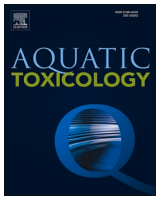
Aquatic Toxicology 251 (2022) 106289

Contents lists available at ScienceDirect

Aquatic Toxicology

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



Effects of the agricultural pollutant 17β-trenbolone on morphology and behaviour of tadpoles (*Limnodynastes tasmaniensis*)

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ARTICLE INFO

Keywords:
Behavioural ecotoxicology
Agricultural contaminant
Amphibian
Hormonal growth promotant
Endocrine disrupting chemical
Trenbolone

ABSTRACT

Pollutants, such as endocrine disrupting chemicals (EDCs), are increasingly being detected in organisms and ecosystems globally. Agricultural activities, including the use of hormonal growth promotants (HGPs), are a major source of EDC contamination. One potent EDC that enters into the environment through the use of HGPs is 17β-trenbolone. Despite EDCs being repeatedly shown to affect reproduction and development, comparatively little is known regarding their effects on behaviour. Amphibians, one of the most imperilled vertebrate taxa globally, are at particular risk of exposure to such pollutants as they often live and breed near agricultural operations. Yet, no previous research on amphibians has explored the effects of 17β-trenbolone exposure on foraging or antipredator behaviour, both of which are key fitness-related behavioural traits. Accordingly, we investigated the impacts of 28-day exposure to two environmentally realistic concentrations of 17β-trenbolone (average measured concentrations: 10 and 66 ng/L) on the behaviour and growth of spotted marsh frog tadpoles (*Limnodynastes tasmaniensis*). Contrary to our predictions, there was no significant effect of 17β-trenbolone exposure on tadpole growth, antipredator response, anxiety-like behaviour, or foraging. We hypothesise that the differences in effects found between this study and those conducted on fish may be due to taxonomic differences and/or the life stage of the animals used, and suggest further research is needed to investigate the potential for delayed manifestation of the effects of 17β-trenbolone exposure.

1. Introduction

Chemical pollution is one of the greatest environmental threats to humans and wildlife worldwide (Naidu et al., 2021). Due to an ever-increasing human population and subsequent escalating urbanisation, chemical pollutants are being detected globally, with locations as remote as Antarctica being impacted (aus der Beek et al., 2016; Corsolini, 2009). Endocrine-disrupting chemicals (EDCs) are one group of chemical pollutants that are of particular concern due to their capacity to disrupt natural hormone functioning at extremely low levels (i.e. ng/L; Gore et al., 2015; Jackson and Klerks, 2019). The potency of EDCs is compounded by their propensity to bioaccumulate and persist temporally in the environment (Diamanti-Kandarakis et al., 2009; Ruhí et al., 2016). The ability of EDCs to disrupt natural hormone function means that for non-target organisms, exposure can result in detrimental impacts on reproduction, development, and behaviour, which may result in population declines (Gore et al., 2015; Hotchkiss et al., 2008; World Health Organization (WHO), 2012). Thus, understanding the effects of EDCs on exposed organisms is crucial for providing greater protection to both humans and wildlife (World Health Organization (WHO), 2012).

Agricultural activity is one of the most significant contributors of EDC pollution into the environment (Kemper, 2008). In the agricultural industry, hormonal growth promotants (HGPs) are used to increase muscle mass and feed conversion in cattle (Preston, 1999). The use of HGPs is banned in certain parts of the world (e.g. the European Union) over concerns for human health (Johnson, 2017). However, they are still extensively used in countries such as the United States and Australia, where in parts of these countries it is estimated that 80–90% of cattle on feedlots receive HGP injections (Hunter, 2010; Johnson, 2017). After

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https://doi.org/10.1016/j.aquatox.2022.106289
Received 8 May 2022; Received in revised form 1 September 2022; Accepted 2 September 2022
Available online 5 September 2022

administration, HGPs are rapidly hydrolysed to various metabolites, which are commonly excreted via urine and faeces (Blackwell et al., 2014). The use of HGPs therefore provides a direct pathway for EDCs to enter the environment, as these biologically active metabolites are often washed into nearby water bodies (Durhan et al., 2006; Geissen et al., 2015; Jones et al., 2014; Soto et al., 2004).

Trenbolone acetate is one of the most commonly used HGPs worldwide (Blackwell et al., 2014; Kolodziej et al., 2013) and has 15–50 times the potency of testosterone (Neumann, 1967). Once administered, trenbolone acetate is hydrolysed to various metabolites, the most biologically active of which is 17β-trenbolone, a high-affinity ligand for the vertebrate androgen receptor (Wilson et al., 2002). Due to the widespread use of trenbolone acetate, 17β-trenbolone has been repeatedly detected in the environment at concentrations ranging from <1–270 ng/L in feedlot discharge (Bartelt-Hunt et al., 2012; Durhan et al., 2006; Khan, Qiao, and Lee, 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 river water upstream and downstream of agricultural areas (Durhan et al., 2006; Soto et al., 2004). This widespread 17β-trenbolone contamination is further compounded by an extensive half-life in wastewater effluent (~260 days; Schiffer et al., 2001), and rapid uptake by aquatic organisms (Schultz et al., 2013). As the primary molecular target of 17β-trenbolone (i.e. the androgen receptor) is evolutionarily conserved across a diverse range of taxa, a wide variety of non-target organisms are potentially vulnerable to 17β-trenbolone exposure (Mcginnis et al., 2002). Indeed, past research has shown that 17β-trenbolone exposure at environmentally-realistic concentrations can have sub-lethal effects in a multitude of species (reviewed in Ankley et al., 2018). Such impacts include changes to morphology (e.g. increased growth; Baumann et al., 2014), reduced fecundity (Ankley et al., 2003; Cripe et al., 2010; Mizukami-Murata et al., 2015), abnormal development of reproductive organs (Olmstead et al., 2012), and skewed sex ratios (Olmstead et al., 2012; Örn et al., 2006). More recently, research has shown that exposure to environmentally-realistic concentrations of 17β-trenbolone can also impact important behaviours in exposed animals, such as risk-taking (Heintz et al., 2015; Lagesson et al., 2019), antipredator responses (Lagesson et al., 2019), activity/exploration (Bertram et al., 2018; Lagesson et al., 2019), sociality (Bertram et al., 2018), foraging (Bertram et al., 2018), and sexual behaviours (Bertram et al., 2019, 2015, 2018; Saaristo et al., 2013; Tan et al., 2021; Tomkins et al., 2016; Tomkins et al., 2018, 2017). Effects of 17β-trenbolone exposure on behaviour are of particular concern as the ability of organisms to perform appropriate behaviours is vital for survival and reproduction (Sih et al., 2004; Smith and Blumstein, 2008). Disruptions to behaviour may also have detrimental ecological and evolutionary consequences, as behavioural modifications play a vital role in the ability of organisms to adapt to changes in the environment (Wong and Candolin, 2015). Furthermore, changes to behaviour caused by chemical pollutants can be detected at lower concentrations than those required to impact traditional toxicological endpoints (e.g. morphology and LC50; the concentration required to kill 50% of the population), thus making behaviour a particularly sensitive biomarker for investigating effects of anthropogenic chemical pollution on wildlife (Melvin and Wilson, 2013; Relyea, 2012; Saaristo et al., 2018).

