Comparative acute toxicity of pesticides to tadpoles of a tropical anuran (*Epipedobates anthonyi*), a North American native anuran (*Lithobates sphenocephalus*) and a standard fish species

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Global amphibian declines have the highest incidence in tropical regions, but most of the ecotoxicological data on amphibians is collected on temperate northern hemisphere anuran species. We tested the hypothesis that tropical anuran larvae (*Epipedobates anthonyi*) would be more sensitive to pesticides than a North American native species (*Lithobates sphenocephalus*). For 12 pesticides, 96-hr range-finding acute toxicity tests were conducted to determine if mortality occurred at environmentally relevant levels. Based on those studies, two substances were selected for additional time-to-event analyses in both species as well as median lethal concentration (LC50) calculations. Time-to-event results indicated that the two species appear to be roughly equivalent in their sensitivity to the two tested pesticides. Significant differences between species were not consistent across concentrations for either the insecticide terbufos or the herbicide pendimethalin. The utility of LC50 data was mixed with one LC50 providing an arbitrarily large standard error around the LC50 precluding informative comparisons across species. However, standard LC50 methods allowed data collection that continues to contribute to our understanding of the protectiveness of fish as surrogates for anuran larvae. While our data set is limited, it appears that testing temperate species would be protective for tropical species in ecological risk assessments. Our data also support the continued use of fish as surrogates for amphibian larvae as none of the species were more sensitive to the tested pesticides than rainbow trout (*Oncorhynchus mykiss*), the standard sensitive fish species used for acute toxicity testing.

Key words: amphibians; plant protection product; poison-arrow frog; rainbow trout; southern leopard frog.

Global amphibian declines have now been well documented (Houlahan *et al.*, 2000) and have highest incidences in the tropics (Stuart *et al.*, 2004). The International Union for Conservation of Nature reports that as many as 41% of amphibian species are threatened globally, and the five countries with the greatest number of

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at-risk amphibians are all Neotropical countries (Leudtke *et al.*, 2023). The causes of amphibian declines are numerous but include introduced diseases, habitat degradation, invasive species, climate change and chemical contamination (Collins & Storfer, 2003). There have been few specific instances in which amphibian declines have been associated with chemical pollution (e.g., Davidson, 2004). However, the lack of direct connections may have two related causes. First, there is significantly less published research on amphibians in the field of ecotoxicology than on mammals, birds, and fish (Sparling *et al.*, 2010). Second, amphibian declines are most prominent in the tropics, while amphibian research is biased towards temperate areas, which could potentially miss relevant sensitive species (Schiesari *et al.*, 2007).

Ecotoxicology often makes use of standard toxicity tests carried out under controlled laboratory conditions, but these standardized tests do not always provide protection for all species (Cairns Jr., 1986), and thus additional assessment factors are applied in the risk assessment to address potentially more sensitive species. Further, no standardized and validated acute toxicity tests exist for amphibians (although this type of study has often been conducted on tadpoles using procedures developed for fish or daphnids), and few researchers in developed countries use tropical species in their experiments. Finally, many of the pesticides that developed countries have banned were used for many more years before being banned in some tropical countries (e.g., Costa Rica, Castillo *et al.*, 1997) and some (e.g., endosulfan) continue to be used in areas where amphibian declines are strongest.

Because amphibians do not have required tests with standardized protocols in regulatory frameworks for ecological risk assessment of pesticides, fish toxicity data are often used as a surrogate when amphibian toxicity data are not available (Ockleford *et al.*, 2018). It has been suggested that fish acute toxicity data generally provide protection for amphibians (Weltje *et al.*, 2013; Ockleford *et al.*, 2018; Ortiz-Santaliestra *et al.*, 2018). However, some authors have called these results into question as much of the data used to compare fish and amphibians are biased towards species from North America, Europe, and the commonly used African clawed frog *Xenopus laevis* (Schiesari *et al.*, 2007; Ghose *et al.*, 2014).

The purpose of this paper is to compare the relative sensitivity of North American and Latin American amphibian species to pesticides of various classes (e.g., insecticides, herbicides, fungicides) using time-to-event analysis and, when possible, median lethal concentrations (LC50s). In addition, the pesticides tested in these experiments have hardly, if ever, been tested on amphibians, which will contribute further to the database of amphibian toxicity data that can be compared to fish toxicity endpoints to continue investigating the hypothesis that fish are adequately sensitive surrogates for aquatic amphibian life stages (Weltje *et al.*, 2013; Ockleford *et al.*, 2018; Glaberman *et al.*, 2019). Assuming there is any connection between pesticide exposures and amphibian declines in the tropics, we hypothesized the tropical species would be more sensitive than the temperate anuran.

Materials and Methods

All methods were regulated and approved by the Institutional Animal Care and Use Committee of Queens University of Charlotte (Protocol Number 4).

