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Effects of Chlorpyrifos Over Reproductive Traits of Three Sympatric Freshwater Crustaceans

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Abstract
The exposure to environmentally relevant chlorpyrifos concentrations (0.03, 0.06 and 0.12 µg chlorpyrifos L^{-1}) causes increases in precopulatory guardian behavior time, amplexus reformulation after exposure and in the number of ovigerous females in the amphipod *Hyalella curvispina*. Effects in incubation period, effective hatching and median lethal concentration on the decapods *Macrobrachium borellii* and *Aegla uruguayana*, both in adults and embryos, were achieved at higher concentrations than those found in the environment. Environmentally relevant chlorpyrifos concentrations appear not to affect decapods but several effects in reproductive traits of amphipods were observed.

Keywords Amphipods · Decapods · Reproduction effects · Pesticides

Chlorpyrifos is an organophosphate pesticide widely used in agricultural activities to minimize the damage produced by pests (Crane et al. 2003). Their mode of action is related with the inactivation of acetylcholinesterase (AChE), causing hyperstimulation of nicotinic and muscarinic receptors, disrupting neurotransmission and finally leading to death (Ghedira et al. 2009). After applications, the pesticides migrate to nearby aquatic ecosystems, mainly by run-off after rain events, and the biota inhabiting in those ecosystems are periodically exposed to xenobiotic inputs (Williman et al. 2017; Etchegoyen et al. 2017).

The crustaceans are a zoological group widely represented in freshwater ecosystems. They occupy an intermediate position in aquatic foodwebs and promote matter and energy exchange between lower and upper links. Amphipod crustaceans are phytobenthos and detritus feeders, and play a key role in nutrient cycling and an important link in food webs as a prey of several vertebrates, such as fishes, amphibians and birds (Saigo et al. 2009). Decapod crustaceans are active predators and detritus feeders, and are an important food source for fish, reptiles, birds, and mammals, even human beings (Carvalho et al. 2016).

Amphipods and decapods are a common part of freshwater aquatic biota. In crop-related areas, they are periodically exposed to xenobiotics, which could produce various effects over them (Gallardo et al. 2019). Beyond lethality, which is the easiest effect to observe, pesticides and other anthropogenic compounds may produce several sublethal effects in reproduction traits (Reduction in mating effectiveness, in the egg number, in effective hatching, in offspring survival, among others), reducing the number of new individuals and eventually the population (De Lange et al. 2006; Lebrun et al. 2020). Effects in reproduction achieved at lowest and environmentally relevant concentration affects in turn populations, community and matter and energy fluxes in the ecosystem (Newman and Unger 2003).

Several effects are caused by chlorpyrifos exposure, as mortality, intoxication, effects in growth, in locomotion, in development, at histological and physiological levels, among others (Huang et al. 2020). However, there are few records of the effects of chlorpyrifos especially in reproductive traits of freshwater crustaceans achieved at sublethal concentrations (Negro et al. 2014; Huang et al. 2020). The objectives of this work were to observe the effects in precopulatory guardian behavior and reproductive effectiveness caused by environmentally relevant sublethal chlorpyrifos concentrations in the amphipod crustacean *Hyalella curvispina*, and in incubation period and effective embryo hatching in the decapod

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crustaceans *Macrobrachium borellii* and *Aegla uruguayana* as crustacean models.

Methods and Materials

Three environmentally relevant chlorpyrifos concentrations (0.03, 0.06 and 0.12 µg chlorpyrifos L^{-1}) were chosen according to the ones found in aquatic ecosystems of the pampean region (Argentina) (Mugni et al. 2012; Williman et al. 2017; Etchegoyen et al. 2017). The effects of chlorpyrifos in precopulatory guardian behavior, i. e. when amphipod males carry their mates until the females are ready to molt and be fertilized (amplexus) (Conlan 1991), and reproductive effectiveness were determined in the amphipod *H. curvispina* because of the natural prolonged precopulary amplexus and the easy maintenance of ovigerous females. The amphipods were obtained from a culture of the Instituto Nacional de Limnología. They were maintained in 130 L aquaria at 25 ± 1 °C, with aquatic plants as substrate and food (*Eichhornia crassipes, Salvinia biloba* and *Pistia stratiotes*), and fish food (Shulet®) was added 3 times a week. Effects in precopulatory guardian behavior were evaluated by exposing the precopulatory pairs to environmentally relevant chlorpyrifos concentrations for 24 h, observing the number of paired/unpaired precopulatory amplexus at 2, 4, 6, 8 and 24 h. The amphipod precopulatory pairs were placed in cylindrical clear plastic (biaxially oriented polystyrene) containers with 50 mL of dechlorinated water (control) or pesticide solutions. PVC tubes (10 mm long, 16 mm diameter) were added as shelters. Forty precopulatory pairs were exposed in every concentration, including control.

