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Exposure to an androgenic agricultural pollutant does not alter metabolic rate, behaviour, or morphology of tadpoles

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ABSTRACT

Globally, amphibian species are experiencing dramatic population declines, and many face the risk of imminent extinction. Endocrine-disrupting chemicals (EDCs) have been recognised as an underappreciated factor contributing to global amphibian declines. In this regard, the use of hormonal growth promotants in the livestock industry provides a direct pathway for EDCs to enter the environment—including the potent anabolic steroid 17β-trenbolone. Emerging evidence suggests that 17β-trenbolone can impact traits related to metabolism, somatic growth, and behaviour in non-target species. However, far less is known about possible effects of 17β-trenbolone on anuran species, particularly during early life stages. Accordingly, in the present study we investigated the effects of 28-day exposure to 17β-trenbolone (mean measured concentrations: 10 and 66 ng/L) on body size, body condition, metabolic rate, and anxiety-related behaviour of tadpoles (*Limnodynastes tasmaniensis*). Specifically, we measured rates of O_2 consumption of individual tadpoles as a proxy for metabolic rate and quantified their swimming activity and their time spent in the upper half of the water column as indicators of anxiety-related behaviour. Counter to our predictions based on effects observed in other taxa, we detected no effect of 17β-trenbolone on body size, metabolic rate, or behaviour of tadpoles; although, we did detect a subtle, but statistically significant decrease in body condition at the highest 17β-trenbolone concentration. We hypothesise that 17β-trenbolone may induce taxa-specific effects on metabolic function, growth, and anxiety-related behaviour, with anurans being less sensitive to disruption than fish, and encourage further cross-taxa investigation to test this hypothesis.

1. Introduction

Amphibian populations have experienced dramatic global declines. Indeed, it is estimated that as much as 40% of all amphibian species face risk of imminent extinction (Stuart et al., 2004; Wake, 2012). This global loss is likely to have dire ecological consequences, as amphibians are a key component of many ecosystems and, in some instances, comprise the highest fraction of vertebrate biomass (Blaustein et al., 1994). As part of the wider 'biodiversity crisis' (Eldredge, 1998), amphibians appear to be disproportionately impacted relative to other vertebrate taxa by anthropogenic change (Stuart et al., 2004). Emerging evidence points to multifarious reasons for this decline, including habitat loss, climate change, and chytridiomycosis (Wake, 2012).

More recently, environmental contamination with endocrine-disrupting chemicals (EDCs) have been recognised as an underappreciated factor contributing to the global decline of amphibians (Orton and Tyler, 2015). Endocrine-disrupting chemicals' are a broad group of pollutants which interfere with hormone action, including industrial chemicals used in manufacturing, pharmaceuticals, and agricultural chemicals (Gore et al., 2015). Through disruption of hormone action, EDCs can alter reproduction, development, and behaviour of exposed organisms, even at minute concentrations (Ankley et al., 2018; Gore et al., 2015). In this regard, the use of hormonal growth promotants (HGPs) in the livestock industry provides a direct pathway for potent EDCs to enter the environment (Ankley et al., 2018). This occurs because a number of chemicals used in HGPs are not completely metabolised by livestock and are therefore excreted either in their parent form (i.e. unchanged) or as a biologically active metabolite (Blackwell et al.,

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2014). Importantly, once excreted by livestock, these potent endocrine-disrupting chemicals can make their way into aquatic environments in and near agricultural landscapes where amphibians are known to persist (Kolok and Sellin, 2008; Lange et al., 2002; Mann et al., 2009).

One of the most common HGP chemicals administered to livestock globally is trenbolone acetate (Kolodziej et al., 2013; Meyer, 2001; Neumann, 1976). Trenbolone is a potent synthetic androgen agonist with 15–50 times the anabolic and androgenic potency of testosterone that causes livestock, such as beef cattle, to gain muscle mass (Neumann, 1976). Once administered, trenbolone acetate is hydrolysed to form a number of metabolites that induce the desired growth-promoting effects by binding to the androgen receptor (Neumann, 1976; Wilson, 2002). Of these metabolites, 17β-trenbolone has, by far, the strongest binding affinity to the androgen receptor (Bauer et al., 2001). Worryingly, 17β-trenbolone has repeatedly been detected in aquatic environments associated with livestock waste runoff (Bartelt-Hunt et al., 2012; Khan and Lee, 2012; Schiffer et al., 2001; Webster et al., 2012). Currently, limited data exist regarding environmental concentrations and fate of 17β-trenbolone, and thus, there is not yet a robust assessment for the exposure risk of aquatic organisms (Ankley et al., 2018). That said, 17β-trenbolone has been detected in feedlot discharge (<1–270 ng/L; Bartelt-Hunt et al., 2012; Durhan et al., 2006; Khan & Lee, 2012; Parker et al., 2012; Schiffer et al., 2001a; Soto et al., 2004; Webster et al., 2012), in river water up and down stream of agricultural operations (<1–8 ng/L; Durhan et al., 2006; Soto et al., 2004), and even in air-borne particulate matter collected adjacent to feedlots (<1–63 ng g^{-1}; Blackwell et al., 2011). Further, 17β-trenbolone is relatively stable in wastewater effluent, with a half-life of approximately 260 days (Schiffer et al., 2001), and appears to have rapid uptake in aquatic taxa, such as fish (e.g. reaching a steady state within 8 h; Schultz et al., 2013).

