Impacts of exposure to UV radiation and an agricultural pollutant on morphology and behaviour of tadpoles (*Limnodynastes tasmaniensis*)

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Abstract

Amphibians are the most threatened vertebrate class globally, with many species at risk of extinction. Multiple factors have been implicated in the global decline of amphibian populations, and it has been hypothesised that interactions between these stressors may be responsible for such rapid declines. Increased ultraviolet (UV) radiation as a result of ozone depletion has been identified as one such stressor. Exposure to UV radiation has been shown to have detrimental effects on amphibian survival, development, and behaviour. UV radiation has also been shown to exacerbate the effects of other stressors, such as chemical pollutants. Chemical pollution has been recognised as another major factor contributing to amphibian declines, particularly, endocrine-disrupting chemicals. 17β-trenbolone is a potent anabolic steroid used in the agricultural industry to increase muscle mass in cattle, and has been repeatedly detected in the environment where amphibians live and breed. At high concentrations, 17β-trenbolone exposure has been shown to impact amphibian survival and gonadal development. In the present study, we investigated the effects of one-month exposure to environmentally realistic levels of UV radiation and 17β-trenbolone, both in isolation and in combination, on the morphology and behaviour of spotted marsh frog tadpoles (*Limnodynastes tasmaniensis*). We found that neither stressor in isolation affected the morphology or behaviour of tadpoles, nor did we find any interactive effects. The results from our 17β-trenbolone treatment are consistent with recent research suggesting that, at environmentally realistic concentrations, tadpoles may be less vulnerable to this pollutant compared to other vertebrate classes (e.g. fish). The absence of UV radiation-induced effects found in this study could be due to species-specific variation in susceptibility to UV radiation,

as well as the UV dosage utilised in the present study. We suggest future research incorporates long-term studies using multiple stressors at environmentally realistic levels to accurately identify the threats, and subsequent consequences, that amphibians face in natural conditions.

Keywords

Agricultural pollutant, Amphibian declines, Behavioural ecotoxicology, Endocrine disrupting chemical, Interactive effects, Multiple stressors, Ultraviolet radiation

Introduction

Up to 41% of amphibian species are currently at risk of extinction, making them the most imperilled vertebrate class globally (IUCN, 2022). The rate at which amphibian populations are declining is more rapid than that of birds or mammals, and potentially over 120 amphibian species have become extinct since 1980 (Stuart et al 2004, Mendelson et al 2006). The loss of amphibian populations globally may lead to further ecological consequences, as amphibians are a key component of trophic dynamics and energy flow within ecosystems, and are often viewed as excellent indicators of environmental health (Quaranta et al., 2009; Whiles et al., 2006).

Various factors have been identified as contributing to the decline of amphibians, including increased exposure to ultraviolet (UV) radiation (reviewed in Alton and Franklin, 2017). The coinciding fall in ozone levels and the beginning of rapid amphibian declines during the late 1970s resulted in an increased effort to understand the potential impacts of increased UV radiation associated with ozone depletion (Blaustein et al., 2001). Such studies have shown that UV exposure can cause increased mortality, reduced growth, slower rates of development, delayed metamorphosis, developmental abnormalities, and behavioural changes in amphibians (reviewed in Alton and Franklin, 2017).

UV exposure has been demonstrated to interact with both abiotic and biotic factors to have compounding negative effects on amphibians (e.g. temperature and predation, respectively; Alton and Franklin, 2017; Bancroft et al., 2008). Here, the interactive effects of multiple stressors can be difficult to predict. On the one hand, they can be additive, that is, the interaction can be equal to the sum of the stressors' independent effects. On the other, evidence suggests that exposure to multiple stressors can have synergistic or even antagonistic effects, meaning that their impacts can be either greater, or less than, the sum of their independent effects, respectively (Folt et al., 1999). Although UV radiation and additional stressors tend to interact synergistically to increase mortality in amphibians (Bancroft et al., 2008), there is evidence that UV can interact both antagonistically and additively with other stressors (Alton and Franklin, 2017; Lundsgaard et al., 2021). Multi-stressor studies have therefore been recognised as a key area of research in investigating amphibian declines (Alton and Franklin, 2017; Bancroft et al., 2008; Sievers et al., 2019), as it is now generally understood that declines are more likely to be due to complex interactions between multiple factors, rather than single factors acting in isolation (Côté et al., 2016; Womack et al., 2022). These studies also help to increase the environmental realism of experiments, and therefore our understanding of the threats that amphibians face in the wild, because in natural settings, amphibians are frequently exposed to more than one stressor simultaneously (Croteau et al., 2008; Sievers et al., 2019; Sih et al., 2004).

