SETAC PRESS

DETECTION OF ENVIRONMENTAL ANDROGENS: A NOVEL METHOD BASED ON ENZYME-LINKED IMMUNOSORBENT ASSAY OF SPIGGIN, THE STICKLEBACK (*GASTEROSTEUS ACULEATUS*) GLUE PROTEIN

IOANNA KATSIADAKI,*† ALEXANDER P. SCOTT,† MARK R. HURST,‡ PETER MATTHIESSEN,§ and IAN MAYER‖
†Centre for Environment, Fisheries and Aquaculture Science, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB, United Kingdom
‡Centre for Environment, Fisheries and Aquaculture Science, Remembrance Avenue, Burnham-on-Crouch, Essex, CM0 8HA,
United Kingdom
§Centre for Ecology and Hydrology, Far Sawrey, Ambleside, Cumbria LA22 0LP, United Kingdom
‖Department of Zoology, Stockholm University, Stockholm 106 91, Sweden

Abstract—We report the development and validation of a novel in vivo biomarker test for waterborne androgens. During breeding, male sticklebacks (*Gasterosteus aculeatus*) manufacture a glue protein, spiggin, in their kidneys that they use to build their nests. Spiggin production is under the control of androgens. Until now, however, it has only been possible to quantify its production by measurement of the height of kidney epithelial cells. In the present study, we report the development of an enzyme-linked immunosorbent assay (ELISA) for spiggin and demonstrate its application to the measurement of spiggin in the kidneys of female sticklebacks that have been exposed to androgens in water. Results from the ELISA procedure revealed a strong correlation with measurement of kidney epithelial cell height ($r^2 = 0.93$). However, the ELISA was much quicker and had a considerably higher response range (100,000-fold vs fourfold). Clear, graded responses in spiggin production were obtained by exposing intact females to increasing concentrations of 17α-methyltestosterone and 5α-dihydrotestosterone over three-week test periods. The lowest effective concentrations for these two steroids were 100 ng/L and 3 µg/L, respectively. Female sticklebacks that were exposed to pulp mill effluent also produced spiggin in their kidneys. Possession of an androgen-regulated protein by the female stickleback makes it a unique bioassay organism for detecting androgenic contamination in the aquatic environment.

Keywords—Stickleback   Spiggin   Androgen   Endocrine disruption

INTRODUCTION

Recently, much research has focused on the hormone-like effects of environmental compounds, such as pesticides and industrial chemicals, in both wildlife and humans. Some of these so-called endocrine-disrupting chemicals (EDCs) mimic or antagonize the effects of the endogenous hormones estradiol and testosterone, either directly (at the steroid receptor) or indirectly (by disrupting the synthesis and metabolism of the endogenous hormones). Chemicals that interact directly with the steroid receptor and either mimic or block the steroid action also can cause effects at extremely low concentrations. Because gonadal steroid hormones control and regulate embryonic development and sex differentiation, the effects of EDCs are particularly severe during these periods.

Much research also has been conducted on estrogenic endocrine disrupters. However, far less research has dealt with compounds that cause androgenic or antiandrogenic effects, despite an increasing concern about the clinical implications of such compounds in humans and mammals [1,2]. One of the clearest observations of androgenicity has been made in female mosquitofish (*Gambusia* sp.) living downstream of kraft mill effluent discharges. These female fish develop anal fin appendages (gonopodia) that are normally found only in male fish [3,4]. The active compounds have been tentatively identified as bacterial degradation products of the plant sterol stigmastanol [5]. Moreover, exposure of white sucker (*Catostomus commersoni*) to bleached kraft pulp mill effluent from North

American plants affects plasma levels of sex-steroid hormones, age at sexual maturity, and gonadal size [6,7]. The situation is similar in Europe: Studies report decreased gonadal size of female fish living near coastal water receiving bleached pulp mill effluent [8] and significantly male-biased eelpout (*Zoarces viviparus*) broods near a large pulp mill [9].

Although researchers are presently exploring the use of *Gambusia* as a biomarker for environmental androgens [10], a sensitive test system to screen for environmental androgens is lacking. This is especially true in Europe, where *Gambusia* is not native. To resolve this problem, we suggest using the three-spined stickleback (*Gasterosteus aculeatus* L.). The kidney of the male stickleback hypertrophies during the breeding season, when it starts to produce a glue protein that is used as a cementing substance for the building of its nest. By injecting castrated males with a range of steroids, followed three weeks later by measurement (in histological sections) of kidney epithelium cell height (KEH), Borg et al. [11] established that this process was controlled by androgens, the most potent compound being 11-ketotestosterone (11-KT). Recently isolated, the glue protein is a glycoprotein with a molecular mass of 203 kDa [12] and is named spiggin (from the Swedish name for the stickleback, the spigg). More recently [13], we succeeded in producing a polyclonal antiserum to spiggin and established a spiggin enzyme-linked immunosorbent assay (ELISA).

The procedures described by Borg et al. [11] provide a good bioassay for androgens, but they are time-consuming. The main objectives of the present study, therefore, were to determine if kidney hypertrophy could be induced by andro-

gens, in a dose-related fashion, in intact females (rather than in castrated males); if the androgen could be added to the water (rather than microinjected daily into individual fish); and if measurement of KEH could be replaced by measurement of the spiggin content in the kidneys. Early studies by Hoar [14] suggested that gonadectomized females responded positively to the synthetic androgen 17α-methyltestosterone (17α-MT) dissolved in water. Accordingly, at the start of the present study, this compound, rather than 11-KT, was selected as the standard androgen. The 11-KT induces kidney hypertrophy in intact females when added to the water [13], but it is less readily available and much more expensive than 17α-MT. Later, however, concerns were expressed that 17α-MT also has estrogenic activity at high concentrations [15,16], so we then chose 5α-dihydrotestosterone (DHT) which cannot be aromatized to 17β-estradiol, as the standard androgen.

MATERIALS AND METHODS

Chemicals

The chemicals DHT, 17α-MT, and 17α-ethinylestradiol (EE_2) were purchased from Sigma-Aldrich (Poole, UK). Rabbit polyclonal antisera to 17α-MT (catalog no. 86809010, batch no. F991130) and DHT (catalog no. 32500106, batch no. 911205A) were purchased from Biogenesis (Poole, UK). Tritiated DHT was purchased from Amersham Pharmacia Biotech (Bucks, UK). Anti-rabbit IgG (whole molecule conjugated to alkaline phosphatase, affinity isolated; catalog no. A-3937), alkaline phosphatase substrate (1 mg/ml of 4-nitrophenyl phosphate [pNPP] in 0.2 M Tris buffer; Sigma Fast™ tablets) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich. Electrophoresis reagents, including precast Tris-HCl polyacrylamide gels, buffer, and staining solutions, were obtained from Bio-Rad (Hempstead, Herts, UK).

Animals and maintenance

Adult three-spined sticklebacks were purchased from a trout farm in Kent (UK National Grid Reference, TQ 861529).