Amphibians are one group of animals that are at increased risk of exposure to agricultural pollutants, such as 17β-trenbolone, as they often inhabit and breed in areas that receive contaminated agricultural runoff (Brand and Snodgrass, 2010; Hazell et al., 2001; Sievers et al., 2018a). Indeed, amphibians are one of the most vulnerable vertebrate taxa globally, with recent estimates placing 41% of amphibian species at risk of extinction (Hoffmann et al., 2010; Monastersky, 2014; IUCN, 2021). Exposure to chemical pollutants has been recognised as a contributing factor to amphibian declines (Egea-Serrano et al., 2012; Orton and Tyler, 2015), and has been linked with impacts on amphibian survival (Baker et al., 2013; Egea-Serrano et al., 2012), morphology (Hegde et al., 2019; Pinelli et al., 2019), physiology (Christin et al., 2004; Karlsson et al., 2021; Strong et al., 2017), and multiple facets of behaviour (e.g. feeding and reproduction; reviewed in Sievers et al., 2019). Previous research has shown that 17β-trenbolone affects amphibian survival (Li et al., 2015; Olmstead et al., 2012; Rozenblut-Kościsty et al., 2019), gonadal development (Haselman et al., 2016; Li et al., 2015; Olmstead et al., 2012; Rozenblut-Kościsty et al., 2019), and sex ratios (Li et al., 2015; Olmstead et al., 2012). However, there is currently a lack of studies investigating the potential effects of 17β-trenbolone on key fitness-related behavioural traits, such as foraging and antipredator behaviour, in amphibians, particularly during vulnerable early life stages (Ankley et al., 2018).

Accordingly, in this study, we tested the impacts of exposure to environmentally realistic 17β-trenbolone concentrations on morphology, antipredator behaviour, and foraging behaviour in spotted marsh frog tadpoles (*Limnodynastes tasmaniensis*)—a species likely to inhabit polluted environments, including those receiving agricultural waste inputs (Hazell et al., 2001; Sievers et al., 2018a). We hypothesised that tadpoles exposed to 17β-trenbolone would decrease anxiety-like behaviours, decrease responsiveness to predatory threats, and increase foraging rates, as this has been reported in other aquatic vertebrates (e.g. fish; Bertram et al., 2018; Heintz et al., 2015; Lagesson et al., 2019). Further, based on results from studies on fish and mammals (Ankley et al., 2003; Bertram et al., 2019; Ye et al., 2014), we predicted 17β-trenbolone would increase the size and body condition of exposed tadpoles.

2. Materials and methods

2.1. Animal collection and housing

Ten partial egg masses of the spotted marsh frog (*L. tasmaniensis*) were collected on 10 March 2020 from Melbourne, Victoria, Australia (37° 50′ 31.6" S, 145° 12′ 43.1" E). Analysis of water samples taken from this site at the time of collection confirmed the absence of 17β-trenbolone pollution (Envirolab Services, $n = 2$, below detection limit; ≤ 2 ng/L, see Supplementary material for details of analysis method). Following collection, egg masses were transported to Monash University, where they were housed in a constant temperature room maintained on a 12:12 h light:dark regime at 19.4 ± 0.1°C (mean ± SD). Egg masses were placed in separate tanks (51 × 32 × 30 cm; length × width × height) filled with ~32 L of aged carbon-filtered water. The eggs, and the tadpoles that subsequently hatched, were housed in these conditions for 10 days, at which point the tadpoles reached Gosner stage 25 (Gosner, 1960) and started feeding. During this period, two partial water changes (30%) were performed using aged carbon-filtered water. Tadpoles were fed *ad libitum* with commercial fish food (Sera Flora Tropical Vegetable Flakes, Heinsberg, Germany) and raw zucchini.

2.2. Experimental exposure

Upon reaching Gosner stage 25 (~10 days post-collection), tadpoles were allocated to one of three exposure treatments: freshwater control (hereafter referred to as control), low-17β-trenbolone (nominal concentration: 10 ng/L), or high-17β-trenbolone (nominal concentration: 100 ng/L). The concentrations chosen for the low- and high-17β-trenbolone treatments were selected to represent levels detected downstream of agricultural operations and in ditches receiving feedlot runoff, respectively (Ankley et al., 2018). Tadpoles were exposed to one of the three exposure treatments using a partial static-renewal system for 28 days (starting on 20 March 2020). This exposure period was chosen as previous research has shown that exposing other aquatic species to 17β-trenbolone for 21–28 days is sufficient to induce both behavioural and morphological changes (Bertram et al., 2015; Saaristo et al., 2013; Sone et al., 2005; Tomkins et al., 2017). It is important to note that the current study and Martin et al. (2022) shared the same exposure system,

