Sex and steroids: the impact of an agricultural contaminant on the mechanisms of sexual selection in the guppy

Patrick Tomkins

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Supervisors: Bob B.M. Wong and Minna Saaristo

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Patrick Tomkins

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School of Biological Sciences

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Abstract

Pollution is one of the most widespread forms of environmental change. One group of environmental pollutants that are of particular concern is the endocrine disrupting chemicals (EDCs), which are compounds that affect the endocrine system, resulting in a divergence from normal hormonal and homeostatic function. EDCs encompass a broad range of natural and synthetic compounds that enter the environment from a variety of sources (eg: agricultural run-off, industrial wastewater), and are particularly insidious as they can affect organisms at minute concentrations. Although studies investigating the environmental impacts of EDCs have traditionally focused on morphological and physiological endpoints, a growing interest in the effects of EDCs on behaviour has revealed that exposure can influence a range of behaviourally important processes. Despite this, we know relatively about the potential impact of EDCs on the mechanisms of sexual selection.

Sexual selection, by influencing the quality and quantity of offspring produced, has important implications for population dynamics, ecosystem function, and broader evolutionary processes. One EDC with the potential to impact sexual selection processes is 17β-trenbolone; a synthetic, androgenic steroid used globally to promote growth in beef cattle, which has been detected in surface waters around the world. Although it is well established that 17β-trenbolone can have severe morphological and physiological effects, it's behavioural impacts remain poorly

understood. This is surprising considering that sexually selected traits (eg: colourful ornaments and sexual behaviours) are under androgenic control, meaning it is highly likely that exposure to 17β-trenbolone will impact the mechanisms of sexual selection.

The guppy, *Poecilia reticulata*, is a species of fish commonly found in EDC-contaminated environments. The mating system of the guppy makes them an ideal candidate for investigating the impacts of EDCs on sexual selection. Males achieve copulations via two alternate mating strategies: courtship, which is employed to solicit consensual copulations from females, and 'sneak' attempts, which involves the male sneaking up behind the female and attempting to mate with her coercively. Importantly, female guppies are choosy and actively associate with preferred males, favouring a number of male traits including increased orange pigmentation, size, and display rate. Accordingly, the aim of this thesis was to provide a comprehensive investigation into the impact of an environmentally realistic concentration of 17β-trenbolone on the mechanisms of sexual selection in the guppy.

My thesis is split into two parts. In the first, I experimentally investigated how exposure to 17β-trenbolone impacted female mate choice in guppies. In Chapter 2, I found that when given a choice between a 17β-trenbolone-exposed and an unexposed male, unexposed females spent more time associating with unexposed males. Conversely, exposed females showed no preference for either male, and also associated with males less overall than unexposed females. In Chapter 3, I found

that when unexposed females were sequentially presented, one after the other, with two males that differed in their level of orange colouration, the time that females spent associating with the second male varied depending on previous male experience. Exposed females, on the other hand, showed no preference for orange colouration throughout behavioural trials, and again associated with males less overall than unexposed females.

In the second part of my thesis, I experimentally investigated how exposure to 17β-trenbolone impacted the reproductive behaviour of male guppies. Specifically, I examined how exposure influenced male-male competition (Chapter 4), as well as the impact of 17β-trenbolone on sequential male mate choice (Chapter 5). In Chapter 4, I found that when in the presence of a rival male and a female, exposed males were more aggressive, conducted significantly more sneak mating attempts, and courted less than unexposed males. In Chapter 5, I compared the response of exposed and unexposed males to sequentially presented large and small females. I found that regardless of the order females were presented in, both exposed and unexposed males performed significantly more courtship bouts and sneak mating attempts towards large females, which suggests that males were basing their mate choice decisions on an absolute preference for large females. Further, 17β-trenbolone-exposed males conducted significantly more sneak mating attempts than unexposed males towards females throughout behavioural trials.

Together, these results show that exposure to 17β-trenbolone can impact female mate choice, male mate choice, and male-male competition, providing a comprehensive demonstration of the impacts of 17β-trenbolone on several key mechanisms of sexual selection. Considering the power of sexual selection as an evolutionary driver, these findings highlight the need for a greater understanding of the potential impacts of EDCs on sexual selection processes.

Publications during enrolment

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes four original papers published in peer reviewed journals (one included in the appendix). The core theme of the thesis is the impact of EDCs on sexual selection. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the school of biological sciences under the supervision of Bob Wong.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. **In the case of *chapter 2,3,4* my contribution to the work involved the following:**

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
2	Exposure to an agricultural contaminant, 17β-trenbolone, impairs female mate choice in a freshwater fish	Accepted	*95%*	Bob Wong (2%): Designing and conceiving experiment, proof reading, contributing to manuscript Minna Saaristo (2%): Designing and conceiving experiment, proof reading, contributing to manuscript Mayumi Allinson (1%): Conducting lab work	No No No
3	An endocrine-disrupting agricultural contaminant impacts sequential female mate choice in fish	Accepted	90%	Bob Wong (2.5%): Designing and conceiving experiment, proof reading, contributing to manuscript Minna Saaristo (2.5%): Designing and conceiving experiment, proof reading, contributing to manuscript Michael Bertram (2.5%): Proof reading, contributing to manuscript Marcus Michelangeli (1.25%): Conducting statistical analysis Raymond Tomkins (1.25%): Conducting lab and field work	No No Yes No No
4	The agricultural contaminant 17β-trenbolone disrupts male-male competition in the guppy (Poecilia reticulata)	Accepted	90%	Bob Wong (2.5%): Designing and conceiving experiment, proof reading, contributing to manuscript Minna Saaristo (2.5%): Designing and conceiving experiment, proof reading, contributing to manuscript Michael Bertram (2.5%): Proof reading, contributing to manuscript Mayumi Allinson (1.5%): Conducting lab work Raymond Tomkins (1%): Conducting lab and field work	No No Yes No No

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] **Date: 20/11/2017**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: [redacted] **Date:** 20/11/2017

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Chapter 1: Introduction

Human-induced environmental change is taking place at an unprecedented pace and scale. Habitat destruction, the spread of invasive species, over-harvesting, pollution, and climate change are all having negative impacts on the world's ecosystems (Rohr et al., 2006; Salo et al., 2007; Fabry et al., 2008; Sih et al., 2011), resulting in global loss of biodiversity (Parmesan and Yohe, 1999; Jackson and Sax, 2010). A recent analysis of the human footprint concluded that 75% of Earth's environments are experiencing measurable human pressures (Venter et al., 2016), which means the vast majority of organisms are living in environments that have been altered in some way by anthropogenic activity. Although humans impact the environment in a myriad of ways, the most common and widespread form of environmental change is pollution.

1.1 Chemical pollution

Pollution is a global problem. Heat, light, noise, chemical waste, and solid waste are all known to impact environments (Clark, 1969; Laist, 1987; Francis et al., 2009), causing species declines, range shifts and, in extreme cases, extinctions (Connell and Miller, 1984; Derraik, 2002; Miller and Rodriguez, 2007). Chemical pollutants are one of the most insidious forms of environmental pollution as they interfere with an organism's physiology (Travis and Hester 1991). They are particularly worrying as they often enter environments undetected, can have additive and synergistic effects,

and are widely dispersed via wind, water, and anthropogenic activity (Zala and Penn, 2004). As a result, chemical pollution has been implicated in the decline of numerous species and populations worldwide (Blaustein and Kiesecker, 2002; Stabenau et al., 2008; Laurance and Useche, 2009), and as anthropogenic production, consumption, and mobility continues to rise globally (Venter et al., 2016), so will the presence and impact of chemical pollutants in the environment.

1.2 EDCs

One group of chemical contaminants that are of particular concern is endocrine disrupting chemicals (hereafter referred to as 'EDCs'). EDCs refer to compounds that affect the endocrine system 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). EDCs are extremely widespread and diverse, including natural hormones found in urine, faeces, and food, and synthetic compounds such as pesticides, plastics, pharmaceutical products and industrial by-products (Cederroth et al., 2012; Diamanti-Kandarakis et al., 2009). They enter the environment via a number of sources including industrial wastewater, agricultural run-off, and domestic wastewater (Johnson and Sumpter, 2001; Thorpe et al., 2009), and have been detected even in Earth's most remote environments (e.g., polar bears in the Arctic; Letcher et al., 2010; amphipods in the Mariana Trench; Jamieson et al., 2017). Once in the environment, EDCs pose a constant and insidious threat to wildlife due to their tendency to persist and bioaccumulate, potential to act transgenerationally,

and ability to impact organisms at minute concentrations (i.e., in the range of ng/L) (Crews et al., 2007; Walker and Gore, 2011). As a result, a vast literature now exists documenting their morphological and physiological impacts (for a review see Mills and Chichester, 2005; Vos et al., 2000; Vandenberg et al., 2012). But what about behaviour?

1.3 EDCs and behaviour

The potential of EDCs to impact behaviour was first identified nearly 60 years ago in a study conducted by Broley (1958) who established a link between exposure to the pesticide DDT and abnormal nesting and courtship behaviour in the Florida bald eagle. Despite this, behavioural studies comprise a relatively small portion of the EDC literature, and it is only recently that they have become a focus of the scientific community. As a result of this recent surge in interest towards understanding the impacts of EDCs on behaviour, it has become apparent that behavioural abnormalities resulting from EDC exposure can manifest at much lower concentrations than those required to induce morphological or physiological change. Further, unlike standard laboratory assays, which typically only target one or (at most) a few morphological or physiological endpoints, behaviour is the manifestation of numerous complex developmental and biochemical processes, meaning that behaviour can be an especially sensitive and comprehensive biomarker of EDC exposure (reviewed in Melvin and Wilson, 2013). Indeed, we now know that EDCs can impact a range of behaviourally important processes (reviewed Clotfelter et al., 2004; Frye et al., 2012), including those under sexual selection.

Sexual selection, by influencing the quality and quantity of offspring produced, has important implications for population dynamics, ecosystem function and broader evolutionary processes (reviewed in Candolin and Wong, 2012). Two of the key mechanisms of sexual selection are female mate choice, which is known to confer a suite of direct (i.e., material) and indirect (i.e., genetic) benefits to choosy individuals (Andersson, 1994; Kokko et al., 2003), and male-male competition, which has important consequences for both male mating success (Moller and Jennions, 2001) and female fitness (Fisher et al., 2006). Although recent research has revealed that the mechanisms of sexual selection are vulnerable to EDC exposure (for a review see Soffker and Tyler, 2012; Gore et al., 2017), the scope of these studies has been limited, with the vast majority focusing on only one group of EDCs, i.e., those with estrogenic properties. In comparison, relatively few studies have investigated the potential impacts of androgenic EDC exposure on sexual selection processes.

1.4 Androgenic EDCs

Androgenic hormones play an essential role in male and female reproductive development, regulating the formation of sexual phenotypes during embryogenesis, sexual maturation at the time of puberty, and the expression of secondary sexual traits (Wilson, 1999). The physiological and morphological impacts of androgenic EDCs are well documented, with exposure known to cause abnormal growth and reproductive development in males (eg: Finch et al., 2013; Brande-Lavridsen et al., 2008; Bertram et al., 2015) and masculinisation in females (eg: Seki et al., 2006; Örn

et al., 2006; Larsen and Baatrup, 2010). Despite this, relatively little is known about the behavioural impacts of androgenic EDC exposure, which is surprising considering that androgens regulate a range of behaviours including aggression, dominance, courtship behaviour, and parental care (Mooradian et al., 1987; Rubinow and Schmidt, 1996; Hotchkiss et al., 2002). As a result, it is highly likely that exposure to exogenous androgenic EDCs will influence behaviour, particularly behavioural processes under sexual selection.

1.5 Hormonal growth promotants and 17β-trenbolone

One group of androgenic EDCs with the potential to influence the mechanisms of sexual selection are hormonal growth promotants (hereafter referred to as 'HGPs'), which are natural and synthetic compounds used to promote growth in beef cattle by specifically targeting the endocrine system. Despite being banned by the European Union (Johnson, 2015), the use of HGPs is common and widespread in many beef-producing countries including the United States, Canada, Mexico, South Africa, Chile, Japan, New Zealand and Australia (Hunter, 2010; Kolodziej et al., 2013). As just one example highlighting the prevelance of HPG use, the USA is the world's leading beef producer, and it is estimated that 20 million cattle (i.e. approximately two thirds of the total livestock in the country) currently receive HGP implants (Johnson 2015).

HGPs commonly include a cocktail of chemical compounds, including androgens (eg: trenbolone acetate), estrogens (eg: 17β-estradiol, zeranol), and progestins (eg:

melengestrol acetate) (Lange et al., 2001). Trenbolone acetate, which is the most common androgen administered to beef cattle (Hunter, 2010), is a powerful synthetic steroid with 15-50 times the androgenic and anabolic potency of testosterone (Neumann, 1976). After implantation, trenbolone acetate is hydrolyzed within the body of cattle to produce the biologically active steroid 17β-trenbolone, which enters the environment via urine and faeces. 17β-trenbolone has a particularly long half-life (~260 days measured in animal waste; Durhan et al., 2006) allowing it to accumulate in the environment. As a result, 17β-trenbolone has been repeatedly detected in waterways associated with cattle feedlots, ranging in concentration from ≤1-20 ng/L in discharge and diffuse run-off (Durhan et al., 2006) to 162 ng/L in tile-drained agroecosystems (Gall et al., 2011).

Aquatic vertebrates are particularly vulnerable to HGP exposure as uptake from waterways occurs readily via multiple pathways. These include direct absorption from water via the gills, skin, and gut, ingestion of contaminated food, and/or dermal contact with contaminated sediment (Soffker and Tyler, 2012; Mills and Chichester, 2005). EDCs also concentrate in the tissues of aquatic organisms and have been found to bioaccumulate in fish that occur at higher trophic levels (Smith and Hill, 2004; Sharma et al., 2009), and can be transferred from exposed adults into developing embryos via the lipid reserves of eggs (Van Der Kraack et al., 2001). Recent research has revealed that 17β-trenbolone can cause severe morphological and physiological abnormalities in fish, ranging from altered gonadal morphology (Örn et al., 2006) and reduced fecundity (Ankley et al., 2003), to irreversible

masculinization (Baumann et al., 2014) and even complete and functional female-to-male sex reversal (Larsen and Baatrup, 2010; Morthorst et al., 2010). More recently, it has been revealed that 17β-trenbolone can also influence the behavior of fish. Specifically, we now know that exposure to environmentally realistic concentrations of 17β-trenbolone can alter the reproductive behavior of mosquitofish (Saaristo et al., 2013) and guppies (Bertram et al., 2015; Tomkins et al., 2016). However, studies investigating the behavioiural impacts of 17β-trenbolone are still in their infancy, and we are yet to investigate the impact of 17β-trenbolone on many of the complex and varied mechanisms of sexual selection.

1.6 Study species: the guppy

The guppy, *Poecilia reticulata*, is a small, viviparous fish native to North-eastern South America that has become invasive throughout the world as a result of numerous deliberate and accidental introductions (Lindholm et al., 2005). Guppies are sexually dimorphic, with females growing significantly larger than males but lacking their vibrant colouration. Males also possess a modified anal fin called a gonopodium, which is used as an intromittent organ (Houde, 1997). The mating system of the guppy makes them an excellent candidate for investigating the impacts of EDCs on sexual selection. Male guppies employ two alternate mating strategies to achieve copulations: sigmoid displays, which are elaborate courtship displays employed to attract females and achieve consensual copulations, and 'sneak' mating attempts, which are when males sneak up behind females and thrust their gonopodium towards the genital pore in an attempt to mate coercively (Houde,

1997). Males compete for the attention of choosy females, with females actively associating with preferred mates and favouring a number of known male traits including increased orange pigmentation, size, and display rate (Endler, 1980; Brooks and Caithness, 1995; Kodric-Brown and Nicoletto, 2001), which are honest indicators of quality. Considering that endogenous androgens play a key role in moderating sexual behaviour (Cunningham et al., 2012), it is highly likely that 17β-trenbolone-exposure will influence sexual selection processes in the guppy. This provides an excellent opportunity to address a poorly understood area of the EDC literature by furthering our understanding of the impacts of androgenic EDCs on the mechanisms of sexual selection.

Accordingly, the aim of this thesis was to investigate, at environmentally relevant concentrations, the impact of 17β-trenbolone on the mechanisms of sexual selection in male and female guppies.

1.7 Thesis structure

My thesis is split into two parts. In the first, I experimentally investigated how exposure to 17β-trenbolone impacted female mate choice, both when females were able to make direct comparisons between potential suitors (Chapter 2), and when males were encountered sequentially (Chapter 3). In the second part of my thesis, I investigated how exposure to 17β-trenbolone influenced sexual selection processes in male guppies. Specifically, I examined how exposure impacted male-male competition (Chapter 4) and sequential male mate choice (Chapter 5). Together,

these results provide a comprehensive demonstration of the impacts of 17β-trenbolone on the mechanisms of sexual selection in the guppy, highlighting the need for a greater understanding of the impacts of EDCs on sexual selection processes.

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Declaration for thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution was the following

Nature of contribution	Extent of contribution (%)
Conceiving/designing the study, conducting fieldwork, laboratory experiments and data analysis, and writing the chapter/manuscript	95%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%)
Bob Wong	Helping design the study, proof reading, and contributing to the manuscript	2%
Minna Saaristo	Helping design the study, proof reading and contributing to the manuscript	2%
Mayumi Allinson	Helping conduct laboratory work	1%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work

Student signature: [redacted] **Date:** 17/11/2017

Main supervisor signature: [redacted] **Date:** 17/11/2017

Chapter 2

Exposure to an agricultural contaminant, 17β-trenbolone, impairs female mate choice in a freshwater fish

Patrick Tomkins[1], Minna Saaristo[1,2], Mayumi Allinson[3], Bob B. M. Wong[1]

Field site: Alligator creek

[1] *School of Biological Sciences, Monash University, Victoria, Australia*

[2] *Department of Biosciences, Åbo Akademi University, Turku, Finland*

[3] *Centre for Aquatic Pollution Identification and Management (CAPIM), School of Chemistry, The University of Melbourne, Victoria, Australia*

2.1 ABSTRACT

Despite the pivotal role sexual selection plays in population dynamics and broader evolutionary processes, the impact of chemical pollution on female mate choice is poorly understood. One group of chemical contaminants with the potential to disrupt the mechanisms of female mate choice is endocrine disrupting chemicals (EDCs); a broad class of environmental pollutants that can interfere with the endocrinology of organisms at extremely low concentrations. Recent research has revealed that estrogenic EDCs can affect female mate choice in fish, but the impact of androgenic EDC exposure has yet to be studied. To address this, we investigated the effects of an environmentally relevant concentration of 17β-trenbolone – an androgenic steroid used as a growth promoter in the cattle industry – on female mate choice in wild-caught guppies (*Poecilia reticulata*). We exposed male and female guppies to 17β-trenbolone for 21 days (measured concentration 4 ng/L) via a flow-through system, and found that 17β-trenbolone-exposed female guppies spent less time associating with males, and were less choosy, compared to unexposed females. In contrast, 17β-trenbolone had no impact on male reproductive behavior or morphology. This is the first study to show that androgenic EDC exposure can disrupt female mate choice, highlighting the need for studies to investigate the behavioural impacts of environmental contaminants on both sexes.

Key words: guppy, 17β-trenbolone, female mate choice, endocrine disrupting chemical, sexual selection

2.2 INTRODUCTION

Sexual selection, by influencing the quality and quantity of offspring produced, has important implications for population dynamics, ecosystem function, and broader evolutionary processes (reviewed in Candolin and Wong, 2012). One of its key mechanisms is female mate choice (Andersson and Simmons, 2006), which is known to confer a suite of direct (i.e. material) and indirect (i.e. genetic) benefits to choosy individuals (Andersson, 1994; Kokko et al., 2002). The traits that females use to evaluate male quality are finely attuned to the local environmental conditions in which they have evolved (Wong and Candolin, 2015). As a result, the mechanisms of female mate choice are particularly vulnerable to anthropogenic, environmental change. Although the majority of studies have focused on how altering the physical and auditory environment can interfere with these mechanisms (eg: Slabbekoorn and Peet, 2003; Wong et al., 2007), changing the chemical environment can also disrupt female mate choice (eg: Fisher et al., 2006). This is not surprising given the profound influence chemical pollution can have on morphology, physiology and behavior (Vos et al., 2000; Clotfelter et al., 2004; Ward et al., 2008; Diamanti-Kandarakis et al., 2009), and the pivotal role chemical communication plays in the reproduction of a wide range of taxa (Johansson and Jones, 2007).

One group of chemical contaminants with the capacity to disrupt female mate choice is endocrine disrupting chemicals (EDCs). EDCs are ubiquitous in the environment and possess several characteristics that make them particularly concerning. They can persist in ecosystems, affect organisms at extremely low concentrations, have a

tendency to bioaccumulate in the environment, and can act transgenerationally (Anway and Skinner, 2006; Crews et al., 2007; Walker and Gore, 2011). There is a plethora of research documenting the environmental impacts of EDCs, with studies traditionally concentrating on morphological and physiological effects. However, with the recognition of behavior as a particularly sensitive and powerful biomarker of EDC contamination (Melvin and Wilson, 2013), an increasing number of studies are also turning their attention to understanding the behavioural impacts of EDCs. Recent research has revealed that EDCs can affect a range of behaviourally important processes, including cognition, boldness, sociality and reproduction (for a review see Clotfelter et al., 2004; Frye et al,. 2012; Soffker and Tyler, 2012).

Surprisingly, despite growing interest in the impacts of EDCs on behaviour, relatively little is known about how EDCs affect sexual selection and, more specifically, female mate choice. Studies have recently revealed, for instance, that EDC exposure can affect female mate choice in fish (Coe et al., 2008; Saaristo et al., 2009a). However, these studies have largely focused on only one group of EDCs – those with estrogenic properties. In comparison, the influence of androgenic EDC exposure on sexual selection has yet to be investigated.

One androgenic EDC with the potential to impact sexual selection is 17β-trenbolone, a synthetic steroid commonly used in many parts of the world to accelerate growth rates in beef cattle (Lange et al., 2002; Khan et al., 2008; Morthorst et al., 2010). 17β-trenbolone enters the environment via urine and faeces, and has been detected

in aquatic environments associated with cattle feedlots at concentrations ranging from <20 ng/L (Durhan et al., 2006) to as high as 162 ng/L (Gall et al., 2011). 17β-trenbolone is an extremely stable compound, with a half-life of up to 260 days measured in animal waste (Durhan et al., 2006), and is particularly potent, binding to androgen receptors with three times the affinity of testosterone (Khan et al., 2008). Exposure to 17β-trenbolone can have severe implications for the sexual morphology of fish (eg: reduced fecundity in fathead minnows, *Pimephales promelas*: Ankley et al., 2003; Jensen et al., 2006; sex reversal in zebrafish, *Danio rerio*: Larsen and Baatrup 2010), but its behavioural consequences are poorly understood. Although recent research has found that 17β-trenbolone can influence the reproductive behaviour of male and female Poecilid fishes (Saaristo et al., 2013; Bertram et al., 2015), it is still unknown whether these behavioural changes may impact female mate choice.

Our study species was the guppy (*Poecilia reticulata*) – a small, viviparous fish commonly found in freshwater environments contaminated with EDCs (López-Rojas and Bonilla-Rivero, 2000; Widianarko, 2000). Guppies are native to North-eastern South America but have become invasive throughout the world as a result of both deliberate and accidental introductions (Lindholm et al., 2005). The mating system of the guppy makes them an ideal candidate for investigating the effects of EDCs on sexual selection. Female guppies are choosy and actively associate with preferred males, which can have a direct influence on mating outcomes (Godin and Briggs, 1996; Shenoy, 2012). Females are known to favor a number of male traits including

increased orange pigmentation, size, and display rate (Endler, 1980; Brooks and Caithness, 1995; Kodric-Brown and Nicoletto, 2001), all of which are honest indicators of male quality.

Here we test the hypothesis that short-term exposure to an environmentally relevant concentration of 17β-trenbolone will impact female mate choice in guppies. The male traits that females use to choose mates are under androgenic control (Wilson, 1999; Emerson, 2000), meaning they are likely to be influenced by 17β-trenbolone. This, combined with the fact that 17β-trenbolone is known to affect reproduction in Poecilids, suggests that exposure should also affect female mate choice.

2.3 METHODS

Collection and housing

Guppies were collected with dip nets from Alligator Creek (19°26'17.94" S, 146°57'1.09" E) in Queensland, Australia. Alligator Creek is a rainforest-fed stream located in the pristine Bowling Green Bay National Park. Water samples taken from this site over consecutive years revealed no contamination from estrogenic or androgenic EDCs (ALS global, unpublished data), thus ensuring that fish used in this study were from an uncontaminated source. Fish were transported to Monash University via airfreight and were acclimated to laboratory conditions (26^{o}C, 12:12h light regime) for 2 months prior to exposure. Fish were fed *ad libitum* once daily with commercial fish pellets (Otohime Hirame larval diet; 580–910 μm).

Exposure

After acclimation, fish were exposed to 17β-trenbolone for 21 days, as previous experiments have shown that exposure periods ranging from 14-28 days are sufficient to induce behavioural changes (Bayley et al., 1999; Bell 2001; Martinovic et al., 2007; Majewski et al., 2002; Maunder et al., 2007; Oshima et al., 2003; Saaristo et al., 2009a,b). Furthermore, EDCs often enter the environment in pulses and may only remain in waterways for a short period of time (Diamanti-Kandarakis et al., 2002), meaning short exposure times are ecologically meaningful.

Guppies were exposed to 17β-trenbolone via a flow-through system based on the design of Saaristo et al., (2013). This system included eight 54L (60cm x 30cm x30cm) tanks: 4 control tanks (containing a solvent control) and 4 17β-trenbolone-exposed tanks (containing 17β-trenbolone). A total of 160 fish (100 males, 60 females) were separated by sex and distributed between these eight tanks (i.e. 2 control and 2 17β-trenbolone-exposed tanks for each sex). Guppies in the 17β-trenbolone-exposed tanks were exposed to 17β-trenbolone at a nominal concentration of 15 ng/L (measured concentration = 4 ng/L; see below for details on how 17β-trenbolone concentrations were monitored), while guppies in the control tanks were exposed to a solvent control (0.000013% ethanol – see below for details). All exposure tanks were maintained at a constant temperature between 25-27^{o}C, and fish were fed daily *ad libitum*.

Water entered these exposure tanks via a mixing tank, which received a constant flow of fresh, filtered tap water and a constant flow of either 17β-trenbolone (in the case of the 17β-trenbolone-exposed tanks) or the solvent solution (in the case of the control tanks) from a stock tank via a peristaltic pump (Watson Marlow 323 U/MC). Water was channeled into the exposure tanks using silicone tubing, and flow rates were kept constant (2.25L/h) using flow meters (BES Flowmeters, MPB Series 1200) and adjustable valves.

Stock solution preparation

The 17β-trenbolone stock solution was created by first dissolving 17β-trenbolone (4,9,11-estratrien-17-ol-3-one; Novachem, Germany) in 100% ethanol (solvent) to create a stock standard of 300mg/L. This stock standard was then diluted to 600ug/L using deionized water, resulting in a solvent concentration of 0.2%. The stock solution was further diluted in the mixing tank in the flow-through system to achieve the desired nominal 17β-trenbolone concentration of 15 ng/L (measured concentration: $\tilde{x}$ = 4 ng/L, SD = 1.4 ng/L, n = 14). A solvent solution of 0.2% was used in the control tanks, which was diluted in the exposure system to a concentration of 0.000013%.

Water analysis

In order to monitor 17β-trenbolone concentrations in the exposure tanks, and to ensure there was no contamination of control tanks, a 100ml water sample was taken from each of the exposure tanks weekly and analysed using enzyme-linked

immunosorbent assay (17β-trenbolone ELISA, EuroProxima, Arnhem, The Netherlands). For a detailed description of the ELISA testing protocol, see Saaristo *et al.* (2013).

Behaviour trials

The impact of 17β-trenbolone exposure on female mate choice was investigated using a dichotomous choice experiment, which is a standard method used for investigating mate choice preferences in guppies (Evans et al., 2004; Pilastro et al., 2004; Jeswiet et al., 2011). A 51L tank (60cm x 30cm x 24cm) was split into three compartments using clear, perforated plastic dividers to allow water flow and full visual and chemical contact between compartments. A single female was placed in the middle compartment in a clear plastic cylinder, while a single male was placed in each of the end compartments. After a 10-minute acclimation period, the cylinder was removed and the fish were allowed to interact for 15 minutes. All trials were filmed using a digital video recorder (Canon Powershot S110).

