

## Use of the Three-Spined Stickleback (*Gasterosteus aculeatus*) As a Sensitive *In Vivo* Test for Detection of Environmental Antiandrogens

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We have previously shown that exposure to exogenous androgens causes female sticklebacks (*Gasterosteus aculeatus*) to produce the glue protein, spiggin, in their kidneys. This protein can be quantified by an enzyme-linked immunosorbent assay developed and validated at the Centre for Environment, Fisheries and Aquaculture Science. Here we report the development of an *in vivo* test for the detection of environmental antiandrogens. The system involves the simultaneous exposure of female sticklebacks to 17 $\alpha$ -methyltestosterone (a model androgen) at 500 ng/L and suspected environmental antiandrogens over a period of 21 days. The spiggin content of the kidneys is then measured, and any antiandrogenic activity is evaluated by comparing the spiggin levels of female fish exposed to antiandrogens to those of female fish exposed solely to the model androgen. The assay detects the antiandrogenic activity of flutamide, vinclozolin (both used at 250  $\mu$ g/L), linuron (at 150  $\mu$ g/L), and fenitrothion (at 15 and 150  $\mu$ g/L). These results provide the first evidence of *in vivo* antiandrogenic activity of both linuron and fenitrothion in teleosts. Although there are other suggested fish species that could be used for this purpose, the stickleback is the only widely available species in which it is now possible to study both estrogenic and antiandrogenic end points in the same individual. Furthermore, the species is endemic and ubiquitous in Europe, and it possesses many ecological traits that make it better suited than other potential species for field research into endocrine disruption. **Key words:** antiandrogens, endocrine disruption, fenitrothion, flutamide, linuron, spiggin, stickleback, vinclozolin. *Environ Health Perspect* 114(suppl 1):115–121 (2006). doi:10.1289/ehp.8063 available via <http://dx.doi.org/> [Online 21 October 2005]

Several field and laboratory studies have shown induction of adverse effects in wildlife species and populations upon exposure to endocrine-disrupting chemicals (EDCs). These effects vary from subtle changes in the physiology and sexual behavior of species to permanently altered sexual differentiation (Vos et al. 2000). The vast majority of endocrine disruption research in fish has focused on estrogenic xenobiotics. This work has used the egg yolk protein precursor, vitellogenin (VTG), as an unambiguous biomarker for estrogens in a variety of model species (Allen et al. 1999; Denslow et al. 1999; Folmar et al. 1996; Heppell et al. 1995; Purdom et al. 1994; Sumpter and Jobling 1995).

The role of environmental androgens and antiandrogens has until recently been overlooked, most likely because of the lack of a sensitive *in vivo* system for the detection of such activity.

Nevertheless, the only androgenic industrial effluent identified to date is pulp mill effluent. Several reports have focused on the masculinizing effect of pulp mill effluent on female mosquitofish (Cody and Bortone 1997; Howell and Denton 1989; Parks et al. 2001), male-biased eelpout embryos (Larsson et al. 2000), and induction of spiggin in female sticklebacks (Katsiadaki et al. 2002b). Although domestic effluent is suspected to have a high content of natural androgens, an advanced level of treatment, such as

percolating filter beds and activated sludge systems, seems to be very efficient in removing the responsible agents (Thomas et al. 2002).

More recently, concerns were expressed that trenbolone acetate, a growth promoter used in livestock, is a strong androgen agonist (Ankley et al. 2003; Wilson et al. 2002).

Although androgens do not appear to be as widespread as estrogens in the environment, a nationwide survey of endocrine activity of final sewage effluents across the United Kingdom, using the yeast androgen screen assay, has revealed significant antiandrogenic activity (Environment Agency, in press).

Antiandrogens in general exert their effects by occupying the androgen receptor (AR) without activating it. Activation of the AR is induced by ligand binding through conformational changes that lead to specific gene expression. Antiandrogens compete with androgens for AR occupancy and subsequently block receptor action. Two classes of antiandrogens are currently recognized: steroidal derivatives, which possess mixed agonistic and antagonistic androgenic activity, and nonsteroidal derivatives or “pure” antiandrogens, exemplified by flutamide, (2-methyl-*N*-[4-nitro-3-(trifluoromethyl)phenyl]propanamide). Flutamide (FL) and its derivatives are the main representatives of the latter category, and they have been studied extensively because of their proven clinical efficacy in the treatment of prostate cancer (Singh et al. 2000). They are

potent AR antagonists that compete with androgens for binding to the AR and prevent AR DNA binding and transcription of androgen-dependent genes.

Several environmental chemicals have been shown to compete with androgens at a receptor level. Kelce et al. (1994) and Gray et al. (1994) identified the first environmental antiandrogens that share the same mechanism of endocrine disruption with FL, as M1 and M2 metabolites of the fungicide vinclozolin. Vinclozolin (VZ) itself has poor affinity for the mammalian AR (Kelce et al. 1994). However, *in vivo*, VZ is hydrolyzed to two open-ringed metabolites, M1 (2-[[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenic acid) and M2 (3',5'-dichloro-2-hydroxy-2-methylbut-3-enamide), which act as AR antagonists by preventing transcription of androgen-dependent genes (Wong et al. 1995).

The same mechanism of antiandrogenic action was identified in *p,p'*-DDE (dichlorodiphenyldichloroethylene), a persistent metabolite of the pesticide DDT (dichlorodiphenyltrichloroethane) that, as in the case of VZ, prevents gene transcription in mammals by binding to the AR (Kelce et al. 1995).

