Aquatic Toxicology 160 (2015) 117–127

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Environmental concentrations of anti-androgenic pharmaceuticals do not impact sexual disruption in fish alone or in combination with steroid oestrogens

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ARTICLE INFO

Article history:
Received 19 September 2014
Received in revised form
28 November 2014
Accepted 29 December 2014
Available online 16 January 2015

Keywords:
Endocrine disruption
Intersex
Predictive modelling
Mixtures
Anti-androgens
Steroid oestrogens

ABSTRACT

Sexual disruption in wild fish has been linked to the contamination of river systems with steroid oestrogens, including the pharmaceutical 17α-ethinylestradiol, originating from domestic wastewaters. As analytical chemistry has advanced, more compounds derived from the human use of pharmaceuticals have been identified in the environment and questions have arisen as to whether these additional pharmaceuticals may also impact sexual disruption in fish. Indeed, pharmaceutical anti-androgens have been shown to induce such effects under laboratory conditions. These are of particular interest since anti-androgenic biological activity has been identified in the aquatic environment and is potentially implicated in sexual disruption alone and in combination with steroid oestrogens. Consequently, predictive modelling was employed to determine the concentrations of two anti-androgenic human pharmaceuticals, bicalutamide and cyproterone acetate, in UK sewage effluents and river catchments and their combined impacts on sexual disruption were then assessed in two fish models. Crucially, fish were also exposed to the anti-androgens in combination with steroid oestrogens to determine whether they had any additional impact on oestrogen induced feminisation. Modelling predicted that the anti-androgenic pharmaceuticals were likely to be widespread in UK river catchments. However, their concentrations were not sufficient to induce significant responses in plasma vitellogenin concentrations, secondary sexual characteristics or gross indices in male fathead minnow or intersex in Japanese medaka alone or in combination with steroid oestrogens. However, environmentally relevant mixtures of oestrone, 17β-oestradiol and 17α-ethinylestradiol did induce vitellogenin and intersex, supporting their role in sexual disruption in wild fish populations. Unexpectedly, a male dominated sex ratio (100% in controls) was induced in medaka and the potential cause and implications are briefly discussed, highlighting the potential of non-chemical modes of action on this endpoint.

1. Introduction

Pharmaceuticals have emerged as contaminants of concern in the aquatic environment, where they are present at nanogram–microgram per litre concentrations globally (Heberer, 2002). They enter river systems primarily through sewage effluent, following patient excretion and incomplete removal during sewage treatment. However, hospitals, manufacturers, landfills and improper disposal by patients can also be prominent sources (Bound and Voulvoulis, 2005; Larsson et al., 2007; Kümmerer, 2009). Some pharmaceuticals have the capacity to induce adverse effects in non-target organisms through their specific interactions with conserved drug targets (Gunnarsson et al., 2008; Rand-Weaver et al., 2013). One of the most notorious examples is 17α-ethinylestradiol (EE2), the active hormone in the contraceptive pill, which has been linked to feminisation of fish downstream of sewage effluent outfalls, alongside the other steroid oestrogens 17β-oestradiol (E2) and oestrone (E1) (Desbrow et al., 1998; Routledge et al., 1998; Jobling et al., 2006). Fish exposed to EE2 in the low nanogram per litre range show dramatic decreases in reproductive success, prompting concerns over the potential effects of this chemical on fish populations (Kidd et al., 2007).

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http://dx.doi.org/10.1016/j.aquatox.2014.12.022

Several studies have demonstrated that sexual disruption, including the induction of vitellogenin and intersex, occurs in fish exposed to STW effluents under laboratory conditions and in the wild (Jobling et al., 1998; Bjerregaard et al., 2006; Jobling et al., 2006; Harris et al., 2011; Lange et al., 2011; Tetreault et al., 2011; Bahamonde et al., 2013). Whilst the steroid oestrogens are clearly significant contributors to intersex through disruption of normal endocrine function, the relative role of other pharmaceuticals that co-occur with these compounds is unclear. Nonetheless, pharmaceuticals that can interact with hormone receptors or affect levels of circulating hormones are in widespread use and many are prescribed in greater amounts than the oestrogens (Runnalls et al., 2010). Of particular interest are anti-androgenic pharmaceuticals. These compounds are designed to disrupt the androgen signalling pathway through the competitive inhibition of androgen binding to the androgen receptor (AR) by direct antagonism, or by reducing concentrations of circulating androgens (Kelce and Wilson, 1997; Hotchkiss et al., 2008). Anti-androgenic activity has also been detected in STW effluents, river water and sediment globally (Conroy et al., 2007; Johnson et al., 2007b; Urbatzka et al., 2007; Weiss et al., 2009; Kumar et al., 2012; Kinani et al., 2010) and there is some evidence of anti-androgenic pharmaceuticals potentially occurring in low nanogram per litre concentrations (Besse and Garric, 2009; Al-Odaini et al., 2012; AZ.com, 2013).

Anti-androgens can cause demasculinisation of male fish under laboratory conditions, inducing similar phenotypes to those observed in the aquatic environment or through exposure to steroid oestrogens. These include intersex and vitellogenin induction, altered gene expression and reduced secondary sexual characteristics, sperm counts and fecundity (Bayley et al., 2002; Kiparissis et al., 2003; Jensen et al., 2004; Filby et al., 2007; Hatef et al., 2012). Furthermore, several studies have shown that the anti-androgenic activity of some STW effluents is equivalent to concentrations of the anti-androgenic pharmaceutical, flutamide, that are capable of causing demasculinisation in fish during laboratory exposures (Jensen et al., 2004; Panter et al., 2004; Johnson et al., 2007b). Anti-androgenic impacts have been detected in wild stickleback *(Gasterosteus aculeatus)* downstream of a STW effluent outfall in the UK (Katsiadaki et al., 2012) and anti-androgenic compounds have been identified in the bile of fish exposed to STW effluent (Hill et al., 2010; Rostkowski et al., 2011). However, the chemical contributors to anti-androgenic activity in the environment are not well characterised and pharmaceutical mixtures have not been investigated from this perspective.

