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

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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

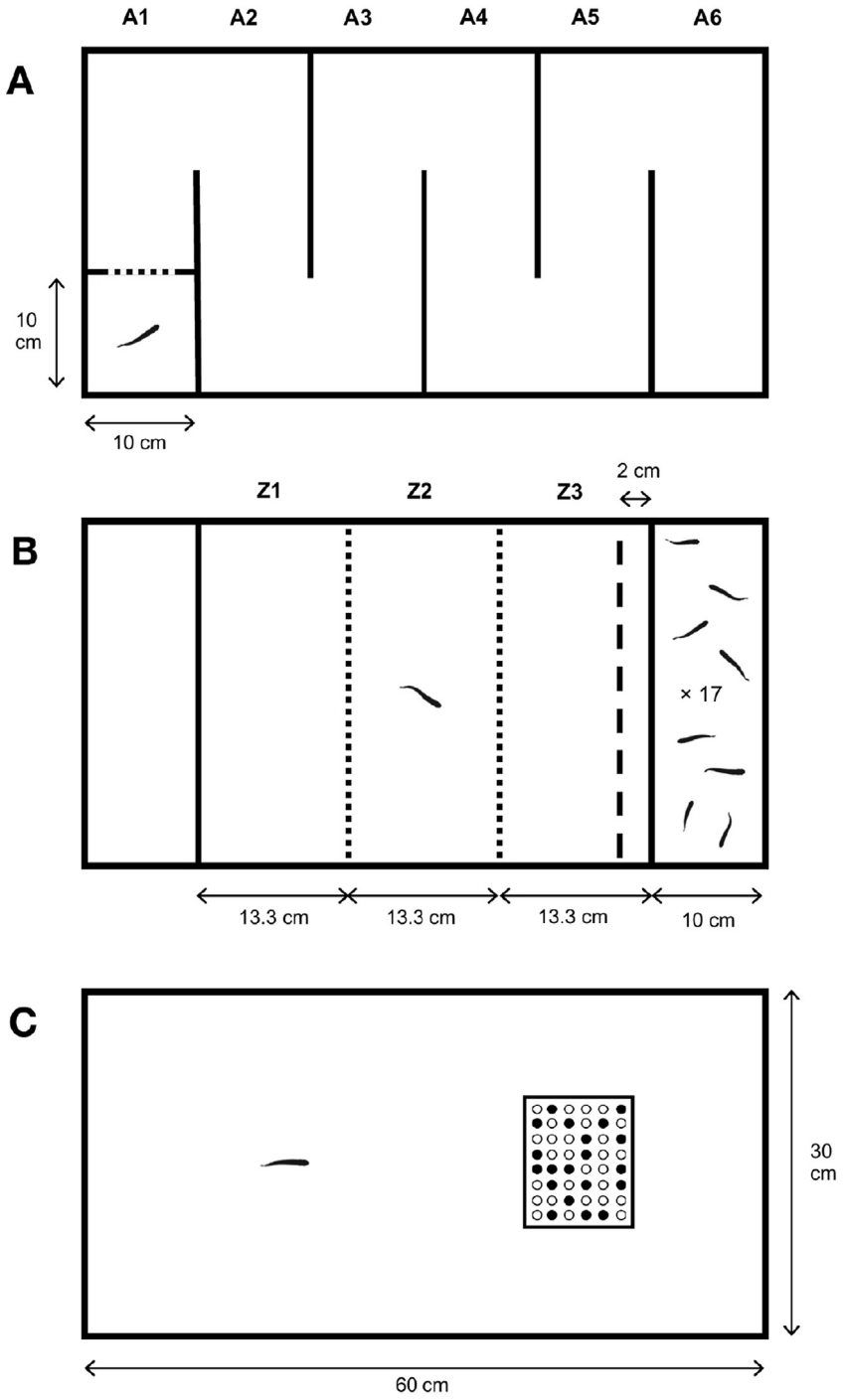

Fig. 1. Aerial views of the (A) maze assay used to test boldness, activity and exploratory behaviour in a novel environment, (B) sociability assay examining tendency to associate with a shoal of 17 stimulus (i.e., unexposed) conspecific females, and (C) foraging assay testing foraging and feeding behaviours. 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

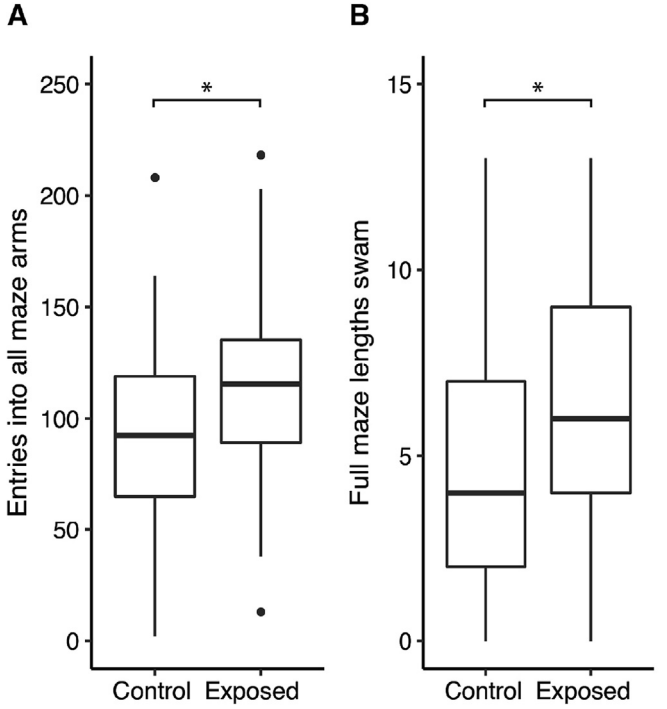

Fig. 2. Number of (A) entries into all maze arms (i.e., A1–A6), and (B) full maze lengths swam, by unexposed ($n = 50$) and 17β-trenbolone-exposed ($n = 46$) fish. Box plots show tenth, twenty-fifth, fiftieth (median), seventy-fifth and ninetieth percentiles with horizontal lines. Whiskers show the range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

