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Environmental chemicals active as human antiandrogens do not activate a stickleback androgen receptor but enhance a feminising effect of oestrogen in roach

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ABSTRACT

Sexual disruption is reported in wild fish populations living in freshwaters receiving discharges of wastewater treatment works (WwTW) effluents and is associated primarily with the feminisation of males by exposure to oestrogenic chemicals. Antiandrogens could also contribute to the feminisation of male fish, but there are far less data supporting this hypothesis and almost nothing is known for the effects of oestrogens in combination with antiandrogens in fish. We conducted a series of *in vivo* exposures in two fish species to investigate the potency on reproductive-relevant endpoints of the antiandrogenic antimicrobials triclosan (TCS), chlorophene (CP) and dichlorophene (DCP) and the resin, abietic acid (AbA), all found widely in WwTW effluents. We also undertook exposures with a mixture of antiandrogens and a mixture of antiandrogens in combination with the oestrogen 17α-ethinyloestradiol (EE2). In stickleback (*Gasterosteus aculeatus*), DCP showed a tendency to reduce spiggin induction in females androgenised by dihydrotestosterone (DHT), but these findings were not conclusive. In roach (*Rutilus rutilus*), exposures to DCP (178 days), or a mixture of TCS, CP and AbA (185 days), or to the model antiandrogen flutamide (FL, 178 days) had no effect on gonadal sex ratio or on the development of the reproductive ducts. Exposure to EE2 (1.5 ng/L, 185 days) induced feminisation of the ducts in 17% of the males and in the mixture of antiandrogens (TCS, CP, AbA) in combination with EE2, almost all (96%) of the males had a feminised reproductive ducts. In stickleback androgen receptor (ARα and ARβ) transactivation assays, the model antiandrogens, FL and procymidone inhibited 11-ketotestosterone (11-KT) induced receptor activation, but none of the human antiandrogens, TCS, CP, DCP and AbA had an effect. These data indicate that antimicrobial antiandrogens in combination can contribute to the feminisation process in exposed males, but they do not appear to act through the androgen receptor in fish.

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Introduction

Endocrine disrupting compounds (EDCs) derive from (primarily) anthropogenic, industrial, agricultural and domestic sources and they have the capacity to interfere with reproductive development and function in a wide range of species. Wildlife associated with freshwater ecosystems is especially at risk of EDC exposure as aquatic environments act as a repository for a wide range of chemical pollutants. Many of these chemicals are discharged *via* effluents from wastewater treatment works (WwTW), and globally exposure to WwTW effluents has been associated with a variety of deleterious effects on reproduction in fish (Jobling et al., 1998; Gravato and Santos, 2003; Mills and Chichester, 2005; Pottinger et al., 2013a; Blazer et al., 2014). To date, most of the focus on EDCs has been on oestrogens and there are now proven links between estrogen

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exposure and a range of feminisation responses in fish. These responses include elevated concentrations of the female egg-yolk precursor vitellogenin (VTG) in males and immature females, development of a female-like ovarian cavity in the testis of males, and intersex characterised by the presence of both male and female sex cells contained within the same gonad. These feminising effects have been linked to reduced gamete quality and there is concern about population level effects (Kidd et al., 2007; Harris et al., 2011; Lange et al., 2011).

Many of the feminised effects seen in wild populations can be induced by controlled exposure to oestrogens and their mixtures. However, in the last decade, antiandrogens have emerged as another class of EDCs that potentially contribute to adverse health effects in human and wildlife. Antiandrogens may cause effects through a variety of different mechanisms, including *via* acting as androgen receptor (AR) antagonists, thus, inhibiting AR-dependent gene expression, or by altering the biosynthesis and/or excretion of natural hormones (Wilson et al., 2008). There is evidence that exposure of rodents to antiandrogens during critical life periods that include sexual differentiation, foetal life and maturation, can have effects on male development (Hotchkiss et al., 2008; Christiansen et al., 2009; Rider et al., 2010). Similarly for fish, there is evidence derived from laboratory-based *in vivo* exposures that some antiandrogens can suppress the effects of androgens in males, thus, contributing to demasculinising/feminising effects. Reported effects include induction of intersexuality in male medaka (*Oryzias latipes*) and ovarian atresia in female medaka, reduced sperm count and reduced secondary sex characteristics in male fathead minnow (*Pimephales promelas*) and male guppy (*Poecilia reticulata*), altered reproductive behaviours in male stickleback (*Gasterosteus aculeatus*) and guppy, and reduced spiggin (an androgen-dependent protein used for nest construction) production in male stickleback (Makynen et al., 2000; Baatrup and Junge, 2001; Bayley et al., 2002; Kinnberg and Toft, 2003; Kiparissis et al., 2003; Jensen et al., 2004; Kang et al., 2006; Martinović et al., 2008; Sebire et al., 2008, 2009). These effects are predominantly derived for exposures to the model antiandrogen flutamide (FL) and to other antiandrogens at concentrations that far exceed those measured in aquatic systems, albeit there is evidence for some effects of antiandrogens in fish for environmentally relevant exposures (*e.g.* Sebire et al., 2009; Sebire et al., 2011; Green et al., 2015).