Study organisms

Southern leopard frog (*Lithobates sphenocephalus*) egg masses were collected from the field in January 2020 during reproductive events. Southern leopard frogs were selected because they are a ubiquitous and common North American species and because they had a strong breeding event at the period when experiments were started. Eggs were collected at the Savannah River Site from wetlands known to have no history of exposure to pesticides. The Savannah River Site has been controlled by the Department of Energy since the 1950s and many of the wetlands have not been impacted by previous contamination and are used as reference locations for multiple studies that occur on the site. Egg masses were brought into the laboratory for hatching and placed into two 37.85 L tanks filled with 5 L of reconstituted moderately hard water that was also used for the experiments. Eggs were allowed to hatch and tadpoles at Gosner stage 25 (Gosner, 1960) were collected for experiments. Tadpoles were not provided with food prior to experimental setup (which was less than 24 hours after hatching). No mortality of hatched tadpoles was observed prior to the start of experiments. Twelve "tricolor" poison-arrow frogs (*Epipedobates anthonyi*) were purchased from a private source and transferred to the laboratory on Queens University of Charlotte campus for culturing and rearing. These frogs readily breed in captivity and are fairly easy to maintain. The adult poison-arrow frogs were grouped with multiple males to one female to induce breeding. Poison-arrow frogs were fed an abundance of wingless fruit flies dusted with a nutrient supplement three times per week. Egg masses were laid every two to four weeks and collected and separated from the adults for hatching and rearing. For both species, embryos were hatched in the laboratory and grown collectively until reaching Gosner stage 25 (Gosner, 1960) at which point they are free-swimming larvae.

Toxicity testing and pesticides

Technical grade pesticides (> 96% purity except for gibberellic acid, which had ~90% purity) were purchased from Chemservice (West Chester, PA) or Fisher Scientific (Hampton, NH, gibberellic acid only). We investigated three herbicides (pendimethalin, triclopyr, haloxyfop-methyl), five insecticides (thiamethoxam, terbufos, bifenthrin, allethrin, flufenoxuron), one fungicide (thiophanate-methyl), one antihelminthic (niclosamide), one molluscicide (metaldehyde), and one plant growth regulator (gibberellic acid). All pesticides were kept in a refrigerator until dosing solutions were prepared. Pesticides that were not readily miscible with water at concentrations necessary for dosing were dissolved in acetone. We ensured that acetone was $\leq$ 1% v/v in the final test solution (e.g., $\leq$ 0.5 mL in 50 mL).

For all experiments, tadpoles were held individually in 125 mL glass jars with either 50 mL (*E. anthonyi*) or 100 mL (*L. sphenocephalus*) of reconstituted moderately hard water. All experiments occurred in a plant growth incubator with a 12:12 hour

light:dark cycle and controlled temperature (set at 20°C, maximum fluctuation was within the range 19-21°C). Jars within the incubator were randomly placed on shelves to ensure that no treatment or species were placed in any specific region of the incubator. Jars were then covered with a large piece of acrylic to prevent water loss and to prevent cross contamination if multiple pesticides were being tested simultaneously.

We first performed a range-finding test with three concentrations spaced by a factor of 10 and three animals per treatment. Default concentrations were 1, 10, and 100 mg/L. Lower concentrations were used if prior amphibian data on similar pesticides suggested higher toxicity or if complete mortality occurred at 1 mg/L in the first test run. For example, previous research on pyrethroids found significant toxicity to amphibians (e.g., VANZETTO *et al.*, 2019). If mortality did not occur at 100 mg/L the definitive tests were not performed and the LC50 was reported as > 100 mg/L. This approach reduced the number of vertebrate animals that would be put through the potential pain/stress of a full experiment when the result have little ecological relevance. Environmental concentrations greater than 100 mg/L for pesticides are exceptionally unlikely and generally not relevant to ecological risk assessment. Further, substances with an LC50 above 100 mg/L are considered "practically nontoxic" for the purpose of classifying aquatic environmental hazards (UN, 2017).

Following the range-finding test, a definitive test was performed with five narrower spaced concentrations (four treatments and one control) and eight tadpoles per concentration. Because *E. anthonyi* lay small egg masses (8 to 20 eggs per mass) and cannot be induced to breed collectively, a traditional LC50 design where all treatments are started simultaneously was not possible. Instead, treatments were started on different days, depending on organism availability, and later combined for analysis. We performed time-to-event analyses (= survival analysis, NEWMAN & MCCLOSKEY, 1996) for assessing and comparing toxicity. Tadpoles were observed at least four times during the first 24 hours, and then at least every 12 hours until the end of exposure at 96 hours. After the experiment, survival curves were statistically compared across species for each concentration and LC50s (the concentration that causes 50% mortality at a particular time) were estimated from the 96-h data when possible and appropriate. Tadpoles were not fed during range-finding or definitive tests.

We collected water quality data (conductivity, dissolved oxygen, pH) on a single jar per concentration at the beginning and at the end of experiments to ensure that water quality parameters were within acceptable ranges and not contributing to toxicity. The U.S. EPA guideline for *Daphnia* acute toxicity tests provides an acceptable pH range of 6.0 to 8.5 and dissolved oxygen at 60% saturation or higher (USEPA, 2016). At 25°C and the elevation of Charlotte, NC (approximately 675 feet), 60% saturation for dissolved oxygen is 4.8 mg/L.

