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Amphibian Dispersal Traits Not Impacted by Triclopyr Exposure during the Juvenile Stage

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Abstract: Exposure to agrochemicals can have lethal and sublethal effects on amphibians. Most toxicology studies only examine exposure during the aquatic larval stage. Survival of the juvenile stage is the most important for population persistence and it is critical to understand the potential impacts of exposure during this life stage. We investigated how short-term exposure to triclopyr, an herbicide commonly used in forestry management, might impact several juvenile traits. To determine if juveniles perceived exposure as an environmental stressor, we measured their release of corticosterone. We also examined dispersal traits by measuring foraging and hopping behavior. We found no evidence that exposure negatively impacted these traits or was a stressor. Our results provide a preliminary assessment of the potential impact of triclopyr on juvenile amphibians, but we recommend additional research on the effects of agrochemicals on juvenile amphibians.

Keywords: Cuban tree frog; corticosterone; frog; metamorph; triclopyr

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1. Introduction

Contamination of the environment from agrochemicals can have lethal [1] and sublethal [2] effects on amphibians and pollution is regularly considered a driver in the global decline of amphibians [3–7]. Sublethal effects of agrochemical exposure on amphibian development [8,9], behavior [10,11], and morphology [11,12] are well documented. Sublethal effects can be challenging to measure, but corticosterone, the primary glucocorticoid stress hormone in amphibians, can be used as a biomarker for amphibian stress [13]. Exposure to chemical contaminants can act as an environmental stressor that results in increased production of corticosterone in amphibian larvae [14–17] and juveniles [18]. A vast majority of research exploring the impacts of agrochemicals on amphibians has examined effects on amphibian larvae and has not considered how exposure affects other stages of development [9,19–24]. Understanding the impacts of agrochemical exposure on juveniles and adults may be especially relevant for amphibians that breed in geographically isolated wetlands because they spend most of their lives in the terrestrial uplands [25,26].

Population models consistently find that survival of the juvenile stage of amphibians is the most important for population persistence [27–30]. Among pond-breeding amphibians, juveniles are known to cross clear-cuts [31], agricultural fields [26,32], and rights-of-way [33] where herbicides are often used. Thus, pronounced exposure to agrochemicals may occur during dispersal. Standard toxicity testing in amphibians typically occurs with early-stage tadpoles [34] and, while efforts have been made to examine the effect of agrochemical exposure through metamorphosis [6,21], few continue into the juvenile stage. When juveniles are studied it is clear they are vulnerable to agrochemicals through both direct and indirect exposure [25,26,35]. For example, exposure to agrochemicals in the larval stage can impact the physiological performance of juveniles and adults [36–38]. In particular, terrestrial amphibians are susceptible to dermal uptake of chemicals resulting from direct

overspray [1,26,39] or contact with contaminated soil [35,40,41]. For example, juvenile pool frogs (*Rana temporaria*) exposed to a fungicide experienced lethal and sublethal effects, including negative effects on locomotion and feeding [42].

Triclopyr is an herbicide commonly used in agricultural and forestry practices. Triclopyr selectively targets broadleaf weeds and brush to keep non-aquatic habitats such as rights-of-way, forests, and fields clear of woody vegetation. Formulations of triclopyr, such as Release® and Garlon 4®, are widely used in forest vegetation management in North America [43,44]. In the southeastern United States, Garlon 4® is recommended for site preparation during longleaf pine restoration efforts [45,46]. The longleaf pine ecosystem is associated with numerous at-risk species [47], making it critical to understand the potential impacts of herbicide usage. An experimental study using triclopyr (Garlon 3A®) to control midstory vegetation had no effect on capture rate of salamanders [48]. Garlon 4® is more toxic than Garlon 3A® to larval amphibians [34] and to our knowledge no studies have examined the effects of exposure to this formulation on terrestrial-stage amphibians. In the aquatic larval stage, exposure to triclopyr formulations can result in lethal [49,50] and sublethal [51] effects. However, triclopyr degrades rapidly in water [52,53] and soil [54,55] and laboratory studies may overestimate toxicity due to the use of static renewal methods maintaining artificially high concentrations [49].

The purpose of our study was to investigate the response of juvenile amphibians exposed to the Garlon 4® formulation of triclopyr. We chose to examine our research questions using Cuban tree frogs (*Osteopilus septentrionalis*) because in the U.S. they can be found in residential, agricultural, and forested areas, where triclopyr is commonly applied. In the U.S., Cuban tree frogs live predominantly in Florida, which has one of the highest triclopyr application rates of any state [56]. Cuban tree frogs are also a highly invasive species with a broad distribution in the U.S. and elsewhere, so understanding the effect that triclopyr might have on them has potential management implications. Our objectives were to determine if exposure of recently metamorphosed Cuban tree frogs to triclopyr for 48 h affects traits associated with dispersal success [57–61].

2. Materials and Methods

2.1. Collection and Rearing of Osteopilus septentrionalis

We collected *O. septentrionalis* tadpoles from Merritt Island in Brevard County, FL on 9 August 2018 and transported them to the University of Georgia Savannah River Ecology Laboratory animal care facility. We reared the tadpoles (n = 10/container) on a 12:12 light:dark cycle at 22 °C in 26 L plastic containers containing ~20 L of laboratory reconstituted water (2.4 g $NaHCO_3$, 1.5 g $CaSO_4$, 1.5 g $MgSO_4$, and 0.1 g KCl dissolved in 50 L of 18 MΩ-cm Milli-Q® water (Millipore, Molsheim, France)). We fed tadpoles boiled kale ad libitum daily and performed $\frac{3}{4}$ water changes weekly. Once tadpoles reached Gosner stage 42 [62], or development of the forelimbs, we removed tadpoles from their tanks and placed them into separate, individually assigned 1 L plastic deli containers with moist paper towel substrate. Upon total tail resorption, we fed juveniles four calcium-dusted crickets daily and removed any uneaten crickets from the previous day's feeding.