Globally, antiandrogenic activities have now been detected in effluents, surface waters and sediments using *in vitro* based receptor AR assays, such as AR transactivation assays or yeast-based transcriptional activation assays (Tollefsen et al., 2007; Urbatzka et al., 2007; Shi et al., 2009; Hill et al., 2010; Rostkowski et al., 2011; Zhao et al., 2011; Bellet et al., 2012; Alvarez-Muñoz et al., 2015). In an extensive survey of WwTW effluents in the UK, significant antiandrogenic activity was identified (between 21.3 and 1231 μg flutamide equivalents L^{-1}) in all samples investigated (Environment Agency, 2007). Furthermore, a modelling study has correlated feminised fish in UK rivers with predicted antiandrogen content both alone and in combination with oestrogens (Jobling et al., 2009). Compounds known to be antiandrogenic include some pesticides (*e.g.* procymidone, vinclozolin, linuron), pharmaceuticals (*e.g.* FL, cyproterone acetate), and some industrial chemicals such as phthalates or polybrominated diphenyl ethers. Our recent studies, however, indicate that these compounds may not be significant contributors to bioavailable antiandrogens in fish living in UK rivers. Using a bioassay-directed analytical approach, we have identified the antimicrobials triclosan (TCS), chlorophene (CP) and dichlorophene (DCP), ingredients in a variety of household and personal care products, together with resin acids, naturally occurring components of wood and bark, as among the antiandrogens in WwTW effluents that bioconcentrate in fish bile at concentrations tens of thousands greater than in the effluent itself (Rostkowski et al., 2011). Due to their occurrence in WwTW effluents and their ability to bioconcentrate, these compounds are considered to be bioavailable to fish. The antimicrobials are present in effluents at ng to low μg/L concentrations and for resin acids from low ng up to mg/L concentrations. All these compounds have been shown to possess similar to higher antiandrogenic potencies *in vitro* on the human AR when compared with the standard antiandrogenic compound FL (Rostkowski et al., 2011).

The aim of this study was to investigate the potency on reproductive-relevant endpoints in fish of some of the antiandrogenic antimicrobials (DCP, CP, TCS) and resin acids present in WwTW effluents, including as mixtures, and in combination with the oestrogen 17α-ethinyloestradiol (EE2). This was done principally through a series of *in vivo* experiments in which fish were exposed to antiandrogens at environmental concentrations not exceeding the maximum antiandrogenic activity identified for WwTW effluents in the UK. In the first study, the ability of DCP to inhibit spiggin induction (a well-established and sensitive biomarker for (anti) androgens) was assessed in female sticklebacks androgenised by exposure to dihydrotestosterone (DHT). Two further experiments investigated the effects of DCP, the model antiandrogen FL, a mixture of TCS, CP and abietic acid (AbA) or of a mixture of antiandrogens (TCS, CP, AbA) in combination with the environmental oestrogen, EE2 on reproductive development in roach (*Rutilus rutilus*), a species that has received some of the most extensive work for understanding the feminising effects of environmental oestrogens. Finally, stickleback AR transactivation assays were applied to support a mechanistic understanding for the effects of the antimicrobial antiandrogens seen in the *in vivo* studies.

Material and Methods

2.1. Fish husbandry and chemical origin

Mixed sex populations of three-spined stickleback were obtained from Priory Fisheries (Cullompton, UK) in November 2008 and maintained in the laboratory under constant water temperature (10–12 °C) and photoperiod (12L:12D) for four months prior to the start of the experiment. The fish were fed daily with frozen gamma-irradiated bloodworm (Tropical Marine Centre, Chorleywood, UK).

Pre-spawning, sexually mature male and female roach were obtained from the Environment Agency's National Coarse Fish Farm (Calverton, Nottinghamshire, UK) in May 2009 and brought to the laboratory. Fish were separated by sex and maintained at 15–16 °C and a photoperiod matching the day length at the time of sampling (16L:8D). Spawning was induced by a single intraperitoneal injection of carp pituitary extract (CPE, Calverton Fish Farm) dissolved in physiological saline, using an established method for inducing spawning of adult fish and ensures synchronous gamete collection (Jobling et al., 2002). 24 h after the injection with CPE, fish were dry stripped of their gametes and eggs fertilised *in vitro*.

Chlorophene (CP, 95% purity), dichlorophene (DCP, 97.5%), 17α-ethinyloestradiol (EE2, »98%), dihydrotestosterone (DHT, ≥97.5%), flutamide (FL, ≥99%), triclosan (TCS, ≥97%), testosterone (T, ≥98%), 11-ketotestosterone (11-KT, ≥98%), bicalutamide (≥98%), bis(2-hydroxyphenyl) methane (98%), oestrone (99%), progesterone (≥99%) and 4-(4-chlorophenoxy) phenol (97%) were obtained from Sigma–Aldrich (Gillingham, UK). Abietic acid (85%) was obtained from Acros Organics (Geel, Belgium) and procymidone (PROCY, >98%) from Fluka. Stock solutions of chemicals were prepared in HPLC grade acetone or ethanol (both Fisher Scientific UK, Loughborough, UK).

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