



Impacts of Exposure to Ultraviolet Radiation and an Agricultural Pollutant on Morphology and Behavior of Tadpoles (*Limnodynastes tasmaniensis*)

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Abstract: Amphibians are the most threatened vertebrate class globally. Multiple factors have been implicated in their global decline, and it has been hypothesized that interactions between stressors may be a major cause. 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 amphibians and can exacerbate the effects of other stressors, such as chemical pollutants. Chemical pollution has likewise been recognized as a major factor contributing to amphibian declines, particularly, endocrine-disrupting chemicals. In this regard, 17 β -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, 17 β -trenbolone has been shown to impact amphibian survival and gonadal development. In the present study, we investigated the effects of environmentally realistic UV radiation and 17 β -trenbolone exposure, both in isolation and in combination, on the morphology and behavior of tadpoles (*Limnodynastes tasmaniensis*). We found that neither stressor in isolation affected tadpoles, nor did we find any interactive effects. The results from our 17 β -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. The absence of UV radiation-induced effects found in the present study could be due to species-specific variation in susceptibility, as well as the dosage utilized. We suggest that future research should incorporate long-term studies with multiple stressors to accurately identify the threats to, and subsequent consequences for, amphibians under natural conditions. *Environ Toxicol Chem* 2024;43:1615–1626. © 2024 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Agricultural pollutant; Amphibian declines; Behavioral 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 imperiled vertebrate class globally (International Union for Conservation of Nature, 2022; Luedtke et al., 2023). The rate at which amphibian populations

are declining is more rapid than that of birds or mammals, and potentially over 222 amphibian species have become extinct since 1980 (Luedtke et al., 2023; Mendelson et al., 2006; Stuart et al., 2004; Womack et al., 2022). The loss of amphibian populations globally may lead to further ecological consequences because 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 potential contributors to the decline of amphibians, including increased exposure to ultraviolet (UV) radiation (reviewed in Alton & Franklin, 2017). Solar UV radiation is a pervasive natural stressor that can cause genetic mutations and cell death by damaging

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nucleic acids, proteins, and lipids (Harm, 1980). Exposure of organisms to solar UV radiation is determined by many factors in the atmosphere (e.g., ozone, clouds, and aerosols) and at the Earth's surface (e.g., vegetation cover and water transparency), and many of these factors are changing as a result of human activities and climate change. For example, the emission of ozone-depleting substances (ODSs) by humans has reduced ozone levels over most of the globe, causing clear-sky erythemal (sunburning) UV radiation to increase by 3% to 9% at mid-latitudes (30–60°) and by >50% in polar regions (80–90°; Bernhard et al., 2023; Herman, 2010). The production and consumption of ODSs have been regulated by the Montreal Protocol and its amendments and adjustments since 1987, and this has been effective at protecting the ozone layer from further damage by ODSs, with signs of ozone recovery now evident over Antarctica (Solomon et al., 2016; World Meteorological Organization, 2022). However, changes in UV radiation at low and mid-latitudes (0–60°) are also attributable to changes in cloud cover and aerosols that are associated partly with greenhouse gas emissions and partly with measures to control tropospheric pollution (Bernhard et al., 2023). Consequently, UV radiation is increasing at some sites and decreasing at others, typically at a rate of <4% per decade (Bernhard et al., 2023). In addition to changes in UV radiation associated with atmospheric factors, exposure of organisms to UV radiation is likely to be changing because of reductions in vegetation cover, melting of snow and ice, changes in water transparency and surface water circulation, and shifts in the geographic distributions and phenology of species that are occurring as a result of human land-use practices (e.g., deforestation) and climate change (explained in detail by Barnes et al., 2023; Downie et al., 2023; Neale et al., 2023). The ecological consequences of such changes in UV radiation are uncertain and complicated by interactions with other factors associated with human-induced global change (explained in detail by Neale et al., 2023; Williamson et al., 2019). However, it is well established that exposure to UV radiation can have negative effects on organismal performance and fitness (Bancroft et al., 2007; Downie et al., 2023; Lladrés et al., 2013; Peng et al., 2017). In amphibians specifically, studies have shown that UV exposure can cause increased mortality, reduced growth, slower rates of development, delayed metamorphosis, developmental abnormalities, and behavioral changes (reviewed in Alton & Franklin, 2017; Downie et al., 2023).

