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Humane acute testing with tadpoles for risk assessment of chemicals: Avoidance instead of lethality

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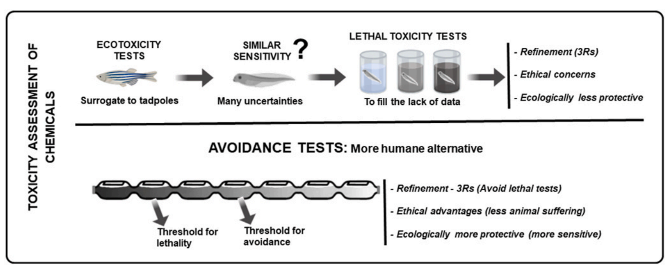
HIGHLIGHTS

- Fish toxicity data are not a secure surrogate to predict the effects on amphibians.
- Avoidance indicates the threshold at which organisms will flee from contamination.
- A reduction in the population density due to emigration is analogous to mortality.
- Test with amphibians based on spatial avoidance is a more humane method.
- Avoidance tests provide an ethical advantage to ecotoxicity tests (Refinement - 3 Rs).

GRAPHICAL ABSTRACT



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ABSTRACT

In spite of the sensitivity of amphibians to contamination, data from fish have been commonly used to predict the effects of chemicals on aquatic life stages. However, recent studies have highlighted that toxicity data derived from fish species may not protect all the aquatic life stages of amphibians. For pesticide toxicity assessment (PTA), EFSA has highlighted that more information on lethal toxicity for the aquatic life stages of amphibians is still needed to reduce uncertainties. The current review aims to propose a test with amphibians based on spatial avoidance, as a more humane alternative method to the lethality tests for chemicals. A review of lethal toxicity tests carried out with amphibians in the period between 2018 and 2021 is presented, then we discuss the suitability of using fish toxicity data as a surrogate to predict the effects on more sensitive amphibian groups. The possible differences in sensitivity to chemicals may justify the need to develop further tests with amphibian embryos and larvae in order to reduce uncertainties. A new test is proposed focused on the avoidance behaviour of organisms fleeing from contamination to replace lethal tests. As avoidance indicates the threshold at which organisms will flee from contamination, a reduction in the population density, or its disappearance, at the local scale due to emigration is expected, with ecological consequences analogous to mortality. Avoidance tests provide an ethical advantage over lethal tests as they respect the concepts of the 3 Rs (mainly Refinement), reducing the suffering of the organisms.

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1. Introduction

Historically, the use of animals in experimentation comes from ancient Greece (500 BCE) as a surrogate for humans (Guerrini, 2003). From that period on, scientific thought about animal experimentation has evolved considerably, so that the lack of intelligence and notions of justice and injustice were initially indicated as reasons for animals not being protected against suffering (Guerrini, 2003). From the 19th century onwards, animal experimentation started to be criticised, which led to the creation of the Society for the Protection of Animals Liable to Vivisection (1875) and the consolidation of the Law of Cruelty to Animals of Britain in 1876 (Rogers, 2007). During the following years, many initiatives that focused on welfare in animal experimentation were launched, such as: the British Cruelty to Animal Act, in the United Kingdom (Pankevich et al., 2012; DGOA, 2014), the Animal Welfare Act, in the USA (AWA, 1966), the Act on Animal Experimentation, in the Netherlands (Bordes, 2005), and the Universal Declaration of Animal Rights by the UNESCO (UNESCO, 1978). In 1986, the Council of the European Communities adopted the Directive 86/609/EEC to guarantee identical legislative, regulatory and administrative provisions in all member states related to the use of animals in experimentation (EEC, 1986). Nowadays, the Directive 2010/63/EU is the legal framework that regulates animal experimentation procedures in the European Union, establishing more humane procedures for animals proven to feel pain: living non-human vertebrate animals (including larval forms with autonomous feeding or reproduction and foetal forms of mammals in the last third of their normal development) and cephalopods (EU, 2010).

The use of animals in experimentation is a practice that deserves special environmental and ethical concern due to the high quantity of organisms used and their suffering. In this sense, alternative methods for animal experimentation have been sought to replace the animals used in laboratory tests (Garthoff, 2005), for instance, computer models, cells and tissue cultures and alternative organisms (the use of invertebrates or microorganisms instead of vertebrates) (Doke and Dahwale, 2015). Similarly, Rai and Kaushik (2018) also proposed in-silico simulation, informatics, 3D cell culture models and organ-on-chips as innovative and alternative methods to animal experimentation. The use of these alternative methods has some disadvantages, which makes it difficult to fully replace animals (Van Norman, 2019). It is important to consider that the extrapolation of the effects from the sub-organismal level to the population and community levels, or even to the individual level, presents many uncertainties, since, from the functional (e.g., genetic and physiological mechanisms) and behavioural (e.g., ecological niche) points of view, organisms are much more than merely a group of cells, just as populations and communities are much more complex than groups of individuals. In the case of ecotoxicity studies, the replacement is very difficult because the aim of these studies is to assess the effects that chemicals cause on biota, considering from the sub-organismal to the community and ecosystem levels. In this cascade of effects, the various genetic, biochemical, physiological, and behavioural disruptions might vary according to the surrounding environment. Thus, it is important to know the chemical processes of the contaminants, like toxicokinetics and toxicodynamics, and how organisms deal with the absorption of contaminants (detoxification: from complexation to the elimination of chemicals). The use of alternative methods can lack relevance if they do not simulate the organismal complexity of the individuals, their arrangement or their functionality accurately.

Probably, one of the most accepted replacement procedures in ecotoxicology occurs among ecological groups, where fish are commonly used as a surrogate for the aquatic life stages of amphibians, although for some chemicals fish are not an accurate surrogate (Ortiz-Santaliestra et al., 2018; Glaberman et al., 2019). The European Food Safety Authority (EFSA) has identified that, for some chemical compounds (namely pyrethroids), fish may not be good alternatives to assess the risk to the aquatic stages of amphibians, as the latter tends to be more sensitive, therefore more data is needed to be able to suggest assessment factors to apply to existing fish data (EFSA, 2007). Although the recommendation of using lethality tests is based on this important ecological reasoning, two inconvenient aspects for animal welfare can be highlighted: i) the concentrations used to detect mortality tend to be very high and might suppose the maximal suffering that an animal can experience and ii) the experimental procedure using a mandatory intensity of exposure for a given period (forced-exposure) supposes a continuous suffering during the experiments. Therefore, the use of mortality as an ecotoxicological response essentially does not meet the recommendation of the Directive 2010/63/EU (EU, 2010) to end the procedures where an animal experiences pain equivalent to or higher than that caused by the introduction of a needle (the humane endpoint). From a regulatory perspective, the endpoints considered of relevance in ecotoxicology are those that may predict a population decline (Ågerstrand et al., 2020); however, the application of more humane experimental procedures that meet this requisite without losing biological relevance should be encouraged. In this regard, the avoidance response, which focuses on the spatial displacement of organisms escaping from contamination (Lopes et al., 2004 and reviews by Araújo et al., 2016; Moreira-Santos et al., 2019), could be considered a more humane alternative to replace lethality tests. This idea is premised on the fact that when the organisms move away from a contaminated area the effect, at the local population level, is identical to mortality (Lopes et al., 2004; Hellou, 2011), even though no long-lasting direct toxic effect occurs regarding the individuals. Therefore, the aim of the present critical review is to propose spatial avoidance tests, using a non-forced multi-compartmented approach, as an alternative to lethality tests. This alternative considerably reduces the animal's suffering as it prevents the continuous exposure to chemicals, given that the animals are not mandatorily exposed to the test concentrations and can move freely among different concentrations (gradient or patchy scenarios), choosing the most favourable one. Initially, we reviewed (from 2018 to July of 2021) how frequently mortality was used as an endpoint to assess the toxicity of chemicals on the larval stages of amphibians, at development stages considered as animal experimentation (Gosner stage ≥25, Nieuwkoop and Faber stage ≥45 and Harrison stage ≥42). This time period was established to give continuity to the review by Ortiz-Santaliestra et al. (2018). Later, the alternative of using fish ecotoxicity data as surrogates for the aquatic stage (tadpoles) of amphibians is discussed. To support the hypothesis that tadpoles avoid contamination, experimental evidence that tadpoles, of many species, are able to detect and escape chemical contamination is provided. Furthermore, the sensitivity of this avoidance response is compared with that of lethality. Finally, we discuss the ecological importance of spatial avoidance when the non-forced exposure approach is used and how humane this method is when considering one of the principles of the 3 Rs: Refinement (reducing suffering and improving the living conditions of animals) (NC3Rs, 2009).

