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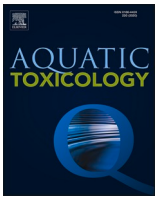

Bigger and bolder: Widespread agricultural pollutant 17β-trenbolone increases growth and alters behaviour in tadpoles (*Litoria ewingii*)

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ABSTRACT

Endocrine-disrupting chemicals—compounds that directly interfere with the endocrine system of exposed animals—are insidious environmental pollutants that can disrupt hormone function, even at very low concentrations. The dramatic impacts that some endocrine-disrupting chemicals can have on the reproductive development of wildlife are well documented. However, the potential of endocrine-disrupting chemicals to disrupt animal behaviour has received far less attention, despite the important links between behavioural processes and population-level fitness. Accordingly, we investigated the impacts of 14 and 21-day exposure to two environmentally realistic levels of 17β-trenbolone (4.6 and 11.2 ng/L), a potent endocrine-disrupting steroid and agricultural pollutant, on growth and behaviour in tadpoles of an anuran amphibian, the southern brown tree frog (*Litoria ewingii*). We found that 17β-trenbolone altered morphology, baseline activity and responses to a predatory threat, but did not affect anxiety-like behaviours in a scototaxis assay. Specifically, we found that tadpoles exposed to our high-17β-trenbolone treatment were significantly longer and heavier at 14 and 21 days. We also found that tadpoles exposed to 17β-trenbolone showed higher levels of baseline activity, and significantly reduced their activity following a simulated predator strike. These results provide insights into the wider repercussions of agricultural pollutants on key developmental and behavioural traits in aquatic species, and demonstrate the importance of behavioural studies in the ecotoxicological field.

1. Introduction

Amphibians are currently the most threatened vertebrate class on Earth, with 41% of species now at risk of extinction (IUCN, 2022). A myriad of threats have been implicated in amphibian population declines, including climate change, disease, and invasive species (Hayes et al., 2010; Olson et al., 2013; Warren et al., 2013). Anthropogenic change appears to be impacting amphibians disproportionately relative to other vertebrate taxa, with habitat alteration and loss being identified as a principal driver of amphibian declines, threatening ~46% of species (Stuart et al., 2004; Womack et al., 2022).

Chemical pollution has also been acknowledged as a major driver of amphibian declines (Egea-Serrano et al., 2012; Orton and Tyler, 2015; Womack et al., 2022). Many amphibian species are particularly vulnerable to water-borne chemical pollutants due to various physical and life-history traits, such as permeable skin, aquatic egg and larval life-stages, and a propensity to inhabit and breed in areas that receive contaminated water or areas where chemicals are intentionally applied (Bókony et al., 2020; Brand and Snodgrass, 2010; Hazell et al., 2001; Orton and Tyler, 2015; Sievers et al., 2018a). One class of chemical pollutants that are of particular concern are endocrine-disrupting chemicals (EDCs; Orton and Tyler, 2015). These chemicals have the capacity to interfere with natural hormonal functioning at extremely low concentrations (i.e. ng/L), and can alter reproduction, development and behaviour in exposed individuals (Ankley et al., 2018; Gore et al., 2015).

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A major pathway of EDCs into the environment is through the use of hormonal growth promotants (HGPs) in the agricultural industry, which are designed to increase the muscle mass of cattle (Ankley et al., 2018; Neumann, 1976). Not all of the chemicals used in HGPs are metabolised by the cattle, and are therefore excreted into the environment through faeces or urine, either unchanged or as a biologically active metabolite (Blackwell et al., 2014). Trenbolone acetate is one of the most commonly used HGPs worldwide, with a potency 15–50 times that of testosterone (Neumann, 1976). Following administration via subcutaneous injection, trenbolone acetate is hydrolysed into various metabolites that bind to androgen receptors, resulting in the desired increase in muscle mass (Neumann, 1976; Wilson et al., 2002). 17β-trenbolone is the most biologically active of these metabolites, and has been repeatedly detected in aquatic environments impacted by agricultural operations (reviewed in Ankley et al., 2018). Environmentally measured concentrations of 17β-trenbolone range from <1–270 ng/L in feedlot discharge (Bartelt-Hunt et al., 2012; Durhan et al., 2006; Khan et al., 2009; Parker et al., 2012; Schiffer et al., 2001; Soto et al., 2004; Webster et al., 2012), 3–162 ng/L in runoff from tile drains (Gall et al., 2011), and <1–8 ng/L in rivers upstream and downstream of agricultural operations (Durhan et al., 2006; Soto et al., 2004). Further compounding the threat posed by this pollutant, 17β-trenbolone has an extensive half-life (~260 days) and is rapidly taken up by aquatic organisms (Schiffer et al., 2001; Schultz et al., 2013). Various sub-lethal effects of exposure to 17β-trenbolone have been reported, including altered morphology (e.g. increased growth), male-skewed sex ratios, impacts on fecundity, and gonadal abnormalities (reviewed in Ankley et al., 2018). In addition, recent studies have reported effects of 17β-trenbolone on key behaviours in fish, such as activity, boldness, antipredator responses, foraging, sociality, and sexual behaviours (Bertram et al., 2020, 2019, M.G. 2018a, M.G. 2018b; Heintz et al., 2015; Lagesson et al., 2019; Tan et al., 2021; Tomkins et al., 2018).

However, in contrast to what we know from studies of fish, there has been far less research investigating the potential impacts of 17β-trenbolone exposure on amphibians, with only a handful of studies being conducted thus far (Ankley et al., 2018). This scarcity of data is surprising given that EDCs have been linked to amphibian population declines (Orton and Tyler, 2015). Further, amphibians are at high risk of exposure, due to inhabitation of areas where 17β-trenbolone is likely to enter the environment (i.e. water bodies in close proximity to agricultural areas) and the aquatic development of early-life stages (Babbitt and Tanner, 2000; Hazell et al., 2001; Melvin et al., 2018). Current research has indicated that 17β-trenbolone can impact amphibian survival and sexual development (Haselman et al., 2016; Li et al., 2015; Olmstead et al., 2012; Rozenblut-Kościsty et al., 2019). However, many of these studies use concentrations of 17β-trenbolone that far exceed those found naturally in the environment. In order to accurately identify the threats posed by chemical pollutants, such as 17β-trenbolone, the environmental realism of ecotoxicological studies must be increased, including through the use of environmentally relevant concentrations of exposure chemicals (Ågerstrand et al., 2011; Bertram et al., 2022; Rudén et al., 2017). Further, despite the important role of behaviour in an organism's ability to adapt to environmental changes (Wong and Candolin, 2015), to our knowledge, research addressing potential effects of 17β-trenbolone exposure on amphibian behaviour is limited to just two studies (Martin et al., 2022; Orford et al., 2022).

