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RESEARCH ARTICLE

Acute, chronic and biochemical effects of chlorothalonil on *Agalychnis callidryas*, *Isthmohyla pseudopuma* and *Smilisca baudinii* tadpoles

Michael Méndez[1] · **Priscilla Obando**[1] · **Margaret Pinnock-Branford**[1] ·
Clemens Ruepert[1] · **Luisa E. Castillo**[1] · **Freylan Mena**[1] iD · **Gilbert Alvarado**[2,3,4]

Abstract Declines of amphibian populations have been a worldwide issue of concern for the scientific community during the last several decades. Efforts are being carried out to elucidate factors related to this phenomenon. Among these factors, pathogens, climate change, and environmental pollution have been suggested as possible causes. Regarding environmental pollutants, some pesticides are persistent in the environment and capable of being transported long distances from their release point. In Costa Rica, some pesticides have been detected in protected areas, at locations where amphibian populations have declined. Information about toxicity of pesticides used in Costa Rican agriculture to amphibians is still scarce, particularly for native species.

Toxicity tests with chlorothalonil, a fungicide intensively used in Costa Rica, were carried out exposing tadpoles of three Costa Rican native species: *Agalychnis callidryas*, *Isthmohyla pseudopuma*, and *Smilisca baudinii* in order to evaluate acute and chronic toxicity as well as the biomarkers

cholinesterase activity (ChE), glutathione-S transferase activity (GST), and lipid peroxidation (LPO).

96-h LC_{50}: 26.6 (18.9–35.8) μg/L to *A. callidryas*, 25.5 (21.3–29.7) μg/L to *I pseudopuma* and 32.3 (26.3–39.7) μg/L to *S. baudinii* were determined for chlorothalonil. These three species of anurans are among the most sensitive to chlorothalonil according to the literature. Besides, GST was induced in *S. baudinii* after exposure to sub-lethal concentrations of chlorothalonil while evisceration occurred in *S. baudinii* and *A. callidryas* tadpoles exposed to lethal concentrations of the fungicide. Chronic exposure to sub-lethal concentrations accelerated development in *S. baudinii* and caused lesions in tail of *S. baudinii* and *I. pseudopuma* tadpoles. Our results demonstrate that chlorothalonil is highly toxic to native amphibian species and that low concentrations can cause biochemical responses related to phase II of biotransformation and effects on development.

Responsible editor: Thomas Braunbeck

✉ Michael Méndez
  michael586@gmail.com

✉ Freylan Mena
  fmena@una.cr

1 Central American Institute for Studies on Toxic Substances/Instituto Regional de Estudios en Sustancias Toxicas (IRET), Universidad Nacional, Heredia, Costa Rica

2 School of Biology, Universidad de Costa Rica, San Pedro, Costa Rica

3 Centro de Investigación en Estructuras Microscópicas (CIEMIC), Universidad de Costa Rica, San Pedro, Costa Rica

4 Department of Pathology, Laboratory of Comparative Wildlife Pathology (LAPCOM), School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil

Introduction

Declines of amphibian populations have been a worldwide issue of concern for the scientific community during the last several decades (Pounds and Crump 1994; Alford and Richards 1999; Lips et al. 2005; Mendelson et al. 2006; Whitfield et al. 2007; Blaustein et al. 2011). Several factors have been suggested as possible causes for these population declines; among them pathogens, specifically the infection caused by *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al. 1998; Lips et al. 2006; Gillespie et al. 2015), climate change (Pounds 2001; Li et al. 2013), habitat loss and fragmentation (Becker et al. 2008), invasive species (Adams

1999; Kats and Ferrer 2003), environmental pollution (Sparling et al. 2001) and the interaction of these factors (Collins and Storfer 2003; Rohr and Palmer 2013).

Costa Rica, with only 51,100 km^2 of terrestrial and 568,000 km^2 marine territories, is among the 20 most biodiverse countries in the word, almost 100 thousand species have been described for the country and it represents 4.75 % of world's biodiversity (SINAC 2009). As of 2009, 192 amphibian species have been described in Costa Rica, making this among the top 13 most species-rich countries for amphibians (Bolaños 2009). Of those amphibian species described, 63 (33 %) are considered from vulnerable to extinct (2) according to IUCN red list. One of the extinct species is the emblematic golden toad (*Incilius periglenes*) which was endemic in Monteverde's cloud forest, Costa Rica (IUCN 2015).

Despite of the rich biodiversity, Costa Rican ecosystems face threats caused by human activities like other regions. Regarding amphibian populations, these pressures include global climate change, habitat loss due to land use conversion to productive or urbanistic activities, pollution, invasive species and diseases (Lips et al. 2005; Whitfield et al. 2009). In a country where agriculture is the main economic activity, pesticides are one of the major risks regarding chemical pollution. Furthermore, the use of pesticides in local agriculture is high compared to developed and even other developing countries and some compounds forbidden in many other places are used here (de la Cruz et al. 2014).

Pesticides are considered among the environmental pollutants related to amphibian declines. Pesticides can cause organism mortalities but also, even at low environmental concentrations they might have negative effects on amphibian's growth and development (Hayes et al. 2006). Besides, some of these compounds are persistent in the environment and capable of being transported long distances from their point of release. In Costa Rica, some pesticides used in agriculture have been detected in air and fog samples collected from protected areas, far from their site of use and at places where amphibian populations have declined (Daly et al. 2007; Shunthirasingham et al. 2011). Information about effects of pesticides and other environmental pollutants on neotropical amphibian species is scarce and necessary. Especially as atmospheric drift might transport pollutants from croplands to protected areas.

