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The agricultural contaminant 17β-trenbolone disrupts male-male competition in the guppy (*Poecilia reticulata*)

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HIGHLIGHTS

- 17β-trenbolone (TB) is a widespread agricultural contaminant used in cattle farming.
- Male guppies were exposed to TB at an environmentally relevant level for 21 days.
- TB increased male aggression towards a rival and decreased courting of a female.
- Males exposed to TB performed more 'sneak' mating attempts towards females.
- First study to show disruption of male-male competition by exposure to TB.

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ABSTRACT

Despite a growing literature highlighting the potential impact of human-induced environmental change on mechanisms of sexual selection, relatively little is known about the effects of chemical pollutants on male-male competition. One class of environmental pollutant likely to impact male competitive interactions is the endocrine-disrupting chemicals (EDCs), a large and heterogeneous group of chemical contaminants with the potential to influence morphology, physiology and behaviour at minute concentrations. One EDC of increasing concern is the synthetic, androgenic steroid 17β-trenbolone, which is used globally to promote growth in beef cattle. Although 17β-trenbolone has been found to cause severe morphological and behavioural abnormalities in fish, its potential impact on male-male competition has yet to be investigated. To address this, we exposed wild male guppies (*Poecilia reticulata*) to an environmentally realistic concentration of 17β-trenbolone (average measured concentration: 8 ng/L) for 21 days using a flow-through system. We found that, in the presence of a competitor, 17β-trenbolone-exposed males carried out more frequent aggressive behaviours towards rival males than did unexposed males, as well as performing less courting behaviour and more sneak (i.e., coercive) mating attempts towards females. Considering that, by influencing mating outcomes, male-male competition has important consequences for population dynamics and broader evolutionary processes, this study highlights the need for greater understanding of the potential impact of EDCs on the mechanisms of sexual selection.

1. Introduction

In many species, competition between males for access to potential mates is a key mechanism of sexual selection (Darwin, 1871). Male-male competition plays a pivotal role in the maintenance and exaggeration of male traits and behaviours (Andersson, 1994; Berglund et al., 1996), and has important consequences for both male mating success (Møller and Jennions, 2001) and female fitness (Fisher et al., 2006). It is now well established that anthropogenic changes to the environment can interfere with male-male

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competition by compromising the transmission and/or reception of male sexual signals (reviewed in Wong and Candolin, 2015). Increased urban noise, for example, is causing male great tits (*Parus major*) to sing at a higher minimum frequency (Slabbekoorn and Peet, 2003), while anthropogenically induced water turbidity is allowing male three-spined sticklebacks (*Gasterosteus aculeatus*) to signal dishonestly, thereby increasing the likelihood of females mating with poor-quality suitors (Wong et al., 2007). However, despite a growing literature documenting the effects of human-induced environmental change on mechanisms of sexual selection, relatively little is known about the potential impacts of an altered chemical environment on male-male competition. This is surprising given the increasing prevalence of chemical pollutants in the environment and the severe impact that chemical pollution can have on morphology, physiology and behaviour (reviewed in Vos et al., 2000; Clotfelter et al., 2004; Frye et al., 2012).

Endocrine-disrupting chemicals (EDCs) are one class of chemical pollutant with the potential to interfere with male-male competition. Endocrine disruptors are a large and highly heterogeneous group of chemicals capable of altering hormonal signalling by blocking, mimicking or modulating the production, release, transport, metabolism, binding, action and/or elimination of natural hormones (Kavlock et al., 1996; Lintelmann et al., 2003; Buchanan and Partecke, 2012). This group includes both natural (e.g., phytoestrogens, Cederroth et al., 2012) and synthetic compounds (e.g., plastics, pesticides and pharmaceuticals, Diamanti-Kandarakis et al., 2009), which enter the environment from a range of sources, including industrial and domestic wastewater, as well as agricultural run-off (Johnson and Sumpter, 2001; Thorpe et al., 2009). Endocrine disruptors pose an insidious threat to wildlife, resulting from their ubiquity in the environment and tendency to bioaccumulate (WHO/UNEP, 2013), potential to act transgenerationally (Anway and Skinner, 2006; Crews et al., 2007; Walker and Gore, 2011) and ability to affect organisms at extremely low concentrations (Diamanti-Kandarakis et al., 2009). Although studies investigating the environmental impacts of EDCs have conventionally focused on their morphological and physiological effects, a growing body of research has begun to highlight the potential behavioural impacts of EDC exposure (reviewed in Clotfelter et al., 2004; Zala and Penn, 2004; Frye et al., 2012). As a result, it is becoming increasingly apparent that behavioural abnormalities induced by exposure to EDCs can often manifest at concentrations that are much lower than those required to induce morphological and physiological change, meaning that behaviour can serve as a particularly sensitive biomarker for EDC contamination (reviewed in Melvin and Wilson, 2013). For example, we now know that exposure to various EDCs at environmentally realistic levels can have severe detrimental impacts on male reproductive behaviour in fish (e.g., Salierno and Kane, 2009; Saaristo et al., 2010; Bertram et al., 2015). However, very few studies have investigated how these behavioural anomalies may manifest in a competitive setting.

Hormonal growth promotants (HGPs) are natural and synthetic chemicals used to stimulate growth in beef cattle by specifically targeting the endocrine system (Johnson, 2015). Hormonal growth promotants are used in many beef-producing countries worldwide, including the United States, Canada, Mexico, South Africa, Chile, Japan, New Zealand and Australia (Hunter, 2010; Kolodziej et al., 2013; Johnson, 2015), and commonly include formulations of androgens, estrogens and/or progestins (Lange et al., 2001; Hunter, 2010). The androgenic steroid most commonly administered in HGP implants is trenbolone acetate (Hunter, 2010), a highly efficient synthetic steroid with 15–50 times the androgenic and anabolic potency of testosterone (Neumann, 1976; Kolodziej et al., 2013). Trenbolone acetate is hydrolysed in the cattle to form various metabolites, including the potent androgen receptor agonist 17β-trenbolone (Khan et al., 2008; Parker et al., 2012), which is detectable in solid dung and liquid manure from implanted cattle, where it is highly persistent (half-life: ~260 days measured in animal waste, Schiffer et al., 2001). After often being allowed to enter the environment, 17β-trenbolone can accumulate in aquatic habitats and has been detected at concentrations ranging from ≤1–20 ng/L in diffuse run-off and discharge (Durhan et al., 2006), to as high as 162 ng/L in ditch networks associated with agricultural fields receiving animal waste (Gall et al., 2011).