Accordingly, we tested the effects of 14- and 21-day exposure to environmentally realistic concentrations of 17β-trenbolone during early-life development on antipredator and anxiety-like behaviours, as well as growth, in tadpoles of an Australian anuran amphibian, the southern brown tree frog (*Litoria ewingii*). To our knowledge, this is the first study to look at the effects of environmentally realistic levels of trenbolone on amphibian growth during early life stages, and the first to look at potential impacts on this species. The 17β-trenbolone concentrations used in this study (nominal concentrations: 10 and 50 ng/L) represent concentrations detected in environments exposed to run-off from agricultural operations and in highly polluted sites located directly on agricultural land, respectively (Durhan et al., 2006; Gall et al., 2011). Based on effects observed in other aquatic vertebrates (e.g. Ankley et al., 2003; M.G. Bertram et al., 2018b; Heintz et al., 2015; Hemmer et al., 2008; Lagesson et al., 2019), we hypothesised that exposure to 17β-trenbolone would increase growth rates (resulting in larger tadpoles), and decrease antipredator and anxiety-like behaviours.

2. Material and methods

2.1. Animal collection

Thirty-six partial egg masses of the southern brown tree frog were collected from six different populations in Tasmania, Australia (i.e. six partial egg masses per population; see Table S1 for site list and coordinates). Egg masses were collected from these locations because the use of hormonal growth promotants are not permitted for use in Tasmania (Hunter, 2010), and egg masses were collected from pristine environments (i.e. no agricultural operations were nearby), thus ensuring that our study population had no prior exposure to 17β-trenbolone. Egg masses were transported to Monash University where they were held separately in containers (16 × 11 × 5 cm; length × width × height) filled with aged carbon-filtered fresh water in an incubator that maintained the temperature to 12.0 ± 0.1 °C (mean ± SD) and had a 12:12 h light:dark regime. Tadpoles were housed in these conditions for approximately 4 months. During this time, 75% water changes were conducted once a week using carbon-filtered water. This 4-month maintenance period with frequent water changes, allowed for the depuration of any potential pollution load from the collection site.

2.2. Experimental exposure

Upon reaching Gosner stage 25 (Gosner, 1960), tadpoles from each egg mass were randomly distributed to individual tanks in one of three exposure treatments for 21 days: a freshwater control treatment (hereafter referred to as control; $n = 63$), a low-17β-trenbolone treatment ($n = 64$; nominal concentration: 10 ng/L) or a high-17β-trenbolone treatment ($n = 63$; nominal concentration: 50 ng/L), with morphological and behavioural measurements occurring after 14 and 21 days. These time points were chosen as previous research has shown that 17β-trenbolone exposures of this duration are sufficient to induce behavioural and morphological changes in fish, including effects on mass, anal fins, and foraging and anxiety-like behaviour (e.g. Ankley et al., 2003; M.G. Bertram et al., 2018b; Heintz et al., 2015; Lagesson et al., 2019; Sone et al., 2005; Tan et al., 2021). Introduction to the exposure system was done in batches staggered over a 5-day period (i.e. 15 tadpoles were introduced to each treatment on each of the first 4 days, and on day 5, 3 tadpoles were introduced to the control treatment, 4 to the low-17-β-trenbolone treatment, and 3 to the high-17β-trenbolone treatment). On the day prior to being introduced to the exposure system, tadpoles were moved from the incubator to a room that maintained the air temperature at 19.4 ± 0.1 °C, in order to acclimate them to the exposure room temperature. The difference in temperature between pre-exposure housing and the exposure itself was due to the egg masses being collected during winter, with the pre-exposure temperature of 12 °C chosen to reflect environmental temperatures during that particular period. When the exposure took place, it was during the spring/summer time, and the increase in temperature to 19 °C reflected the seasonal increase in temperature. Both temperatures are environmentally realistic, and were applied evenly across all tadpoles and treatments. When distributing tadpoles to the three experimental treatments, the number of tadpoles from each population and egg mass was approximately balanced across treatments in order to avoid clutch effects (Gibbons and George, 2013). Due to the staggered introduction, tadpole age at the beginning of the exposure, and subsequently when they underwent behavioural and morphological assays at 14 and 21 days, varied by 1–5

days. Experimental batch (i.e. the day they were introduced to the exposure) was therefore included in statistical models to control for any potential effects on morphology or behaviour this variation may have introduced (Orford et al., 2022; Touchon et al., 2013; see statistical analysis below). During the exposure, tadpoles were held individually in glass tanks (diameter = 11.5 cm, height = 22.6 cm) in 400 mL aged carbon-filtered fresh water, and 120-mL water changes using aged carbon-filtered fresh water were performed weekly. Tadpoles were fed commercial fish food (Sera Flora Tropical Vegetable Flakes, Heinsberg, Germany) three times a week. At the beginning of the exposure, all low- and high-17β-trenbolone tanks received an initial dose of 0.004 μg or 0.04 μg of 17β-trenbolone (CAS: 10161-33-8; Novachem, Germany) dissolved in 1 mL of ethanol (HPLC grade, ≥ 99.99%), respectively. As a solvent control, all tanks in the control treatment received an initial 1-mL dose of ethanol (Tomkins et al., 2016). Based on the results of a 7-day pilot, all low- and high-17β-trenbolone tanks were re-dosed once a week with 0.0024 μg or 0.012 μg of 17β-trenbolone dissolved in 1 mL of ethanol, respectively, in order to maintain 17β-trenbolone concentrations (see Table S2 for full pilot results). Control tanks were re-dosed with 1 mL of ethanol.