This study aimed to determine whether the two of the most prescribed anti-androgenic pharmaceuticals in the UK, bicalutamide and cyproterone acetate, could contribute to feminization of fish at environmentally relevant concentrations. Both drugs are AR antagonists, although unlike bicalutamide, cyproterone acetate can also inhibit androgen secretion by inhibiting gonadotrophin release through its progestagenic activity (Mahler et al., 1998). Both can cause demasculinising effects in male patients during treatment through either intended or adverse effects, such as gynecomastia and reduced libido (Mahler et al., 1998; Bradford, 1999). In fact, the use of cyproterone acetate to treat sexually deviant behaviour in men has been shown to be equivalent to surgical castration (Neumann and Kalmus, 1991). In fish, exposures to these compounds caused similar effects to the steroid oestrogens, including intersex and impacts on reproduction (Kiparissis et al., 2003; Panter et al., 2012). However, although they are likely environmental contaminants due to their prescription levels and patient excretion, there are few available data on their environmental concentrations and corresponding effects in fish. In addition, there has been no assessment of the possibility of combined effects with steroid oestrogens, with which they are likely to co-occur in the aquatic environment. This is warranted since outcomes of modelling of fish fieldwork studies indicated that anti-androgenic chemicals may play a significant role in sexual disruption alone and in combination with steroid oestrogens (Jobling et al., 2009). There is also evidence from rodent studies that two sets of chemicals with different mechanisms of action can act additively on common endpoints (Kortenkamp, 2008; Christiansen et al., 2009), although there may in fact be some commonalities in the mode of action of oestrogens and anti-androgens in fish at a molecular level (Filby et al., 2007).

To address these knowledge gaps, this study used predictive modelling techniques to predict concentrations of bicalutamide and cyproterone acetate in typical UK sewage effluents. This was later supplemented with modelling of concentrations in river catchments in England and Wales to further investigate the wider environmental relevance of the exposure concentrations. Such techniques have already been used to predict concentrations of a range of pharmaceuticals in STW effluents and rivers (Schowanek and Webb, 2002; Johnson et al., 2007a; Besse and Garric, 2009; Johnson and Williams, 2004; Williams et al., 2009; Kugathas et al., 2012) and comparisons of modelled and measured steroid oestrogens have been shown to compare well in two UK river catchments (Williams et al., 2012). The modelled concentrations of bicalutamide and cyproterone acetate from STW effluents were employed in two experiments using two appropriate fish models. These aimed to determine whether the anti-androgenic pharmaceuticals could induce effects associated with feminisation in fish, alone or in combination with environmentally relevant mixtures of steroid oestrogens.

2. Materials and methods

2.1. Prediction of sewage treatment works effluent concentrations of pharmaceutical anti-androgens

The NHS prescriptions cost analysis for England and Wales was used to determine the annual consumption of bicalutamide and cyproterone acetate in 2009 (based on Runnalls et al., 2010). The daily per-capita consumption was then calculated based on the mid-2009 population (estimated as 54,809,100). Excretion rates of 55% for bicalutamide (Goa and Spencer, 1998) and 80.75%, cyproterone acetate (Speck et al., 1976; Humpel et al., 1977; Frith and Phillipou, 1985), were incorporated to determine the microgram per day of each drug excreted per capita as the parent compound in faeces and urine. Effluent concentrations (microgram per litre) were calculated for four UK STWs in the Severn Trent catchment based on the linear emission model:

$$C_{\text{effluent}} = \frac{\text{pc} \times \text{pop}}{Q_{\text{STW}}} \times (1 - R)$$

where the per capita excreted (pc) was multiplied by the population served by the STW (pop) and divided by the flow (L/day) (Q) through the STW (Table S2). Since removal is expected to be low for bicalutamide (Brixham Environmental Laboratory, 1998; AZ.com, 2013) and no data were available for cyproterone acetate, a removal percentage (R) was not incorporated. This provided a worst case scenario where the total anti-androgenic pharmaceutical load arriving at a STW was available to enter the aquatic environment. These data are displayed in Tables S1 and S2.

2.2. Experimental test chemicals

Bicalutamide ≥98% (CAS No. 90357-06-5), cyproterone acetate ≥98% (CAS No. 427-51-0), oestrone (E1) ≥99% (CAS No. 53-16-7), 17β-oestradiol (E2) ≥99% (CAS No. 50-28-2) and 17α-ethinylestradiol (EE2) ≥98% (CAS No. 57-63-6) were purchased from Sigma–Aldrich, Gillingham (UK). Concentrated stock solutions were prepared with *N*,*N*-dimethylformamide (DMF) ≥99.8% (CAS

No. 68-12-2) purchased from Fisher Scientific, UK for tank dosing and Absolute Ethanol 100 AR (CAS No. AR 64-17-5) purchased from Hayman Speciality Products, UK for Petri dish dosing in experiment two.

2.3. Experiment one: vitellogenin induction and secondary sexual characteristics

2.3.1. Experimental design, chemical concentrations and test species

Experiment one was carried out using the experimental system described by (Brian et al., 2005). Pre-spawning, male fathead minnow of approximately seven months of age were exposed to defined mixtures of steroid oestrogens and anti-androgenic pharmaceuticals for 14 days. Fathead minnow were an appropriate experimental model for this experiment since males have a set of well-defined androgen dependent secondary sexual characteristics (Ankley et al., 2004; Ankley et al., 2010). They are also large enough in size to allow for the collection of sufficient blood for vitellogenin analysis (Ankley and Johnson, 2004).

Six treatment groups were employed, including aquarium water and solvent controls, a steroid oestrogen treatment (E1, E2 and EE2), an anti-androgenic pharmaceutical treatment (bicalutamide and cyproterone acetate) and a combined mixture treatment containing both anti-androgenic pharmaceuticals and steroid oestrogens. Concentrations of the anti-androgenic pharmaceuticals were based on the maximum concentrations predicted for untreated effluent (115 ng/L bicalutamide and 74 ng/L cyproterone acetate) using the equation described in Section 2.1. Since the steroid oestrogens occur together in the environment, fish were exposed to an environmentally relevant mixture of 9.6 ng/L E1, 2.1 ng/L E2 and 0.3 ng/L EE2. These were within the range of oestrogen concentrations observed in a survey of UK effluents and their proportions were based on the ratio of their highest detected concentrations (Johnson et al., 2007b). These concentrations had a combined E2 equivalent (EEQ) of 8.3 ng/L, based on PNECs for reproductive endpoints (as described in Young et al., 2004) and were expected to induce an intermediate response in vitellogenin induction based on their estimated EC50's for this endpoint (Panter et al., 1998; Brian et al., 2005). A positive control of 10 ng/L EE2 was also included. All treatments were run in duplicate with eight fish per tank.

