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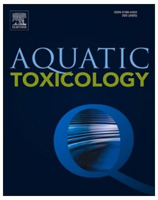

Effects of the insecticide β-endosulfan on tadpoles of *Isthmohyla pseudopuma* (Anura: Hylidae)

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ABSTRACT

Conventional agriculture uses pesticides intensively. Once pesticides are released into the environment, they can be toxic to non-target organisms. Exposure of amphibians to pesticides can be lethal and affect their growth, development and behavior. β-endosulfan is a persistent organochlorine that has been detected in environmental samples within protected sites in Costa Rica, far from agricultural areas. The aim of this study was to evaluate the lethal and sublethal effects, as well as changes in three biomarkers (Cholinesterase activity [ChE], glutathione S-transferase activity [GST] and lipid peroxidation [LPO]) in tadpoles of *Isthmohyla pseudopuma* exposed to β-endosulfan. A 96-h acute test (20, 40, 60, 80, 100 and 200 μg/L) was performed in order to calculate the median lethal concentration (LC_{50}), while effects on growth and development were assessed during a 4-weeks chronic test (10, 20, 30 and 50 μg/L). In addition, we measured the aforementioned biomarkers in tadpoles exposed to concentrations below the LC_{50}. The 96-h LC_{50} for this species was 123.6 μg/L. We found no evidence of β-endosulfan influencing any of the three biomarkers evaluated. At 50 μg/L, both length and total weight of tadpoles decreased with respect to the control. Also, at 30 and 50 μg/L we observed that individuals showed a slower development. Therefore, we demonstrated that at sublethal concentrations, β-endosulfan negatively affects *I. pseudopuma* at early stages causing tadpoles to develop slower and smaller than normal.

1. Introduction

Agriculture has dramatically increased in Latin America in the past decades, and it is predicted to continue to expand further in the forthcoming years (OECD/FAO, 2019). The increase of agriculture has come along with a significant increase in pesticide use. In Costa Rica, over 23% of the country is dedicated to some type of agricultural practice and over 90% of the farmers use pesticides to improve their production (INEC, 2015). The country is one of the biggest importers and users of pesticides in the world (Ramírez et al., 2015); 11.5 kg of active ingredient per hectare were used on average in cropland in 2019 (SFE, 2020). Concern has been raised regarding the risk that agrochemicals may pose to wildlife health (Arias-Andrés et al., 2018; Fournier et al., 2018). Such intensive use of pesticides could seriously threaten non-target biota, since these substances can be toxic to different taxa, including algae, plants, crustaceans, fish and amphibians (de la Cruz et al., 2014).

Amphibians are especially sensitive to agrochemicals because of their highly permeable skin, which is their main route of exposure to contaminants (Brühl et al., 2013). Their complex life cycles may expose them to polluted water, sediment or air during different stages of their life (Smith et al., 2007). Chemical pollution can also act synergistically with other environmental stressors, such as habitat loss, overharvesting, emerging infectious diseases, climate change, ultraviolet radiation, and invasive species, increasing the risk to amphibians (Whitfield et al., 2016). Pesticides like the insecticide endosulfan have been detected in water, soil and air at three protected and pristine highlands of Costa Rica (Daly et al., 2007; Shunthirasingham et al., 2011), suggesting airborne transportation from their application sites and deposition by rain and mist into these areas. Thus, the occurrence of pesticide residues in pristine areas should be considered as another factor that might be

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related with enigmatic amphibian declines or population impacts of endangered and relictual species (Abarca, 2012; García-Rodríguez et al., 2012; Whitfield et al., 2016).

Endosulfan is an organochlorine insecticide that can be bioaccumulated and has a high persistence in the environment, potential for transport over long distances and high toxicity to aquatic organisms (Sparling, 2010; Weber et al., 2010); it has attracted attention because of its presence, even at low concentrations, in soil (0.003 μg/g) and water (0.009 μg/L) samples of protected areas of Costa Rica (Daly et al., 2007; Shunthirasingham et al., 2011). Endosulfan acts as a neurotoxin to vertebrates, inhibiting the Cl^- flux during the gamma amino butyric acid (GABA)-induced activation by $GABA_A$ receptors (reviewed by Ballesteros et al. (2009)). Aside from acute toxicity, the exposure to pesticides can induce biomarker responses related to biochemical or physiological events elicited by the xenobiotic (Amiard-Triquet et al., 2011). Responses of biotransformation like the glutathione S-transferase activity, signs of oxidative stress such as lipid peroxidation, or neurotoxic responses such as the inhibition of cholinesterase activity, represent useful biomarkers to characterize the potential effects of pesticides (Venturino et al., 2003; Sparling, 2010; Méndez et al., 2016).