It is now well established that exposure to 17β-trenbolone can cause severe morphological and physiological abnormalities in fish, including modified gonadal morphology (Örn et al., 2006), altered body condition (Bertram et al., 2015), reduced fecundity (Ankley et al., 2003) and even female-to-male sex reversal (Larsen and Baatrup, 2010; Morthorst et al., 2010). Exposure to 17β-trenbolone can also impact behaviour, with several studies revealing that environmentally realistic exposure levels can alter reproductive behaviour in female mosquitofish (*Gambusia holbrooki*, Saaristo et al., 2013) and disrupt female mate choice in guppies (*Poecilia reticulata*, Tomkins et al., 2016). Further, recent research has shown that exposure to 17β-trenbolone can alter coercive mating behaviour in male guppies individually exposed to females (Bertram et al., 2015). However, the response of males in the presence of a competitor remains to be investigated, despite the fact that the more common (and realistic) scenario in wild animal populations is for males to compete for mating opportunities.

Guppies are a small, viviparous, freshwater fish native to north-eastern South America that have a global distribution as a result of numerous deliberate and accidental introductions (Lindholm et al., 2005). Male guppies possess a modified anal fin known as a gonopodium, which acts as an intromittent organ. Males achieve copulations via two alternate mating strategies: elaborate courtship displays employed to solicit consensual copulations from females, and sneak attempts, which involve the male sneaking up from behind the female and thrusting his gonopodium towards the female's genital pore in an attempt to mate coercively (Luyten and Liley, 1985). Further, male guppies will actively chase and nip at rivals to monopolise potential mates (Gorlick, 1976; Magurran and Seghers, 1991). Female guppies are choosy and can favour a number of male traits, including greater orange colouration (i.e., area and chroma, Endler, 1980; Brooks and Caithness, 1995), as well as increased male body size (Reynolds and Gross, 1992) and courtship display rate (Kodric-Brown and Nicoletto, 2001). In the wild, multiple male guppies often compete for the attention of a single female (Houde, 1997), meaning that investigations into the impact of 17β-trenbolone on male reproductive behaviour in a competitive setting are ecologically meaningful. Guppies are also known to inhabit polluted waterways (e.g., López-Rojas and Bonilla-Rivero, 2000; Widianarko et al., 2000), making them an ideal candidate for investigating the impact of endocrine disruptors on mechanisms of sexual selection.

Here, we test the hypothesis that short-term exposure to an environmentally realistic concentration of 17β-trenbolone will alter male guppy competitive mating interactions by influencing male reproductive behaviour and aggression. Given that, as aforementioned, exposure to 17β-trenbolone has been shown to affect coercive mating behaviour in male guppies when a single male is presented with a single female (i.e., in a one-on-one scenario, Bertram et al., 2015), we expected that 17β-trenbolone exposure would also disrupt male reproductive behaviour in the more environmentally realistic scenario of two males competing for a single female. Further, although the impacts of water-borne exposure to 17β-trenbolone on aggressive behaviour were previously unknown, circulating levels of endogenous androgens are potent mediators of male aggressive behaviour and dominance (Taves

et al., 2009; Nelson, 2011). Therefore, we hypothesised that exposure to 17β-trenbolone would result in an increase in male aggressive behaviours in a competitive setting.

2. Methods

2.1. Animal housing

Guppies were collected with dip nets from Alligator Creek (19° 26′ 18″ S, 146° 57′ 01″ E), a pristine rainforest-fed stream located within Bowling Green Bay National Park, Queensland, Australia. Water samples drawn from this site over consecutive years revealed no contamination with 17β-trenbolone (ALS Group, unpublished data). Fish were acclimated to laboratory conditions (25–27 °C, 12:12 h light:dark cycle) for 2 months prior to exposure and were fed *ad libitum* once daily with commercial fish pellets (Otohime Hirame larval diet, 580–910 μm).

2.2. Chemical exposure

After acclimation to laboratory conditions, male fish were exposed to 17β-trenbolone for 21 days, as previous experiments have shown that EDC exposure periods ranging from 14–28 days are sufficient to induce behavioural changes in a variety of fish species (e.g., Bayley et al., 1999; Bell, 2001; Bjerselius et al., 2001; Majewski et al., 2002; Martinović et al., 2007; Maunder et al., 2007; Oshima et al., 2003; Saaristo et al., 2009a,b), including in guppies (Bertram et al., 2015; Tomkins et al., 2016). Further, EDCs often enter the environment in pulses and may only remain in waterways for short periods of time (Diamanti-Kandarakis et al., 2009), meaning that short-term exposure periods are ecologically meaningful.

Male guppies were exposed to 17β-trenbolone via a flow-through system, based on the design of Saaristo et al. (2013), Bertram et al. (2015) and Martin et al. (2017), with some modifications. This system included four identical aquaria (54 L, 60 × 30 × 30 cm), consisting of two control (unexposed) tanks and two 17β-trenbolone-exposed tanks. A total of 100 sexually mature male guppies were distributed randomly between these four aquaria (25 males per tank). To achieve the desired 17β-trenbolone concentration in the exposure tanks, flow rates were kept constant (2.25 L/h) using flow meters (BES, MPB Series 1200), with 100% of the water in each exposure tank turned over each day. The exposed tanks contained 17β-trenbolone at an average measured concentration of 8 ng/L (see 'Monitoring of 17β-trenbolone' below for details of chemical analyses), while the control tanks contained only fresh water. Exposure tanks were maintained in an identical manner as described for the housing period.

2.3. Monitoring of 17β-trenbolone

A stock solution was created by firstly dissolving 17β-trenbolone (17β-hydroxyestra-4,9,11-trien-3-one; CAS: 10161-33-8; Novachem, Germany) in ethanol (HPLC grade, ≥99.99%) at 300 mg/L, which was then diluted to 300 μg/L using deionised water. This stock solution was further diluted in the flow-through system's mixing tank (162 L, 90 × 45 × 40 cm) to achieve the desired 17β-trenbolone concentration (mean = 7.70 ng/L, SD = 4.40, n = 6). Stock solutions were created weekly to prevent any potential degradation of 17β-trenbolone over the exposure period.

In order to monitor 17β-trenbolone concentrations in the exposure tanks and ensure the absence of contamination of control tanks, a 100 mL water sample was drawn from all tanks weekly and analysed using a commercial enzyme-linked immunosorbent assay (ELISA). Water samples were acidified by adding a mixture of 1% acetic acid in methanol, then loaded onto a conditioned solid-phase cartridge (Strata-X 33 μm, 500 mg/6 mL; Phenomenex, Torrance, CA, USA). The cartridge was then eluted with methanol (2 × 4 mL), with the eluate dried under a nitrogen stream. Samples were reconstituted with 100 μL methanol and 900 μL of deionised water.

