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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.

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).

Table 1
Average measured chemical concentrations in exposure tanks.

Treatment (nominal exposure concentration; μg/L)	Mean measured exposure concentrations (μg/L) ± SEM (*n*)						
	DHT	FL	DCP	AbA	CP	TCS	EE2
Exposure of androgenised female stickleback to DCP							
DWC	≤LOD	≤LOD	≤LOD	n.a.	n.a.	n.a.	n.a.
SC	≤LOD	≤LOD	≤LOD	n.a.	n.a.	n.a.	n.a.
DHT (5)	4.20 ± 0.12 (8)	≤LOD	≤LOD	n.a.	n.a.	n.a.	n.a.
DHT (5) + FL (150)	3.70 ± 0.21 (8)	132.44 ± 2.72 (8)	≤LOD	n.a.	n.a.	n.a.	n.a.
DHT (5) + DCP (0.1)	3.58 ± 0.26 (8)	≤LOD	0.041 ± 0.010 (8)	n.a.	n.a.	n.a.	n.a.
DHT (5) + DCP (1)	3.73 ± 0.22 (10)	≤LOD	0.570 ± 0.060 (8)	n.a.	n.a.	n.a.	n.a.
DHT (5) + DCP (10)	4.05 ± 0.14 (8)	≤LOD	7.020 ± 0.736 (9)	n.a.	n.a.	n.a.	n.a.
Exposure of roach to DCP or FL							
DWC	n.a.	≤LOD	≤LOD	n.a.	n.a.	n.a.	n.a.
SC	n.a.	≤LOD	≤LOD	n.a.	n.a.	n.a.	n.a.
DCP (1)	n.a.	≤LOD	0.36 ± 0.05 (16)	n.a.	n.a.	n.a.	n.a.
DCP (10)	n.a.	≤LOD	3.52 ± 0.88 (16)	n.a.	n.a.	n.a.	n.a.
DCP (30)	n.a.	≤LOD	10.39 ± 2.60 (16)	n.a.	n.a.	n.a.	n.a.
FL (150)	n.a.	136.79 ± 5.77 (16)	≤LOD	n.a.	n.a.	n.a.	n.a.
FL (450)	n.a.	355.14 ± 21.88 (16)	≤LOD	n.a.	n.a.	n.a.	n.a.
Exposure of roach to a mixture of antiandrogens (AAmix) and/or EE2							
DWC	n.a.	n.a.	n.a.	≤LOD	≤LOD	≤LOD	≤LOD
AAmix (50 each)	n.a.	n.a.	n.a.	6.40 ± 0.79 (20)	23.86 ± 1.25 (20)	7.11 ± 1.05 (20)	≤LOD
3 ng/L EE2 (3)	n.a.	n.a.	n.a.	≤LOD	≤LOD	≤LOD	(1.60 ± 0.60) x 10^{-3} (2)
AAmix (50 each) + EE2 (3)	n.a.	n.a.	n.a.	3.87 ± 0.67 (19)	23.18 ± 2.06 (19)	9.10 ± 1.87 (19)	(1.29 ± 0.05) x 10^{-3} (11)

n.a.: not applicable; limit of detection (LOD): 0.12 ng DCP/L; 0.21 ng FL/L; 0.21 ng DHT/L;0.50 ng EE2/L; 0.15 ng CP/L, 0.12 ng TCS/L, 0.22 ng AbA/L. DWC, dilution water control; SC, solvent control; AAmix, Aba + CP + TCS (50 μg/L each).

2.2. Stickleback exposure to DCP

In the stickleback experiment, females were exposed for 21 days, based on the OECD Guidance document 148 for the androgenised female stickleback screen (AFSS) (OECD, 2011). The exposure consisted of seven treatments each in duplicate with each tank containing 10 fish in a volume of 10 L. Fish were maintained in a flow-through system with incoming water at ambient temperature (15 ± 0.5 °C) and a 12L:12LD photoperiod throughout. There was a daily exchange of two tank volumes of water and the chemical dosing solutions were delivered to the tanks using peristaltic pumps.

Fish were simultaneously exposed to DHT (nominal 5 μg/L) and one of three concentrations of DCP (nominal 0.1, 1.0 or 10 μg/L) or the antiandrogen-positive control FL (nominal 150 μg/L). The experiment included a dilution water control (DWC), a solvent control (SC) and an androgen-positive control (DHT alone). Chemical stock solutions were prepared in ethanol and further diluted with dilution water before dosing into the exposure tanks. The final solvent exposure concentration was 0.0003%. The dosing solutions were renewed every 2 days and the flow rates monitored at the same time. Water samples were taken for chemical analysis from each tank on a weekly basis. Fish were fed daily with frozen bloodworm.

2.3. Roach exposures

Two exposures were conducted with roach. Three days post fertilisation (dpf), fertilised eggs were deployed into glass aquaria and exposed under flow-through conditions, in duplicate tanks, as described below. Embryos hatched 7–10 dpf and the fish were provisioned with dietary requirements according to their age (Paull et al., 2008). Briefly, roach were fed with Cyprico Crumble EX dry food (Coppens International bv, Helmond, The Netherlands) and at all life stages, the diet was supplemented initially with freshly hatched brine shrimp (*Artemia* sp.) nauplii, given to satiation and, as the fish grew beyond 80 day post hatch (dph) with frozen gamma-irradiated brine shrimp and bloodworm (*Chironomus* sp.; Tropical Marine Centre, Chorleywood, UK) and TetraMin dry coldwater flake food (Tetra Werke, Melle, Germany). Fish were maintained at ambient water temperatures (18 ± 2 °C May to October, 13 ± 2 °C October to December, 8 ± 2 °C for experiment 1 and 21 ± 2 °C May–October and 17 ± 1 °C October–November for experiment 2). The photoperiod regime was adjusted to stimulate ambient seasonal changes (16L:8D between May and August after which it was reduced in a stepwise manner to 12L:12D). The chemical dosing solutions were delivered to the tanks using peristaltic pumps and the dosing solutions were renewed every 3–4 days and the flow rates monitored at these times. Water samples for chemical analysis were taken regularly from each tank for analysis (once or twice per month).