2. Lethality as an endpoint in amphibian toxicity tests

The amount of data available relating to amphibians in the aquatic system has been considered too limited to make comparisons concerning toxicity with alternative animal groups accurately (EFSA PPR Panel, 2018). Currently, among vertebrates, only fish, birds and mammals are recommended for use in ecological risk assessments (EEC, 1986). However, the EFSA Panel on Plant Protection Products (PPP) and their Residues recognized the need for further data on the acute and chronic effects of active substances or PPPs on amphibians in simple laboratory tests (EFSA PPR Panel, 2018). However, although test data concerning amphibians is not required for the submission of new chemicals (only terrestrial phases are required), all previously available information must be provided to the authorities.

Based on Ortiz-Santaliestra et al. (2017, 2018), Table 1 presents the latest studies using lethality as an endpoint with amphibian larval stages. Briefly, a total of 62 studies were found, in which 36 species

Table 1
Studies on toxicity using amphibian tadpoles from 2018 to 2020, in which mortality was used as the endpoint.

Year	Species (Family; Order)	Stage[a]	Number of organisms without control	Other endpoints	Contaminants	Exposure time (h)	References
2018	*Boana pulchella* (Hylidae: Anura)	GS 36	150	Growth, development, body mass, and morphological effects	Imazethapyr-based herbicide formulation Pivot H	96	Pérez-Iglesias et al. (2018a)
		GS 25	30	Growth development, behaviour, and morphologic abnormalities	Sediment with various contaminants	240	Sansiñena et al. (2018)
		GS 25 and GS 36	110	Behavioural changes, growth inhibition and morphological abnormalities	Pirimicarb-based commercial formulation Aficida	48/96	Natale et al. (2018)
	Ceratophrys ornata (Ceratophridae: Anura)	GS 25 and GS 31	590	Swimming alterations, morphological abnormalities, and growth inhibition	Chlorpyrifos	96	Salgado Costa et al. (2018)
	Euphlyctis cyanophlyctis (Dicroglossidae: Anura)	GS 21-23	900	–	Sodium floride	96	Pal et al. (2018)
	Holobatrachus rugulosus (Dicroglossidae: Anura)	GS ≥ 25	300	–	Neem seed extract (NSAI) and Bioinsecticides derived from *Azadirachta indica*, *Stemona curtisii* and *Mammea siamensis* (SCMS)	96	Saenphet et al. (2018)
	Limnodynastes tasmaniensis (Limnodynastidae: Anura)	GS ≥ 25	660	Avoidance of predators, changes in swimming behaviour and interactive effects of copper and imidacloprid	Copper and imidacloprid	24	Sievers et al. (2018)
	Physalaemus cuvieri (Leptodactylidae: Anura)	GS 24-27	510	Swimming activity	Atrazine (herbicide), cypermethrin (insecticide) and tebuconazole (fungicide)	96	Wrubleswski et al. (2018)
	Physalaemus gracilis (Leptodactylidae: Anura)	GS 19-25	40	Motility and malformations	Herbicide atrazine	96	Rutkoski et al. (2018)
	Rana nigromaculata (Ranidae: Anura)	GS 26	150	–	rac-Cyproconazole, 1-enantiomers, 2-enantiomers, 3-enantiomers and 4-enantiomers	96	Zhang et al. (2018a)
		GS 26	120	Weight, body length and development stages	Triadimefon and triadimenol	96	Zhang et al. (2018b)
	Rhinella arenarum (Bufonidae: Anura)	GS 26-30	150	Morphological alterations	Colloidal silicon dioxide nanoparticles	48	Lajmanovich et al. (2018)
	Rhinella schneideri (Bufonidae: Anura)	GS 30	60	–	Atrazine-based herbicide formulation SIPTRAN 500 SC	48/96	Pérez-Iglesias et al. (2018b)
	Silurana tropicalis (Pipidae: Anura)	NF 49/50	70	Growth inhibition, development retardation, thyroid gland histology, and axial malformations	Seven 1,3,5-triazine (s-triazine) herbicides (ametryn, prometryn, dimethametryn, simazine, atrazine, propazine, cyanazine)	96	Saka et al. (2018)
2019	*Ambystoma gracile* (Ambystomatidae: Caudata)	HS 43	180	Body weight	Diquat dibromide	96	Moreton and Marlatt (2019)
	Ambystoma opacum (Ambystomatidae: Caudata)	HS 46	60	–	Copper	240	Weir et al. (2019)
	Ambystoma talpoideum (Ambystomatidae: Caudata)	HS 46	60	–	Copper	1152	Weir et al. (2019)
	Ambystoma tigrinum (Ambystomatidae: Caudata)	HS 46	130	–	Copper	240	Weir et al. (2019)
	Hypsiboas pardalis (Hylidae: Anura)	GS 25	800	–	Herbicides: acetochlor, ametryn, glyphosate, and metribuzin	96	Daam et al. (2019)
	Leptodactylus fuscus (Leptodactylidae: Anura)	GS 25	120	Energy storage, development, respiration rates, swimming performance and avoidance behaviour	2,4-dichlorophenoxyaceti acid herbicide (DMA 806)	96	Freitas et al. (2019)
	Lithobates catesbeianus (Ranidae: Anura)	GS 25	120	Energy storage, development, respiration rates, swimming performance and avoidance behaviour	2,4-dichlorophenoxyaceti acid herbicide (DMA 806)	96	Freitas et al. (2019)
		GS 25	100	Swimming behaviour and avoidance	Diuron Nortox 500 SC pesticide	96	Moreira et al. (2019)
		GS 25	48	Leukocyte and erythrocyte measurements and erythrocyte nuclear abnormalities	Pyrethrum extract and solid lipid nanoparticles loaded with pyrethrum	48	Oliveira et al. (2019)
	Lithobates clanitans (Ranidae: Anura)	GS 26	120	Body mass	Chloride in a climate change context	96	Green et al. (2019)

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Table 1 (*continued*)