The risk posed by fungicides to amphibian populations should be considered as it is one of the most imported and used groups of pesticides in Costa Rica (de la Cruz et al. 2014). Specifically, chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) is applied in banana, melon, coffee, chayote and other fruit and vegetable crops, along the country (Bravo et al. 2013). Chlorothalonil is a multi-site contact fungicide which causes toxicity by complexing with sulphydryl containing proteins, affecting glutathione reserves. Aside from fungi, this thiol-related mechanism has also been described in fish and aquatic invertebrates (Elskus 2012). Chlorothalonil's toxicity is considered

high for amphibians, fish, and aquatic invertebrates (de la Cruz et al. 2014; Van Scoy and Tjeerdema 2014); acute effects are expected on fish and invertebrates with environmental concentrations above 5.25 and 1.8 μg/L respectively while chronic effects are expected above 3 and 0.6 μg/L respectively for those groups (Elskus 2012). Chlorothalonil's half-life in aquatic environments goes from 2 h to 8 days (Grabuski et al. 2004). In Costa Rica, concentrations as high as 11 μg/L have been documented in surface water samples (Castillo et al. 2000). Daly et al. (2007) reported presence of chlorothalonil in air and soil samples from different sites in Costa Rica, the higher concentrations measured were 17.3 ng/m^3 in air and 1.06 ng/g in soil, both in samples collected at the central area of the country.

Evaluation of the effects of this pollutant on amphibians is necessary in order to suggest and eventually establish protective limits to its use. For toxicity assays with amphibians, early life stages (embryo and larvae) are frequently used; several endpoints can be evaluated in these tests, among them, survival, growth, development, behavior, deformities and several biochemical biomarkers (Sparling et al. 2010). Information on acute and chronic toxicity as well as early warning physiological responses can be obtained. Typically, ecotoxicological information for amphibians is obtained using a few surrogates, well known and laboratory "adapted" species. This might pose an inconvenience as life history and other important ecological traits vary greatly among amphibians and it might affect the way they are exposed to pollutants (Sparling et al. 2010). In this regard, evaluating the toxicity of chlorothalonil on tropical species would contribute to build up information regarding the effects of this relevant pesticide.

In this work, we aimed to undertake the evaluation of one of the factors suspected to interact in the complex causing amphibian population declines: the toxicity and other effects caused by environmental pollutants. Specifically, we evaluated effects caused by chlorothalonil, a fungicide used in Costa Rican agriculture, to tadpoles of three native anurans species.

Material and methods

Toxicity of chlorothalonil was evaluated on tadpoles of three Costa Rican native species: *Agalychnis callidryas*, *Isthmohyla pseudopuma*, and *Smilisca baudinii*. The three species are listed as Least Concern by the IUCN (IUCN 2015). All the tests were carried out at the Laboratory for Ecotoxicology Studies (ECOTOX) of the Central American Institute of Studies on Toxic Substances (IRET) at the Universidad Nacional (UNA) in Heredia.

Tadpole collection and maintenance

All tadpoles were acclimatized to laboratory conditions during at least 1 week before any assay, they were fed ad libitum,

three times per week with TetraVeggie spirulina-enhanced flakes (Tetra®) and water was 50 % renewed weekly. Collection of eggs or tadpoles of each species was carried out according to their reproductive habits.

A. callidryas

Eight egg masses were collected from vegetation surrounding a pond located outside Tirimbina Rainforest Center in Sarapiqui. Egg masses were transported in plastic containers with water from the pond and at the ECOTOX laboratory they were placed in aquariums with a shallow amount of carbon filtered, UV treated tapwater (UV water). Leaves containing the eggs were held with adhesive tape to aquarium walls just above the water surface, until they hatched and the tadpoles fell into the water. All *A. callidryas* tests were started using tadpoles on stages 26–27 (Gosner 1960).

I. pseudopuma

Four egg masses were collected from temporary or permanent natural ponds located in mountain area of San Rafael in Heredia. Organisms were transported in plastic container with site water to the laboratory where they were treated as described for *A. callidryas*. Toxicity tests were started with tadpoles on stages 25–27 (Gosner 1960).

S. baudinii

Two hundred recently hatched tadpoles were collected from the Palo Verde wetland, at Palo Verde National Park in Guanacaste, transported to laboratory in water from the site and then transferred to clean UV treated tapwater. Toxicity tests using *S. baudinii* started with tadpoles on stages 28–34 (Gosner 1960).

Acute test

Acute, static, 96-h toxicity tests were carried out to find chlorothalonil's LC50 for each species. A minimum of six pesticide concentrations were tested in every assay (Table 1), a negative (UV treated tapwater) and a solvent control (methanol added to UV water in a volume equal to the one added to the highest concentration of chlorothalonil stock in the corresponding assay) were also set for every test. Tadpoles were exposed individually in 1 L glass containers with 500 mL of exposure solution. For every treatment, nine replicates were set and tadpoles were randomly distributed among treatments and replicates. Animals were not fed during the assay and mortality was recorded every 24 h.