Chemical stocks were prepared at 50,000× the required exposure concentration in 1 L DMF and were pumped by peristaltic pump through medical grade silicone tubing (Watson Marlow, UK) to mixing vessels at a rate of 0.01 mL/min. This was mixed with dechlorinated and filtered tap water from a header tank flowing at 30 L/h *via* medical grade silicone tubing (VWR, UK), to produce the desired concentrations in the exposure aquaria without exceeding the maximum recommended concentration of 20 μL/L of DMF (Hutchinson et al., 2006). Each tank had its own mixing vessel, although single stock bottles fed both replicates and the negative control ran directly off the header tank. Physical and physicochemical conditions were maintained at 25 ± 1 °C with dissolved oxygen exceeding 70% of air saturation and a photoperiod of 16:8 h light:dark with a 20 min simulated dusk/dawn period of low light. The fish were fed twice daily with flaked food (Tetramin Flake, ZM Fish Food, UK) and once daily with frozen adult brine shrimp (Tropical Marine Centre, Gamma irradiated).

2.3.2. Fish sampling and vitellogenin analysis

After 14 days of chemical exposure, fish were sacrificed by a lethal dose of MS222 (500 mg/L), buffered to pH 7.4, in accordance with UK Home Office Regulations. Blood was collected by caudal fin amputation with heparinised microhematocrit tubes and samples were centrifuged at 7000 × *g* for 5 min at 4 °C to collect plasma, which were stored at −80 °C. Fork length and wet weight were recorded and the livers and gonads were excised and weighed for hepatosomatic index (HSI) and gonadosomatic index (GSI). Secondary sexual characteristics, including the fatpad weight and the tubercle number, were also recorded. Tubercle prominence was then graded (scored 0–5) based on their level of development (based on Smith, 1978). Plasma samples from individual fish were then analysed by enzyme linked immunosorbent assay (ELISA) specific to fathead minnow (Biosense Laboratories AS, Norway).

2.4. Experiment two: intersex induction in Japanese medaka (*Oryzias latipes*)

2.4.1. Experimental design, chemical concentrations and test species

Japanese medaka were exposed from an embryo to early adult stage over 98 days. This species can reach sexual maturity within 100 days, are sexually dimorphic and have well documented process of gonad development (Ankley and Johnson, 2004). As in experiment one, fish were treated with the anti-androgenic pharmaceuticals, the steroid oestrogens and a combination of the two sets of compounds. However, this treatment regime was tested at two different concentrations termed "high" and "low." Concentrations of anti-androgenic pharmaceuticals were based on those predicted from the UK sewage effluents based on the equation described in Section 2.1. In addition, the concentrations of steroid oestrogens predicted to occur at these STWs were also employed, based on the same flow regimens and populations served (from Green et al., 2013). For the high concentrations, the treatment concentrations originated from UK1: 115 ng/L bicaltuamide, 74 ng/L cyproterone acetate, 29 ng/L E1, 4 ng/L E2 and 0.4 ng/L EE2 (17.7 ng/L EEQ). The low concentrations were based on the lowest effluent concentrations predicted for UK3: 53 ng/L bicalutamide, 34 ng/L cyproterone acetate, 13 ng/L E1, 2 ng/L E2 and 0.2 ng/L EE2 (equivalent to 8.3 ng/L EEQ). The steroid oestrogen concentrations were both expected to induce an intermediate incidence of intersex based on a concentration response of E2 in medaka for the induction of intersex (Metcalfe et al., 2001). A positive control was dosed at 50 ng/L E2, a concentration expected to completely reverse the sex of males (Kinoshita et al., 2009), and an aquarium water and solvent control were also employed to give a total of nine treatments.

Each treatment was replicated in duplicate with 25 fish per tank (50 per treatment). Embryos were collected and exposure was initiated at less than six hours post fertilisation. The embryos were maintained under static renewal in covered glass Petri dishes with 15 mL of Modified FETAX (Frog Embryo Teratogenesis Assay-Xenopus) Solution (MFS) (from Woods and Kumar, 2011) in an incubator at 25 ± 1 °C with 16:8 h light:dark ratio. Over the first three days, unfertilised or dead embryos were replaced with new fertilised embryos. Each Petri dish was spiked with 10 μL of a concentrated stock solution in absolute ethanol, which was allowed to evaporate before 15 mL of MFS was added. Nonetheless, the solvent control was still spiked with ethanol only. Embryos were transferred, by pipette, to new Petri dishes with new stocks daily to provide 24 h renewal of the exposure mixtures. As the embryos hatched, larvae were transferred to 30 L exposure aquaria run with the same flow regimens as experiment one and were maintained in mesh baskets for up to 14 days before being released into the aquarium itself. Temperature was maintained at 26 ± 1 °C throughout the majority of the study, although greater fluctuations were observed in some tanks on occasion. A photoperiod of 16:8 h light:dark with a 20 min simulated dusk/dawn period of low light was applied. Fish were fed twice daily to excess with pellet food. This was supplemented with larval artemia and, eventually, frozen brine shrimp.

Dissolved oxygen and various water parameters were monitored throughout the experiment.

2.4.2. Fish sampling and histological analysis

After 98 days exposure, medaka were sacrificed by a lethal dose of neutral buffered MS222 (500 mg/L). However, fish in the positive control treatment were sampled earlier at 90 days due to mortality during the exposure. The heads and tails were removed and the body was fixed in bouins and stored in 70% IMS. The body cavity was then opened from the side to reveal the gonad before being fixed in wax and serial sectioned longitudinally, with 5 μm samples taken every 150 μm across the gonad. For smaller fish, the entire body was serial sectioned and for females with large ovaries, the gonad was excised and split longitudinally into sections before being set in wax and serial sectioned in the same fashion as the males. Intersex incidence was quantified through light microscopy by the presence or absence of oocytes and ovarian cavities and severity was scored for each gonad section based on an intersex index score of 0–6, shown in Table S3 (from Jobling et al., 2006), for the number of oocytes. All slides were blinded prior to microscopic analysis.