Year	Species (Family; Order)	Stage[a]	Number of organisms without control	Other endpoints	Contaminants	Exposure time (h)	References
	Microhyla fissipes (Microhylidae: Anura)	GS 26-28	180	Enzymatic activities, growth, and locomotion	Cadmium	48	Hu et al. (2019)
	Odontophrynus americanus (Ceratophryidae: Anura)	GS 26-30	120	Enzyme activities, hormone levels, cardiac rates, behaviour performance, development rate and growth	Pyriproxyfen insecticide	48	Lajmanovich et al. (2019a)
	Pelophylax perezi (Ranidae: Anura)	GS 25	420	Growth and somatic growth	Natural sea water (SW), sodium chloride (NaCl) and sodium chloride before and after short-term exposure to low levels of salinity ($NaCl_{STE}$)	96	Venâncio et al. (2019)
	Physalaenus albonotatus (Leptodactylidae: Anura)	GS 23-27	120	Morphology, development, body, visceral abnormalities and liver histology	Commercial herbicide formulated with 2,4-D	96	Curi et al. (2019)
	Physalaemus cuvieri (Leptodactylidae: Anura)	GS 25	800	–	Herbicides: acetochlor, ametryn, glyphosate, and metribuzin	96	Daam et al. (2019)
	Physalaenus nattereri (Leptodactylidae: Anura)	GS 25	120	Energy storage, development, respiration rates, swimming performance and avoidance behaviour	2,4-dichlorophenoxyaceti acid herbicide (DMA 806)	96	Freitas et al. (2019)
	Rana catesbeiana (Ranidae: Anura)	GS 25	220	Body mass and development stage	Perfluorooctanesulfonic acid (PFOS), perfluorooctanoic (PFOA) acid and their mixtures	96	Flynn et al. (2019)
	Rana sphenocephala (Ranidae: Anura)	GS 26	280	–	Neonicotinoid insecticide clothianidin	96	Holtswarth et al. (2019)
	Rhinella arenarum (Bufonidae: Anura)	GS 26-30	210	Enzymatic activities, hormone levels, miotic index, DNA damage, development stage, and growth	Glyphosate and arsenic mixture	48	Lajmanovich et al. (2019b)
		GS 26-30	500	Erratic swimming, loss of reflex, loss of balance, irregular swimming, growth and anatomical anomalies.	Raw and treated wine effluents	96	Romero et al. (2019)
		GS 25	72	Locomotor swimming performance and thermal tolerance limits	Chlorpyrifos	96	Quiroga et al. (2019)
		GS 35-37	320	DNA damage	Mixture of commercial formulations of glyphosate and imazethapyr herbicides	96	Carvalho et al. (2019)
2020	*Boana pardalis* (Hylidae: Anura)	GS 26	600	Growth, development, behaviour, and enzymatic activity	Glyphosate, ametryn, 2,4-D, metribuzin, and acetochlor herbicides	552	Moutinho et al. (2020)
	Bombecinus pulchelles	GS 29-42	100	Motility	Five pesticides (cypermethrin, chlorpyrifos, endolsufan, glyphosate and 2,4-dichloro-phenoxyacetic acid)	144/192/216	Agostini et al. (2020)
	Euphlyctis cyanophlyctis (Dicroglossidae: Anura)	GS 25-30	900	–	Arsenic, chromium and herbicide almix 20WP	24/48/72/96	Samanta et al. (2020)
		GS 26-30	720	–	Phenanthrene	96	Bhuyan et al. (2020)
	Leptodactylus latinasus (Leptodactylidae: Anura)	GS 25 and GS 36	160 for GS 25 and 220 for GS 36	Swimming activity, morphological abnormalities, and enzymatic activities	Imazethapyr-based herbicide formulation	96	Pérez-Iglesias et al. (2020)
	Leptodactylus latrans (Leptodactylidae Anura)	GS 29-42	100	Motility	Five pesticides: (cypermethrin, chlorpyrifos, endolsufan, glyphosate and 2,4-dichloro-phenoxyacetic acid)	144/192/216	Agostini et al. (2020)
	Lithobates catesbeianus (Ranidae: Anura)	GS 25	120	Swimming activity; morphological analysis; respirometry and avoidance	Mining tailing	96	Girotto et al. (2020)
		GS 25	200	DNA damage	ZnO NPs and $ZnCl_2$	168	Motta et al. (2020)
	Lithobates sphenocephalus (Ranidae: Anura)	GS 25	144	–	*Bacillus thuringiensis kustaki* biopesticide	96	Weeks and Parris (2020)
	Lithobates sylvaticus (Ranidae: Anura)	GS 26	348	Malformations in metamorphosis	NaCl	96	Green and Salice (2020)
	Physalaemus cuvieri (Leptodactylidae: Anura)	GS 25-26	210	Length, mass and malformations	Glyphosate-based herbicide Roundup Original DI	96	Herek et al. (2020)
	Physalaemus gracilis (Leptodactylidae: Anura)	GS 25-26	210	Length, mass and malformations	Glyphosate-based herbicide Roundup Original DI	96	Herek et al. (2020)
		GS 25	300	Swimming activity and biochemical traits	Chlorpyrifos insecticide	96	Rutkoski et al. (2020a)
		GS 25	600			168	

(*continued on next page*)

(especially belonging to the order Anura and with a preference for the Gosner stages between 25 and 36) and about 70 contaminants (pesticides, herbicides, fungicides and heavy metals, among others) have been studied. Data in Table 1 and the review by Ortiz-Santaliestra et al. (2018) may facilitate future comparisons of the sensitivity for each specific toxicant between amphibians and other animal groups. Furthermore, it may also facilitate comparisons among toxicity endpoints, specially the one here proposed: avoidance assays.

3. Fish as surrogates for amphibians

The problem about the decline of amphibian populations and species started to be of major concern since the 90s (Wake, 1991). Several factors were pointed out as drivers for this loss of biodiversity: habitat change, UV radiation, diseases, introduction of invasive species, and environmental contamination (Alford and Richards, 1999; Stuart et al., 2004; McCallum, 2007; IUCN: https://www.iucnredlist.org). One of the first studies that reviewed ecotoxicological data concerning amphibians was published by Power et al. (1989), which was later completed by Pauli et al. (2000). At first, the comparison of the data on ecotoxicity concerning fish and amphibians justified the use of the former as a substitute for the latter. However, even then, there were already suspicions that fish might not be good amphibian surrogates. However, Bridges et al. (2002) found a good efficiency in the use of fish as

Table 1 (*continued*)

Year	Species (Family; Order)	Stage[a]	Number of organisms without control	Other endpoints	Contaminants	Exposure time (h)	References
				Enzymatic activities, biochemical responses	Cypermethrin- (Cyptrin 250 CE) and fipronil- (TerraForte®) based insecticides		Rutkoski et al. (2020b)
	Rhinella arenarum (Bufonidae: Anura)	GS 29-42	100	Motility	Five pesticides: (cypermethrin, chlorpyrifos, endolsufan, glyphosate and 2,4-dichloro-phenoxyacetic acid)	144/192/216	Agostini et al. (2020)
		GS 25	210	Movement and epidermal pigmentation	Mancozeb fungicide	96	Asparch et al. (2020)
		GS 36	1530	–	Herbicide with glyphosate(GLY)-dicamba (DIC) and glyphosate(GLY)-flurochloridone (FLC)	96	Arcaute et al. (2020)
		GS 26-30	210	Enzymatic activities	Soluble and emulsifiable concentrations of dimethoate	48	Martinuzzi et al. (2020)
		GS 25 and 28	330	Oxidative stress biomarkers	Al2O3, NiO/Al2O3 and Ni/Al2O3	96	Svartz et al. (2020)
	Rhinella fernandezae (Bufonidae: Anura)	GS 29-42	100	Motility	Five pesticides (cypermethrin, chlorpyrifos, endolsufan, glyphosate and 2,4-dichloro-phenoxyacetic acid)	144/192/216	Agostini et al. (2020)
	Xenopus laevis (Pipidae: Anura)	NF 46	390	–	Glyphosate pure (GLY) and glyphosate-based product (Roundup)	96	Turhan et al. (2020)
2021	*Ceratophrys ornata* (Ceratophridae: Anura)	GS 31	590	Swimming alterations, presence of morphological abnormalities, and prey consumption	Chlorpyrifos	96	Costa et al. (2021a)
	Elachistocleis bicolor (Microhylidae: Anura)	GS 30-34	330	Biochemical responses	Herbicide DIC Cowboy Elite SURCOS®	48	Attademo et al. (2021)
	Lithobates catesbeianus (Ranidae: Anura)	GS 31-36	168	Healthiness, haematological profile, and histopathological analysis	herbicide 2,4-D (DMA® 806)	96	Viriato et al. (2021)
	Physalaemus cuvieri (Leptodactylidae: Anura)	GS 27	360	Toxicity biomarker and polyethylene glicol accumulation	Polyethylene glycol	72	Nascimento et al. (2021)
		GS 26	800	Toxicity biomarkers, neurotoxicity and azithromycin and hydroxychloroquine accumulation	Hydroxychloroquine and azithromycin	72	Luz et al. (2021)
	Polypedates maculatus (Rhacophoridae Anura)	GS 19-20	300		Cadmium chloride	24/48	Ojha et al. (2021)
	Rana pipiens (Ranidae: Anura)	GS 25	640	Analysis of RNA-seq data, differential transcript expression analysis	2,4,6-trinitrotoluene (TNT), the new insensitive munition formulation (IMX-101), the insensitive munition constituents nitroguinidine (NQ), and 1-methyl-3-nitro-guanidine (MeNQ)	96	Gust et al. (2021)
	Rhinella arenarum (Bufonidae: Anura)	GS 25	180	Morphological and behavioural alterations	Chlorothalonil fungicide	504	Acquaroni et al. (2021)
	Scinax nasicus (Hyidae: Anura)	GS 30-34	330	Biochemical responses	Herbicide DIC Cowboy Elite SURCOS®	48	Attademo et al. (2021)
	Silurana tropicalis (Pipidae: Anura)	NF 49-50	180	Morphometric and gravimetric data	Four neonicotinoids: (acetamiprid, clothianidin, dinotefuran, and imidacloprid) and fipronil	96	Saka and Tada (2021)
	Xenopus laevis (Pipidae: Anura)	NF 46	180	Feeding, growth and weight gain rates, biochemical responses, avoidance	Gold nanorods	72/12	Costa et al. (2021b)
		NF 45-46	210	Changes in growth, behavioural endpoints, neurotransmitters, antioxidant system and thyroid development	Metamifop	96	Liu et al. (2021)

