Science of the Total Environment 664 (2019) 177–187

Contents lists available at ScienceDirect

Science of the Total Environment

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

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

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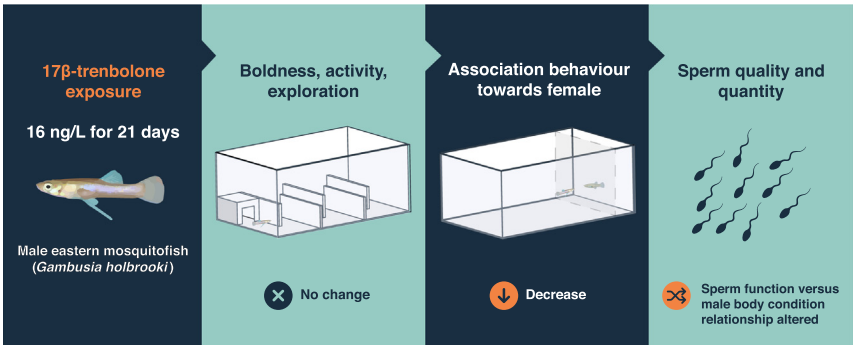
HIGHLIGHTS

- 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



ARTICLE INFO

Article history:
Received 20 November 2018
Received in revised form 28 January 2019
Accepted 28 January 2019
Available online 30 January 2019

Editor: Damia Barcelo

Keywords:
Androgen
Endocrine disrupting chemical
Hormonal growth promotant
Pharmaceutical pollution
Sperm
Trenbolone

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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https://doi.org/10.1016/j.scitotenv.2019.01.382
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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.

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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).

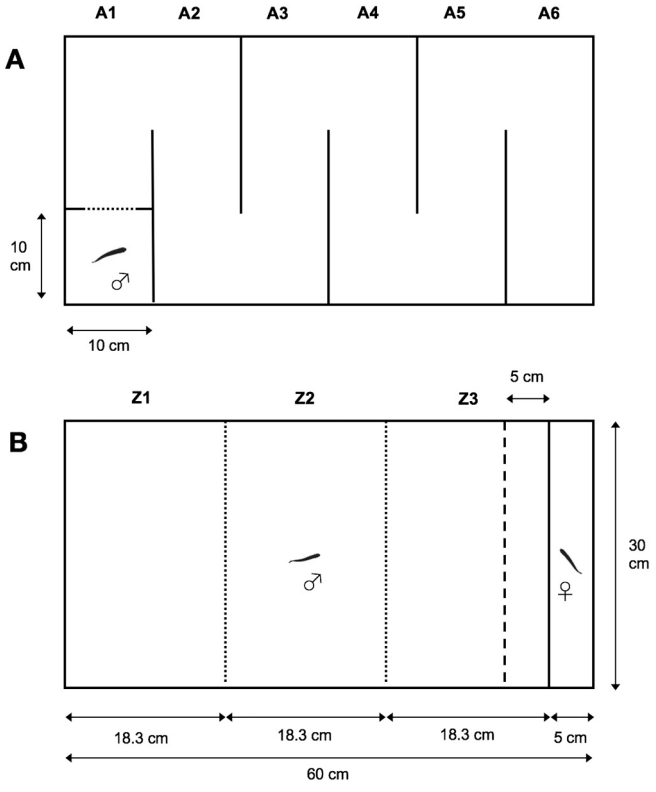

Fig. 1. Aerial view of the (A) maze assay testing boldness, activity, and exploration in a novel environment, and (B) reproductive behaviour assay. The maze arena comprised an enclosed refuge with a door (dotted line) that was opened remotely at the beginning of each trial, as well as six maze arms (A1–6) 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.

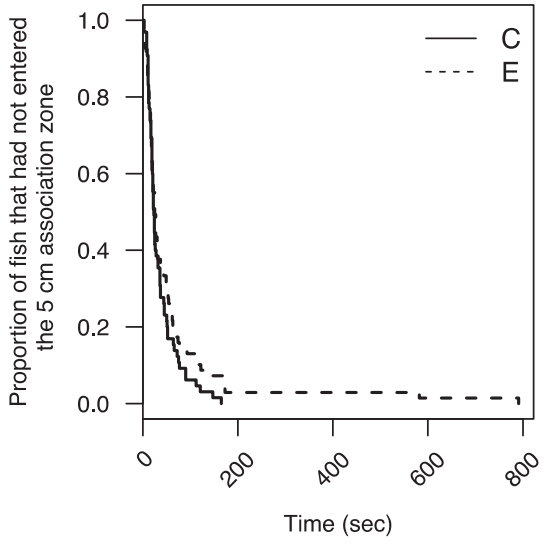

Fig. 2. Kaplan–Meier survival curves showing the time taken for control (C, $n = 65$) and 17β-TB-exposed (E, $n = 70$) males to first enter a 5 cm zone abutting the stimulus (unexposed) female compartment during the reproductive assay (s).