2.5. Chemical analysis

Concentrations of the exposure chemicals were analysed by liquid chromatography tandem mass-spectrometry (LC–MS/MS). The method for analysing steroid oestrogens was taken from the Standing Committee of Analysts book on the detection of steroid oestrogens in water samples by mass spectrometry (Environment Agency, 2008). Methods were developed for detecting cyproterone acetate and bicalutamide and are detailed in the Supporting Information. Briefly, in experiment one, 500 mL samples from treatment tanks were collected on days 1 and 14. In experiment two, 100 mL was collected on days 20, 34, 62 and 90 for solid phase extraction using C18 cartridges (Sep-Pak C18, Waters, UK). Extracts underwent HPLC (Aglient 1100 series) with a C18 column (Aglient ZORBAX Eclipse XDB-C18, 150 mm × 4.6 × 5 μm. Part No. 993967-902). Detection and quantification was carried out using an Applied Biosystems API5000 triple quadrupole mass spectrometer using negative ion electrospray in multiple reaction monitoring mode for bicalutamide and the steroid oestrogens. Positive ion electrospray was employed for cyproterone acetate. The concentrations were then calculated from calibration curves using deuterium labelled internal standard quantitation in Analyst 1.5.1 software (Thermo-Electron Corporation) with d_7-propanolol and d_{10}-carbamazapine for bicalutamide and cyproterone acetate, respectively. Limits of detection (LOD) were determined as 5 ng/L bicalutamide and 10 ng/L cyproterone acetate. The LOD for the steroid oestrogens in experiment one was determined as 0.1 ng/L, compared with 1 ng/L in experiment two. Analysis of samples yielded variable recovery, which in the case of cyproterone acetate could not be recovery corrected using d_{10}-carbamazapine. Consequently, the concentration of cyproterone acetate could not be accurately quantified in samples and had to be predicted from the bicalutamide data using dose ratio information. Concentrations of EE2 in samples could not be reported directly due their low concentration in samples and were estimated from analysed E2 concentrations using dose ratio information.

2.6. Statistical analysis

In experiment one, parametric data underwent one way analysis of variance (ANOVA) followed by *post hoc* all pairwise multiple comparison procedures (Holm–Sidak method). Non-parametric data was assessed by Kruskal–Wallis one way ANOVA on ranks followed by *post hoc* all pairwise multiple comparison procedures (Dunn's method). Vitellogenin data were log transformed prior to assessment and statistical significance was accepted at $P \leq 0.05$ for all tested endpoints. Treatment groups were all compared with the solvent control. In experiment two, binary responses (intersex incidence) were analysed using generalized linear modelling approaches, assuming that the numbers of intersex fish were binomial-distributed. Mean effects were estimated by Maximum likelihood, and treatment-related differences to the controls were analysed by Williams contrast test (Hothorn, 2004). Ordinal responses (intersex severity) were assumed to follow a common slopes model with a cumulative logit link function, and correlations from repeated measurements were estimated according to the principles of generalized estimating equations (Agresti, 1984). Odds ratio estimates were used to express relative differences between treatment means. For the log odds scale, the cumulative logit model is sometimes referred to as the proportional odds model. Analysis of vitellogenin, secondary sexual characteristics and gross indices was performed in SigmaStat 3.5 (Systat Software, Chicago IL), whilst intersex analysis was performed in SAS 9.3 (SAS Institute, Cary NC).

2.7. Environmental relevance

In order to put the exposure concentrations into context and to produce a more extensive picture of anti-androgenic pharmaceuticals in UK rivers, predictive modelling techniques were used to estimate their concentrations in river catchments in the England and Wales. Fourteen major river catchments accounting for approximately 54% of land area and 69% of the population served by STWs were assessed (Figure S3 A&B). The modelling exercise used the Low Flows 2000 (LF2000) WQX (Water Quality eXtension) hydrological modelling platform, which provided a map of the UK's interconnected river reaches with their variable flows and artificial influences incorporated (Williams et al., 2009). In this process, STW's were considered to be the only chemical input based on the per capita excretion and once again a removal percentage was not incorporated. There are no data available on the in-stream degradation of the pharmaceutical anti-androgens, and so the model produced a worst case scenario for their environmental concentrations, only taking into account the effects of dilution of the effluent in downstream river stretches.

3. Results

3.1. Predicted environmental concentrations of anti-androgenic pharmaceuticals

NHS prescriptions of bicalutamide and cyproterone acetate in 2009 were determined as 636 kg and 278 kg, respectively, which produced per capita excretion rates of 17 and 11 μg/day. When these data were input to the linear emission model (Section 2.1), concentrations of 53–115 ng/L for bicalutamide and 34–74 ng/L for cyproterone acetate were predicted to occur in untreated effluent at the four UK STWs. The highest and lowest predicted concentrations from this analysis were used as exposure concentrations for experiments one and two. Subsequent hydrological modelling with LF2000-WQX predicted that the presence of anti-androgenic pharmaceuticals was widespread across river catchments in England and Wales, downstream of STW discharges. The concentrations on a majority of river stretches were below 10 ng/L, although they were much higher on some stretches from both urban and rural areas. Indeed, median concentrations of 9 (0.0002–157) ng/L bicalutamide and 5 (0.0001–86) ng/L of cyproterone acetate were predicted (Figure S3 A&B).

Table 1
Chemical analysis of exposure tanks from experiments one and two versus the nominal. In experiment one, samples analysed at the start and end of the exposure are indicated. In experiment two, the mean measured concentrations (± standard error) are displayed from analysis of up to four samples from throughout the study. Cyproterone acetate was predicted based on bicalutamide and, in experiment two, EE2 was predicted based on E2.