Ultraviolet 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 & Franklin, 2017; Bancroft et al., 2008). 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 hand, 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 & Franklin, 2017; Lundsgaard et al., 2021). Multistressor studies have therefore been recognized as a key area of research in investigating amphibian declines (Alton & Franklin, 2017; Bancroft et al., 2008; Sievers et al., 2019) because 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; Luedtke et al., 2023; Sievers et al., 2019; Sih et al., 2004).

Another stressor that has been recognized as contributing to amphibian declines is chemical pollution (Egea-Serrano et al., 2012; Luedtke et al., 2023; Orton & 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 behavior (reviewed in Baker et al., 2013; Egea-Serrano et al., 2012; Sievers et al., 2019). Alarming, 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 & James, 2003; Hatch & Blaustein, 2003). Research has shown that UV radiation and chemical pollutants can interact synergistically to affect survival, development, morphology, and behavior (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 17 β -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 to 270 ng/L (Ankley et al., 2018). In Australia, many amphibian species are at high risk of exposure to agricultural pollutants, such as 17 β -trenbolone, because they inhabit and breed in areas where agricultural pollution is present, either as runoff or where it is intentionally applied (Brand & Snodgrass, 2010; Hazell et al., 2001; Sievers et al., 2018a). Research has shown that exposure to environmentally realistic concentrations of 17 β -trenbolone is sufficient to induce morphological and behavioral effects in fish (reviewed in Ankley et al., 2018). At high concentrations (e.g., >100 ng/L), exposure to 17 β -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 17 β -trenbolone suggest that, at environmentally realistic

concentrations (i.e., 10–66 ng/L), amphibians may be less vulnerable than fish, with exposure having no effect on behavior or morphology (Martin et al., 2022; Orford et al., 2022). However, the potential for UV radiation to act synergistically with 17 β -trenbolone remains to be investigated.

Accordingly, we set out to examine the effects of UV radiation and 17 β -trenbolone on tadpole morphology and behavior 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 (Australian Radiation Protection and Nuclear Safety Agency [ARPANSA], 2019). For our 17 β -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 behavior 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 (*L. tasmaniensis*) egg masses were collected on October 14, 2020, in Melbourne, Victoria, Australia (37°50'31.6"S, 145°12'43.1"E; Department of Environment, Land, Water, and Planning, permit number 10009162). Water samples taken from this water body confirmed it was free from 17 β -trenbolone contamination ($n = 2$; Envirolab Services, 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 \pm SD). Egg masses were held separately in tanks (60 \times 30 \times 30 cm, length \times width \times height) filled with 32 L of aged carbon-filtered water for approximately 3 weeks prior to chemical exposure to allow the tadpoles to develop to Gosner Stage (GS) 25 (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 GS 25 (Gosner, 1960), tadpoles were exposed to a factorial combination of two UV treatments (UV radiation either present or absent) and two 17 β -trenbolone treatments (17 β -trenbolone either present or absent), such that there were four experimental treatments in total: a freshwater solvent control (17 β -trenbolone and UV radiation both absent; hereafter "control treatment"), a UV treatment (UV radiation present, 17 β -trenbolone absent), a 17 β -trenbolone treatment (17 β -trenbolone present, UV radiation absent; nominal concentration 50 ng/L), and a UV and 17 β -trenbolone combination treatment (17 β -trenbolone and UV radiation both present; nominal concentration 50 ng/L; hereafter "multistressor

treatment"). The exposure length of 1 month was chosen because previous research has shown that exposure to 17 β -trenbolone or UV radiation for similar periods is sufficient to induce effects on morphology or behavior in tadpoles and other aquatic animals (Alton et al., 2012; Alves et al., 2020; Bertram et al., 2015; Lundsgaard et al., 2020, 2022; Saaristo et al., 2013; Sone et al., 2005; Tomkins et al., 2017).