In experiment 1, approximately 400 eggs were placed into each tank and exposed continuously to one of three concentrations of DCP (nominal 1, 10 or 30 μg/L) or one of two concentrations of FL (nominal 150 or 450 μg/L) until 172 days post hatch (dph). Dilution water and solvent-dosed tanks were included as controls. Initially, fertilised eggs were deployed into 20 L of water and the water volume was subsequently increased to 45 L at 30 dph, 70 L at 80 dph and 145 L at 145 dph. At 32 and 136 dph, stocking densities in all tanks were adjusted to ensure the fish biomass was equivalent over the study period. From 136 dph onwards, there were 100 fish per tank ensuring sufficient numbers for biological sampling. At 172 dph, fish were sampled from each tank for biological analyses. Throughout the exposure, there were two tank volume exchanges of water daily. Stock solutions of DCP and FL were prepared in ethanol, diluted in dilution water before supplied to the exposure tanks. The solvent dosing to all the tanks was less than 0.00075% (v/v).

In experiment 2, initially, approximately 200 eggs were placed into each tank exposed continuously to EE2 (at nominal 3 ng/L), a mixture of the three antiandrogens AbA, CP or TCS (each at nominal 50 μg/L), or a mixture of the three antiandrogens (each at nominal 50 μg/L) in combination with EE2 (at nominal 3 ng/L). Fish were maintained in 50 L of water throughout and there was one tank volume exchange of water daily. At 55 dph, stocking densities in all tanks were adjusted to 100 fish per tank and at 185 dph, fish were sampled from each tank for biological analyses. In this experiment, a solvent-free approach was adopted for which chemical stock solutions were prepared in acetone. Solvent-free dosing stocks were prepared every 3–4 days by adding the appropriate volume (<0.5 mL) of acetone stock into glass vessels. The acetone was allowed to evaporate overnight and subsequently, the required volume of water was added before the solution was stirred for 2 h followed by 30 min sonication before connecting into the flow-through system. Controls were maintained in dilution water. The measured chemical concentrations for each of these studies are given in Table 1. At 29 dph, one control tank was lost due to mortalities and replaced using fish from the replicate tank.

2.4. Analytical chemistry

Measured exposure concentrations were determined in water samples from every tank using gas chromatography–mass spectrometry (GC/MS). During each experiment, water samples were collected from the experimental tanks into solvent-cleaned glass flasks to determine the actual exposure concentrations. Immediately after collection, methanol and acetic acid (final concentrations 4 and 1%, respectively) were added to each sample which was also spiked with an equal amount of internal standard (IS) as expected for the test compound in the extracted volume. Internal standards included bicalutamide (for the determination of FL), oestrone (for the determination of EE2 and AbA), progesterone (for the determination of DHT), 4-(4-chlorophenoxy)-phenol (for the determination of TCS and CP) and bis(2-hydroxyphenyl) methane (for the determination of DCP). Water samples were extracted using preconditioned Oasis HLB cartridges (Waters Corporation, Milford, MA, USA), which were then rinsed with water, dried under vacuum and stored at −80 °C until analysis. Prior to analysis, cartridges were defrosted and extracts eluted with methanol. The solvent was removed under vacuum and the sample extracts silylated by the addition of 30 μL bis(trimethylsilyl) trifluoroacetamide (BSTFA) and 30 μL pyridine (65 °C for 30 min). The sample was dried down under nitrogen, re-dissolved in 20 μL BSTFA and 2 μL injected into the GC–MS. Samples were analysed on a Trace GC (Thermoquest, Texas, USA) fitted with a 30 m Zebron ZB-5MS fused silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness) connected to a Polaris-Q ion trap mass spectrometer (Thermo, Texas, USA). The MS was operated in 50–650 *m*/*z* full scan positive ionisation mode with electron ionisation at 70 eV. The *m*/*z* ions with maximum intensity were used as quantifier ions and a calibration curve was used to calculate the absolute amount of each analyte in the sample extract in comparison with the IS response.

2.5. Fish sampling and biological analyses

All fish were sacrificed humanely by terminal anaesthesia with benzocaine followed by cervical dislocation as approved by the UK Home Office (Animals (Scientific Procedures) Act 1986). Total length (stickleback) or standard length (roach) and wet weight were recorded to the nearest 1 mm and 0.01 g, respectively. For sticklebacks (n = 14–20 per treatment), kidneys were dissected out, weighed and stored at −20 °C until the measurement of spiggin

using an ELISA, as described by Katsiadaki et al. (2002). Roach ($n = 15$ from each tank in experiment 1 and $n = 25$ from each tank in experiment 2) were preserved *in toto* in 4% paraformaldehyde for histological analysis of their gonads, embedded in paraffin wax, sectioned to 5–10 μm and stained with hematoxylin and eosin. The sections were analysed for germ cells and gonadal duct formation, according to Paull et al. (2008).

2.6. In vitro studies

To support interpretation of the biological effects data for the *in vivo* exposures, we investigated the activities of the antimicrobial antiandrogens, DCP, TCS, CP, and AbA, and FL in stickleback AR transactivation assays.

HepG2 cells were seeded in 24-well plates at 5×10^4 cells per well in phenol-red free Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% charcoal/dextran treated fetal bovine serum. After 24 h, the cells were transfected with 200 ng of ARα or ARβ, 400 ng of the reporter construct MMTV-Luc and 100 ng of pRL-TK using Fugene HD transfection reagent (Promega). Five hours after transfection, cells were dosed with AR agonists alone, or AR agonists and AR antagonists in combination and incubated for 44 h, when the cells were collected, and the luciferase activity measured using the Dual-Luciferase Reporter Assay System (Promega). Promoter activity was calculated as firefly-/sea pansy-luciferase activities. All chemicals tested were dissolved and diluted in DMSO. The final solvent concentration was 0.1% DMSO for single chemical treatment and 0.2% for simultaneous agonist and antagonist exposure. Full details of the methodology are provided in the Supplementary material.

