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Tadpoles of the horned frog *Ceratophrys ornata* exhibit high sensitivity to chlorpyrifos for conventional ecotoxicological and novel bioacoustic variables☆

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ABSTRACT

Previous studies reported that some species of the family Ceratophryidae are able to produce sounds during premetamorphic tadpole stages. We have now determined the effects of the cholinesterase-inhibiting insecticide chlorpyrifos (CPF) on sounds emitted by tadpoles of *Ceratophrys ornata*. Tadpoles were exposed individually in order to evaluate the progression of effects. Effects on sound production were complemented with common ecotoxicological endpoints (mortality, behavior, abnormalities and growth inhibition). *C. ornata* was found to be more sensitive than other native (= 67%, 50%) and non-native species (= 75%, 100%) considering lethal and sublethal endpoints, respectively. Effects on sounds appear along with alterations in swimming, followed by the presence of mild, then severe abnormalities and finally death. Therefore, sound production may be a good biomarker since it anticipates other endpoints that are also affected by CPF. *Ceratophrys ornata* is a promising new model species in ecotoxicology.

1. Introduction

Ecosystem impacts of human activities depend on their intensity, extent and duration. While all human societies have transformed the environment to suit their needs and demands, in modern societies such transformations have occurred at an accelerated pace, leading to environmental, social and economic consequences. Agriculture is applied to extensive land areas, and there is a growing conflict between agricultural practices, the satisfaction of human needs and environmental sustainability (Leguizamon, 2014; WWF, 2016). Particularly in Argentina, recent use of genetically modified soybeans has led to a major increase in cultivation, occupying almost 57% of the total cultivated area (SAGyP, 2015). With such an expansion, there is not only a consequent increase in the usage of agrochemicals but also a reduction of other crops, lower cattle production, deforestation and habitat loss due to irreversible alteration and/or fragmentation (WWF, 2016). The most commonly used pesticides in descending order are herbicides, insecticides and fungicides. Glyphosate and chlorpyrifos, for example, are each the most commonly used herbicide and insecticide, respectively (CASAFE, 2012). Specifically, chlorpyrifos (CPF; O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate) is a neurotoxic organophosphate insecticide that inhibits acetylcholinesterase to cause accumulation of acetylcholine and nervous system hyperexcitation (Barron and Woodburn, 1995). It may also persist more than two months in water and six months in sediment (Watts, 2012). Although in 2000 the US Environmental Protection Agency (USEPA) regulated its usage, it is the most widely used insecticide in Argentina, being frequently used in the production of vegetables and fruits (Cappello and Fortunato, 2013). Previous studies demonstrated that CPF reaches Argentinian aquatic ecosystems (Marino and Ronco, 2005; De Gerónimo et al., 2014), and thus both larval and adult amphibians may be at risk of exposure.

Amphibians are of special interest in ecotoxicology, allowing not only assessment of potential harmful effects in aquatic environments, but also in terrestrial environments due to their biphasic life cycle. Moreover, most amphibians have highly permeable skin,

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making them particularly sensitive to pollutants, and thus represent good sentinels of local conditions (Stebbins and Cohen, 1995; Guzy et al., 2012). One anuran species that has been used as an experimental model in Argentina is *Rhinella arenarum* (Ferrari et al., 1997; Herkovits and Perez-Coll, 1999a; 1999b; Ferrari et al., 2008; Sotomayor et al., 2012; Nikoloff et al., 2014; Liendro et al., 2015). Considering this is only one of the 168 species of anurans described for Argentina, it is essential to examine others, given the high diversity in life history traits, and perhaps differing sensitivities to environmental contaminants.

In this context, we have been conducting studies with *Ceratophrys ornata* since 2006 (Natale, 2006; Salgado Costa, 2016) and found it easy to breed under controlled laboratory conditions, and its tadpoles are larger than other Argentinian species so they are easy to handle and measurements are more accurate (Salgado Costa, 2016). Most significantly, we discovered for the first time in any vertebrate that the tadpoles of *C. ornata* are able to emit sounds. The ecologically relevant situation when sounds are emitted is during conspecific interactions, and when physically touched under experimental conditions simulating an interaction, perhaps to avoid cannibalism in this aggressive carnivorous species. In marked contrast, *C. ornata* tadpoles never emit sounds when interacting with tadpoles of a prey species (Natale et al., 2011). Three key observations led us to test the effects of chlorpyrifos (CPF) on larval sounds: (1) it is toxic at high levels to amphibians and other non-target vertebrates because it inhibits acetylcholinesterase (Barron and Woodburn, 1995); (2) acetylcholine has a critical role in the development of vocalizations in rats (Krüger and Hanganu-Opatz, 2013), and (3) acetylcholine is a critical neurotransmitter in vertebrate hearing (Puel, 1995; Fritzsch and Elliott, 2017). We therefore hypothesized that CPF may disrupt sounds emitted by *C. ornata* tadpoles. Here, we provide evidence that this unique behavior is disrupted by CPF. In this way, we suggest that tadpole vocalizations are a sensitive non-lethal endpoint. Moreover, we compliment this with well-known ecotoxicological endpoints (mortality, behavior, abnormalities and growth inhibition) and show that sound production is extremely sensitive to waterborne CPF.

2. Materials and methods

2.1. Study species

Scientific names and taxonomic classifications were updated according to Frost (2017). *Ceratophrys ornata*, commonly known as the horned frog, is found in the Pampas region of Argentina (Provinces of Buenos Aires, Cordoba, Entre Rios, La Pampa, Mendoza and Santa Fe) and southern Brazil (Rio Grande do Sul). It inhabits grasslands, highly modified agroecosystems and urban areas. As with other species in the genus, *C. ornata* is an explosive breeder. It is a predatory species with a toad-like, robust and colorful appearance, and is remarkable both for its external characters and aggressive behavior when disturbed (Gallardo, 1974; Cei, 1980). This makes the species a highly desired pet, and is part of the illegal market like many toads of South America (Pistoni and Toledo, 2010). Besides, it is a commonly raised and marketed species in USA, making it easily accessible in different parts of the world. Taking into account the last reports for amphibians in Argentina (Natale and Salgado Costa, 2012), the status of *C. ornata* was raised to “vulnerable”. These anurans are ambush predators characterized for being macrophagic and carnivorous from larval stage to adult (Gallardo, 1974; Cei, 1980). Larvae possess canibbalistic behavior on other larvae and eggs (Cei, 1980), have a maximun body length of 30 mm and reach metamorphosis approximately one month post-hatch (Salgado Costa, 2016). Additionally, tadpoles have the ability to emit sounds as part of an antipredator mechanism which has also been described for a few other related species (Natale et al., 2011; Salgado Costa et al., 2014; Salgado Costa et al., 2016b).