In order to monitor 17β-trenbolone concentrations in the low- and high-17β-trenbolone treatments, and to ensure there were no contaminations in the control treatment, weekly water samples (25 mL) were taken. In the low- and high-17β-trenbolone treatments, six tanks were tested per treatment per week ($n = 18$ per treatment over the three-week exposure period). In the control treatment, two tanks were tested per week ($n = 6$ over the three-week exposure period). Due to the staggered introduction to the exposure, the day (i.e. Monday/Tuesday/Wednesday etc.) that the water sampling occurred on differed between experimental batches, but the amount of time between dosing and sampling was equal across all tanks and treatments. The concentration of 17β-trenbolone was measured using liquid chromatography–tandem mass spectrometry (Shimadzu 8050 LCMSMS), performed by Envirolab Services (MPL Laboratories; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025), with a quantification limit of 5 ng/L. The mean measured concentrations (± SD) for the low- and high-17β-trenbolone treatments during the 21-day exposure were 4.6 ± 3.44 ng/L and 11.2 ± 2.62 ng/L, respectively. For a detailed description of the analytical procedure, see supplementary material.

In addition to water samples, water temperature (19.0 ± 0.2 °C, mean ± SD; $n = 144$) and pH (7.41 ± 0.2, mean ± SD; $n = 144$) were recorded four times per week in order to ensure consistent conditions across tanks (see Table S3 for treatment summaries). Tadpole survival at 14 and 21 days did not differ between treatments (quasi-binomial logistic regression: $\chi^2 = 0.23$, $p = 0.890$ and $\chi^2 = 0.977$, $p = 0.613$, respectively; see Table S4 for sample size summaries).

2.3. Behavioural assays

In order to investigate any potential effects of 17β-trenbolone exposure on anti-predator and anxiety-like behaviours, tadpoles underwent two separate behavioural assays: a simulated predator strike assay conducted after 14 days of exposure, and a scototaxis assay conducted after 21 days of exposure (both detailed below). Immediately following completion of the simulated predator strike assay, tadpoles were returned to their respective exposure treatment tanks until they underwent the scototaxis assay. All trials and subsequent scoring were performed blind to treatment. All trials were video-recorded from above (100 frames per second; Sony FDR-AX33). Trial tanks were emptied and dried between trials in order to prevent residual conspecific cues.

After 14 days of exposure to experimental treatments, tadpoles (control $n = 25$; low-17β-trenbolone $n = 28$; high-17β-trenbolone $n = 27$) underwent a simulated predator strike assay adapted from previously established protocols (Martin et al., 2017). Briefly, trials took place in an observation tank (12 × 12 × 5 cm) filled with aged carbon-filtered fresh water to a depth of 3 cm (18.7 ± 0.1 °C). This water depth was chosen in order to limit vertical movement of the tadpoles, allowing for more accurate tracking of escape behaviour (Langerhans et al., 2004; Martin et al., 2017). Tadpoles were acclimated for 5 min in the observation tank. Tadpole activity (i.e. distance covered) prior to the simulated predatory strike was then recorded for 5 min, after which a metal probe with a rubber stopper (5 mm in diameter) was dropped into the tank to elicit an escape response. An automatic lever system was used to ensure uniform dropping of the probe. Tadpole activity after the strike was then recorded for a further 5 min, after which the trial was complete. Tadpole activity, i.e. the distance covered (cm) in the 5 min pre- and post-strike periods, was extracted by tracking tadpole movement at one-second intervals (i.e. every 100 frames). Tadpole escape response distance (cm) was extracted by tracking tadpole movement at 0.01-second intervals (i.e. every frame) over one second, starting from when the metal probe hit the surface of the water. All data for this assay were extracted using a point of mass tracking software (Tracker V8; Open Source Physics, USA).

After 21 days of exposure to experimental treatments, tadpoles (control $n = 22$; low-17β-trenbolone $n = 20$; high-17β-trenbolone $n = 25$) were then tested in a scototaxis assay following the protocols of Maximino et al. (2010). Scototaxis, a preference for dark over light environments, is a behavioural trait exhibited by many aquatic species (Gouveia Jr et al., 2005; Martin et al., 2020; Maximino et al., 2007; Serra et al., 1999). Behaviour in scototaxis trials reflects a conflict between the preference of an animal for protected areas (i.e. the dark zone) and exploratory behaviour (i.e. the light zone), and scototaxis assays are therefore often used to measure anxiety or fear-like behaviour (Cohen and Putts, 2013; Herculano et al., 2015; Maximino et al., 2010). Trials took place in observation tanks (25 × 15 × 15 cm) filled with aged carbon-filtered fresh water to a depth of 5 cm (18.9 ± 0.2 °C). The tank was divided in half into a white and black zone (where lighting was consistent across both zones but colour varied), using either white or black opaque film applied to the outside of the tank. At the start of the trial, tadpoles were placed in an acclimation chamber (12-cm diameter), positioned in the centre of the tank, for 5 min. The acclimation chamber was then remotely removed using a pulley system to minimise disturbance, and tadpoles were allowed to freely explore the tank for 15 min after which the trial was complete. From the resulting videos, behaviours were scored using the key logging software BORIS (Friard and Gamba, 2016). Specifically, the following behaviours were recorded: (1) latency to transition into the white zone (seconds); (2) total number of transitions between zones; and (3) total time spent in the white zone (seconds).

2.4. Morphological measurements

Immediately prior to being introduced to exposure treatments (i.e. pre-exposure), morphological measurements were taken for all tadpoles. Specifically, tadpoles were laterally photographed (Nikon DSLR D80) for measures of total length (± 0.01 mm), extracted using ImageJ software (Schneider et al., 2012). Tadpoles were then blotted dry and weighed (± 0.0001 g; XS105 Analytical Balance, Mettler Toledo, Melbourne, Australia). The same morphological measurements were then taken at two further timepoints: after 14 days of exposure (i.e. immediately following the simulated predator strike assay), and after 21 days of exposure (i.e. immediately following the scototaxis assay). As a proxy for body condition, a scaled mass index (SMI) was calculated for all tadpoles (*sensu* Peig and Green, 2009). Specifically, we performed a standard major axis regression on the log of body mass (M; mg) and total length of tadpoles (L; cm), and calculated a beta coefficient (β) which was then used to obtain the SMI for each tadpole (Peig and Green, 2009).