Experiment one	Replicate	Bicalutamide (ng/L)		Cyproterone acetate (ng/L)		E1 (ng/L)		E2 (ng/L)		EE2 (ng/L)		EEQ (ng/L)	
Treatment		Nominal	Measured	Nominal	Predicted	Nominal	Measured	Nominal	Measured	Nominal	Measured	Nominal	Predicted
Anti-androgens	A	115	94 (±27)	74.0	60 (±17)	0	<LOD	0	<LOD	0	<LOD	0	<LOD
	B	115	121 (±64)	74.0	78 (±41)	0	<LOD	0	<LOD	0	<LOD	0	<LOD
Steroid oestrogens	A	0	<LOD	0	<LOD	9.6	10.3 (±1.1)	2.1	2.0 (±1.0)	0.3	0.3 (±0.1)	8.3	8.2 (±2.4)
	B	0	<LOD	0	<LOD	9.6	8.7 (±1.9)	2.1	1.7 (±0.6)	0.3	0.2 (±0.1)	8.3	6.9 (±1.9)
Mixture	A	115	94 (±29)	74.0	60 (±19)	9.6	10.6 (±3.1)	2.1	2.0 (±0.2)	0.3	0.3 (±0.1)	8.3	8.4 (±1.8)
	B	115	90 (±17)	74.0	58 (±11)	9.6	7.0 (±4.4)	2.1	1.3 (±0.9)	0.3	0.2 (±0.1)	8.3	5.3 (±3.3)
Positive Control	A	0	<LOD	0	<LOD	0	<LOD	0	<LOD	10.0	9.7 (±2.8)	100.0	97 (±28)
	B	0	<LOD	0	<LOD	0	<LOD	0	<LOD	10.0	9.0 (±2.7)	100.0	90 (±27)

Experiment two	Replicate	Bicalutamide (ng/L)		Cyproterone acetate (ng/L)		E1 (ng/L)		E2 (ng/L)		EE2 (ng/L)		EEQ (ng/L)	
Treatment		Nominal	Measured	Nominal	Predicted	Nominal	Measured	Nominal	Measured	Nominal	Predicted	Nominal	Predicted
Low anti-androgens	A	53	99 (±18)	32	57 (±8)	0	<LOD	0	<LOD	0	<LOD	0	<LOD
	B	53	71 (±7)	32	43 (±4)	0	<LOD	0	<LOD	0	<LOD	0	<LOD
Low oestrogens	A	0	<LOD	0	<LOD	13	24 (±2)	2	3.2 (±0.4)	0.2	0.3 (±0.04)	8	14 (±1)
	B	0	<LOD	0	<LOD	13	27 (±4)	2	3.4 (±0.4)	0.2	0.3 (±0.04)	8	16 (±2)
Low mixture	A	53	107 (±6)	32	64 (±4)	13	33 (±6)	2	3.9 (±0.8)	0.2	0.4 (±0.08)	8	20 (±4)
	B	53	85 (±16)	32	52 (±10)	13	34 (±5)	2	3.7 (±0.8)	0.2	0.4 (±0.08)	8	20 (±4)
High anti-androgens	A	115	133 (±14)	74	85 (±9)	0	<LOD	0	<LOD	0	<LOD	0	<LOD
	B	115	152 (±11)	74	98 (±7)	0	<LOD	0	<LOD	0	<LOD	0	<LOD
High oestrogens	A	0	<LOD	0	<LOD	29	63 (±2)	4	8.3 (±0.7)	0.4	0.8 (±0.07)	18	37 (±2)
	B	0	<LOD	0	<LOD	29	66 (±7)	4	6.8 (±0.4)	0.4	0.7 (±0.04)	18	36 (±3)
High mixture	A	115	99 (±30)	74	64 (±19)	29	64 (±10)	4	8.7 (±1.9)	0.4	0.9 (±0.19)	18	39 (±2)
	B	115	134 (±5)	74	86 (±3)	0	61 (±7)	4	9.5 (±2.2)	0.4	1.0 (±0.22)	18	39 (±3)
Positive control	A	0	<LOD	0	<LOD	0	<LOD	50	76 (±4)	0	<LOD	50	77 (±5)
	B	0	<LOD	0	<LOD	0	<LOD	50	80 (±10)	0	<LOD	50	81 (±11)

3.2. Chemical analysis

The results of chemical analysis are displayed below in Table 1. Concentrations of bicalutamide were successfully determined. However, whilst cyproterone acetate was successfully detected in the anti-androgen and mixture treatment tanks it could not be accurately quantified due to the unreliable quantification of the internal standard. Concentrations of cyproterone acetate were instead predicted based on its expected ratio to bicalutamide, with which it had been combined in the stock solutions. In experiment one, all compounds were close to nominal with good agreement between replicates as well as between the single activity and combined mixture treatments. In experiment two, the concentrations tended to be around 200% of nominal due to issues in maintaining header tank pressure, which caused sporadic reductions the flow and therefore the dilution of the chemicals. Nonetheless, environmentally relevant concentrations were maintained throughout the experiment based on predictive modelling for anti-androgenic pharmaceuticals and reported oestrogen concentrations from UK rivers and effluents (Johnson et al., 2007b; Williams et al., 2009). EE2 was below the 1 ng/L LOD of the method and was instead predicted based on the measured concentration of E2. Good agreement was achieved between replicate tanks and between single activity and mixture treatment tanks. Concentrations of steroid oestrogens in the high and low mixtures differed, whilst the anti-androgens were more similar.

3.3. Experiment one: vitellogenin induction, secondary sexual characteristics and gross indices

Vitellogenin levels in the blood plasma of juvenile male fathead minnows varied significantly between treatments (Fig. 1). Treatment with the anti-androgenic pharmaceutical alone caused a two fold increase in the median vitellogenin concentration in comparison to the solvent control, but this difference was not statistically significant at the 95% level. As expected, the steroid oestrogen mixture significantly induced vitellogenin. The combined mixture of oestrogens and anti-androgens also significantly induced vitellogenin compared with the solvent control and again, although the median was two-fold higher than the oestrogens alone, it was still not statistically significant. GSI significantly varied between treatment groups but there were no statistically significant differences between the treatment groups and the solvent control (Figure S5). In comparison, HSI did not significantly vary. For the secondary sexual characteristics, only tubercle number and prominence were significantly reduced by the EE2 positive control, whereas the fat-pad was unaffected. Apart from this there was no statistically significant impact of the oestrogen, anti-androgen and combination treatment on these endpoints (Figure S6).