The fungicide procymidon alters sexual differentiation in the male rat by also acting as an AR antagonist both *in vitro* and *in vivo* (Ostby et al. 1999; Vinggaard et al. 1999).

Linuron (LN), an herbicide applied to suppress broadleaf and grassy weeds, is a weak competitive AR antagonist *in vitro*, induces a positive response in the immature and adult rat Hershberger assay, and suppresses androgen-dependent gene expression (Cook et al. 1993; Lambright et al. 2000; McIntyre et al. 2000).

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Tamura et al. (2001) were first to demonstrate both *in vitro* and *in vivo* (rats, Hershberger test) the antiandrogenicity of fenitrothion (*O,O*-dimethyl-*O*-4-nitro-*m*-tolyl phosphorothioate), a widely used organophosphate pesticide. However, Sohoni et al. (2001) observed no conclusive antiandrogenic effects of fenitrothion (FN) in either intact or castrated male rats. In addition, Turner et al. (2002) failed to demonstrate significant alteration of androgen-dependent sexual differentiation in male rats exposed *in utero*, and they concluded that FN is only weakly antiandrogenic. Nevertheless, several other reports confirmed an androgen antagonism *in vitro* using a number of different systems (Freyberger and Ahr, 2004; Sohoni et al. 2001).

Antiandrogens may disrupt male sexual differentiation and performance by interfering with androgen signaling at levels other than the AR, such as androgen production, transport, and metabolism.

Inhibitors of the enzyme 5 $\alpha$ -reductase (responsible for the conversion of testosterone in the more potent dihydrotestosterone in mammals), such as finasteride, provide another example of an antiandrogen that produces effects on androgen-regulated sexual differentiation by a mechanism that does not involve AR but, rather, causes interference with androgen production.

Administration of di-(*n*-butyl) phthalate during late gestation in rats also disrupts androgen-regulated male reproductive development (Mylchreest et al. 1999). Di-(*n*-butyl) phthalate and its metabolites do not show any affinity for the AR *in vitro*; thus, the mechanism of action is not by AR interaction.

Interestingly, fetal exposure to high doses of estrogens can produce cryptorchidism and hypospadias (Newbold 1995). *In vitro* studies have also confirmed the affinity of natural estrogens (Sohoni and Sumpter 1998) or environmental estrogens (Lee et al. 2003) for the AR. However, estrogens can also have antiandrogenic effects by acting on feedback mechanisms (inhibition of luteinizing hormone and follicle-stimulating hormone) and by preventing secretion of testosterone from the testes. Other researchers support the view that neonatal treatment of rats with xenoestrogens induces reproductive abnormalities by disturbing the androgen/estrogen balance (Rivas et al. 2002).

Regardless of the mechanism through which a xenobiotic interferes with the endocrine system, its significance depends on the timing of exposure. Indeed, when adult individuals are exposed to EDCs, the effects are reversible once the exposure ceases. In contrast, the consequences of exposure during organogenesis may result in irreversible deleterious developmental effects such as those observed after *in ovo* exposure to the

pesticide DDT in birds (Fry 1995) and those observed after *in utero* exposure to diethylstilbestrol in rodents and humans (Herbst and Anderson 1990; Newbold et al. 1990).

The importance of the issue is highlighted by a large number of studies undertaken to link human adverse effects and exposure to EDCs in the last decade. Evidence for the effect of EDCs in human health includes the increased incidence of idiopathic hypospadias, alterations of the male genitalia, pseudohermaphroditism, decline in sperm counts, and the increasing incidence of breast cancer among females and testicular and prostate cancers among males (Kelce and Wilson 1997; Topari et al. 1996).

The only robust assay for antiandrogens thus far has been that described by Hershberger et al. (1953). The basis of the assay is that castrated sexually mature male rats undergo regression of androgen-sensitive tissues (testes, prostate, epididymis, seminal vesicle, and levator ani muscles). These tissues are restored to their original weight upon treatment with testosterone, and that growth can be blocked by the concomitant administration of an antiandrogen.

In view of the numerous clinical implications in human health and that the only reliable antiandrogen bioassay requires castrated rats, a simple *in vivo* test using intact fish to screen and identify environmental antiandrogens is very important and desirable. The purpose of this study was to test the possibility of adapting the stickleback androgen bioassay, based on spiggin production (Katsiadaki et al. 2002a), for the detection of environmental antiandrogens and to perform a preliminary screen for the antiandrogenic activity of selected environmental contaminants.

## Materials and Methods

**Chemicals, consumables, and equipment.** Dihydrotestosterone (DHT), 17 $\alpha$ -methyltestosterone (17 $\alpha$ -MT), FL, and ethinyl-estradiol (EE<sub>2</sub>) were obtained from Sigma-Aldrich Co. Ltd. (Dorset, UK). VZ, LN, and FN were purchased from QMX Laboratories Ltd (Thaxted, Essex, UK).

**Fish.** Wild three-spined sticklebacks were obtained with hand nets from U.K. rivers in the Kent and Reading areas when they were only a few weeks old, and they were kept in the laboratory for 6 months before the experiments.

**Stock population.** The fish were kept in brackish water at 12°C with a photoperiod of 8:16 hr light:dark, a regimen in which the fish will remain reproductively quiescent. Only fish that had been kept under these conditions, that weighed > 0.8  $\mu$ g, and had no external signs of parasitism were used for the experiments.