Measurement of 17β-trenbolone concentrations was undertaken using commercial ELISA kits, in accordance with the manufacturer's instructions, with a minor modification (Trenbolone ELISA kit; EuroProxima, Arnhem, the Netherlands). In short, a total of thirty samples and trenbolone calibration standards (freshly made in 10% methanol) were dispensed (50 μL) in duplicate into an antibody-coated 96-well plate by an auto dispenser (epMotion 5070; Eppendorf, Hamburg, Germany). Thereafter, 25 μL of HRPO conjugate and 25 μL of antibody were dispensed into the wells. After incubating in darkness for 1 h at room temperature, the plate was washed three times with wash buffer by a microplate washer (Atlantis; ASYS HITECH, Eugendorf, Austria) and 100 μL of substrate was added to all wells. The plate was then incubated for a further 30 min at room temperature in the dark. Finally, 100 μL of stop solution was dispensed into all wells, and the absorbance of the solutions in the wells measured at 450 nm by a microplate reader (UVM 340; ASYS HITECH, Eugendorf, Austria). Calculation of sample concentrations was undertaken by 4 parameter logistics method after creating a calibration curve using a series of standard calibration solutions (0, 0.125, 0.25, 0.5, 1.0, 5.0 μg/L) made up in 10% methanol. In order to verify calibration accuracy, check standards (i.e., standards from the kit run as samples) were run in duplicate on each ELISA plate during each ELISA test. The detection limit of the Trenbolone ELISA kit was 1.8 ng/L. A spike recovery experiment was conducted in triplicate using a 5 ng/L 17β-trenbolone solution. The average recovery was 97%, providing confidence that 17β-trenbolone in the water samples was efficiently extracted, and that measured values were neither under nor over estimates of sample concentrations. The ELISA plate intra- and inter-variability were 0.040 and 0.231, respectively.

2.4. Behavioural trials

After 21 days of exposure, male guppies were taken at random and equally from each exposure tank and allocated to behavioural trials, which were carried out in two stages. In the first, a 27 L tank (30 × 30 × 30 cm) was divided into two compartments using a transparent plastic divider with small holes throughout to allow visual and chemical contact between compartments. Trials in the first stage involved a single stimulus female being placed into the first compartment (10 × 30 × 30 cm), while one exposed and one unexposed male were placed into the second compartment (20 × 30 × 30 cm). All stimulus females were unexposed and sexually mature, and were maintained under the same housing conditions as males, with one stimulus female being used per behavioural trial. After a 5 min acclimation period—during which all fish were isolated in separate containers within their respective zones—fish were released and males were allowed to interact with the stimulus female for 15 min through the divider. At the conclusion of the first stage, the divider was removed remotely and the fish were allowed to interact freely for a further 15 min. This second stage of the experiment allowed us to observe potential differences in male sneaking behaviour, which could not be assessed when the divider was in position. All trials (n = 37) were filmed using a digital video camera (Canon PowerShot S120), with each trial video being watched twice to quantify the behaviour of either male. We were able to distinguish between unexposed and exposed males by noting which holding containers they emerged from after the 5-min acclimation period. Fish were euthanised at the conclusion of the second stage of behavioural trials using an

overdose of anaesthetic clove oil (40 mg/L), before immediately being weighed, measured and photographed for morphological and colouration analysis (see 'Morphological analysis' below).

To quantify male behaviour, we used the event-recording software JWatcher V1.0 (Blumstein and Daniel, 2007). For the first stage of behavioural trials, for either male, we quantified courtship behaviour (i.e., number of sigmoid display bouts, Houde, 1997), aggressive behaviour (i.e., number of chases and fin-nips, Houde, 1997) and the total time spent in the female preference zone (i.e., within 5 cm of the female compartment). For the second stage of behavioural trials, we quantified either male's courtship behaviour (i.e., number of sigmoid display bouts), aggressive behaviour (i.e., total number of chases and fin-nips directed towards either the rival male or the female) and sneak mating attempts (i.e., number of attempted coercive matings).

2.5. Morphological analysis

Male guppies, as well as unexposed stimulus females, were weighed (±0.0001 g) and measured for total length (±0.01 mm) immediately after behavioural trials. Males were also photographed on their right side in a standardised fashion (Nikon D90, shutter speed = 1/250, Nikon AF Micro-Nikkor 60 mm f/2.8D) and the resultant images analysed using Photoshop (CS6 version 13.0 Extended) to determine the percentage of each male's body area containing orange pigmentation. For a detailed description of the colouration analysis method, see Bertram et al. (2015).

2.6. Statistical analysis

Data were analysed using R version 2.13.1 (R Core Team, 2013). Data were checked for normality (Shapiro-Wilk test) and homogeneity of variance (Fligner-Killeen test), and were transformed where necessary in order to approximate normality. Generalised linear models (GLMs) were used to compare the behaviour of exposed and unexposed males using a suite of biologically meaningful predictors, including: male weight (g), male total length (mm) and male area of orange pigmentation (%). Mann-Whitney *U* tests were used to evaluate whether exposure to 17β-trenbolone altered male weight, total length or area of orange colouration (%).

3. Results

3.1. Aggressive behaviour

Exposed males conducted significantly more frequent aggressive behaviours towards rival males than did unexposed males, both when separated from females by a divider ($z = 4.80$, $p < 0.001$, Fig. 1a) and when allowed to interact with females freely ($z = 5.50$, $p < 0.001$, Fig. 1b). However, no significant difference was detected in the frequency of aggressive behaviours carried out by unexposed and exposed males towards females when allowed to interact with females freely ($z = 5.64$, $p = 0.092$, data not shown).

3.2. Mating behaviours

When allowed to interact with females through a partition, no significant difference was detected in the total time spent by unexposed and exposed males in the female preference zone ($z = 7.26$, $p = 0.081$, data not shown). However, unexposed males performed courting behaviour more frequently than exposed males, both when separated from females by a divider ($z = 4.71$, $p < 0.001$, Fig. 2a) and when allowed to interact with females freely ($z = 4.37$, $p < 0.001$, Fig. 2b). Exposed males, on the other hand, conducted significantly more sneak mating attempts than unexposed males when allowed to interact freely with females ($z = 2.87$, $p = 0.004$, Fig. 3). More generally, regardless of the absence or presence of a partition, the number of courting events performed by males towards females was positively associated with both male percentage area of orange coloration ($z = 9.23$, $p < 0.001$, Fig. 4a) and male weight ($z = 6.74$, $p < 0.001$, Fig. 4b).