The primary molecular target of 17β-trenbolone (the androgen receptor) and components of the hypothalamic–pituitary–thyroid (HPT) axis—through which this steroid metabolite induces its growth-promoting effects—are generally conserved across vertebrate taxa (Couderq et al., 2020). This gives 17β-trenbolone the potential to alter metabolism, growth, development, and behaviour of non-target organisms, such as amphibians (Couderq et al., 2020). In addition, many metabolic processes that are not necessarily directly linked to the androgen receptor can also be disrupted by 17β-trenbolone (e.g. lipid metabolism, regulation of protein metabolism, lipoprotein metabolism, cholesterol biosynthesis, and cholesterol transport and metabolism; Brockmeier et al., 2013). Indeed, toxicological data for the effects of 17β-trenbolone on fish indicate that this pollutant may impact metabolism, somatic growth, sexual development, reproduction, and behaviour (reviewed in Ankley et al., 2018). However, far less is known about possible effects of 17β-trenbolone on amphibians (Ankley et al., 2018). This lack of data on amphibians is surprising given that EDC exposure has been implicated in global amphibian declines (Orton and Tyler, 2015), and there is evidence that 17β-trenbolone exposure may be more lethal to amphibians than fish (Li et al., 2015; Olmstead et al., 2012). Further, studies that have addressed the effects of EDCs—including 17β-trenbolone—on anuran species, have focused almost exclusively on impacts during sexual development, at metamorphosis, and on reproduction in adults (e.g. Li et al., 2015; Olmstead et al., 2012; Rozenblut-Kościsty et al., 2019). Comparatively, there is very little known about the direct impacts of EDCs during the early life stages of amphibians (Melvin et al., 2018). This knowledge gap is important as the early-life stage is known to be especially vulnerable to a variety of environmental stressors, such as predation (Werner, 1991), which can be exacerbated by chemical exposure (e.g. Hayden et al., 2015; Sievers et al., 2018).

Accordingly, in the present study, we tested the effects of chronic exposure to environmentally relevant 17β-trenbolone concentrations during early-life development on the metabolic rate, behaviour, size, and body condition of tadpoles of the spotted grass frog (*Limnodynastes tasmaniensis*). The concentrations used in this study (nominal: 10 and 100 ng/L) were selected to represent environments receiving feedlot discharge, and those downstream of agricultural operations (Ankley et al., 2018). We hypothesised that 17β-trenbolone would increase the size and body condition of exposed tadpoles (i.e. body mass relative to length), increase resting metabolic rate (as a result of the increase in metabolic demands of growth), and decrease anxiety-like behavioural phenotypes. Given the paucity of available data on anurans, we based our hypothesis on the growth-promoting effects observed in fish and mammalian models (e.g. Ankley et al., 2003; Baumann et al., 2014; Bertram et al., 2019; Ye et al., 2014), as well as changes in anxiety-related behaviours seen in fish species (e.g. Bertram et al., 2018; Heintz et al., 2015; Lagesson et al., 2018).

2. Methods

2.1. Animal collection, animal maintenance, and experimental treatments

Ten partial egg masses of the spotted grass frog (*Limnodynastes tasmaniensis*) were collected from a pond in Vermont, Victoria, Australia (37° 50′ 31.6″ S, 145° 12′ 43.1″ E) on March 10, 2020. Water samples taken from this site confirmed it was free of 17β-trenbolone contamination (Envirolab Services, $n = 2$, below detection limit; ≤ 2 ng/L, see methods for details). Egg masses were transported to Monash University where they were maintained in a controlled-temperature room (12:12 h light:dark cycle) within separate aquaria (51 × 32 × 30 cm; length × width × height) filled with approximately 32 L of aged, carbon-filtered tap water. The tadpoles were maintained in these conditions until they reached Gosner stage 25 (Gosner, 1960), which is the stage at which tadpoles begin feeding (akin to Nieuwkoop and Faber [NF] stage 45; Faber & Nieuwkoop, 1994).

Once tadpoles had reached Gosner stage 25, they were allocated to one of three 17β-trenbolone exposure treatments for 28 days: freshwater solvent control (hereafter control), low-17β-trenbolone (nominal concentration: 10 ng/L), or high-17β-trenbolone (nominal concentration: 100 ng/L). A 28-day exposure period was selected as similar exposure durations have been shown to alter behavioural phenotypes in fish (e.g. 21 days, Bertram et al., 2018; 21 days Tomkins et al., 2018). The exposure was performed using a static renewal system, consisting of 36 independent exposure tanks (60 × 30 × 30 cm; 12 per treatment), housing 35 tadpoles each ($n = 420$ tadpoles per treatment). The start of the exposure was staggered over a 12-day period, such that each day, for 12 days, one tank per treatment was established with 35 tadpoles. This was done so that all tadpoles had been exposed to treatments for the same duration of time (28 days) before their metabolic rate trials, behavioural testing, and morphological measurements. A stocking density of 35 individuals per tank (~1 individual per L) was selected to minimise the negative effects of high rearing density, which have been observed in tadpoles of other species (Ding et al., 2015; Guadin et al., 2021), while also ensuring tadpoles were reared in a realistic group context as the social context in which individuals are raised can modulate their behaviour (Girish and Saidapur, 2003; Martin and McCallum, 2021). When allocating tadpoles to exposure tanks, the number of tadpoles from each clutch was balanced across tanks. The exposure tanks were filled with 36 L of aged, carbon-filtered fresh water and 12 L (i.e. one-third) water changes were performed weekly for each tank. At the beginning of the exposure period, each low- and high-17-β-trenbolone tank was dosed with 0.72 μg and 7.2 μg of 17β-trenbolone (CAS: 10161–33-8; Novachem, Germany) dissolved in 1 mL of ethanol (HPLC grade, ≥ 99.99%), respectively. After this initial dose, each low- and high-17β-trenbolone tank was dosed twice weekly with 0.216 μg and 1.08 μg of 17β-trenbolone dissolved in 1 mL of ethanol, respectively. To control for any potential solvent effects, all controls tanks were dosed with 1 mL of ethanol at the same time as the low- and high-17-β-trenbolone tanks.

