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Fish on steroids: Temperature-dependent effects of 17β-trenbolone on predator escape, boldness, and exploratory behaviors☆

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ABSTRACT

Hormonal growth promoters (HGPs), widely used in beef cattle production globally, make their way into the environment as agricultural effluent—with potential impacts on aquatic ecosystems. One HPG of particular concern is 17β-trenbolone, which is persistent in freshwater habitats and can affect the development, morphology and reproductive behaviors of aquatic organisms. Despite this, few studies have investigated impacts of 17β-trenbolone on non-reproductive behaviors linked to growth and survival, like boldness and predator avoidance. None consider the interaction between 17β-trenbolone and other environmental stressors, such as temperature, although environmental challenges confronting animals in the wild seldom, if ever, occur in isolation. Accordingly, this study aimed to test the interactive effects of trenbolone and temperature on organismal behavior. To do this, eastern mosquitofish (*Gambusia holbrooki*) were subjected to an environmentally-relevant concentration of 17β-trenbolone (average measured concentration 3.0 ± 0.2 ng/L) or freshwater (i.e. control) for 21 days under one of two temperatures (20 and 30 °C), after which the predator escape, boldness and exploration behavior of fish were tested. Predator escape behavior was assayed by subjecting fish to a simulated predator strike, while boldness and exploration were assessed in a separate maze experiment. We found that trenbolone exposure increased boldness behavior. Interestingly, some behavioral effects of trenbolone depended on temperature, sex, or both. Specifically, significant effects of trenbolone on male predator escape behavior were only noted at 30 °C, with males becoming less reactive to the simulated threat. Further, in the maze experiment, trenbolone-exposed fish explored the maze faster than control fish, but only at 20 °C. We conclude that field detected concentrations of 17β-trenbolone can impact ecologically important behaviors of fish, and such effects can be temperature dependent. Such findings underscore the importance of considering the potentially interactive effects of other environmental stressors when investigating behavioral effects of environmental contaminants.

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1. Introduction

Pharmaceuticals and hormones enter the aquatic environment from various sources with the discharge of sewage effluent, agricultural run-off, and even recycled municipal wastewater for parks and gardens being prime sources of contamination (Diamanti-Kandarakis et al., 2009; Arnold et al., 2014). Pharmaceuticals are highly bioactive compounds, and because the target receptors and endocrine systems of vertebrates are widely conserved across taxa (Campbell et al., 2004; Gunnarsson et al., 2008; Brown et al., 2014), the probability of these compounds affecting exposed wildlife is high. In fact, pharmaceutical compounds can accumulate in aquatic food webs (Lagesson et al., 2016) and a plethora of studies have demonstrated that they can induce a variety of effects on a wide range of organisms (Söffker and Tyler, 2012; Bean et al., 2014; Brodin et al., 2014; Cuthbert et al., 2014; Shore et al., 2014).

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A group of chemical contaminants of particular concern are endocrine disrupting chemicals (EDCs). These compounds are known to alter the hormonal and homeostatic function of organisms and can do so at extremely low concentrations (i.e. ng/L) (reviewed in Diamanti-Kandarakis et al., 2009). Furthermore, EDCs have been shown to bioaccumulate (and even biomagnify) in organisms (Norstrom et al., 1976; Saito et al., 1992; Ross et al., 2000; Takahashi et al., 2003; Porte et al., 2006; Liu et al., 2011; Ruhi et al., 2016), act transgenerationally (Anway and Skinner, 2006; Crews et al., 2007; Guerrero-Bosagna and Skinner, 2009; Walker and Gore, 2011), and persist in the environment (Cooke and Stringer, 1982; Helander et al., 2002; Ying et al., 2003; Diamanti-Kandarakis et al., 2009).

One EDC of major concern entering the environment is trenbolone acetate (Lange et al., 2002; Durhan et al., 2006). Trenbolone acetate is a synthetic androgenic anabolic steroid that is heavily used to promote growth in livestock beef production in many parts of the world (e.g. Australia, USA, South America) (Johnson and Hanrahan, 2010) and is also used illegally by bodybuilders (Perry et al., 1990; Barceloux and Palmer, 2013). Trenbolone acetate is a potent androgenic anabolic steroid binding to androgen receptors with many times the affinity of testosterone (Neumann, 1976; Yarrow et al., 2010; Kolodziej et al., 2013). Its metabolite, 17β-trenbolone (hereafter referred to as trenbolone), is highly stable in animal waste (a half-life in liquid manure around 260 days; Schiffer et al., 2001) and enters aquatic environments through run-off from feedlots or direct discharge of livestock urine and manure (Cavallin et al., 2014). It has been detected at concentrations ranging from 1 to 20 ng/L in discharge and diffuse run-off (Durhan et al., 2006), and up to 162 ng/L in fields directly receiving animal waste (Gall et al., 2011). The effects and accumulation of trenbolone in aquatic wildlife, such as fish, are of particular interest because of their inevitable exposure to contaminated water, direct uptake via gills or skin, and consumption of other exposed organisms. Indeed, in aquatic wildlife, trenbolone exposure has been linked to complete sex reversal (Galvez et al., 1996; Ankley et al., 2003; Orlando et al., 2004; Sone et al., 2005; Seki et al., 2006; Örn et al., 2006; Larsen and Baatrup, 2010; Morthorst et al., 2010; Baumann et al., 2014; Li et al., 2015), irreversible masculinization (Larsen and Baatrup, 2010; Morthorst et al., 2010; Baumann et al., 2014), reduced fecundity (Ankley et al., 2003) and increased mortality (Olmstead et al., 2012; Li et al., 2015).

It is well known that a variety of pharmaceuticals commonly detected in the environment may affect fitness-related behaviors, such as predator avoidance, boldness, sociability and reproductive behavior (reviewed in: Zala and Penn, 2004; Hellou, 2011; Brodin et al., 2014). It is also known that these effects can occur at levels of exposure much lower than those that result in mortality or physiological effects (Gerhardt, 2007; Melvin and Wilson, 2013). Yet, so far, our understanding of the behavioral impacts of trenbolone on fish has been limited to studies of mostly reproductive behavior (Saaristo et al., 2013; Bertram et al., 2015; Tomkins et al., 2016; Tomkins et al. 2017; Tomkins et al. 2018). However, Bertram et al. (2018) recently found that trenbolone exposed female mosquitofish were more active, more exploratory, and less social compared to unexposed females. Interestingly, Heintz et al. (2015) found a contrasting pattern of risk-taking between trenbolone exposed female and male guppies, where females exhibited increased risk-taking behavior compared to unexposed females, males exhibited decreased risk-taking behavior compared to unexposed males, suggesting that behavioral effects may also be sex specific.

Understanding the potential impacts of trenbolone on behaviors, such as predator escape, boldness, and exploration, is important for two reasons. First, both single behaviors and suites of correlated behaviors are known to be directly linked to fitness correlates such as reproduction, growth and survival (Brodin and Johansson, 2004; Sih et al., 2004; Smith and Blumstein, 2008) and can therefore have important ecologically and evolutionary consequences (Sih et al., 2012; Saaristo et al., 2018). For example, a shy individual that is tentative in searching for food may grow more slowly, whereas an individual that is bolder might take too many risks and end up being consumed by a predator (Biro et al., 2006). Second, pharmaceutically induced changes to behavioral traits under controlled laboratory conditions have been shown to correspond to effects seen in full-lake studies whereby shifts in behavior can lead to changes in habitat-use and home range size (Klaminder et al., 2016).

In the wild, a myriad of abiotic stressors can potentially influence and alter behaviors of organisms. Importantly, such stressors have the potential to interact with contaminants to influence behavior. Yet, despite this, surprisingly few behavioral studies consider the impacts of pharmaceuticals and/or EDCs in combination with other environmental stressors. One such stressor is temperature. In this regard, not only can aquatic contaminants affect the temperature tolerance of exposed organisms (Patra et al., 2007; Little and Seebacher, 2015), but temperature can also influence both the effects of the contaminants and the sensitivity of organisms to those contaminants (Cairns et al., 1978; Erickson et al., 1994; Heugens et al., 2001; Li et al., 2014; Noyes and Lema, 2015; DeCourten and Brander, 2017). Further, as the metabolic rate of organisms generally increase with temperature (Gatten, 1974; Dawson et al., 1976; Beaupre et al., 1993), the uptake of contaminants is also likely to be more severe at higher temperatures (Kennedy and Walsh, 1997). This, in turn, can have a direct bearing on certain behaviors, such as foraging, which varies with metabolic rate (Biro et al., 2010; Lans, 2012; Mathot et al., 2015). Given that temperature can alter both the toxicokinetics of chemical pollutants in exposed organisms and the physiological mechanisms and behavior of the organisms themselves (Noyes et al., 2009), it is prudent to assess effects of exposure to contamination under different thermal conditions.