2.2. Breeding and maintenance

Two females (318 and 326 g; 114 and 115 mm) and two males (151 and 158 g; 94 and 96 mm) *C. ornata* adults were collected in the field between 2012 and 2014 in Buenos Aires Province (Collection permit: 22500-14357/11, Decree 209/11 and 14/12) and maintained in the laboratory (25 ± 1 °C, photoperiod16L:8D). Spawning was induced by the Amphiplex method: both male and female were injected i.p. (one couple at a time) with a cocktail of 0.4 μg/g of a GnRH agonist and 10 μg/g of a dopamine antagonist (Trudeau et al., 2010, 2013). A total of approximately 6000 eggs per couple (clutch A and B) were laid and fertilized, with an average hatching rate of 73%. Eggs and tadpoles were reared under the same controlled conditions as adults but in plastic trays ($33.0 \times 23.0 \times 8.5$ cm) with dechlorinated tap water (21 ± 1 °C, pH: 7.6–8.3, hardness: 180–250 mg $CaCO_3$/L) with continuous aeration. Rearing density was 4.20 g of tadpoles/L. They were fed *ad libitum* with several mixed food items: *Tubifex sp.*, tadpoles of *Boana pulchella* and *Rhinella arenarum*, pieces of fish and beef liver (Salgado Costa, 2016). All procedures for the care and use of laboratory animals are in agreement with local guidelines for vertebrate animal welfare (Protocol Number 023-22-15) as well as with US Public Health Service and/or European Union policy on this matter. Collected adults are still alive and are maintained in controlled conditions.

2.3. Experimental design

Desired developmental stages (SD) were selected following Gosner (1960). Stage 25 was selected since it is the first stage when sounds are emitted (Natale et al., 2011; Salgado Costa, 2016) and is also characterized by free-swimming individuals with complete larval morphology (Duellman and Trueb, 1994). At SD25, tadpoles had a snout-to-vent length (SVL) of 6.59 ± 0.78 mm and weighed 0.07 ± 0.01 g ($n = 20$). Stage 31 was also selected since it is characterized by intermediate and larger larvae with developing hind limbs and clear sound emission. At SD31, tadpoles had a SVL of 13.75 ± 1.06 mm and weighed 0.41 ± 0.10 g ($n = 20$).

Standard animal care procedures (USEPA, 1989) were followed with minor modifications for local species (Natale, 2006; Ruiz de Arcaute et al., 2012). A stock solution was prepared from CPF 95.1% (determined by Gleba S.A.; CAS number = 2921-88-2) diluted in absolute ethanol and dilutions were made with dechlorinated tap water. The final concentration of ethanol was less than 0.01%. Four tests (T) were performed under controlled laboratory conditions during acute exposure (96 h) and were then continued to chronic exposure (240/336 h). Two tests were performed with larvae from SD25 (T1 and T2, each test was performed with tadpoles randomly selected from clutch A and B, respectively) and the other two with larvae from SD31 (T3 and T4, each test was performed with tadpoles randomly selected from clutch A and B, respectively). Preliminary tests allowed us to determine a concentration range within which to assess lethal and sublethal effects. The experimental design consisted of exposing organisms individually in 100 ml glass chambers ($4.5 \times 4.5 \times 7$ cm) for SD25 and 1500 ml chambers for SD31 ($13 \times 13 \times 14$ cm). Final nominal CFP concentrations ranged from 0.01 to 0.7 mg/L. Tests included at least 15 replicates (= chambers) per concentration and two negative control groups: (1) a water control without pesticide and a dilution control containing the maximum concentration of ethanol used in dilutions (<0.01%), intended to discard ethanol as a factor (Table 1).

Table 1
Tests (T) performed at two developmental stages (25 and 31) indicating nominal concentrations of chlorpyrifos. T1-2: stage 25; T3-4: stage 31; R: number of replicates per concentration; Dc: dilution control containing the maximum concentration of ethanol used in dilutions (<0.01%).

Tests	R	Concentrations (mg/L)												Dc
T1	15	0.01	0.025	0.05	0.075	0.1	–	0.2	–	0.3	0.4	0.5	–	0.5
T2	15	0.01	0.025	0.05	0.075	0.1	–	0.2	–	0.3	0.4	0.5	–	0.5
T3	20	–	0.025	–	0.075	–	0.15	0.2	0.25	0.3	–	0.5	0.7	0.7
T4	20	–	0.025	–	0.075	0.1	–	0.2	–	0.3	0.4	0.5	0.7	0.7

All solutions were fully renewed every 24 h during acute exposures and every 48 h during chronic exposures. During acute exposures, SD25 larvae were not given food, but were then fed daily during chronic exposure with one larva of *B. pulchella* or *R. arenarum* (each item constitutes approximately 20% of *C. ornata* tadpole weight). Note that food deprivation during acute exposure did not introduce a bias since control groups exhibited no obvious effects (see results section for further details). Stage 31 *C. ornata* tadpoles were fed daily during acute and chronic exposure to avoid potential effects of starvation. As *C. ornata* tadpoles are voracious predators, prey items were instantly consumed.

2.4. Effects of CPF on ecotoxicological endpoints in C. ornata tadpoles

Lethal and sublethal endpoints were recorded every 24 h. Mortality (M) was determined by visual observation of immobility, absence of cardiac activity and rapid decomposition of the body. Possible swimming alterations (SA) were recorded in controls and CPF treated groups after gently swirling the water five times with a glass rod and observing swimming activity of each individual tadpole for 1 min. Morphological abnormalities were determined by visual observation and confirmed under a Wild Heerbrugg M8 binocular stereoscope according to the categories proposed by Bantle et al. (1996). Growth was assessed at the end of acute (96 h) and chronic exposure (240/336 h) by measuring SVL after digital photograph with the ImageJ® program version 1.46r (Rasband, USA). Each tadpole was placed in the center of a millimeter sheet and photographs were taken dorsally with a digital camera Canon® Eos T3i with maximum resolution (= 18 mp). The camera was mounted on a tripod and photographs were taken all the same vertical distance (= 45 cm). Since SVL and weight are highly correlated in this species ($r = 0.906$, $p < .0005$; Salgado Costa, 2016), only the first is detailed. Moreover, dead individuals were fixed for 24 h in a Bouin solution (75% picric acid + 20% formalin 40% + 5% acetic acid) and then stored in 70% ethanol for later examination of possible abnormalities and growth inhibition.