Stock population. The fish were kept in seawater at $10°C$ and a photoperiod of 8:16 h (light:dark). This regime causes the fish to remain reproductively quiescent [17]. Only fish that had been kept under these conditions, that weighed more than 0.4 g, and that had no external signs of parasitism were used for the experiments.

Natural breeders. To induce breeding, sticklebacks were placed in 60-L aquaria with running freshwater at $20°C$ and a 16:8-h photoperiod for approximately one month [17]. The fish were fed daily with live or frozen *Daphnia*, *Artemia*, and finely chopped sand eel and lugworm. The bottoms of the aquaria were covered with gravel, and a filamentous alga was added as nest-building material.

Experimental populations. Groups of 20 to 50 fish were transferred to 40-L glass aquaria containing either brackish water (15 g/L of NaCl) or full seawater (32 g/L of NaCl). The photoperiod was 12:12 h, and the temperature was $15°C$. The water in the tanks was aerated continuously. Seawater was filtered and ultraviolet-sterilized before being added to the tanks. Daily readings were taken from each tank of temperature, dissolved oxygen, pH, conductivity, and salinity. Throughout the exposure period, the fish were fed daily with a combination of dried flakes (Tetramin, Tetra Werke, Melle, Germany) and live *Daphnia*.

Laboratory exposure to androgens

All steroids, dissolved in either methanol or acetone, were applied via the water at different concentrations: 17α-MT, 0.01 to 50 µg/L; DHT, 5 ng/L to 50 µg/L; and EE_2, 20 ng/L. Appropriate volumes of vehicle solvents (100 µl/L) were added to the controls. All treatments were duplicated. The fish were exposed to the steroids for various periods from one to five weeks. However, most of the experiments lasted for three weeks.

A semistatic system was used to administer 17α-MT to the water. This involved half the aquarium water being removed every 48 h and replaced with fresh seawater plus a fresh dose of compound. In later experiments with DHT, the fish were exposed continuously in a through-flow arrangement. Stock solutions of the steroids were introduced into mixing vessels by means of peristaltic pumps. Water flowed into the tanks continuously at a rate of 400 ml/min from the mixing vessels, which were supplied with a predetermined amount of each test steroid. The solvent stock bottles were weighed daily to calculate the exact volume of stock that had been used.

Samples of water (1 L) from each of the tanks were collected and analyzed at least once a week during the experiments. Solid-phase extraction cartridges (SPE; Sep-Pak® C18, Waters chromatography; Millipore, Milford, MA, USA) were preconditioned with 5 ml of methanol followed by 5 ml of distilled water. The water samples were pumped through 0.45-µm filters and then through the preconditioned SPE. After use, the SPE cartridges were kept frozen for no more than 30 d before analysis. After thawing, the cartridges were washed with 5 ml of distilled water, then with 5 ml of methanol. The methanol was evaporated under a stream of N_2 at $45°C$. For measurement of steroids with a radioimmunoassay, the dried residues were redissolved in 1 ml of assay buffer (50 mM sodium phosphate buffer, 140 mM NaCl, 27 mM KCl, 0.1% [w/v] BSA, and 0.05 % [v/v] Tween; overall pH 7.2).

The radioimmunoassay for DHT used exactly the same procedure as that for several teleost steroids [18]. A competitive ELISA, in which plates were coated with 17α-MT conjugated to BSA, was used to measure 17α-MT in water samples. A commercially available antibody was used for this test (technical data sheet for catalog no. 86809010; Biogenesis). The EE_2 was not measured, because it degrades slowly in aquatic systems, with a half-life of several weeks [19].

Light microscopy

Fish used in the experiment were killed by cranial dislocation and then weighed, measured, and laparotomized. Any breeding color or evidence of parasitism was noted. The gonads and kidneys were removed, weighed to the nearest 0.1 mg, and fixed in 10% (v/v) neutral buffered formalin (75 mM sodium phosphate). After rinsing and dehydration in graded concentrations of ethanol (70% for 2 h at room temperature, 90% for 2 h at room temperature, 90% for 1 h at room temperature, and then 90% for 2 h at $4°C$ [v/v]), tissues were embedded in Historesin (Leica, Milton Keynes, UK, and Taab Aldermaston, Berkshire, UK) or paraffin wax. Sections were cut at 5-µm thickness and stained with either Lee's methylene blue-basic fuchsin or hematoxylin-eosin. Ten sections were prepared from the middle part of each kidney, assuring the presence of many secondary proximal tubules. A microscope with an attached image analyzer was used to measure the height of the epithelial cells that lined the secondary proximal convoluted tubules. This measurement, referred to as the KEH,

was made for as many as 60 separate tubules from each fish. Two renal sections from each fish were also stained with periodic acid-Schiff (PAS), which highlights the presence of glycoproteins such as spiggin.

Purification of spiggin by electrophoresis

Starting materials for the isolation and purification of spiggin were the contents of the breeding male's urinary bladder or nest threads. Breeding males, identified by their distinct nuptial coloration and nest-building behavior, were killed and the contents of the urinary bladder squeezed into a collecting tube and frozen in liquid nitrogen. Glue material, teased out from the nests with fine forceps, also was frozen in liquid nitrogen. Urinary bladder contents and nest material were dissolved (1:5 [w/v] and 1:2 [w/v], respectively) in 100 mM Tris-HCl buffer (pH 8.5) containing 10 mM ethylenediaminetetraacetic acid (EDTA), 8 M urea, 2% (w/v) sodium dodecyl sulfate (SDS), and 200 mM β-mercaptoethanol by heating at 70°C for 30 min. Any undigested threads remaining in the nest material were removed, and the solution was filtered. Aliquots of the above solutions were further diluted with Laemmli sample buffer (62.5 mM Tris-HCl, 25 % [v/v] glycerol, 2% [w/v] SDS, and 0.01% [w/v] bromophenol blue; overall pH 6.8) at 1:4 (v/v) and heated at 105°C for 5 min before electrophoresis.

Electrophoresis (SDS-polyacrylamide gel electrophoresis) was performed using precast Tris-HCl polyacrylamide gels (4% stacking gel, 7.5% resolving gel, 2.6% cross-linker [w/v], and 0.375 Tris-HCl; overall pH 8.8). A high-molecular-weight (53–212 kDa) SDS calibration kit was also employed. Electrophoresis was carried out at 200 V (constant voltage) for 30 min with a Bio-Rad Power Pack power supply and a mini-Protean (Bio-Rad) dual slab electrophoresis cell. After separation, the gels were stained with Coomassie brilliant blue for 30 min.

Development of spiggin ELISA

Disposable plastic-ware and equipment included polystyrene plates (high-protein-binding, flat-bottomed, Costar® EIA/RIA 96-well [Corning B. V. Life Sciences, Schiphol-Rijk, The Netherlands]), polypropylene plates (low-binding, round-bottomed), plate covers (mylar sealing tape), a plate washer (MRW, 8-channel plate washer; Dynex Technologies, The Microtiter® Company, Helsinki, Finland), and a plate reader (MRX microplate reader; The Microtiter® Company).