In order to observe the reversibility of the chlorpyrifos effects in time, after the acute exposure all the couples (paired and unpaired) were transferred to 400 mL plastic beakers filled with 200 mL of dechlorinated water and plant pieces (*Ceratophyllum demersum*). To evaluate the reformulation of precopulatory pairs, the unpaired individuals were observed at 2, 4, 6, 24 and 48 h. After that time, males were removed (all the amplexus were unpaired) and females were maintained in order to observe the number of ovigerous females (mating effectiveness). They were fed with fish food (Shulet®) *ad libitum* and half of the dechlorinated water was renewed every 48 h. Females were observed at initial time and every day for the next 8 days. The number of ovigerous/non ovigerous females was recorded.

The effects of chlorpyrifos sublethal concentrations in incubation period and effective hatching were evaluated in the anomuran crab *A. uruguayana* and in the prawn *M. borellii,* because they have big eggs which are easier to manipulate and observe. Ovigerous females were collected in El Espinillo river (31° 47′ S 60° 18′ O; Entre Ríos, Argentina), away from cities and crop areas. They were transported to the laboratory, where they were maintained at 25 ± 1 °C and they were fed ad libitum with fish muscle from fishes obtained from streams and rivers related with the middle Paraná River, until the embryos reached a stage of development in which the eye pigmentation and heartbeats were observed. Once in this stage, the eggs were softly removed from the female abdomen with a brush and placed in 6 mL clear plastic (biaxially oriented polystyrene) containers filled with 5 mL of pesticide solution or dechlorinated water (control), following the techniques previously described in Negro et al. (2014). In total, five concentrations were tested, three environmentally relevant concentrations (0.03, 0.06 and 0.12 µg chlorpyrifos L^{-1}) and two related with their LC_{50} (1/100 and 1/10 of their LC_{50}). Thirty individual in a stage of development in which eye pigmentation and heartbeat were observed, were used in each concentration, including control. The containers were placed in a rocker shaker at 40 rpm inside a 25 ± 1 °C incubator with a 24 h dark cycle. Pesticide solutions and dechlorinated water (control) were renewed at 24 h exposure intervals. Before the solution renewal the embryos were observed with a stereoscopic microscope, and the dead organisms, if any, were counted and removed. The criterion of death was the absence of heartbeat. Assays were performed until embryos hatch.

As there are no records of lethal effects of chlorpyrifos in *A. uruguayana* and *M. borellii*, we calculated the median lethal concentration in order to observe the relation between the LC_{50} and the environmentally relevant chlorpyrifos concentrations, both in adults and embryos. In adults, assays were performed based on the standardized 96-h toxicity test (USEPA 2002). The size of the anomuran crabs used was 27.45 (± 2.08) (mean ± SD) mm. The mean carapace width of the prawns was 21.26 (± 1.41) mm. The largest individuals were less than 1.5 times larger than the smallest individual. Ten individuals were placed in 5 L aquaria filled with 3 L of dechlorinated water (control) or experimental solutions (LC-C series) (Table 1). Three replicates of each concentration were used (n = 30 individuals per concentration). The assays were performed at 25 ± 1 °C with a 12:12 light/darkness photoperiod. The solutions were renewed at 24 h exposure intervals. Before the solution renewal, the dead organisms were counted and removed. The criterion of death was the absence of movement after stimulation. The animals were not fed during the assays.

In 96-h embryo toxicity tests, individuals with eye pigmentation and noticeable heartbeat were used in each concentration, including control. Ten individuals were placed in a Petri dish with dechlorinated water (control) or pesticide solutions (Table 1). Three replicates of every treatment were made. Pesticide solutions and dechlorinated water were replaced daily. The embryos were observed under a stereoscopic microscope. Dead individuals were counted

Table 1 Chlorpyrifos concentrations (nominal and measured) used in LC_{50} tests (LC-CX) of adults and embryos and sublethal concentrations (1/100–1/10 LC50) used in embryos