2.7. Data analyses

Unless stated otherwise, the data are presented as means ± SEM and a probability level of $p < 0.05$ was considered to be statistically significant. General linear models (GLM) were used to analyse for effects of treatment on growth of fish where tank was included as a random factor in the model (nested within treatment) in order to adjust for pseudo-replication occurring due to multiple measurements being taken within one tank. Any significant differences were determined by Tukey's HSD post hoc tests. Although tank effects are accounted for in the full statistical model, a paired *t*-test was also applied if tank effects were found to establish which treatments were responsible for the tank effects. The same analysis was applied to test for effects of DCP on spiggin levels in the stickleback experiment. Estimated marginal means, which are a function of the model parameters and are adjusted for the factors in the model, were calculated and plotted for each group. A paired *t*-test was used to test for differences measured chemical concentrations between replicate tanks. To analyse for relationships between treatment and sex in the roach experiments, chi-square tests were applied. Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 21.0.0

For the *in vitro* experiments, all transfections were performed in triplicate and the assays repeated at three times. Data are presented as mean ± SEM from three separate experiments. Dose–response data using a four-parametric curve fitting, EC_{50} (for agonists) and IC_{50} (for antagonists) were analysed using GraphPad Prism (Graph Pad Software Inc., San Diego, CA, USA). Relative potencies of the agonists were established by comparing their EC_{50} with the EC_{50} of 11-KT and potencies of the antagonists were calculated relative to FL.

Results

3.1. Water chemistry

The mean measured exposure concentrations for the different experiments are shown in Table 1 and the average concentrations for each replicate tank are provided in Supplementary material (Tables S1–3). Recoveries were variable for the individual compounds. For the stickleback exposure, actual measured tank DHT concentrations across the different tanks were between 3.58 ± 0.26 and 4.20 ± 0.12 μg/L (nominal 5 μg/L), and for FL, 132.44 ± 2.72 μg/L (nominal 150 μg/L). For DCP, measured concentrations were 0.041 ± 0.010 μg/L (nominal 0.1 μg/L), 0.570 ± 0.060 μg/L (nominal 1.0 μg/L) and 7.02 ± 0.736 μg/L (nominal 10 μg/L). Measured DCP concentrations differed significantly between the replicate tanks for the medium (*t*-test: $p = 0.028$) and high (*t*-test: $p = 0.016$) DCP treatment groups and for DHT concentrations between the two replicate tanks in the high DCP treatment groups (*t*-test: $p = 0.02$). All other measured chemical concentrations did not differ significantly between the two replicate tanks (*t*-test: $0.102 < p < 0.696$; see Supplementary data).

For the first roach exposure, the measured DCP concentrations were 0.36 ± 0.05 μg DCP/L (nominal 1.0 μg/L), 3.52 ± 0.88 μg DCP/L (nominal 10 μg/L), 10.39 ± 2.60 μg DCP/L (nominal 30 μg/L), and for FL, 136.79 ± 5.77 μg FL/L (nominal 150 μg/L) and 355.14 ± 21.88 μg FL/L (nominal 450 μg/L). There were no statistically significant differences in chemical concentrations between the two replicate tanks (*t*-test: $0.135 < p < 0.978$; see Supplementary data).

In the second roach exposure (mixture experiment), the acetone stocks used for dosing and the stocks of internal standards were analysed and concentrations confirmed as 98% for TCS, 103% for EE2, 108% for AbA, 114% for CP, and for the internal standards as 123% (4-(4-chlorophenoxy) phenol) and 90% (oestrone) of nominal concentrations. In the exposure tanks, recoveries were much lower. The actual measured tank concentrations were between 3.87 ± 0.67 and 6.40 ± 0.792 μg/L for AbA (nominal 50 μg/L), between 23.18 ± 2.06 and 23.86 ± 1.25 μg/L for CP (nominal 50 μg/L), between 7.11 ± 1.05 and 9.10 ± .87 μg/L for TCS (nominal 50 μg/L) and between 1.29 ± 0.05 and 1.60 ± 0.60 ng/L for EE2 (nominal 3 ng/L). Measured CP concentrations differed significantly between the both replicate tanks for both, the mixture of antiandrogens treatment (*t*-test: $p = 0.031$) and the mixture of antiandrogens co-administered with EE2 (*t*-test: $p < 0.001$). All other measured chemical concentrations did not differ significantly between the two replicate tanks (*t*-test: $0.078 < p < 0.896$; see Supplementary; Tables S1–S3).

3.2. Effects of DCP on spiggin production in mature, androgenised female sticklebacks

In total, 125 fish were analysed (14–20 per treatment). The mean length, mass and condition factor of the fish were 4.81 ± 0.04 cm, 1.23 ± 0.03 g and 1.09 ± 0.01, respectively. There were no statistically significant differences between treatment groups for length ($F_{6,7.041} = 0.700$, $p = 0.660$), weight ($F_{6,7.276} = 0.688$, $p = 0.667$) and condition factor ($F_{6,7.008} = 0.573$, $p = 0.743$) (see Supplementary material; Fig. S1). With the exception for fish exposed to 10 μg DCP/L (co-administered with DHT), measured spiggin levels did not differ significantly in fish sampled between the replicate tanks ($0.027 < p < 0.948$; see Supplementary material; Fig. S2). Overall, treatment had significant effects on spiggin levels (ANOVA $F_{6,7.047} = 36.721$, $p < 0.0001$). *Post-hoc* comparisons using the Tukey's HSD test showed no differences in spiggin levels between water control and solvent control. In contrast, DHT exposure significantly induced spiggin levels compared with both