Endosulfan has been used on banana crops in the lowlands of Costa Rica and a high acute toxicity (12-days LC_{50} = 3.26 μg/L) has been reported for tadpoles of *Agalychnis callidryas*, a frog inhabiting these areas (Johnson et al., 2013). However, there is a lack of knowledge about the effects in highland amphibian species that might be exposed to endosulfan. The meadow treefrog, *Isthmohyla pseudopuma*, occurs at middle and high elevations of Costa Rica (1120 - 2340 m above sea level [a.s.l.] of Tilarán, Central and Talamanca mountains) (Savage, 2002), where it has been considered an abundant frog species (Crump, 1989; Abarca, 2012). *I. pseudopuma* is a medium-sized hylid frog, with adult males reaching 37–45 mm and adult females 41–52 mm of snout-vent length, and tadpoles reaching maximum lengths of 31 mm on average. The species reproduces explosively during the rainy season (May to December), with egg masses being deposited in the surrounding vegetation of temporary or permanent ponds; in addition, the species uses a variety of ephemeral breeding sites that includes flooded pastures and roadside ditches (Savage, 2002). Although currently considered as a non-threatened species (IUCN, 2020), some population declines have been reported in part of its range (Pounds et al., 1997). Such characteristics of abundance, availability of egg masses and distribution facilitate the use of *I. pseudopuma* for the assessment of the possible negative outcomes of exposure to endosulfan.

The aim of our study was to investigate lethal and sublethal effects of β-endosulfan on tadpoles of *I. pseudopuma*. Specifically, we assessed i) the acute (96-hour) toxicity of β-endosulfan on tadpoles; ii) the effect of acute exposure to sublethal concentrations of the insecticide on three biomarkers: cholinesterase (ChE), glutathione S-transferase (GST) and lipid peroxidation (LPO); and iii) the effect of chronic exposure (4-week) to sublethal concentrations of β-endosulfan on growth and development of the tadpoles. This evaluation of different endpoints should contribute to the characterization of the risks that this insecticide poses to amphibians.

2. Materials and methods

2.1. Eggs collection and maintenance

Three egg masses of *I. pseudopuma* (permit number: SINAC Costa Rica 022–2014-ACCVC-PI) were collected in February 2014 from a natural pond located in San Rafael de Heredia, Costa Rica (10.07603 N and 84.07706 W). The pond is located on private land with cattle grazing and adjacent to a protected area. We transported the egg masses in plastic containers with pond water. In the laboratory, we placed the egg masses in a 15-L glass tank with aerated, filtered and UV-treated water (Millipore) (10 L). After hatching, tadpoles were held in the glass tank and fed with spirulina-enhanced ground flakes (TetraVeggie®) and, when they reached Gosner stage 26 (around 48 h after hatching) (Gosner, 1960), we started the exposure. All experiments were carried out in the Laboratory of Ecotoxicological Studies (ECOTOX) of the Regional Institute for Studies on Toxic Substances (IRET-UNA, acronym in Spanish), Universidad Nacional, Heredia, Costa Rica.

2.2. Experimental design

2.2.1. Acute toxicity test

We performed a 96-h static acute toxicity test to determine the mean lethal concentration (LC_{50}) of β-endosulfan in tadpoles of *I. pseudopuma*. All assays were conducted at room temperature (24±2 °C). We tested six nominal concentrations of β-endosulfan (20, 40, 60, 80, 100 and 200 μg/L), including the negative control (UV-treated water) and acetone as solvent control (200 μL/L, added in a volume equal to that used in the 200 μg/L treatment). The exposure solutions were prepared by adding an aliquot (taken with micro syringe) from a stock solution to the exposure medium (filtered water). This stock solution (984.57 μg/mL) was prepared from 99% pure β-endosulfan (Dr. Ehrenstorfer®, Augsburg, Germany) dissolved in 99.8% acetone (JT Baker®, PA, USA).

Our experimental unit was one randomly selected tadpole in a 1-L glass container, containing 500 mL of the exposure solution. Nine replicates were used for each treatment. We randomly assigned tadpoles from the clutch of eggs to one of the experimental treatments. Tadpoles were not fed during the trial and mortality was quantified every 24 h. Individuals that survived at the end of the experiment were used to evaluate the effect of β-endosulfan on three biomarkers that may reflect neurotoxicity, biotransformation and oxidative stress (see below).

2.2.2. Biomarker measurements

We examined the influence of β-endosulfan exposure on muscle cholinesterase activity (ChE), liver glutathione-S transferase activity (GST), and liver lipid peroxidation (LPO) from tadpoles that survived after 96-h acute exposure to the insecticide. Tadpoles were dissected to extract their liver to determine GST and LPO. For the analyses, the samples were homogenized in appropriate phosphate (K_2HPO_4 / KH_2PO_4) buffers: buffer 0.1 M with pH=7.2 for tail samples and 0.1 M with pH 7.4 for liver samples. Samples were homogenized using a Branson® SLPt sonicator. All biomarkers were normalized to protein content, with the method by Bradford (1976), using a kit from BioRad® with bovine serum albumin as a protein standard.