[a] Gosner stage – GS (Gosner, 1960); Harrison stage – HS (Harrison, 1969); Nieuwkoop and Faber stage – NF (Nieuwkoop and Faber, 1994).

surrogates for amphibians in their aquatic phases. In that study, the authors compared data on lethality for the southern leopard frog and bored toad tadpoles relating to the contaminants: copper, carbaryl, pentachlorophenol (PCP), permethrin, and 4-nonylphenol. Similarly, at least in the short term, fish were found to be good surrogates for tadpoles when testing pesticides by Aldrich (2009). A study by Fryday and Thompson (2012) provided EFSA with information on the risk assessment of pesticides concerning amphibians. The first part of that study presented recommendations to use fish as surrogates for Anura and Caudata amphibians in toxicity tests. More recently, according to Weltje et al. (2013), the extrapolation of fish sensitivity to amphibians should be done with caution because some contaminants, such as the corticoesteroid hormone dexamethasone, are not detected in fish. Therefore, using fish as surrogates for amphibians for these types of chemicals may not be appropriate. This is due to specific interference with the biochemical pathways involved in the metamorphosis of amphibians. Also, some compounds like pyrethroids have been shown to affect fish and amphibians differently (Ortiz-Santaliestra et al., 2018; Glaberman et al., 2019). The herbicide atrazine also caused different effects on amphibians when compared to fish, namely by impairing metamorphoses, but studies have, so far, allowed no generalisations (Rohr and McCoy, 2010).

Even if, from the perspective of physiological functionality, tadpoles and fish could suffer from contamination in a very similar way, from the perspective of detection and avoidance of contamination, the different sensory mechanisms used by these groups might produce very different responses (Coombs and van Netten, 2005; Saccomanno et al., 2020). The sensory system of fish is generally made up of: (i) inner ears, mechanoreceptor and electroreceptor cells, forming the lateral acoustic system (touch, balance and hearing), (ii) the olfactory system (smell), (iii) the taste buds (taste) and (iv), the optical or visual system (vision) (Moorman, 2001). However, for the detection of contamination, the most important ones to be considered are smell and taste (Tierney, 2016). In their aquatic stages, amphibians depend heavily on their sensory capacity for the chemical detection of the environment (Troyer and Turner, 2015), smell is the most recurrent mechanism (mainly in view of the life stage at which amphibians are considered) (Duellman and Trueb, 1985). Due to the differences in the mechanisms used to perceive the environment, fish should not be considered a safe surrogate to predict the effects of contaminants on amphibians, especially if the avoidance response is to be assessed.

4. Defining avoidance: contamination-driven displacement of organisms

Avoidance response is considered here as the ability of organisms to detect contamination and flee towards a more favourable area. Therefore, an important step to measure this response (and which has contributed to increase its ecological relevance) is the use of a non-forced exposure scenarios, which is the main difference regarding traditional ecotoxicity assays. In these exposure scenarios organisms are able to move among the compartments with different levels of contamination, instead of being exposed exclusively to one concentration. The non-forced exposure approach has existed since the 1940s; when a bi-compartmental system was proposed by Jones (1947). Based on the same goal, many different systems have been proposed to test the ability of organisms to avoid contamination (see review by Jutfelt et al., 2017). An important advance in this approach occurred when the exposure vessel become a multi-compartmented system, making it possible to test many concentrations simultaneously. This has permitted the association of the avoidance response to specific concentrations along a gradient of contamination and to measure the magnitude (% of escape/avoidance) of that response (Lopes et al., 2004). Recently, a more versatile avoidance system (HeMHAS - Heterogeneous Multi-Habitat Assay System) with a two-dimensional design (3 × 6 compartments), able to simulate various scenarios of connections among the compartments, was developed by Araújo et al. (2018).

The avoidance response in a multi-compartmented scenario provides a shift in the paradigm of the exposure approach and the effects observed. When organisms flee from contamination, the potential of the chemical to repel organisms is assessed rather than any direct effects at the sub-individual or individual level (though the initial exposure, before fleeing, may also induce some effects within the organisms). This repellence might bring serious consequences for local populations, similar to the death of organisms (Moreira-Santos et al., 2008). When organisms move to other areas, because contamination makes ecosystems less attractive to inhabit, a decline in the local populations will occur. This response might bring serious consequences to the structure and functioning of the entire ecosystem (Ågerstrand et al., 2020).

Regarding the decline of populations, a relevant approach was presented by Rosa et al. (2008, 2012) whose aim was to verify how avoidance contributes to the downsizing of a population at the local scale. The authors presented the PID index (Population Immediate Decline) that integrates two responses able to cause a reduction in the populations; mortality and avoidance. In many cases, when a contaminant enters a habitat, some individuals are able to detect it and may escape to other areas; but others – the non-avoiders – may not be capable of detecting such contamination and may perish. Hypothetically, avoidance might play a more important role in the decline of the population than mortality, as the latter is only expected to occur at higher concentrations of the contaminant. The ability of tadpoles to avoid contamination and the potential role of avoidance in the decline of amphibian populations has been attested in some studies that are discussed in the next section.

4.1. Avoidance vs lethality in tadpoles

In ecotoxicological studies where mortality and avoidance were simultaneously assessed, it was noticeable that the avoidance response was shown to be more sensitive than mortality.

In the study by Araújo et al. (2014), the avoidance and lethality responses of the tadpoles of three anuran species (*Leptodactylus latrans*, *Lithobates catesbeianus* and *Pelophylax perezi*) exposed to copper were compared. The results showed that avoidance was verified at concentrations lower than those causing mortality: 50% of avoidance occurred at 100 $\mu g.L^{-1}$, while 20% mortality was only observed at 200 $\mu g.L^{-1}$. This result indicates that extinctions of local populations may occur in certain habitats, where such an event is not expected, owing to mortality.

In several studies, avoidance was shown to occur faster and at lower concentrations than mortality, as demonstrated by Vasconcelos et al. (2016) with *L. catesbeianus* GS 21 tadpoles exposed to the pesticide abamectin and by Moreira et al. (2019) with tadpoles of the same anuran species exposed to the herbicide diuron. Vasconcelos et al. (2016) reported a median lethal concentration (96-h LC_{50}) of 55 ± 4 $\mu g.L^{-1}$ and the median avoidance concentration (12-h AC_{50}) of 36 $\mu g.L^{-1}$ while Moreira et al. (2019) described a 96-h LC_{50} of 31 ± 3.7 $mg.L^{-1}$ and about 90% of individuals fleeing concentrations of 2.5–5 $mg.L^{-1}$, during the same exposure period (96 h). An even greater sensitivity of the avoidance response was found in *L. fuscus*, *L. catesbeianus* and *Physalaenus nattereri* tadpoles exposed to the herbicide 2,4-dichlorophenoxyacetic acid (Freitas et al., 2019). In view of the results of these lethality tests, the most sensitive species was *L. fuscus*, whose 96-h LC_{50} value was 28.81 ± 4.18 mgL^{-1}. In the avoidance tests, tadpoles showed a similar behaviour regardless of the species, with an avoidance of 50% of the population at concentrations of 242.5 $\mu g.L^{-1}$ (in a 21-d exposure); two orders of magnitude more sensitive than lethality. More recently, Girotto et al. (2020) exposed the amphibian species *L. catesbeianus* to mining tailings, using lethality and avoidance tests. Although no mortality was recorded in the lethality tests, for a 96-h exposure (even at 100% of a stock solution containing 50 $g.L^{-1}$ of mining tailings), avoidance occurred at the lowest concentrations (10, 25 and 50%;

decreasing with the increasing concentrations) for 16-d and 20-d exposures times.