2.2. Experimental Design

In fall 2018, we assigned 43 of the juveniles to triclopyr (n = 21) or control (n = 22) groups based on the date of metamorphosis to reduce variation experienced in the larval period. Our experiments occurred from 3 December 2018 (Day 1) to 17 December 2018 (Day 16). During this period, we fed frogs as described above on Days 1–7, 10, and 13–16 after any experimental procedures that took place. On Day 1, we collected baseline stress hormones from all individuals using a waterborne corticosterone assay [63]. On Day 8, we initiated a 48 h exposure to the appropriate triclopyr or control treatments (see below). On Day 10 we collected post-exposure stress hormones using a waterborne corticosterone assay. On Day 11, we fasted frogs for one day preceding feeding trials. On Day 12 we performed timed feeding trials, and finally we performed hopping trials on Days 15 and 16.

Two individuals died between Day 10 and 12 and were removed from analyses leaving 20 control and 21 triclopyr-exposed individuals.

2.3. Triclopyr Exposures and Hormone Collection

Our exposure microcosms consisted of glass jars (63.62 cm^2) that were $\frac{3}{4}$ full of sterile soil. To completely moisten the tops of the soil to prevent drying of juvenile frogs, we pipetted 4 mL deionized water evenly on top of the soil. For triclopyr exposures we followed the manufacturer's recommended application of Garlon® 4 Ultra (Dow AgroSciences LLC, Indianapolis, IN, USA) by scaling down the recommended application rate of 6 quarts/acre for the area of a jar (63.62 cm^2). Therefore, 6 µL of Garlon® 4 Ultra was added to the 4 mL of water to mimic the maximum recommended annual forestry site application rate [64]. We added only 4 mL deionized water to control jars. After applying the appropriate solutions to all the jars, we placed one juvenile frog in each jar (assigned randomly within treatment group) and covered the jars with cheesecloth held by a rubber band to allow for aeration but keep frogs from climbing out. We kept the frogs in treatment jars for 48 h then immediately collected waterborne hormones from 1200–1300 h.

We collected hormones secreted in water following previous methods [63] with minor modifications. Briefly, each juvenile frog was placed in a plastic cup with holes in the bottom and this cup was inserted into a second cup containing 90 mL soft water. A third cup, with ventilation holes, was placed on top to prevent frogs from climbing out of the water. The frogs remained in the water for 60 min (from 1200–1300 h) and then we collected the water and stored it at −20 °C until analysis. We conducted these assays on all experimental frogs before and after exposure to soil treatments.

2.4. Behavioral Experiments

To examine the potential impacts of triclopyr exposure on foraging behavior we conducted feeding trials two days after exposures ended from 1100–1300 h. We randomized the order of testing and had two separate observers allowing for two frogs to be assayed at the same time. To begin each feeding trial, we placed the frog under a petri dish covered with black duct tape inside a 1 L deli container arena. This ensured that each trial started with the frog in the same location and allowed crickets to disperse throughout the arena. A non-observer added 10 small crickets to the container while the frog was covered, then removed the petri dish after 60 s. The observer then recorded data for up to 10 min or until all 10 crickets were consumed, whichever came first. We recorded the time that it took frogs to eat one cricket, the time that it took to eat five crickets, and the total number of crickets consumed in a 10 min window.

To examine the potential impacts of triclopyr exposure on physical performance we conducted hopping trials. These began two days after the feeding trials and each frog was tried twice: once each on Days 15 and 16. The hopping arena consisted of a 3 m × 2 m area lined with carpenter's paper and containing 0.3 m high cardboard walls on all sides. Experimental frogs were dipped in food coloring and placed under a taped petri dish (as in the foraging tests) for 60 s to prevent movement before the trial began. To initiate movement, we probed the frog with a padded yardstick and allowed it to hop up to four times. We then measured the distance between each landing spot. A separate observer recorded the time from first to fourth hop and the time it took to complete three and four hops. We measured the straight-line distance between hops with a tape measure. The distance between hops was calculated as the distance from the posterior-most marking of the urostyle between two landing spots marked with food coloring. To reduce variability, we repeated the hopping trials in the same order 24 h later and all trials from both days occurred between 1200–1400 h. Data used in our analyses of hopping were the average of both hopping trials for an individual. We calculated the sprint speed by dividing the hopping distance by the time it took to complete these hops. After all experiments, we recorded the SVL and weight of all frogs.

2.5. Corticosterone Extractions and Quantification

We extracted corticosterone from the collected water samples to quantify hormone secretion before and after exposure to triclopyr. We thawed and passed entire water samples (90 mL/sample) through C18 solid phase extraction cartridges (Sep-Pak Vac 3 cc/500 mg; Waters Inc., Milford, MA, USA) attached to a 20-position extraction manifold (Waters Inc.) following previously published methods [63]. After extraction, we eluted cartridges with 4 mL methanol into 100 mm borosilicate glass tubes (VWR International, Radnor, PA, USA). Methanol was evaporated from each tube with nitrogen gas (~10 psi) through a 24-well heated analytical evaporator (Glas-Col®, Terre Haute, IN, USA) at 37 °C.

