Impacts of pharmaceutical pollution on fitness-related traits and behaviours in fish

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Abstract

Pollution is changing the world around us. Today, all organisms on Earth live in environments impacted by pollution from human activities. This endangers human health, causes wildlife declines and species extinctions, contributes to the degradation and destruction of ecosystems, and threatens planetary health by driving global climate change. What is more, the nature of chemical pollution is changing. This is because, while many established pollutants (e.g. asbestos, dichlorodiphenyltrichloroethane [DDT], lead) are relatively well understood and have seen comparative declines in prevalence, over 140,000 new chemicals have been synthesised since 1950 and many of these compounds, which are considerably less well understood, have become widely dispersed in the environment. Alarmingly, numerous such 'emerging' contaminants have been linked with episodes of environmental degradation, from neonicotinoid insecticide-induced collapses of honey bee colonies to widespread sex-reversal of fish exposed to synthetic estrogens found in the contraceptive pill. In this regard, one major class of emerging contaminants of increasing environmental concern is pharmaceuticals.

Pharmaceuticals are used across the globe in human and veterinary healthcare, as well as for growth promotion in livestock. The number of pharmaceutical doses dispensed per annum is predicted to reach 4.5 trillion by 2020, an increase of 24% from 2015 levels, with this trend being driven by a growing and ageing human population, as well as rapid growth in access to healthcare in emerging markets. Over the last 10–15 years, however, it has been recognised that this increased demand for pharmaceuticals has resulted in an escalation of the quantity and diversity of pharmaceuticals being discharged into the environment. This represents a major hazard because pharmaceuticals are typically designed to have biological effects at low doses, can be highly persistent in the environment, can act on drug targets and physiological pathways that are evolutionarily conserved across diverse taxa—making a broad range of species potentially vulnerable—and can bioconcentrate in organisms and bioaccumulate in food chains.

Despite the prevalence of pharmaceutical pollution in the environment, we still have only a rudimentary appreciation and understanding of how these contaminants might affect complex behavioural processes in wildlife. This is surprising for (at least) four main reasons. First, a wide range of pharmaceuticals are specifically designed to induce behavioural effects (e.g. antidepressants), and still more have known behavioural side-effects. Second, behaviour has been shown to be particularly vulnerable to disruption by exposure to chemical contaminants and is often affected at considerably lower concentrations than more conventional endpoints such as mortality, development, and reproduction. Third, behaviour is the link between an organism's physiological processes and its environment, and is, therefore, fundamental to the ecology of

individuals. Fourth, due to the fundamental role of behaviour in the ecology of individuals, the ability of animals to produce and maintain behaviour appropriate to their environment is intrinsically linked to individual- and population-level fitness, evolution of populations and species, and the ability of animals to respond to environmental change.

Accordingly, in my Ph.D. thesis, I use freshwater poeciliid fish models, the guppy (*Poecilia reticulata*) and the eastern mosquitofish (*Gambusia holbrooki*), to investigate impacts of pharmaceutical exposure on complex behavioural processes and fitness-related traits (e.g. sperm function, morphology) in wildlife. Given that two leading sources of pharmaceutical pollution worldwide are run-off of veterinary pharmaceuticals used in agriculture and inadequate removal of human pharmaceuticals during wastewater treatment processes, my thesis is organised into two distinct sections in which I consider pharmaceutical contaminants originating from each of these sources, in turn.

In section one, which comprises three data chapters, I investigated potential impacts of exposure to field-realistic concentrations of the growth-promoting veterinary pharmaceutical 17β-trenbolone—a potent steroid used in beef production around the world and a widespread endocrine-disrupting agricultural pollutant. First, I found that 17β-trenbolone alters male guppy reproductive behaviour and morphology at the lowest exposure level reported to date (i.e. 4 ng/L), although male preference for greater female size was maintained at this dosage (**Chapter 2**). Second, using mosquitofish as a model, I revealed for the first time that 17β-trenbolone exposure alters activity and exploration, sociability (i.e. shoaling tendency), and foraging behaviour in female fish (**Chapter 3**). Third, I documented how exposure to 17β-trenbolone results in context-specific changes in key fitness-related behaviours in male mosquitofish, disrupts important biological relationships between male morphology and sperm function, and alters male body condition (**Chapter 4**).

In section two, which encompasses one data chapter, I investigated possible consequences of exposure of fish to the widespread human pharmaceutical contaminant fluoxetine (Prozac™)—an antidepressant that is prescribed globally and is frequently detected in aquatic environments. Specifically, using male mosquitofish as a model, I provided the first evidence that fluoxetine exposure at environmentally realistic concentrations can alter mechanisms of both pre- and post-copulatory sexual selection in fish (**Chapter 5**).

Taken together, these studies reveal hitherto unknown sub-lethal effects of exposure to field-realistic levels of two widespread pharmaceutical contaminants. Moreover, these findings highlight the potential for pharmaceutical pollution to disrupt complex behavioural processes in wildlife, with likely ecological and evolutionary implications for exposed populations.

Table of Contents

Publications during enrolment

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Thesis including published works

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 thirteen original papers published in peer-reviewed journals (nine of which are attached in the Appendix) and one submitted manuscript (attached in the Appendix). The core theme of the thesis is investigating impacts of pharmaceutical pollution on fitness-related traits and behaviours in wildlife. The ideas, development and writing-up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the School of Biological Sciences, Monash University, under the supervision of Professor Bob Wong and Dr. Minna Saaristo.

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 chapters 2, 3, 4 and 5, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-authors' names and nature and % of co-authors' contributions	Co-author(s), Monash student Y/N
2	An androgenic endocrine disruptor alters male mating behavior in the guppy (*Poecilia reticulata*)	Published in *Behavioral Ecology*	95%: Conceived and designed the study, conducted the experiments, performed the statistical analysis, wrote and submitted the manuscript.	- Bob B.M. Wong (1.5%): Contributed to experimental design and manuscript preparation. - Minna Saaristo (1.5%): Contributed to experimental design and manuscript preparation. - Tiarne E. Ecker (1%): Assisted with data collection. - John B. Baumgartner (1%): Assisted with statistical analyses. - All authors contributed to manuscript revision and gave final approval for publication.	- BBMW: No - MS: No - TEE: Yes - JBB: No
3	Field-realistic exposure to the androgenic endocrine disruptor 17β-trenbolone alters ecologically important behaviours in female fish across multiple contexts	Published in *Environmental Pollution*	93%: Conceived and designed the study, conducted the experiments, performed the statistical analysis, wrote and submitted the manuscript.	- Bob B.M. Wong (1.5%): Contributed to experimental design and manuscript preparation. - Minna Saaristo (1.5%): Contributed to experimental design and manuscript preparation. - Jake M. Martin (1%): Assisted with data collection. - Tiarne E. Ecker (1%): Assisted with data collection. - Marcus Michelangeli (1%): Assisted with statistical analyses. - Christopher P. Johnstone (1%): Assisted with statistical analyses. - All authors contributed to manuscript revision and gave final approval for publication.	- BBMW: No - MS: No - JMM: Yes - TEE: Yes - MM: Yes - CPJ: No
4	Context-specific behavioural changes induced by exposure to an androgenic endocrine disruptor	Published in *Science of the Total Environment*	91%: Conceived and designed the study, conducted the experiments, performed the statistical analysis,	- Bob B.M. Wong (1.5%): Contributed to experimental design and manuscript preparation. - Jake M. Martin (1.5%): Contributed to experimental design, data collection, and manuscript preparation. - Minna Saaristo (1%): Contributed to experimental design and manuscript preparation.	- BBMW: No - JMM: Yes - MS: No - TEE: Yes - MM: Yes - NDSD: Yes

			wrote and submitted the manuscript.	- Tiarne E. Ecker (1%): Assisted with data collection. - Marcus Michelangeli (1%): Assisted with statistical analyses. - Nicholas D.S. Deal (1%): Assisted with statistical analyses. - Shu Ly Lim (1%): Assisted with data collection. - Moira K. O'Bryan (1%): Coordinated sperm analysis. - All authors contributed to manuscript revision and gave final approval for submission.	- SLL: No - MKOB: No
5	The antidepressant fluoxetine alters mechanisms of pre- and post-copulatory sexual selection in the eastern mosquitofish (*Gambusia holbrooki*)	Published in *Environmental Pollution*	80%: Jointly conceived and designed the study (with TEE), conducted the experiments, performed the statistical analysis, wrote and submitted the manuscript.	- Tiarne E. Ecker (15%): Jointly conceived and designed the study, contributed to data collection, statistical analysis, and drafting the manuscript. - Minna Saaristo (1%): Contributed to experimental design and manuscript preparation. - Bob B.M. Wong (1%): Contributed to experimental design and manuscript preparation. - Moira K. O'Bryan (1%): Coordinated sperm analysis. - John B. Baumgartner (1%): Assisted with statistical analyses. - Jake M. Martin (1%): Assisted with data collection. - All authors contributed to manuscript revision and gave final approval for publication.	- TEE: Yes - MS: No - BBMW: No - MKOB: No - JBB: No - JMM: Yes

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.

Student signature:  Date: 27/11/2018

Main supervisor signature:  Date: 27/11/2018

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Chapter 1

Introduction

INTRODUCTION

Pollution

Pollution is now the leading environmental driver of disease and premature death in humans (Landrigan et al. 2018). In 2015, diseases resulting from exposure to pollution caused more than 9 million premature deaths globally, equating to approximately one of every six premature deaths in that year (GBD 2016a,b). Impacts of pollution are, however, much broader than human health effects, alone. Pollution contributes to wildlife declines and species extinctions (Dulvy et al. 2003; Strayer and Dudgeon 2010), degrades and destroys ecosystems (Carpenter et al. 1998; Andrady 2011), and imperils planetary health by driving global climate change (Ramanathan and Feng 2018). Moreover, recent research has highlighted hitherto unknown and wide-ranging impacts of environmental pollution. For example, human-made objects (e.g. plastic debris) act as 'rafts' on which invasive species hitchhike across oceans (Carlton et al. 2017; Chown 2017), air pollution in megacities has been linked to a range of emerging health concerns, including impaired cognitive performance (Zhang et al. 2018) and irregular menstrual cycles (Mahalingaiah et al. 2018), and antibiotic pollution poses a major threat to natural microbial communities that play a key role in maintaining soil and water quality (Grenni et al. 2018).

Chemical pollution

Among the various forms of pollution (e.g. heat, light, noise), chemical pollution is, perhaps, the most complex and insidious. This is because more than 140,000 new chemicals have been synthesised since 1950, with over 5000 of these having become widespread in the environment (Landrigan et al. 2018). In fact, over the past four decades, increases in production and diversification of synthetic chemicals (e.g. fungicides, herbicides, nanomaterials, pesticides, pharmaceuticals) greatly outpaced all other well-recognised drivers of global change (e.g. biodiversity loss, habitat fragmentation, rising atmospheric CO_2) (Bernhardt et al. 2017). Indeed, the scale of this synthesis of novel chemicals has been recognised as being among the defining features of the Anthropocene epoch (Waters et al. 2016). Contamination of the environment with these chemicals is worrying because, even if impacts of many established chemical pollutants are relatively well understood (e.g. asbestos, copper, dichlorodiphenyltrichloroethane [DDT], lead, polychlorinated biphenyls [PCBs]), synthetic chemicals brought to market worldwide often undergo limited pre-market assessment for safety or toxicity (Landrigan et al. 2018). Furthermore, of these emerging contaminants, pharmaceuticals are of increasing concern to the scientific community given that these compounds are specifically designed to induce physiological and behavioural effects at low concentrations (UNEP 2013; Silva et al. 2015).

Pharmaceutical pollution

Pharmaceuticals are a large and diverse group of human and veterinary drugs used across the globe to diagnose, cure, treat, and prevent disease. The last two decades have seen a rapid expansion of the pharmaceutical industry, due to a growing and ageing human population, rising access to healthcare in emerging markets, and increasing use of veterinary pharmaceuticals in agriculture (MEA 2005; Arnold et al. 2014). Indeed, the number of pharmaceutical doses dispensed per annum is predicted to reach 4.5 trillion by 2020, an increase of 24% from 2015 levels (Aitken and Kleinrock 2015). This increased demand for pharmaceuticals has, consequently, resulted in an escalation of the quantity and diversity of pharmaceuticals being discharged into the environment (Khetan and Collins 2007). In fact, of the approximately 5000 pharmaceutical products currently available on the market, more than 600 have now been detected in the environment worldwide (Küster and Adler 2014), with these detections spanning 71 countries and covering all continents (Küster and Adler 2014; Aus der Beek et al. 2016).

Pharmaceuticals possess a suite of characteristics that make their prevalence in the environment particularly concerning. This includes the fact that pharmaceuticals are typically designed to have biological effects at low doses (Khetan and Collins 2007) and can act on physiological systems that are evolutionarily conserved across diverse taxa (Gunnarsson et al. 2008; Brown et al. 2014), various pharmaceuticals can be highly persistent in the environment (Redshaw et al. 2008; Monteiro and Boxall 2010), and some pharmaceuticals can bioconcentrate in organisms and bioaccumulate in food chains (Bringolf et al. 2010; Richmond et al. 2018). Accordingly, pharmaceutical exposures have been shown to have a range of adverse impacts on wildlife, with particularly well-studied examples being wildlife population crashes. For example, three species of *Gyps* vultures scavenging on livestock carcasses contaminated with the anti-inflammatory drug diclofenac in India and Pakistan suffered dramatic population declines of ≥97.4% between 1993 and 2002 (Oaks et al. 2004). Further, chronic whole-lake exposure of fathead minnows (*Pimephales promelas*) to field-realistic concentrations of the potent contraceptive estrogen 17α-ethinylestradiol (EE2) resulted in sex-reversal (i.e. feminisation) of males and consequent population collapse (Kidd et al. 2007).

Pharmaceuticals and wildlife behaviour

While large-scale mortality events and sex-reversal of wildlife represent a relatively obvious, if rare, sign of pharmaceutical contamination, recent work has shown that animal behaviour can be especially sensitive to disruption by pharmaceuticals at sub-lethal concentrations (Melvin and Wilson 2013; Arnold et al. 2014; Brodin et al. 2014; Saaristo et al. 2018). This is worrying because behaviour is the link between an organism's physiological processes and its environment

(Wong and Candolin 2015). In this regard, animals that forage inefficiently, are more likely to be detected and consumed by predators, and/or are unable to secure fertilisations, will accrue zero fitness. Behaviour is, therefore, vital to individual- and population-level fitness (Smith and Blumstein 2008; Candolin and Wong 2012), the functioning of ecosystems (Woodward 2009), and the evolution of species (Réale and Festa-Bianchet 2003), as well as being critically important in the ability of animals to adapt to environmental change (Candolin and Heuschele 2008; Candolin and Wong 2012; Wong and Candolin 2015). As a result, behaviour represents a sensitive and non-lethal biomarker of pharmaceutical pollutant exposure (Arnold et al. 2014; Brodin et al. 2014), with behavioural studies therefore being increasingly incorporated in assessing impacts of pharmaceuticals in the environment (reviewed in Clotfelter et al. 2004; Zala and Penn 2004; Melvin and Wilson 2013; Brodin et al. 2014; Saaristo et al. 2018). Despite this, relatively few studies to date have comprehensively evaluated impacts of pharmaceutical pollutant exposure at environmentally realistic levels on ecologically important behaviours.

Veterinary pharmaceuticals: 17β-trenbolone

While use of veterinary pharmaceuticals in agriculture is most often for the prevention and treatment of disease, currently, vast amounts of hormonal growth promotants (HGPs) are also administered to beef cattle worldwide to increase rate and extent of growth (APVMA 2003; Bartelt-Hunt 2012). These HGPs are typically administered as slow-release subcutaneous implants that contain a mixture of natural and/or synthetic hormones (Lange et al. 2001; Bartelt-Hunt 2012). Among the most commonly administered HGP implants globally is trenbolone acetate (Neumann 1976a,b; Stephany 2010; Kolodziej et al. 2013), an exceptionally efficient synthetic anabolic steroid with 15–50 times the androgenic and anabolic potency of testosterone (Neumann 1976b). While trenbolone acetate is widely administered in beef production worldwide (Hunter 2010; Johnson 2015), its use is prohibited in various regions. This includes the European Union, where trenbolone (and all HGPs) has been banned since the 1980s, owing to environmental, and human health, concerns (Johnson 2015). A prime example of the widespread use of trenbolone is seen in beef production in the United States of America. The U.S.A. is the world's largest beef producer and currently has approximately 30 million head of cattle, with 60–90% of these animals receiving trenbolone acetate implants (Schiffer et al. 2001; Ankley et al. 2003; Lawrence and Ibarburu 2007).

After being implanted, trenbolone acetate is rapidly broken down to various metabolites, the most biologically active of which is 17β-trenbolone (17β-TB), a powerful androgenic endocrine disruptor (Ankley et al. 2003). After excretion, effluent containing 17β-TB is often allowed to run-off into surrounding environments (reviewed in Ankley et al. 2018). Concentrations de-

tected in this run-off range from <1 to 270 ng/L (Schiffer et al. 2001; Soto et al. 2004; Durhan et al. 2006; Bartelt-Hunt et al. 2012; Khan and Lee 2012; Parker et al. 2012; Webster et al. 2012), with levels of <1 to 20 ng/L having been reported in neighbouring aquatic habitats (Soto et al. 2004; Durhan et al. 2006). In addition, 17β-TB is relatively persistent in effluent (half-life: ~260 days; Schiffer et al. 2001), and is rapidly taken up by (Schultz et al. 2013; Lagesson et al. 2019), and bioconcentrates in (Ankley et al. 2003; Lagesson et al. 2019), fish from diverse taxa. As a high-affinity ligand for the vertebrate androgen receptor (Neumann 1976; Wilson et al. 2002), 17β-TB affects androgen receptor signalling pathways in a wide variety of vertebrate species at relatively low environmentally realistic exposure concentrations. For example, short-term exposure causes changes in endocrine function (e.g. altered sex steroid metabolism, effects on gonadal stage, masculinisation of females), and longer exposures—including during development and reproduction—have been shown to produce a range of adverse apical effects (e.g. skewed sex ratios, impacts on fertility and fecundity) (reviewed in Ankley et al. 2018). What is more, over the last 5 years, it has been revealed that exposure to 17β-TB can disrupt key fitness-related behaviours in non-target species, from altered risk-taking in the presence of a predator (Heintz et al. 2015) to disrupted reproductive behaviours (Saaristo et al. 2013). Despite this, studies investigating impacts of field-realistic exposure to 17β-TB—and pharmaceutical pollutants more generally—on ecologically meaningful behaviours in wildlife are still in their infancy.

Human pharmaceuticals: fluoxetine

Fluoxetine (Prozac™), an antidepressant prescribed to treat mood disorders including depression and anxiety, is among the most commonly prescribed pharmaceuticals globally (Wong et al. 2005; Brijnath et al. 2017). Fluoxetine belongs to a large class of psychotherapeutics known as selective serotonin reuptake inhibitors (SSRIs), which are among the most frequently detected pharmaceutical contaminants in the environment (Silva et al. 2012). Selective serotonin reuptake inhibitors act by limiting reabsorption of the monoamine neurotransmitter serotonin (5-hydroxytryptamine) into the pre-synaptic neuron, therefore elevating levels of extracellular serotonin in the synaptic cleft, resulting in increased binding to post-synaptic neurons (Stahl 1998). After ingestion by human patients, fluoxetine is incompletely metabolised, with as much as 30% of the administered dosage being excreted (van Harten 1993). As a result of this incomplete metabolisation, and insufficient removal during wastewater treatment processes (e.g. Vasskog et al. 2006), fluoxetine frequently enters aquatic environments in wastewater effluent flows. Accordingly, fluoxetine has been detected in aquatic habitats worldwide at levels typically ranging from <1 to 100 ng/L in surface waters (e.g. Kolpin et al. 2002; Vanderford and

Snyder 2006; Schultz and Furlong 2008; Fernández et al. 2010; Schultz et al. 2010; Gardner et al. 2012; Hughes et al. 2013), to as high as 596 ng/L in heavily affected systems (Benotti and Brownawell 2007).

Having entered the environment, fluoxetine can bioaccumulate in tissues of wildlife (e.g. Brooks et al. 2005; David et al. 2018), especially in the brain (Brooks et al. 2005; Schultz et al. 2010). For instance, in an urban wetland receiving treated municipal waste waters and urban storm run-off, of 64 detected pharmaceuticals, fluoxetine was reported to have the highest bioaccumulation factor in wild fish (Muir et al. 2017). What is more, as an SSRI, fluoxetine's primary drug target, the serotonin receptor, is present in all phyla possessing nervous systems (Weiger 1997). Accordingly, fluoxetine has the potential to affect multiple fitness-related processes in highly diverse taxa (Kreke and Dietrich 2008; McDonald 2017). In fact, exposure to fluoxetine has been shown to produce a range of adverse impacts in non-target wildlife, including altered growth and development (Foran et al. 2004; Henry and Black 2008; Conners et al. 2009; Foster et al. 2010), disrupted reproduction (Nentwig 2007; Lister et al. 2009), and reduced survival (Pelli and Connaughton 2015). Further, because fluoxetine acts on the serotonergic system, which is known to play a key role in modulating behaviour (Fent et al. 2006; Kreke and Dietrich 2008; Prasad et al. 2015), recent research has investigated effects of exposure on behavioural processes in wildlife and revealed that a wide range of behaviours are vulnerable to disruption. For example, fluoxetine exposure increases foraging behaviour and activity in estuarine crabs (*Cancer productus*) irrespective of predation risk (Peters et al. 2017), supresses key anti-predator behaviours in eastern mosquitofish (*Gambusia holbrooki*; Martin et al. 2017), and has been linked with impaired learning and memory retention in juvenile cuttlefish (*Sepia officinalis*; Di Poi et al. 2013). Given that fluoxetine's therapeutic use is as an anti-anxiety drug in humans, research on this pharmaceutical to date has mainly focussed on effects on anxiety-related behaviours, such as predator avoidance. However, the serotonin system is involved in a wide variety of physiological processes, including reproduction (Prasad et al. 2015; Dorelle et al. 2017), and potential impacts of fluoxetine on these processes have received relatively little attention.

STUDY SYSTEMS

The guppy

The guppy (*Poecilia reticulata*) is a small, live-bearing freshwater fish indigenous to Trinidad and north-eastern South America (Magurran 2005). Guppies are sexually dimorphic, with males being brightly coloured, while females possess a cryptic beige colour and are relatively larger than

males (Houde 1997). Guppies are an excellent model species for investigating potential impacts of pharmaceutical contaminants on behavioural and reproductive processes in fish for (at least) four main reasons. First, the guppy is prolific and widespread, having successfully established in tropical waters of at least 69 countries outside of its native range, due to numerous accidental and deliberate introductions (Deacon et al. 2016). Second, due to their being a common model organism in behavioural and evolutionary biology, the life-history of guppies is exceptionally well understood (reviewed in Endler 1980, 1983; Houde 1997). Third, guppy mating systems and reproductive behaviour are readily quantifiable, with males either courting females for solicited copulations by performing elaborate 'sigmoid' mating displays or performing coercive 'sneaking' behaviour, which circumvents female choice (Houde 1997). Fourth, guppies are known to inhabit water bodies impacted by human activity, including habitats receiving pharmaceutical pollution (e.g. Phillip 1998; López-Rojas and Bonilla-Rivero 2000; Widianarko et al. 2000; Araújo et al. 2009).

The mosquitofish

Similarly, to the guppy, the eastern mosquitofish (*Gambusia holbrooki*) is a small, sexually dimorphic live-bearing poeciliid (Pyke 2005, 2008). Also like the guppy, mosquitofish are an excellent model for studying impacts of chemical pollution because of a widespread global distribution (Lowe et al. 2000), well-characterised behaviour and life-history (Pyke 2008), and flexibility of habitat use, including a tendency to inhabit systems impacted by human disturbance (Murphy et al. 2015; Lee et al. 2017). Moreover, the mating behaviour of mosquitofish is readily measureable. Unlike guppies, however, mosquitofish have a wholly coercive mating system (Bisazza et al. 2001). Males do not perform courtship behaviour to solicit copulations from female, and instead exclusively inseminate females by performing coercive sneak copulations (McPeek 1992; Bisazza and Marin 1995).

THESIS STRUCTURE

Using the guppy and the eastern mosquitofish as model species, my thesis explores impacts of emerging pharmaceutical pollutants on fitness-related traits and behaviours in fish. The main aims of my thesis were:

Aim 1 — To investigate consequences of exposure to environmentally realistic levels of the veterinary pharmaceutical and agricultural pollutant 17β-trenbolone on fitness-related behaviours and reproductive processes in fish.

Aim 2 — To examine impacts of exposure to field-realistic levels of the widespread human pharmaceutical pollutant fluoxetine on reproductive processes in fish.

These aims are addressed in two sections. In section one, I focussed on impacts of the veterinary pharmaceutical 17β-trenbolone on male mating preferences and reproductive behaviour (**Chapter 2**), female behaviour across multiple non-reproductive contexts (**Chapter 3**), and male behaviour across non-reproductive and reproductive contexts (**Chapter 4**). In section two, I addressed impacts of the human pharmaceutical pollutant fluoxetine on mechanisms of pre- and post-copulatory sexual selection in male fish (**Chapter 5**).

Taken together, the findings reported in my thesis demonstrate the capacity for widespread steroidal and anxiolytic pharmaceutical contaminants to disrupt a range of fitness-related traits and behaviours in fish, with likely ecological and evolutionary implications for exposed populations. Throughout my thesis, I place an emphasis on the application of environmentally realistic treatments, and consideration of ecologically important responses. Such an approach is important and timely because understanding impacts of emerging contaminants on ecological processes has been recognised as a critical knowledge gap (Bernhardt et al. 2017) that threatens humanity's ability to achieve many of the Sustainable Development Goals established by the United Nations to guide global development in the 21st century (UN 2015).

Alongside my own Ph.D. research, over the course of my candidature, I led or contributed to ten additional projects. Eight of these have been published as primary research articles in the journals *Hormones and Behavior*, *Environmental Pollution*, *Chemosphere*, *Science of the Total Environment,* and *Behavioral Ecology*. Further, one review article has been published in the journal *Proceedings of the Royal Society of London B: Biological Sciences*. In addition, one manuscript is currently under review at *Aquatic Invasions*. These projects share a common theme of investigating and addressing impacts of human-induced rapid environmental change on wildlife. These various published articles and manuscripts are attached to the **Appendix** of my thesis.

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SECTION ONE

Chapter 2

An androgenic endocrine disruptor alters male mating behavior in the guppy (*Poecilia reticulata*)

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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**
Conceived and designed the study, conducted the experiments, performed the statistical analysis, wrote and submitted the manuscript.	95%

The following co-authors contributed to the work:

Name	**Nature of contribution**	**Extent of contribution**
Bob B.M. Wong	Contributed to experimental design and manuscript preparation.	1.5%
Minna Saaristo	Contributed to experimental design and manuscript preparation.	1.5%
Tiarne E. Ecker	Assisted with data collection.	1%
John B. Baumgartner	Assisted with statistical analyses.	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:  Date: 27/11/2018

Main supervisor signature:  Date: 27/11/2018

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Original Article

An androgenic endocrine disruptor alters male mating behavior in the guppy (*Poecilia reticulata*)

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Hormonally active chemical pollution threatens human and wildlife populations globally. However, despite the well-established capacity of endocrine-disrupting chemicals (EDCs) to alter reproductive traits, relatively few studies have examined the impacts of EDCs on mechanisms of sexual selection. This study investigated the effects of short-term exposure to an environmentally realistic level of 17β-trenbolone—a potent anabolic steroid used in livestock production worldwide—on male mate preference, reproductive behavior, and morphology in the guppy (*Poecilia reticulata*). Male guppies prefer to mate with larger females because such females are generally more fecund. Hence, males gain direct fitness benefits by being choosy. Here, we found no significant effect of 17β-trenbolone exposure on male courting behavior, with both unexposed and exposed males courting larger females more often. However, exposure to 17β-trenbolone significantly altered the amount of coercive copulatory behavior ("sneak" matings) performed. Specifically, while both unexposed and exposed males demonstrated a preference for larger females by conducting more sneaking attempts toward these females, exposed males carried out a greater number of sneaks toward large females than did unexposed males. Further, exposure resulted in increased male condition index (i.e., mass relative to length). Together, our results show for the first time that 17β-trenbolone can alter reproductive behavior and morphology in male fish at concentrations as low as 4 ng/L, highlighting the potential for disruption of reproductive processes in wildlife exposed to this potent agricultural contaminant.

Key words: **agricultural pollution, endocrine disrupting chemical, pharmaceutical, reproductive behavior, sexual selection, trenbolone.**

INTRODUCTION

Human and wildlife populations worldwide are increasingly being exposed to chemicals capable of altering hormone signaling (WHO-UNEP 2012). Endocrine-disrupting chemicals (EDCs) disturb the natural homeostatic functioning of the endocrine system by interfering with the synthesis, secretion, transport, metabolism, binding, action, and/or elimination of natural hormones (Kavlock et al. 1996; Diamanti-Kandarakis et al. 2009). In this regard, agricultural activity is a leading source of endocrine-disrupting pollution (Horrigan et al. 2002; Yin et al. 2002; Diamanti-Kandarakis et al. 2009; WHO-UNEP 2012). Although many EDCs produce inadvertent effects in nontarget species as an unintended by-product of other functions (Wilson et al. 2002; Clotfelter et al. 2004), endocrine-disrupting pollution from agriculture is concerning given the use of high-potency synthetic chemicals known as hormonal growth promotants (HGPs), which have been specifically developed to act on the endocrine system. Currently, vast amounts of HGPs are administered in beef-producing nations worldwide, including the United States, Canada, Australia, New Zealand, Mexico, Chile, South Africa, and Japan (Hunter 2010; Johnson 2015). For example, approximately 20 million beef cattle per annum receive growth-promoting implants in the United States, representing around two-thirds of beef livestock in the country (Johnson 2015; USDA 2016). This widespread use of HGPs, however, excludes the European Union, where the use of growth hormones in domestic production and imported beef has been banned, dating back to the early 1980s (Johnson 2015).

Chemical compounds used in HGP implants commonly include androgens (e.g., trenbolone acetate), estrogens (e.g., 17β-estradiol, zeranol) and progestins (e.g., melengestrol acetate) (Lange et al. 2001).

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Trenbolone acetate—a powerful synthetic steroid with 15–50 times the androgenic and anabolic potency of testosterone (Neumann 1976)—is the androgen most commonly administered to beef cattle (Hunter 2010). After implantation, trenbolone acetate is rapidly hydrolyzed and excreted in the form of various metabolites, the most biologically potent of which is 17β-trenbolone (Khan et al. 2008; Parker et al. 2012). This excrement can then run off into freshwater systems, where 17β-trenbolone is highly persistent (half-life: ~260 days; Schiffer et al. 2001) and has repeatedly been detected at levels ranging from 1 to 20 ng/L in discharge and diffuse run-off (Durhan et al. 2006) to as high as 162 ng/L in tile-drained agroecosystems (Gall et al. 2011). Rapidly taken up by fish, 17β-trenbolone reaches a steady state at approximately 8 h in fathead minnows (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*) (Schultz et al. 2013). Further, 17β-trenbolone can bioconcentrate in fish—with a bioconcentration factor on the order of 13 reported in fathead minnows exposed for 21 days (Ankley et al. 2003)—and, therefore, potentially also represents a threat to wildlife at higher trophic levels that feed on fish. As a known androgenic endocrine disruptor, reported impacts of 17β-trenbolone exposure on nontarget species are wide-ranging (reviewed in Ankley et al. 2018), including altered gonad morphology (Orn et al. 2006), reduced fecundity (Ankley et al. 2003), decreased fertility (Mizukami-Murata et al. 2016), altered sexual differentiation (Olmstead et al. 2012), skewed sex ratios (Orn et al. 2006; Olmstead et al. 2012), and even complete and functional female-to-male sex reversal (Larsen and Baatrup 2010; Morthorst et al. 2010). Despite this, as is true for many EDCs, relatively little is known about the impacts of exposure to 17β-trenbolone on ecologically important behaviors, including those under sexual selection.

Sexual selection, by influencing reproductive success, is fundamental to individual fitness, population and species viability, as well as broader evolutionary processes (Candolin and Heuschele 2008; Wong and Candolin 2015). Given that sex steroid hormones modulate the expression of a wide range of behaviors under sexual selection (Beyer et al. 1976; Munakata and Kobayashi 2010), these processes are likely to be vulnerable to disruption by hormone-like contaminants. Although endocrine disruptor exposure has been linked to breakdowns in sexual selection processes as a consequence of altered female mate choice (e.g., Saaristo et al. 2009a; Secondi et al. 2009; Partridge et al. 2010; Tomkins et al. 2016, 2018), comparatively little is currently known about the potential for these contaminants to influence male mating preferences and reproductive behavior (but see Saaristo et al. 2009b; Jayasena et al. 2011; Bertram et al. 2015). This is surprising because male mate choice, regarding not only the decision to mate but also the allocation of reproductive investment to each mate or mating, can afford both direct (i.e., material) and indirect (i.e., genetic) benefits to choosy males (Kokko et al. 2003; Edward and Chapman 2011).

The guppy (*Poecilia reticulata*) is a small live-bearing freshwater fish endemic to north-eastern South America that is now established in at least 69 countries outside of its native range due to numerous accidental and deliberate introductions (Deacon et al. 2011). This species' life history and mating behaviors have been exceptionally well studied (Houde 1997; Magurran 2005), which, in combination with a propensity to inhabit freshwater systems receiving agricultural waste (e.g., Phillip 1998; López-Rojas and Bonilla-Rivero 2000; Widianarko et al. 2000; Araújo et al. 2009), means that guppies are an ideal model to study the potential behavioral impacts of endocrine disruptor exposure. Although the guppy mating system is predominantly driven by female choice, with females responding to elaborate male courtship displays and avoiding coercive "sneak" mating attempts (Houde 1997), male guppies can also be choosy and are known to prefer larger females as mates (Dosen and Montgomerie 2004; Herdman et al. 2004). Because female guppy fecundity (brood size) increases with body size (Herdman et al. 2004), males gain direct fitness benefits by mating with larger females. Wild guppies live in mixed-sex shoals, where males routinely encounter multiple females concurrently (Houde 1997), and females vary greatly in body size and fecundity (Reznick and Endler 1982; Kelly 1999), making the fitness benefits of male choosiness particularly pronounced.

Here, we examine the impact of short-term (21-day) exposure to an environmentally realistic concentration (average measured concentration: 4 ng/L) of 17β-trenbolone on male guppy preference for female size, as well as male reproductive behavior (i.e., performance of courtship behavior vs. unsolicited sneaking behavior). In addition to expecting altered behavior, we hypothesized that 17β-trenbolone's potent growth-promoting activity would lead to increased male mass.

MATERIALS AND METHODS

Ethics statement

Methods for animal housing and experimental protocols were approved by the Biological Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2013/09) and observed all relevant State and Federal laws of Australia.

Animal housing

Guppies used in this experiment were laboratory-reared descendants of wild fish collected from Alligator Creek (19°26′79″S, 146°58′65″E), a pristine rainforest-fed stream located in Bowling Green Bay National Park, Queensland, Australia (Queensland permit: WITK07655010). Water samples drawn from this site over consecutive years indicate no contamination from EDCs (ALS Group, unpublished data). Sexually mature male and female guppies were acclimated to laboratory conditions for 2 months in large mixed-sex holding tanks (81 L; 60 cm × 45 cm × 30 cm; 25 to 27 °C; 12:12 h light:dark regime), and were presumed to be nonvirginal considering the incessant mating pressure applied by males toward females (Magurran and Seghers 1994; Houde 1997), with nonvirginal fish being used to simulate mixed-sex wild populations. Fish were fed *ad libitum* once per day (Otohime Hirame larval diet; 580–910 µm).

Exposure set-up

A flow-through system was used to expose male fish to 17β-trenbolone, following previously established methods (Saaristo et al. 2013; Bertram et al. 2015; Tomkins et al. 2016, 2017, 2018), for a period of 21 days. Males were allocated to identical exposure tanks (54 L; 60 cm × 30 cm × 30 cm), which were monitored daily for temperature (mean ± SD = 25.83 ± 0.44 °C) and flow-through rates (mean ± SD = 18.43 ± 0.37 mL/min) maintained using flow meters (BES, MPB Series 1200). Males were randomly allocated to 1 of 4 17β-trenbolone-exposure tanks, or 1 of 4 identical unexposed tanks containing fresh water only (22 fish per tank). Survivorship over the exposure period was 92.0% for unexposed fish and 94.3% for exposed fish (7 and 5 deaths, respectively). This is in line with expected natural mortality rates for adult (i.e., ≥7 weeks; Magurran 2005) male *P. reticulata*, given that males of this

species have an average lifespan of ~24 months (Reznick et al. 2006).

Exposure dosing and GC-MS/MS analysis

The 17β-trenbolone concentration used (mean ± SD = 4.48 ± 1.53 ng/L, n = 16) was attained 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 (400 mg/L). This stock standard was diluted to 4 μg/L with deionized water, and further diluted within the flow-through system, to yield the desired exposure concentration. The exposure level of 4 ng/L was chosen as this concentration falls within the range (1–7 ng/L) of 17β-trenbolone concentrations detected in river water (Durhan et al. 2006).

Levels of 17β-trenbolone were monitored weekly—in exposed tanks, as well as in unexposed tanks (to ensure the absence of contamination)—using gas chromatography−tandem mass spectrometry (7000C Triple Quadrupole GC-MS/MS, Agilent Technologies, Delaware). The analysis was performed by Envirolab Services (MPL Laboratories, Perth; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025). No significant concentration differences were detected across 17β-trenbolone exposure tanks within the flow-through system (ANOVA: $F_{3,12}$ = 0.074, P = 0.973). For additional details on the collection and analysis of water samples, see Tomkins et al. (2018).

Behavior trials

To examine the impact of exposure to 17β-trenbolone on male guppy mate preference and reproductive behavior, fish were tested in 4 treatments: 1) unexposed male paired with large female (hereafter UL; n = 19), 2) unexposed male paired with small female (US; n = 19), 3) exposed male paired with large female (EL; n = 25), and 4) exposed male paired with small female (ES; n = 24). A pair of 1 male and 1 female fish were tested to disentangle impacts of 17β-trenbolone exposure on male mating preferences and reproductive behavior (if any) from potentially interacting factors, such as male–male competition (Jirotkul 1999) and/or audience effects (Makowicz et al. 2010). Further, stimulus females were unexposed, to ensure that potential 17β-trenbolone-induced changes in these stimulus fish did not influence the behavior of males, a technique employed in previous ecotoxicological research (e.g., Tomkins et al. 2017, 2018; Bertram et al. 2018a). Stimulus females comprised large (standard length minimum and maximum cutoffs of 19.00 and 20.00 mm, respectively; mean ± SD = 19.58 ± 0.34 mm, range: 19.05–19.98 mm) and small (standard length minimum and maximum cutoffs of 15.00 and 16.00 mm, respectively; mean ± SD = 15.45 ± 0.33 mm, range: 15.02–15.99 mm) sexually mature fish. Across these size classes, large and small stimulus females differed significantly in standard length (Mann–Whitney U = 1892, P < 0.001).

Behavioral trials involved males being drawn randomly from flow-through exposure tanks and allocated to 1 of 12 observation tanks (54 L; 60 cm × 30 cm × 30 cm) filled with aged carbon-filtered fresh water (mean temperature ± SD = 25.89 ± 0.56 °C) to 20 cm depth, with stimulus females being drawn randomly from 1 of 2 holding aquaria (54 L; 60 cm × 30 cm × 30 cm). Free-swimming behavioral trials (n = 87) were preceded by a 5-min period of acclimation, after which the male and female were released from their respective holding containers and allowed to interact, with their behavior being video-recorded (Canon PowerShot S120). Behavior was recorded for 15 min following Herdman et al. (2004), where the same trial duration was used to demonstrate a preference in male guppies for greater female size. Observation tanks were drained and refilled with aged water upon completion of each trial.

The event-recording software JWatcher V1.0 (Blumstein and Daniel 2007) was used to quantify male reproductive behaviors (as described in Houde 1997) from video recordings. Briefly, the number of courtship bouts performed by males was recorded, involving the male orienting toward the female and performing sigmoid displays (courtship) before moving behind the female for an attempted copulation. The frequency of male sneaking behavior, involving the male surreptitiously approaching a nonreceptive female from behind for a forced copulation attempt, was also quantified. Lastly, male following behavior was recorded as the number of times a male actively pursued a female (within 5 cm).

Experimenters were blind to exposure treatment both during data collection and scoring of video-footage, with all footage being scored by one observer to ensure consistency.

Morphological analysis

Immediately after each trial, fish were euthanized with anesthetic clove oil (40 mg/L). Male guppies were dabbed dry and weighed (±0.0001 g), as well as being measured for standard length (±0.01 mm). An index of male condition was calculated, reflecting the mass of a male relative to that expected for its standard length. Specifically, this male condition index was quantified as the residuals from a linear regression of male mass (g) on standard length (mm) (i.e., weight = −0.211 + 0.019 × length). These measures were also recorded for stimulus females after behavioral trials.

Coloration analysis

Female guppies prefer to mate with males bearing greater orange coloration (i.e., area and chroma; Houde 1997), as has been demonstrated in the source population of fish used in the present study (Brooks and Endler 2001; Gamble et al. 2003). A positive relationship also exists in the laboratory-reared descendants of this population between the area of male orange pigmentation and the number of courting bouts performed toward a female (Bertram et al. 2015). To account for these known relationships in statistical analysis, we quantified the percentage of each male's body area containing orange pigment immediately subsequent to behavioral trials. Briefly, this involved males being photographed on their right side in a standardized fashion (Nikon D90, shutter speed = 1/250, Nikon AF Micro-Nikkor 60 mm f/2.8D), before Photoshop's (CS5 Version 12.0 Extended) Color Range tool was used to sample orange pigmentation from 8 randomly selected reference fish. This orange pigmentation color standard was then used to calculate the area of each male's body surface containing orange pixels (i.e., pixels with colors belonging to the orange pigmentation color standard) as a proportion of the total body area (i.e., the number of pixels forming the body surface). For further details on photographic coloration analysis, see Bertram et al. (2015).

Statistical analysis

Data were analyzed in R version 3.2.3 (R Core Team 2015). Tests of normality (Shapiro–Wilk test; Royston 1995) and homogeneity of variance (Fligner–Killeen test; Conover et al. 1981) were performed, where appropriate. Poisson generalized linear models (GLMs) were used to investigate relationships between counts of recorded behaviors. Vuong tests (*vuong* function, *pscl*

Table 1
Summary of statistical models

Behavioral response	Model	Predictor variables
Number of male courting events	Zero-inflated negative binomial GLM	Treatment Group-means-corrected female standard length Male area of orange pigmentation (%)
Number of male sneaking events	Zero-inflated negative binomial GLM	Treatment Group-means-corrected female standard length Male condition index Male area of orange pigmentation (%)
Number of male following events	Zero-inflated negative binomial GLM	Treatment Group-means-corrected female standard length Male condition index Male area of orange pigmentation (%)

package; Vuong 1989; Jackman 2012) indicated zero-inflation of each behavioral response, with 65.5% of males performing courtship (unexposed = 63.2%, exposed = 67.3%), 35.6% conducting sneak attempts (unexposed = 36.8%, exposed = 34.7%), and 94.3% carrying out following behavior (unexposed = 94.7%, exposed = 93.9%). This zero-inflation was addressed by fitting zero-inflated Poisson (ZIP) GLMs (*zeroinfl* function, *pscl* package; Zeileis et al. 2008). To test for overdispersion of each behavioral response, zero-inflated negative binomial (ZINB) GLMs (*zeroinfl* function) were also fitted and compared with their respective ZIP GLM alternatives using likelihood-ratio tests (*lrtest* function, *lmtest* package; Zeileis and Hothorn 2002). In each case, ZINB GLMs were favored, due to overdispersion of the response variable (Zuur et al. 2009). For all models, predictors were selected based on their biological meaning (Table 1). To represent female standard length within each size class, we calculated group-means-corrected female standard length by subtracting the female groups' means (i.e., the mean of small females, and that of large females) from the lengths of females belonging to the respective groups, and dividing by the groups' standard deviations (SDs). Further, all continuous predictors were centered and standardized to have zero mean and unit variance. General linear hypothesis tests (*glht* function, *multcomp* package; Hothorn et al. 2008) were used for post hoc assessment of differences in the mean response across treatment levels. Assessment of whether coefficients of continuous predictors were significantly different from zero (at $\alpha = 0.05$) was performed using partial Wald tests. Mann–Whitney *U* tests (Mann and Whitney 1947) were used to evaluate whether exposure to 17β-trenbolone altered male condition index, weight, and/or standard length.

RESULTS

Mating behavior

The number of courting bouts performed by males varied significantly with treatment. Specifically, both unexposed and exposed males performed significantly more courting events when paired with large females than with small females (partial Wald test: unexposed males: $z = 2.54$, $P = 0.011$; exposed males: $z = 2.42$, $P = 0.016$; Figure 1a). However, no difference was detected between the number of courting events performed by unexposed versus exposed males towards large (partial Wald test: $z = 0.24$, $P = 0.810$) or small females (partial Wald test: $z = 0.56$, $P = 0.577$) (Figure 1a). The number of courting events performed by males was not significantly impacted by group-means-corrected female standard length (partial Wald test: $z = -1.00$, $P = 0.318$), nor by male area of orange pigmentation (partial Wald test: $z = 1.58$, $P = 0.113$).

The frequency of male sneaking was associated with treatment, group-means-corrected female standard length and male condition index. Firstly, regardless of exposure treatment, males performed more frequent sneaking behavior toward larger females than smaller females (partial Wald test: unexposed males: $z = 2.35$, $P = 0.019$; exposed males: $z = 2.63$, $P = 0.008$; Figure 1b). However, when paired with large females, exposed males carried out significantly more sneaking attempts than did unexposed males (partial Wald test: $z = 2.62$, $P = 0.009$; Figure 1b). Specifically, when paired with large females, exposed males were expected to perform 3.51 [2.18, 5.68] (where values in brackets indicate the mean minus 1 standard error (SE), and the mean plus 1 SE, respectively) times as many copulations as unexposed males. Further, when paired with small females, there was a tendency for 17β-trenbolone-exposed males to perform more frequent sneaking behavior relative to controls, although this result was marginally nonsignificant (partial Wald test: $z = 1.77$, $P = 0.077$; Figure 1b). More generally, there were significant negative relationships between the amount of sneaking behavior performed by males and both group-means-corrected female standard length (partial Wald test: $z = -4.15$, $P < 0.001$) and male condition index (partial Wald test: $z = -3.29$, $P = 0.001$). A 1 SD decrease in female standard length (i.e., 0.33 and 0.34 mm for small and large females, respectively) resulted in 2.59 [2.06, 3.27] times as many male sneaking events. A decrease in male condition index of 1 SD (i.e., 0.012) was predicted to increase the frequency of sneaking behavior by 1.87 [1.55, 2.26] times (Figure 2). Further, a marginally nonsignificant positive trend was observed between the number of sneaking attempts performed and male area of orange pigmentation (partial Wald test: $z = 1.78$, $P = 0.074$).

The number of events of following behavior performed by males did not differ significantly between treatment groups [$\chi^2(3) = 3.33$, $P = 0.342$; Figure 1c]. Further, the number of following events performed by males was not significantly affected by group-means-corrected female standard length (partial Wald test: $z = -1.06$, $P = 0.290$), male condition index (partial Wald test: $z = -0.80$, $P = 0.421$) or male area of orange pigmentation (partial Wald test: $z = 0.92$, $P = 0.356$).

Morphology

Exposure did not affect male weight (Mann–Whitney *U* test: $U = 890$, $P = 0.729$) or standard length (Mann–Whitney *U* test: $U = 1009.5$, $P = 0.504$). However, males exposed to 17β-trenbolone

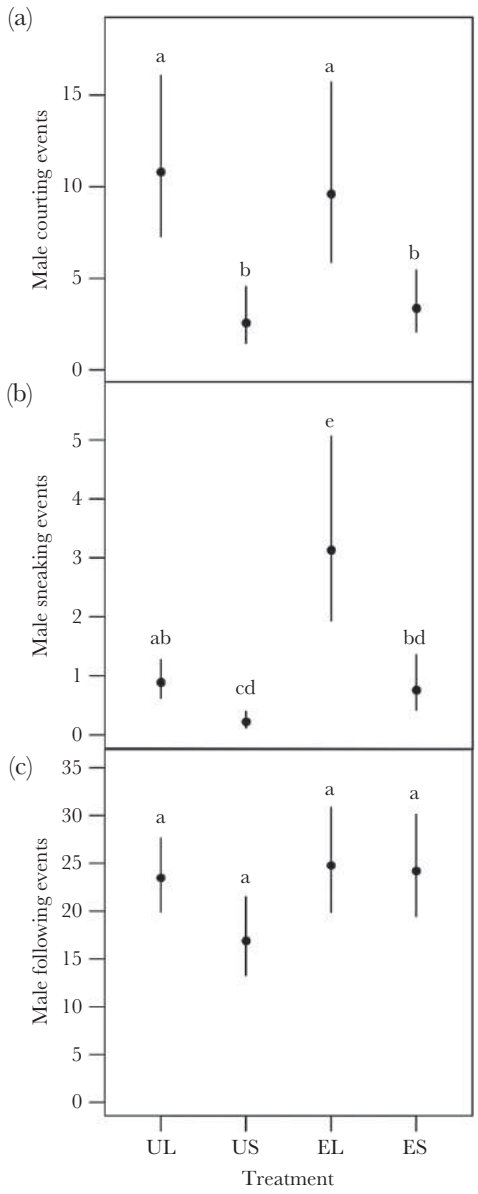

Figure 1
Mean (±SE) number of (a) courting events, (b) sneaking events, and (c) following events performed by males across treatments (UL: $n = 19$; US: $n = 19$; EL: $n = 25$; ES: $n = 24$) when continuous predictors were held at their means. Treatments indicate unexposed (U) or 17β-trenbolone-exposed (E) males, paired with large (L) or small (S) stimulus females. Treatments that do not share lower case letters are significantly different.

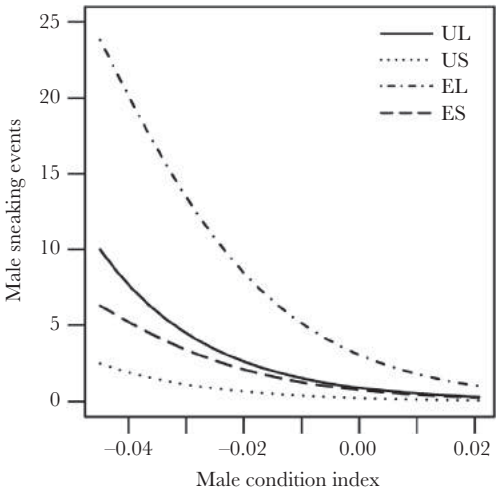

Figure 2
Expected number of male sneaking events given male condition index and treatment (UL: $n = 19$; US: $n = 19$; EL: $n = 25$; ES: $n = 24$), holding group-means-corrected female standard length and male orange pigmentation (% of body area) at their means. Treatments indicate unexposed (U) and exposed (E) males paired with large (L) or small (S) stimulus females.

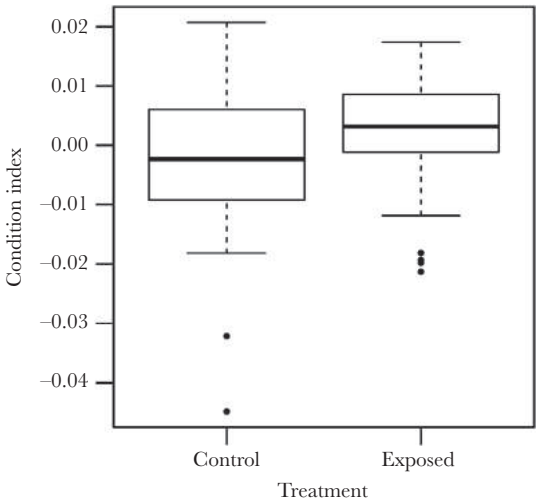

Figure 3
Condition index of unexposed males ($n = 38$) and those exposed to 17β-trenbolone ($n = 49$).

had a significantly higher average condition index than unexposed males (Mann–Whitney U test: $U = 677$, $P = 0.030$; Figure 3).

DISCUSSION

We have demonstrated altered male reproductive behavior and morphology resulting from exposure to an environmentally realistic concentration of the androgenic endocrine disruptor 17β-trenbolone, although male preference for greater female size was maintained at the dosage tested. To date, this is the lowest reported concentration (4 ng/L) of 17β-trenbolone shown to influence reproductive behavior and morphology in male fish. Although the number of courting bouts carried out by males was not impacted by 17β-trenbolone, with both unexposed and exposed males demonstrating a preference for greater female size by courting larger females more often, exposure did significantly affect the amount of coercive copulatory behavior (sneak matings) performed. Specifically, despite both unexposed and exposed males again exhibiting mate preference by performing more frequent sneaking behavior toward larger females, this effect was stronger in exposed males, which performed significantly more sneaking events toward large females than did unexposed males. Males exposed to 17β-trenbolone also showed a significant increase in condition index (i.e., mass relative to length). More generally, male condition index associated negatively with the number of sneaking events performed. This was expected, given that female guppy preference for high-quality males is likely to make courtship behavior more

profitable for those males, while coercive sneaking behavior may be an effective alternative for lower-quality males (Houde 1997).

Hormones control the production and maintenance of sexual behavior in fish (Borg 1994; Munakata and Kobayashi 2010), as in other vertebrates (Rubinow and Schmidt 1996; Cunningham et al. 2012). In most fish species, male sexual behaviors are mediated by testicular androgens (Borg 1994; Munakata and Kobayashi 2010), which bind to androgen receptors (ARs), of which 2 isoforms have been characterized in teleost fish (ARα and ARβ; Harbott et al. 2007). Exogenous androgens such as 17β-trenbolone—which is a high-affinity ligand for the fish AR (Ankley et al. 2003)—can also activate the AR, and can mimic the effect of endogenous androgens (Wilson et al. 2002; Larsen and Baatrup 2010). Further, it has been hypothesized that 17β-trenbolone, which is nonaromatisable (Rogozkin 1991), may also indirectly reduce endogenous 17β-estradiol production by decreasing the production of endogenous androgens (including testosterone), thereby limiting the aromatization of testosterone into 17β-estradiol (Zhang et al. 2008). Given that a vital role of androgens is the development and regulation of male sexual behaviors (Zuloaga et al. 2008; Cunningham et al. 2012), exposure to exogenous androgens may alter these behaviors. Alteration of androgen-dependent reproductive behaviors resulting from exposure to exogenous androgen agonists has, for example, been reported in American kestrels (*Falco sparverius*) exposed to a brominated flame retardant (Marteinson et al. 2015), as well as in methyldihydrotestosterone-exposed African clawed frogs (*Xenopus laevis*) (Hoffmann and Kloas 2012) and cyprinid fish species (Belanger et al. 2010).

Consistent with previous studies on guppies, exposure of males to 17β-trenbolone did not significantly impact the number of courting events performed (4 ng/L, Tomkins et al. 2016; 22 ng/L, Bertram et al. 2015). Instead, both unexposed and exposed males demonstrated a preference for greater female size by courting large females more frequently, probably because fecundity increases with female size (Houde 1997; Herdman et al. 2004). Interestingly, recent research has demonstrated a reduced frequency of courting behavior in male guppies after exposure to 17β-trenbolone at 8 ng/L, when under male–male competition (i.e., with rival males being allowed to freely interact and compete over an unexposed female) (Tomkins et al. 2017). This indicates that 17β-trenbolone-induced reductions in courting behavior in adult guppy males may be context dependent, manifesting only in a competitive setting. This is suggested to result from exposed males exhibiting significantly increased levels of aggression (i.e., chases and fin-nips) toward rival males, thereby limiting the amount of time available for these males to court females (Tomkins et al. 2017).

We show that exposure to 17β-trenbolone at concentrations as low as 4 ng/L can influence the amount of coercive copulatory behavior (sneak mating events) carried out by male guppies toward females. Although unexposed and exposed males both engaged in more frequent sneaking behavior toward larger females, again suggesting that male preference for female size was not impacted by 17β-trenbolone at this exposure concentration, males exposed to 17β-trenbolone performed more sneaking behavior toward large females than did unexposed males. More broadly—that is, independent of male preference for female size—a shift in male reproductive strategy towards coercive mating (sneak behavior) is consistent with previous work on guppies (Bertram et al. 2015; Tomkins et al. 2017) but contrasts with findings reported in another poeciliid, the eastern mosquitofish (*Gambusia holbrooki*), where exposure to 17β-trenbolone at 6 ng/L did not impact the number of gonopodial thrusts performed by males toward females (Saaristo et al. 2013). It is important to point out, however, that Saaristo et al. (2013) paired males and females from the same treatment group (i.e., unexposed or exposed), meaning that their result may have been influenced by the exposure status of the female. Moreover, mosquitofish have a coercive mating system in which males do not court females but, instead, engage exclusively in sneak copulations. Taken together, these results in guppies suggest that, in species that employ both courtship and coercive mating behavior, exposure to 17β-trenbolone can shift relative investment in these key strategies.

The presently observed intensification of sneaking behavior in exposed males has implications for their reproductive fitness. Compared with copulations preceded by courtship, sneak copulations have a lower probability of insemination success (Matthews and Magurran 2000; Russell et al. 2006) and deliver approximately one-third as many sperm into the female's gonoduct (Pilastro and Bisazza 1999). Further, cryptic female choice during or after copulation—via, for example, sperm dumping (Cheng 2004) or biased sperm use (Eberhard 1994; Pizzari and Birkhead 2000)—may disadvantage sneaking males. To our knowledge, cryptic female choice for courting males over sneaking males has not been tested directly in guppies, yet female guppies do show cryptic preference for more colorful males (Pilastro et al. 2004), and cryptic preference for courting males has been documented in various other species (e.g., Edvardsson and Arnqvist 2000; Pizzari and Birkhead 2000). Further, male sneaking is costly to females as it circumvents female mate choice (Pilastro and Bisazza 1999), can physically damage the female's genital pore (Constantz et al. 1989) and may impart costs associated with unnecessary multiple mating, including an increased risk of disease transmission, increased predation risk and reduced foraging efficiency (Bisazza et al. 2001).

A marginally nonsignificant positive association was detected between 17β-trenbolone exposure and frequency of sneaking events performed by males towards small females. In fact, the rate of sneaking by exposed males paired with small females was similar to—that is, not significantly different from—that of unexposed males paired with large females. This suggests that exposure to 17β-trenbolone may be causing males to become somewhat more likely to direct sneak copulations towards small females, despite theory suggesting that male reproductive fitness should be maximized by mating with larger, more fecund females (Houde 1997; Herdman et al. 2004). Ejaculate production in guppies is rate limited (Pilastro and Bisazza 1999) and costly (Wedell et al. 2002), meaning that an increase in ejaculate expenditure toward smaller and less fecund females may have negative implications for male fitness.

Exposure to 17β-trenbolone was associated with a significant increase in male condition index. This was unsurprising given 17β-trenbolone's strong anabolic activity (Neumann 1976) and is consistent with previous research reporting increased condition index in male guppies exposed for the same period to 17β-trenbolone at 22 ng/L (Bertram et al. 2015). The increase in condition index observed in the current study was subtle, however, having not been sufficiently explained by either changed body length or weight alone—with neither of these traits being significantly altered by exposure. Therefore, the detected increase in relative mass was a consequence of exposed males having, on average, slightly increased weights as well as slightly smaller standard lengths. The slight difference in standard length between treatment groups is likely due to natural variation since morphogenesis of skeletal elements is complete in adults and is not, therefore, expected to be vulnerable to chemical exposure (Pandey 1969; Baatrup and Junge 2001). That

exposure did not significantly impact male weight is likely a result of the exposure concentration used (4 ng/L), because studies that have measured weight increases have reported significant differences after exposure at 22 ng/L (Bertram et al. 2015) but not at lower concentrations (4 ng/L, Tomkins et al. 2016; 8 ng/L, Tomkins et al. 2017). Weight gain in guppies has also been reported after exposure at much higher concentrations, with juvenile guppies exhibiting an increased rate of growth after a 60-day dietary exposure to trenbolone acetate—the parent compound of 17β-trenbolone—at a dose of 300 mg/kg in feed (Zamora et al. 2008). Further, exposure of fathead minnows to 17β-trenbolone has been associated with a concentration-dependent increase in female weight (at 0.5, 5, and 50 μg/L), although no significant increase in female body weight was seen at lower concentrations (5 and 50 ng/L) and no effect of exposure was seen on the average weight of males (Ankley et al. 2003). Given these results, including the sex- and species-specific effects reported, additional research is needed to determine the susceptibility of fish to morphological alteration via exposure to 17β-trenbolone at environmentally realistic levels.

CONCLUSION

We report that short-term (21-day) exposure to the pervasive endocrine disruptor 17β-trenbolone at an environmentally realistic level (4 ng/L) altered male reproductive behavior and morphology in the guppy, although male preference for female size was maintained at this concentration. Given that the ability to appropriately perform reproductive behaviors is fundamentally important to the ecology and evolution of wildlife, the presence of hormonally active chemical pollutants in the environment that are capable of disrupting these behaviors is a major concern. More research is clearly needed to reveal the extent to which these contaminants may interfere with behavioral processes, including mating dynamics, in exposed populations (reviewed in Saaristo et al. 2018). In this regard, although our work demonstrates altered mating strategy in male fish when presented with a stimulus (i.e., unexposed) female in a one-on-one context, there is a need to examine potential impacts of 17β-trenbolone—as well as other emerging contaminants—on increasingly complex behavioral interactions, to more closely approximate natural systems. For example, important will be to investigate potential contaminant impacts on complex mating interactions in mixed-sex shoals, including with all shoal members being similarly exposed (i.e., either unexposed or exposed). Moreover, partnering this work with interacting natural stressors—e.g., predation pressure, which could itself be impacted by contamination—will be important in further extending these findings to wild fish populations. Therefore, as behavioral analyses are increasingly being integrated into environmental toxicology research, we emphasize the need to incorporate existing knowledge in behavioral ecology to uncover hitherto unknown impacts of chemical pollution on wildlife.

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Data accessibility: Analyses reported in this article can be reproduced using the data provided by Bertram et al. (2018b).

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Chapter 3

Field-realistic exposure to the androgenic endocrine disruptor 17β-trenbolone alters ecologically important behaviours in female fish across multiple contexts

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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**
Conceived and designed the study, conducted the experiments, performed the statistical analysis, wrote and submitted the manuscript.	93%

The following co-authors contributed to the work:

Name	**Nature of contribution**	**Extent of contribution**
Bob B.M. Wong	Contributed to experimental design and manuscript preparation.	1.5%
Minna Saaristo	Contributed to experimental design and manuscript preparation.	1.5%
Jake M. Martin	Assisted with data collection.	1%
Tiarne E. Ecker	Assisted with data collection.	1%
Marcus Michelangeli	Assisted with statistical analyses.	1%
Christopher P. Johnstone	Assisted with statistical analyses.	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:  Date: 27/11/2018

Main supervisor signature:  Date: 27/11/2018

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ABSTRACT

The capacity of pharmaceutical pollution to alter behaviour in wildlife is of increasing environmental concern. A major pathway of these pollutants into the environment is the treatment of livestock with hormonal growth promotants (HGPs), which are highly potent veterinary pharmaceuticals that enter aquatic ecosystems via effluent runoff. Hormonal growth promotants are designed to exert biological effects at low doses, can act on physiological pathways that are evolutionarily conserved across taxa, and have been detected in ecosystems worldwide. However, despite being shown to alter key fitness-related processes (e.g., development, reproduction) in various non-target species, relatively little is known about the potential for HGPs to alter ecologically important behaviours, especially across multiple contexts. Here, we investigated the effects of exposure to a field-realistic level of the androgenic HGP metabolite 17β-trenbolone—an endocrine-disrupting chemical that has repeatedly been detected in freshwater systems—on a suite of ecologically important behaviours in wild-caught female eastern mosquitofish (*Gambusia holbrooki*). First, we found that 17β-trenbolone-exposed fish were more active and exploratory in a novel environment (i.e., maze arena), while boldness (i.e., refuge use) was not significantly affected. Second, when tested for sociability, exposed fish spent less time in close proximity to a shoal of stimulus (i.e., unexposed) conspecific females and were, again, found to be more active. Third, when assayed for foraging behaviour, exposed fish were faster to reach a foraging zone containing prey items (chironomid larvae), quicker to commence feeding, spent more time foraging, and consumed a greater number of prey items, although the effect of exposure on certain foraging behaviours was dependent on fish size. Taken together, these findings highlight the potential for exposure to sub-lethal levels of veterinary pharmaceuticals to alter sensitive behavioural processes in wildlife across multiple contexts, with potential ecological and evolutionary implications for exposed populations.

1. Introduction

The ability of animals to produce and maintain behaviour appropriate to their environment is fundamental to individual- and population-level fitness (Smith and Blumstein, 2008; Candolin and Wong, 2012), ecosystem function (Woodward, 2009), and the evolution of species (Réale and Festa-Bianchet, 2003). Behaviour, in this regard, appears to be especially sensitive to disruption by chemical pollutant exposure (Melvin and Wilson, 2013; Brodin et al., 2014). Indeed, this sensitivity is among the reasons why behavioural studies are increasingly being recognised as powerful tools for assessing the impacts of environmental contaminants (reviewed in Clotfelter et al., 2004; Zala and Penn, 2004; Melvin and Wilson, 2013; Saaristo et al., 2018). Accordingly, numerous recent studies have shown that exposure to chemical pollutants at environmentally relevant levels can disrupt a broad range of important fitness-related behaviours. For example, bumblebees (*Bombus terrestris*) exposed to a commonly used neonicotinoid

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pesticide (thiamethoxam) display altered foraging behaviour and homing success (Stanley et al., 2016), exposure to polychlorinated biphenyl (PCB) mixtures can disrupt migratory activity and orientation in common starlings (*Sturnus vulgaris*; Flahr et al., 2015), and wild European perch (*Perca fluviatilis*) contaminated with the psychoactive pharmaceutical oxazepam exhibit altered activity, sociality, and feeding rates (Brodin et al., 2013). However, despite an increasing emphasis on ecological realism in behavioural ecotoxicology, relatively few studies have comprehensively evaluated the impacts of environmentally relevant contaminant exposure by testing behaviour across multiple contexts.

One contaminant class with a strong potential to alter behaviour in wildlife is pharmaceuticals. Intake of pharmaceutical products by humans and livestock is escalating globally, a trend driven by a growing and ageing human population, as well as increasingly intensive food production (MEA, 2005; Khetan and Collins, 2007). Indeed, although these contaminants enter the environment via multiple pathways (Arnold et al., 2014), agricultural activity is among the most significant contributors of pharmaceutical pollution worldwide (Kemper, 2008; Geissen et al., 2015). Further, while veterinary pharmaceuticals in agriculture are primarily used for the prevention and treatment of disease (Boxall et al., 2004; Kemper, 2008), livestock are often also treated with hormonal growth promotants (HGPs), which are powerful hormone mixtures used to increase weight gain and feed conversion efficiency (USDA, 2000). Hormonal growth promotants are administered in beef-producing nations worldwide, excluding in the European Union, where hormone-treated meat is banned due to environmental (and human health) concerns (Johnson, 2015). Hormonal growth promotants contain naturally occurring steroids (e.g., 17β-oestradiol, progesterone, testosterone) and/or their synthetic counterparts (e.g., trenbolone acetate, zeranol, melengestrol acetate), and are most often administered to cattle via slow-release subcutaneous ear implants (USDA, 2000). Trenbolone acetate (TBA; 17β-(acetyloxy)estra-4,9,11-trien-3-one), a synthetic androgenic steroid with 15–50 times the androgenic and anabolic potency of testosterone (Neumann, 1976), is amongst the most commonly administered HGPs globally (Hunter, 2010; Kolodziej et al., 2013; Johnson, 2015). In the United States, alone, annual TBA production likely exceeds 5000 kg (Kolodziej et al., 2013), which is administered to approximately 20 million animals (60–90% of beef cattle; Schiffer et al., 2001; Ankley et al., 2003), generating an annual revenue in excess of $1 billion (Lawrence and Ibarburu, 2007).

After implantation, TBA is converted to various biologically active metabolites and excreted. These contaminants then have a direct pathway into the environment because a significant portion (if not all) of this manure is typically applied to agricultural fields as fertiliser (Biswas et al., 2017), allowing TBA metabolites to accumulate in soil, drain into groundwater, and be transported into aquatic ecosystems via runoff from land (Topp et al., 2008; Geissen et al., 2015). Alarmingly, the most biologically active metabolite of TBA, the androgenic endocrine disruptor 17β-trenbolone, has repeatedly been detected in the environment at concentrations ranging from 1 to 20 ng/L in discharge and diffuse run-off (Durhan et al., 2006) to as high as 162 ng/L in tile-drained agroecosystems (Gall et al., 2011). Further, 17β-trenbolone is highly persistent in the environment, with a half-life of approximately 260 days in manure (Schiffer et al., 2001), and likely represents a greater ecological risk than previously recognised due to a newly discovered product-to-parent reversion pathway that increases environmental persistence (Qu et al., 2013). In addition, androgen receptors—for which 17β-trenbolone is a high-affinity ligand (Wilson et al., 2002; Ankley et al., 2003)—are highly evolutionarily conserved and are found in organisms as taxonomically diverse as yeast and humans (McGinnis et al., 2002), making a wide variety of non-target species potentially vulnerable.

There is now substantial evidence that exposure to 17β-trenbolone can have detrimental sub-lethal effects in various aquatic species (reviewed in Ankley et al., 2018). These harmful effects include decreased fertility (Mizukami-Murata et al., 2015) and fecundity (Peterson et al., 2001; Ankley et al., 2003; Mizukami-Murata et al., 2015), developmental abnormalities (Wilson et al., 2002), reduced vitellogenin production (Ankley et al., 2003; Seki et al., 2006; Morthorst et al., 2010), abnormal development of male reproductive organs (Sone et al., 2005) and secondary sexual characteristics (Ankley et al., 2003; Seki et al., 2006), skewed sex ratios (Örn et al., 2006; Olmstead et al., 2012) and even complete and functional female-to-male sex reversal (Larsen and Baatrup, 2010; Morthorst et al., 2010). However, despite the aforementioned sensitivity of animal behaviour to disruption by chemical pollutants, relatively little is known about the potential for environmentally realistic levels of 17β-trenbolone—or endocrine disruptors generally—to influence ecologically meaningful behaviours, especially non-reproductive behaviours.

Here, we set out to test the hypothesis that 21-day exposure to an environmentally relevant level (average measured concentration: 16 ng/L) of 17β-trenbolone would disrupt fitness-related behaviours in wild-caught female eastern mosquitofish (*Gambusia holbrooki*) across multiple contexts. Specifically, in three separate behavioural experiments, we tested the impact of exposure on 1) boldness, activity and exploration in a novel environment, 2) sociability (i.e., shoaling tendency), and 3) foraging behaviour. Boldness (i.e., an individual's location on the continuum from shy to bold temperament, where bolder individuals are those that are more likely to accept a degree of risk in return for potential fitness gains; Wilson et al., 1994), activity, and exploration are fundamentally important in the life-history of individuals (Réale et al., 2007). These traits are often highly stable and consistent over time (Réale et al., 2007; Biro and Stamps, 2008), and are associated with a variety of fitness benefits and consequences (Smith and Blumstein, 2008). For example, in various species, bolder individuals tend to exhibit greater dispersal tendency (Fraser et al., 2001; Cote et al., 2011), while activity and exploration are often positively associated with food intake rates (Werner and Anholt, 1993; Lima, 1998). Further, sociability has important fitness implications as shoaling behaviour is an adaptive response to predation that both provides prey an effective means of defence (Krause and Ruxton, 2002; Ward et al., 2008) and can influence fitness in less direct ways, including by facilitating the transmission of social information (Reader et al., 2003). Lastly, as well as being a key correlate of survival and reproductive success, foraging behaviour involves a complex series of trade-offs between obtaining energy and the time, energy and risk associated with obtaining food (Sih, 1980). Collectively, these behaviours have been shown to be important determinants of invasive potential (Rehage and Sih, 2004), and the ability of wildlife to appropriately modulate these behaviours is known to be crucial in enabling adaptation to environmental change (Sih et al., 2004; Candolin and Wong, 2012; Wong and Candolin, 2015). In addition to behavioural endpoints, across all experiments, 17β-trenbolone-exposed and control fish were measured for differences in morphological characteristics, including weight, length and condition index (mass relative to length).

2. Materials and methods

2.1. Study organism

The eastern mosquitofish is a small freshwater fish native to south-eastern North America (Pyke, 2008) that is receiving

growing research interest as a model for investigating behavioural effects of chemical pollutants (e.g., Saaristo et al., 2013; Magellan et al., 2014; Martin et al., 2017; Melvin et al., 2017; Bertram et al., 2018). Mosquitofish are one of the most prolific and widely distributed freshwater fish in the world (García-Berthou et al., 2005; Pyke, 2008), and are among the world's 100 most invasive species (Lowe et al., 2000). Where they occur, mosquitofish generally exist in high numbers and are often the most abundant species of fish (Arthington et al., 1983; Morton et al., 1988). Further, mosquitofish are flexible in terms of their habitat use and are commonly found in environments that are degraded by human activity (Pyke, 2008), including in water bodies running through agricultural catchments (Murphy et al., 2015; Lee et al., 2017).

2.2. Fish collection and housing

Mosquitofish (female: $n = 350$; male: $n = 350$) were collected with dip nets from the Science Centre Lake (37° 54′ 28" S, 145° 08′ 16" E; 10–12 °C; 10:14 h light:dark), Monash University, Victoria, Australia. Repeated sampling of this site over consecutive years (2015–2018) has indicated no contamination with 17β-trenbolone (Envirolab Services, unpublished data; see below for details of water testing). Fish were acclimated to laboratory conditions for 1 month prior to experimentation in seven mixed-sex glass holding tanks (182 L; 90 cm L × 45 cm W × 45 cm H; 100 fish per tank; 50:50 sex ratio), with 30% water changes performed for each tank once per week. This depuration period allowed for the elimination and/or degradation of any potential body burden of secondary contaminants. However, given that wild fish were used in this study to increase environmental realism, we cannot preclude potential developmental and/or transgenerational effects of previous exposure to secondary contaminants. Throughout the housing period, fish were kept at their preferred temperature range of 24–26 °C (Otto, 1974) and under a 12:12 h light:dark cycle. Both during housing and throughout experimentation, fish were fed *ad libitum* once daily (Otohime Hirame larval diet; 580–910 μm). Females were assumed to be non-virginal by the conclusion of the housing period due to the intense male mating pressure typical of mixed-sex populations (Bisazza et al., 1996; Pilastro et al., 2003).

