The model anti-androgen flutamide suppresses the expression of typical male stickleback reproductive behaviour

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ABSTRACT

Over the past 15 years considerable attention has been given to the presence in the environment of endocrine disrupting chemicals (EDCs) that may have harmful effects on organisms. Specific test guidelines for the detection of EDCs used for short-term fish screening assays have been developed by the Organisation for Economic Cooperation and Development (OECD). Compared to the core species used in the OECD guidelines, the three-spined stickleback (*Gasterosteus aculeatus*) has an additional and unique endpoint for (anti-)androgenic substances through the androgen-dependent glue protein (spiggin) used in the nest building. Here we describe a specific behavioural assay that was developed in parallel to the OECD protocol, utilising unique behavioural features of sticklebacks. In the assay, a photoperiod of 16L:8D (light:dark) and a temperature of $17 \pm 1\,^\circ$C was used to induce breeding in quiescent male sticklebacks that were simultaneously exposed for a 21-day period to the mammalian anti-androgen flutamide (FL) at 100, 500 and 1000 μg/l (plus a water control). Spiggin production and the reproductive behaviour (nest building and courtship) of male sticklebacks were the main measured endpoints. The control fish entered an active breeding cycle including nest building and courtship behaviours as expected due to the stimulating temperature and photoperiodic conditions. The FL-exposed males showed significantly lower spiggin levels at 500 and 1000 μg/l. In addition, there was a significant decrease in the number of nests built by the FL-treated males at 100 μg/l with no nest built at 500 and 1000 μg/l. Finally, FL affected the courtship behaviour of the males with a significant reduction of the number of zigzags towards the female. When the breeding status of the stickleback males is controlled, the behavioural assay developed here is a suitable tool for the detection of androgen antagonists.

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1. Introduction

Over the past two decades, there has been an increasing concern regarding the impact of man-made chemicals released in the environment that are able to interfere with the endocrine system and alter physiological functions in organisms (Colborn et al., 1993; Sumpter, 1998; Vos et al., 2000). A large number of chemicals enter aquatic systems through a variety of direct and indirect release sources; it is therefore not surprising that aquatic animals, and in particular fish are directly affected by chemicals with endocrine disrupting potential (Purdom et al., 1994; Jobling et al., 1998; Tyler et al., 1998; Matthiessen, 2003). However, the issue of risks associated with these chemicals on human reproductive health has generated a lot of controversy (Toppari et al., 1996; Safe, 2000; Fisher, 2004; Waring and Harris, 2005). The processes of screening and testing play therefore a key role in identifying the potential risks a chemical may pose in both wildlife and human health.

Because endocrine disrupting chemicals (EDCs) differ from classic toxicants in the way that affect biological processes, the Organisation for Economic Cooperation and Development (OECD) has taken the initiative of developing harmonised screening assays for the detection of these compounds. A working group on Endocrine Disrupter Testing and Assessment (EDTA) was established in 1997, formed jointly by the OECD Risk Assessment Advisory Board and the National Coordinators of the OECD Test Guidelines Programme. This group developed a tiered, flexible framework for the screening and testing of EDCs consisted of initial assessment, screening and testing (Huet, 2000). The OECD Secretariat subsequently established a Validation Management Group for Ecotoxicological Test Methods for Endocrine Disrupters (VMG-Eco), whose aim was to supervise the work on validation of suitable tests involving fish, amphibian and invertebrate species. A test guideline for a 21-day fish screening assay has been established by the VMG-Eco and performed using three test species—fathead

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minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*) and is currently close to being validated. Essentially, the core endpoints used in this screen are vitellogenin (VTG) concentration and secondary sexual characteristics (SSC). Apical endpoints such as fecundity and gonadal histopathology were judged to be of less diagnostic value. Although VTG induction and SSC are capable of detecting compounds with oestrogenic, and anti-oestrogenic activities, the inter-calibration exercises that were conducted as part of the validation process of the guideline, failed to detect compounds with anti-androgenic properties due to the lack of relevant endpoints (OECD report, 2006). Furthermore, the detection of androgens is achieved via an indirect process, the reduction in VTG in female fish. Although this endpoint was reproducible across all three tested species, it is detectable only for potent, non-aromatisable androgens, limiting the screening capacity of the assay. In addition to the fact that none of the three core species have specific, quantitative, and reproducible endpoint for androgens or anti-androgens, they are all also absent in the European aquatic environment. In order to address these shortfalls, we assessed the potential of the three-spined stickleback (*G. aculeatus*) as an alternative species. To date, the stickleback is the only teleost in which an androgen-specific biomarker exists (Katsiadaki et al., 2002a). During the breeding season, the kidney epithelial cells of male sticklebacks enlarge under androgen control (de Ruiter and Mein, 1982) and produce a glue, a glycoprotein called spiggin, used for building a nest (Jakobsson et al., 1999). We have previously described in detail the development of two *in vivo* assays for the detection of androgens (Katsiadaki et al., 2002a) and anti-androgens using androgen-stimulated females (Katsiadaki et al., 2006) and suggested that the three-spined stickleback should be included in the OECD endocrine disrupter fish screening assay as an additional species. The stickleback has the advantage of being indigenous to UK and other European waters (as well as North America and Asia) and more importantly has fully quantitative endpoints for both (anti-)oestrogens (VTG) and (anti-)androgens (spiggin). We have therefore organised and completed an inter-calibration exercise that followed the OECD guidelines (phase 1a of the VMG-Eco fish screening test protocol) and the results proved highly relevant, sensitive and reproducible (Allen et al., 2008). The need for reliable *in vivo* assays for the detection of anti-androgenicity has become urgent for the UK, with a recent survey revealing that many sewage effluents present a very high *in vitro* anti-androgenic activity (Johnson et al., 2007).

Another advantage of the stickleback is the presence of an additional endpoint for the detection of EDCs with anti-androgenic activity via alterations of its reproductive behaviour, which has been extensively studied and is very well documented (Wootton, 1976; Bell and Foster, 1994; Östlund-Nilsson et al., 2007). There are three distinct phases in the male reproductive cycle, namely nest building – a territorial male secretes spiggin to glue together pieces of vegetation to forming a nest; courtship – the male attracts the female by means of zigzag dancing and leads her to the nest for spawning; and parental care – only the male cares for the eggs by means of fanning and guarding the nest (Wootton, 1976). The associated changes in androgen levels have been determined for the whole reproductive cycle, showing mainly an increase of 11-ketotestosterone during the two first phases followed by a decrease in the parental phase (Wai and Hoar, 1963; Mayer et al., 1990, 2004; Borg and Mayer, 1995; Páll et al., 2002a, 2005; Sebire et al., 2007). Behaviour is a response that integrates biochemical and physiological responses under the influence of hormones. Subtle alterations in hormonal balance may affect distinct measurable changes in behaviour. If normal behaviour patterns are well documented, alterations in these patterns have the potential of providing a sensitive and non-invasive assay for the detection of environmental contaminants. Abnormal behaviour is one of the more obvious endpoints produced by chemicals (including EDCs) and these patterns are relatively easy to evaluate (Jones and Reynolds, 1997; Clotfelter et al., 2004; Scott and Sloman, 2004; Zala and Penn, 2004). A few studies have already used behaviour of the three-spined stickleback successfully as an indicator of pollution (Bernhardt et al., 2006), notably effects of oestrogenic EDCs on sexual behaviour (Bell, 2001; Wibe et al., 2002; Brian et al., 2006).