After extraction, we re-suspended the dried residue with 5% ethanol (95%) and 95% ELISA buffer (Cayman Chemicals Inc., Ann Arbor, MI, USA) for a total volume of 263.16 µL, before shaking the reconstituted samples at 105 rpm on an Innova 4080 incubator shaker (New Brunswick Scientific, Edison, NJ, USA) for 3 h at room temperature. We ran samples in duplicate and read them on a microplate spectrophotometer (BioTek® Eon™, Winooski, VT, USA) set to 420 nm. We calculated the corticosterone concentrations (pg/mL) from the raw OD values for each well using a competitive ELISA analysis tool (Cayman Chemical Inc.). The concentrations (pg/mL) were then multiplied by the resuspension volume to obtain the total corticosterone (pg) retrieved in each well. We did not conduct validations, but the use of corticosterone ELISA kits for measuring waterborne corticosterone for larval *O. septentrionalis* has previously been validated [65].

2.6. Statistical Analyses

We performed all statistical tests using JMP® Pro Ver. 16.0.0 (SAS Institute Inc., Cary, NC, USA). To account for differences in hopping distance and corticosterone release amounts due to size, all distance and corticosterone measurements were standardized by dividing each value by the individual's SVL. We tested the goodness-of-fit of all statistical variables using a Shapiro–Wilk W test ($\alpha = 0.05$) and checked the assumption of homoscedasticity for parametric tests using Levene's test ($\alpha = 0.05$). For statistical variables that were normally distributed (SVL, weight, time to eat five crickets, time to 3rd hop, distance of three and four hops, and sprint speed), we performed a parametric (two-tailed Student's *t*) test to determine if there was an effect of triclopyr exposure. For the variables that were not normally distributed (time to eat one cricket, total number of crickets eaten, and time to 4th hop), we performed a non-parametric (Wilcoxon Rank Sum) test.

3. Results

Because we did not sort the experimental animals by size, we first verified that there was no difference in size between the treatment groups. We were able to verify this for SVL ($t_{39} = -0.6929$, $p = 0.49$) but there was a borderline significant difference in mass ($t_{39} = -2.01$, $p = 0.0514$) with the control individuals beingly slightly heavier (0.39 ± 0.018 g) than the triclopyr-exposed (0.34 ± 0.017 g).

3.1. Corticosterone Production

Overall, corticosterone concentrations measured in ELISA assays were highly variable, even within treatment groups, as has been seen in previous studies of larval Cuban tree frogs [62]. Control corticosterone amounts ranged from 1.10 pg to 160.37 pg while triclopyr-exposed amounts ranged from 1.27 pg to 238.45 pg. There was no difference in pre-exposure release ($z = 0.743$, $p = 0.45$) or post-exposure release of corticosterone ($z = -1.42$, $p = 0.155$) between treatment groups (Figure 1). When we compare the amount of corticosterone released by individuals pre- and post-exposure there is also no difference between the control and triclopyr groups ($z = -1.08$, $p = 0.27$).

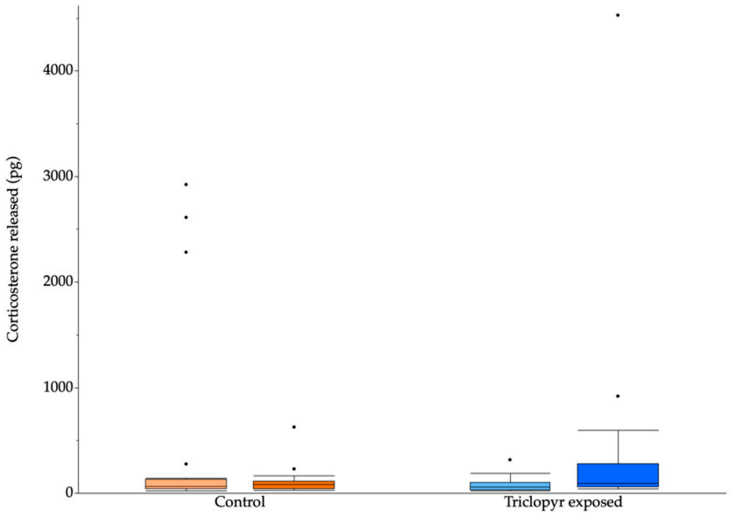

Figure 1. Amount of corticosterone secreted by juvenile Cuban tree frogs assigned to control (orange) and triclopyr-exposed (blue) treatment groups. Corticosterone was measured before (lighter bars) and after (darker bars) exposure to soil.

3.2. Feeding Behavior

As with corticosterone production, we found no effect of triclopyr exposure on feeding behavior. Only two frogs ate all ten crickets in either feeding trial. We found no difference between the treatment groups for the time it took to consume one ($z = -0.139$, $p = 0.89$) or five crickets ($t_{13} = 0.102$, $p = 0.92$). There was also no difference in the total number of crickets consumed ($z = -0.90$, $p = 0.37$; Figure 2). A larger percentage of triclopyr-exposed frogs ate at least one cricket (71.4%) than control frogs (50%), but the difference was not significant (Fisher's exact test $p = 0.21$).

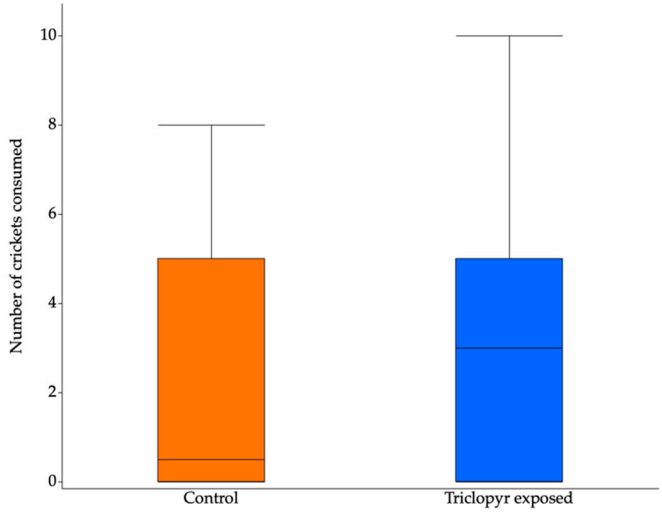

Figure 2. Comparison of the total number of crickets consumed in 10 min by juvenile Cuban tree frogs exposed to soil without (control) or with triclopyr (triclopyr exposed).