**Experimental populations.** Fish (groups of 25 to 30) were transferred to 40-L glass aquaria containing fresh water. The photoperiod was

12:12 hr light:dark, and the temperature was 15°C. Both photoperiod and temperature for the experimental fish were chosen on the basis that these conditions were neither stimulatory nor inhibitory for the reproductive system. Tanks were aerated constantly. Daily readings from each tank were taken for temperature, dissolved oxygen, pH, and conductivity. The fish were fed every other day throughout the exposure period with a combination of frozen shrimp, artemia, and bloodworm.

**Laboratory exposure to androgens/antiandrogens.** The test system we used to assess antiandrogenic activity used female sticklebacks that were simultaneously treated with a model androgen. However, as stickleback sexing outside the breeding season is a difficult task, some males were present in the tanks.

**Semistatic exposure.** For this experiment, we used the synthetic model androgen 17 $\alpha$ -MT at 5 and 0.5  $\mu$ g/L to induce the glue protein spiggin. We chose the semistatic system for administration of the test compounds because of the large volumes of pesticides needed for a flow-through exposure. For this process, two-thirds of the aquarium water was removed every 48 hr and replaced with fresh water plus a fresh dose of compound. Previous exposures to 17 $\alpha$ -MT have indicated that the androgen is stable in water over a period of 48 hr and appropriate to use for semistatic exposures during screening of xenobiotics (Katsiadaki et al. 2002a). The environmental antiandrogens were simultaneously administered via the water, using the same semistatic system. The highest concentrations tested were either 250 or 150  $\mu$ g/L and were based on toxicity data in fish. FL, VZ, LN, and FN were screened for potential antiandrogenic activity.

Both suspected antiandrogens and 17 $\alpha$ -MT were dissolved in methanol and applied via the water as described above. Appropriate volumes of vehicle solvents were added to the controls. All treatments were duplicated. The fish were exposed to the test compounds for 3 weeks. Water samples were taken each week before and immediately after the addition of fresh solutions for the analytical verification of the tested chemicals.

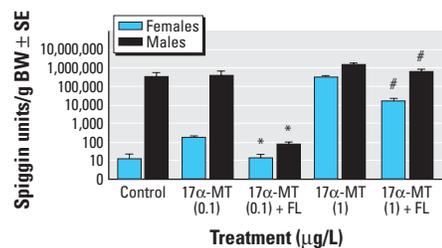
**Flow-through.** We used a continuous flow-through system for exposure to DHT (a nonaromatizable model androgen) at 5  $\mu$ g/L and various concentrations of FL (to construct a dose-response curve). The flow rate was 100 mL/min. Previous unpublished data have indicated that DHT has a low stability in water, so it was not appropriate to use in semistatic exposure. Verification of the test compound concentration took place twice a week over the 3-week period.

**Spiggin measurements.** At the end of the exposure period, all fish were sacrificed by destruction of the brain and then snap frozen in liquid nitrogen. The fish were weighed to

the nearest milligram, and their kidneys were dissected out, placed in individual vials, and labeled. The addition of 200  $\mu$ L of a strong denaturing buffer [100 mM Tris-HCl, 10 mM EDTA, 8 M urea, 2% SDS (wt/vol), and 200 mM  $\beta$ -mercaptoethanol; pH 8.5] and heating of the kidneys at 70°C for 30 min followed. The enzyme-linked immunosorbent assay (ELISA) procedure for spiggin has been described in detail elsewhere (Katsiadaki et al. 2002a).

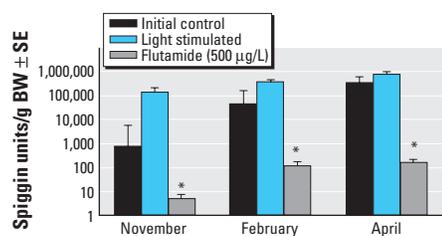
**Histological analysis.** In one experiment, the kidney was divided into two equal parts, one of which was used for the ELISA and the other for histological examination. We have previously described in detail the standard histological protocols used for light microscopy (Katsiadaki et al. 2002a).

**Analytical verification of test concentrations.** We used solvents that were all HPLC grade. Before extraction, a known amount of diuron was added to each tank water sample as an internal (surrogate or recovery) standard. Analytes were extracted from the samples using conditioned C<sub>18</sub> solid phase extraction (SPE) cartridges (Waters Ltd., Watford, UK). Water samples (1-L) were pumped through a prefilter (0.45- $\mu$ m pore size; Pall Life Sciences, Portsmouth, Hampshire, UK) before passing through the SPE cartridge. After elution with methanol and hexane, the final extracts were reduced to near-dryness and prepared in methanol.



**Figure 1.** Amounts of spiggin per gram of body weight (BW;  $\pm$  SE) in female and male sticklebacks exposed for 3 weeks to 17 $\alpha$ -MT with or without flutamide.

\*Significant ( $p < 0.05$ ) inhibition of spiggin induction by flutamide. #Significant ( $p < 0.05$ ) reduction in spiggin production by flutamide.



**Figure 2.** Amounts of spiggin per gram of body weight ( $\pm$  SE) in male sticklebacks (controls and photoperiodically stimulated with or without flutamide).

\*Significant ( $p < 0.05$ ) inhibition of natural spiggin induction by flutamide in comparison to light-stimulated male fish.