The current study describes a stickleback-specific test that was developed within the scope of the 21-day fish screening assay (OECD guidelines) but expanded its relevance to environmental risk assessment by using a sentinel species and by including behavioural endpoints. The latter embraced the first two phases of the male stickleback reproductive cycle (nest building and courtship) but excluded the third (parental phase), as it is not under the control of androgens. The main objective was to assess the ability of the male stickleback bioassay to detect androgen antagonists; hence flutamide was employed as the test compound.

2. Materials and methods

2.1. Fish collection and housing

Prior experience with stickleback husbandry has shown that the male reproductive status is easier controlled during the autumn and winter months when the fish are naturally quiescent. We therefore planned to conduct the 21-day exposure to flutamide in November 2006. For this, wild adult three-spined sticklebacks were collected with nets from a trout farm pond (Golden Spring fish farm) in June 2006. The pond is located in Dorset (UK), is supplied with bore hole water and at the time of collection the pond did not contain any other fish. Upon arrival at the Cefas Weymouth laboratory, the fish were transferred to 120 l tanks in de-chlorinated, UV-treated tap water with no re-circulation (all flow-through). The laboratory's general water quality characteristics were: pH, 7.4–7.6; hardness, 180 ppm; Ca^{2+}, 140 ppm; nitrate, 50 ppm; nitrite, 0 ppm; ammonia, 0 ppm; chlorine (after de-chlorination), less than 0.003 ppm. The fish were kept under a photoperiod of 12L:12D (light:dark hours) and a temperature of $16 \pm 2\,°C$ and under observation for external signs of parasitism (i.e. white spot, *Glugea anomala* cysts, extended abdomen due to *Schistocephalus solidus* plerocercoids) for about a month. In July 2006 and 4 months prior to the scheduled trial, the male population was placed at $10 \pm 2\,°C$ and under a 8L:16D photoperiod in view of inhibiting the onset of breeding prior to the test. In the routine breeding programmes we employ in our laboratory we have demonstrated that male breeding status would be resumed in a matter of days under stimulating photoperiod. Two months (9 weeks) prior to the trials, the photoperiod, under which the female fish were held at, was increased to 16L:8D hours in order to speed up sexual maturation, a desirable condition for the behavioural trials as gravid females provide an extra stimulus for the male (Wootton, 1976; Rowland et al., 2002). The fish were fed daily with frozen bloodworm (Family Chironomidae) that was obtained from Tropical Marine Centre (Chorleywood, Hertfordshire, UK). Only fish weighing ≥0.8 g and showing no external signs of parasitism were used in the experiments.

2.2. Test tank design

The test tanks measured 91.4 cm long × 30.5 cm wide × 25.4 cm deep and were constructed of glass (Fig. 1). The total volume of water in the tanks was 42 l. Chemical and visual cues are stimulating factors for spawning in fish (Chien, 1973; Rowland, 1999). Hence, male and female compartments were separated by a transpar-
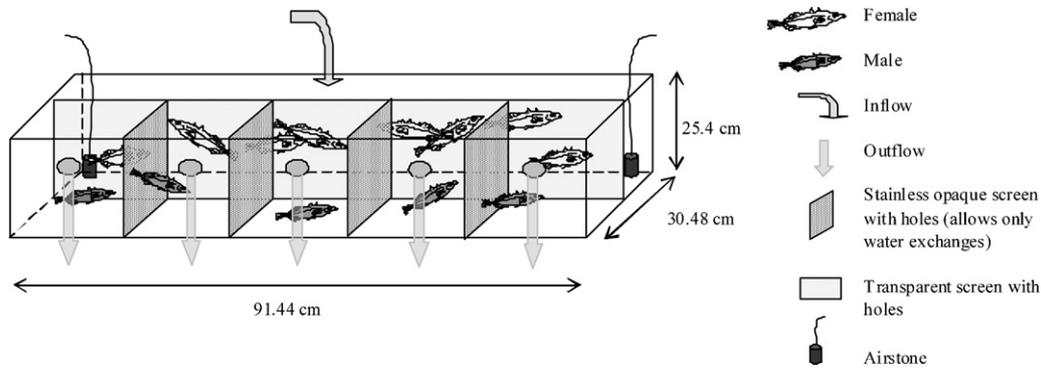
Fig. 1. Design of the exposure test tank used in this study housing 5 male and 8 female three-spined sticklebacks.

ent screen (Methyl methacrylate, Ethyl acrylate, Liteglaze®) drilled with 5 mm diameter holes (20 per male section). Male compartments were separated by stainless steel screens that were similarly drilled with holes (>2000 holes per section, 2-mm diameter). In this way the males could detect the presence of females (via both olfactory and visual cues) and rival males (via olfactory cues only) but the stress that would have otherwise been created by constant visual contact with neighbouring males over the exposure period was avoided. Two air stones were placed into the females' compartment to enhance mixing of the chemical supplied.

2.3. Experiment design

The model androgen antagonist flutamide (FL) was obtained from Sigma Chemical Co Ltd. (Dorset, UK). The FL solutions were supplied in a flow-through system to the experimental tanks by means of peristaltic pumps (Watson-Marlow, UK) 1–2 days prior to the transfer of the fish to ensure chemical saturation. FL is not readily soluble in water. However, the OECD guidelines do not favour the use of solvents hence an elaborate mixing schedule was employed. During this, a stock solution (40 mg/l) was prepared daily by dissolving 0.720 g of FL in 18 l of dilution water (same source than aquaria) with the use of an ultrasonic liquid processor at speed 7 (Sonicator® 3000 by Misonix) and heating (up to 50 °C). From this stock, working solutions of FL were generated every day at 40 mg/l, 20 mg/l and 4 mg/l (pumped at 3.75 ± 0.05 ml/min into the flow-through system) that upon dilution with fresh water in the tank (146.25 ± 0.1 ml/min) provided the target concentrations 1000, 500 and 100 µg/l, respectively, as suggested by the OECD guidelines (OECD report, 2006). One litre of water was sampled from each

tank once a week over the 3-week exposure period to verify the test compound concentrations.