Solutions used were spiggin buffer (100 mM Tris-HCl, 10 mM EDTA, 8 M urea, 2% SDS [w/v], and 200 mM β-mercaptoethanol, pH 8.5), plate-coating buffer (0.05 M sodium bicarbonate-carbonate; overall pH 9.6), plate-washing buffer (0.1 M sodium phosphate [72 mM di-basic salt, 28 mM monobasic salt], 140 mM sodium chloride, 27 mM potassium chloride, and 0.05% Tween 20 [v/v]; overall pH 7.8), and assay buffer (same as plate-washing buffer, but with 0.1% BSA (w/v) and 0.15 mM sodium azide).

Spiggin standard

Spiggin, being a glue protein, is highly hydrophobic. Several attempts were made to prepare a pure, weighable standard by column chromatography. However, even in minor amounts, spiggin reformed into glue. As a temporary solution to this problem, a large number of hypertrophied kidneys and urinary bladders from breeding male sticklebacks were pooled and dissolved in spiggin buffer (1:50 [w/v]). This solution was divided into 100-μl aliquots, which were frozen and assigned an arbitrary value of 10,000 U/ml. To minimize the possibility

that the immunoassay would detect nonspecific kidney or bladder proteins, the coating material for the ELISA plates was prepared from protein derived from nests. A large pool of nest-derived material was collected, treated with the spiggin buffer as noted above, and frozen in aliquots of 100 μl.

Development of the antiserum against spiggin and the preliminary specificity studies have been reported previously [13]. Several experiments were conducted to optimize the ELISA procedure with respect to the amount of coating material, incubation time, and antiserum dilutions. We found that it was not necessary to add 2% (w/v) BSA as a blocking buffer after the coating step. The standard operating procedure is described below.

The kidneys to be assayed were placed in 200 μl of spiggin buffer and heated at 70°C for 30 min. Nest protein was diluted 1:1,000 (v/v) with plate-coating buffer, and 100 μl of this solution were dispensed into each well of the polystyrene plates (one plate per 20 samples). The plates were then sealed, wrapped in moistened paper and a plastic bag, and stored at 4°C overnight. On the same day, 135 μl of assay buffer were dispensed into each well of the polypropylene plates. A vial of spiggin standard was thawed out and diluted 1:10 (1,000 U/ml) (v/v) and 1:50 (200 U/ml) (v/v) in assay buffer. Each of the wells in columns 1 and 5 and wells A to D of column 9 received 15 μl of each sample. Wells E and F of column 9 received 15 μl of the high standard, and wells G and H received 15 μl of the low standard. Using a multipipette set to 15 μl, the contents of the wells in column 1 were mixed thoroughly by pipetting up and down 20 times. Fifteen microliters were then transferred to column 2, and the procedure was repeated up to column 4. The final 15 μl were discarded and the pipette tips changed. This complete procedure was used to prepare the samples/standards that had been added to the wells in columns 5 and 9. The outcome was a set of four, 10-fold dilutions of all samples and standards.

Polyclonal spiggin antiserum (raised in a rabbit) [13] was diluted 1:10,000 (v/v) in assay buffer, and 50 μl were added to each well. The plates were then sealed, wrapped in moistened paper and a plastic bag, and stored at 4°C overnight.

On the morning of day 2, the high-binding plates were rinsed three times with 200 μl of plate-washing buffer. A multipipette was used to transfer 150 μl of the contents of the wells of the low-binding plate into the corresponding wells of the high-binding plate. The transfer was started with column 12 and worked backward. Pipette tips were discarded after every four columns to avoid contamination between samples. The plates were resealed, wrapped, and incubated at room temperature for 4 to 6 h. They were then rinsed three times with wash buffer, followed by the addition of 150 μl of second antibody diluted 1:15,000 (v/v) in assay buffer. The plates were resealed, wrapped, and incubated overnight at 4°C.

On the morning of day 3, the plates were rinsed three times with distilled water; then, 150 μl of pNPP were added to each well. Color development was measured less than 1 h after the pNPP addition with the microplate reader at a wavelength of 405 nm. Samples were expressed as spiggin units/g body weight.

Effect of parasitism

We noted intensive parasitism of some stickleback body cavities by plerocercoids of the cestode *Schistocephalus solidus*. More than 30% of the fish harbored this parasite and were therefore not used for the experiments. However, by col-

lecting the experimental population when a few weeks old and keeping them in the laboratory, the incidence of parasitism fell to less than 2%.

Validation, precision, and reproducibility of the assay

The validation procedure for the spiggin ELISA involved bisection of 160 kidneys into approximately two equal parts (weighed to the nearest μg), one of which was used for the immunoassay and the other for histology. Estimates of intra- and interassay variation, which measure the precision and re-producibility of the assay, respectively, were determined by repeated measurements on the same kidney extracts and then calculation of the coefficient of variation.

Laboratory studies with pulp mill effluent

Adult three-spined sticklebacks were caught on the coast of southern Sweden (56°N) during the winter of 1999/2000 and transported to Stockholm University, where they were kept in a 700-L aquarium containing brackish water (0.5% salinity) that was constantly filtered and aerated. The fish were kept under a simulated natural photoperiod at a water temperature of approximately 5°C; they were fed daily with frozen red mosquito larvae. Primary effluent water from a large modern kraft pulp mill at Mönsterås in Southeast Sweden was sampled in September 1999 and frozen in plastic containers. The mill had been processing softwood (pine and spruce) while using oxygen, hydrogen peroxide, and ozone as bleaching agents. The effluent had a pH of 8.3 and a total organic carbon content of 380 mg/L. A 25-L container of effluent was thawed 12 to 16 h before use.

In March 2000, 64 adult female sticklebacks were randomly divided between eight 50-L aquaria containing brackish water (0.5% salinity) mixed with 10%, 1%, or 0.1% (v/v) pulp mill effluent or brackish water alone (negative controls). Each one of the four experimental groups was tested in duplicate aquaria, each aquarium containing eight fish (the total number of fish in each group [n] was therefore 16). The contents of the aquaria were constantly filtered and aerated and maintained under a photoperiod of 14:10 h. The water temperature was allowed to increase gradually from 5 to 16°C; the fish were fed once daily. Every 5 d, the fish were transferred to new aquaria containing freshly mixed and aerated pulp mill effluent dilu-tions or brackish water.

The experiment concluded after six weeks. Kidneys were quickly dissected out, weighed, and divided into two halves: One half was immediately frozen for spiggin determination, and the other half was fixed for histological evaluation of KEH. Initial controls ($n = 12$) were sampled in the same way.

Statistical analysis

Differences between groups were analyzed by analysis of variance with a post-hoc Duncan's test. Spiggin unit data were logarithmically transformed before analysis. Male and female fish were analyzed separately due to the large differences in spiggin contents between the sexes, particularly in control groups.