Table 1 Chlorothalonil's ranges of concentrations (µg/L) used in definite acute and chronic tests with tadpoles

Test	*Agalychnis callidryas*	*Isthmohyla pseudopuma*	*Smilisca baudinii*
Acute	70	100	60
	35	50	40
	17.5	25	20
	8.75	12.5	10
	4.37	6.25	5
	2.2	3.12	2.5
Chronic	20	20	20
	15	15	10
	10	10	5
	5	5	
		2.5	

Chronic test

Chronic effects (growth and development) were evaluated in tadpoles exposed to sub-lethal concentrations (Table 1) (defined according to the outcome of acute tests) of chlorothalonil in a semi-static assay. At least three concentrations of fungicide plus controls were tested; with nine replicates per treatment and 500 mL of exposure solution. During the test, tadpoles were fed three times per week with approximately 15 mg of tetraVeggie/individual and once a week transferred into containers with freshly prepared solutions. Weight, total length, and developmental stage were registered weekly until tadpoles reached stage 42 or died (Gosner 1960).

For each experiment, exposure solutions were prepared by adding an aliquot of a stock solution to the exposure medium (UV water). Stock solution (1162 µg/mL) was prepared from 97.5 % pure chlorothalonil standard (Dr. Ehrenstorfer, Germany) dissolved in HPLC-grade, 99.97 % methanol (J.T. Baker) and kept at 4 °C. Aliquots were taken using a micro volume syringe (SGE Analytical science).

Concentration of chlorothalonil was evaluated at the beginning and at the end of acute and chronic tests: a 20 mL sample of each exposure solution was extracted (liquid/liquid extraction) with 98 % pure n-hexane (SupraSolv®) and resuspended in a final volume of 99.8 % pure isooctane (SupraSolv®). Determination of chlorothalonil in the extracts was performed using gas chromatography coupled to mass spectrometry (GC-MS) and electron capture detection (GC-ECD). For determination, an external calibration curve of chlorothalonil was processed. Initial concentrations were confirmed with a regression to nominals of $R^2 = 0.86$; no chlorothalonil was detected in samples collected at the end of assays, either acute (96-h) or chronic (7 days). For this

reason, acute tests should be interpreted as static 96-h exposure with an initial pulse of the nominal concentrations and the chronic tests, the exposure to a weekly pulse of the fungicide during the time the assay lasted. A 10 % error associated with the analytical methods should be considered for the quantification.

Four physical-chemical parameters: pH (Corning pH meter 220), dissolved oxygen (WTW Oxi 325), temperature and conductivity (WTW Cond 315i) were monitored in exposure solutions during the tests. Average and standard deviation of these parameters kept during the assays were pH = 6.6 ± 0.25; dissolved oxygen = 5.3 ± 1.35 mg/L; conductivity = 108.8 ± 4.5 μS/cm and temperature = 25.1 ± 0.7 °C.

Biomarkers

Muscle (tail) cholinesterase activity (ChE), liver glutathione-S transferase activity (GST), and liver lipid peroxidation (LPO) were evaluated in tadpoles after 96 h of exposure to sub-lethal concentrations of chlorothalonil. Samples were homogenized in an appropriate buffer and processed as described in Mena et al. (2014) for biomarker determinations. Briefly, protein content in sample homogenates was determined by the method of Bradford (1976) using γ-globulin as standard. ChE activity was measured using the method of Ellman et al. (1961), using 1 mM acetylthiocholine and 0.1 mM 5,5′ dithiobis-2-dinitrobenzoic acid (DTNB) as substrate and conjugate; reaction was measured at 415 nm during 15 min and expressed as nmol/min/mg protein. GST activity was determined as described by Habig et al. (1974), exposing samples to 1 mM CDNB and 1 mM GSH and monitoring the reaction at 340 nm during 3 min.; activity reported as nmol/min/mg protein. Lipid peroxidation was measured by the thiobarbituric reactive species (TBARS) (Oakes and Van Der Kraak 2003) and expressed as nmol TBARS per mg of protein.

Data analysis

Toxicity parameters: (LC_{50}) and 95 % confidence limits were calculated using IBM® SPSS® Statistics 22 (trial version). Concentration data were log-transformed and effect data were Probit-transformed.

To determine the effects of clorothalonil on total length, weight and development stage of tadpoles, we used linear mixed-effect models (LMMs) as suggested by Cox (2010). The total length, weight and development stage of tadpoles were specified as response variables. We used specified treatment as fixed factor and the number of individuals as random effect to account for repeated measures in each tadpole. During the analysis, the variables were classified as significant if the 95 % confidence intervals (CI) did not overlap zero (which represents the negative control).

We constructed linear models for each species to assess the effect of clorothalonil for each biomarker (LPO level, ChE and GST activities). Models were compared with null models (hypothesizes a difference of 0) and ranked according to their Akaike Information Criterion adjusted for small sample sizes (AICc). When a treatment effect was detected, we conducted model-averaged effect sizes (Mazerolle 2006); this is an information-theoretic alternative to multiple comparisons (e.g., Burnham et al. 2011). We log-transformed response variables if they showed non-normality.

The analyses of chronic effects and biomarkers were conducted in R version 3.1.2 (R Development Core Team 2014) using the package "lme4" (Bates et al. 2014) to conduct LMMs, and the package "AICcmodavg" to calculate AICc and model-averaged effect sizes (Mazerolle 2006).

Results

Acute toxicity

The tadpoles of the three native species tested showed a high and very similar sensitivity to chlorothalonil (Table 2, Fig. 1). Additionally, in *A. callidryas* and *S. baudinii* acute assays, it was observed that tadpoles which died during the first 24 h exposed to lethal concentrations of chlorothalonil, a spontaneous rupture of linea alba and posterior evisceration was observed (Fig. 2). In *A callidryas*, after 15 h of exposure, this effect was observed in one out of nine tadpoles exposed to 40 μg/L, two out of nine tadpoles exposed to 50 μg/L and three out of nine individuals exposed to 60 μg/L. During acute tests, we had a hundred percent survival of controls.