Two trial combinations were used to investigate the impacts of 17β-trenbolone on female mate choice: 1) an unexposed female was given a choice between an exposed and an unexposed male (hereafter referred to as 'unexposed female trial'), and 2) an exposed female was given a choice between an exposed and an unexposed male (hereafter referred to as 'exposed female trial'). There was no difference in the weight or length of exposed and unexposed males (weight: $t = 1.35$, $df = 58.95$, $p = 0.18$; length: $t = 1.05$, $df = 59.95$, $p = 0.33$, see Table 1 for details) or exposed and

unexposed females (weight: t = 0.18, df = 28.93, p = 0.86; length: t = 0.46, df = 28.64, p = 0.65, see Table 1 for details) in either of the trial combinations. A total of 31 trials were conducted (n = 15 unexposed female trials, n = 16 exposed female trials).

Female preference was determined by quantifying the amount of time spent within a 5cm 'preference zone' of either male compartment. Association time is commonly used as a measure of female preference in guppies (Kodric-Brown, 1985; Kodric-Brown, 1989; Karino and Shinjo, 2004; Pilastro et al., 2004) and has been shown to be an accurate indicator of female mate choice in Poecilid fishes (Walling et al., 2010). The courtship behaviour (i.e. time spent conducting sigmoid displays and orienting their body towards that of the female) of both exposed and unexposed male fish was also quantified. We calculated the total time that males spent courting as females were in view of the males throughout the entire trial, meaning males courted even if females were not directly associating with them. Male and female behaviours were quantified using JWatcher v 1.0 (Blumstein et al., 2007).

Morphological analysis

Male and female weight and length was measured directly after behavior trials. Males were also photographed on their right side in a standardized fashion (Nikon D90, shutter speed = 1/250, Nikon AF Micro-Nikkor 60 mm f/2.8D), and the resultant pictures analysed using Photoshop (CS6 version 13.0 Extended) to determine the total percentage of the body containing orange pigments. For a detailed description of the colouration analysis method, see Bertram et al. (2015).

Statistical analysis

All data were analysed using the statistical program 'R' (version 2.13.1; R Development Team, 2011). Data was checked for normality and homogeneity of variance, and transformed where necessary. Independent samples t-tests were used to compare the amount of time that unexposed and exposed females spent associating with males. The amount of time that females spent associating with each male, and the courtship behavior of exposed and unexposed males, were compared in each treatment using paired t-tests. The weight and length of males and females was compared using independent samples t-tests, as was the percentage of orange pigmentation of males.

Ethical statement

The research detailed in this paper was approved by the Biological Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2013/09) and complies with all relevant State and Federal laws of Australia.

2.4 RESULTS

Female behavior

Exposed females spent significantly less time associating with males than unexposed females ($t = 4.03$ $df = 28.05$, $p < 0.001$, Figure 1). When they were associating with males, unexposed females spent significantly more time associating with unexposed males than exposed males ($t = 3.14$, $df = 14$, $p < 0.01$, Figure 2),

while exposed females showed no preference for either exposed or unexposed males ($t = 0.44$, $df = 15$, $p = 0.67$, Figure 2).

Male behavior and morphology

There was no difference in the total time that exposed and unexposed males spent courting in both the unexposed female trial ($t = -0.66$, $df = 14$, $p = 0.52$, Table 2) and the exposed female trial ($t = -0.56$, $df = 15$, $p = 0.58$, Table 2). There was also no difference in the percentage of orange pigmentation between exposed and unexposed males in either treatment (unexposed female trial: $t = -0.37$, $df = 23.29$, $p = 0.71$, exposed female trial: $t = 0.1819$, $df = 29.81$, $p = 0.86$, Table 2).

Table 1. Total length and weight of females and males (mean ± SE) used in behavior trials (TB = 17β-trenbolone).

Trial combination	n	Mean length (mm) ± S.E.	Mean weight (mm) ± S.E.
Unexposed female trial			
Unexposed males	15	21.22 ± 0.402	0.09 ± 0.002
TB-exposed males	15	21.58 ± 0.359	0.10 ± 0.004
Unexposed females	15	24.14 ± 0.663	0.16 ± 0.016
Exposed female trial			
Unexposed males	16	21.68 ± 0.365	0.09 ± 0.004
TB-exposed males	16	21.35 ± 0.450	0.10 ± 0.003
TB-exposed females	16	24.61 ± 0.769	0.16 ± 0.016

Table 2. Total percentage of orange pigmentation and total time spent courting by males used in behavior trials (TB = trenbolone).

Trial combination	n	Mean % orange pigmentation ± S.E.	Mean time spent courting ± S.E. (min)
Unexposed female trial			
Unexposed males	15	9.20 ± 0.678	7.12 ± 0.929
TB-exposed males	15	9.66 ± 1.341	7.82 ± 0.578
Exposed female trial			
Unexposed males	16	9.68 ± 1.101	6.58 ± 0.838
TB-exposed males	16	9.37 ± 1.078	7.25 ± 0.981

Figure 1. Mean total time (± SE) that unexposed females (n=15) and exposed (n=16) females (n=16) spent associating with males.

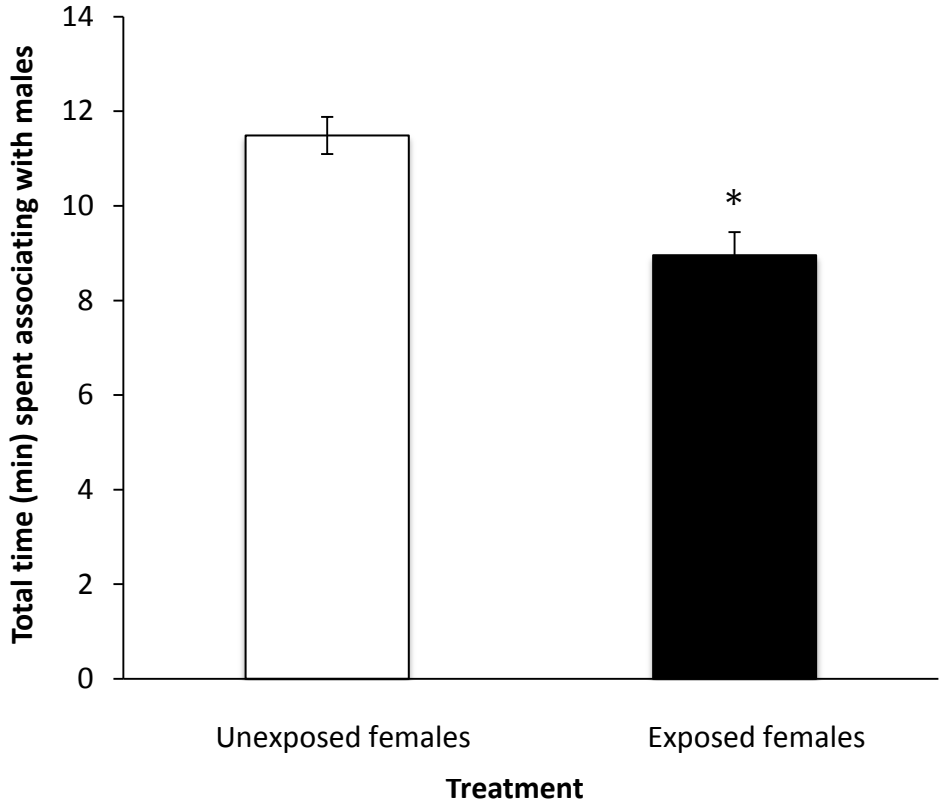
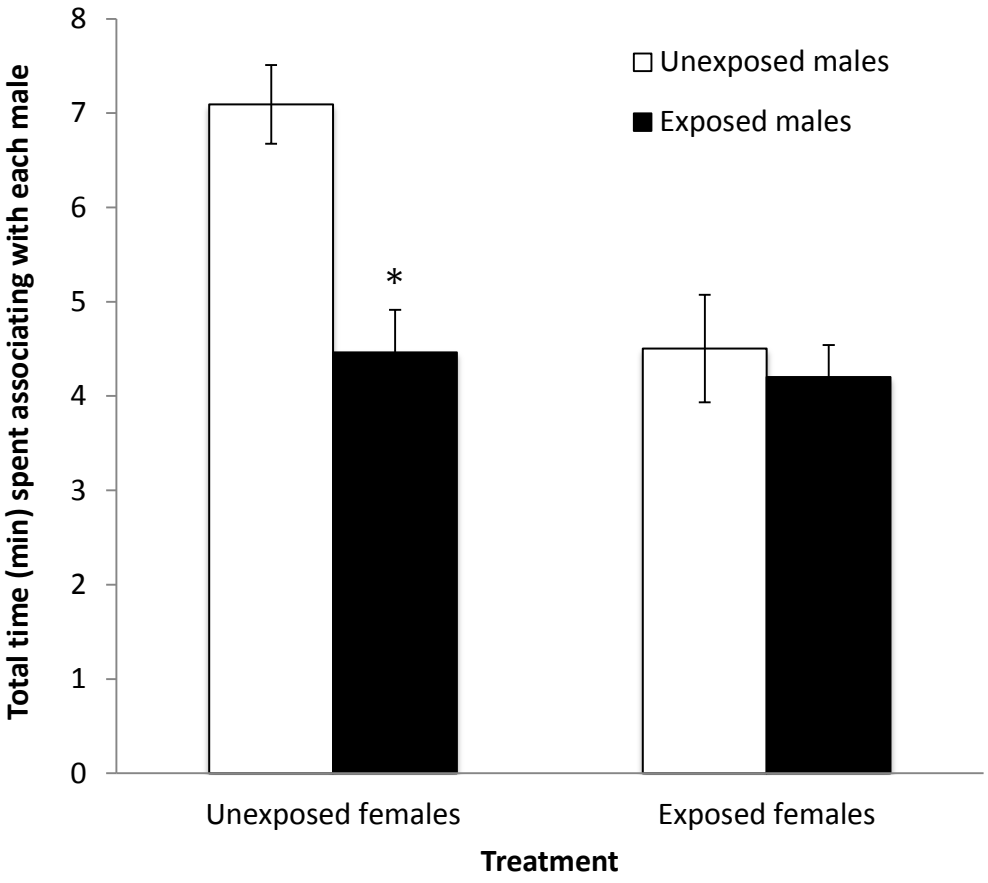


Figure 2. Mean total time (± SE) that unexposed females (n=15) and exposed females (n=16) spent associating with exposed and unexposed males.



2.5 DISCUSSION

This is the first study to show that an androgenic agricultural contaminant can affect female mate choice. We found that unexposed females spent more time associating with males than 17β-trenbolone-exposed females, and also showed a preference for unexposed males over 17β-trenbolone-exposed males. Exposed females, on the other hand, showed no preference for either male. Surprisingly, 17β-trenbolone exposure had no impact on any of the male traits or behaviours examined in this study, with no difference observed in male body size, percentage of orange pigmentation, or courtship behavior of exposed and unexposed fish.

17β-trenbolone-exposed females spent less time associating with males than unexposed females, indicating a decreased desire to mate, and were also less choosy than unexposed females. Previous research has shown that 17β-trenbolone exposure can suppress estrogenic activity in female fish, causing varying levels of masculinization. Specifically, Ankley et al. (2003) observed reduced concentrations of vitellogenin and β-estradiol in 17β-trenbolone-exposed female fathead minnows, which was correlated with the development of male morphological characteristics. 17β-trenbolone-induced masculinization has also been observed in female mosquitofish (*Gambusia holbrooki*; Sone et al., 2003) and zebra fish (Morthorst et al., 2010). Hence, it is conceivable that females in our study were masculinized to some degree, which could have reduced their desire to mate and made them less choosy.

In contrast to the exposed females, unexposed female guppies were choosy, and associated more with unexposed than exposed males. However, we did not find any differences between exposed and unexposed males in traits that have previously been found to influence female mate choice in guppies (i.e. colour: Endler, 1980; display rate: Kodric-Brown and Nicoletto, 2001). Although it is not clear in this experiment what cue(s) unexposed females may have been using to discriminate against exposed males and to preferentially associate with unexposed males, previous research has shown that chemical cues play an important role in Poecilid reproduction, including mate choice in guppies (Guevara-Fiore and Watt, 2009; Guevara-Fiore et al., 2010). It is possible that 17β-trenbolone exposure altered the chemical cues of male guppies in this study, which may explain female preference for unexposed males. This warrants further investigation into the effect of 17β-trenbolone exposure on chemical cues and its subsequent impact on reproductive behavior.

17β-trenbolone is a potent androgen agonist, meaning it has the potential to intensify the expression of male sexual traits by stimulating androgen production (Schiffer et al., 2001; Hotchkiss et al., 2008). This process of xenoandrogen-induced 'hyper-masculinisation' has been observed in multiple species. For example, androgenic EDC-exposed African clawed frogs, *Xenopus laevis*, exhibited increased levels of androgen-dependent male mate calling (Hoffman and Kloas, 2010), while androgenic EDC exposure was found to increase the intensity of male sexual behaviours in several cyprinid fish species (Belanger et al., 2010). Why, then, were

the male sexual traits in this experiment not influenced by 17β-trenbolone exposure? It has been well established that EDCs can influence the percentage of orange colouration in male guppies (Bayley et al., 2002, 2003; Shenoy, 2012; Tian et al., 2012). However, the vast majority of these studies exposed guppies before they became sexually mature, while in this study only mature adults were exposed. Male courtship behavior was also unaffected by 17β-trenbolone exposure, but this result is more difficult to interpret. Recent studies investigating the impact of 17β-trenbolone on the courtship behavior of male Poecilids have yielded contrasting results. Saaristo et al., (2013) found that short-term exposure to an environmentally relevant concentration of 17β-trenbolone (6 ng/L, 21 day exposure) had no impact on the reproductive behavior of male mosquitofish, while Bertram et al., (2015) found that exposure to 22 ng/L of 17β-trenbolone for 21 days had a significant impact on the amount of time that male guppies spent courting and conducting coercive mating behaviours. This suggests that more research is required in order to gain a more comprehensive understanding of the impact of 17β-trenbolone on reproductive behavior.

In conclusion, this is the first study to show that an androgenic EDC can impact sexual selection by disrupting the mechanisms of female mate choice. We found that females exposed to an environmentally relevant concentration of 17β-trenbolone associated with males less than unexposed females and were also less choosy. Female mate choice can have important consequences at the population level by influencing both the quality and quantity of offspring produced (Wong and

Candolin, 2015), highlighting the need for studies to investigate the behavioural impacts of environmental contaminants on both sexes.

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Declaration for thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution was the following:

Nature of contribution	Extent of contribution (%)
Conceiving/designing the study, conducting fieldwork, laboratory experiments and data analysis, and writing the chapter/manuscript	90%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%)
Bob Wong	Helping design the study, proof reading, and contributing to the manuscript	2.5%
Minna Saaristo	Helping design the study, proof reading, and contributing to the manuscript	2.5%
Michael Bertram	Proof reading and contributing to the manuscript	2.5%
Marcus Michelangelli	Helping conduct statistical analysis	1.25%
Raymond Tomkins	Helping with fieldwork and labwork	1.25%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work

Student signature: [redacted] **Date:** 17/11/2017

Main supervisor signature: [redacted] **Date:** 17/11/201

Chapter 3

An endocrine-disrupting agricultural contaminant impacts sequential female mate choice in fish

Patrick Tomkins[a], Minna Saaristo[a,b], Michael G. Bertram[a], Marcus Michelangeli[a], Raymond B. Tomkins[c], Bob B.M. Wong[a]

Raymond Tomkins conducting fieldwork at Alligator

[a] *School of Biological Sciences, Monash University, Victoria, Australia*

[b] *Department of Biosciences, Åbo Akademi University, Turku, Finland*

[c] *Centre for AgriBioscience, Department of Environment and Primary Industries (DEPI), Victoria, Australia*

3.1 ABSTRACT

The environmental impact of endocrine-disrupting chemicals (EDCs)—compounds that interfere with endocrine system function at minute concentrations—is now well established. In recent years, concern has been mounting over a group of endocrine disruptors known as hormonal growth promotants (HGPs), which are natural and synthetic chemicals used to promote growth in livestock by targeting the endocrine system. One of the most potent compounds to enter the environment as a result of HGP use is 17β-trenbolone, which has repeatedly been detected in aquatic habitats. Although recent research has revealed that 17β-trenbolone can interfere with mechanisms of sexual selection, its potential to impact sequential female mate choice remains unknown, as is true for all EDCs. To address this, we exposed female guppies (*Poecilia reticulata*) to 17β-trenbolone at an environmentally relevant level (average measured concentration: 2 ng/L) for 21 days using a flow-through system. We then compared the response of unexposed and exposed females to sequentially presented stimulus (i.e., unexposed) males that varied in their relative body area of orange pigmentation, as female guppies have a known preference for orange colouration in males. We found that, regardless of male orange pigmentation, both unexposed and exposed females associated with males indiscriminately during their first male encounter. However, during the second male presentation, unexposed females significantly reduced the amount of time they spent associating with low-orange males if they had previously encountered a high-orange male. Conversely, 17β-trenbolone-exposed females associated with males indiscriminately (i.e., regardless of orange colouration)

during both their first and second male encounter, and, overall, associated with males significantly less than did unexposed females during both presentations. This is the first study to demonstrate altered sequential female mate choice resulting from exposure to an endocrine disruptor, highlighting the need for a greater understanding of how EDCs may impact complex mechanisms of sexual selection.

Keywords

Agricultural pollution, endocrine-disrupting chemical, *Poecilia reticulata*, reproductive behaviour, sequential female mate choice, trenbolone

3.2 INTRODUCTION

Chemical pollutants are accumulating in environments worldwide at an alarming pace and scale (Kolpin et al., 2002; WHO-UNEP, 2012; Arnold et al., 2014). Of great concern are endocrine-disrupting chemicals (EDCs)—compounds that can alter the endocrine function of organisms at minute concentrations (in the range of ng/L) by interfering with hormonal communication (Kavlock et al., 1996; Lintelmann et al., 2003; Buchanan and Partecke, 2012; Brander, 2013). Endocrine-disrupting chemicals encompass a broad range of both artificial compounds, which include pharmaceuticals, metals, plastics and pesticides (Diamanti-Kandarakis et al., 2009), and natural hormones, such as xenoestrogens (Gore et al., 2015). They can infiltrate ecosystems during their production, use, and/or disposal (WHO-UNEP, 2012), with common sources including wastewater from industry and households, agricultural and suburban run-off, and solid waste (Diamanti-Kandarakis et al., 2009). Once in the environment, many EDCs have a tendency to bioaccumulate (Crews et al., 2007; Walker and Gore, 2011), and have therefore continually been detected at elevated concentrations in wildlife tissues, even in the most remote regions on Earth (e.g., polar bears in the Arctic, Letcher et al., 2010; amphipods in the Mariana Trench, Jamieson et al., 2017).

One group of EDCs with the potential to impact wildlife is hormonal growth promotants (HGPs), which are natural and synthetic chemicals used to promote growth in livestock (Hunter, 2010; Sellin Jeffries et al., 2011; Kolodziej et al., 2013; Johnson, 2015). HGPs are used globally, and their use is particularly widespread in several of the world's leading beef-producing nations. For example, in the USA,

which is the world's leading beef producer, it is estimated that 20 million cattle (i.e. approximately two thirds of the total livestock in the country) currently receive HGP implants (Johnson 2015). Although HGPs generally include mixtures of natural and synthetic hormones (Lange et al., 2001; Hunter, 2010), the most commonly administered androgen in HGP implants is trenbolone acetate (Hunter, 2010), which is a highly efficient and potent synthetic steroid (Neumann, 1976). Trenbolone acetate is hydrolysed within implanted cattle to produce the biologically active steroid hormone 17β-trenbolone, which enters the environment via run-off of urine and faeces. Once present in the aquatic environment, 17β-trenbolone has a tendency to accumulate as a result of its long half-life (~260 days measured in animal waste; Schiffer et al., 2001) and has been detected at concentrations ranging from 1–20 ng/L in waterways upstream and downstream of cattle farm outflow points (Durhan et al., 2006) to 162 ng/L in tile-drained agroecosystems (Gall et al., 2011).

A growing number of studies have demonstrated that exposure to 17β-trenbolone can have alarming impacts on wildlife, particularly in aquatic environments. Exposure has been linked with severe morphological and physiological abnormalities in fish, including abnormal gonadal development (Örn et al., 2006), reduced reproductive output (Ankley et al., 2003), irreversible masculinisation (Baumann et al., 2014), and even complete and functional sex reversal (Larsen and Baatrup, 2010; Morthorst et al., 2010). We now know that 17β-trenbolone can also induce behavioural abnormalities, with recent research revealing that environmentally realistic exposure concentrations can affect risk-taking behaviour in guppies (*Poecilia reticulata*; Heintz et al., 2015), as well as

reproductive behaviour and sexual selection processes in both guppies (Bertram et al., 2015; Tomkins et al., 2016, 2017) and eastern mosquitofish (*Gambusia holbrooki*; Saaristo et al., 2013). However, the potential impacts of 17β-trenbolone on more complex mechanisms of sexual selection remain poorly understood, as is also true for EDCs generally.

Sexual selection, by directly influencing mating outcomes, has important consequences for reproductive success, population dynamics and broader evolutionary processes (Candolin and Wong, 2012). Because sex hormones regulate the expression of a range of behaviours under sexual selection (Beyer et al., 1976; Munakata and Kobayashi, 2010), exposure to endocrine disruptors is likely to influence sexual selection processes. Indeed, recent research has revealed that, in simultaneous mate choice experiments (i.e., when females are presented with two or more males at the same time), exposure to environmentally relevant concentrations of endocrine-disrupting chemicals can impair female mate choice in sand gobies (*Pomatoschistus minutus*; Saaristo et al., 2009) and guppies (Tomkins et al., 2016). However, in nature, opportunities for females to make direct comparisons between suitors are often limited (Jennions and Petrie, 1997). In many species, it is more common for females to encounter mates sequentially (Bradbury and Andersson, 1987), making investigating the effects of EDCs on sequential female mate choice more ecologically relevant.

Guppies are a small, freshwater fish that occur in contaminated environments around the world (e.g., López-Rojas and Bonilla-Rivero, 2000; Widianarko et al., 2000). They are an ideal species for investigating the impacts of

endocrine disruptors on the mechanisms of sexual selection as their mating system is driven primarily by female choice. Males compete for the attention of females, achieving copulations via two contrasting mating strategies. Briefly, males either mate consensually with females following successful courtship displays (termed 'sigmoid displays'), or gain copulations by sneaking up behind females and attempting to mate with them coercively (termed 'sneak' attempts) (Houde, 1997). Previous research investigating female mate choice in guppies has found that females show a strong preference for males with relatively large areas of orange pigmentation on their bodies (e.g., Houde, 1987; Kodric-Brown, 1989; Long and Houde, 1989; Endler, 1995; Grether, 2000; Kodric-Brown and Nicoletto, 2001). Orange colouration is an honest indicator of male quality in guppies, correlating positively with swimming performance (Nicoletto, 1993), foraging ability (Endler, 1980; Karino and Shinjo, 2007; Karino et al., 2007), sperm quality (Locatello et al., 2006; Pitcher et al., 2007) and sperm load size (Pitcher and Evans, 2001; Pitcher et al., 2007), as well as parasite resistance (Houde and Torio, 1992). However, these studies have relied almost exclusively on experimental set-ups in which females are able to make direct comparisons between males. This is true, despite the fact that, in the wild, female guppies will often have to make reproductive decisions based on sequential encounters with potential suitors (Houde, 1997; Pitcher et al., 2003). Guppies, therefore, provide an excellent opportunity to further our understanding of the impacts of EDCs on sexual selection by investigating the hitherto unknown impact of EDCs on sequential female mate choice

Here, we test the hypothesis that short-term exposure to an environmentally realistic concentration of 17β-trenbolone will impact sequential female mate choice in guppies. Given that 17β-trenbolone has been shown to affect reproductive behaviour in guppies and other Poeciliids, we expected exposure to also disrupt female mate choice processes when males are encountered sequentially, which is often the more environmentally realistic scenario.

3.3 METHODS

Fish collection and housing

Guppies were collected from Alligator Creek in Queensland, Australia (19° 26' 17" S, 146° 57' 01" E), where a wild population has established itself as a result of deliberate and/or accidental introductions from the pet trade. The sampling site is located inside the Bowling Green Bay National Park, and is thus thought to be a pristine location. Indeed, we have taken water samples from this site over consecutive years and found no presence of 17β-trenbolone (ALS Group, unpublished data). Fish were actively collected using dip nets and brought back to Monash University in aerated containers, where they were acclimated to laboratory conditions (25–27 °C, 12:12 h light:dark regime) in sex-specific tanks for three months prior to exposure to ensure sexual receptivity during behavioural trials. Fish were fed *ad libitum* once daily with a commercial fish pellet (Otohime Hirame larval diet, 580–910 μm).

Chemical exposure and water testing

Female guppies were exposed to 17β-trenbolone for 21 days via a flow-through system adapted from previous studies (Saaristo et al., 2013; Bertram et al., 2015; Tomkins et al., 2016, 2017). The system was comprised of six 54 L aquaria, consisting of three unexposed tanks and three 17β-trenbolone-exposed tanks. A total of 120 females were randomly distributed between these six tanks (i.e., 20 fish per tank). Fish in the exposed aquaria received an average measured concentration of 2 ng/L of 17β-trenbolone (see below for details), which is consistent with concentrations detected in freshwater systems affected by agricultural activity (Durhan et al., 2006), while the unexposed tanks received fresh water only. Throughout the flow-through exposure, all fish were maintained under the same housing conditions as those described above.

The stock solution was created by dissolving 17β-trenbolone (17β-hydroxyestra-4,9,11-trien-3-one, CAS: 10161-33-8; Novachem, Germany) in ethanol (HPLC grade, ≥99.99%) to create a stock standard of 400 mg/L. This stock solution was diluted to 400 μg/L using deionised water, before being further diluted in the flow-through system to achieve a 17β-trenbolone exposure concentration of 2 ng/L (average measured concentration = 1.67 ng/L, SD = 0.56, n = 9). Water samples (200 mL) were collected from each of the 17β-trenbolone-exposed and unexposed tanks weekly and analysed using gas chromatography–tandem mass spectrometry (7000C Triple Quadrupole GC-MS/MS, Agilent Technologies, Delaware, USA). Analysis was conducted by Envirolab Services (MPL Laboratories, Perth; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025). No contamination with 17β-trenbolone was detected in the unexposed tanks throughout the exposure period

(limit of quantification: 1 ng/L, n = 9). For a detailed description of the collection and analysis of water samples, see 'Supplementary Methods' in Supplementary material.

Behavioural trials

To investigate the impact of exposure to 17β-trenbolone on sequential female mate choice in guppies, a 27 L trial tank (30 × 30 × 30 cm) was separated into two compartments using a transparent perforated divider to allow full visual and chemical communication. A single experimental (i.e., unexposed or 17β-trenbolone-exposed) female was placed into one compartment (20 × 30 x 30 cm) in a 500 mL holding container and a single stimulus (i.e., unexposed) male placed into the other compartment (10 × 30 x 30 cm) in an identical holding container. Stimulus males were not subjected to the flow-through exposure, instead being drawn randomly from one of eight 27 L same-sex holding tanks (30 × 30 × 30 cm), having been housed under the same temperature, light and feeding conditions as females from the flow-through exposure. Stimulus males were unexposed to ensure 17β-trenbolone-induced changes in male behaviour did not influence the behaviour of females (Saaristo et al., 2013). After a 5 min acclimation period, both fish were released from their holding containers into their respective compartments and allowed to interact for 15 min through the divider. The first stimulus male was then removed and replaced with a second stimulus male, which was again subject to a 5 min acclimation period in a holding container before being released and allowed to interact with the female through the divider for a further 15 min.

Our experimental design required two categories of stimulus males, those with a high percentage body area of orange pigmentation (i.e., 'high-orange' males) and those with a low percentage body area of orange pigmentation (i.e., 'low-orange' males). This is because a strong female preference for males with relatively large areas of orange pigmentation on their bodies has been documented in many guppy populations (e.g., Kodric-Brown, 1985; Houde, 1987; Long and Houde, 1989; Endler, 1995; Kodric-Brown and Nicoletto, 2001), including in guppies from the Alligator Creek population used in our study (e.g., Gamble et al., 2003; Bertram et al., 2015). Male percentage body area of orange pigmentation was judged visually at the beginning of the exposure period and males were separated accordingly. Immediately following behavioural trials, males were photographed and the subsequent images used to quantify their percentage body area of orange pigmentation using digital colouration analysis (see 'Morphological analysis' below). Low-orange males possessed a percentage body area of orange pigmentation ranging from 3.16–8.22% (mean = 5.21%, SD = 1.32%), while high-orange males ranged from 12.05–19.66% (mean = 15.31%, SD = 1.75%) (Table S1). These values are comparable to those reported in previous research investigating sequential female mate choice in guppies by Pitcher et al. (2003), both in terms of the mean percentage body area of orange pigmentation in each stimulus male group, as well as the degree of separation between the group means. Further, Karino and Shinjo (2004) demonstrated that female guppies show a preference for males bearing as little as 2.0% more orange colouration than relatively dull males, indicating that the

minimum difference of 3.83% orange pigmentation in our study between low- and high-orange groups is a sufficient gap for females to exercise choice.