The experimental design has been described previously (Katsiadaki et al., 2007). There were two replicate tanks (with a staggered start time of 1 day) at each treatment concentration, plus two control tanks containing only fresh water (8 tanks containing 5 male and 8 female fish each providing a total of 104 fish). Fig. 2 summarises the behavioural procedures carried out during the exposure. On day 0 the fish were placed into the experimental tanks at 16L:8D and 17 ± 1 °C. Until day 10, the observations made were related only to normal feeding and swimming behaviours of the fish. On day 10, nest material (long tread vegetation like *Cladophora* sp.) and gravel were provided to each male, both at equal amounts (see next section). The behavioural trials were undertaken on days 19 and 20.

2.4. Behavioural trial

The behavioural trial has been outlined in Katsiadaki et al. (2007). The main studied behavioural attributes, described in detail in Wootton (1976), are presented in Table 1 and included nest building (digging activity and nest presence), courtship (bites, zigzags, position near to the female, dorsal pricking, leading) and spawning. During mating, activities directly linked to the presence of a nest (i.e. pushing, boring, glueing, fanning, creeping, showing nest entrance, quivering, fertilisation) were recorded but not presented here (except leading) as some males did not built a nest. We have noticed in numerous occasions that male sticklebacks when isolated in breeding tanks and let to interact with a female display leading behaviour even if no nest is present (to an empty pit).
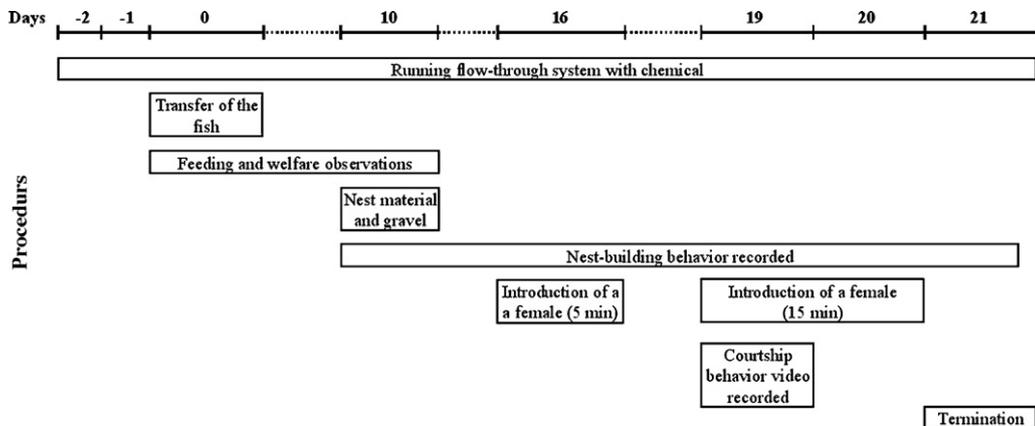

Fig. 2. Schematic representation of the exposure system employed in the study.

Table 1
Main behavioural variables from three-spined sticklebacks measured during flutamide exposure

Variable	Description	PCA code
Nest building phase		
Nest presence	Presence/absence of a nest, even if not completed (i.e. not a visible entrance)	
Digging activity	Presence/absence of movement of gravel in the compartment	
Courtship behaviour		
From the male		
Position near to the female	Time spent (s) within one body length in distance from the female	1
Zigzags	Number of zigzags, i.e. jumps side to side	2
Zigzag bouts	Number of sequences of zigzagging	3
Zigzag duration	Time spent (s) zigzagging	4
Bites	Number of bites towards the female	5
Dorsal pricking	Number of sequences of dorsal pricking	6
Dorsal pricking duration	Time spent (s) pricking	7
Leading	Number of times a male led the female to the nest or to a pit when there is no nest	8
From the female		
Head-up posture	Presence/absence of the head-up posture	
Follow	Presence/absence of the female following the male	
Stationary position	Time spent (s) in a stationary position in the compartment	

Although the study did not focus on the effects of anti-androgens of female reproductive behaviour, the responsiveness of the females was also assessed as Table 1 describes.

To facilitate the expression of all attributes of stickleback reproductive behaviour during the trial, nest material and gravel were provided equally to each male on day 10: the nest material was suspended over the compartment and the gravel was placed at the back half of each male section. In this way, it was possible to determine the movement of gravel, described as digging activity. The progress of nest construction (which unequivocally denotes nest building behaviour by the male) was recorded by daily photographic documentation between days 10 and 21. On day 16, a female, randomly selected from the same tank, was introduced in each male compartment during 5 min to stimulate the male, and then returned to its compartment. This procedure further stimulated males that have not initiated a nest whilst kept the interest high in those that are already guarding a nest (author's observation). On day 19, males were paired with a female taken randomly from the associated tank and their interaction was recorded during a maximum period of 15 min, with a digital camera (Canon MVX25i). The first male from each tank was tested first, followed by the second male from each tank until the fifth male from the last tank. Hence each tank was treated in the same way, with only 45 min difference between tanks for a "same-position" male. In our experience on breeding sticklebacks we have never observed differences in spawning activity between different times of the day, so it is unlikely that the stickleback is similar to zebrafish for example that spawn first thing in the morning. Any spawning event was recorded and the females were then returned to their compartment. In our experience increasing this interaction period does not always improve the chances of successful spawning. Finally, the males for which no spawning occurred on day 19 were given a second chance on day 20 (15-min period, no video records). After removing the fish for termination on the last day, the number of eggs in each nest was recorded. The video footage was subsequently analysed blindfold and the different behavioural parameters were recorded with a personal digital assistant (PDA) running FIT-system software (Flexible Interface Technique, version 3.1©, J. Held 1996–2001) (Held and Manser, 2005), kindly provided by Dr. Iain Barber, Leicester University.

2.5. Physiological biomarkers

At the end of the study, all fish were humanely killed in accordance with UK Home Office regulations. This involved placing them to anaesthetic (MS222) for about a minute before collecting blood (after severing the caudal peduncle) in a pre-heparinised micro-haematocrit capillary tube (Bilbate LTD, Daventry, UK). Plasma was isolated by centrifugation at 13,000 rpm for 5 min at 4 °C and stored at −80 °C until determination of vitellogenin (VTG) by ELISA (Katsiadaki et al., 2002b; Hahlbeck et al., 2004; Andersson et al., 2007). The kidneys were dissected out to allow measurement of spiggin levels by ELISA (Katsiadaki et al., 2002a). Spiggin levels were also assessed in the stock population on day 0 in order to estimate the breeding status of the fish prior to the test. The gonads were also dissected, weighed and stored in Bouin's fixative. For brevity, however, their analysis is not presented here.