2.3. Flow-through exposure

A flow-through system was used to expose female fish to 17β-trenbolone, as described previously (Saaristo et al., 2013; Bertram et al., 2015; Tomkins et al., 2016, 2017, 2018), with some modifications. We focussed on potential impacts of exposure on female fish only, in order to disentangle contaminant-induced effects on female behaviours (if any) from interacting effects on males—including male sexual harassment, which, as aforementioned, is characteristically intense in mixed-sex mosquitofish populations (Bisazza et al., 1996; Pilastro et al., 2003) and increases due to 17β-trenbolone exposure in another poeciliid, the guppy (*Poecilia reticulata*; Bertram et al., 2015; Tomkins et al., 2017)—as well as to avoid potential confounds associated with mixed-sex exposures, including the formation of dominance hierarchies within exposure aquaria (Schultz et al., 2011).

The flow-through exposure regime involved 320 sexually mature female mosquitofish being randomly allocated to identical glass aquaria (54 L; 60 cm × 30 cm × 30 cm) within a flow-through exposure system, where they were housed for 21 days. This period was chosen because prior research has demonstrated that 21 days of exposure to 17β-trenbolone at environmentally realistic levels is sufficient to induce behavioural shifts (e.g., Saaristo et al., 2013; Bertram et al., 2015; Heintz et al., 2015; Tomkins et al., 2016, 2017, 2018), as well as because mosquitofish have relatively small home ranges (Noggle et al., 2004; Pyke, 2005), and are therefore likely to be continuously exposed for prolonged periods. Eight exposure tanks were used (four 17β-trenbolone exposure tanks and four unexposed tanks), each of which housed 40 fish. Each exposure aquaria contained 2 cm of natural gravel substrate, a large stone for refuge, an airstone, and an aquarium heater (Aqua One glass heater, 55W).

Tanks within the flow-through system were monitored daily for temperature (exposed tanks: mean = 24.13 °C, SD = 0.48 °C, $n = 84$; unexposed tanks: mean = 24.03 °C, SD = 0.44 °C, $n = 84$), as well as flow-through rates (exposed tanks: mean = 18.48 mL/min, SD = 0.42 mL/min, $n = 84$; unexposed tanks: mean = 18.55 mL/min, SD = 0.42 mL/min, $n = 84$), which were controlled using flow meters (BES, MPB Series 1200). These parameters were consistent across exposure treatments (temperature: Mann-Whitney $U = 3059$, $p = 0.135$; flow-through rate: Mann-Whitney $U = 3918$, $p = 0.192$). Survivorship over the exposure period was 93.1% for unexposed fish and 94.4% for exposed fish (11 and 9 deaths, respectively), which—given that sexually mature (i.e., ≥8 weeks; Pyke, 2005) female *G. holbrooki* were exposed, having an average lifespan of ~18 months (Pen and Potter, 1991; Pyke, 2005)—is in line with background natural mortality rates in this species.

2.4. Chemical exposure and GC-MS/MS analysis

The exposure level of 17β-trenbolone used (nominal concentration: 25 ng/L; mean measured concentration = 15.94 ng/L, SD = 5.17 ng/L, $n = 16$) was achieved 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%) to produce a stock solution (400 mg/L). This solution was then diluted with deionised water (4 μg/L) and diluted again within the flow-through system, producing the final average exposure concentration of 16 ng/L. The final solvent dilution in the exposure tanks was 0.000028%, a level far below observed no-effect concentrations for fish (Majewski et al., 1978; Yokoto et al., 2001). The observed deviation of the average measured 17β-trenbolone concentration from the nominal level is likely due to the scale and ecological realism of the flow-through system employed—i.e., numerous adult fish having been exposed concurrently in large aquaria fitted with natural substrate and refuges. While these features may have contributed to this divergence, they were used to more closely replicate environmental conditions.

Levels of 17β-trenbolone were measured weekly in exposed tanks, as well as in unexposed tanks to ensure the absence of contamination. This involved water samples (200 mL) being drawn from each tank and stored in amber glass bottles at 4 °C until analysis (performed within 4 days since collection). Water samples were tested using gas chromatography–tandem mass spectrometry (7000C Triple Quadrupole GC-MS/MS, Agilent Technologies, Delaware, USA) by Envirolab Services (MPL Laboratories, Perth; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025). The limit of quantification was 1 ng/L and no contamination of unexposed tanks was detected throughout the exposure period ($n = 16$). For a detailed description of the GC-MS/MS protocol followed, see Tomkins et al. (2018).

2.5. Behavioural trials

To test for potential impacts of exposure to 17β-trenbolone on female fitness-related behaviours, three separate experiments were conducted. First, we characterised boldness, activity and exploratory behaviour in a novel environment using a maze arena. Second, we tested sociability by measuring shoaling tendency, as female mosquitofish exhibit cohesive shoaling in nature (Wilson

et al., 2010). Third, we examined foraging behaviour using a novel assay. Focal fish used in each experiment were drawn randomly from unexposed and 17β-trenbolone-exposed aquaria within the flow-through system and were not reused between behavioural assays to avoid potential effects of trial order on behavioural responses (Díaz-Uriarte, 2002). All trials were conducted in glass aquaria containing aged carbon-filtered fresh water (i.e., water free from 17β-trenbolone) and were video-recorded from above (Canon PowerShot S120), with behaviours then being quantified from this footage using the event-recording software JWatcher V1.0 (Blumstein and Daniel, 2007).

2.6. Boldness, activity and exploration

Fish were tested for boldness, activity and exploratory behaviour in a novel environment using a maze arena (Fig. 1A) adapted from Ward (2012) and Moran et al. (2016). Behavioural trials involved a single fish (unexposed: $n = 50$, exposed: $n = 46$) being allocated to one of four identical maze arenas (60 cm × 30 cm × 60 cm; water depth: 10 cm). The focal fish was initially introduced into an enclosed refuge (10 cm × 10 cm × 10 cm) and allowed 5 min to acclimate. At the commencement of each trial, a door to the refuge (5 cm W × 7.5 cm H) was remotely opened, allowing the fish to enter into the maze and explore the novel environment for 20 min. The maze arena consisted of six arms—each of which was 30 cm long and 10 cm wide—delineated by internal walls of opaque white acrylic. Focal fish were considered to have transitioned into a maze arm if ≥50% of the fish's body had crossed into the arm. Tanks were drained and rinsed between each trial, which was also the case in each of the other behavioural assays.

Recorded behaviours characterising boldness included latency to first exit the refuge at the beginning of the maze (sec), as well as the total time spent inside this refuge (sec). Refuge use is an established measure of boldness in a variety of fish species (e.g., Dowling and Godin, 2002; Hulthén et al., 2017), including in mosquitofish (Rehage and Sih, 2004; Cote et al., 2010; Wilson et al., 2010). Furthermore, the combined number of entries into all maze arms during the trial was assessed as a general measure of fish activity. Lastly, we characterised exploratory behaviour by quantifying both latency to complete the maze (i.e., reach the final maze arm) after having first exited the refuge (sec), and the number of full maze lengths swam (i.e., the number of times an individual swam from the first to the last maze arm, or vice versa).

2.7. Sociability

To investigate possible effects of exposure to 17β-trenbolone on sociability, females were tested for their tendency to associate with a shoal of stimulus (i.e., unexposed) conspecific females following a standard assay (Ward et al., 2004; Cote et al., 2010, 2011), with some modifications. Experimental trials involved a single fish (unexposed: $n = 44$, exposed: $n = 43$) being allocated to one of eight identical shoaling observation tanks (54 L; 60 cm × 30 cm × 30 cm; water depth: 20 cm; Fig. 1B). Each tank was divided into three compartments using transparent perforated dividers (allowing visual and olfactory communication but not physical interaction), one large central compartment (40 cm × 30 cm × 30 cm) and two smaller compartments on either side (each compartment: 30 cm × 10 cm × 30 cm). One of eight shoals of 17 stimulus (i.e., unexposed) adult females—each of which had previously been isolated for 24 h in 9 L (30 cm × 15 cm × 20 cm) holding tanks—was randomly allocated to each trial and introduced into one of the side compartments. Shoals were size-matched for standard length (i.e., snout to caudal peduncle; mean = 22.27 mm, SD = 3.49 mm)—which did not differ significantly across shoals (Kruskal-Wallis test: $\chi^2 = 0.26$, $p = 0.999$, $n = 136$)—as body size influences shoaling behaviour in fish (Hoare et al., 2000). Moreover, stimulus shoals were unexposed, to exclude the possibility that effects of 17β-trenbolone exposure on the behaviour of the focal female (if any) could have been mediated by effects of exposure on the stimulus fish—an approach employed in previous ecotoxicological experiments (e.g., Tomkins et al., 2017; Bertram et al., 2018: Tomkins et al., 2018). Before each behavioural trial, the stimulus shoal was allowed to acclimate within the side compartment for 20 min, with the focal fish acclimated for 5 min in a holding container (500 mL) within the central compartment. Acclimation containers were opaque, precluding any visual and olfactory communication between the focal and stimulus fish during the acclimation period. At the beginning of each trial, the acclimation container holding the focal fish was gently emptied into the middle of the central compartment, before the behaviour of the focal fish was video-recorded for 20 min. This central compartment was delineated transversely into three zones of equal size (each zone: 30 cm × 13.33 cm × 30 cm) using external tank markings, with these zones being used to quantify the position of the focal fish relative to the stimulus shoal compartment—representing 'asocial', 'intermediate' and 'social' behaviour, relative to the position of the shoal. Further, a 2 cm preference zone abutting the stimulus shoal compartment was used to quantify close-proximity shoaling behaviour (i.e., time spent within one body length of the shoal compartment). Shoal position (i.e., left or right compartment) was randomised across trials to control for potential side bias.

Shoaling behaviours quantified included latency to first enter (sec) and total time spent within (sec) the 2 cm shoaling zone. The total time spent by the focal fish in each of the three sociability zones (i.e., the 'asocial, 'intermediate' and 'social' zones) was also used to calculate a weighted sociability score (i.e., [seconds in 'social' zone × 3] + [seconds in 'intermediate' zone × 2] + [seconds in 'asocial' zone × 1]). This weighted sociability score provides a measure of how focal fish used the entire central compartment relative to the position of the stimulus shoal, with a higher score indicating a more social individual (minimum score: 1200, maximum: 3600). Further, the combined number of entries into each of the three sociability zones was investigated as a measure of general activity.

2.8. Foraging behaviour

Potential impacts of exposure to 17β-trenbolone on foraging behaviour were tested using a novel foraging task. This involved a single fish (unexposed: $n = 47$, exposed: $n = 45$) being allocated to one of four identical foraging trial tanks (60 cm × 30 cm × 30 cm, 54 L; water depth: 10 cm; Fig. 1C), each of which had a sand substrate. Fish were acclimated for 5 min behind a perforated transparent partition 10 cm from one end of the tank before the partition was remotely removed, allowing the fish access to the main tank area for 20 min. Located two-thirds of the length of the tank (40 cm) from the acclimation area was the 'foraging zone' (12 cm × 8 cm). This foraging zone consisted of 48 shallow cylindrical wells (well diameter: 17 mm, depth: 5 mm), into which 20 prey items (chironomid larvae) had been randomly placed. Prey items were placed into shallow wells to ensure that fish engaged actively in food discovery and foraging behaviour, in order to simulate natural ecosystem processes. The position of the foraging zone on either side of the tank was randomised across trials to control for possible side bias.

Foraging behaviours investigated included latency to first enter the foraging zone (sec), latency to first consume a prey item (sec), total time spent within the foraging zone (sec), number of entries

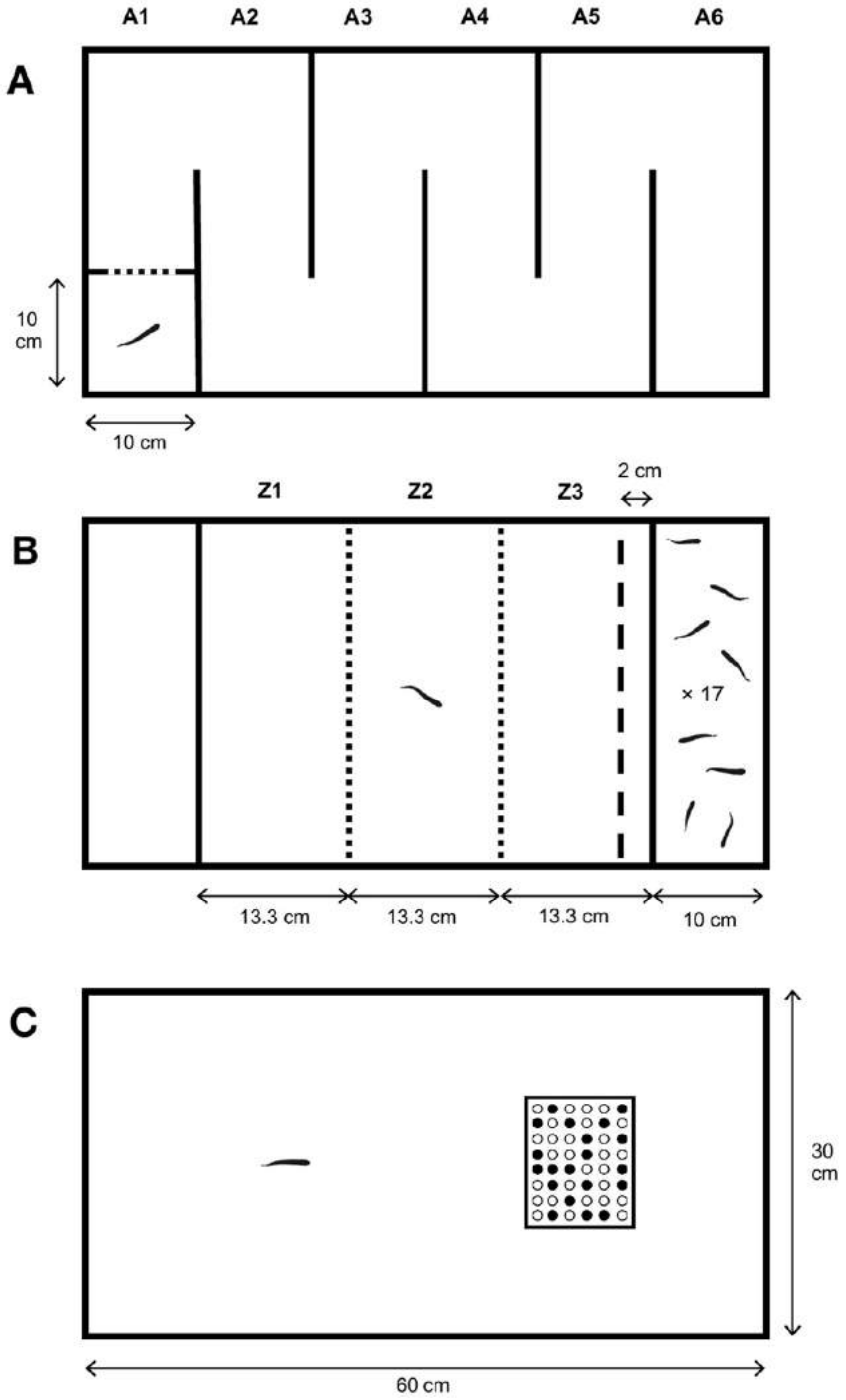

Fig. 1. Aerial views of the (A) maze assay used to test boldness, activity and exploratory behaviour in a novel environment, (B) sociability assay examining tendency to associate with a shoal of 17 stimulus (i.e., unexposed) conspecific females, and (C) foraging assay testing foraging and feeding behaviours. The maze arena contained an enclosed refuge with a door that was remotely opened at the commencement of each trial, allowing the focal fish to enter into the first maze arm (A1) and explore the novel environment (arms A1–A6). The sociability arena included a central compartment into which a focal female was introduced. This focal fish was scored for use of a 2 cm association zone abutting a neighbouring compartment containing 17 stimulus (i.e., unexposed) conspecific females, as well as for use of the entire central compartment relative to the position of the stimulus shoal (i.e., usage of sociability zones; Z1: 'asocial', Z2: 'intermediate', Z3: 'social'). Lastly, the foraging assay involved the focal fish being allowed to forage for 20 chironomid larvae that had been randomly dispersed amongst an array of 48 cylindrical wells (i.e., the 'foraging zone'). Within the foraging zone, prey items are indicated by filled circles.

into the foraging zone, and number of prey items consumed.

2.9. Morphology

Immediately after behavioural trials, morphological measures were recorded for fish pooled from all experiments (unexposed: $n = 141$, exposed: $n = 134$). This involved fish being euthanised with an overdose (40 mg/L) of anaesthetic clove oil and blotted dry, before being measured for standard length (±0.01 mm) and weight (±0.0001 g). Body condition index was then calculated by producing a least-squares regression of the mass (g) of all fish against their standard length (mm) (i.e., weight $= -0.581 + 0.035 \times$ length),

with condition index being calculated as the residuals of this regression line.

2.10. Statistical analysis

All analyses were performed using R version 3.2.3 (R Core Team, 2013), with statistical significance being assigned at $\alpha = 0.05$. Data were checked for normality (Shapiro-Wilk test; Royston, 1995) and homogeneity of variance (Fligner-Killeen test; Conover et al., 1981), where appropriate. For a full description of statistical methods, see 'Statistical procedures' (S1.1) in Supplementary material, as well as Tables S1–S3 for further details of model parameters.

Models used to analyse behavioural responses included one explanatory variable (exposure treatment) and one fixed effect selected for its biological relevance (standard length; see Supplementary material, S2.2, for details of covariate-response relationships). For the sociability assay, shoal ID was also included as a random effect. In order to exercise caution with interpretation of main effects, we investigated interaction terms where they were significant at the $\alpha = 0.1$ level. Where interactions were detected between exposure treatment and standard length—which occurred exclusively in the foraging assay—they were investigated by choosing values of the covariate and comparing treatment groups only at these specific values (as recommended by Quinn and Keough, 2002). Specifically, this involved splitting the data at the median value for the covariate standard length (i.e., 23.04 mm) to form 'small' and 'large' subgroups. Consequently, standard length was removed as a fixed effect in subsequent analyses. Due to the number of unplanned comparisons made at the subgroup level, the Holm-Bonferroni correction method was applied to all *p*-values resulting from this analysis.

For latency data (measuring time to an event), parametric survival models were used where only fixed effects were required in a model. For each dataset, the most suitable hazard distribution was selected using ANOVA. Where mixed-effects were required (i.e., the shoaling assay), a Cox proportional-hazard model was used instead. In the foraging assay, interactions were detected between exposure treatment and standard length in two cases, and were investigated as described above. In a single case where no events were observed in a subgroup (i.e., where no unexposed large fish consumed a prey item), survival curves were compared between subgroups using the *G-rho* family of tests.

For total time data (time spent performing a behaviour) and weighted sociability score, data were rank-normal transformed to approximate normality of the residuals. Where shoal ID was not a consideration, ANCOVA was used to analyse the total time values. For trials where shoal ID was important (i.e., sociability trials), linear mixed-effects (LME) models were used instead, using shoal ID as a random effect.

For count data, a generalised linear model (GLM) approach was taken. As with above, shoal ID was used as a random effect in the sociability assay (i.e., GLMM). We checked models for appropriate link functions, as well as over-dispersion and potential zero-inflation, modifying link functions and using zero-inflated models where appropriate. Interactions were examined using the approach outlined above. For one trial (number of worms eaten), no GLM was found to be suitable, so we used a Kruskal-Wallis rank sum non-parametric test and Dunn's non-parametric *post hoc* test instead.

Mann-Whitney *U* tests (Mann and Whitney, 1947) were used to test whether exposure to 17β-trenbolone altered standard length, weight and/or condition index.

For descriptive statistics of behavioural responses performed in each assay, as well as fish morphology, see 'Supplementary tables' (S1.2) in Supplementary material (Tables S4–S7).

3. Results

3.1. Boldness, activity and exploration

Exposure to 17β-trenbolone did not significantly impact the latency of fish to first exit the refuge at the beginning of the maze (parametric survival regression: $z = 0.12$, $p = 0.902$) nor the total time spent in the refuge (ANCOVA: $F_{1,93} = 0.16$, $p = 0.690$). However, exposed fish showed a significantly reduced latency to complete the maze after having first exited the refuge (parametric survival regression: $z = 1.98$, $p = 0.047$; Fig. S1). Further, exposed fish entered a greater number of maze arms in total (quasi-Poisson GLM: $t = 2.29$, $p = 0.024$; Fig. 2A), and swam a greater number of full maze lengths (zero-inflated negative binomial [ZINB] GLM: $z = 2.05$, $p = 0.041$; Fig. 2B).

3.2. Sociability

No significant effect of exposure to 17β-trenbolone was detected on the latency of fish to first enter the 2 cm shoaling zone (Cox proportional-hazard regression: $z = 0.69$, $p = 0.490$). A negative relationship was, however, identified between 17β-trenbolone-exposure and both the total time spent by fish within the 2 cm shoaling zone (LME: $t = 3.21$, $p = 0.002$; Fig. 3A), and weighted sociability score (LME: $t = 2.27$, $p = 0.026$; Fig. 3B). Further, exposure to 17β-trenbolone was positively associated with the combined number of entries made by fish into all sociability zones (negative binomial GLMM: $z = 2.65$, $p = 0.008$; Fig. 3C).

3.3. Foraging behaviour

A marginally non-significant interaction between treatment

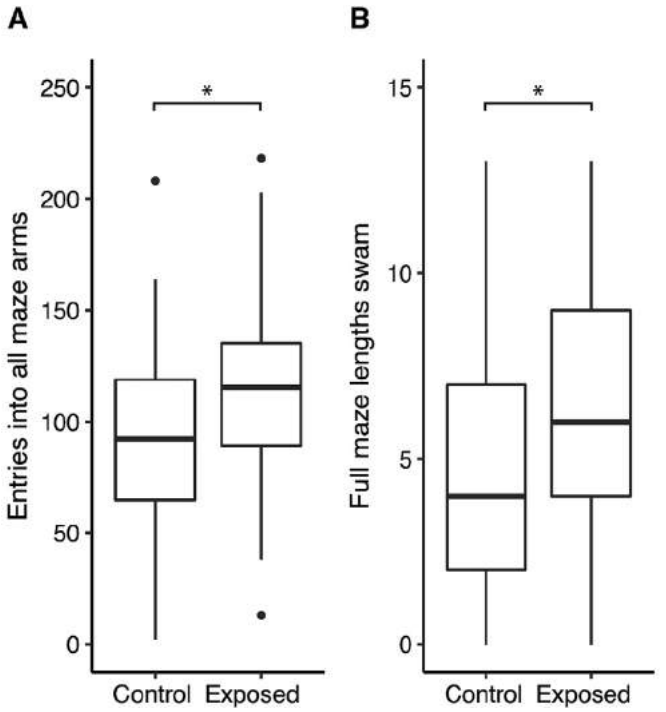

Fig. 2. Number of (A) entries into all maze arms (i.e., A1–A6), and (B) full maze lengths swam, by unexposed ($n = 50$) and 17β-trenbolone-exposed ($n = 46$) fish. Box plots show tenth, twenty-fifth, fiftieth (median), seventy-fifth and ninetieth percentiles with horizontal lines. Whiskers show the range of the data, with outliers being represented by filled circles. $^{*}p < 0.05$.

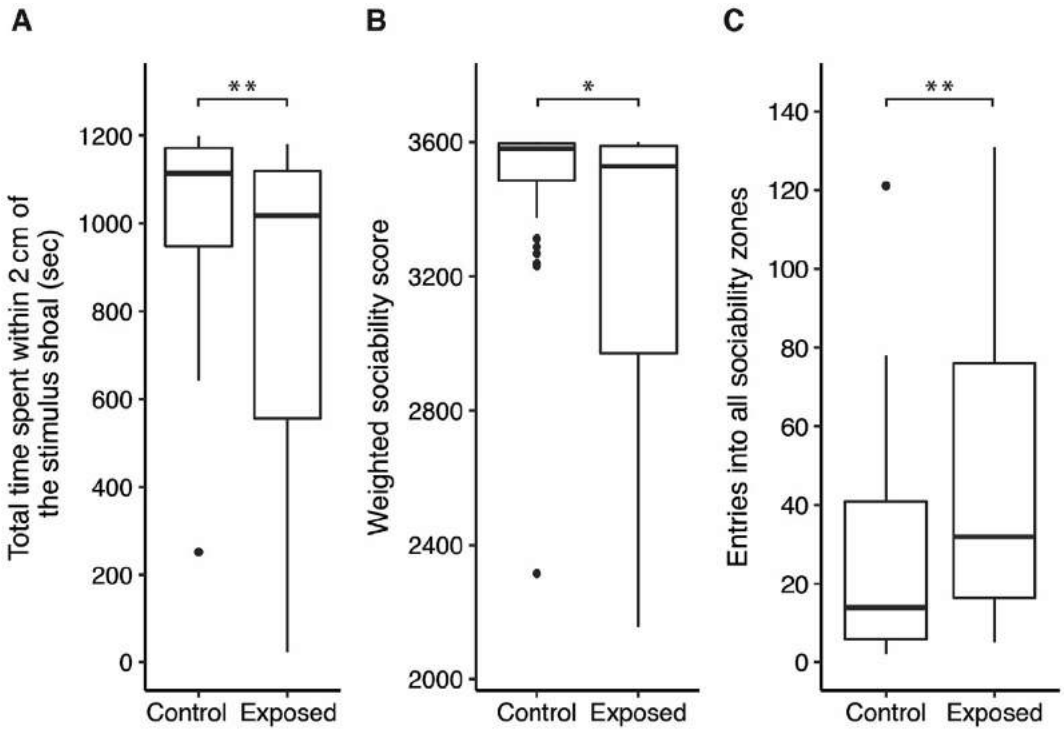

Fig. 3. Sociability of unexposed ($n = 44$) and 17β-trenbolone-exposed ($n = 43$) females towards a shoal of 17 stimulus (i.e., unexposed) conspecific females, in terms of (A) total time spent within 2 cm of the stimulus shoal, (B) weighted sociability score (higher score indicates greater sociability; see Materials and methods), and (C) total number of entries made by fish into all sociability zones (i.e., Z1–Z3). $^{*}p < 0.05$, $^{**}p < 0.01$.

(i.e., 17β-trenbolone exposure status) and standard length was observed for latency of fish to first enter the foraging zone (parametric survival regression: $z = 1.76$, $p = 0.079$). Splitting the data at the median value for the covariate standard length (see 'Statistical procedures' [S1.1] in Supplementary material for more information) revealed that, relative to unexposed large fish, fish in both the unexposed small and exposed large subgroups were quicker to first reach the foraging zone (parametric survival regression: $z = 3.41$, $p = 0.004$ and $z = 2.90$, $p = 0.019$, respectively; Fig. S2A), with no significant differences having been observed between any other subgroups (all $p > 0.05$). A marginally non-significant interaction was also detected between treatment and standard length for the latency of fish to commence feeding (parametric survival regression: $z = 1.86$, $p = 0.064$). An investigation of this interaction revealed that, in large fish, exposure to 17β-trenbolone was associated with a reduced latency to first feed (*G-rho* family of tests: $\chi^2 = 8.27$; $p = 0.020$; Fig. S2B), although no such effect was seen in small fish (*G-rho* family of tests: $\chi^2 = 3.08$; $p = 0.237$; Fig. S2B). In addition, while the time taken to commence feeding did not differ between unexposed-small and exposed-large fish (*G-rho* family of tests: $\chi^2 = 0.76$; $p = 0.612$; Fig. S2B), exposed-small fish were significantly faster than those in the unexposed-large subgroup to first consume a prey item (*G-rho* family of tests: $\chi^2 = 12.25$; $p = 0.003$; Fig. S2B). No significant differences were detected between any other subgroups (all $p > 0.05$).

Exposed fish spent a greater total time within the foraging zone (ANCOVA: $F_{1,89} = 8.30$, $p = 0.005$; Fig. 4A) and entered the foraging zone more frequently than did unexposed fish (negative binomial GLM: $z = 2.38$, $p = 0.017$; Fig. 4B). In addition, a marginally non-significant interaction between treatment and standard length was detected for the total number of prey consumed (zero-inflated Poisson [ZIP] GLM: $z = 1.81$, $p = 0.071$). In large fish, exposed females consumed significantly more prey than did unexposed

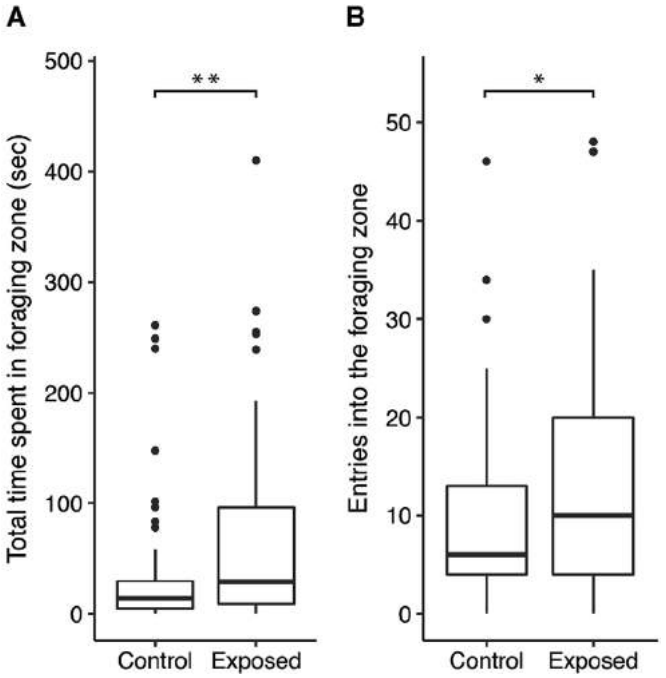

Fig. 4. Foraging behaviour of unexposed ($n = 47$) and 17β-trenbolone-exposed ($n = 45$) fish, in terms of (A) total time spent in the foraging zone, and (B) number of entries into the foraging zone. $^{*}p < 0.05$, $^{**}p < 0.01$.

females (Dunn's test: $z = 2.85$, $p = 0.011$; Fig. S3), while there was no significant effect of treatment on the number of prey consumed by small females (Dunn's test: $z = 1.90$, $p = 0.114$; Fig. S3). In addition, exposed small females consumed significantly more prey than

did unexposed large females (Dunn's test: $z = 3.19$, $p = 0.004$; Fig. S3). No significant differences were detected between the remaining subgroups (all $p > 0.05$).

3.4. Morphology

Exposure to 17β-trenbolone did not significantly impact standard length (Mann-Whitney $U = 8667$, $p = 0.237$), weight (Mann-Whitney $U = 8796$, $p = 0.324$), or condition index (Mann-Whitney $U = 10204$, $p = 0.251$).

4. Discussion

This study investigated whether the endocrine-disrupting veterinary pharmaceutical 17β-trenbolone, via waterborne exposure at a field-realistic level, affects ecologically important behaviours in female fish. We report that exposed fish were more active and exploratory in a novel environment, less social when interacting with a shoal of conspecific females, and generally exhibited increased foraging behaviour (although the effect of exposure on certain foraging behaviours was dependent on female size).

4.1. Boldness, activity and exploration

Fish exposed to 17β-trenbolone displayed increased activity and exploratory behaviour in a novel environment (i.e., maze assay), being faster to first complete the maze, entering a greater number of maze arms, and swimming significantly more full maze lengths. However, no significant effect of exposure was detected on boldness, neither in terms of latency to exit a refuge nor total refuge use.

In general, exposure to chemical pollutants (e.g., pharmaceuticals, heavy metals, herbicides and pesticides) more often results in reductions in swimming activity and locomotor behaviour in aquatic species (reviewed in Little and Finger, 1990). While these behaviours can be influenced by exposure to endocrine disruptors, reported impacts have been relatively mixed due to the highly varied modes of action of these contaminants. For example, developing African clawed frog tadpoles (*Xenopus laevis*) contaminated with a polychlorinated biphenyl mixture (Aroclor 1254) exhibit disrupted swimming behaviour (Jelaso et al., 2002), while goldfish (*Carassius auratus*) exposed to the herbicide atrazine perform significantly increased burst swimming (Saglio and Trijasse, 1998).

To our knowledge, the impact of xenoandrogen exposure on female activity and exploratory behaviour has not previously been tested in a non-reproductive context. However, adult female goldfish implanted with 11-ketotestosterone perform male-typical increased locomotor behaviour in a reproductive setting (Stacey and Kobayashi, 1996) and zebrafish having undergone sex reversal by exposure to 17β-trenbolone from egg until sexual maturity display activity levels during mating (e.g., total path swam, average swimming velocity) that are non-significantly different from genotypic males (Larsen and Baatrup, 2010). Interestingly, the effects of estrogenic endocrine disruptors on non-reproductive activity and swimming behaviour have received relatively more attention, with exposure typically resulting in reductions of these behaviours. For example, zebrafish juveniles exposed to the synthetic estrogen mimic 17α-ethinylestradiol (EE_2) display reduced swimming behaviour (Sária et al., 2011) and EE_2-exposed Siamese fighting fish (*Betta splendens*) spend less time being active both in an empty tank and a novel environment (Dzieweczynski et al., 2014).

4.2. Sociability

Exposure to 17β-trenbolone resulted in fish being less social. Specifically, while latency to first associate with a shoal of stimulus (i.e., unexposed) conspecific females was not significantly affected, exposed fish spent less time within close proximity of a shoal (i.e., within one body length of the shoal compartment), as well as being generally less social (i.e., achieving a lower weighted sociability score). Further, as seen in the maze assay, exposed fish demonstrated increased activity by moving between tank zones more frequently than controls.

Increasingly, exposure to a variety of chemical pollutants is being shown to interfere with conspecific social interactions in fish. For example, exposure to 4-nonylphenol affects social recognition and shoaling in juvenile banded killifish (*Fundulus diaphanous*, Ward et al., 2008), contamination with the anxiolytic pharmaceutical oxazepam reduces sociality in European perch (*Perca fluviatilis*, Brodin et al., 2013), and administration of benzyl butyl phthalate—used in the production of plastic products—depresses shoaling behaviour in mummichog (*Fundulus heteroclitus*, Kaplan et al., 2013). However, as an interesting point of comparison with the present results, exposure of adult male zebrafish (*Danio rerio*) to the estrogen 17α-ethinylestradiol has actually been shown to increase shoaling behaviour, with exposed males being slower to leave a shoal of conspecifics and leaving this shoal fewer times (Reyhanian et al., 2011).

That exposure to 17β-trenbolone decreased shoaling tendency in the present study is broadly consistent with recent research by Heintz et al. (2015), where the effects of exposure for the same period (i.e., 21 days) were tested on guppy risk-taking behaviour in the presence of a predator. Specifically, Heintz et al. (2015) reported that female guppies exposed to 17β-trenbolone (0.25, 2.5 and 25 ng/L), and tested in groups of three—with all fish being similarly exposed, or not—demonstrated reduced shoaling behaviour in the presence of a predatory gold severum cichlid (*Heros severus*). We propose, however, that decreased shoaling behaviour resulting from 17β-trenbolone exposure may be a more general phenomenon than increased risk-taking behaviour in the presence of a predator, given than reduced shoaling was presently observed even in the absence of a predator. However, it is important to note that, in the present study, shoaling tendency of individual 17β-trenbolone-exposed fish was tested in the presence of a shoal of unexposed fish. Therefore, an important avenue of future research will be to investigate impacts of exposure to 17β-trenbolone—as well as other endocrine-disrupting contaminants—on formation and cohesion of large shoals of fish, with all members of a shoal being either unexposed or exposed. Moreover, given that male sexual harassment is known to influence female social group choice and shoaling behaviour in mosquitofish (Agrillo et al., 2005; Dadda et al., 2008), also important will be investigations of contaminant-induced effects on mixed-sex shoaling behaviour.

4.3. Foraging behaviour

Exposure of fish to 17β-trenbolone significantly affected foraging behaviour, although the effect of exposure on certain foraging behaviours was dependent on female size. Specifically, in large fish, those exposed to 17β-trenbolone were faster than controls to first enter a foraging zone and to commence feeding, as well as consuming significantly more prey items in total, with no such significant effects being detected in small fish. Further, regardless of female size, exposed fish spent a greater total amount of time within the foraging zone, as well as entering the foraging zone more frequently than controls—reflecting the general activity increase seen in both the maze and sociability assays.

In general, feeding and foraging behaviours are more often impaired by chemical pollutant exposure, thereby reducing juvenile growth and adult biomass (reviewed in Weis and Candelmo, 2012). Although effects of endocrine disruptors on such endpoints have been relatively understudied, impaired foraging behaviour in fish has been reported after exposure to xenoestrogens. For example, environmentally realistic exposure to EE_2 decreases foraging success in juvenile roach (*Rutilus rutilus*, Hallgren et al., 2014), while a temperature-dependent reduction in foraging ability has been reported in larval fathead minnows (*Pimephales promelas*) exposed to estrone (Ward et al., 2017).

The presently observed increase in female foraging behaviours resulting from exposure to an androgen is an interesting exception to this general trend. Again, this finding is broadly consistent with those of Heintz et al. (2015), where 17β-trenbolone exposure (0.25, 2.5 and 25 ng/L) increased the time spent by female guppies inspecting prey items (*Daphnia magna*) while in the presence of a predator. However, as with shoaling behaviour, our work suggests that intensified foraging behaviour resulting from 17β-trenbolone exposure is likely a broader phenomenon than increased risk-taking behaviour under threat of predation given that this effect was observed even in the absence of a predator. Moreover, as with shoaling, female foraging behaviour in mixed-sex populations is known to be influenced by the behaviour of males. In particular, several studies have highlighted that male sexual harassment can reduce female foraging efficiency in poeciliids (Magurran and Seghers, 1994a; b; Pilastro et al., 2003). Hence, effects of contaminant exposure on sexual conflict is clearly an important area for future research.

As was observed in the present study, Heintz et al. (2015) found that effects of 17β-trenbolone exposure on guppy foraging behaviour (under predation risk) were size-dependent, which was hypothesised to have resulted from differential vulnerabilities of fish of different sizes to predation, thereby causing shifts in behaviour secondary to exposure. Nevertheless, the present findings suggest that this phenomenon may be driven by different inherent vulnerabilities across fish of different sizes to disrupted behaviour by 17β-trenbolone exposure. As well as direct effects of body mass, size-dependent effects of exposure could conceivably be due to age-related differential sensitivity, given that both endogenous hormone levels and vulnerability to endocrine disruption are known to vary greatly between fish at different life stages (Leet et al., 2011), despite all of the fish tested in both studies having been sexually mature adults.

4.4. Physiological and molecular mechanisms

How might exposure to an androgenic endocrine disruptor alter behaviour in female fish? Androgens play important physiological roles in female vertebrates, both indirectly by acting as precursors for estrogen biosynthesis, and directly via activation of the androgen receptor (AR; Borg, 1994; Staub and de Beer, 1997; Munakata and Kobayashi, 2010). Among the assorted mechanisms of androgenic action in female vertebrates are neuronal growth, stimulation of muscle and bone development, lipid metabolism, and immune responses (Staub and de Beer, 1997; Martyniuk and Denslow, 2012). Androgens are also involved in regulating various important female behaviours, including communication and social recognition, aggression, mating behaviour, and cognitive functioning (Staub and de Beer, 1997; Martyniuk and Denslow, 2012). Because endogenous androgens—primarily testosterone and 11-ketotestosterone in female fish (Borg, 1994; Munakata and Kobayashi, 2010)—are involved in modulating these traits and behaviours, they are potentially vulnerable to disruption by exogenous androgens such as 17β-trenbolone.

In addition to being a high-affinity AR ligand (Wilson et al., 2002; Ankley et al., 2003), it has been hypothesised that 17β-trenbolone exposure produces a compensatory response, resulting in a decrease in the production of endogenous androgens such as testosterone (Zhang et al., 2008; Mizukami-Murata et al., 2015). It is proposed that this then indirectly inhibits 17β-estradiol (E_2) production, given that E_2 is converted from testosterone by aromatase (Miracle et al., 2006; Zhang et al., 2008; Mizukami-Murata et al., 2015), while 17β-trenbolone is non-aromatisable (Rogozkin, 1991). Accordingly, recent research has shown that 17β-trenbolone exposure can alter reproductive behaviours in female fish (Saaristo et al., 2013; Bertram et al., 2015; Tomkins et al., 2016, 2018). However, given the findings of the present study, further research is clearly needed to understand potential impacts of 17β-trenbolone exposure on non-sexual behaviours in female fish.

4.5. Morphology

Despite 17β-trenbolone's effectiveness as an anabolic steroid (Ankley et al., 2003), no significant effect of exposure was detected on any of the assessed measures of female morphology—including weight, standard length, or condition index. This finding is in agreement with existing research having investigated the effects of exposure to field-realistic levels of 17β-trenbolone on morphology in female fish. Specifically, the weight and length of female guppies was not significantly impacted after exposure at 2 ng/L (Tomkins et al., 2018), 4 ng/L (Tomkins et al., 2016), 8 ng/L (Tomkins et al., 2017) or 22 ng/L (Bertram et al., 2015). In addition, female fathead minnows (*Pimephales promelas*) showed no appreciable morphological change after exposure at 5 ng/L or 50 ng/L (Ankley et al., 2003), although concentration-dependant female weight increase was reported at higher concentrations (0.5, 5 and 50 μg/L; Ankley et al., 2003). Interestingly, males appear to be more sensitive to 17β-trenbolone-induced morphological change, with exposure of guppies at 4 ng/L being associated with increased male condition index (M.G. Bertram et al., unpublished data), while exposure at 22 ng/L caused an increase in both weight and condition index (Bertram et al., 2015).

5. Conclusion

This study demonstrates behavioural alterations in female fish, across multiple contexts, resulting from exposure to an androgenic endocrine disruptor. Specifically, we found that 21-day exposure to a field-realistic level (average measured concentration: 16 ng/L) of the widely administered veterinary pharmaceutical 17β-trenbolone altered a range of ecologically important behaviours in female mosquitofish. Exposed fish exhibited increased activity and exploratory behaviour in a novel environment (i.e., maze arena), while boldness was not significantly affected. Further, when assayed for sociability, exposed fish spent less time within close proximity of a shoal of stimulus (i.e., unexposed) conspecific females, as well as, again, being more active within the shoaling arena. Lastly, exposed fish demonstrated increased foraging behaviour when presented with a novel foraging task, although the impact of exposure on certain foraging behaviours was dependent on fish size. Taken together, our findings illustrate that environmentally realistic exposure of female fish to a widespread agricultural contaminant is sufficient to alter behaviours that are crucial fitness determinants, with possible ecological and evolutionary implications for exposed populations.

Ethics

All procedures performed for this study were approved by the

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

Authors' contributions

M.G.B., M.S. and B.B.M.W. conceived and designed the study. M.G.B., J.M.M. and T.E.E. collected the data. M.G.B., M.M. and C.P.J. carried out data analysis. M.G.B. wrote the manuscript. All authors contributed to manuscript revisions and gave final approval for publication.

Competing interests

The authors declare that we 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.2018.09.044.

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Supplementary material

1. Materials and methods

1.1. STATISTICAL PROCEDURES

Experiment one: boldness, activity and exploration

Models testing the impact of exposure to 17β-trenbolone on boldness, activity and exploratory behaviour in the maze arena included one explanatory variable (exposure treatment) and one fixed effect selected due to its biological relevance (standard length) (see Table S1).

Parametric survival models (*survreg* function, *survival* package; Kalbfleisch and Prentice, 2002) were used to compare the latency of fish to first exit the enclosed refuge at the beginning of the maze, as well as latency to first complete the maze (i.e., reach the final maze arm) after first exiting the refuge. For both models, a Weibull hazard function was selected as the most suitable distribution, as determined by a comparative analysis of hazard distributions using analysis of variance (ANOVA). This was also the case for other relevant models using this hazard function (see below). Each model met the assumption of proportionality, as was the case for all survival analyses performed, which was ensured by checking the interaction of Schoenfeld residuals and log time (*coxph* and *cox.zph* functions, *survival* package; Grambsch and Therneau, 1994). In addition, the total time spent by fish within the refuge at the beginning of the maze was rank-normal transformed to approximate normality of the residuals (*rntransform* function, *GenABEL* package; Aulchenko et al., 2007) before being compared using analysis of covariance (ANCOVA). Further, the combined number of entries into all maze arms was analysed using a generalised linear model (GLM), fitted with a quasi-Poisson distribution to account for overdispersion of the response variable. Lastly, a Vuong non-nested test (*vuong* function, *pscl* package; Vuong, 1989; Jackman, 2012) indicated an excess of zeroes (i.e., zero-inflation) in the number of full maze lengths swam, which was accommodated by fitting a zero-inflated Poisson (ZIP) GLM (*zeroinfl* function, *pscl* package; Zeileis et al., 2008). To test for overdispersion of the non-zero counts, a zero-inflated negative binomial (ZINB) GLM (*zeroinfl* function) was then also fitted and compared with its ZIP GLM alternative using a likelihood-ratio test (*lrtest* function, *lmtest* package; Zeileis and Hothorn, 2002). This procedure indicated overdispersion, with the ZINB GLM therefore being favoured (Zuur et al., 2009).

Experiment two: sociability

Models examining shoaling behaviours in the sociability assay comprised one explanatory variable (exposure treatment), one fixed effect (standard length) and one random effect (stimulus shoal ID) (see Table S2).

To compare the latency of fish to first enter the 2 cm shoaling zone, a mixed-effects Cox proportional-hazard model was used (*coxme* function, *coxme* package; Ripatti and Palmgren, 2000; Therneau et al., 2003). Total time spent within the 2 cm shoaling zone, as well as weighted sociability score (see Materials and methods), were rank-normal transformed before separate linear mixed-effects (LME) models were applied (*lmer* function, *lme4* package; Bates et al., 2015). Finally, the total number of times a fish crossed any sociability zone boundary (i.e., the 'asocial', 'intermediate' and 'social' zones) was modelled using a generalised linear mixed-effects model (GLMM) with a negative binomial error distribution (*glmer.nb* function, *lme4* package; Bates et al., 2015), as is appropriate for overdispersed count data (Zuur et al., 2013). For all LME and GLMM tests, the random effect of shoal ID always had a percentage of variance explained ≤2.76%.

Experiment three: foraging behaviour

Behavioural responses in the foraging assay were examined using models comprising one explanatory variable (exposure treatment) and one fixed effect (standard length) (see Table S3).

The latency of fish to enter the foraging zone was first investigated using a parametric survival model (*survreg* function) with a Weibull hazard function, which revealed a marginally non-significant interaction between exposure status and standard length (parametric survival regression: z = -1.76, p = 0.079). Where interaction terms are significant, assuming homogeneity of slopes can lead to misleading results (Engqvist, 2005). We therefore investigated interaction terms where they were significant at the α = 0.1 level, as we consider this to be in line with a cautious evaluation of interactions in linear models (however, note that main effects were still only considered significant at the α = 0.05 level). One straightforward approach to examining heterogeneous regression slopes (i.e., significant interaction terms) is to choose values of the covariate and compare treatment groups only at these specific values (Quinn and Keough, 2002). Thus, we investigated interactions by splitting the data at the median value for the covariate standard length (i.e., 23.04 mm). This resulted in 'small' (standard length: 16.61–23.02 mm, mean = 20.59 mm, SD = 1.76 mm, n = 46) and 'large' (standard length: 23.06–32.57 mm, mean = 26.34 mm, SD = 2.59 mm, n = 46) subgroups, with standard length then being removed as a fixed effect. These subgroups were analysed using parametric survival models (each

with a Weibull hazard function).

The latency of fish to consume a prey item was first tested using a parametric survival model with a Weibull hazard function, which, again, revealed a marginally non-significant interaction between exposure status and standard length (parametric survival regression: z = -1.86, p = 0.064). This interaction was investigated by splitting the data at the median value for the covariate standard length, as described above, with the latency of fish in each subgroup to first consume a prey item then being analysed using parametric survival models. However, due to there being no events in the unexposed large subgroup (i.e., no unexposed large fish consumed a prey item), potential differences between subgroups in terms of latency to first consume a prey item were assessed using the *G-rho* family of tests (*survdiff* function, *survival* package; Harrington and Fleming, 1982). In addition, total time spent in the foraging zone was rank-normal transformed and tested using ANCOVA. The total number of entries into the foraging zone was modelled using a negative binomial GLM (*glm.nb* function, *MASS* package; Venables and Ripley, 2002) in order to accommodate overdispersion. The number of prey items consumed was first investigated using a GLM. Due to a Vuong non-nested test indicating zero-inflation, a ZIP GLM was then applied. This revealed a marginally non-significant interaction between exposure status and fish standard length (ZIP GLM: z = -1.81, p = 0.071), which was, again, investigated by splitting fish into subgroups at the median value of the covariate standard length, as above. However, due to one of the subgroups having no variation in the response (i.e., no unexposed large fish consumed a prey item), further analysis with a GLM was unsuitable. We instead applied a Kruskal-Wallis rank sum test followed by a Dunn's multiple comparison test (*dunn.test* function, *dunn.test* package; Dunn, 1961). Non-parametric tests that rely on ranking cannot provide exact p-values where there are ties. Although the use of a non-parametric test here is therefore not ideal, the effect is to inflate Type-II error, which we consider acceptable.

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1.2. SUPPLEMENTARY TABLES

Table S1. Statistical models used to analyse boldness, activity and exploratory behaviour in Experiment 1.

Response variable	Model	Predictor variables
Latency to first exit refuge (sec)	Parametric survival model (Weibull distribution)	Treatment Standard length (mm)
Total time in refuge (sec)	Analysis of covariance (with rank-normal transformation)	Treatment Standard length (mm)
Latency to first complete the maze (i.e., reach A6) after first exiting the refuge (sec)	Parametric survival model (Weibull distribution)	Treatment Standard length (mm)
Combined number of entries into all maze arms (i.e., A1–A6)	Quasi-Poisson generalised linear model	Treatment Standard length (mm)
Number of full maze lengths swam	Zero-inflated negative binomial generalised linear model	Treatment Standard length (mm)

Table S2. Statistical models used to analyse sociability in Experiment 2.

Response variable	Model	Predictor variables
Latency to first enter 2 cm shoaling zone (sec)	Cox mixed-effects proportional hazard model	Treatment Standard length (mm) Shoal ID (random effect)
Total time spent within 2 cm shoaling zone (sec)	Linear mixed-effects model (with rank-normal transformation)	Treatment Standard length (mm) Shoal ID (random effect)
Combined number of entries into all sociability zones (i.e., Z1–Z3)	Negative binomial Generalised Linear Mixed Model	Treatment Standard length (mm) Shoal ID (random effect)
Weighted sociability score	Linear mixed-effects model (with rank-normal transformation)	Treatment Standard length (mm) Shoal ID (random effect)

Table S3. Statistical models used to analyse foraging in Experiment 3.

Response variable	Model	Predictor variables
Latency to first enter foraging zone (sec)	Parametric survival model (Weibull distribution) followed by separate parametric survival models (each with a Weibull distribution) used to explore non-significant marginal interaction	Subgroup (four subgroups comprising fish split by median standard length and exposure status)
Latency to first consume a prey item (sec)	Parametric survival model (Weibull distribution) followed by an investigation of non-significant marginal interaction term, involving testing the difference between survival curves using the *G-rho* family of tests	Subgroup (four subgroups comprising fish split by median standard length and exposure status)
Total time spent within foraging zone (sec)	Analysis of covariance (with rank-normal transformation)	Treatment Standard length (mm)
Number of entries into foraging zone	Negative binomial generalised linear model	Treatment Standard length (mm)
Number of prey items consumed	Kruskal-Wallis rank sum test followed by Dunn's *post hoc* test (all subgroup comparison of fish standard length and exposure status pairs)	Subgroup (four subgroups comprising fish split by median standard length and exposure status)

Table S4. Mean (±SE) behavioural responses of fish in Experiment 1 (unexposed: n = 50; 17β-trenbolone-exposed: n = 46).

Behavioural response	Treatment	
	Unexposed	Exposed
Latency to first exit refuge (sec)	71.24 ± 16.76	67.15 ± 13.11
Total time in refuge (sec)	237.50 ± 37.51	190.48 ± 28.65
Latency to first complete the maze (i.e., reach A6) after first exiting the refuge (sec)	341.86 ± 51.58	230.87 ± 39.32
Combined number of entries into all maze arms (i.e., A1–A6)	92.16 ± 6.06	113.37 ± 6.36
Number of full maze lengths swam	4.72 ± 0.51	6.22 ± 0.49

Table S5. Mean (±SE) behavioural responses of fish in Experiment 2 (unexposed: n = 44; 17β-trenbolone-exposed: n = 43).

Behavioural response	Treatment	
	Unexposed	Exposed
Latency to first enter 2 cm shoaling zone (sec)	26.34 ± 4.25	36.23 ± 9.14
Total time spent within 2 cm shoaling zone (sec)	1031.04 ± 29.56	835.83 ± 52.09
Combined number of entries into all sociability zones (i.e., Z1–Z3)	26.55 ± 4.02	46.67 ± 5.70
Weighted sociability score	3494.73 ± 32.07	3270.88 ± 64.63

Table S6. Mean (±SE) behavioural responses of fish in Experiment 3 (unexposed: *n* = 47; 17β-trenbolone-exposed: *n* = 45).

Behavioural response	Treatment	
	Unexposed	Exposed
Latency to first enter foraging zone (sec)	190.45 ± 55.21	104.40 ± 30.67
Latency to first consume a prey item (sec)	1074.45 ± 50.19	829.47 ± 71.63
Total time spent within foraging zone (sec)	37.70 ± 9.33	70.53 ± 14.05
Number of entries into foraging zone	9.94 ± 1.45	13.51 ± 1.82
Number of prey items consumed	0.23 ± 0.11	1.42 ± 0.35

Table S7. Mean (±SE) morphological traits of fish pooled from all experiments (unexposed: *n* = 141, 17β-trenbolone-exposed: *n* = 134).

Morphological trait	Treatment	
	Unexposed	Exposed
Standard length (mm)	22.76 ± 0.30	23.29 ± 0.32
Weight (g)	0.2235 ± 0.0110	0.2385 ± 0.0125
Condition index	0.0010 ± 0.0036	-0.0029 ± 0.0046

2. Supplementary Results

2.1. SUPPLEMENTARY FIGURES

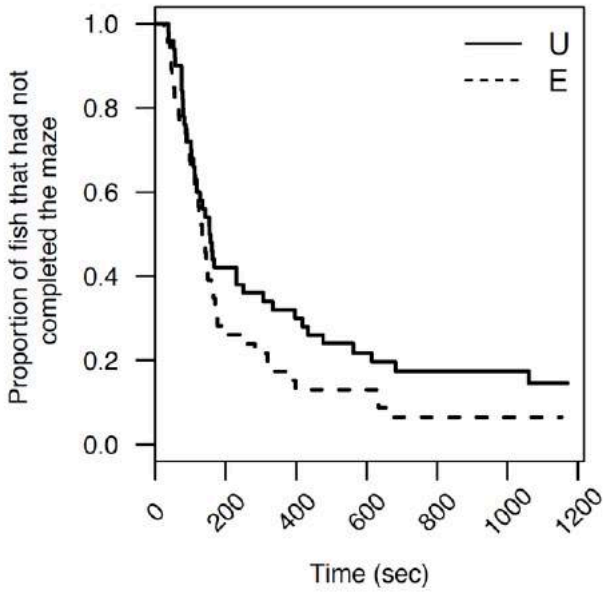

Figure S1. Kaplan–Meier survival curves displaying the latency of unexposed (U, n = 50) and 17β-trenbolone-exposed (E, n = 46) fish to complete the maze (i.e., reach the final maze arm) after having first exited the refuge (sec).

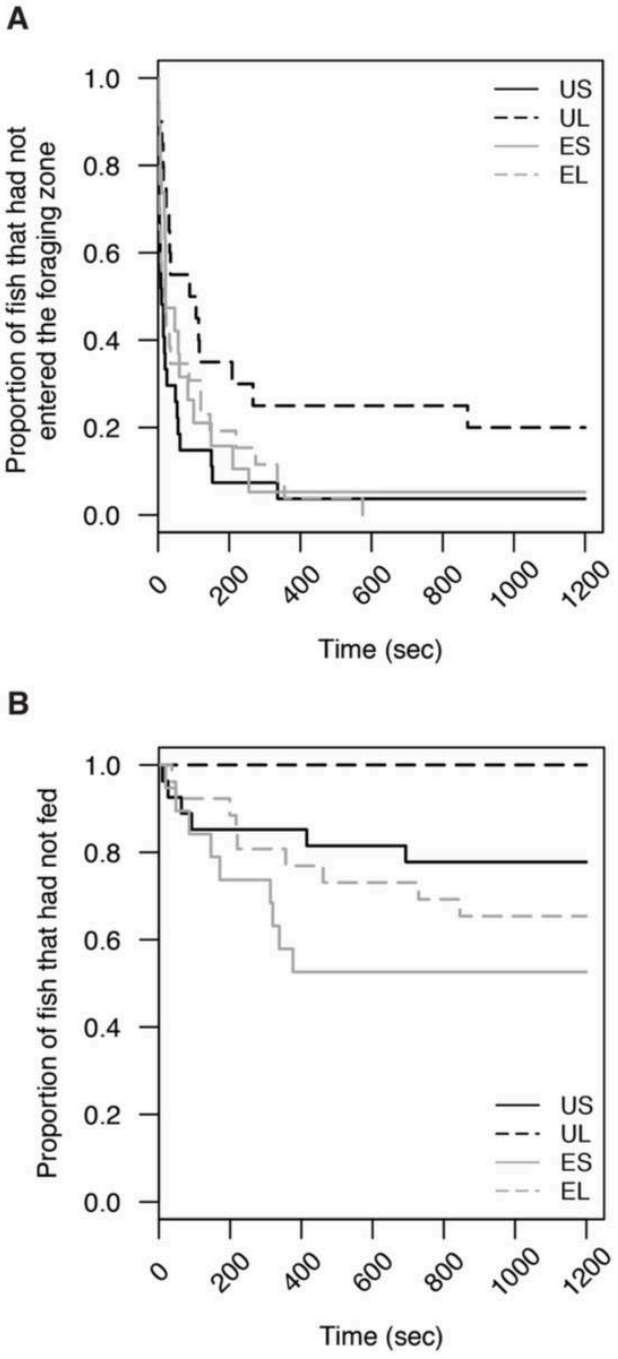

Figure S2. Kaplan–Meier survival curves showing the latency of fish in each treatment group (US: n = 27; UL: n = 20; ES: n = 19; EL: n = 26) to (A) enter the foraging zone, and (B) commence feeding. Due to an interaction between treatment and size, females were split by the median value for standard length (i.e., 23.04 mm), with treatment groups therefore encompassing unexposed (U) or 17β-trenbolone-exposed (E) fish, that were either small (S) or large (L).

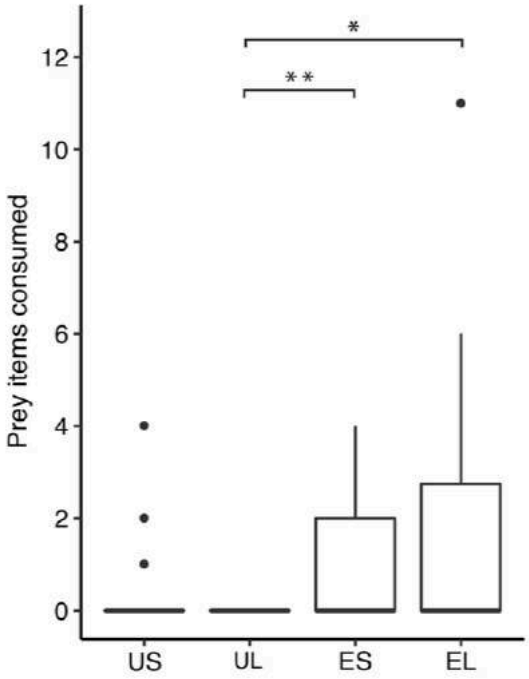

Figure S3. Total number of prey items (i.e., chironomid larvae) consumed during the foraging trial (US: $n = 27$; UL: $n = 20$; ES: $n = 19$; EL: $n = 26$). Treatments groups comprised unexposed (U) and exposed (E) females split by the median value for female standard length (i.e., 23.04 mm) into small (S) and large (L) fish. * $p < 0.05$, ** $p < 0.01$.

2.2. COVARIATE-RESPONSE RELATIONSHIPS

Experiment one: boldness, activity and exploration

In the maze assay, a negative relationship was detected between female standard length and the total number of full maze lengths swam (ZINB GLM: $z = 2.71$, $p = 0.007$). Standard length did not, however, associate significantly with any other behavioural responses recorded in the maze arena (all $p > 0.05$).

Experiment two: sociability

In fish tested for sociability, standard length was positively associated with the total time spent within the 2 cm shoaling zone (LME: $t = 2.67$, $p = 0.009$) and related negatively with the total number of entries made into all sociability zones (negative binomial GLMM: $z = 2.98$, $p = 0.003$). No significant relationship was detected between standard length and the other behavioural responses recorded in the sociability assay (all $p > 0.05$).

Experiment three: foraging behaviour

In general, when tested for foraging behaviour, larger fish (i.e., those with greater standard lengths) took longer to first reach the foraging zone (parametric survival regression: $z = 2.55$, $p = 0.011$) and to commence feeding (parametric survival regression: $z = 2.69$, $p = 0.007$). Further, standard length was negatively associated with the total number of entries made by fish into the foraging zone (negative binomial GLM: $z = 2.82$, $p = 0.005$). However, larger fish spent more time within the foraging zone (ANCOVA: $F_{1,89} = 6.96$, $p = 0.010$) and consumed a greater number of prey items (ZIP GLM: $z = 2.29$, $p = 0.022$).

Chapter 4

Context-specific behavioural changes induced by exposure to an androgenic endocrine disruptor

Michael G. Bertram [a], Jake M. Martin [a], Minna Saaristo [a,b], Tiarne E. Ecker [a], Marcus Michelangeli [a,c], Nicholas D.S. Deal [a], Shu Ly Lim [d], Moira K. O'Bryan [a,d], Bob B.M. Wong [a]

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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
Conceived and designed the study, conducted the experiments, performed the statistical analysis, wrote and submitted the manuscript.	91%

The following co-authors contributed to the work:

Name	Nature of contribution	Extent of contribution
Bob B.M. Wong	Contributed to experimental design and manuscript preparation.	1.5%
Jake M. Martin	Contributed to experimental design, data collection, and manuscript preparation.	1.5%
Minna Saaristo	Contributed to experimental design and manuscript preparation.	1%
Tiarne E. Ecker	Assisted with data collection.	1%
Marcus Michelangeli	Assisted with statistical analyses.	1%
Nicholas D.S. Deal	Assisted with statistical analyses.	1%
Shu Ly Lim	Assisted with data collection.	1%
Moira K. O'Bryan	Coordinated sperm analysis.	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:  Date: 27/11/2018

Main supervisor signature:  Date: 27/11/2018

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Context-specific behavioural changes induced by exposure to an androgenic endocrine disruptor

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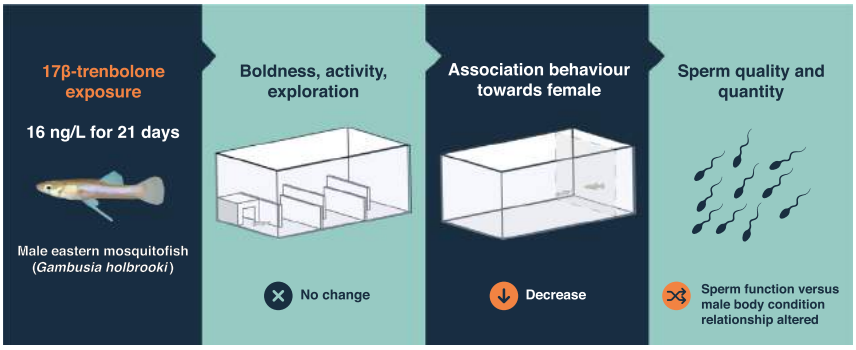
HIGHLIGHTS

- 17β-Trenbolone (17β-TB) is a growth promoter used extensively in beef production.
- Wild-caught male mosquitofish were exposed to a field-realistic level of 17β-TB.
- Exposure resulted in context-specific behavioural changes.
- Effects of 17β-TB on behaviour were observed in a reproductive context.
- 17β-TB altered male morphology–sperm function relationship, and changed morphology.

GRAPHICAL ABSTRACT



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ABSTRACT

Pharmaceutical contaminants are being detected with increased frequency in organisms and ecosystems worldwide. This represents a major environmental concern given that various pharmaceuticals act on drug targets that are evolutionarily conserved across diverse taxa, are often persistent in the environment, and can bioconcentrate in organisms and bioaccumulate in food chains. Despite this, relatively little is known about the potential for pharmaceutical contaminants to affect animal behaviour, especially across multiple fitness-related contexts. Here, we investigated impacts of 21-day exposure of wild-caught male eastern mosquitofish (*Gambusia holbrooki*) to a field-realistic level of the veterinary pharmaceutical 17β-trenbolone—a growth-promoting steroid used extensively in beef production worldwide and a potent androgenic endocrine disruptor repeatedly detected in surface waters affected by livestock effluent run-off. First, we examined male boldness, activity, and exploratory behaviour in a novel environment (maze arena) and found no significant effect of 17β-trenbolone exposure. Second, the same males were tested in a reproductive assay for their tendency to associate with a stimulus (unexposed) female behind a partition. Exposed males exhibited reduced association behaviour, taking longer to first associate with, and spending less time within close proximity to, a female. Third, all males were assayed for sperm function (computer-assisted sperm analysis, sperm viability) or quantity (total sperm count) and, although no significant main effects of 17β-trenbolone were seen on sperm traits, exposure altered the relationship between male morphology and sperm function. Lastly, morphological traits were assessed and exposed males were found to have, on average, increased mass relative to length. In combination, these results

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demonstrate that exposure to a field-realistic level of 17β-trenbolone can produce subtle but important trait alterations in male fish—including context-specific behavioural changes, disruption of key sperm function trade-offs, and altered morphology—with potential impacts on exposed wildlife.

1. Introduction

Intake of pharmaceutical products by humans and livestock is escalating globally. This trend is being driven by a growing and ageing human population, expanding global market availability, and increasingly intensive food production (MEA, 2005; Khetan and Collins, 2007). This rising demand has resulted in a greater discharge and accumulation of pharmaceuticals in the environment (Hughes et al., 2013; IWW, 2014). Indeed, >600 active pharmaceutical substances—or their metabolites and transformation products—have now been detected across 71 countries covering all continents (IWW, 2014; Aus der Beek et al., 2016), with these figures predicted to continue to rise (Hughes et al., 2013; Arnold et al., 2014). In this regard, pharmaceutical residues have now been identified in the tissues of species as taxonomically and spatially diverse as Oriental white-backed vultures (*Gyps bengalensis*) feeding on contaminated livestock in India and Pakistan (Oaks et al., 2004), earthworms (*Eisenia fetida*) living in sewage treatment works in the United Kingdom (Markman et al., 2007), and fish exposed to wastewater treatment plant effluent in the Niagara River (Arnnok et al., 2017). This increased prevalence of pharmaceutical contaminants in the environment is cause for concern, given that various pharmaceuticals are specifically designed to produce physiological effects at low concentrations (Khetan and Collins, 2007). Although these contaminants enter the environment via multiple and diverse pathways (Arnold et al., 2014), agricultural activity is among the most significant contributors of pharmaceutical pollution globally (Kemper, 2008).

While veterinary pharmaceutical use in agriculture is primarily for the prevention and treatment of disease, hormonal growth promotants (HGPs) are also administered in livestock operations worldwide to increase the rate and extent of growth of beef cattle (APVMA, 2003; Bartelt-Hunt et al., 2012). Typically, HGP implants comprise a mixture of natural and/or synthetic steroids, including androgens (e.g. trenbolone acetate), estrogens (e.g. 17β-estradiol, zeranol) and progestins (e.g. melengestrol acetate) (Lange et al., 2001; Bartelt-Hunt et al., 2012). These HGPs are administered to cattle, either alone or in combination, where they mimic endogenous hormones (Bartelt-Hunt et al., 2012). Trenbolone acetate (TBA; 17β-(acetyloxy)estra-4,9,11-trien-3-one), a potent anabolic steroid, is among the most commonly used HGPs worldwide (Neumann, 1976a, 1976b; Kolodziej et al., 2013). This is despite TBA having been banned as a livestock supplement in various regions (e.g. European Union) due to environmental and human health concerns (Johnson, 2015). The scale of TBA use is seen, for example, in the United States—the world's largest beef producer—where over 20 million cattle are implanted annually (Schiffer et al., 2001; Ankley et al., 2003).

After being implanted, TBA is rapidly hydrolysed to various metabolites, the most biologically active of which is 17β-trenbolone (hereafter 17β-TB). As a high-affinity ligand for the vertebrate androgen receptor (Neumann, 1976b; Wilson et al., 2002), 17β-TB has an androgenic and anabolic potency 15–50 times that of testosterone (Neumann, 1976b). What is more, given that the excrement of cattle dosed with TBA is often applied to agricultural fields as fertiliser, 17β-TB has a direct pathway into the environment via run-off of this effluent into neighbouring terrestrial and aquatic habitats (Lange et al., 2002; Kolok and Sellin, 2008). Consequently, 17β-TB has repeatedly been detected in these environments at concentrations ranging from 0.0015 to 270 ng/L in feedlot run-off and lagoon water (Schiffer et al., 2001; Soto et al., 2004; Durhan et al., 2006; Bartelt-Hunt et al., 2012; Khan and Lee, 2012; Parker et al., 2012; Webster et al., 2012), and 0.0013–20 ng/L in river water (Soto et al., 2004; Durhan et al., 2006).

Several characteristics of 17β-TB make its presence in the environment particularly concerning. This includes that 17β-TB is highly temporally persistent (half-life in effluent: ~260 days; Schiffer et al., 2001), is rapidly taken up by, and can be bioconcentrated in, various fish species (Ankley et al., 2003; Schultz et al., 2013; Lagesson et al., 2019), and affects androgen receptor signalling pathways that are evolutionarily conserved across diverse taxa (McGinnis et al., 2002). A large body of evidence now exists suggesting that field-realistic levels of 17β-TB are sufficient to cause adverse biological effects in a wide variety of aquatic species (e.g. amphibians, fish; reviewed in Ankley et al., 2018). Reported impacts of exposure include: reduced fertility and fecundity (e.g. Ankley et al., 2003; Mizukami-Murata et al., 2015), changes in gene expression (e.g. Ekman et al., 2012; Leet et al., 2015), developmental abnormalities (e.g. Wilson et al., 2002), altered sex steroid plasma concentrations (e.g. Ankley et al., 2003; Ekman et al., 2012), malformations in gonad histopathology (e.g. Sone et al., 2005; Cripe et al., 2010), reduced vitellogenin production (e.g. Ankley et al., 2003; Seki et al., 2006), abnormal sexual differentiation resulting in skewed sex ratios (e.g. Örn et al., 2006; Olmstead et al., 2012), and even fully functional female-to-male sex reversal (e.g. Larsen and Baatrup, 2010). Furthermore, relatively recent research has uncovered that exposure to 17β-TB at environmentally realistic levels can alter a range of key fitness-related behaviours in aquatic species, including activity and exploration (Bertram et al., 2018a; Lagesson et al., 2019), feeding and foraging (Bertram et al., 2018a), sociability (Bertram et al., 2018a), risk-taking behaviour (Heintz et al., 2015; Lagesson et al., 2019), and reproductive behaviour (Saaristo et al., 2013; Bertram et al., 2015; Tomkins et al., 2016, 2017; Bertram et al., 2018b; Tomkins et al., 2018).

The capacity of 17β-TB to disrupt behaviour at low dosages is concerning given that the ability of organisms to perform behaviours appropriate to their environment is fundamentally important for individual survival and reproduction (Sih et al., 2004; Smith and Blumstein, 2008), ecosystem function and stability (Woodward, 2009), and species evolution (Réale and Festa-Bianchet, 2003). Indeed, behavioural adjustments are often an organism's first response to altered conditions and can facilitate adaptation to environmental change, meaning that disturbances in behaviour can have dire ecological and evolutionary consequences (reviewed in Candolin and Wong, 2012; Wong and Candolin, 2015). Moreover, altered behaviour reflects multiple physiological changes and links physiological function with ecological processes (reviewed in Saaristo et al., 2018), and behaviour has been shown to be especially sensitive to perturbation by chemical pollution (Melvin and Wilson, 2013), including pharmaceutical exposure (Brodin et al., 2014). To date, however, few studies have tested potential impacts of pharmaceutical exposure on behavioural traits in individuals across multiple ecological contexts (but see Dzieweczynski and Hebert, 2012; McCallum et al., 2017; Martin et al., 2019). This is despite a large body of research having shown that behaviours can correlate across time and/or contexts (i.e. behavioural syndromes, Sih et al., 2004, 2012), which has important implications for individual fitness (Biro and Stamps, 2008; Smith and Blumstein, 2008) and ecological processes (e.g. response to environmental change, Sih et al., 2012; dispersal, Michelangeli et al., 2017).

Accordingly, in this study, we investigated whether 21-day exposure to a field-realistic level of 17β-TB (average exposure concentration: 16 ng/L) would affect male behaviour across two ecologically important

contexts in wild-caught eastern mosquitofish (*Gambusia holbrooki*). First, fish were tested for boldness (i.e. the likelihood of accepting a degree of risk in return for potential fitness gains; Wilson et al., 1994), activity, and exploratory behaviour in a novel environment (maze arena). Second, the same males were tested for reproductive behaviour (association tendency) when presented with a stimulus (unexposed) conspecific female. Third, due to the fundamental importance of sperm function and number to fertilisation success (Parker, 1982, 1998), these males were then tested for either sperm function (via computer-assisted sperm analysis [CASA] and sperm viability assays) or quantity (total sperm count). Lastly, all males were tested for a suite of morphological characteristics, including standard length, weight, and condition index (i.e. weight relative to length). As a potent androgenic steroid, we predicted that 17β-TB exposure would 1) increase male boldness, activity, and exploratory behaviour in a novel environment, 2) increase association behaviour performed towards a stimulus female, 3) increase sperm function and quantity, and 4) increase male relative mass.

2. Materials and methods

2.1. Study species

The eastern mosquitofish is a small sexually dimorphic livebearer that is among the most widely distributed freshwater fish species globally (biology reviewed in Pyke, 2005, 2008). Mosquitofish are known to utilise habitats polluted by human activity (Pyke, 2008; Díez-del-Molino et al., 2018), including systems impacted by agricultural land-use (Murphy et al., 2015; Lee et al., 2017). Moreover, the mating system and reproductive behaviour of *G. holbrooki* are well studied and readily quantifiable. Male *G. holbrooki* do not court females for solicited copulations but instead sneak upon females for coercive copulations (Bisazza et al., 2001). This involves the male approaching the female from behind and forcibly inserting his modified anal fin (i.e. gonopodium) into the female's genital pore for internal fertilisation (Bisazza et al., 2001).