3.3. Hopping Behavior

As can be seen in Figure 3, the frogs that were exposed to triclopyr appeared to cover less total distance (74.33 ± 4.0 cm) in their four hops than the control (86.16 ± 4.1 cm; $t_{39} = -2.05$, $p = 0.047$) frogs. However, this absolute difference was not significantly different between groups when standardized by individual size ($t_{39} = -1.87$, $p = 0.07$; Figure 3). There was also no difference after the first three hops ($t_{39} = -1.17$, $p = 0.25$). There was no difference in sprint speed ($t_{39} = 1.09$, $p = 0.28$).

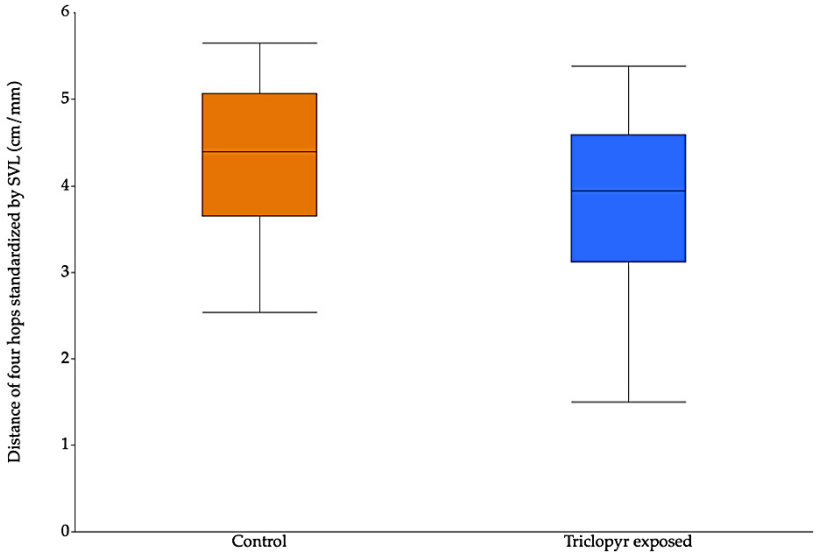

Figure 3. Comparison of the total distance covered (cm) standardized by the snout–vent length (mm) of juvenile Cuban tree frogs exposed to soil without (control) or with triclopyr (triclopyr exposed).

4. Discussion

Overall, we found that short-term exposures to soil with triclopyr did not impact the release of corticosterone, feeding ability, or hopping behavior in juvenile Cuban tree frogs. Other studies have linked elevated corticosterone release to variation in behavior [66–68]. For example, exposure to increased conductivity led to an increase in corticosterone and a decrease in prey consumption in larval *Ambystoma jeffersonianum* salamanders [66]. Given that the Cuban tree frogs did not elevate corticosterone production it is not surprising that we saw no downstream effects on performance. Together, our findings suggest that exposure to triclopyr in the substrate does not induce a significant physiological stress response in juvenile Cuban tree frogs following a 48 h exposure period and may pose no immediate or short-term risk at relevant concentrations.

Previous studies have demonstrated lethal [34,49,50,69] and sublethal [51,70] effects in larval amphibians exposed to triclopyr formulations. Larval mortality has been observed even at concentrations much lower than the expected environmental concentrations in water of 2.56 acid equivalents/liter [49]. However, as mentioned above, laboratory exposures may overestimate toxicity. In particular, because triclopyr degrades rapidly [52,53], when concentrations are held constant through static renewal (e.g., [49,51]) larvae are exposed to higher concentrations than would be experienced in a wetland. Further, there is wide variation in the observed lethal concentrations, and it is evident that early-stage larvae are more sensitive than embryos [69,70]. In one study of late-stage larvae undergoing metamorphic climax, exposure resulted in delayed metamorphosis and potential short-term stress, but no increase in mortality [71]. Moreover, the toxicity risk of pollutants may vary among amphibian species due to variation in skin permeability [72]. Our study was the first to evaluate sublethal effects of triclopyr on juvenile amphibians in a terrestrial environment. Given the amount of variation in sensitivity to triclopyr that has been observed, it would be beneficial to examine additional species, but our results are encouraging regarding potential effects to terrestrial amphibians. This is especially true given that for most of its uses, triclopyr only needs to be applied infrequently, making repeated exposures unlikely.

We chose to use Cuban tree frogs for our study, which are considered invasive to multiple regions of the U.S. Invasive species are thought to be more broadly tolerant to environmental factors than native species [73–75]. Cuban tree frogs appear to be highly tolerant to variation in environmental stressors such as acidic water [76], temperature [77], and elevated salinity [78]. However, they do not appear to be generally more tolerant than native species to chemical contaminants including atrazine [79] and the fungicide

chlorothalonil [14]. Cuban tree frogs exposed to agrochemicals can also experience increased susceptibility to the chytrid fungus well after exposure [80]. Thus, we cannot rule out latent effects of exposure, which are commonly seen in amphibians exposed to chemical contaminants [9,36,80,81].