Stimulus males were presented to females in four combinations (first male/second male): low-orange/low-orange, high-orange/high-orange, low-orange/high-orange, and high-orange/low-orange. These treatments allowed us to disentangle whether females were simply showing an absolute preference for males with increased orange pigmentation, or if their responsiveness to sequentially presented males varied depending on previous male experience. These four presentation combinations were repeated for both unexposed females (low-orange/low-orange: n = 16; high-orange/high-orange: n = 16; low-orange/high-orange: n = 15; high-orange/low-orange: n = 16) and exposed females (low-orange/low-orange: n = 16; high-orange/high-orange: n = 16; low-orange/high-orange: n = 15; high-orange/low-orange: n = 15). All male and female fish were tested once only. Female preference for both the first and second male was determined by quantifying the amount of time spent within a 5 cm 'preference zone' abutting the male compartment. Association time is commonly used as a measure of female mating preference in guppies (e.g., Kodric-Brown, 1985, 1989; Karino and Shinjo, 2004; Pilastro et al., 2004; Tomkins et al., 2016) and has been shown to be an accurate indicator of female mate choice in Poeciliid fish (Walling et al., 2010). Female behaviour was quantified using the event-recording software JWatcher V1.0 (Blumstein and Daniel, 2007).

Morphological analysis

Immediately following behavioural trials, all fish were weighed (±0.0001 g) and measured for total length (±0.01 mm). Stimulus males were also photographed immediately after behavioural trials 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. See Bertram et al. (2015) for details.

Statistical analysis

Data were analysed in R version 3.3.2 (R Core Development Team, 2016). Tests of normality (Shapiro-Wilk test; Royston, 1995) and homogeneity of variance (Fligner-Killeen test; Conover et al., 1981) were performed, where appropriate. Association time was square-root transformed prior to analysis to normalise residual errors. Statistical significance was assigned at $\alpha = 0.05$.

Firstly, we examined whether female association time differed due to treatment (i.e., unexposed versus 17β-trenbolone-exposed) and/or male percentage body area of orange pigmentation during the first presentation using a generalised linear model (GLM). Treatment, male percentage body area of orange pigmentation and the interaction term were treated as fixed effects. Secondly, a linear mixed-effects model (*lme* function, *nlme* package; Pinheiro et al., 2017) with a Gaussian error distribution was used to determine whether females altered their response to males based on previous male experience. Treatment, male percentage body area of

orange pigmentation, presentation order and the interaction terms were entered as fixed effects, with male ID entered as a random effect. Likelihood ratio tests (G^2) were then used to calculate the *p*-values of interaction terms (Bolker et al., 2009). Lastly, another GLM was used to test whether female association time differed due to treatment and/or male percentage body area of orange pigmentation during the second male presentation. In this instance, treatment, presentation order and the interaction term were entered as fixed effects. Presentation order was entered as a fixed effect to account for previous male experience. Mann-Whitney *U* tests were used to evaluate whether exposure to 17β-trenbolone altered female weight or total length, and independent samples *t*-tests were used to compare the orange pigmentation of males.

3.4 RESULTS

Female association time during first male presentation

We found no interaction between treatment and male orange pigmentation on female association time ($F_{3,116}$ = 0.20, p = 0.660). Regardless of their own exposure status, we found no difference in the total time that females spent associating with low- and high-orange males ($F_{1,117}$ = 2.85, p = 0.094). However, in general, unexposed females spent more time associating with males than exposed females, irrespective of male orange pigmentation ($F_{1,117}$ = 45.17, p < 0.001; Fig. 1)

Sequential female choice

We found a significant three-way interaction between treatment, male orange pigmentation and presentation order (G^2 = 9.94, p = 0.019). To account for this complex interaction, we analysed each treatment group separately.

For unexposed females, we found an interaction between male orange pigmentation and presentation order on female association time (G^2 = 31.39, p < 0.001). Specifically, unexposed females that were initially offered a high-orange male reduced their association time when subsequently presented with a low-orange male (t_{28} = 3.49, p < 0.001; Fig. 2A). However, in all other presentation combinations, there were no significant differences in the total time that unexposed females spent associating with the first and second male (low/low: t_{14} = 1.73, p = 0.10; high/high: t_{14} = 1.42, p = 0.178; low/high: t_{14} = -1.99, p = 0.066).

Contrasting with unexposed females, for exposed females we found no interaction between male percentage body area of orange pigmentation and presentation order on female association time (G^2 = 3.81, p = 0.283; Fig. 2B).

Female association time during second male presentation

Overall, unexposed females spent more time associating with males than did exposed females during the second male presentation ($F_{1,112}$ = 33.47, p < 0.001). However, when unexposed females were presented with a low-orange male after having first observed a high-orange male, their association time reduced to a level that was comparable to that of exposed females in all presentation combinations ($F_{1,28}$ = 0.14, p = 0.712; Fig. 3).

Morphology

There was no significant difference in the weight (U = 870, p = 0.605) or total length (U = 587, p = 0.592) of unexposed and exposed females.

Table 1. Orange colouration (mean ± SD) of males used in behaviour trials. The percentage of orange colouration of the first and second male in each presentation combination was compared using independent samples t-tests.

Presentation combination	*n*	First male orange colouration (Mean% ± SD)	Second male orange colouration (Mean% ± SD)	Comparison of first and second male
Unexposed female				
Low/Low	16	6.01 ± 1.02%	5.88 ± 1.03%	t = 0.19, df = 23.26, p = 0.890
High/High	16	14.90 ± 2.02%	14.11 ± 1.98%	t = -0.44, df = 29.22, p = 0.923
Low/High	15	5.80 ± 0.94%	14.15 ± 1.39%	t = -0.76, df = 27.29, p < 0.001
High/Low	16	14.66 ± 1.66%	5.29 ± 1.20%	t = -0.52, df = 29.81, p < 0.001
Exposed female				
Low/Low	16	4.82 ± 1.08%	5.48 ± 1.17%	t = -0.42, df = 28.46, p = 0.668
High/High	16	16.09 ± 1.46%	15.06 ± 1.72%	t = 0.13, df = 27.81, p = 0.861
Low/High	15	5.01 ± 1.45%	15.32 ± 1.80%	t = -0.87, df = 30.46, p < 0.001
High/Low	15	14.76 ± 1.88%	5.53 ± 1.50%	t = -0.76, df = 25.76, p < 0.001

Figure 1. Mean (±SE) time spent by unexposed and 17β-trenbolone-exposed females associating with low- and high-orange males during the first male presentation. The asterisk indicates a significant difference between groups ($p < 0.05$) obtained from ANOVA.

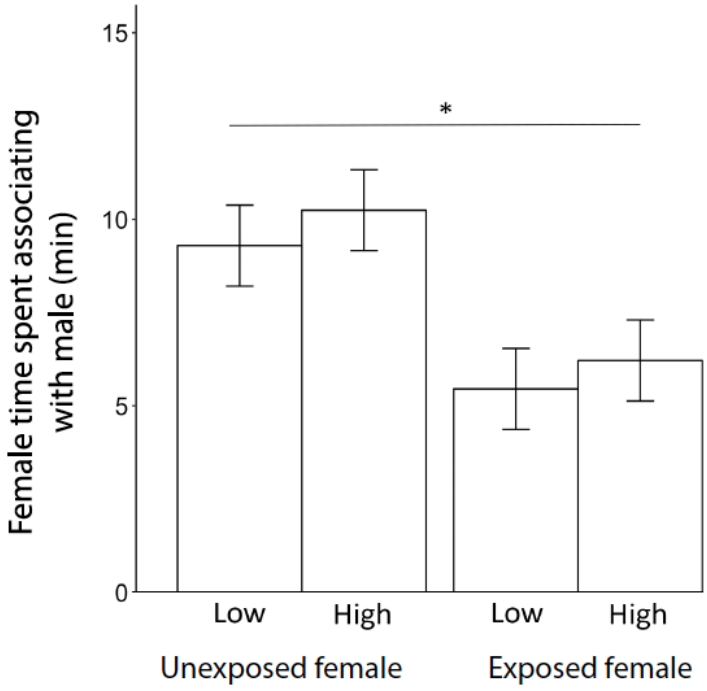


Figure 2. Mean (±SE) time that (A) unexposed and (B) 17β-trenbolone-exposed females spent associating with males in each trial combination. Grey bars represent the first male presentation and white bars represent the second male presentation. The asterisk indicates a significant difference between groups ($p < 0.05$) obtained from Tukey's tests of simplified linear mixed-effects models.

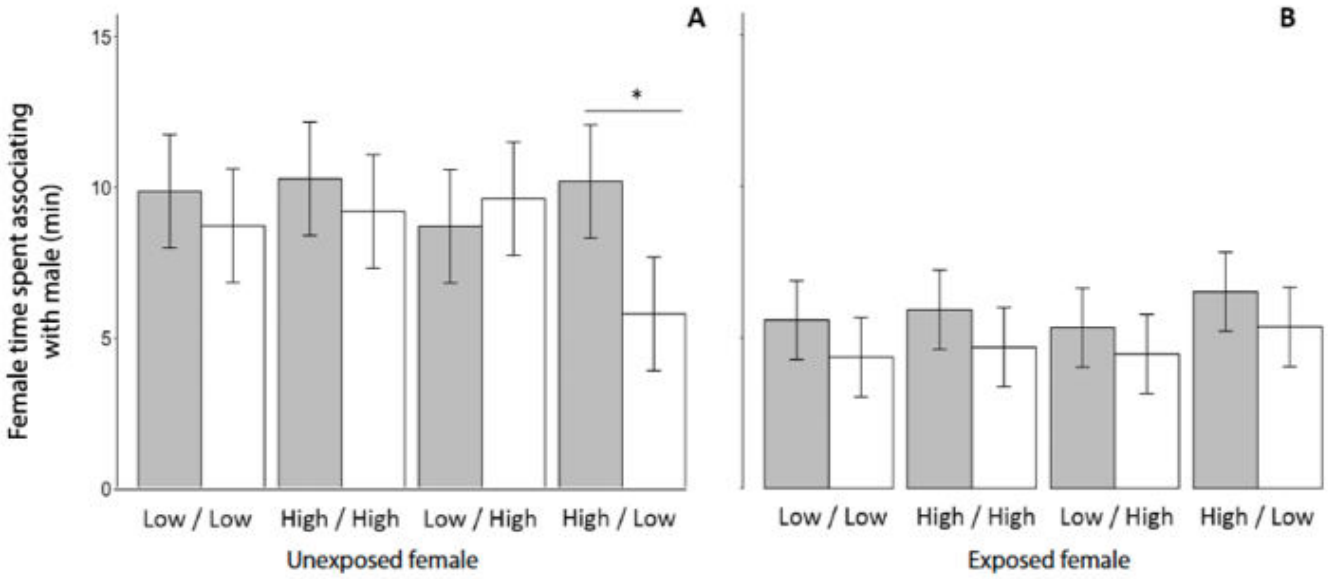
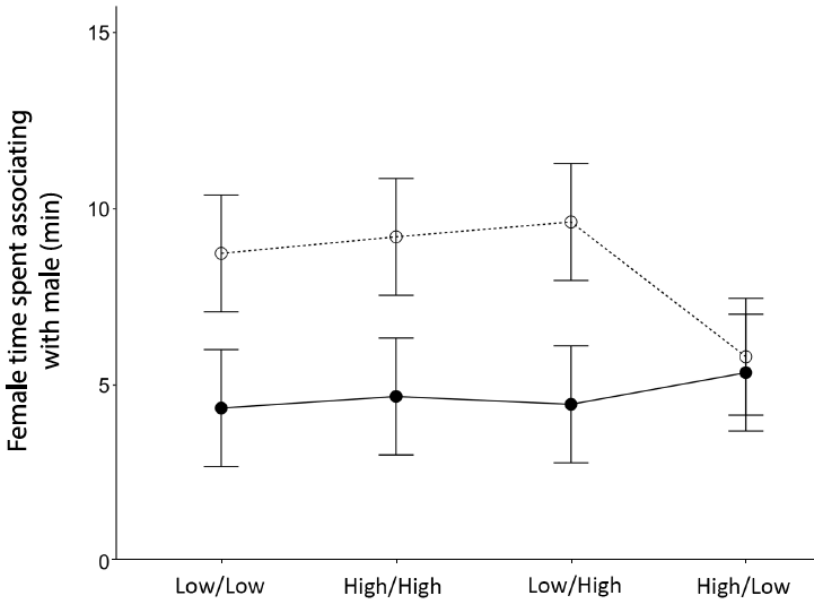


Figure 3. Interaction plot showing the mean (±SE) time that unexposed females (open circles) and 17β-trenbolone exposed females (closed circles) from each presentation combination spent associating with males during the second male encounter. Plot displays the interaction between treatment and presentation order.



3.5 DISCUSSION

This is the first study to demonstrate that exposure to an endocrine-disrupting chemical (EDC) at an environmentally relevant concentration can influence female mate choice when males are encountered sequentially. We found that, during their first male encounter, both unexposed and 17β-trenbolone-exposed females associated with males indiscriminately, although exposed females spent significantly less time associating with males overall than did unexposed females. During their second male encounter, unexposed females that were presented with a low-orange male significantly reduced their association time if they had previously encountered a high-orange male. Conversely, exposed females associated indiscriminately with males during their second male encounter, and again associated with males significantly less overall than did unexposed females. These findings demonstrate the profound influence that a widespread androgenic EDC can have on sexual selection processes at environmentally realistic exposure concentrations.

Both unexposed and exposed female guppies showed no preference for greater orange colouration during their first male encounter. It is well established that female guppies prefer males with increased orange pigmentation (Endler, 1980; Houde, 1997), including in the population used in this research (Gamble et al., 2003; Bertram et al., 2015). However, the vast majority of studies that have investigated female mate choice in guppies have done so using simultaneous choice experiments, where the female is able to make direct comparisons between males. To our knowledge, only one study has investigated female mate choice in guppies

when males are encountered sequentially, which, in accordance with our results, found that virginal female guppies showed no preference for greater orange colouration during their first male encounter (Pitcher et al., 2003). While virgin females were not used in this study, females were sexually isolated for three months prior to exposure, as well as throughout the 21-day exposure period, which likely explains why they associated with males indiscriminately during their first male encounter. Further, although female guppies are able to store sperm for several months (Houde, 1997; Gasparini, 2012; Lopez-Sepulcre et al., 2013), it is possible that the sperm storages of females used in this experiment were diminished during the extended isolation period preceding and during our exposure, which may have contributed to the lack of choosiness observed in females during their first male encounter.

Our results demonstrate that female preference can be influenced by previous male experience. Time spent by unexposed females associating with males during the first and second presentation did not differ in the low-orange/low-orange, low-orange/high-orange and high-orange/high-orange trial combinations. However, when unexposed females were presented with a low-orange male after having first observed a high-orange male, the amount of time they spent associating with the second male reduced significantly. This suggests that females were not simply showing an overall preference for increased orange colouration, but were adjusting their mate choice decisions based on previous experience with potential suitors (i.e., 'previous male effect' *sensu* Bakker and Milinski, 1991). Considering that females increase their reproductive success by maximising the quality of their

mating partners (Bateman, 1948), this strategy reduces the likelihood of females mating with low-quality males in a population of high-quality suitors (Bakker and Milinski, 1991; Milinski, 2001), and has previously been demonstrated in zebra finches (*Taeniopygia guttata*; Collins, 1995), smooth newts (*Lissotriton vulgaris*; Gabor and Halliday, 1997), crickets (*Gryllus bimaculatus*; Bateman et al., 2001) and guppies (Pitcher et al., 2003).

In contrast to unexposed females, we found no evidence of a previous male effect in females exposed to 17β-trenbolone. Moreover, exposed females showed no preference for increased orange colouration in either their first or second male presentation, indicating a breakdown of sexual selection processes. Further, exposed females spent significantly less time associating with males overall than did unexposed females during both their first and second male encounter, indicating that not only were exposed females less choosy, they were also generally less interested in mating. This finding is in agreement with research by Saaristo et al. (2013), where female mosquitofish exposed to 17β-trenbolone at 6 ng/L for 21 days approached males less, and spent more time swimming away from males, than did unexposed females. This result is also consistent with work by Tomkins et al. (2016), where 21-day exposure at 4 ng/L resulted in guppy females being less choosy and performing less association behaviour when presented with two males simultaneously. Interestingly, when unexposed females in the present study exhibited reduced interested in a male (i.e., during the second male presentation in the high-orange/low-orange combination), their association time reduced to a level that was comparable to—i.e., not significantly different from—the time spent by

exposed females associating with males in all presentation combinations. This is important as it demonstrates that, regardless of male quality, females exposed to 17β-trenbolone behave similarly to unexposed females that are relatively disinterested in mating. To understand why 17β-trenbolone impacts choosiness in females, its mode of action must be considered.

The agricultural contaminant 17β-trenbolone is a potent, non-aromatisable androgen receptor agonist (Rogozkin, 1991; Hotchkiss et al., 2008). It binds with high affinity to available androgen receptors, mimicking the effects of androgens such as testosterone and 11-ketotestosterone (Wilson et al., 2002). It is also hypothesised that 17β-trenbolone indirectly inhibits 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). As a result, 17β-trenbolone can suppress estrogenic activity in female fish. Ankley et al. (2003) observed reduced plasma concentrations of vitellogenin and 17β-estradiol in 17β-trenbolone-exposed female fathead minnows (*Pimephales promelas*), which was linked with the development of male morphological characteristics. Exposure to 17β-trenbolone has also been found to cause varying levels of masculinisation in female mosquitofish (Sone et al., 2005; Brockmeier et al., 2013), zebrafish (*Danio rerio*; Morthorst et al., 2010; Baumann et al., 2014) and Japanese medaka (*Oryzias latipes,* Seki et al., 2006). It is likely that, despite our low exposure concentration and relatively short exposure period, 17β-trenbolone-exposed females in our experiment experienced some degree of masculinisation, which may have reduced their desire to mate and, in turn, made them less choosy. Further research in this

area is needed to gain a better understanding of the underlying mode of action of 17β-trenbolone.

We found no effect of 17β-trenbolone exposure on female weight or length, despite the anabolic potency of 17β-trenbolone (Neumann, 1976). This result is consistent with previous research examining the morphological impacts of 17β-trenbolone-exposure at environmentally realistic concentrations. Specifically, 17β-trenbolone had no impact on the weight or length of female guppies at 4 ng/L (Tomkins et al., 2017), 8 ng/L (Tomkins et al., 2016) or 22 ng/L (Bertram et al., 2015), and had no influence on the morphology of female fathead minnows at 5 ng/L or 50 ng/L (Ankley et al., 2003). However, at 22 ng/L, 17β-trenbolone resulted in an increase in the weight and condition index of male guppies (Bertram et al., 2015), while at 4 ng/L, exposure resulted in an increase in male condition index, but not weight (M.G. Bertram et al., unpublished data). This suggests that male morphology is more sensitive to 17β-trenbolone-exposure than female morphology. However, more research is required to disentangle these dose-dependent and sex-specific effects.

In conclusion, this is the first study to show altered sequential female mate choice resulting from exposure to an endocrine disruptor. We found that, during a second male encounter, unexposed females altered the amount of time they spent associating with males depending on the orange colouration of a previously encountered male. Exposed females, on the other hand, associated with males indiscriminately during both the first and second male presentations. Further, exposed females spent less time associating with males overall than did unexposed

females, indicating a decrease in mating interest. Considering that orange colouration is an honest indicator of male quality in guppies (Endler, 1980; Houde and Torio, 1992; Nicoletto, 1993; Pitcher and Evans, 2001; Locatello et al., 2006; Karino and Shinjo, 2007; Karino et al., 2007; Pitcher et al., 2007), the 17β-trenbolone-induced behavioural shifts observed in this study are expected to result in exposed females mating with inferior suitors. In nature, it is often more common for female guppies to encounter males sequentially, meaning the indirect costs associated with this breakdown in sexual selection processes could have population-level impacts by influencing the quality and quantity of offspring produced (reviewed in Candolin and Heuschele, 2008; Candolin and Wong, 2012; Wong and Candolin, 2015). Thus, this study highlights the need for a greater understanding of the potential impacts of EDCs on complex sexual selection processes, and how these changes may, in turn, influence population dynamics, ecosystem function, and broader evolutionary processes.

ETHICS

This study was approved by the Biological Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2013/09) and is compliant with all relevant State and Federal laws of Australia.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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Declaration for thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution was the following:

Nature of contribution	Extent of contribution (%)
Conceiving/designing the study, conducting fieldwork, laboratory experiments and data analysis, and writing the chapter/manuscript	90%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%)
Bob Wong	Helping design the study, proof reading, and contributing to the manuscript	2.5%
Minna Saaristo	Helping design the study, proof reading, and contributing to the manuscript	2.5%
Michael Bertram	Proof reading and contributing to the manuscript	2.5%
Mayumi Allinson	Helping with labwork	1.5%
Raymond Tomkins	Helping with fieldwork and labwork	1%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work

Student signature: [redacted] **Date:** 17/11/2017

Main supervisor signature: [redacted] **Date:** 17/11/2017

Chapter 4

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

Patrick Tomkins [a], Minna Saaristo [a,b], Michael G. Bertram [a], Raymond B. Tomkins [c], Mayumi Allinson [d], Bob B. M. Wong [a]

Guppies (Poecilia reticulata) collected in Alligator Creek

[a] *School of Biological Sciences, Monash University, Victoria, Australia*

[b] *Department of Biosciences, Åbo Akademi University, Turku, Finland*

[c] *Centre for AgriBioscience, Department of Environment and Primary Industries (DEPI), Victoria, Australia*

[d] *Centre for Aquatic Pollution Identification and Management (CAPIM), School of Chemistry, The University of Melbourne, Victoria, Australia*

4.1 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. An 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.

Keywords: 17β-trenbolone, endocrine disrupting chemical, guppy, hormonal growth promotant, sexual selection

4.2 INTRODUCTION

In many species, competition between males for access to potential mates is a key mechanism of sexual selection (Andersson, 1994). Male-male competition plays a pivotal role in the maintenance and exaggeration of male traits and behaviours (Andersson and Simmons, 2006), 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 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 molecules 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, 2012), 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., 2002). 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 Zala and Penn, 2004; Clotfelter et al., 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., Schoenfuss et al., 2008; 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 several 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). Often allowed to enter the environment, 17β-trenbolone can accumulate in the 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 complete and functional 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 that were 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 favour a number of male traits, including greater orange colouration (i.e., area and chroma, Endler, 1980), as well as increased male body size (Reynolds and Gross, 1992) and courtship display rate (Brooks and Caithness, 1995; 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 EDC-contaminated waterways (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 effect 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 (reviewed in Nelson, 2000; Taves et al., 2009). Therefore, we hypothesised that exposure to 17β-trenbolone would result in an increase in male aggressive behaviours in a competitive setting.

4.3 METHODS

Animal housing

Guppies were collected with dip nets from a pristine, rainforest-fed stream located within Alligator Creek, Queensland, Australia (19° 26' 18" S, 146° 57' 01" E). 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).

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; Oshima et al., 2003; Martinovic et al., 2007; Maunder et al., 2007; 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., 2002), 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 concentrations in the exposure tanks, flow rates were kept constant (5.38 L/h) using flow meters (BES Flowmeters, 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.

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 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 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 hr 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 trenbolone in 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.

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 S110), 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).

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).

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 (%).

4.4 RESULTS

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).

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 with 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).

Morphology

No significant difference was detected in weight (Mann-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.

Figure. 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 α = 0.05.

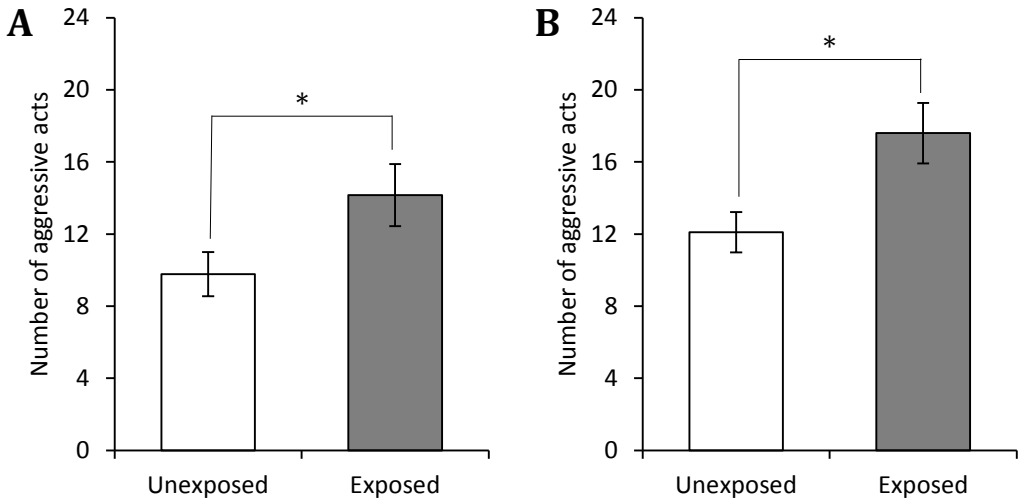


Figure. 2. Mean (±SE) number of courtship events conducted by males when A) males were separated from females by a transparent partition (*n* = 37) and B) males were allowed to interact freely with females (*n* = 37). Asterisks indicate a significant difference between groups at α = 0.05.

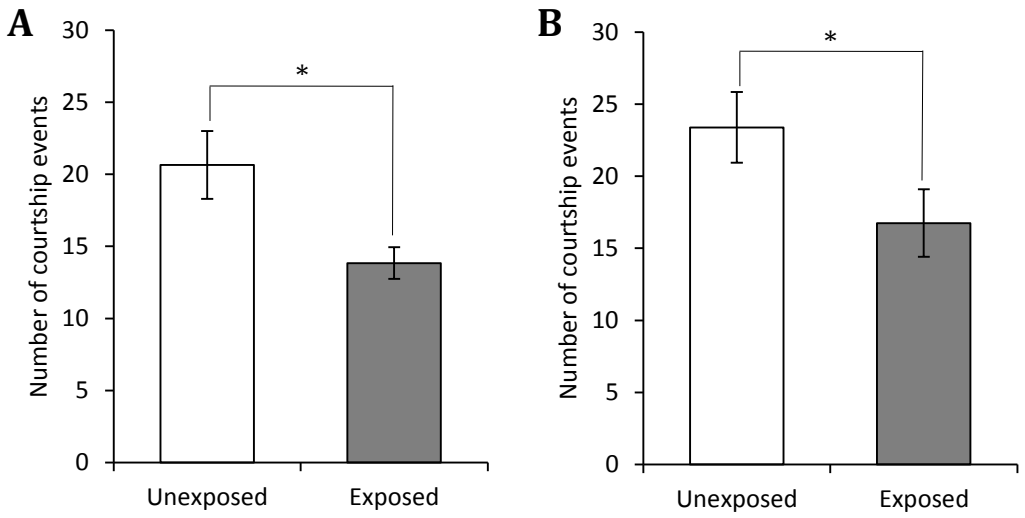


Figure. 3. Mean (±SE) number of sneak mating attempts conducted by males towards females when allowed to freely interact (n = 37). Asterisk indicates a significant difference between groups at $\alpha = 0.05$.

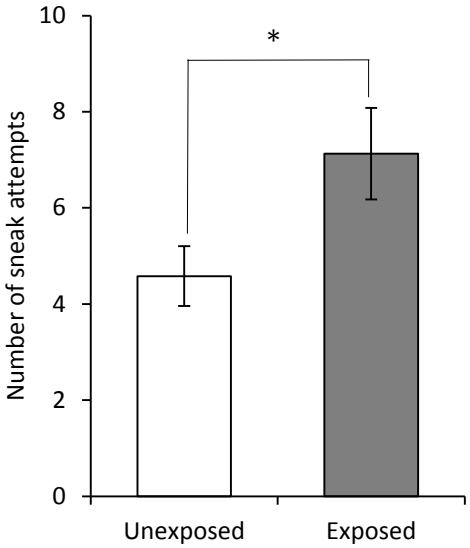
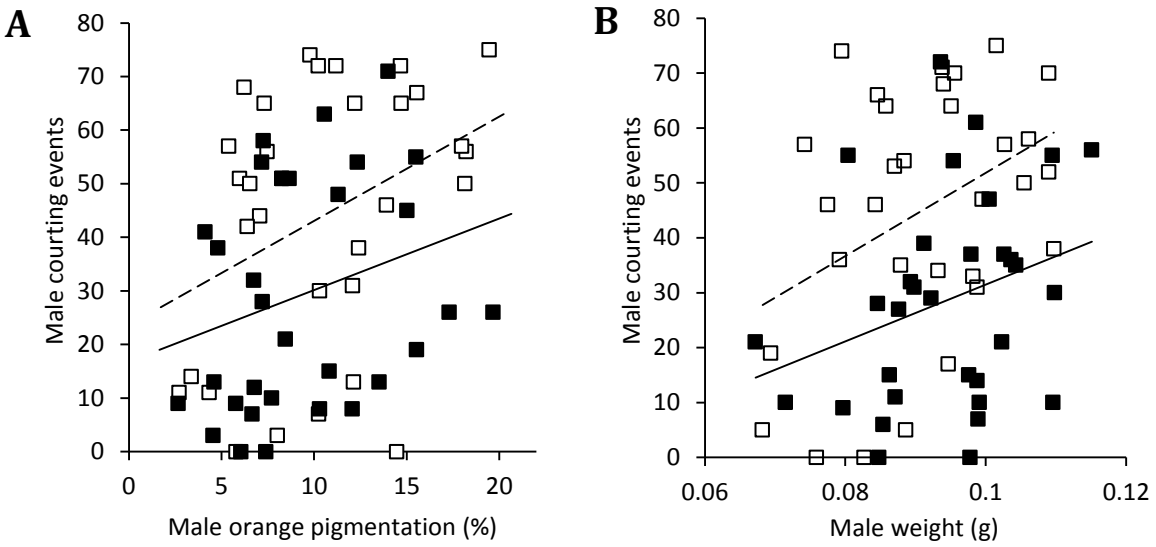


Figure. 4. Number of courtship events performed by unexposed and exposed males as a function of A) male orange pigmentation (% of body area) and B) male weight (g). Figures represent combined data from both behavioural trial stages (i.e., when males were separated from females by a transparent partition 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.