2.6. Analytical chemistry

Concentrations of FL were determined in the stock solution and in water samples from each experimental tank during the 21-day assay (days 0, 9 and 18).

C18 solid-phase extraction (SPE) cartridges containing octadecylsilane (Sep-pak Plus, Waters Ltd., Watford, UK) were first washed with 5 ml of HPLC grade methanol and then 5 ml of distilled water as described previously (Scott and Sorensen, 1994). Water samples (1 l) were passed through a pre-filter (0.45 μm pore-size; Pall Life Sciences, UK) – to remove particulate matter that might block the cartridge – and an activated SPE cartridge by means of a peristaltic pump. Both cartridges and filters were then washed with distilled water, the excess of which was blown out. They were then covered with parafilm and stored at −20 °C until required for analysis.

The analysis took place in the Cefas Burnham laboratory. Filters and SPE cartridges were assembled and rinsed with 5 ml of distilled water and dried by vacuum suction for 2 min. A total volume of 5 ml of HPLC grade methanol was pushed through the filter and this volume of methanol was then drawn through the SPE cartridge at a flow-rate of 2–10 ml/min using vacuum and, without letting the SPE cartridge going to dryness; another 10 ml methanol were drawn through the cartridge. The SPE extracts were combined, the internal standard, Tetrabromobisphenol A (TBBP-A, Sigma Chemical Co. Ltd., Dorset, UK), was added and the mixture was made up to 100 ml with HPLC grade methanol before liquid chromatography–mass spectrometry (LC–MS) analysis. The analytical method has been described in detail before (Katsiadaki et al., 2006). Briefly, sample fractions in methanol were analysed for FL using high performance liquid chromatography (HPLC) (Surveyor® HPLC system, ThermoFinnigan, San Jose, CA, USA) coupled to an ion trap mass spectrometer (LCQ Advantage, ThermoFinnigan, San Jose,

Table 2
Analytical concentrations of flutamide in water and in the stock (μg/l)

	Nominal concentration (μg/l)								
	0		100		500		1000		40,000
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Stock solution
Day 0	0.00	0.00	136	112	431	625	1175	1116	46,079
Day 9	0.00	0.00	72	83	579	502	814	535	45,221
Day 18	0.00	0.00	104	100	446	521	308	340	50,621

Rep, replicate.

CA, USA), equipped with an electrospray (ESI) interface operated in negative ionisation mode.

The HPLC column was a 100 mm × 2.0 mm i.d. (3 μm particle size) Luna C18(2) column protected by a SecuriGuard^{TM} cartridge (all Phenomenex, Macclesfield, Cheshire, UK). A gradient program with a mobile phase starting at 40% methanol and 60% water, and increasing to 95% methanol in 10 min at a flow-rate of 200 μl/min was used. The methanol:water ratio (95:5) was held constant for 5 min before reconditioning the column with 40:60 methanol:water for 10 min. Analyte identification and quantification were achieved in SIM mode with m/z 275.1 ± 0.5 and 543.1 ± 1 for FL and TBBP-A, respectively. Quantification was carried out by internal calibration using a set of 6 calibration standards of known concentrations ranging from ca. 0.1 ng/μl to 50 ng/μl.

2.7. Statistical analyses

The statistical analyses were performed with the software Intercooled Stata, version 9.2, unless stated otherwise. In the present experimental design, the "tanks" were considered to be nested within "treatments" and therefore a two-level nested design ANOVA was tested to determine the importance of the random factor "tanks". All data were tested for normality with the Shapiro–Wilk test and were found non-parametric. The non-parametric analyses of the spiggin and VTG levels, between groups, were carried out using the two-sample Wilcoxon rank-sum (Mann–Whitney) test (MWW), at 95% significance level. The behavioural parameters that were defined by a presence/absence answer were compared between groups using a Fisher exact test (2 × n tables). A principal component analysis (PCA) was performed on the behavioural activities linked to the courtship phase (MVSP software, version 3.1). The variables were standardised and centered prior the test. A one-way analysis of variance (ANOVA) was achieved to test differences between the experimental and control groups' first principal component score. Finally, each behavioural variable was tested using a Generalised Linear Model (GLM) with a Poisson distribution and robust standard errors. Results were considered significant at $p < 0.05$. All data are presented as means (±S.E.) of all male or female fish within each treatment level unless stated otherwise.

3. Results

The two-level nested design ANOVA was carried out for every endpoint, and the tanks on the whole (position, conditions) had no significant effect on the results (data not shown).

3.1. Water analyses

The analytical determination of FL in the three stock solution samples and in the experimental tanks were close to nominal concentrations (Table 2). In general, measured concentrations were close to nominal concentrations with the exception of the highest

FL level. There, a gradual decrease in the measured FL was observed over the 3-week period. Besides a daily cleaning routine, FL at this high concentration precipitated in the tubing, blocking it partially and slowing down the inflow of the chemical.

3.2. Vitellogenin

The plasma levels of VTG measured were negligible, a fact that was expected in male fish that have not been exposed to an oestrogen: 0.062 ± 0.027 (control), 0.027 ± 0.000 (FL at 100 μg/l), 0.046 ± 0.002 (FL at 500 μg/l), 1.071 ± 0.133 (FL at 1000 μg/l) all expressed in μg/ml plasma ± S.E. There was no difference in male VTG concentrations between the treatments (MWW, $p > 0.05$). The VTG levels found in the females (also expressed in μg/ml) were 9176 ± 2104 (control), 7526 ± 1223 (FL at 100 μg/l) showing higher values at 500 and 1000 μg/l, $14,936 \pm 4193$ and $12,945 \pm 4696$, respectively. This increase however was not significant (MWW, $p > 0.05$).