The exposure consisted of 24 independent tanks (60 \times 30 \times 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, to control for any potential clutch effects (Gibbons & George, 2013). For logistical 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 behavioral assays and morphological measurements at the end of the exposure, varied by 1 to 9 days. In addition, this design meant that there was a slight variation in exposure length, with treatment tanks being exposed for 28 to 31 days (for simplicity, exposure duration is referred to as 1 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 behavior (Orford et al., 2022; Touchon et al., 2013; see *Statistical analyses* 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 \pm SD; $n = 96$) and pH (6.94 ± 0.13 ; $n = 96$) checks were performed (data available at Martin et al., 2023). Tadpole survival in each treatment tank was measured at the end of the 1-month exposure and did not differ significantly among treatments (mean \pm SD survival percentage $82.9 \pm 17.3\%$; see Supporting Information S1 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 in the present study. Tadpoles were fed boiled spinach three times a week.

UV radiation exposure

Treatment tanks were exposed to UV-B radiation (UVBR), UV-A radiation (UVAR), and visible light emitted from a 36-W 120-cm linear fluorescent lightbulb (Repti-Glo Exo Terra T8) that was suspended 50 cm above the water surface across the center of three tanks and set on a 12:12-h light:dark photoperiod. The bulbs suspended over the control and 17 β -trenbolone treatment tanks were covered with a UV-blocking filter (Crystal clear window film; Pillar), 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) at seven fixed positions at the level of the water surface (see Supporting Information S1, Methods, for further detail). Three measurements were taken directly below the lightbulb (i.e., one measurement at the center of each tank), and four measurements were taken at the edge of the tanks, to estimate the range of absolute irradiance that the tanks would be receiving. For tanks in the UV and multistressor treatments, the absolute irradiance of UVBR ranged from 4.3 to 8.7 $\mu\text{W cm}^{-2}$ from the edge to the center of the tanks, respectively. For UVAR, the absolute irradiance ranged from 34.7 to 71.8 $\mu\text{W cm}^{-2}$. These absolute irradiances result in a UV index of <1 (see Supporting Information S1, Table S2 and Figure 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). Because 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 & Franklin, 2017; Ceccato et al., 2016). We therefore chose to expose tadpoles to relatively low UV radiation levels because this is more likely to reflect levels that tadpoles would naturally experience in the wild, due to the aforementioned absorbance of UV radiation by dissolved organic matter in the water column, as well as shading by vegetation (both terrestrial and aquatic) and cloud cover (Alton & Franklin, 2017; Diamond et al., 2005; Lundsgaard et al., 2020; Palen & Schindler, 2010; Palen et al., 2002).

17 β -Trenbolone exposure

Tadpoles in the 17 β -trenbolone and multistressor treatments were exposed to 17 β -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 17 β -trenbolone (Chemical Abstracts Service no. 10161–33-8; Novachem, Germany) dissolved in 1 mL of ethanol (high-performance liquid chromatography–grade, $\geq 99.99\%$). Thereafter, to maintain exposure concentrations and replace any 17 β -trenbolone lost to evaporation and water changes, all 17 β -trenbolone and multistressor tanks were dosed twice a week with 2.45 μg of 17 β -trenbolone dissolved in 1 mL of ethanol. 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 17 β -trenbolone and multistressor tanks. To monitor concentrations of 17 β -trenbolone in the 17 β -trenbolone and multistressor treatments, water samples (100 mL) were taken from each exposure tank approximately 24 h after the second of the two weekly doses. Over the 1-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 1-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 17 β -trenbolone and multistressor tanks. The concentration of 17 β -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; National Association of Testing Authorities accreditation 2901; accredited for compliance with International Organization for Standardization/International Electrotechnical Commission [ISO/IEC] 17025), with a quantification limit of 2 ng/L (for a detailed description of the analytical procedure, see Supporting Information S1).