and thus exposure conditions (temperature, pH), 17β-trenbolone analysis, and survival have already been reported in Martin et al. (2022). However, no tadpoles in the present study were used for the behavioural experiments or morphological measurements in Martin et al. (2022), and vice versa— i.e. the two studies used different individuals. The exposure set-up consisted of 36 tanks (60 × 30 × 30 cm; $n = 12$ per treatment) that each held 35 tadpoles ($n = 420$ per treatment). When distributing tadpoles to exposure tanks, the number of tadpoles from each egg mass was balanced across both the individual tanks and exposure treatments, in order to control for any potential clutch effects (Gibbons and George, 2013). Tadpole introduction to experimental tanks was staggered over 12 days (i.e. one tank per treatment was established with 35 tadpoles, per day, for 12 days), thus ensuring all tadpoles had been exposed for the same duration before measuring behavioural and morphological traits. Due to this design, tadpole age at the beginning of the exposure, and subsequently when they underwent behavioural and morphological assays at the end of the exposure varied by 1–12 days. Therefore, the experimental block was included in statistical models to control for any potential effects on morphology or behaviour this variation may have had (Mühlenhaupt et al., 2022; Touchon et al., 2013; see statistical analysis below). Each exposure tank was filled with 36 L of aged carbon-filtered water, and 12 L (i.e. 33%) water changes were performed once a week. At the start of the exposure, all low- and high-17β-trenbolone tanks received an initial dose of 0.72 μg or 7.2 μg of 17β-trenbolone (CAS: 10161–33-8; Novachem, Germany) dissolved in 1 mL of ethanol (HPLC grade, ≥ 99.99%) respectively. These concentrations were selected based on a pilot experiment conducted using the same exposure system. Thereafter, to maintain exposure concentrations and replace any 17β-trenbolone lost to evaporation and water changes, all low- and high-17β-trenbolone tanks were dosed twice a week with 0.216 μg or 1.08 μg of 17β-trenbolone dissolved in 1 mL of ethanol respectively. To control for any potential effects of the ethanol solvent, all tanks in the control treatment received an initial dose of 1 mL ethanol and subsequent 1 mL ethanol doses twice a week, at the same time as the low- and high-17β-trenbolone tanks.

To monitor concentrations of 17β-trenbolone in the low- and high-17β-trenbolone treatments, weekly water samples (130 mL) were taken from each exposure tank 24 h after the second of the two weekly doses. Over the 28-day exposure period, each exposure tank in these two treatments had one sample tested per fortnight, meaning that all tanks were tested twice throughout the exposure period, and a total of 48 samples were tested overall. Each tank in the control treatment was also tested once during the 28-day exposure (12 samples in total), to confirm that contamination had not occurred in the freshwater control tanks. These samples were collected at the same time as those from the low- and high-17β-trenbolone treatments. The concentration of 17β-trenbolone was measured using liquid chromatography–tandem mass spectrometry (Shimadzu 8050 LCMSMS), performed by a commercial environmental testing company, Envirolab Services (MPL Laboratories; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025), with a quantification limit of 2 ng/L (for a detailed description of the analytical procedure, see Supplementary material). A single 17β-trenbolone contamination was detected in 1 of the 12 control tanks (2.7 ng/L in week 4 of exposure). As a result, all data from this tank were excluded from further analysis.

Throughout the exposure period, daily temperature checks were carried out to ensure consistent conditions across tanks (18.77 ± 0.51°C; $n = 1007$; mean ± SD; see Table S1 for summary across tanks, previously published in Martin et al., 2022), and pH was also tested in each tank using a pH probe (7.39 ± 0.12; mean ± SD; $n = 216$; see Table S1 for summary across tanks, previously published in Martin et al., 2022). At the end of the 28-day exposure, tadpole survival was measured in each independent tank. Survival did not significantly differ amongst treatments (mean ± SD survival percentage: 87.5 ± 10.6%, 85.1 ± 10.63%, and 82.2 ± 9.86% for control, low-17β-trenbolone and high-17β-trenbolone, respectively; see Martin et al., 2022).

2.3. Behavioural assays

To test for potential impacts of 17β-trenbolone exposure on behaviour, two separate assays (antipredator and foraging; detailed below) were conducted after 28 days of exposure. Focal tadpoles used in each assay were randomly selected from control, low- and high-17β-trenbolone exposure tanks. Each individual was only subjected to one of the two assays. Tadpoles were allocated to trial tanks (25 × 15 × 15 cm) filled with aged carbon-filtered water (i.e. water free from 17β-trenbolone; 18.74 ± 0.24°C, $n = 360$), and covered on all sides with frosted opaque sheeting to prevent tadpole behaviour being affected by experimenters. Trial tanks were emptied and wiped clean between trials to avoid cross-contamination of 17β-trenbolone and to remove any conspecific chemical cues that may have influenced tadpole behaviour in subsequent trials. Individuals with video tracking percentage scores of < 95% were removed from the analysis (i.e. tadpoles that were detected in < 95% of frames; for final sample sizes, see supplementary material Table S2). All trials and subsequent data extraction were performed blind to experimental treatment.

2.4. Antipredator assay

We measured the behaviour of tadpoles before and after simulated predator strikes using methods adapted from previously established protocols (Arendt, 2003). The simulated predator strike involved gently prodding the tail of the tadpole using a 20 cm blunt glass probe, a common technique used for eliciting escape responses in tadpoles (Arendt, 2003; Sievers et al., 2018b). A water depth of 2 cm was chosen to limit vertical movement of the tadpoles, allowing for more accurate scoring of distance moved (Martin et al., 2017). Prior to behavioural recordings, tadpoles were left to freely acclimate to the trial tank for 10 min. After this acclimation period, tadpole behaviour was recorded for 5 min prior to a simulated predator strike in order to establish a measure of baseline activity (i.e. pre-strike period). Tadpoles were then subjected to a simulated predator strike to elicit an escape response, and subsequent post-strike behaviour was recorded for 5 min. The simulated strike and following 5 min period were repeated twice more to generate three escape responses and three 5 min post-strike behaviour periods. During the assay, all behaviours were recorded from above at 100 frames per second (Sony FDR-AX33). The resulting videos were analysed in line with previously established methods (Henry et al., 2019; Henry and Wlodkowic, 2020). Briefly, videos were reduced to a 5 min pre-strike period (i.e. baseline) and three 5 min post-strike periods using DaVinci Resolve 15 (BlackMagic Design, Australia) video editing software. For each video, the animal tracking software Ethovision XT ver. 15 (Noldus Information Technology, the Netherlands) was used to track the position of the tadpole in the tank (i.e. produced time-stamped x,y coordinate pairs assigned to the tadpole's centroid). This allowed for total distance travelled (mm), and time spent not moving (i.e. freezing behaviour; sec) to be calculated for each tadpole. A tadpole was considered to not be moving if velocity dropped below 5 mm/sec (as previously used in Martin et al., 2017). The distance (mm) of each tadpole's three escape responses was also measured. This was calculated using a point of mass tracking software (TrackerV8; Open Source Physics, USA), and was defined as the distance travelled in 1 sec (i.e. 100 frames) immediately following the simulated predatory strike.

