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Short-term exposure to a treated sewage effluent alters reproductive behaviour in the three-spined stickleback (*Gasterosteus aculeatus*)

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ABSTRACT

Some UK sewage treatment work (STW) effluents have been found to contain high levels of anti-androgenic activity, but the biological significance of this activity to fish has not been determined. The aim of this study was to investigate the effects of exposure to a STW effluent with anti-androgenic activity on the reproductive physiology and behaviour of three-spined sticklebacks (*Gasterosteus aculeatus*). Fish were exposed to a STW effluent (50 and 100%, v/v) with a strong anti-androgenic activity ($328.56 \pm 36.83\ \mu g\,l^{-1}$ flutamide equivalent, as quantified in a recombinant yeast assay containing the human androgen receptor) and a low level of oestrogenic activity ($3.32 \pm 0.66\ ng\,l^{-1}$ oestradiol equivalent, quantified in a recombinant yeast assay containing the human oestrogen receptor) for a period of 21 days in a flow-through system in the laboratory. Levels of spiggin, an androgen-regulated protein, were not affected by the STW effluent exposure, nor were levels of vitellogenin (a biomarker of oestrogen exposure), but the reproductive behaviour of the males was impacted. Males exposed to full strength STW effluent built fewer nests and there was a significant reduction in male courtship behaviour for exposures to both the 50 and 100% STW effluent treatments compared with controls. The effect seen on the reproduction of male sticklebacks may not necessarily have been as a consequence of the endocrine active chemicals present in the STW effluent alone, but could relate to other features of the effluent, such as turbidity that can impair visual signalling important for courtship interactions. Regardless the specific causation, the data presented show that effluents from STW have an impact on reproductive behaviour in male sticklebacks which in turn affects reproductive performance/outcome. The study further highlights the use of fish behaviour as a sensitive endpoint for assessing potential effects of contaminated water bodies on fish reproduction.

1. Introduction

To date, the majority of studies assessing the biological effects of sewage treatment work (STW) effluents on fish have been concerned with their oestrogenic activity. Induction of vitellogenin (a female specific yolk protein precursor) has been shown in a wide range of fish exposed to UK STW effluents, e.g. in rainbow trout (*Oncorhynchus mykiss*, Routledge et al., 1998a), roach (*Rutilus rutilus*, Jobling et al., 1998; Liney et al., 2006; Rodgers-Gray et al., 2000; Routledge et al., 1998a), sand goby (*Pomatoschistus minutus*, Robinson et al., 2003) and gudgeon (*Gobio gobio*, van Aerle et al., 2001). Feminised gonads have also been documented in wild fish (roach and gudgeon) populations living in effluent contaminated UK Rivers (Jobling et al., 1998; van Aerle et al., 2001). Importantly, wild male roach that have been found to be feminised as a consequence of exposure to oestrogenic effluents have a reduced reproductive capability, providing potential for population level effects (Harris et al., 2011; Jobling et al., 2002). STW effluents have also been shown to be oestrogenic in other parts of Europe, in North America, Australia, China and Japan (Batty and Lim, 1999; Flammarion et al., 2000; Folmar et al., 1996; A. Johnson et al., 2007; Knudsen et al., 1997; Larsson et al., 1999; Ma et al., 2007; Nakada et al., 2004; Pettersson et al., 2006; Viganò et al., 2008). The oestrogenic chemicals in STW effluents have been identified and include natural and synthetic steroidal oestrogens, alkylphenol ethoxylates and in some instances natural products, preservatives (parabens) and pesticides (Chen et al., 2002; Desbrow et al., 1998; Holland, 2003; Larsson et al., 1999; McCarthy et al., 2006; Routledge et al., 1998b; Routledge and Sumpter, 1996; Shore and Shemesh, 2003; Soto et al., 1991).

Few studies have investigated hormonal activities in the environment other than for oestrogens, but both androgenic (Chatterjee et al., 2007; Ellis et al., 2003; Katsiadaki et al., 2002a; Parks et al.,

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2001; Svenson and Allard, 2004a; Thomas et al., 2002) and anti-androgenic (I. Johnson et al., 2007; Tollefsen et al., 2007; Urbatzka et al., 2007) activities have been identified. Androgenic activity occurs in both pulp and paper mill effluents and cattle feedlot effluents (in the US) and has been shown to result in masculinisation of wild fish in the receiving waters (Adams et al., 1992; Ankley et al., 2003; Ellis et al., 2003; Jensen et al., 2006; Karels et al., 2001; Orlando et al., 2007; Parks et al., 2001; Soto et al., 2004; Wilson et al., 2002). The androgenic chemical in cattle feedlots was identified as trenbolone (a growth promoter used in beef production, Durhan et al., 2006; Schiffer et al., 2001) whilst the androgenic activity of pulp and paper mills appears to result from wood derived-products (Svenson and Allard, 2004b). Androstenedione has been reported to contribute to the androgenic activity of pulp and paper mills (Allinson et al., 2008; Jenkins et al., 2001) – although this finding remains controversial (Bandelj et al., 2006; Ellis et al., 2003; Hewitt et al., 2006). A report by I. Johnson et al. (2007) showed significant anti-androgenic activity in 41 out of 43 UK STW effluents tested, with potency ranging between 21.3 and 1231 $\mu g\,l^{-1}$ flutamide equivalent (FLeq, as determined in the recombinant yeast androgen screen – rYAS), and in a modelling study, this activity was shown to be statistically associated with some of the feminised responses seen in wild roach living in those rivers (Jobling et al., 2010). Some of these anti-androgens have recently been identified and they include dichlorophene and di(chloromethyl)-anthracene (Hill et al., 2010), although these two chemicals accounted for only a small fraction of the total anti-androgenic activity as determined in the rYAS. *In vitro* anti-androgenic activity (as determined in the rYAS) has also been shown to occur in the produced water of oil production platforms in Norway, at concentrations ranging between 20 and 8000 μg FLeq l^{-1} (Tollefsen et al., 2007), and in the River Lambro, North Italy, at levels between 370 and 4223 μg FLeq l^{-1} (Urbatzka et al., 2007). A study by Thomas et al. (2009) reported that the anti-androgenic fraction in the produced water from offshore oil production platforms in the North Sea was comprised mainly of naphthenic acids and polycyclic aromatic hydrocarbons (PAHs). Studies on effluents discharged in the River Lambro have identified bisphenol A, iprodione, nonylphenol, *p,p′*-DDE and 4-*tert*-octylphenol, chemicals all known to have weak anti-androgenic activity, but the vast majority of the anti-androgenic activity in those effluents has not been chemically ascribed (Urbatzka et al., 2007). Further studies have shown that water extracts from the River Lambro induced feminisation and demasculination in the South African clawed toads *Xenopus laevis* (gonad and aromatase P450 endpoints; Cevasco et al., 2008; Massari et al., 2010) while an early study reported intersex barbel *Barbus plebejus* derived from this river (Viganò et al., 2001). Such effects likely reflect responses to the mixture of oestrogens and anti-androgens present in this river (Urbatzka et al., 2007; Viganò et al., 2008). In studies on mosquitofish (*Gambusia affinis holbrooki*) populations in Lake Apopka (USA), males have been found with shorter gonopodia and reduced sperm counts when compared with fish from reference lakes and contaminants with anti-androgenic potential in this lake have been implicated in causing these effects, notably *p,p′*-DDE (Toft et al., 2003).