Another stressor that has been recognised as contributing to amphibian declines is chemical pollution (Egea-Serrano et al., 2012; Orton and Tyler, 2015). Due to various physical and life-history traits (e.g. permeable skin, aquatic eggs), many amphibian species are particularly vulnerable to toxicants in the environment (Bókony et al., 2020; Hayes et al., 2010). In isolation, chemical pollutants have been shown to impact amphibian survival, morphology, physiology, and behaviour (reviewed in Baker et al., 2013; Egea-Serrano et al., 2012; Sievers et al., 2019). Alarmingly, even chemicals that do not have an effect in isolation have been shown to impact amphibian survival and development when combined with an additional stressor (Boone et al., 2005; Boone and James, 2003; Hatch and Blaustein, 2003). Research has shown that UV radiation and chemical pollutants can interact synergistically to affect survival, development, morphology and behaviour (Bancroft et al., 2008; Blaustein et al., 2003; Mann et al., 2009). However, so far, pollution studies have tended to focus on only a few chemical classes (e.g. carbaryl, nitrate), and often use UV radiation doses and/or concentrations of chemicals above those commonly found in the environment (Ankley et al., 2000; Licht, 2003; Zaga et al., 1998). One pollutant of increasing concern is 17β-trenbolone, a potent endocrine-disrupting chemical used in the agricultural industry to increase meat yield from cattle (Ankley et al., 2018; Hunter, 2010). It enters the environment through excrement of injected animals, and has been measured in environmental concentrations of <1–270 ng/L (Ankley et al., 2018). In Australia, many amphibian species are at high risk of exposure to agricultural pollutants, such as 17β-trenbolone, because they inhabit and breed in areas where agricultural pollution is present, either as run-off or where it is intentionally applied (Brand and Snodgrass, 2010; Hazell et al., 2001; Sievers et al., 2018a). Research has shown that exposure to environmentally realistic concentrations of 17β-trenbolone is sufficient to induce morphological and behavioural effects in fish (reviewed in Ankley et al., 2018). At high concentrations, exposure to 17β-trenbolone has also been shown to affect amphibian survival, gonadal development, and sex ratios (Haselman et al., 2016; Li et al., 2015; Olmstead et al., 2012; Rozenblut-Kościsty et al., 2019). Recent studies of 17β-trenbolone suggest that, at environmentally realistic concentrations, amphibians may be less vulnerable than fish, with exposure having no effect on behaviour or morphology (Martin et al., 2022; Orford et al., 2022). However, the potential for UV radiation to act synergistically with 17β-trenbolone remains to be investigated.

Accordingly, we set out to examine the effects of UV radiation and 17β-trenbolone on tadpole morphology and behaviour of the spotted marsh frog (*Limnodynastes tasmaniensis*). The levels of UV radiation used in the experiment were based on conditions experienced by *L. tasmaniensis* during their breeding season in Melbourne, Australia (ARPANSA 2019). For our 17β-trenbolone exposure, we used an environmentally realistic nominal concentration of 50 ng/L, representing levels detected on a working agricultural farm (Gall et al., 2011). Using a factorial combination of these two stressors, we then investigated the effects of exposure on the morphology and behaviour of tadpoles, specifically, responses to conspecific chemical cues that signal the presence of predators and a simulated predator strike, and assessed whether the ubiquitous environmental stressor UV radiation may intensify or reduce the effects of a potent chemical pollutant on exposed wildlife.

Methods

Animal collection and housing

Twelve spotted marsh frog (*Limnodynastes tasmaniensis*) egg masses were collected on 14 October 2020 in Melbourne, Victoria, Australia (37° 50' 31.6" S, 145° 12' 43.1" E; DELWP permit number 10009162). Water samples taken from this water body confirmed it was free from 17β-trenbolone contamination (Envirolab Services, *n* = 2, unpublished data). 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 held separately in tanks (60 × 30 × 30 cm; length × width × height) filled with 32 L of aged carbon-filtered water for approximately three weeks prior to chemical exposure in order to allow the tadpoles to develop to Gosner stage 25 (GS; Gosner, 1960). During this period, weekly water changes using aged carbon-filtered water (6 L) were performed to maintain water quality.

Experimental exposure

Upon reaching Gosner stage (GS) 25 (Gosner, 1960), tadpoles were exposed to a factorial combination of two UV treatments (UV radiation either present or absent) and two 17β-trenbolone treatments (17β-trenbolone either present or absent), such that there were four experimental treatments in total: a freshwater solvent control (17β-trenbolone and UV radiation both absent; hereafter control treatment), a UV treatment (UV radiation present, 17β-trenbolone absent), a 17β-trenbolone treatment (17β-trenbolone present, UV radiation absent; nominal concentration 50 ng/L), and a UV and 17β-trenbolone combination treatment (17β-trenbolone and UV radiation both present; nominal concentration 50 ng/L; hereafter multi-stressor treatment). The exposure length of one month was chosen as previous research has shown that exposure to 17β-trenbolone or UV radiation for similar periods is sufficient to induce effects on morphology or behaviour in tadpoles and other aquatic animals (Alton et al., 2012; Alves et al., 2020; Bertram et al., 2015; Lundsgaard et al., 2022, 2020; Saaristo et al., 2013; Sone et al., 2005; Tomkins et al., 2017).

The exposure consisted of 24 independent tanks (60 × 30 × 30 cm; *n* = 6 per treatment), with 40 tadpoles in each tank (*n* = 240 per treatment). When allocating individuals to tanks, the number of tadpoles from each egg mass was approximately balanced across both the individual tanks and the four treatment groups, in order to control for any potential clutch effects (Gibbons and George, 2013). For logisitical reasons due to space and equipment constraints, introduction to the exposure system was staggered in three batches over 9 days (i.e. two tanks per treatment were established with 40 tadpoles each on days 1, 5 and 9). As a result of this design, tadpole age at the beginning of the exposure, and subsequently when they underwent behavioural assays and morphological measurements at the end of the exposure, varied by 1–9 days. In addition, this design meant that there was a slight variation in exposure length, with treatment tanks being exposed for 28–31 days (for simplicity, exposure duration is referred to as one month). Importantly, however, tadpole age and the variation in exposure length were balanced equally across treatments, and were included in statistical models to control for any potential effects on morphology or behaviour (Orford et al., 2022; Touchon et al., 2013; see statistical analysis below). Each exposure tank was filled with 18 L of aged carbon-filtered water, and contained 1 cm of natural gravel substrate. Water changes (6 L, i.e. 33%) were performed once a week. To ensure consistent conditions across tanks, weekly temperature (18.56 ± 0.19 °C, mean ± SD; *n* = 96) and pH (6.94 ± 0.13; *n* = 96) checks were performed (see Table S1 for summaries across tanks). Tadpole survival in each treatment tank was measured at the end of the one-month exposure and did not differ significantly among treatments (mean ± SD survival percentage: 82.9 ± 17.3 %; see Supplementary material for survival by treatment comparisons). At day 14 of the exposure, 12 tadpoles per treatment tank were removed for use in a separate experiment that is not discussed here. Tadpoles were fed boiled spinach three times a week.