2.2. Animal collection and housing

Sexually mature mosquitofish used in this study were collected from Monash University Science Centre Lake (male: $n = 200$, female: $n = 200$; 37° 54′ 28″ S, 145° 08′ 16″ E), Victoria, Australia. Repeated water sampling of the collection site both at the time of fish capture and over consecutive years (2015–2018) has revealed no contamination with 17β-TB (Envirolab Services, unpublished data; see details of water testing below). Fish were transported in aerated containers to the laboratory, where they were acclimated for 1 month prior to experimentation in four mixed-sex glass housing tanks (81 L, 60 cm length × 45 cm width × 30 cm height; 24–26 °C; 12:12 h light:dark regime; 100 fish per tank; 50:50 sex ratio), which were cleaned weekly via 30% water changes using reverse osmosis water. During this housing period, and throughout experimentation, fish were fed *ad libitum* once daily with commercial feed (Otohime Hirame larval diet; 580–910 μm).

2.3. Exposure set-up

Male fish were exposed to 17β-TB using a flow-through system adapted from previous experiments (Saaristo et al., 2013; Bertram et al., 2015; Tomkins et al., 2016, 2017; Bertram et al., 2018a, 2018b; Tomkins et al., 2018). This involved a total of 160 males being randomly allocated to one of four glass flow-through 17β-TB-exposure tanks (54 L; 60 cm × 30 cm × 30 cm; water depth: 25 cm) or one of four identical unexposed tanks containing only fresh water (20 fish per tank). All aquaria within the flow-through system were equipped with 2 cm of natural gravel substrate, a large stone to serve as a refuge, an airstone, and a glass heater (Aqua One, 55 W).

Fish were exposed to 17β-TB (or fresh water only) for 21 days because previous research has shown that 21-day exposure to field-realistic levels of 17β-TB is sufficient to elicit a range of behavioural alterations in fish (Bertram et al., 2015; Heintz et al., 2015; Tomkins et al., 2016, 2017; Bertram et al., 2018b; Tomkins et al., 2018), including mosquitofish (Saaristo et al., 2013; Bertram et al., 2018a). Further, mosquitofish typically have small territories (Pyke, 2005), meaning that they are likely to be continuously exposed to contaminants for extended periods.

Throughout the exposure period, flow-through aquaria were monitored daily for temperature (exposed tanks: mean = 23.97 °C, SD = 0.49 °C, $n = 84$; unexposed tanks: mean = 24.16 °C, SD = 0.57 °C, $n = 84$). The amount of water passing through each tank was monitored daily using flow meters (BES, MPB Series 1200; exposed tanks: mean = 18.57 mL/min, SD = 0.40 mL/min, $n = 84$; unexposed tanks: mean = 18.54 mL/min, SD = 0.42 mL/min, $n = 84$). No appreciable difference in these parameters was detected across treatments over the exposure period (temperature: Mann-Whitney $U = 3621$, $p = 0.768$; flow-through rate: Mann-Whitney $U = 3369.5$, $p = 0.594$).

2.4. Exposure dosing and GC–MS/MS analysis

The 17β-TB exposure level (nominal concentration: 25 ng/L; mean measured concentration = 15.75 ng/L, SD = 3.40 ng/L, $n = 16$) was achieved using methods described in Bertram et al. (2018a). Firstly, this involved 17β-TB (17β-hydroxyestra-4,9,11-trien-3-one; CAS: 10161-33-8; Novachem, Germany) being dissolved in ethanol (HPLC grade, ≥99.99%) to create a stock solution (400 mg/L). This solution was then diluted a further two times, first with deionised water (4 μg/L) and then within the flow-through system, which was fed with aged carbon-filtered tap water, to achieve the final average exposure concentration of 16 ng/L. The divergence seen between the nominal and average measured concentrations is most likely a result of the scale and ecological realism of the flow-through system used, including aquaria having been fitted with natural substrate and refuges.

Gas chromatography—tandem mass spectrometry (7000C Triple Quadrupole GC–MS/MS, Agilent Technologies, Delaware, USA) was used to monitor concentrations of 17β-TB in exposure tanks, as well as in unexposed tanks to ensure the absence of contamination. In short, this involved 200 mL water samples being collected from each tank weekly and stored in amber glass bottles at 4 °C for a maximum of 4 days until analysis. Samples were analysed by Envirolab Services (MPL Laboratories, Perth; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025). Protocols followed those described in Tomkins et al. (2018). This analysis yielded a limit of quantification of 1 ng/L. No contamination with 17β-TB was detected in any unexposed aquaria ($n = 12$).

2.5. Experimental design overview

In this study, all males were first tested for potential impacts of exposure to 17β-TB on boldness, activity, and exploratory behaviour in a novel environment (maze arena) (unexposed: $n = 65$, exposed: $n = 70$). Each male was then rested for 30 min before being assessed in a reproductive context (unexposed: $n = 65$, exposed: $n = 70$), where males were tested for their tendency to associate with a stimulus (unexposed) conspecific female behind a transparent partition. Immediately after the reproductive assay, males were randomly selected to be tested for either sperm function (CASA and sperm viability; unexposed: $n = 42$, exposed: $n = 40$) or quantity (total sperm count; unexposed: $n = 22$, exposed: $n = 26$). Males were not tested for both sperm function and quantity due to timing and logistical constraints resulting from the number of individuals tested per day. Lastly, all males were analysed for a suite of morphological traits (i.e. length, weight, and body condition).

2.6. Behavioural trials: boldness, activity, and exploration

All males were first tested for boldness, activity, and exploratory behaviour in a maze arena, following previously established protocols (Bertram et al., 2018a; Martin et al., 2019). This involved fish being collected at random from unexposed and 17β-TB-exposed aquaria within the flow-through system and allocated to one of four identical glass maze arenas (60 cm × 30 cm × 30 cm; water depth: 10 cm; Fig. 1A). Each behavioural trial firstly involved a single focal male being introduced into an enclosed refuge (10 cm × 10 cm × 10 cm) and acclimated within this compartment for 5 min. At the beginning of each trial, a door to the refuge (5 cm W × 7.5 cm H) was opened remotely, allowing the focal fish to exit into the maze, which it was allowed to freely explore for 20 min. This door was left open so that the refuge was accessible throughout the trial. The maze arena was divided transversely into six arms of equal size (30 cm L × 10 cm W) that were delineated with opaque internal walls of white acrylic. Maze trials were conducted with aged carbon-filtered water that did not contain 17β-TB. To avoid chemical cross-contamination between trials, observation tanks were drained and re-filled with aged water upon completion of each trial, as was also done for the reproductive behaviour assay.

Maze trials were filmed from above using video cameras (Canon PowerShot S120) and behaviours were scored from this footage using the event-recording software JWatcher V1.0 (Blumstein and Daniel, 2007). Experimenters were blind to exposure treatment throughout data collection and while scoring behavioural footage, and trial videos were scored by a single observer, as was also the case for reproductive behaviour trials (see below). Behaviours quantified in the maze assay included the time taken for fish to first exit the refuge at the beginning of the maze (s) and the total time spent within this refuge (s). These behaviours are known to characterise boldness in fish (e.g. Dowling and Godin, 2002), including mosquitofish (Rehage and Sih, 2004; Cote et al., 2010). General activity level was quantified as the combined number of entries made by fish into all maze arms throughout the trial. Moreover, exploratory behaviour was quantified as the time taken for fish to first complete the maze (by reaching maze arm 6) after having first exited the refuge at the beginning of the maze (s), as well as the number of full maze lengths swam (i.e. the number of times a fish swam from maze arm 1 to maze arm 6, or vice versa).

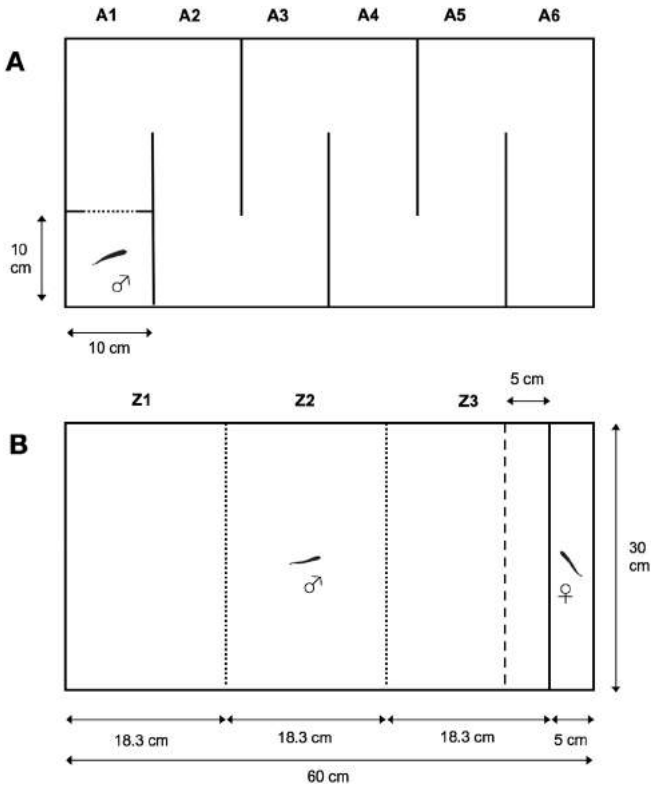

Fig. 1. Aerial view of the (A) maze assay testing boldness, activity, and exploration in a novel environment, and (B) reproductive behaviour assay. The maze arena comprised an enclosed refuge with a door (dotted line) that was opened remotely at the beginning of each trial, as well as six maze arms (A1–6) that the focal fish was allowed to freely explore. Reproductive behaviour trials involved a male being introduced into the larger of two tank compartments and scored for its use of a 5 cm zone abutting the neighbouring compartment, which contained a stimulus (unexposed) conspecific female. Further, external tank markings were used to divide the larger compartment transversely into three zones of equal size (i.e. 'interest' [Z3], 'intermediate' [Z2], and 'disinterest' [Z1] zones), allowing each male to be scored for its use of this entire compartment relative to the stimulus female.

2.7. Behavioural trials: reproductive behaviour

At the conclusion of the maze assay, each male was rested (see details below) and then subjected to a reproductive assay, which was conducted in one of eight trial tanks (54 L; 60 cm × 30 cm × 30 cm; water depth: 20 cm; Fig. 1B). This assay involved males being tested for their tendency to associate with a stimulus (unexposed) conspecific female. Each trial arena was divided transversely into two compartments, a larger central compartment (55 cm × 30 cm × 30 cm) and a smaller compartment (30 cm × 5 cm × 30 cm). The dividing partition was transparent and perforated with small holes throughout to allow for visual and chemical communication, but not physical interaction. This was necessary to prevent males expending their ejaculate prior to sperm analysis, given that the internal mode of fertilisation in mosquitofish means that males must be in close proximity to females in order to copulate (Martin, 1975).

Prior to each trial, the male was rested for 30 min in a 500 mL holding container, in aged tap water not dosed with 17β-TB, within the larger compartment of the trial arena. Then, for 5 min prior to the commencement of the trial, a randomly selected sexually mature stimulus female—previously housed for 24 h in one of four single-sex housing tanks (54 L; 60 cm × 30 cm × 30 cm)—was acclimated in a holding container (500 mL) within the smaller compartment. Stimulus females were not exposed to 17β-TB in order to ensure that contaminant-induced effects on female behaviour (if any) did not interact with potential effects of exposure on the focal male (*sensu* Tomkins et al., 2017; Bertram et al., 2018b, 2018c; Tomkins et al., 2018). Further, stimulus females were size-matched to control for the known preference in male poeciliids for larger females (Arriaga and Schlupp, 2013), and did not differ across treatments in terms of standard length (Mann–Whitney $U = 2198.5$, $p = 0.738$), weight (Mann–Whitney $U = 2185.5$, $p = 0.695$), or condition index (Mann–Whitney $U = 2638$, $p = 0.110$). Both male and female acclimation containers were opaque to prevent any visual or chemical communication between the focal male and stimulus female during the acclimation period, and the stimulus female compartment in each trial was randomly positioned on either the left or the right of the observation tank to control for any potential side-bias.

At the commencement of each trial, the male and female were gently released from their acclimation containers into their respective tank compartments, with the male being released into the centre of the larger compartment and allowed to freely explore over a 20 min video-recorded trial. Using external tank markings, a 5 cm zone abutting the stimulus female's compartment was demarcated, which was used to quantify close-proximity male association behaviour performed towards the female—a commonly used measure of mating intent in poeciliids (e.g. Bierbach et al., 2011; Jeswiet and Godin, 2011), including mosquitofish (Pyke, 2005), which has been shown to reflect mating outcomes (e.g. Kodric-Brown, 1992; Couldridge and Alexander, 2001; Gonçalves and Oliveira, 2005). Further, the larger compartment was delineated transversely into three zones of equal size (each zone: 30 cm × 18.3 cm × 30 cm). These 'interest', 'intermediate', and 'disinterest' main tank zones were used to quantify the position of the focal male relative to the stimulus female compartment.

Behaviours quantified in the reproductive assay include the time taken for males to first reach the 5 cm zone abutting the female compartment (s), and the total time spent within this zone throughout the trial (s). Further, a weighted association score was generated from the total time spent by the focal male within each of the main tank zones (calculated as: [seconds in the 'interest' zone × 3] + [seconds in the 'intermediate' zone × 2] + [seconds in the 'disinterest' zone × 1]). This score represents a fish's use of the entire main tank area relative to the position of the stimulus female compartment, with a higher score indicating a male exhibiting more association behaviour (minimum possible score: 1200, maximum: 3600). Lastly, as a measure of general activity level, the combined number of entries made by males into all main tank zones was quantified.

2.8. Sperm analysis

Immediately after being tested for reproductive behaviour, experimental males were euthanised using an overdose (40 mg/L) of anaesthetic clove oil and analysed for sperm function (CASA and sperm viability) or sperm quantity (total sperm count). All protocols for sperm collection and analysis followed Bertram et al. (2018c). For a full description of each of these protocols, see 'Sperm analysis methods' (S1.1) in Supplementary material. Briefly, for sperm function, a negative phase-contrast microscope coupled with a CASA system (v.14, CEROS, Hamilton-Thorne Biosciences, Beverly, MA) was used to assess a suite of sperm function parameters for each male (see Table S1 for detailed descriptions), including average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), path linearity (LIN, %) and motility (MOT, %). To calculate the proportion of viable sperm in each male's ejaculate, a second sub-sample of ejaculate was collected from each male analysed with CASA, which was tested using a live/dead sperm viability assay (L-7011; Molecular Probes Inc., OR, USA). Lastly, for sperm quantity, separate males were tested for total sperm count using an improved Neubauer haemocytometer.

2.9. Morphological analysis

Subsequent to sperm analysis, all males were measured for standard length (±0.01 mm) and weight (±0.0001 g). Condition index was then calculated as the residuals of a least-squares regression line of each fish's standard length (mm) against its mass (g) (i.e. weight = −0.460 + 0.029 × length).

2.10. Statistical analysis

Statistical analyses were conducted using R version 3.2.3 (R Development Core Team, 2015). Where appropriate, data were tested for normality (Shapiro-Wilk test; Royston, 1995) and homogeneity of variance (Fligner-Killeen test; Conover et al., 1981).

Models generated to test behavioural responses performed in the maze assay included exposure treatment and one additional covariate, condition index, which was chosen due to its biological relevance. Parametric survival models (*survreg* function, *survival* package; Kalbfleisch and Prentice, 2002) were used to analyse the time taken for fish to exit the refuge at the beginning of the maze, and the time taken to first complete the maze (i.e. reach arm 6) after having first exited the refuge. In both cases, a Weibull hazard function was the most suitable distribution, as determined via a comparative analysis of hazard distributions using analysis of variance (ANOVA). Both models met the assumption of proportionality, which was determined by examining the interaction between Schoenfeld residuals and log time (*coxph* and *cox.zph* functions, *survival* package; Grambsch and Therneau, 1994). The total time spent by fish in the enclosed refuge at the beginning of the maze was rank-normal transformed in order to approximate normality of the residuals (*rntransform* function, *GenABEL* package; Aulchenko et al., 2007) before being compared using analysis of covariance (ANCOVA). In addition, the combined number of entries made by the focal fish into all maze arms was examined using a generalised linear model (GLM), which was fitted with a quasi-Poisson distribution due to overdispersion of the response variable. Further, a Vuong non-nested test (*vuong* function, *pscl* package; Vuong, 1989) was used to test for a potential excess of zeroes in the number of full maze lengths swam. This analysis suggested that zero-inflation was present, which was accommodated by fitting a zero-inflated Poisson (ZIP) GLM (*zeroinfl* function, *pscl* package; Zeileis et al., 2008). We then used a likelihood-ratio test (*lrtest* function, *lmtest* package; Zeileis and Hothorn, 2002) to check for potential overdispersion of the non-zero counts by comparing this ZIP GLM with a zero-inflated negative binomial (ZINB) GLM (*zeroinfl* function) alternative. This process indicated overdispersion, with the ZINB GLM therefore being favoured (Zuur et al., 2009).

As with the maze, models testing individual behavioural responses in the reproductive behaviour assay included exposure treatment and one additional covariate, condition index. First, the time taken for males to reach the 5 cm zone abutting the female compartment was analysed using a parametric survival model with a Weibull distribution, with hazard distribution selection and proportionality checks performed as described above. In addition, the total time spent by fish within this 5 cm zone, as well as weighted association score (see Materials and methods), were rank-normal transformed and tested using ANOVA. Last, the combined number of entries made by males into all main tank zones was examined using a GLM, which was fitted with a quasi-Poisson distribution.

To test for potential behavioural correlations across assays, a principal component analysis (PCA; *prcomp* function, *stats* package; Becker et al., 1988) followed by an oblique rotation was first conducted to reduce the variables measured in the maze assay into two principal component (PC) scores. Further, a separate PCA followed by an oblique rotation was conducted to similarly reduce variables measured in the reproductive assay. Prior to running PCAs, rank-normal transformations were applied to all variables in order to approximate normal distributions, as well as to centre and scale variables. The two PCs per behavioural assay were then used to investigate behavioural correlations across the maze assay and reproductive assay. More specifically, within each treatment group (i.e. unexposed and exposed), Pearson's correlation tests were used to investigate the relationship between PC scores across the two behavioural contexts.

Measures of sperm function (i.e. VAP, VSL, VCL, LIN, MOT, viability) and quantity (i.e. total sperm count) were compared across treatments using ANCOVA, with data being rank-normal transformed beforehand, where appropriate, to approximate normality of the residuals. All models analysing sperm function and quantity included both exposure treatment and condition index as covariates, given that male body condition is known to affect sperm number and production rates in mosquitofish (O'Dea et al., 2014). This analysis revealed a significant interaction between exposure treatment and condition index on VCL. Therefore, the relationship between these traits was investigated within each treatment using Spearman's rank-order correlation tests (*cor.test* function, *stats* package; Hollander and Wolfe, 1973).

Male standard length was compared between treatments using a *t*-test, while Mann-Whitney *U* tests (Mann and Whitney, 1947) were used to examine potential impacts of 17β-TB on weight and condition index.

For descriptive statistics of responses performed in each behavioural assay, as well as sperm function and quantity, and fish morphology, see Tables S2–S5. For details of covariate-response relationships, see Supplementary material S2.2 and Table S6.

3. Results

3.1. Behavioural trials: boldness, activity and exploration

No significant effect of exposure to 17β-TB was detected in terms of latency of fish to first exit the refuge at the beginning of the maze (parametric survival regression: $z = -0.57$, $p = 0.570$), total time spent in the refuge (ANCOVA: $F_{1,132} = 0.84$, $p = 0.361$), latency to complete the maze after first exiting the refuge (parametric survival regression: $z = 1.34$, $p = 0.181$), total number of maze arm entries (quasi-Poisson GLM: $t = -0.52$, $p = 0.605$), or number of full maze lengths swam (ZINB GLM: $z = -0.12$, $p = 0.905$).

3.2. Behavioural trials: reproductive behaviour

Males exposed to 17β-TB took significantly longer to first enter the 5 cm zone abutting the stimulus female's compartment (parametric survival regression: $z = 2.91$, $p = 0.004$; Fig. 2) and spent less time within this zone throughout the trial (ANCOVA: $F_{1,131} = 3.95$ $p = 0.049$; Fig. 3A). Furthermore, 17β-TB-exposed males had a lower weighted association score than unexposed males (ANCOVA: $F_{1,131} = 7.85$, $p = 0.006$; Fig. 3B). No effect of exposure was observed, however, on the combined number of entries made by male focal fish into each of the main tank zones (quasi-Poisson GLM: $t = -0.20$, $p = 0.841$).

3.3. Across-context correlations

For each behavioural assay (i.e. maze and reproduction), we retained two Principal Components with Eigenvalues ≥1 (Table 1).

In the maze assay, the first PC (PC1), interpreted as 'activity-exploration score', had a strong negative loading for latency to first complete the maze and strong positive loadings for both entries into all maze arms and directional maze use. This 'activity-exploration score' represents a continuum of fish of which those with a higher score are more exploratory and active, completing the maze more rapidly and frequently, as well as having higher general activity levels. The second PC (PC2), interpreted as 'boldness score', had strong positive loadings for both latency to exit the refuge and total time spent in the refuge. This 'boldness score' represents a continuum of fish of which those with a higher score are shyer, taking longer to first exit the refuge and spending more time in the refuge. The activity-exploration score and the boldness score accounted for 42% and 31% of the variance in the data, respectively.

In the reproductive assay, the first PC (PC1), interpreted as a 'reproductive interest and activity score', had strong positive loadings for both the total time spent in the 5 cm zone closest to the stimulus female and weighted association score, as well as a strong negative loading for total number of zone entries. This reproductive interest and activity score represents a continuum of males with higher scores indicating more intense association behaviour and lower activity levels. The second PC (PC2) in the reproductive assay, interpreted as the 'latency to associate score', had a strong positive loading for the time taken to enter the 5 cm zone abutting the female compartment, with a high score therefore indicating a fish that took longer to first associate with the stimulus female. The reproductive interest and activity score and the latency to associate score accounted for 61% and 26% of the variance in the data, respectively.

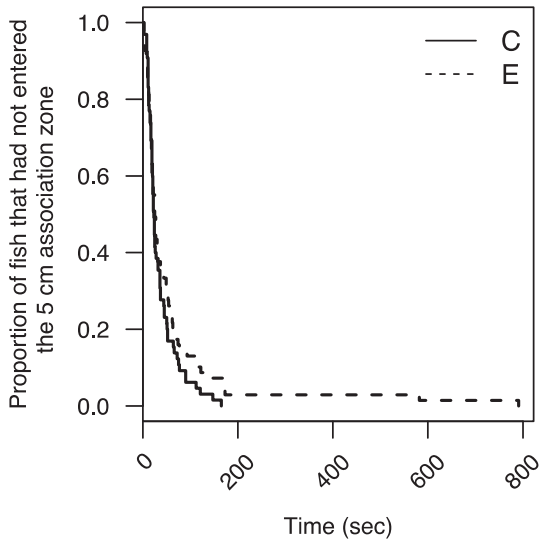

Fig. 2. Kaplan–Meier survival curves showing the time taken for control (C, $n = 65$) and 17β-TB-exposed (E, $n = 70$) males to first enter a 5 cm zone abutting the stimulus (unexposed) female compartment during the reproductive assay (s).

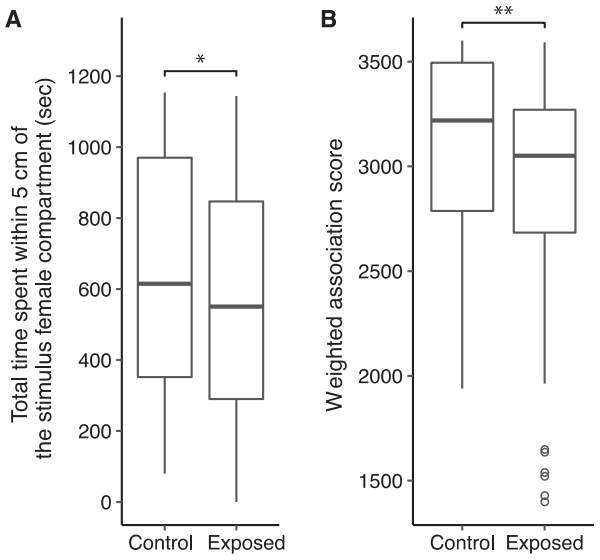

Fig. 3. The (A) total time spent by males within 5 cm of the compartment containing the stimulus (unexposed) female (s) during the reproductive assay, and (B) male weighted association score (representing the use of the entire main tank area relative to the position of the stimulus female, with a higher score indicating a male performing a greater amount of association behaviour) (control: $n = 65$, 17β-TB-exposed: $n = 70$). Box plots show the median (horizontal line), upper and lower quartiles (box length), and the range, with outliers being represented by empty circles. $^{*}p < 0.05$, $^{**}p < 0.01$.

Table 1
Principal component analysis on endpoints measured across the maze and reproductive assays. Strong loadings (i.e. magnitude ≥0.5) appear in bold.

PCA (with oblique rotation)	Loadings	
	PC1	PC2
Maze assay		
Latency to exit refuge	−0.10	**0.81**
Total time in the refuge	0.05	**0.86**
Latency to complete the maze	**−0.80**	−0.26
Entries into all maze arms	**0.77**	−0.24
Directional maze use	**0.90**	−0.06
Eigenvalues	2.08	1.55
Proportion of variance explained	0.42	0.31
Reproductive assay		
Latency to enter 5 cm zone	−0.03	**0.98**
Total time in 5 cm zone	**0.90**	−0.17
Weighted association score	**0.92**	−0.09
Total zone entries	**−0.89**	−0.25
Eigenvalues	2.45	1.06
Proportion of variance explained	0.61	0.26

Table 2
Pearson's correlation tests within each exposure treatment for the first two PCs in each behavioural assay (unexposed: $n = 65$; exposed: $n = 70$).

Maze assay	Reproductive assay	Unexposed		Exposed	
		r	*p*-value	*r*	*p*-value
PC1	PC1	−0.168	0.181	0.170	0.159
PC1	PC2	−0.187	0.135	−0.141	0.246
PC2	PC1	0.016	0.898	−0.082	0.498
PC2	PC2	0.009	0.946	0.072	0.556

For both unexposed and exposed males, no significant correlations were seen between PCs across the maze and reproductive assays (Table 2).

3.4. Sperm analysis

No significant main effect of 17β-TB exposure was seen on any sperm traits assessed with CASA, or sperm viability (ANCOVA: all $p > 0.05$; Table S6). However, exposure was associated with a significant change in the relationship between male condition index and VCL (ANCOVA: $F_{1,78} = 5.96$, $p = 0.017$; Table S6). Specifically, while a significant negative correlation was seen in unexposed fish between condition index and VCL (Spearman's rank correlation: $r_s = -0.30$, $p = 0.050$), this relationship was seen to be positive in males exposed to 17β-TB (Spearman's rank correlation: $r_s = 0.33$, $p = 0.037$) (Fig. 4). Moreover, exposure induced non-significant marginal shifts in the relationship between condition index and VAP (ANCOVA: $F_{1,78} = 3.41$, $p = 0.069$), and the relationship between condition index and VSL (ANCOVA: $F_{1,78} = 3.33$, $p = 0.072$) (Table S6). No significant effect of exposure to 17β-TB was observed on total sperm count (quasi-Poisson GLM: $t = 0.94$, $p = 0.354$).

3.5. Morphology

Exposure to 17β-TB did not significantly affect male standard length (*t*-test: $t = -0.28$, $p = 0.780$) or weight (Mann-Whitney *U* test: $U = 2033$, $p = 0.288$). However, exposure was associated with a significant increase in male condition index (Mann-Whitney *U* test: $U = 1760$, $p = 0.023$; Fig. 5).

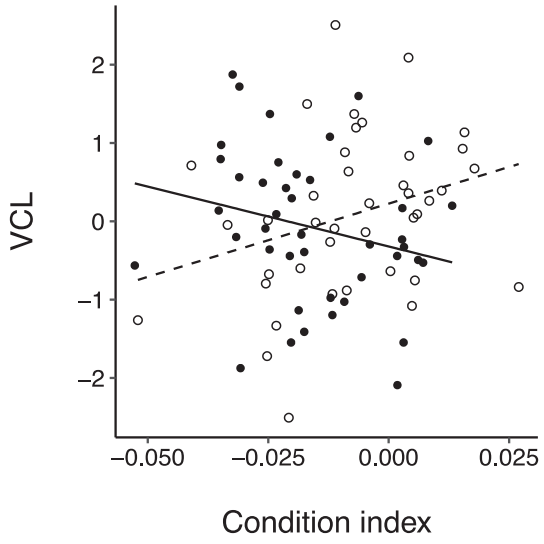

Fig. 4. Sperm curvilinear velocity (VCL, μm/s) as a function of condition index (i.e. relative mass) for males in the control ($n = 42$) and 17β-TB-exposed ($n = 40$) treatments. The filled circles and solid trend line represent unexposed males, while the unfilled circles and dashed trend line represent 17β-TB-exposed males.

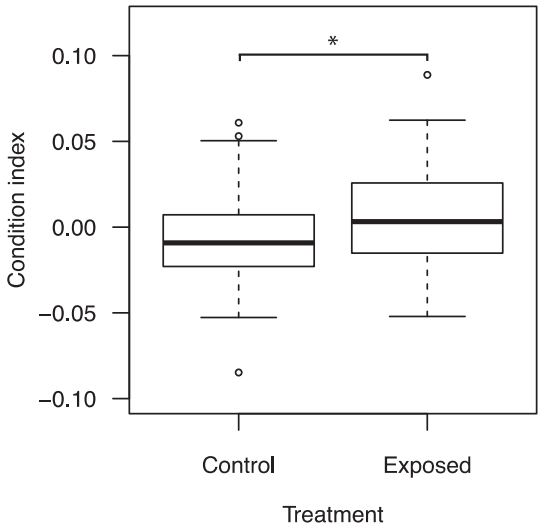

Fig. 5. Boxplots showing the condition index of males in the control ($n = 65$) and 17β-TB-exposed ($n = 70$) treatments. *$p < 0.05$.

4. Discussion

We report that short-term exposure to a field-realistic level of the widespread agricultural pollutant 17β-trenbolone (17β-TB) altered key fitness-related behaviours in male fish, although impacts of exposure were subtle and were only seen in one of two independent behavioural contexts (i.e. where behaviours did not correlate across contexts). No significant effect of exposure was seen on male boldness, activity, or exploratory behaviour in a maze arena, although exposure-induced behavioural changes were seen in the same individuals when tested for reproductive behaviour. Specifically, exposed males took longer to first associate with, and spent less time within close proximity to, a stimulus (unexposed) female. Further, although no significant main effects of exposure were detected when males were assessed for sperm function (CASA and sperm viability) or sperm quantity (total sperm count), exposure was associated with disruption of key relationships between male morphological and sperm function traits. Finally, exposure was associated with a significant increase in male relative mass.

4.1. Boldness, activity and exploration

Contrary to our first hypothesis, we found no significant effect of exposure to 17β-TB on male boldness, activity, or exploratory behaviour in a novel environment (maze arena). Contamination with pharmaceuticals has been associated with altered swimming behaviour and locomotor activity in a variety of aquatic species. For example, using a novel tank diving test, it has been shown that exposure of adult male zebrafish (*Danio rerio*) to the synthetic contraceptive estrogen 17α-ethinylestradiol (EE2) alters swimming behaviour and spatial use of a novel tank (Reyhanian et al., 2011), and contamination with the antidepressant drug citalopram increases locomotor activity in adult female three-spine stickleback (*Gasterosteus aculeatus*) (Kellner et al., 2016). Moreover, although no effect of exposure was seen in the present study, recent research has shown that these behaviours are potentially susceptible to disruption by 17β-TB. Specifically, in recent work investigating interactive effects of 17β-TB exposure and temperature, in which an identical maze assay was used as that employed in the present study, Lagesson et al. (2019) reported an increase in boldness (i.e. reduced time to first exit the refuge) and exploration (i.e. reduced time to first complete the maze) in male *G. holbrooki*, although general activity levels were not assessed. The contrasting results seen between Lagesson et al. (2019) and the present study are most likely due to the different temperatures employed. Specifically, the present study tested impacts of

17β-TB at 24 °C (a standard temperature for housing *G. holbrooki*; Otto, 1974), whereas Lagesson et al. (2019) employed low- and high-temperature treatments of 20 °C and 30 °C. It is also possible that these contrasting findings are due to differences in exposure concentrations employed across studies, with Lagesson et al. (2019) having exposed fish at 2.6 and 3.3 ng/L, while fish in the present study were exposed at 16 ng/L.

Interestingly, impacts of 17β-TB on this suite of behaviours seem to be relatively more consistent across temperatures and exposure concentrations in female fish. Specifically, in an experiment utilising an identical exposure design as was used in the current study, where fish were exposed to the same average 17β-TB concentration (i.e. 16 ng/L), and tested at the same temperature (24 °C) in the same maze assay, Bertram et al. (2018a) showed that 17β-TB exposure alters activity and exploratory behaviour in adult female *G. holbrooki*. Specifically, exposed females demonstrated increased activity (i.e. entered a greater number of maze arms in total) and exploratory behaviour (i.e. were faster to first complete the maze and swam significantly more full maze lengths), while boldness was not significantly affected (Bertram et al., 2018a). Similarly, Lagesson et al. (2019) demonstrated increased exploratory behaviour in female fish, although an increase was also seen in boldness, and general activity levels were not assessed. In combination, these findings indicate that effects of field-realistic concentrations of 17β-TB on this suite of behaviours are likely sex-specific, with females being relatively more vulnerable to disruption than males, although more research is clearly needed to elucidate sex- and temperature-specific effects of exposure.

4.2. Reproductive behaviour

Males exposed to 17β-TB exhibited depressed levels of association behaviour when presented with an unexposed stimulus female, which was counter to our second hypothesis. Specifically, exposed males were, on average, slower to first reach a 5 cm zone abutting a compartment containing a stimulus female, and spent less time overall within this zone, although these behavioural shifts were subtle. Moreover, in terms of spatial use of the entire main tank area, exposed males spent less time in close proximity to the stimulus female. Recent research has demonstrated that exposure to 17β-TB at concentrations reflecting those present in the environment can alter reproductive behaviours in male (and female) fish. For example, field-realistic levels of 17β-TB have consistently been shown to intensify male coercive 'sneaking' copulatory behaviour in another poeciliid, the guppy (2 ng/L, M.G. Bertram, unpublished data; 4 ng/L, Bertram et al., 2018b; 8 ng/L, Tomkins et al., 2017; 22 ng/L, Bertram et al., 2015). Although sneaking behaviour was not tested in this study, given that both guppies and mosquitofish are internal fertilisers, meaning that males must be in close proximity to females to copulate, males in the present study were expected to exhibit increased reproductive behaviour (i.e. be faster to associate with, and spend more time in close proximity to, a female).

This apparent disparity in findings is likely due to behavioural endpoints measured across studies being independent, with coercive copulatory behaviour being differentially affected by exposure than spatial use of a tank relative to a female confined behind a partition. Taken together, these findings merit further investigation as they suggest that 17β-TB-induced increases in male copulatory behaviour are opportunistic, i.e. males increase copulatory behaviour when the opportunity is available (free-swimming interactions with a female), however, when this opportunity is not available, males exhibit disinterest towards females. Such endpoint-specific effects have been reported previously. For example, in the aforementioned studies reporting 17β-TB-increases in male coercive copulatory behaviour in guppies, no significant effect of exposure was seen on male courtship behaviour (Bertram et al., 2015; Bertram et al., 2018b; M.G. Bertram, unpublished data)—except when in the presence of a rival male (Tomkins et al., 2017). Furthermore, although stimulus females were unexposed in the current study to preclude any potential effect of female exposure on male behaviour, male reproductive behaviour appears to be affected by female exposure. When male and female *G. holbrooki* from the same treatment group (i.e. unexposed or exposed to 17β-TB at 6 ng/L) were tested in free-swimming behavioural trials, no effect of exposure was seen on male reproductive behaviours (e.g. copulatory behaviour, orienting, chasing) (Saaristo et al., 2013).

4.3. Sperm analysis

No significant main effects of 17β-TB exposure were detected on assessed measures of sperm function (CASA, viability) or quantity (total sperm count), which was inconsistent with our third hypothesis. Due to the mode of action of 17β-TB, gonads are expected to be a primary target organ (reviewed in Ankley et al., 2018). Indeed, the presence of ovotestes (i.e. intersex tissue) has been reported following developmental 17β-TB exposure in fish (e.g. mosquitofish, *Gambusia affinis affinis*: Sone et al., 2005) and amphibians (e.g. western clawed frog, *Xenopus tropicalis*: Olmstead et al., 2012). Developmental exposure has also been shown to alter testicular growth (e.g. hypertrophy of Wolffian ducts) in amphibians (western clawed frog: Olmstead et al., 2012; African clawed frog, *Xenopus laevis*: Haselman et al., 2016). Moreover, alterations in testicular tissue have been reported following adult exposure to 17β-TB. For example, exposure of adult males resulted in increased numbers of spermatozoa (and fewer spermatogonia) in Japanese medaka (*Oryzias latipes*, Park et al., 2009), thinned germinal epithelia and enlarged sperm-filled lumens in fathead minnow (Ankley et al., 2003), and enlarged testes containing a greater number of spermatozoa in zebrafish (Örn et al., 2006; Baumann et al., 2014). Therefore, given that previous research has reported impacts of 17β-TB exposure on various gonad-related endpoints, that no significant effects of 17β-TB were detected on sperm function or quantity in the present study is likely due to exposure concentration and/or timing. More specifically, effects of 17β-TB on gonad-related endpoints are often seen at higher exposure concentrations than were used in the present study and/or when exposure occurs during active sexual differentiation and development (Ankley and Johnson, 2004). Therefore, it is possible that, in our study, exposure of adult fish to a low environmentally realistic 17β-TB concentration was not sufficient to elicit effects on sperm function or quantity, although we cannot rule out potential differences in species sensitivities.

While no significant main effects of 17β-TB were seen on sperm traits, exposure altered the relationship between male morphology and sperm function. Specifically, a significant negative correlation was seen between male condition index and sperm curvilinear velocity in unexposed males, while a significant positive association was seen between these traits in exposed fish. Moreover, a similar but marginally non-significant interaction was seen in terms of both sperm average path velocity and straight-line velocity. That a negative association was detected between male condition index and sperm function (i.e. curvilinear velocity) in control males suggests a potential trade-off between these traits. Producing numerous fast-swimming sperm is costly (Rahman et al., 2013), meaning that increased investment in this ejaculate trait is expected to result in reduced investment in body condition (Parker et al., 2013). Indeed, elevations in both pre- and post-copulatory investment in reproduction have been shown to have negative effects on body condition (e.g. Mappes et al., 1996) and maintenance (e.g. McNamara et al., 2013) across diverse species. That a positive association between body condition and sperm function was seen in exposed fish indicates a disruption of this trade-off. For males inhabiting contaminated systems, this has broad implications for life-history strategies, particularly in terms of optimisation of investment in reproduction. Clearly, more research is needed to uncover how exposure to sub-lethal levels of 17β-TB—and pharmaceutical contaminants more generally—may influence sperm function in exposed wildlife, including trade-offs between investment in sperm function and other fitness-related traits.

4.4. Morphology

In line with our fourth hypothesis, 17β-TB exposure resulted in increased male condition index. This effect was subtle, however, given that neither standard length nor weight alone was significantly affected by exposure. This means that the observed increase in relative mass was the result of a small increase in weight as well as exposed males having somewhat smaller standard lengths. This relative weight gain is expected to be the result of a slight increase in mass as morphogenesis of skeletal elements is complete in adults and, hence, no effect of 17β-TB on standard length is expected (Pandey, 1969; Baatrup and Junge, 2001). This finding is consistent with previous work investigating impacts of 17β-TB at 2.6 and 3.3 ng/L on mosquitofish (Lagesson et al., 2019), and at 4 ng/L on guppies (Bertram et al., 2018b), which showed that 21-day exposure increases male condition index. Further, exposure at 22 ng/L for the same period caused an increase in both condition index and weight (Bertram et al., 2015), suggesting a more pronounced anabolic effect at this higher dosage. This sensitivity to weight gain seems to be sex-specific given that a range of previous studies have reported no significant change in standard length, weight, or condition index in female guppies exposed for 21 days at 2 ng/L (Tomkins et al., 2018), 4 ng/L (Tomkins et al., 2016), 8 ng/L (Tomkins et al., 2017) or 22 ng/L (Bertram et al., 2015), or in female mosquitofish at 16 ng/L (Bertram et al., 2018a). Further, while no change in morphological characteristics was seen in female fathead minnows (*Pimephales promelas*) exposed to 17β-TB at 5 ng/L or 50 ng/L, concentration-dependant weight increase was observed at higher levels (0.5, 5 and 50 μg/L; Ankley et al., 2003).

5. Conclusion

We report that 21-day exposure to an environmentally realistic level (average exposure concentration: 16 ng/L) of the widely administered veterinary steroid and pervasive agricultural pollutant 17β-TB caused context-specific behavioural shifts in male fish. Specifically, exposure resulted in changes to male behaviour in a reproductive context, while no significant change was seen in terms of boldness, activity, or exploratory behaviour in a novel environment. Observed effects of treatment on reproductive behaviour were subtle and further investigations are warranted to uncover how these trait changes might translate to the field. In addition to behavioural effects, exposure disturbed relationships between male morphology and sperm function, and altered male body condition. Broadly, our results highlight the importance of studies in behavioural ecotoxicology testing behaviour across multiple fitness-related contexts, as behaviours performed in different contexts may be differentially vulnerable to disturbance by contaminant exposure. Further, our findings support a growing body of literature revealing the capacity of pharmaceutical contaminants to alter key traits and behaviours at concentrations that have repeatedly been detected in the environment, with potential implications for individual fitness, population dynamics, and evolutionary processes in exposed wildlife.

Ethical statement

Animal housing and experimental procedures performed for this study were approved by the Biological Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2013/09) and complied with Australian law.

Authors' contributions

M.G.B., J.M.M., M.S. and B.B.M.W. conceived and designed the study. M.G.B., J.M.M. and T.E.E. performed the experiments. M.G.B., M.M. and N.D.S.D. analysed the data. Sperm analysis was coordinated by M.K.O.B. and carried out by M.G.B. and T.E.E., with assistance from S.L.L. The manuscript was drafted by M.G.B. All authors contributed to revising the manuscript and gave their final approval for publication.

Competing interests

The authors declare that we have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.01.382.

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Supplementary material

1. Materials and methods

1.1. SPERM ANALYSIS METHODS

The computer-assisted sperm analysis (CASA) assay firstly involved euthanised males being positioned on their right lateral side on a glass petri dish under a dissection microscope (Leica MZ9.5), before being covered with 500 µL of extender solution (207 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 10 mM Tris, pH 7.5; Locatello et al. 2006), in which sperm remain inactive. The gonopodium was swung forward three times using a probe and gentle pressure was applied to the abdomen, just anterior to the base of the gonopodium, to release the entire ejaculate. A 5 µL aliquot of ejaculate in extender medium was then added to 20 µL of activation solution (150 mM KCl and 2 mg/mL bovine serum albumin) and re-suspended 100 times to break up spermatozeugmata (i.e. sperm bundles). A 4 µL drop of this solution was then loaded onto a 12-cell multitest slide (MP Biomedicals, Aurora, OH, USA)—that had previously been coated with 1% polyvinyl alcohol for 3 min to prevent sperm sticking (Wilson-Leedy and Ingermann 2007)—and a coated coverslip placed gently on top. A negative phase-contrast microscope coupled with a CASA system (v. 14, CEROS, Hamilton-Thorne Biosciences, Beverly, MA) was used to assess sperm function. A minimum of 1000 sperm were tracked per male (mean = 1101.72, SE = 7.27, n = 82).

A fluorescence-based assay (L-7011; Molecular Probes Inc., OR, USA) was used to analyse the sperm viability of each male, following protocols originally described by Evans (2009), with some modifications. This involved staining live sperm cells green using a membrane-permeant nucleic acid stain (SYBR-14), and dead cells red with propidium iodide. Briefly, a 10 µL subsample of ejaculate in extender solution was drawn with a micropipette and gently resuspended 50 times to break up spermatozeugmata. This solution was mixed with 10 µL of live cell dye (1 µl 1 mM SYBR-14 in 50 µl Live Cell Imaging Solution, containing 0.005% bovine serum albumin). The sample was then vortexed and incubated for 10 mins. After incubation, 2 µl of 2.4 mM propidium iodide was added to the sample, before vortexing and an additional 10 min of incubation. A 4 µL subsample of this solution was then transferred to a glass slide, coverslipped, and viewed under a fluorescence microscope (Leica DFC425C). Ten non-overlapping fields were captured at ×40 magnification for each male and the green and red sperm cells counted manually using the image analysis software ImageJ (U.S. National Institutes of Health,

Bethesda, Maryland, USA), providing an average live/dead sperm viability. A minimum of 200 sperm cells were counted per male (mean = 250.04, SE = 27.61, n = 82).

Total sperm count was estimated following protocols originally described by Evans et al. (2003), with some modifications. Males were stripped of ejaculate according to the methods detailed above, although, here, males were covered with 2 mL of activation solution. All sperm bundles were then collected in 500 μL of activation solution, which was gently resuspended 100 times. Trypan blue (5 μL) and 10% formalin (10 μL) were then added—to stain and kill the cells, respectively—before the solution was vortexed for 10 seconds to ensure an even dilution of sperm. A 10 μL subsample of this solution was then diluted with 50 μL of activation solution, with this solution being stored at 4 °C until analysis. At analysis, the solution was again vortexed to ensure a homogenous sample, before two 10 μL aliquots were transferred into each well of an improved Neubauer haemocytometer (100 μm deep). The haemocytometer was then incubated for 10 min to allow cells to settle, before being viewed under a brightfield microscope at ×40 magnification. For each male, 10 images were captured of 200 × 200 μm counting squares in either haemocytometer well. Sperm was then counted automatically using Image-Pro Plus software (Media Cybernetics, Silver Spring, USA), to determine the average number of sperm per square.

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1.2. SUPPLEMENTARY TABLES

Table S1. Definitions of sperm function traits quantified using computer-assisted sperm analysis (CASA).

Sperm trait	Definition
Average path velocity (VAP) (μm/s)	Time-averaged velocity of a sperm along its average path, calculated by smoothing the curvilinear trajectory.
Straight-line velocity (VSL) (μm/s)	Time-averaged velocity along the straight line between a sperm's first and last detected position.
Curvilinear velocity (VCL) (μm/s)	Average point-to-point velocity measured over the actual sperm track.
Path linearity (LIN) (%)	Departure of actual sperm track from the point-to-point sperm track (VSL/VCL*100).
Motility (%)	Sperm cells with VAP, VSL and VCL values above predetermined thresholds. Following Evans (2009), threshold values used for defining static cells were 24.9 μm/s for VAP and VCL, and 15 μm/s for VSL.

Evans, J.P., 2009. No evidence for sperm priming responses under varying sperm competition risk or intensity in guppies. Naturwissenschaften 96, 771–779. http://dx.doi.org/10.1007/s00114-009-0529-6.

2. STATISTICAL ANALYSIS

2.1 Supplementary tables

Table S2. Mean (±SE) behavioural responses of males in the maze assay (unexposed: n = 65; exposed: n = 70).

	Treatment	
Behavioural response	Control	Exposed
Latency to first exit refuge (sec)	38.63 ± 7.25	33.04 ± 5.60
Total time in refuge (sec)	170.51 ± 23.47	193.31 ± 29.25
Latency to first complete the maze (i.e., reach A6) after first exiting the refuge (sec)	224.86 ± 29.34	190.00 ± 22.49
Combined number of entries into all maze arms (i.e., A1–A6)	115.51 ± 5.84	114.33 ± 6.20
Number of full maze lengths swam	6.65 ± 0.52	6.39 ± 0.57

Table S3. Mean (±SE) behavioural responses of fish in the reproductive assay (unexposed: n = 65; exposed: n = 70).

	Treatment	
Behavioural response	Control	Exposed
Time taken to first reach the 5 cm zone abutting the female compartment (sec)	35.85 ± 4.16	59.59 ± 14.09
Total time spent within 5 cm zone (sec)	647.37 ± 42.40	566.96 ± 40.29
Weighted association score	3060.09 ± 61.53	2896.10 ± 67.39
Combined number of entries into all main tank zones (i.e., Z1–Z3)	71.66 ± 6.76	69.49 ± 5.17

Table S4. Mean (±SE) for measures of sperm quality (i.e. sperm average path velocity [VAP], straight-line velocity [VSL], curvilinear velocity [VCL], path linearity [LIN], motility [MOT], viability) and quantity (i.e. total sperm count). Separate males were tested for either sperm quality traits (unexposed: $n = 42$; exposed: $n = 40$) or sperm quantity (unexposed: $n = 22$; exposed: $n = 26$).

	Treatment	
Sperm trait	Control	Exposed
Sperm quality		
VAP (μm/s)	84.80 ± 2.64	87.86 ± 2.54
VSL (μm/s)	78.76 ± 2.61	81.69 ± 2.39
VCL (μm/s)	94.47 ± 2.31	97.00 ± 2.31
LIN (%)	82.33 ± 1.19	83.10 ± 0.66
MOT (%)	86.10 ± 2.31	86.88 ± 1.55
Viability (%)	71.93 ± 4.24	75.49 ± 4.08
Sperm quantity		
Total sperm count ($\times 10^6$)	12.4140 ± 1.1524	13.9185 ± 1.3989

Table S5. Mean (±SE) morphological traits of experimental males (unexposed: $n = 65$; exposed: $n = 70$).

	Treatment	
Morphological trait	Control	Exposed
Standard length (mm)	21.41 ± 0.17	21.48 ± 0.20
Weight (g)	0.1519 ± 0.0052	0.1646 ± 0.0069
Condition index	-0.0055 ± 0.0032	0.0051 ± 0.0033

2.2. COVARIATE-RESPONSE RELATIONSHIPS

Behavioural trials: boldness, activity, and exploration

A positive association was detected between male condition index and both total time spent within the enclosed refuge at the beginning of the maze (ANCOVA: $F_{1,131} = 5.41$, $p = 0.022$) and the number of full maze lengths swam (ZINB GLM: $z = 2.28$, $p = 0.023$). Further, a non-significant marginal trend was detected towards males with higher condition indexes entering a greater number of maze arms (quasi-Poisson GLM: $t = 1.96$, $p = 0.052$). Condition index did not, however, co-vary significantly with any of the other behavioural responses recorded in the maze assay (all $p > 0.05$).

Behavioural trials: reproductive behaviour

In the reproductive assay, males with a higher condition index were, on average, quicker to first enter the 5 cm zone abutting the stimulus female compartment (parametric survival regression: $z = -2.08$, $p = 0.038$). Further, a non-significant marginal trend was detected towards fish with a higher condition index possessing a higher weighted association score (ANCOVA: $F_{1,132} = 2.94$, $p = 0.089$). No other such associations were detected between condition index and the other behavioural responses recorded in the reproductive assay (all $p > 0.05$).

3. Results

3.1 SUPPLEMENTARY TABLES

Table S6. Test statistics from ANCOVA models used to examine potential impacts of exposure to 17β-trenbolone on sperm quality traits in experimental males (unexposed: $n = 42$; exposed: $n = 40$). Measures of sperm quality include average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), path linearity (LIN) and motility (MOT), as well as sperm viability.

Sperm trait	Predictor	*df*	*F value*	Pr(>*F*)
VAP (μm/s)	Treatment	1	0.55	0.462
	Condition index	1	0.78	0.379
	Treatment * Condition index	1	3.41	0.069†
VSL (μm/s)	Treatment	1	0.51	0.478
	Condition index	1	1.01	0.319
	Treatment * Condition index	1	3.33	0.072†
VCL (μm/s)	Treatment	1	0.39	0.533
	Condition index	1	0.37	0.547
	Treatment * Condition index	1	5.96	0.017*
LIN (%)	Treatment	1	0.27	0.603
	Condition index	1	2.35	0.129
	Treatment * Condition index	1	0.54	0.464
MOT (%)	Treatment	1	0.64	0.427
	Condition index	1	0.07	0.796
	Treatment * Condition index	1	0.23	0.632
Viability (%)	Treatment	1	0.05	0.824
	Condition index	1	1.32	0.255
	Treatment * Condition index	1	1.87	0.175

Note: * $p < 0.05$; † $p < 0.1$

SECTION TWO

Chapter 5

The antidepressant fluoxetine alters mechanisms of pre- and post-copulatory sexual selection in the eastern mosquitofish (*Gambusia holbrooki*)

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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**
Jointly conceived and designed the study (with TEE), conducted the experiments, performed the statistical analysis, wrote and submitted the manuscript.	80%

The following co-authors contributed to the work:

Name	**Nature of contribution**	**Extent of contribution**
Tiarne E. Ecker	Jointly conceived and designed the study, contributed to data collection, statistical analysis, and drafting the manuscript.	15%
Minna Saaristo	Contributed to experimental design and manuscript preparation.	1%
Bob B.M. Wong	Contributed to experimental design and manuscript preparation.	1%
Moira K. O'Bryan	Coordinated sperm analysis.	1%
John B. Baumgartner	Assisted with statistical analyses.	1%
Jake M. Martin	Assisted with data collection.	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:  Date: 27/11/2018

Main supervisor signature:  Date: 27/11/2018

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The antidepressant fluoxetine alters mechanisms of pre- and post-copulatory sexual selection in the eastern mosquitofish (*Gambusia holbrooki*)☆

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ABSTRACT

Contamination of aquatic habitats with pharmaceuticals is a major environmental concern. Recent studies have detected pharmaceutical pollutants in a wide array of ecosystems and organisms, with many of these contaminants being highly resistant to biodegradation and capable of eliciting sub-lethal effects in non-target species. One such pollutant is fluoxetine, a widely prescribed antidepressant, which is frequently detected in surface waters globally and can alter physiology and behaviour in aquatic organisms. Despite this, relatively little is known about the potential for fluoxetine to disrupt mechanisms of sexual selection. Here, we investigate the impacts of 30-day exposure to two environmentally realistic levels of fluoxetine (low and high) on mechanisms of pre- and post-copulatory sexual selection in the eastern mosquitofish (*Gambusia holbrooki*). We tested 1) male mating behaviour in the absence or presence of a competitor male, and 2) sperm quality and quantity. We found that high-fluoxetine exposure increased male copulatory behaviour in the absence of a competitor, while no effect was detected under male-male competition. Further, fluoxetine exposure at both concentrations increased total sperm count relative to males from the control group, while no significant change in sperm quality was observed. Lastly, low-fluoxetine males showed a significant reduction in condition index (mass relative to length). Our study is the first to show altered mechanisms of both pre- and post-copulatory sexual selection in an aquatic species resulting from environmentally realistic fluoxetine exposure, highlighting the capacity of pharmaceutical pollution to interfere with sensitive reproductive processes in wildlife.

1. Introduction

Numerous pharmaceutical pollutants are capable of altering ecologically important traits and behaviours in wildlife (Boxall et al., 2012; Arnold et al., 2014; Brodin et al., 2014). Pharmaceutically active compounds enter the environment via multiple pathways, including the excretion of chemicals used for human and veterinary healthcare, discharge from manufacturing and disposal of unused medications (Heberer, 2002). Of the approximately 5000 actively marketed pharmaceutical products, over 600 have now been detected in the environment globally (Küster and Adler, 2014). Worldwide consumption of pharmaceuticals is also increasing due to a growing and ageing human population (Khetan and Collins, 2007; Arnold et al., 2014). Antidepressant pharmaceuticals pose a distinct threat to wildlife as they are specifically designed to induce physiological effects at low concentrations (Khetan and Collins, 2007) and have a particularly strong potential to alter behaviour (Arnold et al., 2014; Brodin et al., 2014). The most frequently

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0269-7491/

prescribed class of antidepressants is the selective serotonin reuptake inhibitors (SSRIs) (Fong and Ford, 2014), which act by inhibiting the reuptake of the monoamine neurotransmitter serotonin (5-hydroxytryptamine) by the pre-synaptic nerve cleft, thereby increasing the effect of serotonin on the post-synaptic nerve (Stahl, 1998). Serotonin is a ubiquitous neurotransmitter, present in all phyla possessing nervous systems (Weiger, 1997). As such, SSRIs have the potential to alter a range of ecologically important traits and behaviours in wildlife.

One SSRI of environmental concern is fluoxetine, which is used to treat major depression and other psychiatric disorders in humans, and is among the most commonly prescribed pharmaceuticals (Wong et al., 2005). Present in aquatic environments globally, fluoxetine has been detected in surface waters at concentrations typically ranging from <1 to 100 ng/L (e.g., Kolpin et al., 2002; Fernández et al., 2010; Gardner et al., 2012; Hughes et al., 2013), although levels as high as 596 ng/L have been reported in systems receiving wastewater discharge (Benotti and Brownawell, 2007). In addition, fluoxetine has been found to bioaccumulate in fish tissues, especially in the brain (Brooks et al., 2005; Schultz et al., 2010). Exposure to fluoxetine can influence a range of ecologically important traits, including development (Japanese medaka, *Oryzias latipes*, Foran et al., 2004; western mosquitofish, *Gambusia affinis*, Henry and Black, 2008), reproduction (zebrafish, *Danio rerio*, Lister et al., 2009) and survival (guppy, *Poecilia reticulata*, Pelli and Connaughton, 2015), as well as various morphological and physiological characteristics (e.g., altered growth in *P. reticulata*, Pelli and Connaughton, 2015; impaired cardiovascular and ventilatory response to hypoxia in Gulf toadfish, *Opsanus beta*, Panlilio et al., 2016). Fluoxetine exposure has also been linked with alterations in a variety of behaviours in fish, such as activity (Siamese fighting fish, *Betta splendens*, Kohlert et al., 2012; Arabian killifish, *Aphanius dispar*, Barry, 2013), feeding and foraging (fathead minnow, *Pimephales promelas*, Stanley et al., 2007; *P. promelas*, Weinberger and Klaper, 2014), aggression (*B. splendens,* Lynn et al., 2007; *B. splendens*, Dzieweczynski and Hebert, 2012; *A. dispar,* Barry, 2013), sociability (*A. dispar*, Barry, 2013; *O. latipes*, Ansai et al., 2016) and antipredator behaviour (*P. reticulata*, Pelli and Connaughton, 2015; eastern mosquitofish, *Gambusia holbrooki*, Martin et al., 2017). However, variability in fluoxetine sensitivity reported across studies, model species and biological responses has made ascertaining what fluoxetine concentrations pose a risk to aquatic wildlife challenging (Stewart et al., 2014; Sumpter et al., 2014), highlighting the need for further research investigating the impacts of environmentally realistic concentrations of fluoxetine on ecologically relevant traits. Having received little attention relative to other endpoints, this is especially true for the effects of exposure to fluoxetine on mechanisms of sexual selection.

Sexual selection can occur both before (i.e., pre-copulatory) and after (i.e., post-copulatory) mating (Andersson and Simmons, 2006), with both of these processes being vulnerable to disruption by pharmaceutical pollution. Studies using pharmacological dosages have demonstrated that treatment with fluoxetine can induce male sexual dysfunction in humans (Gregorian et al., 2002; Serretti and Chiesa, 2009) and rodents (Taylor et al., 1996; Matuszcyk et al., 1998). However, findings from the handful of studies that have examined the impacts of environmentally realistic concentrations of fluoxetine on reproductive behaviour have been mixed. Specifically, while some studies have reported an increase in certain reproductive behaviours following fluoxetine exposure (Weinberger and Klaper, 2014), others have reported a decrease (Forsatkar et al., 2014), or no significant effect (Schultz et al., 2011; Dzieweczynski and Hebert, 2012). Further, the effects of fluoxetine on male mating behaviour under male-male competition, where males compete for the opportunity to reproduce, has received very little attention, despite being a central component of pre-copulatory sexual selection (Andersson, 1994). Clearly, the potential impacts of fluoxetine on mating and reproductive behaviours in wildlife require further investigation.

In species where females mate multiply (polyandry), an important component of post-copulatory sexual selection is sperm competition, where the sperm of multiple males compete to fertilise available ova (Andersson and Simmons, 2006). In polyandrous species, a key predictor of each male's fertilisation success is his proportional contribution to the sperm pool (Parker, 1998), with elevated sperm production allowing males to copulate more often and allocate more sperm to each ejaculate (Parker, 1982). Sperm quality traits such as viability and speed can also influence fertilisation success under sperm competition (Snook, 2005). Because treatment with SSRIs, including fluoxetine, can reduce fertility in human males (reviewed in Brezina et al., 2012; Nørr et al., 2016), considerable attention has been paid to the impacts of fluoxetine at pharmacological levels on fertility in rodent models (e.g., Bataineh and Daradka, 2007; Alzahrani, 2012; Monteiro Filho et al., 2014). In addition, research in aquatic species has reported reproductive dysfunction in species as diverse as male goldfish (*Carassius auratus*, Mennigen et al., 2010) and zebra mussels (*Dreissena polymorpha*, Fong, 1998). Despite this, the potential effects of exposure to environmentally realistic levels of fluoxetine on both sperm quality and quantity remain to be investigated in *any* aquatic vertebrate.

The eastern mosquitofish is a small, internally fertilising poeciliid fish with a widespread geographic distribution (Pyke, 2005, 2008) that is attracting increased interest as a model for investigating the impacts of chemical pollutants (e.g., Saaristo et al., 2013, 2014; Magellan et al., 2014; Martin et al., 2017; Melvin et al., 2017). Mosquitofish have a coercive mating system, where males copulate with females by 'sneaking' from behind and thrusting the tip of their gonopodium—a modified anal fin used for internal fertilisation—into the female's genital pore (Bisazza et al., 2001). No courtship occurs and, although females may exert some control over the outcome of unsolicited mating attempts by spending more time associating with preferred males, male sexual coercion and male-male competition are the primary modes of pre-copulatory sexual selection in this species (Bisazza et al., 2001). Wild mosquitofish females are typically inseminated by multiple males (Zane et al., 1999) and are capable of storing sperm for several months (Evans et al., 2003), putting the sperm of multiple males in direct competition. Further, in this species, approximately ninety-percent of all broods are sired by multiple males, making sperm competition a major source of post-copulatory sexual selection (Zane et al., 1999). These attributes make mosquitofish an excellent system for investigating the effects of pollutants on sexually selected traits and behaviours.

Here, we investigated the effects of 30-day exposure to two environmentally realistic levels of fluoxetine—nominal low and high concentrations of 40 and 400 ng/L, respectively—on mechanisms of pre- and post-copulatory sexual selection in mosquitofish. Utilising two separate flow-through exposures, we experimentally investigated the impact of fluoxetine on 1) male mating behaviour in the absence or presence of a competitor, and 2) total sperm count and sperm quality. In addition, all fluoxetine-exposed and control (i.e., unexposed) males were tested for differences in their morphological characteristics.

2. Materials and methods

2.1. Animal collection and housing

Mosquitofish were wild-caught from the Science Centre Lake at Monash University (37° 54′ 28″ S, 145° 08′ 16″ E), Victoria, Australia.

Analysis of water samples from the site of fish collection indicated no contamination with fluoxetine (Envirolab Services, unpublished data; see below for details of water testing). Sexually mature fish were acclimated to laboratory conditions in single-sex aquaria for 1 month prior to experimentation (12:12 h light:dark cycle; 24–26 °C; 128 L; 80 × 45 × 45 cm). Fish were fed *ad libitum* once daily with commercial fish food (Otohime Hirame larval diet; 580–910 μm).

2.2. Flow-through chemical exposures

Male mosquitofish were exposed to fluoxetine using two separate flow-through systems that were identical in design, comprising fish to be tested for 1) reproductive behaviour and 2) sperm traits. Separate exposures were conducted to ensure that males tested for sperm traits would not have the opportunity to expend ejaculate in free-swimming behavioural trials. In either system, males were randomly allocated to one of three exposure treatments: freshwater control, low fluoxetine or high fluoxetine (see below). Fish were subjected to a 30-day exposure period. This length of exposure was chosen because clinical trials in humans suggest that fluoxetine does not exhibit its full therapeutic (anxiolytic-like) effects for 2–4 weeks after the initiation of treatment (e.g., Gardier et al., 1996; Matuszcyk et al., 1998), fluoxetine exposure periods ranging from 28 to 35 days are sufficient to induce behavioural changes in a variety of fish species (e.g., Pelli and Connaughton, 2015; Martin et al., 2017; McCallum et al., 2017; Saaristo et al., 2017), and 30 days is the duration of one spermatogenic cycle in mosquitofish (Koya and Iwase, 2004). Further, mosquitofish are non-migratory and individuals generally have a relatively small home range (several meters, Noggle et al., 2004; Pyke, 2005), meaning that populations living in contaminated systems are likely to be exposed for prolonged periods. Each exposure involved three identical flow-through systems, one per treatment, which followed the design of previous experiments (Bertram et al., 2015; Martin et al., 2017; Saaristo et al., 2017; Tomkins et al., 2017, 2018), with some modifications. For each treatment, a mixing tank (182 L; 90 × 45 × 45 cm) fed into four exposure tanks (54 L; 60 × 30 × 30 cm), each of which housed 35 males. Exposure aquaria were equipped with 2 cm of natural gravel substrate, a large stone for refuge, an airstone, and an aquarium heater. Exposure tanks were kept on a 12:12 h light:dark cycle and were monitored daily for temperature (first exposure: mean = 25.19 °C, SD = 0.66 °C, $n = 360$; second exposure: mean = 25.21 °C, SD = 0.57 °C, $n = 360$) and flow-through rates (24 h cycling, ~1.67 L/h per tank).

To achieve the nominal low- and high-fluoxetine treatment concentrations—40 and 400 ng/L, respectively—used in each flow-through system, stock solutions were prepared as follows. Every third day, 1 mL of fluoxetine hydrochloride (CAS: 56296-78-7; Sigma-Aldrich, St Louis, MO) dissolved in methanol (HPLC grade, ≥99.9%) (low: 0.1 mg/mL, high: 1 mg/mL) was evaporated to dryness under a gentle nitrogen stream, before being diluted with Milli-Q water to form a 1 L solution. Every 24 h, a 180 mL aliquot of this solution was further diluted to produce a 3 L stock solution for each exposure level. Fluoxetine concentrations in each exposure tank were measured weekly, as well as being randomly sampled in half of the control (i.e., unexposed) aquaria, to ensure the absence of contamination. Analysis was performed by Envirolab Services (MPL Laboratories; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025), using gas chromatography–tandem mass spectrometry (7000C Triple Quadrupole GC-MS/MS, Agilent Technologies, Delaware, USA), following methods adapted from Papoutsis et al. (2012). For additional detail on the collection and analysis of water samples, see electronic supplementary material, 'Supplementary methods'.

2.3. Male reproductive behaviour

Males from the first flow-through exposure were used to test the impacts of fluoxetine on mechanisms of pre-copulatory sexual selection in two separate behavioural experiments. Two days prior to behavioural trials, males in all exposure tanks had a small portion of either the top or bottom of their caudal fin clipped for identification during competitive mating trials—a common method of fish identification (Ricker, 1949). Both non-competitive and competitive mating trials involved males being drawn at random from exposure tanks and allocated to one of 16 observation tanks (54 L; 60 × 30 × 30 cm) filled to a depth of 20 cm with aged water. Unexposed stimulus females were used in both behavioural experiments to avoid any potential influence of female fluoxetine exposure on male behaviour and were drawn randomly from four holding tanks containing fresh water only (54 L; 60 × 30 × 30 cm). Males and stimulus females were tested in one trial only and were not retested across behavioural experiments to control for any potential order effects.

In the first behavioural experiment, the effects of fluoxetine on male reproductive behaviour were tested in a non-competitive setting. This involved quantifying the behaviour of a single control ($n = 33$), low-fluoxetine ($n = 39$) or high-fluoxetine ($n = 37$) male when paired with an unexposed stimulus female. Free-swimming behavioural trials were preceded by a 5 min acclimation period, after which both fish were simultaneously released from their holding containers and allowed to freely interact for 15 min. Behaviours quantified included the number of male copulation attempts performed, involving a male approaching a female from behind and attempting to insert his gonopodium into her gonoduct (Bisazza et al., 2001), as well as the duration of time spent by the male actively following the female (within 5 cm).

In the second behavioural experiment, the impact of fluoxetine on male mating performance was tested under male-male competition. This involved quantifying the sum of the combined reproductive behaviours of two rival males when allowed to freely interact with, and compete over, a single unexposed stimulus female. Each trial was comprised of males both from either the control ($n = 37$), low-fluoxetine ($n = 43$) or high-fluoxetine ($n = 35$) treatments. Males from the same treatment were paired because wild males are likely to experience similar levels of environmental contamination. For each trial, males were drawn from separate exposure tanks within the same treatment to ensure that competing males had no recent experience with one another (i.e., no interaction for ≥31 days prior to behavioural trials). Again, after a 5 min acclimation period, the fish were released into the trial tank and allowed to freely interact for 15 min. The sum of the number of copulation events directed by both males towards the female was quantified, as well as the cumulative amount of time spent by both males following the female.

Subsequent to all trials, males were euthanised with an overdose of anaesthetic clove oil (40 mg/L) and were subject to morphological analysis (see below). Behavioural trials were video-recorded and quantified using the event-recording software JWatcher V1.0 (Blumstein and Daniel, 2007). Quantification of video recordings was performed blind to treatment, with competitive mating trials being scored twice (once per male).

2.4. Sperm traits

Males from the second flow-through exposure were used to investigate the effects of fluoxetine exposure on mechanisms of post-copulatory sexual selection. Here, experimental (control, low-fluoxetine or high-fluoxetine) males were tested for total sperm count and sperm quality traits, with males tested for total sperm

count being separate from those analysed for sperm quality.

Total sperm count was estimated in control ($n = 32$), low-fluoxetine ($n = 29$) and high-fluoxetine ($n = 29$) males following Evans et al. (2003), with some modifications. Briefly, after being euthanised as described above, males were dabbed dry and placed on a glass Petri dish under a dissection microscope (Leica MZ9.5), before being covered with 2 mL of activation solution (150 mM KCl with 2 mg/mL bovine serum albumin). To release spermatozeugmata (sperm bundles), the gonopodium was swung forward three times before gentle pressure was applied to the abdomen, slightly anterior to the base of the gonopodium. After repeating this action to ensure the release of the entire ejaculate, the evacuated spermatozeugmata were immediately recovered using a micropipette and made up to a volume of 1 mL with activation solution. This solution was then gently resuspended 100 times with a pipette to aid in breaking up spermatozeugmata. The sperm were then killed with 20 μL of 35% formalin and stained with 10 μL of trypan blue. Using Milli-Q water, a 3.5-fold dilution was produced to create an appropriate cell concentration for counting. This solution was vortexed to produce a homogeneous suspension, with a 10 μL aliquot being loaded into each well of an improved Neubauer haemocytometer (Blaubrand, Germany). Sperm in ten 200 × 200 μm squares, five per haemocytometer chamber, were counted under × 40 magnification (Olympus B×60). Total sperm count was estimated by multiplying the mean of these ten counts by the sample dilution factor and the initial sample volume. Sperm counts were performed blind of treatment, as is also true for the following assays.

Sperm quality was measured using computer-assisted sperm analysis (CASA) software (v. 14, CEROS, Hamilton-Thorne Biosciences, Beverly, MA) in control ($n = 51$), low-fluoxetine ($n = 50$) and high-fluoxetine ($n = 53$) males. Briefly, this involved euthanised males being covered in 500 μL of extender solution (207 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 10 mM Tris, pH 7.5), in which sperm remain quiescent. Sperm were then extracted (as above) and a 5 μL aliquot of sperm in extender medium collected. The sperm were activated with 20 μL of activation solution and gently resuspended 100 times using pipette action to break up the spermatozeugmata. A 3 μL drop of this solution was placed into the well of a 12-well multitest slide (MP Biomedicals, Irvine, CA) and a coverslip gently placed on top. To avoid sperm sticking, all slides and coverslips were dipped in 1% polyvinyl alcohol (Sigma-Aldrich) solution for 3 min and air-dried prior to use (Wilson-Leedy and Ingermann, 2007), as well as being warmed to 25 °C (LEC Warm Stage). A minimum of 1000 sperm were tracked per male (mean = 1116.54, SE = 6.09, $n = 154$) using a video camera (XC-ST50, Sony, Tokyo, Japan) coupled to a negative phase-contrast microscope (Olympus CX41) with a 10 × objective. Measurements of sperm function included: average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), path linearity (LIN, %) and motility (%) (see electronic supplementary material, Table S1 for detailed descriptions).

A second sub-sample of ejaculate was collected from males analysed using CASA, which was simultaneously tested for the proportion of live sperm (control: $n = 51$, low-fluoxetine: $n = 50$, high-fluoxetine: $n = 53$), following Evans (2009), with some modifications. A live/dead Sperm Viability Kit (L-7011; Molecular Probes Inc., OR, USA) was used, which firstly involved a 10 μL sample of ejaculate in extender solution being collected and gently resuspended 30 times with pipette action to break up the spermatozeugmata. Sperm were stained with 10 μL of a 1:50 dilution of membrane-permeant nucleic acid stain (1 mM SYBR 14), which stains live sperm green under fluorescent light. The sample was then vortexed and incubated at 25 °C in the dark for 10 min, before being counter-stained with 2 μL of 2.4 mM propidium iodide, which stains dead sperm red, and incubated for a further 10 min. After again being vortexed, 4 μL of the solution was placed onto a slide and viewed under a fluorescence microscope (Leica DFC425C). A minimum of 200 cells were counted per male (mean = 256.01, SE = 4.80, $n = 154$).

2.5. Morphological analysis

Males were measured subsequent to both reproductive behaviour and sperm analysis trials. Euthanised males were dabbed dry and measured for standard length (snout to caudal peduncle) (±0.01 mm), weight (±0.0001 g) and gonopodium length (±0.01 mm). An index of male body condition was calculated by plotting mass (g) against standard length (mm) to produce a least-squares regression line (i.e., weight = −0.440 + 0.029 × length). Condition index was calculated as the residuals of this regression line. All relevant morphological measures were also recorded for stimulus females.

2.6. Statistical analysis

Data were analysed in R version 3.2.3 (R Development Core Team, 2015). Where appropriate, data were checked for normality (visual inspection of standard diagnostic plots) and homogeneity of variance (Fligner-Killeen test). Vuong tests (*vuong* function, *pscl* package; Jackman, 2012) indicated zero-inflation of the number of copulation attempts performed by males towards females, both in the absence and presence of a competitor, which was accounted for by fitting zero-inflated Poisson (ZIP) generalised linear models (GLMs) (*zeroinfl* function, *pscl* package; Zeileis et al., 2008). To test for overdispersion in the number of copulation attempts performed, zero-inflated negative binomial (ZINB) GLMs (*zeroinfl* function) were then also fitted and compared with their respective ZIP GLM alternatives using likelihood-ratio tests (*lrtest* function, *lmtest* package; Zeileis and Hothorn, 2002). In both competitive and non-competitive trials, this procedure indicated overdispersion and, thus, ZINB GLMs were favoured (Zuur et al., 2009). For all models, predictors were selected based on their biological relevance (see electronic supplementary material, Table S2 for a summary of model parameters). General linear hypothesis tests (GLHTs; *glht* function, *multcomp* package; Hothorn et al., 2008) were used for post-hoc comparison of mean responses across treatment levels. Partial Wald tests were used to assess whether coefficients, or pairwise differences between treatment levels, were significantly different from zero (at $\alpha = 0.05$). The impact of fluoxetine on the amount of time males spent following females, both in the absence and presence of a competitor, was tested using analysis of covariance (ANCOVA). To approximate normality, following time in non-competitive trials was cube root transformed, while following time in the competitive trials underwent a rank normal transformation.