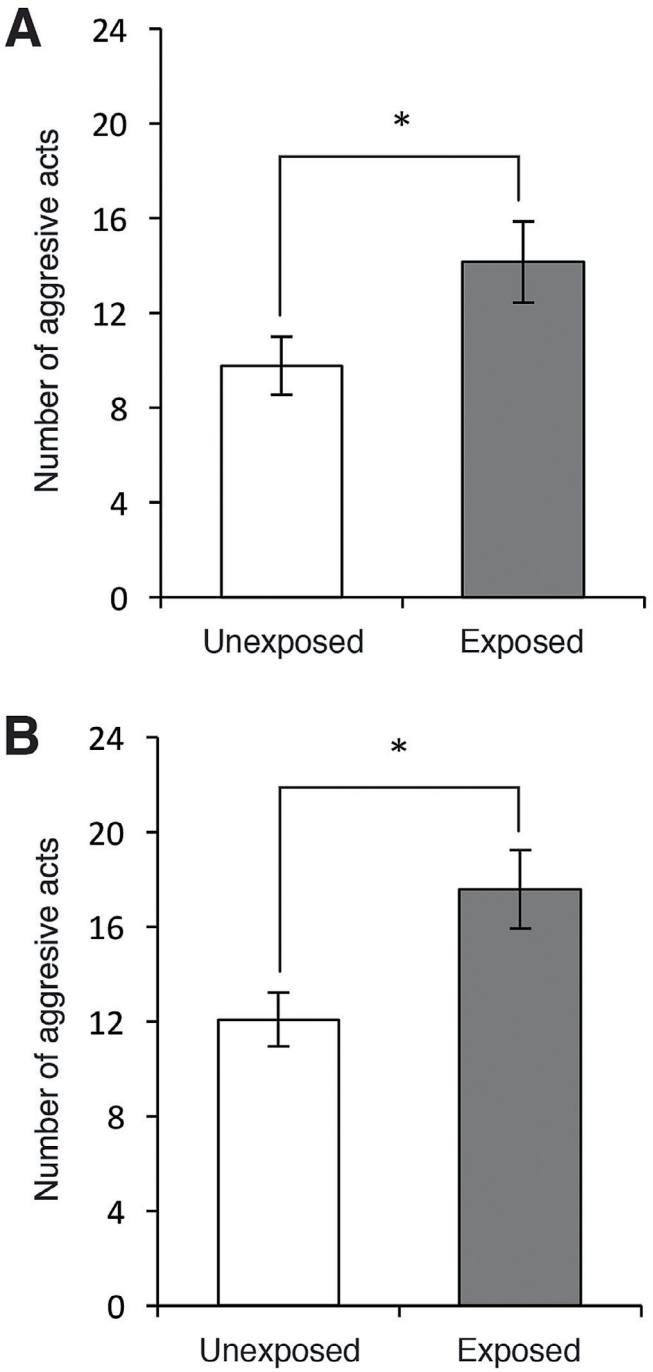

Fig. 1. Mean (±SE) number of aggressive acts (i.e., chases and fin nips) directed by a male towards a rival when A) males were separated from females by a transparent partition ($n = 37$), and B) males were allowed to interact freely with females ($n = 37$). Asterisks indicate a significant difference between groups at $\alpha = 0.05$.

3.3. Morphology

No significant difference was detected in weight (Mann-

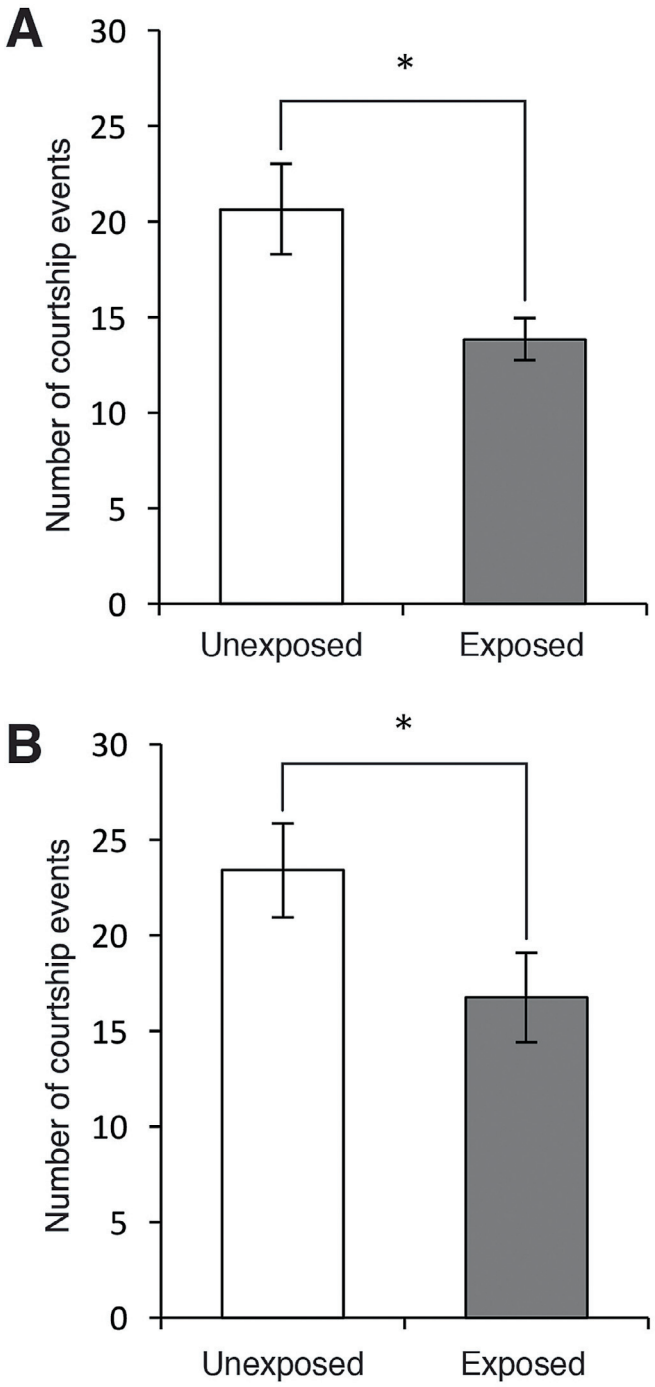

Fig. 2. Mean (±SE) number of courtship events conducted by males when A) males were separated from females by a transparent partition ($n = 37$), and B) males were allowed to interact freely with females ($n = 37$). Asterisks indicate a significant difference between groups at $\alpha = 0.05$.