Although the number of studies with tadpoles in which mortality (under forced exposure) and avoidance (non-forced exposure) have been simultaneously assessed is relatively small, all the evidence points to the higher sensitivity of avoidance when compared to mortality, also for other organisms (reviewed by Moreira-Santos et al., 2019).

5. Conclusions

(1) If test organisms are mobile and able to detect contamination, then avoidance tests, in a non-forced exposure approach, involve a much lower sensation of anxiety and pain than mortality tests, and therefore represent an alternative in accordance with the evolution of animal experimentation.
(2) The use of avoidance as an ecotoxicity endpoint is also important from an ecological perspective. Even at concentrations that do not cause the death of organisms, if they flee to adjacent habitats, the loss of amphibian diversity can be critical for the location avoided. This dynamic of contamination-driven migration allows us not only to study the environmental conditions that trigger avoidance, but also to include new concepts related to habitat colonisation. Thus, the role of contamination may now be seen from a broader spatial perspective, in which the connectivity among habitats should be considered.
(3) The avoidance/(re)colonisation approach makes it possible to integrate landscape ecology with ecotoxicology and study habitats not simply as isolated environmental compartments, but, instead, as part of a whole. This approach draws attention to a new perspective to the risk of contaminants related to the disturbance of spatial dynamics, which goes beyond merely considering classical toxic effects, at the physiological level.
(4) Another important point to be highlighted about avoidance testing is its feasibility and rapidity of results. Avoidance experiments usually take very short times of exposure, from 3 to 24 h, although more extended time periods may also be used.
(5) The present study proposes an alternative methodology for toxicity tests with tadpoles, making animal experimentation with amphibians more humane. Taking into account an evident need to perform toxicity tests on amphibians, for the purpose of pesticide toxicity assessment, the avoidance methodology proposed here potentially contributes to performing them in an ethically more effective way, reducing suffering and distress, as required by current legislation.

Author statement

All authors conceived the ideas, contributed in data collection and analysis of data, and participated in the development, drafting and editing of the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Acquaroni, M., Svartz, G., Coll, C.P., 2021. Developmental toxicity assessment of a chlorothalonil-based fungicide in a native amphibian species. Arch. Environ. Contam. Toxicol. 80, 680–690.
Ågerstrand, M., Arnold, K., Balshine, S., Brodin, T., Brooks, B.W., Maack, G., McCallum, E.S., Pyle, G., Saaristo, M., Ford, A.T., 2020. Emerging investigator series: use of behavioural endpoints in the regulation of chemicals. Environ. Sci. Process. Impacts 22, 49–65.
Agostini, M.G., Roesler, I., Bonetto, C., Ronco, A.E., Bilenca, D., 2020. Pesticides in the real world: the consequences of GMO-based intensive agriculture on native amphibians. Biol. Conserv. 241, 108355.
Aldrich, A.P., 2009. Empfindlichkeit von Amphibien gegenüber Pflanzenschutzmitteln. Agrarforschung 16, 466–471.
Alford, R.A., Richards, S.J., 1999. Global amphibian declines: a problem in applied ecology. Annu. Rev. Ecol. Systemat. 30, 133–165.
Araújo, C.V.M., Shinn, C., Moreira-Santos, M., Lopes, I., Espíndola, E.L.G., Ribeiro, R., 2014. Copper-driven avoidance and mortality in temperate and tropical tadpoles. Aquat. Toxicol. 146, 70–75.
Araújo, C.V.M., Moreira-Santos, M., Ribeiro, R., 2016. Active and passive spatial avoidance by aquatic organisms from environmental stressors: a complementary perspective and a critical review. Environ. Int. 92–93, 405–415.
Araújo, C.V.M., Roque, D., Blasco, J., Ribeiro, R., Moreira-Santos, M., Toribio, A., Aguirre, E., Barro, S., 2018. Stress-driven emigration in complex field scenarios of habitat disturbance: the heterogeneous multi-habitat assay system (HeMHAS). Sci. Total Environ. 644, 31–36.
Arcaute, C.R., Brodeur, J.C., Soloneski, S., Larramendy, M.L., 2020. Toxicity to *Rhinella arenarum* tadpoles (Anura, Bufonidae) of herbicide mixtures commonly used to treat fallow containing resistant weeds: glyphosate-dicamba and glyphosate-flurochloridone. Chemosphere 245, 125623.
Asparch, Y., Svartz, G., Coll, C.P., 2020. Toxicity characterization and environmental risk assessment of Mancozeb on the South American common toad *Rhinella arenarum*. Environ. Sci. Pollut. Res. 27, 3034–3042.
Attademo, A.M., Lajmanovich, R.C., Peltzer, P.M., Boccioni, A.P.C., Martinuzzi, C., Simonielo, F., Repetti, M.R., 2021. Effects of the emulsifiable herbicide Dicamba on amphibian tadpoles: an underestimated toxicity risk? Environ. Sci. Pollut. Res. 28, 31962–31974.
AWA, 1966. Animal Welfare Act. National Agricultural Library – US Department of Agriculture. Consulted on 24/04/2020. https://www.nal.usda.gov/awic/animal-welfare-act.
Bhuyan, K., Patar, A., Singha, U., Giri, S., Giri, A., 2020. Phenanthrene alters oxidative stress parameters in tadpoles of *Euphlyctis cyanophlyctis* (Anura, Dicroglossidae) and induces genotoxicity assessed by micronucleus and comet assay. Environ. Sci. Pollut. Res. 27, 20962–20971.
Bordes, E.C., 2005. Animal protection legislation in The Netherlands: past and present. In: Freek de Jonge, Van den Bos, R. (Eds.), The Human-Animal Relationship: Forever and a Day, first ed. (Gorcum b.v., Koninklijke Van).
Bridges, C.M., Dwyer, F.J., Hardesty, D.K., Whites, D.W., 2002. Comparative contaminant toxicity: are amphibian larvae more sensitive than fish? Environ. Toxicol. Chem. 69, 562–569.
Carvalho, W.F., Arcaute, C.R., Pérez-Iglesias, J.M., Laborde, M.R.R., Soloneski, S., Larramendy, M.L., 2019. DNA damage exerted by mixtures of commercial formulations of glyphosate and imazethapyr herbicides in *Rhinella arenarum* (Anura, Bufonidae) tadpoles. Ecotoxicology 28, 367–377.
Coombs, S., van Netten, S., 2005. The hydrodynamics and structural mechanics of the lateral line system. Fish Physiol. 23, 103–139.
Costa, C.S., Rimoldi, F., Saralegui, M.J.P., Puzzo, M.L.R., Trudeau, V.L., Natale, G.S., 2021a. Disruptive effects of chlorpyrifos on predator-prey interactions of *Ceratophrys ornata* tadpoles: consequences at the population level using computational modeling. Environ. Pollut. 285, 117344.
Costa, B., Quintaneiro, C., Daniel-da-Silva, A.L., Trindade, T., Soares, A.M.V.M., Lopes, I., 2021b. An integrated approach to assess the sublethal effects of colloidal gold nanorods in tadpoles of *Xenopus laevis*. J. Hazard Mater. 400, 123237.
Curi, L.M., Peltzer, P.M., Sandoval, M.T., Lajmanovich, R.C., 2019. Acute toxicity and sublethal effects caused by a commercial herbicide formulated with 2,4-D on *Physalaemus albonotatus* tadpoles. Water Air Soil Pollut. 230, 22.
Daam, M.A., Moutinho, M.F., Espíndola, E.L.G., Schiesari, L., 2019. Lethal toxicity of the herbicides acetochlor, ametryn, glyphosate and metribuzin to tropical frog larvae. Ecotoxicology 28, 707–715.