3.3. Spiggin

The males that were sacrificed on day 0 of the test showed very low levels (644 units/g bw, body weight) (Fig. 3). This result confirmed that the photoperiodic manipulations employed prior to the commencement of the exposure trials were successful in providing male fish in optimum reproductive status. There was a marked inhibition in spiggin induction (brought up by the stimulating temperature and photoperiod in control fish) by FL exposure in a dose response manner, significant at 500 and 1000 μg/l (MWW, $N = 10$, $z = 3.70$, $p = 0.000$ and $N = 10$, $z = 3.63$, $p = 0.000$, respectively; Fig. 3).
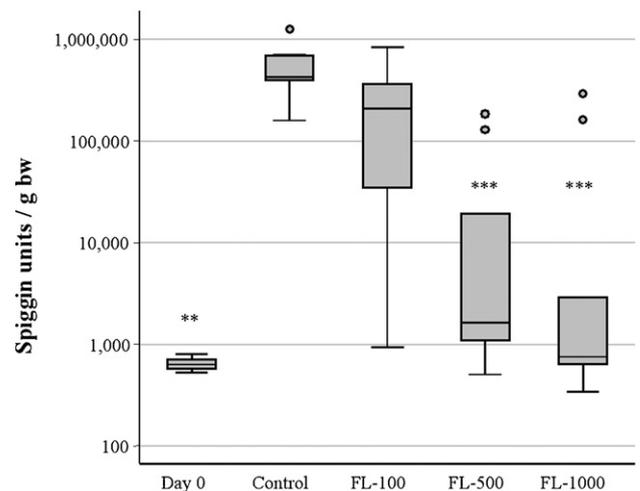

Fig. 3. Spiggin units/g body weight in male three-spined sticklebacks exposed to flutamide. $N = 5$ males on day 0; $N = 10$ males in all treatments except for FL-100 ($N = 9$). FL-100, FL-500 and FL-1000, flutamide at 100, 500 and 1000 μg/l, respectively. Box–Whisker plots represent percentiles. Significant difference from the control, $**p < 0.01$ and $***p < 0.001$ (MWW).

Day10 Day13 Day16 Day19 Day21

Control

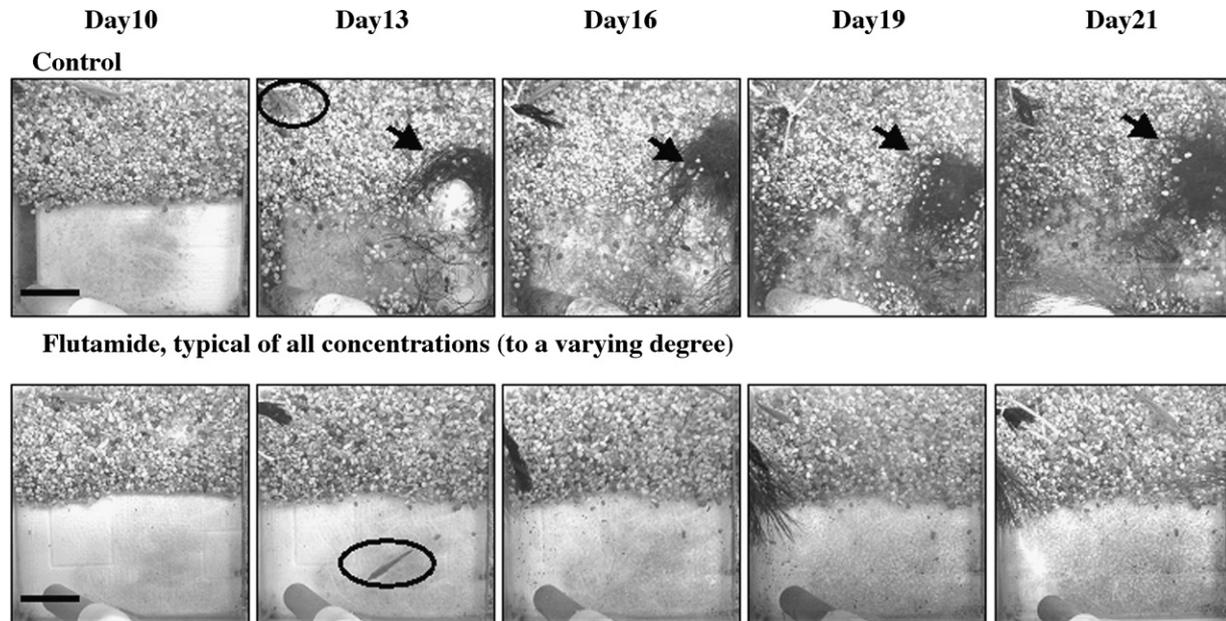**Flutamide, typical of all concentrations (to a varying degree)**

Fig. 4. Photographic documentation of the nest building behaviour of male three-spined sticklebacks exposed to flutamide. Arrows indicate a nest presence and circles the male stickleback (showed once). Bar scale = 4 cm.

In females, the spiggin levels were within baseline levels across all treatments (MWW, $p > 0.05$), indicating that FL is not androgenic, as was expected.

3.4. Nest building behaviour from the male

Fig. 4 provides an example of the photographic documentation of nest building behaviour of male stickleback. There was a clear reduction in the number of males digging, significant at 500 and 1000 μg/l (Fisher exact test, $p < 0.001$; Fig. 5). Eight out of ten males built a nest in the control groups while only one male built a nest in the FL-treated groups at 100 μg/l (Fisher exact test, $p < 0.01$) and none at 500 and 1000 μg/l (Fisher exact test, $p < 0.001$). Most of the control males started building nest within 4 days after the introduction of nest material and gravel while the only exposed male that built a nest started after 9 days.

3.5. Female behaviour during courtship

The number of females displaying the head-up posture decreased, albeit non-significantly, as the amount of FL increased, from 10 out of 10 in control groups to 6 out of 10 in the higher FL 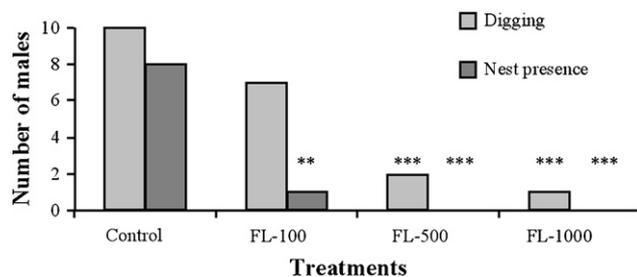concentration (data not shown). The exposed females spent significantly more time in a stationary position ($23 \pm 10\%$ overall) during their 15-min encounter with the males than the control ones (around 7%; GLM, $p < 0.01$).

In all the treatments, when the male displayed the leading behaviour the female followed him. Four spawning actions occurred in the control groups (none for the only exposed male that had a nest) and the average number of eggs was 177 ± 66, for three of the control males (the fourth male ate his eggs before we were able to count them).