2.5. Effects of CFP on novel bioacoustic variables in vocalizing C. ornata tadpoles

Tadpoles from T2 and T3 were recorded while emitting sounds at the end of acute (96 h) and chronic exposure (240/336 h). Bioacoustic variables determined were sound duration (Sd) in seconds (s), number of pulses (Np) and dominant frequency (Df) in Hertz (Hz). Sounds were recorded and analyzed following validated procedures we established previously (Salgado Costa, 2016). In brief, each larva was recorded individually at random for 30 s. Considering that tadpoles emit sounds repeatedly when taken out of water, we analyzed three sounds per tadpole selecting those emitted at 5, 10 and 20 s of recording representing those times the beginning, middle and final part of recording. Each selected sound was trimmed from its original and saved as a single file. In case that there were no recorded sounds at the indicated times, we selected the closest to each time either before or after it. This methodology ensures having the same amount of sounds per larva and takes into account individual variability. Sound post-processing and analysis were made with a computational tool we specifically developed in a numerical environment. Concisely, the duration of each sound was defined considering the duration of the file and the frequency sampling; pulses were determined as the highest local maximum between a minimum time interval; and dominant frequency was determined by applying a filter of constant bandwidth (Fast Fourier Transformation) and associating it with the higher level component (Salgado Costa et al., 2016a).

2.6. Comparisons between all variables selected

We tested the hypothesis that there is a progression of negative effects with exposure duration as proposed by Ruiz de Arcaute et al. (2012), from the appearance of SA, the presence of mild and then severe abnormalities to death. In order to contrast that hypothesis, each larva was assigned a particular state at the end of acute and chronic exposure of each test. Such states (0 = alive larva without negative effects, 1 = larva with SA, 2 = larva with mild abnormalities, 3 = larva with severe abnormalities, 4 = dead larva) were allocated taking into account the maximum state that the larva possessed at the time of measuring (e.g., a living larva with SA and mild abnormalities was assigned state 2). Also, the location of affected sounds on that progression of negative effects, the correlation between conventional (frequently used variables) and bioacoustic variables, and cross-correlations between each sublethal conventional variable were evaluated.

2.7. Statistical analysis

The level of significance set was 0.05 for all tests. Homogeneity of variances and normality were corroborated with Levene's and Shapiro-Wilk's test, respectively. The LC-50/EC-50 and 95% confidence limits were obtained by Probit analysis version 1.5 and following the method of linear intersection to estimate concentration-response curves (USEPA, 1989). The lowest observable effects concentration (LOEC) was calculated by ANOVA followed by Dunnett's test.

A species sensitivity distribution (SSD) was built, taking into account the 96 h LC-50 data published for other species of anuran tadpoles at SD25, and performed following the Aldenberg and Jaworska (2000) method using 2.0 ETX software (van Vlaardingen et al., 2005). The cutoff value of 5% of species at the lower tail of the distribution (hazardous concentrations 5%, HC5) has been traditionally used for potential environmental protection, under the assumption that ecosystems can tolerate a certain low degree of chemical stress (Posthuma et al., 2002). The exposure scenario considered environmental concentrations of CPF measured in the Pampas region of Argentina (Jergentz et al., 2005; Marino and Ronco, 2005; Mugni et al., 2011).

Comparisons between SVL and treatments were performed, with Dunnett's test or Fisher's test to compare treatments with water control group or with each other, respectively. Correlations between SVL and concentration values were evaluated by a Pearson

correlation coefficient (r) or a Spearman rank correlation coefficient (r_s), as applicable. On the other hand, comparisons between bioacoustic variables (Sd, Np, Df) of sounds emitted by larvae of the water control and dilution control groups were performed to discard ethanol as a factor (Student *t*-test or Mann-Whitney *U* test, as applicable). Also, multiple comparison tests (ANOVA or Kruskal-Wallis tests, as applicable) were performed between each bioacoustic variable and concentration values, in order to compare each treatment with water control group by a Dunnett's test or Multiple Comparison of Mean Ranks for all groups, respectively. Moreover, correlation between bioacoustic variables and concentration values were also evaluated by a Pearson correlation coefficient or a Spearman rank correlation coefficient, as applicable. Note that analyses were performed without summarizing the information corresponding to the sounds emitted by each larva (n = 3) so not to lose individual variability.

In order to evaluate the negative effects progression hypothesis, a contingency table was constructed between the various treatments and states that imply presence/absence of progression. Then, a correlation analysis between the frequency of states that indicate a progression of negative effects and logarithm of the corresponding concentrations where effects appear was performed at a constant time of exposure (end of acute/chronic test). Correlation analyses between the novel bioacoustic and conventional ecotoxicological endpoints were performed by a Spearman rank correlation coefficient. The same applies for the cross-correlations performed between each sublethal conventional variable. Also, a comparison between bioacoustic (Sd, Np, Df) and conventional endpoints (SA, MFLT, SLFT, SVL) and the time of exposure (end of acute and chronic exposure) was performed for each stage of development (Student *t*-test or Mann-Whitney *U* test, as applicable). Then, correlation analyses were performed by a Spearman rank correlation coefficient.

2.8. Analysis of CPF concentrations

Water samples (n = 12) were taken before and after renewal of the exposure solutions and stored in glass bottles at −18 °C before the analysis. Samples were passed through 0.45 μm filters and CPF concentrations measured by LC-MS (detection limit, 0.001 mg/L; quantification limit, 0.003 mg/L). For the analysis, a liquid chromatograph model Agilent 1100, coupled to an Agilent mass spectrometer model VL was used. Chromatographic separation was run in isocratic condition of acetonitrile (HPLC grade, J.T. Baker, USA): water (formic acid 0.1%, analytical quality, Merck, Germany) at 80:20 ratio and a flow of 0.5 ml/ml on a C18 X-SELECT ™ column (75 mm × 4.6 mm and 3 mm pore size, from Waters Corp., Milford, USA). For the ionization, an electrospray source was used in positive mode with selective ions m/z = 350, 352 and 198. The analytical quality and molecular identity criteria were those proposed by SANTE 11945/2015 (European Commission, 2015). Nominal and measured concentrations were compared by factorial ANOVA taking into account the time and expected/observed values.