4.5 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.

Androgens are essential to the development and maintenance of male traits (Mooradian et al., 1987; 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), thereby resulting in the up- or down-regulation of genes essential to reproductive function (Hotchkiss et al., 2008; Zhenghong et al., 2010). As a potent

and non-aromatisable androgen receptor agonist (Rogozkin, 1991; Schiffer et al., 2001; Hotchkiss et al., 2008), 17β-trenbolone binds with high affinity to available androgen receptors, mimicking the effects of testosterone and 11-ketotestosterone (Wilson et al., 2002). Further, it is hypothesised that 17β-trenbolone indirectly inhibits 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). Recent research has demonstrated that exposure to 17β-trenbolone can influence plasma concentrations of 17β-estradiol, testosterone and vitellogenin (Ankley et al., 2003; Zhenghong et al., 2011), and cause varying levels of masculinisation in females, including vaginal agenesis, increased anogenital distance and induced male sex accessory tissues in rats (Hotchkiss and Nelson, 2007), and the development of male morphological characteristics in female fathead minnows (e.g., nuptial tubercules and dorsal fat pads, 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. Compared to standard laboratory assays, which typically 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 17β-trenbolone-exposed behavioural changes are not wholly understood (Larsen and Baatrup, 2010), it is highly likely that, as a potent AR agonist (Rogozkin, 1991;

Schiffer et al., 2001), exposure to 17β-trenbolone will result in the intensification of 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'. Considering that male reproductive behaviours, including courtship (Nelson 2000), are under androgenic control, 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 an increase in sneaky 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.

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Declaration for thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution was the following:

Nature of contribution	Extent of contribution (%)
Conceiving/designing the study, conducting fieldwork, laboratory experiments and data analysis, and writing the chapter/manuscript	90%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%)
Bob Wong	Helping design the study, proof reading and contributing to the manuscript	2.5
Minna Saaristo	Helping design the study, proof reading and contributing to the manuscript	2.5
Michael Bertram	Proof reading and contributing to the manuscript	2.5%
Marcus Michelangelli	Helping with statistical analysis	1.25
Raymond Tomkins	Helping with fieldwork and laboratory work	1.25

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work

Student signature: [redacted] **Date:** 17/11/2017

Main supervisor signature: [redacted] **Date:** 17/11/2017

Chapter 5

The impact of an agricultural contaminant, 17β-trenbolone, on sequential male mate choice in the guppy

Patrick Tomkins [a], Minna Saaristo [a,b], Michael G. Bertram [a], Raymond B. Tomkins [c], Bob B. M. Wong [a]

Guppy habitat, Alligator Creek

[a] *School of Biological Sciences, Monash University, Victoria, Australia*

[b] *Department of Biosciences, Åbo Akademi University, Turku, Finland*

[c] *Centre for AgriBioscience, Department of Environment and Primary Industries (DEPI), Victoria, Australia*

5.1 ABSTRACT

Endocrine disrupting chemicals (EDCs) are an insidious class of chemical pollutant. Of growing concern are a group of EDCs known as hormonal growth promotants (HGPs), which are natural and synthetic compounds used globally to promote growth in the livestock industry. One of the most common and potent compounds to enter the environment as a result of HGP use is 17β-trenbolone, which has repeatedly been detected in waterways associated with cattle feedlots. Although recent research has revealed that exposure to 17β-trenbolone can have a severe impact on female mate choice processes in fish, comparatively little is known about the effect of exposure on male mate choice, which is surprising considering that males of many species are also choosy. In particular, due to a positive size-fecundity relationship, larger females often represent better reproductive value and are often preferred by males. Further, the potential of EDCs to impact male reproductive behaviour when females are encountered sequentially remains to be investigated, even though, in nature, mates are often encountered in a sequential manner. To address this, we exposed male guppies to an environmentally relevant concentration of 17β-trenbolone (average measured concentration: 1.67 ng/L) and compared the response of exposed and unexposed males to sequentially presented large and small females. We found that, regardless of the order of female presentation, both exposed and unexposed males performed significantly more courtship bouts and sneak mating attempts towards large females, which suggests that males were basing their mate choice decisions on an absolute preference for large females. More generally, irrespective of female size, 17β-trenbolone-exposed

males conducted significantly more sneak mating attempts than unexposed males. Considering the pivotal role mate choice mechanisms play in population dynamics and broader evolutionary processes, this study highlights the need for a greater understanding of the impacts of EDCs on sexual selection processes.

Key words: sequential male mate choice, endocrine disrupting chemical, 17β-trenbolone, guppy

5.2 INTRODUCTION

Endocrine disrupting chemicals (EDCs) are a class of chemical pollutant that has received widespread attention from the scientific community. EDCs describe compounds that interfere with the normal hormonal and homeostatic function of organisms, and include a diverse range of natural and synthetic compounds. They enter the environment from a variety of sources, including domestic wastewater, agricultural run-off, and industrial wastewater (Johnson and Sumpter, 2001; Thorpe et al., 2009), and are extremely widespread, having been detected even in the most remote environments on Earth (e.g., amphipods in the Mariana Trench; Jamieson et al., 2017). EDCs are particularly concerning as they can affect organisms at minute concentrations (Diamanti-Kandarakis, 2009), and a vast literature now exists documenting their morphological, physiological, and behavioural impacts (reviewed in Mills and Chichester, 2005; Vos et al., 2000; Vandenberg et al., 2012).

In recent years, concern has been mounting over a group of EDCs known as hormonal growth promotants (HGPs), which are natural and synthetic compounds used to promote growth in livestock. Despite being banned by the European Union (Johnson, 2015), the use of HGPs is common and widespread in many of the world's leading beef-producing countries (Hunter, 2010; Kolodziej et al., 2013; Johnson, 2015). As just one example highlighting the prevelance of HPG use, the USA is the world's leading beef producer, and it is estimated that 96% of US cattle currently receive HGP implants (Biswas et al., 2016). Predictions based on typical hormone excretion rates suggest that, in the 15 million mG of manure generated by cattle in

the US each year, 43 mG of hormones can be released into the environment (Lange et al., 2002; Biswas et al., 2016).

HGPs commonly include a cocktail of chemical compounds, including androgens (eg: trenbolone acetate), estrogens (eg: 17β-estradiol, zeranol), and progestins (eg: melengestrol acetate) (Lange et al. 2001). Trenbolone acetate, which is the most common androgen administered to beef cattle (Hunter 2010), is a powerful synthetic steroid with several times the androgenic and anabolic potency of testosterone (Neumann, 1976). After implantation, trenbolone acetate is hydrolyzed within the body to produce the biologically active steroid 17β-trenbolone, which enters the environment via urine and faeces. 17β-trenbolone has a particularly long half-life (~260 days measured in animal waste; Durhan et al., 2006) allowing it to accumulate in the environment. As a result, 17β-trenbolone has been repeatedly detected in waterways associated with cattle feedlots, ranging in concentration from ≤1-20 ng/L in discharge and diffuse run-off (Durhan et al., 2006) to 162 ng/L in tile-drained agroecosystems (Gall et al., 2011).

Once in the environment, 17β-trenbolone can act as a powerful endocrine disruptor, and we now know that the reproductive morphology of fish is particularly sensitive to exposure. Specifically, 17β-trenbolone has been linked with altered gonadal morphology (Örn et al., 2006), decreased fertility (Mizukami-Murata et al., 2015), altered sexual differentiation (Olmstead et al., 2012), reduced fecundity (Ankley et al., 2003), skewed sex ratios (Örn et al., 2006; Olmstead et al., 2012), irreversible

masculinization (Baumann et al., 2014) and complete and functional female-to-male sex reversal (Larsen and Baatrup, 2010; Morthorst et al., 2010). More recently, it has been revealed that 17β-trenbolone can also influence the behavior of fish, particularly behaviours under sexual selection.

Anthropogenic changes to sexually selected behaviors can affect ecological and evolutionary processes by influencing the quality and quantity of offspring produced (Wong and Candolin, 2015). Considering that sex hormones mediate the expression of a range of sexually selected behaviours (Rubinow and Schmidt, 1996; Cunningham et al., 2012), it is not surprising that exposure to exogenous steroids influences mechanisms of sexual selection. Specifically, recent research has revealed that exposure to environmentally realistic concentrations of 17β-trenbolone can impact female mate choice processes in mosquitofish, (*Gambusia holbrooki*; Saaristo et al., 2013) and guppies (*Poecilia reticulata;* Tomkins et al., 2016). However, mate choice is not restricted to females, and in many species, males can also be choosy. In this regard, males may strategically allocate their reproductive effort if females differ in reproductive value and/or if investment into current mating attempts reduces future mating opportunities (Wong et al., 2004). This is especially true in internally fertilized species, where significant costs of ejaculate production can amplify male choosiness as sperm depletion increases (Galvani and Johnstone, 1998; Byrne and Rice, 2006). Although the vast majority of studies investigating mate choice in males have focused on simultaneous choice experiments (i.e. when males are able to make direct comparisons between females), males of many species

often encounter females sequentially (Real, 1990). Despite this, few studies have specifically investigated sequential mate choice in males, and the potential of EDCs to impact male sequential mate choice remains unknown.

The guppy is a small, viviparous fish native to North-eastern South America that is likely to inhabit EDC-contaminated environments (Lopez-Rojas and Bonilla-Rivero, 2000). The mating system of the guppy makes them an ideal candidate for investigating the impact of EDCs on sexual selection. Males utilise two alternate mating strategies. Specifically, males can engage in elaborate sigmoid courtship displays, which are employed to gain consensual copulations from females. Alternatively, males can also partake in 'sneak' mating attempts, which involve males sneaking up behind females and thrusting their modified anal fins (gonopodium) towards the female genital pore in an attempt to mate coercively (Houde, 1997). Although the mating system of the guppy is primarily driven by female mate choice, males can also be choosy and are known to show a strong preference for larger females (Dosen and Montgomerie, 2004), which are also more fecund (Herdman et al., 2004). Previous research investigating mate choice in guppies has focused exclusively on simultaneous choice experiments, which is surprising considering that, in the wild, male guppies typically encounter mates sequentially (Houde, 1997). Guppies, therefore, provide an excellent opportunity to further our understanding of both sequential male mate choice and the effects of EDCs on sexual selection by investigating the hitherto unknown impact of an endocrine disruptor on sequential male mate choice in the guppy.

Accordingly, the aims of this experiment were twofold. Firstly, we tested whether male guppies exercise mate choice when females are encountered sequentially, and secondly, whether the impact of an environmentally realistic concentration of 17β-trenbolone affects patterns (if any) of sequential male mate choice.

5.3 METHODS

Fish collection and housing

Guppies were collected from Alligator Creek, Queensland, Australia (19° 26' 17" S, 146° 57' 01" E); a pristine, rainforest-fed stream located within the Bowling Green Bay National Park. Water samples taken from this site over consecutive years revealed no contamination from 17β-trenbolone (ALS Group, unpublished data). Fish were collected using dip nets and transported to Monash University in aerated tanks, where they were acclimated to laboratory conditions in sex-specific tanks (26° C, 12:12 h light:dark regime) for three months prior to exposure to ensure sexual receptivity during behavioural trials. Fish were fed *ad libitum* once daily with commercial fish pellets (Otohime Hirame larval diet; 580–910 μ m).

Exposure and water testing

Male fish were exposed to 17β-trenbolone for 21 days via a flow-through system, which is consistent with previous studies investigating the impacts of 17β-trenbolone on Poecilid behaviour (Saaristo et al., 2013; Bertram et al., 2015; Tomkins et al., 2016). The system included six 54 L tanks consisting of three control

tanks and three 17β-trenbolone-exposed tanks. A total of 120 males were separated by sex and randomly distributed between these six tanks (i.e. 20 fish per tank). Males in the 17β-trenbolone-exposed tanks were exposed to 17β-trenbolone at a nominal concentration of 8 ng/L (see below for details), which is consistent with 17β-trenbolone concentrations detected in the environment (Durhan et al., 2006), while the control tanks contained only fresh water. All exposure tanks were maintained under the same light, temperature, and feeding regime as described above.

The 17β-trenbolone stock solution was created by dissolving 17β-trenbolone (17β-hydroxyestra-4,9,11-trien-3-one; CAS: 10161-33-8; Novachem, Germany) in ethanol (HPLC grade, ≥99.99%) to create a stock standard of 400 mg/L. This stock solution was diluted to 400 μg/L using deionized water, which was further diluted in the flow-through system to achieve the desired nominal concentration of 8 ng/L (average measured concentration = 1.67 ng/L, SD = 0.56, n= 9). 100mL water samples were taken from each of the 17β-trenbolone-exposed and control tanks weekly and analysed by a commercial water testing company (EnviroLab, Australia) using gas chromatography–tandem mass spectrometry (7000C Triple Quadrupole GC-MS/MS, Agilent Technologies, Delaware, USA). The concentration of 17β-trenbolone in the control tanks was below the detection limit (< 1 ng/L, n = 9) throughout the exposure period.

Behavioural trials

To investigate the impact of 17β-trenbolone on sequential male mate choice in guppies, we carried out behavioural trials in two stages. In the first, a single male and a single stimulus (unexposed) female were placed in a 21 L tank (30 cm × 30 cm × 24 cm) in separate, 500ml holding containers. After a 5 min acclimation period, the fish were released from their respective holding containers and allowed to interact freely for 15 min. The stimulus female was then removed and replaced with a second (unexposed) stimulus female. After another 5 min acclimation period, the second female was released and she was then allowed to interact freely for 15 minutes with the focal male. Stimulus females used in both stages of each trial were drawn randomly from one of eight 21 L holding tanks (30 × 30 × 24 cm) that were housed under the same temperature, light and feeding conditions as the experimental males (Bertram et al., 2017). Stimulus females were unexposed to ensure 17β-trenbolone-induced changes in female reproductive behaviour did not influence male behaviour. Males and females were allowed to interact freely during behavioural trials (as opposed to being separated by a divider) to ensure males could conduct their full array of reproductive behaviours (i.e., courtships and sneaks) towards the stimulus females.

Due to a known male preference for larger (i.e. more fecund) females (Dosen and Montgomerie, 2004), focal males were presented sequentially with 'small' and/or 'large' females. The length of small females ranged from 16.59 mm - 22.95 mm (mean = 19.52, SD = 1.86, see Table 1), while the length of large females ranged from

24.1 mm – 29.94 mm (mean = 27.13, SD = 1.77, see Table 1). Females were presented to males in four different combinations (first female/second female): small/small, large/large, small/large, and large/small. These treatments allowed us to disentangle whether males were showing an absolute preference for large females, or if their responsiveness to sequentially presented females varied depending on previous female experience (Wong et al., 2004; Wong and Svensson, 2009). These four treatments were repeated for both exposed males (small/small: n = 16; large/large: n=16; small/large: n = 15; and large/small: n = 15) and unexposed males (small/small: n = 16; large/large: n=16; small/large: n = 15; and large/small: n = 15). All male and female fish were tested only once.

All behavioral trials were video-recorded (Canon PowerShot S120) and the subsequent videos analysed using JWatcher V1.0 (Blumstein and Daniel, 2007). As a measure of male preference, we quantified the total number of courtship bouts and sneak mating attempts performed towards each female. Previous research investigating male mate choice in guppies has found that, when males and females are allowed to interact freely, both courtship and sneak mating behaviour are accurate indicators of male preference in guppies (Herdman et al., 2004). Courtship bouts describe a male orienting his body towards the female while performing sigmoid displays, while sneak attempts involve the male surreptitiously approaching a non-receptive female from behind and attempting to mate with her coercively (as described in Houde, 1997).

Morphological analysis

Immediately after each behavioural trial, all fish were euthanized with anaesthetic clove oil (40mg/L) before being weighed (±0.0001g) and measured for total length (±0.01 mm). 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).

Statistical analysis

Data analysis was conducted in R version 3.3.2 (R Core Development Team, 2016). Tests of normality (Shapiro-Wilk test) and homogeneity of variance (Fligner-Killeen test; Conover et al., 1981) were performed where appropriate. Statistical significance was assigned at $\alpha = 0.05$.

First, we examined whether the total number of male courtship events and sneak mating attempts differed due to treatment (i.e., unexposed and 17β-trenbolone-exposed) and/or female body length during the first female presentation using a generalised linear model (GLM) with a poisson error distribution after checking for overdispersion. Treatment, female body length and the interaction term were treated as fixed effects. Secondly, generalized linear mixed-effects models (*lme* function, *nlme* package; Pinheiro et al., 2017) with poisson error distributions were used to determine whether males altered their

courtship or sneak mating behaviour based on previous female experience. Treatment, female body length, presentation order and the interaction terms were entered as fixed effects, with male ID entered as a random effect. Likelihood ratio tests (G^2) were then used to calculate the *p*-values of interaction terms (Bolker et al., 2009). Lastly, another GLM was used to test whether the total number of male courtship bouts and sneak mating attempts differed due to treatment and/or female body length during the second female presentation. In this instance, treatment, presentation order and the interaction term were entered as fixed effects. Presentation order was entered as a fixed effect to account for any previous male effect.

Mann-Whitney *U* tests were used to evaluate whether exposure to 17β-trenbolone altered female weight or total length.

5.4 RESULTS

First female presentation

We found no interaction between treatment and female body length on total number of male courtship attempts (z = 1.335, p = 0.182) or total number of sneak mating attempts (z = 0.510, p = 0.610). Both unexposed and exposed males conducted significantly more courtship bouts and sneak mating attempts towards large females than small females (courtship bouts: z = 7.311; p <0.001; sneak attempts: z = 3.134, p = 0.002). However, exposed males conducted significantly more sneak mating attempts than unexposed males towards both large females (z = 3.853, p < 0.001; Figure 1) and small females (z = 2.486, p < 0.001; Figure 1).

Courtship behaviour, on the other hand, was not impacted by 17β-trenbolone exposure (z = 1.417, p = 0.156).

Second female presentation

Both unexposed and exposed males conducted significantly more courtship bouts and sneak mating attempts towards large females than small females (courtship bouts: z = 5.630, p <0.001, Figure 2; sneak mating attempts: z = 4.071, p <0.001, Figure 3). However, we found no three-way interaction between treatment, female length, and presentation order on the total number of courtship events (G^2 = 0.265, p = 0.966) or sneak mating attempts (G^2 = 0.622, p = 0.891). We also found no two-way interaction between presentation order and female length on the total number of courtship bouts (G^2 = 0.591, p = 0.898) or sneak mating attempts (G^2 = 0.236, p = 0.627). This suggests that males were basing their mate choice decisions on an absolute preference for larger females, as opposed to adjusting their response to females depending on previous female experience. Further, exposed males conducted significantly more sneak mating attempts than unexposed males towards both large (z =-5.276, p <0.001; Figure 3) and small females (z =2.648, p <0.001; Figure 4) in the second female presentation. Courtship behaviour, on the other hand, was not impacted by 17β-trenbolone exposure (z = 3.525, p = 0.246).

Morphology

There was no significant difference in the weight (U = 650, p = 0.709) or total length (U = 772, p = 0.681) of unexposed and exposed males.

Table 1. Size (mean ±SD) of females used in behavioural trials. The mean length of the first and second female in every presentation combination was compared using independent samples t-tests.

Presentation combination	*n*	Mean length of first female ± SD	Mean length of second female ± SD	Comparison of first and second female
Unexposed male trials				
Small/Small	15	18.87 ± 2.01	19.34 ± 1.54	t = 0.76, df = 25.46, p = 0.776
Large/Large	15	27.28 ± 1.78	26.83 ± 1.94	t = 0.49 , df = 27.83 , p = 0.554
Small/Large	15	19.67 ± 1.86	27.78 ± 1.66	t = -0.31 , df = 25.02, p < 0.001
Large/Small	16	27.33 ± 1.97	20.01 ± 1.46	t = 0.87, df = 23.22, p < 0.001
Exposed male trials				
Small/Small	15	19.20 ± 1.72	18.29 ± 1.98	t = -0.21 , df = 29.89, p = 0.653
Large/Large	16	27.18 ± 1.90	26.55 ± 1.37	t = -0.53, df = 20.64, p = 0.621
Small/Large	15	19.32 ± 1.88	26.24 ± 1.58	t = 0.71, df = 22.56, p < 0.001
Large/Small	15	27.92 ± 1.61	19.46 ± 2.50	t = -0.43, df = 25.54, p < 0.001

Figure 1. Mean (±SE) total number of sneak mating attempts performed by unexposed and 17β-trenbolone-exposed males towards small and large females during the first female presentation. Asterisks indicate a significant difference between groups at $\alpha = 0.05$.

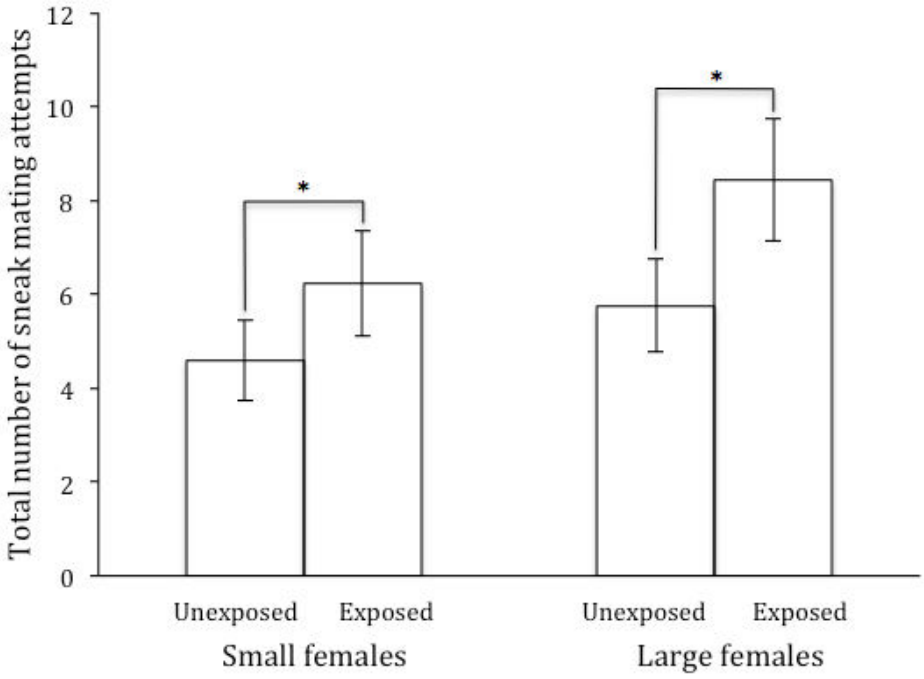


Figure 2. Mean (±SE) total number of courtship bouts performed by (A) unexposed and (B) 17β-trenbolone-exposed males towards females. Asterisks indicate a significant difference between groups at $\alpha = 0.05$.

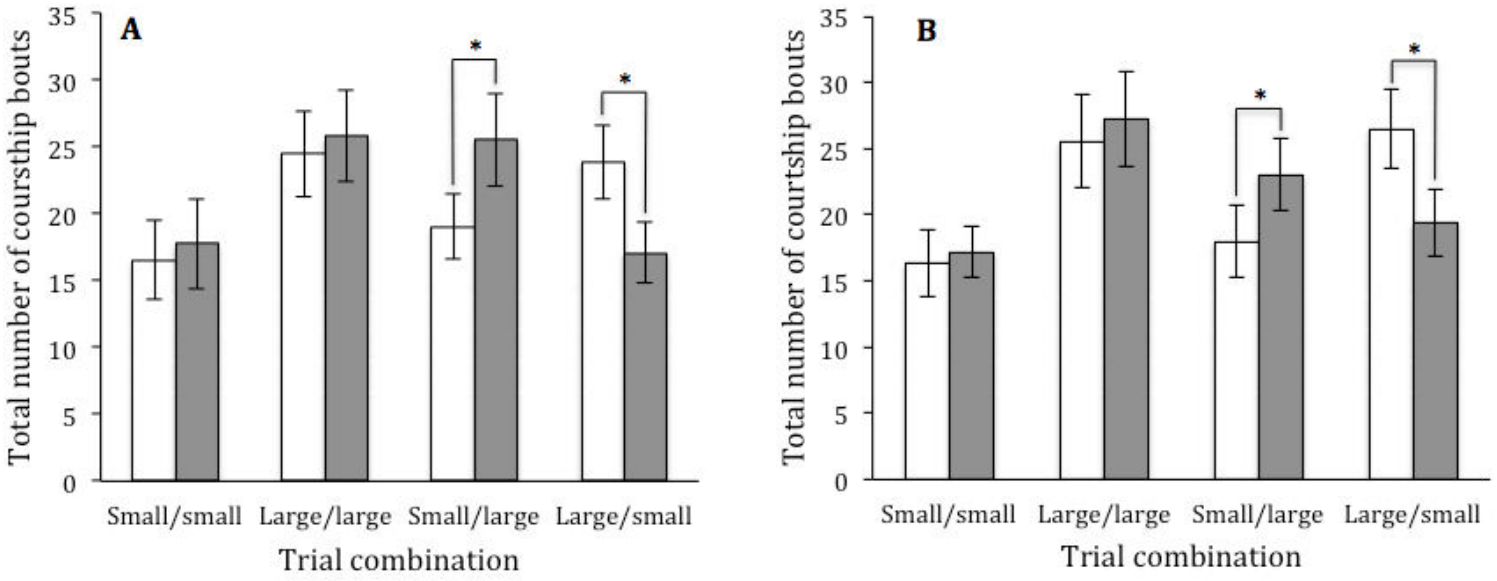


Figure 3. Mean (±SE) total number of sneak mating attempts performed by (A) unexposed and (B) 17β-trenbolone-exposed males towards females. Asterisks indicate a significant difference between groups at $\alpha = 0.05$.