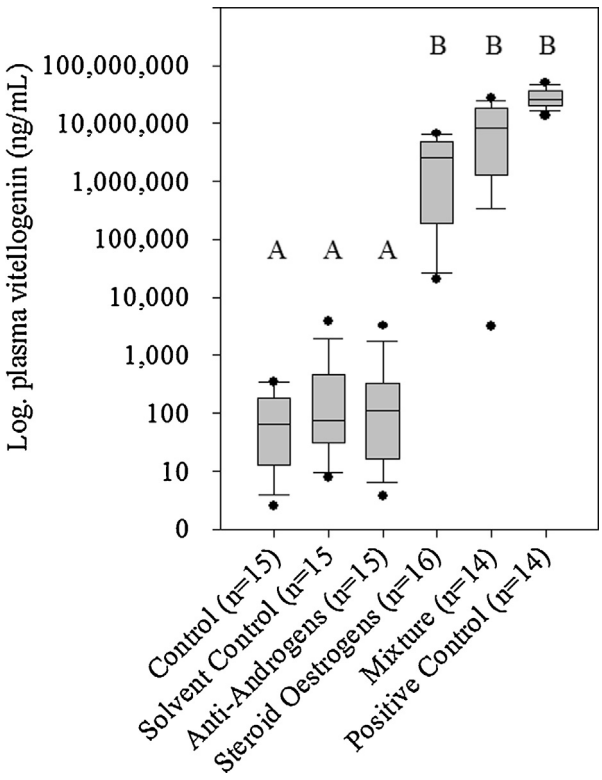

Fig. 1. Log. vitellogenin concentrations in the blood plasma of fathead minnow from each treatment group. Vitellogenin was not quantified in some cases when values were outside of the working range of the assay (*N* number of samples is indicated out of 16 fish). Boxes represent the median with 25th and 75th percentiles and error bars extending to the 95th percentile with outliers shown as dots. The labels A and B denote a statistically significant difference between groups at $p \leq 0.05$.

3.4. Experiment two: intersex induction and severity

Due to the long term nature of this study, some mortality was unavoidable. This occurred in all treatment groups during the experiment. On average, survival was over 80%. However, lower survival was observed in one water control tank (56%) and a solvent control tank (72%). Survival was also lower in the two positive control tanks (60% and 68%), which were sampled early after 91 days as a result. The exact cause for mortality was unknown, although in the control tank temperature fluctuations could be implicated. Indeed, this tank experienced a greater temperature range (5.7 °C) than the other tanks in this experiment. In the case of the positive controls, previous studies have observed mortality in fish associated with very high vitellogenin induction, which would be expected

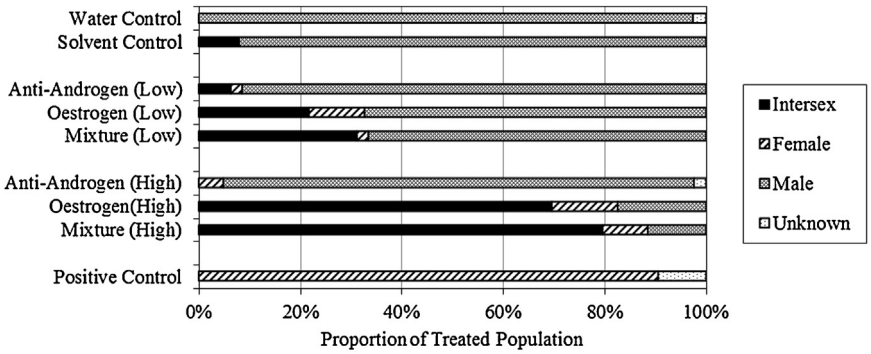

Fig. 2. The proportions of intersex, male, female and unknown Japanese medaka within the treated populations based on gonad histology.

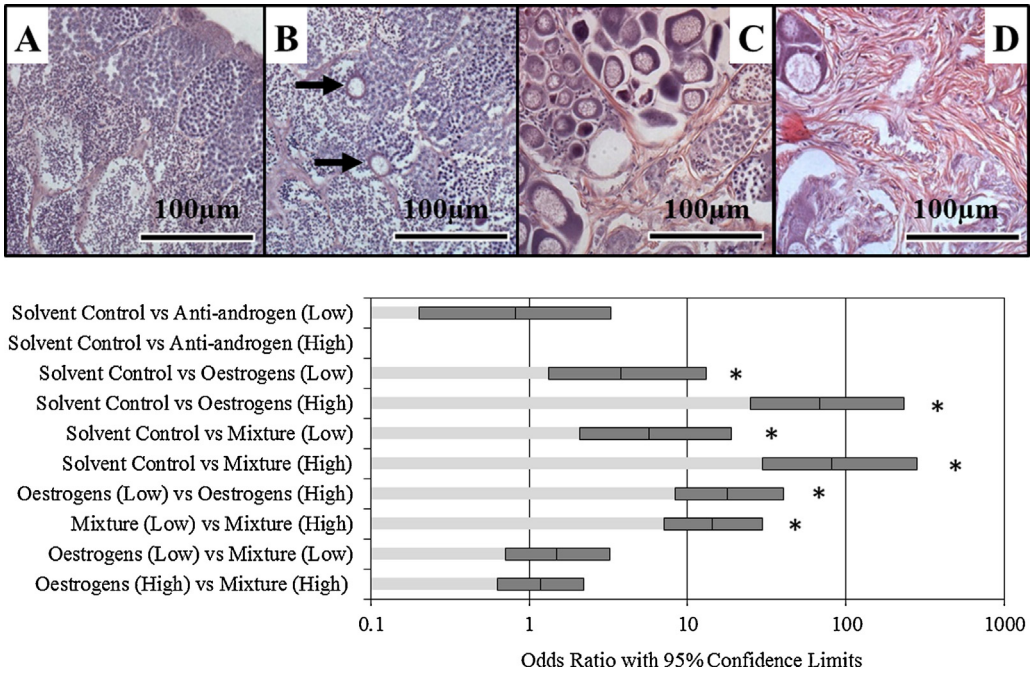

Fig. 3. Images show a normal male gonad from the solvent control (A), sporadic oocytes in the testes (shown with arrows) typical of the low oestrogen and low mixture treatment (B), increased abundance and clustering of oocytes in the testes typical of the high oestrogen and high mixture (C) and the extensive fibrosis which occurred in the testes of some high oestrogen and high mixture treated fish (D). The graph below shows statistical comparisons of the intersex severity between treatment groups. The boxes represent the odds ratio value and extend to the 95% confidence limits where a significant difference is judged when the boxes exceed the value of 1 (indicated by *).

in the sex reversed males observed in this treatment (Herman and Kincaid, 1988).

At the time of sampling, whilst all Japanese medaka in the positive control population appeared phenotypically female characteristics, the water and solvent controls appeared exclusively male and a male majority was observed in all other treatments based on their external appearance. This unexpected result was confirmed with gonad histology.