UV radiation exposure

Treatment tanks were exposed to ultraviolet-B radiation (UVBR), ultraviolet-A radiation (UVAR) and visible light emitted from a 36 W 120-cm linear fluorescent light bulb (Repti-Glo Exo Terra T8, Montreal, QC, Canada) that was suspended 50 cm above the water surface across the centre of three tanks and set on a 12:12 h light:dark photoperiod. The bulbs suspended over the control and 17β-trenbolone treatment tanks were covered with a UV-blocking filter (Crystal clear window film, Pillar, Melbourne, Australia), whereas the bulbs over the UV and multi-stressor treatment tanks were uncovered.The absolute irradiance of UVBR and UVAR for all treatments was measured using a spectrometer (USB2000+ Miniature Fiber Optic Spectrometer, Ocean Optics, Dunedin, FL, USA) at 7 fixed positions at the level of the water surface (see Supplementary Methods for further detail). Three measurements were taken directly below the light bulb (i.e., one measurement at the centre of each tank) and four measurements were taken at the edge of the tanks, in order to estimate the range of absolute irradiance that the tanks would be receiving. For tanks in the UV and multi-stressor treatments, the absolute irradiance of UVBR ranged from 4.3 to 8.7 μW cm^{-2} from the edge to the centre of the tanks, respectively. For UVAR, the absolute irradiance ranged from 34.7 to 71.8 μW cm^{-2}.

These absolute irradiances result in a UV index of < 1 (see Table S2 and Fig S1 for measurements from all experimental treatments). The UV index during the breeding season of *L. tasmaniensis* in Melbourne, Australia, falls below 1 in the morning (i.e. before 09.00 h) and in the evening (i.e. after 18.00 h), with the average daily maximum UV index between October and December 2019 being 6.5 (ARPANSA, 2019). As UV penetration into water is attenuated by suspended particulate matter and dissolved carbon, it is likely that tadpoles in the wild would experience only a small fraction of the total solar irradiance measured at water surface level (Alton and Franklin, 2017; Ceccato et al., 2016). We therefore chose to expose tadpoles to relatively low UVR levels as this is more likely to reflect levels that tadpoles would naturally experience in the wild, due to the aforementioned absorbance of UVR by dissolved organic matter in the water column, as well as shading by vegetation (both terrestrial and aquatic) and cloud cover (Alton and Franklin, 2017; Diamond et al., 2005; Lundsgaard et al., 2020; Palen et al., 2002; Palen and Schindler, 2010).

17β-Trenbolone exposure

Tadpoles in the 17β-trenbolone and multi-stressor treatments were exposed to 17β-trenbolone using a static renewal system. At the start of the exposure, all tanks in these treatments received an initial dose of 16.36 µg of 17β-trenbolone (CAS: 10161–33-8; Novachem, Germany) dissolved in 1 mL of ethanol (HPLC grade, ≥ 99.99%) respectively. Thereafter, to maintain exposure concentrations and replace any 17β-trenbolone lost to evaporation and water changes, all 17β-trenbolone and multi-stressor tanks were dosed twice a week with 2.45 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 and UV treatments received an initial dose of 1 mL ethanol and subsequent 1 mL ethanol doses twice a week, at the same time as the 17β-trenbolone and multi-stressor tanks. To monitor concentrations of 17β-trenbolone in the 17β-trenbolone and multi-stressor treatments, water samples (100 mL) were taken from each exposure tank approximately 24 h after the second of the two weekly doses. Over the one-month exposure period, each exposure tank in these two treatments was tested six times (*n* = 36 per treatment). Each tank in the control and UV treatments was tested three times during the one-month exposure (*n* = 18 per treatment) to confirm that contamination had not occurred in these tanks. These samples were collected at the same time as those from the 17β-trenbolone and multi-stressor tanks. The concentration of 17β-trenbolone in all samples 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).

Behavioural assays

The effects of experimental treatments on behaviour were tested in two separate assays (conspecific cue and simulated predator strike; detailed below) that were conducted after one month of exposure. These behavioural assays were chosen as UV radiation and 17β-trenbolone have previously been shown to affect anxiety-like behaviour (i.e. activity) and antipredator behaviour (Alton and Franklin, 2017; Bertram et al., 2018; Heintz et al., 2015; Lagesson et al., 2019). Upon completion of the one month exposure period, for each batch of exposure tanks (3 batches total; 8 tanks per batch) eight individuals were randomly selected per tank each day over a 2-day trial period. Half of the individuals performed the conspecific cue experiment (*n* = 16 per day), and the other half underwent the simulated predator strike experiment (*n* = 16 per day). This process was repeated for all three batches. Any variation in exposure period due to this design was accounted for in statistical analyses. Trial tanks were covered on all sides with frosted opaque sheeting to prevent tadpole behaviour being affected by external stimuli. 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. All trials and subsequent data extraction were performed blind to experimental treatment. During the trials, all behaviours were recorded from above at 60 frames per second (Panasonic HC-V180) for the conspecific cue assay, and 100 frames per second (Sony FDR-AX33) for the simulated predator strike assay. From the resulting videos, the animal tracking software Ethovision XT V16 (Noldus Information Technology, the Netherlands) was used to track tadpoles. This allowed for total distance moved (cm), and time spent stationary (i.e. freezing behaviour; s) before and after either the addition of conspecific chemical cues or the simulated predator strike to be calculated for each tadpole. A tadpole was considered to not be moving if velocity dropped below 5 mm s^{-1} (as previously used in Orford et al., 2022). All trials had a tracking efficiency of > 99%.