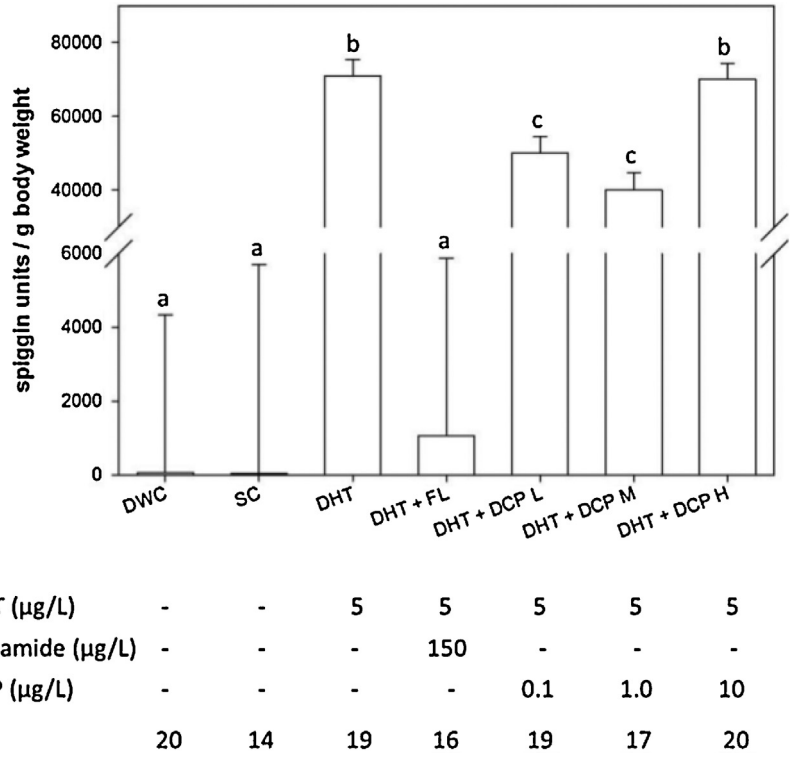

Fig. 1. Spiggin units/g body weight in androgenised female three-spined stickleback exposed to DCP. Fish were simultaneously exposed to DHT (nominal 5 μg/L) and one of three concentrations of DCP (nominal 0.1, 1.0 or 10 μg/L) or the antiandrogen-positive control FL (nominal 150 μg/L). The experiment included a dilution water control (DWC), a solvent control (SC) and an androgen-positive control (DHT alone). Data are presented as estimated marginal means ± SEM (n = 14–20 fish for each treatment). Statistically significant differences between experimental groups are denoted by different letters ($p < 0.05$; GLM followed by Tukey's HSD test). DWC, dilution water control; SC, solvent control; DCP L, 0.1 μg DCP/L; DCP M, 1.0 μg DCP/L; DCP H, 10 μg DCP/L.

controls as expected. Co-administration of FL with DHT significantly reduced spiggin levels compared with exposure to DHT alone as expected. Spiggin levels in the low (0.1 μg/L) and medium (1.0 μg/L) DCP treatment groups (co-administered with DHT) were significantly lower compared with the DHT group, but there were no differences for the high (10 μg/L) DCP treatment group (Fig. 1). There were no tank effects on spiggin levels or on growth, but there was a tank effect on condition factor (ANOVA $F_{7,111} = 8.353$, $p < 0.0001$) (see Supplementary material; Table S4). Although tank effects are accounted for in the full statistical model, a paired t-test was also applied to test for differences between the replicate tanks if tank effects were seen. The observed tank effects for condition factor were driven by only two treatments whereas there were no tank effects for the remaining five treatments ($0.234 < p < 0.611$). The tank effects were accounted for in the model and do not mask the effects of treatment on condition factor.

3.3. *In vivo potency of FL and DCP on gonadal sexual differentiation of roach*

The mean length, mass and condition factor of male and female fish were 4.15 ± 0.04 cm/0.76 ± 0.02 g/1.05 ± 0.01 and 4.17 ± 0.04 cm/0.77 ± 0.03 g/1.05 ± 0.01, respectively. There were no statistically significant differences between treatment groups for length, weight and condition factor (see Supplementary material; Fig. S3). There were no tank related effects on growth, but there was a tank effect for condition factor in female roach (ANOVA $F_{4,35} = 5.631$, $p = 0.001$) (see Supplementary material; Table S4). At 172 dph, there were no effects of the highest exposure concentrations for DCP (30 μg/L) or FL (450 μg/L) on the sex ratio of exposed roach or on development of the reproductive duct, as determined by gonad histopathology. In the DWC, 57% and 43% of the fish could be assigned as males and females, respectively, and in the SC, 73% and 27% as males and females, respectively ($n = 30$ fish analysed from each treatment). In the FL exposure (nominal 450 μg/L), 70% of the fish were males and 30% females ($n = 30$). In the exposure to DCP (nominal 30 μg/L), 45% were males and 55% females ($n = 29$). No relationship was found between treatment and the sex of fish (χ^2 (3, $n = 119$) = 3.278 ($p = 0.351$)). No differences were observed for duct formation in any of the treatments (χ^2 (3, $n = 114$) = 5.487 ($p = 0.139$)); duct development was unclear for 5 histological samples due to technical problems with tissue processing.

3.4. *Effects of antiandrogens alone and in combination with EE2 on development of the reproductive duct in roach*

The mean length, mass and condition factor of male and female fish were 3.93 ± 0.06 cm/0.92 ± 0.04 g/1.42 ± 0.02 and 3.85 ± 0.07 cm/0.87 ± 0.04 g/1.42 ± 0.02, respectively. There were no statistically significant differences between treatment groups for length, weight and condition factor (see Supplementary material; Fig. S4). There were no tank effects on morphometric endpoints measured in male roach, but there were tank effects for growth (length (ANOVA $F_{4,78} = 3.316$, $p < 0.05$)), weight (ANOVA $F_{4,78} = 4.722$, $p < 0.01$) and condition factor ($F_{4,78} = 6.396$, $p < 0.001$) in female roach (see Supplementary material; Table S4). Feminising effects of treatment on reproductive ducts were analysed for male fish (Fig. 2). There was a significant relationship between treatment and feminised ducts in male roach (χ^2 (6, $n = 109$) = 88.345 ($p < 0.001$)), *i.e.* feminisation of reproductive ducts in male roach was associated with treatment. A significant difference between replicate tanks was observed in the development of reproductive ducts in control males only (χ^2 (1, $n = 38$) = 4.479 ($p < 0.05$)) with no significant differences between replicate tanks for the other treatments