Cholinesterase (ChE) activity was assessed according to the method of Ellman et al. (1961), adapted to microplate by Guilhermino et al. (1996). Briefly, samples were tested with a reaction mixture containing the synthetic substrate, acetylthiocholine (1 mM) and the conjugate 5, 5′-dithiobis-2-dinitrobenzoic acid (DTNB) (0.1 mM); the reaction was measured at 415 nm during 15 min and expressed as nanomoles of substrate metabolized per minute per milligram of protein. Glutathione S-transferase (GST) activity was measured according to Habig et al. (1974), exposing samples to a mixture containing 1 mM of 1-chloro-2, 4-dinitrobenzene (CDNB), and 1 mM of reduced glutathione (GSH). The reaction was followed for 3 min at 340 nm and the activity was reported as nanomoles per minute per milligram of protein. Lipid peroxidation (LPO) was measured by the thiobarbituric acid reactive species (TBARS) assay (Oakes and Van der Kraak, 2003) and expressed as nanomoles of TBARS per milligram of protein.

2.2.3. Chronic toxicity test

We conducted a chronic toxicity test to evaluate the effects of sublethal concentrations of β-endosulfan on body weight, total length and developmental stage of tadpoles. Based on the LC_{50}-value obtained from acute testing (96-h), we used four sublethal concentrations of β-endosulfan (10, 20, 30 and 50 μg/L) as the nominal concentrations of the experiment. Solutions and the negative and solvent controls were prepared as mentioned in Section 2.2.1. *Acute toxicity test*. Actual concentrations of the pesticide were quantified at the Laboratory of Pesticide

Residue Analysis-IRET; for this purpose, we replicated the acute and the chronic test and the concentrations of β-endosulfan at 0 h, 96 h and 168 h were measured (See Supplementary Data, Table S2). For the analysis, water samples (25 mL) were extracted twice using 2 mL n-hexane (JT Baker®, pesticide residue analysis, USA). The identification and quantification of β-endosulfan in the hexane extracts was achieved by capillary gas chromatography with a mass detector (Agilent 7890A/5975C, Agilent Technologies, Inc. USA).

The tests were conducted with one tadpole, selected randomly, in a 1-L glass container filled with 500 mL of the exposure solution (i.e., experimental unit), and seven replicates were used for each treatment. All tadpoles at the beginning of the experiment were at Gosner stage 26 and had similar body weight and total length. We adopted a semi-static design, in which every 7-days tadpoles were transferred to freshly prepared exposure solution, to ensure the initial dose of β-endosulfan, since it hydrolyzes with half-life of approximately 19 days (US EPA, 2012) and is susceptible to adsorption to food and biodegradation. Each tadpole was fed three times a week with approximately 15 mg of spirulina (TetraVeggie®). Trials were carried out at room temperature (24±2 °C). Weekly, we measured body weight to the nearest 0.001 g, total length to the nearest 0.1 cm and developmental stage over a 4-week period. We checked for dead animals every 24 h and removed them from the experiment.

2.3. Statistical analysis

Statistical analyses were performed in R (version 3.1.2), unless otherwise stated. To estimate the median lethal concentration (LC_{50}) value at 96-h period, we used a probit analysis to fit a probit regression of binomial variables using β-endosulfan concentration as the independent variable. Probit analysis was performed in SPSS (version 22). Parameters from the Probit method were obtained: a = intercept ± SE (standard error), b = slope ± SE, z = z test.

Since we used acetone as a carrier solvent in our experiment, we first compared the solvent control to the negative control to detect statistical differences for each independent variable analysis. We found no significant differences in the responses of the three biomarkers analyzed, between the negative control and the solvent control (Generalized Linear Models with gaussian distribution; ChE: $X^2 = 1.42, p = 0.23$; GST: $X^2 = 0.09, p = 0.76$; LPO: $X^2 = 2.21, p = 0.14$). We found no difference in body condition over the 4-week period between the negative control and the solvent control (Generalized Linear Mixed Effect Models (GLMM) with Gaussian distribution; $X^2 = 3.71$, $p = 0.44$). However, we found significant differences in total length and body weight of tadpoles between the negative control and the solvent control (Generalized Linear Mixed Effect Models (GLMM) with Gaussian distribution; total length: $X^2 = 4.98$, $p = 0.02$; body weight: $X^2 = 12.88$, $p < 0.001$). Thus, the solvent control was used as the basis of comparison in further analyses.

To evaluate the effect of β-endosulfan treatment on tadpole's total length, body weight and body condition through time (4-week period) we used GLMMs with Gaussian distribution. We implemented the tadpole identity as a random factor. Then, we performed treatment contrasts to identify if treatments significantly differed from solvent control (Venables and Ripley, 2002). The latter was conducted by performing Dunnett's multiple comparison tests using the R package "emmeans" as suggested by Landis et al., (2018). We estimated marginal means and 95% confidence intervals (95% CI) for the GLMMs using "emmeans" and plotted using the R package "gpplot2". We used residuals from a least square regression of body weight and total length as an index of body condition (Anthony et al., 2008; Schulte-Hostedde et al., 2005).