Conspecific cue assay

We measured the behaviour of tadpoles (control *n* = 48, UV *n* = 46, 17β-trenbolone *n* = 48, multi-stressor *n* = 48) before and after the addition of conspecific chemical cues using methods adapted from previously established protocols (Crossland et al., 2019; Gonzalo et al., 2007; Hagman et al., 2009; McCann et al., 2020). Conspecific chemical cues were used in this experiment as a variety of taxa (including amphibians and fish) respond to chemical cues from injured conspecifics because they act as a reliable indicator of an immediate threat, such as the presence of a predator (Chivers and Smith, 1998; Hagman et al., 2009; Rajchard, 2006). Trials took place in observation tanks (25 × 15 × 15 cm) filled with aged carbon-filtered water to a depth of 2 cm (17.8 ± 0.1 °C). Tadpoles were introduced to the observation tank and given a 10 min acclimation period. After the acclimation period, 5 mL of control water (i.e. aged carbon-filtered water free of any chemical cues) was slowly added to the tank at either end via syringes (i.e. 10 mL added to the tank in total). A dye infusion test (using green food dye) confirmed that this method resulted in a rapid and equal dispersion of stimulus throughout the tank. The addition of control water to the tanks was to control for any impact on tadpole behaviour that the manual addition of cues may have had. Tadpoles were then filmed for 5 min (i.e. pre-cue period). After the 5 min period, 10 mL of conspecific cue mix was added in the same manner (i.e. 5 mL added from each end of the tank). To prepare the conspecific cue mix, 2 g of tadpoles (sourced from the same egg clutches as the tadpoles in the exposure system but not exposed to any experimental treatments) were macerated in 2 L of water which was then filtered. A 10 mL aliquot of this mix was then used in our trials, resulting in the same final concentration of cue mix employed by Hagman et al. (2009). For further details regarding conspecific cue preparation, see Supplementary methods. Tadpoles were then filmed for another 5 min (i.e. post-cue period).

Simulated predator strike assay

In a separate assay, we measured the behaviour of tadpoles (*n* = 48 per treatment) before and after a simulated predator strike using methods adapted from previously established protocols (Arendt, 2003; Orford et al., 2022). Trials took place in observation tanks (diameter = 15 cm, height = 14.5 cm) filled with aged carbon-filtered water to a depth of 2 cm (18.2 ± 0.2 °C). 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. This 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). Subsequent post-strike behaviour was then recorded for 5 min (i.e. post-strike period).

Morphology

Upon completion of the behavioural assays, tadpoles were photographed dorsally (Canon Powershot S120) and laterally (Nikon DSLR D80) for measures of total length, body length, body width and body height (cm; ± 0.01 mm). Data were extracted from the photographs using ImageJ software (Schneider et al., 2012). Tadpoles were then blotted dry and weighed (g; ± 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 (*sensu* Peig and Green, 2009). Specifically, we performed a standard major axis regression on the base-e log of body mass (M; g) and body 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). 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 = 0.972).

Statistical Analyses

Statistical analyses were conducted using *R* version 4.1.1 (R Core Team, 2022). Where appropriate, data were transformed to approximate Gaussian error distributions and continuous predictors were scaled to aid model fitting (see Tables S3–9 for descriptions). F-tests for linear mixed-effects models (LME, *lme4* package; Bates et al., 2015) with Kenward-Roger Degrees of Freedom Approximation were used to calculate *p*-values of fixed effects. The interactive effects of UV radiation radiation and 17β-trenbolone on tadpole morphology and behaviour were analysed. All models included an interaction term between the categorical factors of UV and 17β-trenbolone, each of which had two levels (i.e. UV+/UV-, TB+/TB-), indicating presence or absence of exposure to UV radiation and/or 17β-trenbolone. The significance of the interaction term was tested using type-III tests.

Data from both the conspecific cue and simulated predator strike assays (distance moved and freezing behaviour) were analysed using LMEs. For both endpoints, models included a 3-way interaction between UV, 17β-trenbolone, and trial period (i.e. pre-cue/strike, post-cue/strike). Tadpole mass (mg), exposure length (i.e. 28–31 days of exposure), and time of day were also included as fixed effects. Treatment tank (i.e. what exposure tank the individual was housed in) nested within experimental group (i.e. tadpole age when it was introduced to the exposure) and tadpole ID were included as random intercepts.

All morphological endpoints (total length, body length, body width, body height and mass) were shown to be highly correlated with each other (≥ 0.86), so analyses proceeded with total length and mass as the chosen morphological measurements of interest. Morphological data (total length, mass and body condition) were analysed using LMEs. Models included a 2-way interaction between UV and 17β-trenbolone. Exposure length was included as a fixed effect. Treatment tank nested within experimental group was included as a random intercept.

Results

Analytical verification of 17β-trenbolone analysis

To account for left-censoring of 17β-trenbolone due to the method detection limit (MDL = 2 ng/L), all trenbolone samples that fell below the MDL (17β-trenbolone treatment *n* = 9) were included in the analysis as the MDL divided by 2, following Antweiler and Taylor (2015). The mean measured concentrations (± SD) for the 17β-trenbolone and multi-stressor treatments during the one-month exposure were 33.6 ± 56 ng/L and 24.9 ± 37.3 ng/L, respectively. No 17β-trenbolone was detected in the control or UV treatment tanks.

Conspecific cue assay

For both distance moved and freezing behaviour during the conspecific cue assay, there was no significant interaction between UV radiation, 17β-trenbolone, and trial period ($F_{1,186.0} = 0.03$, $p = 0.873$; $F_{1,186.0} = 1.47$, $p = 0.226$, respectively), nor were there significant main effects of UV radiation or 17β-trenbolone (see Tables S3–4 for full model outputs). Irrespective of treatment, trial period had a significant effect on both distance moved and freezing behaviour ($F_{1,186.0} = 30.23$, $p < 0.001$; $F_{1,186.0} = 14.43$, $p < 0.001$, respectively), with tadpoles travelling shorter distances and spending more time freezing after the addition of conspecific cues (Fig. 1).