Chronic effects

Exposure to chlorothalonil did not affect the variables associated with growth and development measured in *I. pseudopuma* individuals compared to controls (Fig. 3a–c). In contrast, development of *S. baudinii* tadpoles exposed to 20 μg/L of the fungicide showed a positive difference compared to controls (95 % CI 0.69, 3.43) (Fig. 3f); while total

Table 2 Acute 96-h toxicity (median lethal concentration values [LC_{50}] with 95 % confidence intervals) in μg/L of chlorothalonil to tadpoles of three amphibian species

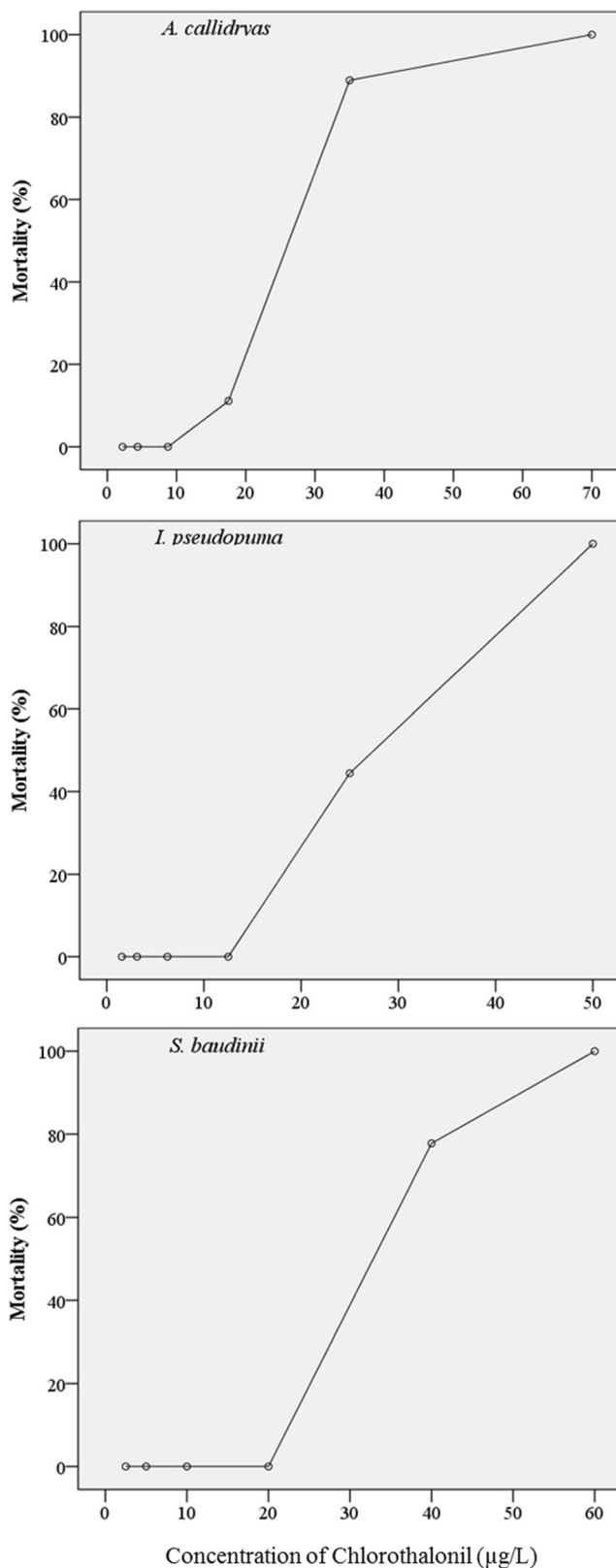| Species	Gosner stage	LC50	Lower 95 % confidence limit	Upper 95 % confidence limit
A. callidryas	26–27	26,6	18,9	35,8
I.pseudopuma	25–27	25,5	21,3	29,7
S. baudinii	28–31	32,3	26,3	39,7

Fig. 1 Mortality in tadpoles of three frog species, 96 h after exposure to a range of concentrations of chlorothalonil

length and body weight were not affected in this species (Fig. 3d, e). In the case of *S. baudinii*, the solvent control affected significantly the body weight of the tadpoles, therefore, for animals exposed to chlorothalonil, this variable was tested against the solvent control (Fig. 3e).

As an additional observation, during chronic exposure, lesions were observed on tails of *S. baudinii* and *I. pseudopuma* tadpoles exposed to the fungicide but not in the controls (Fig. 4). In *S. baudinii*, this effect was observed in five out of nine tadpoles exposed to 5 µg/L, six out of nine exposed to 10 µg/L and six out of nine exposed to 20 µg/L.

During chronic tests with *I. pseudopuma* no mortalities occurred in the negative control. In the case of *S. baudinii*, three individuals in the control died during the experiment before reaching stage 42, their data was analyzed for the period they lived. Chronic tests with *A. callidryas* were ended because of mortality of more than four replicates in controls during the first week of exposure. In this case, the batch of organisms used to start chronic test came from different egg masses than the ones used for acute assays.

Biomarkers

The analysis of model selection identified an effect of chlorothalonil treatments for *I. pseudopuma* ChE activity and *S. baudinii* GST activity (Table 3).

ChE activity was not affected in tadpoles of *A. callidryas* and *S. baudinii* exposed to chlorothalonil (Fig. 5a, c). In the case of *I. pseudopuma*, tadpoles exposed to 3 µg/L had a lower ChE activity compared to tadpoles exposed to higher concentrations of the pesticide (Fig. 5b).