We calculated body condition index for each individual at the beginning of the experiment, and per week separately to avoid pseudo-replication. Due to the small sample size to fit a statistical model with developmental stage (categorical variable), we only describe the changing patterns of Gosner stages between and among endosulfan concentrations over time, by plotting 1) the frequency of observed development stages and 2) the mean developmental stage using "ggplot2". Thus, we carried out GLMs with Gaussian distribution to evaluate the effect of β-endosulfan concentrations on the biomarkers (ChE, GST, LPO). We assessed the significance of the treatment effect using the *Anova* function of the R package "car" and conducted treatment contrasts as mentioned above.

3. Results

3.1. Acute toxicity test

The 96-h LC_{50} value (95% confidence limits) of β-endosulfan to *I. pseudopuma* was 123.6 μg/L (93.9 – 206.8) ($a = -7.6 \pm 2.0$; $b = 3.7 \pm 1.0$; $z = 3.6$). No mortality of tadpoles was recorded in the negative and solvent controls. Furthermore, zero mortality was observed in the two low concentrations of β-endosulfan tested (20 and 40 μg/L), while the experiment showed an increasing mortality related to increasing concentrations of the insecticide, reaching 77% of mortality in the highest concentration tested (Fig. 1). Actual concentrations were measured and are shown in Supplementary Data, Table S2.

3.2. Biomarker measurements

After the 96-h exposure, none of the concentrations of β-endosulfan tested, caused a significant change in ChE activity ($X^2 = 0.81, p = 0.98$), GST activity ($X^2 = 4.31, p = 0.50$) and LPO levels ($X^2 = 5.48, p = 0.36$) of tadpoles of *I. pseudopuma* (Table 1).

3.3. Chronic toxicity test

Exposure to sub-lethal concentrations of β-endosulfan altered the growth and development of *I. pseudopuma* tadpoles. The tadpoles exposed to 50 μg/L of the insecticide remained shorter (23%) than those in the solvent control along the 4-week period (pairwise contrasts: $p < 0.05$; Fig. 2). In addition, the tadpoles in the 50 μg/L treatment showed a significantly lower body weight compared to those in the solvent control over the 4-week period (pairwise contrasts: $p < 0.05$; Fig. 3). The effect of the insecticide on both, length and weight of the tadpoles, increased with time. As well, a dose response was obvious after week three in both

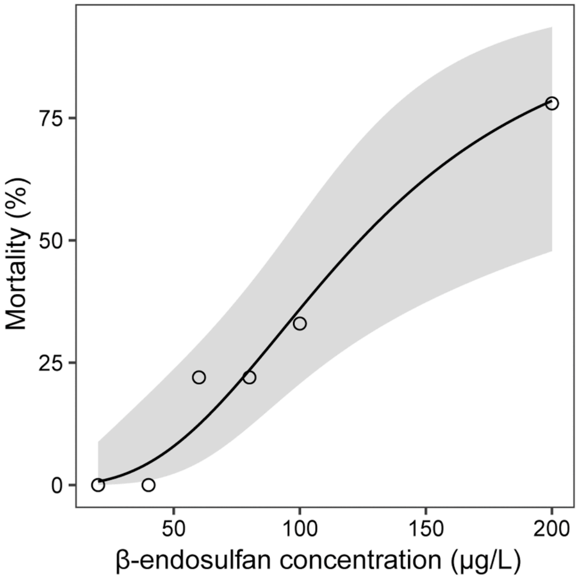

Fig. 1. Mortality in tadpoles of *I. pseudopuma*, after 96-h exposure to β-endosulfan. The continuous line is the predicted fit and shaded areas are 95% confidence intervals (CIs). Circles indicate observations.

Table 1
Mean (±standard deviation) of muscle (tail) cholinesterase activity (ChE) (nmol $\min^{-1}$mg-^{1} protein), liver glutathione-S transferase activity (GST) (nmol $\min^{-1}$ mg-^{1} protein), and liver lipid peroxidation (LPO) (nmol TBARs mg^{-1} protein) from tadpoles of *I. pseudopuma* exposed to β-endosulfan concentrations for 96 h.