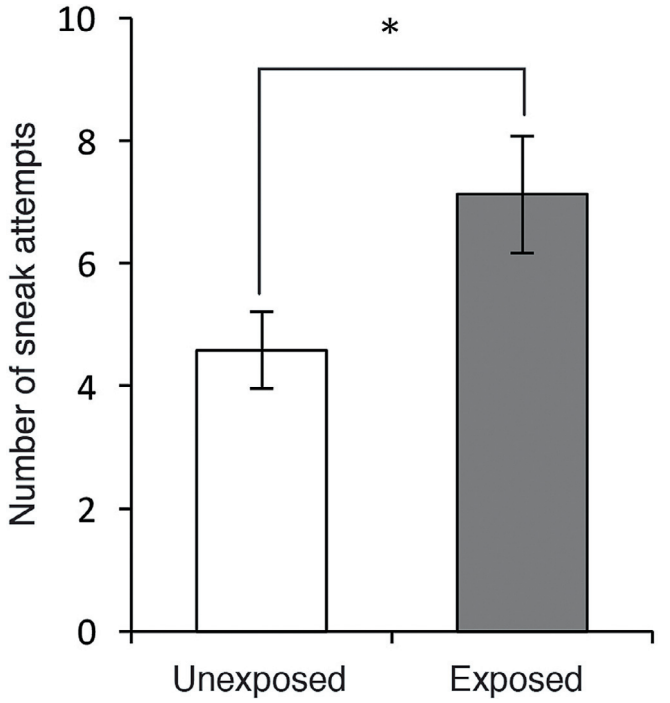

Fig. 3. Mean (±SE) number of sneak mating attempts conducted by males towards females when allowed to freely interact ($n = 37$). Asterisk indicates a significant difference between groups at $\alpha = 0.05$.

Whitney $U = 848$, $p = 0.544$), total length ($U = 897$, $p = 0.391$) or percentage area of orange pigmentation ($U = 1001$, $p = 0.811$) between unexposed and exposed males.

4. Discussion

We found that exposure to an environmentally realistic concentration of 17β-trenbolone significantly altered competitive mating behaviour in male guppies. When separated from a stimulus female by a divider, exposed males were more aggressive towards rival males and courted less than unexposed males. When allowed to interact freely with a stimulus female, exposed males were again more aggressive and courted less than unexposed males, as well as performing significantly more frequent sneak mating attempts towards females. More generally, we found that the number of courtship events performed by males was positively associated with both male percentage area of orange colouration and weight. This was not surprising, as previous research conducted on this guppy population has shown that an increase in male courtship behaviour was correlated with both increased male percentage area of orange pigmentation and condition index (Bertram et al., 2015). Here, we show for the first time that exposure to an androgenic EDC at concentrations present in aquatic ecosystems can impact male reproductive behaviour in a competitive setting.

In teleost fish, androgens are essential to the development and maintenance of male traits (Borg, 1994; Munakata and Kobayashi, 2010). The androgen receptor (AR) is activated via binding of natural hormones, such as testosterone, which influence the hypothalamic-pituitary-gonadal axis (Borg, 1994; Munakata and Kobayashi, 2010). As a potent androgen receptor agonist (Rogozkin, 1991), 17β-trenbolone binds with high affinity to available androgen receptors, mimicking the effects of endogenous androgens (Wilson et al., 2002). Further, 17β-trenbolone—which is non-aromatisable—may indirectly inhibit the production of 17β-estradiol by limiting the production of testosterone and, thus, restricting the aromatisation of testosterone to 17β-estradiol (Zhang et al., 2008). Concordantly, in females, exposure to 17β-trenbolone can influence concentrations of plasma steroids (testosterone and β-estradiol) and vitellogenin (Ankley et al., 2003), cause vaginal agenesis, increased anogenital distance and the induction of male sex accessory tissues (Hotchkiss et al., 2008), as well as stimulate the development of male morphological

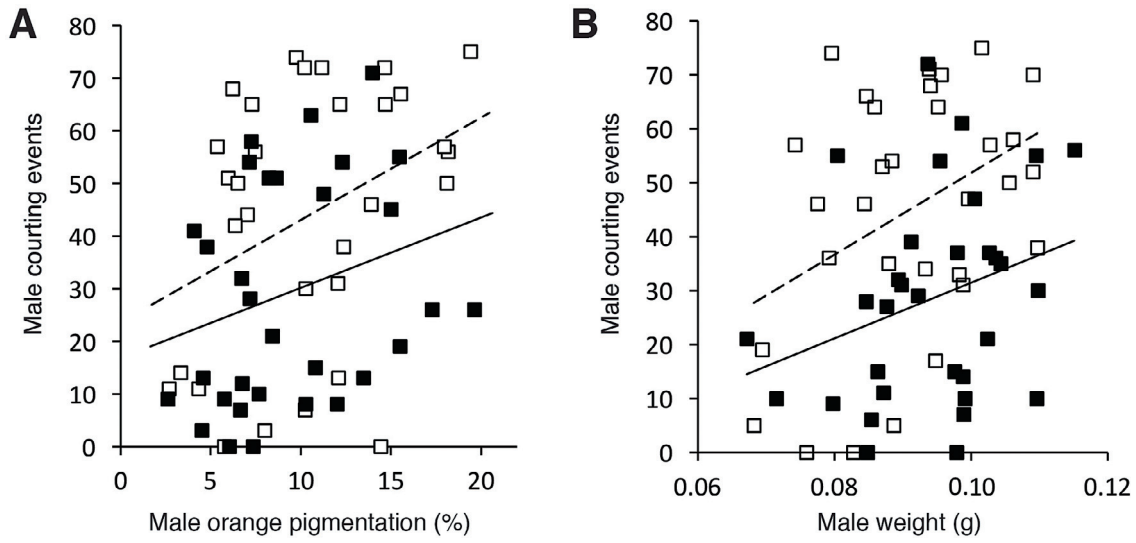

Fig. 4. Number of courtship events performed by unexposed and exposed males as a function of A) male orange pigmentation (% of body area) and B) male weight (g). Figures represent combined data from both behavioural trial stages (i.e., when males were separated from females by a transparent partition and when allowed to interact freely with females). Unfilled squares and dashed trend lines represent unexposed males, while filled squares and solid trend lines represent exposed males.

characteristics (Ankley et al., 2003). But how might exposure to 17β-trenbolone influence behaviour?

As emphasised in several reviews (Clotfelter et al., 2004; Zala and Penn, 2004; Melvin and Wilson, 2013), behaviour can be an especially sensitive and comprehensive biomarker of EDC exposure. In contrast to standard laboratory assays, which often target a small suite of morphological and/or physiological endpoints, behaviour is the manifestation of numerous complex developmental and biochemical processes. Although the exact mechanisms underpinning the presently observed behavioural changes in 17β-trenbolone-exposed males are not yet wholly understood, exposure to 17β-trenbolone is likely to intensify behaviours under androgenic control. Indeed, exposure to other androgenic endocrine disruptors has been found to increase androgen-dependent male mate calling behaviour in African clawed frogs (*Xenopus laevis*, Hoffmann and Kloas, 2012) and intensify male sexual behaviour in various cyprinid fish species (Belanger et al., 2010). In the present study, 17β-trenbolone-exposed male guppies were more aggressive towards rivals than were unexposed males, which is likely a result of 17β-trenbolone-induced 'hyper-masculinisation'. Further, considering that virtually all male reproductive-related behaviours are under androgenic control (Rubinow and Schmidt, 1996; Cunningham et al., 2012), we would expect 17β-trenbolone exposure to have also resulted in increased male courtship behaviour, but this was not the case.