A significant increase in GST activity was observed in livers of *S. baudinii* tadpoles exposed to 10 and 20 µg/L of chlorothalonil compared to controls and lower concentrations of the pesticide (Fig. 5f). A positive dose-response relationship was observed in this species (Fig. 5f), indicating an induction of phase II biotransformation process caused by the fungicide.

We measured LPO liver levels as a possible sign of oxidative stress induced by exposure of tadpoles to the fungicide; however, no significant increase of this biomarker was observed in any of the three species tested (Fig. 5g–i).

Discussion

The three species tested showed a similar sensitivity to chlorothalonil and this level of sensitivity agrees with observations made on other amphibian species (Yu et al. 2013). Also, this data confirms that larval stages of amphibians are more sensitive to chlorothalonil than other aquatic organisms (invertebrates and fish) (Yu et al. 2013). Compared to databases reports and literature, the three species tested are among

Fig. 2 Exposed viscera in tadpoles of *S. baudinii* (**a**) and *A. callidryas* (**b**) which died during first 24 h of exposure to lethal concentrations of chlorothalonil
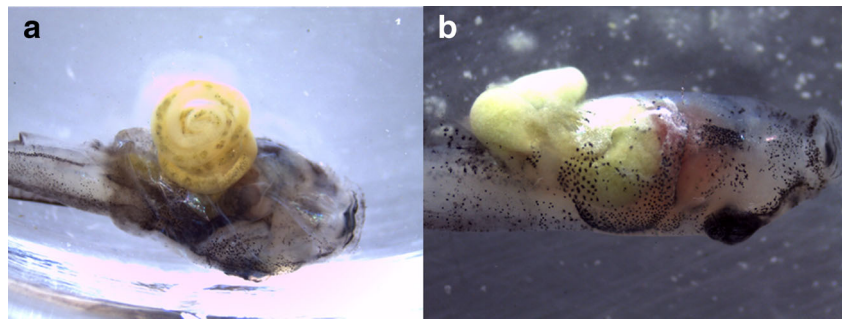
the most sensitive to chlorothalonil (US-EPA 2015; Kegley et al. 2015). Previous works developed in Costa Rica found also a high toxicity of chlorothalonil to *A. callidryas* tadpoles (Johnson et al. 2013; Ghose et al. 2014). The studies mentioned, used formulations of the fungicide and evaluated acute and chronic endpoints also. The acute 96-h LC$_{50}$ reported by them is higher than the one reported in this study, but still agrees with the fact that compared to other species, *A. callidryas* is very sensitive to chlorothalonil. According to

our data, this species is more sensitive to the pure compound than to the formulation. Regarding the toxicokinetics of pesticides, some research shows that through dermal exposure, the process of absorption of chemicals in amphibians is two orders faster than mammals (Brühl et al. 2013) which could be related with the higher sensitivity observed.

Considering other anurans, a great variation of sensitivity among species has been reported. For instance, McMahon et al. (2011) observed significant mortality in tadpoles of
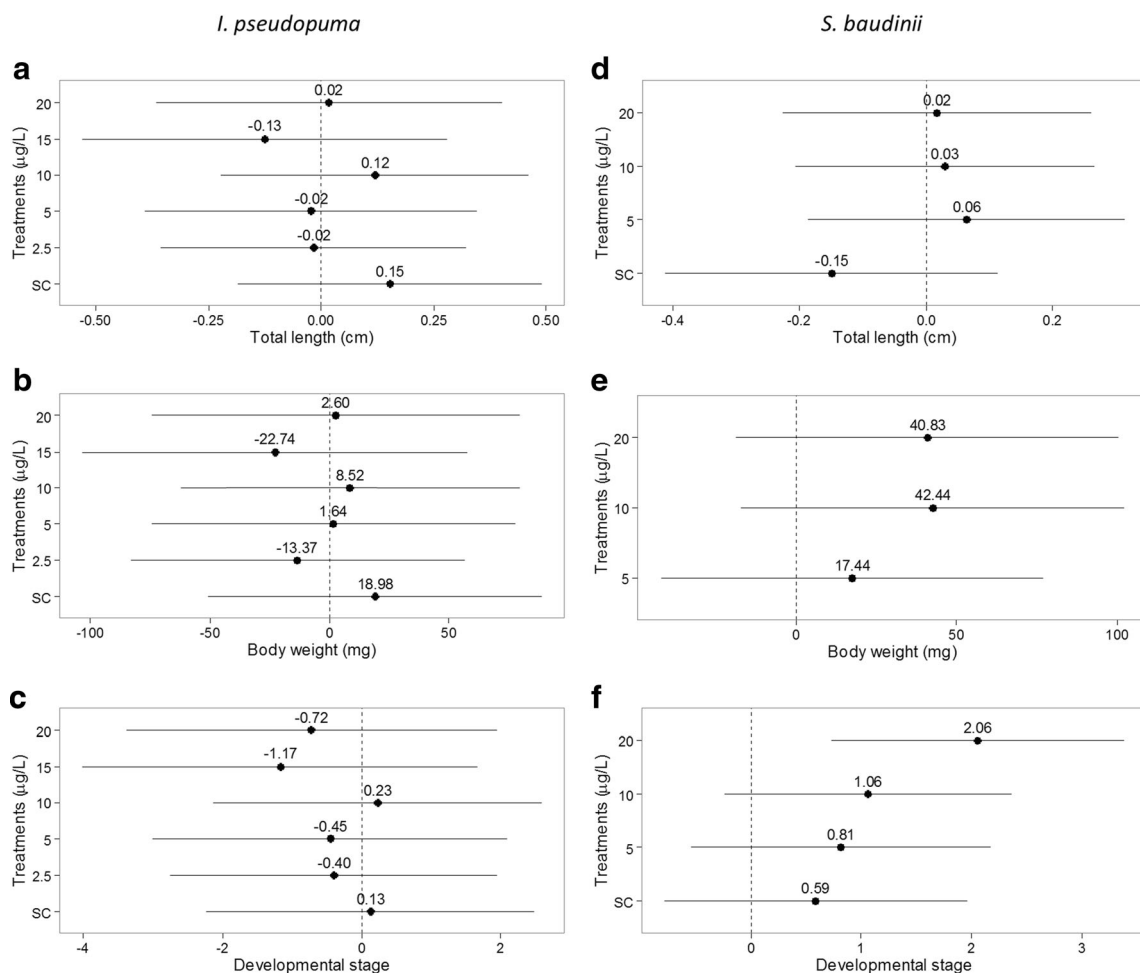