3. Results

3.1. Chemical analysis

All the LC-50/EC-50 and LOEC values were calculated using the measured concentrations in the exposure solutions at the initial time of testing (0 h). An average loss of CPF of 43.4% and 19.8% was observed at 24 h and 48 h of exposure, respectively. The comparison of nominal and measured concentrations taking into account the time and expected/observed values revealed no significant differences ($F(2, 18) = 0.547$, $p = .588$; assumptions were corroborated: $F = 1.728$, $p = .179$; $W = 0.829$, $p = .015$).

3.2. Effects of CPF on ecotoxicological endpoints in C. ornata tadpoles

Both control groups exhibited a total absence of any abnormalities. The LC-50/EC-50 and LOEC of each evaluated endpoint (M, SA, morphological abnormalities) under acute and chronic exposure for each test (T1-4) are summarized in Table 2.

The SSD for lethal endpoints was estimated with 13 LC-50 (arithmetic mean = −0.308, standard deviation = 0.771) values obtained from the published literature (Abbasi and Soni, 1991; Cowman and Mazanti, 2000; Richards and Kendall, 2002; El-Merhibi et al., 2004; Kerby, 2006; Yin et al., 2009; Bernabò et al., 2011; Ruiz de Arcaute et al., 2012; Natale et al., 2013) and is presented in Supplementary Figure 1. Estimated HC5 was 0.025 mg/L (lower limit at 95% = 0.004 mg/L − upper limit at 95% = 0.072 mg/L).

Two types of abnormalities were observed in animals exposed to CPF. These were mild lateral flexure of the tail (MLFT), consisting of less than 45° bending relative to the longitudinal axis, and severe lateral flexure of the tail (SLFT) consisting in a 90° bending (Table 2). They were more frequently recorded for SD25 (MLFT: 82%, SLFT: 39%) than for SD31 (MLFT: 51%, SLFT: 0.08%).

We observed that CPF decreased SVL (see Supplementary

Table 2
Lethal and sublethal evaluated endpoints on two stages of development (SD) and their corresponding lethal and effective concentration (LC-50 and EC-50, respectively), and LOEC values in mg/L of each test (T) at different times (in hours). M: mortality; SA: swimming alterations; MLFT: mild lateral flexure of the tail; SLFT: severe lateral flexure of the tail.

SD	Endpoint	Test	Time	LC/EC-50	LOEC
25	M	T1	96	0.185	0.100
25	M	T2	96	0.173	0.100
25	M	T1	240	0.013	0.010
25	M	T2	240	0.007	0.010
25	SA	T1	96	0.02	0.025
25	SA	T2	96	0.02	0.025
25	SA	T1	240	–	0.025
25	SA	T2	240	–	0.025
25	MLFT	T1	96	0.128	0.075
25	MLFT	T2	96	0.067	0.05
25	MLFT	T1	240	0.021	–
25	MLFT	T2	240	0.011	0.01
25	SLFT	T1	96	0.112	–
25	SLFT	T2	96	–	–
25	SLFT	T1	240	0.024	–
25	SLFT	T2	240	–	–
31	M	T3	96	0.121	0.075
31	M	T4	96	0.267	0.2
31	M	T3	240	0.102	0.075
31	M	T4	240	0.112	0.075
31	M	T3	336	0.100	0.075
31	M	T4	336	0.029	0.025
31	SA	T3	96	0.069	0.025
31	SA	T4	96	0.027	0.025
31	SA	T3	240	0.066	–
31	SA	T4	240	–	–
31	SA	T3	336	0.065	–
31	SA	T4	336	–	–
31	MLFT	T3	96	0.219	–
31	MLFT	T4	96	–	–
31	MLFT	T3	240	–	–
31	MLFT	T4	240	0.150	–
31	MLFT	T3	336	–	–
31	MLFT	T4	336	–	–

Figure 2). From SD25, there were significant differences between concentration (C) 0.4 mg/L CPF (= C8) and the water control group at 96 h (T1: $F(8, 118) = 7.314$, $p < .0005$; assumptions: $F = 1.745$, $p = .095$; $W = 0.927$, $p < .0005$) and concentration 0.3 mg/L (= C7) and the water control group at 240 h (T1: $H(7, 96) = 17.589$, $p = .014$; assumptions: $F = 3.934$, $p < .0005$; $W = 0.990$, $p = .729$). No significant differences were found for T2 (assumptions: T2-96 h: $F = 1.581$, $p = .141$; $W = 0.970$, $p = .009$; T2-240 h: $F = 4.419$, $p = .050$; $W = 0.971$, $p = .542$). However, correlation analysis for both tests (T1, T2) showed a negative correlation between concentration values and SVL (T1-96 h: $r_s = -0.476$, $p < .05$; T2-240 h: $r = -0.570$, $p = .007$). Moreover, analysis performed for SD31 showed significant differences at 96 h (T3: $F(7, 152) = 3.253$, $p = .003$) but only between higher (0.25, 0.3, 0.5 and 0.7 mg/L CPF) and lower (0.075, 0.15 and 0.2 mg/L CPF) concentrations (Fisher's test). At 336 h, significant differences in SVL were found between 0.5 mg/L CPF (= C7) and 0.7 mg/L CPF (= C8) with water control group (T3: $H(8, 111) = 17.558$, $p = .025$). Assumptions for each test were corroborated: T3-96 h: $F = 1.681$, $p = .118$; $W = 0.981$, $p = .028$; T3-336 h: $F = 2.393$, $p = .020$; $W = 0.981$, $p = .197$. Moreover, there was a negative correlation between concentration values and SVL (T3-96 h: $r_s = -0.327$, $p < .05$; T3-336 h: $r = -0.361$, $p < .0005$). Neither significant differences nor correlations were evident for T4.