Water samples (130 mL) were drawn from each tank weekly. To

measure the concentration of 17β-trenbolone in low and high exposure tanks, two water samples were tested for each tank over the four-week exposure period—i.e. each tank was tested every other week, with a total of 48 water samples tested. To confirm that contamination had not occurred in control tanks, each control tank was tested once during the four-week exposure period, with a total of 12 control water samples tested. The concentration of 17β-trenbolone was measured using liquid chromatography–tandem mass spectrometry (Shimadzu 8050 LC–MS/MS), performed by a commercial environmental testing company, Envirolab Services (MPL Laboratories; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025). The limit of quantification for this procedure was 2 ng/L. In one of the 12 control tanks, a 17β-trenbolone contamination was detected (2.7 ng/L in week 4 of the exposure period) and, as a result, all data from this tank were excluded from further analysis.

In addition to water samples, daily temperature checks were carried out to ensure consistent conditions among tanks (18.77 ± 0.51 °C, mean ± SD; $n = 1007$; see Table S1 for summary across tanks), and weekly pH was tested in each tank using a pH probe (7.39 ± 0.12; $n = 216$; see Table S1 for summary across tanks). Tadpole survival in each independent exposure tank was measured at the end of the 28-day exposure and did not differ significantly among treatments (mean ± SD survival percentage: 84.82 ± 10.28%; see Supplementary material methods and results for survival by treatment comparisons).

2.2. Metabolic rate

On day 28 of exposure to experimental treatments, a six-channel closed aquatic respirometry system was used to measure the rates of O_2 consumption ($\dot{V}_{O_2}$, mL h^{-1}) of individual tadpoles as a proxy for metabolic rate ($n = 50$, 55, and 55 tadpoles for control, low, and high, respectively). Each channel of the respirometry system was used to measure the decline in oxygen concentration inside a sealed glass respirometry chamber (empty volume of 35 mL) filled with aerated, aged, carbon-filtered tap water that was submerged in a water bath (Fig. 1). Each respirometry chamber had three water-tight silicon tubes inserted into the lid (Fig. 1). Two of the tubes were connected to a peristaltic pump (Watson Marlow 323 U/MC) that was used to create gentle circulation of water within the closed system (flow rate of 2.5 mL min^{-1}). The third tube allowed a fluorescence-based oxygen sensor (PreSens Oxygen Dipping Probe, DP-PSt7, Regensburg, Germany) to be inserted into the respirometry chamber. The oxygen sensor was connected to a PreSens OXY-4 ST (G2) oxygen meter, which was connected to a PC running PreSens Measurement Studio 2 (v2.2.2.943, PreSens, Regensburg, Germany), which recorded the oxygen concentration (% air-saturation) every 30 s for 150 min. The average (±SE) oxygen concentration recorded at the end of the 150-min measurement period was 87.08 ± 0.48%.

For each 150-min measurement block, five of the six respirometry chambers contained an individual tadpole and the remaining chamber without a tadpole was used to measure background respiration rates, which will be referred to as the blank measurement. The oxygen sensor used to measure background respiration was systematically changed for each measurement block such that all sensors had the same number of background respiration measurements balanced over time. The oxygen sensors used to measure tadpoles were selected at random, and all measurements were performed blind to experimental treatment. Measurements were conducted over a period of 12 days with one measurement block conducted each day. Prior to measurement, food was removed from tadpole holding tanks to minimise the variation in metabolic rate associated with digestion. The day prior to the 12-day measurement period, the oxygen sensors were calibrated with aerated, aged, carbon-filtered tap water (i.e. an oxygen concentration of 100% air-saturation) and water containing 2% sodium sulfite (an oxygen concentration of 0% air-saturation). All assays were conducted under red lighting in a temperature-controlled room that maintained water temperature at 18 ± 1 °C.