Chlorpyrifos concentrations (μg L^{-1})					
LC_{50} tests					
Adults	*A.uruguayana*		*M. borellii*		
	Nominal	Measured		Nominal	Measured
LC-C1	0.192	0.1	LC-C1	1.28	1.2
LC-C2	0.272	0.2	LC-C2	1.6	1.5
LC-C3	0.536	0.5	LC-C3	1.92	1.8
LC-C4	1.097	0.9	LC-C4	2.4	2.2
LC-C5	1.568	1.4	LC-C5	3.05	2.9
LC-C6	3.194	3.1	LC-C6	4.8	4.7
LC-C7	4.571	4.4			
Embryos	Both species		Sublethal concentrations		
LC-C1	4800	4567	*A.uruguayana*	Nominal	Measured
LC-C2	9600	9145	1/100 LC50	257.97	223.8
LC-C3	19200	17730	1/10 LC50	2579.7	2354.6
LC-C4	36000	34672	*M. borellii*		
LC-C5	72000	70232	1/100 LC50	319.83	286.3
LC-C6	144000	137495	1/10 LC50	3198.3	2786.5

and removed. The criterion of death was the absence of heartbeat.

The pesticide product tested was Clorpi® (Red Surcos, Argentina), a commercial product containing 48% of chlorpyrifos. All the solutions were prepared the day they were used. Chlorpyrifos concentrations were measured by gas chromatography fitted with a standard electron capture and flame photometric detectors, according to Goncalvez and Alpendurada (2002), with minor modifications. Pesticide concentrations were renewed daily and measured by duplicate at initial time (one time only for every concentration). As we always used the same methodology, we assume that the concentrations were similar in all days. It was suggested that a constant pesticide concentration in test solution could be kept during the exposure by the method of daily renewal (Li et al. 2006). Of the environmentally relevant concentrations only C3 was measured (nominal: 0.12 μg chlorpyrifos L^{-1}; measured: 0.1 μg chlorpyrifos L^{-1}) C1 and C2 were not measured because they were below the detection limit (0.1 μg L^{-1}).The concentration ranges used in the acute toxicity tests were determined after several range finding tests. A probit analysis was used to estimate the LC_{50} and the 95% confidence limits, based on measured concentrations, with Abbot's correction for mortality control. The differences in the LC_{50} were considered to be significant when the higher LC_{50}/lower LC_{50} ratio exceeded the critical value. Kruskal–Wallis tests followed by Dunn's method were performed to determine the significant effects of chlorpyrifos in incubation period. The pesticide effects on the percentage of effective hatching of decapods, the disruption of the precopulatory amplexus, in the reformulation of precopulatory pairs and in the number of ovigerous females in amphipods were determined using the chi-square method ($p < 0.05$) (Zar 1996).

Results and Discussion

The water quality did not significantly vary during the tests. The temperature, dissolved oxygen, pH and conductivity were 25 ± 1 °C, 6.48 ± 1.37 mg L^{-1}, 7.04 ± 0.86 and 1148.68 ± 34.47 μS cm^{-1}, respectively. The results of the 96-h toxicity tests showed that in both species the embryos were more resistant than adults ($p < 0.05$). Comparing both species, there were no significant differences at the embryo stage, but at adult stages *A. uruguayana* was more sensitive to chlorpyrifos than *M. borellii* ($p < 0.05$) (Table 2).

In some species, such as the crabs *Eriocher sinensis*, the embryonic stage is sensitive, with a median chlorpyrifos lethal concentration lower than adults (Li et al. 2006). However, in some crustacean species resistance to pesticides is higher in embryos than in adults, as was observed in the freshwater crab *Zilchiopsis collastinensis* exposed to chlorpyrifos and endosulfan (Negro et al. 2014), in the grass shrimp *Palaemonetes pugio* exposed to endosulfan (Key et al. 2003) and in this case. Low embryo toxicity could be explained by the presence of the embryonic coat, which partially isolates them from the environment and helps to

Table 2 Median lethal concentrations and 95% confidence limits of chlorpyrifos in adults and embryos of *Aegla uruguayana* and *Macrobrachium borellii*

Median lethal concentration (ug L^{-1})						
	A.uruguayana			*M. borellii*		
	LC_{50}	Lower	Upper	LC_{50}	Lower	Upper
Adults	1.4	1.1	1.8	1.8	1.7	1.9
Embryos	25797.4	15352.5	36752.1	31983.2	15714.7	66866.5