RESULTS

Establishing a concentration-response curve for 17α-MT

Male and female sticklebacks were exposed for three weeks, under semistatic conditions, to four concentrations of 17α-MT ranging from 10 ng/L to 10 μg/L. The KEH for female sticklebacks at 10 ng/L was not significantly different ($p <$
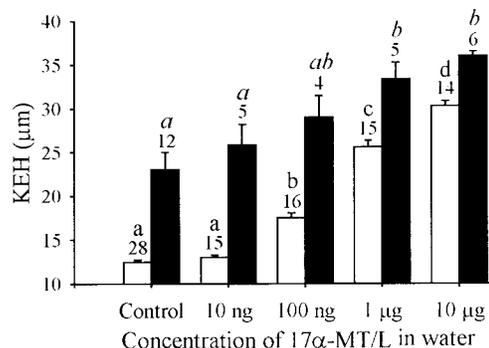

Fig. 1. Kidney epithelium cell height (KEH; ± standard error) in female (open bars) and male (black bars) sticklebacks exposed for three weeks to four concentrations of 17α-methyltestosterone (17α-MT) or to solvent only (control) under semistatic conditions (first experiment). The numbers refer to the number of fish in each treat-ment. Treatments shown with the same letter do not differ significantly from each other ($p < 0.05$). Males and females were analyzed sep-arately.

0.01) from that of the controls, but the KEH for females at 10 μg/L was as high as that found in reproductively mature males (Fig. 1). The KEH of male sticklebacks also was significantly increased by 17α-MT treatment at 100 ng/L. Based on these results, the lowest-observed effect concentration (LOEC) for 17α-MT (based on a three-week exposure) was 100 ng/L, and the no-observed-effect concentration (NOEC) was 10 ng/L.

On staining with PAS, we observed the increasing presence of a carbohydrate-containing protein in the secondary tubule segment of female fish treated with increasing concentrations of 17α-MT (Fig. 2a–e). No PAS-positive material was detected when using 17α-MT at concentrations lower than 100 ng/L. The ELISA for 17α-MT in water (Table 1) showed that this steroid did not accumulate appreciably in the aquarium water. Only the group that was treated with a nominal 10 μg/L ex-perienced steroid concentrations that were slightly higher than the nominal concentration after fresh test solution had been added.

Comparison between KEH and spiggin measurements in fish treated with 17α-MT

The KEH measurements provided a clear dose-response curve for 17α-MT, but the histological processing of the sam-ples and the measurement of KEH was time-consuming. Fol-lowing development of the spiggin ELISA, sticklebacks were again exposed to a range of concentrations of 17α-MT for three weeks. This time, when the fish were killed, their kidneys were divided in two: One half was processed histologically for KEH measurement, and the other half, after weighing, was digested, frozen, and assayed for spiggin. Differences in KEH at different concentrations of 17α-MT closely matched those observed in the first experiment (and are therefore not shown). The spiggin content of males in all the groups was very high, but it still showed a concentration-related trend (Fig. 3). The greatest differences, however, were found in the females (Fig. 3). A comparison of KEHs of all fish that were exposed to different concentrations of 17α-MT (as measured on histolog-ical sections of half the kidney) with their kidney spiggin units (as measured in the other half of the kidney) yielded a strong, positive correlation ($r^2 = 0.93$, $y = 9.5x^{0.09}$) (Fig. 4). The spiggin assay was completed within 3 d of killing the fish; the histological method required four weeks to complete. Spiggin
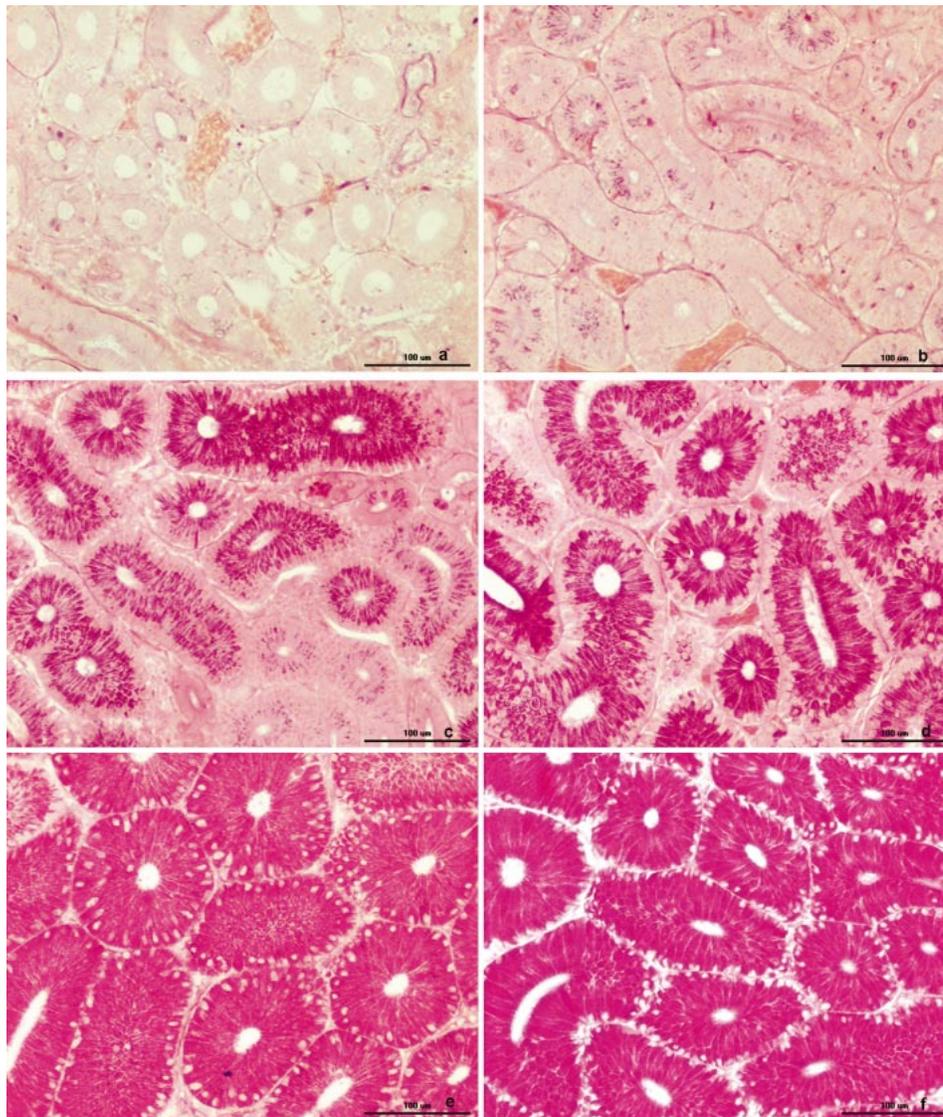
Fig. 2. Kidney sections of female sticklebacks treated with increasing concentrations of 17α-methyltestosterone (**a–e**) and of a naturally breeding male (**f**). Sections were stained with periodic acid-Schiff; all photomicrographs were taken under the same magnification. The bar at the bottom right corner of each plate is 100 µm. (**a**) Control; kidney epithelium cell height (KEH) = 13.5 µm. (**b**) 100 ng/L; KEH = 18 µm. (**c**) 1 µg/L; KEH = 24.2 µm. (**d**) 10 µg/L; KEH = 29.7 µm. (**e**) 500 µg/L; KEH = 36.2 µm. (**f**) Breeding male; KEH = 34.5 µm.