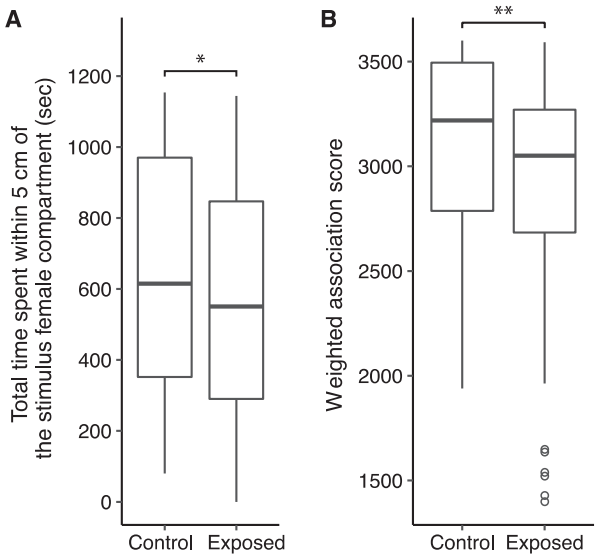

Fig. 3. The (A) total time spent by males within 5 cm of the compartment containing the stimulus (unexposed) female (s) during the reproductive assay, and (B) male weighted association score (representing the use of the entire main tank area relative to the position of the stimulus female, with a higher 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).

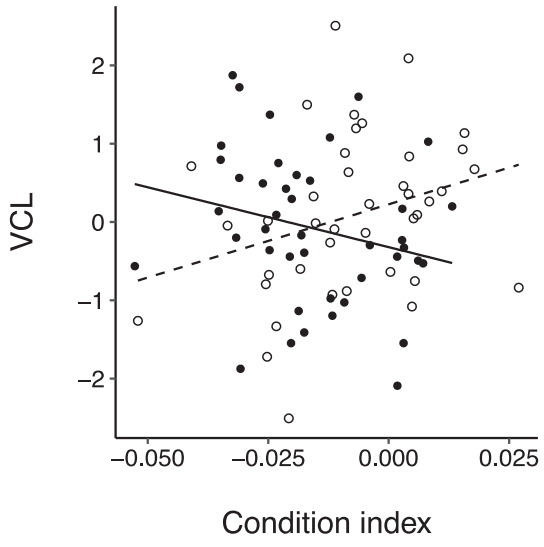

Fig. 4. Sperm curvilinear velocity (VCL, μm/s) as a function of condition index (i.e. relative mass) for males in the control ($n = 42$) and 17β-TB-exposed ($n = 40$) treatments. The filled circles and solid trend line represent unexposed males, while the unfilled circles and dashed trend line represent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

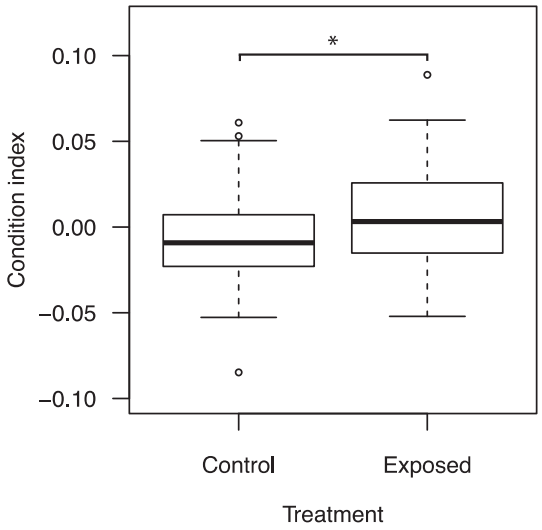

Fig. 5. Boxplots showing the condition index of males in the control ($n = 65$) and 17β-TB-exposed ($n = 70$) treatments. $^*p < 0.05$.

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.

Funding statement

Funding for this research was provided by two Australian Postgraduate Award scholarships (to M.G.B. and J.M.M.), a Postgraduate Publications Award from Monash University, a university student grant from the Australian Wildlife Society, a Postgraduate Global Environmental Sustainability Award from the Rotary Clubs of Balwyn and Geelong, the Australian Society For Fish Biology's Barry Jonassen Award, student research grants from the Australasian Society for the Study of Animal Behaviour, a student research award from the Ecological Society of Australia (all to M.G.B.), an Academy of Finland Postdoctoral Researcher Fellowship (265629) (to M.S.), a fellowship from the National Health and Medical Research Council of Australia (APP1058356) (to M.K.O.B.), and Discovery Grants from the Australian Research Council (DP130100385 and DP160100372) (both to B.B.M.W.).

Acknowledgements

We thank David Williams and the teams at Envirolab Services in Perth and Melbourne. We are also grateful to Jessica Dunleavy, Jo Merriner and Anne O'Connor for their assistance in the lab, as well as Clelia Gasparini, Susi Zajitschek and Palestina Guevara-Fiore for their helpful advice on sperm analysis techniques.

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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