DGOA, 2014. Guidance on the operation of the animals (scientific procedures) Act 1986. United Kingdom home office. https://www.gov.uk/guidance/research-and-testing-using-animals#history.
Doke, S.K., Dhawale, S.C., 2015. Alternatives to animal testing: a review. Saudi Pharmaceut. J. 23, 223–229.
Duellman, W.E., Trueb, L., 1985. Biology of Amphibians. Mc Graw-Hill, New York.
EEC, 1986. European Economic Community. Council directive on the approximation of laws, regulations and administrative provisions of member states regarding the protection of animals used for experimental and other scientific purposes. Off. J. Eur. Commun. No. L 358, 18. 12. 86).
EFSA, 2007. European Food safety authority. Opinion of the scientific Panel on plant protection products and their Residues on a request from the commission related to the revision of annexes II and III to Council directive 91/414/EEC concerning the placing of plant protection products on the market – ecotoxicological studies. European Food safety authority. EFSA J. 5 (3), 44–461.
EU, 2010. European Union. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes. Official Journal of the European Union No. L 276/33 (20. 10. 2010.
Flynn, R.W., Chislock, M.F., Gannon, M.E., Bauer, S.J., Tornabene, B.J., Hoverman, J.T., Sepúlveda, M.S., 2019. Acute and chronic effects of perfluoroalkyl substance mixtures on larval American bullfrogs (*Rana catesbeiana*). Chemosphere 236, 124350.
Freitas, J.S., Girotto, L., Goulart, B.V., Alho, L.O.G., Gebara, R.C., Montagner, C.C., Schiesari, L., Espíndola, E.L.G., 2019. Effects of 2,4-D-based herbicide (DMA® 806) on sensitivity, respiration rates, energy reserves and behavior of tadpoles. Ecotoxicol. Environ. Saf. 182, 109446.
Fryday, S., Thompson, H., 2012. Toxicity of Pesticides to Aquatic and Terrestrial Life Stages of Amphibians and Occurrence, Habitat Use and Exposure of Amphibian Species in Agricultural Environments. EFSA Supporting Publications. EN-343.
Garthoff, B., 2005. Alternatives to animal experimentation: the regulatory background. Toxicol. Appl. Pharmacol. 207, S388–S392.
Girotto, L., Espíndola, E.L.G., Gebara, R.C., Freitas, J.S., 2020. Acute and chronic Effects on tadpoles (*Lithobates catesbeianus*) exposed to mining tailings from the dam rupture in Mariana, MG (Brazil). Water Air Soil Pollut. 231, 325.
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.
Green, F.B., Salice, C.J., 2020. Increased temperature and lower resource quality exacerbate chloride toxicity to larval *Lithobates sylvaticus* (wood frog). Environ. Pollut. 266, 115188.
Green, F.B., East, A.G., Salice, C.J., 2019. Will temperature increases associated with climate change potentiate toxicity of environmentally relevant concentrations of chloride on larval green frogs (*Lithobates clamitans*)? Sci. Total Environ. 682, 282–290.
Guerrini, A., 2003. Experimenting with Humans and Animals: from Galen to Animal Rights. Johns Hopkins University Press, Baltimore, MD, p. 165.
Gust, K.A., Indest, K.J., Lotufo, G., Everman, S.J., Jung, C.M., Ballentine, M.L., Hoke, A. V., Sowe, B., Gautam, A., Hammamieh, R., Ji, Q., Barker, N.D., 2021. Genomic investigations of acute munitions exposures on the health and skin microbiome composition of leopard frog (*Rana pipiens*) tadpoles. Environ. Res. 192, 110245.
Harrison, R., 1969. Harrison Stage and Description of Normal Development of the Spotted Salamander, *Ambystoma Punctatum*. Yale University Press, New Haven.
Hellou, J., 2011. Behavioural ecotoxicology, an "early warning" signal to assess environmental quality. Environ. Sci. Pollut. Res. 18, 1–11.
Herek, J.S., Vargas, L., Trindade, S.A.R., Rutkoski, C.F., Macagnan, N., Hartmann, P.A., Hartmann, M.T., 2020. Can environmental concentrations of glyphosate affect survival and cause malformation in amphibians? Effects from a glyphosate-based herbicide on *Physalaemus cuvieri* and *P. gracilis* (Anura: Leptodactylidae). Environ. Sci. Pollut. Res. 27, 22619–22630.
Holtswarth, J.N., Rowland, F.E., Puglis, H.J., Hladik, M.L., Webb, E.B., 2019. Effects of the neonicotinoid insecticide clothianidin on southern Leopard Frog (*Rana sphenocephala*) tadpole behavior. Bull. Environ. Contam. Toxicol. 103, 717–722.
Hu, Y.C., Tang, Y., Chen, Z.Q., Chen, J.Y., Ding, G.H., 2019. Evaluation of the sensitivity of *Microhyla fissipes* tadpoles to aqueous cadmium. Ecotoxicology 28, 1150–1159.
Jones, J.R.E., 1947. The reaction of *Pygosteus pungitius* L. to toxic solutions. J. Exp. Biol. 24, 110–122.
Jutfelt, F., Sundin, J., Raby, G.D., Krang, A.-S., Clark, T.D., 2017. Two-current choice flumes for testing avoidance and preference in aquatic animals. Methods Ecol. Evol. 8, 379–390.
Lajmanovich, R.C., Peltzer, P.M., Martinuzzi, C.S., Attademo, A.M., Colussi, C.L., Bassó, A., 2018. Acute toxicity of colloidal silicon dioxide nanoparticles on amphibian larvae: emerging environmental concern. Int. J. Environ. Res. 12, 269–278.
Lajmanovich, R.C., Peltzer, P.M., Martinuzzi, C.S., Attademo, A.M., Bassó, A., Colussi, C. L., 2019a. Insecticide pyriproxyfen (Dragón®) damage biotransformation, thyroid hormones, heart rate, and swimming performance of *Odontophrynus americanus* tadpoles. Chemosphere 220, 714–722.
Lajmanovich, R.C., Peltzer, P.M., Attademo, A.M., Martinuzzi, C.S., Simoniello, M.F., Colussi, C.L., Boccioni, A.P.C., Sigrist, M., 2019b. First evaluation of novel potential synergistic effects of glyphosate and arsenic mixture on *Rhinella arenarum* (Anura: Bufonidae) tadpoles. Heliyon 5, 02601.
Liu, R., Qin, Y., Diao, J., Zhang, H., 2021. *Xenopus laevis* tadpoles exposed to metamifop: changes in growth, behavioral endpoints, neurotransmitters, antioxidant system and thyroid development. Ecotoxicol. Environ. Saf. 220, 112417.
Lopes, I., Baird, D.J., Ribeiro, R., 2004. Avoidance of copper contamination by field populations of *Daphnia longispina*. Environ. Toxicol. Chem. 23, 1702–1708.
Luz, T.M., Araújo, A.P.C., Estrela, F.N., Braz, H.L.B., Jorge, R.J.B., Charlie-Silva, I., Malafaia, G., 2021. Can use of hydroxychloroquine and azithromycin as a treatment of COVID-19 affect aquatic wildlife? A study conducted with neotropical tadpole. Sci. Total Environ. 780, 146553.
Martinuzzi, C.S., Attademo, A.M., Peltzer, P.M., Loughlin, T.M.M., Marino, D.J.G., Lajmanovich, R.C., 2020. Comparative toxicity of two different dimethoate formulations in the common toad (*Rhinella arenarum*) tadpoles. Bull. Environ. Contam. Toxicol. 104, 35–40.
McCallum, M.L., 2007. Amphibian decline or extinction? Current declines dwarf background extinction rate. J. Herpetol. 41, 483–491.
Moorman, J.S., 2001. Development of sensory systems in Zebrafish (*Danio rerio*). ILAR J. 42, 15–21.
Moreira, R.A., Freitas, J.S., Pinto, T.J.S., Schiesari, L., Daam, M.A., Montagner, C.C., Goulart, B.V., Espindola, E.L.G., 2019. Mortality, spatial avoidance and swimming behavior of bullfrog tadpoles (*Lithobates catesbeianus*) exposed to the herbicide diuron. Water Air Soil Pollut. 230, 125.
Moreira-Santos, M., Donato, C., Lopes, I., Ribeiro, R., 2008. Avoidance tests with small fish: determination of the median avoidance concentration and of the lowest-observed-effect gradient. Environ. Toxicol. Chem. 27, 1576–1582.
Moreira-Santos, M., Ribeiro, R., Araújo, C.V.M., 2019. What if aquatic animals move away from pesticide-contaminated habitats before suffering adverse physiological effects? A critical review. Crit. Rev. Environ. Sci. Technol. 49, 989–1025.
Moreton, M.L., Marlatt, V.L., 2019. Toxicity of the aquatic herbicide, reward, to the north western salamander. Environ. Sci. Pollut. Control Ser. 26, 31077–31085.
Motta, A.G.C., do Amaral, D.F., Benvindo-Souza, M., Rocha, T.L., de Melo e Silva, D., 2020. Genotoxic and mutagenic effects of zinc oxide nanoparticles and zinc chloride on tadpoles of *Lithobates catesbeianus* (Anura: Ranidae). Environ. Nanotechnol. Monit. Manag. 14, 100356.
Moutinho, M.F., Almeida, E.A., Espíndola, E.L.G., Daam, M.A., Schiesari, L., 2020. Herbicides employed in sugarcane plantations have lethal and sublethal effects to larval *Boana pardalis* (Amphibia, Hylidae). Ecotoxicology 29, 1043–1051.
Nascimento, I.F., Guimarães, A.T.B., Ribeiro, F., Rofrigues, A.S.L., Estrela, F.N., Luz, T. M., Malafaia, G., 2021. Polyethylene glycol acute and sub-lethal toxicity in neotropical *Physalaemus cuvieri* tadpoles (Anura, Leptodactylidae). Environ. Pollut. 283, 117054.
Natale, G.S., Vera-Candioti, J., Ruiz de Arcaute, C., Soloneski, S., Larramendy, M.L., Ronco, A.E., 2018. Lethal and sublethal effects of the pirimicarb-based formulation Aficida® on *Boana pulchella* (Duméril and Bibron, 1841) tadpoles (Anura, Hylidae). Ecotoxicol. Environ. Saf. 147, 471–479.
NC3Rs, 2009. National Centre for the replacement, refinement and reduction of animals in research. The challenge of animal research. https://www.parsemus.org/wp-content/uploads/2012/11/NC3Rs_leaflet_2009.pdf.
Nieuwkoop, P.D., Faber, J., 1994. Normal Table of *Xenopus laevis* (Daudin): A Systematical and Chronological Survey of the Development from the Fertilized Egg till the End of Metamorphosis. Garland, New York.
Ojha, S., Roy, A., Mohapatra, A.K., 2021. Environmentally relevant concentrations of cadmium impair morpho-physiological development and metamorphosis in *Polypedates maculatus* (Anura, Rhacophoridae) tadpoles. Environ. Chem. Ecotoxicol. 3, 133–141.
Oliveira, C.R., Garcia, T.D., Franco-Belussi, L., Salla, R.F., Souza, B.F.S., Melo, N.F.S., Irazusta, S.P., Jones-Costa, M., Silva-Zacarin, E.C.M., Fraceto, L.F., 2019. Pyrethrum extract encapsulated in nanoparticles: toxicity studies based on genotoxic and hematological effects in bullfrog tadpoles. Environ. Pollut. 253, 1009–1020.
Ortiz-Santaliestra, M.E., Maia, J.P., Egea-Serrano, A., Brühl, C.A., Lopes, I., 2017. Biological Relevance of the Magnitude of Effects (Considering Mortality, Sub-lethal and Reproductive Effects) Observed in Studies with Amphibians and Reptiles in View of Population Level Impacts on Amphibians and Reptiles. EFSA Supporting Publications. EN-12512017.
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.
Pal, S., Samanta, P., Kole, D., Mukherjee, A.K., Ghosh, A.R., 2018. Acute toxicity and oxidative stress responses in tadpole of skittering frog, *Euphlyctis cyanophlyctis* (Schneider, 1799) to sodium fluoride exposure. Bull. Environ. Contam. Toxicol. 100, 202–207.
Pankevich, D.E., Wizemann, T.M., Mazza, A.M., Altevogt, B.M., 2012. International Animal Research Regulations: Impact on Neuroscience Research. The National Academies Press, Washington DC.
Pauli, B.D., Perrault, J.A., Money, S.L., 2000. RATL: A Database of Reptile and Amphibian Toxicology Literature. Technical Report Series Nº 357. Canadian Wildlife Service, Environment Canada.
Pérez-Iglesias, J.M., Nataleb, G.S., Soloneskia, S., Larramendy, M.L., 2018a. Are the damaging effects induced by the imazethapyr formulation Pivot® H in *Boana pulchella* (Anura) reversible upon ceasing exposure? Ecotoxicol. Environ. Saf. 148, 1–10.
Pérez-Iglesias, J.M., Franco-Belussi, L., Natale, G.S., de Oliveira, C., 2018b. Biomarkers at different levels of organisation after atrazine formulation (SIPTRAN 500SC®) exposure in *Rhinella schineideri* (Anura: Bufonidae) Neotropical tadpoles. Environ. Pollut. 244, 733–746.
Pérez-Iglesias, J.M., Brodeur, J.C., Larramendy, M.L., 2020. An imazethapyr-based herbicide formulation induces genotoxic, biochemical, and individual organizational effects in *Leptodactylus latinasus* tadpoles (Anura: Leptodactylidae). Environ. Sci. Pollut. Res. 27, 2131–2143.