5. Conclusions

In general, it is evident that although terrestrial amphibians can experience lethal and sublethal effects from dermal exposure to pesticides there are insufficient data for accurate risk assessments (reviewed in [25,26,32]). We encourage additional research to explore the potential sublethal effects of triclopyr given that its worldwide use for agriculture, site preparation, and forest management [82] continues to grow [56]. Further, we were limited by animal numbers and thus could not evaluate multiple exposure levels. For some analyses our design resulted in relatively low power and thus we ran the risk of falsely rejecting that triclopyr did not have effects on the juveniles. Future studies should expand the range of exposures and species studied to examine for possible effects. In addition, the effects of triclopyr may be stronger when combined with other stressors [49]. Given the importance of juvenile survival to population persistence [27–30], it is imperative we gain a broad understanding of what types of agrochemicals and exposure methods may or may not negatively impact dispersing juvenile amphibians.

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Data Availability Statement: The data presented in this study are openly available in the University of Georgia Savannah River Ecology Laboratory's data archive. Requests for access should be addressed to the corresponding author.

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References

1. Relyea, R.A. The lethal impact of roundup on aquatic and terrestrial amphibians. *Ecol. Appl.* **2005**, *15*, 1118–1124.
2. Moutinho, M.F.; de Almeida, E.A.; Espíndola, E.L.G.; Daam, M.A.; Schiesari, L. Herbicides employed in sugarcane plantations have lethal and sublethal effects to larval *Boana pardalis* (Amphibia, Hylidae). *Ecotoxicology* **2020**, *29*, 1043–1051. [CrossRef]
3. Wake, D.B. Declining amphibian populations. *Science* **1991**, *253*, 860. [CrossRef]
4. Alford, R.A.; Richards, S.J. Global amphibian declines: A problem in applied ecology. *Annu. Rev. Ecol. Evol. Syst.* **1999**, *30*, 133–165.
5. Blaustein, A.R.; Kiesecker, J.M. Complexity in conservation: Lessons from the global decline of amphibian populations. *Ecol. Lett.* **2002**, *5*, 597–608. [CrossRef]
6. Boone, M.D.; Semlitsch, R.D.; Little, E.E.; Doyle, M.C. Multiple stressors in amphibian communities: Effects of chemical contamination, bullfrogs, and fish. *Ecol. Appl.* **2007**, *17*, 291–301.
7. Hussain, Q.A.; Ashok, K.P. Global amphibian declines: A review. *Int. J. Biodivers. Conserv.* **2012**, *4*, 348–357.
8. Boone, M.D.; Bridges, C.M. Effects of carbaryl on green frog (*Rana clamitans*) tadpoles: Timing of exposure versus multiple exposures. *Environ. Toxicol. Chem.* **2003**, *22*, 2695–2702. [CrossRef]
9. Rohr, J.R.; Raffel, T.R.; Halstead, N.T.; McMahon, T.A.; Johnson, S.A.; Boughton, R.K.; Martin, L.B. Early-life exposure to a herbicide has enduring effects on pathogen-induced mortality. *Proc. R. Soc. B Biol. Sci.* **2013**, *280*, 20131502. [CrossRef] [PubMed]