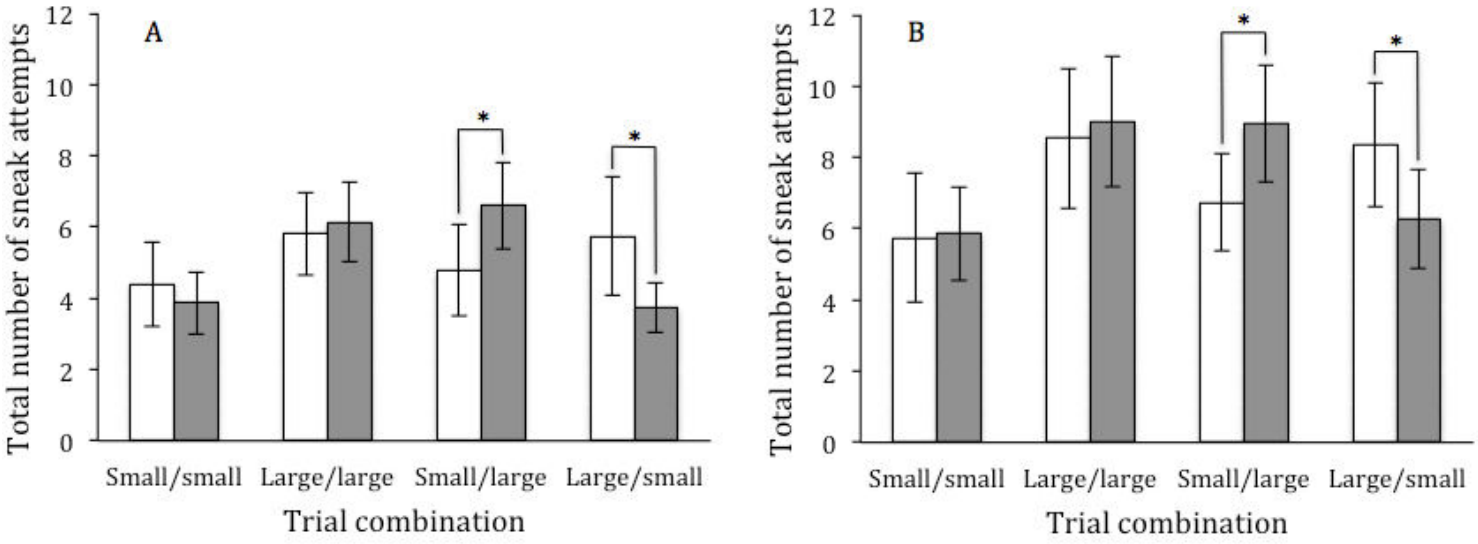
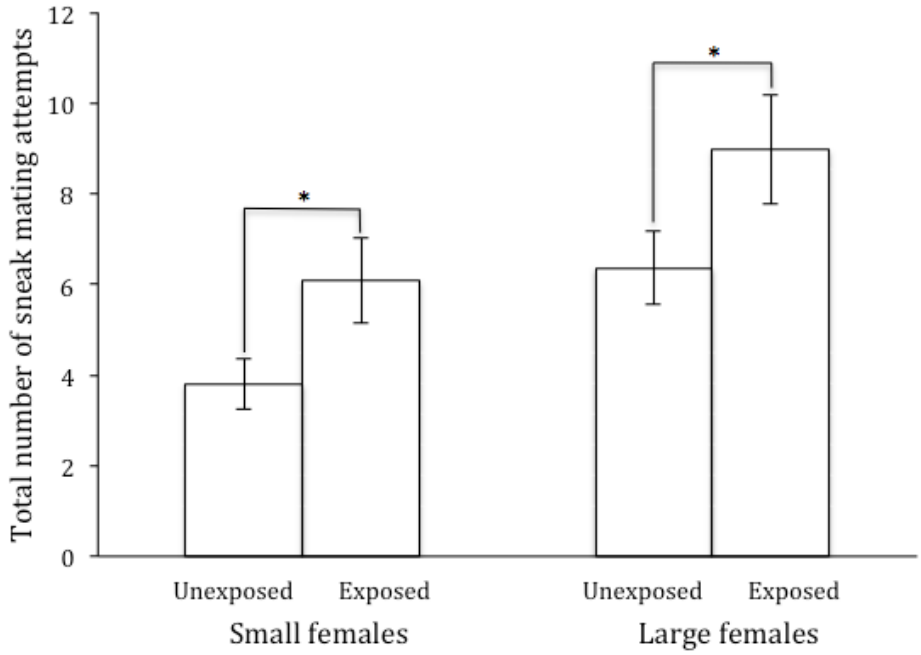


Figure 4. Mean (±SE) total number of sneak mating attempts performed by unexposed and 17β-trenbolone-exposed males towards small and large females during the second female presentation. Asterisks indicate a significant difference between groups at $\alpha = 0.05$.



5.5 DISCUSSION

This is the first study to demonstrate sequential male mate choice in guppies, while highlighting the potential of EDCs to impact mate choice mechanisms when females are encountered sequentially. We found that all males, regardless of exposure status, conducted significantly more courtship bouts and sneak mating attempts when presented with large females in both the first and second female presentation. However, we found no impact of presentation order on male behaviour, which suggests that males were exercising mate choice based on an absolute preference for large females. When comparing the behaviour of 17β-trenbolone-exposed and unexposed males, we found that exposed males conducted significantly more sneak mating attempts towards both large and small females in the first and second female presentation, but we observed no effect of exposure on courtship behaviour. Exposure to 17β-trenbolone also had no impact on male weight or length, which is consistent with previous studies investigating the impact of 17β-trenbolone on male guppy morphology at similar concentrations (e.g. 8 ng/L: Tomkins et al., 2016; 4 ng/L: Tomkins et al., 2017).

Both exposed and unexposed males showed a preference for large females during the first and second female presentation. Although male preference for larger females has previously been demonstrated in guppies using simultaneous choice experiments (i.e., when males are able to make direct comparisons between potential suitors - Dosen and Montgomerie 2004; Herdman et al. 2004), this is the first study, to our knowledge, to show that male guppies can exercise mate choice

when females are encountered sequentially. We found that all males, regardless of exposure status, performed significantly more courtship bouts and sneak mating attempts towards large females in both the first and second female presentation. However, we found no effect of presentation order on the behaviour of exposed and unexposed males, which suggests that males were exercising mate choice based on an absolute preference for larger females, as opposed to altering their response to females based on previous female experience (Wong et al., 2004).

Exposure to 17β-trenbolone had no effect on the courtship behaviour of exposed and unexposed males in either the first or second female presentation. Although previous research has shown that exposure to androgenic EDCs can amplify the expression of male sexual behaviours (eg: Belanger et al., 2010; Hoffman and Kloas, 2012; Marteinson et al., 2015), this result is consistent with the majority of previous studies investigating the impacts of 17β-trenbolone on the reproductive behaviour of male guppies. Specifically, Bertram et al. (2015) found no effect of 17β-trenbolone on male courtship behaviour in a one-on-one scenario (i.e. when a single male is placed with a single female) and Tomkins et al. (2016) observed no differences in the courtship behaviour of exposed and unexposed males in a dichotomous choice experiment. However, a recent study has revealed that 17β-trenbolone can influence the courtship behaviour of male guppies in a competitive setting. Tomkins et al. (2017) found that 17β-trenbolone-exposed males courted females less than unexposed males when in the presence of a rival male. This decrease in courtship behaviour was also associated with an increase in aggression in exposed males,

which may have limited the amount of time available for these males to court. This suggests that 17β-trenbolone-induced changes in male courtship behaviour may be context dependent, manifesting only under male-male competition. However, this does not appear to be the case for sneak behaviour.

Males exposed to 17β-trenbolone conducted significantly more sneak mating attempts than unexposed males during both the first and second male presentation. This is consistent with the results of Bertram et al. (2015), who observed an increase in unsolicicted male mating behaviour amongst 17β-trenbolone-exposed males in a one-on-one situation (i.e., a single male paired with a single female), and Tomkins et al. (2017), who found that exposed males performed significantly more sneak mating attempts towards females when in the presence of a rival male. As a potent androgen-receptor agonist (Rogozkin, 1991), 17β-trenbolone binds with high affinity to available androgen receptors, mimicking the effects of endogenous androgens (eg: testosterone; Wilson et al., 2002). As a result, it is not surprising that exposure resulted in an increase in unsolicited male mating behaviour. The amplification of male sexual behaviours resulting from androgenic EDC exposure has also been demonstrated in American kestrels (*Falco sparverius*; Marteinson et al., 2015), African clawed frogs (*Xenopus laevis*; Hoffman and Kloas, 2012), and cyprinid fishes (Belanger et al., 2010). But how might this behavioural shift impact reproductive fitness?

An increase in coercive male mating behaviour is likely to impact both male reproductive success and female fitness. Previous research has shown that successful sneaks transfer approximately one third as many sperm into the female's gonoduct compared to consensual matings (Pilastro and Bisazza, 1999), which means sneak copulations have a significantly lower probability of insemination compared to copulations preceded by courtship. Further, sexual selection processes that occur either during or after copulation (i.e., cryptic female choice) may also disadvantage sneaking males. A study conducted by Pilastro et al. (2004) found that female guppies preferentially transfer sperm from males they perceive as more desirable (i.e., more colourful males). Although female cryptic preference for courting over sneaking males has not been specifically tested in guppies, cryptic preference for courting males has been documented in various other species (e.g., Evardsson and Arnqvist, 2000; Pizzari and Birkhead, 2000), which, combined with evidence of cryptic female choice in guppies, suggests that female guppies may also preferentially transfer sperm from courting over sneaking males. An increase in sneaking behaviour is also likely to impact female fitness, as previous research has shown that coercive mating behaviour in guppies can physically damage the female genital pore (Constantz et al., 1989), increase the risk of disease transmission (Bisazza et al., 2001), and reduce female foraging efficiency (Pilastro et al., 2003).

In conclusion, this is the first study to demonstrate the potential of EDCs to impact male reproductive behaviour when females are encountered sequentially. In the wild, males of many species encounter potential mates one at a time, meaning the

behavioural shifts observed in this study as a result of exposure to an environmentally realistic concentration of 17β-trenbolone are ecologically relevant. The increased sneaking behaviour observed in exposed males is likely to impact both female fitness and male reproductive success, which, combined with the circumvention of female mate choice that occurs as a result of increased unsolicited copulations, may have consequences at the population level. To explore the potential population-level impacts of 17β-trenbolone further, future studies would benefit from utilizing long-term, multigenerational exposures in large, natural mesocosms, as these types of studies would provide greater insights into how 17β-trenbolone-exposure may impact fish populations in the wild.

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Chapter 6: Discussion

My thesis aimed to investigate the effect of an environmentally realistic concentration of 17β-trenbolone on sexual selection processes in the guppy, *Poecilia reticulata*. My thesis is split into two sections: i) the impacts of 17β-trenbolone on female reproductive behaviours and ii) the effects of exposure on male reproductive behaviours. I found that, at concentrations present in the environment, 17β-trenbolone influenced several key mechanisms of sexual selection, including female mate choice, male mate choice, and male-male competition. Together, these findings demonstrate the profound impact androgenic EDCs can have on the varied mechanisms of sexual selection in both male and female fish, highlighting the need for a greater understanding of the effect of endocrine disruptors on sexual selection processes.

6.1 FINDINGS AND IMPLICATIONS

In the first part of my thesis, I found that exposure to an environmentally realistic concentration of 17β-trenbolone disrupted female mate choice processes, both when females were able to make direct comparisons between potential suitors (Chapter 2), and when males were encountered sequentially (Chapter 3). In both chapters, females exposed to 17β-trenbolone were less choosy than unexposed females, and also associated with males less overall, indicating a decreased desire to mate. To understand the potential impacts of this behavioural shift, we must

consider the mating system of the guppy, which is driven primarily by female choice (Houde, 1997). Female guppies preferentially associate with attractive males, favouring a number of known male traits including increased orange colouration (Endler, 1980; Brooks and Caithness, 1995), size (Reynolds and Gross, 1992), and display rate (Kodric-Brown and Nicoletto, 2001), which are all honest indicators of male quality. Because copulations preceded by courtship are significantly more successful than coercive mating attempts (Pilastro and Bisazza, 1999), attractive males court more and sneak less (Reynolds, 1993), which ensures that attractive males achieve a higher proportion of successful copulations compared to less desirable conspecifics. However, if females are no longer exercising mate choice, attractive males lose their competitive advantage, which may allow less desirable males – who are more likely to employ coercive mating strategies (Bertram et al., 2015; Tomkins et al., 2017) – to gain a higher percentage of successful copulations. Previous studies of guppies have shown that attractive males sire sons that grow faster (Reynolds and Gross, 1992), are more fecund (Moore, 1994), produce more or higher quality sperm (Pilastro et al., 2002; Pitcher and Evans, 2001; Locatello et al., 2006), or are more attractive (Houde and Endler, 1990; Houde, 1992), meaning females may incur indirect genetic costs by mating with less desirable males.

In the second part of my thesis, I demonstrated the potential of 17β-trenbolone to disrupt male mechanisms of sexual selection. I found that, when in the presence of a rival male, exposed males conducted significantly more sneak mating attempts, courted less, and were more aggressive than unexposed males (Chapter 4), as well

as conducting significantly more sneak mating attempts when females were encountered sequentially (Chapter 5). As I alluded to above, female choosiness in guppies ensures that attractive males, who invest significantly more time into courtship compared to less desirable conspecifics (Reynolds, 1993), achieve a higher percentage of successful copulations. However, if 17β-trenbolone-exposed males are courting less and sneaking more, female influence over mating outcomes may decrease, allowing less attractive males to mate more successfully. This is particularly true when considering the results from the first part of my thesis, as the decreased choosiness observed in exposed females is likely to facilitate the circumvention of female choice that occurs as a result of increased sneaking behaviour (Luttbeg, 2004). In addition to the indirect genetic costs associated with this circumvention of female choice, an increase in sneaking behaviour may directly affect female fitness, as sneak mating attempts can physically damage the female genital pore (Constantz et al., 1989), increase the risk of disease transmission (Bisazza et al., 2001), and reduce the foraging efficiency of females (Pilastro et al., 2003). But how might the behavioural shifts observed in my thesis impact at the population level?

There is growing evidence to suggest that disruption of sexual selection mechanisms as a result of EDC exposure can have far reaching evolutionary consequences. For example, a study conducted by Ward and Blum (2012) found that exposure to the endocrine disruptor bisphenol A (BPA) prevented females of two closely related, sympatric fish species from differentiating between conspecific and

heterospecific males, increasing the potential of interspecific hybridization. Further, quantitative modeling has shown that disruption of male guppy signals as a result of EDC exposure may lead to a long-term loss of female preference for those signals within a few generations, which may coincide with a reduction in mean population fitness (Senior et al., 2012). Although there is very little (if any) empirical evidence of the population level effects of EDC-induced behavioural change, other studies have shown that anthropogenic disruption of sexual selection processes can have devastating evolutionary consequences in the wild (eg: Seehausen et al., 1997). This emphasizes the importance of understanding the potential of EDCs to disrupt the mechanisms of sexual selection, and highlights the need for a greater understanding of their potential population-level impacts.

6.2 FUTURE DIRECTIONS AND CONCLUDING REMARKS

This thesis is consistent with the majority of studies investigating the behavioural impacts of EDCs in that the effects of a single compound were investigated (i.e. 17β-trenbolone). However, environments contaminated with EDCs commonly contain a mixture of chemical contaminants (Gore, 2008; Diamanti-Kandarakis *et al.,* 2009), and recent research has revealed that EDCs can have synergistic and additive effects (Crews et al., 2000; Sarria et al., 2011). For example, hormonal growth promotants commonly include a cocktail of chemical compounds (Lange et al., 2001), meaning waterways associated with cattle feedlots are likely to be contaminated with multiple endocrine disruptors. Indeed, 17β-trenbolone has been found to co-occur

with 17β-estradiol – a potent, estrogenic endocrine disruptor with well-documented environmental effects (Panter et al., 1998; Vos et al., 2000; Söffker and Tyler, 2012) – in surface waters receiving livestock effluent run-off (Soto et al., 2004). Despite the potency of these two compounds, and their likelihood to co-occur in the environment, their potential synergistic effects remains uninvestigated. Studies exploring the effects of these two compounds in mixture would, therefore, provide valuable insights into the environmental impacts of HGP implants. However, to improve the ecological relevance of such data, researchers must move beyond traditional laboratory assays.

The ultimate goal of ecotoxicological studies is to assess the environmental risk of aquatic contaminants. Although laboratory-based, behavioural studies provide important insights into the potential environmental impact of EDCs, the disparity in ecological complexity between laboratory systems and natural environments often makes it difficult to extrapolate these results to the wild (Hellstrom et al., 2015). Future studies would benefit from utilizing long-term exposures in large, natural mesocosms, as these types of experiments are likely to provide more ecologically realistic data. Indeed, a recent study investigating the environmental effects of various pharmaceuticals in large, semi-natural ponds provided valuable insights into the accumulation and dispersion of pharmaceuticals in aquatic food webs (Lagesson et al., 2016). However, the cost and logistics involved with these large-scale, longitudinal studies means they are rare, despite their obvious potential to provide meaningful ecological data.

One way for scientists to collect ecologically relevant data in a cost-effective manner is to conduct behavioural EDC studies in the field. This has recently been made possible through the development of acoustic telemetry, which is a recent advance in technology that allows researchers to gather high-resolution, behavioural data of organisms in their natural environment (for a review of acoustic telemetry and its potential use in ecotoxicology, see Hellstrom et al., 2015). This technology provides ecotoxicologists with the opportunity to quantify endpoints that are difficult to assess in the laboratory, including predator-prey dynamics, social networks, schooling, and competition (Donaldson et al., 2014; Newman, 2015). The use of acoustic telemetry in ecotoxicology is still in its infancy; however, it has the potential to bridge the gap between laboratory assays and behaviours in a more ecologically-realistic setting when assessing the potential environmental impact of EDCs.

Although my research focused on effects on wildlife, it is also worth pointing out that there are also concerns over the impact of 17β-trenbolone exposure in humans. Surprisingly, however, there are very few (if any) studies investigating the impact of 17β-trenbolone-exposure on humans at environmentally relevant concentrations. This is true despite the fact that hormonal growth promoters were banned in the European Union in 1981 based on concerns that hormone residues in bovine meat could have adverse effects on human health, including endocrine, developmental, neurobiological, immunological, carcinogenic, genotoxic, and immunotoxicological

effects (Norris and Carr, 2005). More recently, Russia banned the import of Australian beef after detecting traces of 17β-trenbolone in Australian cattle products, highlighting concerns that still remain over the use of hormonal growth promoters. Clearly, further research is warranted.

In conclusion, this thesis demonstrates the profound influence that exposure to 17β-trenbolone can have on sexual selection processes. I found that, at concentrations found in the environment, 17β-trenbolone impacted female mate choice, male mate choice, and male-male competition. Disruption of these mechanisms can influence both reproductive success and output, which, in turn, may have impacts at the population level. Thus, this thesis highlights the importance of understanding the potential of EDCs to influence the complex and varied mechanisms of sexual selection.

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Appendix

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An Androgenic Agricultural Contaminant Impairs Female Reproductive Behaviour in a Freshwater Fish

Minna Saaristo[1]*, Patrick Tomkins[1], Mayumi Allinson[2], Graeme Allinson[2,3], Bob B. M. Wong[1]

1 School of Biological Sciences, Monash University, Melbourne, Victoria, Australia, 2 Centre for Aquatic Pollution Identification and Management (CAPIM), The University of Melbourne, Bio21 Institute, Melbourne, Victoria, Australia, 3 DPI Queenscliff, DPI, Melbourne, Victoria, Australia

Abstract

Endocrine disrupting chemicals (EDCs) are a large group of environmental pollutants that can interfere with the endocrine system function of organisms at very low levels. One compound of great concern is trenbolone, which is widely used as a growth promoter in the cattle industry in many parts of the world. The aim of this study was to test how short-term (21-day) exposure to an environmentally relevant concentration of 17b-trenbolone (measured concentration 6 ng/L) affects reproductive behaviour and fin morphology in the eastern mosquitofish (Gambusia holbrooki). The mosquitofish is a sexually dimorphic livebearer with males inseminating females using their modified anal fin, the gonopodium, as an intromittent organ. Although the species has a coercive mating system, females are able to exert some control over the success of male mating attempts by selectively associating with, or avoiding, certain males over others. We found that females exposed to trenbolone approached males less and spent more time swimming away from males than non-exposed (control) females. By contrast, we found no difference in the behaviour of exposed and non-exposed males. Furthermore, exposure did not affect the anal fin morphology of males or females. This is the first study to demonstrate that exposure to an androgenic EDC can impair female (but not male) behaviour. Our study illustrates how anthropogenic contaminants can have sex-specific effects, and highlights the need to examine the behavioural responses of environmental contaminants in both sexes.

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* E-mail: minna.saaristo@monash.edu

Introduction

Over the last few decades, concern has been mounting over a group of environmental contaminants known as endocrine disrupting chemicals (EDCs). EDCs are causing concern because they disturb the endocrine function of organisms, often at very low concentrations (nanograms per litre levels), with potentially catastrophic effects. Infamous examples include eggshell thinning in birds [1], developmental abnormalities in alligators [2], and birth defects, gametogenesis and cervical cancer in humans [3,4]. However, until now, studies have focussed mostly on estrogenic EDCs, with far less attention given to understanding the impacts of EDC pollutants with androgenic effects [5–9].

The androgenic steroid trenbolone acetate is widely used as a growth promoter in the beef industry in many parts of the world. In vivo, the compound is rapidly converted to the biologically active steroid 17b-trenbolone (hereafter referred to as trenbolone), which is an extremely stable compound, with a half-life up to 267 days measured in animal waste [10]. Trenbolone enters the environment through livestock urine and manure, and has been detected at levels from ,5 ng/L to 20 ng/L in run off from cattle feedlots [11,12] and up to 162 ng/L in fields receiving animal waste [13]. The morphological impacts of trenbolone on aquatic organisms, particularly fish, have been well documented, with effects ranging from reduced fecundity [6,7] to complete sex reversal resulting in an all-male population [8,9]. Our understanding of the behavioural effects of trenbolone exposure, however, is limited, even though behaviour has the potential to be a much more sensitive (and powerful) indicator of aquatic pollution than morphological biomarkers [14–19].

Trenbolone is known to bind to androgen receptors with three times the affinity of testosterone [20] and is therefore an extremely potent androgenic steroid in the environment. Considering its potency and the fact that androgens are known to affect the expression of sexual and agonistic behaviours, we would expect trenbolone to influence behaviour. So far, however, only two studies have specifically looked at the behavioural effects of trenbolone – and the results have been equivocal. Specifically, while embryonic exposure (50 ug) was observed to suppress copulatory behaviour in Japanese quail [21], trenbolone exposure (20 ng/L) had no effect on zebrafish courtship behaviour [8]. EDC-studies, to date, have also tended to focus only on male behaviour. In nature, however, both sexes are likely to be exposed to the same pollutants simultaneously and the effects on one sex could be very different in the other. As a result, it is important to

investigate how EDCs might affect both males and females contemporaneously.

Recent studies have found that exposure to environmentally relevant concentrations of trenbolone can also induce morphological changes in fish. Ankley et al. [6], for example, found that female fathead minnows Pimephales promelas, exposed to trenbolone for 21 days developed dorsal tubercules – structures normally present on mature males. Also, trenbolone concentrations as low as 9.2 ng/L were found to cause irreversible masculinisation of zebrafish after 60 days of exposure [9]. Whether trenbolone exposure induces similar morphological changes in other species, however, remain unknown.

The eastern mosquitofish, Gambusia holbrooki, is an excellent model organism for studying the effects of androgenic EDCs because of its widespread, cosmopolitan distribution in shallow freshwater habitats in both urban and agricultural areas [22]. The mosquitofish is a sexually dimorphic livebearer, with males inseminating females using their gonopodium, as an intromittant organ [23]. Male mosquitofish do not court females but, instead, attempt forced copulations by thrusting their gonopodia towards the female's genital pore [24,25]. Despite the coercive mating system, evidence suggests that female mosquitofish are choosy [26– 28] and may be able to exert some control over the success of male mating attempts by, for example, selectively approaching certain males over others [26]. Due to their internal mode of fertilisation, male mosquitofish need to be in close proximity to females before any mating attempts can be made, and both sexes clearly associate with each other during the breeding season [29]. Thus, as with other poeciliids [30–32], the time spent by females associating with males can have a direct bearing on mating outcomes and is a widely used measure of mating intentions in behavioural studies [28,31,33] Morphologically, previous research on mosquitofish has also found that embryonic exposure to androgenic hormones can increase the length of the modified anal fin (i.e. gonopodium) of males in relation to body size [34,35], and induce gonopodial development in females [34,36–37]. However, it is unknown whether EDCs might affect anal fin morphology once fish have reached maturity.

Accordingly, the aim of our study was to investigate the impact of trenbolone on male and female reproductive behaviour and fin morphology. In particular, we were interested in effects arising from short-term exposure to an environmentally relevant concentration (6 ng/L). This is ecologically important because agricultural pollutants enter the environment in pulses and previous work suggests that exposure to EDCs need not to be permanent to have long-lasting, detrimental effects [6–9,38].

Materials and Methods

Ethical Statement

The methods for animal housing, handling and experimental protocols were assessed and approved by the Biological Sciences Animal Ethics Committee at Monash University (permit number: BSCI/2011/07). Because mosquitofish are a noxious species under State laws, the terms of the collecting permit (Department of Primary Industries Victoria, permit number NP191) did not allow them to be returned to the wild and hence fish were euthanised.

Exposure Set up

Mosquitofish were collected from Brodies Lake in Victoria, Australia. This is a relatively pristine site located adjacent to a reservoir that supplies drinking water to parts of suburban Melbourne. Fish were caught during the breeding season (February) using dip nets and transported in coolers back to the laboratory. In total, 280 fish were collected, of which 140 were females and 140 males. Fish were separated by sex and acclimated to laboratory conditions (12:12 h light regime) for 10 days in 54 L tanks (20 fish per tank). After acclimation, fish were randomly placed into separate-sex 'exposure' tanks (60 cm630 cm624 cm; 20 fish per tank), the set up of which followed the design of Saaristo et al. [17] with a few modifications. Briefly, 14 tanks were assigned to one of two treatments, namely, (1) a 17b-trenbolone exposed treatment (TB), and (2) a freshwater control. In total, 280 fish were exposed: seven tanks were allocated to the TB treatment (4 tanks for males and 3 tanks for females) and seven tanks were allocated to the control treatment (4 tanks for males and 3 tanks for females). We randomly took four fish from each of the holding tanks and placed them into each of the exposure tanks. This was continued until all of the fish from the holding tanks had been assigned to an exposure tank. Thus, each exposure tank had fish from several holding tanks. Male and female mosquitofish tanks in the TB treatment were exposed to trenbolone at a nominal concentration of 15 ng/L (measured concentration = 6 ng/L; see below for details on how trenbolone levels were monitored) via a flow-through system for 21-days. Mosquitofish tanks in the control treatment were connected to an identical, but separate, flow through system over the same period but, in contrast to the TB tanks, the flow through system supplied only freshwater to the fish during the exposure period. The water supplied to these fish tanks was fed through a mixing tank into which either trenbolone from a stock solution (in the case of the TB treatment) or freshwater (in the case of the control treatment) was pumped using a peristaltic pump (Watson Marlow 323 U/MC). From the mixing tanks, the water was channelled into the fish tanks using silicon tubing. The flow rate was kept constant (2.25 L/h) or all tanks using flow meters (BES Flowmeters, MPB Series 1200) and adjustable valves. For the trenbolone exposure, a fresh stock solution was prepared once a week and the stock solution tank was changed every third day to minimize the possible deterioration of TB. Water temperature in the tanks was monitored daily and ranged from 19–23*u*C. Fish were fed ab litium with commercial fish flakes (Otohime Hirame, Aquasonic) once a day during the exposure period.

Monitoring of Trenbolone

The level of trenbolone used was achieved by firstly dissolving 17b-trenbolone (4,9,11-estratrien-17-ol-3-one; Novachem, Germany) in 100% ethanol (600 ug/L, 1% of ethanol) to create a stock solution, which was then diluted in the flow-through system to achieve the desired concentration. The final solvent concentration was 0.00006% in the exposure tanks.

The concentration of trenbolone in the exposure and control tanks was monitored by enzyme-linked immunosorbent assay (ELISA). To do this, a 100 mL water sample was taken from each exposure tank once a week. Water samples were acidified by adding a mixture of 1% acetic acid methanol, then loaded onto a conditioned solid phase cartridges (Strata633 u, 500 mg,/6 mL; Phenomenex, Torrance, CA, USA). The cartridge was then eluted with methanol (264 mL), with the eluate dried under nitrogen stream. Samples were reconstituted with 100 uL methanol and 900 uL of deionised water.

Measurement of trenbolone 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 water) were dispensed (50 uL) in duplicate into an antibody coated 96 well plate by an auto dispenser (epMotion 5070, eppendorf,

Hamburg, Germany). Thereafter, 25 uL of HRPO conjugate and 25 uL of antibody were dispensed into the wells. After 1 hour incubation at room temperature in the dark, the plate was washed three times with wash buffer by a microplate washer (Atlantis, ASYS HITECH, Eugendorf, Austria), and 100 uL of substrate was added to all wells. The plate was then incubated for a further 30 minutes at room temperature in the dark. Finally, 100 uL 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 (UVM40, 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 ug/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 trenbolone ELISA was 2.0 ng/L. The ratio of nominal concentrations and measured values were 90%, which indicates that the calibration curve provided good (accurate and precise) sample concentration values provided the ELISA response was within the upper and lower bounds of the calibration curve. A spike recovery experiment was conducted in triplicate using a 5 ng/L 17b-trenbolone solution. The average recovery was 97%, providing confidence that trenbolone in water samples was efficiently extracted, and that measured values were neither under nor over estimates of sample concentrations.