Males exposed to 17β-trenbolone courted less than unexposed males, both when separated from a female by a transparent divider and when allowed to interact freely with the female. This is surprising, as recent research investigating the effects of exposure to 17β-trenbolone on reproductive behaviour in guppies has reported that exposure did not significantly impact the total number of courtship events performed by males (Bertram et al., 2015) or the total time males spent courting (Tomkins et al., 2016). However, these studies both tested the impact of exposure to 17β-trenbolone on male behaviour in the absence of a rival male, suggesting that 17β-trenbolone-induced differences in male courtship may only manifest in a competitive setting. Further, we found that exposed males conducted more aggressive behaviour towards rival males, but not towards females. This suggests that the presence of a sexual competitor may incite heightened levels of aggression amongst 17β-trenbolone-exposed males, which may, in turn, limit the amount of time spent by these males courting (Kangas and Lindström, 2001; Wong, 2004). This finding highlights the importance of utilising competitive scenarios when investigating the potential impact of EDCs on male reproductive behaviour.

When allowed to interact freely with a female, exposed males conducted significantly more sneak mating attempts than unexposed males. This is consistent with previous research conducted by Bertram et al. (2015), where 17β-trenbolone exposure was linked with an increase in this unsolicited male mating behaviour in a one-on-one situation (i.e., a single male paired with a single female). Previous research has shown that male guppies transfer approximately one third as much sperm during sneak copulations compared to copulations preceded by courtship (Pilastro and Bisazza, 1999), meaning that an increase in sneak mating behaviour is likely to impact male mating success. This behavioural shift could also have consequences for female fitness, as increased male sexual harassment has been found to negatively impact the foraging efficiency of female poeciliids (Pilastro et al., 2003). Further, the increased coercive mating attempts and decreased courtship behaviour observed amongst 17β-trenbolone-exposed males could have consequences at the population level, as this circumvention of female mate choice can have a direct impact on both the quality and quantity of offspring produced (Wong and Candolin, 2005).

In conclusion, this is the first study to demonstrate that exposure to an androgenic endocrine disruptor can alter male-male competition. We found that males exposed to an environmentally realistic concentration of 17β-trenbolone performed less courting behaviour and attempted more sneak copulation attempts than unexposed males, as well as conducting more frequent aggressive behaviours towards a rival male. Competitive interactions between males have important consequences for population dynamics and broader evolutionary process, highlighting the importance of understanding the potential impact of EDCs on male-male competition.

Ethics

This study was conducted with the approval of the Biological

Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2013/09) and observed all relevant State and Federal laws of Australia.

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Conflict of interest statement

The authors declare no competing interests.