2.5. Foraging assay

A separate subset of tadpoles underwent a foraging assay modified from previously established protocols (Bertram et al., 2018; Brand et al., 2021). All tadpoles were fasted for approximately three days prior to foraging trials to standardise hunger levels. Trial tanks were filled to 4 cm with aged carbon-filtered fresh water. At the start of the assay, tadpoles were acclimated behind a clear partition positioned 7 cm from one edge of the tank for 10 min. A food item (i.e. slice of raw zucchini) of

standard weight and size (4.90 ± 0.88 g; $n = 168$) was placed in the middle of the tank 1 cm away from the other end of the tank. After the 10-min acclimation period, the partition was remotely removed and tadpoles were allowed to freely explore the tank for 50 min, after which the trial was complete. During the trial, tadpole behaviour was recorded from above at 30 frames per second (Panasonic HC-V180). For each tadpole, latency to forage and total time spent foraging were scored using the open-source event-logging software BORIS (v. 7.4.7; Friard and Gamba 2016). Total distance moved (mm) during the trial was scored using Ethovision XT V 15 (following methods described above).

2.6. Tadpole morphology

Upon completion of behavioural assays, tadpoles were humanely killed using an MS-222 solution (500 mg/L), buffered with sodium carbonate. Dorsal (Canon Powershot S120) and lateral photographs (Nikon DSLR D80) of all tadpoles that underwent assays were taken for measures of total and body length (± 0.01 mm). Data were extracted from the photographs using ImageJ software (Schneider et al., 2012). Tadpoles were then blotted dry and weighed (± 0.0001 g; ZSA210 Analytical Balance, Scientech, Melbourne, Australia). As a proxy for body condition, a scaled mass index (SMI) was calculated for all tadpoles. Specifically, we performed a standard major axis regression on the log of body mass and body length of tadpoles (*smatr* package; Warton et al., 2012), and calculated a beta coefficient which was then used (with body length) to obtain the SMI for each tadpole (Peig and Green, 2009).

2.7. Statistical analyses

Statistical analyses were conducted using *R* version 4.1.1 (*R* Core Team, 2020). Where appropriate, data were transformed to approximate Gaussian error distributions (see Tables S3–11 for descriptions), and continuous predictors were mean centred and scaled to aid in model fitting. Models were selected using information theoretic model selection based on Akaike's Information Criterion (AIC) estimates (see Tables S3–11 for final models). Type-II F-tests (for LME models), or Type-II Wald's χ^2 tests (for GLME models), with Kenward-Roger Degrees of Freedom Approximation were used to calculate the *p*-values of fixed effects and interaction terms (*car* package; Fox and Weisberg, 2019; *lme4* package; Bates et al., 2015). Where significant main effects were detected, pair-wise comparisons using Tukey's and Sidak's *p*-adjustments were performed (*emmeans* package; Lenth et al., 2022).

Data from the antipredator assay (distance moved, freezing behaviour and escape response distance) were analysed using linear mixed-effects (LME) models. For all antipredator endpoints, global models included exposure treatment (control, low-17β-trenbolone, or high-17β-trenbolone), strike period (i.e. pre-strike, after the first strike, after the second strike, after the third strike), tadpole mass (mg), tracking percentage, experimental block, trial tank temperature, time of day, and an interaction between treatment and strike period, as fixed effects. Treatment tank and tadpole ID were included as random intercepts to account for variation among individual treatment tanks and tadpoles.

For all foraging endpoints (distance moved, latency to forage and total time spent foraging), global models included exposure treatment, tadpole mass, tracking percentage, experimental block, trial tank temperature, and time of day as fixed effects. For distance moved during the foraging assay, total time spent foraging was also included as a fixed effect to control for any potential effects of time spent foraging on activity during the trial. Treatment tank was included as a random intercept in all models. A LME model was used to investigate distance moved during the foraging assay. For latency to forage, a Cox Proportional Hazards Mixed Effects survival analysis was used (*coxme* package; Therneau, 2022). A generalised linear mixed-effects (GLME) zero-inflated poisson model with a negative binomial type I distribution was used for total time spent foraging, as there was an excess number of zeros in the data (*glmmTMB* package; Brooks et al., 2017).

Morphological data (total length, mass and body condition) were analysed using LME models. The global models included exposure treatment and experimental block as fixed effects. In addition, exposure tank was included as a random intercept.

3. Results

3.1. Analytical verification of 17β-trenbolone concentrations

The mean measured concentrations (±SD) for the low- and high-17β trenbolone treatments during the 28-day exposure were 9.8 ±3.7 ng/L ($n = 24$) and 65.7 ± ± 21.7 ng/L ($n = 24$), respectively (previously published in Martin et al., 2022).

3.2. Antipredator assay

There was no significant effect of 17β-treatment on the distance moved by tadpoles during the antipredator assay ($F_{2,56.2} = 0.16$, $p = 0.855$; Fig. 1a). However, independent of the effects of 17β-trenbolone, there was a significant positive effect of mass ($F_{1,144.8} = 22.23$, $p < 0.001$), with larger tadpoles moving more. There was also a significant negative effect of tracking percentage on distance moved ($F_{1,586.7} = 17.06$, $p < 0.001$), meaning tadpoles with lower tracking efficiency moved more. Strike period (i.e. pre-strike, after the first strike, after the second strike, after the third strike) also significantly influenced distance moved ($F_{3,509.0} = 3.05$, $p = 0.028$; Fig. S1). Specifically, across all treatments, distance moved by tadpoles was significantly lower in post-strike period one ($t = 3.82$, $df = 509$, $p < 0.001$), post-strike period two ($t = 2.78$, $df = 509$, $p = 0.029$), and post-strike period three ($t = 2.90$, $df = 509$, $p = 0.020$) compared to the pre-strike period, indicating that tadpoles reduced their activity following perceived predation threat. There were no significant differences between the post-strike periods (for full list of pairwise comparisons see Table S12).