The separation and detection of LN, FL (including 2-hydroxyflutamide), and VZ (including metabolites M1 and M2) were performed by a HP1050 liquid chromatograph (Agilent Technologies Ltd., Wokingham, Berkshire, UK) coupled to a Platform II mass spectrometer (Micromass, Altrincham, Cheshire, UK). Analysis was conducted in the negative ionization mode, using an electrospray interface. For FN, an atmospheric pressure chemical ionization interface was used, and detection was performed in the positive mode. An acetonitrile:water gradient was applied, and separation was achieved by using either phenyl-hexyl or C<sub>8</sub> minibore analytical columns (Phenomenex, Torrance, CA, USA). Selective ion monitoring (SIM) was then performed in time-scheduled events.

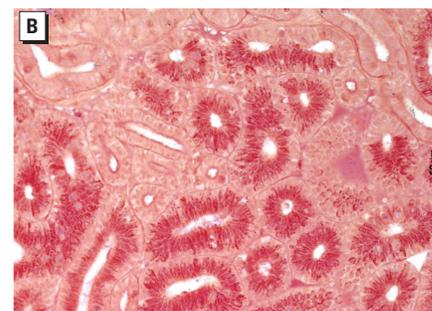
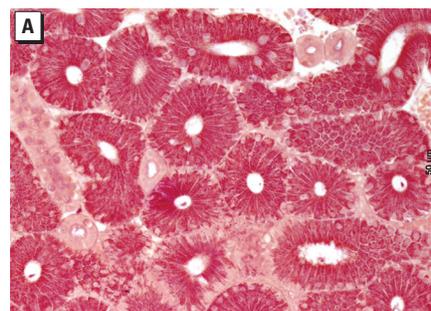
Quantification of LN, FL, and VZ was achieved internally and relative to diuron; hydroxyflutamide was determined relative to the response of FL. Concentration data for FN were derived from external calibration curves.

A competitive ELISA and a radioimmunoassay were used to measure 17 $\alpha$ -MT and DHT in water samples, respectively. We used commercially available antibodies as described previously (Katsiadaki et al. 2002a).

**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 because of the large differences in spiggin contents between the sexes, particularly in control groups.

## Results

**In vivo tests.** During the early experiments, the amounts of FL added to the aquaria were 500  $\mu$ g/L of ambient water (Figures 1, 2). Both the histological analysis of kidney epithelium height (Figure 3) and the immunoassay developed for spiggin demonstrated that FL is highly effective at inhibiting or reducing (depending on the androgen dose used) spiggin induction in androgen-treated sticklebacks or in photoperiodically stimulated male sticklebacks.



**Figure 3.** Kidney sections of female sticklebacks treated with 17 $\alpha$ -MT (10  $\mu$ g/L) alone (A), and with 17 $\alpha$ -MT (10  $\mu$ g/L) plus flutamide at 500  $\mu$ g/L (B). Sections were stained with periodic acid-Schiff. Both microphotographs were taken under the same magnification. Scale bar = 50  $\mu$ m. (A) Kidney epithelium height, 28.5  $\mu$ m; (B) kidney epithelium height, 20.01  $\mu$ m.

These early results suggested that the antiandrogenic effect of FL at a given dose depends on the ligand's concentration, its binding affinity for the AR in the stickleback kidney, and on the presence of competing natural ligands (in the case of male fish). The antagonizing effect is not observed when large doses of androgens are used (i.e., 17 $\alpha$ -MT at 500  $\mu$ g/L, results not shown), is moderate at 1  $\mu$ g/L 17 $\alpha$ -MT, and is complete at 100 ng/L 17 $\alpha$ -MT. The inhibitory effect of FL with the simultaneous administration of 17 $\alpha$ -MT at 100 ng/L is evident in male fish only because 17 $\alpha$ -MT at this concentration did not show much induction of spiggin in females (Figure 1).

Remarkably, inhibition of natural spiggin production in photoperiodically stimulated male sticklebacks was complete (Figure 2). It should be noted that in males, the magnitude of response to a temperature/photoperiod stimulus is dependent on the time of the year that the experiment is taking place. Generally, the response increases from January to April, where a maximum response is observed, remains high until August, and then decreases during November and December. The spiggin levels of control fish in November are statistically lower than those of the light-stimulated fish, whereas this difference was not observed in the control fish during February and April (Figure 2). This observation is in line with previous studies that have shown that extraretinal photoreception and strong circadian rhythms are operating in the male sticklebacks (Borg 1982; Bornestaf and Borg 2000). Social hierarchies also influence the magnitude of secondary sexual characters such as kidney hypertrophy, and therefore, a sound knowledge of stickleback reproductive biology is needed for the design of successful experiments using male fish. Although it is possible that the time of the year and the reproductive status of the female fish might influence the magnitude of response to androgen treatment, more data are needed to establish this effect.

The inhibitory effect of FL on the androgen-induced kidney hypertrophy in the stickleback was confirmed histologically (Figure 3).

We followed this interesting finding by selecting two environmental antiandrogens, namely, VZ at 250 and 25  $\mu\text{g/L}$  and LN at 150 and 15  $\mu\text{g/L}$  for screening. In addition, we tested FL at 250 and 25  $\mu\text{g/L}$  as a positive control. Because of the large volumes of environmental antiandrogens used (i.e., 150–250  $\mu\text{g/L}$ ), it was decided that at least the preliminary screening test should be run in a semi-static manner. We have previously reported inhibition of DHT-induced (at 5  $\mu\text{g/L}$ ) kidney hypertrophy in female fish by FL at 500  $\mu\text{g/L}$  (Katsiadaki et al. 2002b). We therefore chose the same concentration for 17 $\alpha$ -MT for the screening of suspected environmental antiandrogens. The results are presented in Figure 4.