Total sperm count was compared between treatments using a GLM with a quasipoisson distribution to accommodate overdispersion, after which post-hoc comparisons were made using partial Wald tests through a GLHT, with *p*-values adjusted based on the joint normal distribution of the linear function. The effect of fluoxetine on sperm quality was assessed using ANCOVAs. To approximate normality, a rank normal transformation was applied to sperm path linearity (LIN) and the proportion of live sperm, while a folded root transformation was applied to the proportion of motile sperm. Male condition index and standard length were included as predictors in all models analysing sperm quality and quantity, as male body size is known to affect sperm traits in mosquitofish (O'Dea et al., 2014; see electronic supplementary material, Table S2 for further details).

The impact of fluoxetine on male morphology was assessed using ANCOVA, with post-hoc GLHT evaluation across fluoxetine treatments where appropriate, and with *p*-values adjusted as above. Standard length, weight and condition index were rank normal transformed, while gonopodium length was cube root transformed, in order to approximate normality. For all models, preliminary ANCOVAs revealed no significant interaction between fluoxetine treatment and chemical exposure system (i.e., males tested for either reproductive behaviour or sperm analysis) (ANCOVA: standard length: $F_{2,577} = 0.52$, $p = 0.594$; weight: $F_{2,577} = 0.79$, $p = 0.457$; condition index: $F_{2,577} = 0.37$, $p = 0.693$; gonopodium length: $F_{2,577} = 0.25$, $p = 0.776$). Morphological measurements from males across reproductive behaviour and sperm analysis experiments were therefore pooled within treatment levels ($n = 583$).

3. Results

3.1. Chemical analyses

During the first flow-through exposure—comprising males to be tested for reproductive behaviour—mean measured exposure concentrations in the low- and high-fluoxetine treatments were 41.68 ng/L (SD = 25.87, $n = 20$) and 478.50 ng/L (SD = 121.71, $n = 20$), respectively. Mean exposure concentrations within the second flow-through system—comprising males to be examined for sperm traits—were 29.51 ng/L (SD = 6.22, $n = 20$) and 379.50 ng/L (SD = 69.01, $n = 20$) in the low- and high-fluoxetine treatments, respectively. Fluoxetine concentrations measured within both flow-through exposures are environmentally realistic, with each of the low concentrations falling within the range of levels detected in surface waters (e.g., Fernández et al., 2010; Gardner et al., 2012; Hughes et al., 2013), while each of the high concentrations are within the range of levels measured in receiving waters (Benotti and Brownawell, 2007; Lara-Martín et al., 2015). The observed variation in measured exposure concentrations from the nominal levels for each of the low- and high-fluoxetine treatments—40 and 400 ng/L, respectively—is likely explained by the scale and ecological realism of the flow-through systems used, with numerous adult fish being exposed simultaneously in large aquaria containing a gravel substrate and stones for refuge. While these factors likely contributed somewhat to the observed variability in exposure concentrations, they were utilised to more closely reflect environmental conditions.

3.2. Male reproductive behaviour

Fluoxetine impacted the number of copulation attempts performed by male mosquitofish. In the absence of a competitor, high-fluoxetine males attempted to mate with females more often than did control (i.e., unexposed) males ($z = 2.02$, $p = 0.043$; Fig. 1). Specifically, high-fluoxetine males performed an average of 3.22 [1.81, 5.75] (where values in brackets indicate one standard error below, and one standard error above the mean, respectively) times the number of copulation attempts performed by control males. No significant differences were detected in the number of copulation events performed by control and low-fluoxetine males ($z = 1.07$, $p = 0.284$), nor by low- and high-fluoxetine males ($z = 1.14$, $p = 0.255$). More generally, male condition index was positively associated with the number of copulation attempts performed ($z = 2.22$, $p = 0.026$), with a one standard deviation (i.e., 0.012) increase in condition index resulting in, on average, 1.80 [1.38, 2.35] times as many copulations. A non-significant positive trend was also detected between the number of copulation attempts performed by males and female standard length ($z = 1.70$, $p = 0.089$).

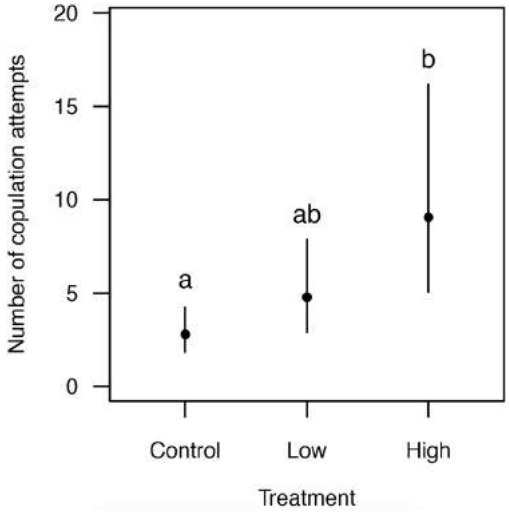

Fig. 1. Mean (±SE) number of copulation attempts performed by control (0 ng/L; $n = 33$), low-fluoxetine (42 ng/L, $n = 39$) and high-fluoxetine (479 ng/L, $n = 37$) males towards a stimulus (i.e., unexposed) female in the absence of a competitor male.

The amount of time males spent following females was not affected by treatment ($F_{2,103} = 0.94$, $p = 0.395$).

Under male-male competition, no significant effect of fluoxetine was detected on the total number of copulation attempts performed by rival males (all $p > 0.05$; Fig. 2). The combined number of copulation attempts performed by competing males was not significantly affected by the absolute difference in their condition index, nor standard length ($z = -0.10$, $p = 0.920$ and $z = 0.24$, $p = 0.813$, respectively). Longer females attracted more copulation attempts ($z = 2.49$, $p = 0.013$), with males together performing an average of 1.36 [1.20, 1.54] times as many attempts per standard deviation (i.e., 1.91 mm) increase in female length. Similar to the results of the non-competitive mating trials, no impact of treatment was detected on the combined amount of time males spent following females ($F_{2,108} = 1.22$, $p = 0.299$). Further, following time was not significantly affected by the absolute difference in condition index, nor standard length, between rival males ($F_{1,108} = 1.23$,

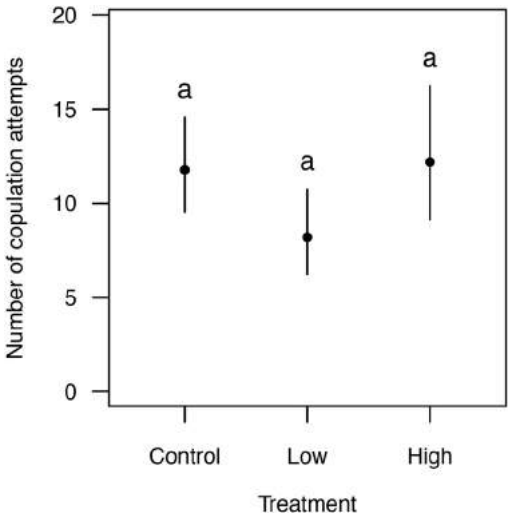

Fig. 2. Mean (±SE) of the combined number of copulation attempts performed by two competing males in the control (0 ng/L; $n = 37$), low-fluoxetine (42 ng/L, $n = 43$) and high-fluoxetine (479 ng/L, $n = 35$) treatments, towards a stimulus (i.e., unexposed) female.

$p = 0.270$ and $F_{1,108} = 2.34$, $p = 0.129$, respectively). Following time was, however, affected by female standard length, with males spending more time following longer females ($F_{1,108} = 13.80$, $p < 0.001$).

3.3. Sperm traits

Fluoxetine significantly affected sperm count ($F_{2,85} = 8.61$, $p < 0.001$), with males from both the low- and high-fluoxetine treatments having higher sperm counts than control fish (Fig. 3). For a given condition and standard length, low-fluoxetine males were predicted to have 1.45 [1.32, 1.59] times as many sperm as control males ($z = 3.98$, $p < 0.001$). High-fluoxetine males were predicted to have 1.31 [1.19, 1.44] times as many sperm as control males with similar condition and length ($z = 2.77$, $p = 0.016$). Standard length was positively associated with sperm count ($t = 3.74$, $p < 0.001$; Fig. S1), with a one standard deviation (i.e., 1.42 mm) increase in standard length corresponding to 1.14 [1.10, 1.18] times as many sperm.

Fluoxetine did not significantly impact any CASA parameters or sperm viability (all $p > 0.05$; electronic supplementary material, Table S3). Further, no measures of sperm quality were influenced by male standard length, condition index or weight (all $p > 0.05$).

3.4. Morphological analysis

Male standard length, weight and gonopodium length were not significantly affected by fluoxetine ($F_{2,579} = 1.04$, $p = 0.355$, $F_{2,579} = 2.93$, $p = 0.054$ and $F_{2,579} = 0.81$, $p = 0.447$, respectively). However, fluoxetine did impact condition index ($F_{2,579} = 5.16$, $p = 0.006$; Fig. 4). Specifically, low-fluoxetine males showed a significant reduction in condition index compared to control males ($t = -3.18$, $p = 0.004$). There was, however, no difference in condition index between males in low-fluoxetine and high-fluoxetine treatments ($t = -1.92$, $p = 0.135$), or between males in high-fluoxetine and control treatments ($t = -1.25$, $p = 0.423$, respectively).

4. Discussion

We found that exposure to environmentally realistic levels of fluoxetine can alter sexually selected traits and behaviours in male mosquitofish. Fluoxetine influenced mechanisms of pre-copulatory sexual selection through changed male mating behaviour, although these effects were dependent on both exposure concentration and the absence or presence of a competitor. Further, fluoxetine influenced mechanisms of post-copulatory sexual selection through increased sperm counts, while sperm quality was unaffected. Finally, exposure to fluoxetine resulted in reduced body condition in males from the low treatment.

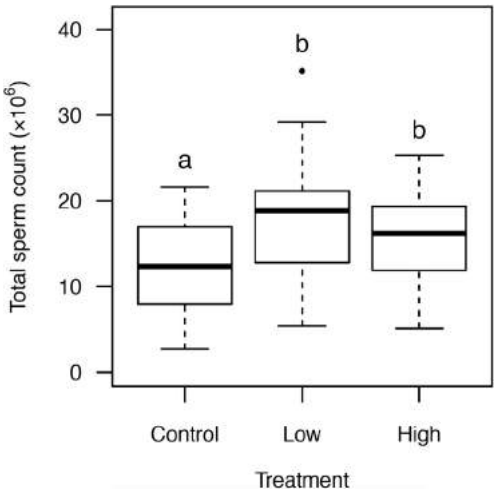

Fig. 3. Total sperm count ($\times 10^6$) of control (0 ng/L; $n = 32$), low-fluoxetine (30 ng/L, $n = 29$) and high-fluoxetine (380 ng/L, $n = 29$) males.

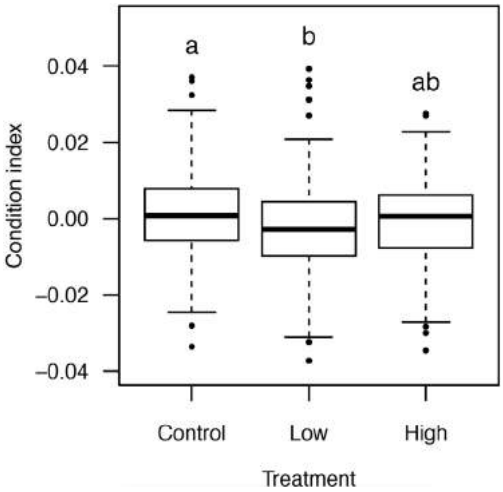

Fig. 4. Condition index of control ($n = 184$), low-fluoxetine ($n = 209$) and high-fluoxetine ($n = 190$) males. Males within each treatment were pooled across chemical exposures.

4.1. Pre-copulatory sexual selection: male reproductive behaviour

Fluoxetine affected the number of copulation attempts performed by males in the absence of a competitor. Specifically, while low-fluoxetine exposure (42 ng/L) did not significantly alter the number of mating attempts performed, high-fluoxetine (479 ng/L) males performed a greater number of attempts than males in the control treatment. To date, this is the lowest measured concentration of waterborne fluoxetine shown to alter reproductive behaviour in fish. This increase in copulatory behaviour in high-fluoxetine males can be expected to result in enhanced reproductive fitness as higher mating effort (i.e., number of mating attempts made) by eastern mosquitofish males has been shown to increase the likelihood of successful insemination (Evans et al., 2003). Further, mating effort is likely to be a strong predictor of actual reproductive success, with Deaton (2008) reporting that the number of mating attempts performed by male western mosquitofish explained approximately 67% of the variation in the proportion of offspring sired. However, the increased frequency of male sexual acts seen in high-fluoxetine males is expected to increase sexual conflict, as male sexual harassment is known to interfere with female foraging efficiency in eastern mosquitofish (Pilastro et al., 2003). Therefore, high-fluoxetine males may also experience reduced reproductive success if females employ strategies to minimise the costs of male sexual harassment, including, for example, by preferentially associating with males performing less frequent copulatory attempts (Pilastro et al., 2003).

Through what pathways may fluoxetine be altering male mating behaviour in fish? Although the effects of SSRIs—including fluoxetine—on reproductive behaviour in fish are not yet wholly understood (reviewed in Fent et al., 2006; Prasad et al., 2015), it is established that serotonin has an important role in modulating

various reproductive functions in fish and does so via multiple pathways, at both central (i.e., preoptic-hypothalamic area and pituitary) and peripheral (i.e., gonads) levels (Prasad et al., 2015; Dorelle et al., 2017). One key pathway by which SSRIs can alter reproductive behaviour in fish is by influencing the hypothalamus-pituitary-gonadal (HPG) and/or hypothalamus-pituitary-interrenal (HPI) axes (reviewed in Kreke and Dietrich, 2008), including by affecting the secretion of gonadotropin-releasing hormone (GnRH) and luteinising hormone (LH) from the hypothalamus and pituitary, respectively (Yaron and Sivan, 2006; Kreke and Dietrich, 2008). Further, SSRIs may disrupt reproductive function in fish by influencing the production of androgens, which are known to regulate sexual behaviours in fish (Borg, 1994; Munakata and Kobayashi, 2010) and are influenced by fluoxetine treatment (Mennigen et al., 2010, 2011; Fernandes et al., 2011). To date, few studies have tested the effects of environmentally realistic concentrations of fluoxetine on reproductive behaviour. However, consistent with the present findings, research on fathead minnows has reported increased reproductive behaviour (i.e., nest maintenance) in fluoxetine-exposed fish (at 1 μg/L and 100 μg/L; Weinberger and Klaper, 2014), with no effect being observed at lower levels (2.5 ng/L and 28 ng/L, Schultz et al., 2011; 100 ng/L, Weinberger and Klaper, 2014). Further, as was also seen in the present study, fluoxetine exposure (0.1 μg/L and 1 μg/L) did not affect the amount of following behaviour performed by male fathead minnows towards females (Weinberger and Klaper, 2014). In contrast, Siamese fighting fish exposed to fluoxetine were found to decrease male territorial defence during parental care (540 ng/L; Forsatkar et al., 2014). Overall, the differential sensitivities of reproductive behaviours between these studies may be due to differences in the reproductive behaviours tested (e.g., territorial defence during parental care and mating behaviours), dissimilar methods of reproduction (e.g., internal versus external fertilisation), incompatible exposure dosages and durations, or, perhaps, interspecific differences in fluoxetine sensitivity (e.g., differences in fluoxetine metabolism or bioavailability) (Gust et al., 2009).

No effect of fluoxetine was detected on the total number of copulation attempts performed by males under male-male competition, at either dosage (42 ng/L or 479 ng/L). Copulating multiple times with individual females is a means by which males can adjust their sperm allocation in a competitive mating situation (Parker, 1998). Indeed, male mosquitofish are known to adjust their mating effort based on perceived sperm competition risk and should generally perform higher levels of mating activity with increasing competition (Evans et al., 2003). This result, therefore, indicates that males exposed to fluoxetine were able to appropriately adjust their reproductive behaviour in the presence of a rival. Considering the seemingly limited scope of male eastern mosquitofish to adjust the size of individual ejaculates between copulations (Evans et al., 2003), fluoxetine exposure at the dosages tested is also not expected to alter the amount of sperm transferred to the female by competing males. To date, the effects of environmentally realistic concentrations of fluoxetine on male mating behaviour under male-male competition have been examined in only one other study. Concordant with our findings, Dzieweczynski and Hebert (2012) reported that 3-day exposure of male Siamese fighting fish to fluoxetine (540 ng/L) did not affect the amount of time spent by males performing female-directed courtship behaviour when encountering models of a male and female conspecific simultaneously. However, fluoxetine exposure also decreased male-directed aggressive behaviours in Siamese fighting fish, which was not the case in mosquitofish, with very few overtly aggressive interactions (e.g., fin nips) observed in our study, regardless of treatment.

4.2. Post-copulatory sexual selection: sperm traits

Exposed males from both low- and high-fluoxetine treatments (30 ng/L and 380 ng/L, respectively) had higher average sperm counts than control males. Sperm number is the strongest predictor of the outcome of sperm competition in poeciliids (Boschetto et al., 2011) and, as aforementioned, male mosquitofish likely have minimal scope for adjusting the size of ejaculates between copulations (Evans et al., 2003). However, males with larger sperm reserves may be able to increase their number of sperm allocations (over multiple copulations) and, thereby, fertilise a greater number of females (O'Dea et al., 2014). While our study is the first to test the effects of environmentally realistic levels of fluoxetine on total sperm count in an aquatic organism, previous studies have, for example, demonstrated that exposure to fluoxetine can reduce basal milt volume (54 μg/L) and decrease pheromone-stimulated milt volume in goldfish (540 ng/L and 54 μg/L; Mennigen et al., 2010), as well as decrease spermatozoan density in zebra mussels (20 ng/L and 200 ng/L; Lazzara et al., 2012). However, exposure to lower levels of fluoxetine did not significantly impact spermatogenesis in fathead minnows (2.5 ng/L and 28 ng/L; Schultz et al., 2011). Nevertheless, in fiddler crabs (*Uca pugilator*), administration of fluoxetine at pharmacological levels can stimulate testicular development (Sarojini et al., 1993).

The divergence in observed impacts of fluoxetine on sperm production and performance may be due to contrasting serotonergic regulation of reproductive processes in different species. For example, in female fish, administration of serotonin prevents steroid-induced maturation of oocytes in mummichog (*Fundulus heteroclitus*, Cerdà et al., 1998) but induces oocyte maturation in Japanese medaka (Iwamatsu et al., 1993). These alterations are predicted to reduce fecundity, and increase reproductive output, respectively. In the present study, increased sperm production resulting from fluoxetine exposure is likely driven by changes to androgen signalling within the HPG axis, as androgens—namely 11-ketotestosterone—are responsible for the induction of spermatogenesis in mosquitofish (Edwards et al., 2013). However, the specific mechanisms by which fluoxetine increases sperm counts require further investigation. More generally, across all treatments, standard length was positively associated with sperm count, as is an established relationship in mosquitofish (O'Dea et al., 2014).

This is the first study to have tested the effects of fluoxetine at environmentally relevant levels on sperm quality in an aquatic organism, and found no significant effect of exposure. This result contrasts with studies on rodents, where fluoxetine administration has been associated with impaired sperm motility (Bataineh and Daradka, 2007; Alzahrani, 2012). Further, although it has been suggested that fluoxetine exhibits spermicidal properties (Kumar et al., 2006; Alzahrani, 2012), these toxic effects may only be seen in response to exposure at higher dosages and were, therefore, not presently observed.

4.3. Morphology

Low-fluoxetine males showed a reduction in condition index, while the body condition of high-fluoxetine males did not differ significantly from the control. This represents a non-monotonic dose-response relationship, a phenomenon commonly reported in fluoxetine exposures (e.g., Guler and Ford, 2010; Bossus et al., 2014; Martin et al., 2017), as well as in pharmaceutical exposures more generally (reviewed in Vandenberg et al., 2012; Fong and Ford, 2014; Wilkinson et al., 2016). Reduced body condition in response to fluoxetine exposure has also been reported in various fish species, including convict cichlids (*Amatitlania nigrofasciata*, Latifi et al., 2015), goldfish (Mennigen et al., 2009) and hybrid

striped bass (*Morone saxatilis* × *M. chrysops*, Gaworecki and Klaine, 2008), albeit at higher concentrations than those used in the present study. Reduced body condition may be explained by a reduction in food intake, an effect previously observed in fluoxetine-exposed fish (Gaworecki and Klaine, 2008; Mennigen et al., 2009; Weinberger and Klaper, 2014). This suppression of appetite may be driven by fluoxetine-induced neuroendocrine disruption of the hypothalamic-pituitary-adrenal axis or through direct action on liver metabolism (Mennigen et al., 2009).

5. Conclusion

Here, we report that 30-day exposure to environmentally realistic concentrations of the pervasive pharmaceutical contaminant fluoxetine altered mechanisms of both pre- and post-copulatory sexual selection in mosquitofish. Further research is needed to better understand the governing mechanisms underpinning these effects as well as the potential for fluoxetine at environmentally realistic exposure concentrations to influence pre- and post-copulatory reproductive processes in other species. Taken together, the present findings highlight the complex and ecologically important effects of psychotherapeutic drugs on aquatic organisms and emphasise the need for continued investigation into their potential sub-lethal impacts.

Ethics

This research was approved by the Biological Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2015/02) and complied with all relevant State and Federal laws of Australia.

Authors' contributions

M.G.B., T.E.E., B.B.M.W. and M.S. conceived and designed the experiments, which M.G.B., T.E.E. and J.M.M. conducted. M.G.B., T.E.E. and J.B.B. carried out statistical analysis. M.K.O.B. coordinated all sperm analysis, which M.G.B., T.E.E. and J.M.M. performed. M.G.B. and T.E.E. drafted the manuscript. All authors contributed to manuscript preparation and gave final approval for publication.

Conflicts of interest

The authors declare that we 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.2018.03.006.

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Supplementary material

1. SUPPLEMENTARY METHODS

Gas chromatography–tandem mass spectrometry

Water samples (200 mL) were drawn from each tank within the low- and high-fluoxetine flow-through exposure systems, as well as randomly from half of the control (i.e., unexposed) aquaria, weekly. Samples were stored at 4 °C in amber glass bottles and analysed within 4 days since collection. Analysis firstly involved 39 mL of each sample being acidified to pH 6, with 20 µL of 1 µg/mL norfluoxetine (CAS: 56161-73-0; Novachem, Germany) in methanol (HPLC grade, ≥99.9%) being added to each sample, as well as to all calibration standards, to serve as a surrogate standard. The samples were then extracted onto ion exchange solid-phase extraction (SPE) cartridges (CSDAU203 clean screen extraction columns, 200 mg/3 mL; United Chemical Technologies, purchased from PM Separations, QLD, Australia). These were then washed with de-ionised water followed by methanol (HPLC grade, ≥99.9%), before being dried under vacuum. The fluoxetine and norfluoxetine surrogate were eluted off the SPE cartridges using dichloromethane:isopropanol:ammonium hydroxide (78:20:2, v/v/v; 3 mL) at approximately 1.2 mL/min. Each extract was then evaporated to approximately 200 µL and quantitatively transferred into a 300 µL GC vial, before being evaporated to dryness under a gentle stream of argon at ≤40 °C. When just dry, the vials were left for a further 10 seconds, before being inspected to ensure the complete absence of moisture. Samples were then dried and derivatised in their respective GC vials to maximise recovery.

The fluoxetine was reconstituted in 100 µL of ethyl acetate (HPLC grade, ≥99.8%) using sonication. To each vial, 50 µL of heptafluorobutyric anhydride derivatising agent (United Chemical Technologies, purchased from PM Separations, QLD, Australia) was added. The fluoxetine and norfluoxetine were both derivatised at 65 °C for 30 min to form their respective heptafluorobutyryl derivatives. The contents of each vial were then evaporated to dryness and left to sit for a further 10 sec. To each vial, 100 µL of ethyl acetate saturated with ammonium, as well as 10 µL of semi-volatile organic compound internal standard solution (EPA 8270), were added. The vials were capped and vortexed briefly to re-constitute the fluoxetine and surrogates, before the samples were analysed using GC-MS/MS. Injection of 2 µL was carried out in pulsed splitless mode at 50 psi until 0.75 min at an injector temperature of 230 °C, ramped up to 280 °C at 100 °C/min, onto a DB5 equivalent capillary column (30 m, 0.25 mm ID, 0.25 µm film thickness).

The temperature gradient commenced at an initial temperature of 100 °C for 0.5 min, before being increased by 40 °C/min to 220 °C. The temperature was then increased by 30 °C/min to a final temperature of 325 °C, and was kept at this temperature for 0.2 min. Two transitions were monitored each for fluoxetine (quantification ion: 344 → 117.1, collision energy 30; confirmatory ion: 240 → 69, collision energy: 30) and the norfluoxetine surrogate standard (quantification ion: 225.9 → 169, collision energy 5; confirmatory ion: 225.9 → 69, collision energy: 30).

We used a five-point calibration curve, with calibration standards having been extracted and analysed alongside experimental samples. To achieve this, a stock solution of 1000 µg/mL of fluoxetine was first prepared in methanol (HPLC grade, ≥99.9%), with a working standard then being made in methanol at 5 µg/L. Calibration standards were prepared in unexposed flow-through tank water and were treated identically to experimental samples. No contaminations with fluoxetine were detected in any unexposed aquaria throughout the exposure period. Fluoxetine recovery was 98.03% (RSD 6.72%, $n = 47$), while recovery of the norfluoxetine surrogate was 98.94% (RSD: 9.13%; $n = 201$). With this method, a limit of quantification (LOQ) of 2 ng/L was obtained for fluoxetine.

2. SUPPLEMENTARY TABLES

Table S1. Sperm quality parameters measured with Computer Assisted Sperm Analysis (CASA).

Average path velocity (VAP) (µm/s)	Time-averaged velocity of a sperm along its average path, calculated by smoothing the curvilinear trajectory.
Straight line velocity (VSL) (µm/s)	Time-averaged velocity along the straight line between a sperm's first and last detected position.
Curvilinear velocity (VCL) (µm/s)	Average point-to-point velocity measured over the actual sperm track.
Path linearity (LIN) (%)	Departure of actual sperm track from the point-to-point sperm track (VSL/VCL*100).
Motility (%)	Sperm cells with VAP, VSL and VCL values above predetermined thresholds. Following Evans (2009), threshold values used for defining static cells were 24.9 µm/s for VAP and VCL, and 15 µm/s for VSL.

Table S2. Summary of statistical models.

Behavioural response	Model	Predictor variables
Number of male copulation attempts performed towards a stimulus female in the absence of a rival male	Zero-inflated negative binomial GLM	Treatment Male condition index Female condition index Female standard length (mm)
Time spent following stimulus female in the absence of a rival male (sec)	ANCOVA	Treatment Male condition index Female condition index Female standard length (mm)
Cumulative number of male copulation attempts performed towards a stimulus female by both males under male-male competition	Zero-inflated negative binomial GLM	Treatment Absolute difference in condition index between rival males Absolute difference in standard length between rival males (mm) Female condition index Female standard length (mm)
Cumulative time spent following a stimulus female by both males under male-male competition (sec)	ANCOVA	Treatment Absolute difference in condition index between rival males Absolute difference in standard length between rival males (mm) Female condition index Female standard length (mm)
Total sperm count	quasi-Poisson GLM	Treatment Male condition index Male standard length (mm)
VAP (μm/s), VSL (μm/s), VCL (μm/s), LIN (%), Motility (%), Live sperm (%)	ANCOVA	Treatment Male condition index Male standard length (mm)
Male standard length (mm), weight (g), condition index and gonopodium length (mm)	ANCOVA	Treatment Chemical exposure (i.e., males tested for either reproductive behaviour or sperm analysis)

Table S3. Sperm quality measures in control (0 ng/L; n = 51), low-fluoxetine-exposed (30 ng/L; n = 50) and high-fluoxetine-exposed (380 ng/L; n = 53) males. ANCOVA results presented are for the effect of fluoxetine treatment on each response variable.

	Control		Low		High		ANCOVA	
	Mean	SE	Mean	SE	Mean	SE	*F*	*p*
VAP (μm/s)	80.05	1.98	81.03	2.52	82.52	2.44	0.29	0.752
VSL (μm/s)	65.56	1.67	66.22	2.20	67.13	2.05	0.16	0.854
VCL (μm/s)	108.58	1.81	110.03	2.22	108.41	2.09	0.19	0.830
LIN (%)	61.35	0.95	60.90	1.28	62.32	1.10	0.55	0.579
Motility (%)	82.71	1.75	82.18	2.25	85.00	1.59	1.06	0.348
Viability (%)	62.82	4.73	69.51	3.24	71.74	3.48	0.97	0.382

VAP: average path velocity, VSL: straight line velocity, VCL: curvilinear velocity, LIN: path linearity

3. SUPPLEMENTARY FIGURE

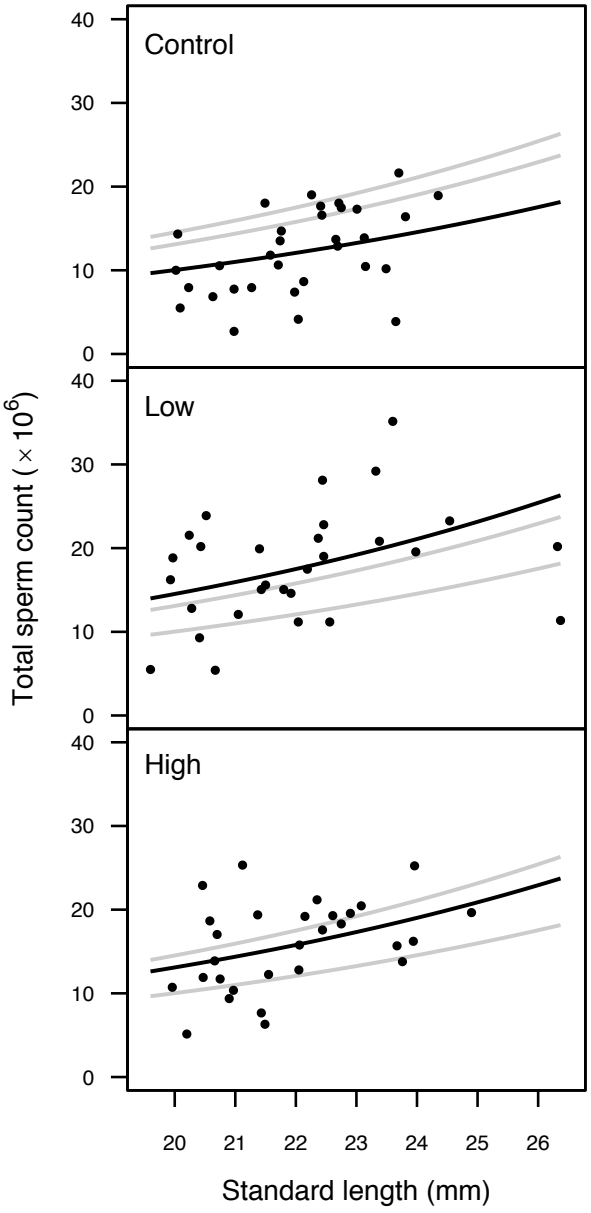

Figure S1. Relationship between total sperm count ($\times 10^6$) and standard length (mm) of control (0 ng/L; n = 32), low-fluoxetine (30 ng/L, n = 29) and high-fluoxetine (380 ng/L, n = 29) males. In each panel, points indicate measurements for individual fish within the corresponding treatment group. Lines indicate the fitted relationship between standard length and sperm count for the focal treatment (black line) and, for reference, the other treatments (grey lines).

Chapter 6

Discussion

DISCUSSION

The over-arching goal of the studies described in this thesis was to investigate impacts of environmentally realistic exposure to pharmaceutical pollution on fitness-related traits and behaviours in fish. I used freshwater poeciliid fish models, the guppy and the eastern mosquitofish, to examine effects of waterborne exposure to widespread pharmaceutical contaminants. My thesis explores the impact of the two leading sources of pharmaceutical pollution globally: run-off of veterinary pharmaceuticals used in agriculture, and insufficient removal of human pharmaceuticals by sewage treatment plants. Accordingly, the thesis is split into two sections, and addresses the following two aims, in turn:

> **Aim 1** — To investigate consequences of exposure to environmentally realistic levels of the veterinary pharmaceutical and agricultural pollutant 17β-trenbolone on fitness-related behaviours and reproductive processes in fish.
>
> **Aim 2** — To examine impacts of exposure to field-realistic levels of the widespread human pharmaceutical pollutant fluoxetine on reproductive processes in fish.

In section one, I uncovered that, at concentrations present in the environment, 17β-TB can alter a range of key traits and behaviours in both male (e.g. mating strategy, reproductive behaviour, sperm function trade-offs, morphology) and female (e.g. activity and exploratory behaviour, sociability, foraging) fish. Then, in section two, I provide evidence that exposure to fluoxetine at field-realistic levels can disrupt reproductive behaviour, sperm production, and morphology in male fish. In combination, this collection of studies provides valuable insights into a range of previously unknown and ecologically important impacts of exposure to sub-lethal levels of two pervasive pharmaceutical pollutants. More generally, these studies underscore the capacity of emerging pharmaceutical contaminants to disturb key behavioural, physiological, and morphological traits in wildlife, with important implications for ecological and evolutionary processes in contaminated wildlife populations.

Key findings and implications

In **Chapter 2**, I found that, at an exposure concentration that is representative of low environmental levels (i.e. 4 ng/L), 17β-TB alters male reproductive behaviour and morphology in fish. More specifically, I found no significant effect of exposure on male courtship behaviour, with males from both the unexposed and exposed treatments courting larger females more often. However, exposure did affect the amount of coercive copulatory behaviour performed by males. This is because, while males from both treatment groups exhibited a preference for larger females by performing a greater number of sneak attempts toward these females, males

that were exposed to 17β-TB conducted more frequent sneaking behaviour towards large females than did unexposed males. In addition, exposure produced an increase in male mass relative to length.

That exposure to 17β-TB increased male coercive copulatory behaviour performed towards females is consistent with three separate studies reporting this effect in guppies (i.e. 2 ng/L, M.G. Bertram, unpublished data; 8 ng/L, Tomkins et al. 2017; 22 ng/L, Bertram et al. 2015). This includes an exposure scenario where both males and females were exposed (Bertram et al. 2015), although no such effect was seen under this exposure scenario in mosquitofish (6 ng/L, Saaristo et al. 2013), indicating potential species-specific sensitivity differences. While, superficially, increased copulatory behaviour in contaminated male guppies may appear to be a 'positive' side-effect of exposure, to assess potential population-level impacts of this effect, we must consider the mating system of the species. The guppy mating system is primarily driven by female choice (Houde 1997), with females exercising this choice by preferentially associating with, and being receptive towards, males possessing a variety of traits that are known to honestly signal fitness (e.g. area and chroma of orange pigmentation, Brooks and Caithness 1995; courtship display rate, Kodric-Brown and Nicoletto 2001). As a result of female receptivity towards attractive males, copulations preceded by courtship deliver approximately three times as many sperm into the female's gonoduct (Pilastro and Bisazza 1999) and so have a higher probability of successful insemination (Matthews and Magurran 2000; Russell et al. 2006). Therefore, attractive males, on average, court more and sneak less (Reynolds 1993), with females then exercising mate choice, ensuring that relatively attractive males achieve higher reproductive success.

In an ecosystem contaminated with 17β-TB, where males shift towards coercive copulatory behaviour, circumvention of female mate choice may result in high-quality males losing their competitive advantage, potentially allowing males of lower quality to secure a greater number of fertilisations. This has implications for fitness at the population level, given that relatively attractive males have been shown to sire offspring that have higher growth rates (Reynolds and Gross 1992), as well as being more fecund (Moore 1994) and more attractive (Houde and Endler 1990; Houde 1992). What is more, increased male sneaking imposes a direct fitness cost on females, as gonopodial thrusts can damage the female genital pore (Constantz et al. 1989), and because more frequent mating increases disease transmission risk, increases likelihood of detection by predators, and reduces female foraging efficiency (Bisazza et al. 2001).

In **Chapter 3**, I assessed impacts of exposure to 17β-TB on behaviour in female mosquitofish across three key fitness-related contexts. First, I found that exposed females exhibited

increased activity and exploratory behaviour in a novel environment, although no significant effect was seen on boldness (i.e. refuge use). Second, exposed females spent less time associating with a shoal of stimulus conspecific females and were, again, seen to be more active than control fish. Third, when tested for feeding and foraging behaviour, relative to females in the control treatment, 17β-TB-exposed females were quicker to first enter a foraging zone containing prey items, were faster to commence feeding, spent more time foraging, and consumed a greater number of prey, although some exposure-induced effects on feeding and foraging were dependent on female size.

In predicting potential population-level impacts of changes in behaviours performed across these three contexts, an important consideration is whether other fish species inhabiting systems impacted by agricultural activity, and, thereby, exposed to 17β-TB, will be similarly affected by exposure. For example, if female mosquitofish are especially susceptible to 17β-TB-induced increases in feeding and foraging behaviours, they may be expected to outcompete other species in obtaining prey, particularly given that mosquitofish are adaptable generalist predators (Pyke 2008). However, as aforementioned, the androgen receptor binding targets of 17β-TB are highly evolutionarily conserved across diverse taxa (McGinnis et al. 2002), meaning that similar behaviours in other fish species are also likely to be vulnerable to disruption. This is supported by the increase in foraging behaviour in 17β-TB-exposed guppies under simulated predation risk reported by Heintz et al. (2015). Therefore, altered foraging behaviours in exposed fish species could result in flow-on effects for populations and communities, potentially including altered interspecies interactions between relatively susceptible and resistant species and/or cascading indirect effects on species at other trophic levels (discussed in Saaristo et al. 2018). In addition, foraging behaviour involves a complex series of trade-offs between obtaining energy and the time, energy and risk associated with obtaining food (Schoener 1971; Werner 1974). Therefore, any potential benefits conferred to exposed fish via increased prey consumption will be contingent on the ability to appropriately adjust foraging behaviour depending on environmental conditions (e.g. threat of predation; Verdolin 2006).

In **Chapter 4**, I examined impacts of 17β-TB exposure on behaviour in male mosquitofish across both non-reproductive and reproductive contexts, as well as effects of exposure on sperm quality and quantity, and fish morphology. In a non-reproductive context, no significant effect of exposure was seen on male boldness, activity or exploratory behaviour in a novel environment. However, when tested in the presence of a stimulus (i.e. unexposed) female, exposed males exhibited reduced association behaviour. Specifically, males exposed to 17β-TB were slower to first associate with, and spent less time within close proximity to, a female. All males were then tested for sperm quality or quantity and, while no significant main effects of expo-

sure were seen on sperm function (i.e. computer-assisted sperm analysis, sperm viability) or total sperm count, exposure altered the relationship between male body condition and sperm function. Finally, males were tested for a suite of morphological traits and exposed males were found to have increased mass relative to length.

That 17β-TB exposure resulted in context-specific behavioural shifts in the same individuals when behaviours were independent (i.e. did not correlate across contexts) highlights that behaviours performed in different contexts can be differentially vulnerable to contaminant exposure. This underscores the importance of research in behavioural ecotoxicology testing impacts of exposure to pharmaceuticals—and chemical contaminants more generally—across multiple fitness-related contexts. To date, however, this approach has been relatively rare, with the majority of existing studies having tested behaviour in one context only. What is more, although behaviours performed in this study were found to be independent across contexts, in cases where such correlations are seen, this multiple context approach will be necessary to reveal the potential for contaminants to influence animal personality (i.e. across-context consistency in single behaviours) and associated behavioural syndromes (i.e. across-context correlations between behaviours) (Sih et al. 2004, 2012). Currently, research investigating impacts of pharmaceutical exposure on these phenomena is still in its infancy (but see Dzieweczynski et al. 2014; Hebert et al. 2014; Dzieweczynski et al. 2016a,b; Martin et al. 2019). However, this will be an important avenue for future research given that animal personalities and behavioural syndromes can have important implications for population dynamics, interspecies interactions, species distributions and networks, invasive potential, and response to environmental change (Sih et al. 2011, 2012; Bestion et al. 2015; Spiegel et al. 2015). Furthermore, given the potential for animal personality to constrain behavioural plasticity (Sih et al. 2004), potential disruption of personality by exposure to pharmaceutical contaminants may influence the ability of exposed wildlife to adjust to rapid environmental change. Clearly, disruption of animal personalities by exposure to pharmaceutical pollution could have significant, yet overlooked, consequences for fitness.

Finally, in **Chapter 5**, I expanded my investigation of potential impacts of pharmaceutical pollution to include the human antidepressant medication fluoxetine. Male mosquitofish were exposed to fluoxetine at two environmentally realistic levels (low and high). I revealed that exposure to fluoxetine at the higher concentration increased the amount of copulatory behaviour performed by males in the absence of a competitor, while no such increase was seen when in the presence of a rival male (i.e. under male-male competition). Moreover, exposure to fluoxetine at either level increased total sperm count relative to control males. Lastly, relative to males in the control treatment, low-fluoxetine-exposed males had significantly reduced condition index (i.e. mass relative to length).

Given that males in the high-fluoxetine exposure treatment performed increased copulatory behaviour towards females—as has been supported by two subsequent studies in both mosquitofish (Martin et al. 2019) and guppies (Fursdon et al. 2019)—and were also found to have increased sperm counts, this would suggest that, in nature, males inhabiting contaminated habitats may be more successful at securing fertilisations. However, given the complexity of natural systems, this possibility requires further investigation. For example, in mosquitofish, females have been shown to actively avoid males performing excessive copulation attempts (Agrillo et al. 2005), meaning that any potential increase in reproductive fitness in contaminated males is likely to be contingent on their ability to appropriately adjust their behaviour to suit their environment. What is more, as aforementioned, increased male copulatory behaviour is likely to result in direct costs to female fitness by, for example, damaging the female genital pore (Constantz et al. 1989), increasing the risk of disease transmission, increasing detectability by predators, and interrupting female foraging behaviour (Bisazza et al. 2001).

Considering the findings reported in this thesis, how are pharmaceutical-induced changes in behaviours seen in the laboratory expected to manifest in wild fish populations? The answer is that predicting population-level effects is highly complex because pharmaceuticals—as with other forms of chemical pollution—vary in concentration (and thus exposure) over time and space, and can interact with other abiotic stressors (e.g. temperature, water chemistry) (Saaristo et al. 2018). What is more, pharmaceutical exposure can affect organisms in different ways, producing a diverse range of direct and indirect effects at multiple levels of biological organisation (reviewed in Saaristo et al. 2018). Using the example of 17β-TB, as well as behavioural anomalies, exposure has been shown to cause a wide range of biological effects, both mechanistic and apical (reviewed in Ankley et al. 2018), including altered sex steroid synthesis and/or plasma steroid concentrations (Ankley et al. 2003; Zhang et al. 2008; Garcia-Reyero et al. 2009), decreased fertility and fecundity (e.g. Ankley et al. 2003; Mizukami-Murata et al. 2015), delayed and/or abnormal sexual differentiation (e.g. Örn et al. 2006; Olmstead et al. 2012), and female-to-male sex-reversal resulting in phenotypic sex ratio skews (Larsen and Baatrup 2010; Morthorst et al. 2010; Boettcher 2011). What is more, as a result of exposure-induced declines in fecundity in female fish, population modelling indicates that continual exposure to 17β-TB at slightly higher concentrations than have been detected in aquatic habitats would result in population crashes and possible future population-level extinctions (Miller and Ankley 2004). More specifically, after two years of exposure, fathead minnow (*Pimephales promelas*) populations exposed at 27 ng/L exhibited a 51% projected decrease in average population size, while populations exposed at ≥266 ng/L exhibited a 93% projected decline (Miller and Ankley 2004). Hence, it is important to recognise that, in natural systems, behavioural effects of 17β-TB expo-

sure will occur in a context where organisms are (likely differentially) experiencing a multitude of additional direct and indirect contaminant-induced effects at multiple levels of biological organisation, as is also likely to be true for many pharmaceutical contaminants.

While existing research has reported effects of pharmaceutical exposure on a range of biological endpoints, understanding effects of exposure on behavioural processes is important for (at least) three main reasons. First, a growing body of research has demonstrated that behavioural endpoints can be particularly sensitive to disruption by exposure to pharmaceuticals (reviewed in Arnold et al. 2014; Brodin et al. 2014), and chemical contaminants more generally (reviewed in Clotfelter et al. 2004; Zala and Penn 2004; Melvin and Wilson 2013). Again, using the example of 17β-TB, lowest observable effect concentrations for mechanistic and apical endpoints are most often in the range of 10 to 10,000 ng/L (reviewed in Ankley et al. 2018). Meanwhile, recent research has shown that a range of key behavioural traits can be disrupted at low environmentally realistic concentrations of between 2 and 25 ng/L (i.e. Saaristo et al. 2013; Bertram et al. 2015; Heintz et al. 2015; Tomkins et al. 2016; Tomkins et al. 2017; Bertram et al. 2018a,b; Tomkins et al. 2018; Lagesson et al. 2019). This has important implications because dilution in aquatic environments means that, spatially, the majority of contamination at watershed-scales will occur at relatively low concentrations (Durhan et al. 2006). Second, many pharmaceutical contaminants produce their most pronounced effects when exposure occurs during critical windows of organismal development (e.g. Koger et al. 2000; Ankley and Johnson 2004; Maack and Segner 2004). For example, male fish exposed to the synthetic birth control estrogen 17α-ethynylestradiol are more sensitive to induction of vitellogenin synthesis and alterations of gonadal development when exposed during early life-stages (van Aerle et al. 2002). However, mounting evidence, including the studies presented in this thesis, indicates that behavioural traits are highly sensitive to disruption by pharmaceuticals even when exposure occurs at sexual maturity. Lastly, and more fundamentally, given that the ultimate goal of ecotoxicology is to uncover effects of pollution within the context of all other environmental factors in natural systems, investigating potential behavioural impacts of pharmaceutical pollutant exposure is critically important because of the profound role of behaviour in the ecology of individuals, as well as the evolution of populations and species.

Future directions and concluding remarks

By uncovering a range of hitherto unknown sub-lethal effects of pharmaceutical exposure on fitness-related traits and behaviours in wildlife, this thesis underscores the importance of investigating impacts of emerging contaminants at environmentally realistic exposure concentrations and on ecologically important responses. Through laboratory experiments integrating ecotoxi-

cology, behavioural ecology, and physiology, the multidisciplinary approach utilised in this thesis also highlights the need for testing impacts of pharmaceutical exposure on increasingly complex biological processes in order to more accurately assess ecological and evolutionary impacts of pharmaceuticals in natural systems. However, although laboratory-based behavioural studies deliver important insights into potential ecological effects of contaminants, the considerable gap in complexity between laboratory-based studies and natural environments can make extrapolating these results challenging (discussed in Hellström et al. 2016; Saaristo et al. 2018). Hence, an important avenue for future research will be scaling up behavioural ecotoxicology experiments to the field, to allow for collection of data on impacts of pharmaceutical contaminants while accounting for the chemical and biological complexity of natural systems.

While field-based behavioural ecotoxicology studies have conventionally been considered expensive and logistically challenging, making obtaining high-quality *in situ* data difficult (Newman 2015), recent advancements in acoustic telemetry technology have made performing detailed behavioural studies in the field increasingly accessible. This technology allows for simultaneous tracking of multiple individuals in their natural environment with high temporal and spatial resolution, and offers the option to incorporate sensors to measure a wide variety of important physiological and environmental parameters (Hellström et al. 2016). Therefore, telemetry represents a state-of-the-art tool for behavioural ecotoxicologists seeking to quantify impacts of pharmaceutical pollution on ecological processes in complex, large-scale and dynamic natural environments, including fitness-related behaviours, interspecies dynamics, predator-prey interactions, social networks and collective movement, etc. (Donaldson et al., 2014; Hellström et al. 2016; Newman, 2015). While, to date, adoption of telemetry technology in behavioural ecotoxicology research has been slow, this technology has great potential to increase our understanding of how behavioural effects of pharmaceutical exposures observed in the laboratory setting translate into real ecological effects on organisms inhabiting contaminated systems.

To conclude, in this thesis, I demonstrate a range of previously unknown impacts of veterinary and human pharmaceutical exposure on wildlife. Waterborne exposure to environmentally realistic pharmaceutical concentrations disrupted a range of traits and behaviours in fish that are fundamentally important for survival and reproductive fitness. These findings highlight the potential for widespread pharmaceutical pollutants to disrupt key ecological and evolutionary processes in wildlife at exposure concentrations reflecting those present in the environment. Given the increasing pressure on wildlife and ecosystems from chemical pollution—including contaminants of emerging concern, such as pharmaceuticals—this research demonstrates the importance of considering ecologically meaningful sub-lethal endpoints in assessing the risks posed by these contaminants.

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APPENDIX

List of Appendices

This Appendix documents additional papers that I have led, or been involved with, during my candidature. These are not an examinable component of my thesis.

Appendix A

Bertram, M.G., Saaristo, M., Baumgartner, J.B., Johnstone, C.P., Allinson, M., Allinson, G., Wong, B.B.M., 2015. Sex in troubled waters: Widespread agricultural contaminant disrupts reproductive behaviour in fish. Horm. Behav. 70, 85–91. https://doi.org/10.1016/j.yhbeh.2015.03.002

Appendix B

Martin, J.M., Saaristo, M., **Bertram, M.G.**, Lewis, P.J., Coggan, T.L., Clarke, B.O., Wong, B.B.M., 2017. The psychoactive pollutant fluoxetine compromises antipredator behaviour in fish. Environ. Pollut. 222, 592–599. https://doi.org/10.1016/j.envpol.2016.10.010

Appendix C

Tomkins, P., Saaristo, M., **Bertram, M.G.**, Tomkins, R.B., Allinson, M., Wong, B.B.M., 2017. The agricultural contaminant 17β-trenbolone disrupts male-male competition in the guppy (*Poecilia reticulata*). Chemosphere 187, 286–293. https://doi.org/10.1016/j.chemosphere.2017.08.125

Appendix D

Tomkins, P., Saaristo, M., **Bertram, M.G.**, Michelangeli, M., Tomkins, R.B., Wong, B.B.M., 2018. An endocrine-disrupting agricultural contaminant impacts sequential female mate choice in fish. Environ. Pollut. 237, 103–110. https://doi.org/10.1016/j.envpol.2018.02.046

Appendix E

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Appendix G

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Appendix I

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Appendix J

Michelangeli, M., Cote, J., Chapple, D.G., Sih, A., Brodin, T., Fogarty, S., **Bertram, M.G.**, Eades, J., Wong, B.B.M. In review. Sex-dependent personality in two invasive species of mosquitofish. Aquat. Invasions.

Appendix A

Sex in troubled waters: Widespread agricultural contaminant disrupts reproductive behaviour in fish

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Sex in troubled waters: Widespread agricultural contaminant disrupts reproductive behaviour in fish

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ABSTRACT

Chemical pollution is a pervasive and insidious agent of environmental change. One class of chemical pollutant threatening ecosystems globally is the endocrine disrupting chemicals (EDCs). The capacity of EDCs to disrupt development and reproduction is well established, but their effects on behaviour have received far less attention. Here, we investigate the impact of a widespread androgenic EDC on reproductive behaviour in the guppy, *Poecilia reticulata*. We found that short-term exposure of male guppies to an environmentally relevant concentration of 17β-trenbolone—a common environmental pollutant associated with livestock production—influenced the amount of male courtship and forced copulatory behaviour (sneaking) performed toward females, as well as the receptivity of females toward exposed males. Exposure to 17β-trenbolone was also associated with greater male mass. However, no effect of female exposure to 17β-trenbolone was detected on female reproductive behaviour, indicating sex-specific vulnerability at this dosage. Our study is the first to show altered male reproductive behaviour following exposure to an environmentally realistic concentration of 17β-trenbolone, demonstrating the possibility of widespread disruption of mating systems of aquatic organisms by common agricultural contaminants.

Introduction

Chemical pollutants have accumulated in ecosystems globally, endangering wildlife, ecosystem function and human health (Schwarzenbach et al., 2006). One class of chemical pollutant, known as endocrine disrupting chemicals (EDCs), comprises environmental contaminants with the capacity to disrupt the natural hormonal functioning of organisms (Colborn et al., 1993). Endocrine disruptors are of particular concern given their extreme potency, with exposure to concentrations as low as nanograms per litre having deleterious effects, as well as the propensity of some EDCs to bioaccumulate, persist temporally and act transgenerationally (Diamanti-Kandarakis et al., 2009). Conventionally, studies in ecotoxicology have focussed on direct mortality and chronic sub-lethal effects of EDCs on development and reproduction (Melvin and Wilson, 2013). However, EDCs can also induce alarming changes in behaviour. Indeed, the particular sensitivity of behaviour to EDCs has driven recent interest in behavioural ecotoxicology as a tool for investigating endocrine disruption at environmentally relevant pollutant concentrations (reviewed in Melvin and Wilson, 2013). Existing studies in behavioural ecotoxicology typically focus on EDCs that disrupt gonadal steroid signalling by interacting with vertebrate estrogen or androgen receptors, as chemical interference with this pathway has the potential to disrupt sexual selection (e.g., Saaristo et al., 2009). However, the vast majority of these efforts have concentrated on EDCs with estrogenic activity. This is surprising because the handful of studies that have considered androgenic EDCs suggest that they are also capable of markedly altering animal behaviour (e.g., Hoffmann and Kloas, 2012).

An androgenic EDC of particular concern is 17β-trenbolone, the most bioactive metabolite of trenbolone acetate, a hormonal growth promotant used extensively in livestock production around the world (Kolodziej et al., 2013). Trenbolone acetate is a powerful steroid, with androgenic and anabolic potency 15–50 times greater than testosterone (Kolodziej et al., 2013; Neumann, 1976). Its metabolite 17β-trenbolone acts as a powerful androgen receptor agonist in the environment, is highly temporally persistent (with a half-life of approximately 260 days;

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0018-506X/

Schiffer et al., 2001) and has been repeatedly detected in aquatic environments associated with feedlot operations. Detected concentrations of 17β-trenbolone range from ≤20 ng/L in diffuse run-off (Durhan et al., 2006), to as high as 162 ng/L in fields directly receiving effluent (Gall et al., 2011). Recent studies report that exposure to 17β-trenbolone adversely impacts physiological and morphological endpoints in fish species (e.g., Morthorst et al., 2010). However, despite the potency and widespread global use of 17β-trenbolone, very little is known about its effects on behaviour. This is concerning as the ability of animals to produce and maintain behaviour appropriate to their environment is fundamental for survival and reproduction, so that disruption of these behaviours can have dire ecological and evolutionary consequences (reviewed in Candolin and Wong, 2012).

The mating system of the guppy, *Poecilia reticulata*, is ideal for investigating the effects of 17β-trenbolone on reproductive behaviour. The guppy is a small, live-bearing freshwater fish native to north-eastern South America that has a widespread global distribution, precipitated by numerous deliberate and accidental introductions (Lindholm et al., 2005). Importantly, throughout their range, guppies have the potential to be exposed to 17β-trenbolone, as they are known to inhabit water bodies receiving agricultural waste (e.g., Araújo et al., 2009; López-Rojas and Bonilla-Rivero, 2000; Widianarko et al., 2000). Male guppies employ two alternate mating strategies, either soliciting copulations from females through courtship ('sigmoid displays') or coercing copulations through unsolicited 'sneaking' behaviour (Luyten and Liley, 1991). The latter involves males surreptitiously approaching females from behind to insert their gonopodium (a modified anal fin serving as an intromittent organ) into the female's genital pore (Luyten and Liley, 1991). Female guppies are choosy and are known, for example, to prefer males possessing greater orange pigmentation (Houde, 1987). By preferentially associating with certain males over others, females are able to directly influence mating outcomes (Shohet and Watt, 2004).

Here we test the hypothesis that short-term (21-day) exposure to an environmentally relevant concentration of 17β-trenbolone (22 ng/L) alters male and female reproductive behaviour in guppies. A short-term exposure duration was employed as agricultural pollutants often enter aquatic habitats in pulses and these temporally discrete contamination events can have persistent consequences (García et al., 2011; Morthorst et al., 2010).

Materials and methods

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.

Animal housing

This study used laboratory-reared descendants of wild caught guppies from Alligator Creek (19° 26 18 S, 146° 57 01 E), Queensland, Australia. Sexually mature guppies reared in large mixed-sex holding tanks (90 cm × 45 cm × 45 cm) were assumed to be non-virginal given the near-constant mating pressure exerted by males in mixed-sex populations (Houde, 1997; Magurran and Seghers, 1994). Non-virgin fish were used to simulate mixed-sex wild populations, and because mate choice in virgin females can be indiscriminate (Pitcher et al., 2003). Fish were separated by sex into 81 L housing tanks (60 cm × 45 cm × 30 cm) and acclimated to laboratory conditions (25–27 °C; 12:12 h light regime) for 2 months. Fish were fed once daily (Otohime Hirame larval diet; 580–910 μm).

Exposure set-up and monitoring

A flow-through exposure design was used, as described by Saaristo et al. (2013). Fish were assigned to identical 54 L separate-sex aquaria (60 cm × 30 cm × 30 cm), which were monitored for temperature ($\bar{x}$ = 26.38 °C, SD = 0.52 °C) and flow-through rates ($\bar{x}$ = 18.88 mL/min, SD = 0.59 mL/min). In total, 308 fish were randomly assigned to one of seven 17β-trenbolone-exposure tanks, or one of seven unexposed tanks containing fresh water (22 fish per tank). Exposed and unexposed aquaria each comprised four male tanks and three female tanks, with a surplus of fish exposed to ensure adequate sample sizes for each treatment.

The concentration of 17β-trenbolone used ($\bar{x}$ = 22 ng/L, SD = 14.55 ng/L, n = 28) was monitored following Saaristo et al. (2013), with some modifications, using a commercial enzyme-linked immunosorbent assay (ELISA). Weekly water samples were drawn according to the protocol detailed by Saaristo et al. (2013).

Behaviour trials

To investigate the impact of 17β-trenbolone on the reproductive behaviour of guppies, four treatments were employed: (1) unexposed male paired with unexposed female (control; hereafter UU; n = 18), (2) unexposed male with exposed female (UE; n = 19), (3) exposed male with unexposed female (EU; n = 18), and (4) exposed male with exposed female (EE; n = 20). Fish were taken at random and equally from each exposure tank and allocated to behavioural trials (n = 75), which took place in 54 L observation tanks (60 cm × 30 cm × 30 cm) containing fresh water (i.e. water free from 17β-trenbolone). Trials involved a 5-minute period of acclimation, before both fish were released from holding containers and allowed to freely interact, while their behaviour was video-recorded for 15 min. Fish were euthanized immediately after trials using an overdose (40 mg/L) of anaesthetic clove oil, following which morphological and colouration analyses were conducted.

Reproductive behaviours (see Supplementary materials Table S1 for detailed descriptions) were quantified from video recordings using the event-recording software JWatcher V1.0 (Blumstein and Daniel, 2007). Briefly, for males, we counted the number of courtship bouts performed (i.e., male orienting in front of the female and performing courtship displays), as well as the number of sneaking attempts (i.e., male surreptitiously approaching the female from behind for forced copulation). For females, we counted the number of times that a female actively associated with the male, a frequently used measure of mating intent in poeciliid fishes (e.g., Kahn et al., 2010), including guppies (e.g., Shohet and Watt, 2004).

Morphological analysis

After behavioural trials, we measured the length of males and females (±0.01 mm). Males were also weighed (±0.0001 g), and an index of male condition was derived from a regression of the mass (g) of all males against their standard length (mm). This male Condition Index was calculated as the residuals from the least squares regression line (i.e., weight = −0.164 + 0.016 × length).

Colouration analysis

Because female guppies typically prefer males with greater orange pigmentation (e.g., Houde, 1987)—including in the population from which fish were sourced for the present study (Brooks and Endler, 2001; Gamble et al., 2003)—the percentage of males' body surface containing orange pigments was assessed using photographic colouration analysis, performed immediately after behavioural trials. After euthanasia, fish were photographed on the right side in a

standardised fashion (Nikon D90, shutter speed = 1/250, Nikon AF Micro-Nikkor 60 mm f/2.8D).

Colouration analysis involved using Photoshop (CS5 Version 12.0 Extended) to isolate the fishes' body surface, from snout to caudal peduncle (i.e., excluding fins). Eight reference specimens were randomly selected (4 exposed, 4 unexposed). Photoshop's Colour Range tool was used to sample the orange pigmentation of the reference fish to create an orange pigmentation colour standard, which was applied to all photographs. For each fish, the extent of orange pigmentation was calculated as the number of orange pixels (i.e., pixels with colours belonging to the orange pigmentation colour standard) as a proportion of the total body area (i.e., the number of pixels forming the body surface).

Statistical analysis

Data were analysed in R version 3.0.2 (R Core Team, 2013). Where appropriate, data were tested for normality (Shapiro–Wilk test, *shapiro.test* function; Royston, 1995) and homogeneity of variance (Fligner–Killeen test, *fligner.test* function; Conover et al., 1981). To assess whether exposure to 17β-trenbolone impacted male Condition Index, a two-sample *t*-test was used. Relationships were examined between behavioural responses and a small suite of predictors by fitting Poisson Generalised Linear Models (GLMs) (detailed in Supplementary materials Tables S2–S5). Vuong tests (*vuong* function, *pscl* package; Jackman, 2012; Vuong, 1989) indicated zero-inflation of count data used for the latter, which was accommodated by fitting Poisson models with the *zeroinfl* function (*pscl* package; Zeileis et al., 2008). Continuous predictors were centred and standardised to have zero mean and unit variance to enable a direct comparison of their coefficients. Post-hoc evaluation of the differences in the mean response across factor levels (holding other predictors at their means) was performed through General Linear Hypothesis Testing (GLHT) (*glht* function, *multcomp* package; Hothorn et al., 2008). Partial Wald tests were used to assess whether coefficients were equal to zero.

Results

Male behaviour

The number of courting events performed by males was associated with treatment, male orange pigmentation, male Condition Index and female length. Relative to males in the control treatment (i.e., unexposed males paired with unexposed females), both unexposed and exposed males performed fewer courting events when paired with exposed females (partial Wald test: $z = -2.219$, $p = 0.027$ and $z = -2.409$, $p = 0.016$, respectively; Fig. 1A). Increased male orange pigmentation was associated with an increase in courting event occurrence, with an average of 7.6% (±SE: 5.4–9.8%) more courting attempts for each one standard deviation (4.73%) increase in percent orange pigmentation (partial Wald test: $z = 3.633$, $p < 0.001$; Fig. 2). Male Condition Index was also positively associated with number of male courting events (partial Wald test: $z = 7.509$, $p < 0.001$), with a one standard deviation increase in male Condition Index (i.e., 0.012) yielding an 18.8% (±SE: 16.1–21.6%) increase in the number of courting events. Males performed fewer courting events when paired with longer females (partial Wald test: $z = -2.447$, $p = 0.014$), with a 4.9% (±SE: 2.9–6.9%) reduction in the number of courting events for every one standard deviation (i.e., 5.6 mm) increase in female length.

The number of sneaking attempts performed by males varied significantly with treatment, male Condition Index and female length. The number of sneaking attempts performed was significantly different between all pairs of treatments (Fig. 1B). Males in the control treatment performed fewer sneaking attempts than males in any other treatment group (partial Wald test: all $z \geq 3.932$, all $p < 0.001$; Fig. 1B). Unexposed males paired with exposed females snuck more than exposed males paired with exposed females (partial Wald test: $z = 2.704$, $p = 0.007$), with males from both of these treatments performing more sneaking attempts than exposed males paired with unexposed females (partial Wald test: $z = 5.127$, $p < 0.001$ and $z = 2.777$, $p = 0.005$, respectively; Fig. 1B). Male Condition Index was negatively associated with the number of sneaking attempts performed by males (partial Wald test: $z = -5.261$, $p < 0.001$), with a one standard deviation increase in Condition Index (i.e., 0.012) associated with 20.0% (±SE: 16.5–23.3%) fewer sneaking attempts per trial. Female total length also related negatively with the number of male sneaking attempts performed (partial Wald test: $z = -6.159$, $p < 0.001$), with a one standard deviation increase in female length (i.e., 5.6 mm) yielding 28.9% (±SE: 24.8–32.7%) fewer sneaking attempts.

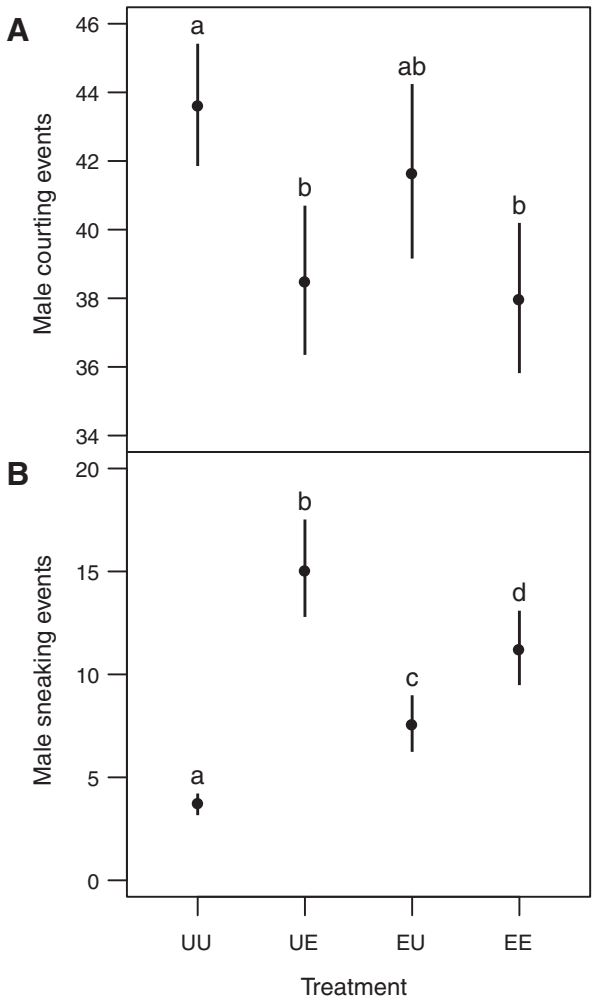

Fig. 1. Mean (±SE) of the (A) number of male courting events (orienting and sigmoid behaviours) and (B) number of male sneaking attempts across treatment groups ($n = 18, 19, 18$ and 20, for UU, UE, EU and EE, respectively). Treatments indicate unexposed (U) and exposed (E) fish, with male treatment followed by female treatment. Treatments without lower case letters in common are significantly different.

Female behaviour

Female association behaviour varied with male treatment. On average, unexposed females associated approximately 18.9% (±SE: 10.1–28.5%) more frequently with exposed males than with unexposed males (partial Wald test: $z = 2.239$, $p = 0.025$; Fig. 3). There was a significant positive effect of male orange pigmentation (partial Wald test: $z = 7.967$, $p < 0.001$) and a negative effect of female length (partial Wald test: $z = -13.296$, $p < 0.001$) on female association behaviour,

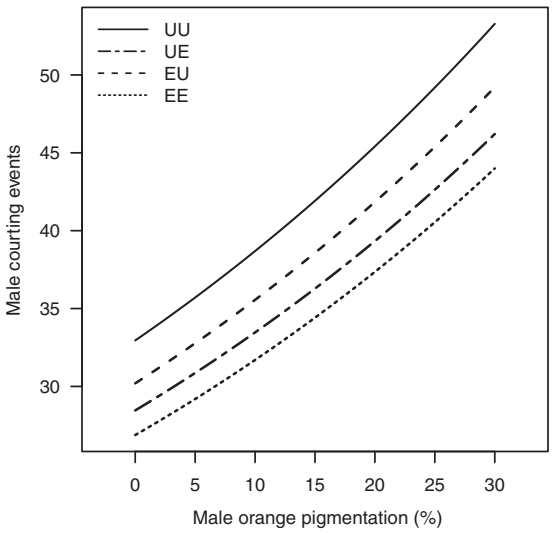

Fig. 2. Expected number of male courting events given male orange pigmentation (% of body area) and treatment ($n = 18$, 19, 18 and 20, for UU, UE, EU and EE, respectively), holding female total length and male Condition Index at their means. Treatments indicate unexposed (U) and exposed (E) fish, with male treatment followed by female treatment.

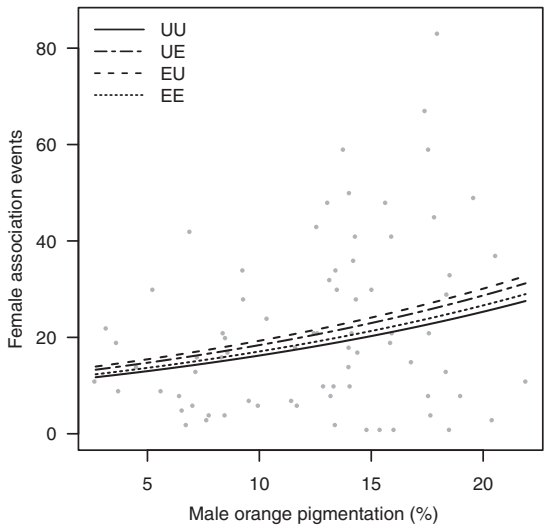

Fig. 4. Expected number of female association events given male orange pigmentation (% of body area) for each treatment ($n = 18$, 19, 18 and 20, for UU, UE, EU and EE, respectively), holding female total length and male Condition Index at their means. Points indicate observed data.

but no effect of male Condition Index (partial Wald test: $z = -1.898$, $p = 0.058$). An increase in male orange pigmentation of one standard deviation (i.e., 4.73%) corresponded with a 23.4% (±SE: 20.2–26.7%) increase in the number of associations (Fig. 4). A one standard deviation increase in total female length (i.e., 5.6 mm) corresponded to a decrease in the number of female association events of 30.7% (±SE: 28.7–32.6%).

Morphology

Exposed males had, on average, a significantly higher Condition Index than unexposed males (two-sample *t*-test: $t = 2.454$, df = 70.174, $p = 0.017$; Fig. 5). This was due to exposed males being heavier (two-sample *t*-test: $t = 2.296$, df = 72.985, $p = 0.025$), while male length was unaffected by exposure (two-sample *t*-test: $t = 1.231$, df = 72.926, $p = 0.222$).

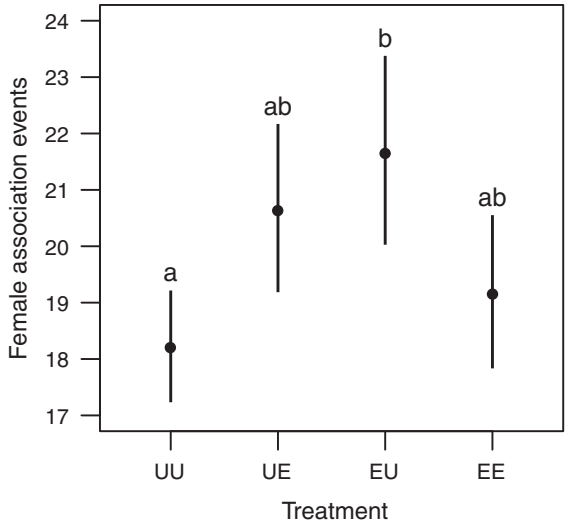

Fig. 3. Mean (±SE) number of female association events across treatment groups ($n = 18$, 19, 18 and 20, for UU, UE, EU and EE, respectively). Treatments indicate unexposed (U) and exposed (E) fish, with male treatment followed by female treatment. Treatments without lower case letters in common are significantly different.

Discussion

This research is the first to document altered male reproductive behaviour following exposure to 17β-trenbolone at an environmentally relevant concentration. Males paired with exposed females performed fewer courtship bouts than did males in the control treatment. Male exposure to 17β-trenbolone led to an increase in sneaking behaviour when paired with unexposed females. However, this finding was reversed when males were paired with exposed females, with unexposed males sneaking more than exposed males. In addition, regardless of male exposure status, males performed more sneaking behaviour when paired with exposed females. Exposed males paired with unexposed females also attracted more female association behaviour than males in the control treatment. More generally, males possessing greater areas of orange pigmentation performed more courting bouts toward females than less-colourful males. This correlation was anticipated, as orange pigmentation and display rate are both honest signals of male condition (Kodric-Brown and Nicoletto, 2001; Nicoletto, 1993).

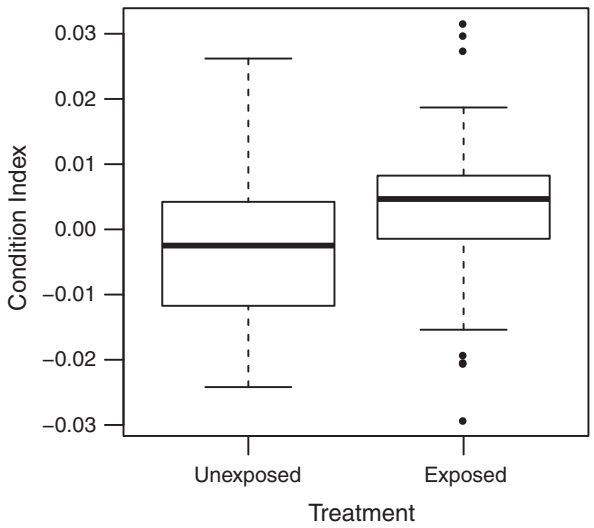

Fig. 5. Boxplots of male Condition Index for unexposed males ($n = 37$) and those exposed to 17β-trenbolone ($n = 38$).