Assessing for anti-androgenic effects in fish requires specific biomarkers. Many fish species have androgen dependent sex traits (such as secondary sexual characters), although most of these can also be affected (inhibited) by exposure to oestrogens (e.g. Ankley et al., 2010). Sex hormone dependent behaviours have been used for assessing the impacts of individual EDCs (Jones and Reynolds, 1997; Kane et al., 2005; Scott and Sloman, 2004). Examples of this include a study on mosquitofish where exposure of male fish to a paper mill effluent in Florida resulted in greater aggressiveness (an androgen dependent behaviour) in their courtship display (Howell et al., 1980). Contrasting with that study, Bortone et al. (1989) found no difference in behaviour of mosquitofish exposed to a kraft mill effluent, even though females showed morphological signs of masculinisation. The same observation (morphological changes but no effects on behaviour changes) were reported for mosquitofish living in pollutant contaminated lakes in Florida (Toft et al., 2003). In a study with fathead minnows (*Pimephales promelas*), males exposed to an oestrogenic STW effluent for three weeks failed to compete for nesting sites and females against control males, but they spawned successfully when no competition was involved (Martinović et al., 2007).

The three-spined stickleback (*Gasterosteus aculeatus*) offers a number of useful features for studying the effects of (anti-) androgenic EDCs. The first of these is the production of a glue protein, spiggin, that is specifically dependent on androgens (Björkblom et al., 2009; Hahlbeck et al., 2004; Jolly et al., 2006; Katsiadaki et al., 2002a,b; Sanchez et al., 2008) and inhibited by anti-androgens (Jolly et al., 2006; Katsiadaki et al., 2006; Sebire et al., 2008, 2009). In addition to spiggin production, the reproductive behaviour in male sticklebacks is well described and under androgen control (Borg and Mayer, 1995; Mayer et al., 2004; Sebire et al., 2007; Wai and Hoar, 1963). A series of previous studies have successfully employed reproductive behaviour of male sticklebacks to assess effects of thyroid disrupting chemicals (Bernhardt and von Hippel, 2008), oestrogens (Bell, 2001; Brian et al., 2006; Wibe et al., 2002) and anti-androgens (Sebire et al., 2008, 2009). In the two latter studies, exposure to the model anti-androgen, flutamide, and to the anti-androgenic pesticide, fenitrothion, resulted in reduced nest building activity and reduced male courtship behaviour towards the female. In this study we investigated the effect of exposure to an anti-androgenic (and weakly oestrogenic) treated sewage effluent on the reproductive physiology and behaviour of sticklebacks.

2. Materials and methods

2.1. Animals

The parents of the experimental broods were originally obtained from a wild population from a site in Dorset (Winterbourne Houghton). We regularly use parents from this site as the water supply is of high environmental quality with very low levels of metals, PAHs and no endocrine activity. Two broods were used for this study (brood 1 and brood 2) and were raised to adulthood in the laboratory under a photoperiod of 12L:12D and a temperature of $16 \pm 2\,^{\circ}C$. For the breeding test employed in this study, the separation of adult fish into males and females is vital as the holding conditions prior to the test are different for the sexes. At the start of the test the females need to be gravid and ready to spawn, while the males need to be in a non-breeding condition. Since sticklebacks lack sexual dimorphism outside the breeding season, the sex was determined using a molecular sex probe.

Genetically identified males from brood 1 were moved to a holding tank, under winter conditions to avoid the onset of breeding (8L:16D and $10 \pm 2\,^{\circ}C$) and kept for 4 months prior to the exposure. The females, originating from brood 2 – in order to avoid sibling effects on reproductive behaviour (Frommen and Bakker, 2006), were kept at $16 \pm 2\,^{\circ}C$ under a summer photoperiod of 16L:8D hours during the same period. The fish were fed daily with frozen bloodworm and any remaining debris was removed from the tanks. Only fish weighing $\geq$0.8 g were used in the experiment.

2.2. Sex determination of the fish

When the fish were six months old, and before the development of any external secondary sexual characters, the sex of the fish was identified by the use of sex-linked DNA-markers (Peichel