Table 1. Analytical verification of the concentration of 17α-methyltestosterone (17α-MT) in aquarium water before and after addition of fresh steroid

Nominal concentration	Measured concentration (by ELISA)[a]	
	Before adding fresh 17α-MT	After adding fresh 17α-MT
Control	ND[b]	ND
10 ng/L	ND	8.9 ng/L
100 ng/L	ND	80.4 ng/L
1 µg/L	79 ng/L	780 ng/L
10 µg/L	11.8 µg/L	17.7 µg/L

[a] ELISA = enzyme-linked immunosorbent assays.
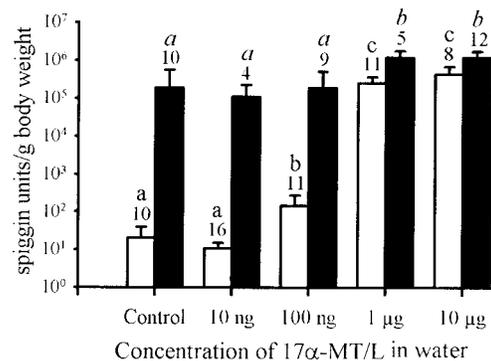[b] ND = nondetectable; steroid refreshed every 2 d; measurements made at 10 d.

Fig. 3. Amounts of spiggin per gram of body weight (± standard error) in female and male sticklebacks exposed for three weeks to 17α-methyltestosterone (17α-MT) under semistatic conditions (second experiment). Refer to Figure 1 for explanation of numbers and letters.
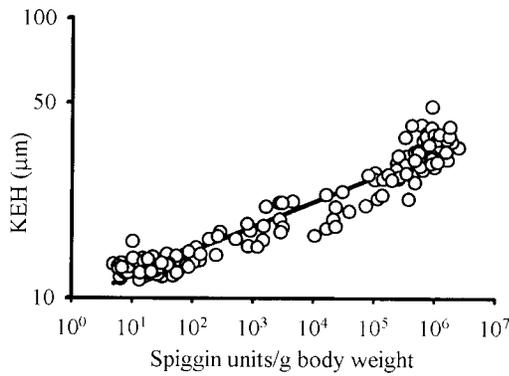
Fig. 4. Scatter plot of kidney epithelium height (KEH) versus spiggin units per gram of body weight of sticklebacks treated with 17α-methyltestosterone (from the same data as plotted in Fig. 3) plus some untreated male and female sticklebacks from the same stock of fish.

Fig. 6. Amount of spiggin per gram of body weight ($\pm$ standard error) in female sticklebacks treated for three (open bars) and five weeks (black bars) with 5α-dihydrotestosterone (DHT) at concentrations between 1 and 5 μg/L, with ethinylestradiol (EE$_2$) at 20 ng/L, or with solvent only (control). Refer to Figure 1 for explanation of numbers and letters.

concentrations showed a 100,000-fold variation, whereas KEH values varied only fourfold.

The intraassay coefficient of variation of the spiggin ELISA was 9%, regardless of whether the samples contained 10^6 or 10^3 spiggin units/g body weight. The interassay coefficient of variation with the same samples was 13 %.

Establishing a concentration-response curve for DHT

During an initial screening test, sticklebacks were exposed to solvent only; to DHT at concentrations of 5 ng/L, 50 ng/L, 500 ng/L, 5 μg/L, or 50 μg/L, and to 17α-MT at concentrations of 1 and 10 μg/L (positive controls). The amounts of spiggin per gram of body weight after three weeks of exposure were 37, 41, 49, 49, 1.82×10^5, 1.90×10^5, 2.31×10^5, and 2.82×10^5, respectively. Clearly, the response threshold for DHT lay between 500 ng/L and 5 μg/L.

During another experiment, male and female sticklebacks were exposed for as long as five weeks to DHT while at least 10 fish were sampled at weekly intervals. Unfortunately, the fish present in the tanks with a DHT concentration at 5 μg/L developed a bacterial infection and died after 14 d. Nevertheless, this dose had caused significant ($p < 0.01$) induction of spiggin at 7 and 14 d (Fig. 5). One female fish showed spiggin induction at a DHT concentration of 1.5 μg/L, but only after 35 d exposure; no spiggin-positive female sticklebacks were observed at 500 ng/L.
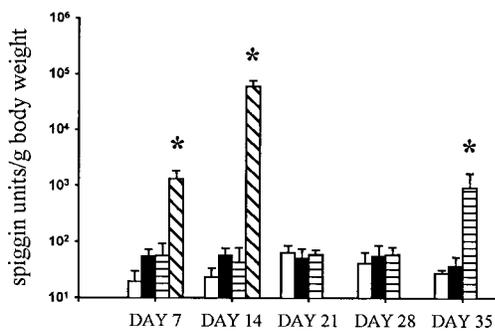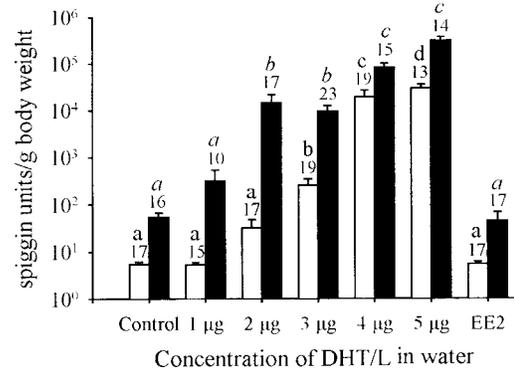

Fig. 5. Amount of spiggin per gram of body weight ($\pm$ standard error) in female sticklebacks treated for varying lengths of time (7–35 d) with solvent only (open bars) or three concentrations of 5α-dihydrotestosterone: 500 ng/L (black bars), 1.5 μg/L (horizontally hatched bars), or 5 μg/L (diagonally hatched bars). The numbers of fish in the treatments varied from four to 12. Asterisks indicate groups that were significantly different ($p < 0.05$) from controls on the same sampling day.

During the final experiment with DHT, sticklebacks were exposed to a narrower range of concentrations (1–5 μg/L) and also to EE$_2$ at 20 ng/L. Some fish were exposed for three weeks and others for five weeks. After three weeks of exposure (Fig. 6), no spiggin-positive female sticklebacks were found with DHT at 1 μg/L. However, two of 17 females had low amounts of spiggin at 2 μg/L, four of 19 females contained low amounts of spiggin at 3 μg/L; 17 of 19 females were spiggin positive at 4 μg/L, and all 13 females treated at 5 μg/L were spiggin positive. No spiggin-positive female sticklebacks were found in the tanks containing EE$_2$ or methanol only. The NOEC and LOEC for three-week exposure to DHT were approximately 2 and 3 μg/L, respectively.