Accordingly, in this study, we test if an environmentally relevant concentration of 17β-trenbolone affects ecologically important behaviors in fish and whether effects (if any) are influenced by water temperature. Specifically, we investigated if short-term (21 day) exposure to an environmentally relevant concentration of 17β-trenbolone at two different temperatures (+20 and 30 °C) alters predator escape, boldness, and exploratory behaviors in wild-caught mosquitofish (*Gambusia holbrooki*). Overall, we hypothesized, based on previous studies (Heintz et al., 2015; Bertram et al., 2018) that trenbolone exposure, would result in fish becoming less reactive to a predator strike, take more risks, and engage in more exploratory behavior. Further, we hypothesized that the strength of these predicted effects of trenbolone could be temperature dependent, with evidence suggesting that exposure to multiple stressors could result in additive, antagonistic or even synergistic effects compared to the impacts that each stressor may otherwise induce in isolation (Heugens et al., 2001; Coors and De Meester, 2008; Alton and Franklin, 2017).

2. Materials and methods

2.1. Study organism

The eastern mosquitofish (*Gambusia holbrooki*) is an excellent organism for studying the effects of androgenic EDCs due to its widespread distribution in a variety of freshwater aquatic habitats, including in agricultural and urban areas that are likely to be contaminated by a range of anthropogenic chemicals (Saaristo

et al., 2014). The mosquitofish is a small, sexually dimorphic livebearer (Family Poeciliidae), with mature males being distinctly smaller (≤3.5 cm) than mature females (≤6 cm). The species have been successfully introduced to many parts of the world and is extremely widespread and abundant (Welcomme, 1992; Lowe et al., 2000). Mosquitofish are highly adaptable and have been found in temperatures ranging from 0 to 45 °C, however, they prefer shallow and warm (~30 °C) waters (Pyke, 2005).

2.2. Animal collection and housing

Sexually mature mosquitofish were caught, using dip nets, from the Science Centre Lake (37° 54′ 28″ S, 145° 08′ 16″ E), Monash University, Victoria, Australia in March 2016. Water temperature at the time of collection ranged from 14 to 24 °C. Fish were separated by sex and acclimated to laboratory conditions (12:12 h light:dark regime, 20–23 °C) for four weeks prior to exposure. During the acclimatization period, fish were kept in same sex glass holding tanks (80 × 45 × 45 cm, 40 fish per tank) and fed once daily with commercial fish food (Otohime Hirame larval diet; 580–910 μm). Water samples taken from the Science Centre Lake confirmed no 17β-trenbolone contamination (unpublished).

2.3. Exposure set up

After acclimation, fish were randomly distributed to one of the four treatments: trenbolone exposure at 20 °C (**T20**; measured concentration 2.6 ± 0.2 ng/L), trenbolone exposure at 30 °C (**T30**; measured concentration 3.3 ± 0.3 ng/L), freshwater control at 20 °C (**C20**), or freshwater control at 30 °C (**C30**). The exposure systems were comprised of 16 tanks (54 L; 60 × 30 × 30 cm), with four tanks per treatment. The sexes were separated and distributed between these tanks, resulting in two tanks per treatment and sex (33 females per tank and 30 males per tank). Fish were exposed for 21 days in a flow-through system following previously published methods (Saaristo et al., 2013; Bertram et al., 2015, 2018; Tomkins et al., 2017, 2018). Briefly, the set-up consisted of two identical, but separate, flow-through systems with glass mixing tanks above the exposure tanks that supplied the glass exposure tanks with either carbon-filtered freshwater (control treatments) or trenbolone spiked water from a stock solution (trenbolone treatments) via a peristaltic pump (Watson Marlow 323 U/MC). During the exposure period, water samples from each of the exposure tanks were collected weekly. For a detailed description of the analysis of water samples, see 'Supplementary Methods' in Supplementary material.

The light-dark regime was kept at 12:12 h and fish were fed *ad libitum* once daily with commercial fish food (Otohime Hirame larval diet; 580–910 μm). Temperatures (**T20**: 20.4 ± 0.9 °C, **T30**: 28.7 ± 1.2 °C, **C20**: 20.5 ± 0.7 °C, **C30**: 28.5 ± 1.2 °C), and flow-through rates (14.48 ± 0.4 mL/min) were monitored daily. Flow-through rates were maintained at on a 24 h cycle (~1.67 L/h per tank).

2.4. Behavioral trials

2.4.1. Experiment 1: predator escape

Mosquitofish were exposed to a simulated predator strike to assess the impacts of 17β-trenbolone and temperature on a predator escape behavior. The experiment followed the design of Martin et al. (2017). Briefly, each trial consisted of a 0.72 L test arena (12 × 12 × 5 cm), containing 3 cm temperature treatment-matched (i.e. 20 or 30 °C) aged carbon-filtered freshwater, and a cylindrical metal probe (5 mm in diameter) with a rubber stopper aimed to simulate the predator strike. After the 21 day exposure, each focal individual (**T20**: females $n = 29$, male $n = 31$, **T30:** females $n = 33$, males $n = 28$, **C20:** females $n = 29$, males $n = 27$, **C30**: females $n = 30$, males $n = 28$) was individually introduced to the test arena and allowed to settle for 5 min. After 5 min of acclimation, the metal probe was remotely dropped within 3 cm of the fish to provoke an escape response (see Fig. S1). The escape response was video-recorded from above (Canon Powershot S110) for 1 min. After the completion of the trial, the arena was emptied and re-filled with temperature treatment-matched aged carbon-filtered freshwater to avoid trenbolone cross-contamination and conspecific chemical cues from previously tested individuals.

From each video recording, we measured the C-start escape response and general activity (measured as the total distance covered over the trial) of each focal fish. The C-start is an escape response initiated by a fear stimulus (e.g. a predator strike) that is used by many fish and aquatic amphibian species to evade predators (Domenici and Blake, 1997; Hale et al., 2002). The response is characterized by three distinct stages: rest (just before the deployment of the fear stimulus), C-bend (the individual turns away from the stimulus bending into a C-shape) and propulsion (burst acceleration swimming away from the stimulus) (Hale et al., 2002). Each stage was represented by a single camera frame captured at 30 frames per second, with frame one representing the rest stage, frame two captured the C-bend, and frame three captured the propulsion stage. A point of mass tracking software (Tracker 4.97; Open Source Physics, USA) was used to analyze the fish movements. C-start speed (cm/sec) was calculated as the distance travelled between frame two and frame three. Propulsion (cm/sec) was calculated as the distance travelled between frame three and four. A fish was considered to have performed a C-start only if it achieved the C-bend by frame two; those that performed a delayed C-bend were not included in speed measures, following protocols of Martin et al. (2017). The total distance (time × speed for each frame and then the sum of all frames) was recorded for 1 min following the simulated predator strike.

2.4.2. Experiment 2: boldness and exploration

To assess the combined impacts of 17β-trenbolone and temperature on boldness and exploration, mosquitofish were tested in a maze experiment following the design of Bertram et al. (2018) with minor modifications. For this purpose, trials were carried out in 54 L (60 × 30 × 30 cm) maze arenas. The arenas were filled with 10 cm of aged, temperature treatment-matched carbon-filtered freshwater. The mazes comprised an enclosed square opaque refuge (10 × 10 cm) with a removable door, and six "corridors", defined by opaque white acrylic internal walls, extending two-thirds of the width of the aquarium (see Fig. S2). The external walls of the aquaria were frosted to prevent external visual disturbances. The trials were video-recorded from above (Canon Powershot S110). The test individual was randomly drawn from one of the four treatments (**T20**: females $n = 25$, male $n = 14$, **T30**: females $n = 22$, males $n = 22$, **C20**: females $n = 19$, males $n = 16$, **C30**: females $n = 22$, males $n = 21$) and placed in the small box in the test arenas. After 5 min of acclimation inside the refuge, the door was remotely lifted using a pulley system, and the fish was allowed to enter the arena. Each fish was then given 20 min (from the moment the door was opened) to explore the maze. From each video recording, we quantified the time (i.e. latency) taken for fish to fully exit the refuge, as well as the time taken to pass by each maze corridor, with a shorter time indicating a bolder and more exploratory individual. After every trial, the arenas were emptied and re-filled with aged, temperature-matched carbon-filtered freshwater to avoid trenbolone cross-contamination and conspecific chemical cues from previously tested individuals.