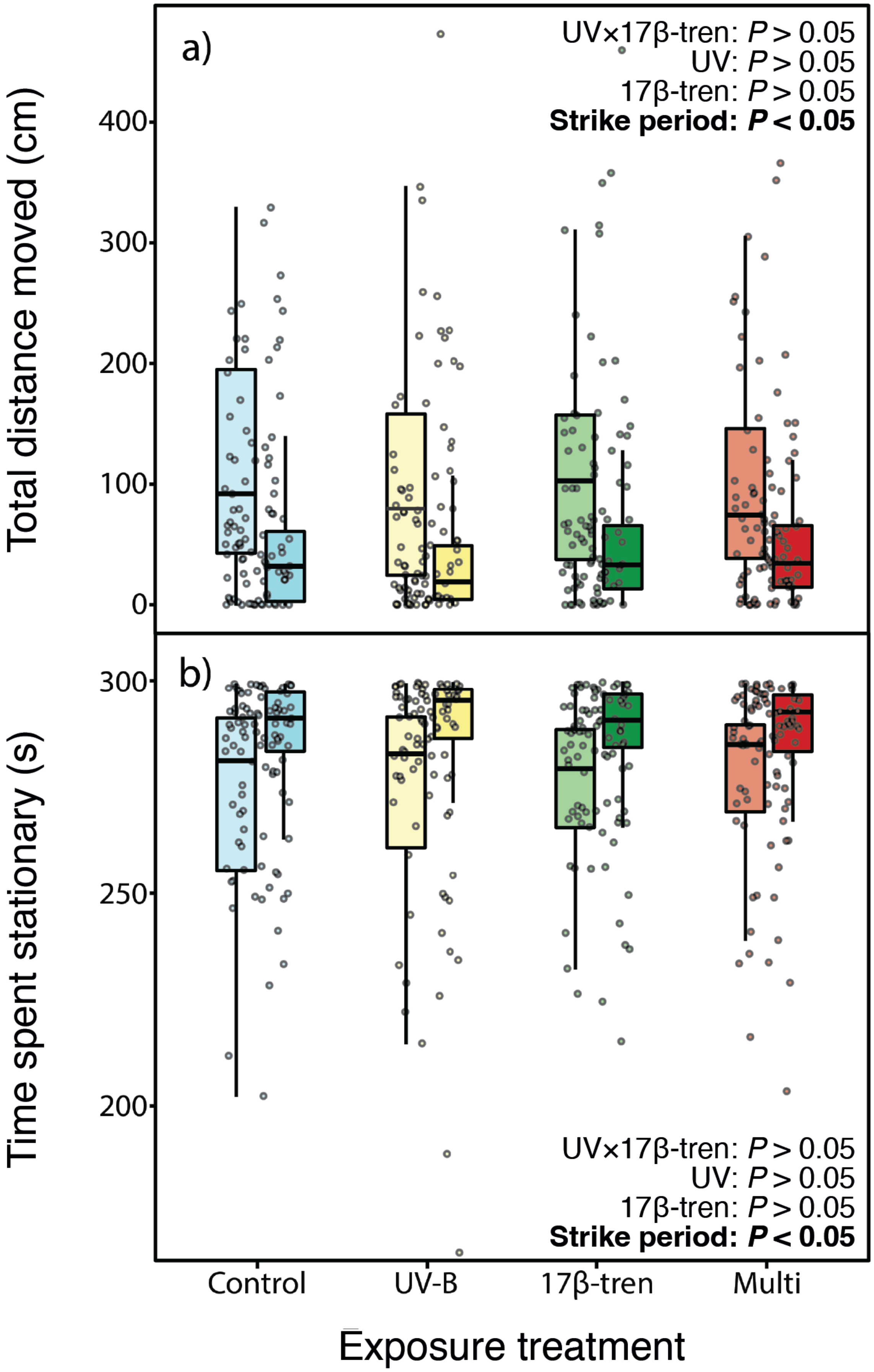
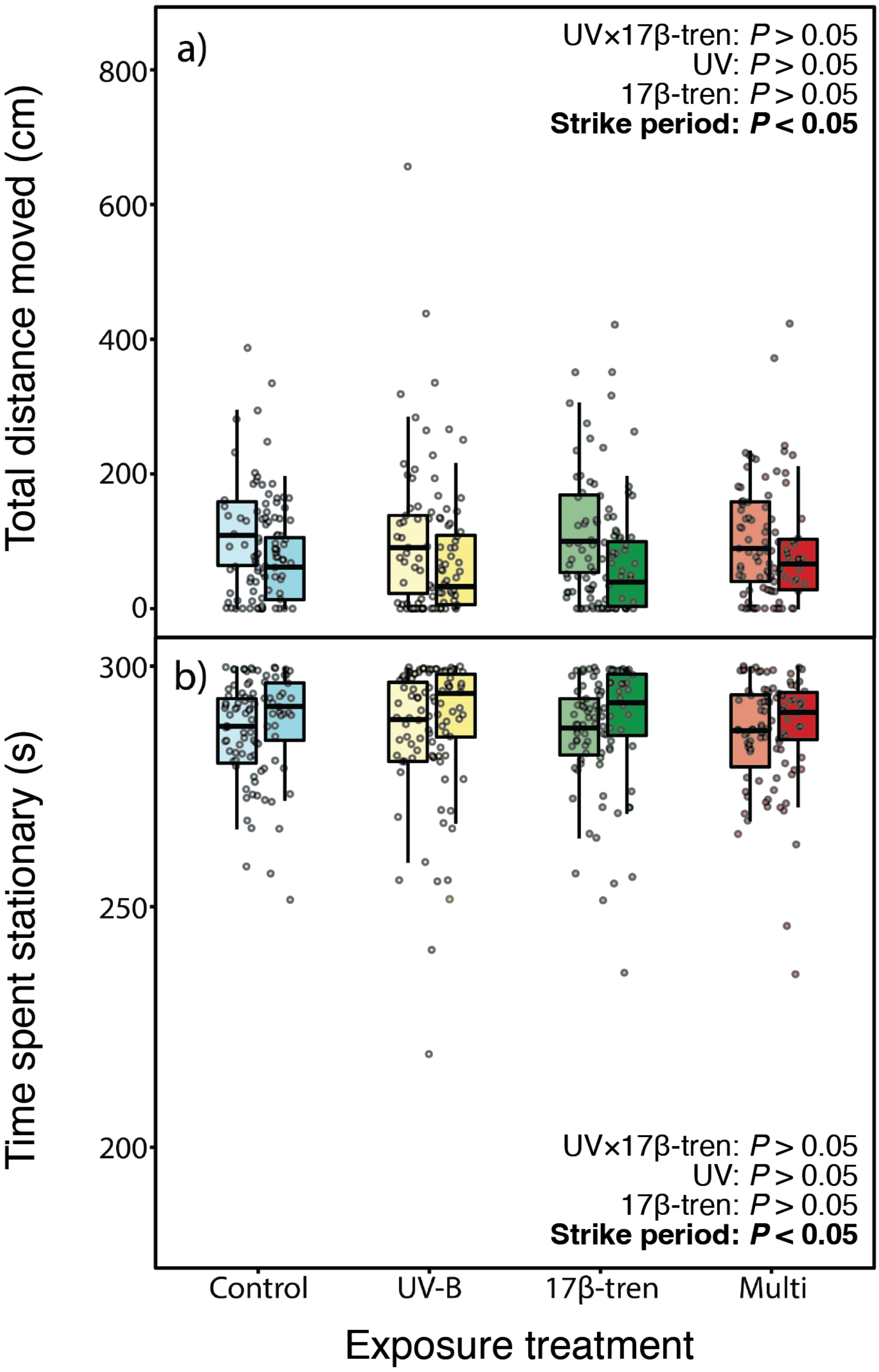