2.5. Statistical analyses

Statistical analyses were conducted using *R* version 4.1.1 (R Core Team, 2023). Where appropriate, data were transformed to approximate

Gaussian error distributions and continuous predictors were scaled to aid model fitting. Models were selected using Akaike's Information Criterion (AIC; see Tables S5–15 for final models). F-tests (for linear mixed-effects models; *lme4* package; Bates et al., 2015) and Wald's χ^2 tests (for cox proportional hazards mixed-effect models; *coxme* package; Therneau, 2022) with Kenward-Roger Degrees of Freedom Approximation were used to calculate *p*-values of fixed effects (type-III tests were used where interaction terms were included in models, if no interaction terms were included type-II tests were used). Where significant main effects were detected, pair-wise comparisons using Tukey's *p*-adjustments were performed (where this was not appropriate, Sidak's *p*-adjustments were used instead; *emmeans* package; Lenth et al., 2022).

Morphological data (total length, mass and body condition) were analysed using linear mixed-effects (LME) models. All models included exposure treatment (control, low-17β-trenbolone, high-17β-trenbolone) and exposure timepoint (i.e. days 0, 14 and 21 of the exposure), and an interaction between the two, as fixed effects. In addition, individual ID and experimental batch (i.e. the day an individual was introduced to the exposure system) were included as random intercepts.

Data from the simulated predator strike assay (tadpole activity and escape response distance) were analysed using LME models. For both endpoints, global models included exposure treatment (control, low-17β-trenbolone, or high-17β-trenbolone), tadpole mass (mg), time of day, trial tank (i.e. which tank the trial took place in), and trial tank temperature as fixed effects. Experimental batch was included as a random intercept. For tadpole activity, strike period (i.e. pre-strike and post-strike), as well as an interaction between treatment and strike period, were also included as fixed effects, and individual ID as a random intercept. For escape response, the distance between the tadpole and the striker when it hit the surface of the water was included as a fixed effect.

Data from the scototaxis assay (latency to transition into the white zone, number of transitions between zones, and total time spent in white zone) were analysed using LME models. For all measured endpoints, global models included exposure treatment (control, low-17β-trenbolone, or high-17β-trenbolone), tadpole mass (mg), time of day, trial tank, and trial tank temperature as fixed effects. For the number of transitions between zones and total time spent in the white zone data, the zone that a tadpole initially entered after being released from the acclimation chamber (white or black) was also included as a fixed effect. Experimental batch was included as a random intercept for all endpoints. For latency to transition into the white zone, a Cox Proportional Hazards mixed-effects survival analysis was used.

3. Results

3.1. Analytical verification of 17β-trenbolone concentrations

To account for left-censoring of 17β-trenbolone due to the method detection limit (MDL: 5 ng/L), all trenbolone samples that fell below the MDL (low-17β-trenbolone $n = 12$; high-17β-trenbolone $n = 8$) were included in the analysis as the MDL divided by 2, following Antweiler and Taylor (2015). The mean measured concentrations (± SD) for the low- and high-17β-trenbolone treatments during the 21-day exposure were 4.6 ± 3.44 ng/L and 11.2 ± 2.62 ng/L, respectively (see Table S16 for a full list of 17β-trenbolone concentration results).

3.2. Simulated predator strike assay

The best supported model included a marginally non-significant interaction between 17β-trenbolone treatment and the strike period (i.e., pre- or post-strike) on tadpole activity (i.e. distance moved) during the simulated predator strike assay ($F_{2,77} = 2.72$, $p = 0.072$). Specifically, we found that the distance moved by tadpoles prior to the simulated predator strike (i.e. the baseline activity of tadpoles) was significantly greater in those exposed to low- and high-17β-trenbolone concentrations compared to tadpoles from the control treatment ($t = -3.45$, $df = 101.0$, $p = 0.007$; $t = -3.77$, $df = 98.5$, $p = 0.003$, respectively; Fig. 1). However, tadpoles exposed to low- and high-17β-trenbolone concentrations did not differ significantly in their baseline activity levels prior to the simulated predator strike ($t = -0.56$ $df = 98.6$, $p = 0.999$; Fig. 1).

Following the simulated predator strike, the distance moved by tadpoles from the control treatment was not significantly different from that prior to the strike ($t = 0.68$, $df = 77.0$, $p = 0.998$; Fig. 1). However, the distance moved by tadpoles exposed to both low- and high-17β-trenbolone concentrations was significantly reduced following the simulated predator strike ($t = 4.02$, $df = 77.0$, $p = 0.001$; $t = 3.07$, $df = 77.0$, $p = 0.026$, respectively; Fig. 1), and was not significantly different from that of control tadpoles ($t = -1.59$, $df = 101.0$, $p = 0.669$; $t = -2.49$, $df = 98.5$, $p = 0.123$, respectively; Fig. 1). That is, exposed tadpoles moved significantly less after the simulated strike whereas unexposed tadpoles did not alter their activity.

The best supported model indicated that there was no significant effect of treatment on escape response distance ($F_{2,69.7} = 2.52$, $p = 0.088$; Fig. S1). There was, however, a significant effect of distance between a tadpole and the striker on escape response ($F_{1,71.7} = 23.0$, $p < 0.001$; Fig. S2), with tadpoles that were closer to the striker when it hit the water's surface moving further during the escape response. There was also a marginally non-significant effect of mass on escape response ($F_{1,9.0} = 4.76$, $p = 0.057$; Fig. S3), with heavier tadpoles moving greater distances.

3.3. Scototaxis assay

The best supported model indicated that there was no significant effect of 17β-trenbolone treatment on the time taken for tadpoles to enter the white zone ($\chi^2 = 4.04$, $p = 0.133$; Fig. S4), nor on the number of transitions between zones in the scototaxis assay ($F_{2,48.6} = 0.97$, $p = 0.386$; Fig. S5). There were no significant impacts of any other fixed effects on either endpoint (see Tables S8 and S9).