Statistical analysis

All statistical analyses were performed using the R environment (version 3.4.2, R

CORE TEAM, 2017). Time-to-event analyses were performed using the MASS (VENABLES & RIPLEY, 2002) and Survival (THERNEAU & LUMLEY, 2009) packages for R. The logrank test (i.e. Mantel-Cox test) was used to compare survival curves between species, which computes observed and expected number of events at each time point for each group. The expected values are subtracted from observed values for each time point and then summed across all time points. A chi-square (χ^2) statistic is computed from the sum of the observed minus expected values and tests the hypothesis that there is no difference between treatment survival curves. Survival curves were compared between species at each concentration tested for all pesticides for which a definitive test was performed. LC50 values were estimated using one of two methods. In the first method, we used the "drm" function in the "drc" package to build a dose-response model with a log-logit four parameter model. Multiple models available with the "drm" function were tested and the log-logit provided the best fit. The second method used a log-logit model in the "glm" function of the "MASS" package in R (VENABLES & RIPLEY, 2002). We used two different methods because mathematical constructs in the data sometimes result in arbitrarily inflated error values. Our goal was to acquire the most robust estimation of the LC50 as possible. An arbitrarily large error value around an LC50 is inherently less useful than a small one. Significant differences between LC50 values were determined by comparing 95% confidence intervals calculated for each LC50 from the standard error provided by the logit model. For all statistical analyses, we used $\alpha = 0.05$ for assessing significant differences between species.

Fish toxicity data

To compare the sensitivity of amphibians with fish, LC50 values obtained from the experiments with tadpoles described above were compared with regulatory fish data. Standard acute toxicity data, i.e. 96-h LC50 values, for rainbow trout (*Oncorhynchus mykiss*) were collected from the Pesticides Properties Database (PPDB, http://sitem.herts.ac.uk/aeru/ppdb/), which contains regulatory data from registration dossiers. Studies were conducted according to OECD TG 203: Fish Acute Toxicity Test. In case an LC50 value was not available in the PPDB, other sources, such as the USEPA ECOTOX database (https://cfpub.epa.gov/ecotox/) were consulted. In addition, we extracted linear interspecies correlation estimations (ICE) for fish-amphibian sensitivity relationships from the US-EPA's WebICE program (https://www3.epa.gov/webice/).

RESULTS

Range-finding toxicity Tests

We performed range-finding toxicity tests on at least one amphibian species for 12 pesticides. Five of the pesticides did not result in significant mortality to the anurans tested (thiamethoxam, metaldehyde, triclopyr, gibberellic acid, and thiophanate -methyl) while the remaining seven caused mortality to at least one anuran species (Table 1). In general, insecticides were more likely to cause mortality (n = 4 caused toxicity) than other classes of pesticides (two herbicides and one molluscicide

Table 1: Percent mortality per treatment recorded during range-finding tests for poison-arrow frog (*Epipedobates anthonyi*) and southern leopard frog (*Lithobates sphenocephalus*). Three tadpoles were tested at each concentration. Asterisks (*) indicate pesticides for which definitive tests with time-to-event analyses were performed. NT: not tested.

Pesticide	Concentration (mg/L)	Poison-arrow frog mortality	Southern leopard frog mortality
Pendimethalin*	1	0	NT
	10	100	NT
	100	100	NT
Thiamethoxam	1	0	NT
	10	0	NT
	100	0	NT
Terbufos*	1	0	NT
	10	100	NT
	100	100	NT
Metaldehyde	1	0	NT
	10	33	NT
	100	0	NT
Bifenthrin	0.1	0	NT
	1	100	NT
	10	100	NT
Triclopyr	1	0	NT
	10	0	NT
	100	0	NT
Allethrin	0.1	0	NT
	1	100	NT
	10	100	NT
Gibberellic Acid	1	0	NT
	10	0	NT
	100	0	NT
Thiophanate-methyl	1	0	NT
	10	0	NT
	100	0	NT
Flufenoxuron	1	0	0
	10	0	0
	100	0	66.7
Niclosamide	0.01	NT	0
	0.1	NT	100
	1	NT	100
Haloxyfop-methyl	1	NT	0
	10	NT	100
	100	NT	100

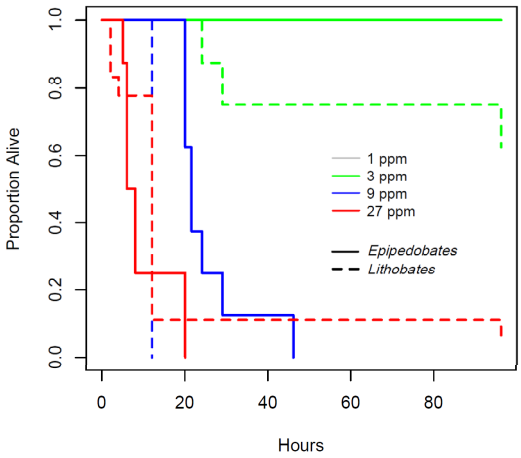

Figure 1: Time-to-event analysis of survival curves for southern leopard frogs (*L. sphenocephalus*) and poison-arrow frogs (*E. anthonyi*) following exposure to the insecticide terbufos. Control survival was 100% throughout the experiments. The 1 mg/L lines are not visible for both species as survival was 100% and is covered by the 3 mg/L line for *E. anthonyi*. At 9 mg/L, southern leopard frogs responded significantly faster (i.e., were more sensitive) compared to the poison-arrow frogs (χ^2 = 15, df = 1, p < 0.001). There were no other significant differences between the two species at any concentration tested (all $\chi^2 \leq 3.5$, all $p \geq 0.06$ with df = 1).