3.6. Male behaviour during courtship

The PCA, performed on 8 behavioural variables and 39 males, summarised the effect of FL on the courtship behaviour of the male stickleback (Fig. 6). The two components explained 78% of the variability in the dataset (55% and 23% for the axis 1 and 2, respectively). Results of the analysis showed a clear difference between the con-
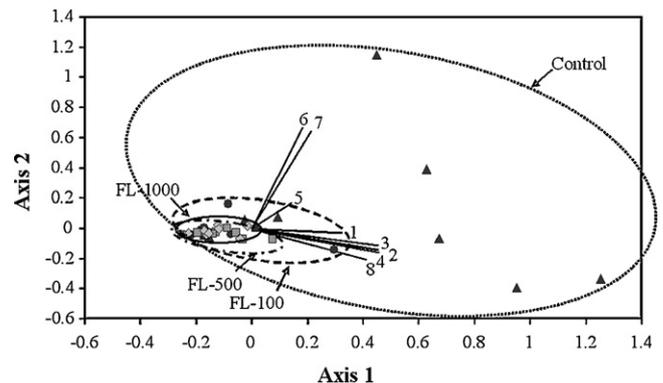

Fig. 5. Nest building activity by male three-spined sticklebacks after 21 days of exposure to flutamide. Number of males showing digging activity (light grey) and/or had a nest (dark grey). $N = 10$ males in all treatments except for FL-100 ($N = 9$). FL-100, FL-500 and FL-1000, flutamide at 100, 500 and 1000 μg/l, respectively. Significant difference from the control, $^{**}p < 0.01$ and $^{***}p < 0.001$ (Fisher exact test).

Fig. 6. Biplot of the behavioural variables from male three-spined sticklebacks during the courtship phase on day 19. The control groups are indicated by the triangles and the flutamide-exposed groups at 100, 500 and 1000 μg/l are indicated by the circles, squares and lozenges, respectively. FL-100, FL-500 and FL-1000, flutamide at 100, 500 and 1000 μg/l, respectively. Numbers at the end of each vector correspond to the variables' code in Table 1. The vectors indicate the relationship between each of the behavioural variables and the principal axes.
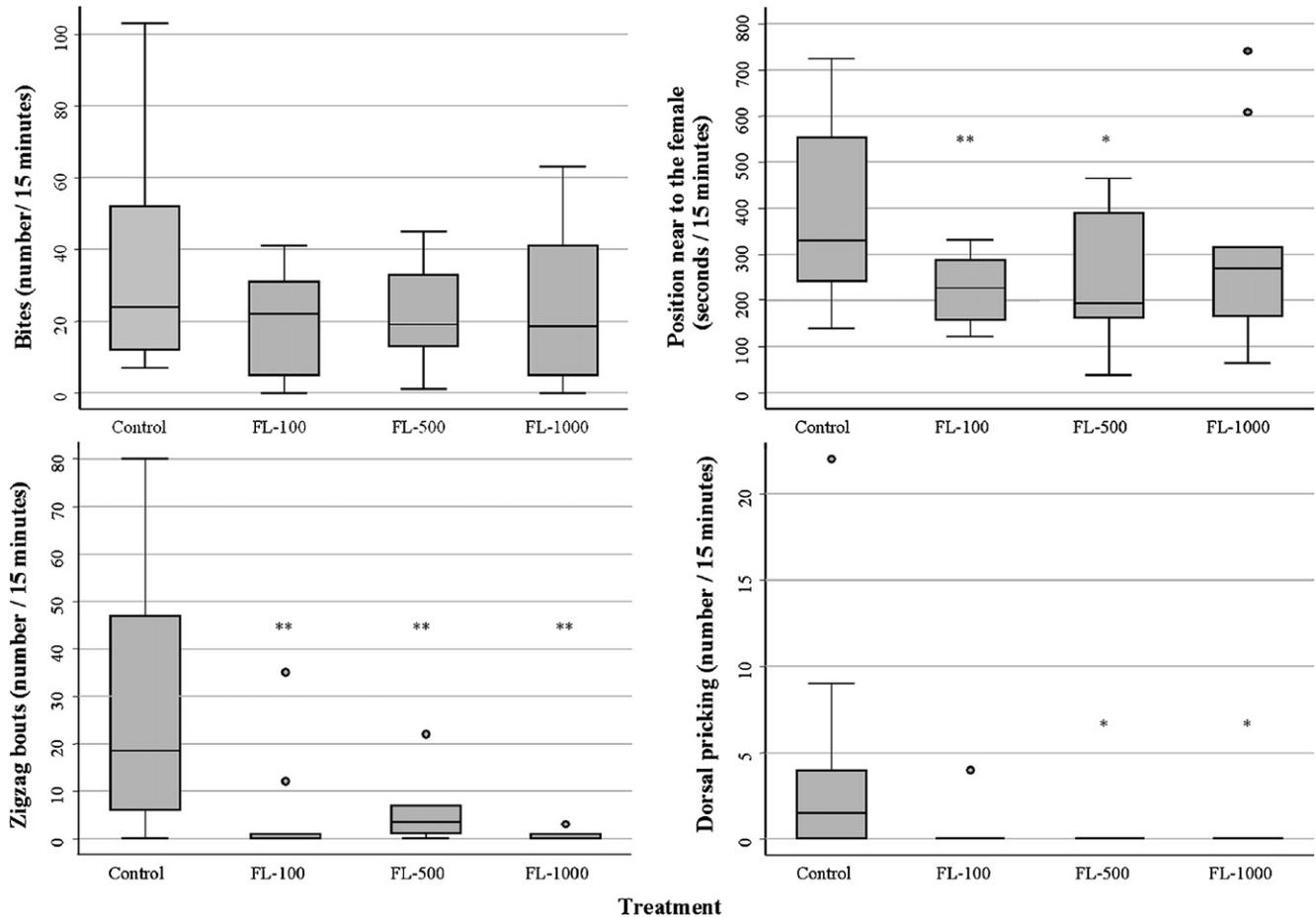
Fig. 7. Courtship behaviour towards a gravid female of male three-spined sticklebacks exposed to flutamide (day 19). $N = 10$ males in all treatments except for FL-100 ($N = 9$). FL-100, FL-500 and FL-1000, flutamide at 100, 500 and 1000 μg/l, respectively. Box–Whisker plots represent percentiles. Significant difference from the control, $^*p < 0.05$ and $^{**}p < 0.01$ (GLM).

trol and treated groups. Control males showed a greater variability in their courtship displays than the FL-treated ones, which showed a marked reduction of each of the behavioural variables. Interestingly the males that performed dorsal pricking tended to decrease their zigzag movements. The variables bites and dorsal pricking were closely linked while position near to the female, zigzag features and leading were closely related. The results from the ANOVA using the first component scores showed a significant reduction of all the behavioural variables ($p < 0.001$). This meant that FL affected the behavioural attributes in their whole. Fig. 7 shows the results for four attributes individually. The FL-exposed males bite the females less than the control males but this was not significant (GLM, $p > 0.05$), while they spent significant less time near them at 100 (GLM, $p = 0.008$), 500 (GLM, $p = 0.032$) and 1000 μg/l (GLM, $p > 0.05$ but excluding two outliers $p = 0.007$). They also reduced their zigzag movements at each treatment level (GLM, $p < 0.01$ overall the zigzag variables) and their dorsal pricking display at 500 and 1000 μg/l (GLM, $p < 0.05$ overall dorsal pricking variables). Finally, only the control males lead the females to their nest or a pit in the two cases that no nest was present.