Overall, tadpoles spent more time in the white zone (741.93 secs ± 175.83; mean ± SD) than the black zone (156.05 secs ± 176.32);

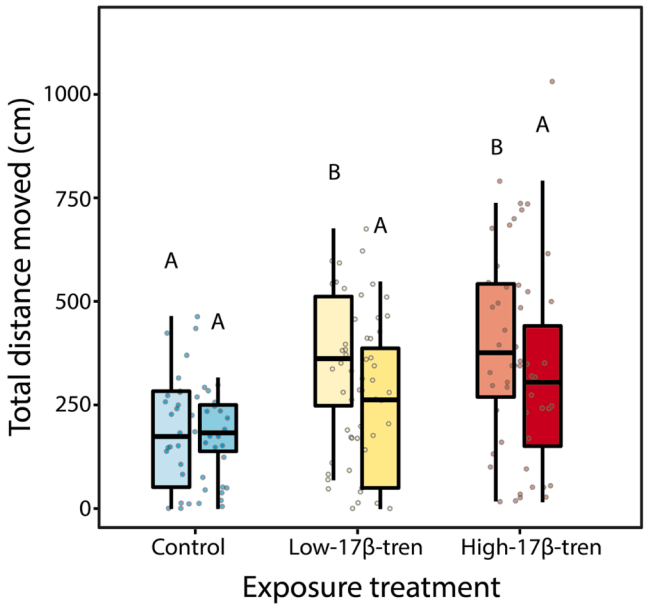

Fig. 1. Total distance moved (cm) by tadpoles prior to the simulated predator strike (lighter coloured boxes on the left for each treatment) and post-simulated predator strike (darker coloured boxes on the right for each treatment), for the control (blue; $n = 25$), low-17β-trenbolone (yellow; $n = 28$), and high-17β-trenbolone (red; $n = 27$) treatments. Treatment groups that do not share upper case letters are significantly different ($p < 0.05$).

however, this did not differ amongst 17β-trenbolone treatments ($F_{2,50.8} = 1.72$, $p = 0.190$; Fig. S6). The total time tadpoles spent in the white zone was, however, significantly impacted by what zone the tadpole initially entered after being released from the acclimation chamber ($F_{1,51.5} = 11.56$, $p = 0.001$; Fig. S7). That is, tadpoles that immediately entered the white zone subsequently spent more time in that zone than tadpoles that immediately entered the black zone.

3.4. Morphology

When investigating the effect of 17β-trenbolone treatment on morphology, the best supported models indicated a marginally non-significant interaction between exposure treatment and exposure timepoint (i.e. days 0, 14 and 21 of the exposure) on total length ($F_{2,145.6} = 2.90$, $p = 0.058$), and a significant interaction between exposure treatment and exposure timepoint for mass ($F_{2,146.5} = 3.99$, $p = 0.021$). Pairwise comparisons of the slope of the relationship between mass and time and length and time revealed that tadpoles in the high-17β-trenbolone treatment showed a slightly greater increase in their total length and mass over time (i.e. tadpole growth rate; Table 1, Figs. 2a and 3a, respectively) compared to tadpoles in the control treatment. There were no significant differences in how either total length or mass changed over time between tadpoles in the control and low-17β-trenbolone treatments, or between tadpoles in the low- and high-17β-trenbolone treatments (Table 1).

With the interactions between treatment and exposure timepoint in mind, the difference in length and mass between treatments was compared at 14 and 21 days (i.e. model comparison-centred at 14 and 21 days). We also confirmed there were no treatment differences in the total length or mass of tadpoles pre-exposure (i.e. experimental day 0; see Tables S12 and S14, Figs. 2b and 3b). At both 14 and 21 days, there was a significant difference in the total length (Table 1, Fig. 2b) and mass (Table 1, Fig. 3b) of tadpoles in the control and high-17β-trenbolone treatments. However, there were no differences in either total length or mass between the control and low-17β-trenbolone treatments at both 14 and 21 days (Table 1). There was also no significant difference in either total length or mass between the low- and high-17β-trenbolone treatments at 14 days (Table 1). However, there was a significant difference between the low- and high-17β-trenbolone treatments at 21 days for both total length (Table 1, Fig. 2b) and mass (Table 1, Fig. 3b).

For body condition, there was no significant effect of treatment ($F_{2,195.4} = 1.29$, $p = 0.279$; see Fig. S8), nor was there an interaction between 17β-treatment and exposure timepoint ($F_{2,149.2} = 1.51$, $p = 0.225$).

Table 1
Pairwise comparisons for the effects of 17β-trenbolone treatment (control, low, high) on tadpole total length (mm) and mass (mg). Tukey's Post Hoc testing was used to calculate *p*-values. Significant *p*-values are indicated in bold. Degrees of freedom are denoted by "d.f." and t-statistics by "t".

Length slope comparison (i.e. length growth rate)			
17β-trenbolone treatment	d.f.	t	*P* value
Control – Low	146.0	–0.70	0.763
Control – High	145.0	–2.34	0.054
Low – High	146.0	–1.62	0.239
14-day length comparison			
17β-trenbolone treatment	d.f.	t	*P* value
Control – Low	78.3	–0.81	0.701
Control – High	76.3	–2.85	0.016
Low – High	78.5	–2.08	0.101
21-day length comparison			
17β-trenbolone treatment	d.f.	t	*P* value
Control – Low	110.0	–0.94	0.615
Control – High	105.0	–3.30	0.004
Low – High	110.0	–2.37	0.050
Mass slope comparison (i.e. mass growth rate)			
17β-trenbolone treatment	d.f.	t	*P* value
Control – Low	147.0	–0.37	0.926
Control – High	146.0	–2.59	0.028
Low – High	147.0	–2.22	0.071
14-day mass comparison			
17β-trenbolone treatment	d.f.	t	*P* value
Control – Low	79.8	–1.17	0.473
Control – High	76.8	–3.41	0.003
Low – High	80.0	–2.27	0.067
21-day mass comparison			
17β-trenbolone treatment	d.f.	t	*P* value
Control – Low	128.0	–1.15	0.483
Control – High	121.0	–3.92	<0.001
Low – High	128.0	–2.76	0.018

