in the anal fin of female mosquitofish also can be induced with androgens like methyltestosterone (Rodriquez-Sierra and Rosa-Molinar, 1990).

In addition to the "androgenic" hypothesis, Orlando *et al*., (1999) hypothesized that inhibition of the ovarian aromatase by

PME could result in masculinization of the female fish by causing an accumulation of testosterone (T), the precursor of estradiol an aromatized steroid hormone. Hence, until now, it was not certain if PME was directly androgenic or if the observed masculinization was caused by alterations in steroid metabolism.

Our data indicate that PME does not masculinize female mosquitofish by displaying cortisol-like activity, as no GR-like activity was detected *in vitro*, although GR agonists could induce luciferase activity, because hGR and hAR transcriptional complexes both bind the hormone response element (HRE) on the MMTV promoter gene when activated by a ligand. The HRE for these steroids is a 15-bp palindromic consensus hormone-response element that binds the highly conserved amino-acid sequence in the DNA-binding domain of the receptor homodimer complex. As these receptors, and the progesterone receptor as well, are all from the same steroid hormone superfamily, even though separate endocrine functions have evolved, they all bind the same HRE on DNA (Martinez and Wahli, 1991). The lack of any hGR-mediated induction of luciferase, along with the ability of the well characterized antiandrogen hydroxyflutamide to block the induction of luciferase caused by PME binding supports the hypothesis that PME is not acting via GR. Even if GR-like activity was present in PME, it is unlikely that this would induce masculinization of female fish. In general, the corticosteroids antagonize the action of androgens *in vivo* via nonreceptor-mediated mechanisms and are more likely to demasculinize males than masculinize females (Konagaya and Max, 1986).

Masculinization of female fish by Kraft mill effluent is not limited to Florida rivers. Morphological alterations consistent with exposure to androgens have been reported in other fish exposed to effluent from other pulp and paper mills and from other plants (sugar-beet processing plant; Hegrenes, 1999). On Jackfish Bay, Lake Superior, male and female white sucker fish (*Catostomus commersoni*) exposed to PME in the field displayed a variety of reproductive effects, including an increase in size and number of reproductive tubercles, which are androgen-dependent sex traits of males of this species (Wells *et al*., 1999). Wells and Van Der Kraak (2000) found that substances in PME competed for binding to the androgen receptor (AR) isolated in cytosolic preparations of fish testes, gonad, and brain. They also identified several known PME constituents that bound fish AR.

Due to the fact that some plant sterols and PME have been correlated with the masculinization of female fish, it is tempting to hypothesize that these substances could also be responsible for some of the reproductive impairments identified in other fish species (fathead minnow, brown trout, white sucker, and rainbow trout) exposed to PME (Lehtinen *et al*., 1999; Munkittrick *et al*., 1998; NCASI, 1997; Tremblay and Van Der Kraak, 1999). However, PME contains thousands of toxicants, including dioxins, which likely alter reproduction and development via alternative (none AR-mediated) mechanisms. Future PME-exposure studies should incorporate an assessment of androgen-dependent traits appropriate for the fish species of concern, along with standard measures of reproductive fitness. Only carefully integrated laboratory and field studies will be able to sort out which toxicants in PME are responsible for the different reproductive effects in exposed populations.

It is informative to contrast the methods used by Wells and Van Der Kraak (2000), with those employed herein. Wells and Van der Kraak examined the ability of PME constituents to bind fish AR isolated from cytosolic preparations from several tissues, whereas we examined the ability of PME to bind human AR in a mammalian whole-cell assay. The approach of Wells and Van Der Kraak has advantages, because it allows for AR binding to be evaluated in the vertebrate class and even in the species of concern. This could be a significant advantage if fish AR display different affinities for toxicants as compared to mammalian AR, as has been suggested (Makynen *et al*., 2000; Wells and Van der Kraak, 2000). However, cytosolic preparations from tissues contain many different receptors in addition to AR, so it is possible that other factors in the cytosol could affect the results. In addition, binding data alone do not allow one to discrminate androgens from antiandrogens. In contrast, our approach, using mammalian AR and cell lines, allows us to discriminate between AR agonists and antagonists, which is not possible at this time with AR assays for most lower vertebrates. However, both approaches have merit and should be utilized until *in vitro* binding and gene expression assays for nonmammalian vertebrates are developed. When sufficiently standardized and validated, they can replace or augment the methods used herein.

Identification of an environmental mixture with androgenic activity resulting from anthropogenic activity is a novel observation. Over the last few years, several environmental antiandrogens that act as AR antagonists have been detected, including the fungicides vinclozolin and procymidone, the herbicide linuron (Gray *et al*., 1999), the pesticide fenitrothion (Tamura *et al*., 2001) and metabolites of DDT, especially p,p′ DDE (Gray *et al*., 1999; Kelce *et al*., 1997). However, until now, based upon the preponderance of observed AR antagonism, it had been suspected that most, if not all, environmental toxicants that bound AR would be antiandrogenic. As we gather more data about endocrine-disrupting chemicals, unexpected effects exerted via additional mechanisms of action will almost certainly be identified.

The detection of chemicals in the environment with androgenic activity raises questions about the potential for these substance(s) to bioaccumulate and move through the food chain. Androgenic hormones are important for reproduction in all vertebrates. Since the biochemical and molecular mechanisms of hormone action are highly conserved among the vertebrates (Norris, 1996), exposure to significant levels of androgenic substances could adversely affect reproduction in all species, including humans. In fact, the adverse effects of

androgenic drugs, taken during human pregnancy are well established, and are to be avoided (Schardein, 2000). For these reasons, it is important that the androgenic chemicals in PME be identified and their potential to adversely affect other vertebrates determined. In summary, we report here that some component(s) in water collected from sites downstream from a Kraft pulp and paper mill on the Fenholloway River displays androgenic activity. It appears that the *in vitro* potency of PME from the Fenholloway River may be sufficient to account for masculinization of the female mosquitofish (90% affected) that we collected from Site 3 on this river (Fig. 1).

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