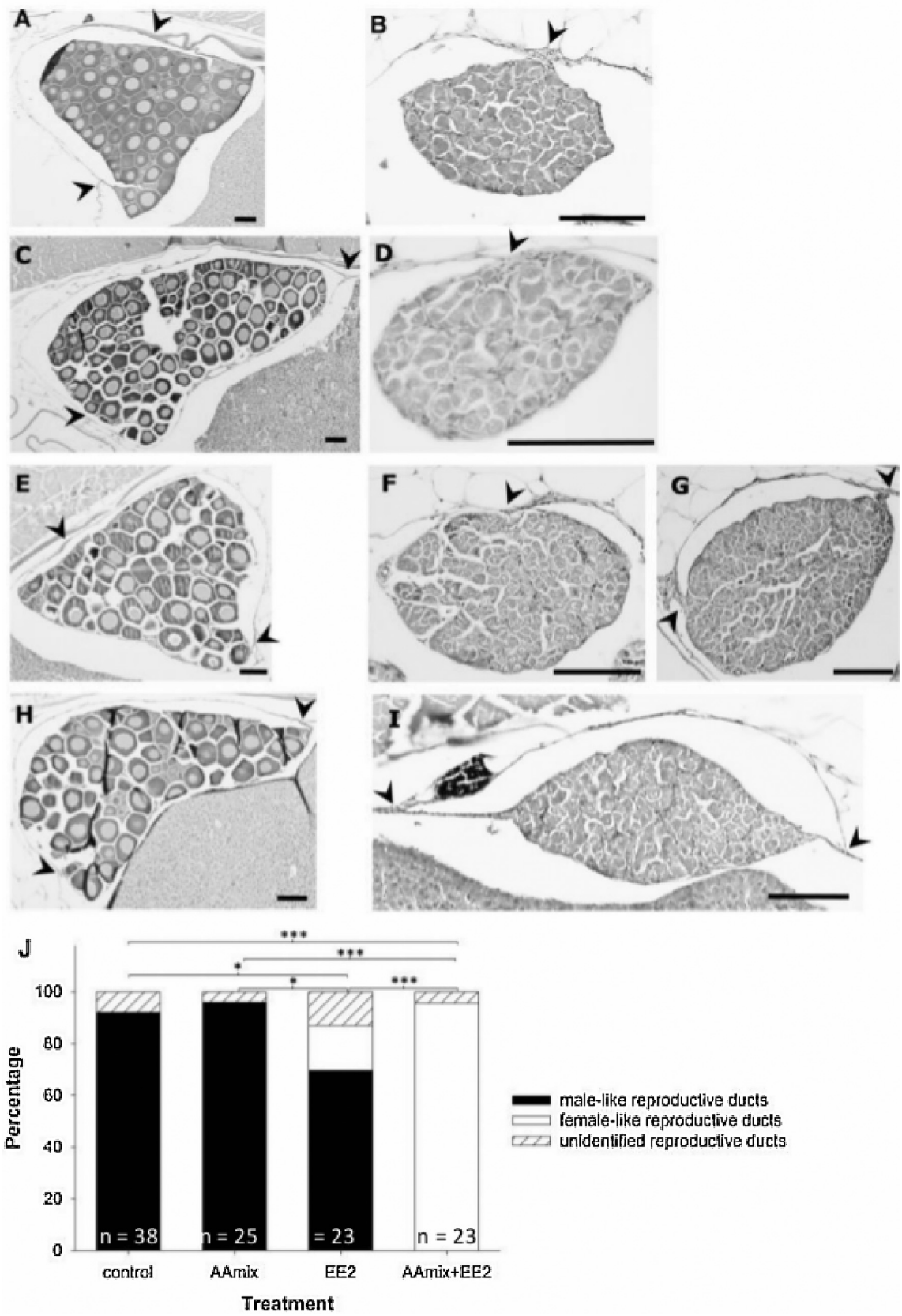

Fig. 2. Effects of early life exposure to a mixture of antiandrogens, EE2, or a mixture of antiandrogens together with EE2, on the development of reproductive ducts in roach. (A–I) Representative histological sections for treatment groups: (A) ovary of control female, (B) testis of control male, (C) ovary of female exposed to the mixture of antiandrogens, (D) testis of male exposed to the mixture of antiandrogens, with a normal duct, (E) ovary of female exposed to EE2, (F), testis of male exposed to EE2, with a normal duct, (G) testis exposed to EE2, with a feminised duct, (H) ovary of female exposed to the mixture of antiandrogens and EE2, and (I) testis in male exposed to the mixture of antiandrogens and EE2, with a feminised duct. Scale bars: 100 μm. (J) Proportions of male- and female-like reproductive ducts in male roach exposed to a mixture of antiandrogens, EE2, or a mixture of antiandrogens together with EE2. Relationships between treatment and feminisation of reproductive ducts in male roach were analysed by chi-square test and significant relationships are indicated ($^{*}p < 0.05$ and $^{***}p < 0.001$). Full statistical results for chi-squared tests and relationships between treatment and feminisation of reproductive ducts in male roach are provided in Supplementary material (Table S6). Numbers in each bar represent the number of fish analysed.

(see Supplementary material; Fig. S5 & Table S5). The difference for ducts between replicate control tanks resulted because it was it was not possible to clearly identify the ducts in in all roach in this treatments even though we could clearly define males based on their gonad morphology and germ cells. (see Supplementary material; Fig. S5 & Table S5).

The reproductive ducts of 38 of 41 males sampled from the control tanks had developed normally (it was not possible to assign the status of the reproductive ducts for the few remaining male fish). All males ($n = 25$) sampled after exposure to the mixture of TCS, CP and AbA had normal, male-like reproductive ducts. There were statistically significant differences in the proportions of feminised ducts in male roach exposed to EE2 alone or in combination antiandrogens compared to roach the treatments not including EE2.