Vertebrate male sexual behaviours are reliant on androgens for their production and maintenance (Cunningham et al., 2012). As a potent non-aromatizable androgen receptor agonist (Rogozkin, 1991), 17β-trenbolone has the capacity to disturb gonadal steroid signalling pathways by disrupting the hypothalamic–pituitary–gonadal (HPG) axis. Although the varied mechanisms behind the masculinising effects of 17β-trenbolone are not yet wholly understood (Larsen and Baatrup, 2010), it is known to bind with high affinity to available androgen receptors, mimicking the effects of testosterone (Wilson et al., 2002), and is hypothesised to indirectly inhibit the production of 17β-estradiol by limiting the production of testosterone, and thereby limiting the aromatisation of testosterone to 17β-estradiol (Zhang et al., 2008). Given that a central role of androgens is the modulation of male sexual and aggressive behaviours (Cunningham et al., 2012), the anomalous presence of androgenic EDCs may result in the 'hyper-masculinisation' of these traits in males. This phenomenon has been documented previously. For example, exposure of African clawed frogs (*Xenopus laevis*) to androgenic endocrine disruptors intensified androgen-dependent male mate calling (Hoffmann and Kloas, 2012), and increased the intensity of male sexual behaviours in various cyprinid fish species (Belanger et al., 2010). The present results demonstrate that, in guppies, the relative use of alternate reproductive strategies by males can be altered by exposure to 17β-trenbolone. Specifically, despite 17β-trenbolone having no significant effect on mate solicitation by males (i.e., courtship), the increased number of sneaking attempts performed by exposed males toward unexposed females (relative to the control group) suggests that exposed males may favour this coercive reproductive strategy. Interestingly, this finding was reversed given female exposure, with unexposed males sneaking upon exposed females more than exposed males, possibly indicating a greater capacity of unexposed males to take advantage of female exposure (although the mechanisms underlying this possible phenomenon are not presently considered).

Disruption of the relative usage of alternative male reproductive strategies has implications for male reproductive success, as sneaking behaviour is associated with reduced insemination efficiency relative to copulations preceded by courtship (Pilastro and Bisazza, 1999). Although sneaking behaviour is a viable sperm transfer method, sperm transfer rates are approximately three times higher when delivered after courtship (Pilastro and Bisazza, 1999). Further, postcopulatory female choice may hamper the average reproductive success of males engaging in sneaking behaviour. Such directional postcopulatory sexual selection has been documented in female guppies, which have been shown to bias fertilisation in favour of more colourful males (Pilastro et al., 2004).

Female exposure to 17β-trenbolone led to a decrease in the frequency of male courtship behaviour, and an increase in male sneaking behaviour (relative to the control group). Male guppies typically have very high levels of sexual activity, meaning that females receive continual mating attempts that are mostly unwanted (Houde, 1997; Magurran and Seghers, 1994). Typically, females actively avoid these incessant mating attempts by swimming away from pursuing males (Houde, 1997). Despite the males in the control group showing differential courtship behaviour relative to unexposed males paired with exposed females, the present findings suggest that the impact of female exposure to 17β-trenbolone on male reproductive behaviour was primarily male-driven, as female association behaviour (a reproductive behaviour indicative of receptivity) was not influenced by female exposure. In addition, other metrics of female behaviour (i.e. swimming away from a pursuing male, non-reproductive behaviour, and stressed behaviour) also did not differ significantly between exposed and unexposed females (unpublished data). Unexposed females were, however, more likely to associate with exposed males than unexposed males. Alternatively, it is possible that 17β-trenbolone-exposure impacted females in a manner not presently investigated, driving the observed changes in male behaviour. Regarding courtship-initiated mating, females are able to exercise mating preferences—via both precopulatory and postcopulatory mechanisms—by choosing which males to mate with, and which sperm to use for fertilisation (Pilastro et al., 2004; Shohet and Watt, 2004). Sneaking behaviour, however, circumvents female precopulatory mate choice, thus exposure to 17β-trenbolone may directly interfere with sexual selection in guppies.

No significant effect of female exposure to 17β-trenbolone was detected on female reproductive behaviour. This result was unexpected as androgen receptors are not sex-specific and endogenous androgens, as well as having an essential role in the development and maintenance of male traits, serve important functions in female vertebrates (Staub and De Beer, 1997). Androgens are involved in the regulation of female sexual and aggressive behaviours (Staub and De Beer, 1997), meaning that the mechanisms controlling these behaviours are particularly vulnerable to endocrine disruption. Female exposure to androgenic EDCs has previously been linked with physical and behavioural masculinisation, including the expression of male reproductive behaviour (Howell et al., 1980). Exposure of fathead minnow (*Pimephales promelas*) to 17β-trenbolone has been shown to severely alter female reproductive biology and suppress the production of endogenous sex steroid hormones, indicating masculinisation (Ankley et al., 2003). As such, 17β-trenbolone was expected to reduce female receptivity in the present study, but this was not observed. The resilience of the metrics of female reproductive behaviour presently considered to the concentration of 17β-trenbolone employed suggests a differential vulnerability to this EDC between sexes, a phenomenon previously documented in response to other EDCs (e.g., Kundakovic et al., 2012). Consistent with prior research, females exhibited a strong preference for males possessing greater orange pigmentation (e.g., Pilastro et al., 2004).

This study found that exposure to 17β-trenbolone was associated with an increase in male Condition Index, due to exposed males being heavier, despite there being no significant difference in length between exposed and unexposed males. This weight gain was anticipated, given the potent growth-promoting activity of 17β-trenbolone. A similar finding was reported in a study that exposed juvenile guppies to trenbolone acetate for 60 days (Zamora et al., 2008). In that study, however, fish were exposed to 300 mg/kg of trenbolone acetate, a level far higher than those having been reported in the environment. The present research, however, indicates that environmentally realistic levels of contamination are sufficient to cause weight gain, even with short-term exposure. For various taxa, including fish, heavier males have greater reproductive success in competitive breeding scenarios (e.g., Jacob et al., 2009). Although the present study did not test for the effect of 17β-trenbolone on male competitive ability, the increased weights of males exposed to 17β-trenbolone may confer an advantage in jockeying for contested fertilisations. This potential scenario holds ecological relevance as EDC concentrations are typically spatially and temporally variable (e.g., Grover et al., 2011; Lee et al., 2014), and guppies have the capacity to move considerable distances between habitats (Croft et al., 2003), making interactions between exposed and unexposed individuals likely.

Conclusion

This study reports that short-term (21-day) exposure to an environmentally relevant concentration (22 ng/L) of the androgenic endocrine disruptor 17β-trenbolone can alter reproductive behaviour and morphology in the guppy. This is the first study to show altered reproductive behaviours in male animals resulting from an environmentally realistic exposure to 17β-trenbolone. Given the prevalence and potent biological activity of 17β-trenbolone, the ongoing multidisciplinary scrutiny of this EDC is necessary to reveal the consequences of its presence in the environment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yhbeh.2015.03.002.

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Appendix B

The psychoactive pollutant fluoxetine compromises antipredator behaviour in fish

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The psychoactive pollutant fluoxetine compromises antipredator behaviour in fish☆

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ABSTRACT

Pharmaceuticals are increasingly being detected in aquatic ecosystems worldwide. Particularly concerning are pharmaceutical pollutants that can adversely impact exposed wildlife, even at extremely low concentrations. One such contaminant is the widely prescribed antidepressant fluoxetine, which can disrupt neurotransmission and behavioural pathways in wildlife. Despite this, relatively limited research has addressed the behavioural impacts of fluoxetine at ecologically realistic exposure concentrations. Here, we show that 28-day fluoxetine exposure at two ecologically relevant dosages—one representing low surface water concentrations and another representing high effluent flow concentrations—alters antipredator behaviour in Eastern mosquitofish (*Gambusia holbrooki*). We found that fluoxetine exposure at the lower dosage resulted in increased activity levels irrespective of the presence or absence of a predatory dragonfly nymph (*Hemianax papuensis*). Additionally, irrespective of exposure concentration, fluoxetine-exposed fish entered the predator 'strike zone' more rapidly. In a separate experiment, fluoxetine exposure reduced mosquitofish freezing behaviour—a common antipredator strategy—following a simulated predator strike, although, in females, this reduction in behaviour was seen only at the lower dosage. Together, our findings suggest that fluoxetine can cause both non-monotonic and sex-dependent shifts in behaviour. Further, they demonstrate that exposure to fluoxetine at environmentally realistic concentrations can alter antipredator behaviour, with important repercussions for organismal fitness.

1. Introduction

Pharmaceuticals are increasingly being detected in the environment, with approximately 600 of the 5000 actively manufactured pharmaceuticals having been reported in ecosystems worldwide (Küster and Adler, 2014). Indeed, pharmaceutical pollution has recently been recognised as an emerging environmental problem (Boxall et al., 2012; Arnold et al., 2014). One group of pharmaceuticals of particular concern is the selective serotonin re-uptake inhibitors (SSRIs), a class of antidepressants. These compounds (e.g., citalopram, sertraline and fluoxetine) have been repeatedly detected in the environment. In particular, fluoxetine has been detected in aquatic environments worldwide, with surface water detections typically ranging from <1 to 66 ng/L (e.g., Kolpin et al., 2002; Metcalfe et al., 2003; Glassmeyer et al., 2005; Fernández et al., 2010; González Alonso et al., 2010; Metcalfe et al., 2010; Yoon et al., 2010; Birch et al., 2015), to as high as 929 ng/L in direct sewage effluent (Bueno et al., 2007). Fluoxetine exhibits its primary pharmacological action on the serotonergic system, which is thought to play a key role in regulating a number of important behavioural and physiological functions, including, but not limited to feeding, locomotion, reproduction, aggression, fear and anxiety (Lucki, 1998; Lillesaar, 2011). Importantly, fluoxetine has the potential to impact non-target species, with its primary target molecule (serotonin transporter, 5-HTT)—along with other potential targets—being present in a wide variety of taxa (Ford and Fong, 2015), including in many fish species (e.g., Wang et al., 2006; Gould et al., 2007).

☆ This paper has been recommended for acceptance by Klaus Kummerer.

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Despite increasing concern surrounding the ecological effects of fluoxetine, it remains unclear whether exposure at environmentally realistic concentrations can alter the behaviour of wildlife (Sumpter et al., 2014). While recent studies have reported behavioural alterations in aquatic organisms resulting from acute exposure to environmentally realistic fluoxetine concentrations (e.g., De Lange et al., 2006; Painter et al., 2009; Barry, 2012; Winder et al., 2012; Bossus et al., 2014), studies employing exposure durations greater than 2 weeks are relatively uncommon. This is surprising given that the long-term therapeutic (anxiolytic-like) effects of fluoxetine are thought to be driven by adaptive changes within neurons (altered expression of 5-HT receptors), a process which can take up to 2–4 weeks (Gardier et al., 1996; Hensler, 2003). Therefore, it is possible that the anxiolytic-like effects of fluoxetine on non-target species are similarly time dependent (Stewart et al., 2014).

From an ecological perspective, understanding the potential impacts of fluoxetine and other widespread pharmaceutical pollutants on animal behaviour is crucial. Behaviour is the link between an organism's internal physiological processes and its environment, with alterations in behaviour having the potential to directly impact fitness (reviewed in Candolin and Wong, 2012; Sih, 2013; Wong and Candolin, 2014). In this regard, it is important that we address the effects of fluoxetine, as well as other pharmaceutical pollutants, from an ecological perspective, using behaviours with a direct bearing on individual and population-level fitness (Brodin et al., 2014)—such as the ability to avoid, and escape from, predators (Lima, 1998).

Here, using two separate experiments, we test the effects of 28-day fluoxetine exposure on antipredator behaviours of Eastern mosquitofish (*Gambusia holbrooki*) using environmentally realistic concentrations. The lower exposure treatment reflected levels typically reported in environmental surface water, whereas the higher exposure treatment reflected levels reported in and around wastewater effluent flow (see below). In the first experiment, we tested the impact of fluoxetine exposure on the performance of predator avoidance behaviour in the presence of a sympatric dragonfly nymph predator. In the second, we tested the effects of fluoxetine exposure on the predator escape behaviour of fish in response to a simulated predator strike.

2. Materials and methods

2.1. Animal collection and housing

Mosquitofish used in this study were wild-caught from the Science Centre Lake (37° 54′ 28″ S, 145° 08′ 16″ E), Monash University, Victoria, Australia. Water samples drawn from the lake revealed no fluoxetine contamination (Envirolab Services, unpublished data). Prior to experimentation, fish were acclimated to laboratory conditions (24–26 °C; 12:12 h light:dark cycle) for 3 months in mixed-sex holding tanks (80 × 45 × 45 cm, 128 L; stocking density: 100 fish per tank). Fish were fed *ad libitum* once daily with commercial fish food (Otohime Hirame larval diet; 580–910 μm).

2.2. Chemical exposure and monitoring

A 28-day fluoxetine exposure was performed using a flow-through system, following the design of Saaristo et al. (2013) and Bertram et al. (2015). Briefly, fish were randomly assigned to one of three exposure treatments: freshwater control, low fluoxetine and high fluoxetine. For each treatment, a large glass mixing tank (182 L) fed water into four identical separate-sex aquaria housing 30 fish (two tanks per sex; 60 × 30 × 30 cm, 54 L). During the exposure, fish were kept under a 12:12 h light:dark cycle and temperatures maintained at 24.4 ± 0.8 °C (±SD). Flow-through rates were maintained at 24 h cycling (~1.67 L/h per tank).

For the low- and high-fluoxetine treatments, a stock solution of fluoxetine was continuously added to the mixing tank (1.95 mL/min). The stock solutions (3 L) were prepared and changed daily. To achieve this, fluoxetine hydrochloride (Sigma-Aldrich; Product Number: F132, CAS: 56296-78-7) was dissolved in advance in 1 mL of methanol (32.1 μg/mL for high treatment and 321.0 μg/mL for low treatment). Then, on the day that the stock solutions were required, the methanol solvent was evaporated under a gentle nitrogen flow for 15 min before being diluted with 2999 mL of Milli-Q water. During the 28-day exposure period, 1 L water samples were periodically drawn from all exposure tanks to monitor fluoxetine concentrations (see below for measured concentrations). Specifically, following Anumol et al. (2013), the concentration of fluoxetine in each sample was analysed using ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). Compound separation was achieved using an Agilent 1210 binary pump (Palo Alto, CA) equipped with a ZORBAX Eclipse Plus reverse phase column (2.1 × 50 mm). The analysis was performed using an Agilent 1210 UHPLC connected to an Agilent 6410 triple quadruple mass spectrometer (QQQ). Blank and laboratory control samples (LCS) used as quality control samples were analysed with each batch of nine samples. There was no background contamination present in blank samples and LCS recoveries were in an acceptable range (fluoxetine recovery: 70–100%; $n = 6$).

2.3. Experiment one: predator avoidance

To investigate the effects of fluoxetine exposure on predator avoidance behaviour, a 3 × 2 factorial design was used, incorporating exposure treatment (unexposed, low fluoxetine and high fluoxetine) and predation risk (presence versus absence of dragonfly nymph). Measured fluoxetine concentrations in the low and high treatments were 25 ± 18 ng/L (mean ± SD, $n = 12$) and 226 ± 172 ng/L ($n = 12$), respectively.

Australian emperor dragonfly nymphs (*Hemianax papuensis*) were used as a predator stimulus, having been sourced from water bodies surrounding Geelong (Victoria, Australia). All nymphs were captured from the wild 14 days before experimental trials, during which time they were not fed, in order to standardise their hunger levels. A dragonfly nymph was selected as the predator model because large nymphs (like those of *H. papuensis*) are known to predate upon small fish (Pritchard, 1964) and have been used as a predatory stimulus in similar experiments (Squires et al., 2008; Barry, 2012, 2014). Additionally, *G. holbrooki* and *H. papuensis* share similar habitat preferences (Rowe, 1987; Pyke, 2005) and have been recorded sympatrically over a significant portion of their range in Australia (ALA, 2016a,b), including the source population of mosquitofish used in this study (*pers. obs.*).

Fish behaviour in the presence or absence of a dragonfly nymph was recorded in an observation tank (60 × 30 × 30 cm, 54 L), with 5 cm grid lines dividing the bottom of the arena. For each trial, focal fish were selected at random from exposure tanks and allocated to one of three observation tanks. Observation tanks were filled to a depth of 5 cm with aged water, with all tanks being emptied and dried between trials to control for any potential cross-contamination of chemical cues. In the predator-exposure trials, unexposed (male: $n = 19$, female: $n = 19$), low-fluoxetine exposed (male: $n = 16$, female: $n = 20$) and high-fluoxetine exposed (male: $n = 20$, female: $n = 19$) fish were individually presented with the visual and chemical cues of dragonfly nymphs. This was achieved by confining a nymph to one side of the observation tank in a small glass cage (6 × 2 × 2 cm) with a mesh net opening at one end

(2 × 2 cm) that allowed predator chemical cues to enter the tank environment. Trials in the absence of a predator were also conducted, where unexposed (male: $n = 19$, female: $n = 20$), low-fluoxetine exposed (male: $n = 16$, female: $n = 20$) and high-fluoxetine exposed (male: $n = 20$, female: $n = 20$) fish were presented with an identical, but empty, glass cage. Before the beginning of each trial, fish were acclimated for 5 min behind a clear partition positioned 10 cm from the edge of the tank on the opposite side to the predator. The partition was remotely lifted after acclimation, allowing the focal fish to freely explore the tank while its behaviour was recorded from above (Canon PowerShot S120).

Over the 5 min trial, we measured three behaviours: activity level, latency to enter the 'strike zone' and the total number of entries into the 'strike zone'. Activity level was measured by counting the total number of 5 cm grid lines crossed throughout the trial. Latency to enter the 'strike zone' and number of entries into the 'strike zone' were measured by observing the time taken for the fish to first enter the 1 cm zone around the perimeter of the predator cage and, subsequently, the number of times the fish ventured into this zone during the course of the trial. This zone represented the predator's striking range (i.e., 'strike zone') and was based on previously reported hunting tactics of *H. papuensis* nymphs (Rowe, 1987). Specifically, this strike zone was based on the length of the nymphs' striking mouth appendage or labium (mean length ± SD = 1.01 ± 0.14 cm, $n = 30$). All behaviours were quantified from video recordings using the event-recording software JWatcher V1.0 (Blumstein and Daniel, 2007).

2.4. Experiment two: predator escape

To investigate the impacts of fluoxetine exposure on predator escape behaviour, mosquitofish were subjected to a simulated predator strike. In this experiment, the measured concentrations of fluoxetine in the low and high exposures were 8 ± 7 ng/L (mean ± SD, $n = 10$) and 97 ± 45 ng/L ($n = 12$), respectively. As with Experiment 1, behavioural trials were performed using randomly selected fish from unexposed (male: $n = 35$, female: $n = 34$), low-fluoxetine (male: $n = 33$, female: $n = 33$) and high-fluoxetine (male: $n = 34$, female: $n = 34$) exposure tanks.

Trials were conducted in an observation tank (12 × 12 × 5 cm, 0.72 L) filled to 3 cm with aged water. Trials took place in shallow water to limit the vertical displacement of fish during the escape response, to more accurately measure horizontal escape velocity (Langerhans et al., 2004). Prior to behavioural recordings, fish were acclimated for 5 min in the observation tank, before a cylindrical metal probe with a rubber stopper (5 mm in diameter) was dropped into the tank to elicit an escape response. The probe was dropped within 3 cm of the fish, following Langerhans et al. (2004). To ensure the probe was dropped in a consistent manner, an automated lever system was used (see Fig. S1). The escape response of the focal fish was recorded from above (Canon PowerShot S120). Between trials, observation tanks were emptied and dried.

For each fish, two predator escape behaviours were measured after the simulated predator strike was delivered: the C-start escape response and the freezing response. Firstly, C-start escape response is a reflexive escape behaviour, initiated in response to a fear stimulus (e.g., a predator strike). The C-start escape is common among many species of fish (as well as amphibians) and is characterised by three distinct stages: rest, C-bend and propulsion (Hale et al., 2002). Each of these three stages were represented by a single camera frame captured at 30 frames per second, following Langerhans et al. (2004) and Grigaltchik et al. (2012). Specifically, frame one represented the rest stage prior to the probe being dropped. Frame two captured the C-bend, immediately after the probe was dropped. Lastly, frame three captured the propulsion stage, where the focal fish moved rapidly away from the fear stimulus. The C-start velocity (cm/sec) was calculated as the distance travelled between frame two and frame three using a point of mass tracking software (TrackerV8; Open Source Physics, USA). Not all fish exhibited C-start behaviour, with the performance of the C-start therefore being recorded as a binary response. Fish that did not perform a C-bend by frame two were given a score of zero.

Secondly, the amount of time spent by fish exhibiting freezing behaviour was recorded for 1 min following the deployment of the simulated predator strike. Freezing behaviour is another common antipredator behaviour in fish, in which the fish ceases all movement (except for those involved in respiration) after a fear-inducing stimulus (Godin, 2002; Brown and Magnavacca, 2003). Freezing behaviour is a strategy of crypsis, used to avoid detection when a predator is within close proximity or has already launched a strike (Godin, 2002). The total time spent exhibiting freezing behaviour was calculated using velocity data from each frame over 1 min (frame duration: $^1/_3$ sec). Specifically, total freezing time reflected the total amount of time that a fish spent moving less than 0.5 cm/s.

2.5. Morphological analysis

In both experiments, immediately after behavioural trials, fish were euthanised with an overdose of anaesthetic clove oil (40 mg/L) and morphological measurements were taken. Fish were weighed (±0.0001 g) and their standard length measured (±0.01 mm). An index of fish condition was then derived from a regression of fish mass (g) against standard length (mm). This condition index was calculated as the residuals from the least squares regression line and was calculated for males and females separately. Condition index was used as a proxy for health and was compared to investigate any possible shifts in fish heath as a result of fluoxetine exposure.

2.6. Statistical analysis

Data were analysed in R version 3.2.2 (R Development Core Team, 2015) and were checked for normality (Shapiro-Wilk test; *shapiro.test* function; Royston, 1995) and homogeneity of variance (Fligner-Killeen test; *fligner.test* function; Conover et al., 1981) as appropriate. Additionally, exposure tank number—as a measure of tank effects—was initially included in all models, but did not significantly affect any of the response variables. Therefore, exposure tank was excluded from all final models to increase the predictive power of our analyses.

For experiment one, models testing the impacts of fluoxetine exposure on predator avoidance behaviours included three predictors (fluoxetine treatment, predator presence or absence and fish sex), as well as one covariate (condition index). Activity (number of 5 cm grid lines crossed) was analysed using a three-way Analysis of Covariance (ANCOVA) (*aov* function; Chambers and Hastie, 1992). Tukey's Post Hoc testing (*glht* function, *multcomp* package; Hothorn et al., 2008) was used to further investigate relationships between treatment groups. A Cox Proportional Hazards Survival Analysis (*survreg* function, *survival* package; Therneau and Grambsch, 2000) was used to compare the latency of fish to enter the strike zone. A *Weibull* hazard function was selected as the most appropriate distribution for the model, determined by a comparative analysis of multiple hazard distributions using an ANOVA. The model met the assumption of proportionality, as tested by examining the interaction between Schoenfeld residuals and log time (*coxph* and *cox.zph* functions, *survival* package; Grambsch and Therneau, 1994). Lastly, the number of entries by fish into the strike zone was analysed using a Generalised Linear Model (GLM). Vuong tests (*vuong* function, *pscl* package; Vuong, 1989) indicated

zero-inflation of count data. To address this, a zero-inflated Poisson GLM (ZIP GLM) was used (*zeroinfl* function, *pscl* package; Zeileis et al., 2008).

In experiment two, the number of C-starts performed was compared among treatments using a chi-square test (*chisq.test* function; Patefield, 1981). C-start velocity data were square root transformed and compared among treatments using a two-way ANCOVA. The ANCOVA included two predictors (treatment and fish sex), as well as one covariate (condition index). Total time spent performing freezing behaviour following the fear stimulus (sec) was rank-transformed and tested using a two-way ANCOVA. This model revealed a significant interaction between treatment and sex. As a result, the sexes were subsequently analysed separately to identify the main effect of fluoxetine. Tukey's Post Hoc testing was used to further examine the relationships between treatments.

For each experiment, the condition index of fish was also compared among treatments using three-way ANOVAs. To meet assumptions of normality, condition index was transformed using a rank normal function.

3. Results

3.1. Experiment one: predator avoidance

In the predator avoidance experiment, fluoxetine exposure significantly impacted fish activity levels (ANOVA: $F_{2,222} = 3.78$, $p = 0.025$), though no difference was detected in activity levels in the presence versus absence of a predator (ANOVA: $F_{1,222} = 0.34$, $p = 0.560$; Table S1), or between the sexes (ANOVA: $F_{1,222} = 0.90$, $p = 0.344$; Table S1). Fluoxetine exposure caused a non-monotonic shift in activity. Specifically, fish in the low-fluoxetine treatment were significantly more active than unexposed fish (Tukey's HSD: $t = 2.66$, $p = 0.023$; Fig. 1), while there was no difference in activity between high versus unexposed, or low versus high, fluoxetine treatments (Tukey's HSD: $t = 1.90$, $p = 0.144$ and $t = 0.82$, $p = 0.692$, respectively; Fig. 1). Latency to enter the strike zone was significantly reduced in both fluoxetine treatments compared to unexposed fish (Cox Regression Survival Analysis: low treatment, $z = -2.20$, $p = 0.028$; high treatment, $z = -2.94$, $p = 0.003$; Fig. 2). However, the latency to enter the strike zone was consistent between trials with and without a predator (Cox Regression Survival Analysis: $z = 0.70$, $p = 0.482$; Table S1). Additionally, across all treatments (regardless of exposure), male fish more rapidly entered the strike zone compared to females (Cox Regression Survival Analysis: $z = -4.06$, $p < 0.001$; Table S1). There was no difference in the number of strike zone entries between fluoxetine-exposed and unexposed fish, with an average of 3.31 ± 0.30 (mean ± SE) for unexposed, 3.61 ± 0.34 for low-exposed and 3.80 ± 0.43 for high-exposed fish (ZIP GLM: comparison between control and low, $z = 0.20$, $p = 0.839$; control and high, $z = -0.26$, $p = 0.795$; low and high, $z = -1.29$, $p = 0.199$). Similarly, there was no difference in strike zone entries for trials with and without a predator (ZIP GLM: $z = -0.02$, $p = 0.982$; Table S1), although, across trials, males were found to enter the strike zone significantly more often than females (ZIP GLM: $z = 4.72$, $p < 0.001$; Table S1).

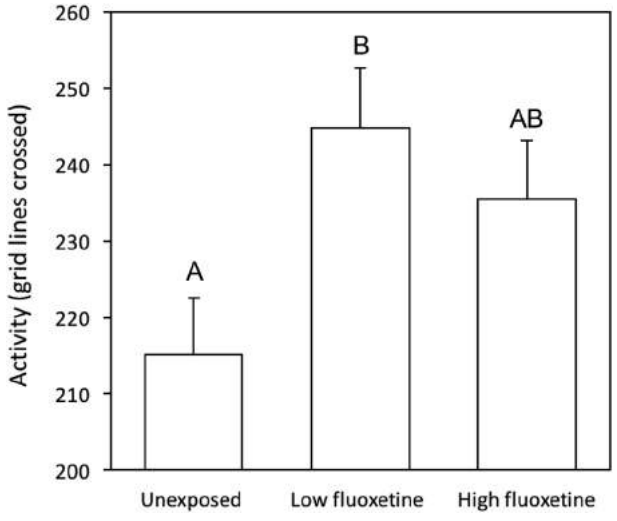

Fig. 1. Mean (+SE) activity levels (i.e., number of 5 cm grid lines crossed) for unexposed ($n = 77$), low-fluoxetine ($n = 72$) and high-fluoxetine ($n = 79$) treatments. Treatments without letters in common are significantly different.

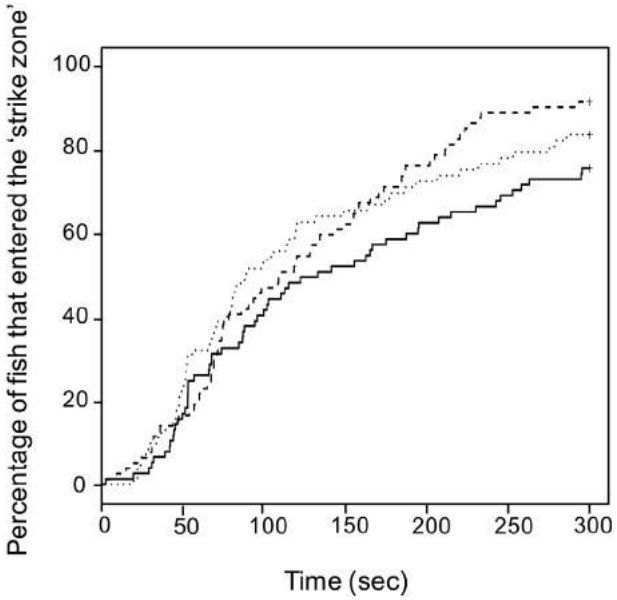

Fig. 2. Percentage of fish that entered the 'strike zone' over time, with the unexposed treatment represented by a solid line ($n = 77$), low-fluoxetine treatment by a long dash ($n = 72$) and high-fluoxetine treatment by a short dash ($n = 79$).

3.2. Experiment two: predator escape

The number of fish that performed a C-start response did not differ significantly between treatments (unexposed: 79.71%, low-fluoxetine exposed: 78.79%, high-fluoxetine exposed: 78.26%; Chi-squared test: $\chi^2 = 0.040$, df = 2, $p = 0.980$). Likewise, C-start escape velocity (cm/sec) did not differ across treatments, with a mean ± SE escape velocity of 32.19 ± 1.68 for unexposed fish, 32.75 ± 1.94 for low-exposed fish and 30.36 ± 1.80 for high-exposed fish (ANOVA: $F_{2,156} = 0.48$ $p = 0.620$). In addition, there was no effect of sex on C-start velocity (ANOVA: $F_{1,156} = 0.23$, $p = 0.635$), with the average C-start velocity of males and females being 32.68 ± 1.49 and 30.96 ± 1.44, respectively. There was, however, a significant interaction between treatment and sex on freezing behaviour (sec) following the simulated strike (ANOVA: $F_{2,192} = 5.16$, $p = 0.006$). Freezing behaviour of both male and female fish was altered by fluoxetine exposure (ANOVA: males $F_{2,99} = 6.92$ $p = 0.001$ and females $F_{2,97} = 9.10$, $p < 0.001$), although the effects of fluoxetine were sex-dependent. Specifically, males exposed to both low and high treatments exhibited significantly reduced freezing behaviour compared to unexposed fish (Tukey's HSD: $t = -2.52$, $p = 0.035$ and $t = -3.63$, $p = 0.001$, respectively; Fig. 3). For females, fluoxetine exposure had a non-monotonic effect

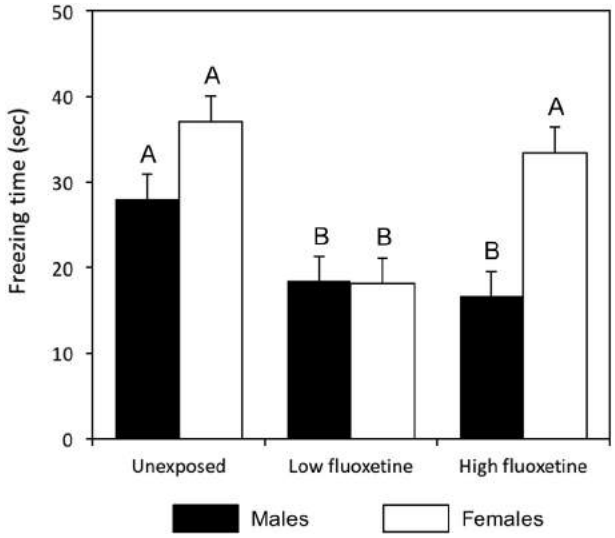

Fig. 3. Mean (+SE) freezing time (sec) over 1 min following a simulated predator strike for unexposed (males: $n = 35$; females: $n = 34$), low-fluoxetine (males: $n = 33$; females: $n = 33$) and high-fluoxetine (males: $n = 35$; females: $n = 34$) treatments. Treatments without letters in common are significantly different.

on freezing behaviour, with low-fluoxetine exposure resulting in a reduction of freezing behaviour compared to both unexposed and high-exposed fish (Tukey's HSD: $t = -4.02$, $p < 0.001$ and $t = -3.36$. $p = 0.003$, respectively; Fig. 3), while no difference was detected between unexposed and high-exposed fish (Tukey's HSD: $t = -0.66$, $p = 0.786$; Fig. 3). Additionally, males (regardless of exposure) generally spent less time exhibiting freezing behaviour, with a mean ± SE of 29.60 ± 1.90 s, compared to females at 21.12 ± 1.73 s ($F_{1,192} = 14.28$, $p < 0.001$).

3.3. Morphology

No significant differences were detected in condition index between unexposed, low-exposed and high-exposed treatments for fish used in the predator avoidance experiment (ANOVA: $F_{2,225} = 0.06$, $p = 0.940$; Table S2). Similarly, there were no differences in condition index across unexposed, low-exposed and high-exposed treatments for fish used in the predator escape experiment (ANOVA: $F_{2,201} = 0.13$, $p = 0.880$; Table S2).

4. Discussion

We found that exposure to environmentally relevant concentrations of fluoxetine increased mosquitofish locomotor activity regardless of the presence or absence of a predator, as well as decreasing the time taken to enter a predator's 'strike zone'. Fluoxetine exposure, however, did not affect the number of times fish entered the striking range of a predator. Additionally, while there was no effect of fluoxetine on C-start escape performance, fluoxetine-exposed fish showed a reduction in freezing behaviour following a simulated predator strike. Further, the behavioural shifts seen in both the predator avoidance and predator escape experiments occurred at comparatively lower fluoxetine concentrations (25 and 8 ng/L, respectively) than have previously been reported to have induced behavioural effects in vertebrates.

The general increase in activity levels—in both the presence and absence of the predator—seen at the low-fluoxetine dosage (i.e., 25 ng/L) contrasts with previous studies that have tested the effects of environmentally relevant fluoxetine exposure in other taxa (Barry, 2012, 2014; Winder et al., 2012). Specifically, Barry (2012) found that Arabian killifish (*Aphanius dispar*) showed a decrease in activity after exposure to fluoxetine at 300 ng/L and in the presence of conspecific alarm cues, with no difference between the lower dosage and control treatment (30 ng/L). Additionally, Barry (2014) found that tadpoles of the Arabian toad (*Bufo arabicus*) reduced their activity when exposed to 300 and 3000 ng/L of fluoxetine, though, again, no effect was reported at a lower dosage (30 ng/L). The differences between these findings could potentially be explained by differences in exposure scenarios, with Barry (2012, 2014) employing a shorter duration of exposure (7 and 14 days, respectively) in comparison to the present study (28 days). Acute and chronic fluoxetine exposures have been shown to produce different, and even conflicting, behavioural effects. Specifically, acute SSRI exposure seems to increase anxiety-like behaviour, while chronic exposure reduces the occurrence of such behaviour (Herculano and Maximino, 2014). The acute effects of fluoxetine are predominately the result of enhanced serotonergic neurotransmission, while fluoxetine's therapeutic (anxiolytic-like) effects—which can take 2–4 weeks to manifest—are driven by altered expression of serotonin receptors (Gardier et al., 1996; Hensler, 2003; Stewart et al., 2014). Therefore, it is possible that the increased activity levels seen in the present study are a result of the chronic effects of fluoxetine through neuronal adaptation (i.e., therapeutic effects), whereas the reduced activity observed by Barry (2012, 2014) may have been a result of the anxiety-inducing effects of acute exposure. More broadly, our findings are concordant with the recent study of Kellner et al. (2016), where 21-day exposure to another SSRI, citalopram, increased swimming activity in three-spined sticklebacks (*Gasterosteus aculeatus*) (Kellner et al., 2016).

In addition, we found evidence of a non-monotonic dose-response relationship, a phenomenon that has increasingly been detected as a result of low level antidepressant exposure (reviewed in Ford and Fong, 2015). Specifically, only fish exposed to the lower fluoxetine dosage (and not the higher dosage) showed a significant increase in activity levels (number of grid lines crossed) compared to unexposed fish. Broadly, these results are concordant with a number of studies that have similarly reported non-monotonic dose-response relationships to fluoxetine in various freshwater species (e.g., De Lange et al., 2006; Painter et al., 2009; Sánchez-Argüello et al., 2009; Guler and Ford, 2010; Di Poi et al., 2013; Bossus et al., 2014). These non-linear behavioural shifts resulting from low-dosage fluoxetine exposures seem to parallel those reported in endocrine disrupting chemicals (Vandenberg et al., 2012; Ford and Fong, 2015). Such findings underscore the fact that many of the potential causes for non-monotonic responses described for endocrine disrupting chemicals (reviewed in Vandenberg et al., 2012) may also be relevant to neurotransmission and neuroendocrine disruptors, such as fluoxetine.

Fluoxetine exposure reduced the average time taken for fish to enter the predator 'strike zone', as was evident in both fluoxetine treatments. In this respect, we rule out the possibility that reduced latency to enter the strike zone was merely a result of heightened activity, given that fish exposed to the higher treatment did not significantly differ in activity levels from control fish and yet, despite this, ventured into the strike zone more rapidly. Instead, it would appear that both low- and high-exposed fish are actively investigating and approaching the predator strike zone, irrespective of whether the predator was present or absent. Given that mosquitofish responded similarly to both the dragonfly predator and the empty glass cage (predatory control), it is possible that they did not perceive the dragonfly nymphs as a predatory threat. In this regard, a lack of dietary cues (i.e., alarm cues from recently consumed prey)—with nymphs having not been fed for a week to

standardise hunger levels—may have contributed to them having been perceived as less threatening (Smith and Belk, 2001; Ward and Mehner, 2010). Despite this, the nymphs used in this experiment would be expected to pose a predatory threat, particularly given that starved predators are likely to have the highest motivation to feed (e.g., Altwegg, 2003). Conversely, it is possible that the presence of both the predator and the empty glass cage resulted in similar levels of avoidance or, alternatively, inspection-like behaviour. In many fish species, including mosquitofish, individuals will actively approach novel objects or predators to gain information about potential resources and threats ('inspection behaviour') (Godin and Davis, 1995; Smith and Belk, 2001). Active inspection behaviour would explain why both low- and high-exposed fish demonstrated reduced latency to enter the 'strike zone', regardless of activity levels. Further, citalopram exposure (1.5 and 15 μg/L for 21 days) has previously been shown to increase inspection-like behaviour of a novel object in three-spined sticklebacks (Kellner et al., 2016). Critically, irrespective of why the fish responded similarly across trials with and without the predator, the fact that fluoxetine-exposed fish were more active and more readily entered the 'strike zone', could, in turn, increase their vulnerability to predation (Skelly, 1994; Johansson, 1995).

For the predator escape experiment, we found that fluoxetine exposure did not affect the C-start escape ability of fish. Exposed fish performed a similar number of C-start escapes, and did so at a similar velocity, compared with unexposed fish. This was surprising given that serotonin has been reported to have an inhibitory effect on Mauthner cells, neurons thought to be responsible for initiating C-start behaviour (Korn and Faber, 2005). Despite this, our results are comparable to those of Painter et al. (2009), where no difference was detected in the C-start ability of the adult fathead minnow (*Pimephales promelas*) following a 2-week fluoxetine exposure (at 25, 125 and 250 ng/L). The similar results reported in the present study and by Painter et al. (2009) suggest that 12–28 day exposure to environmentally relevant levels of fluoxetine (9–250 ng/L) may not be sufficient to significantly impact C-start escape ability in adult fish.

Fluoxetine exposure decreased the amount of freezing behaviour performed by fish subsequent to a simulated predator strike. This result may again be mediated by the anxiolytic effects of fluoxetine, causing a reduction in fear-like responsiveness to a potentially threatening situation. More generally, other SSRIs have also been shown to reduce fear-like responsiveness. Specifically, Endler guppies (*Poecilia wingei*) exposed to citalopram (2.3 and 15 μg/L for 21 days) reduced their freezing behaviour (among other anxiety-related behaviours) in a novel diving test (Olsén et al., 2014). In the present study, the effect of fluoxetine on freezing behaviour also appears to be sex-dependent. Females, but not males, demonstrated a non-monotonic dose-response relationship in freezing behaviour, exhibiting a reduction in freezing behaviour in the low-fluoxetine treatment only (i.e, 8 ng/L). By contrast, males showed a significant decrease in freezing behaviour at both low and high dosages (i.e, 8 and 97 ng/L). Sex-specific fluoxetine sensitivities have been reported previously in clinical studies using human and animal models (Dalla et al., 2010; Kercmar and Majdic, 2014). The sex differences observed in this study emphasise the importance of testing the ecological impacts of fluoxetine exposure on both males and females. Intriguingly, in both experiments, we also saw evidence of sex differences in behaviours regardless of exposure. Although this was not the focus of our study, these findings suggest that male and female mosquitofish differ in their behavioural responses to predatory stimuli.

In summary, we report that 28-day exposure to fluoxetine at environmentally relevant concentrations resulted in altered antipredator behaviour of adult fish. Further, some of these behavioural shifts seem to reflect non-monotonic and sex-dependent dose-responses. The behavioural shifts seen here could affect prey vulnerability, since active individuals that readily approach potential predators are more likely to be detected and captured (Skelly, 1994; Johansson, 1995). Moreover, the observed reduction in freezing behaviour in fluoxetine-exposed fish may increase the likelihood of detection (or re-detection) and capture by a predator following an initial strike. Indeed, our results suggest that fluoxetine exposure at environmentally realistic concentrations can alter antipredator behaviour and, in doing so, compromise the fitness of exposed wildlife. More broadly, such findings highlight the potential for pharmaceutical contaminants to affect ecosystem function and stability by altering the behavioural dynamics of predator-prey interactions (Brodin et al., 2014; Wong and Candolin, 2014).

Ethics

The present research was approved by the Biological Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2015/02) and complies with all relevant State and Federal laws of Australia.

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Conflict of interest statement

The authors declare the inexistence of any conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.10.010.

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Appendix C

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

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HIGHLIGHTS

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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

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

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

2. Methods

2.1. Animal housing

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

2.2. Chemical exposure

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

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

2.3. Monitoring of 17β-trenbolone

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

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

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

2.4. Behavioural trials

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

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

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

2.5. Morphological analysis

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

2.6. Statistical analysis

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

3. Results

3.1. Aggressive behaviour

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

3.2. Mating behaviours

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

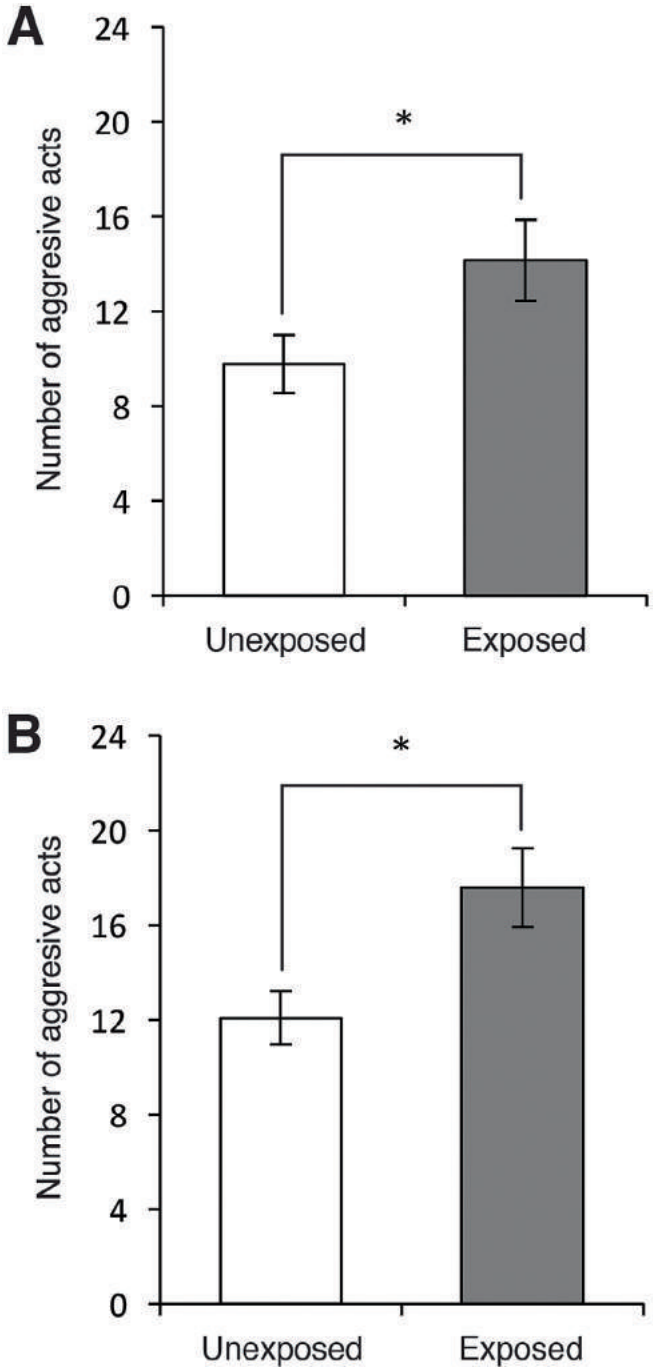

Fig. 1. Mean (±SE) number of aggressive acts (i.e., chases and fin nips) directed by a male towards a rival when A) males were separated from females by a transparent partition ($n = 37$), and B) males were allowed to interact freely with females ($n = 37$). Asterisks indicate a significant difference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

3.3. Morphology

No significant difference was detected in weight (Mann-

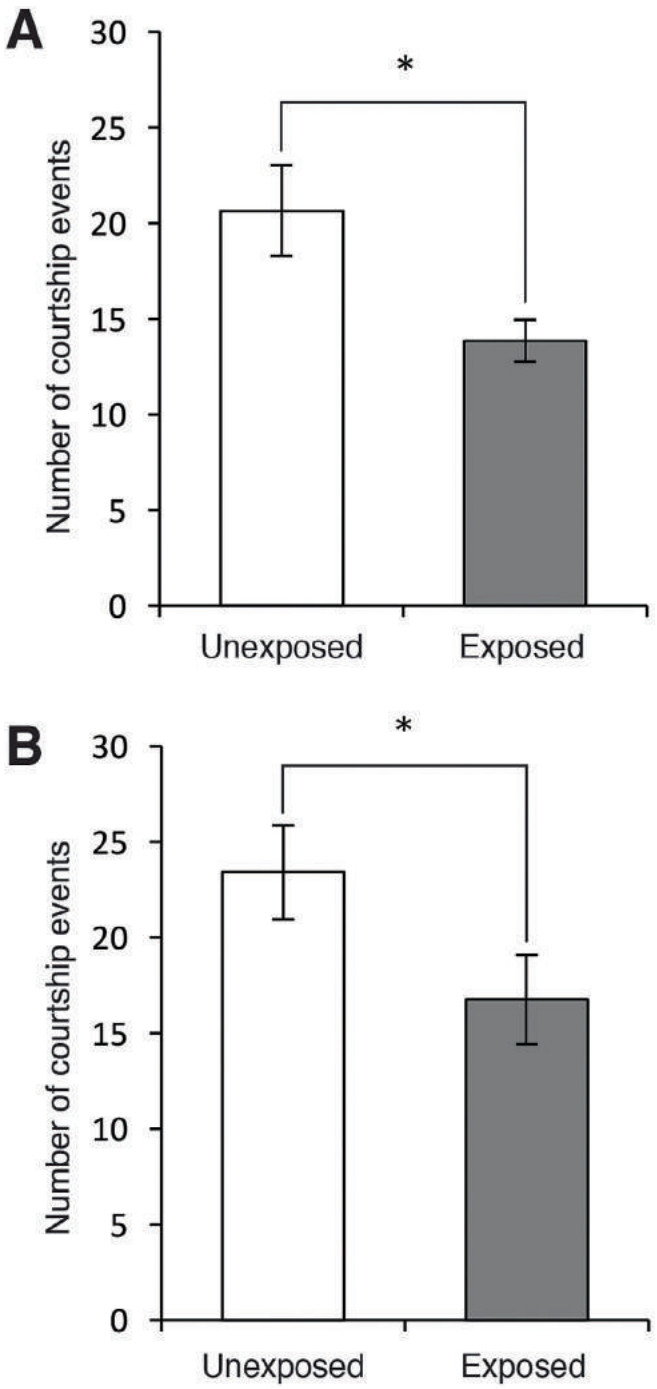

Fig. 2. Mean (±SE) number of courtship events conducted by males when A) males were separated from females by a transparent partition ($n = 37$), and B) males were allowed to interact freely with females ($n = 37$). Asterisks indicate a significant difference between groups at $\alpha = 0.05$.

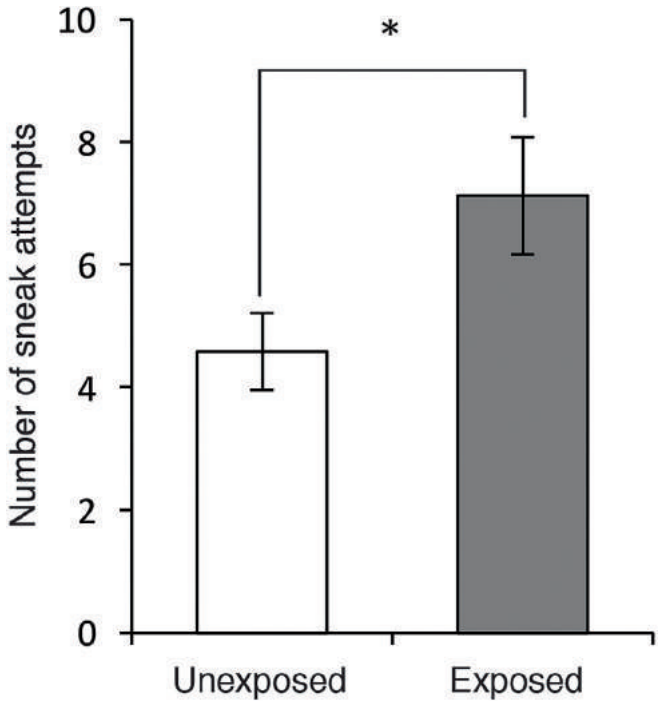

Fig. 3. Mean (±SE) number of sneak mating attempts conducted by males towards females when allowed to freely interact ($n = 37$). Asterisk indicates a significant difference between groups at $\alpha = 0.05$.

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

4. Discussion

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

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

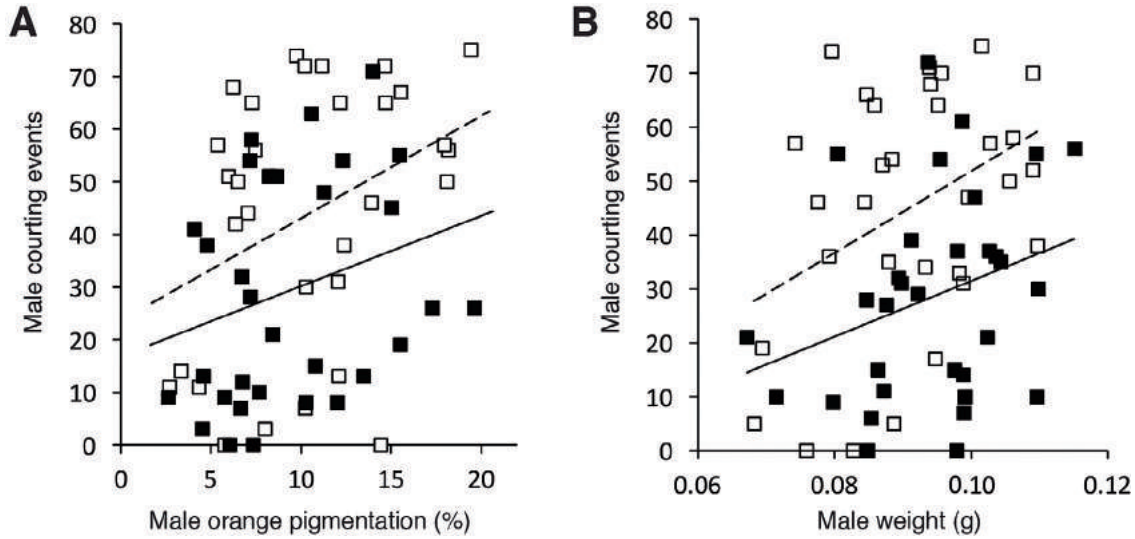

Fig. 4. Number of courtship events performed by unexposed and exposed males as a function of A) male orange pigmentation (% of body area) and B) male weight (g). Figures represent combined data from both behavioural trial stages (i.e., when males were separated from females by a transparent partition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

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

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

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

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

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

Ethics

This study was conducted with the approval of the Biological

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

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Conflict of interest statement

The authors declare no competing interests.

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Appendix D

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

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

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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.

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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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 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 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 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 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.

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 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).

2.2. 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.

2.3. 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 × 30 cm) in a 500 mL holding container and a single stimulus (i.e., unexposed) male placed into the other compartment (10 × 30 × 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 to 8.22% (mean = 5.21%, SD = 1.32%), while high-orange males ranged from 12.05 to 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).

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 = 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 $\alpha = 0.05$.

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. Results

3.1. Female association time during first male presentation

We found no interaction between treatment and male orange pigmentation on female association time ($F_{3,116} = 0.20$, $p = 0.660$). 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).

3.2. 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).

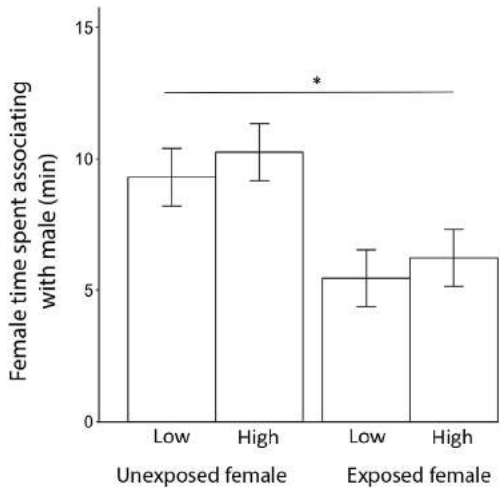

Fig. 1. Mean (±SE) time spent by unexposed and 17β-trenbolone-exposed females associating with low- and high-orange males during the first male presentation. The asterisk indicates a significant difference between groups ($p < 0.05$) obtained from ANOVA.

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 ($F_{1,112} = 33.47$, $p < 0.001$). 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).

3.4. 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.

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 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

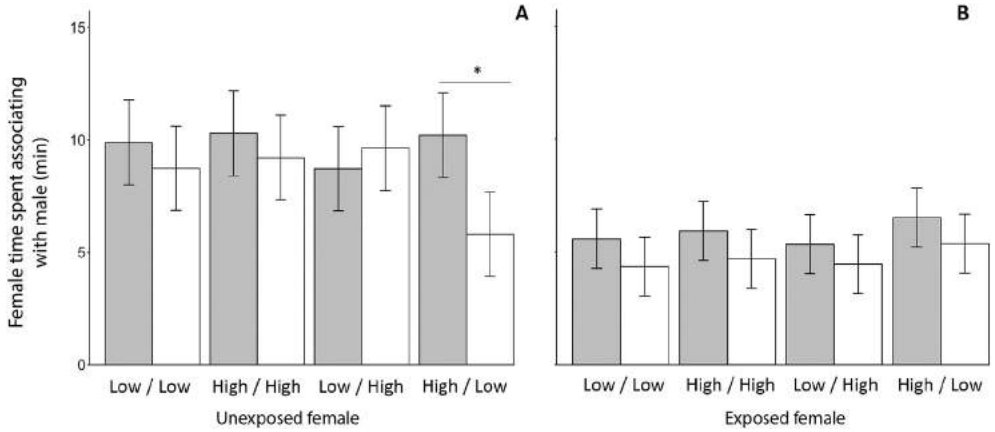

Fig. 2. Mean (±SE) time that (A) unexposed and (B) 17β-trenbolone-exposed females spent associating with males in each trial combination. Grey bars represent the first male presentation and white bars represent the second male presentation. The asterisk indicates a significant difference between groups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

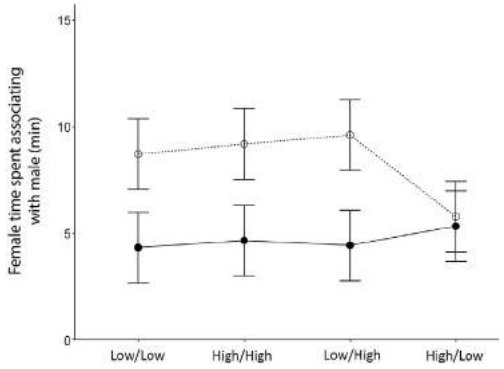

Fig. 3. Interaction plot showing the mean (±SE) time that unexposed females (open circles) and 17β-trenbolone exposed females (closed circles) from each presentation combination spent associating with males during the second male encounter. Plot displays the interaction between treatment and presentation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

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 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., 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, 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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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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Appendix E

Direct and indirect effects of chemical contaminants on the behaviour, ecology and evolution of wildlife

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Direct and indirect effects of chemical contaminants on the behaviour, ecology and evolution of wildlife

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Chemical contaminants (e.g. metals, pesticides, pharmaceuticals) are changing ecosystems via effects on wildlife. Indeed, recent work explicitly performed under environmentally realistic conditions reveals that chemical contaminants can have both direct and indirect effects at multiple levels of organization by influencing animal behaviour. Altered behaviour reflects multiple physiological changes and links individual- to population-level processes, thereby representing a sensitive tool for holistically assessing impacts of environmentally relevant contaminant concentrations. Here, we show that even if direct effects of contaminants on behavioural responses are reasonably well documented, there are significant knowledge gaps in understanding both the plasticity (i.e. individual variation) and evolution of contaminant-induced behavioural changes. We explore implications of multi-level processes by developing a conceptual framework that integrates direct and indirect effects on behaviour under environmentally realistic contexts. Our framework illustrates how sublethal behavioural effects of contaminants can be both negative and positive, varying dynamically within the same individuals and populations. This is because linkages within communities will act indirectly to alter and even magnify contaminant-induced effects. Given the increasing pressure on wildlife and ecosystems from chemical pollution, we argue there is a need to incorporate existing knowledge in ecology and evolution to improve ecological hazard and risk assessments.

1. Introduction

Contamination of the environment with diverse inorganic and organic compounds, such as pesticides, pharmaceuticals and metals, represents one of the main environmental challenges driven by anthropogenic activity. In 2010, the global chemical industry's value was US$4.12 trillion, having risen 54% over a decade [1]. In addition, the trend towards global urbanization is concentrating chemical consumption in cities faster than environmental interventions and remediation systems can be implemented, including in developing countries near biodiversity hotspots [2]. The increasing production and release of chemicals means that wildlife, humans and ecosystems are continuously exposed to chemical contaminants. While large-scale mortality events of wildlife represent an

obvious, if rare, sign of chemical releases, chemical contaminants can elicit more subtle but nevertheless important and harmful ecological impacts [3]. Further, chemical contamination of the environment is certainly not limited to short-term, acute exposures. Effects of long-term, low-level chronic exposures can be equally deleterious, though less obvious for human observers. In this review, we develop a conceptual framework that integrates concepts and approaches from multiple disciplines to investigate how chemical contaminants can alter animal behaviour, with resultant impacts on short- (e.g. individual and community) and long-term (e.g. evolutionary) responses, potentially leading to population declines.

Research on chemical contaminants conventionally recorded a limited range of endpoints, most commonly by studying mortality following exposure in the laboratory and/or by testing the impact of a single contaminant on a single species under standardized laboratory conditions ([4], but see [5]). These approaches are logistically tractable and repeatable but are criticized for their simplicity, particularly when such experiments neither take chemical nor biological complexity into account [6]. Behaviour, on the other hand, is the result of numerous complex developmental and physiological processes, and so connects physiological function and ecological processes [7]. Thus, behavioural change provides a comprehensive measure of both direct and indirect effects of chemical contaminants on individuals, linking to population-level processes [8–10] and, importantly, is often impacted at much lower contaminant concentrations than are traditional toxicological endpoints [11]. Here, we illustrate how behavioural responses can represent a powerful, highly quantifiable and biologically relevant indicator of environmental impacts.

Chemical contaminants can affect animal behaviour both directly and indirectly. Direct effects on behaviour in wildlife—here, we focus mostly on vertebrates—are caused by contaminants acting on the physiology of an animal (e.g. impaired sensory or cognitive abilities, altered endocrine/neural signalling, metabolic dysfunction). To date, research in behavioural ecotoxicology has largely focused on direct effects of contaminants on individuals (e.g. activity) (see §2). In contrast, indirect effects, when contaminant-induced changes to animal behaviour in one organism or species have cascading effects on other organisms and species in the exposed system, have received far less attention [12–15]. Indirect effects are most pronounced when a contaminant affects exposed organisms differentially, such as when one species is more sensitive and another more resistant (i.e. asymmetrical effects; [12,14,16]). While the importance of investigating both direct and indirect effects of contaminants is evident, this multi-directional approach has rarely been applied in ecotoxicology (but see [15,17]).

In this review, we focus exclusively on studies conducted under 'natural' conditions, specifically measuring behavioural responses following contaminant exposures in the wild or at environmentally relevant concentrations in the laboratory. We first critically examine existing literature on the role of chemical contaminants in mediating direct effects on individual behaviour (§2). In contrast with previous reviews [14,17], our focus centres on sublethal effects, particularly those induced by emerging contaminants, such as pharmaceuticals. Moreover, as well as considering short-term, mean behavioural responses to exposure, we discuss how chemical contaminants can alter trait variance (i.e. plasticity) and act as potent evolutionary forces. Moving from effects on individuals, we investigate how chemical contaminants can alter interspecific interactions indirectly via changes in behaviour of susceptible species (§3). By integrating these collective effects, we develop a conceptual framework to identify ways in which animal behaviour can be affected by chemical contaminants (§4). In doing so, we use predator–prey interactions as a case study to demonstrate how our conceptual framework has real-world impact. While we highlight the challenges of scale and complexity involved with predicting ecological effects of chemical contaminants (§5), we also provide directions for future research (§6). Finally, the overarching aim of this review is to improve research practices by increasing the ecological relevance of research approaches employed, in order to uncover global hazards and risks posed by chemical contaminants.

2. Direct effects on individual behaviour

Here, we discuss why, in a rapidly changing world, we need to expand our concept of direct effects—perhaps more accurately 'mean behavioural responses'—to incorporate the potential for chemical contaminants to affect both plasticity in, and evolution of, behavioural responses.

(a) Direct effects

Exposure to chemical contaminants can result in direct effects on a range of both 'general' behaviours (e.g. activity levels)—changes in which can have knock-on effects on multiple fitness-related traits—and specific mechanisms underpinning specific behaviours. Given that behaviour is the product of interconnected physiological, anatomical and neurological processes, and, in the wild, organisms are usually exposed to chemical cocktails rather than single contaminants, pinpointing mechanistic pathways between exposure to a contaminant and a behavioural change can be challenging. For example, round gobies (*Neogobius melanostomus*) collected from heavily contaminated industrial sites (e.g. polychlorinated biphenyls (PCBs), PAHs, metals) [18] or exposed to municipal wastewater effluent [19] both showed reduced aggression, even though the contaminant mixtures were very different.

Disruption of reproductive behaviours resulting from exposure to chemical contaminants has been increasingly studied in both laboratory and field settings because of the obvious population-level consequences [8]. Mechanisms underlying such behavioural changes include contaminant actions on endocrine and neural signalling, via changes to receptors, enzymes and/or transporters [20–22]. For instance, environmental exposures to organochlorine pesticides reduce parental care behaviour in predatory birds [23]. Studies on fish have demonstrated that exposure to municipal wastewater treatment plant effluent (e.g. [19]), and the active ingredients in (and metabolites of) the oral contraceptive pill, reduce nest building and courtship behaviours (reviewed in [20]). Furthermore, exposure to the insecticide endosulfan disrupts pheromonal communication between the sexes in red-spotted newts (*Notophthalmus viridescens*), leading to disrupted mate choice and depressed mating success [24]. Apparently subtle changes in reproductive behaviour could potentially be as devastating for fitness as major malformations of reproductive morphology, because an animal that fails to attract a mate or care for offspring appropriately will accrue zero fitness.

Changes in animal movement (e.g. frequency and speed) following contaminant exposure are common behavioural endpoints in ecotoxicological studies [25,26]. For example,

small-scale activity, which is often measured in the laboratory, has high ecological importance because it increases encounter rates with both resources (e.g. food, potential mates) and risks (e.g. predators, diseases). Activity also underlies individual dispersal and migration tendencies [27,28], although smaller scale movements measured in the laboratory do not automatically reflect larger scale movements in the field. Chemical contaminants can alter these movement behaviours by disrupting either sensory capabilities used to locate suitable environments and resources (e.g. inability to detect chemical cues [29–31]) or physiological pathways governing and supporting movement (e.g. neural/endocrine disruption, metabolic dysfunction [32,33]). Contaminants can, for instance, directly impair movement, making animals less adept at capturing prey and/or escaping predators, as has been noted in vertebrates exposed to acetylcholinesterase-inhibiting pesticides [34]. So far, only a handful of studies have connected these measures to dispersal or migration in the wild. One such study showed that Atlantic salmon (*Salmo salar*) smolts exposed to the anxiolytic pharmaceutical oxazepam migrate faster both in laboratory migration pools and down a river [35]. By contrast, while round gobies collected from heavily contaminated environments dispersed more slowly in a laboratory maze, there was no evidence that dispersal was affected in the wild [36]. Recent work has also demonstrated that exposure of European starlings (*Sturnus vulgaris*) to a PCB mixture in the laboratory resulted in reduced activity and incorrect orientation for migration [37], indicating that exposed birds might migrate later and less accurately in the wild. Overall, activity seems to be a sensitive and relatively easily measured endpoint, but its potential to indicate individual fitness or population-level processes is assumed rather than proven, in most cases.

Chemical contaminants can also interfere with complex behaviours, such as predator-avoidance, grouping and aggression, which have direct implications for fitness and population dynamics. By acting on the sensory system, contaminants can affect an organism's responses to conspecifics or predators by, for example, reducing their ability to detect stimuli, but also rendering them less active or motivated to respond [29]. If receivers are unable to detect prey, predators or signals from conspecifics, or alternatively if signallers emit altered signals, this could lead to ineffective communication [38]. The resulting disruption of group interactions and coordination could potentially reduce the anti-predator and food-location benefits of grouping [39]. By impacting conspecific detection pathways, chemical contaminants can also alter aggression and dominance hierarchies among individuals. For example, captive rainbow trout (*Oncorhynchus mykiss*) exposed to cadmium, which damages the olfactory epithelium, were less aggressive towards an unexposed rival and, therefore, formed dominance hierarchies faster [40].

Interestingly, some chemicals, such as psychoactive pharmaceuticals, have actually been designed to modulate adaptive stress or fear responses. Thus, they have great potential to impact foraging and anti-predator responses of wild animals (e.g. [41–44]). Indeed, recent studies have shown that exposure of fish to environmentally relevant concentrations of the antidepressant fluoxetine can extend the duration of 'freezing' behaviour [44] after predatory attack and increase activity levels regardless of the presence of a predator [43]. Because natural selection favours individuals that can quickly and accurately detect and assess risk, any disruption of this fine-tuned system is likely to have important implications for individual fitness [45] (see electronic supplementary material for more on predator–prey effects).

(b) Plasticity

Individuals can adjust their behaviour in response to chemical contaminants, i.e. they show phenotypic plasticity [7]. This 'plasticity' in behaviours has been the subject of much interest in behavioural ecology, because of its role in enabling species to cope with rapid environmental change [46,47]. However, most ecotoxicological studies so far have focused primarily on the mean behavioural responses of the contaminated population, with little to no mention of the variance in the trait. To date, we are unaware of any research explicitly investigating how contaminants can modulate behavioural plasticity or flexibility (i.e. how responsive individuals are to environmental variation) (but see [41]; §5). Predictions as to how plasticity will be modulated by chemical contaminants are not straightforward. If a behaviour is attenuated by a contaminant by, for example, all individuals becoming inactive regardless of environmental conditions, this could erode plasticity. Thus, there would be no benefit to individuals having variable responses to environmental changes, because they would never be expressed. Consequently, over time, this could decrease the intensity of selection for plasticity. In turn, this could reduce population variation in responsiveness to environmental change, reflecting a decrease in variance in behavioural responsiveness of all individuals. Conversely, one study found that exposure of jumping spiders (*Eris militaris*) to pesticides led to an increase in within-individual behavioural variability, while not changing the population's average level of predatory behaviour [48]. There is a clear need to integrate new experimental designs, technologies and statistical approaches (e.g. [35,47–50]) from behavioural ecology to measure individual behavioural responses under varying environmental conditions, such as, for example, multi-stressor studies, to better understand the consequences of contaminant exposure.

(c) Chemical contamination drives evolution

There is growing interest in the long-term, multi-generational consequences of chemical contamination and how contaminants might modulate population persistence and evolutionary trajectories. Our current focus is on how selection can act directly on exposed organisms, although it is important to acknowledge that selection may also operate indirectly via impacts of chemical contaminants on, for example, a species' prey, or competitors (see §4).

It is established that exposure to chemical contaminants can result in the evolution of physiological resistance, with perhaps the best-studied example being the micro-evolution of resistance in populations exposed to metal pollution (see [51,52]). By contrast, far less is known about how this resistance might affect the subsequent behavioural responses of exposed organisms. Adaptive physiological adjustments could reduce the likelihood that downstream behaviours are maladaptive. On the other hand, changes in physiology can also have negative effects on behaviour and life histories via the reallocation of resources required for growth and reproduction. For example, laboratory selection for cadmium resistance in least killifish (*Heterandria formosa*) resulted in decreased fecundity, female life expectancy and brood size [53]. Whether such trade-offs also impinge on behaviour remains to be tested.

Even in the absence of physiological resistance, organisms can simply change their behaviour, for example altering their diet, to avoid contaminants. However, it is often unclear whether these behavioural changes reflect plasticity or evolved responses [54,55]. Studies have shown spatial avoidance of contaminated sediments and water by aquatic invertebrates [55] and vertebrates [54,55], as well as adjustment of migration routes by salmon in response to metal pollution [56]. Other species show temporal avoidance of potential contaminant exposure by employing a faster life history or changing reproductive timing [52]. An interesting hypothesis is that the adaptive potential of an organism to respond rapidly to strong selection favouring earlier maturation and reproduction could, in turn, facilitate adaptations to novel stressors, such as chemical contaminants [57].

If organisms have neither evolved physiological tolerance nor behavioural compensation, exposure to chemical contaminants can result in drastic population declines [58]. This potentially creates a destructive feedback loop where a reduction in population size leads to further loss of genetic diversity, thus restricting the adaptive potential of populations [59,60], including adaptive behavioural responses. Chemical contaminants (e.g. persistent organic pollutants) can also affect mutation rate (e.g. [61]), which may either compensate for the loss of genetic diversity during population bottlenecks (e.g. marsh frogs, *Rana ridibunda* [62]) or otherwise alter population responses to contaminants [63]. However, most contaminant-induced mutations are likely to be deleterious [64]. Thus, adaptive behaviour that shields genotypes from otherwise harsh selection imposed by chemical contaminants could allow for population persistence and the maintenance of adequate levels of standing genetic variation crucial for further adaptation [65].

Chemical contaminants can also impact the strength and targets of selection via their direct effects on behaviour. For example, because sexually selected behaviours can affect the rate and trajectory of evolution (e.g. [66]), contaminants that interfere with sexual selection (e.g. endocrine-disrupting chemicals, EDCs; [67]) have considerable potential to affect subsequent evolution. For example, in European starlings, treatment with an EDC mixture resulted in males producing longer and more complex songs that are preferred by females, despite exposed males also having suppressed immune responses [68]. Whereas, in guppies (*Poecilia reticulata*), exposure to the agricultural contaminant 17β-trenbolone increased the occurrence of coercive copulatory behaviour in males, thus circumventing female mate choice [69]. While such changes that weaken sexual selection could further contribute to population decline [70], some studies find the opposite effect, whereby sexual selection enhances the evolution of mechanisms to cope with contaminants, presumably resulting in population growth. For example, flour beetles (*Tribolium castaneum*) evolved resistance to a pyrethroid pesticide faster when sexual selection was allowed to occur compared with when it was experimentally precluded [71].

Given the importance of evolution in facilitating population persistence, a key question is: what might limit the ability of organisms to evolve adaptive physiological or behavioural responses to contaminants? One possibility is that it may be difficult to adaptively respond simultaneously to multiple contaminants, or, more broadly, multiple stressors that exert conflicting selection pressures [72]. Resistance to a single class of contaminants, such as pesticides, can evolve very quickly, but evolving resistance to cocktails of contaminants with different modes of action is likely to be much slower. Here, the ability to cope with a particular contaminant could make it more difficult to deal with another [63]. A complementary idea emphasizes the role of evolutionary history—i.e. the notion that organisms often have greater difficulty coping with stressors that are truly 'novel', as opposed to those that are mechanistically similar to those that are familiar [73]. Clearly, there is a need for a deeper mechanistic understanding of when and why plastic or evolutionary responses to one contaminant should facilitate or conflict with responses to another.

3. Indirect effects of chemical contaminants on behaviour via interspecies interactions

Contaminants can, as outlined above, exert direct effects on the behaviour of species, which often results in decreases in organism abundance. However, species and their behaviours can also be altered *indirectly* because changes in behaviour (or abundance) of susceptible species will lead to cascading indirect effects—even on resistant species—at all trophic levels within a community. One of the most commonly documented indirect effects of contamination is predator responses to reduced prey abundance caused by contaminant-induced direct lethality or reproductive failure in their prey species. A population crash of fathead minnows (*Pimephales promelas*), caused by experimental EE2-exposure of a whole lake, led to cascading indirect effects: zooplankton populations in the exposed lake increased without minnow predation, while the biomass of larger lake trout (*Salvelinus namaycush*) decreased without minnows as a prey item [14]. Indirect effects can also reduce the efficacy of ecosystem services provided by wildlife. For instance, population crashes of *Gyps* vultures in India due to diclofenac toxicity resulted in an increase in feral dogs scavenging on decaying carcasses and a consequent increase in human rabies infections from dog bites [74]. By contrast, examples of indirect effects caused specifically by changes to animal behaviour are rare in the literature [16]. For example, mummichog (*Fundulus heteroclitus*) from industrial sites were less active and less adept at capturing prey grass shrimp (*Palaemonetes paludosus*) than were fish at pristine sites, allowing these prey to grow larger and become more abundant [75]. We predict that contaminant-induced increases in boldness or aggression in one species, for example, will change the competition and predation pressures on, and thus alter the behaviour of, other species within a community (figure 1). Contaminant-disrupted courtship leading to declines in abundance is predicted to have cascading effects on the interspecies interactions across a community. Here, we use cascading effects as a tool to illustrate the importance of indirect effects in ecological risk assessment, although other indirect effects such as keystone predator effects and exploitative competition can also be locally important [76]. The key point, here, is the need to understand the mechanism, i.e. the contaminant-induced change in behaviour(s), initiating the cascade.