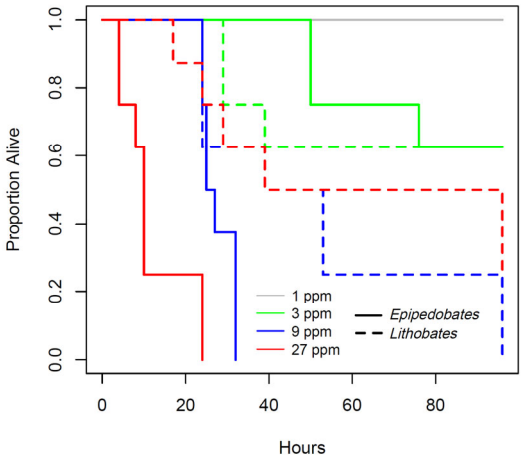

Figure 2: Time-to-event analysis of survival curves for southern leopard frogs (*L. sphenocephalus*) and poison-arrow frogs (*E. anthonyi*) following exposure to the herbicide pendimethalin. Control survival was 100% throughout the experiments. At 27 mg/L poison-arrow frogs responded significantly faster to toxicity (i.e., were more sensitive) compared to the leopard frogs (χ^2 = 11.2, df = 1, p < 0.001). There were no other significant differences between the two species at all concentrations tested (all $\chi^2 \leq 3.3$, all $p \geq 0.07$ with df = 1).

caused mortality). Terbufos (insecticide) and pendimethalin (herbicide) were selected for time-to-event analysis in both species.

Time-to-event analysis

For terbufos, there were no consistent patterns to relative sensitivity. For example, at 9 mg/L there were significant differences between the survival curves of the two species (χ^2 = 15, df = 1, p < 0.001) where leopard frogs responded faster than the poison-arrow frogs suggesting they were more sensitive (Fig. 1). However, at 27 mg/L, the poison-arrow frog curves suggested faster responses than leopard frogs, but this result was not significant (χ^2 = 2.2, df = 1, p = 0.1). Pendimethalin results were more consistent in which the poison-arrow frogs responded more quickly indicating greater sensitivity when significant mortality occurred (Fig. 2). The only significant difference between the species occurred at 27 mg/L where the poison-arrow frog responded faster than the leopard frog (χ^2 = 11.2, df = 1, p < 0.001). Pro-

nounced differences were also seen for 9 mg/L but this result was not significant (χ^2 = 3.3, df = 1, p = 0.07).

LC50 data

LC50 data were less informative than the time-to-event analysis for the purpose of comparing sensitivity (response over time) of species. The method used to estimate LC50s provided poor accuracy of the LC50 for *L. sphenocephalus* and terbufos resulting in the standard error around the LC50 being arbitrarily large and not useful for comparing species (Table 2). Comparing 95% confidence intervals around LC50s for pendimethalin found no significant differences between the two species (Table 2). Terbufos sensitivity between species could not be statistically assessed because of the arbitrarily large standard error around the LC50 for terbufos and *L. sphenocephalus*, so we are not able to make a statement about the significance of the differences in the LC50s of these two species (Table 2).

Table 2: Summary of LC50 data for both pesticides and both species. Eight individuals were tested at each concentration for each species. CI is the 95% confidence interval calculated from the standard error (SE) assuming a normal distribution.

Species	Chemical	Concentration (mg/L)	96-h mortality	LC50 (mg/L)	SE	CI
E. anthonyi	Terbufos	0	0	5.22	0.034	5.15-5.29
		1	0			
		3	0			
		9	8			
		27	8			
	Pendimethalin	0	0	2.99	0.170	2.66-3.32
		1	0			
		3	3			
		9	8			
		27	8			
L. sphenocephalus	Terbufos	0	0	3.53	3.53[a]	-3.39-10.45
		1	0			
		3	2			
		9	8			
		27	8			
	Pendimethalin	0	0	3.10	0.45	2.22-3.98
		1	0			
		3	3			
		9	8			
		27	6			

[a]The error values for the terbufos test run on *L. sphenocephalus* represent an arbitrarily large range that is a mathematical result of the model estimation and were not used for statistical assessment.

Water quality

Water quality data did not indicate any contribution to toxicity and were within levels that are acceptable for anuran larvae. Conductivity ranged from 324 to 473 μS/cm, pH ranged from 6.45 to 8.08, and dissolved oxygen was always greater than 4.5 mg/L (N = 27 measurements).

Discussion

While our data set is limited, and only two pesticides have been fully tested for acute toxicity, there was no clear indication that the tropical poison-arrow frog was more sensitive than the North American species. This ran counter to our hypothesis. We predicted that if pesticides were part of the cause of amphibian declines in the tropics, a tropical amphibian would be more sensitive to a range of pesticides than a North American anuran. There are very few direct comparisons of sensitivity between temperate and tropical anuran species. For example, an investigation into relative sensitivity of a North American (*Rana*), European (*Rana*), and South American (*Leptodactylus*) tropical anuran species reported that the tropical species had the greatest LC50 (though not significantly different than the temperate species) among the three species tested, which indicates it is the least sensitive species (Araújo *et al.*, 2014). Ghose *et al.* (2014) collected some temperate anuran data from the US EPA database ECOTOX (USEPA, 2019) to compare to their own toxicity testing with a tropical species, the red-eyed tree frog (*Agalychnis callidryas*), though they provide no formal analysis of the two datasets.