No significant difference was observed in the development of reproductive ducts between control males and males exposed to a mixture of TCS, CP and AbA (χ^2 (1, $n = 63$) = 0.385 ($p = 0.535$)). After exposure to 1.6 ng EE2 /L (average measured concentration), 23 of the sampled fish were males. Of these, 70% of male gonads had developed normal ducts, whereas 17% of male gonads had developed feminised ducts (it was not possible to assign the status of the reproductive ducts for the few remaining male fish). This finding was statistically significant different from control males (χ^2 (2, $n = 61$) = 7.866 ($p = 0.02$)) and males exposed to a mixture of TCS, CP and AbA (χ^2 (2, $n = 48$) = 6.528 ($p = 0.038$)).

For the combination of three antiandrogens with EE2, 23 of the sampled fish were males of which 96% had developed feminised reproductive ducts (it was not possible to assign the status of the reproductive ducts for the few remaining male fish). This finding was significantly different from males in the other three treatment groups (control: (χ^2 (2, $n = 61$) = 57.807 ($p = 2.801 \times 10^{-13}$)); mixture of TCS, CP and AbA: (χ^2 (2, $n = 48$) = 45.997 ($p = 1.028 \times 10^{-10}$)) or EE2 alone: ($\chi^2$ (2, $n = 46$) = 29.462 ($p = 4.004 \times 10^{-7}$))). The results of all statistical analyses for the development of feminised ducts in male roach are summarised in Supplementary material (Tables S5 & S6).

3.5. *In vitro* inhibition of androgen receptor (AR) activation by environmental antiandrogens

Both model antiandrogens, FL and PROCY inhibited the activation of both stickleback ARs activated by 11-KT or DHT. The IC_{50} values were 1.85×10^{-7} M FL (11-KT, ARα), 2.25×10^{-7} M FL (11-KT, ARβ), 5.24×10^{-7} M FL (DHT, ARα), 1.78×10^{-7} M FL (DHT, ARβ), 2.72×10^{-6} M PROCY (11-KT, ARα), 7.33×10^{-6} M PROCY (11-KT, ARβ), 1.52×10^{-6} M PROCY (DHT, ARα) and 5.69×10^{-6} M PROCY (DHT, ARβ) (Fig. 3 A & B). In contrast, the environmental antiandrogens TCS, CP, DCP and AbA did not inhibit the 11-KT induced activation of stickleback ARα (Fig. 3C) or ARβ (Fig. 3D).

4. Discussion

Whilst the effects of environmental oestrogens on sexual development in fish have been well studied, little attention has thus far been paid to antiandrogens that are taken up and bioconcentrate in fish, including for environmentally relevant mixtures or for combinations with relevant environmental oestrogens. Here, we exposed fish to compounds identified in WwTW effluents and shown to be antiandrogenic on the human AR *in vitro*, to establish their effects *in vivo* (in stickleback and roach). Furthermore, we applied stickleback AR transactivation assays in an attempt to support a mechanistic understanding for the effects of the antimicrobial antiandrogens seen *in vivo*.

For the *in vivo* exposures, concentrations of the chemical stocks (in solvent) measured prior to the start of the second roach experiment, were consistent with nominals. In the tank exposures where solvent was used as a carrier the recoveries of the different chemicals were 72–84% for DHT, 88% for FL and 47–70% for DCP in the stickleback experiment and between 79 and 91% for FL and 35–36% for DCP in the roach experiment, but they were consistent within any one treatment. In the solvent free exposure (roach mixture experiment) recoveries of the chemicals were much lower at 8–13%, 14–18% and 46–48% for AbA, TCS and CP, respectively. Possible reasons for the lower measured concentrations of the antiandrogenic chemicals in the exposure tanks include those relating to chemical stability, adsorption and losses during the extraction process. TCS, CP and DCP have all been shown previously to be photodegradable in the aquatic environment, for TCS even under low light intensities (Werner et al., 1983; Mansfield and Richard, 1996; Latch et al., 2005; Lores et al., 2005; Aranami and Readman, 2007; Fang et al., 2010). Some of the chemical loss could be accounted for by adsorption to the tank walls as all three antiandrogens have high predicted log K_{ow} values (4.41CP, 5.17 TCS, 6.51 AbA, ACD/Percepta software—Classic edition, ACD Labs, Toronto, Canada). For TCS and CP, they are also known to adsorb to suspended solids (Hazardous Substances Data Bank, 2006, 2012) and this could have occurred due to the presence of uneaten food and faeces in the exposure tanks. Losses of the chemicals during the extraction process are less likely as the same protocol and equipment was used for all three *in vivo* experiments and the recoveries for DHT (76%) and FL (79–91%) were much higher and similar to previously published ranges (Panter et al., 2004; Sebire et al., 2008; Pottinger et al., 2013b). Despite the low recoveries for some chemicals the measured concentrations were relatively consistent over the long exposure times and they provided appropriate dosing regimens for the purpose of these studies. For some chemicals (stickleback experiment: DCP in the 1 and 10 μg/L exposure groups and DHT in the 10 μg DCP/L exposure group; roach experiment 2: CP in the mixture of CP, TCS and AbA groups), there were differences in measured concentrations between replicate tanks. Although this was not directly taken into account in the statistical model, comparisons of biological effects in replicate tanks suggest that (with the exception for stickleback exposed to 10 μl DCP/L) biological effects seen were as a consequence of the chemical treatments.

In the stickleback *in vivo* exposures basal levels of spiggin were <70 spiggin units/g body weight in the control groups, spiggin was induced by exposure to DHT and the induction was inhibited in fish co-exposed to DHT and FL, as expected (OECD, 2011). Co-exposure of female stickleback to DHT and DCP caused a significant decrease in spiggin levels for the low (0.1 μg/L) and medium (1.0 μg/L) concentration of DCP, supporting the findings from studies with recombinant yeast hAR assays (Rostkowski et al., 2011) that DCP can act as an antiandrogen. In contrast, however, exposure to the high concentration of DCP (10 μg/L), did not inhibit spiggin production. Antiandrogens that act through the AR have been shown to induce a monotonic concentration-dependent inhibition of spiggin in the AFSS (OECD, 2010; Knag et al., 2013; Pottinger et al., 2013b), and it is hard to conclude therefore on whether DCP can convincingly be ascribed as antiandrogen in the stickleback spiggin bioassay. Taking into account the chemistry results for the high DCP treatment group, the concentration of DHT on average appears to be higher compared with that dosed in the low and medium DCP combination treatments which could explain why these two DCP treatments resulted in a slightly lower induction of spiggin. No mortalities were observed in the high dose DCP treatment, so it is unlikely that the lack of spiggin inhibition was caused by cytotoxicity, supported by the condition factor of fish which did not differ between fish exposed to DHT alone and fish exposed to DHT together with the high dose of DCP.