a)
UV×17β-tren: $P > 0.05$
UV: $P > 0.05$
17β-tren: $P > 0.05$
Strike period: $P < 0.05$
Total distance moved (cm)
0
100
200
300
400
b)
UV×17β-tren: $P > 0.05$
UV: $P > 0.05$
17β-tren: $P > 0.05$
Strike period: $P < 0.05$
Time spent stationary (s)
200
250
300
Control
UV-B
17β-tren
Multi
Exposure treatment

Figure 1. Boxplots showing (a) the distance moved (cm) and (b) time spent stationary (s) by tadpoles before (lighter coloured boxes on the left for each treatment) and after (darker coloured boxes on the right for each treatment) the addition of conspecific cues for all exposure treatments (control $n = 48$, UV $n = 46$, 17β-trenbolone $n = 48$, multi-stressor $n = 48$). Treatment groups that do not share upper case letters are significantly different ($p < 0.05$).

Simulated predator strike assay

For both distance moved and freezing behaviour during the simulated predator strike assay there was no significant interaction between UV radiation, 17β-trenbolone, and trial period ($F_{1,188.0} = 0.99$, $p = 0.321$; $F_{1,188.0} = 1.37$, $p = 0.243$, respectively), nor were there significant main effects of UV radiation or 17β-trenbolone (see Tables S5–6 for full model outputs). Irrespective of treatment, trial period had a significant effect on both distance moved and freezing behaviour ($F_{1,188.0} = 10.13$, $p < 0.001$; $F_{1,188.0} = 7.33$, $p < 0.001$, respectively), with tadpoles travelling shorter distances and spending more time freezing after the simulated predator strike (Fig. 2). There was also a significant effect of mass on both distance moved and freezing behaviour ($F_{1,164.1} = 11.09$, $p = 0.001$; $F_{1,169.3} = 4.88$, $p = 0.028$, respectively), with heavier tadpoles moving greater distances and spending more time moving overall (Fig S2 and S3).



a)
UV×17β-tren: P > 0.05
UV: P > 0.05
17β-tren: P > 0.05
Strike period: P < 0.05
Total distance moved (cm)
0
200
400
600
800
b)
UV×17β-tren: P > 0.05
UV: P > 0.05
17β-tren: P > 0.05
Strike period: P < 0.05
Time spent stationary (s)
200
250
300
Control
UV-B
17β-tren
Multi
Exposure treatment

Figure 2. Boxplots showing (a) the distance moved (cm) and (b) time spent stationary (s) by tadpoles before (lighter coloured boxes on the left for each treatment) and after (darker coloured boxes on the right for each treatment) the simulated predator strike for all exposure treatments (*n* = 48 per treatment). Treatment groups that do not share upper case letters are significantly different ($p < 0.05$).

Morphology

For total length, mass, and body condition, we found no significant interaction between UV radiation and 17β-trenbolone ($F_{1,17.3} = 0.56$, $p = 0.464$; $F_{1,17.3} = 0.89$, $p = 0.358$; $F_{1,17.1} = 0.02$, $p = 0.902$, respectively), nor any significant main effects of either stressor (Fig. 3; see Tables S7–9 for full model outputs).