2.4.3. Trenbolone uptake and morphological analysis

In order to assess at what time point during exposure trenbolone could be measured in whole body samples of mosquitofish, a subset of three fish were randomly sampled from one exposure holding tank per treatment (T20 females $n = 27$, T20 males $n = 27$, T30 females $n = 27$, T30 males $n = 27$) after 12 h, 16 h, 24 h, 48 h, 3 d, and 7 d of exposure (**20°C**: 2.6 ± 0.3 ng/L, **30°C**: 3.6 ± 0.5 ng/L). Fish were euthanized with an overdose of MS-222 (Ethyl 3-aminobenzoate methanesulfonate), weighed (±0.0001 g) and measured from the tip of the snout to the end of the caudal fin (±0.01 mm) before they were put in a −20 °C freezer for later internal concentration analysis. Water was also sampled from the same tanks as the fish was taken from at each time point. For a detailed description of the analysis of whole body and water samples, see 'Supplementary Methods' in Supplementary material.

Following the behavioral trials, the fish were euthanized, weighed and measured in the same way as previously mentioned. We used the Fulton's condition factor (Heincke, 1908; Nash et al., 2006) as a measure of physiological condition. As our fish were of similar size and of the same species, we use it as a relative measure of physiological states between treatments (Froese, 2006). The euthanized fish were, after measurements, placed in a −20 °C freezer for future trenbolone analysis. We used whole body concentrations (ng/kg) of the fish divided with measured water concentrations (ng/L) of trenbolone to calculate the bioconcentration factor (BCF) for each fish (Arnot and Gobas, 2006).

2.4.4. Statistical analyses

Data were checked for normality using a Kolmogorov-Smirnov test with a Lilliefors Significance Correction. If not normally distributed, data were log transformed to allow for parametric tests, where possible. If the requirements were not met after log transformation, non-parametric tests were used. All final models induced exposure, temperature and sex as predictors. Initial models also included body condition as a predictor and exposure tank as a random effect (as a measure of tank effect), however, in all models both were removed, as they did not significantly improve model fit and did not significantly influence the response variable ($p > 0.05$). When models revealed a significant interaction between exposure and/or temperature and sex, the sexes or temperatures were subsequently analyzed separately to identify the separate effects of trenbolone on each sex or temperature. All means are presented as ± 1 standard error (mean ± 1 S.E.). All statistical analyses were conducted using the statistical software IBM SPSS Statistics 24 (IBM Corp., 2016).

For the first experiment (i.e. the predator escape), the number of C-starts performed was compared among treatments using a chi-square test. To test for differences in C-start and propulsion speed between the different treatments, generalized linear models (GLM) with gamma distribution and log-link (due to positive skewed data) were used. Differences in activity levels (i.e. total distance swam) for 1 min following the simulated predator strike were compared across treatments using a linear model (LM).

For the second experiment (i.e. the boldness and exploration), the effect of treatments on boldness (i.e. time to exit the refuge) in the maze was tested with a gamma distribution GLM with log-link. A LM was used to compare exploration (i.e. time to reach first corridor) of fish in the maze across treatments. Time to reach the last corridor of the maze was analyzed using a gamma distribution GLM with log-link.

The nature of trenbolone (i.e. 17β-trenbolone) uptake (BCF) was tested with a LM, combining fish from both behavioral experiments (included as a predictor). We found an equivalent pattern for body condition in the two behavioral experiments, hence fish from both experiments were all analyzed in the same model. The internal concentration was compared among treatments using a two-way ANOVA.

3. Results

3.1. Experiment 1: predator escape

In the first experiment, we found that 82% of the control fish at 20 °C, and 81% of the control fish at 30 °C performed a C-start after the simulated predator strike. A similar fraction (78%) of the exposed fish at 20 °C performed C-starts (Chi squared: $\chi^2 = 0.315$, $df = 1$, $p = 0.575$) but at 30 °C, significantly fewer individuals (only 55%) performed a C-start when exposed to trenbolone (Chi squared: $\chi^2 = 9.156$, $df = 1$, $p = 0.002$; Fig. S4). Furthermore, in the control fish at 20 °C, more females (93%) than males (70%) performed a C-start (Chi squared: $\chi^2 = 5.180$, $df = 1$, $p = 0.023$).

There was a significantly three-way interaction detected between trenbolone exposure, temperature and sex on C-start speed (Table 1). When considering males at 20 °C, there was no significant effect of exposure treatment (GLM: $z = 1.40$, $df = 1$, $p = 0.16$; Fig. 1a). Similarly, for females at 20 °C, there was no significant effect of exposure treatment (GLM: $z = 0.035$, $df = 1$, $p = 0.970$; Fig. 1a). Focusing on males at 30 °C, there was a significant effect of exposure treatment (GLM: $z = 1.88$, $df = 1$, $p = 0.050$), with trenbolone-exposed males having a significantly slower C-start speed (Fig. 1b), whereas, for females at 30 °C, there was no significant effect of exposure treatment (GLM: $z = 1.37$, $df = 1$, $p = 0.170$; Fig. 1b). Control fish at 20 °C and 30 °C, showed no significant difference between the sexes (GLM: both $p > 0.600$; Fig. 1a and b). By contrast, for trenbolone-exposed fish at 20 °C, there was no effect of sex (GLM: $z = 1.76$, $df = 1$, $p = 0.080$), however, at 30 °C, there was a significant effect of sex (GLM: $z = 2.73$, $df = 1$, $p = 0.006$); with males having a significantly lower C-start speed than females (Fig. 1b).

For propulsion speed, there was also a significantly three-way interaction detected between trenbolone exposure, temperature and sex (Table 1). When considering males at 20 °C, there was no significant effect of exposure treatment (GLM: $z = 1.39$, $df = 1$, $p = 0.170$; Fig. 1c). Similarly, for females at 20 °C, there was no significant effect of exposure treatment (GLM: $z = 0.26$, $df = 1$, $p = 0.790$; Fig. 1c). Focusing on males at 30 °C, there was a non-significant trend that exposure affected propulsion speed (GLM: $z = 1.80$, $df = 1$, $p = 0.070$), with trenbolone-exposed males being slower (Fig. 1d). By contrast, for females at 30 °C, there was no significant effect of exposure treatment (GLM: $z = 1.34$, $df = 1$, $p = 0.180$). Control fish at 20 °C and 30 °C, showed no significant difference between the sexes (GLM: both $p > 0.050$; Fig. 1c and d). For trenbolone-exposed fish at 20 °C, there was also no effect of sex (GLM: $z = 1.04$, $df = 1$, $p = 0.300$), however, at 30 °C, there was a significant effect of sex (GLM: $z = 3.02$, $df = 1$, $p = 0.003$), with males having a significantly lower propulsion speed than females (Fig. 1d).

There was a significant two-way interaction between exposure and temperature treatment on total distance swam after the simulated predatory strike (Table 1). At 20 °C, trenbolone-exposed fish swam significantly longer distances than control fish (104 ± 5, and 81 ± 6 cm, respectively; LM: $F_{1,113} = 9.268$, $p = 0.003$) whereas at 30 °C, there was no difference between control and exposed fish (131 ± 7, and 124 ± 8 cm, respectively; LM: $F_{1,116} = 0.531$, $p = 0.468$; Fig. S3). Fish at 30 °C swam longer than fish at 20 °C (127 ± 5, and 93 ± 4 cm; Table 1). Further, we found no difference between males and females in total distance swam (female: 105 ± 5, and male: 115 ± 5 cm, LM: $F_{1,227} = 2.615$, $p = 0.107$).

Table 1
Predator escape response, boldness and exploration behavior in mosquitofish. Generalized linear model (z) and general linear model (F) results indicating the effect of trenbolone exposure (Exp) and sex on predator escape response behaviors; C-start speed, Propulsion speed, Total distance, boldness; Time to exit refuge, and exploration; Time to reach first and last maze corridor, at two different temperatures (Temp). * indicates a significant difference at $p \leq 0.05$. For all comparisons, control fish at 20 °C are the reference group.