Behavioural Trials

All behavioural trials were conducted in tanks (60 cm630 cm624 cm) containing freshwater with a 2 cm layer of gravel on the bottom as substrate. One male and one female from the same treatment group (i.e. either TB or control) were randomly assigned to an experimental tank and allowed to freely interact. We specifically paired fish from the same treatment groups because, in the wild, both sexes would typically be exposed to the same environmental contaminants simultaneously. Male and female behaviours were recorded with a video camera. Filming began when the male and female were released into the tank. Fish were filmed for 15 minutes and the behaviour of each sex was analyzed using JWatcher software, which calculates the total time and frequency of each quantified behaviour during this period. For females, we quantified whether or not the female was interacting with the male and, if so, whether she was actively associating with the male (i.e. swimming towards him), exhibiting aggressive behaviour (i.e. biting and performing tale beats), or trying to avoid the male by swimming away from him. For males, we quantified whether or not the male was showing an interest in the female. If so, we noted whether the male was orienting towards the female (within 5 cm of her body), chasing her, or engaging in gonopodial thrusts. We also noted the time the male took to perform the first chase. Trials were replicated 19 times for the trenbolone treatment and 18 times for the control. Each trial had a new pair of fish. We used 12 tanks for behavioural trials and ran 12 trials per day (66TB and 66control). For each tank, we alternated between TB and control trials to avoid tank effects.

Morphological Measurements

After each behavioural trial, fish were euthanized with an overdose (40 mg/L) of anaesthetic clove oil [39]. Fish were then weighted and measured from the tip of the snout to the end of the caudal fin, and preserved in 70% ethanol for further anal fin measurements. The male gonopodium and female anal fin were analysed using the morphometric analysis described by Angus et al. [34]. The anal fin was photographed using a moticam 3.0 mounted on a Motic SMZ-168 stereomicroscope. From these images, ray 4 and ray 6 were measured to the nearest 0.001 mm using Motic Digilab II (Motic Instruments Inc., Hong Kong). The R4:R6 ratio is known to be influenced by EDCs in both the male gonopodium [40,41] and the female anal fin [35], and was thus calculated (dividing length of R4 by R6) for both sexes.

Statistical Treatment of Data

Data was checked for normality and heterogeneity of variance. In our analyses of female and male behaviour, the data did not conform to a normal distribution and we were unable to render them normal with transformation. Therefore, the effect of treatment on behavioural variables were analysed using Mann-Whitney tests. For the morphological (length and anal fin) data, independent-sample t-tests were used to test differences between TB-exposed and control fish. SE = standard error of mean. All statistical analyses were performed using SPSS (19.0).

Results

Female Behaviour

Trenbolone-exposed females spent less time associating with the males (duration of time (ms): Mann-Whitney: U = 106.000, p = 0.048, n = 37; number of times: U = 102.000, p = 0.035, n = 37; Fig. 1a,b). Females, instead, spent more time swimming away from the males (duration of time (ms): Mann-Whitney: U = 102.000, p = 0.036, n = 37; Fig. 2a), although the frequency of this behaviour did not differ between the treatments (Mann-Whitney: U = 127.000, p = 0.181, n = 37; Fig. 2b). Aggressive behaviours were not significantly affected (all p.0.05).

Male Behaviour

Trenbolone-exposed males did not differ in behaviour from control males (Table 1). Males chased females in all of the trials and there was no difference in the time they took to perform the first chase between treatments (Mann-Whitney: U = 146.000, p = 0.417, n = 37; Mean 6 SE: Control = 1.37 min 60.454; TB = 1.67 min 60.450).

Morphological Analysis

The standard length of females and males (mean 6 SE) did not differ between treatments (Females: two sample t-test: t = 21.367, df = 38, p = 0.180; Mean 6 SE: control = 32.65 mm 61.296, TB = 35.65 mm 61.772; Males: t = 20.264, df = 38, p = 0.793; Mean 6 SE: control = 26.53 mm 60.498, TB = 26.28 mm 60.812). Moreover, the R4:R6 fin ray length ratio did not differ between treatments for either male or female fish (two sample t-test: Males: t = 0.217, df = 38, p = 0.828, Mean 6 SE: 2.2860.055; Females: t = 0.208, df = 38, p = 0.701, Mean 6 SE: 1.1560.017).

Trenbolone Measurements

The concentration of TB in the exposure tanks was 6 ng/L (SE = 2.6, n = 21). The concentration of TB in the control tanks was below detection limit throughout the exposure period. Details regarding nominal and actual water concentration of trenbolone are presented in Table S1.

Discussion

We found that female and male mosquitofish responded differently to trenbolone. Trenbolone-exposed females approached males less and spent more time swimming away from males. This was true even though exposed males did not differ in

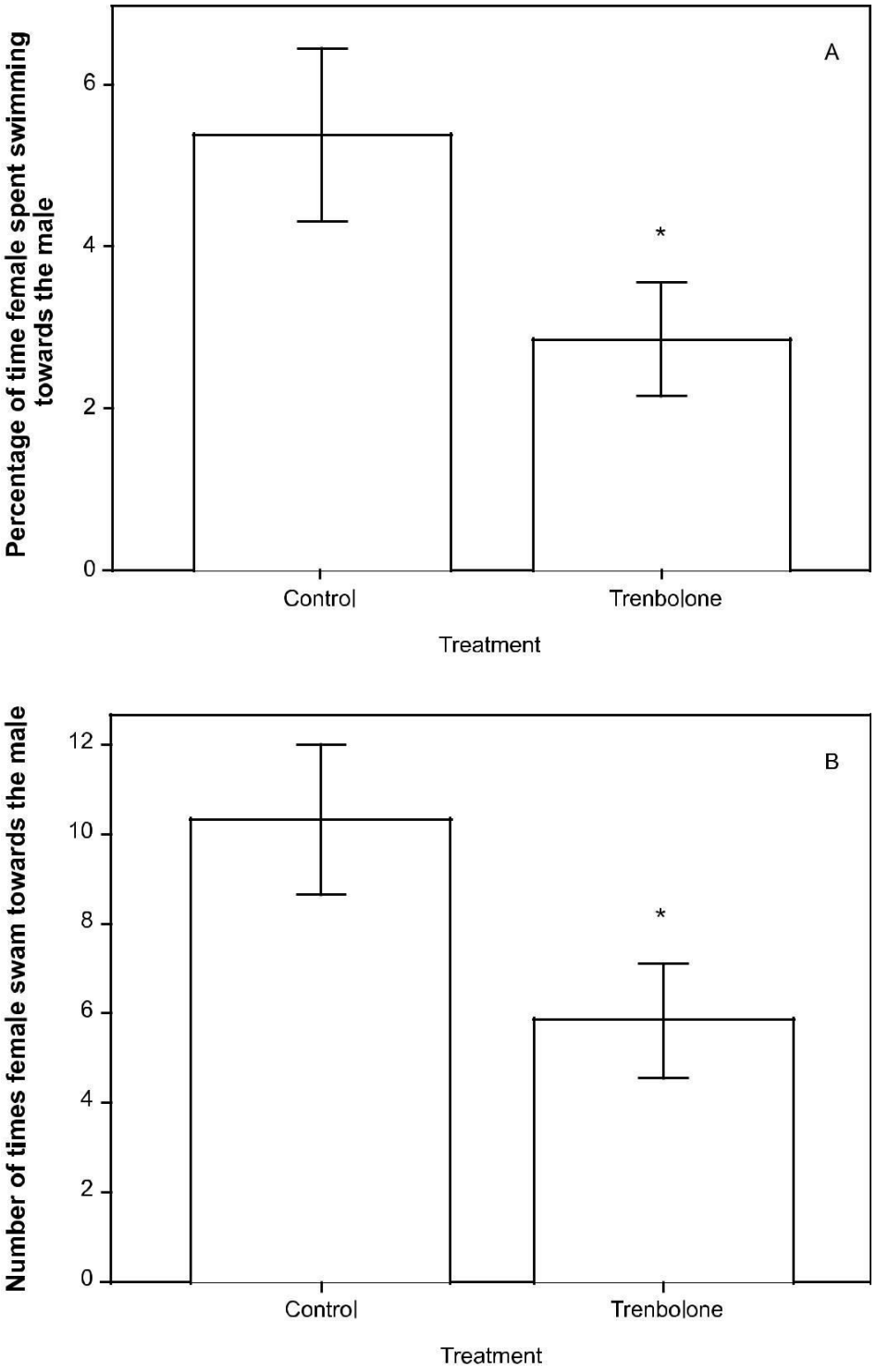

Figure 1. Mean percentage (6 SE) of (A) time and (B) frequency females spent swimming towards the male during each trial. The two treatments are: Control = fish exposed to freshwater (n = 18), and Trenbolone = fish exposed to 6 ng/L of 17b-trenbolone (n = 19); Asterisk indicates a significant difference (p,0.05) between the TB treatment and control.
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their behaviour from control males. Because short-term exposure affected only female behaviour, this finding suggests that females may be more sensitive to trenbolone than males. To our knowledge, this is the first study to not only show that exposure to an environmentally relevant concentration of androgenic EDC can impair female reproductive behaviour, but that the behavioural consequences of EDC exposure can differ between the sexes.

When females were exposed to trenbolone, they approached males less often. A previous study by Toft et al. [42] showed that lifetime exposure to androgenic paper mill effluent decreased the time mosquitofish females stayed close to the male. The authors of

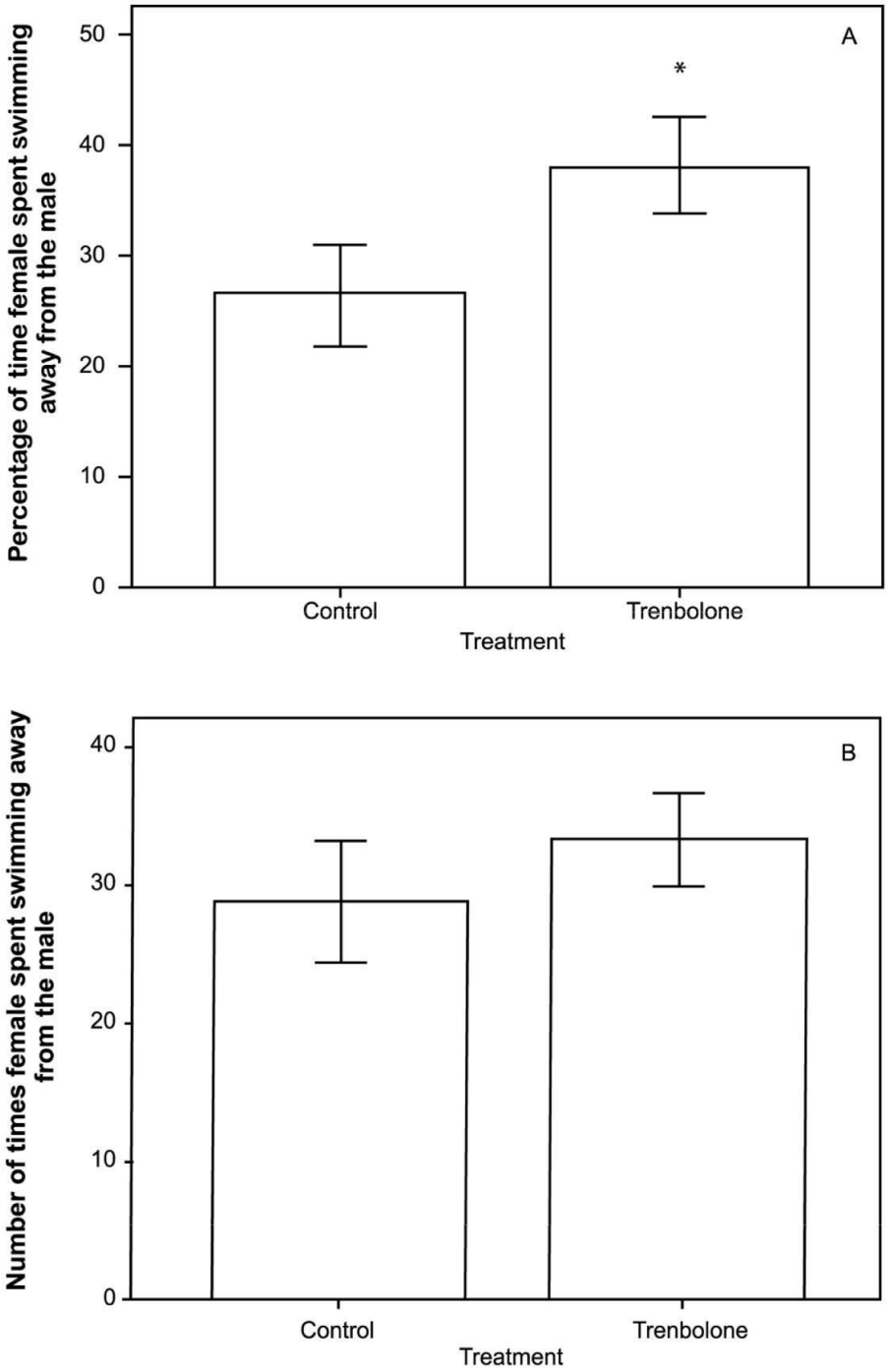

Figure 2. Mean percentage (6 SE) of (A) time and (B) frequency females spent swimming away from the male during each trial. The two treatments are: Control = fish exposed to freshwater (n = 18), and Trenbolone = fish exposed to 6 ng/L of 17b-trenbolone (n = 19); Asterisk indicates a significant difference (p,0.05) between the TB treatment and control. doi:10.1371/journal.pone.0062782.g002

that study described this as social 'attending' behaviour, but in the light of our findings, females might also have actively avoided the close distance of males. Although mosquitofish breed via a coercive mating system, females can nevertheless exert some control over fertilisation success and skew copulations by actively approaching and associating with certain males over others [26]. Hence, exposure to trenbolone could have implications for females by affecting their motivation to mate. Lack of interest is supported by the fact that exposed females actually spent more time swimming away from males even though trenbolone males did not harass females more. The impact of trenbolone on male mating behaviours, however, was less clear.

Testosterone is known to mediate male aggression and courtship behaviour [43]. Therefore, we hypothesised that trenbolone, which is more potent than testosterone, would increase male harassment (e.g. chasing, or engaging in gonopodial thrusts) of

Table 1. Effect of treatment on male behaviours.

	Mean 6 S.E.		Test	
			Two sample t-test	
Length (mm)	Trenbolone	Control	t	p
Male	26.2860.812	26.5360.498	20.264	0.793
Female	36.4461.874	32.7461.363	0.116	1.613
			Mann-Whitney test	
Male behaviours	Trenbolone	Control	U	p
Proportion of time				
Orientation	0.63060.050	0.52460.078	150.0	0.523
Chasing	0.01660.002	0.01560.003	149.0	0.504
Gonopodium thrust	0.00460.001	0.00360.001	144.0	0.412
No display	0.34560.050	0.45660.080	152.0	0.564
Frequency				
Orientation	46.6764.506	43.1665.962	149.0	0.504
Chasing	38.7264.417	34.1166.139	135.5	0.280
Gonopodium thrust	14.7262.266	13.2162.412	148.5	0.493
No display	9.8961.188	11.8961.644	144.0	0.411

The two treatments are: Control = fish exposed to freshwater (n = 18), and Trenbolone = fish exposed to 6 ng/L of 17b-trenbolone (n = 19). S.E. = standard error of mean.
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females. However, we did not find this to be the case. Why? Androgens, such as testosterone, are typically converted to estrogens in target tissues [44]. This increased concentration of estrogenic hormones can lead to a down-regulation of androgen production [43], which can in turn influence behaviour. However, this scenario is unlikely to occur with trenbolone. Previous work has shown that trenbolone is relatively non-estrogenic, because it is not a substrate for the aromatase enzyme that converts androgens to estrogens [20]. Therefore, exposure to trenbolone is likely to impact behaviours that are controlled by androgens rather than estrogens. It is unknown what impact trenbolone might have at higher concentrations on males but in our study, 6 ng/L was not sufficient to induce any significant behavioural changes. Clearly, further investigation into the behavioural impacts of trenbolone is required.

We did not observe any abnormal anal fin development amongst trenbolone-exposed males or females. Hormonally-dependent processes, such as anal fin development, are known to be particularly sensitive to EDC exposure [34,36–37,40,45–46] Recent research has revealed that trenbolone can also influence gonopodial development in mosquitofish, with Brockmeier et al. [47] and Sone et al. [48] observing masculinisation of the female anal fin amongst trenbolone-exposed mosquitofish after 21 and 28 days of exposure respectively. However, in contrast to previously published studies, there are two possible reasons why we found no effect of trenbolone exposure on gonopodial morphology. First, we used a particularly low exposure concentration (6 ng/L compared to 10 *m*g/L used by Brockmeier et al. [47] and Sone et al. [48]). Second, we only exposed adult fish to the hormone. The male gonopodium, which is under androgenic control [36], forms via elongation of the anal fin during sexual development [22]. Therefore future studies may benefit from exposing fish to trenbolone from birth through to sexual maturity.

What are the potential population-level consequences of trenbolone-induced changes to behaviour? Recent studies have suggested that altered behaviours could have important population effects [16–19,49–53]. At the beginning of the breeding season, mosquitofish densities are typically low, and sex ratios are female-biased [54]. Moreover, despite the persistence of males in trying to secure matings, actual copulatory success is extremely low [55]. Thus, selective female association could play an important role in reproductive success, particularly at low densities. As we have shown, trenbolone-exposed females not only approached males less than control fish, but they also actively avoided them more. This suggests that at times of low population density, trenbolone exposure has the potential to impact reproductive success and overall population viability. Such a possibility warrants further investigation.

In conclusion, we showed that exposure to an environmentally relevant concentration of trenbolone affected female reproductive behaviour. During the last decade, research has demonstrated that estrogenic EDCs can weaken reproduction and reproductive behaviour in a wide range of species [18,50–52,56–57]. Andro-gens, such as trenbolone, however, have been neglected, even though laboratory and field studies have demonstrated severe morphological effects [5–7,58]. Not only does our study uncover a previously unknown behavioural impact of exposure to androgenic EDCs, but highlights how anthropogenic contaminants can have sex-specific effects, thus underscoring the need to examine both female and male responses contemporaneously.

Supporting Information

Table S1 Trenbolone concentration in the control and exposure tanks. ELISA = enzyme-linked immuno sor-bent assay. LOR = limit of reporting.
(DOC)

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Author Contributions

Conceived and designed the experiments: MS BBMW. Performed the experiments: MS PT. Analyzed the data: MS. Contributed reagents/ materials/analysis tools: MA GA. Wrote the paper: MS BBMW.

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Exposure to an agricultural contaminant, 17β-trenbolone, impairs female mate choice in a freshwater fish

Patrick Tomkins[a,*], Minna Saaristo[a,b], Mayumi Allinson[c], Bob B.M. Wong[a]

[a] School of Biological Sciences, Monash University, Victoria, Australia
[b] Department of Biosciences, Åbo Akademi University, Turku, Finland
[c] Centre for Aquatic Pollution Identification and Management (CAPIM), School of Chemistry, The University of Melbourne, Victoria, Australia

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abstract

Despite the pivotal role sexual selection plays in population dynamics and broader evolutionary processes, the impact of chemical pollution on female mate choice is poorly understood. One group of chemical contaminants with the potential to disrupt the mechanisms of female mate choice is endocrine disrupting chemicals (EDCs); a broad class of environmental pollutants that can interfere with the endocrinology of organisms at extremely low concentrations. Recent research has revealed that estrogenic EDCs can affect female mate choice in fish, but the impact of androgenic EDC exposure is yet to be studied. To address this, we investigated the effects of an environmentally relevant concentration of trenbolone – an androgenic steroid used as a growth promoter in the cattle industry – on female mate choice in wild-caught guppies (Poecilia reticulata). We exposed male and female guppies to 17β-trenbolone for 21 days (measured concentration 4 ng/L) via a flow-through system, and found that trenbolone-exposed female guppies spent less time associating with males, and were less choosy, compared to unexposed females. In contrast, trenbolone had no impact on male reproductive behavior or morphology. This is the first study to show that androgenic EDC exposure can disrupt female mate choice, highlighting the need for studies to investigate the behavioral impacts of environmental contaminants on both sexes.

1. Introduction

Sexual selection, by influencing the quality and quantity of offspring produced, has important implications for population dynamics, ecosystem function, and broader evolutionary processes (reviewed in Candolin and Wong, 2012). One of its key mechanisms is female mate choice (Andersson and Simmons, 2006), which is known to confer a suite of direct (i.e., material) and indirect (i.e., genetic) benefits to choosy individuals (Andersson, 1994; Kokko et al., 2003). The traits that females use to evaluate male quality are finely attuned to the local environmental conditions in which they have evolved (Wong and Candolin, 2015). As a result, the mechanisms of female mate choice are particularly vulnerable to anthropogenic, environmental change. Although, the majority of studies have focused on how altering the physical and auditory environment can interfere with these mechanisms (e.g., Slabbekoorn and Peet, 2003; Wong et al., 2007), changing the chemical environment can also disrupt female mate choice (e.g., Fisher et al., 2006). This is not surprising given the profound influence chemical pollution can have on morphology, physiology and behavior (Clotfelter et al., 2004; Ward et al., 2008; Diamanti-Kandarakis et al., 2009), and the pivotal role chemical communication plays in the reproduction of a wide range of taxa (Johannson and Jones, 2007).

One group of chemical contaminants with the capacity to disrupt female mate choice is endocrine disrupting chemicals (EDCs). EDCs are ubiquitous in the environment and possess several characteristics that make them particularly concerning. They can persist in ecosystems, affect organisms at extremely low concentrations, have a tendency to bioaccumulate in the environment, and can act transgenerationally (Anway and Skinner, 2006; Crews et al., 2007; Walker and Gore, 2011). There is a plethora of research documenting the environmental impacts of EDCs, with studies traditionally concentrating on morphological and physiological effects. However, with the recognition of behavior as a particularly sensitive and powerful biomarker of EDC contamination (Melvin and Wilson, 2013), an increasing number of studies are also turning their attention to understand the behavioral impacts of EDCs. Recent research has revealed that EDCs can affect a range of behaviorally important

* Corresponding author at: School of Biological Sciences, Monash University Clayton Campus, Victoria, Australia.
E-mail address: patrick.tomkins@monash.edu (P. Tomkins).

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processes, including cognition, boldness, sociality and reproduction (for a review see Clotfelter et al., 2004; Frye et al., 2012; Soffker and Tyler, 2012).

Surprisingly, despite growing interest in the impacts of EDCs on behavior, relatively little is known about how EDCs affect sexual selection and, more specifically, female mate choice. Studies have recently revealed, for instance, that EDC exposure can affect female mate choice in fish (Coe et al., 2008; Saaristo et al., 2009a). However, these studies have largely focused on only one group of EDCs—those with estrogenic properties. In comparison, the influence of androgenic EDC exposure on sexual selection has yet to be investigated.

One androgenic EDC with the potential to impact sexual selection is trenbolone, a synthetic steroid commonly used in many parts of the world to accelerate growth rates in beef cattle (Lange et al., 2002; Khan et al., 2008; Morthorst et al., 2010). Trenbolone enters the environment as 17β-trenbolone, and has been detected in aquatic environments associated with cattle feedlots at concentrations ranging from <20 ng/L (Durhan et al., 2006) to as high as 162 ng/L (Gall et al., 2011). 17β-trenbolone is an extremely stable compound, with a half-life of up to 260 days measured in animal waste (Durhan et al., 2006), and is particularly potent, binding to androgen receptors with three times the affinity of testosterone (Khan et al., 2008). Exposure to 17β-trenbolone can have severe implications for the sexual morphology of fish (eg: reduced fecundity in fathead minnows, Pimephales promelas: Ankley et al., 2003; Jensen et al., 2006; sex reversal in zebrafish, Danio rerio: Larsen and Baatrup, 2010), but its behavioral consequences are poorly understood. Although, recent research has found that trenbolone can influence the reproductive behavior of male and female Poecilid fishes (Saaristo et al., 2013; Bertram et al., 2015), it is still unknown whether these behavioral changes may impact the mechanisms of sexual selection and female mate choice.

Our study species was the guppy (Poecilia reticulata)—a small, viviparous fish commonly found in freshwater environments contaminated with EDCs (López-Rojas and Bonilla-Rivero, 2000; Widianarko et al., 2000). Guppies are native to north-eastern South America but have become invasive throughout the world as a result of both deliberate and accidental introductions (Lindholm et al., 2005). The mating system of the guppy makes them an ideal candidate for investigating the effects of EDCs on sexual selection. Female guppies are choosy and actively associate with preferred males, which can have a direct influence on mating outcomes (Godin and Briggs, 1996; Shenoy, 2012). Females are known to favor a number of male traits including increased orange pigmentation, size, and display rate (Endler, 1980; Brooks and Caithness, 1995; Kodric-Brown and Nicoletto, 2001), all of which are honest indicators of male quality.

Here we test the hypothesis that short-term exposure to an environmentally relevant concentration of trenbolone will impact female mate choice in guppies. The male traits that females use to choose mates are under androgenic control (Wilson, 1999; Emerson, 2000), meaning they are likely to be influenced by trenbolone. This, combined with the fact that trenbolone is known to affect reproduction in Poecilids, suggests that exposure should also affect female mate choice.

2. Methods

2.1. Collection and housing

Guppies were collected with dip nets from Alligator Creek (19° 26′ 17.94″ S, 146° 57′ 1.09″ E) in Queensland, Australia. Alligator Creek is a rainforest-fed stream located in the pristine Bowling Green Bay National Park. Water samples taken from this site over consecutive years revealed no contamination from estrogenic or androgenic EDCs (ALS global, unpublished data), thus ensuring that fish used in this study were from an uncontaminated source. Fish were transported to Monash University via airfreight and were acclimated to laboratory conditions (26 °C, 12:12 h light regime) for 2 months prior to exposure. Fish were fed ad libitum once daily with commercial fish pellets (Otohime Hirame larval diet; 580–910 μm).

2.2. Exposure

After acclimation, fish were exposed to trenbolone for 21 days, as previous experiments have shown that exposure periods ranging from 14 to 28 days are sufficient to induce behavioral changes (Bayley et al., 1999; Bell, 2001; Martinović et al., 2007; Majewski et al., 2002; Maunder et al., 2007; Oshima et al., 2003; Saaristo et al., 2009a,b). Furthermore, EDCs often enter the environment in pulses and may only remain in waterways for a short period of time (Diamanti-Kandarakis et al., 2009), meaning short exposure times are ecologically meaningful.

Guppies were exposed to trenbolone via a flow-through system based on the design of Saaristo et al. (2013). This system included eight 54 L (60 cm × 30 cm × 30 cm) tanks: 4 control tanks (containing a solvent control) and 4 trenbolone-exposed tanks (containing 17β-trenbolone). A total of 160 fish (100 males, 60 females) were separated by sex and distributed between these eight tanks (i.e., 2 control and 2 trenbolone-exposed tanks for each sex). Guppies in the trenbolone-exposed tanks were exposed to trenbolone at a nominal concentration of 15 ng/L (measured concentration = 4 ng/L; see below for details on how trenbolone concentrations were monitored), while guppies in the control tanks were exposed to a solvent control (0.000013% ethanol—see below for details). All exposure tanks were maintained at a constant temperature between 25 and 27 °C, and fish were fed daily ad libitum.

Water entered these exposure tanks via a mixing tank, which received a constant flow of fresh, filtered tap water and a constant flow of either trenbolone (in the case of the trenbolone-exposed tanks) or the solvent solution (in the case of the control tanks) from a stock tank via a peristaltic pump (Watson Marlow 323 U/MC). Water was channeled into the exposure tanks using silicone tubing, and flow rates were kept constant (2.25 L/h) using flow meters (BES Flowmeters, MPB Series 1200) and adjustable valves.

2.3. Stock solution preparation

The trenbolone stock solution was created by first dissolving 17β-trenbolone (4,9,11-estratrien-17-ol-3-one; Novachem, Germany) in 100% ethanol (solvent) to create a stock standard of 300 mg/L. This stock standard was then diluted to 600 μg/L using deionized water, resulting in a solvent concentration of 0.2%. The stock solution was further diluted in the mixing tank in the flow-through system to achieve the desired nominal 17β-trenbolone concentration of 15 ng/L (measured concentration: $\tilde{x} = 4$ ng/L, SD = 1.4 ng/L, n = 14). A solvent solution of 0.2% was used in the control tanks, which was diluted in the exposure system to a concentration of 0.000013%.

2.4. Water analysis

In order to monitor trenbolone concentrations in the exposure tanks, and to ensure there was no contamination of control tanks, a 100 mL water sample was taken from each of the exposure tanks weekly and analysed using enzyme-linked immunosorbent assay (Trenbolone ELISA, EuroProxima, Arnhem, The Netherlands). For a detailed description of the ELISA testing protocol, see Saaristo et al. (2013).

2.5. Behavior trials

The impact of trenbolone exposure on female mate choice was investigated using a dichotomous choice experiment, which is a standard method used for investigating mate choice preferences in guppies (Pilastro et al., 2004; Jeswiet et al., 2012). A 51 L tank (60 cm × 30 cm × 24 cm) was split into three compartments using clear, perforated plastic dividers to allow water flow and full visual and chemical contact between compartments. A single female was placed in the middle compartment in a clear plastic cylinder, while a single male was placed in each of the end compartments. After a 10-min acclimation period, the cylinder was removed and the fish were allowed to interact for 15 min. All trials were filmed using a digital video recorder (Canon Powershot S110).