Behavioral assays

The effects of experimental treatments on behavior were tested in two separate assays (conspecific cue and simulated predator strike; detailed below) that were conducted after 1 month of exposure. These behavioral assays were chosen because UV radiation and 17 β -trenbolone have previously been shown to affect anxiety-like behavior (i.e., activity) and anti-predator behavior (Alton & Franklin, 2017; Bertram et al., 2018; Heintz et al., 2015; Lagesson et al., 2019). On completion of the 1-month exposure period, for each batch of exposure tanks (three batches total, eight 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 behavior being affected by external stimuli. Tanks were emptied and wiped clean between trials to avoid cross-contamination of 17 β -trenbolone and to remove any conspecific chemical cues that may have influenced tadpole behavior in subsequent trials. All trials and subsequent data extraction were performed blind to experimental treatment. During the trials, all behaviors 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 (Ver. 16) was used to track tadpoles. This allowed for total distance moved (centimeters) and time spent stationary (i.e., freezing behavior; seconds) 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 behavior of tadpoles (control $n = 48$, UV $n = 46$, 17 β -trenbolone $n = 48$, multistressor $n = 48$) 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 because 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 & Smith, 1998; Hagman et al., 2009; Rajchard, 2006). Trials took place in observation tanks (25 \times 15 \times 15 cm) filled with aged carbon-filtered water to a depth of 2 cm (17.8 \pm 0.1 $^{\circ}$ 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 behavior that the manual addition of cues may have had. Tadpoles were then filmed for 5 min (i.e., precue 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 Supporting Information S1, Methods. Tadpoles were then filmed for another 5 min (i.e., postcue period).

Simulated predator strike assay

In a separate assay, we measured the behavior 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 \pm 0.2 $^{\circ}$ C). Prior to behavioral recordings, tadpoles were left to freely acclimate to the trial tank for 10 min. After this acclimation period, tadpole behavior was recorded for 5 min prior to a simulated predator strike to establish a measure of baseline activity (i.e., prestrike 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 poststrike behavior was then recorded for 5 min (i.e., poststrike period).

Morphology

On completion of the behavioral 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 (centimeters; \pm 0.01 mm). Data

were extracted from the photographs using ImageJ software (Schneider et al., 2012). Tadpoles were then blotted dry and weighed (grams; \pm 0.0001 g; ZSA210 Analytical Balance, Scientech). As a proxy for body condition, a scaled mass index (SMI) was calculated for all tadpoles (sensu Peig & Green, 2009). Specifically, we performed a standard major axis regression on the base-e log of body mass (M ; grams) and body length of tadpoles (L ; centimeters) and calculated a beta coefficient, which was then used to obtain the SMI for each tadpole (Peig & Green, 2009). Body length was selected as the length metric for condition estimates because 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* (Ver. 4.1.1). Where appropriate, data were transformed to approximate Gaussian error distributions, and continuous predictors were scaled to aid model fitting (see Supporting Information S1, Tables S2–S4, 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 and 17 β -trenbolone on tadpole morphology and behavior were analyzed. All models included an interaction term between the categorical factors of UV and 17 β -trenbolone, each of which had two levels (i.e., UV+/UV–, TB+/TB–), indicating presence or absence of exposure to UV radiation and/or 17 β -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 behavior) were analyzed using LMEs. For both endpoints, models included a three-way interaction between UV, 17 β -trenbolone, and trial period (i.e., precue/strike, postcue/strike). Tadpole mass (milligrams), 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 identifier 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 analyzed using LMEs. Models included a two-way interaction between UV and 17 β -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 17 β -trenbolone analysis