Source	C-start velocity			Propulsion velocity			Total distance			Time to exit refuge			Time to reach first corridor			Time to reach last corridor		
	z	*df*	*p*	*z*	*df*	*p*	*F*	*df*	*p*	*z*	*df*	*p*	*F*	*df*	*p*	*z*	*df*	*p*
Exp	1.438	1	0.151	1.501	1	0.133	1.574	1	0.211	1.648	1	0.002*	4.301	1	0.040*	3.472	1	<0.001*
Temp	1.467	1	0.142	2.142	1	0.032*	30.676	1	<0.001*	0.266	1	0.149	3.709	1	0.056	1.465	1	0.410
Sex	2.088	1	0.037*	2.696	1	0.007*	2.615	1	0.107	1.966	1	0.001*	4.758	1	0.031*	1.684	1	0.439
Exp × temp	1.855	1	0.064	1.977	1	0.048*	5.476	1	0.020*	0.051	1	0.136	0.388	1	0.534	0.694	1	0.841
Exp × sex	1.872	1	0.061	2.157	1	0.031*	0.076	1	0.784	1.007	1	0.949	0.407	1	0.524	0.766	1	0.835
Temp × sex	2.583	1	0.010*	2.725	1	0.006*	0.488	1	0.485	1.387	1	0.341	2.063	1	0.153	2.957	1	0.004*
Exp x temp × sex	1.966	1	0.049*	2.363	1	0.018*	0.569	1	0.452	1.414	1	0.157	1.865	1	0.174	1.234	1	0.217

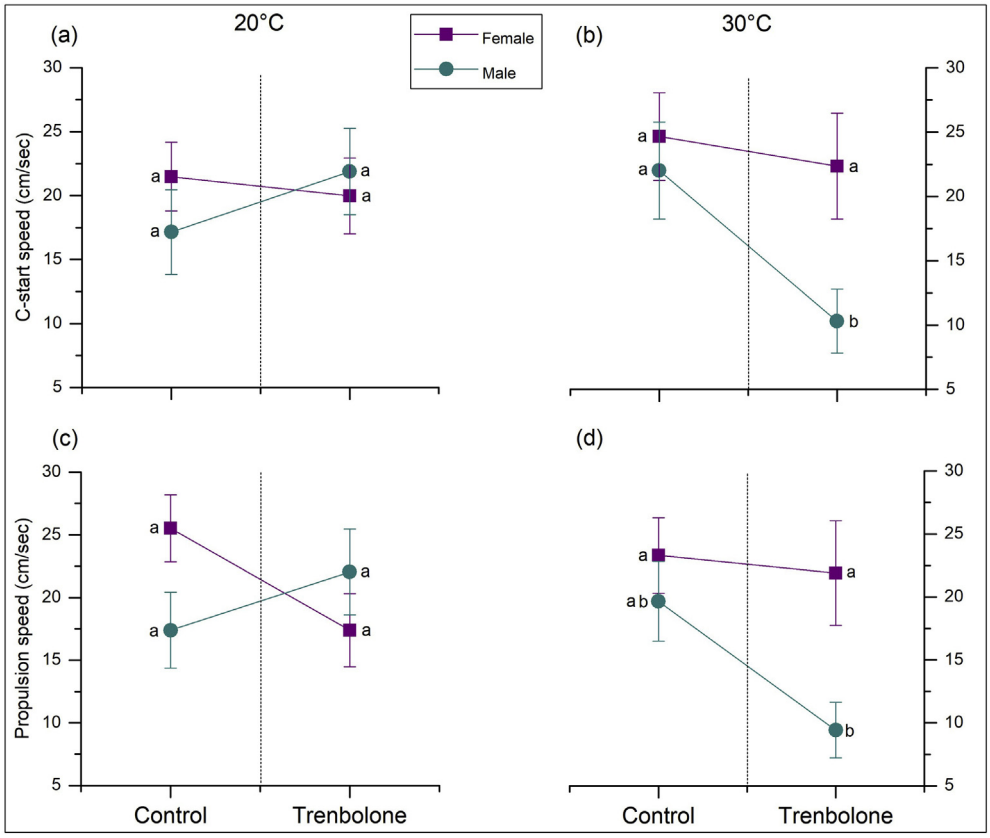

Fig. 1. Predator escape response. C-start speed of fish in control treatment versus trenbolone-exposure at **a)** 20 °C, and **b)** 30 °C, and propulsion speed of fish in control treatment versus trenbolone-exposure at **c)** 20 °C, and **d)** 30 °C. Squares indicate female and circles indicate male. Error bars represent ±1 S.E. and different letters indicates a significant difference at $p \leq 0.05$.

3.2. Experiment 2: boldness and exploration

There was no significant interaction between any of our predictors on our measure of boldness, i.e. the time to exit the refuge (sec; Table 1). In addition, there was no significant effect of temperature treatment (GLM: $z = 0.266$, $df = 1$, $p = 0.149$). There was however, a significant effect of both exposure (GLM: $z = 1.648$, $df = 1$, $p = 0.002$) and sex (GLM: $z = 1.966$, $df = 1$, $p < 0.001$), with trenbolone-exposed fish being on average, bolder than control fish (Fig. 2), and also males being significantly bolder than females (time to exit refuge: 64.3 ± 11.8 and 126.2 ± 21.6 sec, respectively).

There was no significant interaction between any of our predictors on time to reach the first maze corridor (sec; Table 1). There was a non-significant trend for fish at the higher temperature to be faster than fish at the lower temperature (132.2 ± 26.7 and 177.0 ± 32.9 sec, respectively; LM: $F_{1,152} = 3.709$, $p = 0.056$). There was a significant effect of both exposure (LM: $F_{1,152} = 4.301$, $p = 0.040$) and sex (LM: $F_{1,152} = 4.758$, $p = 0.031$), where, on average, trenbolone-exposed fish reached the first corridor faster than control fish (115.1 ± 24.2 and 193.0 ± 34.2 sec, respectively), and males were significantly faster than females (117.7 ± 27.2 and 181.9 ± 30.7 sec, respectively).

There was a significant interaction between temperature and sex on time to reach the last corridor (sec; Table 1). At 20 °C, males—averaged across both exposure treatments—reached the last corridor significantly more rapidly than females (GLM: $z = 2.20$,

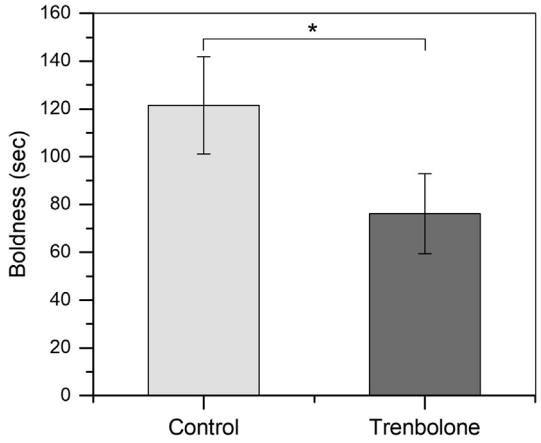

Fig. 2. Boldness of male and female mosquitofish, averaged across temperature treatments and plotted against exposure treatment. Boldness, is measured as the latency to enter a novel environment (total trial duration: 1200 sec), with a lower latency indicating a bolder individual. Error bars represent ±1 S.E. and * indicates a significant difference at $p \leq 0.05$.

$df = 1$, $p = 0.028$; Fig. 3), whereas, at 30 °C, males and females did not differ in exploration (GLM: $z = 1.23$, $df = 1$, $p = 0.22$; Fig. 3). There was a significant effect of exposure, with trenbolone-exposed fish being faster to reach the last corridor than control fish (338.4 ± 42.0 and 531.5 ± 51.6 sec, respectively; GLM: $z = 3.472$, $df = 1$, $p < 0.001$).

3.3. Trenbolone uptake and body condition

Whole body samples showed that trenbolone (ng/kg) could be detected in females held at 20 °C and males held at 30 °C from 24 h (662 ± 270 and 511 ± 209, respectively), and in females held at 30 °C and males held at 20 °C from 48 h (639 ± 261 and 659 ± 312, respectively; for more details of measured internal concentrations and water concentrations, see Table S4 and Table S5). Exposed fish at 30 °C had, at the time of the behavioral trials, higher BCFs than fish at 20 °C (LM: $F_{1,193} = 5.074$, $p = 0.025$), and females had higher BCFs than males (LM: $F_{1,193} = 36.490$, $p < 0.001$; see Table S3). Notably, the internal concentration did not differ between the temperature treatments ($F_{1,197} = 0.225$, $p = 0.636$) or the sexes ($F_{1,197} = 2.105$, $p = 0.148$).

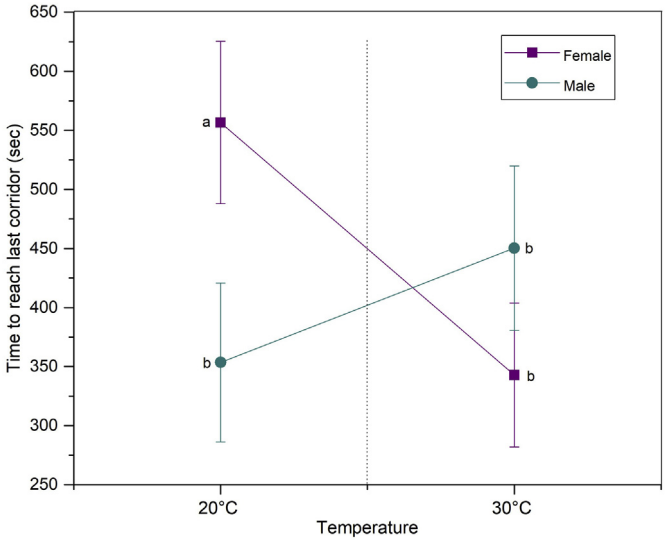

Fig. 3. Exploration of mosquitofish, averaged across exposrue treatment, plotted by temperature and split by sex. Explorations is measured as time to reach last corridor (sec; total duration 1200 sec) with a lower time indicating a more exploratory individual. Error bars represent ±1 S.E. and different letters indicates a significant difference at $p \leq 0.05$.