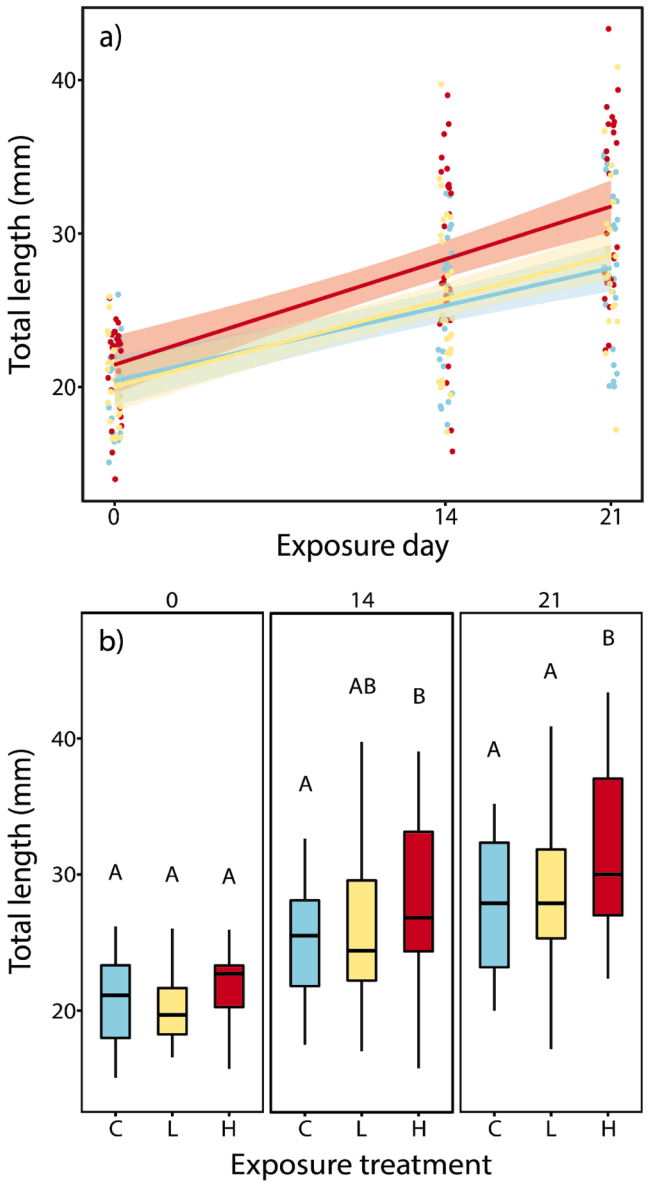

Fig. 2. Plot showing (a) the linear relationship between total length (mm) and exposure day with shaded areas around lines representing 95% confidence intervals, and (b) the median tadpole total length (mm) at each time point, plotted for control (C; blue), low-17β-trenbolone (L; yellow) and high-17β-trenbolone (H; red) treatments, where the box plots show 25th (Q1), 50th (median), and 75th (Q3) percentiles, and the whiskers represent the Q1–1.5 × IQR (interquartile range) to Q3+1.5 × IQR. Treatment groups that do not share upper case letters are significantly different ($p < 0.05$).

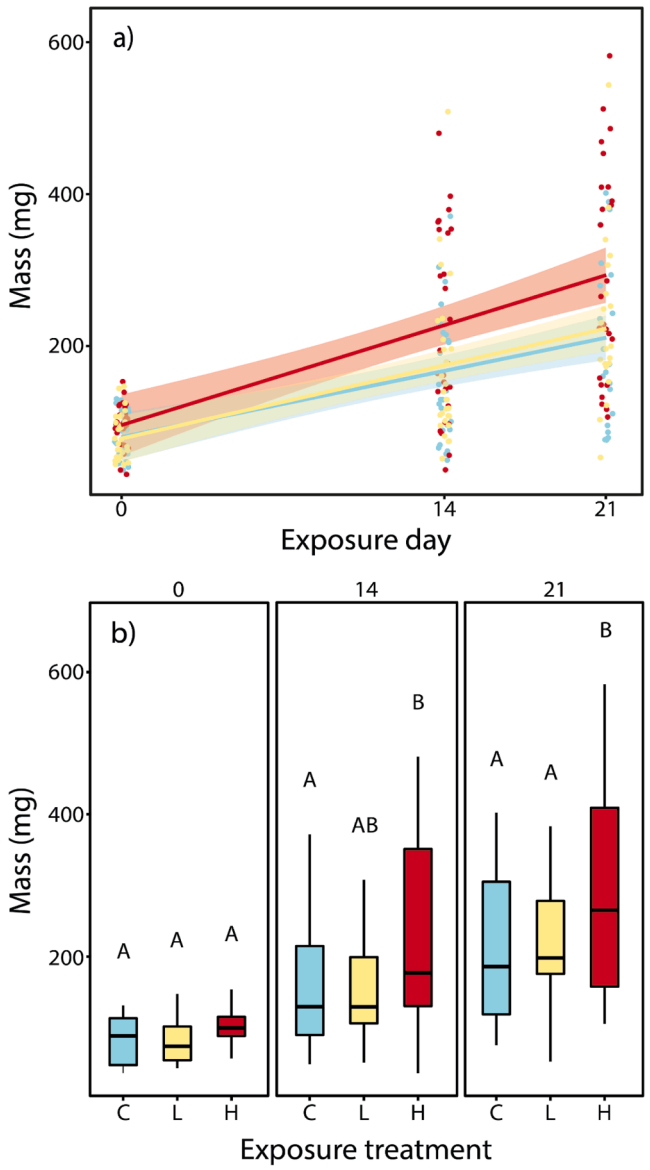

Fig. 3. Plot showing (a) the linear relationship between mass (mg) and exposure day with shaded areas around lines representing 95% confidence intervals, and (b) the median tadpole mass (mg) at each time point, plotted for control (C; blue), low-17β-trenbolone (L; yellow) and high-17β-trenbolone (H; red) treatments, where the box plots show 25th (Q1), 50th (median), and 75th (Q3) percentiles, and the whiskers represent the Q1–1.5 × IQR (interquartile range) to Q3+1.5 × IQR. Treatment groups that do not share upper case letters are significantly different ($p < 0.05$).

4. Discussion

Here, we investigated the effects of 14 and 21 day exposure to environmentally realistic levels of the agricultural pollutant 17β-trenbolone on tadpole morphology and behaviour. We found that tadpoles exposed to the high-17β-trenbolone treatment had higher growth rates than unexposed tadpoles, resulting in longer and heavier tadpoles at 14 and 21 days. Despite recent reported evidence that exposure to 17β-trenbolone may not affect tadpole behaviour (Martin et al., 2022; Orford et al., 2022), here we show that 17β-trenbolone exposure resulted in significantly altered baseline activity, as well as impacting how tadpoles responded to a simulated predatory threat. However, no significant effect of exposure was seen on anxiety-like behaviours in the scototaxis assay.