Exposure to 17β-treatment did not have a significant impact on tadpole freezing behaviour ($F_{2,46.5} = 0.12$, $p = 0.886$; Fig. 1b). However, irrespective of trenbolone exposure, there was a significant negative effect of mass on freezing behaviour ($F_{1,133.7} = 24.91$, $p < 0.001$), that is, larger tadpoles spent more time moving. The final model also indicated a significant interaction between treatment and strike-period ($F_{6,509.0} = 2.19$, $p = 0.043$). However, planned pairwise comparisons between treatments and within periods were not statistically significantly (Table S13). There was also a significant effect of strike period on freezing behaviour ($F_{3,509.0} = 12.64$, $p < 0.001$; Fig. S2). Across all treatments, tadpoles spent more time freezing in post-strike period one ($t = –4.76$, $df = 509$, $p < 0.001$), post-strike period two ($t = –6.26$, $df = 509$, $p < 0.001$), and post-strike period three ($t = –6.10$, $df = 509$, $p < 0.001$) compared to the pre-strike period, indicating that tadpoles spent less time moving following a perceived predation threat. There were no significant differences between the post-strike periods (for full list of pairwise comparisons see Table S14).

There was no significant effect of 17β-trenbolone treatment on tadpole escape response ($F_{2,62.5} = 2.80$, $p = 0.068$; Fig. 1c). However, regardless of 17β-treatment, there were significant positive effects of both mass ($F_{1,121.4} = 6.69$, $p = 0.011$) and experimental block ($F_{1,33.5} = 6.67$, $p = 0.014$) on escape response, with larger tadpoles, and those introduced in later blocks, travelling further when responding to the simulated predator strike. This effect of experimental block is likely because tadpoles that were introduced into the exposure in later blocks were older.

3.3. Foraging assay

In our foraging assay, we found that there was no significant effect of 17β-treatment on the distance moved by the tadpole ($F_{2,32.2} = 2.88$, $p = 0.071$; Fig. 2a), their latency to forage ($\chi^2 = 3.20$, $p = 0.202$; Fig. 3), or the total time they spent foraging ($\chi^2 = 4.50$, $p = 0.105$; Fig. 2b).

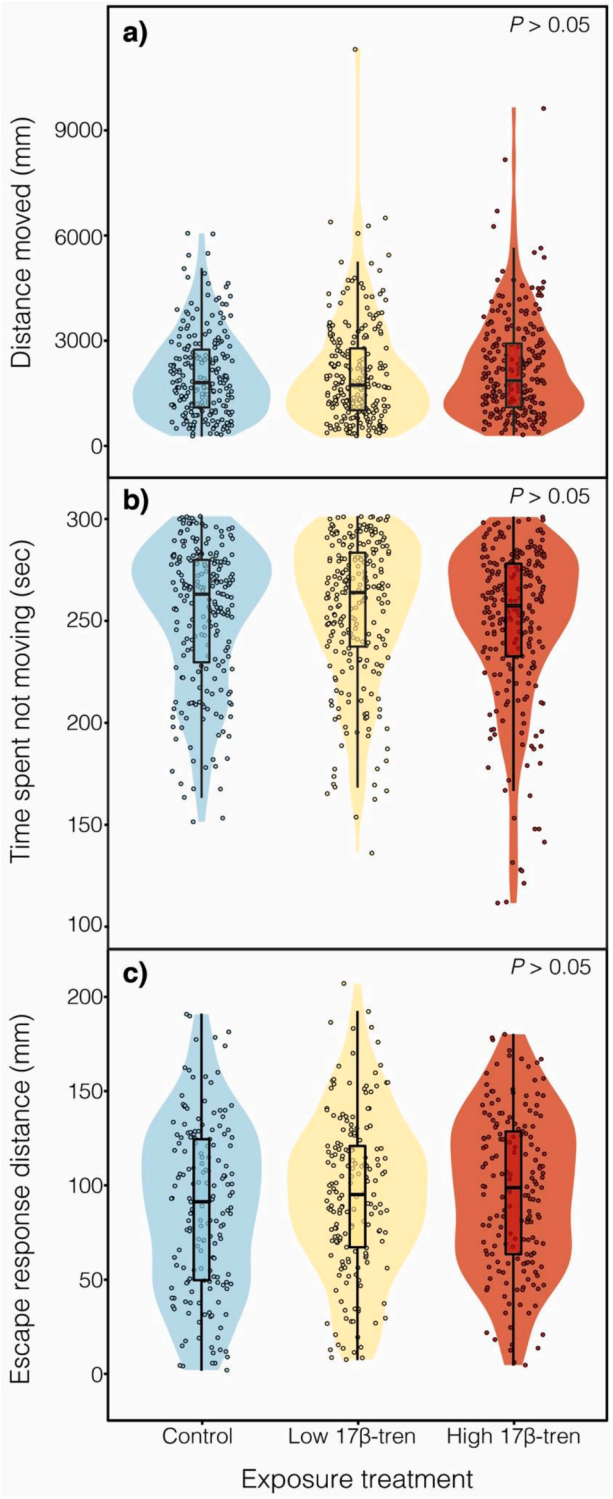

Fig. 1. Violin plots and boxplots showing the (a) distance moved, (b) time spent not moving, and (c) escape response distance by tadpoles in the anti-predator assay, plotted for control (blue; n = 55), low-17β-trenbolone (yellow; n = 59), and high-17β-trenbolone (red; n = 59) tadpoles. Fig. 1a and b include all data across time periods (i.e. pre-strike, post-strike 1, post-strike 2, and post-strike 3). Fig. 1c shows escape response data for the three post-strike periods. Box plots show 25th (Q1), 50th (median), and 75th (Q3) percentiles. The whiskers represent the Q1–1.5*IQR (interquartile range) to Q3+1.5*IQR. The coloured area surrounding the boxplot (violin plot) shows the probability density at different values smoothed by a kernel density estimator.