4. Discussion

The objective of this study was to assess the ability of the male stickleback bioassay, a test designed as close a possible to the OECD guidelines as an EDC screen but incorporating novel behavioural endpoints, to detect anti-androgens. The results we obtained were significantly different between flutamide-treated and control fish in all endpoints assessed (spiggin, nest building and courtship behaviour) suggesting that the assay is sensitive. The environmental risk posed by anti-androgens in the aquatic environment appears to be significant in view of a recent nationwide survey in the UK, which reported that more than 53% of the final sewage effluents displayed anti-androgenic activity higher than 100 μl/l flutamide equivalent and 9% higher than 500 μl/l flutamide equivalent (Johnson et al., 2007). The level of disruption observed in vital components of male reproductive success (sexual maturation and behaviour) in our study, indicates that there is a serious risk to fish populations (at least sticklebacks) associated with anti-androgenic activities found in the environment.

4.1. Physiological biomarkers for the detection of anti-androgens

So far, the only widely accepted *in vivo* assay for anti-androgens has been described by Hershberger et al. (1953), is using rats (*Rattus norvegicus*) and is currently close to validation by the OECD program (Owens et al., 2006, 2007). The few studies involving FL exposures in adult male fish, report alteration of testicular histology—spermatocyte degeneration and necrosis in the fathead minnow (Jensen et al., 2004), reduced number of spermatogenetic cysts and an increased number of spermatozeugmata in the ducts of guppies (*Poecilia reticulate*) (Kinnberg and Toft, 2003), smaller

testes and significant reduction in the number of ejaculated sperm cells in guppies (Baatrup and Junge, 2001) and no concentration-related testis–ova in male medaka (Kang et al., 2006). Other affected endpoints were fecundity, embryo hatchability, elevated concentrations of oestradiol and VTG in the fathead minnow (Jensen et al., 2004) and nuptial coloration and male courtship behaviour in the guppy (Baatrup and Junge, 2001). However, none of the above endpoints has a specific diagnostic value. Embryo hatchability and fecundity for example are apical endpoints that could have been altered by any chemical with reproductive toxicity, not specifically an anti-androgen. The same holds true for the increased levels of VTG, which are diagnostic for oestrogens but not specific to FL. VTG response was not altered by exposure of adult medaka to FL at the measured concentrations of 90.4 and 1470 μg/l (Nozaka et al., 2004). Male fathead minnows exposed to 320 μg FL/l have neither significant higher levels in plasma VTG nor hepatic expression of VTG mRNA (Filby et al., 2007). In this study and others (Katsiadaki et al., 2006, unpublished) where male stickleback were exposed to FL, no elevation of plasma VTG has been found so far. On the other hand, increasing levels of VTG were detected in female fish exposed to FL (Chikae et al., 2004; Jensen et al., 2004; Filby et al., 2007). Our results were similar although the increase in female VTG by FL was not significant.

Female fathead minnows develop nuptial tubercles in response to exogenous androgens and so the number of tubercles in males has been suggested as an endpoint for anti-androgen exposure. During phase 1b, in only one study, FL inhibited secondary sex characteristics of male fathead minnow at the highest concentration, 1000 μg/l (OECD report, 2006). Panter et al. (2004) reported that the FL exposure at the nominal concentration of 1000 μg/l for 3 weeks significantly reduced the nuptial tubercles number in male fathead minnow. However, FL exposure at 50 and 500 μg/l did not alter any external male secondary sex characters, including tubercles number (Jensen et al., 2004) as well as FL exposure at 320 μg/l (Filby et al., 2007). The sensitivity of this endpoint remains therefore questionable. Various androgens affected the formation of papillary processes on the anal fin rays in the female medaka, included methyltestosterone (Asahina et al., 1989; Seki et al., 2004); this characteristic presents the only androgen endpoint (other than spiggin in the stickleback) that appears to have a great potential among the core OECD species. However, to our knowledge, no study on this secondary sex characteristic of medaka exposed to an anti-androgen has been published to date. In the present study, as FL concentrations increased, the spiggin contents in male sticklebacks decreased significantly, particularly at 500 and 1000 μg/l, confirming the known anti-androgen effect of this compound. Katsiadaki et al. (2006) treated male and female three-spined sticklebacks with 5 μg/l of dihydrotestosterone (DHT) simultaneously with increasing concentrations of FL, in a flow-through exposure and found that after a 21-day exposure, the inhibition of spiggin production was total at 125 μg/l of FL and reduced at 10 μg/l in the females, while total inhibition was observed at 250 μg/l of FL and a reduction at 50 μg/l in the males. The current data are within similar ranges concerning spiggin levels in males. Using the androgenised female stickleback assay (Katsiadaki et al., 2006) we have demonstrated that a number of environmental anti-androgens (based on mammalian or *in vitro* data) inhibit spiggin induction by DHT. We therefore suggest that stickleback kidney spiggin level is so far the only reliable and quantifiable biomarker for (anti)-androgens.

4.2. Behavioural endpoints for the detection of anti-androgens

Reproductive behaviour is essential in the mating success of most animals and therefore any impairment of it may have harmful consequences on the reproductive output of the animals. In the stickleback, the head-up posture is the characteristic of a female sexually mature; the lordosis posture, which is the head-up posture, combined with a raised tail and concave back, is strongly positively associated to the spawning readiness (Rowland et al., 2002). In this paper the distinction between the two postures has not been done because our primary purpose was to describe the effects of FL on male fish. Nevertheless, we observed that fewer females displayed head-up posture as the concentrations of FL increased. One could argue that this observation was random and associated with the fact that the treated females were not sexually mature and this was confirmed by histological analysis (data no shown). The majority of females in each of the treatments had late-development stage gonads, although in the FL-exposed groups a few females were less advanced showing a stage 0 (pre-vitellogenic) or stage 1 (early development) gonads. Providing that the overall effect expected by an anti-androgen such as FL is feminising (see also Filby et al., 2007) the lack of spawning readiness in the FL-treated females is most likely a random occurrence.