We know of no systematic assessment of family-level sensitivity analysis in anurans. Early phylogenetic trees of amphibians placed dendrobatid (like *E. anthonyi*) species in the same clade as Ranidae (which includes *Lithobates*, Duellman & Trueb, 1994). However, more recent analyses suggest dendrobatid anurans are more closely related to bufonid and hylid species of North American anurans, while *Lithobates* species remain in the ranid group (Hay *et al.*, 1995; Zhang *et al.*, 2013). However, more broadly, all of these taxonomic groups are within the suborder Neobatrachia, which contains most anuran species. Comparisons are scattered across contaminants and taxonomic groups. In addition to the work of Araújo *et al.* (2014), an analysis of endosulfan across nine species reported that bufonids were the least sensitive while ranid frogs were more sensitive (Jones *et al.*, 2009). Following copper exposure, ranids were much less sensitive (Lance *et al.*, 2012) than either a toad (Lance *et al.*, 2013) or *G. carolinensis* (Flynn *et al.*, 2015). Birge *et al.*, (2000) provide an extensive database of toxicity values, but the particular family that is most sensitive tends to be inconsistent across different contaminants. However, when combining all data across inorganic and organic compounds, the more sensitive species tended to be ranid, hylid, or *G. carolinensis*, compared to bufonids generally being listed as tolerant (Table 14a-1 in Birge *et al.*, 2000). As previously stated, it is unlikely that any particular group will be the most sensitive to all compounds (Cairns Jr., 1986). What will be more interesting (with greater data collection) is to study patterns of sensitivity across taxa. Perhaps dendrobatids have an

Table 3: Comparison of anuran toxicity data to standard fish toxicity values for the pesticides tested. NT: not tested. Values for rainbow trout (*Oncorhynchus mykiss*) were compiled from the Pesticide Properties Database (http://sitem.herts.ac.uk/aeru/ppdb/), unless noted otherwise.

Pesticide active substance	Class/Use	*Epipedobates anthonyi*	*Lithobates sphenocephalus*	*Oncorhynchus mykiss*
Pendimethalin	Herbicide	2.99	3.1	0.196
Thiamethoxam	Insecticide	>100	NT	>125
Terbufos	Insecticide	5.22	3.53	0.0094[b]
Metaldehyde	Molluscicide	>100	NT	75
Bifenthrin	Insecticide	0.32[3]	NT	0.00026
Triclopyr	Herbicide	>100	NT	117
Allethrin	Insecticide	0.32[3]	NT	0.0097[c]
Gibberellic acid	Plant Growth Regulator	>100	NT	>120
Thiophanate-methyl	Fungicide	>100	NT	11
Flufenoxuron	Insecticide	>100	32[a]	>0.0049
Niclosamide	Anthelmintic/Molluscicide	NT	0.032[a]	0.03[d]
Haloxyfop-methyl	Herbicide	NT	3.2[a]	0.46[e]

[a] LC50 estimate according to OECD (2019) by calculating the geometric mean of the concentration causing no mortality and the next higher concentration causing 100% mortality from the range-finder tests. [b] Retrieved from USEPA (1982). [c] Retrieved from USEPA (2019). [d] Retrieved from USEPA (2017). [e] Retrieved from EFSA (2009).

innate tolerance of some neurotoxic pesticides because their systems are already adapted to prevent toxicity from the neurotoxic compounds they use for defense. An example mechanism for increased tolerance might be altered acetylcholine receptors (TARVIN *et al.*, 2017).

Our pesticide toxicity data are similar to previous research on the same or closely related pesticides. For example, previous research has investigated the herbicide trifluralin, which is structurally similar to pendimethalin (i.e., they are both dinitroaniline herbicides, WEIR *et al.*, 2012). The LC50 for green frog tadpoles (*Lithobates clamitans*) exposed to technical grade trifluralin (9.76 mg/L) was approximately three times higher than our results for *E. anthonyi* and *L. sphenocephalus* exposed to pendimethalin. GHOSE *et al.*, (2014) exposed red-eyed treefrog tadpoles (*A. callidryas*) to formulated pesticides including a formulation of the insecticide terbufos. The 8-day LC50 for the terbufos formulation was estimated as 2.66 mg/L (GHOSE *et al.*, 2014), which is approximately a factor of two lower than both estimates in the current study. Differences may be related to exposure duration, the use of technical grade pesticides versus formulated products (which may include surfactants and other additives) and species' sensitivity.

Time-to-event analysis continues to be a useful, statistically robust, way to compare sensitivity among organisms

Table 4: Linear relationships using rainbow trout (*Oncorhynchus mykiss*) toxicity data to predict amphibian genus and family group toxicity data. Analyses obtained from the U.S. Environmental Protection Agency's WebICE program (https://www3.epa.gov/webice/).

Predicted taxonomic group	Taxonomic level	Slope estimate	R^2	P-value
Bufonidae	Family	0.54	0.47	< 0.001
Ranidae	Family	0.89	0.98	< 0.001
Hylidae	Family	0.34	0.37	0.047
Dicroglossidae	Family	0.97	0.85	0.001
Anaxyrus	Genus	0.54	0.45	0.003
Lithobates	Genus	0.90	0.98	< 0.001
Pseudacris	Genus	0.34	0.37	0.047

(NEWMAN & MCCLOSKEY, 1996). The difficulties we encountered with estimating LC50s from our time-to-event analysis may have been a function of the relatively low number of concentrations tested (four) or the relatively small sample size within each concentration (eight). For better resolution of relative species sensitivity, future experiments could consider increasing the number of test concentrations or the number of individuals in each concentration (which should be balanced against national/regional regulatory requirements regarding vertebrate animal use). In addition, because our goal was to compare species it is difficult to test species of varying sensitivity to the same concentrations, while ensuring ideal dose-response curve data. The ideal data for one species (creating low, moderate, and high mortality) might cause an "all or nothing" response in the other species. The goal of the experiment should drive these considerations. Ultimately, we wanted to compare the sensitivity of these two species, and acquiring robust LC50 data was considered a secondary goal.