No inhibition of spiggin production was apparent even by FL at 250  $\mu\text{g/L}$  when female fish were exposed to 5  $\mu\text{g/L}$  17 $\alpha$ -MT (Figure 4). Although we failed to demonstrate antagonism by the test compounds, our results provided further evidence that 17 $\alpha$ -MT strongly induces spiggin, as reported earlier (Katsiadaki et al. 2002a). However, in the followup experiment where 17 $\alpha$ -MT was used at 0.5  $\mu\text{g/L}$ , the conclusions are different. This study included the organophosphate pesticide FN at concentrations of 150 and 15  $\mu\text{g/L}$  (Figure 5).

All tested antiandrogens inhibited or significantly reduced spiggin production by androgen-treated female fish. In particular, the inhibitory effects of FN are very impressive, implying an even higher antiandrogenic potency than the model FL.

When concerns were expressed that 17 $\alpha$ -MT, which is aromatizable to an estrogen, was not an ideal androgen model, we decided to replace it with DHT, which cannot be converted to an estrogen. We therefore designed another semistatic system of exposure using DHT as a model androgen at 5  $\mu\text{g/L}$ . We tested LN at 150  $\mu\text{g/L}$ , procymidon at 150  $\mu\text{g/L}$ , VZ at 250  $\mu\text{g/L}$ , and *p,p'*-DDE at 250  $\mu\text{g/L}$ . These results are not presented because only 20% of the female fish in the androgen control group were spiggin positive, thereby precluding any assessment

on inhibition. Chemical analysis of DHT in the aquaria water confirmed the lack of DHT at the nominal concentration, thus suggesting that this model androgen is very unstable in water.

To confirm this observation we designed another flow-through test using DHT alone at 5  $\mu\text{g/L}$  (positive control), DHT along with FL at 500  $\mu\text{g/L}$ , and EE<sub>2</sub> at 20 ng/L (negative control).

We were able to reproduce our previous data using the flow-through system (Figure 6). FL antagonized DHT more successfully than 17 $\alpha$ -MT because the inhibition was complete at 5  $\mu\text{g/L}$  DHT, thereby imposing a higher androgenic potency of 17 $\alpha$ -MT over DHT, which was also indicated through the levels of spiggin units induced by the androgens alone. In addition, it appears that EE<sub>2</sub> might have an antiandrogenic effect itself because there were no males in breeding condition in this group (Figure 6).

The next step involved in adapting the spiggin bioassay for antiandrogens was to obtain a dose–response curve for FL-induced inhibition of spiggin production. Indeed, all flow-through experiments up to this point used FL 500  $\mu\text{g/L}$  at a single concentration only. The results of this study indicated that FL successfully antagonized DHT at much lower concentrations than 500  $\mu\text{g/L}$  (Figure 7).

The very smooth standard curve confirmed the suitability of the stickleback bioassay as a test for antiandrogens. Inhibition of DHT-induced spiggin production in females was complete at 125  $\mu\text{g/L}$  FL and above and was significant at 10  $\mu\text{g/L}$ . For the male fish, total inhibition was observed at 250  $\mu\text{g/L}$  of FL, and significant reduction was noted at 50  $\mu\text{g/L}$ .

**Analytical verification of test concentrations.** Table 1 presents the recoveries of test compounds in aquaria water. Analysis of methanol washings from the interior walls of the sample collection bottles as well as the pre-SPE filters indicated negligible (< 0.5  $\mu\text{g/L}$ ) concentrations per analyte. The recoveries of diuron from the extracts ranged between 80 and 93% in all preparations.

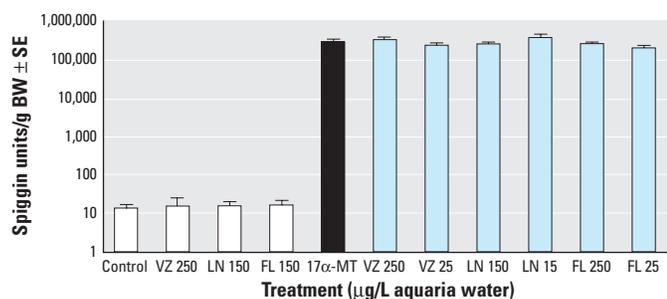
## Discussion

The present study provides further evidence that the stickleback is a unique bioindicator species that can be invaluable in the evaluation of antiandrogenic chemicals, EDCs that appear to pose a significant threat of adverse effects to both wildlife and humans.

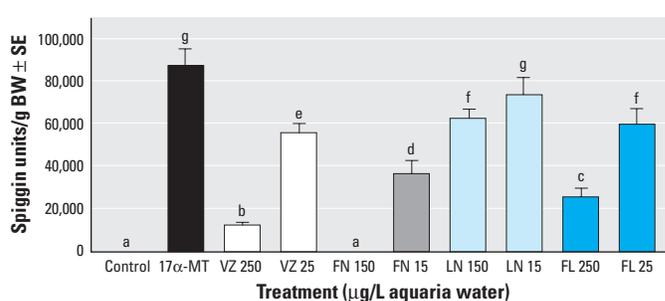
In the test we describe here, antiandrogenic activity is detected by comparing the kidney content of spiggin in female sticklebacks simultaneously exposed to a model androgen (17 $\alpha$ -MT or DHT) and known antiandrogens, to female sticklebacks exposed solely to the model androgen. This bioassay represents the adaptation of the established stickleback androgen bioassay in which the androgen specific biomarker spiggin is measured using an ELISA (Katsiadaki et al. 2002a).