Given the complexity of studying multi-species responses to contaminants [12], it is not surprising that indirect community effects, particularly those acting via changed behaviours, have not yet been broadly studied and quantified. First, multiple organisms must be studied simultaneously in real time using environmentally realistic mesocosms or field-based studies. Second, the system often must be studied for longer durations than are typical of laboratory exposures (i.e. several

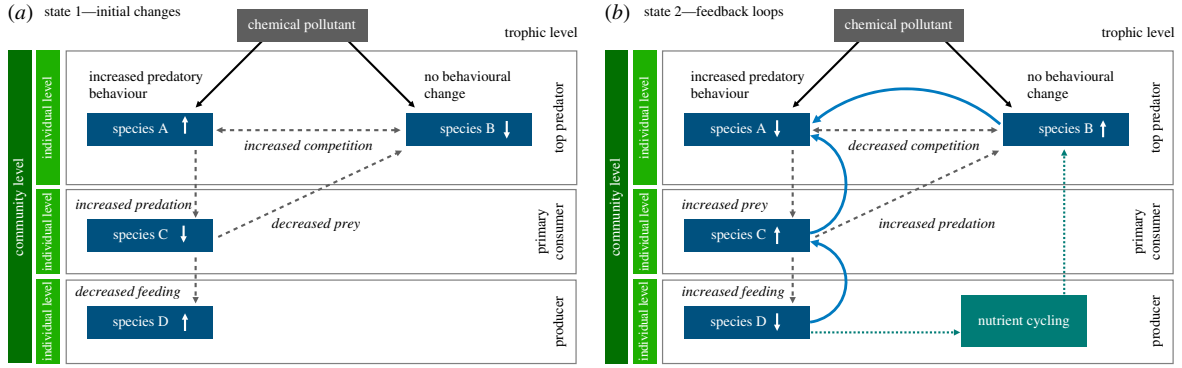

Figure 1. Outline of our conceptual framework modelling the direct and indirect effects of a chemical contaminant using predator–prey dynamics as a case study. Two predatory species (A and B) are exposed to a chemical contaminant. (*a*) State 1 shows initial changes to species in the food web at the individual and community levels; (*b*) state 2 includes feedback loops, which show dynamic interactions between species in time and space. Increases and decreases in population size for each species are indicated by arrows. The solid arrows indicate direct effects, dashed arrows indirect effects, dotted arrows nutrient cycling and blue arrows species interactions.

months to years). One might argue that studying indirect effects is redundant because the net effect on the community is the ultimate endpoint. However, because species compositions differ between most environments and reactions to contaminants can be highly species-specific, the net effect on a mesocosm community will only provide the outcome for that particular community. Without a mechanistic understanding of which behaviours in which species are affected and how, the generality, and, as such, the predictive power of mesocosm studies for risk assessment of particular contaminants, is limited at best. Knowledge of indirect effects is also crucial for modelling ecological risk, a promising and cost-effective tool that will help to reduce the number of animals required for ecotoxicological testing.

4. Conceptual framework for understanding the ecological and evolutionary impacts of chemical contaminants

Here, we have developed a conceptual framework that can be used by researchers aiming to design experiments or research programmes that move away from the 'one chemical–one species–one (usually lethal) endpoint' style of ecotoxicology (but see [71]) towards a more holistic approach. Specifically, our framework demonstrates the direct and indirect effects of chemical contaminants on the behaviour of individuals within a population, and of species within communities. We draw upon knowledge and literature from ecology and lay out potential scenarios of community-level effects caused by chemical contaminants (figure 1). As communities are composed of interconnected populations overlapping in time and space, the effects of chemical contaminants on communities necessarily manifest in the interactions within and among populations [72]. For example, some of the most salient interactions shaping ecological communities worldwide are between prey and their predators [72,73]. All animals are either prey or predators at some point in their lives and this interaction often has considerable consequences on individual fitness and population size [74].

Imagine that a chemical contaminant is introduced into an ecosystem. This chemical does not change the behaviour of top predator 'species B', but does increase the boldness of a second top predator 'species A', resulting in species A taking more risks, spending longer foraging and less time avoiding predators. 'Species C', the prey of species A, which is resistant to the contaminant, is indirectly affected because of the increased time and energy spent on anti-predator behaviours, but it is still consumed at a higher rate than when the ecosystem was uncontaminated. Thus, prey species C decreases in numbers, which, in turn, causes its own plant prey 'species D' to proliferate, thereby shifting the nutrient cycling and changing the ecosystem for all species (figure 1*a*). Notably, if the contaminant's action was conserved across taxa, such that species C also became bolder, its population would rapidly decline by predation-induced mortality from species A. Further, the decreased numbers of prey species C could potentially result in predator species B changing its foraging preference to alternative prey. The risky behaviour of species A will increase its own probability of being preyed upon, attacked by competitor species B and/or eating novel but toxic or infected foods. This would, in turn, decrease the predation pressure from predator species A on species C, and could potentially decrease competition between species A and B (figure 1*b*) [72]. We have included dynamic feedback loops to magnify the actions of the chemical contaminant on both directly and indirectly affected species, which, in turn, have community-level consequences and can alter ecosystem functioning (figure 1*b*).

Importantly, indirect effects due to contaminant-induced behavioural shifts could cause systems to respond far more strongly and quickly than an assessment of direct effects alone, or simply monitoring changes in the abundance of key predators, would predict [73]. Moreover, contaminant-mediated effects could yield novel forms of ecological interactions by, for example, inducing prey-switching due to changes in predatory behaviour and/or changes in prey abundance or quality, or by differentially altering the vulnerability of individuals or species to parasites [75]. Also, we have focused on the top-down effects, but some contaminants will affect primary productivity and so will have bottom-up impacts. These can be difficult to predict but, again, could have indirect, sublethal effects by increasing competition for food and/or necessitating greater foraging distances. Such a framework allows us to integrate and go beyond individual experiments and encourages researchers to assess behavioural

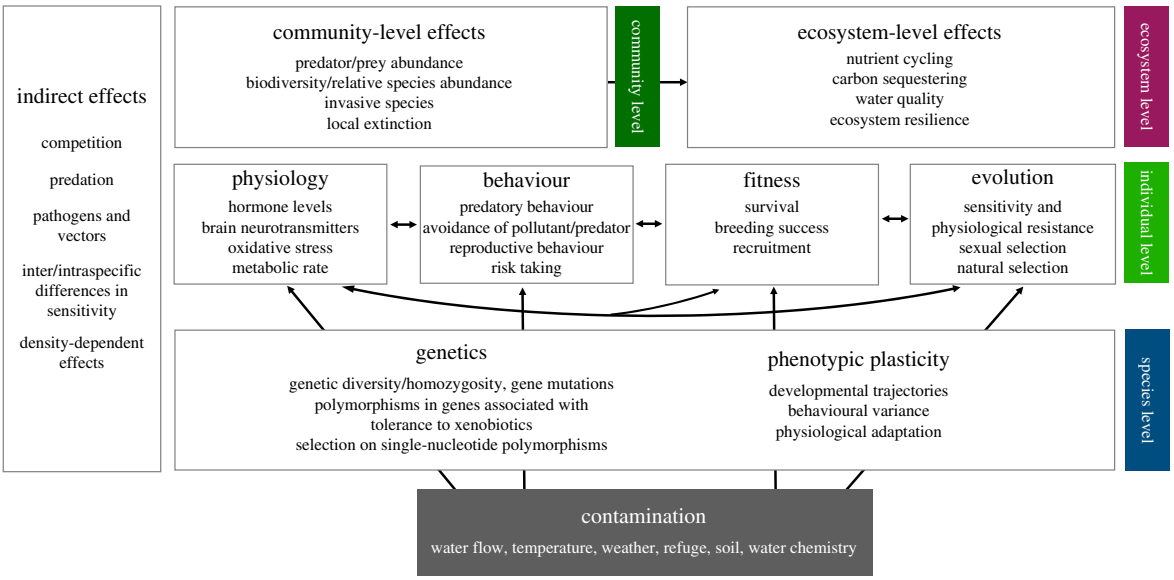

Figure 2. Implementation plan suggesting methodological approaches for utilizing our conceptual framework to identify the routes by which animal behaviour is affected by chemical contaminants. For each level of biological organization (individual, species, community and ecosystem), we highlight some of the factors that should or could be quantified or experimentally manipulated.

change within its environmental context. By understanding the behavioural mechanism underpinning multi-level changes, modelling, for example, can be used to predict the impacts of contaminants with similar modes of action for enhanced environmental risk assessments [77]. As an implementation plan, we provide figure 2, which directs researchers to consider which experimental design (laboratory, mesocosm or whole ecosystem manipulations) and level (individual, species or community) or modelling approaches are required, and which endpoints should or could be tested. Our basic framework can, therefore, be applied to specific behaviours and/or interspecific interactions, as well as to different levels of organisation, as required.

5. Problems of scale and complexity: predicting effects in the wild from effects in the laboratory

Predicting the ecological effects and behavioural perturbations caused by chemical contaminants is valuable for guiding legislation and policy to protect wildlife, but it is also challenging for many reasons. Behaviour is inherently variable—although so are many of the physiological endpoints currently measured—and how organisms respond to any given contaminant may vary across an individual's lifetime, between sexes, among individuals of the same species, and across species with different life histories, habitat use, trophic position and/or physiology [7,10,33,75,78].

Most earlier standardized ecotoxicological tests used model species that are easily cultured with simple, measurable endpoints [4], which allowed direct comparisons of toxicity among different compounds. This long-used approach has efficiently generated hazard and risk assessments for many chemical contaminants under the premise that similar species are equally affected by the contaminant. Of course, the 'all species are the same' argument does not hold for the effects of many contaminants (e.g. pharmaceuticals [79]). Inter- and intraspecies differences in physiology, behaviour and life history, when coupled with differential metabolism, generate substantial differences among species and individuals in susceptibility and responses to chemical contaminants. Unfortunately, our understanding of comparative mechanistic responses to contaminants still remains quite limited, even for model laboratory organisms.

Susceptibility differences between species are one of the key challenges in ecotoxicology. For example, studies have shown that small wild-caught prey fish are more sensitive to the anxiolytic effects of the pharmaceutical oxazepam than are larger predatory fish or laboratory-reared fish [5,80,81]. This could be due to species differences in the rate and extent of pharmaceuticals being taken up, metabolized and concentrated. Indeed, bioconcentration of pharmaceuticals in fish tissues can differ by several orders of magnitude between species [82], and even across life history stages [83]. Therefore, two species inhabiting the same polluted system can be exposed to very different internal concentrations of contaminants [81]. Moreover, tests including a less vulnerable life-stage might underestimate ecological risk [83]. Such differential exposures, and the associated effects, make it very difficult to predict the ecological effects of chemical contaminants in the environment [16].

Differential behavioural responses to chemical contaminants in laboratory-reared versus wild species have also been explained by the lack of predation risk or high competition in laboratory environments, which selects for inherited behavioural phenotypes that are often bolder, more aggressive and less responsive to predators than wild-type individuals [84]. For example, in assessing the risk of chemicals that potentially modify anti-predator behaviour, using a laboratory fish model that may exhibit a suppressed basal behavioural response to predators may greatly underestimate actual risk in the field (figure 3). Also, the distribution of behavioural traits studied

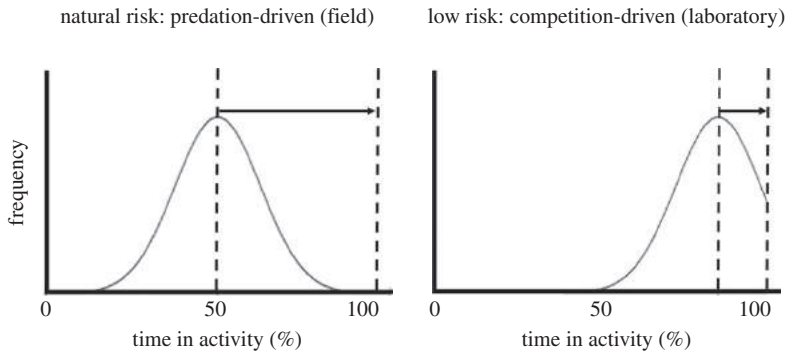

Figure 3. The distribution of expressions of a trait (here, activity) in two populations from environments with different levels of predation risk. (*a*) Population collected from the field (high predation); (*b*) laboratory-bred population (low predation). Black arrows illustrate the potential for contaminant-induced increases in activity in the populations (the longer the arrow, the greater the potential change).

should be characterized within each test group [83]. This consideration is critically important because a contaminant that acts to increase activity and/or boldness will more probably generate behavioural change in individuals originating from a (wild-type) population of low competition/high predation, compared with a (laboratory-reared) high-competition/low-predation population that contains many active and bold individuals (figure 3). Even in the wild, populations of the same species under different predation pressures are known to have evolved different physiology, morphology and behaviours [84]. In terms of our conceptual framework, such population-level differences in behavioural responses will alter both the state of a community before contamination, and the magnitude of feedback loops triggered by a contaminant. Such differences between populations, generated by differing selection regimes, have received very little attention despite clearly being important considerations when assessing contaminant vulnerability.

6. Future directions

The use of behavioural studies enables us to link the effects of contaminants at multiple levels of organization, from individual to ecosystem. This is an invaluable asset, because chemical contaminants have a wide range of actions and effects. At the individual level, the fields of behavioural ecology and so-called 'personalized medicine' are increasingly realizing the need to analyse inter-individual variation in responses, not just population means [46]. Far from being 'noise', plasticity in responses in itself represents a trait that can shape the capacity of individuals and populations to cope with environmental change in the short term. In this review, we illustrate that chemical contaminants can impact the capacity of populations to persist into the future by altering the strength and targets of evolutionary selection, for example, via direct effects of behaviour. To date, a mechanistic understanding of how evolutionary and plastic responses interact to facilitate population persistence is lacking. This also limits our ability to predict how populations will respond if legislation succeeds in reducing concentrations of specific chemical contaminants. Consequently, we have identified avenues to fill the knowledge gaps and challenge the often simplistic assessment of direct effects of contaminants, specifically in terms of how behaviour and other endpoints should be measured, analysed and interpreted.

With the rise in emerging contaminants, many of which are designed to exert sublethal effects on evolutionarily conserved physiological systems at ecologically realistic concentrations, it is important to update existing frameworks for studying their short- and long-term consequences. Sublethal behavioural effects can be both 'positive' and 'negative' for individuals, populations and communities. As illustrated by our conceptual framework (figure 1), effects can vary dynamically within the same individuals and populations. Indeed, this could be described as a key feature of emerging or dilute contaminants. Importantly, behavioural effects can lead to top-down and/or bottom-up effects. For example, changes at a lower trophic level could have sublethal effects by increasing competition for food and/or necessitating greater foraging distances. This is because linkages within communities will act indirectly to alter and even magnify contaminant-induced effects. Future work, integrating modelling, remote sensors and tracking technologies and statistical analyses, should focus on quantifying changes on the individual level and how the linkages within these networks are affected by contaminants. We argue that understanding the behavioural and ecological mechanisms underpinning contaminant-induced population changes will greatly increase the accuracy and power of environmental risk assessment to protect wildlife and ecosystems from disturbance by chemical contaminants.

Data accessibility. This article has no additional data.

Authors' contributions. M.S., T.B. and K.E.A. organized the symposia on which this paper is based, developed the conceptual framework, edited the manuscript and created figures. All authors contributed to publication writing. All authors gave final approval for publication.

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Appendix F

The pharmaceutical pollutant fluoxetine alters reproductive behaviour in a fish independent of predation risk

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The pharmaceutical pollutant fluoxetine alters reproductive behaviour in a fish independent of predation risk

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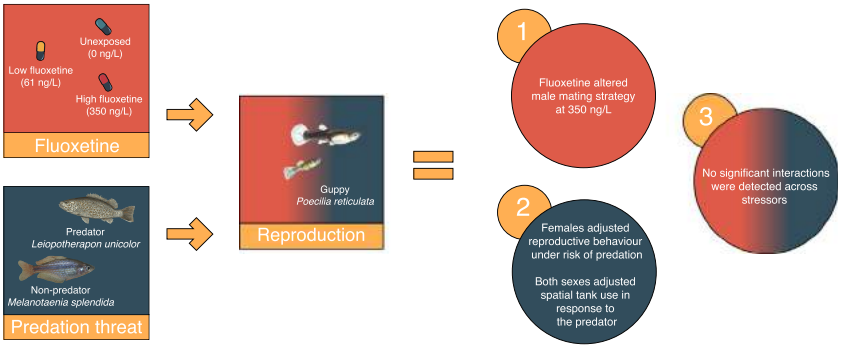
HIGHLIGHTS

- Pharmaceutical pollution represents a major global threat to wildlife and ecosystems.
- Guppies (*P. reticulata*) were exposed to fluoxetine at two field-realistic levels.
- Male and female guppy reproductive behaviour was assessed under predation risk.
- High fluoxetine (350 ng/L) increased male coercive mating behaviour, independent of a predatory threat.
- Highlights importance of considering interactions between natural stressors and pharmaceutical pollutants.

GRAPHICAL ABSTRACT



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ABSTRACT

Pharmaceutical pollutants constitute a major threat to wildlife because of their capacity to induce biological effects at low doses. One such pollutant is the antidepressant fluoxetine, which has been detected in surface waters globally at levels that recent studies suggest can alter physiology and behaviour in aquatic organisms. However, wildlife exposed to pharmaceutical contaminants are typically confronted with multiple stressors simultaneously, including predation risk, which is a particularly important natural stressor that can have direct (e.g. mortality) and indirect (e.g. changed prey behaviour) fitness effects. Accordingly, we investigated potential impacts of environmentally realistic fluoxetine exposure on reproductive behaviour in the guppy (*Poecilia reticulata*) under predation risk. Specifically, we tested whether fluoxetine exposure altered mating behaviour in male and female guppies in the presence of either a predatory spangled perch (*Leiopotherapon unicolor*) or a non-predatory rainbowfish (*Melanotaenia splendida*) control. We found that fluoxetine and the presence of a predatory spangled perch did not interact to affect reproductive behaviour. We also found that, independent of a predatory threat, fluoxetine exposure altered male mating strategy, with males in the high treatment conducting significantly more coercive 'sneak' copulations, whereas the number of courtship displays performed was not significantly affected. Moreover, while fluoxetine exposure did not significantly affect the amount of time that males and females spent following one another, we found that females, but not males, followed a potential partner less when in the presence of the predatory fish. Finally, both sexes reacted to the risk of predation by spending less time in close proximity to a predator than a non-predator. In combination, our findings highlight the capacity of fluoxetine to influence processes of sexual selection at field-realistic concentrations and emphasise the importance of considering multiple stressors when assessing impacts of pharmaceutical pollutants on the behaviour of wildlife.

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1. Introduction

Pharmaceutical pollution represents a major global threat to humans and wildlife (Arnold et al., 2014; Bernhardt et al., 2017; Boxall et al., 2012; Saaristo et al., 2018). Indeed, in excess of 600 different pharmaceutical contaminants (or their transformation products) have now been detected in the environment across >71 countries spanning all continents (Aus der Beek et al., 2016; Küster and Adler, 2014). In this regard, selective serotonin reuptake inhibitors (SSRIs), a widely prescribed class of antidepressants, are among the most commonly detected pharmaceutical pollutants in the environment (Silva et al., 2012). Acting by limiting reabsorption of the neurotransmitter serotonin into the pre-synaptic nerve cell, SSRIs elevate levels of extracellular serotonin in the synaptic cleft, leading to increased activation of post-synaptic receptors (Stahl, 1998). Serotonin is ubiquitous in all animal phyla possessing nervous systems and is known to play a key role in regulating a range of physiological and behavioural processes (Fent et al., 2006; Weiger, 1997).

One SSRI of particular environmental concern is fluoxetine, which is among the most commonly prescribed antidepressants in the world (Brijnath et al., 2017; Wong et al., 2005). Fluoxetine enters and remains in the environment as a result of excretion by human patients and insufficient removal during wastewater treatment processes (Arnold et al., 2014; Mennigen et al., 2011), with many countries worldwide not presently having regulatory frameworks in place for restricting the discharge of, or monitoring, fluoxetine in drinking water and wastewater flow (e.g. Australia: Department of Agriculture and Water Resources, 2016; European Union: The Council of the European Communities, 2018; New Zealand: Ministry of Health, 2018; United States of America: Environmental Protection Agency, 2016). In this regard, fluoxetine has been detected in surface waters globally, at concentrations typically ranging from <1–100 ng/L (e.g. Batt et al., 2015; Birch et al., 2015; Hughes et al., 2013; Kolpin et al., 2002; Meador et al., 2016; Paíga et al., 2016; Wu et al., 2017), and up to 596 ng/L in systems receiving wastewater discharge (Benotti and Brownawell, 2007). Moreover, levels as high as 929 ng/L have been reported in direct effluent flow (Bueno et al., 2007; Metcalfe et al., 2010).

While levels of fluoxetine found in the environment are not sufficient to induce lethal effects (e.g. 2.89 mg/L LC_{50} for juvenile topmouth gudgeon, *Pseudorasbora parva*: Chen et al., 2018; 198 μg/L LC_{50} for fathead minnow, *Pimephales promelas*: Stanley et al., 2007), many recent studies have found that fluoxetine exposure at close to, and at, environmental concentrations can alter a range of ecologically important traits in non-target species. Reported effects include altered development (Japanese medaka, *Oryzias latipes*: Foran et al., 2004; Northern Leopard Frog, *Rana pipiens*: Foster et al., 2010; western mosquitofish, *Gambusia affinis*: Henry and Black, 2008), growth (guppy, *Poecilia reticulata*: Pelli and Connaughton, 2015; California mussel, *Mytilus californianus*: Peters and Granek, 2016) and survival (guppy: Pelli and Connaughton, 2015). Fluoxetine exposure has also been linked to alterations in various key fitness-related behaviours, including feeding rate (fathead minnow: Weinberger and Klaper, 2014), sociability (Japanese medaka: Ansai et al., 2016; Arabian killifish, *Aphanius dispar*: Barry, 2013), aggression (Arabian killifish: Barry, 2013; Siamese fighting fish, *Betta splendens*: Dzieweczynski and Hebert, 2012), phototaxis (an amphipod, *Echinogammarus marinus*: Guler and Ford, 2010; water flea, *Daphnia magna*: Rivetti et al., 2016), boldness (Siamese fighting fish: Dzieweczynski et al., 2016a, 2016b) and activity (Arabian killifish: Barry, 2013; an amphipod, *Gammarus pulex*: De Lange et al., 2006; Siamese fighting fish: Kohlert et al., 2012), as well as learning and memory retention (common cuttlefish, *Sepia officinalis*: Di Poi et al., 2013). To date, however, investigations of behavioural shifts caused by fluoxetine have focussed on testing effects of exposure independently from other stressors typically found in the environment—as is also true for pharmaceutical pollutants more generally. In nature, however, complex interactions between multiple stressors are likely to be the norm rather than the exception (Blaustein and Kiesecker, 2002; Slocum and Mendelssohn, 2008). Moreover, of the studies that have considered such interactive effects, most have focussed on other abiotic factors (e.g. mixture effects with other pharmaceuticals, see De Castro-Català et al., 2017; Painter et al., 2009), with surprisingly few having examined potential effects of pharmaceutical pollutants in combination with biotic stressors.

Predation is a ubiquitous biotic stressor that can impact fitness directly via mortality or indirectly by producing changes in prey morphology, life-history and/or behaviour (Creel and Christianson, 2008; Sih et al., 1985). Previous studies have shown that fluoxetine can alter behavioural responses of fish to visual (e.g. Martin et al., 2017; Pelli and Connaughton, 2015; Saaristo et al., 2017) and chemical (e.g. Barry, 2014) predator cues. However, to date, potential interactive effects of fluoxetine exposure and predation risk on reproductive behaviours have not been investigated. Such behaviours include conspicuous mating displays, which often communicate an individual's phenotypic and genetic quality, such as health, ability to sire young, and quality of parental care (Barber et al., 2001; Hoikkala et al., 1998; Lindström et al., 2006; Sargent, 1982). However, conspicuous sexual displays can also be costly, as they often elevate an individual's vulnerability to predators by increasing detectability and rate of predator-prey encounters (Hoefler et al., 2008; reviewed in Lima and Dill, 1990), and by limiting escape potential from would-be predators (Cooper, 1999; Killian et al., 2006). In light of such costs, individuals often adjust their reproductive behaviour according to perceived predation risk (Sih, 1994). For example, to minimise the likelihood of detection, male cross-banded tree frogs (*Smilisca sila*) reduce their calling rate—a behaviour used to attract females—when in the presence of a predator (Tuttle and Ryan, 1982). Therefore, it is important to consider potential interactions between pharmaceutical pollutant exposure and predation risk on reproductive behaviour in wildlife (reviewed in Saaristo et al., 2018).

The guppy (*Poecilia reticulata*) is a small, internally fertilising poeciliid native to north-eastern South America (Rosen and Bailey, 1963) that is now found in over 69 countries around the world (Deacon et al., 2011). Guppies inhabit freshwater habitats, many of which are exposed to wastewater contaminants (Araújo et al., 2009; reviewed in Magurran, 2005), such as fluoxetine (Hughes et al., 2013). Guppies have also been the focus of extensive behavioural research examining mating tactics under predation risk (reviewed in Houde, 1997), which, in combination with their presence in polluted environments, makes them an ideal model for investigating potential effects of fluoxetine contamination and predation risk on reproductive behaviour. Indeed, guppies have recently received increasing attention as a model species in behavioural ecotoxicology (Bertram et al., 2015; Holmberg et al., 2011; Pelli and Connaughton, 2015; Saaristo et al., 2017; Tomkins et al., 2017). Male guppies engage in two alternative mating strategies, either soliciting copulations from females by performing elaborate courtship displays or engaging in surreptitious 'sneak' copulations without first courting the female (Houde, 1997). When under threat of predation, males typically favour sneaking behaviour as the conspicuous nature of courtship displays increases the likelihood of detection by predators (Endler, 1987). Moreover, sneak copulations circumvent some of the energetic costs associated with courtship displays, although sneaking also carries a relatively low probability of successful insemination, with approximately one third as many sperm being transferred during sneak copulations compared to copulations following courtship (Matthews and Magurran, 2000; Pilastro and Bisazza, 1999; Pilastro et al., 2007). Given these trade-offs, males should favour sneaking in situations where courtship displays are less effective or are relatively costly, such as in environments with high predation risk (reviewed in Houde, 1997).

Here, we examined impacts of short-term (28-day) exposure to two environmentally relevant levels of fluoxetine—nominal low and high concentrations of 40 and 400 ng/L, respectively—on male and female guppy reproductive behaviour in the presence or absence of a predatory

threat. We tested for individual effects of fluoxetine exposure and predation risk, as well as their potential interactive effects, on reproductive behaviour. Given that fluoxetine exposure at environmentally realistic levels has been shown to reduce various antipredator behaviours (e.g. Martin et al., 2017; Pelli and Connaughton, 2015), we predicted that, when subjected to an increase in perceived predation risk, exposed individuals would adjust their mating behaviour to a lesser extent than controls.

2. Materials and methods

2.1. Animal collection and housing

Guppies used in this study were laboratory-reared descendants of a wild population from Alligator Creek (19° 26′ 18″ S, 146° 57′ 01″ E), which is a rainforest-fed stream located within Bowling Green Bay National Park in Queensland, Australia (collection permit: WITK07655010). Analysis of water samples from the collection site indicated no contamination with fluoxetine (Envirolab Services, unpublished data; see Section 2.3 Chemical exposure and analyses for details of water testing). Sexually mature male and female guppies were acclimated to laboratory conditions (24 °C; 12:12 h light:dark cycle) for 5 weeks in mixed-sex holding tanks (54 L, 60 cm length × 30 cm width × 30 cm height). Guppies were fed a daily diet of commercially prepared fish pellets (Otohime Hirame larval diet; 580–910 μm).

The spangled perch (*Leiopotherapon unicolor*) and rainbowfish (*Melanotaenia splendida*) used in behavioural trials (see Section 2.2 Experimental design for details) were wild-caught specimens purchased from commercial suppliers (Australian Native Fish Enterprises in Sydney and AquaGreen in Darwin, respectively). These stimulus fish were housed for 6 weeks prior to the start of experiments, under the same laboratory conditions described above, in species-specific holding tanks (54 L, 60 cm × 30 cm × 30 cm, 5 per tank). Spangled perch and rainbowfish were fed daily with chironomid larvae (Hikari frozen bloodworms).

2.2. Experimental design

To investigate potential effects of fluoxetine exposure on male and female guppy reproductive behaviour under predation risk, a 3 × 2 factorial design was used, with 3 fluoxetine treatments (unexposed, low-fluoxetine or high-fluoxetine) and 2 levels of perceived predation risk (a predator or non-predator stimulus fish).

Stimulus fish used in behavioural trials were either predatory spangled perch (total length: mean = 7.41 cm, SD = 0.61 cm, range = 6.56–8.34 cm, $n = 7$) or non-predatory rainbowfish (total length: mean = 6.33 cm, SD = 0.91 cm, range = 5.03–7.74 cm, $n = 10$). The spangled perch is an aggressive and opportunistic omnivore, which is known to prey on crustaceans and small fish, including guppies (Davis et al., 2011). The rainbowfish, by contrast, feeds exclusively on invertebrates and plant material (Davis et al., 2011). Importantly, both species are known to co-occur with guppies from the Alligator Creek source population (Allen et al., 2002). A non-predator control treatment was included to ensure that behavioural changes caused by the predator treatment (if any) were, in fact, due to a perceived threat of predation, rather than simply the presence of a stimulus fish per se (Michelangeli and Wong, 2014). We used unexposed spangled perch and rainbowfish to exclude the possibility that fluoxetine-induced effects on guppy behaviour (if any) could have been mediated by effects on the stimulus fish, a technique employed in previous ecotoxicological experiments (e.g. Bertram et al., 2018; Tomkins et al., 2017, 2018).

2.3. Chemical exposure and analyses

Male and female guppies in this experiment were randomly allocated to one of three fluoxetine treatments—unexposed (i.e. fresh water only), low fluoxetine or high fluoxetine—and exposed using established protocols (Bertram et al., 2018; Martin et al., 2017; Saaristo et al., 2017), with some modifications (see below). Guppies were subjected to a 28-day exposure period as previous studies suggest that fluoxetine can take 2–4 weeks to exhibit its full therapeutic effects in humans (Gardier et al., 1996; Matuszcyk et al., 1998), and because recent research indicates that similar exposure periods (i.e. 21–35 days) can alter a wide range of behaviours in fish (e.g. Bertram et al., 2018; Martin et al., 2017; McCallum et al., 2017; Pelli and Connaughton, 2015; Saaristo et al., 2017). Flow-through systems were used to expose guppies from each of the three fluoxetine treatments, with two identical systems being used per treatment. Each system was comprised of a large mixing tank (81 L, 60 cm × 45 cm × 30 cm) which fed two identical sex-specific exposure tanks (54 L, 60 cm × 30 cm × 30 cm), containing 40 fish each. Exposure aquaria were equipped with 2 cm of natural gravel substrate, a large stone for refuge, a heater and an airstone. All aquaria were maintained under a 12:12 h light:dark cycle and monitored daily for temperature (mean = 24.4 °C, SD = 0.5 °C, $n = 336$), as well as being maintained at a flow-through rate of ~1.67 L/h per tank (i.e. water in each exposure tank was fully cycled once per day).

To achieve the nominal low- and high-fluoxetine treatment concentrations—40 and 400 ng/L, respectively—stock solutions were prepared daily. This involved firstly dissolving fluoxetine hydrochloride (Sigma Aldrich; Product Number: F132, CAS: 56296-78-7) in methanol (low: 18 μg/mL, high: 180 μg/mL). Then, for each exposure system, a 1 mL aliquot of this solution was evaporated to dryness under a gentle nitrogen gas stream, before being diluted to a 3 L stock solution using reverse osmosis water. Unexposed stock solutions contained 3 L of reverse osmosis water only. For each exposure system, stock solutions were continuously fed into each of the mixing tanks (1.95 mL/min) using a peristaltic pump (Watson Marlow 323 U/MC).

Fluoxetine concentrations were measured weekly in all exposure tanks within the low and high treatments, as well as in half of the unexposed aquaria to ensure the absence of contamination. This involved water samples (40 mL) being drawn from each tank using a serological pipette (Macroman, Gilson), which were then stored at 4 °C in amber glass bottles, and analysed within 4 days of collection. Samples were analysed by Envirolab Services (MPL Laboratories; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025), where 39 mL of each sample was acidified to pH 6, with 20 μL of 1 μg/mL norfluoxetine (CAS: 56161-73-0; Novachem, Germany) in methanol being added to each sample to serve as a surrogate standard. The fluoxetine and norfluoxetine surrogate were then eluted using dichloromethane: isopropanol:ammonium hydroxide (78:20:2, *v*/*v*/*v*; 3 mL) and evaporated to dryness under a gentle stream of argon. The fluoxetine was reconstituted in 100 μL of ethyl acetate, and 50 μL of heptafluorobutyric anhydride derivatising agent (United Chemical Technologies, purchased from PM Separations, QLD, Australia) was added and evaporated. The samples were then analysed using gas chromatography-tandem mass spectrometry (7000C Triple Quadrupole GC-MS/MS, Agilent Technologies, Delaware, USA) with a limit of quantification of 2 ng/L. A detailed description of the chemical analysis protocol is provided in Bertram et al. (2018).

The measured average fluoxetine concentrations in the low- and high-fluoxetine treatments were 60.51 ng/L (SD = 21.54 ng/L, $n = 16$) and 349.85 ng/L (SD = 158.64 ng/L, $n = 16$), respectively. Both of these exposure treatments are environmentally realistic, with the lower level falling within the range of concentrations detected in surface waters (e.g. Hughes et al., 2013; Kolpin et al., 2002; Wu et al., 2017), while the higher level is within the upper ranges reported in aquatic systems heavily impacted by wastewater discharge (Benotti and Brownawell, 2007; Lara-Martín et al., 2015). No contamination with fluoxetine was detected in any of the unexposed aquaria across the 28-day exposure ($n = 8$ measurements).

2.4. Behavioural trials

Guppy behaviour was recorded in 54 L observation tanks (60 cm × 30 cm × 30 cm; water depth: 20 cm), which were separated into two compartments (40 cm × 30 cm × 30 cm and 20 cm × 30 cm × 30 cm) using a transparent perforated divider (see electronic supplementary material, Fig. S1 for a schematic diagram of the tank set-up). For each trial, the transparent partition was randomly allocated to the left or right of the observation tank to account for any potential side bias. Fifteen minutes before the trial, a randomly selected stimulus fish (i.e. a predator or non-predator of known identity) was placed into the smaller compartment. In addition to this visual stimulus, chemical cues of the stimulus fish (250 mL of holding-tank water) were added to the larger compartment—where guppies would be located—as guppies can use both visual and chemical cues to detect predators (Bleakley et al., 2006). A 5 cm zone abutting the transparent partition was used to measure the total time male and female guppies spent in the area closest to the stimulus fish. This 5 cm zone represented the spangled perch's striking range (i.e. 'strike zone') and was based on previous literature investigating fast-start performance and strike distance in closely related teleost fish (Domenici and Blake, 1997; Webb, 1978).

Each behavioural trial involved one male and one female guppy being randomly collected from the same exposure treatment (unexposed, low-fluoxetine or high-fluoxetine). This was done because wild guppies that inhabit the same body of water are likely to experience similar levels of fluoxetine contamination, especially due to their strong schooling preference (Houde, 1997). Pairs consisting of one male and one female were used to disentangle effects caused by fluoxetine exposure and predation risk on reproductive behaviour (if any) from additional interacting stressors, such as audience effects (Makowicz et al., 2010) and male-male competition (Jirotkul, 1999). The two guppies were placed in separate opaque containers (300 mL) filled with trial tank water, and floated on the water's surface within the larger trial tank compartment for a 5 min acclimation period. Opaque containers were used to prevent the guppies from interacting with visual and chemical cues of the stimulus fish before the trial. After acclimation, both guppies were simultaneously released into the larger compartment, with their behaviour then being video-recorded for 15 min (Canon PowerShot S120). After each trial, observation tanks were drained and refilled. Each stimulus fish was also returned to its holding tank in preparation for subsequent trials.

Behaviours performed by guppies were quantified from trial videos using the event-recording software JWatcher V1.0 (Blumstein and Daniel, 2007). Each video was watched four times to measure male and female reproductive behaviour, as well as zone use in either sex. Specifically, to examine possible effects of fluoxetine on male mating strategy, we measured the number of male courtship bouts (i.e. male orienting towards the female and performing stereotyped 'sigmoid' courtship displays before attempting a copulation) and coercive sneak copulation attempts (i.e. male surreptitiously approaching the female from behind to copulate) directed towards females (Houde, 1997). In addition, to test the potential for fluoxetine exposure to alter reproductive interest in guppies under predation risk, we measured the total time spent by males and females actively following their potential mate. Male guppies frequently and persistently follow females in their pursuit of mating opportunities and, in female guppies, actively following potential suitors is a strong predictor of actual mating intent (Houde, 1997). Lastly, we monitored the total time spent by male and female guppies within 5 cm (i.e. the strike zone) of the stimulus fish (Kramer and Bonenfant, 1997). This was also used to determine whether guppies perceived the difference in threat posed by the predatory spangled perch and non-predatory rainbowfish.

2.5. Morphology and colouration analysis

Subsequent to behavioural trials, males ($n = 156$) and females ($n = 156$) were euthanised with an overdose of anaesthetic clove oil (40 mg/L) and dabbed dry, before being measured for standard length (i.e. snout to caudal peduncle; Kincrome digital calipers, ±0.01 mm). The wet weights of males and females were also measured using an electronic balance (Scientech ZSA-210 digital analytical scale, ± 0.0001 g). A sex-specific index of body condition was calculated by plotting weight (g) against standard length (mm) to produce a least-squares regression line. Sex-specific condition index was calculated as the residuals of this regression line (i.e. males: weight $= -0.128 + 0.012 \times$ length; females: weight $= -0.644 + 0.041 \times$ length). Additionally, the percentage area of orange pigmentation on the body of each male was analysed using photographic colouration analysis, following Bertram et al. (2015). Briefly, this involved photographing each male's right side in a standard position (Nikon D90, shutter speed = 1/250, Nikon AF Micro-Nikkor 60 mm f/2.8D). Photoshop (version 2017.0.1) was then used to isolate the body surface (excluding fins) of each fish and calculate percentage area of orange pigmentation using the colour range tool. Orange colouration was initially included in all models of guppy behaviour as female guppies show a strong preference for males that possess greater orange pigmentation (Houde, 1987), including fish from the population used in the present study (Bertram et al., 2015; Gamble et al., 2003).

2.6. Statistical analysis

Data were analysed in R version 3.2.3 (R Development Core Team, 2015). Where appropriate, response variables were checked for normality (Shapiro-Wilk test, *shapiro.test* function; Royston, 1995) and homogeneity of variance (Bartlett test, *bartlett.test* function; Bartlett, 1937). In analysing behavioural responses, a small suite of biologically meaningful predictors (based on previously established relationships) were initially included in all models, consisting of male standard length (mm), male condition index, male orange pigmentation (%), female standard length (mm) and female condition index. For each of these models, covariates were selected by performing backward stepwise elimination (i.e. covariates were sequentially excluded based on their impact on the Akaike Information Criterion [AIC]; see electronic supplementary material, Table S1 for model summaries). Where appropriate, a rank-normal transformation (*rntransform* function, *GenABEL* package; Aulchenko et al., 2007) was applied to approximate normality of the residuals. All models analysing behavioural responses included stimulus fish ID as a random effect to account for potential variation in guppy behaviour caused by the presence of specific stimulus individuals. Additionally, exposure system ID—as a measure of tank effects—was initially included in all behavioural models as a random effect but did not significantly affect any of the response variables, explaining <1% of the variation in the data, and was, therefore, excluded in each case to increase predictive power. In all behavioural models detailed below, where relevant, third- and second-order interactions between fixed effects were removed using reverse stepwise elimination, leaving only those that were statistically significant (at $\alpha = 0.05$).

To both address zero-inflation and incorporate a random effect, separate zero-inflated generalised linear mixed-effect models (*glmmADMB* function, *glmmADMB* package; Fournier et al., 2012) were used to compare the number of courtship events, as well as the number of sneak attempts, performed by males towards females. The model generated to investigate the number of courtship displays had two fixed predictors (fluoxetine treatment and stimulus fish type), one continuous covariate (female condition index) and one random effect (stimulus fish identity). The model used to examine the number of male sneaking events included two fixed predictors (fluoxetine treatment and stimulus fish type), three continuous covariates (male standard length, male condition index and female condition index) and one random effect

(stimulus fish identity). Zero-inflated models were used, as Vuong tests (*vuong* function, *pscl* package; Vuong, 1989) indicated that the data were zero-inflated in both cases. A negative binomial distribution was selected as the most appropriate family for both models, as it accounted for over-dispersion of the count component (Zuur et al., 2009). For both courting and sneaking behaviours, general linear hypothesis tests (GLHTs; *glht* function, *multcomp* package; Hothorn et al., 2008) were used to compare mean responses across treatment levels.

Two separate linear mixed-effects models (LME; *lme* function, *nlme* package; Pinheiro et al., 2018), one per sex, were used to analyse male and female guppy reproductive interest (i.e. total time males and females spent following each other). Each of these models had two fixed effects (fluoxetine treatment and stimulus fish type), two continuous covariates (female model: male orange pigmentation and female standard length; male model: male standard length and female condition index) and one random effect (stimulus fish identity). Males and females were investigated using separate models because male guppies are known to perform more intense following behaviour than females (Houde, 1997).

A LME was used to test the total time spent by guppies within 5 cm of the stimulus fish and included three fixed effects (fluoxetine treatment, stimulus fish type, and sex), one continuous covariate (standard length) and one random effect (stimulus fish identity). Sex was included as a fixed effect to investigate whether males and females spent different amounts of time within the vicinity of the predator or non-predator.

Finally, impacts of fluoxetine on male and female morphology (i.e. standard length, weight, and condition index in both sexes, as well as male area of orange pigmentation) were assessed using separate sex-specific ANOVA models. For males, weight was square root transformed before analysis, in order to approximate normality of the residuals. Likewise, for females, data for each morphological trait were rank-normal transformed before analysis.

3. Results

3.1. Reproductive behaviour

Regarding the number of courtship displays performed by males towards females, interactions between fluoxetine exposure treatment and stimulus fish type were non-significant (glmmADMB; $p \geq 0.213$) and were removed. Further, number of courtship events was not significantly affected by fluoxetine treatment (glmmADMB; all $p \geq 0.199$; Fig. 1a) or stimulus fish type (glmmADMB; $z = 0.76$, $p = 0.445$; Fig. 2a). However, a non-significant positive trend was detected between female condition index and the number of courtship displays performed by males (glmmADMB; $z = 1.89$, $p = 0.058$).

For the total number of sneak attempts carried out by males towards females, no significant interactions between fluoxetine exposure treatment and stimulus fish type were found (glmmADMB; all $p \geq 0.180$), with the interaction terms therefore being removed. Fluoxetine treatment significantly affected the number of male sneak attempts performed towards females, with high-fluoxetine males engaging more frequently in sneak attempts than unexposed males (glmmADMB; $z = 2.08$, $p = 0.038$; Fig. 1b). However, the number of sneaks did not differ significantly between unexposed and low-fluoxetine males (glmmADMB; $z = 0.96$, $p = 0.339$; Fig. 1b) or low- and high-fluoxetine males (glmmADMB; $z = 0.84$, $p = 0.400$; Fig. 1b). Stimulus fish type did not significantly affect the number of sneak attempts performed by males towards females (glmmADMB; $z = 1.64$, $p = 0.101$; Fig. 2b). Additionally, the number of sneaks performed by males did not associate significantly with male standard length (glmmADMB; $z = 1.08$, $p = 0.281$), male condition index (glmmADMB; $z = 0.74$, $p = 0.457$) or female condition index (glmmADMB; $z = 1.45$, $p = 0.147$).

In terms of the total time spent by males and females following their potential partner, interactions between fluoxetine exposure treatment and stimulus fish type were non-significant (LME, males: $p = 0.343$; females: $p = 0.401$) and were removed from the model. The refitted models indicated no significant main effects of fluoxetine treatment (LME; males: $F = 0.47$, $p = 0.629$, Fig. 3a; females: $F = 0.40$, $p = 0.669$, Fig. 3b). Further, the total time spent by males following females did not differ significantly across stimulus fish type (LME; $F = 0.39$, $p = 0.539$; Fig. 4a). However, females spent significantly less time following males when in the presence of a predatory spangled perch than a non-predatory rainbowfish (LME; $F = 6.52$, $p = 0.022$; Fig. 4b). In addition, while area of orange pigmentation in males had no significant effect on the total time spent by females performing following behaviour (LME; $F = 2.18$, $p = 0.143$), female standard length associated negatively with total time spent following males (LME; $F = 18.51$, $p < 0.001$). Lastly, male standard length and female condition index were not significant indicators of the total time males followed

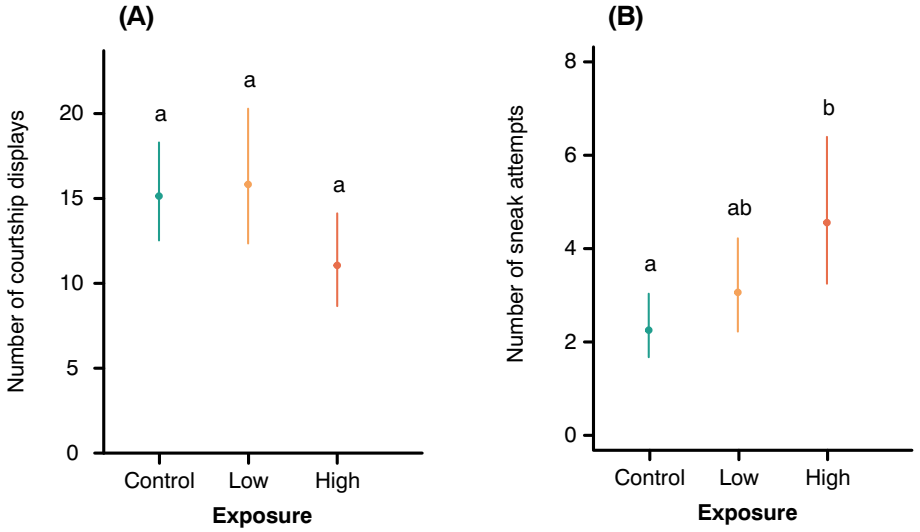

Fig. 1. Mean (±SE) number of (a) courtship displays, and (b) sneak attempts, performed by males towards females when both guppies were subject to either unexposed (0 ng/L, $n = 51$), low-fluoxetine (61 ng/L, $n = 52$) or high-fluoxetine (350 ng/L, $n = 53$) treatment, with continuous predictors being held at their means. Treatments without lower case letters in common are significantly different.

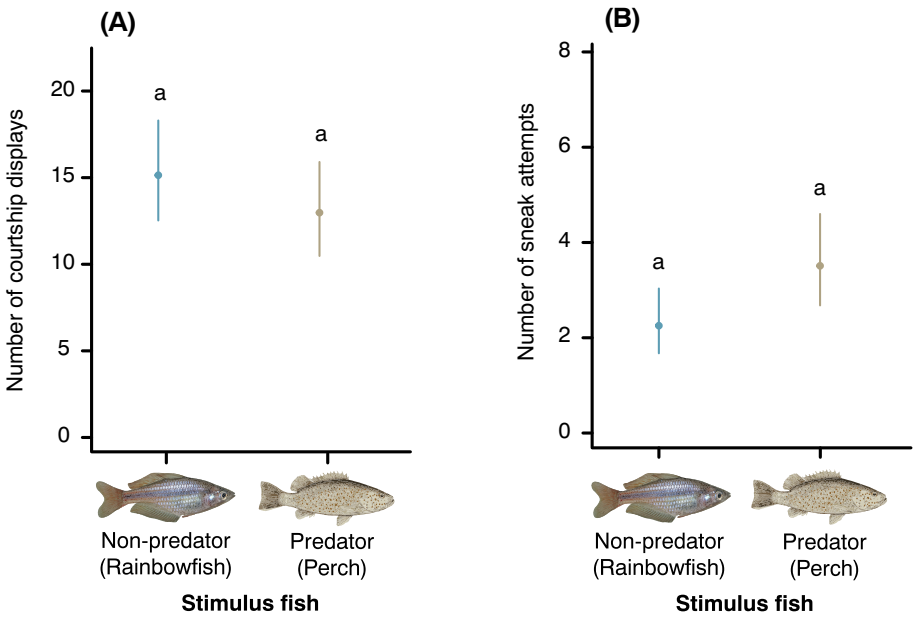

Fig. 2. Mean (±SE) number of (a) courtship displays, and (b) sneak attempts, performed by males towards females when in the presence of a non-predatory ($n = 66$) or predatory ($n = 90$) stimulus fish, with continuous predictors being held at their means. Treatments without lower case letters in common are significantly different.

females (LME; $F = 0.06$, $p = 0.812$ and $F = 2.55$, $p = 0.113$, respectively).

3.2. Predator avoidance behaviour

In analysing the total time spent by males and females within 5 cm of the stimulus fish, no significant interactions were detected between the categorical predictors (i.e. fluoxetine exposure treatment, stimulus fish type, and sex) (LME; three-way interaction, $p = 0.332$; refitted model two-way interactions, $p \geq 0.245$), and were thus removed. Main effects of fluoxetine exposure (LME; $F = 1.13$, $p = 0.324$, Fig. 5a) and sex (LME; $F = 0.01$, $p = 0.921$) were also non-significant. However, guppies spent significantly less time within the 5 cm zone when in the presence of a predatory spangled perch compared to a non-predatory rainbowfish (LME; $F = 8.01$, $p = 0.013$, Fig. 5b). Total time spent within the 5 cm zone was also positively associated with guppy standard length, with larger individuals spending more time in this area (LME; $F = 27.56$, $p < 0.001$).

3.3. Morphology and colouration analysis

Fluoxetine treatment did not significantly impact male standard length (ANOVA; $F_{2,153} = 0.70$, $p = 0.497$), weight (ANOVA; $F_{2,153} = 0.28$, $p = 0.760$) or condition index (ANOVA; $F_{2,153} = 0.60$, $p = 0.550$). Additionally, area of orange pigmentation in males did not differ significantly between fluoxetine treatments (ANOVA; $F_{2,153} = 0.60$, $p = 0.551$). Similarly, in females, fluoxetine exposure levels did not significantly impact standard length (ANOVA; $F_{2,153} = 0.24$, $p = 0.787$), weight (ANOVA; $F_{2,153} = 0.31$, $p = 0.732$) or condition index (ANOVA; $F_{2,153} = 1.69$, $p = 0.188$).

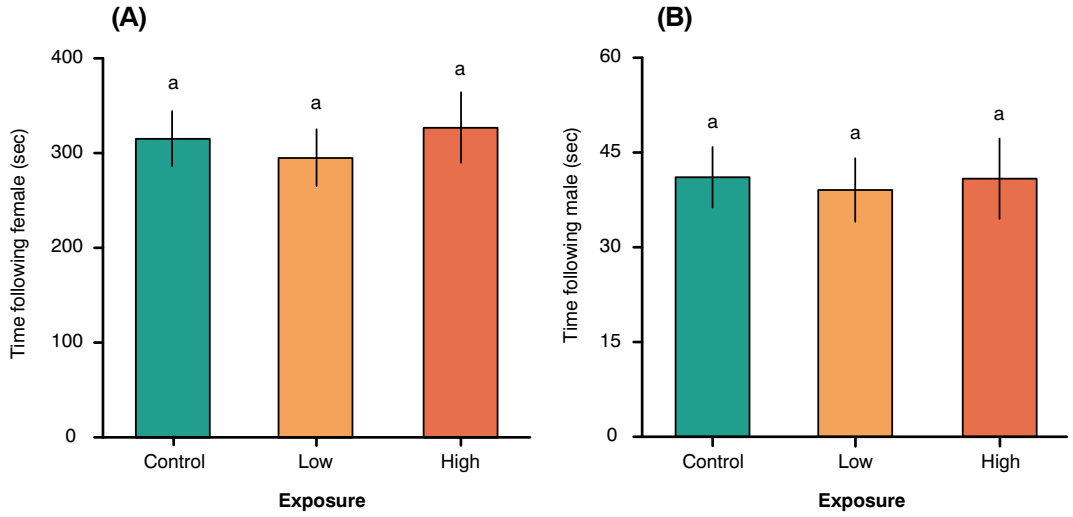

Fig. 3. Mean (±SE) amount of time spent by (a) male, and (b) female, guppies performing following behaviour towards their potential partner, in unexposed (0 ng/L, $n = 51$ per sex), low-fluoxetine (61 ng/L, $n = 52$ per sex) and high-fluoxetine (350 ng/L, $n = 53$ per sex) treatments. Treatments without lower case letters in common are significantly different.

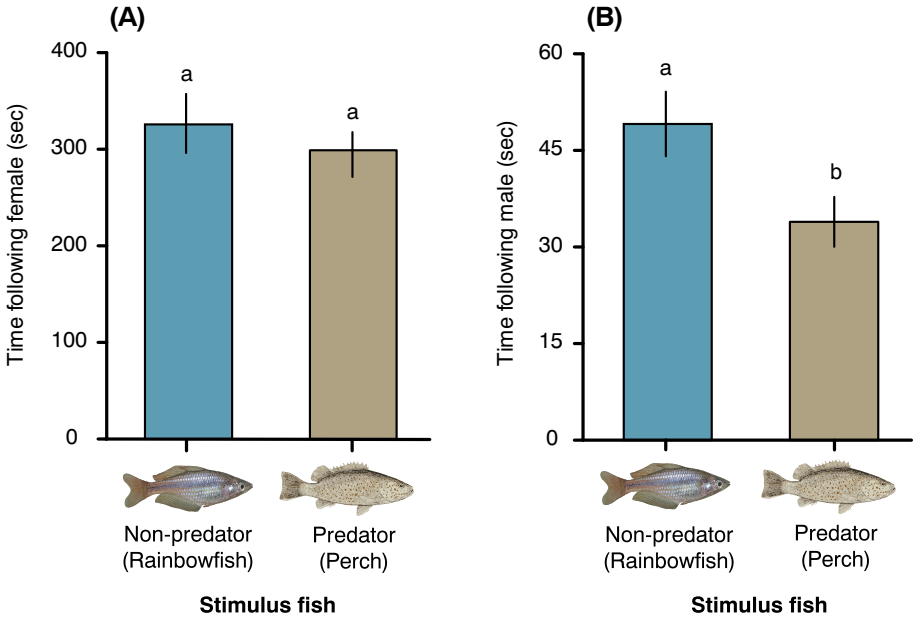

Fig. 4. Mean (±SE) amount of time spent by (a) male, and (b) female, guppies performing following behaviour towards their potential partner when in the presence of a non-predatory ($n = 66$ per sex) or predatory ($n = 90$ per sex) stimulus fish. Treatments without lower case letters in common are significantly different.

4. Discussion

In this study, exposure to an environmentally realistic level of fluoxetine altered the mating strategy of male guppies, which was true independent of the presence of a predator. Specifically, fluoxetine increased male coercive 'sneak' copulations in the high-exposed treatment, relative to unexposed males, while courtship displays and following behaviour were not significantly affected. The total time spent by females following males was also unaffected by fluoxetine treatment. Moreover, fluoxetine exposure did not significantly affect male or female predator avoidance behaviour. This was the case despite guppies in this study demonstrating a capacity to perceive differences in predation risk posed by stimulus predatory spangled perch and non-predatory rainbowfish, with both males and females spending less time in the vicinity of the predatory stimulus, and with females also following males less in the predator treatment.

Fluoxetine exposure affected the frequency of male sneaking behaviour performed towards females, with high-fluoxetine (350 ng/L) males performing a greater number of sneak attempts than males in the unexposed treatment. This shift in the use of male alternative mating strategies towards coercive sneaking behaviour is likely to have implications for male fitness. Specifically, although an increase in sneak copulations could potentially improve male reproductive success due to a general increase in mating attempts, sneaking is associated with reduced insemination efficiency given that successful sneaks deliver approximately one third as many sperm as copulations preceded by courtship

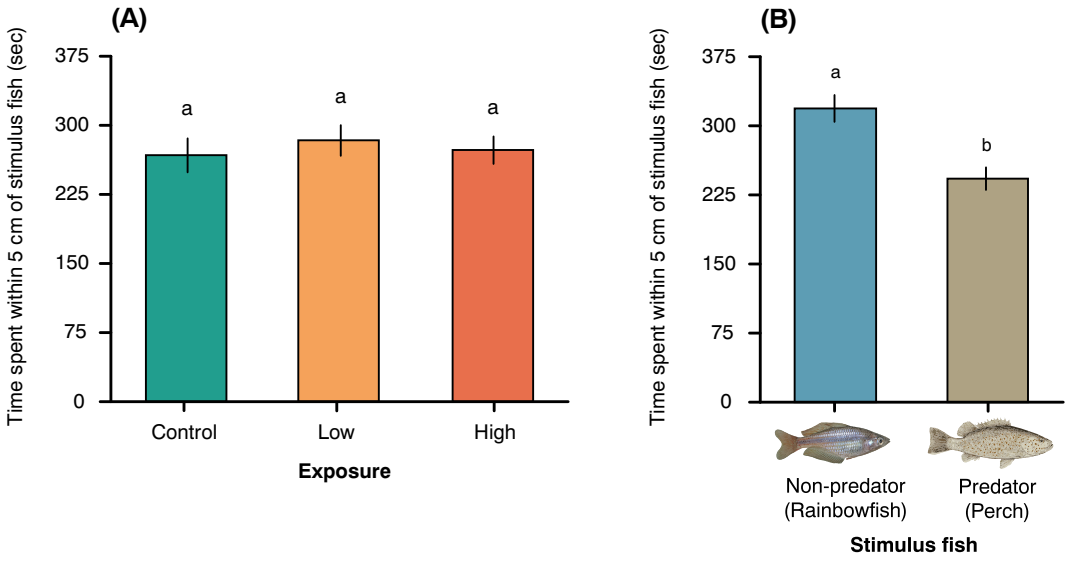

Fig. 5. Mean (±SE) amount of time spent by male and female guppies within 5 cm of the stimulus fish when (a) from the unexposed (0 ng/L, $n = 51$ pairs), low-fluoxetine (61 ng/L, $n = 52$ pairs) and high-fluoxetine (350 ng/L, $n = 53$ pairs) treatments, and (b) in the presence of a non-predatory ($n = 66$ pairs) or predatory ($n = 90$ pairs) stimulus fish. Treatments without lower case letters in common are significantly different.

displays (Pilastro and Bisazza, 1999). Females may also avoid males performing excessive sneak copulations (i.e. harassment) (Houde, 1997; Magurran and Seghers, 1994), thereby disadvantaging fluoxetine-exposed males. In addition, increased male sexual harassment could have indirect implications for female fitness by, for example, increasing predator exposure (Pocklington and Dill, 1995) and/or reducing foraging efficiency (Magurran and Seghers, 1994; Pilastro et al., 2003). More broadly, a fluoxetine-induced shift towards male mating strategies that circumvent female mate choice could have population-level consequences by impacting the quality and quantity of offspring produced (Candolin and Heuschele, 2008; Candolin and Wong, 2012; Wong and Candolin, 2015).

Consistent with our results, several studies have similarly reported fluoxetine-induced changes to male reproductive behaviours in fish. In particular, Weinberger and Klaper (2014) found that fluoxetine exposure increased nest-tending behaviour in male fathead minnows (1000 ng/L for 28 days). Likewise, Bertram et al. (2018) found an increase in male copulatory behaviour in eastern mosquitofish (*Gambusia holbrooki*) following a 30-day exposure at 479 ng/L. However, in contrast to those studies, Schultz et al. (2011) and Dzieweczynski and Hebert (2012) reported no impact of fluoxetine exposure on reproductive behaviour in fathead minnows (2.5–28 ng/L for 21 days) or Siamese fighting fish (540 ng/L for 3 days), respectively. One possible reason for the differences observed across studies is social context. For example, in the study by Bertram et al. (2018), the increase in male copulatory behaviour was only observed in the absence of male-male competition while no such effect was seen when a rival was present. Thus, reproductive responses could be affected by whether males are allowed to directly interact with each other (e.g. Dzieweczynski and Hebert, 2012; Schultz et al., 2011) or whether males are tested in the absence of competitors (e.g. Weinberger and Klaper, 2014). In this regard, an important avenue for future research will be examining potential effects of fluoxetine on increasingly complex behavioural interactions and across different social contexts (e.g. audience effects: Makowicz et al., 2010; male-male competition: Jirotkul, 1999).

Certain reproductive behaviours may also be more sensitive to disruption by fluoxetine exposure than others. Indeed, evidence of endpoint-specific sensitivity was apparent in the current study, with a fluoxetine-induced increase in male sneak copulations but no significant change in courtship displays or following behaviour. In this respect, the physiological mechanisms by which fluoxetine affects male mating strategies warrants further investigation. Indeed, in general, the mechanisms through which SSRI exposure can alter male sexual behaviours are not fully understood (Fent et al., 2006; Prasad et al., 2015). Fluoxetine has the ability to bioaccumulate in fish tissues, including the brain (e.g. Brooks et al., 2005; David et al., 2018; Ramirez et al., 2009), reaching a steady state at approximately 4 days, and possessing a bioconcentration rate of 20–240 L/kg depending on the species (Boström et al., 2017; Silva et al., 2016). Once in the body, fluoxetine can influence extracellular levels of serotonin, which is known to play a key role in regulating reproductive function in fish via both central (i.e. preoptic-hypothalamic area and pituitary) and peripheral (i.e. gonadal) pathways (Dorelle et al., 2017; Prasad et al., 2015). Specifically, through the central pathway, serotonin interacts with the hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-interrenal (HPI) axes (Kreke and Dietrich, 2008), affecting the production of gonadotropin-releasing hormone and luteinising hormone (Kreke and Dietrich, 2008; McDonald, 2017; Yaron and Sivan, 2006). Fluoxetine affects peripheral pathways by influencing the production of testosterone and other androgens (Fernandes et al., 2011; Mennigen et al., 2010a, 2011), which are known to mediate reproductive behaviour (Munakata and Kobayashi, 2010).

Fluoxetine exposure did not significantly affect the total time spent by male or female guppies within 5 cm of the stimulus fish (predatory or non-predatory). This is surprising given that fluoxetine-induced shifts in serotonin could—through its effects on the HPI axis—alter the synthesis of adrenocorticotropic hormone and, accordingly, cortisol, which is important for mediating stress response in fish (reviewed in McDonald, 2017). Indeed, a number of earlier studies have reported altered antipredator behaviours (i.e. predator-related stress behaviour) as a result of fluoxetine exposure (e.g. Martin et al., 2017; Pelli and Connaughton, 2015; Saaristo et al., 2017; Weinberger and Klaper, 2014). However, to date, the effects of environmentally relevant fluoxetine exposure on antipredator behaviour have been mixed. For example, Martin et al. (2017), using eastern mosquitofish, and Pelli and Connaughton (2015), using guppies, both reported a decrease in antipredator behaviour as a result of environmentally realistic fluoxetine exposure (8 ng/L for 28 days and 30 ng/L for 21 days, respectively). By contrast, Weinberger and Klaper (2014) reported no effect of fluoxetine on antipredator behaviour of fathead minnows at environmentally relevant concentrations but did observe a decrease at concentrations exceeding those detected in the environment (>1000 ng/L for 28 days). In addition, Saaristo et al. (2017) observed an increase in antipredator behaviour in guppies (16 ng/L for 28 days). In this regard, differences in reported effects could be due to differences in the types of predatory stimulus used (and, thus, the perceived level of threat), species-specific differences in sensitivity to fluoxetine exposure, or a combination of both. Future research could, therefore, investigate how different predatory threats may mediate behavioural responses of prey to fluoxetine exposure.

While the total time males and females spent within 5 cm of the stimulus fish was not affected by fluoxetine, both sexes spent less time within this zone in the presence of the predatory spangled perch than that of the non-predatory rainbowfish. This indicates that males and females did, in fact, recognise the chemical and/or visual cues from the spangled perch as a threat (Sih et al., 1985; Swaney et al., 2015). It is worth noting, however, that the similarity of responses across the two sexes may also have been driven by male following behaviour, with males often remaining in close proximity to females for the majority of the trial and, hence, matching the females' use of the 5 cm zone. In addition, guppy following behaviour was altered by the presence of a predator, independent of fluoxetine exposure. Specifically, female guppies followed males significantly less in the presence of a predator than a non-predator, while males were unaffected by stimulus fish type. These results are supported by earlier investigations of wild guppy populations, where females have been shown to have reduced sexual interest and follow males less often when under threat of predation (Godin and Briggs, 1996), while males remain risk insensitive (Magurran and Nowak, 1991; Magurran and Seghers, 1990). One reason for this pattern is the considerable sexual size dimorphism seen in guppies, which affects predator strategy (Dill et al., 1999). Indeed, some predators have been found to preferentially target females over males, which are smaller (Pocklington and Dill, 1995). Hence, females could potentially decrease their own risk by reducing interactions with males (Dill et al., 1999), whereas males may be more likely to disregard predation risk in favour of increased potential mating opportunities (Dill et al., 1999; Magurran and Nowak, 1991).

In the present study, we found no interactive effects of fluoxetine exposure and predation threat, with each stressor acting on different behaviours independently. To date, only two studies have explicitly investigated interactions between natural environmental stressors and fluoxetine exposure in freshwater biota. In estuarine crabs (*Cancer productus*), Peters et al. (2017) found an antagonist interaction between predation threat and fluoxetine exposure, with unexposed crabs reducing foraging behaviour under risk of predation and fluoxetine-exposed crabs increasing foraging behaviour regardless of predation risk. In contrast, Barbosa et al. (2017) reported a synergistic effect of water temperature and fluoxetine exposure in water fleas, with the interaction of both stressors resulting in higher fitness costs than when in isolation. Hence, while previous studies have shown that fluoxetine exposure in combination with other environmental stressors does have the potential to induce interactive effects on behaviour, our findings suggest

that this is not always inevitable. In this regard, sertraline (another SSRI) and predation stressors have been shown to have no interactive effect on activity or boldness in freshwater snails (*Radix balthica*), although this was due to sertraline having no effect on either behaviour (Hedgespeth et al., 2018), making uncovering potential interactions challenging. Further research is therefore necessary to determine how fluoxetine and other antidepressants may interact with additional stressors such as predation risk, and how these potential interactions may affect wild populations.

We found no significant morphological effects of fluoxetine on either male or female guppies, in terms of standard length, weight or condition index. In contrast, previous studies in other fish species have reported a decline in body condition after fluoxetine exposure (e.g. convict cichlid, *Amatitlania nigrofasciata*: Latifi et al., 2015; goldfish, *Carassius auratus*: Mennigen et al., 2010b), including at field-detected concentrations (e.g. eastern mosquitofish: Bertram et al., 2018). In goldfish, fluoxetine exposure (5 μg/g body weight for 13 days) resulted in both reduced food intake and weight gain by increasing the expression of potent inhibitory feeding neuropeptides in the brain (Mennigen et al., 2009). Whether this is also the case in guppies remains to be tested. Clearly, further research is needed to elucidate the mechanisms underpinning morphological changes (if any) induced by fluoxetine, both within and between species.

5. Conclusion

We report that short-term (28-day) exposure to an environmentally relevant concentration of the widespread pharmaceutical contaminant fluoxetine altered reproductive behaviour in male, but not female, guppies. More specifically, males in the high-fluoxetine treatment (350 ng/L) exhibited an altered mating strategy, performing a higher number of coercive sneaking copulations than unexposed males, regardless of perceived predation risk (i.e. in the presence of both a predator and non-predator). Contamination of the environment with pharmaceuticals, such as fluoxetine, that are capable of disrupting key fitness-related behaviours, such as mating strategy, is a major concern. Therefore, although we found no interactive effects between fluoxetine and perceived predation risk, further research on co-effects between pharmaceuticals and other environmental stressors is certainly needed to better understand potential impacts of these contaminants on ecological and evolutionary processes in wildlife.

Ethics

The research detailed in this paper was approved by the Biological Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2016/21) and complied with all relevant State and Federal laws of Australia.

Authors' contributions

All authors conceived and designed the experiments, which JBF and JMM conducted. JBF, JMM and MGB carried out statistical analysis and wrote the manuscript. All authors contributed to manuscript preparation and gave final approval for publication.

Competing interests

The authors declare that we have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.09.046.

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Appendix G

Impact of the widespread pharmaceutical pollutant fluoxetine on behaviour and sperm traits in a freshwater fish

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Impact of the widespread pharmaceutical pollutant fluoxetine on behaviour and sperm traits in a freshwater fish

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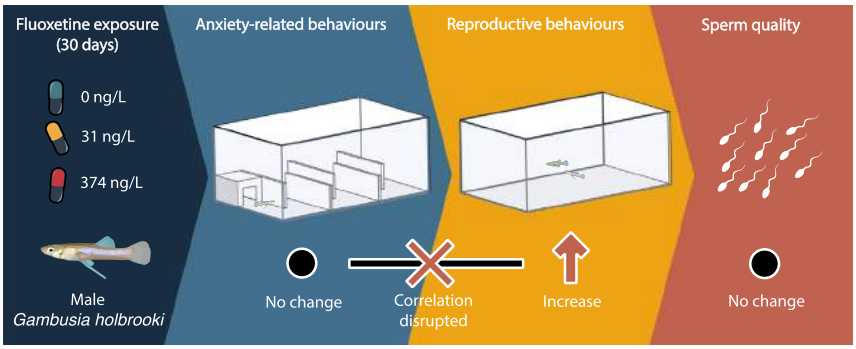
HIGHLIGHTS

- Male mosquitofish (*G. holbrooki*) exposed to fluoxetine at two realistic levels.
- Fluoxetine did not impact anxiety-related behaviours.
- Fluoxetine increased reproductive behaviour.
- Fluoxetine disrupted an across contexts correlation.
- Fluoxetine did not affect sperm quality

GRAPHICAL ABSTRACT



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ABSTRACT

Pharmaceutical pollutants are detected in aquatic habitats and wildlife tissues globally. One widespread contaminant of major concern is the antidepressant fluoxetine, which can affect behavioural and physiological processes in non-target species. Despite this, effects of fluoxetine on wildlife behaviour have seldom been investigated across multiple fitness-related contexts, especially at environmentally realistic concentrations. Accordingly, we examined impacts of 35-day fluoxetine exposure at two environmentally relevant concentrations (31 and 374 ng/L) across a suite of fitness-related contexts in wild-caught male mosquitofish (*Gambusia holbrooki*). First, we investigated anxiety-related behaviours (boldness, exploration and activity) in a novel environment (maze arena) and found no significant impacts of exposure. Second, we tested effects of fluoxetine in a reproductive context, including mating behaviour and sperm quality. We found that, relative to controls, fluoxetine exposure resulted in males spending a greater amount of time pursuing females. Further, low-exposed males were more likely to attempt copulation than unexposed males. Lastly, we investigated across-context behavioural correlations, and how fluoxetine exposure might affect such relationships. A significant positive correlation was detected in control fish between activity levels in the maze and time spent pursuing females in the reproductive assay. This relationship was disrupted by fluoxetine at both exposure levels. This is the first evidence that field-detected concentrations of a pharmaceutical pollutant can disturb across-context behavioural correlations in wildlife. Our findings provide clear evidence that fluoxetine can produce context-specific behavioural effects in fish and underscore how pharmaceutical exposure at field-detected concentrations can induce important shifts in wildlife behaviour.

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1. Introduction

Pharmaceutical pollution is a major threat to aquatic ecosystems globally (Arnold et al., 2014; Bernhardt et al., 2017; Saaristo et al., 2018). Hundreds of human and veterinary pharmaceuticals have now been detected in aquatic ecosystems and wildlife tissues around the world (Hughes et al., 2013; Küster and Adler, 2014). One pharmaceutical pollutant of environmental concern is the antidepressant fluoxetine. As with most pharmaceuticals, fluoxetine typically enters the environment via human consumption and excretion (Schultz et al., 2010). Indeed, up to 30% of administered fluoxetine can remain unmetabolised when excreted (van Harten, 1993). This incomplete metabolisation, coupled with insufficient removal by sewage treatment plants (e.g. Vasskog et al., 2006), results in fluoxetine entering aquatic environments in wastewater effluent flows. Consequently, fluoxetine (as well as its primary metabolite norfluoxetine) has been detected in surface waters worldwide at levels ranging from <1–100 ng/L, to as high as 596 ng/L in systems directly receiving wastewater discharge (Hughes et al., 2013; Schultz and Furlong, 2008; Schultz et al., 2010; Vanderford and Snyder, 2006). Once in the environment, fluoxetine can bioaccumulate in wildlife tissues (e.g. Brooks et al., 2005; David et al., 2018; Muir et al., 2017). For example, in an urban wetland receiving treated municipal wastewaters, fluoxetine—relative to 64 other pharmaceuticals present—showed the highest level of bioaccumulation in wild fish (Muir et al., 2017).

In addition to fluoxetine's prevalence in aquatic habitats, its primary pharmacological target, the serotonin transporter molecule, is conserved across a variety of taxa (Gunnarsson et al., 2008; Wang and Tsai, 2006). Consequently, fluoxetine may affect wildlife through its pharmacological action at lower concentrations than are required to induce general toxicity (McDonald, 2017). Moreover, by altering the serotonin system and associated neuroendocrine pathways, fluoxetine can influence multiple fitness-related processes (Kreke and Dietrich, 2008; McDonald, 2017). For example, in fish, pharmacologically relevant dosages of fluoxetine (i.e. ≥100 μg/L) have repeatedly been shown to reduce anxiety-like behaviours (Ansai et al., 2016; Cachat et al., 2010; Wong et al., 2013). By extension, fluoxetine exposure in wildlife could result in alterations to ecologically important behaviours linked to anxiety, such as boldness (i.e. the propensity to take risks), exploration, and activity, which are directly related to fitness and are associated with a range of important processes, such as dispersal (e.g. Cote et al., 2010; Michelangeli et al., 2017) and migration (e.g. Chapman et al., 2011). Moreover, fluoxetine exposure can also disrupt reproduction (reviewed in Kreke and Dietrich, 2008; McDonald, 2017). For example, in aquatic species, fluoxetine has been shown to induce gamete release in mussels (Bringolf et al., 2010; Fong, 1998) increase ovarian growth in crayfish (Kulkarni et al., 1992), and cause shifts in the release of sex hormones in fish species (Foran et al., 2004; Khan and Thomas, 1992; Mennigen et al., 2010).

Despite fluoxetine's capacity to influence a range of biological processes, few studies have investigated the effects of environmentally realistic fluoxetine exposure on non-reproductive and reproductive behaviours concomitantly—which is also true for pharmaceutical pollutants more generally. Fewer still have considered the importance that behavioural and physiological alterations can have on individuals across multiple ecological contexts, despite growing appreciation that functionally unrelated behaviours are often correlated, whereby a shift in one trait can correspond with a shift in another (i.e. behavioural syndromes, Sih et al., 2004, 2012).