Treatment	ChE	GST	LPO
Control	42.1 ± 3.7	78.5 ± 4.5	1.2 ± 0.4
Solvent control	47.8 ± 8.1	127.9 ± 57.5	1.32 ± 0.4
20 μg/L	41.6 ± 5.4	109.9 ± 16.1	1.2 ± 0.2
40 μg/L	41.2 ± 11.7	77.4 ± 16.2	1.9 ± 0.6
60 μg/L	40.6 ± 8.0	120.6 ± 32.9	0.9 ± 0.1
80 μg/L	42.9 ± 11.6	113.0 ± 74.4	1.5 ± 1.2
100 μg/L	44.8 ± 5.4	119.9 ± 5.5	1.7 ± 0.1

cases. We found no effect of endosulfan on the body condition over the 4-week period ($X^2 = 2.10$, $p = 0.71$). Regarding development, we observed that individuals exposed to 30 μg/L and 50 μg/L of β-endosulfan, showed a delay in development (reaching maximum stages 34 and 32, respectively) by the time at least one of the individuals from the negative control reached Gosner stage 37 (Figs. 4, 5), which is when the appearance of individual toes occurs (Gosner, 1960). The same was true for the solvent control (Figs. 4, 5). Actual concentrations were measured and are shown in Supplementary Data, Table S2.

4. Discussion

Environmental risk assessment for pesticides has drawn attention to amphibians and reptiles as vertebrate taxa of particular interest because of their high susceptibility to such contaminants (EFSA PPR Panel,

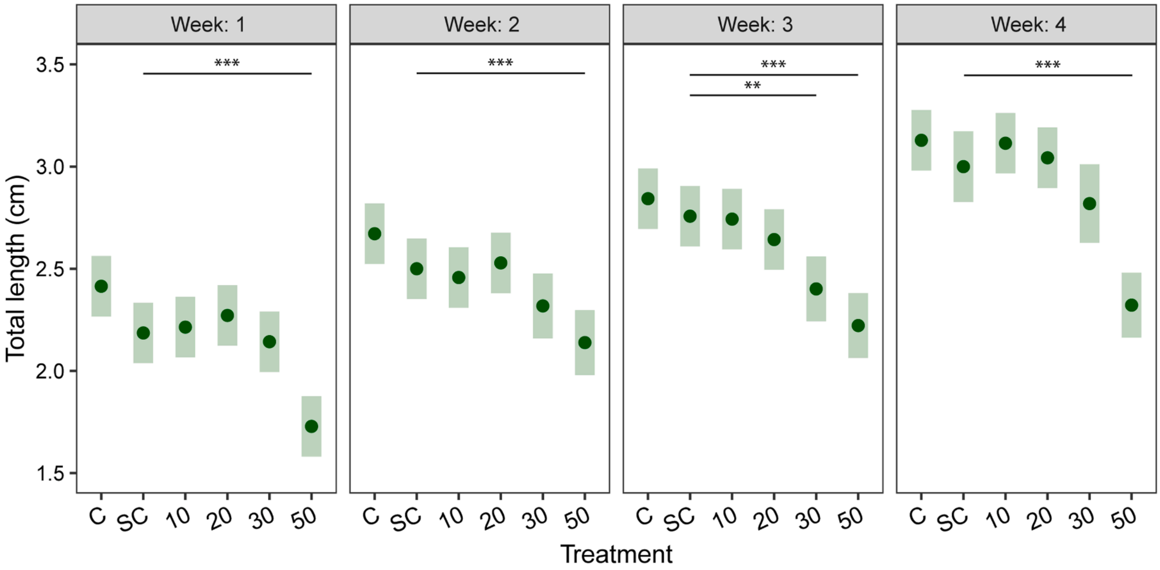

Fig. 2. Effects of β-endosulfan concentrations on the total length (cm) of tadpoles of *I. pseudopuma* along a 4-week period. Circles are estimated marginal means and shaded areas are 95% CIs. C = control; SC = solvent control. Statistical differences between SC and β-endosulfan concentrations: *** *p*-value < 0.0001; ** *p*-value < 0.001.