Male sticklebacks can also be used for the *in vivo* detection of antiandrogenic activity; female sticklebacks, however, provide more consistent results because of the absence of endogenous androgens. The bioassay can use male sticklebacks only under extremely controlled photoperiodic conditions before and during the experiment. The reproductive status must be fully synchronized to allow comparison between groups. Social hierarchies within tanks can preclude any conclusions, as dominant males can suppress the onset of breeding in repressed males in the control groups. In addition, the endogenous levels of androgens in the dominant males are extremely high (Mayer et al. 1990), thereby masking the antiandrogenic effect of the test compounds. The development of strict protocols to ensure that the fine balance between environment and reproductive status of male fish is achieved is certainly one area of further work that would be highly beneficial. In general, it appears to be easier to manipulate the reproductive status of male fish during the autumn and winter months when the levels of endogenous androgens and AR numbers are low.

At 5  $\mu\text{g/L}$ , 17 $\alpha$ -MT effectively overwhelmed the antiandrogenic activity of the test compounds at the concentration they were administered. Using this information we modified the experimental design for the second



**Figure 4.** Amounts of spiggin per gram of body weight ( $\pm$  SE) in female sticklebacks (control and 17 $\alpha$ -MT–treated with simultaneous exposure to flutamide (FL), vinclozolin (VZ), and linuron (LN).

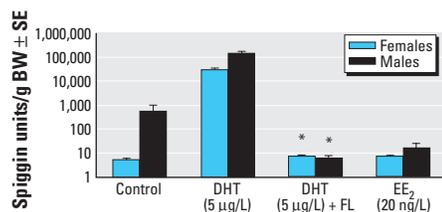


**Figure 5.** Amounts of spiggin per gram of body weight ( $\pm$  SE) in female sticklebacks (control and 17 $\alpha$ -MT–treated with simultaneous exposure to flutamide (FL), vinclozolin (VZ), fenitrothion (FN), and linuron (LN). Treatments shown with the same letter do not differ significantly from each other ( $p < 0.05$ ).

exposure experiment, during which  $17\alpha$ -MT was applied at a concentration of  $0.5 \mu\text{g/L}$ . We believed that this concentration would be sufficient to induce spiggin production but would not be so high as to mask any antiandrogenic activity.

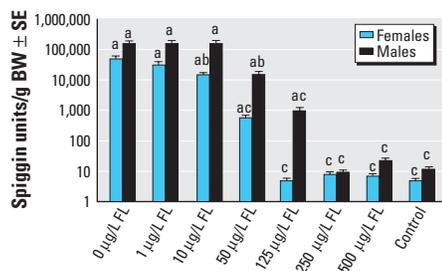
In the second semistatic exposure, the average spiggin content in the androgen-exposed females was approximately one-third of that for the first exposure experiment. FL, VZ, and LN significantly antagonized  $17\alpha$ -MT-induced spiggin production in the female stickleback (Figure 5). The additional antiandrogen FN proved to be exceptionally potent *in vivo*. At its lowest administered concentration, only  $15 \mu\text{g/L}$ , its antiandrogenic activity was analogous to the activity of the pharmaceutical antiandrogen FL at  $250 \mu\text{g/L}$ . Furthermore, at the highest administered concentration,  $150 \mu\text{g/L}$ , FN completely inhibited spiggin production. The spiggin content at this concentration is statistically the same as that obtained for the water and solvent control groups. These results provide the first *in vivo* evidence of antiandrogenic activity for both LN and FN in teleosts.

When the administration of test compounds is waterborne, one should consider the differences in solubility, stability, and means of transport to target tissues that exist between the test compounds and that influence the magnitude of biological response. In this case the differences between the two androgens ( $17\alpha$ -MT and DHT) could have more than one cause. Our own unpublished data suggest



**Figure 6.** Amounts of spiggin per gram of body weight ( $\pm$  SE) in male and female sticklebacks treated with DHT alone or in combination with flutamide.  $\text{EE}_2$  was used as a negative control.

\*Significant ( $p < 0.05$ ) inhibition of DHT-induced spiggin induction by flutamide.



**Figure 7.** Inhibition of DHT-induced spiggin production by flutamide in a dose-response manner. All groups except controls received  $5 \mu\text{g/L}$  of DHT.

Treatments shown with the same letter do not differ significantly from each other ( $p < 0.05$ ).

that  $17\alpha$ -MT induces higher kidney hypertrophy than DHT when used at the same concentration in both flow-through and semistatic exposure systems. Furthermore, measurement of the model androgens in the aquaria water under semistatic conditions suggests that  $17\alpha$ -MT is far more stable than DHT.

Unfortunately, the number of studies on antiandrogens in fish is limited, and as far as the authors are concerned, this current study is only the third time that FL, a classic mammalian antiandrogen, has been shown to be antiandrogenic in teleosts.

Sower et al. (1983) showed that administration of FL along with  $17\alpha$ -MT to juvenile steelhead trout (incorporated in food pellets) prevented epidermal thickening and reduced growth rate, characteristics that were both caused by  $17\alpha$ -MT alone.