Regarding the possibility of the negative progression of effects, chi-square tests between treatments and the presence/absence of progression showed significant differences for SD25 at 96 h (T1: $X^2 = 40.255$, $df = 8$, $p < .0005$; T2: $X^2 = 78.116$, $df = 8$, $p < .0005$) and 240 h (T1: $X^2 = 41.114$, $df = 8$, $p < .0005$; T2: $X^2 = 24.545$, $df = 8$, $p < .0005$), and for SD31 at 96 h (T3: $X^2 = 21.217$, $df = 7$, $p = .004$; T4: $X^2 = 40.847$, $df = 8$, $p < .0005$) and 336 h (T3: $X^2 = 31.668$, $df = 7$, $p < .0005$; T4: $X^2 = 16.364$, $df = 8$, $p = .038$). Therefore, there is an association between treatments and the presence/absence of progression. In the same way, the chi-square test performed between treatments and all states (without classifying them in presence/absence of progression) showed significant differences for SD25 at 96 h (T1: $X^2 = 220.085$, $df = 72$, $p < .0005$; T2: $X^2 = 254.091$, $df = 56$, $p < .0005$) and 240 h (T1: $X^2 = 265.896$, $df = 72$, $p < .0005$; T2: $X^2 = 77.071$, $df = 32$, $p < .0005$), and for SD31 at 96 h (T3: $X^2 = 162.583$, $df = 35$, $p < .0005$; T4: $X^2 = 123.718$, $df = 40$, $p < .0005$) and 336 h (T3: $X^2 = 185.336$, $df = 36$, $p < .0005$; T4: $X^2 = 68.605$, $df = 40$, $p = .003$). Hence, both variables are dependent, showing a progression of negative effects. In this sense, a positive correlation was found between the frequency of states that indicate a progression of negative effects (0, 1, 2, 3, 4) and the logarithm of the corresponding concentration where those effects appear, both for SD25 (T1-96 h: $r = 0.832$, $p = .005$; T1-240 h: $r = 0.795$, $p = .010$; T2-96 h: $r = 0.942$, $p < .0005$) and SD31 (T3-96 h: 0.809, $p = .015$; T3-336 h: $r = 0.945$, $p < .0005$; T4-96 h: $r = 0.876$, $p = .002$; T4-336 h: $r = 0.847$, $p = .004$). Note that normality of data was previously corroborated before performing previous tests (T1-96 h: $W = 0.897$, $p = .237$; T3-96 h: $W = 0.968$, $p = .879$; T3-336 h: $W = 0.983$, $p = .975$; T4-96 h: $W = 0.887$, $p = .187$; T4-336 h: $W = 0.836$, $p = .052$).

3.3. Effects of CFP on novel bioacoustic variables of sounds emitted by C. ornata tadpoles

Comparison of bioacoustic variables for larvae from SD25 and SD31 in the water control and dilution control groups did not show significant differences for 96 h or 240 h (see Supplementary Table 1), so we did not detect any effects of the ethanol vehicle. Exposure to CPF affected sounds in SD25 and SD31 tadpoles (see Supplementary Material). Shown in Fig. 1 are typical oscillograms and sonograms generated from recording tadpole sound emissions.

From SD25 (Fig. 2), significant differences were found at 240 h between water control group and treatments, considering sound duration (Sd: $H(2, 66) = 26.953$, $p < .0005$; $F = 4.042$, $p < .05$; $W = 0.928$, $p < .005$), number of pulses (Np: $H(2, 66) = 22.877$, $p < .0005$; $F = 3.783$, $p < .05$; $W = 0.935$, $p < .005$), and dominant frequency (Df: $H(2, 66) = 14.886$, $p < .005$; $F = 8.917$, $p < .0005$; $W = 0.620$, $p < .0005$). These analyses revealed shorter duration and higher frequency of sounds for CPF-exposed groups. Analysis performed between bioacoustic variables and concentration values showed a negative correlation ($p < .05$) for Sd ($r_s = -0.637$) and Np ($r_s = -0.574$), and a positive correlation ($p < .05$) for Df ($r_s = 0.478$). Note that no effects were found in sounds emitted at the end of acute exposure (= 96 h).

From SD31, sounds emitted by tadpoles at the end of acute exposure to CPF showed a decreased in the number of pulses. Significant differences were found in Np ($H(8, 301) = 29.078$, $p < .0005$; $F = 4.596$, $p < .0005$; $W = 0.905$, $p < .0005$) between water control and both 0.025 mg/L (= C1) and 0.5 mg/L (= C7) CPF groups (Fig. 3). Analysis performed between bioacoustic variables and concentration values showed a negative correlation for Np ($r_s = -0.161$, $p < .05$). Moreover, significant differences were found at the end of chronic exposure (= 336 h) between water control and different treatments considering Sd ($H(8, 253) = 58.796$, $p < .005$; $F = 2.170$, $p < .05$; $W = 0.970$, $p < .0005$), Np ($H(8, 253) = 97.179$, $p < .005$; $F = 3.884$, $p < .0005$; $W = 0.957$, $p < .0005$), and Df ($H(8, 253) = 37.765$, $p < .005$; $F = 7.652$, $p < .005$; $W = 0.903$, $p < .0005$). Analysis performed between bioacoustic variables and concentration values showed a negative correlation ($p < .05$) for Sd ($r_s = -0.446$) and Np ($r_s = -0.584$), and a positive correlation for Df ($r_s = 0.331$).

Correlation analysis performed between sublethal conventional variables (SA, MLFT, SLFT, and SVL) and bioacoustic variables (Ds, Np, and Df) are shown in Table 3. Also, cross-correlations between each sublethal conventional variable were performed (Table 3). From this, the following pattern emerges: as the length of each individual decreases, the proportion of individuals with swimming alterations and abnormalities increases, the duration and number of pulses of sounds emitted by those individuals decreases, while the dominant frequency increases. These results indicate that tadpoles exposed to CPF exhibited an increase of negative effects.

4. Discussion

To our knowledge, this is the first investigation using *C. ornata* to assess the effects of an environmental contaminant. We studied CPF, one of the most frequently used insecticides in Argentina (Cappello and Fortunato, 2013) that contaminates aquatic ecosystems (Marino and Ronco, 2005; De Gerónimo et al., 2014), with a high persistence in water and sediment (Watts, 2012). We are proposing that *C. ornata* may be a relevant test species for the environmental conditions of the extensive Pampas regions of Argentina, Uruguay and Brazil. This is the natural range of the species and where CPF is heavily used. The population status of *C. ornata* is 'Near Threatened' according to IUCN (IUCN, 2017), but was raised to "vulnerable" considering last assessment of amphibians for Argentina (Natale and Salgado Costa, 2012). This presents two issues: (1) it may be sensitive to other organophosphate insecticides frequently used in the Pampas, which could explain some aspects of the declining population status, and (2) eggs or wild tadpoles should therefore not be collected for ecotoxicological testing. However, this species does very well in captivity: it is easy to breed using our established hormone induction protocol (Trudeau et al., 2010), and importantly for ecotoxicology, it exhibits extremely rapid morphological development, high reproductive potential, and tadpole, juveniles and adult care is relatively easy