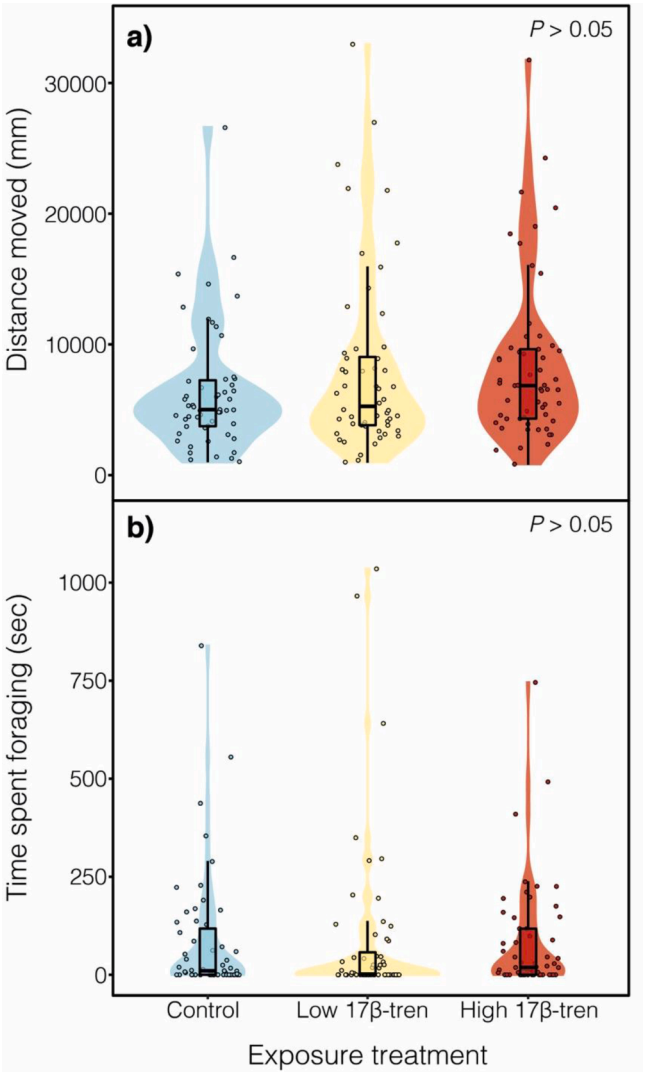

Fig. 2. Violin plots and boxplots showing (a) the distance moved, and (b) the total time spent foraging by tadpoles in the foraging assay, plotted for control (blue; n = 55), low-17β-trenbolone (yellow; n = 56), and high-17β-trenbolone (red; n = 57) tadpoles. Box plots show 25th (Q1), 50th (median), and 75th (Q3) percentiles. The whiskers represent the Q1–1.5*IQR (interquartile range) to Q3+1.5*IQR. The coloured area surrounding the boxplot (violin plot) shows the probability density at different values smoothed by a kernel density estimator.

However, irrespective of 17β-trenbolone treatment, there was a significant positive relationship between mass and all three behavioural responses (distance moved: $F_{1,160.5} = 6.89$, $p = 0.009$; foraging latency: $\chi^2 = 24.66$, $p < 0.001$; time spent foraging: $\chi^2 = 52.04$, $p < 0.001$, respectively), and a significant negative relationship between tracking percentage and all three response variables ($F_{1,158.9} = 20.62$, $p < 0.001$; $\chi^2 = 18.77$, $p < 0.001$; $\chi^2 = 13.22$, $p < 0.001$, respectively), meaning larger tadpoles, or those with lower tracking efficiency, moved more, began foraging earlier, and spent more time foraging. Further, experimental block had a significant negative relationship with latency to begin foraging ($\chi^2 = 5.53$, $p = 0.019$) and total time spent foraging ($\chi^2 = 12.01$, $p < 0.001$), that is, tadpoles introduced in an earlier block began foraging earlier and spent more time foraging.

3.4. Morphology

For morphology, 17β-trenbolone exposure did not significantly impact tadpole total length ($F_{2,32.0} = 0.90$, $p = 0.417$; Fig. 4a), mass

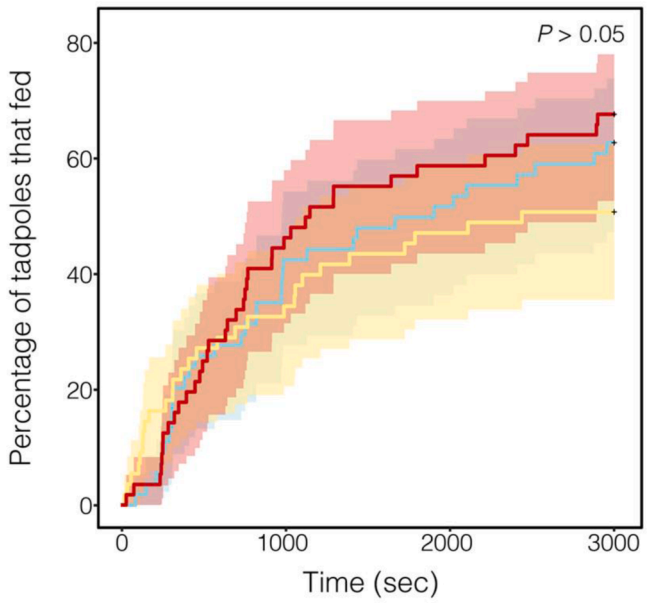

Fig. 3. Percentage of tadpoles that fed plotted by the average time taken to begin foraging during the foraging assay, with shaded areas around each line representing the 95% confidence interval for each treatment. Plotted for control (blue; n = 55), low-17β-trenbolone (yellow; n = 56), and high-17β-trenbolone (red; n = 57) tadpoles. Latency to forage data was tested using cox proportional hazard mixed effect models.

($F_{2,31.0} = 0.84$, $p = 0.441$; Fig. 4b), or body condition ($F_{2,32.0} = 2.46$, $p = 0.102$; Fig. 4c).

4. Discussion

This study investigated whether 28-day exposure to environmentally realistic concentrations of the agricultural pollutant 17β-trenbolone would impact the morphology and behaviour of tadpoles. As a metabolite of a powerful anabolic steroid, we predicted that 17β-trenbolone would increase size and body condition in exposed tadpoles, decrease antipredator responses and anxiety-like behaviour, and increase foraging. However, contrary to our predictions, 17β-trenbolone did not significantly affect tadpole morphology or behaviour.

In the present study, we did not detect a significant effect of 17β-trenbolone on the foraging, anxiety-like, or antipredator behaviour of tadpoles, nor on any measured aspect of growth. The results presented here provide an interesting contrast to previous studies on fish, which have found that exposure to 17β-trenbolone can result in changes to both behaviour and morphology (e.g. Baumann et al., 2014; Bertram et al., 2019, 2018, 2015; Saaristo et al., 2013; Tan et al., 2021; Tomkins et al., 2016). Specifically, in regards to foraging and anxiety-like behaviour, both Bertram et al. (2018) and Heintz et al. (2015) reported that 17β-trenbolone exposure increased foraging behaviour (e.g. time spent inspecting prey, time spent in foraging zone) and decreased anxiety-like behaviour (e.g. time spent shoaling, time taken to complete a maze) in female guppies (*Poecilia reticulata*; 0.25, 2.5 and 25 ng/L for 21 days) and mosquitofish (*Gambusia holbrooki*; 15.94 ng/L for 21 days). Additionally, Lagesson et al. (2019) reported that 17β-trenbolone exposure in mosquitofish (3 ng/L for 21 days) decreased anxiety-like behaviour (time taken to complete a maze, time taken to exit a refuge) and antipredator response, with exposed fish becoming less reactive to a simulated predator strike. Further, as to be expected from an anabolic steroid, past research has also found 17β-trenbolone exposure to increase various aspects of growth in fish, including body size, mass and condition index (Ankley et al., 2003; Baumann et al., 2014; Bertram et al., 2019, 2015; Hemmer et al., 2008).