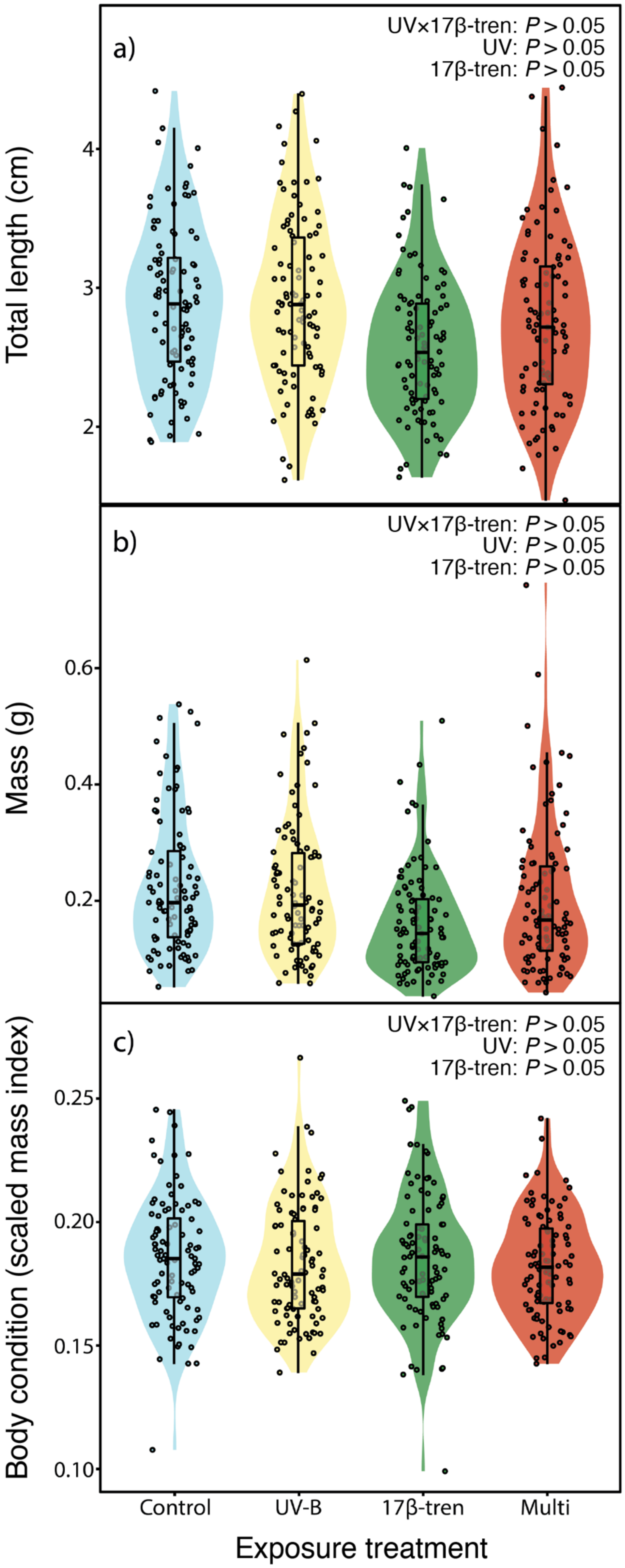

a)
UV×17β-tren: $P > 0.05$
UV: $P > 0.05$
17β-tren: $P > 0.05$
Total length (cm)
4
3
2
b)
UV×17β-tren: $P > 0.05$
UV: $P > 0.05$
17β-tren: $P > 0.05$
Mass (g)
0.6
0.4
0.2
c)
UV×17β-tren: $P > 0.05$
UV: $P > 0.05$
17β-tren: $P > 0.05$
Body condition (scaled mass index)
0.25
0.20
0.15
0.10
Control
UV-B
17β-tren
Multi
Exposure treatment

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Figure 3. Violin plots and boxplots showing tadpole (a) total length, (b) mass, and (c) body condition. Control (*n* = 96) is shown here in blue, UV (*n* = 94) in yellow, 17β-trenbolone (*n* = 96) in green, and multi stressor (*n* = 96) 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 box-plot (violin plot) shows the probability density at different values smoothed by a kernel density estimator. The *p*-values for the interaction between UV radiation and 17β-trenbolone, and main effects of both stressors, are included.

Discussion

Here we investigated whether exposure to environmentally realistic levels of UV radiation and the agricultural pollutant 17β-trenbolone for one month would impact the behaviour and morphology of tadpoles. We found no significant interactions between UV radiation and 17β-trenbolone, and no significant main effects of either stressor, on tadpole morphology or behaviour.

We found no significant effect of 17β-trenbolone on *L. tasmaniensis* tadpole morphology, or anxiety-like behaviour during the conspecific cue and simulated predator strike assays. Currently, research investigating the effects of environmentally-realistic concentrations of 17β-trenbolone on tadpoles is scarce (Ankley et al., 2018). However, the findings presented here are concordant with our previous studies on *L. tasmaniensis*, where 28-day exposure to comparable concentrations of 17β-trenbolone (10 and 66 ng/L for 28 days) had no impact on tadpole length and mass, or on anxiety-like behaviours across multiple assays (i.e., freezing behaviour, distance moved, and time spent in upper half of water column;

Martin et al., 2022; Orford et al., 2022). The results presented here do, however, contrast with Li et al. (2015), who reported *Pelophylax nigromaculatus* tadpoles exposed to 17β-trenbolone exhibited decreased growth. One potential explanation for the differences between this study and the present experiment is the dosage of 17β-trenbolone used, with Li et al. (2015) exposing tadpoles to 10,000 ng/L, a concentration far exceeding those recorded in the environment (Ankley et al., 2018). Another potential explanation is that species-specific responses to chemical pollutants have been repeatedly observed in amphibians, with behavioural responses of different species following exposure to the same concentration of chemical pollutant differing in terms of both magnitude and direction (discussed further in Orford et al., 2023). The results in this experiment also contrast with existing literature on fish, which found that exposure to 17β-trenbolone can result in changes to both morphology and behaviour (e.g. Baumann et al., 2014; Bertram et al., 2018; Tan et al., 2021). We have previously hypothesised that these differences may be due to the life-stage of the animals used, with fish studies often using sexually mature animals (see Orford et al., 2022). 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). However, tadpole development had not reached this stage in the present study, and therefore tadpoles were not exposed to 17β-trenbolone during this period of increased sensitivity.