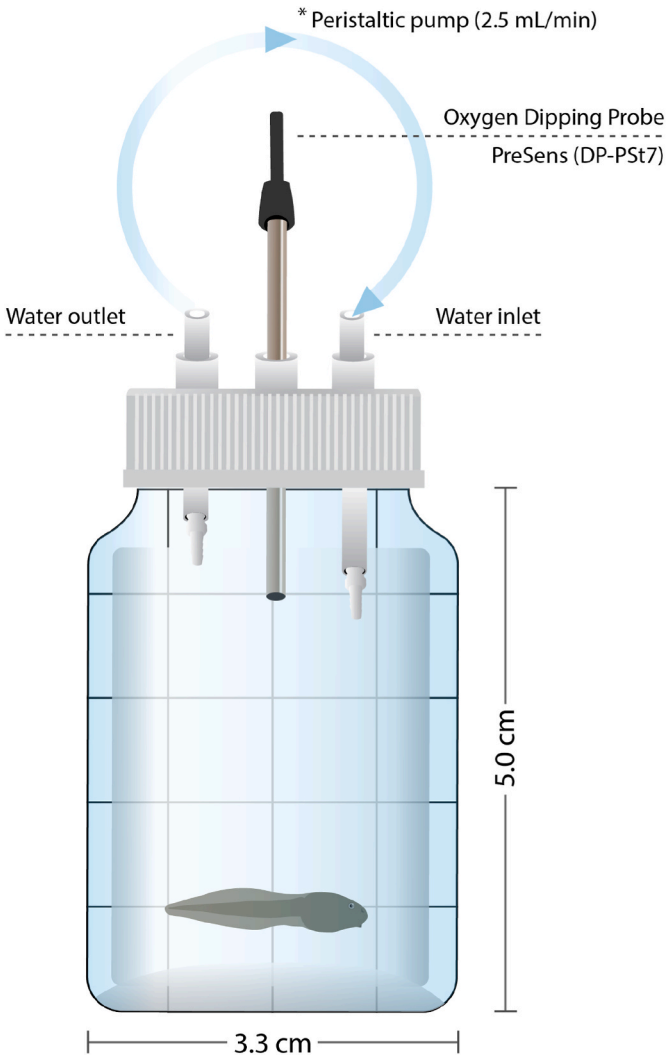

Fig. 1. Aquatic respirometry set-up used to estimate the metabolic rate of tadpoles.

The rate of oxygen consumption was estimated for each tadpole using the linear slope from the O_2-concentration time series (i.e. linear rate of change of O_2 concentration) over the 150-min measurement period. To do this in a statistically robust and reproducible way, the R package *LoLinR* (Olito et al., 2017) was used to build multiple local linear regressions with a minimum of 50% of the data (11,325 regressions per time series). Each local linear regression was then ranked using the $L_{\%}$ weighting method (Olito et al., 2017), and the slope from the best fitting regression (m, % h^{-1}) was used to calculate the rate of oxygen consumption ($\dot{V}_{O_2}$, mL h^{-1}) following the formula (Alton et al., 2012, 2007),

$$\dot{V}_{O_2} = \frac{-(m_a - m_b)}{100} \times V \times \beta_{O_2}$$

where, m_a and m_b are the calculated slopes for the tadpole and the block-matched blank (see Supplementary methods 'blank selection and estimation' for details), respectively (note that the difference between m_a and m_b is divided by 100 to convert the percentage of oxygen in the water to a fraction). β_{O_2} is the solubility of oxygen in freshwater at standard atmospheric pressure (101.3 kPa) at 18 °C (6.67 mL L^{-1}; Cameron, 1986), and V is the volume of water (L) in each system (i.e. the volume of water and tubing minus the mass of the tadpole; Svendsen

et al., 2016).

For a detailed list of methodological information following the proposed guidelines of Killen et al. (2021) for reporting methods of aquatic respirometry, see Table S2.

2.3. Behavioural traits

During metabolic rate measurements, swimming activity and time spent in the upper half of the water column were quantified for each tadpole. Activity levels and relative time spent near the water surface are commonly measured indicators of anxiety-like behaviour (i.e. boldness) in aquatic organisms (Cachat et al., 2011; Haghani et al., 2019). Further, altered activity levels and anxiety behaviour in tadpoles have previously been linked to changes in antipredator responses and increased mortality (Hayden et al., 2015; Squires et al., 2008). For example, Hayden et al. (2015) demonstrated that tadpoles exposed to a sublethal concentration of copper (1.85 μg L^{-1}) became less active and spent more time at the water surface, and subsequently, were more likely to be attacked by a predator. Tadpole behaviour was measured using video recordings (Panasonic HC-V180) captured from the side of the respirometry tank, with the quality of the recordings facilitated by covering the back half of the respirometry tank with opaque white frosting (to improve contrast). The swimming activity (i.e. distance travelled; cm) and time spent in the upper half of the water column were spot-sampled using three 5 min sections. Specifically, the activity levels of tadpoles were recorded from 30–35, 80–85 and 125–130 min. These time periods were selected as they represent the average lower-bound point, mid-point, and upper-bound point used to calculate metabolic rate from the O_2-concentration time series. Swimming activity and time spent in the upper half of the water column were measured using the open source video analysis package and modelling tool Tracker (v. 5.1.5; open source physics, USA), adapted from previously established protocols (Lagesson et al., 2018; Martin et al., 2017). Tadpoles were tracked at 1 fps and, from this, the total distance each tadpole moved was calculated, using 1 cm gridlines marked on the inside of the respirometry tank as a distance calibration. For positional data, the y-axis was centred in the middle of the tank; thus, the number of frames with positive y-scores was summed to calculate the number of seconds the tadpole was located in the upper half of the water column.