Power, T., Clark, K.L., Harfenist, A., Peakall, D.B., 1989. A Review and Evaluation of the Amphibian Toxicological Literature. Technical Report No. 61. Canadian Wildlife Service, Headquarters, Ottawa, ON, Canada.

EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues), Ockleford, C., Adriaanse, P., Berny, P., Brock, T., Duqyesne, S., Grilli, S., Hernandez-Jerez, A.F., Bennekou, S.H., Klein, M., Khul, T., Laskowski, R., Machera, K., Pelkonen, O., Pieper, S., Stemmer, M., Sundh, I., Teodorovic, I., Tiktak, A., Topping, C.J., Wolterink, G., Aldrich, A., Berg, C., Ortiz-Santaliestra, M., Weir, S., Streissl, F., Smith, R.H., 2018. Scientific Opinion on the state of the science on pesticide risk assessment for amphibians and reptiles. EFSA J. 16 (2), 301–5125.

Quiroga, L.B., Sanabria, E.A., Fornés, M.W., Bustos, D.A., Tejedo, M., 2019. Sublethal concentrations of chlorpyrifos induce changes in the thermal sensitivity and tolerance of anuran tadpoles in the toad *Rhinella arenarum*? Chemosphere 219, 671–677.

Rai, J., Kaushik, K., 2018. Reduction of animal sacrifice in biomedical science & research through alternative design of animal experiments. Saudi Pharmaceut. J. 26, 896–902.

Rogers, K., 2007. Scientific Alternatives to Animal Testing: A Progress Report," britannica.Com. Sep. 17.

Rohr, J.R., McCoy, K.A., 2010. A Qualitative meta-analysis reveals consistent effects of atrazine on freshwater fish and amphibians. Environ. Health Perspect. 118, 20–32.

Romero, A.L.N., Moratta, M.A.H., Rodríguez, M.R., Quiroga, L.B., Echegaray, M., Sanabria, E.A., 2019. Toxicity of wine effluents and assessment of a depuration system for their control: assay with tadpoles of *Rhinella arenarum* (Bufonidae). Ecotoxicology 28, 48–61.

Rosa, R., Moreira-Santos, M., Lopes, I., Picado, A., Mendonça, F., Ribeiro, R., 2008. Development and sensitivity of a 12-h laboratory test with *Daphnia magna* Straus based on avoidance of pulp mill effluents. Bull. Environ. Contam. Toxicol. 81, 464–469.