Fig. 3 Model estimates and 95 % CI for total length, body weight and development of *I. pseudopuma* and *S. baudinii* tadpoles exposed to sublethal concentrations of chlorothalonil. Confidence intervals below or above zero denote a difference with regard to control. *SC* = solvent control

Fig. 4 Lesions on *S. baudinii* (**a**) and *I. pseudopuma* (**b**) tadpoles tails during chronic exposure to chlorothalonil
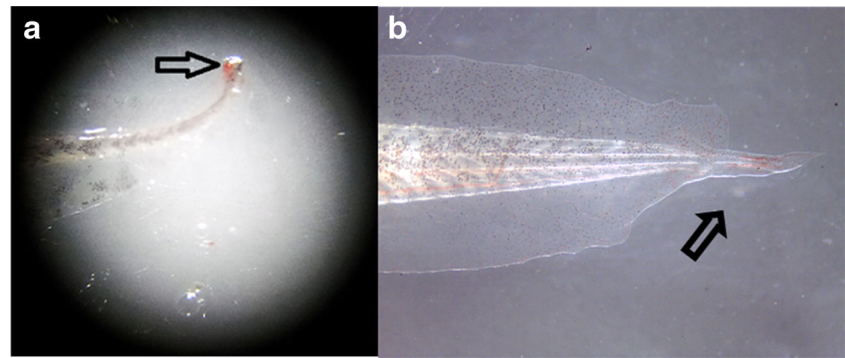
two frog species exposed to chlorothalonil at a concentration below the range tested in this work (16.4 ng/L) while the exposure of *Osteopilus septentrionalis* tadpoles to the a concentration thousand times higher (17.6 µg/L) did not cause mortality (McMahon et al. 2013). This last report agrees with our results regarding sub-lethal concentrations. In other example, McMahon et al. (2012) applied chlorothalonil in concentrations above 164 µg/L to a mesocosm where *Osteopilus septentrionalis* and *Rana sphenocephala* tadpoles among aquatic organisms were present. The treatment with the fungicide in this case increased mortality of the tadpoles and other

Table 3 Candidate set of linear models for the biomarkers GST, LPO and ChE of three Costa Rican anuran native species, with their respective treatment and null model. Number of parameters (K), Akaike's Information Criterion values (AICc), difference in AICc regard to best model (ΔAICc), and AICc weights (w)

Model	K	AICc	ΔAICc	w
A. callidryas				
GST~1	2	194	0	0.99
GST~treatment	7	203	9	0.01
LPO~1	2	20.8	0	0.99
LPO~treatment	7	29.6	8.7	0.01
ChE~1	2	-14.2	0	0.99
ChE~treatment	7	-5.3	8.9	0.01
I. pseudopuma				
GST~1	2	174	0	1
GST~treatment	7	193	19.4	<0.00
LPO~1	2	37.1	0	1
LPO~treatment	7	55.2	18	<0.00
ChE~treatment	7	53.4	0	0.968
ChE~1	2	60.2	6.8	0.032
S. baudinii				
GST~treatment	7	138	0	1
GST~1	2	153	14.9	<0.00
LPO~1	2	22.7	0	0.976
LPO~treatment	7	30.1	7.4	0.024
ChE~1	2	46	0	0.9942
ChE~treatment	7	56.3	10.3	0.0058

groups. For the species we tested, this concentration would be lethal to a hundred percent of the animals.

Chlorothalonil is a multi-site contact fungicide, which in aquatic organisms is known to affect immune responses and interfere with glutathione-related and oxidase-like enzymes (Elskus 2012; Van Scoy and Tjeerdema 2014). In humans, the occurrence of dermatitis has been reported in individuals exposed to this compound (Penagos 2002) and this effect has been attributed to irritant properties of the substance (Lensen et al. 2007). During acute assays with *A. callidryas* and *S. baudinii*, evisceration was observed in tadpoles exposed to lethal concentrations and we hypothesize that this effect might be attributed to a rupture of the linea alba because of the pesticide irritant properties. It would be necessary to confirm our findings with histological analysis. In this case, due to overnight mortality, some decomposition had already occurred in the samples and it was not possible to properly process them.

During chronic exposure, damage in tails was observed in *S. baudinii* and *I. pseudopuma* tadpoles. Yu et al. (2013) reported a similar effect of chlorothalonil on amphibians and they suggest that it might be related with an apoptotic process. It has been observed that xenobiotics have the capacity to produce skin lesions: recently it was found that after a short exposure to endosulfan, morphology and structure of the skin of *Bufo bufo* tadpoles suffered alterations and degenerative processes (Bernabò et al. 2013). Other pesticides may produce similar effects, in Sri Lanka, tadpoles of an endemic species were exposed chronically to chlorpyrifos, dimethoate, glyphosate and propanil. They developed malformations at high frequencies, mainly kyphosis, scoliosis, skin ulcers and edema (Jayawardena et al. 2010).