2.4. Tadpole morphology and body condition

Morphology measurements were taken for a total of 174 individuals ($n = 56$, 58 and 60 for control, low- and high-17β-trenbolone treatments, respectively). More specifically, from dorsal (i.e. top down) and ventral (i.e. side on) photographs, the following morphological measures were collected: body length, tail length, body depth, and body width (see Fig. S1 for a visual representation of how each measurement was taken). In addition, each tadpole was blotted dry and weighed to the nearest 0.0001 g using an analytical balance (ZSA210 Analytical Balance, Scientech). A scaled mass index (SMI) was calculated using mass and body length as a proxy for body condition, following established protocols (Peig and Green, 2009). Specifically, the β coefficient was calculated from a standard major axis regression (SMA) of log body mass and log body length using all tadpoles (β = 2.970). The β coefficient was then used with mean body length of all tadpoles (mean body length = 0.7285 cm) to calculate the SMI for each individual tadpole (Peig and Green, 2009). Body length was selected as the length metric for condition estimates as it had the strongest correlation with mass on a log-log scale (Pearson's correlation: $R = 0.960$; see Table S3 for all correlations). The SMI value for each tadpole represents their measured mass relative to the expected mass for a tadpole of their size.

3. Statistics

Data were analysed using R v. 4.0.4 (R Core Team, 2020). Across all models, data were transformed where necessary to approximate a Gaussian error distribution (see Table S4–8 for descriptions), and continuous predictors were mean centred and scaled to improve the interpretability of main effects. Final models (i.e. well-supported models) were selected using information theoretic (IT) model selection based on Akaike's Information Criterion (AIC) estimates (see Table S4–8 for final models). For all models, Wald's F-tests with Kenward-Roger Degrees of Freedom Approximation were used to calculate the *p*-values of fixed effects and interaction terms. Where a significant main effect of exposure treatment was indicated by the F-test, Tukey's *p*-adjustments were used to investigate pair-wise contrasts.

Metabolic rate and behavioural data were analysed using a linear mixed-effects (LME) model. The global model included tadpole mass (mg), swimming activity during the trial (cm), exposure treatment (control, low-17β-trenbolone, or high-17β-trenbolone), and the two-way interaction between exposure treatment and tadpole mass, as well as exposure treatment and swimming activity as fixed effects. In addition, measurement channel (1–6), measurement block (1–36) and exposure tank (1–35) were included as random intercepts. The global models for behavioural traits (i.e. swimming activity, and time spent in the upper half of the water column) included the same fixed structure as that listed above but included only measurement block and exposure tank as random intercepts. The inclusion of these clustering/hierarchical factors (i.e. channel, measurement block, and exposure tank) as random intercepts in the models allows us to account for the amount of variation attributed within and among these factors.

The effect of chronic exposure to 17β-trenbolone on tadpole morphology and body condition were also analysed using LME models. The four measured morphological traits (i.e. body length, tail length, body depth, and body width) were first collapsed into a single composite value using a principal component analysis (PCA) followed by oblique rotation. The morphological traits were centred and scaled prior to the PCA. Principal components (PC) were retained based on the Kaiser–Guttman criterion (i.e. an eigenvalue >1; Jackson, 1993), resulting in only PC1 being retained (supplementary material, Table S9). Principle component one accounted for 91% of the total trait variation and had strong positive loadings for all morphological traits (see Table S9 for all loadings). Thus, PC1 one essentially represents a continuum of 'size', with higher scores indicating larger tadpole size. Principal component 1 and scaled mass index (i.e. body condition) data were analysed in models with exposure treatment as the sole fixed effect and a random intercept for exposure tank.

4. Results

4.1. Analytical verification of 17β-trenbolone concentrations

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

4.2. Metabolic rate

There was no significant effect of 17β-trenbolone exposure on the metabolic rate of tadpoles (LME; $F_{2,\ 20.6} = 1.10$, $P = 0.353$; Fig. 2), but there was a significant positive relationship between metabolic rate and mass (LME; $F_{1,\ 127.0} = 39.80$, $P < 0.001$) and metabolic rate and activity (LME; $F_{1,\ 158.0} = 11.70$, $P = 0.001$; Fig. S3). There was also a marginally non-significant interaction between 17β-trenbolone exposure and tadpole mass (LME; $F_{2,93.5} = 2.59$, $P = 0.080$). For estimates from the random structure of the model, see Table S4.

4.3. Tadpole behaviour

There was no significant effect of 17β-trenbolone exposure on swimming activity (LME; $F_{2,\ 28.1} = 0.52$, $P = 0.598$; Fig. 3a) or the time