Wells and Van Der Kraak (2000) provided the first evidence that certain environmental

chemicals bind to AR in fish but exhibit interspecies and tissue differences in their binding profile. The lack of AR binding of some classic antiandrogens such as FL and VZ (and its metabolites M1 and M2) in rainbow trout, goldfish, and fathead minnow must be considered, especially when these species are used as models for endocrine research purposes.

The induction of nuptial tubercles in female fathead minnows has been proposed as a biomarker of androgen exposure. The concentrations of  $17\alpha$ -MT used to induce the formation of these tubercles range from 200 to  $2,000 \mu\text{g/L}$  (Ankley et al. 2001; Smith 1974), which is 400–4,000 times the amount required to induce spiggin production in female sticklebacks. Furthermore, administration of  $17\alpha$ -MT at high concentrations resulted in high mortalities in the study conducted by Ankley et al. (2001). The antiandrogenic activity of FL has been detected in male fathead minnows

**Table 1.** Analytical verification of test compounds in water (relevant to Figures 5 and 7).

Compound	Nominal concentration ( $\mu\text{g/L}$ )	Determined concentration ( $\mu\text{g/L}$ )					
		D3b <sup>a</sup>	D3a <sup>b</sup>	D10b	D10a	D17b	D17a
Linuron (R1 <sup>c</sup> )	150	91	116	63	167	107	111
Linuron (R2 <sup>d</sup> )	150	91	94	61	177	94	101
Linuron (R1)	15	5.4	6.1	3.4	11	5.9	8.5
Linuron (R2)	15	5.9	6.3	3.9	12	6.6	8.2
Control (R1)	0	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Control (R2)	0	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Flutamide (R1)	250	231	243	176	251	195	212
Flutamide (R2)	250	232	248	178	256	191	210
Flutamide (R1)	25	17	18	13	18	16	18
Flutamide (R2)	25	18	17	11	18	13	15
Control (R1)	0	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Control (R2)	0	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Vinclozolin (R1)	250	3.5	177	3.8	127	1.9	178
Vinclozolin (R2)	250	3.5	210	4.5	92	2.7	103
Vinclozolin (R1)	25	< 0.5	11	< 0.5	8.8	< 0.5	10
Vinclozolin (R2)	25	< 0.5	7	< 0.5	7.3	< 0.5	7.2
Control (R1)	0	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Control (R2)	0	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Fenitrothion (R1)	150	119	141	127	133	129	135
Fenitrothion (R2)	150	137	140	134	137	124	131
Fenitrothion (R1)	15	12	13	11	12	9	10
Fenitrothion (R2)	15	8.5	10	12	13	11	12
Control (R1)	0	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Control (R2)	0	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
$17\alpha$ -MT (R1)	0.5	0.04	0.45	0.03	0.46	0.02	0.47
$17\alpha$ -MT (R2)	0.5	0.03	0.46	0.02	0.49	0.03	0.48
Control (R1)	0	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Control (R2)	0	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

Compound	Nominal concentration ( $\mu\text{g/L}$ )	Determined concentration ( $\mu\text{g/L}$ )					
		W1a <sup>e</sup>	W1b <sup>f</sup>	W2a	W2b	W3a	W3b
FL (flow-through)	500	396	394	361	335	380	392
FL (flow-through)	250	205	194	202	205	200	201
FL (flow-through)	125	99	114	104	96	101	103
FL (flow-through)	50	40	40	39	49	41	42
FL (flow-through)	10	6.7	8.1	8.5	8.2	7.5	7.9
FL (flow-through)	1	< 0.1	3.3	3.2	3.3	2.1	2
FL (flow-through)	0	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Control (FL)	0	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
DHT (flow-through) <sup>g</sup>	5	4.99	4.92	4.95	4.93	4.96	4.95
Control (DHT)	0	0	0	0	0	0	0

Abbreviations: D, day; R, replicate; W, week. <sup>a</sup>Day 3 before addition of fresh solution in the semistatic system. <sup>b</sup>Day 3 after addition in the semistatic system. <sup>c</sup>Tank replicate 1. <sup>d</sup>Tank replicate 2. <sup>e</sup>Week 1 first sample. <sup>f</sup>Week 1 second sample. <sup>g</sup>The recovered concentrations of DHT from the tanks were very similar and close to the nominal concentration; thus we report only average values from all tanks on each sampling occasion.

through a reduction in the number of nuptial tubercles (Panter et al. 2004). However, a FL concentration of 1,000 µg/L (4 times greater than that used in this study) was required to elicit this response. In addition, exposure of fathead minnow embryos to VZ at concentrations ranging from 90 to 1,200 µg/L did not result in any adverse effects on sexual differentiation or reproductive health (Makynen et al. 2000). These findings draw into question the suitability of fathead minnow bioassays for the detection of EDCs with antiandrogenic activity.

More recently, Bayley et al. (2002) exposed juvenile guppies to FL, VZ, and *p,p'*-DDE via the food from birth to adulthood and concluded that all three chemicals had a clear demasculinizing effect (reduction of orange display coloration, gonopodium development, reduction in sperm count, and suppressed courtship behavior). However, the length of the study and the nonwaterborne exposure preclude any comparisons of the sensitivity of the bioassay with the stickleback assay.

In addition, Kinnberg and Toft (2003) exposed sexually mature male guppies to a number of estrogenic and antiandrogenic compounds. Although FL, *p,p'*-DDE, and estrogens blocked spermatogonial mitosis, VZ did not have any adverse effects.