Regarding sub-lethal chronic effects, in most of the cases reported so far, exposure to pesticides has been observed to affect negatively the growth and development of tadpoles (Brunelli et al. 2009; Sparling and Fellers 2009; Baker et al. 2013). In the specific case of chlorothalonil, its sub lethal toxicity to *A. callidryas* has been studied and it resulted in a reduction of biomass in tadpoles exposed to the chemical for periods of 8 or 12 days (Johnson et al. 2013; Ghose et al. 2014; Alza et al. 2016). Also, Yu et al. (2013) found that
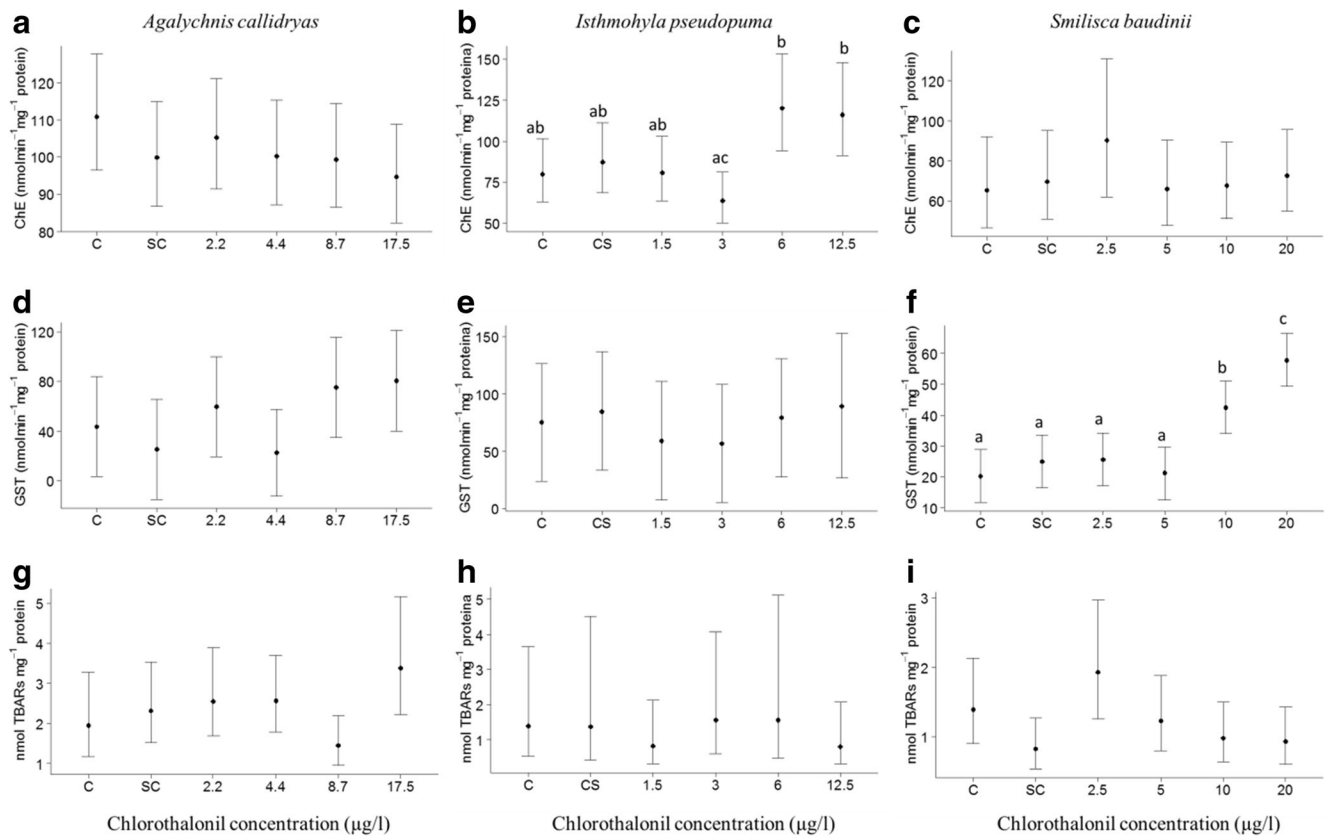
Fig. 5 Biomarkers measured in tadpoles exposed to sub-lethal concentrations of chlorothalonil: muscle ChE activity (**a–c**), liver GST activity (**d–f**) and liver LPO (**g–i**). *Bars* represent mean ± 95 % CI of the biomarker measured for each treatment. Differences among treatments in an experiment are indicated with *lowercase letters*. C = negative control, SC = solvent control.

96-h exposure to chlorothalonil reduced length of *Xenopus leavis* tadpoles.

The study we conducted maintained a prolonged exposure of tadpoles to the fungicide. In these conditions, we had no effect on *I. pseudopuma* growth or development; but in *S. baudinii*, development was positively affected by the exposure. This response has already been observed with the herbicide acetochlor in the presence of an exogenous dose of 3, 3′, 5-triiodothyronine (T_3), which is the active form of thyroxine (T_4) hormone, both involved in the metamorphosis activation. It produced an accelerated development of *Rana pipiens* forelimbs and this was attributed to an enhancement of the hormone action caused by the presence of the pesticide (Cheek et al. 1999). In *X. laevis*, when exposed to acetochlor and T_3, the metamorphosis was accelerated. The authors relate the course of action of acetochlor with gene transcription during metamorphosis (Crump et al. 2002).