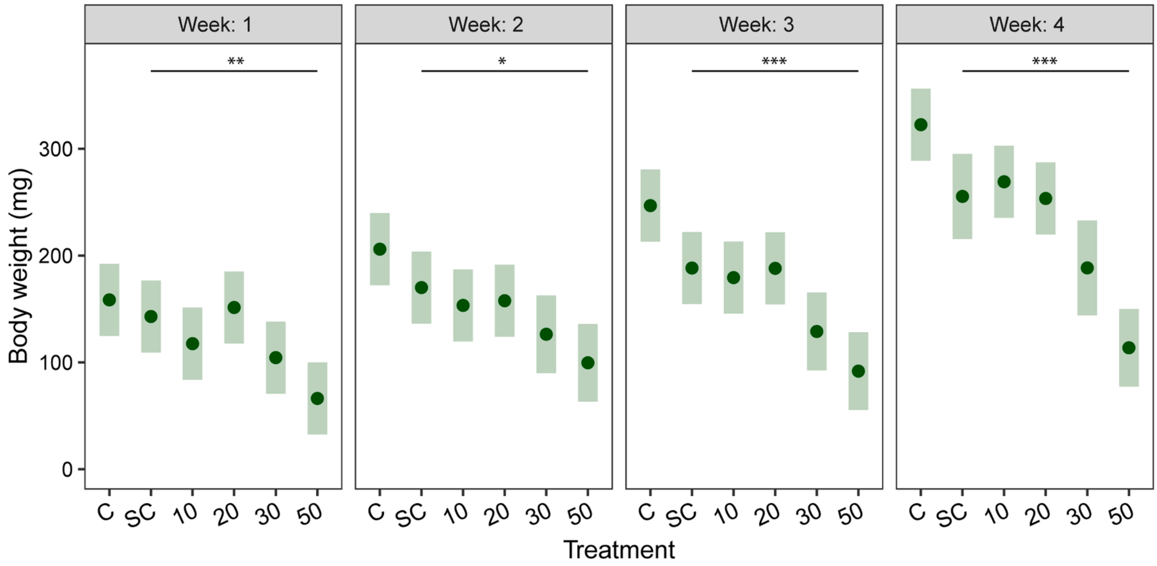

Fig. 3. Effects of β-endosulfan concentrations on the body weight (mg) of tadpoles of *I. pseudopuma* along a 4-week period. Circles are estimated marginal means and shaded areas are 95% CIs. C = control; SC = solvent control. Statistical differences between SC and β-endosulfan concentrations: *** *p*-value < 0.0001; ** *p*-value < 0.001, * *p*-value < 0.05.

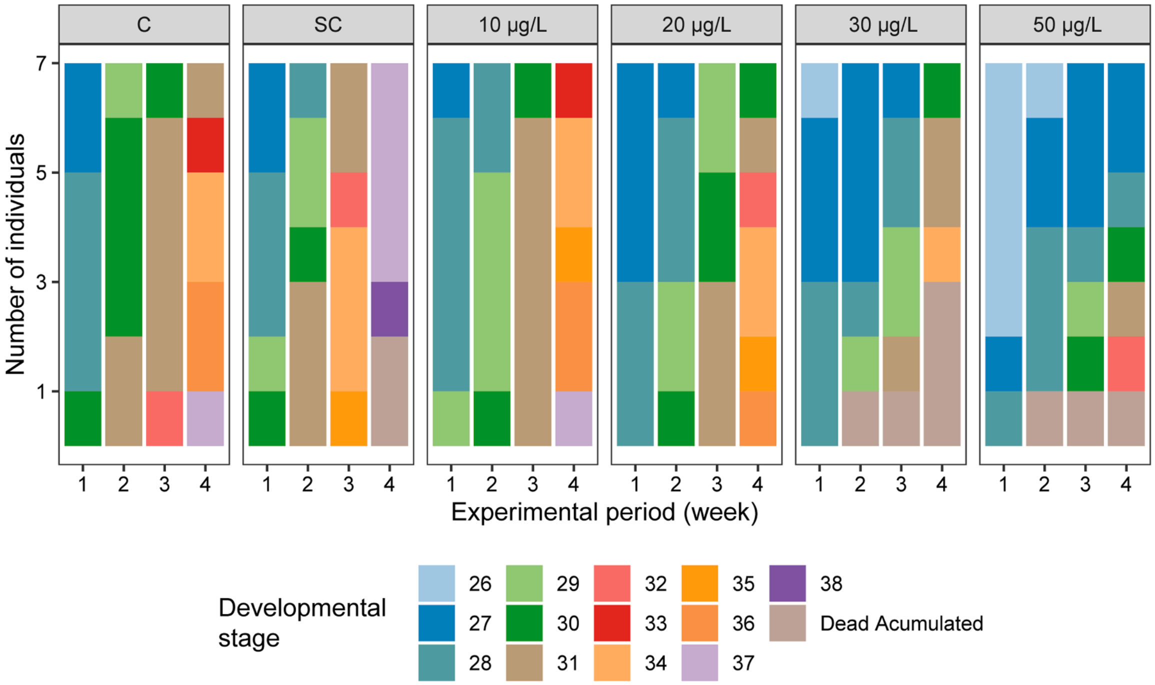

Fig. 4. Developmental stages (Gosner stage) of tadpoles of *I. pseudopuma* exposed to sublethal concentrations of β-endosulfan along a 4-week period. C = control; SC = solvent control. Every sub-panel represents a treatment with the four weeks of observation indicated in the X axis. Each stage was assigned a different color and the seven individuals are represented for every treatment.