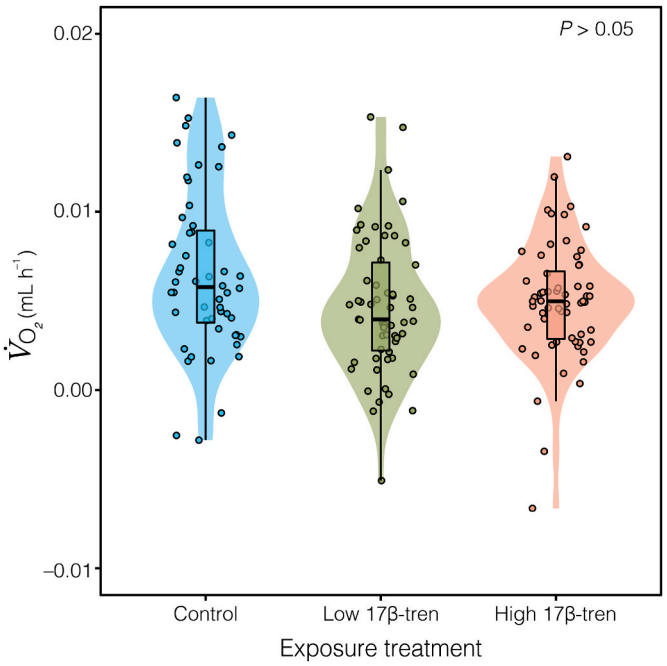

Fig. 2. Violin plots and boxplots showing the rate of oxygen consumption ($\dot{V}_{O_2}$, mL h^{-1}), unadjusted for weight and activity, for control (blue; $n = 50$), low-17β-trenbolone (green; $n = 55$) and high-17β-trenbolone (red; $n = 55$) tadpoles. Boxplots show the 25th, 50th (median) and 75th percentiles. The coloured area surrounding the boxplot (violin plot) shows the probability density at different values smoothed by a kernel density estimator. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

spent in the upper half of the water column (LME; $F_{2,\ 27.7} = 0.18$, $P = 0.837$; Fig. 3b). There was also no effect of tadpole mass on swimming activity (LME; $F_{1,\ 94.0} = 0.80$, $P = 0.375$) or the time spent in the upper half of the water column (LME; $F_{1,\ 118.0} = 1.19$, $P = 0.278$). For estimates from the random structure of the model, see Table S5–6.

4.4. Tadpole morphology and condition

Exposure to 17β-trenbolone did not significantly affect tadpole size (LME; $F_{2,\ 32.0} = 0.47$, $P = 0.628$; Fig. 4a), but had a significant effect on body condition (LME; $F_{2,\ 31.8} = 5.04$, $P = 0.013$; Fig. 4b). Specifically, the body condition of tadpoles exposed to the high-17β-trenbolone treatment was significantly lower than that of control tadpoles (Tukey's p-adjustment: $\beta = 6.73$, $t = 3.02$, $P = 0.013$), whereas the body condition of tadpoles exposed to the low-17β-trenbolone treatment was similar to that of control tadpoles (Tukey's p-adjustment: $\beta = 1.71$, $t = 0.77$, $P = 0.727$). Tadpoles exposed to the low- and high-17β-trenbolone treatments also had similar body conditions (Tukey's p-adjustment: $\beta = 5.01$, $t = 2.31$, $P = 0.070$).

5. Discussion

Due to the anabolic potency of 17β-trenbolone, we predicted that exposure would increase muscle accretion and somatic growth, and thus increase the energetic demand for metabolism (Ankley et al., 2018), resulting in larger tadpoles with higher body conditions, and higher resting metabolic rates. However, counter to our predictions, 17β-trenbolone did not significantly alter tadpole mass, size, or resting metabolic rate, and, at the higher dosage (i.e. 65 ng/L), actually resulted in a subtle decrease in body condition.

The effects reported within the present study contrast those in fish, which have reported changes in metabolism through altered gene expression involved in cellular and metabolic processes (Hook et al., 2006; Martyniuk et al., 2009). For example, following a 7-day exposure to 17β-trenbolone at 1000 ng/L, Hook et al. (2006) reported significant upregulation of genes in the liver of rainbow trout (*Oncorhynchus mykiss*) involved in protein binding, mitochondrial electron transport, lipid binding, protein biosynthesis, metabolism, glutamine metabolism, fatty acid biosynthesis, and development. Similar effects on metabolism have also been found in mammalian models (e.g. Mittal et al., 2021). In addition, past studies using fish have reported increased body size and mass gain as a result of 17β-trenbolone exposure, as would be expected from its intended growth-promoting effects (Ankley et al., 2003; Baumann et al., 2014; Bertram et al., 2015, 2018, 2019, 2020; Herrera et al.,