Both trenbolone exposure and temperature affected body condition ($K = 100(W/L^3)$). Fish exposed to trenbolone had significantly higher body conditions compared to control fish (mean × 1000 ± 1 S.E: 1.70 ± 0.014 versus 1.62 ± 0.015, respectively; LM: $F_{1,388} = 18.610$, $p < 0.001$) and fish at the higher temperature (30 °C) had significantly higher body conditions compared to fish kept at 20 °C (1.72 ± 0.013 versus 1.59 ± 0.014, respectively; LM: $F_{1,388} = 58.268$, $p < 0.001$). Further, females had higher body conditions compared to males (1.71 ± 0.014 versus 1.60 ± 0.013, respectively; LM: $F_{1,388} = 40.909$, $p < 0.001$).

4. Discussion

This is the first study to show that temperature may regulate the strength of behavioral effects induced by exposure to environmentally relevant levels of trenbolone. Further, our results reveal that some behavioral effects are sex-specific. We found significant effects of trenbolone on predator escape behavior in trials conducted at 30 °C, with trenbolone-exposed individuals performing fewer and slower predator escapes (i.e. becoming less reactive). We also found that trenbolone-exposed fish increased their boldness compared to control fish (regardless of sex and temperature), and further, had a higher activity level (i.e. total distance swam), at 20 °C (regardless of sex), and males explored the maze faster at 20 °C.

4.1. Effects of trenbolone on behaviors

We found that trenbolone-exposed mosquitofish (regardless of sex and temperature) were significantly bolder (i.e. time to exit refuge) than control fish, and that mosquitofish exposed to trenbolone (regardless of sex) explored the maze (i.e. reached the last corridor) faster than control fish at 20 °C. This was in line with our hypothesis that trenbolone would make fish take greater risks (i.e. high boldness and exploration is associated with increased risks of predator encounters), and our hypothesis that the effects of trenbolone could potentially be influenced by temperature. In this respect, a recent study found that trenbolone exposure also affected activity and exploratory behaviors (but not boldness) in female mosquitofish (Bertram et al., 2018). However, in that study, the impact of temperature was not specifically investigated. A recent study by McCormick et al. (2018) showed that, amongst a broad array of attributes (morphological, performance and behavioral), in free-living juvenile damselfish (*Pomacentrus chrysurus*), fast-start escape response latency, followed by boldness, were the most important factors explaining survival. Increased boldness and exploration behavior comes with greater risks (Houston et al., 1993), and a failed predator escape is likely to be fatal. Therefore, it is even more important for a bold individual to have a fast escape response since high survivability of bold individuals is associated with a high responsiveness to threats (the most important factor for survival; McCormick et al., 2018). In our study, we found that trenbolone altered the predator escape response in mosquitofish negatively at 30 °C, with significantly fewer individuals performing C-starts when confronted with a simulated predator compared to control fish at 30 °C. Exposed males also performed slower escape responses compared to control males at 30 °C. Thus, because individuals base many of their decisions and trade-offs on risk perception, even small changes in the perception of risk can lead to individuals behaving suboptimally in risky situations, such as during predator encounters, and thereby risking their fitness and

survival (Slabbekoorn et al., 2010; Ferrari et al., 2012; Halfwerk and Slabbekoorn, 2015).

Sex hormones are likely to affect the sexes differently. In our study, trenbolone-exposed males performed slower predator escapes than females at 30 °C, and also explored the maze faster than females at 20 °C. In contrast, Heintz et al. (2015) found that trenbolone increased risk-taking behavior in guppy females, whereas exposed males decreased their risk-taking behavior. Similarly, trenbolone has also been shown to affect reproductive behaviors in fish. For example, trenbolone exposed male guppies, increase their aggression and reduce their courting behavior (Tomkins et al., 2017), and exposed females have been found to be less choosy and reduce their time associating with males (Tomkins et al., 2016; Tomkins et al., 2018). There are several potential explanations for these contrasting results, including different exposure concentrations and regimes, and/or inter-specific differences (Saaristo et al., 2018). A greater understanding of sex-specific effects is important because differences in the behavioral response of males and females have the potential to cause differential mortality resulting in skewed sex ratios that could impact reproductive output.

4.2. Importance of water temperature

Most species experience changes in temperature on varying time-scales, ranging from predictable large-scale variation over a year (e.g. seasonal changes) to small-scale variation over the course of a day (or shorter). Importantly, temperature extremes can also act as a formidable environmental stressor. As such, it is important to assess if effects of exposure to EDCs are temperature dependent.

In line with our hypothesis, we found that several effects of trenbolone did, indeed, interact with temperature. Specifically, we found that effects on predator escape behavior was less pronounced at lower (20 °C) compared to higher temperatures (30 °C), which might be linked to higher metabolic demands and, therefore, higher motivation for fish to take risks in search for food (Biro et al., 2007). However, in the maze trial, trenbolone-exposed fish were significantly faster at exploring the whole maze than control fish only at 20 °C, with males also being generally faster than females. Further, exposed fish at 20 °C were also more active (i.e. increased the total distance covered over the trial) compared to control fish, an effect not seen at the higher temperature. Interestingly, fish at the lower temperature treatments also had lower body condition. Thus, given that the temperature optimum for mosquitofish is closer to 30 °C than 20 °C (Pyke, 2005), one possible explanation is that fish at 20 °C may be under a higher physiological stress, resulting in a higher food searching motivation (i.e. a higher boldness and exploratory behavior) due to higher energy demands. Support for such a possibility comes from a study of juvenile sea bass (*Dicentrarchus labrax*), which found an increase in risk-taking behavior after food deprivation, with the magnitude of this increase correlated with the rate of individual mass lost, which, itself, was strongly correlated with metabolic rate (Killen et al., 2011).

In regard to the behavioral assays examined in our study, the predator escape behavior can be described as a reflex response to an immediate perceived risk (i.e. the strike), whereas the maze experiment represents a situation that might be dangerous, but without direct information about risk-level. Thus, it would appear that the former actually measures an acute risk-response, while the latter is measuring the chronic level of boldness behavior of the fish. Our results indicate that the effects of trenbolone on these two types of behaviors/reactions are dependent on temperature, with a stronger effect on the acute predator escape behavior at 30 °C and a stronger effect on the chronic boldness at 20 °C. Consequently, our findings highlight that the impact of trenbolone-exposure is not only dependent on temperature and sex, but also on context. The predator escape behaviors following the simulated predator strike and the boldness behavior measured in the maze are both ecologically important, but compromising the predator escape behavior will be more directly devastating for the individual if exposed to a predator attack. Variation in escape response among individuals has been linked to differences in predator avoidance and, hence, survival (Walker et al., 2005). In this regard, risk-taking behavior (i.e. behavioral type) might result in death if predators are present, but can also lead to benefits if predators are absent. An example of this is seen in the study of Biro et al. (2006), where a domestic strain of rainbow trout preformed more risky behaviors than wild trout and, consequently, experienced lower survival compared to wild trout when predators were present. However, the domestic strain also grew faster and experienced greater survival in the absence of predators. Scharf et al. (2003) that individuals that are the most adept at evading predation are not necessarily the ones with the highest escape speed but, rather, those with the shortest reaction distance. Such findings highlight the fact that escape success is very much the product of both behavior and locomotor performance. In any case, alteration of both boldness and predator-escape behaviors can have negative population effects in the long run if fish are getting slower at escaping predators and/or taking more risks in general, with possible population declines that can result in changes in both community and food web structures (Kidd et al., 2014).

4.3. Trenbolone uptake and body condition

Trenbolone was detected in all sampled fish after 24–48 h. The morphology and tissue analyses from the behavioral trials (>21 days of exposure) showed that trenbolone-exposed fish, in general, had higher body conditions than fish in the control treatment. Trenbolone-exposed fish at 30 °C had higher BCFs and better body conditions than fish in 20 °C and females bioconcentrated more and had better body conditions compared to males in general. The higher body condition of trenbolone-exposed fish, compared to control fish, was expected given the role of trenbolone as a potent growth-promotor. Indeed, our result is consistent with earlier studies, which have similarly reported an increase in body condition of trenbolone-exposed guppies (Peña, 2008; Bertram et al., 2015). The difference in uptake between the sexes can potentially be explained by their difference in size and physiology. For instance, due to their larger size, females would have larger gill areas that might promote faster uptake of trenbolone, as well as a higher lipid content that would mediate storage of contaminants. Such factors could have potentially contributed to the observed higher bioconcentration of trenbolone in exposed females.