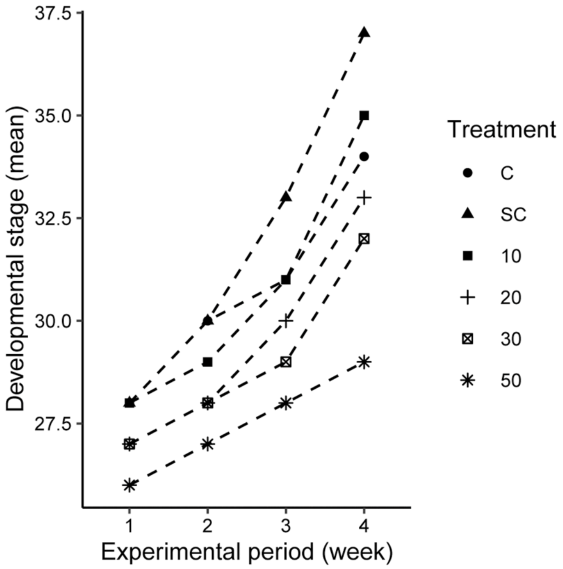

Fig. 5. Mean developmental stages (Gosner stage) of tadpoles of *I. pseudopuma* exposed to sublethal concentrations of β-endosulfan along a 4-week period. C = control; SC = solvent control.

2018). Our study provides evidence of the effects of a commonly used pesticide, endosulfan, on the survival, growth and development of amphibian species native to the middle and highlands of Costa Rica, where population declines have occurred. However, effects were observed at concentrations of β-endosulfan that were much higher than those registered in the natural habitats of this species.

4.1. Acute effects

We demonstrated that β-endosulfan, a pesticide that is highly persistent and highly toxic to aquatic organisms (Weber et al., 2010), has a median lethal concentration of 123.6 µg/L to *I. pseudopuma*. According to the criterium for aquatic organisms followed by the Environmental Protection Agency in an Ecological Effects Characterization (required to the Ecological Risk Assessment), an acute toxicity value between 0.1–1 mg/L classifies a pesticide as highly toxic to amphibians (US EPA, 2017); such is the case of β-endosulfan in our research. In Central America, only one study had reported the toxicity of endosulfan on native frog species. Johnson et al. (2013) found a lower LC_{50} of 8.39 µg/L to the red-eyed tree frog (*A. callidryas*), compared to the value reported in this study (Supplementary Data, Table S1). LC_{50} values for amphibians from American temperate regions exposed to endosulfan were also lower than that reported in this study, for example *Hypsiboas pulchellus* (0.13 µg/L, Agostini et al., 2009), *Rana boylii* and *Pseudacris regilla* (0.55 µg/L and 15.6 µg/L respectively, Sparling and Fellers, 2009) and *Rana catesbeiana* (1.3 µg/L), *Rana clamitans* (3.2 µg/L), *Hyla versicolor* (9 µg/L), *Rana cascadae* (15 µg/L), *P. regilla* (21.4 µg/L), *Bufo boreas* (76.1 µg/L) and *Pseudacris crucifer* (120 µg/L) (the latter seven species were all reported by Jones et al., [2009]) (Supplementary Data, Table S1). Outside of the Americas, lethality values have been reported for *Rana dalmatina* (74 µg/L, Lavorato et al., 2013) and *Bufo bufo* (430 µg/L, Brunelli et al., 2009). Therefore, *I. pseudopuma* tadpoles seem to be more tolerant to endosulfan than other frogs, except for *B. bufo* (Supplementary Data, Table S1). However, it is known that sensitivity to pesticides is variable among amphibian species (Boone and James, 2003; Baker et al., 2013).

4.2. Biomarkers

The evaluation of biomarkers has allowed the identification of early physiological responses associated with the exposure of *Chaunus schneideri* to pesticides in rice fields (Attademo et al., 2007), *Scinax fuscovarius* exposed to fipronil at concentrations of 5, 20 and 100 µg/L (Margarido et al., 2013) and *Smilisca baudinii* exposed to chlorothalonil at concentrations of 10 and 20 µg/L (Méndez et al., 2016). In our study, none of these biomarkers showed significant responses. This might be related with a low activity of the phase I of biotransformation and related oxidative reactions (Venturino et al., 2003; Sparling, 2010) and suggest no relevant role of GST in the metabolism of the insecticide for this stage of *I. pseudopuma*. As well, no neurotoxic effect associated with ChE inhibition was observed. Regarding endosulfan, increased GST activity was observed in tadpoles of *Bufo regularis* exposed to the insecticide (Ezemonye and Tongo, 2010). In other vertebrates such as fish, the exposure to this compound has been related with induction of LPO (Pandey et al., 2001) or ChE inhibition (Ballesteros et al., 2009; Maynart et al., 2012).