UV radiation has previously been shown to impact both behaviour and morphology across a number of anuran species, including species within the *Limnodynastes* genus (reviewed in Alton and Franklin, 2017). For example, exposure to environmentally-realistic levels of UV radiation (i.e. UV index scores of <1) for 7–9 days resulted in significantly reduced mass and size in *Limnodynastes peronii* tadpoles, as well as causing individuals to significantly reduce their activity (Alton et al., 2012, 2010; Bernal et al., 2011). UV radiation exposure has also been shown to affect anuran responses to both conspecific and predator chemical cues. Kats et al. (2000) found that *Bufo boreas* metamorphs exposed to UV radiation exhibited less avoidance behaviour to conspecific cues compared with unexposed toads, and *Rana cascadae* tadpoles exposed to UV radiation did not reduce their movement in response to predatory cues as much as unexposed tadpoles. The lack of effect that UV radiation had on tadpole behaviour and morphology in the present study was therefore surprising, but could potentially be explained by variation in sensitivity and exposure to UV radiation that occurs across amphibian species, populations, and life stages (Alton and Franklin, 2017; Blaustein et al., 1999; Palen et al., 2005). The extent to which survival, development, and behaviour are affected by UV exposure has been shown to vary across taxa, with some species seemingly unaffected by exposure (Long et al., 1995; Pahkala et al., 2003), whilst others suffer increased mortality, developmental abnormalities, extended development times, and various changes to behaviour (reviewed in Alton and Franklin, 2017). Susceptibility to UV radiation can also differ between different populations of the same species, with high-altitude populations seemingly having an increased tolerance compared to lowland populations (Marquis and Miaud, 2008). These species- and population-specific effects of UV radiation are largely associated with variation in DNA repair rates and melanin synthesis (see Alton and Franklin, 2017). In addition, as amphibians develop, they may utilise behavioural strategies to a greater degree, compared to earlier life-stages, in order to avoid UV exposure, for example swimming in shaded areas or at greater depths (Alton and Franklin, 2017; Belden et al., 2000; Blaustein and Belden, 2003). In an experimental setting, these behaviours may affect the amount of UV radiation that a study organism receives. Indeed, Bridges and Boone (2003) suggested that the lack of effects seen in their UV exposure study may have been due to tadpoles swimming into leaf litter and depths where less UV radiation penetrated. This variation in exposure to UV radiation between life-stages means that results from other early-life studies may not be consistent across the life-stages of tadpoles covered in the present study. It should also be noted that some effects observed as a result of UV exposure utilised non-environmentally realistic levels of UV radiation (e.g. Grant and Licht, 1995; Licht, 2003; M. Pahkala et al., 2003; Pahkala et al., 2001), with one study exposing eggs to an absolute irradiance of 340 µW cm^{-2} (Grant and Licht, 1995), which is over 40 times higher than the absolute irradiances employed in the present study. Increasing environmental realism in such studies is paramount to accurately identifying the threats that UV radiation may have on animals under natural conditions (Ankley et al., 2000; Cramp and Franklin, 2018; Peterson et al., 2002), and the use of environmentally realistic levels in this study reflects this. Overall, the literature suggests that the results observed in the present study may be influenced by the UV dosage, species, life stage and behaviour of the study organism.

The literature investigating the effects of combined UV radiation and chemical pollutant exposure is currently limited (reviewed in Bancroft et al., 2008; Blaustein et al., 2003; Mann et al., 2009). Whilst most of the existing studies suggest that UV radiation and chemicals interact synergistically to result in detrimental effects greater than those caused by either stressor alone (e.g. Baud and Beck, 2005; Hatch and Blaustein, 2003; Macías et al., 2007), there is evidence to suggest that combined exposure can result in either no effect (Bridges and Boone, 2003; Croteau et al., 2008), or an effect in the opposite direction to what would commonly be seen as detrimental (e.g. increased growth, faster development; Crump et al., 2002). To our knowledge, there are currently no studies that have used 17β-trenbolone as part of a multi-stressor study. However, based on the results of this study, it appears that at environmentally realistic concentrations there is no interaction between 17β-trenbolone and UV radiation. The combined effects of UV exposure and chemical pollutants are also understudied in tadpoles, with many studies using only the embryo life-stage (Bancroft et al., 2008). Further, very few studies investigating the combined effects of UV radiation and chemical pollutants on amphibians have investigated the potential for early-life exposure to have long-lasting effects that might, potentially, carry over to impact metamorphs and adults (Bancroft et al., 2008; Lundsgaard et al., 2023; Sievers et al., 2019). This is despite research showing that exposure during the tadpole life-stage to either UV radiation or chemical pollutants can result in carryover effects that impact adult fitness (Ceccato et al., 2016; Lundsgaard et al., 2022; Pahkala et al., 2003; Rohr et al., 2013). Increasing environmental realism through the use of multiple stressors, environmentally realistic concentrations, and long-term exposures enables the gap between field and laboratory studies to be bridged, and advances our understanding of the real-world impacts that such stressors have on wildlife (Bertram et al., 2022; Mehdi et al., 2019).

Conclusion

In summary, we did not detect a significant effect of UV radiation or 17β-trenbolone, either alone or in combination, on the morphology or behaviour of tadpoles. In comparison to past research, our study lends support to the idea that there is taxon-specific differences in susceptibility to 17β-trenbolone (Martin et al., 2022; Orford et al., 2022). Given that the effects of UV exposure on tadpoles varies depending on numerous factors, and that exposure can result in no effects, this research adds information on *L. tasmaniensis* to an extensive body of existing work. As this is the first study of its kind to utilise 17β-trenbolone as part of a multi-stressor study, and given the current state of research on UV radiation and chemical pollutants, future studies should aim to investigate the long-term (i.e. across multiple life-stages) effects of environmentally realistic levels of UV radiation and chemical pollutants on amphibians. Studies with high levels of environmental realism can help to further our understanding of the threats that this imperilled taxa face in the wild, which, in turn, can assist with the implementation of effective conservation efforts.

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).

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