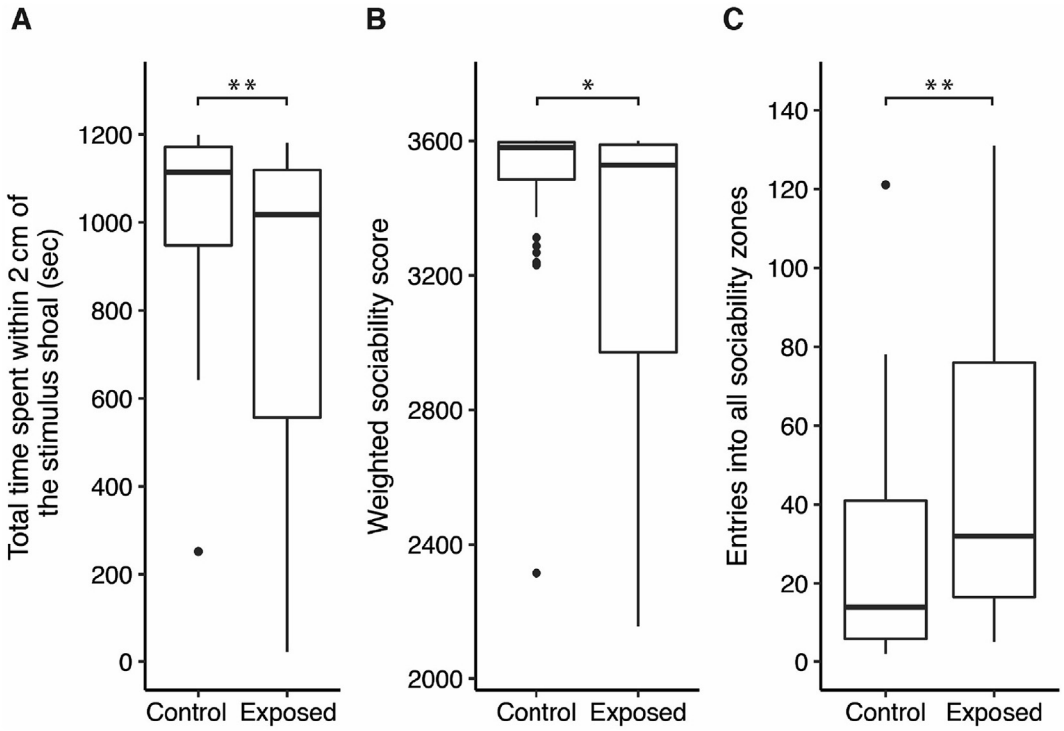

Fig. 3. Sociability of unexposed ($n = 44$) and 17β-trenbolone-exposed ($n = 43$) females towards a shoal of 17 stimulus (i.e., unexposed) conspecific females, in terms of (A) total time spent within 2 cm of the stimulus shoal, (B) weighted sociability score (higher score indicates greater sociability; 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

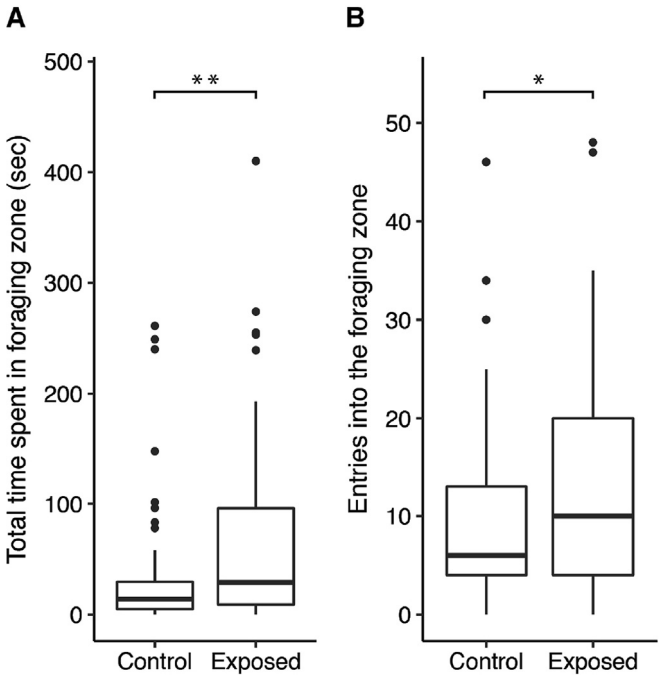

Fig. 4. Foraging behaviour of unexposed ($n = 47$) and 17β-trenbolone-exposed ($n = 45$) fish, in terms of (A) total time spent in the foraging zone, and (B) number of entries into the foraging zone. $^{*}p < 0.05$, $^{**}p < 0.01$.

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 (Sárria 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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