10. Freitas, J.S.; Girotto, L.; Goulart, B.V.; de Oliveira Gonçalves Alho, L.; Gebara, R.C.; Montagner, C.C.; Schiesari, L.; Espíndola, E.L.G. Effects of 2,4-D-based herbicide (DMA® 806) on sensitivity, respiration rates, energy reserves and behavior of tadpoles. *Ecotoxicol. Environ. Saf.* **2019**, *182*, 109446. [CrossRef]
11. Pavan, F.A.; Samojeden, C.G.; Rutkoski, C.F.; Folador, A.; Da Fré, S.P.; Müller, C.; Hartmann, P.A.; Hartmann, M.T. Morphological, behavioral and genotoxic effects of glyphosate and 2,4-D mixture in tadpoles of two native species of South American amphibians. *Environ. Toxicol. Pharmacol.* **2021**, *85*, 103637.
12. Relyea, R.A. New effects of Roundup on amphibians: Predators reduce herbicide mortality; herbicides induce antipredator morphology. *Ecol. Appl.* **2012**, *22*, 634–647. [CrossRef]
13. Gabor, C.R.; Fisher, M.C.; Bosch, J. Elevated Corticosterone Levels and Changes in Amphibian Behavior Are Associated with *Batrachochytrium dendrobatidis* (Bd) Infection and Bd Lineage. *PLoS ONE* **2015**, *10*, e0122685. [CrossRef]
14. McMahon, T.A.; Halstead, N.T.; Johnson, S.; Raffel, T.R.; Roman, J.M.; Crumrine, P.W.; Boughton, R.K.; Martin, L.B.; Rohr, J.R. The fungicide chlorothalonil is nonlinearly associated with corticosterone levels, immunity, and mortality in amphibians. *Environ. Health Perspect.* **2011**, *119*, 1098–1103. [CrossRef]
15. McMahon, T.A.; Boughton, R.K.; Martin, L.B.; Rohr, J. Exposure to the herbicide atrazine nonlinearly affects tadpole corticosterone levels. *J. Herpetol.* **2017**, *51*, 270–273. [CrossRef]
16. Burraco, P.; Gomez-Mestre, I. Physiological stress responses in amphibian larvae to multiple stressors reveal marked anthropogenic effects even below lethal levels. *Physiol. Biochem. Zool.* **2016**, *89*, 462–472. [CrossRef] [PubMed]
17. Adelizzi, R.; Portmann, J.; Van Meter, R. Effect of Individual and Combined Treatments of Pesticide, Fertilizer, and Salt on Growth and Corticosterone Levels of Larval Southern Leopard Frogs (*Lithobates sphenocephala*). *Arch. Environ. Contam. Toxicol.* **2019**, *77*, 29–39. [CrossRef] [PubMed]
18. Van Meter, R.J.; Adelizzi, R.; Glinski, D.A.; Henderson, W.M. Agrochemical mixtures and amphibians: The combined effects of pesticides and fertilizer on stress, acetylcholinesterase activity, and bioaccumulation in a terrestrial environment. *Environ. Toxicol. Chem.* **2019**, *38*, 1052–1061. [CrossRef]
19. Mann, R.M.; Bidwell, J.R. The toxicity of glyphosate and several glyphosate formulations to four species of southwestern Australian frogs. *Arch. Environ. Contam. Toxicol.* **1999**, *36*, 193–199. [CrossRef]
20. Allran, J.W.; Karasov, W.H. Effects of atrazine on embryos, larvae, and adults of anuran amphibians. *Environ. Toxicol. Chem.* **2001**, *20*, 769–775. [CrossRef]
21. Hayes, T.B.; Collins, A.; Lee, M.; Mendoza, M.; Noriega, N.; Stuart, A.A.; Vonk, A. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 5476–5480. [CrossRef]
22. Wojtaszek, B.F.; Staznik, B.; Chartrand, D.T.; Stephenson, G.R.; Thompson, D.G. Effects of Vision herbicide on mortality, avoidance response, and growth of amphibian larvae in two forest wetlands. *Environ. Toxicol. Chem.* **2004**, *23*, 832–842. [CrossRef]
23. Wagner, N.; Reichenbecher, W.; Teichmann, H.; Tappeser, B.; Lötters, S. Questions concerning the potential impact of glypho-sate-based herbicides on amphibians. *Environ. Toxicol. Chem.* **2013**, *32*, 1688–1700.
24. Sievers, M.; Hale, R.; Parris, K.M.; Melvin, S.D.; Lanctôt, C.M.; Swearer, S.E. Contaminant-induced behavioural changes in amphibians: A meta-analysis. *Sci. Total Environ.* **2019**, *693*, 133570.
25. Brühl, C.A.; Pieper, S.; Weber, B. Amphibians at risk? Susceptibility of terrestrial amphibian life stages to pesticides. *Environ. Toxicol. Chem.* **2011**, *30*, 2465–2472. [CrossRef]
26. Brühl, C.A.; Schmidt, T.; Pieper, S.; Alscher, A. Terrestrial pesticide exposure of amphibians: An underestimated cause of global decline? *Sci. Rep.* **2013**, *3*, 1135. [CrossRef] [PubMed]
27. Hels, T.; Nachman, G. Simulating viability of a spadefoot toad *Pelobates fuscus* metapopulation in a landscape fragmented by a road. *Ecography* **2002**, *25*, 730–744. [CrossRef]
28. Vonesh, J.R.; De la Cruz, O. Complex life cycles and density dependence: Assessing the contribution of egg mortality to amphibian declines. *Oecologia* **2002**, *133*, 325–333. [CrossRef] [PubMed]
29. Salice, C.J. Multiple stressors and amphibians: Contributions of adverse health effects and altered hydroperiod to population decline and extinction. *J. Herpetol.* **2012**, *46*, 675–681.
30. Weir, S.M.; Scott, D.E.; Salice, C.J.; Lance, S.L. Integrating copper toxicity and climate change to understand extinction risk to two species of pond-breeding anurans. *Ecol. Appl.* **2016**, *26*, 1721–1732.
31. Graeter, G.J.; Rothermel, B.B.; Gibbons, J.W. Habitat selection and movement of pond-breeding amphibians in experimentally fragmented pine forests. *J. Wildl. Manag.* **2008**, *72*, 473–482. [CrossRef]
32. Fryday, S.; Thompson, H. Toxicity of pesticides to aquatic and terrestrial life stages of amphibians and occurrence, habitat use and exposure of amphibian species in agricultural environments. *EFSA Support. Publ.* **2012**, *9*, EN343. [CrossRef]
33. Stiles, R.M.; Swan, J.W.; Klemish, J.L.; Lannoo, M.J. Amphibian habitat creation on postindustrial landscapes: A case study in a reclaimed coal strip-mine area. *Can. J. Zool.* **2017**, *95*, 67–73.
34. Perkins, P.J.; Boermans, H.J.; Stephenson, G.R. Toxicity of glyphosate and triclopyr using the frog embryo teratogenesis as-say-Xenopus. *Environ. Toxicol. Chem.* **2000**, *19*, 940–945.
35. Van Meter, R.J.; Glinski, D.A.; Henderson, W.M.; Garrison, A.W.; Cyterski, M.; Purucker, S.T. Pesticide uptake across the amphibian dermis through soil and overspray exposures. *Arch. Environ. Contam. Toxicol.* **2015**, *69*, 545–556. [CrossRef]
36. Rumrill, C.T.; Scott, D.E.; Lance, S.L. Delayed effects and complex life cycles: How the larval aquatic environment influences terrestrial performance and survival. *Environ. Toxicol. Chem.* **2018**, *37*, 2660–2669. [CrossRef]