After five weeks of exposure to DHT (Fig. 6), two of 10 female sticklebacks gave a low but positive spiggin signal after treatment at 1 μg/L, 10 of 14 females gave a high spiggin signal at 2 μg/L, and all females had high levels of spiggin at 3, 4, and 5 μg/L. No spiggin-positive females were found in the tanks containing EE$_2$ or methanol only. The NOEC and LOEC for DHT were established as 1 and 2 μg/L, respectively.

Analysis of DHT in water showed that measured concentrations of DHT were close to the nominal concentrations (Table 2).

Testing of pulp mill effluent

The laboratory exposure of female sticklebacks to three dilutions of pulp mill effluent for a period of six weeks con-

Table 2. Analytical verification of 5α-dihydrotestosterone concentrations in aquaria as measured by a specific radioimmunoassay

Nominal concentration (ng/L)	Measured concentration (ng/L)
Control	1[a]
5	8
50	59
500	577
1,000	1,122
1,500	1,434
5,000	4,993
50,000	53,636

[a] This small signal could be due either to the assay's background or to a cross-reaction of the dihydrotestosterone antibody with natural androgens produced by male fish present in the tanks.

Table 3. Quantity of spiggin (units/g body wt $\pm$ standard error [SE]) and kidney epithelium height (KEH; μm $\pm$ SE) in female sticklebacks treated for six weeks with different dilutions of pulp mill effluent (PME)[a]

Treatment	Spiggin	KEH
Initial control ($n = 12$)	22 ± 2.1	12.87 ± 0.13
Control ($n = 14$)	24 ± 1.2	13.24 ± 0.17
0.1% dilution of PME ($n = 15$)	30 ± 1.5	13.98 ± 0.14
1% dilution of PME ($n = 14$)	41 ± 2.7	14.48 ± 0.23
10% dilution of PME ($n = 13$)	$39,190 \pm 6,877$	26.91 ± 0.56

[a] All experimental groups were tested in duplicate aquaria; the total number of fish in each group (n) was between 12 and 15.

firmed the ability of the stickleback to detect environmental androgens. Both assays indicated significant kidney stimulation, revealing on average 40,000 spiggin units/g body weight and a KEH of 27 μm (Table 3). On regression analysis of the data obtained from the two assays, the relationship between KEH and spiggin units was very similar to that obtained during the validation experiment with 17α-MT ($r^2 = 0.96$, $y = 9.3x^{0.09}$).

DISCUSSION

The present study describes a new in vivo test for measuring exposure to androgens and their mimics in the aquatic environment that is based on the induction of spiggin in the kidneys of female sticklebacks, which normally do not synthesize this protein. The test is similar in principle to the widespread in vivo test [20,21] that is used for measuring exposure to estrogens and their mimics in the aquatic environment and that is based on the induction of vitellogenin (the major precursor of the egg yolk in oviparous vertebrates) in the blood plasma or body extracts of male fish, which normally do not synthesize vitellogenin.

Van Oordt [22] first measured the height of the kidney tubule epithelial cells as an index of sexual maturity in the male nine spine stickleback (*Pungitius pungitius* L). The foundation for the present study, however, was laid by Borg et al. [11], who developed an elegant in vivo bioassay with which they could obtain dose-response curves for a range of steroids and clearly demonstrate that stickleback kidneys had a highly specific response to androgens. From the point of view of a test that can be used in ecotoxicological studies, however, their bioassay has several drawbacks. It requires that male sticklebacks be castrated before use and the fish injected with the test compound on a daily basis for three weeks. The kidneys of the males then must be processed histologically and KEH measurements made on each section. Clearly, all three procedures are time-consuming. Also, the first and second procedures rule out the possibility of using the bioassay for testing androgen exposure in the field. However, the present study demonstrates that females are a practical substitute for castrated males, that androgen exposure can be via the water rather than via injection, and that KEH measurement can be replaced, with large gains in resolution, by spiggin ELISA.

The kidneys of male sticklebacks also produce more spiggin in response to waterborne androgens. However, because for much of the year they are able to come into maturity, control male fish tend to have high background levels of spiggin. Males are likely to prove more useful for pinpointing anti-androgenic activity.

If one assumes that, under steady-state exposure conditions, the concentrations of actively available steroid in the blood plasma are at the same concentrations as those in water, one could conclude that the kidneys are stimulated by concentrations of 17α-MT and DHT that are much lower than the normal range of 11-KT concentrations found in males. For example, DHT induced full stimulation when administered at 5 μg/L. Peak concentrations of 11-KT in plasma during the breeding season are between 30 and 70 μg/L [23].

In the recent study by Jones et al. [24], spiggin induction was quantified by measurement of mRNA in castrated male sticklebacks. In that study, responses were found only to one steroid: 11-ketoandrostenedione (11-KA), which is converted to 11-KT in vivo. Dihydrotestosterone, at a concentration 25-fold greater than that of 11-KA, was inactive. The reasons for the differences between this and the present study are not yet known. However, the method of dosing was entirely different. In the study described by Jones et al. [24], the steroids were administered via implants made from cocoa butter and silicon, with no data given on how much of each steroid was able to diffuse from the implants.

With their original bioassay, Borg et al. [11] showed that the most potent androgen was 11-KT (11-KT is 40-fold more potent than DHT and 300-fold more potent than testosterone). Unfortunately, their study did not include 17α-MT. However, De Ruiter and Mein [25] showed that 11-KT was more potent than 17α-MT in stimulating the stickleback kidney in vitro. In the present study, we did not test for a concentration-response relationship for 11-KT administered in the water, but earlier histological observations (unpublished observations) suggest that 11-KT has a higher potency than the other androgens. Results from the present study also indicate that 17α-MT (LOEC = 100 ng/L) is more potent than DHT (LOEC = 3 μg/L). On average, 17α-MT induced 2.30×10^5 spiggin units/g body weight at 1 μg/L, whereas DHT was ineffective at 1 μg/L. At 10 μg/L, 17α-MT also induced 2.80×10^5 units, whereas DHT induced only 1.90×10^5 units at 50 μg/L. Yet, a difference in the potency of androgens is not always related to a higher affinity for the relevant receptor (in this case, the putative androgen receptor [AR] in the stickleback kidney); a difference in potency also could reflect a different degree of binding to steroid serum-binding proteins, which is not mediated via the receptor.

The NOEC for DHT was 2 μg/L for a three-week exposure and 1 μg/L for a five-week exposure. In this case, the determination of NOEC and LOEC was more accurate than those for 17α-MT (because the differences between the DHT concentrations were narrower). After five weeks of exposure, we noted that the spiggin levels at 2 μg/L were slightly higher than those at 3 μg/L. This difference, however, was not statistically significant.