To account for left-censoring of 17 β -trenbolone due to the method detection limit (MDL = 2 ng/L), all trenbolone samples

that fell below the MDL (17 β -trenbolone treatment $n=9$) were included in the analysis as the MDL divided by 2, following Antweiler & Taylor (2015). The mean measured concentrations (\pm SD) for the 17 β -trenbolone and multistressor treatments during the 1-month exposure were 33.6 ± 56 ng/L and 24.9 ± 37.3 ng/L, respectively, with the difference likely due to increased phototransformation caused by UV exposure (*sensu* Qu et al., 2013). No 17 β -trenbolone was detected in the control or UV treatment tanks.

Conspecific cue assay

For both distance moved and freezing behavior during the conspecific cue assay, there was no significant interaction between UV radiation, 17 β -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 17 β -trenbolone (see Supporting Information S1, Table S2, for full model outputs). Irrespective of treatment, trial period had a significant effect on both distance moved and freezing behavior ($F_{(1,186.0)}=30.23$, $p<0.001$; $F_{(1,186.0)}=14.43$, $p<0.001$, respectively), with tadpoles traveling shorter distances and spending more time freezing after the addition of conspecific cues (Figure 1).

Simulated predator strike assay

For both distance moved and freezing behavior during the simulated predator strike assay there was no significant interaction between UV radiation, 17 β -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 17 β -trenbolone (see Supporting Information S1, Table S3, for full model outputs). Irrespective of treatment, trial period had a significant effect on both distance moved and freezing behavior ($F_{(1,188.0)}=10.13$, $p<0.001$; $F_{(1,188.0)}=7.33$, $p<0.001$, respectively), with tadpoles traveling shorter distances and spending more time freezing after the simulated predator strike (Figure 2). There was also a significant effect of mass on both distance moved and freezing behavior ($F_{(1,164.1)}=11.09$, $p=0.001$; $F_{(1,169.3)}=4.88$, $p=0.028$, respectively), with heavier tadpoles moving greater distances and spending more time moving overall (Supporting Information S1, Figure S2).

Morphology

For total length, mass, and body condition, we found no significant interaction between UV radiation and 17 β -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) or any significant main effects of either stressor (Figure 3; see Supporting Information S1, Table S4, for full model outputs).