4.1. Simulated predator strike assay

Although we found no effect of 17β-trenbolone exposure on escape response distance, exposure did result in significantly higher levels of activity prior to a predator strike (i.e. baseline activity), and altered activity following a simulated predatory threat, with exposed tadpoles moving significantly less after a simulated strike whilst unexposed tadpoles did not alter their activity. While we found an effect of 17β-trenbolone on tadpole morphology, we did not find an effect of morphology on distance moved pre- or post-strike in this assay (i.e., morphology was not driving the changes in activity between treatments). The lack of response to a simulated predator strike by control tadpoles could be a result of anxiety-like behaviour. The lower levels of baseline activity exhibited by tadpoles in the control treatment compared to exposed tadpoles suggests control tadpoles may have been showing increased anxiety after being introduced to a novel environment, which may explain why they had no response to an anxiety-inducing stimulus (i.e. the simulated predator strike). Exposed tadpoles, however, were initially bolder (as exhibited by higher levels of baseline activity), and therefore showed a greater response to the simulated strike, reducing their activity post-strike to the level of the controls. Given that the 17β-trenbolone exposed tadpoles only demonstrated higher baseline activity, and all tadpoles had similar levels of activity post-strike, we can assume that exposure to 17β-trenbolone reduced baseline levels of anxiety-like behaviour. Indeed, fish exposed to 17β-trenbolone have exhibited reduced anxiety-like behaviour, with exposed individuals exiting refuges and completing mazes faster, exploring more sections of mazes, and swimming further while doing so, thereby suggesting that 17β-trenbolone can impact activity and anxiety-like behaviour (M.G. Bertram et al., 2018b; Lagesson et al., 2019).

The literature investigating the effects of 17β-trenbolone on tadpole behaviour is currently limited, making cross-species comparisons difficult (Ankley et al., 2018). However, the findings presented here do contrast with our previous study (Orford et al., 2022), in which we found that exposure to 17β-trenbolone had no effect on *Limnodynastes tasmaniensis* baseline activity, and that regardless of exposure treatment, tadpoles moved significantly less following a simulated predator strike. Furthermore, in our previous study we found that there were no significant differences in post-strike activity between exposed and unexposed tadpoles of L. *tasmaniensis*. Given this disparity in results, we would recommend that further study is needed to investigate the effects of 17β-trenbolone on amphibian behaviour before we can make any definitive conclusions about the impacts of this pollutant on amphibians, more generally. However, whilst literature investigating the effects of 17β-trenbolone on tadpole behaviour is scarce, other studies have, in agreement with the present study, found that tadpoles exposed to chemical pollutants (including EDCs) can exhibit higher levels of activity, and following a predator threat, reduce activity and swimming speed (Ehrsam et al., 2016; Gabor et al., 2019; Rohr and McCoy, 2010; Sievers et al., 2018b). The differences between our present and previous study (Orford et al., 2022) could therefore be due to species-specific responses to chemical exposure. For example, Bókony et al. (2020) reported that exposure of agile frogs (*Rana dalmatina*) and common toads (*Bufo bufo*) to the same concentration of chemical (0.3 ug/L terbuthylazine) resulted in different behavioural outcomes—*R. dalmatina* tadpoles exhibited significantly lower swimming activity compared to controls after exposure, whereas *B. bufo* tadpoles increased swimming activity. In the same study, this pattern was also seen at 50 ug/L carbamazepine. Relyea and Edwards (2010) also found that, compared to controls, exposure to 0.1 mg/L carbaryl significantly increased tadpole activity in grey treefrogs (*Hyla versicolor*), but significantly decreased activity in green frogs (*Rana clamitans*) and American bullfrogs (*Rana catesbeiana*). They did, however, find that exposure to 0.1 and 1 mg/L malathion resulted in decreased swimming activity across all three species, but to varying degrees. Collectively, this suggests that behavioural responses following chemical exposure may differ between

species in both direction and magnitude. One outcome of the higher levels of baseline activity seen in this study could be increased susceptibility to predators, as increased activity in tadpoles has been linked to greater risk of predation (Skelly, 1994).

4.2. Scototaxis assay

We found no effect of 17β-trenbolone exposure on tadpole anxiety-like behaviour in the scototaxis assay. In agreement with the present study, Martin et al. (2022) and Orford et al. (2022) found no effect of 17β-trenbolone exposure on endpoints used to measure anxiety-like behaviour in tadpoles and other aquatic organisms (i.e. the time tadpoles spent in the upper half of the water column and freezing behaviour, respectively; Cachat et al., 2011; Haghani et al., 2019; Sievers et al., 2019). However, this contrasts with the existing literature on fish, which has found 17β-trenbolone to affect anxiety-like behaviours, such as increasing exploration of a maze, decreasing time spent shoaling, and decreasing time taken to exit a refuge (M.G. Bertram et al., 2018b; Heintz et al., 2015; Lagesson et al., 2019). In our previous study (Orford et al., 2022), we postulated that these contrasting results between tadpoles and fish could be due to taxonomic differences in sensitivity to 17β-trenbolone, which may be caused by differing levels of androgen receptors present in the study organism as a result of the life stages of the animals used.

In regards to the wider existing literature on behaviour in scototaxis trials, it has repeatedly been found that fish across different taxa show a preference for the black zone over the white zone (Maximino et al., 2007). In regards to amphibians, research has found that *Ranitomeya imitator* tadpoles show a preference for the black zone of the tank (Butler et al., 2022). We therefore predicted that tadpoles would spend more time in the black zone, with exposure to 17β-trenbolone reducing anxiety-like behaviour, resulting in more time spent in the white zone. In contrast to our predictions, we found that regardless of treatment, tadpoles spent more time in the white zone than the black zone. Interestingly, it appears that white/black zone preference may be species-specific, as *Xenopus laevis* tadpoles have been shown to prefer white areas over black (Moriya et al., 1996; Viczian et al., 2009). Preference may also change over development, as both Moriya et al. (1996) and Butler et al. (2022) found that the strength of preference for a white/black zone changed over time. The reasons behind these preferences could be thermoregulatory, as individuals may be attracted to lighter areas because illumination could indicate water exposed to sunlight, which is likely to be warmer than water that has less illumination (Alton and Franklin, 2017; Bancroft et al., 2008). White/black zone preference may also be related to the conditions experienced during development, as Moriya et al. (1996) and Bertolesi et al. (2021) both found that individuals raised in white containers showed a preference for the white zone during trials. As tadpoles in this study were raised in tanks containing light-coloured gravel (i.e. white and light grey), and the tanks were well-illuminated during the day, this may have influenced tadpole preference in our assay. Future studies are therefore warranted in order to establish the effect of conditions experienced during development on light preference in scototaxis assays.