The amounts of spiggin in the kidneys of females treated with 5 μg/L of DHT were 10-fold greater at five weeks than at three weeks—and even greater at lower DHT concentrations. However, the three-week treatment yielded a clearer concentration-response curve.

As expected, EE_2 at a concentration greater than that needed to induce vitellogenin production in a wide range of fish did not induce spiggin production in female sticklebacks.

Spiggin, which was first characterized by Jakobsson et al. [12], is localized in the tubules of the secondary proximal segment, as shown by staining of androgen-treated female and breeding male kidney sections with PAS (Fig. 2). Recent evidence suggests that spiggin is a protein complex rather than

a single protein, and that it is assembled in the urinary bladder from three distinct subunits synthesized in the kidney [24]. The ELISA that we developed for spiggin had strong correlation with the histological method in the present study. It also operated over a considerably higher range (100,000-fold vs fourfold for KEH), which accentuated the differences between treatments. Several improvements can still be made to the spiggin ELISA. For example, weighable amounts of purified spiggin need to be prepared for absolute standardization of the assay. Several attempts were made to purify spiggin by simple column chromatography, but the activity disappeared in all cases. We were unable to determine whether the spiggin had been degraded or, once it had been diluted in column buffer, whether it regained its properties as a glue protein and coagulated. The latter seems possible but is difficult to resolve, at least by chromatography. However, in view of the recent molecular characterization of spiggin [24], recombinant protein may soon become available.

By creating a pool of nest material for coating the plates as well as a spiggin standard pool from hypertrophied kidneys, we obtained a steep standard curve, which allowed the examination and comparison of numerous samples with the same reference material. The reason for using nest material to coat the plates was that it was thought likely not to contain tissue proteins that might interfere in the assays.

Our results provide few clues about the mechanism of action of the androgens. However, preliminary results show that spiggin induction by 17α-MT and DHT can be inhibited by the antiandrogenic drug flutamide [26], suggesting that these steroids are acting directly on a receptor (or receptors). In their studies on androgen binding by stickleback kidneys, Jakobsson et al. [27] found no binding of 11-KT or testosterone to the cytosolic and nuclear fractions, which is where the receptors in mammals are found. However, they did observe binding of 11-KT to the membrane fraction, suggesting this as the location of the AR, but this suggestion contrasts with findings reported for other fish [28–30]. Very recently, it has become clear that most fish possess at least two different ARs that differ in their binding affinities, physical characteristics, tissue distribution, and abundance. Sperry and Thomas [31] found nuclear ARs with different binding specificities in the brain and ovary of Atlantic croaker (*Micropogonias undulatus*). In the same year, two AR subtypes were found by cDNA cloning in the gonads of Japanese eel (*Anguilla japonica*) [32,33] and rainbow trout (*Oncorhynchus mykiss*) [34]. The two types have been termed AR1 (or ARα) and AR2 (or ARβ). Both types bind strongly to testosterone. However, only AR2 binds to 11-KT; AR2 also binds well to DHT [35]. Such differences might explain the different recorded physiological actions of testosterone and 11-KT in teleosts (reviewed by Borg [36]). In addition, AR2 has a higher affinity than AR1 for a wide range of natural and synthetic steroids (along with their metabolites) as well as for steroidal and nonsteroidal androgens [35]. In this sense, the teleostean AR2 is similar to mammalian AR.

Several studies with xenoandrogens in teleosts, both in vivo and in vitro, have proven to be either negative [27,37] or positive in one tissue but not in another [38], further demonstrating substantial differences in AR-binding specificities between species.

The androgenic properties of pulp mill effluent, demonstrated mainly via a masculinizing effect on female mosquitofish [3,4] and, more recently, by male-biased eelpout embryos [9] are well known. We confirmed a similar effect on female sticklebacks in the present study. Howell and Denton [3] suggested that such effects are caused by the presence in the effluent of relatively high concentrations of plant-derived sterols that are probably converted into androgens by bacteria. In a recent paper, androstenedione, a natural steroid in many fish, has been identified as a key active component of pulp mill effluent [39]. Furthermore, Parks et al. [40] confirmed that there are compounds in pulp mill effluent that bind agonistically to human AR in a mammalian whole-cell assay.

CONCLUSION

In response to exposure to synthetic androgens in water and also to pulp mill effluent (a known source of environmental androgens), the kidneys of female sticklebacks produce a glue protein (spiggin) in a concentration- and time-dependent manner. The spiggin can be reliably measured by ELISA. The present results support the contention that spiggin induction by the stickleback kidney could be used as a test for androgenic compounds in water, much like vitellogenin induction by the fish liver is used as a test for estrogenic compounds.

The possession of an androgen biomarker, which can potentially be adapted to detect antiandrogens, and a variety of other advantageous traits [26] make the stickleback potentially very useful for the testing of endocrine disrupters, especially in Europe, which is presently reliant on nonendemic species.

REFERENCES

1. Kelce WR, Gray LE, Wilson EM. 1998. Antiandrogens as environmental endocrine disruptors. *Reprod Fertil Dev* 10:105–111.
2. Sohoni P, Lefevre PA, Ashby J, Sumpter JP. 2001. Possible androgenic/antiandrogenic activity of the insecticide fenitrothion. *J Appl Toxicol* 21:173–178.
3. Howell WM, Denton TE. 1989. Gonopodial morphogenesis in female mosquitofish, *Gambusia affinis affinis*, masculinized by exposure to degradation products from plant sterols. *Environ Biol Fish* 24:43–51.
4. Cody RP, Bortone SA. 1997. Masculinization of mosquitofish as an indicator of exposure to kraft mill effluent. *Bull Environ Contam Toxicol* 58:429–436.
5. Denton TE, Howell WM, McCollum CJ, Marks EB, Allison JJ. 1985. Masculinization of female mosquitofish by exposure to plant sterols and *Mycobacterium smegmatis*. *Bull Environ Contam Toxicol* 35:627–632.
6. Munkittrick KR, Van Der Kraak GJ, McMaster ME, Portt CB, van den Heuvel MR, Servos MR. 1994. Survey of receiving-water environmental impacts associated with discharges from pulp mills. 2. Gonad size, liver size, hepatic EROD activity, and plasma sex steroid levels in white sucker. *Environ Toxicol Chem* 13:1089–1101.
7. Van Der Kraak GJ, Munkittrick KR, McMaster ME, Portt CB, Chang JP. 1992. Exposure to bleached kraft pulp mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. *Toxicol Appl Pharmacol* 115:224–233.
8. Andersson T, Förlin L, Härdig J, Larsson A. 1988. Physiological disturbances in fish living in coastal water polluted with bleached kraft pulp mill effluents. *Can J Fish Aquat Sci* 45:1525–1536.
9. Larsson DGJ, Hällman H, Förlin L. 2000. More male fish embryos near a pulp mill. *Environ Toxicol Chem* 19:2911–2917.
10. Angus RA, McNatt HB, Howell WM, Peoples SD. 2001. Gonopodium development in normal male and 11-ketotestosterone-treated female mosquitofish (*Gambusia affinis*): A quantitative study using computer image analysis. *Gen Comp Endocrinol* 123: 222–234.
11. Borg B, Antonopoulou E, Andersson E, Carlberg T, Mayer I. 1993. Effectiveness of several androgens in stimulating kidney hypertrophy, a secondary sexual character, in castrated male three-

spined sticklebacks, *Gasterosteus aculeatus. Can J Zool* 71: 2327–2329.