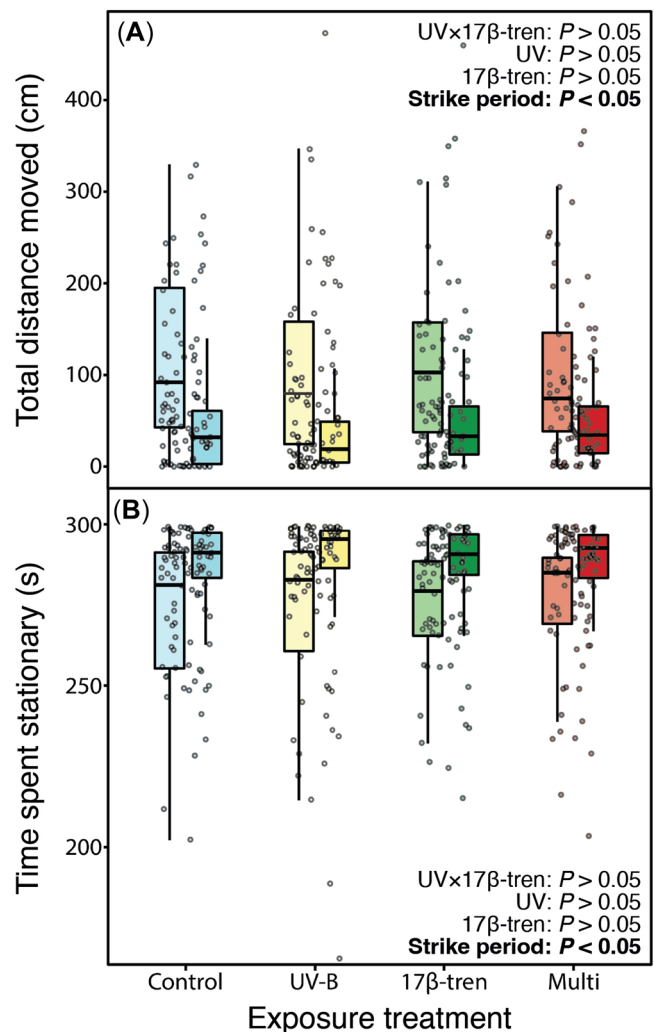


FIGURE 1: Boxplots showing (A) the distance moved and (B) time spent stationary by tadpoles before (lighter-colored boxes on the left for each treatment) and after (darker-colored boxes on the right for each treatment) the addition of conspecific cues for all exposure treatments (control $n=48$, UV $n=46$, 17 β -trenbolone $n=48$, multistressor $n=48$). Treatment groups that do not share uppercase letters are significantly different ($p<0.05$). UV = ultraviolet; 17 β -tren = 17 β -trenbolone; Multi = multistressor.

DISCUSSION

In the present study, we investigated whether exposure to environmentally realistic levels of UV radiation and the agricultural pollutant 17 β -trenbolone for 1 month would impact the behavior and morphology of tadpoles. We found no significant interactions between UV radiation and 17 β -trenbolone and no significant main effects of either stressor on tadpole morphology or behavior.

We found no significant effect of 17 β -trenbolone on *L. tasmaniensis* tadpole morphology or anxiety-like behavior during the conspecific cue and simulated predator strike assays. Research investigating the effects of environmentally realistic concentrations of 17 β -trenbolone on tadpoles is scarce (Ankley et al., 2018), and thus there are few studies to which we can compare our results. With that said, the findings of the present study are concordant with our previous studies

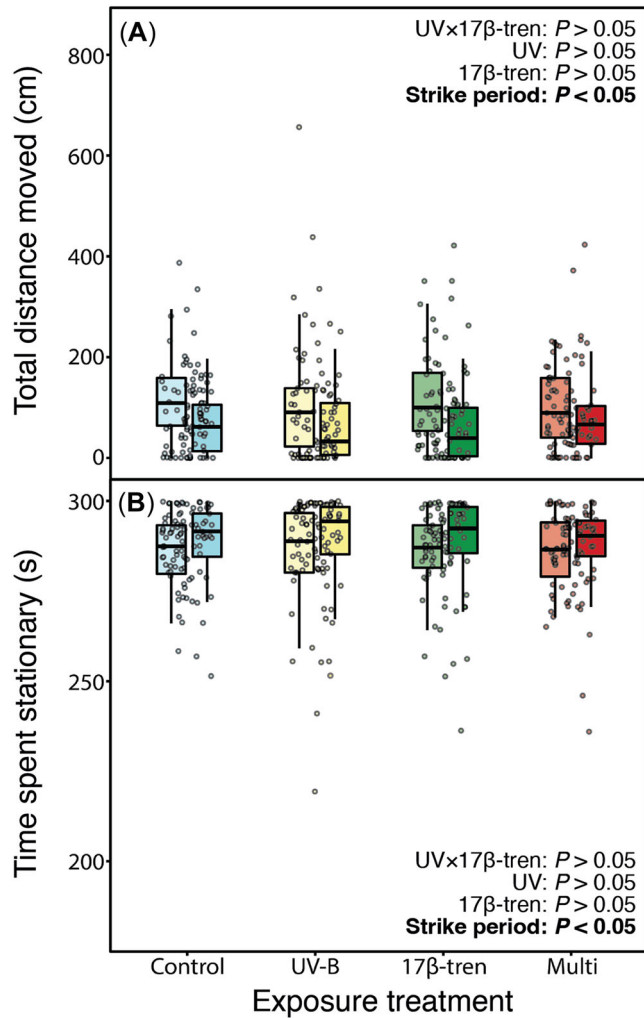


FIGURE 2: Boxplots showing (A) the distance moved and (B) time spent stationary by tadpoles before (lighter-colored boxes on the left for each treatment) and after (darker-colored boxes on the right for each treatment) the simulated predator strike for all exposure treatments ($n=48$ per treatment). Treatment groups that do not share uppercase letters are significantly different ($p < 0.05$). UV = ultraviolet; 17 β -tren = 17 β -trenbolone; Multi = multistressor.