5. Conclusions

Based on our results, it is clear that trenbolone is highly potent at inducing behavioral alterations even at very low concentrations (≤5 ng/L). We found significant effects on ecologically important behaviors at a concentration much lower (4–7 times) than what has been measured in aquatic environments.

This study is the first to show that exposure to environmentally relevant concentrations of 17β-trenbolone interact with temperature to affect predator avoidance, boldness and exploration behavior in fish, with potentially dire consequences for individual fitness. The net ecosystem effects of these behavioral modifications remain uncertain, but according to ecological theory, they may lead to altered species interactions, population dynamics, and changed community structures (Candolin and Wong, 2012; Wong and Candolin, 2015). Further, our results show that behavioral changes caused by trenbolone in combination with temperature

are also sex specific, and such factors need to be more carefully taken into account when doing risk assessments. Together with the results of previous studies, our findings emphasize the importance of studying multiple biotic and abiotic factors associated with individual fitness and survival, and further strengthens the motivation for including behavioral endpoints in ecotoxicological studies.

Ethics

The present research was approved by the Biological Sciences Animal Ethics Committee of Monash University (permit number: BSCI/2016/05) and complies with all relevant State and Federal laws of Australia.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2018.10.116.

References

Alton, L.A., Franklin, C.E., 2017. Drivers of amphibian declines: effects of ultraviolet radiation and interactions with other environmental factors. Clim. Change Responses 4, 6.
Ankley, G.T., Jensen, K.M., Makynen, E.A., Kahl, M.D., Korte, J.J., Hornung, M.W., Henry, T.R., Denny, J.S., Leino, R.L., Wilson, V.S., 2003. Effects of the androgenic growth promoter 17-β-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. Environ. Toxicol. Chem. 22, 1350–1360.
Anway, M.D., Skinner, M.K., 2006. Epigenetic transgenerational actions of endocrine disruptors. Endocrinology 147, 43–49.
Arnold, K.E., Brown, A.R., Ankley, G.T., Sumpter, J.P., 2014. Medicating the environment: assessing risks of pharmaceuticals to wildlife and ecosystems. Phil. Trans. Biol. Sci. 369, 11.
Arnot, J.A., Gobas, F., 2006. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. Environ. Rev. 14, 257–297.
Barceloux, D.G., Palmer, R.B., 2013. Anabolic—androgenic steroids. Disease-a-Month 59, 226–248.
Baumann, L., Knörr, S., Keiter, S., Rehberger, K., Volz, S., Schiller, V., Fenske, M., Holbech, H., Segner, H., Braunbeck, T., 2014. Reversibility of endocrine disruption in zebrafish (Danio rerio) after discontinued exposure to the estrogen 17α-ethinylestradiol. Toxicol. Appl. Pharmacol. 278, 230–237.
Bean, T.G., Boxall, A.B., Lane, J., Herborn, K.A., Pietravalle, S., Arnold, K.E., 2014. Behavioural and physiological responses of birds to environmentally relevant concentrations of an antidepressant. Phil. Trans. Biol. Sci. 369, 20130575.
Beaupre, S.J., Dunham, A.E., Overall, K.L., 1993. Metabolism of a desert lizard: the effects of mass, sex, population of origin, temperature, time of day, and feeding on oxygen consumption of Sceloporus merriami. Physiol. Zool. 66, 128–147.
Bertram, M.G., Saaristo, M., Baumgartner, J.B., Johnstone, C.P., Allinson, M., Allinson, G., Wong, B.B.M., 2015. Sex in troubled waters: widespread agricultural contaminant disrupts reproductive behaviour in fish. Horm. Behav. 70, 85–91.
Bertram, M.G., Saaristo, M., Martin, J.M., Ecker, T.E., Michelangeli, M., Johnstone, C.P., Wong, B.B.M., 2018. Field-realistic exposure to the androgenic endocrine disruptor 17β-trenbolone alters ecologically important behaviours in female fish across multiple contexts. Environ. Pollut. (in press).
Biro, P.A., Abrahams, M.V., Post, J.R., Parkinson, E.A., 2006. Behavioural trade-offs between growth and mortality explain evolution of submaximal growth rates. J. Anim. Ecol. 75, 1165–1171.
Biro, P.A., Post, J.R., Booth, D.J., 2007. Mechanisms for climate-induced mortality of fish populations in whole-lake experiments. Proc. Natl. Acad. Sci. Unit. States Am. 104, 9715–9719.
Biro, P.A., Beckmann, C., Stamps, J.A., 2010. Small within-day increases in temperature affects boldness and alters personality in coral reef fish. Phil. Trans. Biol. Sci. 277, 71–77.
Brodin, T., Johansson, F., 2004. Conflicting selection pressures on the growth/predation risk trade-off in a damselfly. Ecology 85, 2927–2932.
Brodin, T., Piovano, S., Fick, J., Klaminder, J., Heynen, M., Jonsson, M., 2014. Ecological effects of pharmaceuticals in aquatic systems-impacts through behavioural alterations. Phil. Trans. Biol. Sci. 369, 10.
Brown, A.R., Gunnarsson, L., Kristiansson, E., Tyler, C.R., 2014. Assessing variation in the potential susceptibility of fish to pharmaceuticals, considering evolutionary differences in their physiology and ecology. Phil. Trans. Biol. Sci. 369, 20130576.
Cairns, J., Buikema, A.L., Heath, A.G., Parker, B.C., 1978. Effects of temperature on aquatic organism sensitivity to selected chemicals. Va. Water Resour. Res. Cent. Bull. 106, 1–88.
Campbell, R.K., Satoh, N., Degnan, B.M., 2004. Piecing together evolution of the vertebrate endocrine system. Trends Genet. 20, 359–366.
Candolin, U., Wong, B.B.M., 2012. Behavioural Responses to a Changing World: Mechanisms and Consequences. OUP, Oxford.
Cavallin, J.E., Durhan, E.J., Evans, N., Jensen, K.M., Kahl, M.D., Kolpin, D.W., Kolodziej, E.P., Foreman, W.T., LaLone, C.A., Makynen, E.A., 2014. Integrated assessment of runoff from livestock farming operations: analytical chemistry, in vitro bioassays, and in vivo fish exposures. Environ. Toxicol. Chem. 33, 1849–1857.
Cooke, B.K., Stringer, A., 1982. Distribution and breakdown of DDT in orchard soil. Pest Manag. Sci. 13, 545–551.
Coors, A., De Meester, L., 2008. Synergistic, antagonistic and additive effects of multiple stressors: predation threat, parasitism and pesticide exposure in Daphnia magna. J. Appl. Ecol. 45, 1820–1828.
Crews, D., Gore, A.C., Hsu, T.S., Dangleben, N.L., Spinetta, M., Schallert, T., Anway, M.D., Skinner, M.K., 2007. Transgenerational epigenetic imprints on mate preference. Proc. Natl. Acad. Sci. Unit. States Am. 104, 5942–5946.
Cuthbert, R.J., Taggart, M.A., Prakash, V., Chakraborty, S.S., Deori, P., Galligan, T., Kulkarni, M., Ranade, S., Saini, M., Sharma, A.K., 2014. Avian scavengers and the threat from veterinary pharmaceuticals. Phil. Trans. Biol. Sci. 369, 20130574.
Dawson, W.R., Bennett, A.F., Hudson, J.W., 1976. Metabolism and thermoregulation in hatchling ring-billed gulls. Condor 78, 49–60.
DeCourten, B.M., Brander, S.M., 2017. Combined effects of increased temperature and endocrine disrupting pollutants on sex determination, survival, and development across generations. Sci. Rep. 7, 9310.
Diamanti-Kandarakis, E., Bourguignon, J.-P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T., Gore, A.C., 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr. Rev. 30, 293–342.
Domenici, P., Blake, R., 1997. The kinematics and performance of fish fast-start swimming. J. Exp. Biol. 200, 1165–1178.
Durhan, E.J., Lambright, C.S., Makynen, E.A., Lazorchak, J., Hartig, P.C., Wilson, V.S., Gray, L.E., Ankley, G.T., 2006. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. Environ. Health Perspect. 114, 65.
Erickson, R.J., Bills, T.D., Clark, J.R., Hansen, D.J., Knezovich, J., Mayer Jr., F.L., McElroy, A.E., 1994. Synopsis of Discussion Session on Physicochemical Factors Affecting Toxicity. Bioavailability: Physical, Chemical, and Biological Interactions. Lewis, Boca Raton, pp. 31–38.
Ferrari, M.C., McCormick, M.I., Munday, P.L., Meekan, M.G., Dixson, D.L., Lönnstedt, O., Chivers, D.P., 2012. Effects of ocean acidification on visual risk assessment in coral reef fishes. Funct. Ecol. 26, 553–558.
Froese, R., 2006. Cube law, condition factor and weight–length relationships: history, meta-analysis and recommendations. J. Appl. Ichthyol. 22, 241–253.
Gall, H.E., Sassman, S.A., Lee, L.S., Jafvert, C.T., 2011. Hormone discharges from a midwest tile-drained agroecosystem receiving animal wastes. Environ. Sci. Technol. 45, 8755–8764.
Galvez, J.I., Morrison, J.R., Phelps, R.P., 1996. Efficacy of trenbolone acetate in sex inversion of the blue tilapia Oreochromis aureus. J. World Aquacult. Soc. 27, 483–486.
Gatten Jr., R.E., 1974. Effects of temperature and activity on aerobic and anaerobic metabolism and heart rate in the turtles Pseudemys scripta and Terrapene ornata. Comp. Biochem. Physiol. Physiol. 48, 619–648.
Gerhardt, A., 2007. Aquatic behavioral ecotoxicology - prospects and limitations. Hum. Ecol. Risk Assess. 13, 481–491.
Guerrero-Bosagna, C.M., Skinner, M.K., 2009. Epigenetic transgenerational effects of endocrine disruptors on male reproduction. In: Seminars in Reproductive Medicine. © Thieme Medical Publishers, pp. 403–408.
Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O., Larsson, D.G.J., 2008. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. Environ. Sci. Technol. 42, 5807–5813.
Hale, M.E., Long, J., John, H., McHenry, M.J., Westneat, M.W., 2002. Evolution of behavior and neural control of the fast-start escape response. Evolution 56, 993–1007.
Halfwerk, W., Slabbekoorn, H., 2015. Pollution going multimodal: the complex impact of the human-altered sensory environment on animal perception and performance. Biol. Lett. 11, 20141051.
Heincke, F., 1908. Bericht über die Untersuchungen der Biologischen Anstalt auf Helgoland zur Naturgeschichte der Nutzfische (1. April 1905 bis 1. Oktober 1907).