The period of sex differentiation is when the interplay between oestrogens and androgens is critical in determining sexual

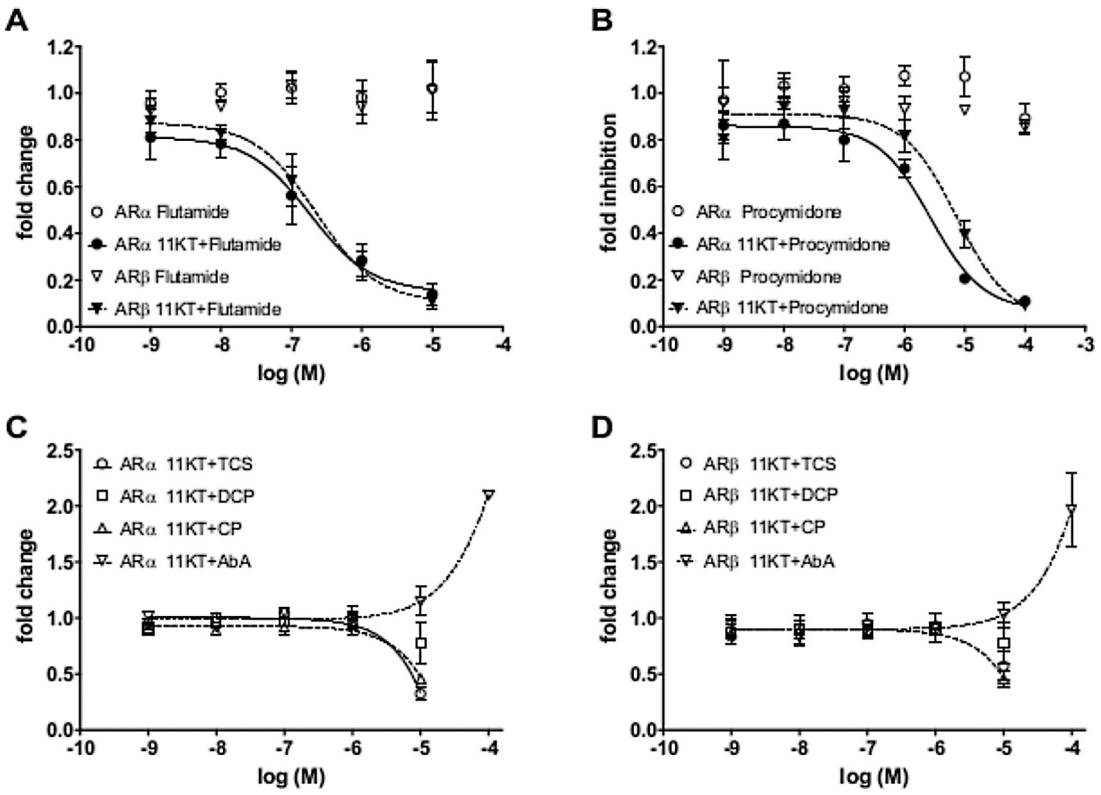

Fig. 3. Dose–response curves for exposures to (A) flutamide, (B) procymidone and (C & D) the environmental antiandrogens TCS, DCP, CP and AbA, in stickleback AR reporter assays. Results are expressed as means ± SEM, $n = 3$. The cells were co-exposed to 11-KT at a concentration of EC_{65} for each receptor and the *Y*-axis indicates fold-change compared to the activity of 11-KT alone.

development and this period is thus most likely to be susceptible to effects of EDCs that act though the oestrogen and androgen axes. In fish, whilst the role of oestrogens in ovarian differentiation is well established, controversy exists about the role of androgens in the regulation of gonadal sex differentiation (Ijiri et al., 2008). In the roach experiments, the endpoints of gonadal sex and feminisation of reproductive ducts were chosen for study based on their known responses to oestrogen exposure. Exposure to the model antiandrogen FL had no effect on sex ratios of fish compared to controls, which compares well with a study on steelhead trout exposed to FL (Sower et al., 1983), but contrasts with a study on guppies exposed to FL (Bayley et al., 2002). FL has been shown also to inhibit testicular growth and/or spermatogenesis in other fish species (Baatrup and Junge, 2001; Jensen et al., 2004; León et al., 2007). In our study, we were unable to identify fine scale differences in germ cells and/or somatic cell development in males due to a technical problem with the gonad fixing conducted in an automated tissue processing system. Collectively, however, from the published data, responses to FL in fish appear to differ between fish species which may relate to differences in the importance of androgens in fish. Species such as guppy and stickleback for instance, show strong androgen-related reproductive behaviours and as such might be more susceptible to the effects of antiandrogens on behaviour than for some other fish species. In mammals, the hydroxy metabolite of FL is active on the AR and differences in metabolism between fish may account for some of the differences in their responses to FL. Similarly, the antiandrogens (DCP or the mixture of CP, TCS and AbA) had no effect on the sex ratio or feminisation of the reproductive ducts in roach. This finding aligns with a recent study finding that pharmaceutical antiandrogens at environmental concentrations had no effects on secondary sexual characteristics in fathead minnow and do not induce intersex in Japanese medaka (Green et al., 2015).