37. Zamora-Camacho, F.J.; Zambrano-Fernández, S.; Aragón, P. Carryover effects of chronic exposure to ammonium during the larval stage on post-metamorphic frogs. *Aquat. Toxicol.* **2022**, *248*, 106196. [CrossRef]
38. Zamora-Camacho, F.J.; Zambrano-Fernández, S.; Aragón, P. Long-term sex-dependent inflammatory response of adult frogs to ammonium exposure during the larval stage. *Chemosphere* **2022**, *307*, 136202. [CrossRef]
39. Belden, J.; McMurry, S.; Smith, L.; Reilley, P. Acute toxicity of fungicide formulations to amphibians at environmentally relevant concentrations. *Environ. Toxicol. Chem.* **2010**, *29*, 2477–2480. [CrossRef]
40. Storrs Méndez, S.I.; Tillitt, D.E.; Rittenhouse, T.A.G.; Semlitsch, R.D. Behavioral response and kinetics of terrestrial atrazine exposure in American toads (*Bufo americanus*). *Arch. Environ. Contam. Toxicol.* **2009**, *57*, 590–597.
41. Mitchkash, M.G.; McPeek, T.; Boone, M.D. The effects of 24-h exposure to carbaryl or atrazine on the locomotor performance and overwinter growth and survival of juvenile spotted salamanders (*Ambystoma maculatum*). *Environ. Toxicol. Chem.* **2014**, *33*, 548–552. [PubMed]
42. Adams, E.; Gerstle, V.; Brühl, C.A. Dermal fungicide exposure at realistic field rates induces lethal and sublethal effects on juvenile European common frogs (*Rana temporaria*). *Environ. Toxicol. Chem.* **2021**, *40*, 1289–1297. [CrossRef]
43. Thompson, D.G.; Pitt, D.G. A review of Canadian forest vegetation management research and practice. *Ann. For. Sci.* **2003**, *60*, 559–572. [CrossRef]
44. Shepard, J.P.; Creighton, J.; Duzan, H. Forestry herbicides in the United States: An overview. *Wildl. Soc. Bull.* **2004**, *32*, 1020–1027.
45. Franklin, R.M. *Stewardship of Longleaf Pine Forests: A Guide for Landowners*; Solon Dixon Forestry Education Center: Andalusia, AL, USA, 1997; p. 41.
46. Minogue, P.J.; Bohn, K.; Williams, R. *Controlling Hardwoods in Longleaf Pine Restoration*; EDIS; University of Florida: Gainesville, FL, USA, 2007; Volume 20, pp. 1–5.
47. Means, D.B. Vertebrate Faunal Diversity of Longleaf Pine Ecosystems. In *The Longleaf Pine Ecosystem: Ecology, Silviculture, and Restoration*; Jose, S., Jokela, E.J., Miller, D.L., Eds.; Springer: Greer, SC, USA, 2006; pp. 157–213.
48. Greenberg, C.H.; Moorman, C.E.; Raybuck, A.L.; Sundol, C.; Keyser, T.L.; Bush, J.; Simon, D.M.; Warburton, G.S. Reptile and amphibian response to oak regeneration treatments in productive southern Appalachian hardwood forest. *For. Ecol. Manag.* **2016**, *377*, 139–149.
49. Chen, C.Y.; Hathaway, K.M.; Thompson, D.G.; Folt, C.L. Multiple stressor effects of herbicide, pH, and food on wetland zoo-plankton and a larval amphibian. *Ecotoxicol. Environ. Saf.* **2008**, *71*, 209–218. [CrossRef] [PubMed]
50. Wojtaszek, B.F.; Buscarini, T.M.; Chartrand, D.T.; Stephenson, G.R.; Thompson, D.G. Effect of release® herbicide on mortality, avoidance response, and growth of amphibian larvae in two forest wetlands. *Environ. Toxicol. Chem.* **2005**, *24*, 2533–2544. [CrossRef] [PubMed]
51. Curtis, A.N.; Bidart, M.G. Increased temperature influenced growth and development of *Lithobates pipiens* tadpoles exposed to leachates of the invasive plant European buckthorn (*Rhamnus cathartica*) and a triclopyr herbicide. *Environ. Toxicol. Chem.* **2021**, *40*, 2547–2558. [CrossRef] [PubMed]
52. Kreutzweiser, D.P.; Holmes, S.B.; Eichenberg, D.C. Influence of exposure duration on the toxicity of triclopyr ester to fish and aquatic insects. *Arch. Environ. Contam. Toxicol.* **1994**, *26*, 124–129. [CrossRef]
53. Thompson, D.G.; Kreutzweiser, D.P.; Capell, S.S.; Thomas, D.R.; Staznik, B.; Viinikka, T. Fate and effects of triclopyr ester in a first-order forest stream. *Environ. Toxicol. Chem.* **1995**, *14*, 1307–1317. [CrossRef]
54. Senseman, S.A. (Ed.) *Herbicide Handbook*, 9th ed.; Weed Science Society of America: Lawrence, KS, USA, 2007.
55. Douglass, C.H.; Nissen, S.J.; Meiman, P.J.; Kniss, A.R. Impacts of imazapyr and triclopyr soil residues on the growth of several restoration species. *Rangel. Ecol. Manag.* **2016**, *69*, 199–205. [CrossRef]
56. U.S. Geological Survey (USGS). Pesticide National Synthesis Project. 2017. Available online: https://water.usgs.gov/nawqa/pnsp//usage/maps/show_map.php?year=2017&map=TRICLOPYR&hilo=L&disp=Triclopyr (accessed on 12 October 2021).
57. Russell, A.P.; Bauer, A.M.; Johnson, M.K. Migration in amphibians and reptiles: An overview of patterns and orientation mechanisms in relation to life history strategies. In *Migration of Organisms: Climate Geography Ecology*; Elewa, A.M.T., Ed.; Springer: Berlin/Heidelberg, Germany, 2005; pp. 151–203.
58. Llewelyn, J.; Phillips, B.L.; Alford, R.A.; Schwarzkopf, L.; Shine, R. Locomotor performance in an invasive species: Cane toads from the invasion front have greater endurance, but not speed, compared to conspecifics from a long-colonised area. *Oecologia* **2010**, *162*, 343–348. [CrossRef]
59. Janin, A.; Léna, J.P.; Deblois, S.; Joly, P. Use of stress-hormone levels and habitat selection to assess functional connectivity of a landscape for an amphibian. *Conserv. Biol.* **2012**, *26*, 923–931. [CrossRef]
60. Rivers, J.W.; Liebl, A.L.; Owen, J.C.; Martin, L.B.; Betts, M.G. Baseline corticosterone is positively related to juvenile survival in a migrant passerine bird. *Funct. Ecol.* **2012**, *26*, 1127–1134. [CrossRef]
61. Louppe, V.; Courant, J.; Videlier, M.; Herrel, A. Differences in standard metabolic rate at the range edge versus the center of an expanding invasive population of *Xenopus laevis* in the West of France. *J. Zool.* **2018**, *305*, 163–172. [CrossRef]
62. Gosner, K.L. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **1960**, *16*, 183–190.
63. Gabor, C.R.; Bosch, J.; Fries, J.N.; Davis, D.R. A non-invasive water-borne hormone assay for amphibians. *Amphib. Reptil.* **2013**, *34*, 151–162. [CrossRef]
64. Dow AgroSciences. *Garlon®4 Ultra Specimen Label*; Dow AgroSciences: Midland, MI, USA, 2019.

