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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.

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 $16 \pm 2\,^{\circ}C$ 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 $10 \pm 2\,^{\circ}C$ 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 $\geq$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 $\times$ 30.5 cm wide $\times$ 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-