12. Jakobsson S, Borg B, Haux C, Hyllner SJ. 1999. An 11-ketotestosterone induced kidney-secreted protein: The nest building glue from male three-spined stickleback, *Gasterosteus aculeatus. Fish Physiol Biochem* 20:79–85.

13. Katsiadaki I, Scott AP, Matthiessen P. 2000. The use of the three-spined stickleback as a potential biomarker for androgenic xenobiotics. In Norberg B, Kjesbu OS, Taranger GL, Andersson E, Stefansson SO, eds, *Proceedings,* 6th International Symposium on the Reproductive Physiology of Fish, Bergen, Norway, July 4–9, 1999, pp 359–361.

14. Hoar WS. 1963. Hormones and the reproductive behavior of the male three-spined stickleback, *Gasterosteus aculeatus. Anim Behav* 10:247–266.

15. Mori T, Matsumoto H, Yokota H. 1998. Androgen-induced vitellogenin gene expression in primary cultures of rainbow trout hepatocytes. *J Steroid Biochem Mol Biol* 67:133–141.

16. Ankley GT, Jensen KM, Kahl MD, Korte JJ, Makynen EA. 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 20:1276–1290.

17. Baggerman B. 1972. Photoperiodic responses in the stickleback and their control by a daily rhythm of photosensitivity. *Gen Comp Endocrinol* 3(Suppl.):466–476.

18. Scott AP, Mackenzie DN, Stacey NE. 1984. Endocrine changes during natural spawning of the white sucker (*Catostomus commersoni*). II. Steroid hormones. *Gen Comp Endocrinol* 56:349–359.

19. Williams RJ, Jurgens MD, Johnson AC. 1999. Initial predictions of the concentrations and distribution of 17 beta-estradiol, estrone and ethinyl estradiol in three English rivers. *Water Res* 33:1663–1671.

20. Sumpter JP, Jobling S. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ Health Perspect* 103(Suppl. 7):173–178.

21. Heppell SA, Denslow ND, Folmar LC, Sullivan CV. 1995. Universal assay of vitellogenin as a biomarker for environmental estrogens. *Environ Health Perspect* 103(Suppl. 7):9–15.

22. Van Oordt GJ. 1924. Die veranderungen des hodens wahrend des auftretens der sekundaren geschlechtiesmerkmale bei fischen. I. *Gasterosteus pungitious* L. *Archiv fur Mikroskopische Anatomie und Entwicklungsmechanik* 102:379–405.

23. Mayer I, Borg B, Schulz R. 1990. Seasonal changes in and effect of castration/androgen replacement on the plasma levels of five androgens in the male three-spined stickleback, *Gasterosteus aculeatus* L. *Gen Comp Endocrinol* 79:23–30.

24. Jones I, Lindberg C, Jakobsson S, Hellqvist A, Hellman U, Borg B, Olsson P-E. 2001. Molecular cloning and characterization of spiggin: An androgen-regulated extraorganismal adhesive with structural similarities to von Willebrand factor-related proteins. *J Biol Chem* 276:17857–17863.

25. De Ruiter AJH, Mein CG. 1982. Testosterone-dependent trans-

26. formation of nephronic tubule cells into serous and mucous gland cells in stickleback kidneys in vivo and in vitro. *Gen Comp Endocrinol* 47:70–83.

26. Katsiadaki I, Scott AP, Mayer I. Potential of the stickleback as a combined biomarker for estrogens and androgens in European waters. *Mar Environ Res* (in press).

27. Jakobsson S, Mayer I, Schulz RW, Blankenstein MA, Borg B. 1996. Specific binding of 11-ketotestosterone in an androgen target organ, the kidney of the male three-spined stickleback, *Gasterosteus aculeatus. Fish Physiol Biochem* 15:459–467.

28. Pottinger TG. 1987. Androgen binding in the skin of mature male brown trout, *Salmo trutta* L. *Gen Comp Endocrinol* 66:224–232.

29. Pasmanik M, Callard GV. 1988. A high abundance androgen receptor in goldfish brain: Characteristics and seasonal change. *Endocrinology* 123:1162–1171.

30. Fitzpatrick MS, Gale WL, Schreck CB. 1994. Binding characteristics of an androgen receptor in the ovaries of coho salmon, *Oncorhynchus kisutch. Gen Comp Endocrinol* 95:399–408.

31. Sperry TS, Thomas P. 1999. Characterization of two nuclear androgen receptors in Atlantic croaker: Comparison of their biochemical properties and binding specificities. *Endocrinology* 140:1602–1611.

32. Todo T, Ikeuchi T, Kobayashi T, Nagahama Y. 1999. Fish androgen receptor: cDNA cloning, steroid activation of transcription in transfected mammalian cells, and tissue mRNA levels. *Biochem Biophys Res Commun* 254:378–383.

33. Ikeuchi T, Todo T, Kobayashi T, Nagahama Y. 1999. cDNA cloning of a novel androgen receptor subtype. *J Biol Chem* 274:25205–25209.

34. Takeo J, Yamashita S. 1999. Two distinct isoforms of cDNA encoding rainbow trout androgen receptors. *J Biol Chem* 274:5674–5680.

35. Sperry TS, Thomas P. 2000. Androgen binding profiles of two distinct nuclear androgen receptors in Atlantic croaker (*Micropogonias undulatus*). *J Steroid Biochem Mol Biol* 73:93–103.

36. Borg B. 1994. Androgens in teleost fishes. *Comp Biochem Physiol C* 109:219–245.

37. Makynen EA, Kahl MD, Jensen KM, Tietge JE, Wells KL, Van Der Kraak G, Ankley GT. 2000. Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the fathead minnow (*Pimephales promelas*). *Aquat Toxicol* 48:461–475.

38. Wells K, Van Der Kraak G. 2000. Differential binding of endogenous steroids and chemicals to androgen receptors in rainbow trout and goldfish. *Environ Toxicol Chem* 19:2059–2065.

39. Jenkins R, Angus RA, McNatt H, Howell WM, Kemppainen JA, Kirk M, Wilson EM. 2001. Identification of androstenedione in a river containing paper mill effluent. *Environ Toxicol Chem* 20:1325–1331.

40. Parks LG, Lambright CS, Orlando EF, Guillette LJ Jr, Ankley GT, Gray LE Jr. 2001. Masculinization of female mosquitofish in kraft mill effluent–contaminated Fenholloway River water is associated with androgen receptor agonist activity. *Toxicol Sci* 62:257–267.