on *L. tasmaniensis*, where 28-day exposure to comparable concentrations of 17 β -trenbolone (10 and 66 ng/L for 28 days) had no impact on tadpole length and mass or on anxiety-like behaviors across multiple assays (i.e., freezing behavior, distance moved, and time spent in upper half of water column; Martin et al., 2022; Orford et al., 2022). The present results do, however, contrast with those of Li et al. (2015), who reported that *Pelophylax nigromaculatus* tadpoles exposed to 17 β -trenbolone exhibited decreased growth. One potential explanation for the differences between their study and the present study is the dosage of 17 β -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 behavioral

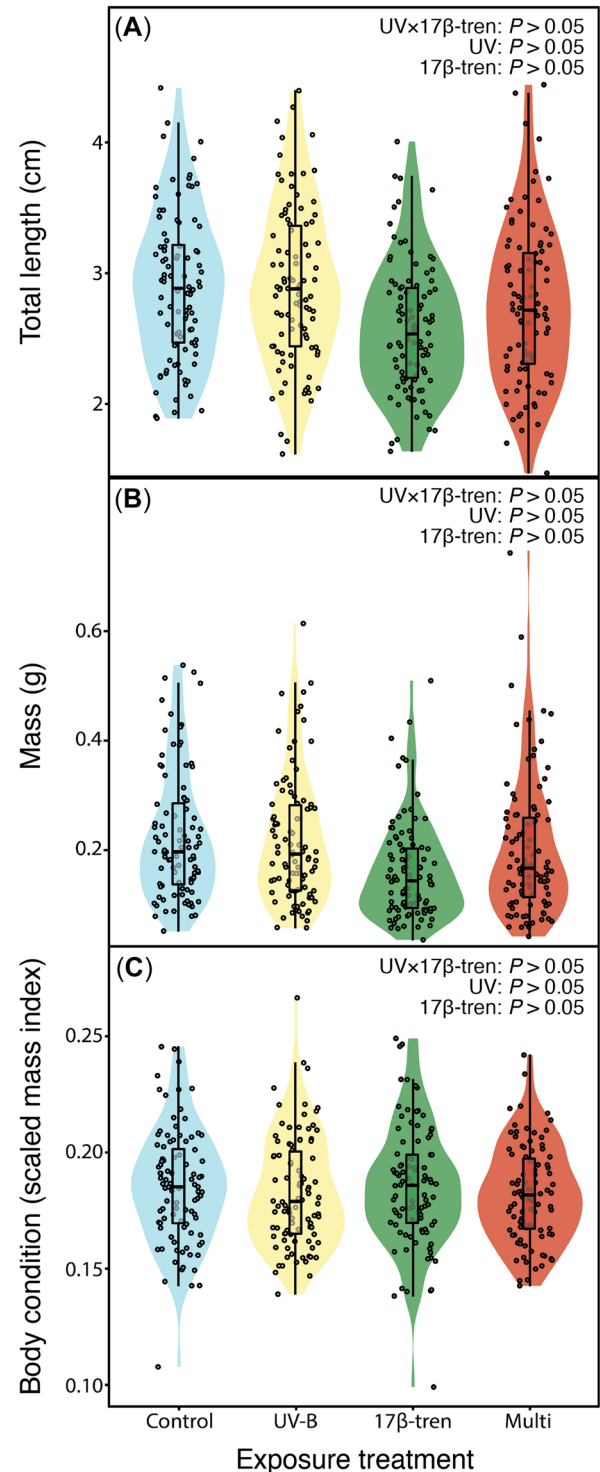


FIGURE 3: Violin plots and boxplots showing tadpole (A) total length, (B) mass, and (C) body condition. Control ($n=96$) is shown here in blue, UV ($n=94$) in yellow, 17 β -trenbolone ($n=96$) in green, and multistressor ($n=96$) in red. Boxplots show 25th (Q1), 50th (median), and 75th (Q3) percentiles. Whiskers represent the $(Q1 - 1.5) \times IQR$ to $(Q3 + 1.5) \times IQR$. The colored area surrounding the boxplot (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 17 β -trenbolone and for the main effects of both stressors are included. UV = ultraviolet; 17 β -tren = 17 β -trenbolone; Multi = multistressor.

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 of the present study also contrast with existing literature on fish, which found that exposure to 17 β -trenbolone can result in changes to both morphology and behavior (see Baumann et al., 2014; Bertram et al., 2018; Tan et al., 2021). We have previously hypothesized 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, 17 β -trenbolone may have greater effects during this developmental period because of 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 17 β -trenbolone during this period of increased sensitivity.

Previously UV radiation has been shown to impact both behavior and morphology across a number of anuran species, including species within the *Limnodynastes* genus (reviewed in Alton & Franklin, 2017). For example, exposure to environmentally realistic levels of UV radiation (i.e., UV index scores of <1) for 7 to 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. 2010, 2012; Bernal et al., 2011). Ultraviolet 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 behavior 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 behavior 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 & Franklin, 2017; Blaustein et al., 1999; Palen et al., 2005). The extent to which survival, development, and behavior are affected by UV exposure has been shown to vary across taxa, with some species seemingly unaffected by exposure (Long et al., 1995; Pakkala et al., 2003), while others suffer increased mortality, developmental abnormalities, extended