4.3. Chronic effects

We determined that after a prolonged period of exposure to sublethal dose of β-endosulfan (50 μg/L) caused tadpoles to be smaller, lighter, and less developed than unexposed individuals. Tadpoles that were not exposed to the pesticide developed faster than those that were exposed to the highest concentration, indicating that a pulse contact with sublethal concentrations delayed their development. Comparable effects have been observed in other amphibians exposed to endosulfan. For example, exposure to endosulfan at concentrations between 10 and 100 μg/L caused significant developmental delay and reduction of body mass and body length in tadpoles of *B. bufo* (Brunelli et al., 2009), *P. regilla, Rana boylii* (Sparling and Fellers, 2009), *R. dalmatina* (Lavorato et al., 2013), *Xenopus laevis* (Yu et al., 2015), and *Ambystoma barbouri* (Rohr et al., 2003). Even exposure to lower concentrations of endosulfan can have significant chronic effects. For instance, a 96-h exposure of tadpoles of *Litoria freycineti* to 0.03 and 1.3 μg/L resulted in size reduction of individuals (Broomhall and Shine, 2003).

Endosulfan concentrations that induced chronic effects on *I. pseudopuma* tadpoles are in the same order of magnitude than maximum historical sampling values from Costa Rica. Earlier studies conducted in Costa Rica reported concentrations of 0.47 – 9.3 μg/L of α-endosulfan and 0.11 – 8.9 μg/L of β-endosulfan, while concentrations in sediment were between 0.21 and 9 μg/kg of α-endosulfan and 0.12 - 46 μg/kg of β-endosulfan (de la Cruz et al., 2004; Chin et al., 2012; Carazo-Rojas et al., 2018). Water is the main route of exposure to pesticides for *I. pseudopuma* tadpoles, although their benthic feeding habits (Savage, 2002) increase the possibility of contact with contaminated sediment particles as well.

Lower feeding rate (Broomhall, 2004), mouth deformations (Brunelli et al., 2009), and abnormal swimming behavior (Bernabò et al., 2013; Denoël et al., 2013) have been reported in tadpoles of different species after exposure to endosulfan (0.03 - 200 μg/L), interfering with the feeding capacity and energy procurement necessary for their normal growth and development (Broomhall and Shine, 2003; Rohr et al., 2003; Mann et al., 2009). The induction of such effects by the insecticide might be related with our results and this possibility should be further assessed. Likewise, the delay in development of *I. pseudopuma* tadpoles also suggests a possible effect of endosulfan as an endocrine disruptor. Such an effect has been reported in other species like *B. bufo* at a concentration of 50–100 μg/L (Brunelli et al., 2009) and to *X. laevis* tadpoles at a concentration of 50 μg/L. In these studies, endosulfan was shown to inhibit reabsorption of the tail and to decrease the production of triiodothyronine (T_3) and thyroxine (T_4) (Fort et al., 2000). Although the mechanism of pesticides interfering with endocrine processes is not fully known, there is evidence of inhibition in the synthesis of these hormones, morphological changes of glands, conjugation of contaminants with hormone receptors in target tissues, and effects on the expression of receptor genes (Fort et al., 2000; Miyata and Keiko, 2012). Further research is required to determine the specific effect of β-endosulfan on endocrine processes and development of individuals of *I. pseudopuma*.

Our research made relevant observations related to a delay in the growth and development of *I. pseudopuma* tadpoles exposed to β-endosulfan. Despite the lack of observation of effects at concentrations of endosulfan measured in pristine and protected areas (Daly et al., 2007), there are remaining uncertainties and risks with regard to the exposure of other native species. Also, to understand the contamination of these conservation sites, research such as those carried out in protected mountains in Costa Rica (Daly et al., 2007; Shunthirasingham et al., 2011) are very important to get measurements of pesticide exposure in such areas. Currently, it is clear that amphibian diversity is threatened by multiple factors, including exposure to contaminants (Whitfield et al., 2016); national and regional efforts should be directed towards ensuring good agricultural practices, restricting the use of pesticides that might cause severe and irreversible impacts on local ecosystems, and monitoring bioindicator species such as amphibians.

5. Conclusions

We report the acute toxicity and chronic effects caused by the organochlorine β-endosulfan on tadpoles of *I. pseudopuma*, a frog species that inhabits mountains of Costa Rica. The mortality caused by a pulse exposure to the insecticide indicates that the insecticide is highly toxic to this species, although such acute exposure did not induce significant responses of biochemical biomarkers. Furthermore, the exposure to concentrations below half of the LC_{50} induced a delay in the growth and development of *I. pseudopuma* tadpoles, which might affect the ecological fitness of the species. Our data reflects the importance of assessing different endpoints regarding the effects that a pollutant can produce on a species. In this case, the impairment in the development after a chronic sublethal exposure should be a relevant endpoint to consider when the risk of the substance should be assessed.

CRediT authorship contribution statement

Michael Méndez-Rivera: Writing – original draft, Methodology, Investigation, Visualization, Supervision. **Freylan Mena:** Methodology, Investigation, Writing – review & editing. **Margaret Pinnock-Branford:** Conceptualization. **Clemens Ruepert:** Methodology. **Marco D. Barquero:** Writing – review & editing. **Randall R. Jiménez:** Formal analysis, Visualization. **Gilbert Alvarado:** Funding acquisition.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Supplementary materials

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