Intersex was based only on the presence of oocytes in testicular tissue since ovarian cavities could not be properly quantified in the longitudinal sections. Treatment of Japanese medaka with the low and high steroid oestrogen concentrations induced intersex in 22% and 70% (Fig. 2) of the respective treatment populations. However, only the high oestrogen treatment induced what was considered a significant induction of intersex in comparison to the solvent control, where intersex was identified in 6% of the treatment population. A small proportion of fish in the low anti-androgen treatment were also found to be intersex (6%), which did not significantly differ from the solvent control and no intersex fish were found in the high anti-androgen treatment. Interestingly, in both the low and high mixture treatments, the number of intersex individuals was 9% and 10% higher, respectively, than their corresponding oestrogen treatments alone. However, whilst intersex incidence in these treatments was significantly higher than the solvent control, there was no significant difference in intersex induction between the mixture treatments and their respective oestrogen treatments.

Fig. 3 summarises the outcomes from the data analysis when considering the severity of intersex. Intersex fish in the low anti-androgen treatment was found to be at a low severity (index 1), which did not significantly differ from that occurring in the solvent control. In comparison, severity in the low oestrogen and the low mixture treatments was statistically similar with an index around 1–2. Intersex index in these treatments was significantly higher than the solvent control. No intersex fish were identified in the high anti-androgen treatment and severity of intersex in the high oestrogen and high mixture treatments was significantly higher than in the lower treatments. Histological similarities were observed in these two treatment groups with fish identified across all bands of severity, with a tendency towards higher severity (4–6). In addition, fish in these treatments had increased levels of lobular disorganisation in both intersex and non-intersex males. Fibrotic tissue was also identified and, in some cases, it was severe enough to make up a majority of the gonad tissue. Furthermore, intersex fish within both treatment groups showed reductions in spermatozoa in the sperm ducts in comparison to solvent control fish. In some fish, particularly those with extensive fibrosis, the ducts were empty (Figure S7). As a result, intersex fish with testes made up primarily of fibrotic tissue were classified as index 6 in statistical analysis. The few female fish which were identified in the treatment groups were confirmed as histologically female.

4. Discussion

This study aimed to determine the biological significance of the presence of anti-androgenic pharmaceuticals in the aquatic environment by assessing their impacts on sexual disruption in fish, alone and in combination with co-occurring steroid oestrogens. The results indicated that environmentally relevant concentrations of up to 157 ng/L bicalutamide and 86 ng/L cyproterone acetate were not sufficient to induce significant effects in fish either alone or in combination with the steroid oestrogens. However, this study clearly demonstrated that environmentally relevant mixtures of steroid oestrogens were capable of causing sexual disruption in fish, in terms of both vitellogenin induction and intersex. However, secondary sexual characteristics, GSI and HSI in fathead minnow were unaffected, and previous studies suggest that higher concentrations are required to impact these endpoints (Miles-Richardson et al., 1999; Filby et al., 2007; Salierno and Kane, 2009). Indeed, in this study only 10 ng/L EE2 in the positive control significantly reduced tubercle number and prominence, although a longer

exposure to this concentration may have been required to affect gross indices (Filby et al., 2007).

The induction of intersex by a mixture of steroid oestrogens invites the question of whether this was an effect of a single chemical or a combination of all three steroid oestrogens. Additive effects of steroid oestrogens are well documented and have been observed both *in vitro* and *in vivo*, on endpoints including vitellogenin production, fecundity and secondary sexual characteristics in combination with other xenoestrogens (Thorpe et al., 2001; Rajapakse et al., 2002; Silva et al., 2002; Brian et al., 2005, 2007). However, additive effects have not been demonstrated for intersex, although they are plausible. It should be noted that the concentrations of E2 and EE2 in the low oestrogen treatment were below their reported LOECs of 0.75 ng/L EE2 (Zhao et al., 2014) and 10 ng/L E2 for intersex induction (Metcalfe et al., 2001). In comparison, E1 was detected above its LOEC of 10 ng/L (Metcalfe et al., 2001). However, the 10 ng/L E1 exposure of Japanese medaka during early life only induced 5% intersex, whilst 100 ng/L induced 7% intersex in the same study. In comparison, a much greater intersex incidence of 24% of male fish was observed in experiment two in this study. However, without detailed concentration response curves from single chemical exposures it is not possible to mathematically prove additive effects for this endpoint in this study. Nonetheless, it remains clear that environmentally relevant concentrations of steroid oestrogens are major drivers of intersex induction.

In comparison, the environmentally relevant concentrations of anti-androgenic pharmaceuticals had no significant impact on the measured endpoints alone, when dosed without the addition of steroid oestrogens. Previous studies have found that these endpoints can be affected by anti-androgenic pharmaceuticals in single chemical exposure scenarios. However, the concentrations required to induce significant effects were in the microgram per litre range, 1000 times higher than the exposure concentrations in this study. For example, bicalutamide reduced tubercle prominence at 100 μg/L (Panter et al., 2012), whilst cyproterone acetate at concentrations up to 200 μg/L failed to have any significant impact on this endpoint (Ankley et al., 2010). Vitellogenin induction by anti-androgens has also been documented. One study induced around a six fold increase in male fathead minnow following exposed to 500 μg/L of the pure AR antagonist flutamide (Jensen et al., 2004). In the current study, vitellogenin appeared slightly elevated above the solvent control in the anti-androgen treatment and in the mixture treatment in comparison to oestrogens alone, but this was not statistically significantly higher. There are no other studies of bicalutamide available for comparison with this endpoint. However, there is some evidence to suggest that it may act at similar concentrations to flutamide in rodent studies and it is therefore plausible that concentrations in the microgram per litre range may be required to produce significant changes in vitellogenin in male fish (Chandolia et al., 1991; Maucher and Von Angerer, 1993). In contrast, cyproterone acetate reduced vitellogenin in female fathead minnow at 200 μg/L, possibly due to a reduction in endogenous sex steroids caused by its anti-gonadotrophic effects in addition to its AR antagonism (Ankley et al., 2010). By acting through both mechanisms, cyproterone acetate caused more pronounced effects than flutamide in mammalian models and in human trials for prostate cancer treatment at similar doses (Barradell and Faulds, 1994). Indeed, in fish studies, flutamide caused intersex in medaka above 200 μg/L (Kang et al., 2006; León et al., 2007) in comparison to cyproterone acetate at 1 and 10 μg/L (Kiparissis et al., 2003). At the concentrations employed in this study, cyproterone acetate has also been shown to reduce both the testicular synthesis and plasma concentrations of testosterone and 11-ketotestosterone in mummichog (Sharpe et al., 2004). However, this was not measured in this study.