development times, and various changes to behavior (reviewed in Alton & 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 & 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 & Franklin, 2017). In addition, as amphibians develop, they may utilize behavioral strategies to a greater degree, compared to earlier life-stages, to avoid UV exposure, for example, swimming in shaded areas or at greater depths (Alton & Franklin, 2017; Belden et al., 2000; Blaustein &

Belden, 2003). In an experimental setting, these behaviors may affect the amount of UV radiation that a study organism receives. Indeed, Bridges & 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 utilized nonenvironmentally realistic levels of UV radiation (see Grant & Licht, 1995; Licht, 2003; Pakkala et al., 2001, 2003), with one study exposing eggs to an absolute irradiance of 340 $\mu\text{W cm}^{-2}$ (Grant & Licht, 1995), which is >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 & Franklin, 2018; Peterson et al., 2002), and the use of environmentally realistic levels in the present 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 behavior 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). While 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 (see Baud & Beck, 2005; Hatch & Blaustein, 2003; Macías et al., 2007), there is evidence to suggest that combined exposure can result in either no effect (Bridges & 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 17 β -trenbolone as part of a multistressor study. However, based on the results of the present study, it appears that at environmentally realistic concentrations there is no interaction between 17 β -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; Pakkala 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 17 β -trenbolone, either alone or in combination, on the morphology or behavior of tadpoles. In comparison to past research, our study lends support to the idea that there are taxon-specific differences in susceptibility to 17 β -trenbolone (Martin et al., 2022; Orford et al., 2022). Given that the effects of UV exposure on tadpoles vary depending on numerous factors and that exposure can result in no effects, the present study adds information on *L. tasmaniensis* to an extensive body of existing work. Because this is the first study of its kind to utilize 17 β -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 imperiled taxa face in the wild, which in turn can assist with the implementation of effective conservation efforts.

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 This article has earned both an Open Data and an Open Materials badge for making publicly available the digitally shareable data necessary to reproduce the reported results. The data are available at <https://doi.org/10.17605/OSF.IO/XYG63>. Learn more about

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