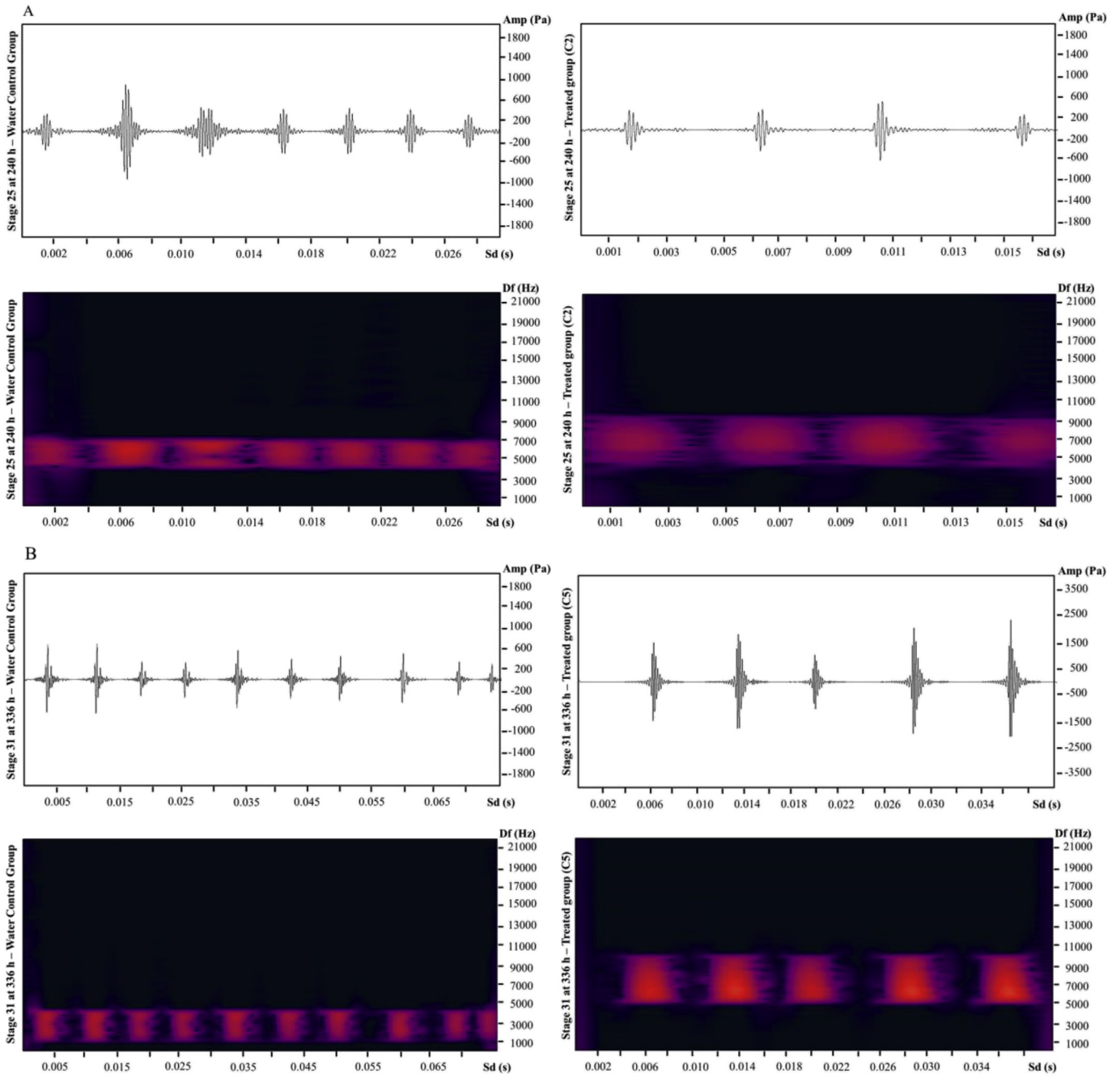

Fig. 1. Oscillograms, sound duration in seconds (Sd, s)/amplitude in pascals (Amp, Pa) and sonograms, sound duration in seconds/dominant frequency in Hertz (Df, Hz) of sounds emitted by tadpoles of water control group (no CPF) and treated groups (C2: 0.025 mg/L CPF; C5: 0.25 mg/L CPF) from stage 25 (A) and 31 (B). Each graph corresponds to a sound emitted by a single tadpole of a particular replicate (= chamber).

and inexpensive (Salgado Costa, 2016). Moreover, the relatively large size of the tadpoles provides ample material to develop biochemical studies identifying toxic modes of action of chemicals. All these characteristics meet the criteria proposed by Segner and Baumann (2016) and indicate that *C. ornata* is an amenable model species relevant to South America. Moreover, *C. ornata* is the first documented vertebrate with underwater acoustic communication by larvae (Natale et al., 2011). Here we use this characteristic to explore disruption of tadpole sound production by CPF. Acoustic communication is an ecologically-relevant behavior because tadpoles in the genus *Ceratophrys* (*C. aurita*, *C. cranwelli*, *C. ornata*) emit sounds as part of an antipredator mechanism (Salgado Costa, 2016; Natale et al., 2011). Until now, the only other vertebrate known to produce larval sounds is the tadpole of a Madagascar mantellid frog (*Gephyromantis azzurrae*) during competitive feeding (Reeve et al., 2011). However, underwater acoustic communication has also been described in the adult stage of a variety of marine mammals, anurans, fishes, and crustaceans (Duellman and Trueb, 1994; Myrberg, 1997; Popper et al., 2001; Ladich and Bass, 2003; Ladich, 2015). Also, acoustic communication is well established in terrestrial vertebrates and invertebrates such as mammals, birds, reptiles, anurans, and insects (Drosopoulos and Claridge, 2005; Suthers et al., 2016), so sound production is a generalizable trait across diverse taxa and beyond amphibians.