Using traditional toxicity methods was adequate to collect data for anurans to compare to fish. Nothing in our data would suggest that the relationship between fish and amphibians previously reported (WELTJE *et al.*, 2013; ORTIZ-SANTALIESTRA *et al.*, 2018) is any less valid. It appears that fish provide an adequate surrogate for aquatic life stages of amphibians for ecological risk assessment. For terbufos, the fish LC50s (96 hours, n = 37) range from very low LC50s of 0.00077 to 1.8 mg/L and all but one were less than 0.39 mg/L, which is much lower than *L. sphenocephalus* (3.53 mg/L) and the poison-arrow frog (5.22 mg/L). Similarly, pendimethalin 96-hr LC50s (n = 4) ranged from 0.138 to 4.92 mg/L (USEPA, 2019). Our two LC50s (2.99 and 3.10 mg/L) are at the upper end of this range. The take home message is that neither of the toxicity estimates (nor the data from range-finding tests) that were collected in this study are likely to be significantly lower than the reported fish values (see Table 3 for a comparison of our LC50 values with the regulatory values for rainbow trout) and do not provide evidence that tropical amphibian testing will have any effect on the decision to use fish as a surrogate for anuran larvae. It is important to point out that our data set is limited, but as more toxicity data are collected, we expect the relationship to con-

tinue in the same direction.

Any inferences taken from these data must be considered in light of the limited data that we have collected so far. That said, there appear to be clear indications that there are significant linear relationships between fish and anuran toxicity data. For example, every family and genus relationship that can be produced from the US EPA's WebICE program finds a significant linear relationship between rainbow trout and the amphibian family or genus predicted from the trout data, although some of the significant relationships provide fairly weak R^2 values (Table 4). Future research could include multiple directions such as increasing the number of compounds to investigate and increasing the diversity of anuran groups that are tested for each pesticide. Ecological risk assessment should be based on the best available and most accurate data possible. As the database on species and pesticides expands, our confidence in using fish (or a linear relationship between fish and anuran toxicity data) as a surrogate for anurans will continue to increase as data continue to support the use of the surrogate.

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REFERENCES

ARAÚJO, C.V.; SHINN, C.; MOREIRA-SANTOS, M.; LOPES, I.; ESPÍNDOLA, E.L. & RIBEIRO, R. (2014). Copper-driven avoidance and mortality in temperate and tropical tadpoles. *Aquatic Toxicology* 146: 70-75.

BIRGE, W.J.; WESTERMAN, A.G. & SPROMBERG, J.A. (2000). Comparative toxicology and risk assessment of amphibians, *In* D.W. Sparling, G. Linder & C.A. Bishop (eds.) *Ecotoxicology of Amphibians and Reptiles, 1st ed*. SETAC Press, Pensacola, Florida, USA, pp. 727-791.

CAIRNS JR., J. (1986). The myth of the most sensitive species. *Bioscience* 36: 670-672.

CASTILLO, L.E.; DE LA CRUZ, E. & RUEPERT, C. (1997). Ecotoxicology and pesticides in tropical aquatic ecosystems of Central America. *Environmental Toxicology and Chemistry* 16: 41-51.

COLLINS, J.P. & STORFER, A. (2003). Global amphibian declines: sorting the hypotheses. *Diversity and Distributions* 9: 89-98.

DAVIDSON, C. (2004). Declining downwind: amphibian population declines in California and historical pesticide use. *Ecological Applications* 14: 1892-1902.

DUELLMAN, W.E. & TRUEB, L. (1994). *Biology of Amphibians*. John Hopkins University Press, Baltimore Maryland, USA.

EFSA (2009) Conclusion on the peer review of the pesticide risk assessment of the active substance haloxyfop-P (haloxyfop-R) on request from the European Commission. *EFSA Journal* 7: 1348.

FLYNN, R.W.; SCOTT, D.E.; KUHNE, W.; SOTEROPOULOS, D. & LANCE, S.L. (2015). Lethal and sublethal measures of chronic copper toxicity in the eastern narrowmouth toad, *Gastrophryne carolinensis*. *Environmental Toxicology and Chemistry* 34: 575-582.

GHOSE, S.L.; DONNELLY, M.A.; KERBY, J. & WHITFIELD, S.M. (2014). Acute toxicity tests and meta-analysis identify gaps in tropical ecotoxicology for amphibians. *Environmental*

Toxicology and Chemistry 33: 2114-2119.

GLABERMAN, S.; KIWIET, J. & AUBEE, C.B. (2019). Evaluating the role of fish as surrogates for amphibians in pesticide ecological risk assessment. *Chemosphere* 235: 952-958.

GOSNER, K.L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16: 183-190.