The *in vivo* studies that failed to demonstrate androgen antagonism by mammalian and environmental antiandrogens in teleosts suggest profound differences in the AR binding specificities between species.

In the stickleback kidney, previous studies have shown that no specific binding of 11-ketotestosterone (11-KT) or testosterone was detected in either cytosolic or nuclear fractions, although displacement of tritiated 11-KT with unlabeled 11-KT was observed in the kidney membrane fraction (Jakobsson et al. 1996). More recently, molecular cloning of a nuclear AR in the stickleback kidney has revealed that it is the classic mammalian type, AR2 or AR beta (GenBank accession no. AA083572/3; <http://ncbi.nlm.nih.gov/Genbank>), as we have previously speculated on the basis of androgen potency (Katsiadaki et al. 2002a). The presence of a nuclear AR homologous to the mammalian AR in the stickleback kidney has important implications regarding the use of the species as a model organism for EDC research.

The most promising mammalian assay for androgens and antiandrogens is the castrated male rat assay, widely known as the Hershberger assay (Ashby and Lefevre 2000; Stocker et al. 2000). However, the prerequisite for surgical castration of rats makes it a rather laborious test for chemical screening. The need for a more rapid test for antiandrogens is reflected by the increasing number of alternative tests such as the weaning male rat assay (Ashby and

Lefevre 1997), the intact young male rat assay (O'Connor et al. 1999), the peripubertal intact male assay (Stocker et al. 2000), the use of androgen-stimulated immature intact male rats (Ashby et al. 2002), *in utero* exposure (Shultz et al. 2001), and the use of gonadotrophin release hormone-inhibited rats (Nellemann et al. 2003).

Although surgical castration is not required in these alternative tests, the majority of these assays rely on the same principle changes in the reproductive or accessory sex gland weights upon treatment. This end point has received criticism because chemical treatment may affect growth rate; thus the relationship between body weight and/or accessory gland weight is problematic (Marty et al. 2003).

It should be added that although *in utero* and *in vivo* exposure (You et al. 1998) of weaning rats to *p,p'*-DDE confirmed the antiandrogenic activity observed *in vitro* by Kelce et al. (1995), exposure of adult male rodents has failed to identify any antiandrogenic effects (Leavens et al. 2002). With the adaptation of the spiggin assay described here, a dramatic inhibition of photoperiodically or androgen-induced kidney hypertrophy is detectable in intact adult sticklebacks within 21 days of exposure, which highlights the advantages that the assay presents. The exposure period of 21 days was chosen because it is used as the standard period in the fish screening assay for the detection of endocrine-active substances (Organisation for Economic Cooperation and Development 2004). In our experience, a 3-week exposure period is sufficient for detecting changes in biomarkers (i.e., spiggin and VTG).

EE<sub>2</sub> was used in one exposure as a negative control (Figure 6). As expected, EE<sub>2</sub> at a concentration that is effective at inducing VTG production in a wide range of fish did not induce spiggin in females. Indeed, there is a suggestion that it inhibited/reduced spiggin production in males. Both findings are in line with previous observations by Oguro (1957), who first reported that estrogens do not stimulate kidney hypertrophy in the stickleback but do result in regression of kidney hypertrophy. Exposure to high concentrations of estradiol also results in lower spiggin levels in male fish (unpublished data). We are currently testing a number of estrogens in androgen-treated female fish to determine whether they display the same antiandrogenic effect as in the males.

It is as yet unclear whether the antiandrogenic effect of estrogens is exerted through their binding to the AR in a genomic way (Kelce and Wilson 1997; Sohoni and Sumpter 1998); in a nongenomic way, mediated by an estrogen membrane receptor (Loomis and Thomas 2000); or via other mechanism(s) involving feedback control of sex steroid levels to gonadotrophins (Shultz et al. 2001).

One of the great advantages of the stickleback as a model organism is that the simultaneous assessment of an androgen and estrogen end point (we have also developed a homologous ELISA for stickleback VTG) can provide vital clues regarding the mechanisms responsible for endocrine adverse effects. We applied the VTG ELISA to detect estrogenicity of the test compounds and found that none of the tested environmental antiandrogens induced VTG in male fish or increased the VTG content in female fish (results not shown). As the two protein markers are produced and stored in different organs (kidney for spiggin, plasma or liver for VTG), a single fish can be analyzed for androgenic and estrogenic activity. This versatility reduces the number of test organisms needed, which is of great importance both ethically and economically.

There are several other reasons why the stickleback is an ideal European bioindicator species, as discussed elsewhere (Katsiadaki et al. 2002b).

## Conclusions

The stickleback androgen bioassay can be adapted to detect antiandrogens in two ways:

- Simultaneous treatment of females with an androgen
- Photoperiodic manipulation of male stickleback reproductive status

The degree of antagonism by a given compound depends on the following:

- Type and concentration of model androgen used (17 $\alpha$ -MT being more potent than DHT).
- Stability/solubility of the androgen/antiandrogen in water (particularly when semistatic exposure is employed).
- Reproductive status of fish/experimental conditions (competition with natural ligands in male fish).
- On the basis of the results of the present study, we propose the use of DHT at 5 µg/L for flow-through systems and 17 $\alpha$ -MT at 0.5 µg/L for either flow-through or semistatic exposures in order to stimulate spiggin production in female fish.
- This work represents the first study in teleosts to demonstrate that linuron is an androgen antagonist.
- Fenitrothion is an exceptionally potent androgen antagonist *in vivo* (also first evidence in teleosts).

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