References

Andersson, M., 1994. Sexual Selection. Princeton University Press, USA.
Ankley, G.T., Jensen, K.M., Makynen, E.A., Kahl, M.D., Korte, J.J., Hornung, M.W., Henry, T.R., Denny, J.S., Leino, R.L., Wilson, V.S., Cardon, M.C., Hartig, P.C., Gray, L.E., 2003. Effects of the androgenic growth promoter 17-β-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. Environ. Toxicol. Chem. 22, 1350–1360.
Anway, M.D., Skinner, M.K., 2006. Epigenetic transgenerational actions of endocrine disruptors. Endocrinology 147, 43–49.
Bayley, M., Nielsen, J.R., Baatrup, E., 1999. Guppy sexual behaviour as an effect biomarker of estrogen mimics. Ecotoxicol. Environ. Saf. 43, 68–73.
Belanger, R.M., Pachkowski, M.D., Stacey, N.E., 2010. Methyltestosterone-induced changes in electro-olfactogram responses and courtship behaviors of cyprinids. Chem. Senses 35, 65–74.
Bell, A.M., 2001. Effects of an endocrine disrupter on courtship and aggressive behaviour of male three-spined stickleback, *Gasterosteus aculeatus*. Anim. Behav. 62, 775–780.
Berglund, A., Bisazza, A., Pilastro, A., 1996. Armaments and ornaments: an evolutionary explanation of traits of dual utility. Biol. J. Linn. Soc. 58, 385–399.
Bertram, M.G., Saaristo, M., Baumgartner, J.B., Johnstone, C.P., Allinson, M., Allinson, G., Wong, B.B.M., 2015. Sex in troubled waters: widespread agricultural contaminant disrupts reproductive behaviour in fish. Horm. Behav. 70, 85–91.
Bjerselius, R., Lundstedt-Enkel, K., Olsén, H., Mayer, I., Dimberg, K., 2001. Male goldfish reproductive behaviour and physiology are severely affected by exogenous exposure to 17β-estradiol. Aquat. Toxicol. 53, 139–152.
Blumstein, D.T., Daniel, J.C., 2007. Quantifying Behaviour the JWatcher Way. Sinauer Associates, USA.
Borg, B., 1994. Androgens in teleost fishes. Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol. 109, 219–245.
Brooks, R., Caithness, N., 1995. Female choice in a feral guppy population: are there multiple cues? Anim. Behav. 50, 301–307.
Buchanan, K.L., Partecke, J., 2012. The endocrine system: can homeostasis be maintained in a changing world? In: Candolin, U., Wong, B.B.M. (Eds.), Behavioural Responses to a Changing World. Oxford University Press, UK, pp. 159–174.
Cederroth, C.R., Zimmermann, C., Nef, S., 2012. Soy, phytoestrogens and their impact on reproductive health. Mol. Cell. Endocrinol. 355, 192–200.
Clotfelter, E.D., Bell, A.M., Levering, K.R., 2004. The role of animal behaviour in the study of endocrine-disrupting chemicals. Anim. Behav. 68, 665–676.
Crews, D., Gore, A.C., Hsu, T.S., Dangleben, N.L., Spinetta, M., Schallert, T., Anway, M.D., Skinner, M.K., 2007. Transgenerational epigenetic imprints on mate preference. Proc. Natl. Acad. Sci. U.S.A. 104, 5942–5946.
Cunningham, R.L., Lumia, A.R., McGinnis, M.Y., 2012. Androgen receptors, sex behaviour, and aggression. Neuroendocrinology 96, 131–140.
Darwin, C., 1871. The Descent of Man, and Selection in Relation to Sex. John Murray, UK.
Diamanti-Kandarakis, E., Bourguignon, J.P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T., Gore, A.C., 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr. Rev. 30, 293–342.
Durhan, E.J., Lambright, C.S., Makynen, E.A., Lazorchak, J., Hartig, P.C., Wilson, V.S., Gray, L.E., Ankley, G.T., 2006. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. Environ. Health Perspect. 114, 65–68.
Endler, J.A., 1980. Natural selection on color patterns in *Poecilia reticulata*. Evolution 34, 76–91.
Fisher, D.O., Double, M.C., Blomberg, S.P., Jennions, M.D., Cockburn, A., 2006. Post-mating sexual selection increases lifetime fitness of polyandrous females in the wild. Nature 444, 89–92.
Frye, C.A., Bo, E., Calamandrei, G., Calzà, L., Dessì-Fulgheri, F., Fernández, M., Fusani, L., Kah, O., Kajta, M., Le Page, Y., Patisaul, H.B., Venerosi, A., Wojtowicz, A.K., Panzica, G.C., 2012. Endocrine disrupters: a review of some sources, effects, and mechanisms of actions on behaviour and neuroendocrine systems. J. Neuroendocrinol. 24, 144–159.
Gall, H.E., Sassman, S.A., Lee, L.S., Jafvert, C.T., 2011. Hormone discharges from a midwest tile-drained agroecosystem receiving animal wastes. Environ. Sci. Technol. 45, 8755–8764.
Gorlick, D.L., 1976. Dominance hierarchies and factors influencing dominance in the guppy *Poecilia reticulata* (Peters). Anim. Behav. 24, 336–346.
Hoffmann, F., Kloas, W., 2012. Effects of environmentally relevant concentrations of the xeno-androgen, methyldihydrotestosterone, on male and female mating behavior in *Xenopus laevis*. Chemosphere 87, 1246–1253.
Hotchkiss, A.K., Ankley, G.T., Wilson, V.S., Hartig, P.C., Durhan, E.J., Jensen, K.M., Martinovi, D., Gray, L.E., 2008. Of mice and men (and mosquitofish): anti-androgens and androgens in the environment. BioScience 58, 1037–1050.
Houde, A.E., 1997. Sex, Color, and Mate Choice in Guppies. Princeton University Press, USA.
Hunter, R.A., 2010. Hormonal growth promotant use in the Australian beef industry. Anim. Prod. Sci. 50, 637–659.
Johnson, A.C., Sumpter, J.P., 2001. Removal of endocrine-disrupting chemicals in activated sludge treatment works. Environ. Sci. Technol. 35, 4697–4703.
Johnson, R., 2015. The U.S.-EU Beef Hormone Dispute. Congressional Research Service. Available at: http://fas.org/sgp/crs/row/R40449.pdf.
Kangas, N., Lindström, K., 2001. Male interactions and female mate choice in the sand goby, *Pomatoschistus minutus*. Anim. Behav. 61, 425–430.
Kavlock, R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S., Lucier, G., Luster, M., Mac, M.J., Maczka, C., Miller, R., Moore, J., Rolland, R., Scott, G., Sheehan, D.M., Sinks, T., Tilson, H.A., 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. Environ. Health. Perspect. 104, 715–740.
Khan, S.J., Roser, D.J., Davies, C.M., Peters, G.M., Stuetz, R.M., Tucker, R., Ashbolt, N.J., 2008. Chemical contaminants in feedlot wastes: concentrations, effects and attenuation. Environ. Int. 34, 839–859.
Kodric-Brown, A., Nicoletto, P.F., 2001. Female choice in the guppy (*Poecilia reticulata*): the interaction between male color and display. Behav. Ecol. Sociobiol. 50, 346–351.
Kolodziej, E.P., Qu, S., Forsgren, K.L., Long, S.A., Gloer, J.B., Jones, G.D., Schlenk, D., Baltrusaitis, J., Cwiertny, D.M., 2013. Identification and environmental implications of photo-transformation products of trenbolone acetate metabolites. Environ. Sci. Technol. 47, 5031–5041.
Lange, I.G., Daxenberger, A., Meyer, H.H.D., 2001. Hormone contents in peripheral tissues after correct and off-label use of growth promoting hormones in cattle: effect of the implant preparations Filaplix-H, Raglo, Synovex-H and Synovex Plus. APMIS 109, 53–65.
Larsen, M.G., Baatrup, E., 2010. Functional behavior and reproduction in androgenic sex reversed zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 29, 1828–1833.
Lindholm, A.K., Breden, F., Alexander, H.J., Chan, W.K., Thakurta, S.G., Brooks, R., 2005. Invasion success and genetic diversity of introduced populations of guppies *Poecilia reticulata* in Australia. Mol. Ecol. 14, 3671–3682.
Lintelmann, J., Katayama, A., Kurihara, N., Shore, L., Wenzel, A., 2003. Endocrine disruptors in the environment (IUPAC technical report). Pure Appl. Chem. 75, 631–681.
López-Rojas, H., Bonilla-Rivero, A.L., 2000. Anthropogenically induced fish diversity reduction in Lake Valencia Basin, Venezuela. Biodivers. Conserv. 9, 757–765.
Luyten, P.H., Liley, N.R., 1985. Geographic variation in the sexual behaviour of the guppy, *Poecilia reticulata* (Peters). Behaviour 95, 164–179.
Magurran, A.E., Seghers, B.H., 1991. Variation in schooling and aggression amongst guppy (*Poecilia reticulata*) populations in Trinidad. Behaviour 118, 214–234.
Majewski, A.R., Blanchfield, P.J., Palace, V.P., Wautier, K., 2002. Waterborne 17α-ethynylestradiol affects aggressive behaviour of male fathead minnows (*Pimephales promelas*) under artificial spawning conditions. Water Qual. Res. J. Can. 37, 697–710.
Martin, J.M., Saaristo, M., Bertram, M.G., Lewis, P.J., Coggan, T.L., Clarke, B.O., Wong, B.B.M., 2017. The psychoactive pollutant fluoxetine compromises anti-predator behaviour in fish. Environ. Pollut. 222, 592–599.
Martinović, D., Hogarth, W.T., Jones, R.E., Sorensen, P.W., 2007. Environmental estrogens suppress hormones, behavior, and reproductive fitness in male fathead minnows. Environ. Toxicol. Chem. 26, 271–278.
Maunder, R.J., Matthiessen, P., Sumpter, J.P., Pottinger, T.G., 2007. Impaired reproduction in three-spined sticklebacks exposed to ethinyl estradiol as juveniles. Biol. Reprod. 77, 999–1006.
Melvin, S.D., Wilson, S.P., 2013. The utility of behavioral studies for aquatic toxicology testing: a meta-analysis. Chemosphere 93, 2217–2223.
Møller, A.P., Jennions, M.D., 2001. How important are direct fitness benefits of sexual selection? Naturwissenschaften 88, 401–415.
Morthorst, J.E., Holbech, H., Bjerregaard, P., 2010. Trenbolone causes irreversible masculinization of zebrafish at environmentally relevant concentrations. Aquat. Toxicol. 98, 336–343.
Munakata, A., Kobayashi, M., 2010. Endocrine control of sexual behavior in teleost fish. Gen. Comp. Endocrinol. 165, 456–468.
Nelson, R.J., 2011. An Introduction to Behavioral Endocrinology. Sinauer Associates, USA.
Neumann, F., 1976. Pharmacological and endocrinological studies on anabolic