Heintz, M.M., Brander, S.M., White, J.W., 2015. Endocrine disrupting compounds alter risk-taking behavior in guppies (*Poecilia reticulata*). Ethology 121, 480–491.
Helander, B., Olsson, A., Bignert, A., Asplund, L., Litzén, K., 2002. The role of DDE, PCB, coplanar PCB and eggshell parameters for reproduction in the white-tailed sea eagle (*Haliaeetus albicilla*) in Sweden. AMBIO A J. Hum. Environ. 31, 386–403.
Hellou, J., 2011. Behavioural ecotoxicology, an "early warning" signal to assess environmental quality. Environ. Sci. Pollut. Control Ser. 18, 1–11.
Heugens, E.H., Hendriks, A.J., Dekker, T., Straalen, N. M. v., Admiraal, W., 2001. A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. Crit. Rev. Toxicol. 31, 247–284.
Houston, A.I., McNamara, J.M., Hutchinson, J.M., 1993. General results concerning the trade-off between gaining energy and avoiding predation. Phil. Trans. Biol. Sci. 341, 375–397.
Johnson, R., Hanrahan, C.E., 2010. The US-EU beef hormone dispute. In: CRS Report for Congress. Congressional Research Service.
Kennedy, C.J., Walsh, P.J., 1997. Effects of temperature on xenobiotic metabolism. In: Seminar Series - Society for Experimental Biology. Cambridge University Press, pp. 303–324.
Kidd, K.A., Paterson, M.J., Rennie, M.D., Podemski, C.L., Findlay, D.L., Blanchfield, P.J., Liber, K., 2014. Direct and indirect responses of a freshwater food web to a potent synthetic oestrogen. Phil. Trans. Biol. Sci. 369, 20130578.
Killen, S.S., Marras, S., McKenzie, D.J., 2011. Fuel, fasting, fear: routine metabolic rate and food deprivation exert synergistic effects on risk-taking in individual juvenile European sea bass. J. Anim. Ecol. 80, 1024–1033.
Klaminder, J., Hellström, G., Fahlman, J., Jonsson, M., Fick, J., Lagesson, A., Bergman, E., Brodin, T., 2016. Drug-induced behavioral changes: using laboratory observations to predict field observations. Front. Environ. Sci. 4, 81.
Kolodziej, E.P., Qu, S., Forsgren, K.L., Long, S.A., Gloer, J.B., Jones, G.D., Schlenk, D., Baltrusaitis, J., Cwiertny, D.M., 2013. Identification and environmental implications of photo-transformation products of trenbolone acetate metabolites. Environ. Sci. Technol. 47, 5031–5041.
Lagesson, A., Fahlman, J., Brodin, T., Fick, J., Jonsson, M., Byström, P., Klaminder, J., 2016. Bioaccumulation of five pharmaceuticals at multiple trophic levels in an aquatic food web-Insights from a field experiment. Sci. Total Environ. 568, 208–215.
Lange, I.G., Daxenberger, A., Schiffer, B., Witters, H., Ibarreta, D., Meyer, H.H., 2002. Sex hormones originating from different livestock production systems: fate and potential disrupting activity in the environment. Anal. Chim. Acta 473, 27–37.
Lans, L., 2012. Behaviour and Metabolic Rates of Brown Trout and Atlantic Salmon: Influence of Food, Environment and Social Interactions. Karlstad University.
Larsen, M.G., Baatrup, E., 2010. Functional behavior and reproduction in androgenic sex reversed zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 29, 1828–1833.
Li, A.J., Leung, P.T., Bao, V.W., Andy, X., Leung, K.M., 2014. Temperature-dependent toxicities of four common chemical pollutants to the marine medaka fish, copepod and rotifer. Ecotoxicology 23, 1564–1573.
Li, Y.-Y., Xu, W., Chen, X.-R., Lou, Q.-Q., Wei, W.-J., Qin, Z.-F., 2015. Low concentrations of 17β-trenbolone induce female-to-male reversal and mortality in the frog *Pelophylax nigromaculatus*. Aquat. Toxicol. 158, 230–237.
Little, A.G., Seebacher, F., 2015. Temperature determines toxicity: bisphenol A reduces thermal tolerance in fish. Environ. Pollut. 197, 84–89.
Liu, J., Wang, R., Huang, B., Lin, C., Wang, Y., Pan, X., 2011. Distribution and bioaccumulation of steroidal and phenolic endocrine disrupting chemicals in wild fish species from Dianchi Lake, China. Environ. Pollut. 159, 2815–2822.
Lowe, S., Browne, M., Boudjelas, S., De Poorter, M., 2000. 100 of the World's Worst Invasive Alien Species: a Selection from the Global Invasive Species Database. Invasive Species Specialist Group Auckland.
Martin, J.M., Saaristo, M., Bertram, M.G., Lewis, P.J., Coggan, T.L., Clarke, B.O., Wong, B.B.M., 2017. The psychoactive pollutant fluoxetine compromises antipredator behaviour in fish. Environ. Pollut. 222, 592–599.
Mathot, K.J., Nicolaus, M., Araya-Ajoy, Y.G., Dingemanse, N.J., Kempenaers, B., 2015. Does metabolic rate predict risk-taking behaviour? A field experiment in a wild passerine bird. Funct. Ecol. 29, 239–249.
McCormick, M.I., Fakan, E., Allan, B.J., 2018. Behavioural measures determine survivorship within the hierarchy of whole-organism phenotypic traits. Funct. Ecol. 32, 958–969.
Melvin, S.D., Wilson, S.P., 2013. The utility of behavioral studies for aquatic toxicology testing: a meta-analysis. Chemosphere 93, 2217–2223.
Morthorst, J.E., Holbech, H., Bjerregaard, P., 2010. Trenbolone causes irreversible masculinization of zebrafish at environmentally relevant concentrations. Aquat. Toxicol. 98, 336–343.
Nash, R.D., Valencia, A.H., Geffen, A.J., 2006. The origin of Fulton's condition factor—setting the record straight. Fisheries 31, 236–238.
Neumann, F., 1976. Pharmacological and endocrinological studies on anabolic agents. Environ. Qual. Saf. Suppl. 253–264.
Norstrom, R., Risebrough, R., Cartwright, D., 1976. Elimination of chlorinated dibenzofurans associated with polychoorinated biphenyls fed to mallards (*Anas platyrhynchos*). Toxicol. Appl. Pharmacol. 37, 217–228.
Noyes, P.D., Lema, S.C., 2015. Forecasting the impacts of chemical pollution and climate change interactions on the health of wildlife. Current Zool. 61, 669–689.
Noyes, P.D., McElwee, M.K., Miller, H.D., Clark, B.W., Van Tiem, L.A., Walcott, K.C., Erwin, K.N., Levin, E.D., 2009. The toxicology of climate change: environmental contaminants in a warming world. Environ. Int. 35, 971–986.
Olmstead, A.W., Kosian, P.A., Johnson, R., Blackshear, P.E., Haselman, J., Blanksma, C., Korte, J.J., Holcombe, G.W., Burgess, E., Lindberg-Livingston, A., 2012. Trenbolone causes mortality and altered sexual differentiation in *Xenopus tropicalis* during larval development. Environ. Toxicol. Chem. 31, 2391–2398.
Orlando, E.F., Kolok, A.S., Binzcik, G.A., Gates, J.L., Horton, M.K., Lambright, C.S., Gray Jr., L.E., Soto, A.M., Guillette Jr., L.J., 2004. Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. Environ. Health Perspect. 112, 353.
Örn, S., Yamani, S., Norrgren, L., 2006. Comparison of vitellogenin induction, sex ratio, and gonad morphology between zebrafish and Japanese medaka after exposure to 17α-ethinylestradiol and 17β-trenbolone. Arch. Environ. Contam. Toxicol. 51, 237–243.
Patra, R.W., Chapman, J.C., Lim, R.P., Gehrke, P.C., 2007. The effects of three organic chemicals on the upper thermal tolerances of four freshwater fishes. Environ. Toxicol. Chem. 26, 1454–1459.
Peña, E.M., 2008. Anabolic and androgenic effect of steroid trenbolone acetate on guppy (*Poecilia reticulata*). Vet. Mex. 39, 269–277.
Perry, P.J., Andersen, K.H., Yates, W.R., 1990. Illicit anabolic steroid use in athletes: a case series analysis. Am. J. Sports Med. 18, 422–428.
Porte, C., Janer, G., Lorusso, L., Ortiz-Zarragoitia, M., Cajaraville, M., Fossi, M., Canesi, L., 2006. Endocrine disruptors in marine organisms: approaches and perspectives. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 143, 303–315.
Pyke, G.H., 2005. A review of the biology of *Gambusia affinis* and *G. holbrooki*. Rev. Fish Biol. Fish. 15, 339–365.
IBM Corp, 2016. IBM SPSS Statistics for Windows, Version 24.0. IBM Corp, Armonk, NY.
Ross, P.S., Ellis, G., Ikonomou, M., Barrett-Lennard, L., Addison, R., 2000. High PCB concentrations in free-ranging Pacific killer whales, Orcinus orca: effects of age, sex and dietary preference. Mar. Pollut. Bull. 40, 504–515.
Ruhi, A., Acuna, V., Barcelo, D., Huerta, B., Mor, J.-R., Rodriguez-Mozaz, S., Sabater, S., 2016. Bioaccumulation and trophic magnification of pharmaceuticals and endocrine disruptors in a Mediterranean river food web. Sci. Total Environ. 540, 250–259.
Saaristo, M., Tomkins, P., Allinson, M., Allinson, G., Wong, B.B.M., 2013. An androgenic agricultural contaminant impairs female reproductive behaviour in a freshwater fish. PloS One 8, e62782.
Saaristo, M., Myers, J., Jacques-Hamilton, R., Allinson, M., Yamamoto, A., Allinson, G., Pettigrove, V., Wong, B.B.M., 2014. Altered reproductive behaviours in male mosquitofish living downstream from a sewage treatment plant. Aquat. Toxicol. 149, 58–64.
Saaristo, M., Brodin, T., Balshine, S., Bertram, M.G., Brooks, B.W., Ehlman, S.M., McCallum, E.S., Sih, A., Sundin, J., Wong, B.B.M., Arnold, K.E., 2018. Direct and indirect effects of chemical contaminants on the behaviour, ecology and evolution of wildlife. Proc. Biol. Sci. 285 (1885), 20181297.
Saito, S., Tanoue, A., Matsuo, M., 1992. Applicability of the i/o-characters to a quantitative description of bioconcentration of organic chemicals in fish. Chemosphere 24, 81–87.
Scharf, F.S., Buckel, J.A., McGinn, P.A., Juanes, F., 2003. Vulnerability of marine forage fishes to piscivory: effects of prey behavior on susceptibility to attack and capture. J. Exp. Mar. Biol. Ecol. 294, 41–59.
Schiffer, B., Daxenberger, A., Meyer, K., Meyer, H., 2001. The fate of trenbolone acetate and melengestrol acetate after application as growth promoters in cattle: environmental studies. Environ. Health Perspect. 109, 1145.
Seki, M., Fujishima, S., Nozaka, T., Maeda, M., Kobayashi, K., 2006. Comparison of response to 17β-estradiol and 17β-trenbolone among three small fish species. Environ. Toxicol. Chem. 25, 2742–2752.
Shore, R.F., Taggart, M.A., Smits, J., Mateo, R., Richards, N.L., Fryday, S., 2014. Detection and drivers of exposure and effects of pharmaceuticals in higher vertebrates. Phil. Trans. Biol. Sci. 369, 20130570.
Sih, A., Bell, A., Johnson, J.C., 2004. Behavioral syndromes: an ecological and evolutionary overview. Trends Ecol. Evol. 19, 372–378.
Sih, A., Cote, J., Evans, M., Fogarty, S., Pruitt, J., 2012. Ecological implications of behavioural syndromes. Ecol. Lett. 15, 278–289.
Slabbekoorn, H., Bouton, N., van Opzeeland, I., Coers, A., ten Cate, C., Popper, A.N., 2010. A noisy spring: the impact of globally rising underwater sound levels on fish. Trends Ecol. Evol. 25, 419–427.
Smith, B.R., Blumstein, D.T., 2008. Fitness consequences of personality: a meta-analysis. Behav. Ecol. 19, 448–455.
Söffker, M., Tyler, C.R., 2012. Endocrine disrupting chemicals and sexual behaviors in fish—a critical review on effects and possible consequences. Crit. Rev. Toxicol. 42, 653–668.
Sone, K., Hinago, M., Itamoto, M., Katsu, Y., Watanabe, H., Urushitani, H., Tooi, O., Guillette, L.J., Iguchi, T., 2005. Effects of an androgenic growth promoter 17β-trenbolone on masculinization of mosquitofish (*Gambusia affinis affinis*). Gen. Comp. Endocrinol. 143, 151–160.
Takahashi, A., Higashitani, T., Yakou, Y., Saitou, M., Tamamoto, H., Tanaka, H., 2003. Evaluating bioaccumulation of suspected endocrine disruptors into periphytons and benthos in the Tama River. Water Sci. Technol. 47, 71–76.
Tomkins, P., Saaristo, M., Allinson, M., Wong, B.B.M., 2016. Exposure to an agricultural contaminant, 17β-trenbolone, impairs female mate choice in a freshwater fish. Aquat. Toxicol. 170, 365–370.
Tomkins, P., Saaristo, M., Bertram, M.G., Tomkins, R.B., Allinson, M., Wong, B.B.M., 2017. The agricultural contaminant 17β-trenbolone disrupts male-male competition in the guppy (*Poecilia reticulata*). Chemosphere 187, 286–293.
Tomkins, P., Saaristo, M., Bertram, M.G., Michelangeli, M., Tomkins, R.B., Wong, B.B.M., 2018. An endocrine-disrupting agricultural contaminant impacts