FL altered the reproductive behaviour of male sticklebacks at two levels, the nest building and the courtship. Anti-androgens have been shown to affect reproductive behaviour in other male fish species (Rouse et al., 1977; Baatrup and Junge, 2001; Bayley et al., 2002, 2003). In two studies, male adult guppies were exposed to three anti-androgenic compounds, the fungicide vinclozolin, the insecticide DDT metabolite *p,p'*-DDE and FL via food (Baatrup and Junge, 2001; Bayley et al., 2002). Their courtship was severely disturbed, with the sigmoid display, a suitable measure of the male's mating interest, strongly affected. Here, FL severely reduced the number of male sticklebacks that constructed a nest at 100 μg/l and completely inhibited nest building at 500 and 1000 μg/l. The nest is an important element for the species reproduction as it is the place where the female spawns and fertilisation occurs. The absence of a nest is therefore a major disadvantage in the reproductive success of a male. In another study, Rouse et al. (1977) observed a delay of the start of the nest-building phase in male three-spined sticklebacks, which have been exposed to cyproterone acetate (an anti-androgen of a different type to FL) for 21 days. Other studies used the nest-building behaviour of the three-spined stickleback to detect the effect of oestrogenic compounds (Wibe et al., 2002; Brian et al., 2006). Again a delay in the onset of the nest building was found with males exposed to 17β-oestradiol at 2 μg/g bw, by intraperitoneal injection (Wibe et al., 2002) and to 17β-ethinyl oestradiol (EE$_2$) at 10 ng/l (Brian et al., 2006) but not a total abolishment of the nest building process as a whole as in the case of FL.

FL affected all aspects of the male reproductive behaviour. Both nest building and courtship behaviours were completely abolished in castrated mature males prior the start of the reproductive cycle (Páll et al., 2002b). However, males that have been castrated after they entered the reproductive cycle (i.e. with a nest) still displayed courtship behaviour (Páll et al., 2002b). This suggests the importance of androgens in the initiation of the mechanisms controlling the reproductive behaviour (particularly territoriality, aggressiveness, nest building) but not necessarily its maintenance during courtship (Páll et al., 2002b; Mayer et al., 2004). In our study, maturing males were exposed to FL prior the onset of the reproductive behaviour, hence FL acted as a "castration" factor by binding to the androgen receptor. Androgens have been shown to be involved in the control of aggressiveness and of the sexual behaviour during the courtship phase (Hoar, 1962; Wai and Hoar, 1963; Wootton, 1976; Rouse et al., 1977; Bakker and Sevenster, 1989; Baggerman, 1990). Here, the variables bites and dorsal pricking have been closely related by the PCA analysis. This is not surprising as dorsal pricking may be considered as an aggressive display towards the female, preventing her to come close to the nest (Wilz, 1970). Therefore the

group "bites and dorsal pricking" defines the aggressive composite of the courtship behaviour and the group "position near to the female, zigzag features and leading" formed the sexual composite; and both behavioural composites were affected by FL exposure in a negative way. Bell (2001) showed that male three-spined sticklebacks exposed to EE$_2$ at 15 ng/l decreased their aggressive response to a conspecific male over time, whereas control males increased theirs. However, no difference was found in the courtship behaviour (zigzag dance) towards a conspecific female. In the present study, FL impaired both sexual and aggressive behaviours of the male towards the female during courtship; similar results were found with cyproterone acetate (Rouse et al., 1977).

4.3. The importance of timing in the assay

Mature males are less sensitive in detecting the anti-androgenic effects of FL (compared to the androgen-stimulated females), due to high levels of natural androgens (Katsiadaki et al., 2006). Therefore, an important pre-requisite of the male stickleback screening assay for anti-androgenic activity is that the males should not be actively breeding. To this end, males were maintained under quiescent conditions (short photoperiod and low temperature) until the start of the test to ensure that the onset of exposure coincides with regressed kidneys and therefore baseline levels of spiggin. In addition, sticklebacks appear to have a very strong circannual rhythm (see further), which means that during their normal breeding season it is very difficult to keep them quiescent in the laboratory besides the employment of low photoperiod and temperature regimes. Using the same experimental set up we described here in spring, the assay failed to detect any changes in the spiggin levels and in the reproductive behaviour due to FL exposure (own unpublished data). The spiggin content of the male fish on day 0 in spring were very high (451,739 units/g bw) while in November (the present study) were very low (644 units/g bw).

All members of the stickleback family are typical spring breeders, between March at the earliest (usually April) and August at the latest (Craig-Bennett, 1931; Baggerman, 1990; Sokolowska and Kulczykowska, 2006). In the three-spined stickleback, as in many other teleosts, endogenous circadian and circannual rhythms occurred in co-operation (Baggerman, 1985; Baggerman, 1990; Bornestaf and Borg, 2000). Baggerman exposed underyearling three-spined sticklebacks caught at two different times of the year (January and November) to three different photoperiods (16L:8D, 12L:12D and 8L:16D) at 20 °C (Baggerman, 1990). The aim of this study was to define the photoreactivity threshold being the threshold from when 50% of the fish will become mature. The experiment showed that by late January/early February, the photoreactivity threshold was in such low level that even short day lengths could induce maturity in the fish. In addition, Baggerman showed that fish continuously kept under short photoperiod at a lower temperature (15 °C) were able to show alternating reproductive and non-reproductive periods, just like those maintained under 16L:8D at 20 °C (Baggerman, 1990). In this respect it is not surprising that the conditions employed prior to the experiments that took place in the middle of the natural breeding period, were not sufficient to override their endogenous clock. Conversely, in the present experiment, the fish were caught in June and maintained under a short photoperiod from July until November, a regime that mimicked their natural winter season. The low spiggin contents at the commencement of the test (day 0) revealed the quiescence (non-breeding) status of the males, and allowed a clear effect to be seen between control and FL-exposed photoperiodically stimulated males. Hence the results presented here and those of other studies conducted in our laboratory (Katsiadaki et al., 2006; unpublished results), demonstrate the importance of timing for the male stick-

leback bioassay; the optimum period for conducting the test is between September and January (before the onset of the natural breeding period and when full control of the reproductive status of the fish can be achieved).

Finally, in view of the value of the described test as a screening assay, we propose that *in vitro* assays such as kidney cell culture assays (Jolly et al., 2006; Bjorkblom et al., 2007) or the *in vivo* assay using androgen-stimulated female sticklebacks (Katsiadaki et al., 2006) present the advantages of being quicker, easier to conduct in laboratories with no prior experience in stickleback husbandry, and more cost effective.

However, both spiggin inhibition and abolition of reproductive behaviour of mature males are endpoints that are of diagnostic value for disruption of the androgen axis mediated through antagonism of the androgen receptor and can inform better environmental risk assessment. In addition, the lack of nest building and courtship behaviours observed as a result of FL exposure is an easily recorded system that can provide valuable information on the effects of anti-androgens on fish populations.

5. Conclusion

In conclusion, the present study demonstrated that FL (and hence other androgen receptor antagonists) has the potential to impair male sexual maturation both at physiological and behavioural level after a 21-day screening assay. When the status of reproductive development has been finely tuned (quiescent males), the bioassay described here combining spiggin levels and reproductive behaviour as endpoints is of great diagnostic value in terms of the impact of EDCs on fish populations.

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