agents. Environ. Qual. Saf. Suppl. 5, 253–264.
Örn, S., Yamani, S., Norrgren, L., 2006. Comparison of vitellogenin induction, sex ratio, and gonad morphology between zebrafish and Japanese medaka after exposure to 17α-ethinylestradiol and 17β-trenbolone. Arch. Environ. Contam. Toxicol. 51, 237–243.
Oshima, Y., Kang, I.J., Kobayashi, M., Nakayama, K., Imada, N., Honjo, T., 2003. Suppression of sexual behavior in male Japanese medaka (*Oryzias latipes*) exposed to 17β-estradiol. Chemosphere 50, 429–436.
Parker, J.A., Webster, J.P., Kover, S.C., Kolodziej, E.P., 2012. Analysis of trenbolone acetate metabolites and melengestrol in environmental matrices using gas chromatography-tandem mass spectrometry. Talanta 99, 238–246.
Pilastro, A., Bisazza, A., 1999. Insemination efficiency of two alternative male mating tactics in the guppy *Poecilia reticulata*. Proc. R. Soc. Lond. B 266, 1887–1891.
Pilastro, A., Benetton, S., Bisazza, A., 2003. Female aggregation and male competition reduce costs of sexual harassment in the mosquitofish *Gambusia holbrooki*. Anim. Behav. 65, 1161–1167.
R Core Team, 2013. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Reynolds, J.D., Gross, M.R., 1992. Female mate preference enhances offspring growth and reproduction in a fish, *Poecilia reticulata*. Proc. R. Soc. Lond. B 250, 57–62.
Rogozkin, V.A., 1991. Metabolism of Anabolic-androgenic Steroids. CRC Press, USA.
Rubinow, D.R., Schmidt, P.J., 1996. Androgens, brain, and behavior. Am. J. Psychiatry 153, 974–984.
Saaristo, M., Craft, J.A., Lehtonen, K.K., Björk, H., Lindström, K., 2009a. Disruption of sexual selection in sand gobies (*Pomatoschistus minutus*) by 17alpha-ethinyl estradiol, an endocrine disruptor. Horm. Behav. 55, 530–537.
Saaristo, M., Craft, J.A., Lehtonen, K.K., Lindström, K., 2009b. Sand goby (*Pomatoschistus minutus*) males exposed to an endocrine disrupting chemical fail in nest and mate competition. Horm. Behav. 56, 315–321.
Saaristo, M., Craft, J.A., Lehtonen, K.K., Lindström, K., 2010. An endocrine disrupting chemical changes courtship and parental care in the sand goby. Aquat. Toxicol. 97, 285–292.
Saaristo, M., Tomkins, P., Allinson, M., Allinson, G., Wong, B.B.M., 2013. An androgenic agricultural contaminant impairs female reproductive behaviour in a freshwater fish. PLoS One 8, e62782.
Salierno, J.D., Kane, A.S., 2009. 17α-Ethinylestradiol alters reproductive behaviors, circulating hormones, and sexual morphology in male fathead minnows (*Pimephales promelas*). Environ. Toxicol. Chem. 28, 953–961.
Schiffer, B., Daxenberger, A., Meyer, K., Meyer, H.H., 2001. The fate of trenbolone acetate and melengestrol acetate after application as growth promoters in cattle: environmental studies. Environ. Health. Perspect. 109, 1145–1151.
Slabbekoorn, H., Peet, M., 2003. Ecology: birds sing at a higher pitch in urban noise – great tits hit the high notes to ensure that their mating calls are heard above the city's din. Nature 424, 267.
Taves, M.D., Desjardins, J.K., Mishra, S., Balshine, S., 2009. Androgens and dominance: sex-specific patterns in a highly social fish (*Neolamprologus pulcher*). Gen. Comp. Endocrinol. 161, 202–207.
Thorpe, K.L., Maack, G., Benstead, R., Tyler, C.R., 2009. Estrogenic wastewater treatment works effluents reduce egg production in fish. Environ. Sci. Technol. 43, 2976–2982.
Tomkins, P., Saaristo, M., Allinson, M., Wong, B.B.M., 2016. Exposure to an agricultural contaminant, 17β-trenbolone, impairs female mate choice in a freshwater fish. Aquat. Toxicol. 170, 365–370.
Vos, J.G., Dybing, E., Greim, H.A., Ladefoged, O., Lambré, C., Tarazona, J.V., Brandt, I., Vethaak, A.D., 2000. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. Crit. Rev. Toxicol. 30, 71–133.
Walker, D.M., Gore, A.C., 2011. Transgenerational neuroendocrine disruption of reproduction. Nat. Rev. Endocrinol. 7, 197–207.
Widianarko, B., Van Gestel, C.A.M., Verweij, R.A., Van Straalen, N.M., 2000. Associations between trace metals in sediment, water, and guppy, *Poecilia reticulata* (Peters), from urban streams of Semarang, Indonesia. Ecotoxicol. Environ. Saf. 46, 101–107.
Wilson, V.S., Lambright, C., Ostby, J., Gray, L.E., 2002. *In vitro* and *in vivo* effects of 17β- trenbolone: a feedlot effluent contaminant. Toxicol. Sci. 70, 202–211.
Wong, B.B.M., 2004. Male competition is disruptive to courtship in the Pacific blue-eye. J. Fish. Biol. 65, 333–341.
Wong, B.B.M., Candolin, U., 2005. How is female mate choice affected by male competition? Biol. Rev. Camb. Philos. Soc. 80, 559–571.
Wong, B.B.M., Candolin, U., Lindström, K., 2007. Environmental deterioration compromises socially enforced signals of male quality in three-spined sticklebacks. Am. Nat. 170, 184–189.
Wong, B.B.M., Candolin, U., 2015. Behavioral responses to changing environments. Behav. Ecol. 26, 665–673.
World Health Organization, 2013. State of the science of endocrine disrupting chemicals — 2012. In: Bergman, Å., Heindel, J.J., Jobling, S., Kidd, K.A., Zoeller, R.T. (Eds.), An Assessment of the State of the Science of Endocrine Disruptors Prepared by a Group of Experts for the United Nations Environment Programme (UNEP) and WHO. WHO Press, Switzerland.
Zala, S.M., Penn, D.J., 2004. Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. Anim. Behav. 68, 649–664.
Zhang, X., Hecker, M., Park, J.W., Tompsett, A.R., Newsted, J., Nakayama, K., Jones, P.D., Au, D., Kong, R., Wu, R.S., Giesy, J.P., 2008. Real-time PCR array to study effects of chemicals on the Hypothalamic-Pituitary-Gonadal axis of the Japanese medaka. Aquat. Toxicol. 88, 173–182.