sequential female mate choice in fish. Environ. Pollut. 237, 103–110.
Walker, D.M., Gore, A.C., 2011. Transgenerational neuroendocrine disruption of reproduction. Nat. Rev. Endocrinol. 7, 197–207.
Walker, J., Ghalambor, C., Griset, O., McKenney, D., Reznick, D., 2005. Do faster starts increase the probability of evading predators? Funct. Ecol. 19, 808–815.
Welcomme, R., 1992. A History of International Introductions of Inland Aquatic Species. In: ICES Marine Science Symposium, pp. 3–14.
Wong, B.B.M., Candolin, U., 2015. Behavioral responses to changing environments. Behav. Ecol. 26, 665–673.
Yarrow, J.F., McCoy, S.C., Borst, S.E., 2010. Tissue selectivity and potential clinical applications of trenbolone (17β-hydroxyestra-4, 9, 11-trien-3-one): a potent anabolic steroid with reduced androgenic and estrogenic activity. Steroids 75, 377–389.
Ying, G.-G., Kookana, R.S., Dillon, P., 2003. Sorption and degradation of selected five endocrine disrupting chemicals in aquifer material. Water Res. 37, 3785–3791.
Zala, S.M., Penn, D.J., 2004. Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. Anim. Behav. 68, 649–664.