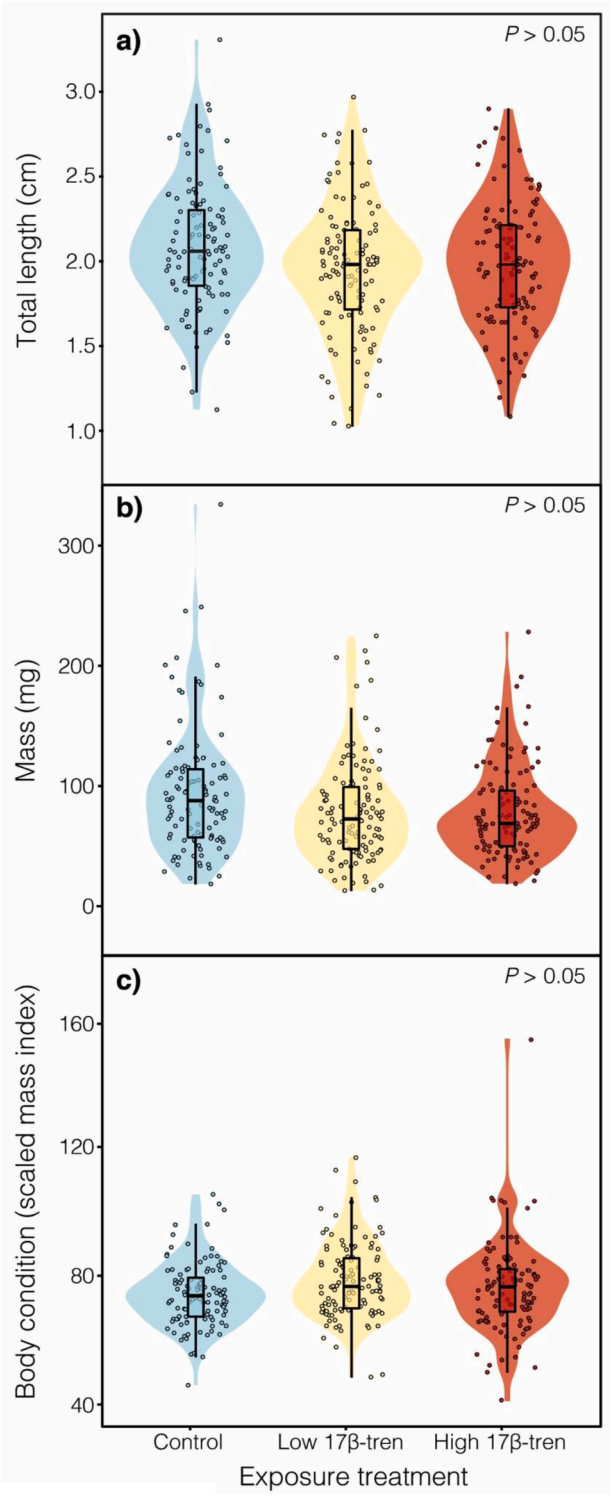

Fig. 4. Violin plots and boxplots showing tadpole (a) total length (control n = 110, low-17β-trenbolone n = 118, high-17β-trenbolone n = 120), (b) mass (control n = 110, low-17β-trenbolone n = 120, high-17β-trenbolone n = 120), and (c) body condition (control n = 110, low-17β-trenbolone n = 118, high-17β-trenbolone n = 120). Control is shown here in blue, low-17β-trenbolone in yellow, and high-17β-trenbolone in red. Box plots show 25th (Q1), 50th (median), and 75th (Q3) percentiles. The whiskers represent the Q1–1.5*IQR (interquartile range) to Q3+1.5*IQR. The coloured area surrounding the boxplot (violin plot) shows the probability density at different values smoothed by a kernel density estimator.

In contrast to fish, there has been far less research investigating the effects of 17β-trenbolone on amphibians (Ankley et al., 2018). To our knowledge, this study is among the first to investigate the effects of 17β-trenbolone on tadpole behaviour. In the only other existing study of its kind, Martin et al. (2022) found no effect of exposure on spotted marsh frog tadpole anxiety-like behaviour (*L. tasmaniensis;* 9.8 and 65.7 ng/L for 28 days). This is in agreement with the present study, which found no effect of 17β-trenbolone on swimming activity during either the foraging or antipredator assay, as well as no effect on freezing behaviour (both of which have been repeatedly used as measures of anxiety-like behaviour in tadpoles; Sievers et al., 2019). Although there are currently no other studies that have investigated the effects of 17β-trenbolone on foraging or antipredator behaviour in amphibians, previous research has shown exposure to other chemical pollutants can affect both of these endpoints. Indeed, in a recent meta-analysis investigating the effects of chemical pollutant exposure on amphibian behaviour, Sievers et al. (2019) found a 43% reduction in feeding rates and a 176% increase in predation for exposed individuals. In line with the results presented here, studies investigating the effects of 17β-trenbolone on growth in amphibians have found either a decrease in growth, or no effect of exposure (Haselman et al., 2016; Li et al., 2015; Martin et al., 2022; Olmstead et al., 2012). Using comparable concentrations to the present study, Haselman et al. (2016) and Olmstead et al. (2012) found no effect of exposure on growth at metamorphosis (*Xenopus laevis* exposed to 9–79.9 ng/L and *Xenopus tropicalis* exposed to 3–102 ng/L, respectively). Similarly, Martin et al. (2022) also found no effect of exposure on body length, but did report a significant decrease in body condition (65.7 ng/L for 28 days). However, Li et al. (2015) did report an effect of 17β-trenbolone exposure on mass, with *Pelophylax nigromaculatus* tadpoles exposed to 10,000 ng/L exhibiting decreased growth. Therefore, the effects of 17β-trenbolone on growth appear to differ between fish and amphibians.