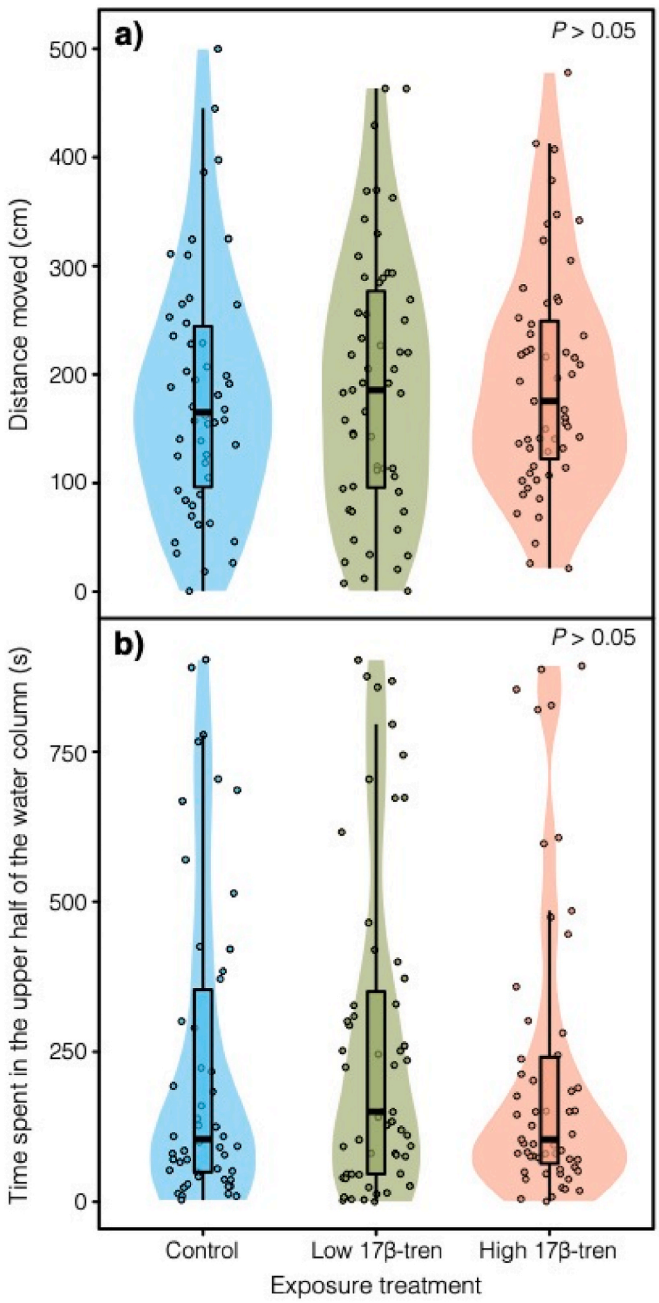

Fig. 3. Violin plots and boxplots showing (a) the swimming activity of tadpoles, and (b) the time tadpoles spent in the upper half of the water column, plotted for control (blue; $n = 50$), low-17β-trenbolone (green; $n = 55$) and high-17β-trenbolone (red; $n = 55$) tadpoles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

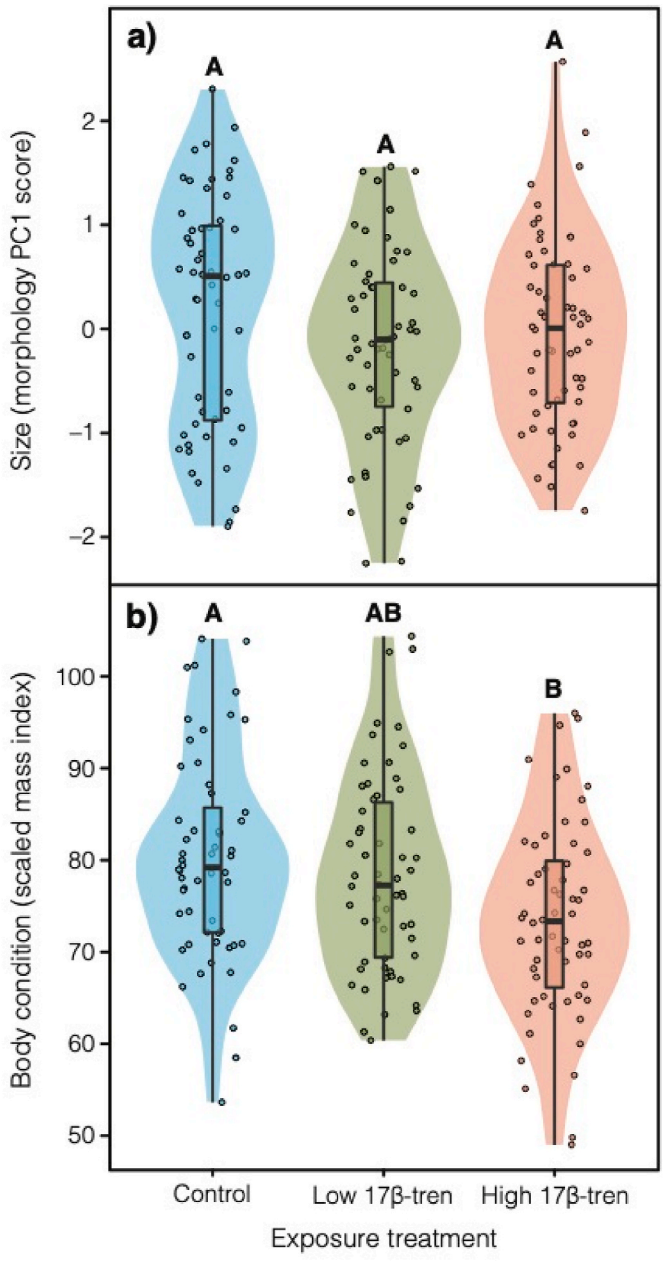

Fig. 4. Violin plots and boxplots showing (a) tadpole size (morphology PC1 score), and (b) tadpole scaled mass index (i.e. body condition) plotted for control (blue; $n = 53$), low-17β-trenbolone (green; $n = 60$) and high-17β-trenbolone (red; $n = 58$) tadpoles. Groups that share a capital letter are not significantly different from one another ($\alpha = 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2008). For example, Ankley et al. (2003) found that 17β-trenbolone concentrations as low as 5 ng/L increased the weight of adult fathead minnows (*Pimephales promelas*) following a 21-day exposure. Likewise, Bertram et al. (2019) reported an increase in body condition in adult mosquitofish (*Gambusia holbrooki*) after a 21-day exposure at 15 ng/L.