4.3. Morphology

The effects on morphology reported here are in agreement with previous studies on fish, that, as to be expected from exposure to a growth-promoting hormone, have found increases in various morphological endpoints as a result of exposure to 17β-trenbolone (Ankley et al., 2003; Baumann et al., 2014; Bertram et al., 2019, M.G. 2018a, 2015; Hemmer et al., 2008). In regards to mass, both Ankley et al. (2003) and Hemmer et al. (2008) found that 21 day exposure to 17β-trenbolone resulted in increased mass in female fathead minnows (*Pimephales promelas*) exposed to 5 ng/L, and female sheepshead minnows (*Cyprinodon variegatus*) exposed to 5000 ng/L, respectively. Baumann et al. (2014) also reported increased mass and length for zebrafish (*Danio rerio*) exposed to 3 and 10 ng/L for 60 days. Further, increased body condition after 21 days of 17β-trenbolone exposure has been recorded for male guppies and male eastern mosquitofish (*Poecilia reticulata* at 4 ng/L and *Gambusia holbrooki* at 15 ng/L, respectively; M.G. Bertram et al., 2018a, 2019). However, the literature investigating the effects of 17β-trenbolone on tadpole morphology is more inconclusive, making generalisations about the effects of 17β-trenbolone on amphibian morphology difficult. To date, there have been five published studies investigating the effects of 17β-trenbolone on tadpole/metamorph morphology, conducted across a variety of life stages, species, and concentrations (see Table S17 for a summary of available data). The majority of these studies (3 out of 5) report no effect of 17β-trenbolone exposure on tadpole and metamorph morphology. The differing effects of exposure on morphology and growth between fish and amphibians could be due to taxa-specific effects of 17β-trenbolone. One potential driver of these differences is that amphibians have complex multiphasic developmental stages compared to fish (Martin et al., 2022). These different development stages often result in drastic changes to metabolic requirements, and the effects of this on morphology are not yet fully understood (Zhu et al., 2020). Another potential contributor to these apparent taxa-specific effects could be the levels of androgen receptors present in the study organism (Orford et al., 2022). As an androgen agonist, the effects of 17β-trenbolone may be greater during developmental periods where high levels of androgen receptors are available (i.e. during sexual development; Ankley et al., 2018; Fujii et al., 2014; Oike et al., 2017). As many studies conducted on fish use sexually mature animals (e.g. M.G. Bertram et al., 2018b; Heintz et al., 2015; Lagesson et al., 2019), while the studies on amphibians mentioned above have either exclusively or partly used pre-sexual development tadpoles, it is likely that the levels of androgen receptors available for 17β-trenbolone to bind to differed between taxa, resulting in the observed differences in effects on morphology and growth. However, as some level of androgen receptors are still likely to be present prior to sexual development in tadpoles, effects of 17β-trenbolone on morphology may still be observed (Ohtani et al., 2003; Yokoyama et al., 2009). For example, a decrease in body mass at very high concentrations (10,000 ng/L) and a subtle decrease in body condition at environmentally-realistic concentrations (66 ng/L) have both previously been reported in amphibians, suggesting that 17β-trenbolone can impact tadpole morphology, though the authors reporting the latter have suggested that this result should be interpreted with caution given the subtlety of the effect (Li et al., 2015; Martin et al., 2022). Interestingly, although Olmstead et al. (2012) found no effect of 17β-trenbolone exposure on western clawed frog mass or length upon completion of metamorphosis (*Xenopus tropicalis;* 3–102 ng/L; Table S17), at 6 weeks post-metamorphosis both of these endpoints were significantly lower in individuals that had been exposed during the tadpole life stage compared to unexposed individuals. However, no morphological measurements were taken when tadpoles were at early stages of development (i.e. Gosner stages covered by the current study). Depending on their research aims, studies can differ in both the length of time that they expose animals for, and the times at which morphological measurements are taken. For tadpoles, variability in exposure length means that the animals may be exposed over different developmental stages. As levels of available androgen receptors changes over these stages, this may partly explain the differences in effects of exposure on morphology. While we found that exposure to 17β-trenbolone resulted in larger tadpoles during early life stages, we do not know if this will have lasting effects in later life (i.e. post-metamorphosis). As other studies have shown that size at metamorphosis is indicative of future size and survival (Székely et al., 2020; Tarvin et al., 2015), based on our findings, 17β-trenbolone exposure might actually give tadpoles an advantage in later life stages. Hence, there should be further study investigating potential carryover effects of early life exposure to trenbolone on frog fitness.

5. Conclusions

We report that exposure to environmentally realistic concentrations of the agricultural pollutant 17β-trenbolone altered morphology and behaviour in southern brown tree frog tadpoles. Specifically, exposure increased tadpole growth rates, resulting in longer and heavier individuals, increased baseline activity, and altered how tadpoles responded to a predator threat. Given there is a lack of studies investigating the behavioural effects of 17β-trenbolone on tadpoles, and the results from the present study appear to contrast with the few existing studies, we believe it is currently not possible to make broad generalisations regarding the potential for 17β-trenbolone exposure to affect tadpole behaviour in natural populations. Our findings contribute to a growing body of literature illuminating the potential of 17β-trenbolone to disrupt various aspects of development and behaviour at environmentally-realistic concentrations. This study also highlights the importance of behavioural studies in ecotoxicology, and illustrates the need for continued research into the potential effects of this potent chemical pollutant on exposed wildlife.

Ethical statement

The research detailed in this paper was approved by the Biological Sciences Animal Ethics Committee of Monash University (AEC approval number 20799) and complies with all relevant State and Federal laws of Australia. Eggs were collected in compliance with the Nature Conservation Act 2002 (Department of Primary Industries, Parks, Water and Environment Authority permit number: FA 19089).

CRediT authorship contribution statement

Jack T. Orford: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Hung Tan:** Investigation, Data curation, Writing – review & editing. **Reid Tingley:** Resources, Methodology, Writing – review & editing. **Lesley A. Alton:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. **Bob B.M. Wong:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. **Jake M. Martin:** Conceptualization, Investigation, Methodology, Data curation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2023.106577.

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