Overall, then, the effects of 17β-trenbolone exposure on tadpole antipredator response, anxiety-like behaviour, foraging and growth seem to differ from the larger existing body of literature for fish. One potential explanation for the differences in results could be the 17β-trenbolone concentrations employed. In relation to effects seen on tadpole growth, Li et al. (2015) found effects at 10,000 ng/L, a concentration over one hundred and fifty times higher than the high-17-β-trenbolone treatment used in this study, and one far exceeding those found in the environment (Ankley et al., 2018). A difference in concentration of this magnitude could explain why there was no effect of 17β-trenbolone on growth in this study, as past research has shown 17β-trenbolone concentration-dependent increases in measured endpoints (Ankley et al., 2003; Baumann et al., 2014). However, it is important to emphasise that studies investigating the effects of 17β-trenbolone on foraging, antipredator and anxiety-like behaviour in fish have, in some instances, employed concentrations lower than those used in this study, but have nevertheless found various effects on behaviour (Bertram et al., 2018; Heintz et al., 2015; Lagesson et al., 2019). This suggests that the non-significant effects of 17β-trenbolone on behaviour seen in this study may not necessarily be a result of the concentrations used *per se*.

Another potential explanation for the difference in effects of 17β-trenbolone exposure between the present study and previous research could be due to the life stage of the organisms used. Indeed, the vulnerability of amphibians to the effects of chemical pollutants has been shown to differ across life stages (Egea-Serrano et al., 2012). Whereas studies on fish tend to use sexually mature animals (Bertram et al., 2018, 2015; Heintz et al., 2015; Lagesson et al., 2019), the present study used the juvenile life stage (i.e. tadpole). In fish, the effects of endocrine-active chemicals, such as 17β-trenbolone, are typically most pronounced during early sexual development (Ankley and Johnson, 2004). As an androgen agonist, 17β-trenbolone may have greater effects during this developmental period due to the high levels of androgen receptors available (Ankley et al., 2018; Fujii et al., 2014; Leet et al., 2011; Oike et al., 2017). As disrupted sexual development has been repeatedly observed in amphibians exposed to 17β-trenbolone—for example, male-skewed cohorts and intersex tissue (Li et al., 2015; Olmstead et al., 2012)—it would appear that amphibians, like fish, may also have this period of increased sensitivity during sexual development. As gonadal differentiation occurs at Gosner stage 30–38 in *L. tasmaniensis* (Horton, 1982), but tadpole development had not reached this stage in the present study, it is likely that androgen receptors were not present in high densities and thus individuals were not exposed to 17β-trenbolone during this period of increased sensitivity, potentially explaining the lack of effects of 17β-trenbolone on growth and behaviour. With that said, evidence does suggest that androgen receptors are expressed prior to this stage across a number of anuran species (Ohtani et al., 2003; Yokoyama et al., 2009), including species within the *Limnodynastes* genus (Melvin et al., 2018). Indeed, previous research by Melvin et al. (2018) showed that chemicals acting on androgen receptors (in that case, the fungicide vinclozolin) can alter physiological processes in *Limnodynastes* tadpoles at GS 25 even after a very short period of exposure.

For amphibians, the effects of exposure as a juvenile may also not manifest until a later life stage (reviewed in Pechenik, 2006). Indeed, whilst Olmstead et al. (2012) found no effect of 17β-trenbolone exposure on body length or mass in tadpoles, at 6 weeks post metamorphosis, both of these endpoints were significantly lower in animals that had been exposed during the tadpole life stage. Further, Robert et al. (2019) found that exposure as tadpoles to a mixture of chemicals with EDC activity resulted in a significant decrease in body mass upon completion of metamorphosis, as well as a weakened antiviral immune response in adult frogs (*Xenopus laevis*; 0.1 and 1 μg/L for 3 weeks). However, the present study did not raise tadpoles up to, and beyond, metamorphosis. Therefore, potential carryover effects of 17β-trenbolone exposure during the tadpole life stage were beyond the scope of the current study. As the vast majority of studies investigating the effects of chemical pollutants on amphibians solely use the tadpole life stage (81%; Sievers et al., 2019), it is imperative that future research raises tadpoles through metamorphosis so that a more complete picture of the impacts of early-life exposure to chemical pollutants can be provided.

5. Conclusion

In conclusion, we did not detect a significant effect of 17β-trenbolone exposure on the morphology or behaviour of tadpoles. When compared to previous research, the results of our study suggest that tadpole growth, antipredator responses, anxiety-like behaviour, and foraging are less sensitive to 17β-trenbolone disruption than in fish. This may be driven partly by concentration-dependent effects, but also by the life stage of the exposed animals, as effects of 17β-trenbolone may be dependent on the number of androgen receptors present. In this regard, 17β-trenbolone has previously been shown to affect survival at metamorphosis, as well as endpoints related to sexual development, both of which were not covered in the scope of the present study. Additionally, there has been very little research into the effects of 17β-trenbolone on behavioural endpoints in amphibians, despite the vital role of the endocrine system in determining behaviour (Ankley et al., 2018; Norris and Carr, 2014). 17β-trenbolone could therefore be impacting tadpole behaviours that are yet to be studied, or the effects of exposure could be manifesting at a later life stage. Further study is therefore warranted in order to investigate these potential effects of 17β-trenbolone on amphibians.

Ethical statement

The research detailed in this study 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 Wildlife Act 1975

(DELWP permit number 10009162).

CRediT authorship contribution statement

Jack T. Orford: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Shiho Ozeki:** Investigation, Data curation, Writing – review & editing. **Jack A. Brand:** Investigation, Data curation, Writing – review & editing. **Jason Henry:** Data curation, Writing – review & editing. **Donald Wlodkowic:** Supervision, Writing – review & editing. **Lesley A. Alton:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition. **Jake M. Martin:** Conceptualization, Investigation, Methodology, Data curation, Writing – review & editing. **Bob B.M. Wong:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

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.

Acknowledgments

This research was supported by funding from the Holsworth Wildlife Research Endowment Fund and The Ecological Society of Australia (both to JTO), as well as the Australian Research Council (DP190100642 to BBMW and LAA, and FT190100014 and DP220100245 to BBMW). The authors would also like to thank Murray Littlejohn, Madeleine Sanders and Frogs Victoria for advice on animal collection and husbandry, and Rachel Mason and Hung Tan for laboratory assistance.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2022.106289.

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