HAY, J.M.; RUVINSKY, I.; HEDGES, S.B. & MAXSON, L.R. (1995). Phylogenetic relationships of amphibian families inferred from DNA sequences of mitochondrial 12S and 16S ribosomal RNA genes. *Molecular Biology and Evolution* 12: 928-937.

HOULAHAN, J.E.; FINDLAY, C.S.; SCHMIDT, B.R.; MEYER, A.H. & KUZMIN, S.L. (2004). Quantitative evidence for global amphibian population declines. *Nature* 404: 752-755.

JONES, D.K.; HAMMOND, J.I. & RELYEA, R.A. (2009). Very highly toxic effects of endosulfan across nine species of tadpoles: Lag effects and family-level sensitivity. *Environmental Toxicology and Chemistry* 28: 1939-1945.

LANCE, S.L.; ERICKSON, M.R.; FLYNN, R.W.; MILLS, G.L.; TUBERVILLE, T.D. & SCOTT, D.E. (2012). Effects of chronic copper exposure on development and survival in the southern leopard frog (*Lithobates* [*Rana*] *sphenocephalus*). *Environmental Toxicology and Chemistry* 31: 1587-1594.

LANCE, S.L.; FLYNN, R.W.; ERICKSON, M.R. & SCOTT, D.E. (2013). Within-and among-population level differences in response to chronic copper exposure in southern toads, *Anaxyrus terrestris*. *Environmental Pollution* 177: 135-142.

LEUDTKE, J.A.; CHANSON, J.; NEAM, K.; HOBIN, L.; MACIEL, A.O.; CATENAZZI, A.; BORZEE, A.; HAMIDY, A.; AOWPHOL, A.; JEAN, A.; SOSA-BARTUANO, A.; FONG G., A.; DE SILVA, A.; FOUQUET, A.; ANGULO, A.; KIDOV, A.A.; SARAVIA, A.M.; DIESMOS, A.C.; TOMINAGA, A.; SHRESTHA, B.; GRATWICKE, B.; TJATURADI, B.; MARTINEZ RIVERA, C.C.; VASQUEZ ALMAZAN, C.R.; SENARIS, C.; CHANDRAMOULI, S.R.; CORTEZ FERNANDEZ, C.F.; AZAT, C.; HOSKIN, C.J.; HILTON-TAYLOR, C.; WHYTE, D.L.; GOWER, D.J.; OLSON, D.H.; CISNEROS-HEREDIA, D.F.; SANTANA, D.J.; NAGOMBI, E.; NAJAFI-MAJD, E.; QUAH, E.S.H.; BOLANOS, F.; XIE, F.; BRUSQUETTI, F.; ALVAREZ, F.S.; ANDREONE, F.; GLAW, F.; CASTANEDA, F.E.; KRAUS, F.; PARRA-OLEA, G.; CHAVES, G.; MEDINA-RANGEL, G.F.; GONZALEZ-DURAN, G.; ORTEGA-ANDRADE, H.M.; MACHADO, I.F.; DAS, I.; DIAS, I.R.; URBINA-CARDONA, J.N.; CRNOBRNJA-ISAILOVIC, J.; YANG, J-H.; JIANPING, J.; WANGYAL, J.T.; ROWLEY, J.J.L.; MEASEY, J.; VASUDEVAN, K.; CHAN, K.O.; GURURAJA, K.V.; OVASKA, K.; WARR, L.C.; CANSECO-MARQUEZ, L.; TOLEDO, L.F.; DIAZ, L.M.; KHAN, M.M.H.; MEEGASKUMBURA, M.; ACEVEDO, M.E.; NAPOLI, M.F.; PONCE, M.A.; VAIRA, M.; LAMPO, M.; YANEZ-MUNOZ, M.H.; SCHERZ, M.D.; RODEL, M-O.; MATSUI, M.; FILDOR, M.; KUSRINI, M.D.; AGMED, M.F.; RAIS, M.; KOUAME, N.G.; GARCIA, N.; GONWOUO, N.L.; BURROWES, P.A.; IMBUN, P.Y.; WAGNER, P.; KOK, P.J.R.; JOGLAR, R.L.; AUGUSTE, R.J.; BRANDAO, R.A.; IBANEZ, R.; VON MAY, R.; HEDGES, S.B.; BIJU, S.D.; GANESH, S.R.; WREN, S.; DAS, S.; FLECHAS, S.V.; ASHPOLE, S.L.; ROBLETO-HERNANDEZ, S.; LOADER, S.P.; INCHAUSTEGUI S.J.; GARG, S.; PHIMMACHAK, S.; RICHARDS, S.J.; SLIMANI, T.; OSBORNE-NAITATINI, T.; ABREU-JARDIM, T.P.F.; CONDEZ, T.H.; DE CARVALHO, T.R.; CUTAJAR, T.P.; PIERSON, T.W.; NGUYEN, T.Q.; KAYA, U.; YUAN, Z.; LONG, B.; LANGHAMMER, P. & STUART, S.N. (2023) Ongoing declines for the world's amphibians in the face of emerging threats. *Nature* 622: 308-314.

NEWMAN, M.C. & MCCLOSKEY, J.T. (1996). Time-to-event analyses of ecotoxicity data. *Ecotoxicology* 5: 187-196.