Two trial combinations were used to investigate the impacts of trenbolone on female mate choice: (1) an unexposed female was given a choice between an exposed and an unexposed male (hereafter referred to as 'unexposed female trial'), and (2) an exposed female was given a choice between an exposed and an unexposed male (hereafter referred to as 'exposed female trial'). There was no difference in the weight or length of exposed and unexposed males (weight: $t = 1.35$, $df = 58.95$, $p = 0.18$; length: $t = 1.05$, $df = 59.95$, $p = 0.33$, see Table 1 for details) or exposed and unexposed females (weight: $t = 0.18$, $df = 28.93$, $p = 0.86$; length: $t = 0.46$, $df = 28.64$, $p = 0.65$, see Table 1 for details) in either of the trial combinations. A total of 31 trials were conducted ($n = 15$ unexposed female trials, $n = 16$ exposed female trials).

Female preference was determined by quantifying the amount of time spent within a 5 cm 'preference zone' of either male compartment. Association time is commonly used as a measure of female preference in guppies (Kodric-Brown, 1985, 1989; Karino and Shinjo, 2004; Pilastro et al., 2004) and has been shown to be an accurate indicator of female mate choice in Poecilid fishes (Walling et al., 2010). The courtship behavior (i.e., time spent conducting sigmoid displays and orienting their body toward that of the female) of both exposed and unexposed male fish was also quantified. We calculated the total time that males spent courting as females were in view of the males throughout the entire trial, meaning males courted even if females were not directly associating with them. Male and female behaviors were quantified using JWatcher v 1.0.

2.6. Morphological analysis

Male and female weight and length was measured directly after behavior trials. Males were also photographed on their right side in a standardized fashion (Nikon D90, shutter speed = 1/250, Nikon AF Micro-Nikkor 60 mm f/2.8D), and the resultant pictures analysed using Photoshop (CS6 version 13.0 Extended) to determine the total percentage of the body containing orange pigments. For a detailed description of the coloration analysis method, see Bertram et al. (2015).

2.7. Statistical analysis

All data were analyzed using the statistical program 'R' (version 2.13.1; R Development Team, 2011). Data was checked for normality and homogeneity of variance, and transformed where necessary. Independent samples t-tests were used to compare the amount of time that unexposed and exposed females spent associating with males. The amount of time that females spent associating with each male, and the courtship behavior of exposed and unexposed males, were compared in each treatment using paired t-tests. The weight and length of males and females was compared using independent samples t-tests, as was the percentage of orange pigmentation of males.

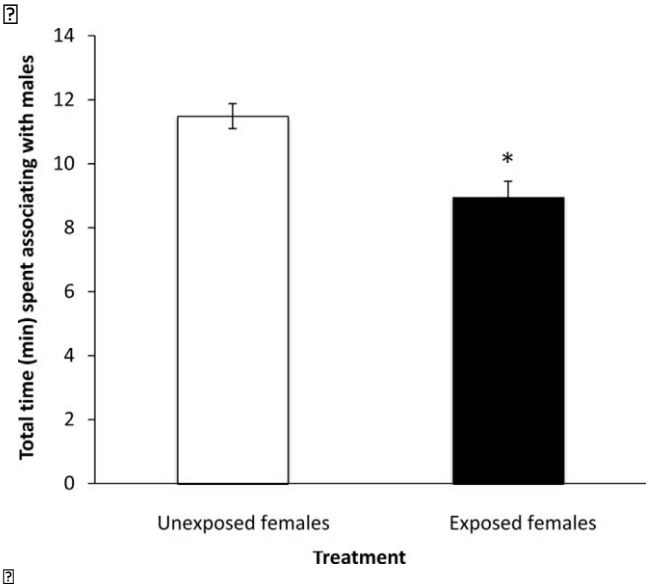

Fig 1. Mean total time (±S.E.) that unexposed females (n = 15) and exposed (n = 16) females (n = 16) spent associating with males.

2.8. Ethical statement

The research detailed in this paper was approved by the Biological Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2013/09) and complies with all relevant State and Federal laws of Australia.

3. Results

3.1. Female behavior

Exposed females spent significantly less time associating with males than unexposed females ($t = 4.03$, $df = 28.05$, $p < 0.001$, Fig. 1). When they were associating with males, unexposed females spent significantly more time associating with unexposed males than exposed males ($t = 3.14$, $df = 14$, $p < 0.01$, Fig. 2), while exposed females showed no preference for either exposed or unexposed males ($t = 0.44$, $df = 15$, $p = 0.67$, Fig. 2).

3.2. Male behavior and morphology

There was no difference in the total time that exposed and unexposed males spent courting in both the unexposed female trial ($t = -0.66$, $df = 14$, $p = 0.52$, Table 2) and the exposed female trial ($t = -0.56$, $df = 15$, $p = 0.58$, Table 2). There was also no difference in the percentage of orange pigmentation between exposed and unexposed males in either treatment (unexposed female trial: $t = -0.37$, $df = 23.29$, $p = 0.71$, exposed female trial: $t = 0.1819$, $df = 29.81$, $p = 0.86$, Table 2).

4. Discussion

This is the first study to show that an androgenic agricultural contaminant can affect female mate choice. We found that unexposed females spent more time associating with males than trenbolone-exposed females, and also showed a preference for unexposed males over trenbolone-exposed males. Exposed females, on the other hand, showed no preference for either male. Surprisingly, trenbolone exposure had no impact on any of the male traits or behaviors examined in this study, with no difference observed in male body size, percentage of orange pigmentation, or courtship behavior of exposed and unexposed fish.

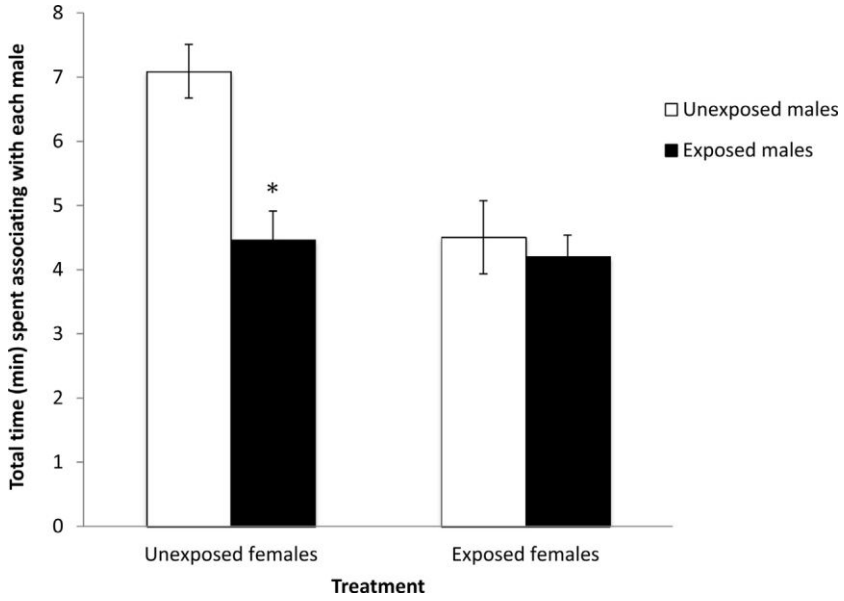

Fig. 2. Mean total time (±S.E.) that unexposed females (n = 15) and exposed females (n = 16) spent associating with exposed and unexposed males.

Trenbolone-exposed females spent less time associating with males than unexposed females, indicating a decreased desire to mate, and were also less choosy than unexposed females. Previous research has shown that trenbolone exposure can suppress estrogenic activity in female fish, causing varying levels of masculinization. Specifically, Ankley et al. (2003) observed reduced concentrations of vitellogenin and 𝛽-estradiol in trenbolone-exposed female fathead minnows, which was correlated with the development of male morphological characteristics. Trenbolone-induced masculinization has also been observed in female mosquitofish (Gambusia holbrooki; Sone et al., 2005) and zebra fish (Morthorst et al., 2010). Hence, it is conceivable that females in our study were masculinized to some degree, which could have reduced their desire to mate and made them less choosy.

In contrast to the exposed females, unexposed female guppies were choosy, and associated more with unexposed than exposed males. However, we did not find any differences between exposed and unexposed males in traits that have previously been found to influence female mate choice in guppies (i.e., color: Endler, 1980; display rate: Kodric-Brown and Nicoletto, 2001). Although, it is not clear in this experiment what cue(s) unexposed females may have been using to discriminate against exposed males and to preferentially associate with unexposed males, previous research has shown that chemical cues play an important role in Poecilid reproduction, including mate choice in guppies (Guevara-Fiore et al., 2009, 2010). It is possible that trenbolone exposure altered the chemical cues of male guppies in this study, which may explain female preference for unexposed males. This warrants further investigation into the effect of trenbolone exposure on chemical cues and its subsequent impact on reproductive behavior.

Trenbolone is a potent androgen agonist, meaning it has the potential to intensify the expression of male sexual traits by stimulating androgen production (Schiffer et al., 2001; Hotchkiss et al., 2008). This process of xenoandrogen-induced 'hypermasculinization' has been observed in multiple species. For example, androgenic EDC-exposed African clawed frogs, Xenopus laevis, exhibited increased levels of androgen-dependent male mate calling (Hoffmann and Kloas, 2010), while androgenic EDC

Table 1
Total length and weight of females and males (mean ± S.E.) used in behavior trials (TB—trenbolone).

Trial combination	n	Mean length (mm) ± S.E.	Mean weight (mm) ± S.E.
Unexposed female trial			
Unexposed males	15	21.22 ± 0.402	0.09 ± 0.002
TB-exposed males	15	21.58 ± 0.359	0.10 ± 0.004
Unexposed females	15	24.14 ± 0.663	0.16 ± 0.016
Exposed female trial			
Unexposed males	16	21.68 ± 0.365	0.09 ± 0.004
TB-exposed males	16	21.35 ± 0.450	0.10 ± 0.003
TB-exposed females	16	24.61 ± 0.769	0.16 ± 0.016

Table 2
Total percentage of orange pigmentation and total time spent courting by males used in behavior trials (TB—trenbolone).

Trial combination	n	Mean% orange pigmentation ± S.E.	Mean time spent courting ± S.E. (min)
Unexposed female trial			
Unexposed males	15	9.20 ± 0.678	7.12 ± 0.929
TB-exposed males	15	9.66 ± 1.341	7.82 ± 0.578
Exposed female trial			
Unexposed males	16	9.68 ± 1.101	6.58 ± 0.838
TB-exposed males	16	9.37 ± 1.078	7.25 ± 0.981

exposure was found to increase the intensity of male sexual behaviors in several cyprinid fish species (Belanger et al., 2010). Why, then, were the male sexual traits in this experiment not influenced by trenbolone exposure? It has been well established that EDCs can influence the percentage of orange coloration in male guppies (Bayley et al., 2002, 2003; Shenoy 2012; Tian et al., 2012). However, the vast majority of these studies exposed guppies before they became sexually mature, while in this study only mature adults were exposed. Male courtship behavior was also unaffected by trenbolone exposure, but this result is more difficult to interpret. Recent studies investigating the impact of trenbolone on the courtship behavior of male Poecilids have yielded contrasting results. Saaristo et al. (2013) found that short-term exposure to an environmentally relevant concentration of trenbolone (6 ng/L, 21 day exposure) had no impact on the reproductive behavior of male mosquitofish, while Bertram et al. (2015) found that exposure to 22 ng/L of trenbolone for 21 days had a significant impact on the amount of time that male guppies spent courting and conducting coercive mating behaviors. This suggests that more research is required in order to gain a more comprehensive understanding of the impact of trenbolone on reproductive behavior.

In conclusion, this is the first study to show that an androgenic EDC can impact sexual selection by disrupting the mechanisms of female mate choice. We found that females exposed to an environmentally relevant concentration of trenbolone associated with males less than unexposed females and were also less choosy. Female mate choice can have important consequences at the population level by influencing both the quality and quantity of offspring produced (Wong and Candolin, 2015), highlighting the need for studies to investigate the behavioral impacts of environmental contaminants on both sexes.

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

Patrick Tomkins [a, *], Minna Saaristo [a, b], Michael G. Bertram [a], Raymond B. Tomkins [c], Mayumi Allinson [d], Bob B.M. Wong [a]

[a] School of Biological Sciences, Monash University, Victoria, Australia
[b] Department of Biosciences, Åbo Akademi University, Turku, Finland
[c] Centre for AgriBioscience, Department of Environment and Primary Industries (DEPI), Victoria, Australia
[d] Centre for Aquatic Pollution Identification and Management (CAPIM), School of Chemistry, The University of Melbourne, Victoria, Australia

highlights

- 17b-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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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 17b-trenbolone, which is used globally to promote growth in beef cattle. Although 17b-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 17b-trenbolone (average measured concentration: 8 ng/L) for 21 days using a flow-through system. We found that, in the presence of a competitor, 17b-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

* Corresponding author. School of Biological Sciences, Monash University, Melbourne, Victoria, 3800, Australia.
E-mail address: patrick.tomkins@monash.edu (P. Tomkins).

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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 15e50 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 17b-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, 17b-trenbolone can accumulate in aquatic habitats and has been detected at concentrations ranging from ::: 1e20 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 17b-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 17b-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 17b-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 17b-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 17b-trenbolone will alter male guppy competitive mating interactions by influencing male reproductive behaviour and aggression. Given that, as aforementioned, exposure to 17b-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 17b-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 17b-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 17b-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 17b-trenbolone (ALS Group, unpublished data). Fish were acclimated to laboratory conditions (25e27 °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, 580e910 mm).

2.2. Chemical exposure

After acclimation to laboratory conditions, male fish were exposed to 17b-trenbolone for 21 days, as previous experiments have shown that EDC exposure periods ranging from 14e28 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; Martinovič 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 17b-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 x 30 x 30 cm), consisting of two control (unexposed) tanks and two 17b-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 17b-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 17b-trenbolone at an average measured concentration of 8 ng/L (see 'Monitoring of 17b-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 17b-trenbolone

A stock solution was created by firstly dissolving 17b-trenbolone (17b-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 mg/L using deionised water. This stock solution was further diluted in the flow-through system's mixing tank (162 L, 90 x 45 x 40 cm) to achieve the desired 17b-trenbolone concentration (mean ¼ 7.70 ng/L, SD ¼ 4.40, n ¼ 6). Stock solutions were created weekly to prevent any potential degradation of 17b-trenbolone over the exposure period.

In order to monitor 17b-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 mm, 500 mg/6 mL; Phenomenex, Torrance, CA, USA). The cartridge was then eluted with methanol (2 x 4 mL), with the eluate dried under a nitrogen stream. Samples were reconstituted with 100 mL methanol and 900 mL of deionised water.

Measurement of 17b-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 mL) in duplicate into an antibody-coated 96-well plate by an auto dispenser (epMotion 5070; Eppendorf, Hamburg, Germany). Thereafter, 25 mL of HRPO conjugate and 25 mL 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 mL 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 mL 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 mg/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 17b-trenbolone solution. The average recovery was 97%, providing confidence that 17b-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 x 30 x 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 x 30 x 30 cm), while one exposed and one unexposed male were placed into the second compartment (20 x 30 x 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 ¼ 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 ¼ 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 17b-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

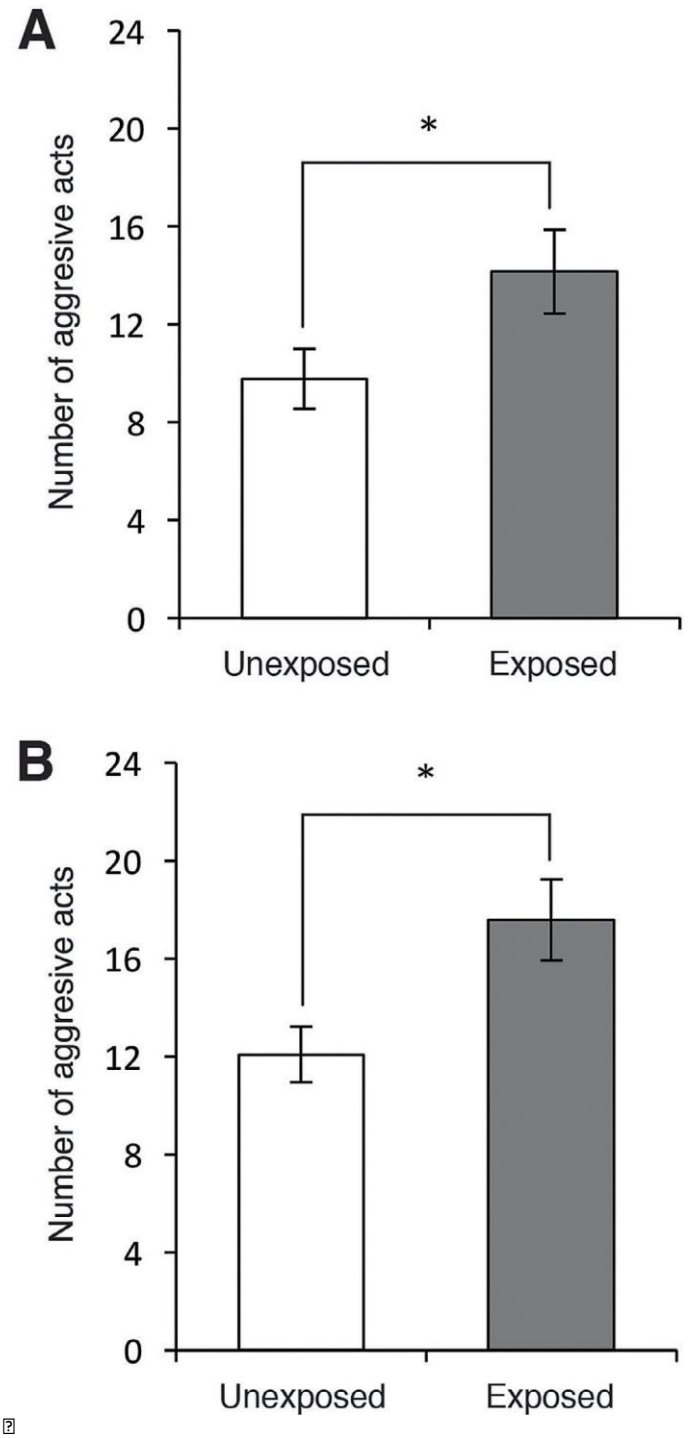

Fig. 1. Mean (±SE) number of aggressive acts (i.e., chases and fin nips) directed by a male towards a rival when A) males were separated from females by a transparent partition (n = 37), and B) males were allowed to interact freely with females (n = 37). Asterisks indicate a significant difference between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

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).

3.3. Morphology

No significant difference was detected in weight (Mann-

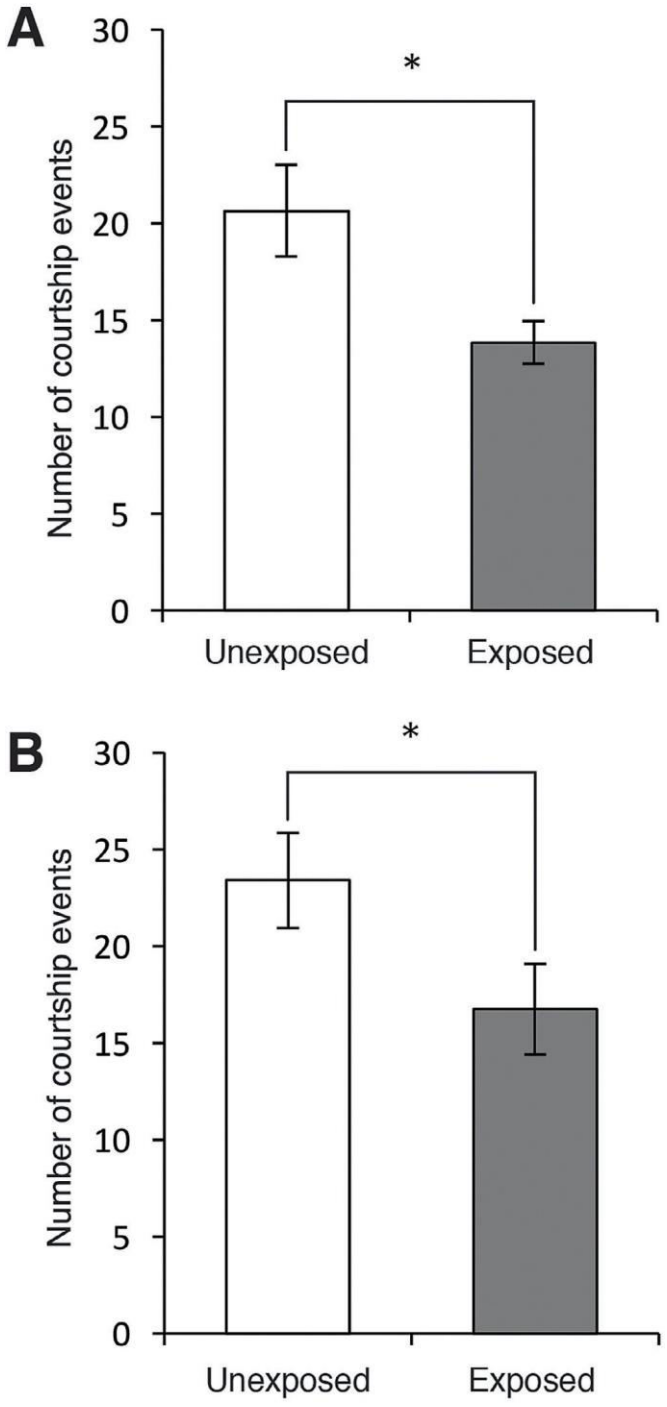

Fig. 2. Mean (±SE) number of courtship events conducted by males when A) males were separated from females by a transparent partition (n ¼ 37), and B) males were allowed to interact freely with females (n ¼ 37). Asterisks indicate a significant difference between groups at a ¼ 0.05.

Whitney U ¼ 848, p ¼ 0.544), total length (U ¼ 897, p ¼ 0.391) or percentage area of orange pigmentation (U ¼ 1001, p ¼ 0.811) between unexposed and exposed males.

4. Discussion

We found that exposure to an environmentally realistic concentration of 17b-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.

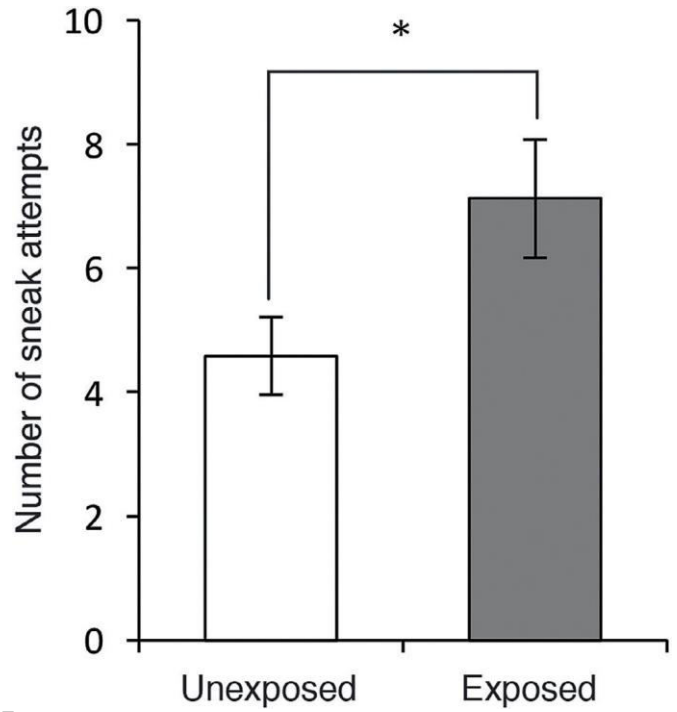

Fig. 3. Mean (±SE) number of sneak mating attempts conducted by males towards females when allowed to freely interact (n ¼ 37). Asterisk indicates a significant difference between groups at a ¼ 0.05.

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), 17b-trenbolone binds with high affinity to available androgen receptors, mimicking the effects of endogenous androgens (Wilson et al., 2002). Further, 17b-trenbolone which is non-aromatisable may indirectly inhibit the production of 17b-estradiol by limiting the production of testosterone and, thus, restricting the aromatisation of testosterone to 17b-estradiol (Zhang et al., 2008). Concordantly, in females, exposure to 17b-trenbolone can influence concentrations of plasma steroids (testosterone and b-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

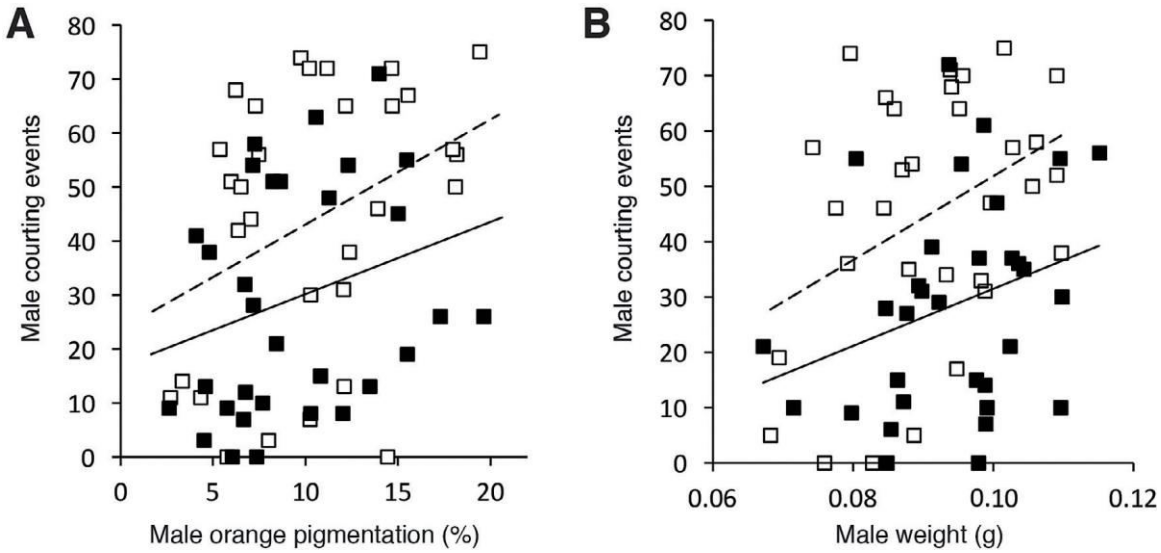

Fig. 4. Number of courtship events performed by unexposed and exposed males as a function of A) male orange pigmentation (% of body area) and B) male weight (g). Figures represent combined data from both behavioural trial stages (i.e., when males were separated from females by a transparent partition 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 17b-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 17b-trenbolone-exposed males are not yet wholly understood, exposure to 17b-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, 17b-trenbolone-exposed male guppies were more aggressive towards rivals than were unexposed males, which is likely a result of 17b-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 17b-trenbolone exposure to have also resulted in increased male courtship behaviour, but this was not the case.

Males exposed to 17b-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 17b-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 17b-trenbolone on male behaviour in the absence of a rival male, suggesting that 17b-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 17b-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 17b-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 17b-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 17b-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.

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An endocrine-disrupting agricultural contaminant impacts sequential female mate choice in fish☆

Patrick Tomkins [a, *], Minna Saaristo [a, b], Michael G. Bertram [a], Marcus Michelangeli [a], Raymond B. Tomkins [c], Bob B.M. Wong [a]

[a] School of Biological Sciences, Monash University, Victoria, Australia
[b] Department of Biosciences, Åbo Akademi University, Turku, Finland
[c] Centre for AgriBioscience, Department of Environment and Primary Industries (DEPI), Victoria, Australia

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The environmental impact of endocrine-disrupting chemicals (EDCs)—compounds that interfere with endocrine system function at minute concentrations—is now well established. In recent years, concern has been mounting over a group of endocrine disruptors known as hormonal growth promotants (HGPs), which are natural and synthetic chemicals used to promote growth in livestock by targeting the endocrine system. One of the most potent compounds to enter the environment as a result of HGP use is 17b-trenbolone, which has repeatedly been detected in aquatic habitats. Although recent research has revealed that 17b-trenbolone can interfere with mechanisms of sexual selection, its potential to impact sequential female mate choice remains unknown, as is true for all EDCs. To address this, we exposed female guppies (Poecilia reticulata) to 17b-trenbolone at an environmentally relevant level (average measured concentration: 2 ng/L) for 21 days using a flow-through system. We then compared the response of unexposed and exposed females to sequentially presented stimulus (i.e., unexposed) males that varied in their relative body area of orange pigmentation, as female guppies have a known preference for orange colouration in males. We found that, regardless of male orange pigmentation, both unexposed and exposed females associated with males indiscriminately during their first male encounter. However, during the second male presentation, unexposed females significantly reduced the amount of time they spent associating with low-orange males if they had previously encountered a high-orange male. Conversely, 17b-trenbolone-exposed females associated with males indiscriminately (i.e., regardless of orange colouration) during both their first and second male encounter, and, overall, associated with males significantly less than did unexposed females during both presentations. This is the first study to demonstrate altered sequential female mate choice resulting from exposure to an endocrine disruptor, highlighting the need for a greater understanding of how EDCs may impact complex mechanisms of sexual selection.