Here, we set out to test the hypothesis that 35-day fluoxetine exposure at two environmentally realistic levels (average measured concentrations: 31 and 374 ng/L) would disrupt behaviour across two ecologically important contexts in wild-caught male mosquitofish (*Gambusia holbrooki*). First, we tested the effect of fluoxetine on anxiety-related behaviours (boldness, exploration, and activity) in a novel environment (maze arena). Second, using the same males, we tested the impact of fluoxetine exposure in a reproductive context, in terms of both reproductive behaviour and sperm quality. Lastly, we tested for potential across-context behavioural correlations and the effects of fluoxetine on such relationships.

2. Methods

2.1. Animal collection and housing

The present research was approved by the Biological Sciences Animal Ethics Committee of Monash University (BSCI/2015/2). Sexually mature male (mean weight: 0.1999 ± 0.0406 g, mean length: 22.31 ± 1.26 mm; $n = 105$) and female (mean weight: 0.4036 ± 0.1990 g, mean length: 26.14 ± 3.81 mm; $n = 105$) mosquitofish were collected from a wild population at Science Centre Lake (37°54′28″ S, 145°08′16″ E), Monash University, Australia. Water samples taken from the site over consecutive years indicated no fluoxetine contamination (unpublished data). Before experimentation, fish were acclimated to laboratory conditions (24–26 °C; 12:12 h light:dark cycle) in single-sex holding tanks (80 × 45 × 45 cm, water depth: 30 cm) for 1 month. Fish were fed daily on an *ad libitum* diet of commercial fish food (Otohime Hirame). The mosquitofish was selected as a model because its life-history is well characterised, including reproductive behaviour and sperm traits (Bisazza et al., 2001; Locatello et al., 2008; McPeek, 1992). Mosquitofish have a largely coercive polyandrous mating system and internal fertilisation, with males using a modified anal fin as an intromittent organ during copulation (McPeek, 1992). Due to the species' coercive mating system (Bisazza et al., 2001), and capacity for females to store sperm (Locatello et al., 2008), sperm quality is likely to play an important role in predicting reproductive success of male mosquitofish under sperm competition (Locatello et al., 2008).

2.2. Chemical exposure and monitoring

Male mosquitofish were randomly allocated to three treatment groups for 35 days: unexposed (i.e. fresh water), low fluoxetine and high fluoxetine. A 35-day exposure duration was selected because the full therapeutic effects of fluoxetine typically take 2–4 weeks to manifest in humans (Gardier et al., 1996; Hensler, 2003), and the spermatogenic cycle of *G. holbrooki* takes 30 days (Koya and Iwase, 2004). The nominal fluoxetine exposure concentration of the low treatment (40 ng/L) was selected to represent levels repeatedly detected in surface waters, while the nominal high concentration (400 ng/L) was selected to represent the higher end of surface water detections (reviewed in Hughes et al., 2013). The design of the chemical exposure followed previously published protocols (Bertram et al., 2018a; Martin et al., 2017). Briefly, exposure involved three identical flow-through systems (24 h cycling), one per treatment, with each system comprising 4 aquaria (60 × 30 × 30 cm, water depth: 25 cm), housing 30 fish each. The low- and high-fluoxetine exposure systems both received a constant supply of fluoxetine stock solution (replaced daily) and fresh water, whereas the unexposed system received fresh water only. The low- and high-fluoxetine stock solutions (6 and 60 μg/L, respectively) were prepared following methods described in Bertram et al. (2018a). Weekly water samples (200 mL) were taken from all of the low and high exposure tanks to measure fluoxetine concentrations. Additionally, water samples were collected from each unexposed tank fortnightly to ensure the absence of fluoxetine. Water samples were analysed by Envirolab Services using gas chromatography coupled to tandem mass spectrometry (7000C Triple Quadrupole GC–MS/MS, Agilent Technologies, Delaware, USA), based on methods described in Bertram et al. (2018a). Mean measured concentrations for the low- and high-fluoxetine treatments were 30.61 ng/L (SD = 6.28, $n = 24$) and 374.50 ng/L (SD = 62.91, $n = 24$). No fluoxetine contamination was detected in the unexposed system ($n = 12$), with the limit of quantification for fluoxetine being 2 ng/L.

2.3. Behavioural assays

A total of 105 males were used in maze and mating behaviour assays (unexposed: $n = 37$, low-fluoxetine: $n = 32$, high-fluoxetine: $n = 36$). Each male was first tested in the maze assay, which was followed by a 1 h rest period, after which each fish was tested in the reproductive assay. This design minimised any potential carryover effects that may have influenced behaviour in the maze trials (Bell, 2013). Behavioural assays were filmed with a digital camera, with behavioural endpoints quantified from the footage using JWatcher v1.0 (Blumstein and Daniel, 2007). During video quantification, observers were blind to treatment. All trials were conducted in aged fresh water (i.e. no fluoxetine) and, after each trial, tanks were drained and refilled to avoid any potential influence of conspecific chemical cues on the behaviour of focal fish.

The maze assay employed was adapted from Ward (2012) and followed the design of Bertram et al. (2018b). Specifically, each maze arena (60 × 30 × 30 cm, water depth: 10 cm) had a refuge box (10 × 10 × 10 cm) at its beginning, as well as five internal opaque walls that obscured the swimming path of fish and delineated six maze arms (Fig. 1a). The floor of the maze was divided by 5 cm gridlines used to measure activity levels (see below). At the beginning of each trial, a focal fish was first introduced into the refuge and allowed to acclimate for 5 min. After acclimation, a door to the refuge was remotely opened, allowing the fish to exit and explore the maze. Over a 20 min trial, we quantified three behavioural traits: (1) boldness, measured as the time taken to emerge from the refuge (emergence test), (2) exploration, measured as the time taken to complete the maze after the fish had exited the refuge (novel environment test), and (3) activity, measured as the total number of gridlines crossed (novel environment test). Trials concluded after 20 min, irrespective of whether or not the fish had completed the maze. After the trial, males were transported to individual temporary holding tanks (30 × 15 × 15 cm, water depth: 10 cm) where they were rested for 1 h.

Following this rest period, males were tested in a reproductive behaviour assay. In these trials, males were paired randomly with a novel unexposed stimulus female in an observation tank (60 × 30 × 30 cm, water depth: 10 cm; Fig. 1b). Stimulus females were unexposed to disentangle the indirect effects that fluoxetine-induced behavioural changes in one sex might have on the other, a technique employed in previous ecotoxicological studies (e.g. Saaristo et al., 2013; Tomkins et al., 2017, 2018). In addition, each stimulus female was only used in a single trial to avoid any potential carryover effects on behaviour. Before the commencement of the reproductive assay, the focal male and stimulus female were acclimated to trial water for 5 min in separate transparent containers (6 × 5 × 3 cm) within the observation tank. At the beginning of the trial, both fish were released and allowed to freely interact for 20 min, during which time we quantified the total time spent by males actively following the female within 5 cm (i.e. association behaviour), and the total number of male copulation attempts.

2.4. Sperm quality

To test for potential effects of fluoxetine on sperm quality, we measured both sperm performance and viability (i.e. the proportion of live sperm) immediately after behavioural trials. Both traits are important predictors of fertilisation success, especially under sperm competition (reviewed in Snook, 2005).

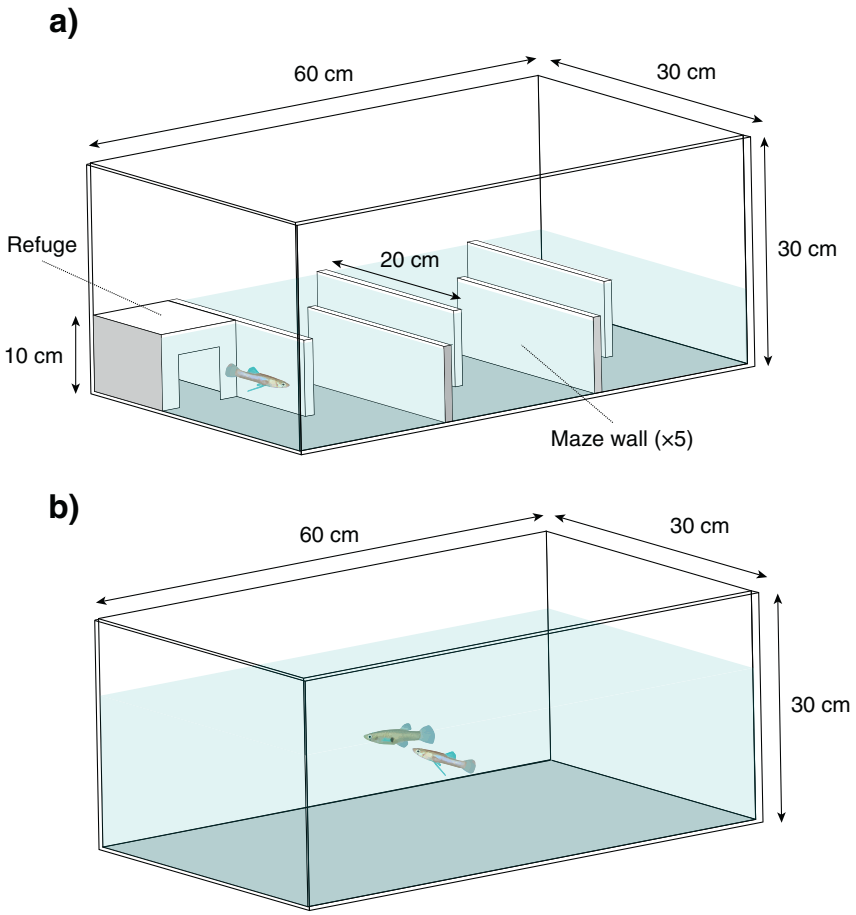

Fig. 1. (a) Maze assay, in which males were assessed for boldness, exploration, and activity. (b) Reproductive behaviour assay, in which males were paired with a single unexposed stimulus female and assessed for association behaviour and copulation attempts.

All sperm analyses were conducted blind to treatment and followed the protocols of Bertram et al. (2018a). Firstly, fish were euthanised (clove oil, 40 mg/L) and a sample of their ejaculate collected. Sperm performance was then measured using computer-assisted sperm analysis (CASA) software (v.14, CEROS, Hamilton-Thorne Biosciences, Beverly, MA, USA) for 96 males (unexposed: $n = 33$, low fluoxetine: $n = 28$, high fluoxetine: $n = 35$). A total of 9 males (4 unexposed, 4 low fluoxetine, 1 high fluoxetine) tested in behavioural trials did not provide sperm and were hence excluded from analyses. A minimum of 1000 sperm were tracked per male (mean = 1136.17, SE = 7.40) using a video camera (XC-ST50, Sony, Japan) coupled to a negative phase-contrast microscope (CX41, Olympus, 10× objective). These sperm tracks were used to obtain five measures of sperm performance (Table S1 for definitions): (1) average velocity of sperm along its average path (VAP, μm/s), (2) straight-line velocity from the first detection to the last detection (VSL, μm/s), (3) average point-to-point velocity along its path (VCL, μm/s), (4) linearity of the sperm path (LIN, %), and (5) percentage of motile sperm (MOT).

A second aliquot of sperm was collected from each male and analysed for the proportion of live sperm using a live/dead sperm viability kit (L-7011; Molecular Probes Inc., USA). For 2 males (1 unexposed, 1 high-fluoxetine), an insufficient volume of ejaculate was extracted to adequately perform viability counts in conjunction with CASA, with these males being excluded from further analyses. In total, sperm viability was assessed for 89 males (unexposed: $n = 32$, low-fluoxetine: $n = 27$, high-fluoxetine: $n = 30$). Sperm samples were first stained with a fluorescent membrane-permeant nucleic acid stain (SYBR-14), which stains live sperm green under fluorescent light. The sample was then counter-stained with propidium iodide, which stains dead sperm red. Using a fluorescence microscope (Leica DFC425C, Leica Microsystems, Germany), 12 non-overlapping fields were then photographed. Subsequently, the proportion of live sperm was calculated by counting a minimum of 150 sperm per male (mean = 320.73, SE = 13.28).

2.5. Morphological analysis

Immediately after sperm analysis, euthanised males were measured (standard length; ±0.01 mm) and weighed (±0.0001 g), and condition index calculated following previously published protocols (Bertram et al., 2015; Martin et al., 2017). These morphological traits were also recorded for stimulus females. The relative size of the male to the stimulus female (i.e. male size minus female size) was not statically different across treatment groups (ANOVA: $F_{2,102} = 1.87$, $p = 0.159$).

2.6. Statistical analysis

Data were analysed in R v3.2.2 (R Development Core Team, 2015) and checked for normality (Shapiro-Wilk test) and homogeneity of variance (Fligner-Killeen test), where appropriate. All models included treatment (unexposed, low-fluoxetine, and high-fluoxetine) as a predictor, and male length as a covariate. Models used to assess behavioural parameters in the reproductive assay also included female length as a covariate. Across all models, continuous covariates were centred to improve the interpretability of main effects. In addition, fish ID and exposure tank number were treated as random effects in all models.

Time taken to exit the refuge at the start of the maze arena, and time taken to complete the maze, were each compared across treatments using Cox mixed-effect proportional hazards models (*coxme* function, *survival* package). For all models, fish were right-censored (i.e. scored as incomplete) if they did not perform the event during the 20 min assay. Both models met the assumption of proportionality, as tested by examining the interaction between Schoenfeld residuals and log time (*coxph* and cox.*zph* functions, *survival* package). A linear mixed-effect model (LME; *lme* function, *nlme* package) was used to compare the total number of 5 cm gridlines crossed in the maze across treatments.

Total time spent by males associating with females in the reproductive assay was compared across treatments using an LME. Copulation attempts were compared across treatments using a generalised mixed-effect model (GLMM; *glmer* function, *lme4* package) with a binomial distribution (i.e. 'attempted' or 'did not attempt'). This was done because an insufficient number of fish conducted the behaviour (<15% across all groups) for it to be analysed as a count variable.

Within each treatment group, a series of Spearman's rank-order correlation tests were used to investigate potential across-context relationships between behaviours in the maze assay (boldness, exploration, and activity) and reproductive behaviour (total time males spent following females).

Sperm performance measures (VAP, VSL, VCL, LIN, MOT), sperm viability, and male morphological traits (length, weight, and condition index) were compared across treatments using LME models. To meet assumptions of normality, a square root folded transformation was applied to sperm motility, a rank-normal transformation was applied to sperm viability, and both male length and weight were cube-root transformed.

3. Results

3.1. Behavioural assays

No significant effect of treatment was detected on the time taken for fish to exit the refuge (boldness) in the maze arena, the time taken to complete the maze (exploration) or activity levels in the maze (coxme: all $p > 0.05$; Table S2–S3). In addition, male length did not significantly affect any of the measured behaviours (coxme: all $p > 0.05$; Table S2).

Total time spent by males associating with females in the reproductive assay was affected by fluoxetine treatment (LME: $F_{2,100} = 4.30$, $p = 0.016$; Fig. 2). Specifically, males in the low- and high-fluoxetine treatments spent significantly longer associating with females than did unexposed males ($t = 2.64$, $df = 100$, $p = 0.001$, and $t = 2.42$, $df = 100$, $p = 0.018$, respectively). There was, however, no significant difference in association behaviour between low- and high-fluoxetine exposed males ($t = -0.31$, $df = 100$, $p = 0.753$). More generally, a marginally

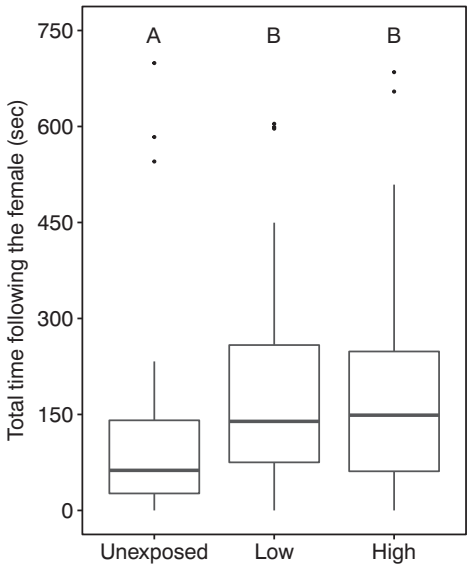

Fig. 2. Total time males spent actively following a female (i.e. associating) across unexposed ($n = 37$), low-fluoxetine ($n = 32$) and high-fluoxetine ($n = 36$) treatments. Box plots show 25th, 50th (median) and 75th percentiles. Groups that share a capital letter are not significantly different from one another.

non-significant effect of stimulus female length was detected on the total time spent by males associating with females ($t = 1.94$, $df = 100$, $p = 0.054$). Male length did not affect the total time spent associating with females ($t = 1.00$, $df = 100$, $p = 0.319$).

Fluoxetine exposure also impacted the likelihood of males to perform a copulation attempt. Specifically, a greater proportion of males from the low treatment attempted to copulate than did unexposed males (GLMM: $z = 2.38$, $df = 100$, $p = 0.017$), with 22% of low-exposed males attempting at least one copulation, as opposed to 3% of unexposed males. There was a similar, but marginally non-significant difference in the proportion of males that attempted to copulate between the high-fluoxetine treatment (13%) and the unexposed treatment ($z = 1.85$, $p = 0.065$), while no significant difference was observed between the low- and high-fluoxetine treatments ($z = -0.47$, $p = 0.882$). More generally, female length positively associated with the likelihood of males performing copulatory behaviour ($z = 2.35$, $p = 0.047$). Male length, however, did not significantly affect the likelihood of copulation ($z = -0.51$, $p = 0.611$).

For unexposed fish, there was a significant positive correlation between total time spent by males actively following females and male activity levels in the maze (Spearman's correlation: $r_s = 0.34$, $p = 0.040$; Fig. 3). However, this relationship was not seen in either low- or high-fluoxetine exposed males ($r_s = 0.16$, $p = 0.377$, and $r_s = 0.06$, $p = 0.740$, respectively; Fig. 3). Further, no significant correlation was detected between the total time males spent associating with females and boldness, or exploration, for any of the treatment groups (all $p > 0.05$; Table S4).

3.2. Sperm quality

Fluoxetine, irrespective of exposure level, did not significantly affect any measure of sperm performance or viability (LME: all $p > 0.05$; Table S5–S6). More generally, male length was positively associated with sperm motility ($F_{1,95} = 4.90$, $p = 0.029$) but did not associate significantly with any other measured sperm traits (Table S5).

3.3. Morphology

Fluoxetine exposure had no significant effect on male length (LME: $F_{2,102} = 0.37$, $p = 0.689$) or weight (LME: $F_{2,102} = 1.51$, $p = 0.225$). Additionally, a marginally non-significant effect of fluoxetine exposure was detected on condition index ($F_{2,102} = 2.95$, $p = 0.057$), with low-fluoxetine exposed males having a significantly lower condition than high-fluoxetine exposed males ($t = -2.38$, $df = 102$, $p = 0.019$). By contrast, control males showed an intermediate condition that was not significantly different from either low- or high-fluoxetine exposed males ($t = -1.67$, $df = 102$, $p = 0.099$ and $t = 0.45$, $df = 102$, $p = 0.752$, respectively).

4. Discussion

We found that fluoxetine did not significantly impact latency to emerge from a refuge (i.e. boldness), time to complete a maze after exiting the refuge (i.e. exploration), or the number of 5 cm gridlines crossed (i.e. activity). To date, only three other studies have employed environmentally realistic dosages (<1–596 ng/L) to investigate impacts of fluoxetine on anxiety-related behaviour in fish (Dzieweczynski et al., 2016a, 2016b; Margiotta-Casaluci et al., 2014). In concordance with our study, Margiotta-Casaluci et al. (2014) reported no effect of fluoxetine on boldness and exploration in fathead minnow (*Pimephales promelas*) at environmentally realistic exposure levels, although they did see an increase in boldness and exploration at concentrations exceeding those detected in the environment (72,000 ng/L for 28 days). By contrast, Dzieweczynski et al. (2016a, 2016b) reported that exposure to an environmentally relevant level of fluoxetine (500 ng/L for 1–15 days) significantly reduced boldness in Siamese fighting fish (*Betta splendens*). Such differences between studies could be due to different exposure durations and/or species-specific sensitivities. We suggest that further investigations at multiple time points in a range of species are warranted to elucidate the impacts of environmentally realistic fluoxetine exposure on anxiety-related behaviour.

In the reproductive behaviour assay, both low- and high-fluoxetine males spent more time associating with a female than did controls. For mosquitofish—and poeciliid fish more generally—the propensity of males to associate closely with females is a reliable and biologically meaningful estimate of male mating intent (Dosen and Montgomerie, 2004; Wong et al., 2005). Furthermore, since mosquitofish have internal fertilisation, actively following (i.e. being in close proximity to) females is essential for males to successfully mate (Bisazza et al., 2001). Low-fluoxetine exposure also increased the likelihood of males attempting copulation in comparison to controls. Further, a similar, but marginally non-significant, trend was seen towards high-fluoxetine-exposed males being more likely to copulate than unexposed males. An increase in copulatory behaviour is likely to result in increased mating success. For example, Evans et al. (2003) reported that male mosquitofish that perform more frequent copulation attempts are more likely to successfully transfer sperm. Moreover, in male western mosquitofish (*Gambusia affinis*), number of copulation attempts associates positively with proportion of offspring sired (Deaton, 2008). Taken together, the behavioural changes seen here are expected to result in increased

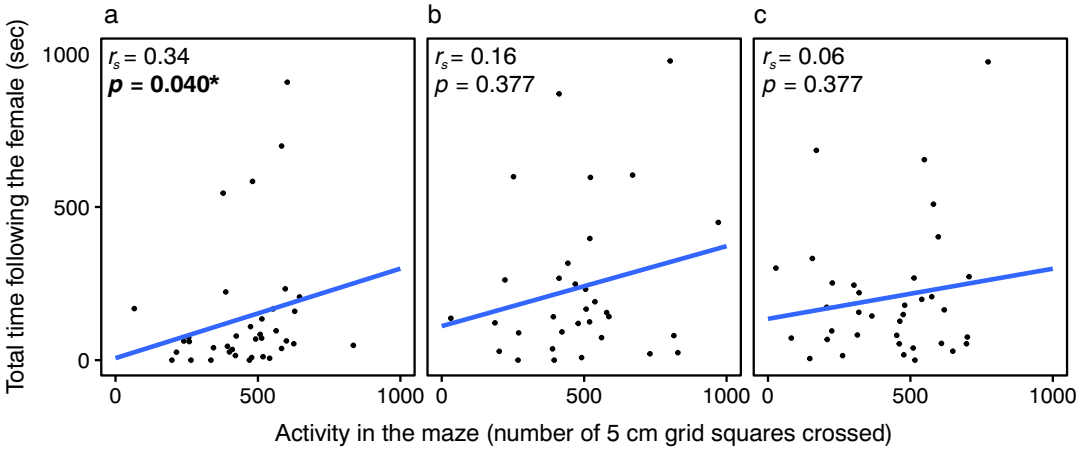

Fig. 3. Across-context behavioural correlations between activity in the maze assay and total time spent following a female in the reproductive assay for males in the unexposed (a; $n = 37$), low-fluoxetine (b; $n = 32$) and high-fluoxetine (c; $n = 36$) treatments.

male mating success in exposed fish. However, from the perspective of females, an increase in mating effort by exposed males could also be costly, with male sexual harassment previously shown to impinge on female foraging efficiency (Pilastro et al., 2003). The resulting increase in sexual conflict could ultimately lead to shifts in the strength and direction of sexual selection, which, in turn, can influence population demography by affecting the quality and quantity of offspring produced (Wong and Candolin, 2014).

The increase in reproductive behaviour observed in fluoxetine exposed fish could be caused by an increase in serotonin concentrations and, consequently, shifts in the hypothalamic–pituitary–gonadal (HGP) axis (reviewed in Kreke and Dietrich, 2008; McDonald, 2017). In fish, increases in extracellular serotonin concentrations have been shown to stimulate the release of gonadotropin-releasing hormones (GnRHs) and gonadotropic hormones (GTHs; reviewed in Kreke and Dietrich, 2008; McDonald, 2017), as well as androgens, which are known to regulate sexual behaviours (Borg, 1994; Munakata and Kobayashi, 2010). Thus, it is possible that the increased reproductive behaviour of male mosquitofish is due to a serotonin-induced increase in hormones responsible for mediating sexual behaviours. In humans, chronic fluoxetine exposure should ultimately lead to a return to pre-treatment serotonin levels, driven by compensatory responses of the brain to perturbed serotonin concentrations, which can take several weeks (reviewed in Andrews et al., 2015). Therefore, it is interesting that, after a 35-day exposure, we saw an increase in reproductive behaviour, which would be expected with increased serotonin concentrations. Perhaps, at fluoxetine concentrations as low as those used here, compensatory responses to perturbed serotonin concentrations are less pronounced or rapid. Such a possibility could be addressed by directly measuring serotonin levels in the brain. It is also worth highlighting that, even for humans, the tolerability, efficacy, and mechanism of action of SSRIs have all been the subject of controversy and debate (reviewed in Walker, 2013).

To date, only a handful of studies have addressed impacts of environmentally realistic fluoxetine exposure on reproductive behaviour in fish (Bertram et al., 2018a; Dzieweczynski and Hebert, 2012; Forsatkar et al., 2014; Fursdon et al., 2018; Schultz et al., 2011; Weinberger and Klaper, 2014). In concordance with the present study, both Bertram et al. (2018a) and Fursdon et al. (2018) reported an increase in copulatory behaviour in male poeciliid fish (*Gambusia holbrooki* and *Poecilia reticulata*, respectively) following ecologically relevant fluoxetine exposure (479 ng/L for 30 days, and 350 ng/L for 28 days, respectively). Similarly, Weinberger and Klaper (2014) reported an increase in reproductive behaviour (i.e. nest tending) in male fathead minnows after 28-day fluoxetine exposure, although this was only seen at 1000 ng/L and not at the lower concentration tested (100 ng/L). In contrast, no effect of fluoxetine exposure was detected on the reproductive behaviour of fathead minnows (2.3 and 28 ng/L for 21 days; Schultz et al., 2011) or Siamese fighting fish (540 ng/L for 6 days; Dzieweczynski and Hebert, 2012). In a separate study using Siamese fighting fish, however, a decrease in reproductive behaviour has also been reported (540 ng/L for 3 days; Forsatkar et al., 2014). Differences in fluoxetine-induced effects across these studies may be a result of different exposure durations and species-specific sensitivities. Indeed, the modulatory function of serotonin on the HPG axis seems to vary considerably across fish species (Kreke and Dietrich, 2008). This disparity may also be a result of the different reproductive behaviours assessed. For example, some of the studies incorporated male-male competition in their measure of reproductive behaviour while others did not. Reproductive behaviours in the presence of male-male competition, may, therefore, not be impacted by fluoxetine exposure to the same degree as reproductive behaviour performed in the absence of such aggression. Indeed, as mentioned above, Bertram et al. (2018a) reported an increase in copulatory behaviour in male mosquitofish in the absence of male-male competition, however, in a separate assay under direct male-male competition, this effect was not evident.

Interestingly, we found a positive across-context correlation in unexposed fish between reproductive behaviour (i.e. time spent by males following females) and activity in the maze, although this was not present in fluoxetine-exposed fish. Evidence of a behavioural correlation between reproduction and activity levels in unexposed fish suggests that these two traits are either directly coupled through some kind of causal (e.g. a gene or hormone that affects both behaviours) and/or an indirect link (e.g. shaped by individual experience and learning feedback loops; reviewed Sih et al., 2004). Since this relationship was absent in fluoxetine-exposed fish, we suggest that fluoxetine-induced effects on neuroendocrine pathways like the HPG and hypothalamic-pituitary-adrenal axes disrupted this behavioural correlation. Given that fluoxetine exposure also impacted reproductive behaviour in this study, we hypothesise that the absence of across-context behavioural correlation is a result of shifts in the HPG axis of exposed fish. To the best of our knowledge, this is the first evidence that field-detected concentrations of a pharmaceutical pollutant may cause a breakdown in across-context behavioural correlations (i.e. behavioural syndromes). In light of this, future research may wish to employ pre- and post-exposure behavioural tests across contexts, in combination with endocrine measures (e.g. plasma hormone levels), to identify the extent to which fluoxetine exposure may disrupt the presence of behavioural syndromes in wildlife. Given that behavioural syndromes have been linked with the ability of species to respond to environmental change and invasive potential (reviewed in Sih et al., 2012), pollution-induced disruption of behavioural correlations could have significant, yet overlooked, consequences for fitness.

We did not find evidence of fluoxetine-induced effects on any measured sperm traits. To date, only the present study and that of Bertram et al. (2018a) have examined effects of environmentally realistic concentrations of fluoxetine on sperm quality in fish, both of which reported no effect. However, Bertram et al. (2018a) did report an increase in the total sperm count of fluoxetine-exposed fish, an endpoint not measured in the present study. Other studies have addressed impacts of exposure on different gonad-related endpoints. For example, in adult Japanese medaka (*Oryzias latipes*), gonadal somatic index and gonadal steroidogenesis were unaffected by 4 weeks of fluoxetine treatment at a range of concentrations (i.e. 0–5000 ng/L; Foran et al., 2004). In addition, Mennigen et al. (2010) reported that 14-day exposure to fluoxetine at 540 ng/L did not affect basal milt volume in male goldfish, although a reduction was observed at 54,000 ng/L. More broadly, in humans and rodents, sexual dysfunction and decreased sperm motility has been reported as a side effect of fluoxetine treatment (reviewed in Nørr et al., 2016). Given that the dosages used in these studies are much higher than were used in the present study, it is possible that spermicidal effects of fluoxetine might only be seen at higher dosages than those used here.

While neither male length nor weight was affected by fluoxetine exposure, a marginally non-significant impact of exposure was detected on condition index, which was driven by a decrease in the condition of low-exposed fish relative to those in the high-exposed treatment. Previous studies have reported a decrease in condition index as a result of fluoxetine exposure (Bertram et al., 2018a; Gaworecki and Klaine, 2008; Latifi et al., 2015), although, with the exception of Bertram et al. (2018a), these effects were seen at higher concentrations than those found in the environment. Given the marginal nature of our results, we suggest that the impacts of environmentally realistic fluoxetine exposure on morphological traits, like condition index, warrant further investigation.

5. Conclusions

In summary, fluoxetine exposure for 35 days at 31 and 374 ng/L impacted male reproductive behaviour, while sperm traits and anxiety-related behaviour in the same individuals were unaffected. Additionally, fluoxetine at both dosages disrupted the presence of an across-context

behavioural correlation (i.e. behavioural syndrome), with a positive correlation being detected between reproductive behaviour and boldness in unexposed fish only. Taken together, these findings suggest that fluoxetine exposure can induce context-specific effects, thus highlighting the need to address the impacts of pharmaceutical exposure over multiple ecologically important contexts. More broadly, shifts in reproductive behaviour support a growing body of evidence that psychoactive pharmaceuticals at field-detected concentrations can induce subtle—but important—changes in wildlife behaviour. The next step in identifying the risk posed by fluoxetine (and other psychoactive pollutants) is to address the potential for synergistic or antagonistic effects during exposure to pharmaceutical mixtures, using a combination of pollutants that are both readily detected in the environment and act via similar mechanisms.

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Authors' contributions

JMM, MGB, MS and BBMW conceived and designed the experiments, which JMM, MGB and TEE carried out. Video and statistical analysis were performed by JMM, MGB, JLT and MM. All sperm analysis was coordinated by MKOB, which JMM, MGB and SLH conducted. The manuscript was drafted by JMM. All authors contributed critically to the drafts and gave final approval for publication.

Data accessibility

Data deposited in the Dryad Digital Repository.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.09.294.

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Appendix H

Behavioural effects of psychoactive pharmaceutical exposure on European perch (*Perca fluviatilis*) in a multi-stressor environment

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Behavioural effects of psychoactive pharmaceutical exposure on European perch (*Perca fluviatilis*) in a multi-stressor environment

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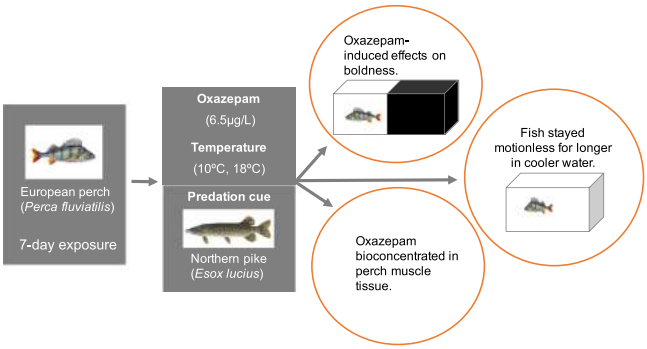
HIGHLIGHTS

- Juvenile European perch were exposed to oxazepam at two temperatures and under two predation risk regimes.
- Exposure altered anxiety-related behaviour and boldness of fish.
- Fish in the low temperature treatments froze for longer. Predator cue treatment affected perch risk-taking behaviour.
- We found no interaction effects of oxazepam and temperature on the studied behaviours.
- Highlights ecologically important sub-lethal effects of pharmaceutical contamination.

GRAPHICAL ABSTRACT



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ABSTRACT

With the ability to resist biodegradation and exert therapeutic effects at low concentrations, pharmaceutical contaminants have become environmental stressors for wildlife. One such contaminant is the anxiolytic oxazepam, a psychoactive pharmaceutical that is frequently detected in surface waters globally. Despite growing interest in understanding how wildlife respond to anxiolytics, synergistic effects of pharmaceuticals and other abiotic (e.g. temperature) and biotic (e.g. predation risk) stressors remain unclear. Here, using a multi-stressor approach, we investigated effects of 7-day oxazepam exposure (6.5 µg/L) on anxiety-related behaviours in juvenile European perch (*Perca fluviatilis*). The multi-stressor approach was achieved by exposing perch to oxazepam at two temperatures (10 °C and 18 °C), and at two predation risk regimes—generated using chemical cues from the northern pike (*Esox lucius*). Our exposures resulted in a successful uptake of the drug from the water, i.e., oxazepam was measured in perch muscle tissue at 50 ± 17 ng/g (mean ± SD). We found significant oxazepam-induced effects on boldness, with 76.7% of the treated fish entering the white background (i.e. 'exposed' area where exposure to presumed risks are higher) within the first 5 min, compared to 66.6% of the control fish. We also found a significant effect of temperature on total time spent freezing (i.e. staying motionless). Specifically, fish in the low temperature treatments (oxazepam, predation) froze for longer than fish in high temperatures. Our multi-stressor study is the first to uncover how anxiety-related behaviours in wild juvenile fish are altered by changes in water temperature and perceived predation risk. Importantly, our findings highlight the

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need to focus on multiple stressors to improve understanding of how organisms not only survive, but adapt to, human-induced environmental change.

1. Introduction

Pharmaceutical contaminants, which enter environments via wastewater effluents, are a major environmental concern due to rising pharmaceutical consumption with a growing and ageing human population (Boxall, 2004; Arnold et al., 2014). In fact, >600 pharmaceutical substances have now been detected in the environment worldwide (aus der Beek et al., 2016). Despite increasing research interest in understanding how wildlife responds to pharmaceutical contaminants (reviewed in Brodin et al., 2014; Saaristo et al., 2018), the synergistic fitness effects of pharmaceuticals and their interaction with temperature remain unclear. In particular, in aquatic environments, changing temperature due to anthropogenic activity (e.g. seasonal extremes due to climate change), impacts ecosystems at multiple levels of biological organisation.

At the individual level, temperature affects physiology, from metabolic activity to protein damage and organ function (Hochachka and Somero, 2002). Moreover, increased water temperature is known to stimulate metabolism and, thus, uptake of chemical contaminants (Pörtner, 2002). Indeed, elevated temperature changes toxicokinetics by enhancing bioavailability and toxicity of chemical contaminants (Buchwalter et al., 2003; Lydy et al., 1999; Maruya et al., 2005). For example, increased water temperature (from 15 °C to 25 °C) magnified acute toxicity of the pain reliever acetaminophen 8.3-fold in a freshwater invertebrate (*Daphnia magna*, Kim et al., 2010). As changing water temperature affects thermoregulation, the associated metabolic costs are directly linked to resources available for growth, reproduction, and development (Kooijman, 2001; Angilletta, 2009). These energetic trade-offs become especially costly when individuals are chronically exposed to a combination of environmental stressors (Gaw et al., 2014). Not all individuals are equally affected, however, and species differ in their ability to adjust their physiology with increasing water temperature. For example, mid-intertidal crabs are more tolerant to rising temperatures than their subtidal congeners (Tomanek and Somero, 1999; Stillman, 2002). Susceptibility to thermal pollution also depends on ontogenic stage (Pörtner, 2002) and thermal tolerance of the organism (Patra et al., 2007; Noyes et al., 2009). Because synergistic effects between rising water temperatures and chemical contaminants are likely to exacerbate these changes (Noyes et al., 2009), it is not surprising that a growing number of studies suggest that, in aquatic habitats, alterations in chemistry may be more important than changes in water temperature alone for the performance and survival of wildlife (Harvey et al., 2006; Gaw et al., 2014).

Due to the conservative nature of physiological processes among vertebrates, pharmaceutical contaminants, which remain biochemically active in the environment, are a threat to non-target organisms. Among pharmaceuticals, psychoactive drugs—such as benzodiazepines—are the most commonly used globally, being prescribed to treat stress and anxiety-related disorders, as well as chronic insomnia (Rieman et al., 2015; Kurko et al., 2018). In the central nervous system, benzodiazepines bind to the gamma amino butyric acid $(GABA)_A$ receptor, which is highly conserved across animal taxa (Gunnarsson et al., 2008), and increase natural activity of the inhibitory neurotransmitter GABA (Pritchett et al., 1989). This, in turn, enhances inhibitory products to all of the major cell groups in the brainstem and hypothalamus that would otherwise stimulate arousal (Revel et al., 2009). One of the most widely used benzodiazepines is oxazepam. As a result of inadequate removal during wastewater treatment processes, oxazepam is commonly detected in surface (up to 61 ng/L) and effluent (up to 1.8 μg/L) waters around the world (Loos et al., 2013; aus der Beek et al., 2016; Fick et al., 2017). The estimated half-life of oxazepam in laboratory conditions is approximately 50–60 days (Loffler et al., 2005; Patterson et al., 2011), but it can withstand microbial degradation and exist in its bioactive form in lake sediment for decades (Klaminder et al., 2015). Furthermore, since psychoactive drugs, such as oxazepam, have been designed to treat behavioural disorders, it is not surprising that laboratory and field studies have found oxazepam to alter behaviour and survival of non-target animals at field-relevant concentrations (European perch, *Perca fluviatilis*: Brodin et al., 2013; Klaminder et al., 2014; Atlantic salmon, *Salmo salar*: Hellström et al., 2016).

The first response of an organism to environmental change is often to alter its behaviour (Nagelkerken and Munday, 2016). In this regard, behaviour is the result of numerous complex developmental and physiological processes (Wong and Candolin, 2015) and, thus, provides a comprehensive measure of exposure to multiple stressors. Importantly, behaviour has proven to be a sensitive early warning sign of contamination (e.g. Bell, 2001; Martinovic et al., 2007; Saaristo et al., 2009a, 2009b; Hallgren et al., 2011; Tomkins et al., 2016; Martin et al., 2017; Bertram et al., 2018a, 2018b) that can detect effects at much lower concentrations than traditional ecotoxicological endpoints (reviewed in Scott and Sloman, 2004; Söffker and Tyler, 2012; Melvin and Wilson, 2013; Arnold et al., 2014). One such behaviour is anxiety, which can be quantified using a well-established behavioural assay called scototaxis or 'white/dark assay' (Maximino et al., 2010). In short, this test utilises time spent in a white (i.e. 'exposed' environment) versus dark (i.e. protected black substrata environment) compartment to evaluate anxiety versus anti-anxiety behavioural attendance of the fish. This is particularly relevant when examining impacts of a drug prescribed to treat anxiety in human patients, such as oxazepam. In addition, the scototaxis assay tests for exploratory tendency and boldness in fish, because the white compartments represent an environment where the fish lacks camouflage and is 'exposed' to heightened predation risk (Maximino et al., 2010; Brodin et al., 2017).

In addition to predation, warmer temperatures have often been associated with increased activity and boldness in fish (e.g. Biro et al., 2010; Ojanguren and Braña, 2000; Forsatkar et al., 2016). However, this is not always the case and more recent studies have reported that elevated temperatures can actually decrease swimming speed, activity, boldness and foraging (Johansen and Jones, 2011; Nowicki et al., 2012; Colchen et al., 2017; Davies et al., 2017). Very few behavioural studies to date have investigated interactions between temperature and chemical contamination (see Lagesson et al., 2018), and even fewer in an ecologically realistic context, such as under predation pressure.

To date, no study has investigated interactions between temperature and oxazepam contamination. Therefore, we investigated responses of fish behaviour to multiple stressors, not only including oxazepam and temperature treatments, but also simulated predation pressure. More specifically, the objectives of this study were to 1) examine how exposure to a combination of temperature (10 °C or 18 °C) and oxazepam impacts the behaviour of European perch (*Perca fluviatilis*), 2) unravel if exposure to multiple stressors, such as temperature and predation threat, would increase or decrease the impact (if any) of oxazepam exposure, and 3) determine if exposure to multiple stressors impacts the level of bioaccumulation of oxazepam in fish muscle tissue. We hypothesised that, 1) oxazepam would reduce anxiety and make fish bolder, and 2) colder temperature would cause reduced activity due to decreased metabolism. With the multi-stressor (oxazepam, temperature, and predation) approach, we expected the behavioural outcome

to either strengthen the single stressor treatment due to additive and/or synergistic effects or to be less than the sum of the independent effects (i.e. antagonistic effect) (Folt et al., 1999; Alton and Franklin, 2017).

2. Methods

2.1. Study species

The European perch is a common freshwater fish native to Europe and Asia that has been introduced in Australia, New Zealand, and South Africa (Thorpe, 1977). It is an eurythermal species (i.e. tolerates wide range of temperatures) that inhabits clear rivers and lakes (Thorpe, 1977). Importantly for their use as a relevant species in behavioural ecotoxicology, the biology and behaviour of perch is well understood (Thorpe, 1977; Christensen and Persson, 1993) and previous studies have reported that perch are exposed to oxazepam in their natural habitats (Brodin et al., 2013).

2.2. Animal collection and housing

One-year-old perch ranging from 5.5 to 7 cm were collected in Bjännsjön, 16 km southeast of Umeå, Sweden, during the first week of June 2016, using a fine-meshed (5 mm mesh size) beach seine. The fish were transported in oxygenated containers, within 2 h of capture, to a holding tank (150 × 85 × 150 cm; length × width × height) at the Department of Ecology and Environmental Science, Umeå University. In the holding tank, the perch were held in oxygenated, aged tap water at ~13 °C, with a constant flow-through of water, and under a light:dark regime of 12:12 h. They were fed thawed chironomid larvae (~10% of perch body weight) daily during an acclimation period of 3 weeks prior to the behavioural trials.

2.3. Exposure design

Prior to the application of treatments, behavioural trials (see details in Section 2.4) were conducted to gain a baseline of behaviours across all individuals. To do that, fish were randomly taken from their holding tanks and individually placed into containers (28 × 19 × 14 cm) with a starting water temperature of 13 °C. Then, each container was placed in one of the temperature-controlled rooms letting the temperature of the water slowly increase or decrease to the corresponding treatment temperature (10 or 18 °C). Note that fish were not exposed to oxazepam or predation cues during these baseline trials. After 12 h in individual containers fish were introduced into the experimental arenas and allowed an acclimation period of 5 min before they were video-recorded from above using a single camera for 30 min (see details in Section 2.4).

Straight after the baseline trials, the same fish were randomly assigned, using a 'random number-draw-method' on excel, to one of eight treatments: freshwater control at 18 °C ($n = 30$), freshwater control at 10 °C ($n = 30$), freshwater control at 18 °C with predator cues ($n = 30$), freshwater control at 10 °C with predator cues ($n = 30$), oxazepam exposure at 18 °C ($n = 30$), oxazepam exposure at 10 °C ($n = 30$), oxazepam exposure at 18 °C with predator cues ($n = 30$), or oxazepam exposure at 10 °C with predator cues ($n = 30$). Temperatures were selected as they represent water temperatures of two different seasons (18 °C = summer, 10 °C = fall) in the area from which the fish were collected (Thorpe, 1977). Fish from each treatment were housed individually in identical exposure containers (28 × 19 × 14 cm) filled with 4 L of aged tap water, and with an air-stone for aeration. As with baseline behavioural trials, each container had a starting water temperature of 13 °C. Fish were acclimated to their specific temperature treatment by placing each container in one of the temperature-controlled rooms letting the temperature in the water slowly increase or decrease to the corresponding treatment temperature (10 or 18 °C). We staggered the exposure to enable us to collect behavioural data for 240 fish (each fish tested before and after exposure). Specifically, a set of 48 fish (1–48) were introduced into their individual exposure container on day one, the next set of 48 fish (49–96) on day two, and so on, until the total number of 240 fish had been introduced.

A stock solution with a nominal concentration of 10 μg/L oxazepam (Sigma-Aldrich, O5254, CAS 604-75-1) was prepared in dissolved water before being added to exposure containers at the start of the experiment. Exposure containers were spiked only once because oxazepam concentration in the water has previously been found to remain stable at least for 7 days (Brodin et al., 2014; Klaminder et al., 2014; Heynen et al., 2016a). We selected 10 μg/L oxazepam as an experimental exposure concentration since it represents a level high enough to generate behavioural effects in perch at 18 °C (Brodin et al., 2013; Klaminder et al., 2014), and our main objective was to see if behavioural effects are modified by temperature and/or predator regime. The predator cue treatment was achieved by adding 12 mL of water daily (3 mL added to each corner of the individual containers), which was taken from a 1 m^3 holding tank (150 × 85 × 150 cm) housing two 40 cm long pike (*Esox lucius*) that were fed perch every third day. Two temperature-controlled rooms were used, with 50% of the containers being stored at a low temperature, resulting in a 10 °C ± 0.32 °C (mean ± SD) water temperature, and 50% of the containers stored at a higher temperature, resulting in a water temperature of 18 °C ± 0.36 °C (mean ± SD). Each perch was fed 10 thawed chironomid larvae every second day during the exposure period. We monitored key water properties in exposure tanks throughout the exposure (ammonium [NH4], <0.004 mg/L; hardness, 2.8 ° dH; iron [Fe], <0.010 mg/L; nitrite [NO2], <0.003 mg/L; pH, 8.0).

2.4. Behavioural trials

After seven days of exposure, each perch was tested in a second set of behavioural trials, which followed the design of Brodin et al. (2017). Briefly, a scototaxis assay (light/dark preference; Maximino et al., 2010) was used to assess effects of oxazepam, temperature and predator cues on boldness. The behavioural trait 'boldness' is a well-established measure that defines how individuals respond to risky situations with direct fitness consequences (Frost et al., 2013). The scototaxis arena (80 × 50 × 42 cm) was divided equally into black (40 × 50 × 42 cm) and white (40 × 50 × 42 cm) halves. The depth of the water—which was clean, aged, and temperature treatment-matched—was 10 cm, yielding a volume of 40 L. Each test individual was introduced into the centre of the experimental arena and then given 30 min to swim freely, while its behaviour was video recorded (SONY Handycam HDR-PJ50VE) from above. Following behavioural trials, fish were euthanised with an overdose of MS-222 (Ethyl 3-aminobenzoate methanesulfonate) and stored at −20 °C for later tissue analysis of oxazepam.

The following behaviours were quantified: total time spent moving within, and frequency of visits to, the white compartment; total time spent moving within, and frequency of visits to, the black compartment; as well as the total time spent performing 'freezing' behaviour (i.e. staying motionless), total duration of the first freezing behaviour, and frequency of freezing events. The preceding behavioural variables were recorded with the intention of compiling them into an analysis that uses a multivariate synthetic axes approach. Additionally, we were interested in initial movement of the fish and measured the time fish took to cross onto the white and/or black background for the first time, and a binary score of whether the fish moved into a white or black region (i.e. recorded in a form suitable for a survival analysis). The behaviours were quantified blind to treatment from the 30 min video recordings using the event-recording software JWatcher V1.0.

2.5. Chemical analyses

Oxazepam concentrations in exposure water and perch muscle tissue (exposed and controls) were determined with a triple stage

Table 1
Standard deviations, proportion of variance and cumulative proportion of variance explained, and individual variable loadings for the behavioural PCA.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
SD	1.91	1.11	0.95	0.78	0.58	0.43	0.32
Proportion of variance	0.52	0.18	0.13	0.09	0.05	0.03	0.01
Cumulative proportion of variance	0.52	0.7	0.82	0.91	0.96	0.99	1
Loadings							
Total time on white background (s)	−0.27	0.46	−0.7	0.1	−0.28	0.06	0.37
Total time on black background (s)	−0.41	0.12	0.59	0.15	−0.12	−0.32	0.58
Total time freezing (s)	0.44	−0.31	−0.17	−0.35	0.3	−0.02	0.69
Frequency of moving onto white	−0.41	0.23	−0.11	−0.53	0.56	−0.38	−0.17
Frequency of moving onto black	−0.44	−0.18	0.13	−0.46	−0.14	0.72	0.08
Frequency of freezing bouts	−0.28	−0.67	−0.26	−0.13	−0.43	−0.45	−0.08
Time elapsed until first freezing bout (s)	0.36	0.38	0.19	−0.58	−0.56	−0.19	−0.08

quadrupole MS/MS TSQ Quantum Ultra EMR (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela and a Surveyor LC pump (Thermo Fisher Scientific, San Jose, CA, USA) and a PAL HTC autosampler (CTC Analytics AG, Zwingen, Switzerland). For a detailed description of the pre-treatment and analysis see Brodin et al. (2013, 2014). For the muscle tissue samples, we measured bioconcentration factor (BCF) (Arnot and Gobas, 2006).

2.6. Statistical analysis

When examining the baseline behavioural assays (i.e. before exposure started) we used a non-parametric univariate approach. Specifically, univariate Kruskal-Wallis tests were performed for the six fundamental behaviours: 1) time spent on white background (s), 2) events moving onto the white background, 3) time spent on black background (s), 4) events moving onto the black background, 5) time spent freezing (s), and 6) number of freezing events. Because testing the baseline behaviours is intended to reveal potential confounding problems, Type II error should be controlled (i.e the β, not the α, is the prime concern) (Quinn and Keyough, 2002). We set $\beta = 0.9$ (i.e. $\alpha = 0.1$), which should be viewed as exploratory rather than a level suitable for hypothesis testing. Also, it is worth noting that the univariate approach inflates Type I error through multiple comparisons, but it would be misguided to view this as a concern. When checking for potential biases, the preference should always be to intentionally inflate Type I error in favor of restricting Type II error.

To examine effects of oxazepam, temperature (High/Low) and predator cue (Yes/No), we used a multivariate synthetic axis approach. This involved using a Principal Component Analysis (*prcomp* in R *stats* (R Core Team, 2018) to identify synthetic axes of positive and negatively correlated variation among measured behaviours (e.g. total time on white background (s), total time on black background (s), total time freezing (s), frequency of movement onto the white background, frequency of movement on the black background, frequency of freezing bouts, total time elapsed until the first freezing bout (s)). We used the standard method of accepting axes with Eigenvalues >1 as our threshold for variable reduction (Eigenvalues for axes PC1 = 3.6; PC2 = 1.2; PC3 = 0.9; PC4 = 0.6; PC5 = 0.3; PC6 = 0.2; PC7 = 0.1). The PCA axes were then used as synthetic behavioural responses in three-way ANOVAs with oxazepam, temperature, and predation, as two-level categorical predictors. As this is a hypothesis testing step, we set $\alpha = 0.05$, in line with standard methodology (Quinn and Keyough, 2002). However, the interpretation of a linear model can be misleading if the slopes of treatment groups are not equal (i.e. if significant interactions are present in the model). Therefore, we looked for significant interactions, although none were identified. Because the original hypothesis included the interaction of temperature × oxazepam, we retained this interaction term in our final models, despite it not showing any significance. Higher order interactions that did not specifically relate to a hypothesis, and were non-significant, were removed.

Finally, we were interested in initial movement of the fish, which was the time until a fish crossed onto the white and/or black background for the first time. These data were not suitable for inclusion in a PCA, but were suitable for survival analysis. We used a survival regression analysis (*survreg* in R library *survival*). We checked for the best

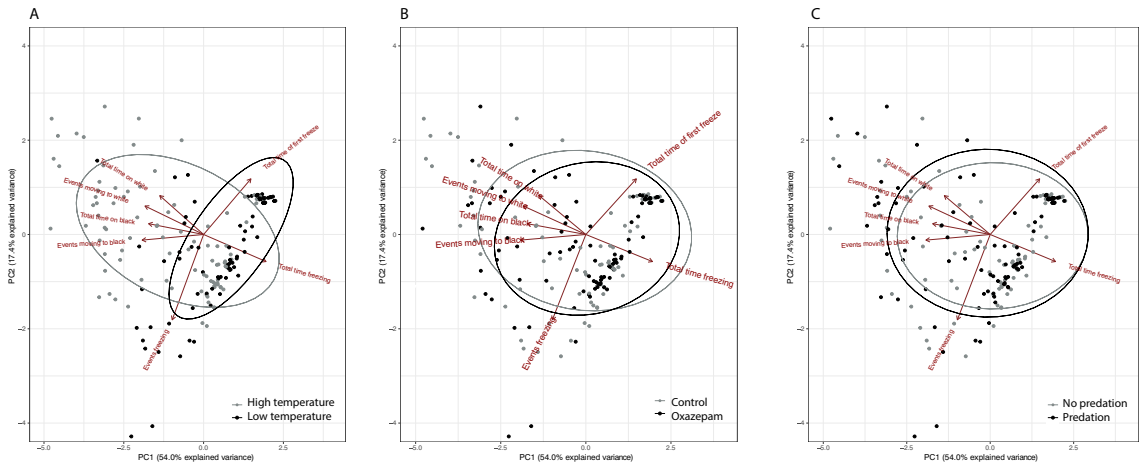


Fig. 1. Principal component analysis (PCA) of the selected behaviours across different treatment groups. PCA presents the level of similarity/dissimilarity between (A) high and low temperature, (B) control and oxazepam, and (C) no predation cue and predation cue. Total black = total time (sec) spent moving in the black background; Event black = number of times fish visited the black blackground; Total white = total time (sec) spent moving in the white blackground; Event white = number of times fish visited the white blackground; Total freezing = total time (sec) spent in staying motionless; Event freezing = total number of freezing events; Duration first freeze = length of the first freezing event (sec).

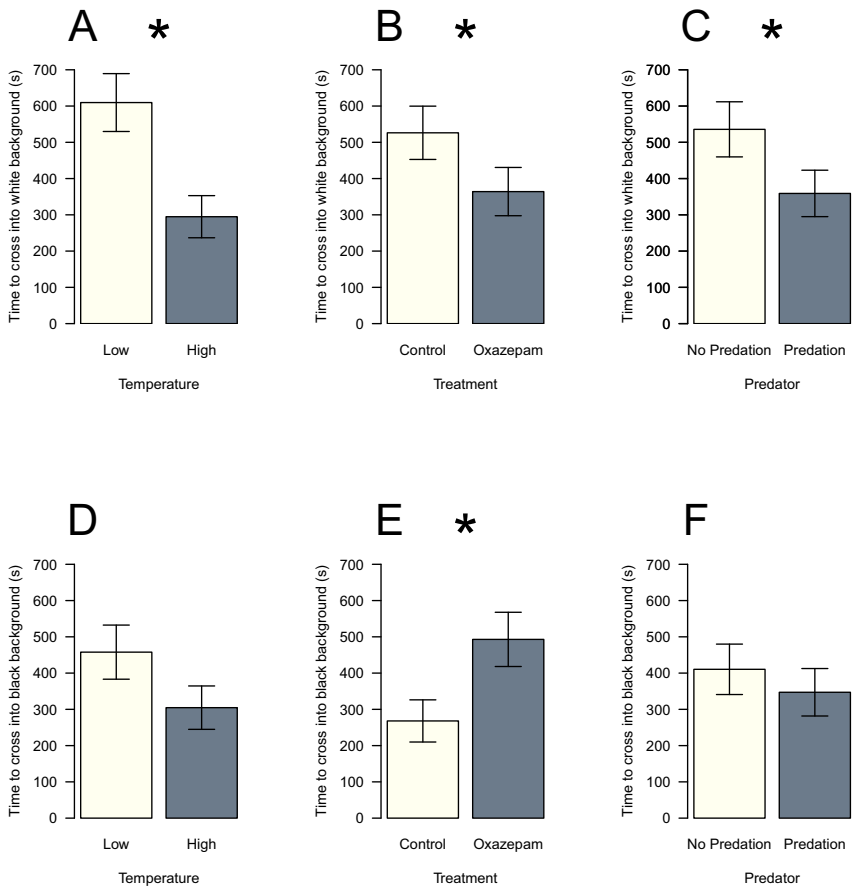


Fig. 2. The effect of temperature on the time (s) taken for fish to cross into (A-C) white background and (D-F) black background. Means and standard errors are shown. High = fish exposed to high temperature (18 °C); Low = fish exposed to low temperature (10 °C); Control = control treatment (freshwater); Oxazepam = oxazepam treatment (6.5 μg/L); No predation = no predation cue; Predation = predation cue. Significant differences ($p < 0.05$) are indicated by an asterisk (*).

distribution among Weibull, exponential, extreme, Gaussian and logistic options, but Weibull was always preferred (i.e. Weibull always had the lowest −2*LL; −2 multiplied by the log likelihood, as an indicator of model fit). As with the above ANOVAs, we checked for significant interaction terms, but no interaction terms were significant, and, therefore, we report main effects only.

To examine patterns of PCA response to the treatments, we used ANOVA. Specifically, we investigated how oxazepam, temperature and predator cue impacted fish behaviour (axis 1 and 2 of the behaviour PCA). All ANOVA assumptions were checked using standard diagnostic plots (i.e. residuals versus fitted, qq-plot) and no data transformations were needed. All significance levels (alpha) were 0.05 and all statistical analyses were conducted using R version 3.5.0 (R Core Team, 2018) and R Studio version 1.0.153 (R Studio Team, 2016).

3. Results

3.1. Baseline behavioural data

Before the exposures, there were no significant differences in any of the assayed baseline behaviours among the oxazepam and predation cue treatment groups (Kruskal-Wallis: $p > 0.130$ for all tests). There was, however, an effect of temperature on behaviour, which is not surprising given that the fish had been moved to the low and high temperature treatment tanks 12 h before the baseline assay and as such already had time to adjust their behaviour to the new environmental condition (Supplementary Figs. 1, 2, 3; Supplementary Table 1).

3.2. Oxazepam exposure

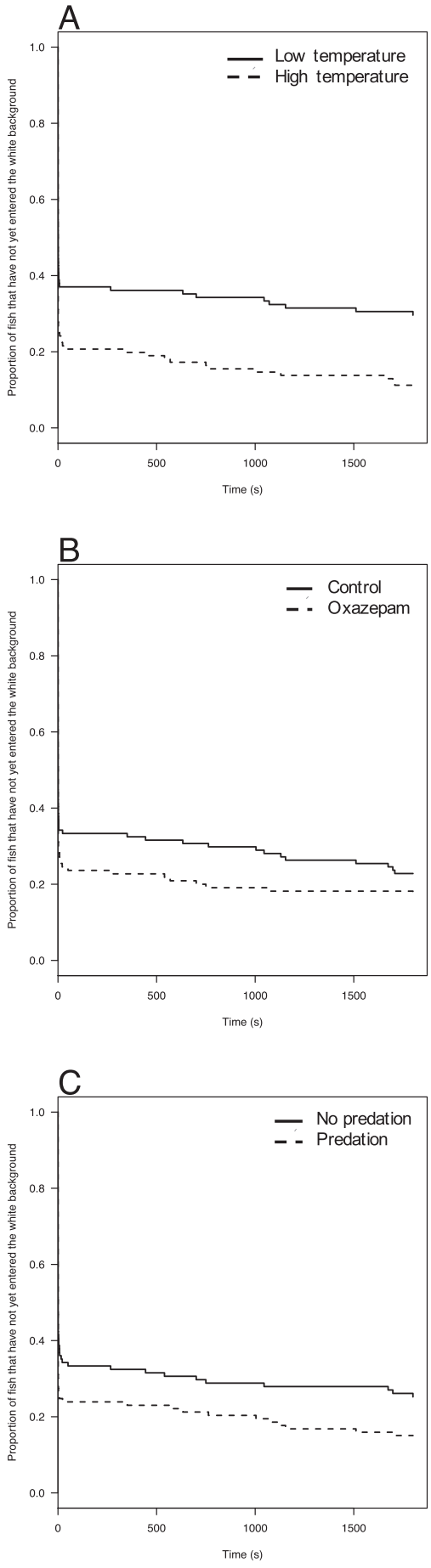
Measured oxazepam concentrations in our exposure treatments were as follows (mean ± SD): oxazepam high temperature = 6.1 ± 0.3 μg/L ($n = 14$), oxazepam high predation = 6.2 ± 0.5 μg/L ($n = 15$), oxazepam low temperature = 6.7 ± 0.7 μg/L ($n = 15$), and oxazepam low predation = 6.9 ± 0.8 μg/L ($n = 15$), respectively. No oxazepam was detected in the control tanks (<1 ng/L; $n = 40$). Further, oxazepam bioconcentrated in perch muscle tissue (mean ng/g ± SD ng/g), as follows: oxazepam high temperature = 49 ± 14.1 ($n = 31$), oxazepam high predation = 50 ± 11.5 ($n = 27$), oxazepam low temperature = 47 ± 14.8 ($n = 29$), and oxazepam low predation = 54 ± 23.0 ($n = 28$). All samples in the control group had concentrations below the method detection limits (<LOQ, $n = 40$) (Supplementary Table 2).

3.3. Behavioural effects: interactions and main effects

After 7-day exposure, we found a number of significant main effects for oxazepam and predation cue treatments. Because there were no statistical interactions among the predictors (oxazepam, temperature or predation cue treatments), interpretation of these main effects is most sensibly presented and interpreted independently for each predictor.

3.3.1. Multivariate synthetic axes

Using the standard threshold of keeping axes with Eigenvalues >1, we identified two potential axes of importance. Loadings that contributed >0.3 are reported for each principal component axis that we



examined (Table 1). The first axis (PCA1, variance explained = 54.0%) was contributed to negatively by total time spent on white (loading = −0.315) and black (loading = −0.395) backgrounds, and frequency of entering white (loading = −0.421) and black (loading = −0.442) backgrounds, implying a significant axis of variation associated with movement around the tanks as a whole. In comparison, total time spent freezing (loading = +0.449) and total time of first freezing event (loading = 0.345) associated positively with PCA1 (Fig. 1a, b, c). We found that there was a strong negative temperature effect for PCA axis 1 (i.e. PC1 values were lower for the high temperature treatment; ANOVA: $F_{1,223} = 49.9$, $p < 0.001$). This implies that the higher temperature treatment associated with less freezing and greater time spent moving around the tank (i.e. time spent on white and black backgrounds, and frequency of entering black and white backgrounds). The second axis (PC2, variance explained = 17.4%), on the other hand, was negatively associated with frequency of freezing events (loading = −0.734). Total time spent on white background (+0.335) and total time of first freezing event (loading = +0.470), associated positively with PC2. There were no significant treatment interactions or main effects on PC2 (all $F < 1.3$, all $p > 0.260$).

3.3.2. Time to enter white/black background

We found significant main effects in the initial movements of fish, but no significant interactions. Therefore, main effects of the temperature, oxazepam, and predation treatments are reported individually, for clarity. Regarding movements to the white background, there were significant temperature, oxazepam, and predation effects (Fig. 2a,b,c). Specifically, in the high temperature treatments, 79.4% of fish entered white background within the first 5 min while only 63.9% did so in the low temperature ($z = 4.15$, $p < 0.001$) (Fig. 3a). In the oxazepam treatment, 76.7% of the treated fish entered white background within 5 min compared to 66.6% control fish ($z = -2.10$, $p = 0.036$) (Fig. 3b). In the predation treatment, 76.2% of fish exposed to predation cues entered the white background within the first 5 min ($z = -2.77$, $p = 0.006$) compared to 67.1% of control fish (Fig. 3c). Regarding movements to the black background, there was only significant effect on oxazepam treatment ($z = 2.73$, $p = 0.006$) (Fig. 2e), with 70.8% oxazepam-exposed fish entering black background compared to 83.2% of control fish (Fig. 4a,b,c). Note that we are reporting the percentage of fish that moved onto a background in the first 5 min, rather than across the whole trial, because the drop in all survival curves is quite steep (Figs. 3a,b,c and 4a,b,c), implying that most of the difference in behaviour develops within the first 5 min of the start of a trial.

4. Discussion

Here, using a multi-stressor approach, we report that exposure to low levels of oxazepam affects the initial movements of perch. Specifically, although we found no significant interaction effects of oxazepam and temperature on the behaviours studied, we found that fish in the low temperature treatments (oxazepam, predation) froze for significantly longer than fish in the high temperature treatment. In addition, we found oxazepam-induced effects on boldness and a significant effect of predation cue on perch entering the white compartment. Finally, we provide evidence supporting earlier studies showing that oxazepam exposure can significantly bioconcentrate in perch muscle tissue (Brodin et al., 2013; Heynen et al., 2016b).

Fig. 3. Survival analysis presenting the time to first cross into the white background. (A) Temperature low = fish exposed to low temperature (10 °C); Temperature high = fish exposed to high temperature (18 °C); (B) Control = unexposed control fish; Oxazepam = fish exposed to oxazepam (6.5 μg/L); (C) No predation = no predation cue; Predation = predation cue.

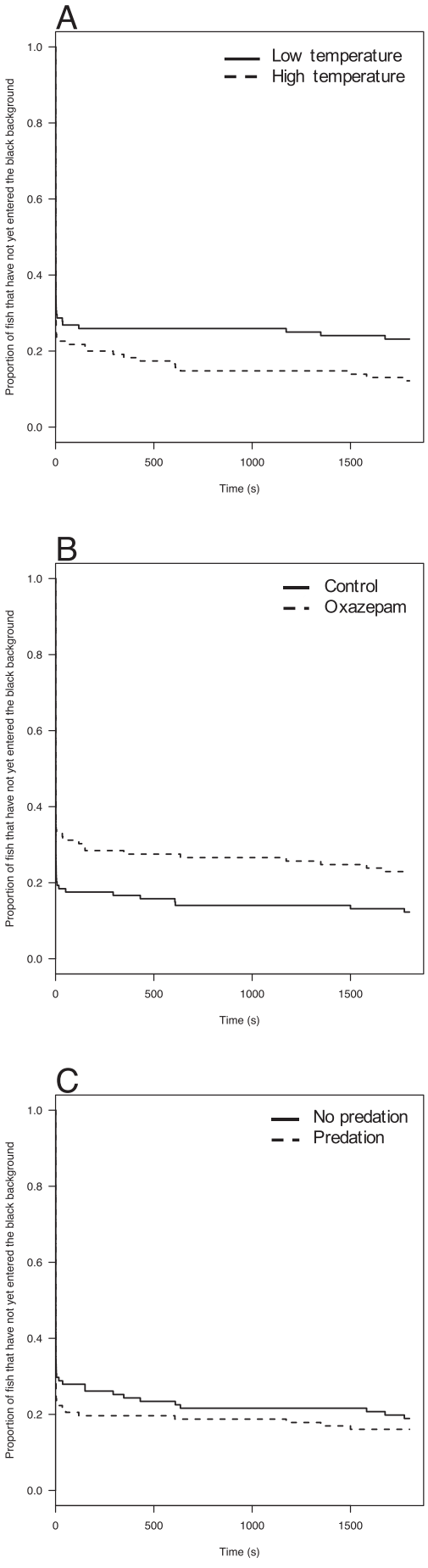


Fig. 4. Survival analysis showing the time taken to first cross into the black background. (A) Temperature low = fish exposed to low temperature (10 °C); Temperature high = fish exposed to high temperature (18 °C); (B) Control = unexposed control fish; Oxazepam = fish exposed to oxazepam (6.5 μg/L); (C) No predation = no predation cue; Predation = predation cue.

4.1. Behavioural effects induced by oxazepam and temperature

In line with our first hypothesis, we found that the initial movements of fish were significantly affected by oxazepam treatment. Specifically, within the first 5 min, 60–93% of the oxazepam-treated fish entered the white background, compared to 59–79% control fish. The white background represents a riskier environment (Maximino et al., 2010) and thus demonstrates boldness, because fish are entering into an environment perceived as more dangerous (Brodin et al., 2017). Previous studies have shown that fish exposed to oxazepam become bolder (roach, *Rutilus rutilus*: Brodin et al., 2017), more active (European perch: Brodin et al., 2013; Klaminder et al., 2014; Chiffre et al., 2016) and migrate faster (Atlantic salmon, *Salmo salar*: Hellström et al., 2016). Our study provides further evidence that oxazepam increases boldness of fish, because a higher proportion of perch exposed to oxazepam entered the white 'more vulnerable' environment compared to controls, and took longer to enter the black 'protected' environment. This enhanced boldness, however, usually comes with a cost. Bolder individuals take more risks (Smith and Blumstein, 2008; Sih and Del Giudice, 2012) and are therefore more exposed to, and more likely to be targeted by, predators (Hulthén et al., 2017). Indeed, recent studies found that migrating salmon smolt exposed to oxazepam migrated faster (Hellström et al., 2016) but were also predated upon to a greater extent (Klaminder et al., in prep). Even though some studies have found that bolder individuals make decisions faster without compromising accuracy (Mamuneas et al., 2015), others have reported high activity to be tempered by poor accuracy (Raoult et al., 2017). Therefore, increased boldness caused by anxiolytic exposure might not lead to better decision-making, as was shown by Klaminder et al. (in prep), where bolder Atlantic salmon smolt seemed unable to respond plastically to the anxiolytic-induced increase in boldness and, hence, suffered increased predation.

Although it is clear that oxazepam is readily taken up by fish, as indicated by higher concentrations in the fish muscle than in the water, the results of the present study highlight that exposed fish are likely to be metabolically compromised, not only via a modified behaviour, but also via detoxification processes driving the depuration of the drug. This increased energy expenditure could, in turn, hamper the ability of individuals to respond adaptively to additional stressors (Vasseur et al., 2014; Barbosa et al., 2017). Future studies should explore this possibility further by examining if the metabolic rate of fish is also affected by multiple stressors.

At the lower temperature, fish spent more time in their first freezing event and, overall, froze for longer, regardless of the predation or oxazepam treatment—hence, supporting our second hypothesis. Staying motionless after being introduced to an unfamiliar environment is a well-established measure of anxiety in both rodents and fish (reviewed in Stewart et al., 2012). Activity, on the other hand, can be temperature-dependent (Fukuhara, 1990; Farrell, 1997; Pörtner, 2002; Biro et al., 2010) and colder temperatures are known to reduce metabolism, heart rate, respiration, and digestion (Farrell, 1997; Pörtner, 2002; Jensen et al., 2017). This could explain why fish in the cooler temperature reduced their activity and stayed motionless for longer. Indeed, a recent study reported that the winter-dormant fish cunner (*Tautogolabrus adspersus*) reduced activity under the cold treatment (0.6 °C), which translated into a reduced metabolic rate (Speers-Roesch et al., 2018). Thermal tolerance, however, is species-specific, and species living at the lowest and/or highest range of their temperature tolerance are more vulnerable and have limited adaptive capacity in a changing climate (Sandblom et al., 2016). In the current study, we

can rule out anxiety and/or stress caused by temperature because the chosen low temperature (+10 °C) treatment is not at the lowest thermal range for the European perch (~5–27 °C, Fiogbé and Kestemont, 2003; Jensen et al., 2017). Also, we used an extended acclimation period (3 weeks) to minimise stress caused by altered thermal environment. Overall, increased time staying motionless is likely to have fitness-related consequences because it translates to time not spent feeding and finding mates, thus underscoring the importance of determining the impacts of multiple stressors on behavioural endpoints.

We found that water temperature also affected the initial movements of fish when entering the white/black background, which gives further support to our second hypothesis. Specifically, in the high temperature treatment, 79% of fish entered the white background within the first 5 min, while only 64% did so in the low temperature. A recent study demonstrated that, in coral reef fish, individual boldness increases 2.5-fold as a function of temperature (Biro et al., 2010). The authors of that study observed dramatically increased levels of activity, boldness and even aggressiveness after only a few degrees increase in water temperature. Similarly, exposure to elevated temperature of 4 °C for ten days increased activity level of Siamese fighting fish (*Betta splendens*, Forsatkar et al., 2016). In the current study, low and high temperature treatments had a difference of 8 °C, which, according to Biro et al. (2010), would be very likely to demonstrate an increase in behaviours, such as boldness. Given that individuals will vary in their contextual plasticity—some will show the same levels of activity at all temperatures (Biro et al., 2010, 2013; Wong and Candolin, 2015; Killen et al., 2016)—and thermal tolerances vary from species to species (Pörtner, 2002), further studies are needed to confirm that increased temperature translates to boldness in European perch. However, it is important to point out that individual variation in responsiveness to temperature is likely to represent an adaptation to variable temperatures, and thus be subject to selection (Chevin et al., 2010; Reed et al., 2010; Biro et al., 2013). Future studies should, therefore, strive to incorporate variation in responsiveness to temperature when investigating impact of chemical contaminants.

4.2. Importance of predator cues

Our study revealed a significant effect of predation cue on perch entering the white compartment. Specifically, we found that up to 93% of the perch exposed to the predation cue treatment entered the white compartment within the first 5 min, compared to, an average of 67% of control fish, suggesting an effect on boldness. Indeed, the prolonged exposure to predators may have habituated or primed fish to cope better with risky situations (Brown and Braithwaite, 2004; Brown et al., 2007). As animals need to trade-off between predator avoidance, foraging, and reproduction to maximise their fitness (Sih, 1980; McNamara and Houston, 1987; Verdolin, 2006), by choosing a more vulnerable white background, perch chose the riskier environment. On one hand, this bolder behaviour could be explained by habituation (i.e., a loss of response in the absence of an actual predation threat; Ferrari et al., 2010; Imre et al., 2016). On the other hand, bolder behaviour could be due to variation in prey personality (Sih et al., 2004; Wolf and Weissing, 2012; Belgrad and Griffen, 2016). Indeed, a recent study showed that mortality of prey was dependent on its personality, with blue crab (*Callineuctes sapidus)* consuming primarily bold mud crabs (*Panopeus herbstii*), while toadfish (*Opsanus tau*) primarily predated upon shy mud crabs (Belgrad and Griffen, 2016). The predator used in the current study was the northern pike, which is an ambush predator (Savino and Stein, 1989). As bolder perch are more likely to be occupying front positions (Bumann and Krause, 1993; Ward et al., 2004) and keeping a greater distance to the school (Budaev, 1997; Wilson et al., 1993), it has been suggested that pike would be more selective, as well as, successful with bolder perch. A recent study, however, found that pike selected prey based on the prey's morphological traits (i.e., shallow bodied) rather than on their behavioural characteristics, such as boldness (Heynen et al., 2017). Interestingly, it has been surprisingly difficult to link the increased boldness induced by oxazepam to increased predation of perch by Northern pike (*Esox lucius*) in pond (Lagesson et al., 2018) and whole lake experiments (Klaminder et al., 2016). Lagesson et al. (2018) hypothesised that this mismatch between laboratory prediction and field verifications were most likely due to increasing abiotic factors (e.g. temperature). Our study is the first to support this idea as our assays suggest that temperature has a profound impact on behaviour, which is independent from exposure to oxazepam. Overall, while it is clear that oxazepam exposure is affecting prey behaviour, to determine whether or not oxazepam is also impacting their survival, further studies should explore the direct (e.g. predators less adept at capturing prey) and indirect effects (e.g. prey-switching due to changes in predatory behaviour) of chemical contaminants on predator-prey interactions (reviewed in Saaristo et al., 2018).