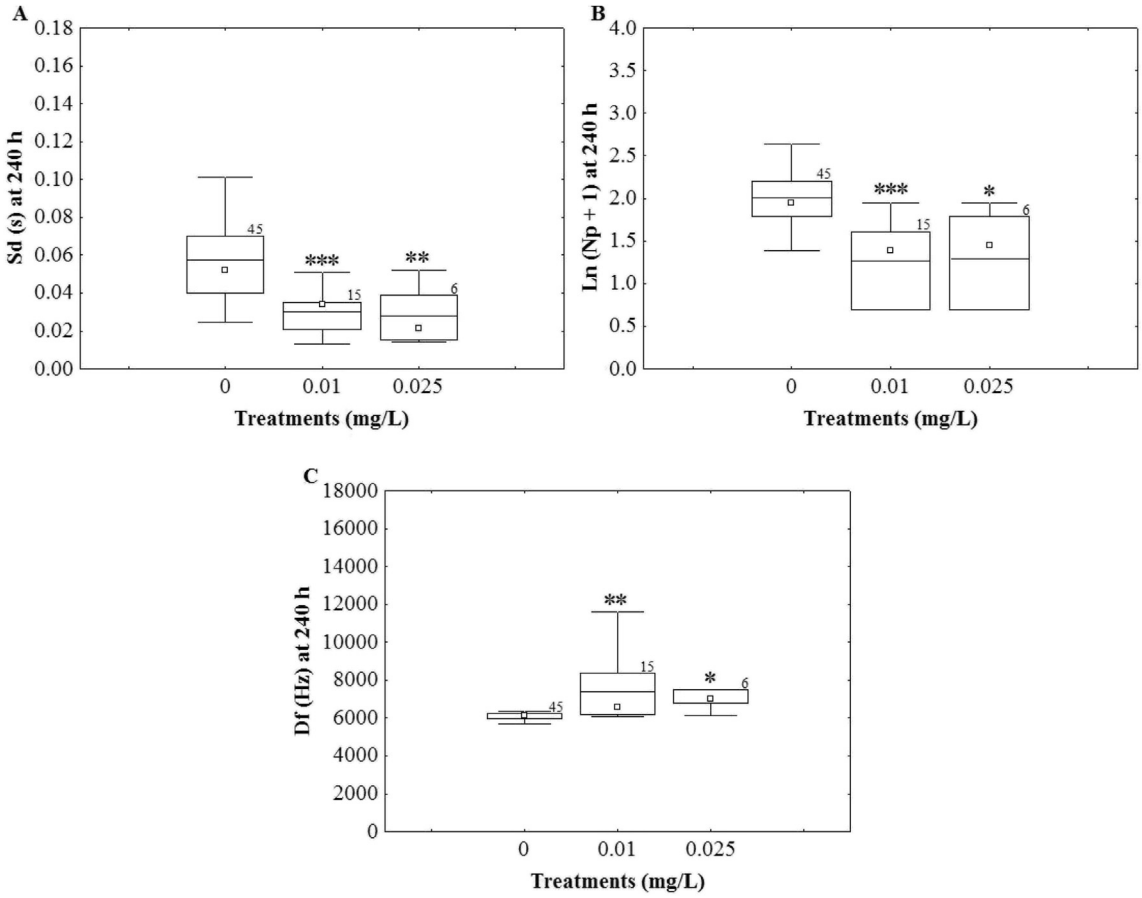

Fig. 2. Box (median ± percentiles) and whisker (non-outlier range) plots showing significant differences between water control group (0) and treatments (in mg chlorpyrifos/L) considering: (A) sound duration (Sd) in seconds, (B) number of pulses (Np, variable previously transformed) and (C) dominant frequency (Df) in Hertz, of sounds emitted by tadpoles from stage 25 at 240 h of exposure. Each treatment summarizes the characteristics of three sounds emitted by each of the 15 larvae (= replicates per treatment). The number of data per treatment is indicated next to the box. Median = point; mean = line; *p < .05; **p < .005; ***p < .0005.

Here we report on the dose-response effects of CPF on typical ecotoxicological endpoints such as mortality, swimming alterations, presence of abnormalities (LFLC and SFLC) and growth inhibition, and additional effects on key bioacoustic variables (Sd, Np, Df) at two developmental stages of *C. ornata*. Considering lethal endpoints, we found that *C. ornata* is more sensitive (= 67%) than other native species and most non-native species (= 75%) noted in the published literature. When considering the LC-50 data for SD25 tadpoles at 96 h of exposure we found that *C. ornata* is close to the 29th percentile of published anuran sensitivities (Abbasi and Soni, 1991; Cowman and Mazanti, 2000; Richards and Kendall, 2002; El-Merhibi et al., 2004; Kerby, 2006; Yin et al., 2009; Bernabò et al., 2011; Ruiz de Arcaute et al., 2012). Chlorpyrifos negatively impacted all common sublethal endpoints (SA, MLFT, SLFT, SVL) we measured, and *C. ornata* was more sensitive that 50% of other native species and 100% of non-native species for which we could find data. When considering the EC-50 data for SD25 tadpoles at 96 h of exposure we found that *C. ornata* is close to the 12th percentile of published anuran sensitivities (Richards, 2000; Richards and Kendall, 2002; Wacksman et al., 2006; Ruiz de Arcaute et al., 2012). Particularly, this species is useful for evaluating swimming alterations (behavioral endpoint), which is a good indicator of other effects. Since a total absence of abnormalities was recorded in control groups, it can be concluded that they are specific responses to CPF exposure as reported by other authors (Bernabò et al., 2011; Sotomayor et al., 2012). Moreover, the EC-50 we calculated for *C. ornata* tadpoles at SD25 is within the 95% confidence interval of the HC5 of CPF.

Effects on sounds were evident on SD25 tadpoles at concentrations 20 times lower (0.01 mg/L CPF) than the LC-50 at 96 h, and 2 and 10 times lower than the EC-50 at 96 h considering swimming alterations (0.02 mg/L) and mild lateral flexure of the tail (0.1 mg/L). Moreover, taking into account LC-50/EC-50, LOEC values and correlation analysis performed to evaluate the existence of a progression of negative effects, it can be concluded that the effects on sound production appear along with swimming alterations followed by the presence of abnormalities (MLFT, SLFT), and lastly mortality. In the same sense, when comparing effects on tadpoles from the same stage between acute and chronic exposure (considering all evaluated lethal and sublethal endpoints), it can be concluded that tadpoles get worse (See Supplementary Table 2); also, younger tadpoles (SD25) are more sensitive to CPF than older ones (SD31) based on LC-50/EC-50 values (Table 2). Therefore, effects on sounds are a very good sublethal endpoint and a promising possible biomarker considering that a good biomarker should be specific and detectable early in the response (Handy et al., 2003; Denoël et al., 2012), effects on sound production in tadpoles meets numerous criteria.