To our knowledge, the current study is the first to investigate the effects 17β-trenbolone on the metabolic function of anurans, making within-taxa comparisons difficult. However, there has been a handful of studies addressing the effects of 17β-trenbolone on anuran growth (Haselman et al., 2016; Li et al., 2015; Olmstead et al., 2012). Interestingly, the results of past research using anurans also contradict the effects observed in fish, reporting either no change, or a decrease in body size and body condition (Haselman et al., 2016; Li et al., 2015; Olmstead et al., 2012). For example, Haselman et al. (2016) reported no change in pre-metamorphic growth of African clawed frogs (*Xenopus laevis*) exposed to 17β-trenbolone (9.0–79.9 ng/L from NF stage 15–62). Similarly, Olmstead et al. (2012) found no change in body mass and body length of Western clawed frog tadpoles (*Xenopus tropicalis*) at metamorphosis following 17β-trenbolone exposure (3–102 ng/L exposure from NF stage 35–66). In the same study, Olmstead et al. (2012) also reported that exposure to 17β-trenbolone (76 ng/L exposure from NF stage 35–66) caused a significant decrease in the body length of juvenile frogs 6-weeks post-metamorphosis. A similar reduction in the body mass of tadpoles (*Pelophylax nigromaculatus*) at metamorphosis was uncovered by Li et al. (2015), following 17β-trenbolone exposure at 10,000 ng/L, with no effect reported at the lower concentrations used (100 and 1000 ng/L). Thus, it appears that the sub-lethal effects of 17β-trenbolone on metabolism and somatic growth may not be consistent between fish and anurans.

In the present study, we did not detect a significant effect of 17β-trenbolone on the anxiety-related behaviour of tadpoles. This contrasts with past studies that have uncovered effects of 17β-trenbolone exposure on the behaviour of non-target species, with the majority of this research focusing on reproductive behaviours of fish (Bertram et al., 2015; Bertram et al., 2019; Bertram et al., 2020; Heintz et al., 2015; Lagesson et al., 2018; Tan et al., 2021; Tomkins et al., 2017; Tomkins et al., 2018). Indeed, as far as we are aware, only four studies have investigated the impacts of 17β-trenbolone pollution on anxiety-related endpoints of non-target species, all of which have been in fish (Bertram et al., 2018, 2019; Heintz et al., 2015; Lagesson et al., 2018). Of these, Lagesson et al. (2018) reported a decrease in anxiety-related behaviour in male and female mosquitofish (*Gambusia holbrooki*; 3 ng/L for 21 days), Bertram et al. (2018b) reported a decrease in anxiety-related behaviour in female guppies (*Poecilia reticulata*; 16 ng/L for 21 days), and Heintz et al. (2015) reported a decrease in anxiety-like behaviour of female guppies. In the same study, Heintz et al. (2015) did not detect a significant shift in anxiety-related behaviour of male guppies (0.25–25 ng/L for 21 days), and in a separate study, Bertram et al. (2019) also reported no effect of 17β-trenbolone on anxiety-related behaviour of male mosquitofish (16 ng/L for 21 days). Thus, as with the effects on metabolism and somatic growth (discussed above), the effects of 17β-trenbolone on anxiety-related behaviour of tadpoles seem to contrast those previously reported in fish—although the results in fish appear to be sex-specific in some cases.

Considering the results of the current study in combination with past research, we hypothesise that there may be taxa-specific effects of 17β-trenbolone on metabolic function, growth, and behaviour with anurans possibly being less sensitive to disruption—at least at the tadpole life stage. These differences across taxa are potentially driven by the complex multiphase developmental stages of anurans compared to fish, which is accompanied by drastic metabolic reorganisation and changes in metabolic requirements (Zhu et al., 2020). The metabolic adjustments preceding morphological and functional transformation of tadpoles are not yet wholly understood (Zhu et al., 2020), and the impacts of 17β-trenbolone on the specific manner by which tadpoles coordinate the requirements of energy production and anabolism at the pre-metamorphic stage was beyond the scope of the present study. We encourage further cross-species investigation of the impacts of 17β-trenbolone, and other androgenic pollutants, on metabolic function to identify potential mechanisms driving the observed differences between fish and anuran.

6. Conclusions

In summary, we did not detect a significant effect of 17β-trenbolone on the metabolic rate, behaviour, or morphology of tadpoles although we did see a subtle but statistically significant decrease in tadpole body condition (i.e. scaled mass index) at the highest 17β-trenbolone

concentration. In comparison to past results reported in fish, our study suggests that tadpole metabolism and somatic growth, as well as anxiety-like behaviour, are less sensitive to 17β-trenbolone disruption. It is important to highlight that despite a lack of reported effects of 17β-trenbolone at early tadpole developmental stages, there is evidence that effects could manifest at later stages of development and life stages (e.g. after NF stage 58; Li et al., 2015; Olmstead et al., 2012). Further, 17β-trenbolone impacts have also been reported on the sexual development of anurans exposed to concentrations as low as 100 ng/L (Li et al., 2015). Thus, despite the present study reporting no effect of 17β-trenbolone on metabolic rate, body size, or behaviour, and a subtle negative effect on body condition, 17β-trenbolone exposure may still result in adverse outcomes in anuran populations.

Author statement

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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.

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Appendix A. Supplementary data

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