5. Conclusions

Here, we report that short-term exposure to oxazepam affects behaviour of juvenile perch. We found the initial movements of fish to be significantly affected by treatment, and fish became bolder (i.e. entered the white background) in the oxazepam, high temperature, and predation treatments. Importantly, we found no interaction effects of oxazepam and temperature on the studied behaviours, which suggests that temperature has a profound impact on behaviour that is independent from exposure to oxazepam. As wildlife are facing an increasing range of biotic and abiotic pressures, it is vital to investigate how organisms adapt and persist under chemically-induced environmental change. More generally, our study highlights the need to focus on multiple stressors to improve understanding of the risks and hazards posed by chemical contaminants.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.11.228.

Ethics

This research was approved by the Ethical Committee on Animal Experiments in Umeå (dnr: A18-15) and complied with Swedish law.

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Appendix I

Behavioral syndromes vary among geographically distinct populations in a reptile

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Original Article

Behavioral syndromes vary among geographically distinct populations in a reptile

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A key goal in the study of animal personalities is to determine their adaptive potential and importance for behavioral evolution. Behavioral syndromes are evolutionarily intriguing because they suggest that an adaptive change in one behavior requires a concomitant shift in another. Within species, behavioral syndromes might be evolutionarily constrained by intrinsic mechanisms that restrict behaviors from evolving independently. Alternatively, behavioral correlations might easily be decoupled over short evolutionary time scales due to variation in selective pressures between environments. In this regard, comparative studies that explore differences in diverse aspects of personality between geographically distinct populations can provide valuable insights into the evolutionary processes acting on different behavioral tendencies. Accordingly, we investigated how behavioral types and behavioral syndromes differed across four geographically distinct populations of the delicate skink, *Lampropholis delicata*. We found strong evidence of mean trait-level variation in activity, exploration, and boldness across populations, suggesting adaptation to local environmental conditions. Similarly, we found that within-population correlations involving boldness varied substantially between populations. However, we did find a consistent within- and among-population correlation between activity and exploration, suggesting that this behavioral syndrome is relatively stable and could explain behavioral divergence in activity and exploration between populations. We suggest that there may be thermal physiological mechanisms that could be limiting the adaptive potential of an activity-exploration correlation in the delicate skink. Broadly, we argue that some behavioral correlations may be more adaptive than others, and that this should be more regularly considered within the animal personality framework.

Key words: **correlated evolution, geographic variation, lizard, local adaptation, repeatability.**

INTRODUCTION

It has been well documented across a range of taxonomic groups that individuals within populations often show consistent differences in a range of behaviors [i.e., behavioral types: Sih et al. (2004) and Reale et al. (2010)] and that these consistent behaviors often correlate with functionally unrelated behaviors across time and context [i.e., behavioral syndromes: Sih et al. (2004) and Reale et al. (2010)]. Together, these phenomena are commonly referred to as "animal personalities" (Roche et al. 2016). A key goal in the study of animal personalities is to determine their adaptive potential and importance for behavioral evolution. At an individual-level, personalities are evolutionarily intriguing because they place a limit on behavioral plasticity and thus mediate an individual's response to a given ecological situation with consequences for individual fitness (Sih et al. 2004). At a population- or species-level, animal personalities can affect higher-order ecological and evolutionary processes, such as social networks and movement ecology (Spiegel et al. 2017), dispersal events (Cote et al. 2010), and biological invasions (Duckworth and Badyaev 2007). However, to understand the evolutionary potential of animal personalities, we must first determine to what extent multifarious personality traits are consistent across space and time.

Population-level characteristics are often shaped by their local biotic and abiotic conditions, but these environmental factors are rarely homogeneous between populations (Foster 1999). Indeed, there is some evidence that animal personalities are strongly influenced by both short- and long-term environmental effects, and that behavioral correlations are quite unstable and can be easily formed and broken down over relatively short evolutionary time scales [i.e., the "adaptive hypothesis": Wilson (1998), Bell (2005), Bell and Sih (2007), and Dingemanse et al. (2007)]. For example, 3-spined sticklebacks, *Gasterosteus aculeatus*, bred under high predation risk have a bold-aggressive behavioral syndrome, whereas those bred in low predation risk lose this syndrome Bell and Sih 2007. In contrast, if two or more behaviors share a proximal association (e.g., physiological, hormonal, or genetic), then we might predict that behavioral

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correlates would remain rigid against strong environmental selection and thus be a consistent population- or species-level trait. Such behavioral syndromes would be evolutionarily constrained, as an intrinsic change in one behavioral trait requires an intrinsic change to another, and thus both behavioral traits cannot evolve independently [i.e., the "constraint hypothesis": Pruitt et al. (2010) and Dochtermann and Dingemanse (2013)]. In these circumstances, a shared behavioral syndrome can also hold explanatory power for personality variation between populations. For instance, Pruitt et al. (2010) found a consistent negative correlation between sociability and boldness both within and among 18 different populations of a spider, *Anelosimus studiosus*, separated by as much as 36° latitude, suggesting a lack of evolutionary independence and support for the constraint hypothesis. Based on this shared within- and among-population behavioral syndrome, the authors could predict that if population "A" is more social than population "B," then population "A" must also be less bold than population "B" (i.e., because a positive shift in sociability corresponds to a negative shift in boldness).

Although comparisons of geographically distinct populations cannot unequivocally determine the evolutionary causations of personality without appropriate genetic- or environment-dependent data, such contrasts are still a vital first step towards identifying the adaptive or nonadaptive significance of animal personalities (Foster 1999, Herczeg et al. 2009). Most studies investigating animal personalities either 1) focus on multiple traits within 1 population or 2) focus on only 1 or 2 traits between multiple populations. Although such studies have provided tremendous insights into animal personality, neither approach addresses how adaptive or constrained different suites of behaviors might be amongst multiple populations. This is an important consideration because diverse aspects of animal personality may be less or more adaptive than others, and this can provide valuable insights into the evolutionary potential of different behavioral tendencies.

In the present study, we sought to compare personality traits among four populations of a widespread reptile, delicate skink, *Lampropholis delicata*. The delicate skink is a small, diurnal, group-living lizard species (adult snout-vent length [SVL] 34–55 mm) that is native, and abundant throughout south-eastern Australia. This species offers an ideal study system to examine geographical variation in animal personality. First, we know the phylogenetic history of the species (Chapple et al. 2011), providing pivotal information for the interpretation of comparative behavioral research on geographically distinct populations (Blomberg and Garland 2002). Second, we have previously found a behavioral syndrome between activity, exploration tendency, and sociability within the species (Michelangeli et al. 2016a), which is consistent between the sexes (Michelangeli et al. 2016b). Third, we recently demonstrated a robust link between thermal physiology and personality traits in the delicate skink. Briefly, "hot" thermal-type lizards perform optimally at higher body temperatures, have faster sprint speeds, are more active, explorative, social, and bold relative to "cold" thermal type lizards, which have the opposite set of characteristics (Goulet et al. 2017a, 2017b; Goulet et al. 2018; Michelangeli et al. 2018). In these studies, we suggest that differences in thermal physiological requirements could provide an intrinsic mechanism that maintains stable behavioral syndromes across geographically distinct populations, particularly in ectothermic organisms that rely on behavioral thermoregulation for ecological performance. This is because an individual's specific thermal physiological demands (i.e., "hot" or "cold") likely constrain them along a predictable behavioral continuum due to behavior's dependence on inherently stable biomechanical processes that are regulated by body temperature. If consistent differences in thermal physiology maintain consistent differences in behavior as predicted, we would expect to find that within-population behavioral syndromes are similar across populations of the delicate skink.

Accordingly, the aims of our study were to 1) test for potential mean trait-level differences in multiple behavioral types between populations of the delicate skink, 2) identify possible behavioral syndromes and determine whether they are consistent between populations, and 3) determine whether population differences in mean trait-level behavior are predicted by their within-population behavioral syndromes (sensu Pruitt et al. 2010).

METHODS

Ethical note

Research was conducted in accordance with appropriate collection and research permits (Queensland: WISP16338615, New South Wales: SL101600, Victoria: 1006866) and was approved by the Monash University Animal Welfare Committee (BSCI/2014/26).

Study sites

Delicate skinks were collected from 4 populations across eastern Australia: 29 lizards from Sydney (Sydney Park, 33°54S, 151°11E), 31 lizards from Coffs Harbour (Boambee Bay park, 30°21S, 153°05E), 30 lizards from Tenterfield (Bald Rock National Park, 28°51S, 152°03E), and 27 lizards from Brisbane (Ithaca Creek Parklands, 27°27S, 152°58E). We selected these sites because they are phylogeographically distinct (Chapple et al. 2011).

Animal collection and husbandry

Lizards were collected from all locations in November 2015, just after the species breeding season. Only adult (SVL > 34 mm), full-tailed (tail length > SVL) male lizards were retained in order to avoid the potential confounding influence of tail loss (Cromie and Chapple 2012) and gravidity (Shine 2003) on behavior. We used hand capture and mealworm fishing capture techniques as both methods have previously been shown not to retain any sampling bias towards particular behavioral types in delicate skinks (Michelangeli et al. 2016a).

Lizards were transported back to Monash University for behavioral experiments and, on arrival, individuals were given a minimally invasive unique permanent identification code using different color combinations of Visual Implant Elastomer (VIE, Northwest Marine Technology, WA). Focal lizards were housed in groups of 5 individuals within large plastic containers (300 × 230 × 370 mm). A basking area, consisting of a heat lamp over 2 terracotta tiles, was provided at one end of each housing container. This created a thermal gradient in the housing container (22−32 °C) allowing thermoregulation from 08:00 to 17:00 h. Small plastic pots were added to provide shelter. UV lighting was placed above the containers and was activated from 08:00 to 18:00 h. All housing containers were located in a temperature-controlled room with an ambient temperature of approximately 22–23 °C and room lighting from 07:00 to 21:00 h daily. Skinks were fed a diet of crickets, *Acheta domesticus*, dusted in a vitamin supplement (Reptivite™), 3 times per week, and water was made available ad libitum.

Behavioral experiments

We conducted a series of behavioral assays to examine behavioral variation and correlation within and among populations. Lizards

had been held in captivity for 2 weeks before behavioral assays commenced. Individuals were tested in each behavioral assay twice in order to assess behavioral repeatability (or consistency). Each retest was done 1 week apart to examine short-term repeatability and reduce any effects arising from potential developmental changes within individuals (Bell et al. 2009). An individual was therefore only ever exposed to 1 behavioral assay per week, for a total of 8 weeks (i.e., 4 behavioral assays, 2 trials per assay, and 1 trial per week). Assays were carried out in a fixed order (in the same order detailed below) where assays that could have the greatest influence on behavior were carried out last to reduce potential carry-over effects (Bell 2012). All behavioral assays were conducted between 09:00 and 15:00, in opaque-walled experimental arenas (550 × 320 × 240 mm) within temperature-controlled rooms which matched the lizard housing temperature (22−23 °C). The setup of the experimental arena was modified to accommodate the assay being conducted. Skinks were allowed to acclimate under transparent containers for 10 min prior to the start of each trial. All trials were recorded using JVC Everio GZ-E100 video cameras. Equipment was thoroughly washed between trials with hot water and scentless dishwashing detergent to prevent scent contamination. Since *Lampropholis* skinks are known to modify their behaviors after large meals (Shine 2003), we ensured that lizards were not fed in the 24 h prior to each behavioral trial.

Nondirected activity test

To measure activity levels, skinks were allowed to move freely in an experimental arena marked with 20 equal grid squares over a 20-min period. Activity was scored based on the number of transitions between grid squares made by the skink, and the mean time taken to transition across grid squares after the skink's initial transition. We took the latter measurement to control for those lizards that took a long time to initially move but were very active after their initial movement (sensu Chapple et al. 2011; Michelangeli et al. 2016a, 2016b; Michelangeli et al. 2017; Michelangeli et al. 2018).

Obstacle test

To measure an individual's propensity to explore a novel environment, skinks were placed into a test arena containing an obstacle in the form of a trapezium-shaped barrier, which divided the test arena into 2 compartments. Lizards commenced the trial in compartment 1 and could only reach compartment 2 by finding, and squeezing through, small gaps at either end of the barrier. This assay aimed to measure an individual's willingness to 1) approach a novel obstacle, 2) examine/explore the obstacle, and 3) cross the obstacle to explore an unknown environment. Over a 20-min trial, we recorded the time spent by lizards inspecting the barrier itself, the time lizards spent stationary, and whether the lizard reached compartment 2 over 20 min (sensu Chapple et al. 2011; Michelangeli et al. 2016a, 2016b, Chung et al. 2017).

Sociability test

We conducted a dichotomous choice experiment to measure the social behavior of skinks. Delicate skinks are often observed either basking in small groups (~2−10 individuals) or alone in the wild (our unpublished data). Thus, we offered individual lizards a choice between basking with a group of conspecifics and basking alone (sensu Michelangeli et al. 2016a, 2016b; Michelangeli et al. 2017). This was achieved by splitting the test arena into 3 zones: a social zone, an asocial zone, and an intermediate neutral zone. Both the social and asocial zones were comprised of a basking site that was divided in half by a transparent Perspex™ barrier that spanned the length of the test arena. In the social zone, 3 stimulus lizards were placed behind the partition, whereas the asocial zone was left bare. Focal lizards could see, but not physically interact with the stimulus lizards. Stimulus lizards comprised individuals that were from the same population but were unfamiliar to the focal lizards (i.e., they were not housed together) and were caught during the collecting trip in November 2015. Stimulus lizards were not used for any other behavioral assay and no focal lizards were used as stimuli. Over a 20-min trial, we recorded the total amount of time spent by lizards basking in the social zone, as well as the mean amount of time a lizard spent within the social zone before transitioning into another zone.

Predator-response test

An individual's boldness is typically measured as their risk-taking response after a threatening situation. In reptiles, basking is considered to be a risky behavior as it exposes individuals to potential predators (Downes and Hoefer 2004). We therefore recorded a lizard's reemergence time from a shelter site and their subsequent basking behavior after a simulated predatory attack, as measures of risk-taking. To achieve this, skinks were placed at the center of a test arena with a basking site on one end, and a shelter site on the other. The basking site was positioned under a 40-W heating lamp so that the temperature of the basking site (~35 °C) was substantially higher than the ambient temperature (~22−23 °C). After the acclimation period, an observer would simulate a predatory attack by prodding the lizard close to its tail with a rod until the lizard entered the shelter site (sensu Rodríguez-Prieto et al. 2011). We then allowed the lizard 30 min to reemerge from the shelter site, recording its time of reemergence. After reemergence, we then recorded the total time spent by the lizard basking over an additional 5-min period. Because we gave all lizards an additional 5 min to bask after reemergence from the shelter site, we treated both behavioral measures as independent behavioral traits.

Statistical analyses

Data were analyzed using R version 3.3.2 (R Core Development Team 2016). Data were checked for normality [Shapiro–Wilk test: Royston (1995)] and homogeneity of variance [Fligner–Killeen test: Conover et al. (1981)] where appropriate. Several variables required data transformations prior to analysis to approximate Gaussian error distributions (see Table 1 for specific transformations).

For each behavioral assay, we used principal component analysis (PCA) followed by varimax rotation to reduce related behavioral variables into single standardized personality scores (Table 1). All PCAs were implemented using a correlation matrix that standardized all variables (Tabachnick and Fidell 2013). Bartlett tests were significant, indicating that correlation matrices were significantly different from identity matrices. Principal components were retained based on the Kaiser–Guttman criterion (eigenvalue > 1; Jackson 1993). Because these PCAs combined data for both trial 1 and trial 2 (i.e., they did not consider repeated measures) and each population (i.e., they did not consider variation within populations), we also ran PCAs on the data for each trial and population separately. These separate trial and population PCAs produced very similar results to the combined data PCAs (Supplementary Tables S1 and S2). Due to the minimal difference, we used the 4 personality scores resulting from the combined data PCAs for the rest of the analysis (Table 1).

Table 1

Principal component scores on the behavioral variables for each behavioral assay

Behavioral assay	Principal component
1. Non-directed activity	PC1 (Activity score)
Behavior (transformation)	
Number of grid transitions	0.92
Mean transition time (log)	−0.92
Eigenvalue	1.69
%Variance explained	0.85
2. Obstacle Test	PC1 (Exploration score)
Behavior (transformation)	
Time spent inspecting barrier (sqrt)	0.89
Number of barrier passes	0.72
Time spent stationary	−0.90
Eigenvalue	2.11
% Variance explained	0.70
3. Sociability Test	PC1 (Sociability score)
Behavior (transformation)	
Time spent in social zone	0.91
Mean time in social zone (sqrt)	0.91
Eigenvalue	1.66
% Variance explained	0.83
4. Predator–response test	PC1 (Boldness score)
Behavior (transformation)	
Time to taken to reemerge (rank)	−0.80
Time spent basking (rank)	0.82
Eigenvalue	1.27
% Variance explained	0.63

Eigenvalue and explained variances are also provided for each component.

Behavioral repeatability

We used linear mixed-effects models (LMM, *lme4* package: Bates et al. 2015) with Gaussian error distributions to assess behavioral repeatability. Behavioral repeatability is calculated as the ratio of between-individual variance (BIV) to total phenotypic variance (BIV + within-individual variance [WIV]). Variance components were extracted from univariate mixed-effects models using restricted maximum likelihood with individual ID as a random factor. We ran separate models for each population and a model containing data from all populations. The model containing data from all populations included population as a fixed factor to consider variation between populations, and thus represents an adjusted repeatability estimate (Nakagawa and Schielzeth 2010). Confidence intervals were calculated by parametric bootstrapping using the package *rptR* (Stoffel et al. 2017).

Mean-trait level population differences

We compared LMM models with and without the fixed effects of population, trial number, population x trial number interaction, and SVL, to examine mean-trait population differences. Individual ID was assigned as a random factor in all models. *P* values were obtained from likelihood ratio tests (Bolker et al. 2009). After model selection, we used post hoc tests to determine to what extent populations differed from one another. Holm–Bonferroni correction method was applied to all *P* values resulting from multiple comparison post hoc tests.

Behavioral syndromes

To estimate within- and among-population correlations, we first calculated Pearson-product moment correlation coefficients between each of the personality scores. Holm–Bonferroni corrections were applied to account for multiple testing. We then used LMM models to assess syndrome similarity between populations. Within these models, we used one personality score as the response variable, and the other personality score as the covariate. Population x covariate interaction and trial x covariate interaction were fixed factors. Skink ID was incorporated as a random factor to consider the repeated measures. A significant interaction suggests that the magnitude and direction of the correlation differ between the levels of the main effect (i.e., population or trial). On the other hand, no interaction, but a significant covariate, suggests that the behavioral correlation is similar in magnitude and direction across population and trial. Sociability score was omitted from this part of the analysis due to its generally low effect size (Table 2).

Table 2

Effect size for each behavioral correlation estimated using Pearson product-moment correlation coefficients (*r*) within each population

	Exploration	Sociability	Boldness
Activity			
Sydney	0.24*	0.09	**−0.34**
Coffs Harbour	**0.42**	−0.01	0.29*
Tenterfield	0.33*	−0.04	−0.09
Brisbane	0.32*	0.08	0.08
Total	**0.45**	0.03	−0.07
Exploration			
Sydney	—	−0.01	−0.15
Coffs Harbour	—	0.16	0.25*
Tenterfield	—	−0.06	**−0.37**
Brisbane	—	−0.16	0.03
Total	—	−0.03	−0.13
Sociability			
Sydney	—	—	−0.15
Coffs Harbour	—	—	0.02
Tenterfield	—	—	−0.01
Brisbane	—	—	−0.01
Total	—	—	−0.01

Total refers to data for all populations combined.
Bold font indicates significant effect size after Bonferroni correction ($P < 0.008$).
*Indicates significant effect size without Bonferroni correction ($P < 0.05$).
Also see Table 5.

RESULTS

Behavioral repeatability

All behaviors were repeatable (i.e., confidence intervals do not overlap zero) when data from all populations were pooled (i.e., species-level repeatability). When considering populations individually, repeatability estimates varied between populations and different behaviors (Table 3). However, most repeatability estimates would be considered relatively high ($r > 0.37$; see meta-analysis by Bell et al. 2009), although the uncertainty (i.e., confidence intervals) around these estimates is also quite large. Sociability, in general, was not repeatable within populations (Table 3).

Mean-trait level population differences

We found evidence of mean behavioral-type differences amongst populations (Table 4). Firstly, the Sydney population was significantly more active in the nondirected activity assay than the other 3 populations (Sydney—Coffs Harbour: $z = 2.75$, $P = 0.023$; Sydney—Tenterfield: $z = 5.31$, $P < 0.001$; Sydney—Brisbane:

Table 3

Repeatability (*r*) with associated confidence intervals (CIs) and variance components (between-individual variance: BIV, and within-individual variance: WIV) of behavioral traits measured in male delicate skinks (*L. delicata*) from four populations in Eastern Australia

	Sydney			Coffs Harbour			Tenterfield			Brisbane			Total		
Behavior	BIV	WIV	*r* (95% CIs)	BIV	WIV	*r* (95% CIs)	BIV	WIV	*r* (95% CIs)	BIV	WIV	*r* (95% CIs)	BIV	WIV	*r* (95% CIs)
Activity	0.33	0.33	**0.50 (0.19, 0.72)**	0.73	0.25	**0.75 (0.55, 0.87)**	0.35	0.39	**0.48 (0.14, 0.72)**	0.64	0.22	**0.75 (0.51, 0.87)**	0.52	0.30	**0.63 (0.52, 0.74)**
Exploration	0.29	0.63	0.32 (0, 0.60)	0.42	0.36	**0.54 (0.23, 0.74)**	0.27	0.32	**0.46 (0.15, 0.70)**	0.65	0.29	**0.69 (0.42, 0.85)**	0.41	0.40	**0.50 (0.38, 0.63)**
Sociability	0.21	0.45	0.32 (0, 0.62)	0.13	0.51	0.20 (0, 0.54)	0.46	0.81	0.36 (0, 0.63)	0.22	0.97	0.18 (0, 0.52)	0.26	0.75	**0.25 (0.09, 0.42)**
Boldness	0.27	0.47	**0.37 (0.02, 0.63)**	0.58	0.45	**0.57 (0.27, 0.76)**	0.40	0.50	**0.45 (0.10, 0.68)**	0.61	0.48	**0.56 (0.20, 0.78)**	0.44	0.51	**0.46 (0.40, 0.65)**

Total refers to data for all populations pooled and represents an adjusted repeatability estimate controlling for between-population variance. Bold scores refer to significant repeatability estimates.

Table 4

Results from LMM model selection comparing behavioral traits

Behaviour	ΔAIC	χ^2	*P* value
Activity	–	–	–
Population x trial number	+3.63	2.371	0.499
Population	**-25.29**	**31.291**	**<0.001**
Trial number	+2.00	0.001	0.972
SVL	+1.72	0.275	0.599
Exploration	–	–	–
Population x trial number	+2.7	3.302	0.347
Population	**-27.95**	**33.95**	**<0.001**
Trial number	**-5.43**	**7.423**	**0.006**
SVL	+1.96	0.044	0.834
Sociability	–	–	–
Population x trial number	+3.95	2.059	0.562
Population	+2.56	3.440	0.329
Trial number	+1.99	0.011	0.915
SVL	+1.49	0.506	0.477
Boldness	–	–	–
Population x trial number	+5.75	0.246	0.970
Population	**-6.80**	**12.750**	**0.005**
Trial number	**-12.42**	**14.365**	**<0.001**
SVL	+1.94	0.056	0.813

Models containing the fixed effects (population, trial number, population x trial number interaction, and SVL) are compared with a model without the fixed effects. All models contained individual ID as a random factor. ΔAIC values refer to differences in AIC when specific fixed factors were included vs. excluded from the initial model.
SVL = snout-vent length; AIC = Aikake information criterion; χ^2 = chi-square value. Bold refers to significant terms at $P < 0.05$.

$z = 4.92$, $P < 0.001$). Lizards from Coffs Harbour were more active than lizards from Tenterfield ($z = 2.62$, $P = 0.026$) and Brisbane ($z = 2.29$, $P = 0.044$). Tenterfield and Brisbane lizards did not differ in their level of activity ($z = -0.25$, $P = 0.800$).

Sydney lizards were also more explorative during the obstacle test (i.e., were more likely to explore and pass the barrier) than the other 3 populations (Sydney—Coffs Harbour: $z = 3.54$, $P = 0.002$; Sydney—Tenterfield: $z = 6.22$, $P < 0.001$; Sydney—Brisbane: $z = 3.79$, $P < 0.001$). Brisbane and Coffs Harbour lizards were also significantly more explorative than Tenterfield lizards (Brisbane—Tenterfield: $z = 2.29$, $P = 0.044$; Coffs Harbour—Tenterfield: $z = 2.76$, $P = 0.018$). Coffs Harbour and Brisbane lizards did not differ in their exploration score ($z = 0.38$, $P = 0.707$).

Finally, lizards from the Sydney population were less bold than lizards from Coffs Harbour ($z = -3.50$, $P = 0.003$) and Tenterfield ($z = -2.73$, $P = 0.032$), but not Brisbane lizards ($z = -2.16$, $P = 0.123$). There was no difference in boldness scores amongst the other populations (Coffs Harbour—Tenterfield: $z = 0.76$, $P = 0.898$; Tenterfield—Brisbane: $z = 0.50$, $P = 0.898$; Coffs Harbour—Brisbane: $z = 1.24$, $P = 0.641$).

We also found that lizards from all populations became more explorative and bolder between trial 1 and trial 2 (Table 4), suggesting potential habituation to the experimental procedure of both the obstacle test and predator-response test, respectively. We found no population differences in sociability (Table 4).

Behavioral syndromes

Overall, we found variation in behavioral syndromes between populations. First, we found a similar activity and exploration correlation within each population (Table 2). Exploration was a significant

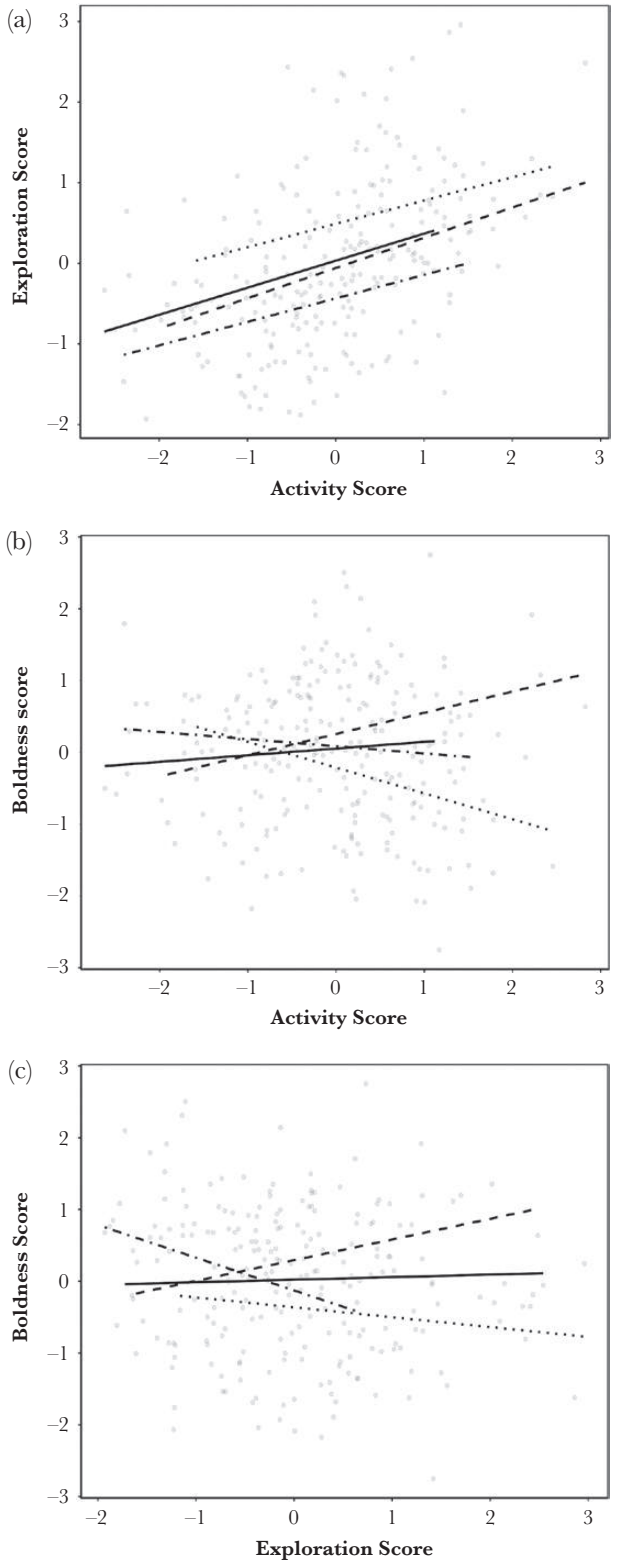

Figure 1
Within-population regression lines for relationships between (a) activity and exploration, (b) activity and boldness, and (c) exploration and boldness. Sydney = dotted line, Coffs Harbour = dashed line, Tenterfield = dotted–dashed line, and Brisbane = solid line.

covariate in the mixed-effects model containing activity as a response variable. Importantly, this within-population activity-exploration syndrome was consistent in magnitude and direction across populations

Table 5
Linear mixed effects models testing the behavioral relationships between traits of interest (i.e., traits that were found to have a significant effect size; Table 2) across populations and trial

Model	Term	df	χ^2	*P* value
Y: Activity ~ X: Exploration	Population	**3**	**19.956**	**<0.001**
	Exploration	**1**	**18.062**	**<0.001**
	Trial	1	0.727	0.494
	Population x exploration	3	1.794	0.616
	Trial x exploration	1	0.070	0.791
Y: Activity ~ X: Boldness	Population	**3**	**30.673**	**<0.001**
	Boldness	1	0.001	0.992
	Trial	1	0.001	0.971
	Population x boldness	**3**	**9.535**	**0.023**
	Trial x boldness	1	0.121	0.727
Y: Exploration ~ X: Boldness	Population	**3**	**31.896**	**<0.001**
	Boldness	1	0.324	0.569
	Trial	**1**	**6.355**	**0.011**
	Population x boldness	**3**	**8.851**	**0.031**
	Trial x boldness	1	0.030	0.863

Significant interaction terms suggest differences in the magnitude and direction of behavioral relationships (see Figure 1).
Bold refers to significant terms in the full model.

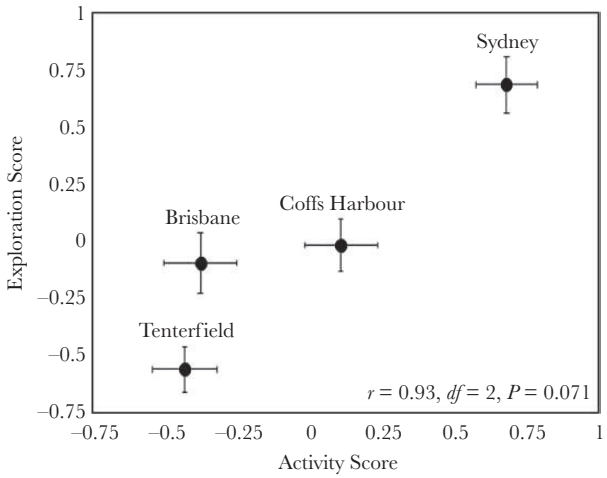

Figure 2
A positive correlation in mean-trait values for activity and exploration scores among 4 populations of the delicate skink.

(Figure 1a), as we found no interaction between the covariate and population in the mixed-effects model (Table 5). There is also suggestion of an among-population correlation in mean level activity and exploration, but this effect is marginally nonsignificant ($r = 0.93$, df = 2, $P = 0.071$, Figure 2). The activity–exploration correlation did not differ between trials (Table 5, also Supplementary Figure S1).

Second, activity and boldness were correlated within the Sydney and Coffs Harbor populations, but not in the Tenterfield or Brisbane populations (Table 2). However, the direction of the activity–boldness correlation differed between Sydney and Coffs Harbour, being negative in Sydney but positive in Coffs Harbour. In line with this result, we also found a significant interaction between population and the covariate (Table 5). This result suggests that the magnitude and direction of the activity–boldness relationship differ between populations (Figure 1b). There is also no suggestion of an among-population correlation ($r = -0.69$, df = 2, $P = 0.312$).

Third, exploration and boldness were significantly correlated in the Tenterfield and Coffs Harbour populations, but not in the Sydney or Brisbane populations. Again, the direction of this exploration–boldness correlation differed between the 2 populations, being positive in Coffs Harbour, but negative in Tenterfield. Indeed, we also found a significant interaction between population and the covariate (Table 5), suggesting population differences in the direction and magnitude of the exploration–boldness relationship (Figure 1c). There is also no suggestion of an among population correlation ($r = -0.79$, df = 2, $P = 0.208$).

DISCUSSION

We found markedly diverse personality traits across four native-range populations of the delicate skink. Briefly, the Sydney population was comprised of consistently more active and explorative behavioral types compared with the Coffs Harbour, Tenterfield, and Brisbane populations. Sydney lizards were also consistently less bold than lizards from Tenterfield and Coffs Harbour. Tenterfield lizards were consistently less explorative than all other populations. Importantly, we also found geographical variation in the magnitude and direction of different behavioral syndromes within populations. First, we found a common activity–exploration correlation within each population, and that this correlation was also in the same direction at the among-population level. This suggests that this syndrome is relatively stable and can help explain population-level variation in behavior (sensu Pruitt et al. 2010). Second, we found that the direction and magnitude of an activity–boldness correlation and exploration–boldness correlation varied greatly between populations. Taken together, our results suggest that variation in average behavioral types may be a product of adaptation to local environmental conditions, but when considering behavioral syndromes, some behavioral correlations are more evolutionarily stable and likely constrained by intrinsic factors, whereas other correlations hold greater adaptive potential.

The Sydney population was behaviorally distinct and was comprised of mainly active, explorative, and shy lizards, relative to the other populations. Conversely, lizards from Tenterfield were noticeably less explorative than all other populations, and less active than both Sydney and Coffs Harbour lizards. It is well recognized that divergence between populations, whether through behavior, physiology, or morphology, is often a result of adaptation to local ecological conditions, such as predation pressure (Michelangeli and Wong 2014), resource availability (Snekser et al. 2008), and population density (Nicolaus et al. 2016). The behavioral differences observed between Tenterfield lizards and the other populations (particularly Sydney lizards) could be explained by the fact that Tenterfield lizards were sourced from a relatively pristine environment (i.e., a national park: Bald Rock NP), whereas the other 3 populations were sourced from urbanized environments. Urbanized environments are often dramatically modified and thus expose their inhabitants to multiple novel selective pressures (e.g., human disturbance, pollution, and novel predators) not frequently encountered by inhabitants of natural environments, often requiring urban-dwellers to make drastic behavioral changes in order to persist and survive (Lowry et al. 2013; Sol et al. 2013). Several studies have documented diverse personalities between urban and natural populations (Sol et al. 2011; Bokony et al. 2012; Lapiedra et al. 2017). For instance, it was hypothesized that lower predation risk in urban environments may allow urban common mynas, *Acridotheres tristis*, to be more explorative and thus more readily accept novel food resources that mynas from nonurban environments (Sol et al. 2011). However, although urbanization could explain the observed differences between the urban populations and Tenterfield lizards, it does not necessarily explain the personality differences between Sydney, Coffs Harbour, and Brisbane lizards. We suggest that these differences could be due to some unmeasured ecological factors (e.g., predation pressure, habitat availability, and competition) and/or due to the fact that populations might fall differently along the urban gradient (i.e., some populations may be more or less urbanized than others; Lowry et al. 2013). For example, previous work on the delicate skink has even found behavioral differences between 2 urbanized populations within suburban Sydney (Moule et al. 2016). Indeed, the results of our study are limited by the fact that we only tested four populations and did not measure any environment-dependent variables (e.g., level of urbanization); thus, we can only speculate as to reasons for the observed population differences. Future studies would benefit from adopting a replicated study design that measures multiple populations, and targets specific ecological factors of interest that could underlie population differences in personality (e.g., different levels of urbanization or thermal regimes). Such an approach would then allow for a more robust interpretation of the ecological and evolutionary factors underpinning consistent individual differences in behavior across populations (Dall and Griffith 2014).

We found evidence to suggest that some behavioral syndromes may be more stable and less adaptive than others. Most comparative studies on behavioral correlations have revealed remarkable population variation in behavioral syndromes, particularly syndromes consisting of traits related to boldness (Bell 2005; Bell and Sih 2007; Dingemanse et al. 2007; Brydges et al. 2008; Herczeg et al. 2009). These studies provide support that some syndromes develop under particular selective environments and can become decoupled over relatively short evolutionary time scales [i.e., the "adaptive hypothesis"; Wilson (1998)]. In this study, we also found evidence that syndromes which contained boldness varied greatly between populations, whereby correlations were either present or absent within populations, and if present, they differed in direction. Boldness is typically measured as the tendency to take risk under threatening situations (Reale et al. 2010). Thus, differences in boldness between populations are typically the result of population differences in the level of predation pressure (Bell 2005; Bell and Sih 2007; Dingemanse et al. 2007; Brydges et al. 2008; Herczeg et al. 2009). It is likely that these divergent correlations are a product of an interplay between predation pressure and other habitat-specific characteristics. For instance, Brydges et al. 2008 found in sticklebacks that boldness and activity were correlated in high-predation river populations, but not in high-predation pond populations, suggesting that predation pressure and habitat complexity interact to influence personality in multifarious ways.

In contrast to behaviors correlated with boldness, we found that activity and exploration were consistently correlated both at the within- and among-population level. Although the magnitude of the among-population correlation was not statistically significant, it had a large effect size (i.e., $r = 0.93$), and it was identical in sign and direction as the within-population correlations (see discussion by Sih and Bell 2008). Furthermore, we have previously found strong correlations between activity and exploration in delicate

skinks from Sydney (Michelangeli et al. 2016a; Moule et al. 2016; Michelangeli et al. 2018) and within both sexes (Michelangeli et al. 2016b). This among-population correlation between activity and exploration implies a lack of evolutionary independence between these traits (i.e., because a shift in one trait corresponds with a shift in the other), and that this result is suggestive of a species-level trait (Martins and Bhat 2014). Our study is one of the few to find empirical evidence that a behavioral correlation might be constrained by some intrinsic mechanism (e.g., genes, physiology, or hormones) across geographically distinct populations [but see Pruitt et al. (2010) and Alcalay et al. (2015)]. However, to test whether this activity–exploration syndrome is in fact evolutionarily constrained, we would need to investigate the genetics underlying these behaviors and correlations using appropriate pedigree information [e.g., test personality of F1 generation: Herzceg et al. (2009) and Dochtermann and Dingemanse (2013)].

We contend that one possible underlying mechanism maintaining this consistent activity–exploration syndrome is thermal physiology. We recently found evidence that thermal physiology drives behavior in delicate skinks and that an individual's personality corresponds with their position on a thermal gradient [i.e., along a cold–hot axis: Goulet et al. (2017a), (2017b), Goulet et al. (2018), and Michelangeli et al. (2018)]. Briefly, individuals that prefer and perform optimally at high body temperatures tend to be faster sprinters, more active, explorative, and bold than individuals that prefer and perform optimally at lower body temperatures. We suggest that animals that need to behaviorally thermoregulate (i.e., ectotherms) in temporally variable environments are more likely to be constrained by their thermal physiological needs, particularly those behaviors that rely more heavily on locomotion (like activity and exploration), the mechanics of which are mediated by an individual's body temperature (Biro and Stamps 2010, Careau and Garland 2012). Indeed, various other studies have documented links between behavior, temperature, and physiology in ectothermic organisms (Stapley 2006, Rey et al. 2015, Cerquiera et al. 2016, Gilbert and Miles 2016). Thus, due to the inherent relationship between temperature, physiology, and behavior, even when traits related to thermal physiology are plastic or consistently vary between populations, we would expect a concomitant shift in personality. For example, a shift from a population largely composed of individuals with higher thermal physiological requirements (i.e., hot thermal types) to a population largely composed of individuals with lower thermal physiological requirements (i.e., cold thermal types) results in a corresponding shift towards an average behavioral profile that is less active and explorative [e.g., Gilbert and Miles (2016)]. Indeed, different thermal types (e.g., hot or cold) are likely linked to different life-history strategies [e.g., pace-of-life: Reale et al. (2010)] that are generated by genetic correlations and environmental effects (e.g., microclimates, early life experience, or incubation temperature). Detailed comparative studies that explore population differences in the covariation between thermal physiological traits, genetics, and personality are first needed to support these hypotheses.

To conclude, we found common and uncommon aspects of personality across four populations of the delicate skink. We found that populations differed in their frequency and composition of behavioral types and how boldness covaried with other behavioral traits. However, we did find a shared activity–exploration syndrome within and among populations. These results suggest that populations are modified by their environmental settings, but there are also likely intrinsic mechanisms that maintain some behavioral correlations across even geographically distinct populations. We propose here that one such mechanism, particularly in ectotherms, could be thermal physiology in which individual differences in personality are inhibited by individual differences in thermal and metabolic requirements (Cerqueira et al. 2016, Goulet et al. 2017b, Michelangeli et al. 2018). We contend that some personality traits may be more adaptive than others and that this needs to be more regularly considered within the animal personality framework.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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Appendix J

Sex-dependent personality in two invasive species of mosquitofish

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Abstract

A key challenge in invasion biology is identifying characteristics that allow some species to be repeatedly successful at invading novel environments. Invasions can often be disproportionately driven by a single sex, with differences in behavioural mechanisms between the sexes potentially underlying sex-biased invasiveness. Here, we took an animal personality approach to study the behaviour of two repeatedly successful congeneric invasive species, the western mosquitofish, *Gambusia affinis*, and the eastern mosquitofish, *Gambusia holbrooki*. In each species, we investigated whether males and females shared common personality traits (i.e. behavioural types and behavioural syndromes), with the aim of identifying possible behavioural mechanisms that could help explain why mosquitofish invasions are often characterised by sex-biased founder populations. We found sex-dependent personality, although sex differences varied between species. Specifically, male *G. affinis* were bolder and less social than female *G. affinis*, whereas we found no behavioural type differences between the sexes in *G. holbrooki*. We also found a consistent correlation between boldness and exploration in both sexes within *G. affinis*, but this correlation was weak in *G. holbrooki*. Finally, exploration was also correlated with sociability in male *G. affinis*, but not in females. Our results suggest that behavioural tendencies may diverge, both among species and between the sexes, because of adaptation experienced during different invasion pathways. Broadly, identifying the behavioural mechanisms that predict an individual's 'invasiveness' may be difficult to tease apart between species because each invasion is characterised by different abiotic and biotic interactions that likely require different suites of behaviours. Future studies are needed to elucidate whether, in fact, personality variation between the sexes can mediate the occurrence of sex-biased invasions.

KEYWORDS

Individual variation; Animal personalities; Invasion syndrome; Invasion process; Life-history; Sex ratio; Risk-taking; Sex differences.

INTRODUCTION

Individuals that successfully invade and establish into new areas often represent a non-random subset of the population, and typically consist of individuals that possess a certain suite of characteristics that differs from those of non-invaders (Blackburn & Duncan 2001, Tingley et al. 2010, Renault et al. 2018). For instance, it has been proposed that these individuals often have behavioural and life-history traits that increases their propensity to be transported to new environments, exploit novel resources, establish viable populations, and spread and colonise new habitats (Holway & Suarez 1999, Chapple et al. 2012, Chapple & Wong 2016, Rehage et al. 2016). These same phenotypic traits also mediate how invaders interact with the local environment and native biota, and thus play a pivotal role in determining the ecological and evolutionary impacts of an invasion (Phillips & Suarez 2012). Hence, a key challenge in invasion biology is identifying the characteristics that allow some species to be repeatedly successful at invading and colonising novel environments (Chapple et al. 2012).

Evidence is starting to accumulate that biological invasions can be often be disproportionately driven by a single sex (Gutowsky & Fox 2011, Miller et al. 2013, Rebrina et al. 2015). Skewed sex ratios at the leading edge of an invasion have been shown to have profound consequences for population growth and persistence (Miller et al. 2013, Shaw et al. 2018), and can lead to greater adverse impacts on native communities than non-skewed sex ratios (Fryxell et al. 2015). For instance, female-biased invasion front populations can exponentially increase the pace of an invasion by speeding up population growth, resulting in a higher probability of colonisation success (Miller et al. 2013), whereas male-biased invasions may be more likely to competitively exclude native species, creating new opportunities for habitats and resources to be exploited (Duckworth & Baydeav 2007, Gutowsky & Fox 2011). However, despite the prevalence of sex-biased invasions, the mechanisms that lead to biased sex ratios at the front of an invasion have rarely been studied.

Sex-biased invasions likely occur because males and females often differ considerably in life-history and behavioural traits related to invasion (Shaw et al. 2018). For example, dispersal is the mechanism that allows invaders to spread from the point of introduction into new areas and thus is a pivotal component of the invasion process (Cote et al. 2010a), but males and females often differ in their propensity to disperse (Trochet et al. 2016), and in traits related to dispersal (e.g. morphology: Llewelyn et al. 2010; behaviour: Marentette et al. 2011). Such sex-dependent traits (e.g. sex-biased dispersal) may enhance the invasiveness of a single sex, leading to biased sex ratios (Miller & Inouye 2013, Fryxell et al. 2015, Shaw et al. 2018). Alternatively, limited behavioural variation between the sexes would be less vulnerable to selective filtering by the invasion process, and thus leading-edge populations would not be expected to

be disproportionately skewed towards a particular sex (Michelangeli et al. 2016a, Gruber et al. 2017).

One relatively new approach to investigating the role of behaviour in invasions is through the study of animal personalities (see reviews: Cote et al. 2010a, Chapple et al. 2012, Sih et al. 2012, Juette et al. 2014). Animal personality refers to the concept that individuals within populations often show consistent differences in a range of behaviours (i.e. behavioural types: Sih et al. 2004), and these behaviours can covary across time/and or context (i.e. behavioural syndrome; Sih et al. 2004). Personality traits are often linked to life-history (reproduction and growth rates: Biro & Stamps 2008), ecological processes (habitat specialisation: Michelangeli et al. 2018a), and social roles within populations and communities (e.g. innovation & cultural transmission: Aplin et al. 2015). Given its direct bearing on fitness, an individual's personality should also influence its probability of transitioning through the invasion process, with different behavioural types being advantageous at different stages of invasion (Cote et al. 2010a, Fogarty et al. 2011, Chapple et al. 2012, Chapple & Wong 2016). Indeed, mounting evidence suggests that invasive individuals may exhibit combinations of behaviours that are beneficial in outcompeting native species (Pintor et al. 2009), dispersing into new habitats (Michelangeli et al. 2017), and avoiding novel predators (Mennen & Laskowski 2018). In this regard, personality differences between the sexes could underlie differences in sex-biased dispersal and sex-biased invasiveness (Michelangeli et al. 2016a, Mishra et al. 2018). Sex differences in the direction and magnitude of behavioural syndromes could arise due to divergent selection pressures and life-histories after maturation. This may be particularly true for sexually dimorphic species, as marked differences in morphology (e.g. body size) can induce variance in behaviour (Shine 1989, Fairbairn et al. 2007). For instance, larger body size requires higher energetic input and, thus, personality traits that are associated with an increase in feeding rate (Biro & Stamps 2008). If personality influences an individual's level of 'invasiveness' and, hence, their potential impact on the environment, it is important to consider how the sexes might differ in personality to better understand the behavioural mechanisms involved in successful invasions.

In this study, we compared the personality traits of males and females in congeneric invasive species, the western mosquitofish, *Gambusia affinis* (Baird & Girard 1853) and the eastern mosquitofish, *Gambusia holbrooki* (Girard 1859). These species provide an ideal opportunity to explore sex differences in personality traits related to invasion for several reasons. First, *Gambusia* are small live-bearing freshwater fish that show pronounced sexual size dimorphism, whereby females are commonly much larger than males (Pyke 2005). Second, both species have undergone numerous deliberate (i.e. introduced as a biocontrol tool for mosquitoes) and accidental introductions, and have now spread and become invasive globally, placing them

within the top 100 of the world's most invasive species (Pyke 2008). Third, invasive populations are often characterised by demographic differences in sex ratios that can either be skewed towards males or females (Fryxell et al. 2015). Fourth, mosquitofish are having tremendous adverse impacts on native insect, amphibian and fish communities worldwide (Pyke 2008, Schluse et al. 2013). Importantly, some studies suggest that the magnitude of these impacts are dependent upon both the sex ratio (Fryxell et al. 2015), and the personality composition of invading populations (Cote et al. 2017). Thus, understanding the behavioural mechanisms driving *Gambusia* invasions is an issue of immediate importance.

The approach used in this study allowed us to determine if each species and sex share common behavioural syndromes and, in so doing, provides insights into the behavioural traits that might contribute to invasiveness. We hypothesised that males and females would differ in a range of behaviours related to invasion, but that these differences would vary among species due to the divergent introduction pathways and local environmental conditions experienced by each species.

MATERIALS AND METHODS

Species collection and husbandry

Gambusia holbrooki (female: $n = 25$; male: $n = 25$) were collected from the Science Centre Lake (37° 54' 28'' S, 145° 08' 16'' E; 10:14 h light:dark), Monash University, Victoria, Australia on 22 January 2014. All fish were caught via seine netting to minimise potential personality-biased sampling (Michelangeli et al. 2016b). Fish were housed individually in glass holding tanks (30 cm length × 15 cm width × 20 cm height) and acclimated to laboratory conditions for 1 month prior to experimentation. We housed fish individually in order to keep track of their identity during behavioural assays. Throughout the housing period, fish were kept at a temperature of 24–26 °C, and under a 12:12 h light:dark cycle. Both during housing and throughout experimentation, fish were fed *ad libitum* with commercial fish food.

Gambusia affinis (female: $n = 110$; male: $n = 112$) were supplied by the Sacramento-Yolo Mosquito and Vector Control District. These fish represent a mix of hatchery-reared and field-collected fish. Fish were transported to the Centre for Aquatic Biology and Aquaculture (CABA), University of California Davis on 18 March 2008, and housed in groups of ~60 in 80 L flow-through fibreglass tanks, and acclimated to laboratory conditions for 1 month prior to experimentation. All individuals were marked with a minimally invasive elastomer tag (north-

west Marine Technologies, Shaw Island, WA, USA) under a low dose (5 mg L^{-1}) of anaesthetic (MS-222). Each individual received a randomly assigned unique identifier by injecting one of four colours subcutaneously into four locations on the caudal peduncle (two on each side). Throughout the housing period, fish were kept at a constant temperature (22–23 °C) on a natural photoperiod (14:10 h light:dark), and were fed commercial fish food *ad libitum*.

Behavioural assays

For both species, in order to characterise personality types of each sex, we ran two behavioural assays, each separated by 1 h. First, we tested sociability by quantifying the tendency of individuals to shoal. Second, we tested individual boldness and exploratory behaviours. The former was characterised by the latency of fish to exit from a refuge and enter a novel environment, and the latter was quantified by recording the movement and space use of fish after exiting the refuge. These behaviours represent an individual's reaction to a social context and to a novel environment, respectively. Both sets of behaviours are hypothesised to play an important role in colonising new environments (Chapple et al. 2012, Sih et al. 2012, Chapple & Wong 2016). Behavioural assays were repeated for *G. holbrooki* a day later. We consider both repeats of the *G. holbrooki* behavioural assays in this study because it adds more precision to the dataset given the relatively small sample size when compared to the *G. affinis* dataset. We do not calculate repeatability in this study, but these behaviours have previously been found to be repeatable in both species (*G. affinis*: Cote et al. 2010, 2011, 2013; *G. holbrooki*: Wilson et al. 2010, Polverino et al. 2018).

Tendency to shoal (sociability)

To measure social behaviour, we recorded the amount of time an individual spent near a shoal of conspecifics (*sensu* Ward et al. 2004, Bertram et al. 2018). The experimental aquarium (50 cm length × 25 cm width × 30 cm height) was divided lengthwise into three compartments (two small and one large central compartment) using two transparent glass partitions 12.5 cm from each end of the tank. The partitions allowed visual, but not physical or olfactory, interaction between the shoal and the focal individual. A randomly designated stimulus shoal was introduced to one of the smaller compartments 1 h before the experiment began, while the other small compartment was left empty as a control. Stimulus shoals were comprised of 14 mosquitofish (seven conspecific males and seven conspecific females) that had no previous experience with the focal individual. After 1 h, the focal fish was introduced into an opaque cylinder in the centre of the larger central compartment and given 10 min to acclimate. At the end of the acclimation period, the cylinder surrounding the focal fish was remotely removed

to allow the fish access to the central compartment with minimal disturbance. The position of the focal fish was continuously recorded for 10 min. The large compartment was divided with vertical marks every 2 cm, and the time spent by the focal fish within the 2 cm closest to the stimulus shoal was recorded. At the conclusion of the trial, individuals were returned to their holding aquaria.

Boldness and exploration in a novel environment

One hour after the sociability assay, boldness and exploration were assessed by recording behaviour in a novel environment. The experimental arenas differed slightly for data collected on each species. For *G. affinis*, the experimental arena was an opaque, white plastic tank (80 cm length × 80 cm width × 20 cm height) filled with 10 cm of water, and furnished with half flower pots in two corners, which served as additional refuges. For *G. holbrooki*, the experimental arena consisted of a glass aquarium (60 cm length × 30 cm width × 30 cm height), filled with 15 cm of water, with 72 equal grid squares marked on its base. For both species, focal fish were added gently to an upright, cylindrical (9–10 cm diameter) opaque PVC pipe refuge on one side of the experimental arena. After 10 min, a 4 cm wide door to the refuge chamber was remotely opened, allowing fish access to the experimental arena. We then allowed the fish 45 min to leave the refuge, recording the time to exit. After the fish left the refuge, we then allowed an additional 5 min to explore the novel environment. Because we gave fish an additional 5 min to explore the novel environment after it left the refuge, we treated both behavioural measures as independent behavioural traits. Trials ended either 5 min after fish left the refuge or after 45 min (2700 s) if animals did not leave the refuge.

For both species, boldness was measured as the maximum time allowed for fish to exit the refuge (2700 s) minus the latency (s) to exit from the refuge, and to stay for greater than 10 consecutive seconds out of the refuge. Shorter latency to exit the refuge indicates a higher boldness and is regularly used as a metric for boldness in studies of fish (Moran et al. 2016, Hulthén et al. 2017), including mosquitofish (Wilson et al. 2010, Bertram et al. 2018, Polverino et al. 2018). Exploratory behaviour was quantified by measuring how much of the experimental arena the focal individual covered. For *G. affinis*, the area explored incorporated both the distance an individual moved and the spatial pattern of those movements. Given x – y coordinates from each video frame, each individual's continuous path was tracked, and the area an individual explored was calculated as the percentage of the arena that fell within 5 cm of the fish's path. For *G. holbrooki*, the area explored was calculated by dividing the total number of unique grid-squares an individual entered by the total number of grid squares (n = 72).

Morphological measurements

All fish were weighed and measured before and after the behavioural assays. *G. affinis* were larger than *G. holbrooki* for both sexes (mean male total body length (TBL) ± standard error (SE): *G. affinis*: 23.07 ± 0.25 mm, *G. holbrooki*: 21.45 ± 0.28 mm, Mann-Whitney test: $U = 1858$, $p < 0.001$; mean female TBL ± SE: *G. affinis*: 29.34 ± 0.56 mm, *G. holbrooki*: 25.78 ± 0.37 mm; $U = 1890$, $p < 0.001$).

Statistical analysis

Data were analysed in R version 3.3.2 (R Core Development Team 2016). Residuals were checked for normality (Shapiro-Wilk test: Royston 1995) and homogeneity of variance (Fligner-Killeen test: Conover et al. 1981). Prior to analysis, time spent in the 2 cm social zone (i.e. sociability) was rank-transformed, and latency to exit the refuge was log-transformed, to approximate Gaussian error distributions. Because each species was reared under different conditions and there were slight differences in the design of behavioural assays, we ran separate statistical tests for each species. Thus, any species-level comparison is based upon a comparison of two separate models and not statistically computed. Statistical significance was assigned at $\alpha = 0.05$.

We first tested whether the sexes differed in the individual behavioural traits studied using linear models for the *G. affinis* dataset, and linear mixed-effects models (LMM; package *lme4*, Bates et al. 2015) for the *G. holbrooki* dataset. Models contained the fixed effects of sex, body length and a sex × body length interaction. We also included trial number and sex × trial number interaction as fixed factors, and individual ID as a random factor within the mixed-effects models in order to consider the repeated measures design of the *G. holbrooki* dataset. *P*-values of interaction terms were calculated using likelihood ratio tests (G^2) for LMM's (Bolker et al. 2009) and Wald's *F*-tests were used for linear models. If interaction terms were non-significant they were removed from the final models.

We assessed trait correlations within species and sex to determine the presence of behavioural syndromes. To do this, we estimated the magnitude of pairwise relationships between behavioural traits using spearman-rank correlations and compared the correlation coefficients using the Fisher *z*-transformations.

RESULTS

1. Behavioural types

1a) Gambusia holbrooki

We found no effect of sex, trial or body length on *G. holbrooki* shoaling behaviour or time taken to re-emerge from the refuge (p <0.05, Table 1; Figure 1). Regardless of sex, fish explored more of the novel environment in trial 2 compared to trial 1, suggesting habituation to the experimental arena ($t_{1,49}$ = 2.77, p = 0.008; Table 1). However, there was no effect of sex or body length on the exploratory behaviour of *G. holbrooki* (p >0.05, Table 1, Figure 1).

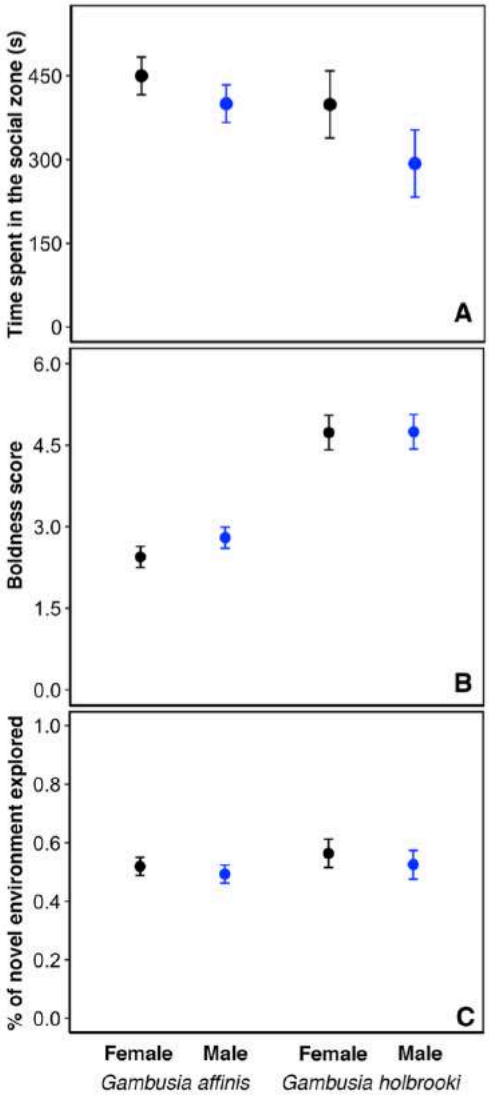

Figure 1. Mean (± standard error) trait-level differences of females (black) and males (blue) across two *Gambusia* species (*G. affinis* and *G. holbrooki*) in A) tendency to shoal (i.e. time spent within 2 cm social zone; sociability), B) boldness score (i.e. log maximum time allowed for fish to exit the refuge (2700 s) minus the log latency (s) to exit from the refuge; boldness) and C) tendency to explore a novel environment (% of novel environment explored; exploration). Sample sizes differed between species; *G. affinis* (female: n = 112, male: n = 111), *G. holbrooki* (female: n = 25, male: n = 25).

Table 1. Main effects of sex, body length and trial on a) time spent shoaling with conspecifics, b) time to re-emerge from a refuge, and c) percentage of novel environment explored, in *Gambusia holbrooki* (female: $n = 25$; male: $n = 25$). Models were first compared with and without the interaction terms using Likelihood ratio tests (G^2). Interaction terms were removed from the final models if were non-significant. Results were obtained from linear mixed effects models (LMM) and contained individual ID as a random factor.

Behaviour	Fixed effects	G^2	β	t	p
a) Time spent shoaling	Sex	–	0.498	1.474	0.147
	Body length	–	0.004	-0.085	0.932
	Trial	–	0.170	1.393	0.170
	Sex × trial	1.392	–	–	0.238
	Sex × body length	0.150	–	–	0.698
b) Time to re-emerge from refuge	Sex	–	0.270	0.795	0.431
	Body length	–	0.059	1.085	0.284
	Trial	–	0.277	1.440	0.156
	Sex × trial	3.368	–	–	0.066
	Sex × body length	0.071	–	–	0.790
c) % of novel environment explored	Sex	–	0.022	0.382	0.704
	Body length	–	0.014	1.543	0.129
	Trial	–	**0.093**	**2.772**	**0.008**
	Sex × trial	0.031	–	–	0.860
	Sex × body length	0.281	–	–	0.596

G^2 = chi-squared value. β = co-efficient. Bold refers to significant terms at $P < 0.05$. Note that each species differed substantially in sample size.

1b) Gambusia affinis

Female *G. affinis* spent more time shoaling than male *G. affinis* ($t_{1,219} = 2.632$, $p = 0.009$; Table 2, Figure 1). Regardless of sex, larger fish spent less time shoaling with conspecifics than smaller fish, although the effect of body length was marginally non-significant ($t_{1,219} = -1.917$, $p = 0.057$; Table 2). Males re-emerged from the refuge faster than females ($t_{1,219} = 2.483$, $p = 0.014$; Table 2, Figure 1), but this effect was dependent on body length (sex × body length interaction: $F_{1,218} = 5.394$, $p = 0.021$; Table 2), with smaller males re-emerging faster from the refuge than larger males ($t_{1,110} = -2.326$, $p = 0.022$). We found no effect of sex or body length on the tendency to explore the novel environment ($p > 0.05$, Table 2).

Table 2. Main effects of sex and body length on a) time spent shoaling with conspecifics, b) time to re-emerge from a refuge, and c) percentage of novel environment explored, in *Gambusia affinis* (female: n = 110; male: n = 112). Results were obtained from linear models. Bold terms indicate significant results.

Behaviour	Fixed effects	F	β	t	p
a) Time spent shoaling	**Sex**	–	**0.612**	**2.632**	**0.009**
	Body length	–	**-0.052**	**-1.917**	**0.057**
	Sex × body length	0.214	–	–	0.644
b) Time to re-emerge from refuge	**Sex**	–	**3.356**	**2.483**	**0.014**
	Body length	–	0.008	0.326	0.745
	Sex × body length	**5.394**	**0.128**	**2.323**	**0.021**
c) % of novel environment explored	Sex	–	0.022	0.800	0.425
	Body length	–	<0.001	0.164	0.870
	Sex × body length	1.931	–	–	0.166

2. Behavioural syndromes

2a) Gambusia holbrooki

We found weak evidence of a behavioural syndrome in *G. holbrooki*. There was a marginal positive correlation between time to re-emerge from the refuge (boldness) and tendency to explore the novel environment (exploration) in females, but this correlation was negative in males (Table 3; Figure 2).

2b) Gambusia affinis

We found a significant positive correlation between the time taken to exit the refuge (boldness) and tendency to explore the novel environment (exploration) in both male and female *G. affinis* (Table 3; Figure 2). There was also evidence that time spent shoaling with conspecifics (sociability) and tendency to explore the novel environment (exploration) were positively correlated in male *G. affinis* (Table 3). This correlation was not present in females, but the correlation coefficients did not significantly differ between the sexes (Table 3).

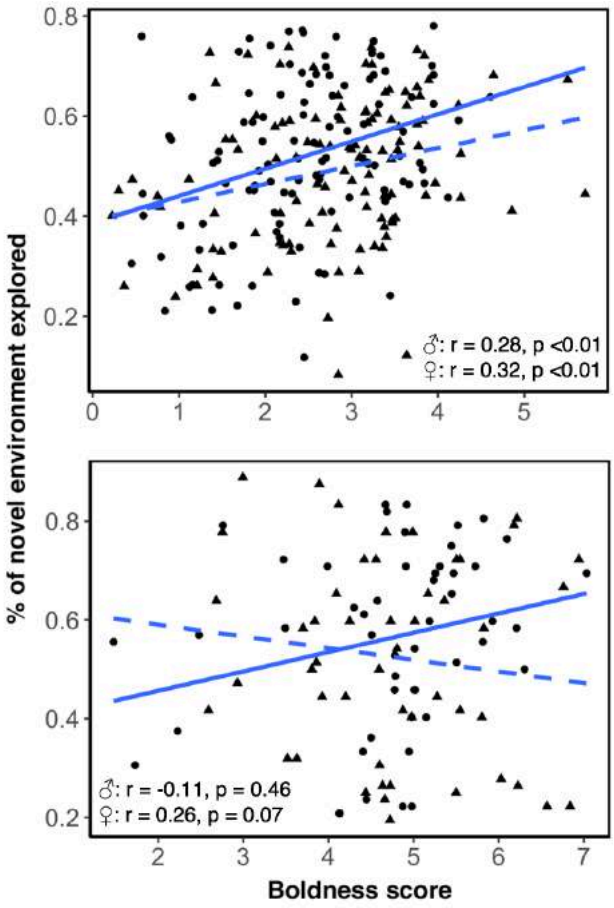

Figure 2. Sex regression lines for relationship between tendency to explore a novel environment (% of novel environment explored) and boldness score (i.e. log maximum time allowed for fish to exit a refuge (2700 s) minus the log latency (s) to exit from the refuge) within two mosquitofish species, *Gambusia affinis* (top; female: n = 112, male: n = 111) and *Gambusia holbrooki* (bottom; female: n = 25, male: n = 25). Males = dashed lines, triangles, females = solid line, circles.

Table 3. Correlation coefficients (r) for each behavioural correlation estimated using Spearman rank correlation tests and Fisher z statistic comparing the sex-specific effect sizes. Bold scores refer to significant correlation coefficients.

		♂		♀			Total (♂ + ♀)	
Species	Correlation	r	p	r	p	Fisher z	r	p
G. holbrooki	Boldness - Exploration	-0.11	0.46	0.26	0.07	z = -1.82, p = 0.07	0.08	0.40
	Sociability - Exploration	-0.03	0.84	0.11	0.43	z = -0.70, p = 0.48	0.05	0.59
	Sociability - Boldness	0.01	0.99	0.03	0.86	z = -0.12, p = 0.90	0.01	0.93
G. affinis	Boldness - Exploration	**0.28**	**<0.01**	**0.32**	**< 0.01**	z = -0.33, p = 0.74	**0.26**	**< 0.01**
	Sociability - Exploration	**0.21**	**0.02**	0.05	0.61	z = 1.19, p = 0.23	**0.14**	**0.04**
	Sociability - Boldness	0.10	0.30	0.10	0.30	z = 0.01, p = 0.99	0.08	0.24

DISCUSSION

We found evidence for sex-specific personality in invasive mosquitofish, but that these sex differences varied depending on species. Specifically, male *G. affinis* were bolder and less social than female *G. affinis*, but we found no behavioural type differences between the sexes in *G. holbrooki*. There was also a positive correlation between boldness and exploration within *G. affinis*, which was consistent in magnitude and direction in both males and females. Notably, however, we also found that sociability was correlated to exploration in male *G. affinis*, but not in females. There was only a weak positive correlation between boldness and exploration in female *G. holbrooki*, but this correlation was negative in males. The absence of a common behavioural syndrome between *Gambusia* species is inconsistent with the hypothesis that there is a specific suite of behaviours that might help to explain both species' successful invasion history. Instead, our results suggest that behavioural tendencies may diverge among species and between the sexes because of adaptation experienced during different invasion pathways.

Sex differences in boldness and sociability in *G. affinis* are likely a product of disparate reproductive and life-history strategies. Females often bear a higher cost of reproduction than males, resulting in females having life-history and behavioural traits associated with a slower pace-of-life that maximises fecundity and reproductive output (e.g. longer life span, less risk-taking; Debecker et al. 2016). Indeed, fecundity selection is a major evolutionary force selecting for larger body size in females in sexually size-dimorphic species (Shine 1989), and higher levels of risk-taking have previously been linked to lower fecundity in female mosquitofish (Wilson et al. 2010). Females also tend to be more social than males and utilise the anti-predator benefits of group shoaling (i.e. dilution effects: Foster & Treherne 1981, increased vigilance: Hoare et al. 2000) as a risk-avoidance strategy. Shoaling has also been shown to reduce the foraging and reproductive costs of sexual harassment by males of female mosquitofish (Pilastro et al. 2003). In several aquatic organisms, females have also been shown to be less bold than males (Harris et al. 2010, King et al. 2013, Biro et al. 2014, Debecker et al. 2016). Such low risk-taking behaviour may be particularly important for female mosquitofish, which may be preferentially targeted by predators as they are larger than males, and consequently represent more profitable prey (Britton & Moser 1982). Interestingly, we also found in the current study that larger males took longer to re-emerge from the refuge than smaller males. This result corroborates with the idea that being larger makes you more vulnerable to predators, thus larger individuals adopt less risky behavioural strategies. On the other hand, smaller male *G. affinis* may have been faster to exit the refuge because the costs associated with hiding, such as the loss of reproductive opportunities (Martín et al. 2003), outweigh the benefits of such risk-avoidance behaviours. Indeed, male mating success is typically highly variable, particularly for smaller males who are often

perceived as lower quality mates by females (Tomkins et al. 2018), which likely encourages a 'high risk, high reward' behavioural strategy in these smaller males (King et al. 2013).

It is somewhat surprising, then, that we did not find the same differences in boldness and sociability between male and female *G. holbrooki*. A possible explanation for this lack of divergence in boldness and social traits is that *G. holbrooki*, in this study, were sourced from an environment with low predation pressure, and thus the risk of emerging from a refuge and the benefits of shoaling in a group were perceived by females to be low, resulting in females being equally likely to take 'risks' as males. An alternative reason for a lack of sex differences in *G. holbrooki* more broadly is that the body range size of our study population was different to the natural variation in body size observed in other wild populations (e.g. McPeek 1992). The size differences between males and females in *G. holbrooki* was comparatively smaller than *G. affinis*, thus the costs associated with having larger body size may not be as robust in our *G. holbrooki* population, favouring selection towards similar behavioural tendencies between the sexes (Fairbairn et al. 2007).

We observed markedly different personality traits between species. A consistent behavioural correlation between boldness and exploration was present in male and female *G. affinis*, but this correlation was weak in *G. holbrooki*. Indeed, observed behavioural differences between species are limited by the fact that we only compared one population of each species with unequal sample sizes. Thus, these results should be interpreted with much caution as our study does not offer a robust test of species differences, which was not the main aim of this research. However, past studies have found differences in behavioural traits between *G. affinis* and *G. holbrooki* (e.g. dispersal: Rehage & Sih 2004; antipredator response: Rehage et al. 2005). In this study, behavioural differences between species could be a result of differences in the level of predation pressure along the introduction pathway and/or the local environment. Our finding that a boldness-exploration behavioural syndrome differed between species is consistent with most comparative studies on behavioural correlations, which have found remarkable variation in syndromes, particularly those related to boldness (Bell & Sih 2007, Dingemanse et al. 2007; Michelangeli et al. in press). These studies suggest that high-consistency in behavioural syndromes are often linked to high-predation sites that place consistent selection on groups of behaviours, particularly behaviours linked to risk taking, compared to more benign environments which favour variable behavioural strategies (Heinen-Kay et al. 2016). For example, in three-spine sticklebacks, *Gasterosteus aculeatus* (Linnaeus, 1758), populations raised in high predation risk environments exhibit a boldness-aggression syndrome, whereas populations raised in low predation environments lost this syndrome (Bell & Sih 2007). It should also be noted, however, that differences between species could be a result of differences in how each

species were reared in our study; as *G. holbrooki* were housed in isolation during the experimental period rather than in groups, and this could have had an influence on their behaviour (Gómez-Laplaza & Morgan 2000, Bevan et al. 2018).

Interestingly, we found evidence that sociability is correlated to exploration in male *G. affinis*, but not females. Sociability has previously been found to be linked to dispersal in *G. affinis*, whereby asocial individuals tend to disperse further, faster and more frequently than social individuals when population densities are high (Cote et al. 2010b, 2011, 2013). Sociability-dependent dispersal in *G. affinis* has also been found to generate more severe impacts on native aquatic insect communities compared to random dispersal (Cote et al. 2017). Furthermore, dispersal propensity is higher in males compared to females (Cote et al. 2010b, 2011). Our results, together with these earlier studies, suggest that males (i.e. the more dispersive sex), that are asocial and bold, would be more likely to disperse away from established populations (i.e. high-density populations) and lead the invasion front. On the other hand, sociability appears to be independent of exploration and boldness in females. These sex-specific differences in personality and dispersal may thus have important implications for the spread and invasion of western mosquitofish, and for their impacts on native ecosystems, as the behavioural composition of range-front populations may be sex-dependent. Conversely, due to a lack of behavioural differences between sexes, *G. holbrooki* invasions may be less prone to skewed sex ratios at the invasion front. A future study that explores the interaction between sex- and behavioural-dependent dispersal, and its implications for founder populations, would yield interesting insights into the spread dynamics of invasive mosquitofish populations.

To conclude, our results suggest that different mosquitofish invasions have required different behavioural tendencies to succeed, and that some of these behaviours are likely sex-dependent. We found limited evidence of sex-specific personality in *G. holbrooki*, suggesting that both sexes have an equal invasion potential. In contrast, differences in syndromes between male and female *G. affinis* could be a mechanism that leads to sex-dependent dispersal in this species, and thus unequal sex ratios at the leading edge, but future studies are needed to test the validity of these hypotheses. Overall, identifying the behavioural mechanisms that predict an individual's 'invasiveness' is difficult to tease apart between species because each invasion is characterised by different abiotic and biotic interactions that likely require different suites of behaviours (Felden et al. 2018, Mennan & Laskowski 2018). Future studies are needed to elucidate whether, in fact, personality variation between the sexes can mediate the occurrence of sex-biased invasions.

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