OCKLEFORD, C.; ADRIAANSE, P.; BERNY, P.; BROCK, T.; DUQUESNE, S.; GRILLI, S.; HERNANDEZ-JEREZ, A.F.; BENNEKOU, S.H.; KLEIN, M.; KUHL, T.; LASKOWSKI, R.; MACHERA, K.; PELKONEN, O.; PIEPER, S.; STEMMER, M.; SUNDH, I.; TEODOROVIC, I.; TIKTAK, A.; TOP-

PING, C.J.; WOLTERINK, G.; ALDRICH, A.; BERG, C.; ORTIZ, M.S.; WEIR, S.; STREISSL, F. & SMITH, R.H. (2018). Scientific opinion on the state of the science of pesticide risk assessment for amphibians and reptiles. *EFSA Journal* 16: e05125.

OECD (2019) *Test No. 203: Fish, Acute Toxicity Test*. OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, France.

ORTIZ-SANTALIESTRA, M.E.; MAIA, J.P.; EGEA-SERRANO, A. & LOPES, I. (2018). Validity of fish, birds and mammals as surrogates for amphibians and reptiles in pesticide toxicity assessment. *Ecotoxicology* 27: 819-833.

R CORE TEAM (2017). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available at https://www.R-project.org/. Retrieved on 1 December 2017.

SCHIESARI, L.; GRILLITSCH, B. & GRILLITSCH, H. (2007). Biogeographic biases in research and their consequences for linking amphibian declines to pollution. *Conservation Biology* 21: 465-471.

SPARLING, D.W.; LINDER, G.; BISHOP, C.A. & KREST, S.K. (2010). Recent advancements in amphibian and reptile ecotoxicology, *In* Sparling D.W., G. Linder, C.A. Bishop & S.K. Krest (eds.) *Ecotoxicology of Amphibians and Reptiles, 2nd ed*. SETAC Press, Pensacola, Florida, USA, pp. 1-11.

STUART, S.N.; CHANSON, J.S.; COX, N.A.; YOUNG, B.E.; RODRIGUES, A.S.L.; FISCHMAN, D.L. & WALLER, R.W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* 306: 1783-1786.

TARVIN, R.D.; BORGHESE, C.M.; SACHS, W.; SANTOS, J.C.; LU, Y.; O'CONNELL, L.A.; CANNATELLA, D.C.; HARRIS, R.A. & ZAKON, H.H. (2017). Interacting amino acid replacements allow poison frogs to evolve epibatidine resistance. *Science* 357: 1261-1266.

THERNEAU, T. & LUMLEY, T. (2009). Survival: Survival analysis, including penalized likelihood. R package version 2.35–2.4. Available at http://CRAN.R-project.org/package=survival. Retrieved on 13 February 2024.

UN (2017). Annex 9, Guidance on Hazards to the Aquatic Environment. Available at: https://unece.org/DAM/trans/danger/publi/ghs/ghs_rev07/English/12e_annex9.pdf. Retrieved on 08 March 2023.

USEPA (1982). *MRID 00037483*. Available at https://www3.epa.gov/pesticides/chem_search/cleared_reviews/csr_PC-105001_8-Dec-82_d.pdf. Retrieved on 13 February 2024.

USEPA (2016). *Ecological Effects Test Guidelines OCSPP 850.1010: Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids*. EPA 712-C-16-013. Office of Chemical Safety and Pollution Prevention, United States Environmental Protection Agency, Washington, DC, USA.

USEPA (2017) *Preliminary Environmental Fate and Ecological Risk Assessment in Support of the Registration Review of 3-Trifluoro-Methyl-4-Nitro-Phenol (TFM) and Niclosamide*. Available at https://www.regulations.gov/document/EPA-HQ-OPP-2013-0137-0016. Retrieved on 13 February 2024.

USEPA (2019). *ECOTOX Database*. National Health and Environmental Effects Laboratory, Mid-Continent Ecology Division, United States Environmental Protection Agency, Duluth, MN, USA. Available at https://cfpub.epa.gov/ecotox/. Retrieved on 02 December 2019.

VANZETTO, G.V.; SLAVIERO, J.G.; STURZA, P.F.; RUTKOSKI, C.F.; MACAGNAN, N.; KOLCENTI, C.; HARTMANN, P.A.; FERREIRA, C.M. & HARTMANN, M.T. (2019). Toxic effects of pyrethroids in tadpoles of *Physalaemus gracilis* (Anura: Leptodactylidae). *Ecotoxicology* 28: 1105-1114.

VENABLES, W.N. & RIPLEY, B.D. (2002). *Modern Applied Statistics with S, 4th ed*. Springer, New York, New York, USA.

WEIR, S.M.; YU, S. & SALICE, C.J. (2012). Acute toxicity of herbicide formulations and

chronic toxicity of technical-grade trifluralin to larval green frogs (*Lithobates clamitans*). *Environmental Toxicology and Chemistry* 31: 2029-2034.

WELTJE, L.; SIMPSON, P.; GROSS, M.; CRANE, M. & WHEELER, J.R. (2013). Comparative acute and chronic sensitivity of fish and amphibians: a critical review of data. *Environmental Toxicology and Chemistry* 32: 984-994.

ZHANG, P.; LIANG, D.; MAO, R.L.; HILLIS, D.M.; WAKE, D.B. & CANNATELLA, D.C. (2013). Efficient sequencing of anuran mtDNAs and a mitogenomic exploration of the phylogeny and evolution of frogs. *Molecular Biology and Evolution* 30: 1899-1915.