An unexpected finding of experiment two was the heavily male biased sex ratio in the controls and all chemical treatments apart from the positive control. Sex ratio can be altered by chemical exposure (Örn et al., 2006) and it is used as an endpoint within the OECD's fish sexual development test (Test 234) (OECD, 2011). It has gained interest for regulatory purposes as an endpoint for identifying endocrine disruptors, since it can be considered to demonstrate both an adverse effect and an endocrine mode of action, particularly when used in conjunction with other endpoints, such as vitellogenin induction (Crane et al., 2010; Munn and Goumenou, 2013; Dang, 2014). However, sexual differentiation in fish can also be driven by environmental factors, including temperature, hypoxia and even changes in social hierarchy, depending on the species (Devlin and Nagahama, 2002). In medaka, thermal manipulation during the larval and embryo stage has been shown to induce masculinisation of genotypic XX females with exposure to increased temperature (Sato et al., 2005; Hattori et al., 2007; Kitano et al., 2012). This is thought to occur through an increase in cortisol in response to external temperature change, which was shown to cause suppression of germ cell proliferation, follicle stimulating hormone receptor expression and ovarian-type aromatase (cyp19a1), as well as inhibition of oogenesis (Hayashi et al., 2010; Kitano et al., 2012). However, the masculinisation effect induced by high temperature or cortisol treatment was shown to be prevented by co-exposure with E2, (Kitano et al., 2012). This provides a good explanation for male dominated sex ratios in this study, which could have been caused by temperature changes during media renewal in the embryonic phase, where MFS water was kept at room temperature and not under incubation. However, this could not be proven. Since E2 can challenge the masculinisation response to temperature changes (Kitano et al., 2012), statistical analysis of intersex induction and severity was rerun with female histopathology considered as an experimental response, with an intersex index of seven. However, the results were still no different from the original analysis.

Phenotypic males produced by temperature induced sex reversal have been found to have histologically normal, male testes and were capable of successfully reproducing (Hattori et al., 2007). Control fish in this study were also found to have histologically normal testes. This suggests that they still provide a sufficient platform for the induction of intersex by exogenous chemicals. Indeed, future experiments could even use temperature in a more controlled fashion to increase the *n* number of phenotypically male or female fish for assessment of sex reversal or intersex induction. Nonetheless, the male dominated sex ratios highlight the importance of maintaining experimental conditions during chemical exposure studies and recognising the possibility of non-chemical effects on this endpoint. It also supports the role of medaka in the fish sexual development test, since it has a well-defined genotypic sex determining gene (DMY) (Matsuda, 2005), which could be used to assess non-chemical sex reversal in controls.

Whilst we found no evidence that the anti-androgenic pharmaceuticals could enhance or diminish a response to oestrogen exposure, there still remains a possibility that oestrogenic and anti-androgenic contaminants could act on fish in combination in the environment, as indicated by Jobling et al. (2009). Indeed, compounds with similar mechanisms of action, such as AR antagonists and steroidogenesis inhibitors, have been shown to act additively or even synergistically *in vivo* on common endpoints in rodent models (Christiansen et al., 2009; Hotchkiss et al., 2010). In fish, flutamide has also been shown to increase the expression of oestrogen receptors β and γ in fathead minnow (Filby et al., 2007) and oestrogen receptor α in Murray rainbowfish (Bhatia et al., 2014), suggesting that there may be some common modes of action between AR antagonists and oestrogens. Crucially, preliminary data from one study has reported an increase in the incidence of ovarian cavities

in juvenile roach following exposure to a combination of anti-androgens identified in STW effluents (by Rostkowski et al., 2011) and steroid oestrogens (Lange et al., 2012). It may be that the total anti-androgenic activity present in the environment, representing a larger number of compounds, could cause sexual disruption in fish in combination with oestrogens. However, so far attempts to characterise anti-androgenic activity in environmental samples has found that the identified anti-androgenic compounds cannot explain the total activity detected (Urbatzka et al., 2007; Kinani et al., 2010). It may be that a large number of compounds are contributing, of which the anti-androgenic pharmaceuticals are a factor. Indeed, preliminary results from the Tox21 programme found that almost 10% of the 1,462 compounds tested were androgen receptor antagonists *in vitro,* which supports the possibility of a large number of contributors to environmental activity (Tice et al., 2013). Consequently, further study is warranted to identify significant environmental anti-androgens and to determine their environmental impacts, particularly with respect to the possibility of a multi-causal aetiology to sexual disruption observed in wild fish.

5. Conclusions

Hydrological modelling predicted that bicalutamide and cyproterone acetate are likely to be widespread contaminants in rivers in England and Wales. In a majority of cases, their concentrations are likely to occur below 10 ng/L, but at many "hot spots" concentrations are likely to be higher, in some cases exceeding 100 ng/L for bicalutamide. However, exposures of fish to these high environmental concentrations suggested that they are not likely to be a threat to fish reproductive health. Indeed, it is likely that concentrations one magnitude higher than those predicted to occur in the environment are required to induce significant responses associated with feminisation of wild fish. However, given the evidence for additive effects of anti-androgenic chemicals in whole organisms, these environmental contaminants should be considered as part of the wider issue of anti-androgenic activity in the environment. Critically, this study demonstrates that a mixture of steroid oestrogens, at concentrations present in the aquatic environment, can induce intersex at a rate comparable with that observed in UK and European rivers. Additional exposures to anti-androgenic pharmaceuticals known to co-occur with these oestrogens did not exacerbate the incidence or severity of intersex. Taken together, these data support the role of steroid oestrogens as major contributors to intersex in wild fish.

Acknowledgements

The authors thank Brunel University, DEFRA and the Commonwealth Scientific and Industrial Research Organisation for funding as part of the UK-Australian collaboration on Endocrine Disruptors. Thanks are also extended to Professor Taisen Iguchi and Dr Norihisa Tatarazako for the provision of Japanese medaka, as well as Nicola Beresford and the animal husbandry team at Brunel University and Emilie Cope of Severn Trent Water for their assistance in this project.

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.2014.12.022.

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