1. Introduction

Chemical pollutants are accumulating in environments worldwide at an alarming pace and scale (Kolpin et al., 2002; WHO-UNEP, 2012; Arnold et al., 2014). Of great concern are endocrine-disrupting chemicals (EDCs)—compounds that can alter the endocrine function of organisms at minute concentrations (in the low ng/L range) by interfering with hormonal communication (Kavlock and Ankley, 1996; Lintelmann et al., 2003; Buchanan and Partecke, 2012; Brander, 2013). Endocrine-disrupting chemicals encompass a broad range of both artificial compounds, which include pharmaceuticals, metals, plastics and pesticides (Diamanti-Kandarakis et al., 2009), and natural hormones, such as xenoestrogens (Gore et al., 2015). They can infiltrate ecosystems during their production, use, and/or disposal (WHO-UNEP, 2012), with common sources including wastewater from industry and households, agricultural and suburban run-off, and solid waste (Diamanti-Kandarakis et al., 2009). Once in the environment, many EDCs have a tendency to bioaccumulate (Crews et al., 2007; Walker

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* Corresponding author. School of Biological Sciences, Monash University, Melbourne, Victoria, 3800, Australia.
E-mail address: patrick.tomkins@monash.edu (P. Tomkins).

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and Gore, 2011), and have therefore continually been detected at elevated concentrations in wildlife tissues, even in the most remote regions on Earth (e.g., polar bears in the Arctic, Letcher et al., 2010; amphipods in the Mariana Trench, Jamieson et al., 2017).

One group of EDCs with the potential to impact wildlife is hormonal growth promotants (HGPs), which are natural and synthetic chemicals used to promote growth in livestock (Hunter, 2010; Sellin Jeffries et al., 2011; Kolodziej et al., 2013; Johnson, 2015). HGPs are used globally, and their use is particularly widespread in several of the world's leading beef-producing nations. For example, in the USA, which is the world's leading beef producer, it is estimated that 20 million cattle (i.e., approximately two thirds of the total livestock in the country) currently receive HGP implants (Johnson, 2015). Although HGPs generally include mixtures of natural and synthetic hormones (Lange et al., 2001; Hunter, 2010), the most commonly administered androgen in HGP implants is trenbolone acetate (Hunter, 2010), which is a highly efficient and potent synthetic steroid (Neumann, 1976). Trenbolone acetate is hydrolysed within implanted cattle to produce the biologically active steroid hormone 17b-trenbolone, which enters the environment via run-off of urine and faeces. Once present in the aquatic environment, 17b-trenbolone has a tendency to accumulate as a result of its long half-life (~260 days measured in animal waste; Schiffer et al., 2001) and has been detected at concentrations ranging from 1 to 20 ng/L in waterways upstream and downstream of cattle farm outflow points (Durhan et al., 2006) to 162 ng/L in tile-drained agroecosystems (Gall et al., 2011).

A growing number of studies have demonstrated that exposure to 17b-trenbolone can have alarming impacts on wildlife, particularly in aquatic environments. Exposure has been linked with severe morphological and physiological abnormalities in fish, including abnormal gonadal development (Örn et al., 2006), reduced reproductive output (Ankley et al., 2003), irreversible masculinisation (Baumann et al., 2014), and even complete and functional sex reversal (Larsen and Baatrup, 2010; Morthorst et al., 2010). We now know that 17b-trenbolone can also induce behavioural abnormalities, with recent research revealing that environmentally realistic exposure concentrations can affect risk-taking behaviour in guppies (Poecilia reticulata; Heintz et al., 2015), as well as reproductive behaviour and sexual selection processes in both guppies (Bertram et al., 2015; Tomkins et al., 2016, 2017) and eastern mosquitofish (Gambusia holbrooki; Saaristo et al., 2013). However, the potential impacts of 17b-trenbolone on more complex mechanisms of sexual selection remain poorly understood, as is also true for EDCs generally.

Sexual selection, by directly influencing mating outcomes, has important consequences for reproductive success, population dynamics and broader evolutionary processes (Candolin and Wong, 2012). Because sex hormones regulate the expression of a range of behaviours under sexual selection (Beyer et al., 1976; Munakata and Kobayashi, 2010), exposure to endocrine disruptors is likely to influence sexual selection processes. Indeed, recent research has revealed that, in simultaneous mate choice experiments (i.e., when females are presented with two or more males at the same time), exposure to environmentally relevant concentrations of endocrine-disrupting chemicals can impair female mate choice in sand gobies (Pomatoschistus minutus; Saaristo et al., 2009) and guppies (Tomkins et al., 2016). However, in nature, opportunities for females to make direct comparisons between suitors are often limited (Jennions and Petrie, 1997). In many species, it is more common for females to encounter mates sequentially (Bradbury and Andersson, 1987), making investigating the effects of EDCs on sequential female mate choice more ecologically relevant.

Guppies are a small, freshwater fish that occur in contaminated environments around the world (e.g., López-Rojas and Bonilla-Rivero, 2000; Widianarko et al., 2000). They are an ideal species for investigating the impacts of endocrine disruptors on the mechanisms of sexual selection as their mating system is driven primarily by female choice. Males compete for the attention of females, achieving copulations via two contrasting mating strategies. Briefly, males either mate consensually with females following successful courtship displays (termed 'sigmoid displays'), or gain copulations by sneaking up behind females and attempting to mate with them coercively (termed 'sneak' attempts) (Houde, 1997). Previous research investigating female mate choice in guppies has demonstrated that females show a strong preference for males with relatively large areas of orange pigmentation on their bodies (e.g., Houde, 1987; Kodric-Brown, 1989; Long and Houde, 1989; Endler, 1995; Grether, 2000; Kodric-Brown and Nicoletto, 2001). Orange colouration is an honest indicator of male quality in guppies, correlating positively with swimming performance (Nicoletto, 1993), foraging ability (Endler, 1980; Karino and Shinjo, 2007; Karino et al., 2007), sperm quality (Locatello et al., 2006; Pitcher et al., 2007) and sperm load size (Pitcher and Evans, 2001; Pitcher et al., 2007), as well as parasite resistance (Houde and Torio, 1992). However, these studies have relied almost exclusively on experimental set-ups in which females are able to make direct comparisons between males. This is true, despite the fact that, in the wild, female guppies will often have to make reproductive decisions based on sequential encounters with potential suitors (Houde, 1997; Pitcher et al., 2003). Guppies, therefore, provide an excellent opportunity to further our understanding of the impacts of EDCs on sexual selection by investigating the hitherto unknown impact of EDCs on sequential female mate choice.

Here, we test the hypothesis that short-term exposure to an environmentally realistic concentration of 17b-trenbolone will impact sequential female mate choice in guppies. Given that 17b-trenbolone has been shown to affect reproductive behaviour in guppies and other Poeciliids, we expected exposure to also disrupt female mate choice processes when males are encountered sequentially, which is often the more environmentally realistic scenario.

2. Methods

2.1. Fish collection and housing

Guppies were collected from Alligator Creek in Queensland, Australia (19° 26′ 17″ S, 146° 57′ 01″ E), where a wild population has established itself as a result of deliberate and/or accidental introductions from the pet trade. The sampling site is located inside the Bowling Green Bay National Park, and is thus thought to be a pristine location. Indeed, we have taken water samples from this site over consecutive years and found no presence of 17b-trenbolone (ALS Group, unpublished data). Fish were actively collected using dip nets and brought back to Monash University in aerated containers, where they were acclimated to laboratory conditions (25–27 °C, 12:12 h light:dark regime) in sex-specific tanks for three months prior to exposure to ensure sexual receptivity during behavioural trials. Fish were fed ad libitum once daily with a commercial fish pellet (Otohime Hirame larval diet, 580–910 μm).

2.2. Chemical exposure and water testing

Female guppies were exposed to 17b-trenbolone for 21 days via a flow-through system adapted from previous studies (Saaristo et al., 2013; Bertram et al., 2015; Tomkins et al., 2016, 2017). The system was comprised of six 54 L aquaria, consisting of three unexposed tanks and three 17b-trenbolone-exposed tanks. A total of 120 females were randomly distributed between these six tanks

(i.e., 20 fish per tank). Fish in the exposed aquaria received an average measured concentration of 2 ng/L of 17b-trenbolone (see below for details), which is consistent with concentrations detected in freshwater systems affected by agricultural activity (Durhan et al., 2006), while the unexposed tanks received fresh water only. Throughout the flow-through exposure, all fish were maintained under the same housing conditions as those described above.

The stock solution was created by dissolving 17b-trenbolone (17b-hydroxyestra-4,9,11-trien-3-one, CAS: 10161-33-8; Novachem, Germany) in ethanol (HPLC grade, ≥99.99%) to create a stock standard of 400 mg/L. This stock solution was diluted to 400 mg/L using deionised water, before being further diluted in the flow-through system to achieve a 17b-trenbolone exposure concentration of 2 ng/L (average measured concentration ¼ 1.67 ng/L, SD ¼ 0.56, n ¼ 9). Water samples (200 mL) were collected from each of the 17b-trenbolone-exposed and unexposed tanks weekly and analysed using gas chromatography–tandem mass spectrometry (7000C Triple Quadrupole GC-MS/MS, Agilent Technologies, Delaware, USA). Analysis was conducted by Envirolab Services (MPL Laboratories, Perth; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025). No contamination with 17b-trenbolone was detected in the unexposed tanks throughout the exposure period (limit of quantification: 1 ng/L, n ¼ 9). For a detailed description of the collection and analysis of water samples, see 'Supplementary Methods' in Supplementary material.

2.3. Behavioural trials

To investigate the impact of exposure to 17b-trenbolone on sequential female mate choice in guppies, a 27 L trial tank (30 x 30 x 30 cm) was separated into two compartments using a transparent perforated divider to allow full visual and chemical communication. A single experimental (i.e., unexposed or 17b-trenbolone-exposed) female was placed into one compartment (20 x 30 x 30 cm) in a 500 mL holding container and a single stimulus (i.e., unexposed) male placed into the other compartment (10 x 30 x 30 cm) in an identical holding container. Stimulus males were not subjected to the flow-through exposure, instead being drawn randomly from one of eight 27 L same-sex holding tanks (30 x 30 x 30 cm), having been housed under the same temperature, light and feeding conditions as females from the flow-through exposure. Stimulus males were unexposed to ensure 17b-trenbolone-induced changes in male behaviour did not influence the behaviour of females (Saaristo et al., 2013). After a 5 min acclimation period, both fish were released from their holding containers into their respective compartments and allowed to interact for 15 min through the divider. The first stimulus male was then removed and replaced with a second stimulus male, which was again subject to a 5 min acclimation period in a holding container before being released and allowed to interact with the female through the divider for a further 15 min.

Our experimental design required two categories of stimulus males, those with a high percentage body area of orange pigmentation (i.e., 'high-orange' males) and those with a low percentage body area of orange pigmentation (i.e., 'low-orange' males). This is because a strong female preference for males with relatively large areas of orange pigmentation on their bodies has been documented in many guppy populations (e.g., Kodric-Brown, 1985; Houde, 1987; Long and Houde, 1989; Endler, 1995; Kodric-Brown and Nicoletto, 2001), including in guppies from the Alligator Creek population used in our study (e.g., Gamble et al., 2003; Bertram et al., 2015). Male percentage body area of orange pigmentation was judged visually at the beginning of the exposure period and males were separated accordingly. Immediately following behavioural trials, males were photographed and the subsequent images used to quantify their percentage body area of orange pigmentation using digital colouration analysis (see 'Morphological analysis' below). Low-orange males possessed a percentage body area of orange pigmentation ranging from 3.16 to 8.22% (mean ¼ 5.21%, SD ¼ 1.32%), while high-orange males ranged from 12.05 to 19.66% (mean ¼ 15.31%, SD ¼ 1.75%) (Table S1). These values are comparable to those reported in previous research investigating sequential female mate choice in guppies by Pitcher et al. (2003), both in terms of the mean percentage body area of orange pigmentation in each stimulus male group, as well as the degree of separation between the group means. Further, Karino and Shinjo (2004) demonstrated that female guppies show a preference for males bearing as little as 2.0% more orange colouration than relatively dull males, indicating that the minimum difference of 3.83% orange pigmentation in our study between low- and high-orange groups is a sufficient gap for females to exercise choice.

Stimulus males were presented to females in four combinations (first male/second male): low-orange/low-orange, high-orange/high-orange, low-orange/high-orange, and high-orange/low-orange. These treatments allowed us to disentangle whether females were simply showing an absolute preference for males with increased orange pigmentation, or if their responsiveness to sequentially presented males varied depending on previous male experience. These four presentation combinations were repeated for both unexposed females (low-orange/low-orange: n ¼ 16; high-orange/high-orange: n ¼ 16; low-orange/high-orange: n ¼ 15; high-orange/low-orange: n ¼ 16) and exposed females (low-orange/low-orange: n ¼ 16; high-orange/high-orange: n ¼ 16; low-orange/high-orange: n ¼ 15; high-orange/low-orange: n ¼ 15). All male and female fish were tested once only. Female preference for both the first and second male was determined by quantifying the amount of time spent within a 5 cm 'preference zone' abutting the male compartment. Association time is commonly used as a measure of female mating preference in guppies (e.g., Kodric-Brown, 1985, 1989; Karino and Shinjo, 2004; Pilastro et al., 2004; Tomkins et al., 2016) and has been shown to be an accurate indicator of female mate choice in Poeciliid fish (Walling et al., 2010). Female behaviour was quantified using the event-recording software JWatcher V1.0 (Blumstein and Daniel, 2007).

2.4. Morphological analysis

Immediately following behavioural trials, all fish were weighed (±0.0001 g) and measured for total length (±0.01 mm). Stimulus males were also photographed immediately after behavioural trials on their right side in a standardised fashion (Nikon D90, shutter speed ¼ 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. See Bertram et al. (2015) for details.

2.5. Statistical analysis

Data were analysed in R version 3.3.2 (R Core Development Team, 2016). Tests of normality (Shapiro-Wilk test; Royston, 1995) and homogeneity of variance (Fligner-Killeen test; Conover et al., 1981) were performed, where appropriate. Association time was square-root transformed prior to analysis to normalise residual errors. Statistical significance was assigned at a ¼ 0.05.

Firstly, we examined whether female association time differed due to treatment (i.e., unexposed versus 17b-trenbolone-exposed) and/or male percentage body area of orange pigmentation during the first presentation using a generalised linear model (GLM).

Treatment, male percentage body area of orange pigmentation and the interaction term were treated as fixed effects. Secondly, a linear mixed-effects model (lme function, nlme package; Pinheiro et al., 2017) with a Gaussian error distribution was used to determine whether females altered their response to males based on previous male experience. Treatment, male percentage body area of orange pigmentation, presentation order and the interaction terms were entered as fixed effects, with male ID entered as a random effect. Likelihood ratio tests (G^2) were then used to calculate the p-values of interaction terms (Bolker et al., 2009). Lastly, another GLM was used to test whether female association time differed due to treatment and/or male percentage body area of orange pigmentation during the second male presentation. In this instance, treatment, presentation order and the interaction term were entered as fixed effects. Presentation order was entered as a fixed effect to account for previous male experience. Mann-Whitney U tests were used to evaluate whether exposure to 17b-trenbolone altered female weight or total length, and independent samples t-tests were used to compare the orange pigmentation of males.

3. Results

3.1. Female association time during first male presentation

We found no interaction between treatment and male orange pigmentation on female association time ($F_{3,116}$ ¼ 0.20, p ¼ 0.660). Regardless of their own exposure status, we found no difference in the total time that females spent associating with low- and high-orange males ($F_{1,117}$ ¼ 2.85, p ¼ 0.094). However, in general, unexposed females spent more time associating with males than exposed females, irrespective of male orange pigmentation ($F_{1,117}$ ¼ 45.17, p < 0.001; Fig. 1).

3.2. Sequential female choice

We found a significant three-way interaction between treatment, male orange pigmentation and presentation order (G^2 ¼ 9.94, p ¼ 0.019). To account for this complex interaction, we analysed each treatment group separately.

For unexposed females, we found an interaction between male orange pigmentation and presentation order on female association time (G^2 ¼ 31.39, p < 0.001). Specifically, unexposed females that were initially offered a high-orange male reduced their association time when subsequently presented with a low-orange male (t_{28} ¼ 3.49, p < 0.001; Fig. 2A). However, in all other presentation combinations, there were no significant differences in the total time that unexposed females spent associating with the first and second male (low/low: t_{14} ¼ 1.73, p ¼ 0.10; high/high: t_{14} ¼ 1.42, p ¼ 0.178; low/high: t_{14} ¼ −1.99, p ¼ 0.066).

Contrasting with unexposed females, for exposed females we found no interaction between male percentage body area of orange pigmentation and presentation order on female association time (G^2 ¼ 3.81, p ¼ 0.283; Fig. 2B).

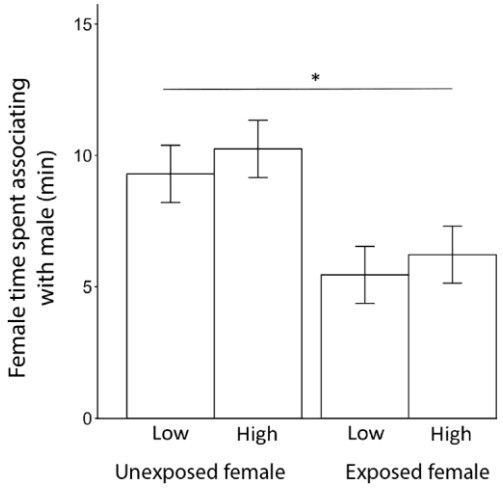

Fig. 1. Mean (±SE) time spent by unexposed and 17b-trenbolone-exposed females associating with low- and high-orange males during the first male presentation. The asterisk indicates a significant difference between groups (p < 0.05) obtained from ANOVA.

3.3. Female association time during second male presentation

Overall, unexposed females spent more time associating with males than did exposed females during the second male presentation ($F_{1,112}$ ¼ 33.47, p < 0.001). However, when unexposed females were presented with a low-orange male after having first observed a high-orange male, their association time reduced to a level that was comparable to that of exposed females in all presentation combinations ($F_{1,28}$ ¼ 0.14, p ¼ 0.712; Fig. 3).

3.4. Morphology

There was no significant difference in the weight (U ¼ 870, p ¼ 0.605) or total length (U ¼ 587, p ¼ 0.592) of unexposed and exposed females.

4. Discussion

This is the first study to demonstrate that exposure to an endocrine-disrupting chemical (EDC) at an environmentally relevant concentration can influence female mate choice when males are encountered sequentially. We found that, during their first male encounter, both unexposed and 17b-trenbolone-exposed females associated with males indiscriminately, although exposed females spent significantly less time associating with males overall than did unexposed females. During their second male encounter, unexposed females that were presented with a low-orange male significantly reduced their association time if they had previously encountered a high-orange male. Conversely, exposed females associated indiscriminately with males during their second male encounter, and again associated with males significantly less overall than did unexposed females. These findings demonstrate the profound influence that a widespread androgenic EDC can have on sexual selection processes at environmentally realistic exposure concentrations.

Both unexposed and exposed female guppies showed no preference for greater orange colouration during their first male encounter. It is well established that female guppies prefer males with increased orange pigmentation (Endler, 1980; Houde, 1997), including in the population used in this research (Gamble et al., 2003; Bertram et al., 2015). However, the vast majority of studies that have investigated female mate choice in guppies have done so using simultaneous choice experiments, where the female is able to make direct comparisons between males. To our knowledge, only one study has investigated female mate choice in guppies when males are encountered sequentially, which, in accordance with our results, found that virginal female guppies showed no preference for greater orange colouration during their first male encounter (Pitcher et al., 2003). While virgin females were not used in this study, females were sexually isolated for three months prior to exposure, as well as throughout the 21-day exposure period, which likely explains why they associated with males indiscriminately

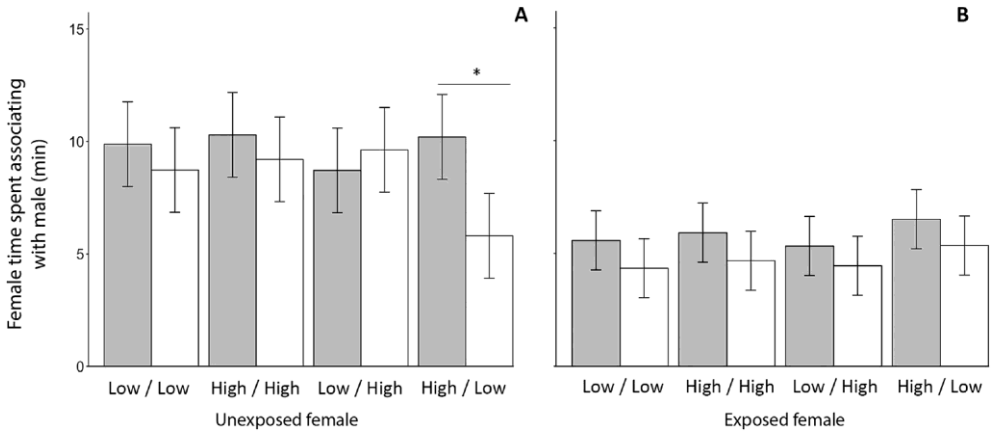

Fig. 2. Mean (±SE) time that (A) unexposed and (B) 17b-trenbolone-exposed females spent associating with males in each trial combination. Grey bars represent the first male presentation and white bars represent the second male presentation. The asterisk indicates a significant difference between groups ($p < 0.05$) obtained from Tukey's tests of simplified linear mixed-effects models.

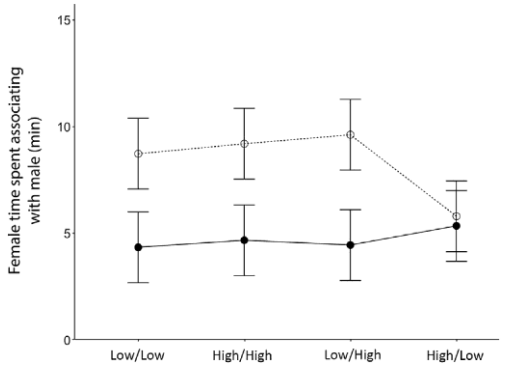

Fig. 3. Interaction plot showing the mean (±SE) time that unexposed females (open circles) and 17b-trenbolone exposed females (closed circles) from each presentation combination spent associating with males during the second male encounter. Plot displays the interaction between treatment and presentation order.

during their first male encounter. Further, although female guppies are able to store sperm for several months (Houde, 1997; Gasparini et al., 2012; López-Sepulcre et al., 2013), it is possible that the sperm storages of females used in this experiment were diminished during the extended isolation period preceding and during our exposure, which may have contributed to the lack of choosiness observed in females during their first male encounter.

Our results demonstrate that female preference can be influenced by previous male experience. Time spent by unexposed females associating with males during the first and second presentation did not differ in the low-orange/low-orange, low-orange/high-orange and high-orange/high-orange trial combinations. However, when unexposed females were presented with a low-orange male after having first observed a high-orange male, the amount of time they spent associating with the second male reduced significantly. This suggests that females were not simply showing an overall preference for increased orange colouration, but were adjusting their mate choice decisions based on previous experience with potential suitors (i.e., 'previous male effect' sensu Bakker and Milinski, 1991). Considering that females increase their reproductive success by maximising the quality of their mating partners (Bateman, 1948), this strategy reduces the likelihood of females mating with low-quality males in a population containing males that differ in quality (Bakker and Milinski, 1991; Milinski, 2001), and has previously been demonstrated in zebra finches (Taeniopygia guttata; Collins, 1995), smooth newts (Lissotriton vulgaris; Gabor and Halliday, 1997), crickets (Gryllus bimaculatus; Bateman et al., 2001) and guppies (Pitcher et al., 2003).

In contrast to unexposed females, we found no evidence of a previous male effect in females exposed to 17b-trenbolone. Moreover, exposed females showed no preference for increased orange colouration in either their first or second male presentation, indicating a breakdown of sexual selection processes. Further, exposed females spent significantly less time associating with males overall than did unexposed females during both their first and second male encounter, indicating that not only were exposed females less choosy, they were also generally less interested in mating. This finding is in agreement with research by Saaristo et al. (2013), where female mosquitofish exposed to 17b-trenbolone at 6 ng/L for 21 days approached males less, and spent more time swimming away from males, than did unexposed females. This result is also consistent with work by Tomkins et al. (2016), where 21-day exposure at 4 ng/L resulted in guppy females being less choosy and performing less association behaviour when presented with two males simultaneously. Interestingly, when unexposed females in the present study exhibited reduced interested in a male (i.e., during the second male presentation in the high-orange/low-orange combination), their association time reduced to a level that was comparable to—i.e., not significantly different from—the time spent by exposed females associating with males in all presentation combinations. This is important as it demonstrates that, regardless of male quality, females exposed to 17b-trenbolone behave similarly to unexposed females that are relatively disinterested in mating. To understand why 17b-trenbolone impacts choosiness in females, its mode of action must be considered.

The agricultural contaminant 17b-trenbolone is a potent, non-aromatisable androgen receptor agonist (Rogozkin, 1991; Hotchkiss et al., 2008). It binds with high affinity to available androgen receptors, mimicking the effects of androgens such as testosterone and 11-ketotestosterone (Wilson et al., 2002). It is also hypothesised that 17b-trenbolone indirectly inhibits the production of 17b-estradiol by limiting the production of testosterone and, thus, restricting the aromatisation of testosterone to 17b-estradiol (Zhang et al., 2008). As a result, 17b-trenbolone can suppress estrogenic activity in female fish. Ankley et al. (2003) observed reduced plasma concentrations of vitellogenin and 17b-estradiol in 17b-trenbolone-exposed female fathead minnows (Pimephales promelas), which was linked with the development of male morphological characteristics. Exposure to 17b-trenbolone has also

been found to cause varying levels of masculinisation in female mosquitofish (Sone et al., 2005; Brockmeier et al., 2012), zebrafish (Danio rerio; Morthorst et al., 2010; Baumann et al., 2014) and Japanese medaka (Oryzias latipes, Seki et al., 2006). It is likely that, despite our low exposure concentration and relatively short exposure period, 17b-trenbolone-exposed females in our experiment experienced some degree of masculinisation, which may have reduced their desire to mate and, in turn, made them less choosy. Further research in this area is needed to gain a better understanding of the underlying mode of action of 17b-trenbolone.

We found no effect of 17b-trenbolone exposure on female weight or length, despite the anabolic potency of 17b-trenbolone (Neumann, 1976). This result is consistent with previous research examining the morphological impacts of 17b-trenbolone-exposure at environmentally realistic concentrations. Specifically, 17b-trenbolone had no impact on the weight or length of female guppies at 4 ng/L (Tomkins et al., 2017), 8 ng/L (Tomkins et al., 2016) or 22 ng/L (Bertram et al., 2015), and had no influence on the morphology of female fathead minnows at 5 ng/L or 50 ng/L (Ankley et al., 2003). However, at 22 ng/L, 17b-trenbolone resulted in an increase in the weight and condition index of male guppies (Bertram et al., 2015), while at 4 ng/L, exposure resulted in an increase in male condition index, but not weight (M.G. Bertram et al., unpublished data). This suggests that male morphology is more sensitive to 17b-trenbolone-exposure than female morphology. However, more research is required to disentangle these dose-dependent and sex-specific effects.

In conclusion, this is the first study to show altered sequential female mate choice resulting from exposure to an endocrine disruptor. We found that, during a second male encounter, unexposed females altered the amount of time they spent associating with males depending on the orange colouration of a previously encountered male. Exposed females, on the other hand, associated with males indiscriminately during both the first and second male presentations. Further, exposed females spent less time associating with males overall than did unexposed females, indicating a decrease in mating interest. Considering that orange colouration is an honest indicator of male quality in guppies (Endler, 1980; Houde and Torio, 1992; Nicoletto, 1993; Pitcher and Evans, 2001; Locatello et al., 2006; Karino and Shinjo, 2007; Karino et al., 2007; Pitcher et al., 2007), the 17b-trenbolone-induced behavioural shifts observed in this study are expected to result in exposed females mating with inferior suitors. In nature, it is often more common for female guppies to encounter males sequentially, meaning the indirect costs associated with this breakdown in sexual selection processes could have population-level impacts by influencing the quality and quantity of offspring produced (reviewed in Candolin and Heuschele, 2008; Candolin and Wong, 2012; Wong and Candolin, 2015). Thus, this study highlights the need for a greater understanding of the potential impacts of EDCs on complex sexual selection processes, and how these changes may, in turn, influence population dynamics, ecosystem function, and broader evolutionary processes.

Ethics

This study was approved by the Biological Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2013/09) and is compliant with all relevant State and Federal laws of Australia.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.02.046.

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