65. Gabor, C.R.; Knutie, S.A.; Roznik, E.A.; Rohr, J.R. Are the adverse effects of stressors on amphibians mediated by their effects on stress hormones? *Oecologia* **2018**, *186*, 393–404. [CrossRef] [PubMed]
66. Chambers, D.L. Increased conductivity affects corticosterone levels and prey consumption in larval amphibians. *J. Herpetol.* **2011**, *45*, 219–223. [CrossRef]
67. Bliley, J.M.; Woodley, S.K. The effects of repeated handling and corticosterone treatment on behavior in an amphibian (Ocoee salamander: *Desmognathus ocoee*). *Physiol. Behav.* **2012**, *105*, 1132–1139. [CrossRef]
68. Narayan, E.J.; Cockrem, J.F.; Hero, J.M. Sight of a predator induces a corticosterone stress response and generates fear in an amphibian. *PLoS ONE* **2013**, *8*, e73564. [CrossRef] [PubMed]
69. Edginton, A.N.; Stephenson, G.R.; Sheridan, P.M.; Thompson, D.G.; Boermans, H.J. Effect of pH and release® on two life stages of four anuran amphibians. *Environ. Toxicol. Chem.* **2003**, *22*, 2673–2678. [CrossRef] [PubMed]
70. Berrill, M.; Bertram, S.; McGilliray, L.; Kolohon, M.; Pauli, B. Effects of low concentrations of forest-use pesticides on frog embryos and tadpoles. *Environ. Toxicol. Chem.* **1994**, *13*, 657–664. [CrossRef]
71. Yahnke, A.E.; Grue, C.E.; Hayes, M.P.; Pearman-Gillman, S. Effects of the herbicide triclopyr on metamorphic northern red-legged frogs. *Environ. Toxicol. Chem.* **2017**, *36*, 2316–2326. [CrossRef]
72. Van Meter, R.J.; Glinski, D.A.; Hong, T.; Cyterski, M.; Henderson, W.M.; Purucker, S.T. Estimating terrestrial amphibian pesticide body burden through dermal exposure. *Environ. Pollut.* **2014**, *193*, 262–268. [CrossRef] [PubMed]
73. Baker, H. Characteristics and modes of origin of weeds. In *The Genetics of Colonizing Species*; Baker, H.G., Stebbins, G.L., Eds.; Academic Press: New York, NY, USA, 1965; pp. 147–168.
74. Wolff, W.J. Recent human-induced invasions of fresh waters by saltwater animals? *Aquat. Ecol.* **2000**, *34*, 319–321. [CrossRef]
75. Sorte, C.J.; Williams, S.L.; Zerebecki, R.A. Ocean warming increases threat of invasive species in a marine fouling community. *Ecology* **2010**, *91*, 2198–2204. [CrossRef]
76. Peck, M.R.; Wilcoxen, T.E. Effects of water acidification and pathogen exposure in innate immunity in Cuban tree frog (*Osteopilus septentrionalis*) tadpoles. *J. Zool. Res.* **2018**, *2*, 13–20.
77. Going, K.; Wilcoxen, T.E. Effects of night time temperate zone temperatures on survival, skin, antioxidant capacity, and corticosterone in two subtropical *Hylid* species. *BIOS* **2018**, *89*, 185–191. [CrossRef]
78. Lukens, E.; Wilcoxen, T.E. Effects of elevated salinity on Cuban treefrog *Osteopilus septontrionalis* aldosterone levels, growth, and development. *Mar. Freshw. Behav. Physiol.* **2020**, *53*, 99–111. [CrossRef]
79. Ehrsam, M.; Knutie, S.A.; Rohr, J.R. The herbicide atrazine induces hyperactivity and compromises tadpole detection of predator chemical cues. *Environ. Toxicol. Chem.* **2016**, *35*, 2239–2244. [CrossRef]
80. Rohr, J.R.; Brown, J.; Battaglin, W.A.; McMahon, T.A.; Relyea, R.A. A pesticide paradox: Fungicides indirectly increase fungal infections. *Ecol. Appl.* **2017**, *27*, 2290–2302. [CrossRef]
81. Budischak, S.A.; Belden, L.K.; Hopkins, W.A. Effects of malathion on embryonic development and latent susceptibility to trematode parasites in ranid tadpoles. *Environ. Toxicol. Chem.* **2008**, *27*, 2496–2500. [CrossRef] [PubMed]
82. Guilherme, S.; Santos, M.A.; Gaivão, I.; Pacheco, M. Genotoxicity evaluation of the herbicide Garlon(®) and its active ingredient (triclopyr) in fish (*Anguilla anguilla* L.) using the comet assay. *Environ. Toxicol.* **2015**, *30*, 1073–1081. [CrossRef] [PubMed]