The CPF concentrations that induce sublethal effects in *C. ornata* tadpoles in the laboratory are within the range of levels measured in local environments (Jergentz et al., 2005; Marino and Ronco, 2005; Mugni et al., 2011). Specifically, the highest concentration measured in water is within the 95% confidence interval of the EC-50 for CPF that induced swimming alterations at SD25 (96 h) and

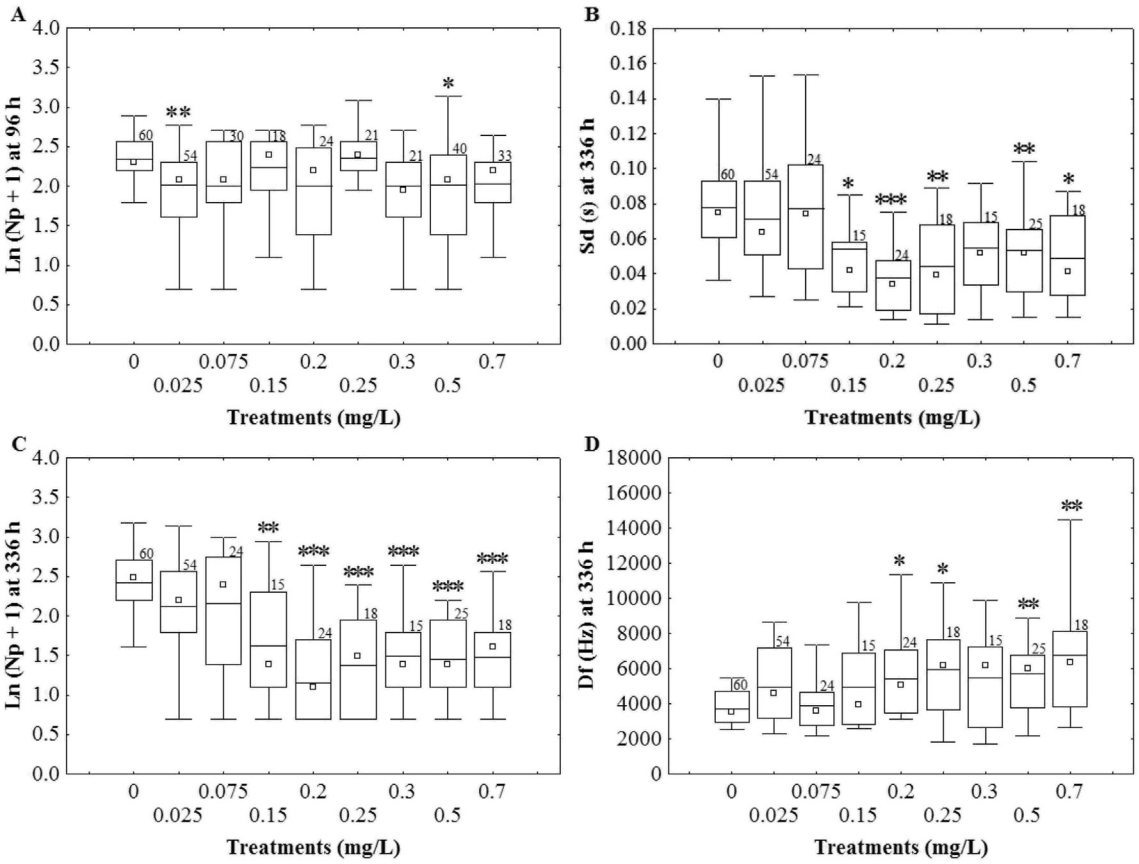

Fig. 3. Box (median ± percentiles) and whisker (non-outlier range) plots showing significant differences between water control group (0) and treatments (in mg chlorpyrifos/L) considering sounds emitted by tadpoles from stage 31 at 96 h (A) and 336 h of exposure (B–D). Np: number of pulses, variable previously transformed; Sd: sound duration in seconds; Df: dominant frequency in Hertz. Each treatment summarizes the characteristics of three sounds emitted by each of the 20 larvae (= replicates per treatment). The number of data per treatment is indicated next to the box. Median = point; mean = line; *p < .05; **p < .005; ***p < .0005.

Table 3
Correlation analysis performed between bioacoustic (Sd, Np, Df) and conventional ecotoxicological endpoints (SA, MFLT, SLFT, SVL), and cross-correlations performed between each sublethal conventional variable, considering two tests (T2, T3) under acute and chronic exposure. Provided values are the Spearman rank correlation coefficients. SD: stage of development; Sd: sound duration; Np: number of pulses; Df: dominant frequency; SA: swimming alterations; MLFT: mild lateral flexure of the tail; SLFT: severe lateral flexure of the tail; SVL: snout-to-vent length; *p < .05; **p < .005.

		Sd	Np	Df	SA	MLFT
T2 SD25 96 h	SA	−0.301**	−0.239**	0.161**	–	0.645**
	MLFT	−0.204**	−0.130*	0.172**	0.645**	–
	SLFT	−0.247**	−0.244**	0.232**	0.558**	0.535**
	SVL	0.220**	−0.315**	−0.359**	−0.092	0.073
T2 SD25 240 h	SA	−0.577**	−0.575**	0.431**	–	0.922**
	MLFT	−0.479**	−0.500**	0.388**	0.922**	–
	SLFT	−0.364**	−0.427**	0.265*	0.870**	0.899**
	SVL	0.174	−0.100	−0.302*	−0.216	0.190
T3 SD31 96 h	SA	−0.078	−0.084	0.029	–	0.287**
	MLFT	−0.036	−0.012	0.013	0.287**	–
	SLFT	−0.040	−0.016	0.014	0.232**	0.912**
	SVL	0.029	−0.358**	−0.159*	−0.174**	0.032
T3 SD31 336 h	SA	−0.386**	−0.519**	0.241**	–	0.526**
	MLFT	−0.057	−0.158*	0.052	0.526**	–
	SLFT	−0.016	−0.127*	0.044	0.495**	0.924**
	SVL	0.198**	−0.264**	−0.161	−0.227**	0.154*

affected sound production at SD25 (240 h). Also, the highest concentration measured in suspended particles (Jergentz et al., 2005) is within the 95% confidence interval of the LC-50 for SD25 tadpoles, and is an order of magnitude higher than the concentrations that induce swimming alterations. Such high concentrations (Jergentz et al., 2005) are within the 95% confidence interval of the EC-50 for abnormalities in tadpoles from SD25 and SD31, and is 22 times higher than the minimum concentration that induces effects in sounds from SD25.

The sounds emitted by *C. ornata* tadpoles are part of an anti-predator mechanism that diminishes the frequency of predation between conspecifics in the presence of prey (Salgado Costa, 2016), and locomotion is a behavior critical to numerous survival strategies (Duellman and Trueb, 1994). Therefore, it appears that low, environmentally-relevant CPF concentrations are disrupting key behaviors in *C. ornata*. If sound emission plays an important role in population dynamics and survival, future studies should evaluate predator-prey interactions in the presence of CPF and other pesticides.

5. Dedication

We dedicate this article to the memory of our colleague and friend Professor Alicia E. Ronco, who committed her life to studying the environment and unfortunately passed away suddenly before the completion of this work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2017.12.096.

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