Acceleration of development associated with pollutants interacting with thyroid hormones is an endpoint already considered in toxicity testing (OECD 2009). In this regard, Grabuski et al. (2004) suggested the potential of chlorothalonil as an endocrine dusruptor as it can potentially interfere with endogenous hormones. In mammals, it has been reported that this fungicide can be an agonist of the aryl hydrocarbon receptor (AhR) (Elskus 2012), which can be a molecular pathway to endocrine disrupting effects. Considering the outcome of our test with *S. baudinii*, we suggest that further investigation should be aimed to elucidate this issue. Based on our observations, we recommend the evaluation of malformations and behavioral effects as well as the continuation of the assays post-metamorphosis in order to identify possible alterations caused by the compound during juvenile and adult stages. This would be especially valuable considering that *I. pseudopuma* inhabits areas where declines have occurred (Abarca 2012) and the life cycle stage and parameters we evaluated did not reveal significant chronic effects on this species.

No significant ChE inhibition was observed in muscle samples of *A. callidryas* or *S. baudinii*, this is reasonable as this fungicide is not associated with a mechanism of ChE inhibition. It is still important to consider and measure ChE activity as its inhibition has been related to several pollutants others than organophosphates and carbamates (Lionetto et al. 2011). Tadpoles of *I. pseudopuma* exposed to 3 µg/L of chlorothalonil had a lower ChE activity compared to individuals exposed to higher concentrations of the pesticide but not lower compared to controls. We consider this difference unrelated with a possible inhibition caused by the pesticide as ChE

inhibitors are known to show a clear dose-response relationship (Thomson 1999; Pathiratne et al. 2008; Mena et al. 2012). However, nonmonotonic responses like this have been observed in the dose-mortality relationship of three amphibian species exposed to chlorothalonil (McMahon et al. 2011). Also, immunological parameters of fish exposed to chlorothalonil have shown nonmonotonic responses (Shelley et al. 2009).

Chlorothalonil's mode of action is related to interference with glutathione and cellular sulfhydryl enzymes (Van Scoy and Tjeerdema 2014), which are involved in phase II of biotransformation. In this work, we found evidence of biotransformation phase II induction in tadpoles of *S. baudinii* exposed to sub-lethal concentrations of chlorothalonil. The role of GST in biotransformation of chlorothalonil has been broadly described in bacteria (Wang et al. 2011) but also in aquatic vertebrates (fish) (Davies 1985). Ezemonye and Tongo (2010) reported the induction of GST in amphibians (adult toads) exposed to endosulfan and diazinon, furthermore they found that the liver is a good tissue to measure this activity. On the other hand, we did not find signs of oxidative stress evidenced as lipid peroxidation. This absence of oxidative stress has been observed in amphibians exposed to pesticides (Venturino et al. 2003), even when phase II responses are evident, like in our case. This might be attributed to the reported low activity of the mixed-function oxidases belonging to the cytochrome P450 family in amphibians (Huang et al. 1998; Venturino et al. 2003; Jung et al. 2004), being xenobiotics metabolized via phase II without a significant induction of phase I reactions and a lower oxidative process.

This work represents one of the few efforts in order to characterize the sensitivity of tropical amphibian species to pesticides. Two studies carried out in Costa Rica (Daly et al. 2007; Shunthirasingham et al. 2011) evidenced that pesticides used in agriculture in the lowlands of the country (including chlorothalonil) are atmospherically transported and found at places where habitat degradation is not evident. Although the three species tested in this work are listed as Least Concern by the IUCN (IUCN 2015), there is already evidence of the presence of pesticides, including chlorothalonil, in areas they inhabit: Cordillera Volcanica Central (Daly et al. 2007), the Caribbean Region (Castillo et al. 2000) and the Pacific Region (Mena et al. 2014). Accordingly, these and other amphibians are exposed to pollution and consequences as those shown in this work, during their life cycle (aquatic and terrestrial). Declines of some amphibian populations have been documented in pristine areas where *Bd* and temperature variability have been identified as causes for those events (Lips et al. 2008; Rohr and Raffel 2010). At the same time, La Marca et al. (2005) warn about the lack of information regarding environmental pollutants released by human activities and their effects on neotropical amphibian species. In this regard, efforts should be undertaken in order to elucidate the role of pollutants and hopefully their interaction with other environmental stressors as part of the factors influencing amphibian declines.

Conclusions

In the present study, we report that amphibian species that inhabit pristine areas of Costa Rica are sensitive to chlorothalonil, a pesticide that has been found to be transported atmospherically and that is intensively used in the country. Thus, efforts should be made in order to reduce the use of pesticides in the country and also focus on the protection of amphibian populations that are currently affected by declines.

Induction of phase II biotransformation indicates that relevant environmental concentration of chlorothalonil can be inducing biological responses that are related to its mode of action.

This study evaluated the chronic effects of a prolonged exposure to chlorothalonil on anurans. Because the tadpoles of *S. baudinii* showed impaired growth and development, it is recommended to investigate the possible role of this pesticide as an endocrine disruptor. It is also suggested to assess effects on juveniles and adults of the species tested.

Spontaneous rupture of linea alba and posterior evisceration was observed in the tadpoles exposed to lethal concentrations of chlorothalonil as well as lesions in tail at sub-lethal concentrations, these effects could be related to histological alterations of the skin, but further studies are needed to confirm this.

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