Rosa, R., Materatski, P., Moreira-Santos, M., Sousa, J.P., Ribeiro, R., 2012. A scaled-up system to evaluate zooplankton spatial avoidance and population immediate decline concentration. Environ. Toxicol. Chem. 31, 1301–1305.

Rutkoski, C.F., Macagnan, N., Kolcenti, C., Vanzetto, G.V., Sturza, P.F., Hartmann, P.A., Hartmann, M.T., 2018. Lethal and sublethal effects of the herbicide atrazine in the early stages of development of *Physalaemus gracilis* (Anura: Leptodactylidae). Arch. Environ. Contam. Toxicol. 74, 587–593.

Rutkoski, C.F., Macagnan, N., Folador, A., Skovronski, V.J., do Amaral, A.M.B., Leitemperger, J., Dorneles, M., Hartmann, P.A., Müller, C., Loro, V.L., Hartmann, M. T., 2020a. Morphological and biochemical traits and mortality in *Physalaemus gracilis* (Anura: Leptodactylidae) tadpoles exposed to the insecticide chlorpyrifos. Chemosphere 250, 126162.

Rutkoski, C.F., Macagnan, N., Folador, A., Skovronski, V.J., do Amaral, A.M.B., Leitemperger, J.W., Costa, M.D., Hartmann, P.A., Müller, C., Loro, V.L., Hartmann, T., 2020b. Cypermethrin- and fipronil-based insecticides cause biochemical changes in *Physalaemus gracilis* tadpoles. Environ. Sci. Pollut. Res. 28, 4377–4387.

Saccomanno, V., Love, H., Sylvester, A., Li, W., 2020. The early development and physiology of *Xenopus laevis* tadpole lateral line system. Cold Spring Harbor Lab. BioRxiv.

Saenphet, K., Saenphet, S., Intamong, J., Nakas, T., Buncharoen, W., 2018. Acute toxicity and histopathological changes in livers of frog tadpoles (*Hoplobatrachus rugulosus*) exposed to bioinsecticides derived from *Azadirachta indica* A. Juss., *Stemona curtisii* Hook.F., and *Mammea siamensis*. Comp. Clin. Pathol. 27, 939–946.

Saka, M., Tada, N., 2021. Acute and chronic toxicity tests of systemic insecticides, four neonicotinoids and fipronil, using the tadpoles of the western clawed frog *Silurana tropicalis*. Chemosphere 270, 129418.

Saka, M., Tada, N., Kamata, Y., 2018. Chronic toxicity of 1,3,5-triazine herbicides in the postembryonic development of the western clawed frog *Silurana tropicalis*. Ecotoxicol. Environ. Saf. 147, 373–381.

Salgado Costa, C., Ronco, A.E., Trudeau, V.L., Marino, D., Natale, G.S., 2018. Tadpoles of the horned frog *Ceratophrys ornata* exhibit high sensitivity to chlorpyrifos for conventional ecotoxicological and novel bioacoustics variables. Environ. Pollut. 235, 938–947.

Samanta, P., Pal, S., Mukherjee, A.K., Ghosh, A.R., 2020. Acute toxicity assessment of arsenic, chromium and almix 20WP in *Euphlyctis cyanophlyctis* tadpoles. Ecotoxicol. Environ. Saf. 191, 110209.

Sansiñenaa, J.A., Pelusoa, L., Salgado Costa, C., Demetrio, P.M., Loughlina, T.M.M., Marinoa, D.J.G., Alcaldeb, L., Natale, G.S., 2018. Evaluation of the toxicity of the sediments from an agroecosystem to two native species, *Hyalella curvispina* (CRUSTACEA: AMPHIPODA) and *Boana pulchella* (AMPHIBIA: ANURA), as potential environmental indicators. Ecol. Indicat. 93, 100–110.

Sievers, M., Hale, R., Swearer, S.E., Parris, K.M., 2018. Contaminant mixtures interact to impair predator-avoidance behaviours and survival in a larval amphibian. Ecotoxicol. Environ. Saf. 161, 482–488.

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.

Svartz, G., Aronzon, C., Pérez Catán, S., Soloneski, S., Pérez Coll, C., 2020. Oxidative stress and genotoxicity in *Rhinella arenarum* (Anura: Bufonidae) tadpoles after acute exposure to Ni-Al nanoceramics. Environ. Toxicol. Pharmacol. 80, 103508.

Tierney, K.B., 2016. Chemical avoidance responses of fish. Aquat. Toxicol. 174, 228–241.

Troyer, R.R., Turner, A.M., 2015. Chemosensory perception of predators by larval amphibians depends on water quality. PLoS One 10, e0131516.

Turhan, D.O., Güngördü, A., Ozmen, M., 2020. Developmental and lethal effects of glyphosate and a glyphosate-based product on *Xenopus laevis* embryos and tadpoles. Bull. Environ. Contam. Toxicol. 104, 173–179.

UNESCO, 1978. Universal Declaration of Animal Rights. Solemnly Proclaimed in Paris on 15 October 1978 at the UNESCO Headquarters. The United Nations Educational, Scientific and Cultural Organization, Paris France.

Van Norman, M.D.G.A., 2019. Limitations of animal studies for predicting toxicity in clinical trials. In: Is it time to rethink our current approach? College of Cardiology Foundation, 4 nº 7.

Vasconcelos, A.M., Daam, M.A., dos Santos, L.R.A., Sanches, A.L.M., Araújo, C.V.M., Espíndola, E.L.G., 2016. Acute and chronic sensitivity, avoidance behavior and sensitive life stages of bullfrog tadpoles exposed to the biopesticide abamectin. Ecotoxicology 25, 500–509.

Venâncio, C., Castro, B.B., Ribeiro, R., Antunes, S.C., Lopes, I., 2019. Sensitivity to salinization and acclimation potential of amphibian (*Pelophylax perezi*) and fish (*Lepomis gibbosus*) models. Ecotoxicol. Environ. Saf. 172, 348–355.

Viriato, C., França, F.M., Santos, D.S., Marcantonio, A.S., Badaró-Pedroso, C., Ferreira, C. M., 2021. Evaluation of the potential teratogenic and toxic effect of the herbicide 2,4-D (DMA® 806) in bullfrog embryos and tadpoles (*Lithobates catesbeianus*). Chemosphere 266, 129018.

Wake, D.B., 1991. Declining amphibian populations. Science 253, 860.

Weeks, D.M., Parris, M.J., 2020. A *Bacillus thuringiensis kurstaki* biopesticide does not reduce hatching success or tadpole survival at environmentally relevant concentrations in southern leopard frogs (*Lithobates sphenocephalus*). Environ. Toxicol. Chem. 39, 155–161.

Weir, S.M., Yu, S., Scott, D.E., Lance, S.L., 2019. Acute toxicity of copper to the larval stage of three species of ambystomatid salamanders. Ecotoxicology 28, 1023–1031.

Weltje, L., Simpson, P., Gross, M., Crane, M., Wheeler, J.R., 2013. Comparative acute and chronic sensitivity of fish and amphibians: a critical review data. Environ. Toxicol. Chem. 32, 984–994.

Wrubleswski, J., Reichert Jr., F.W., Galon, L., Hartmann, P.A., Hartmann, M.T., 2018. Acute and chronic toxicity of pesticides on tadpoles of *Physalaemus cuvieri* (Anura, Leptodactylidae). Ecotoxicology 27, 360–368.

Zhang, W., Cheng, C., Chen, L., Deng, Y., Zhang, L., Li, Y., Qin, Y., Diao, J., Zhou, Z., 2018a. Enantioselective toxic effects of cyproconazole enantiomers against *Rana nigromaculata*. Environ. Pollut. 243, 1825–1832.

Zhang, W., Luc, Y., Huang, L., Cheng, C., Di, S., Chen, L., Zhou, Z., Diao, J., 2018b. Comparison of triadimefon and its metabolite on acute toxicity and chronic effects during the early development of *Rana nigromaculata* tadpoles. Ecotoxicol. Environ. Saf. 156, 247–254.