When roach were exposed to EE2 alone at an exposure level reported for some UK WwTW effluents or surface waters (Williams et al., 2003, 2012), 17% of male gonads had developed feminised ducts which aligns with the fact that the development of ovarian cavities is a mechanism driven by oestrogens (Rodgers-Gray et al., 2001). Perhaps the most intriguing finding from our studies is that exposure to a mixture of three potential antiandrogens and EE2 together resulted in a considerably enhanced feminising effect of the oestrogen on duct development in males (96% of the exposed male roach) despite the fact that this combination of antiandrogens had no effect on the male ducts in the absence of EE2. This finding is in contrast with a recent report which showed no effects of co-exposure of two pharmaceutical antiandrogens together with oestrogens, each at their predicted environmental concentrations, on feminisation of fish (Green et al., 2015). However, we used a different set of antiandrogenic chemicals to that used in the work reported by Green et al. (2015), and their actions as potential antiandrogens may differ (see later). In our study, we exposed roach to a mixture of three antiandrogenic chemicals at concentrations which reflected the total levels of antiandrogen activity commonly measured in WwTW effluents (Environment Agency, 2007). This resulted in a measured concentration of total antiandrogenic activity in the combination experiment of 360 μg flutamide equivalents/L (calculated on potency of individual chemicals in a yeast receptor transcription assay, YAS). As discussed by Green et al. (2015), the likelihood is that the suite of antiandrogenic compounds in the environment collectively result in potentiation of oestrogen-induced feminisation of fish rather than a few selected contaminants. Interestingly, another study recently found that

co-treatment of juvenile Murray rainbowfish (*Melanotaenia fluviatilis*) with FL and E2 did not lead to additive reproductive impairment in this species (Bhatia et al., 2015).

Although the bioavailable 'antiandrogens' tested have been shown to inhibit the human AR *in vitro* (Rostkowski et al., 2011), they did not appear to be antiandrogenic in fish *in vivo*, to inhibit spiggin induction or affect sex, even as a mixture. This was supported by the transactivation assays using stickleback ARs where none of the tested compounds inhibited the activation of AR. It could be argued that hepatocellular carcinoma cells of the transactivation system might potentially metabolise the test compounds resulting in non-antiandrogenic metabolites, but this is unlikely as HepG2 cells possess very low to no drug-metabolising activity (Fukuda et al., 2002; Rodríguez-Antona et al., 2002; Wilkening et al., 2003). It is possible that differences in antiandrogenic activity between human and fish relate to a relatively low sequence identity between the human and teleost AR, which is around 40% (Olsson et al., 2005; Hossain et al., 2008). In the ligand binding domain, the sequence similarities are only 62–73% between human and various fish species (Touhata et al., 1999; Olsson et al., 2005; Hossain et al., 2008; Ogino et al., 2009). Species differences in binding specificities of (anti) androgens to the AR have previously been shown in studies comparing different species including rainbow trout and goldfish ARs (Wells and Van Der Kraak, 2000) and rainbow trout, fathead minnow and human AR (Wilson et al., 2007). Interestingly, the latter study reported differences in the relative order of binding of compounds to the ARs and therefore concluded that species-specific EDCs exist (Wilson et al., 2007). More work is clearly required to better establish whether chemicals more widely identified as antiandrogens on the mammalian AR are also active on fish ARs.

Given that we show that the antiandrogens tested did not appear to interact with fish ARs in the *in vitro* transactivation assays, they are most likely to have contributed to the feminisation of duct development induced by EE2 *in vivo* through another mechanism. One possibility would be through effects on steroidogenesis. TCS has been shown to impair the pituitary–gonadal pathway in male rats, *via* reduction of LH and cholesterol production, depressed StAR expression, and down-regulation of several key steroidogenic enzymes (CYP11a, CYP17, 3β-HSD, and 17β-HSD) (Kumar et al., 2009) resulting in an inhibition of androgen production. Interestingly, studies on rats have shown that TCS potentiates the estrogenic effect of EE2 on uterine growth in the uterotrophic assay, whereas TCS alone had no effects (Stoker et al., 2010; Louis et al., 2013). Two possible mechanisms were proposed for the potentiation of the oestrogenic effect of TCS, either through enhancing the interaction of oestrogens with the oestrogen receptor (ER) or increasing endogenous oestrogen levels by inhibiting oestrogen metabolism (Stoker et al., 2010). Both mechanisms are possible explanations for the feminising effects of the test compounds on reproductive duct development observed in our study on roach.

AbA has previously been described as an inhibitor of 5α-reductase (Roh et al., 2010), the enzyme converting testosterone into DHT, and inhibition of 5α-reductase in fathead minnows (using dutasteride) results in effects on reproductive functions which are consistent with an antiandrogenic mode-of-action (Margiotta-Casaluci et al., 2013). To the best of our knowledge, no data are available for the effects of DCP and CP on steroidogenesis.

Given that some of the chemicals investigated here are likely to have an antiandrogenic mode-of-action through acting on the steroidogenic pathway, the ratio of oestrogens to androgens might play a role in the observed induction feminised ducts in male roach. Exposure to the mixture of antiandrogens could alter the balance of the circulating levels of androgens and oestrogens (as androgens are the precursors of oestrogens), reducing the overall levels of circulating steroids, but not their ratio. Exposure to a low concentration of exogenous oestrogen (EE2), would shift this ratio towards higher oestrogens resulting in feminised ducts. Co-exposure to antiandrogens and oestrogen, might reduce circulating levels of androgens whilst increasing levels of oestrogens such shifting the androgen:oestrogen ratio in favour of oestrogen activity to induce feminisation of reproductive ducts in the males. This however is a hypothesis only at this time and further work is needed to confirm the effect of antiandrogens on potentiation of feminisation of fish and their modes of action. Equally it is possible that the effects seen are manifested through alterations to feedback mechanisms on the hypothalamus–pituitary–gonad (HPG) axis affecting the dynamics for testosterone production and aromatisation (to oestrogen).

In conclusion, our results show that compounds that are contained in effluents and are antiandrogenic to the human AR might not necessarily be antagonists to fish ARs despite their potency to bioaccumulate in fish. Nevertheless, a mixture of these 'antiandrogens' together with EE2 induced an enhanced feminising effect of duct development in male roach showing a combination effect in this species. These data add further to the hypothesis that oestrogens and antiandrogens act in combination to feminise males and impair reproductive health in wild fish populations. Our data also highlight the need for homologous assays for extrapolating between effects *in vitro* and *in vivo*. Furthermore, it is important to consider effects across multiple interaction sites in order to truly understand mixture effects of chemicals on animal health.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2015.09.014.

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