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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).