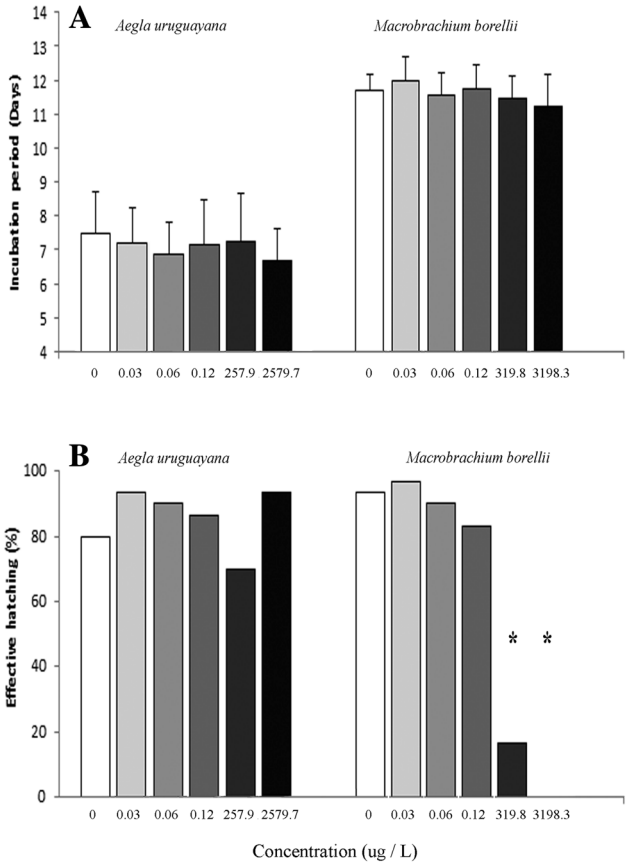

Fig. 1 **a** Incubation period (mean value ± SD) and **b** effective hatching (%) of *Aegla uruguayana* and *Macrobrachium borellii* exposed to sublethal concentrations of chlorpyrifos. n = 30 individuals by species and concentration. C4: 1/100 of the LC_{50}, C5: 1/10 LC_{50}. *significant differences ($p < 0.05$) in the proportion of individuals that accomplished hatching, regarding C0 (Chi-square test)

protect the embryos during development from potentially harmful conditions, as pesticide presence (Glas et al. 1997; Key et al. 2003).

Environmentally relevant chlorpyrifos concentrations did not cause effects in incubation period and effective hatching of decapods (Fig. 1), as there were not significant differences between control and exposed crabs (although some concentrations were too low to be measured and are only expressed as nominal concentrations). Same results were observed in the crab *Z. collastinensis* (Negro et al. 2014). Also, the exposure to sublethal chlorpyrifos concentration (1/10 and 1/100 of the LC_{50}) did not cause effects on the incubation period of *M. borellii* and *A. uruguayana*. However, decreases in effective hatching were observed in *M. borellii* (Fig. 1). The effective hatching might be affected in *M. borellii* because in the final incubation stages the chorion becomes thinner, mediated by proteolytic enzymes released by the embryos, in order to facilitate the hatching (Glas et al. 1997). This thinner or broken chorion allows the embryo to contact the pesticide solutions, causing death mainly at the final stages or during the hatching. In the crab *Z. collastinensis* the exposure to 360 and 720 µg chlorpyrifos L^{-1} did not cause effects in incubation period but causes significant decreases in effective hatching (Negro et al. 2014), as observed in *M. borellii*.

On the other hand, amphipods were affected by environmentally relevant chlorpyrifos concentrations in different ways. Beyond our hypothesis, pesticide exposure did not cause the disruption of the precopulatory amplexus, as observed in *H. azteca* and *Gammarus pulex* exposed to different biocides (Malbouisson et al. 1994; Blockwell et al. 1998; Pedersen et al. 2013). There was a decay in precopulatory amplexus in amphipods of the control group through time, related with the natural end of the reproduction event, but precopulatory amplexus continues longer in the amphipods exposed to the two higher chlorpyrifos concentrations than in control and in those exposed to the lowest concentration groups ($p < 0.05$). Also, after the exposure there was an increase in the re-form of the precopuatory amplexus compared to the control group ($p < 0.05$) and the number of ovigerous females increase in the two higher concentrations ($p < 0.05$) (Fig. 2). In *G. pulex* and *H. azteca* exposed to a short pulse of the pyrethroids esfenvalerate and permethrin respectively, a decrease in re-pairing was observed and also amplexus re-form took longer in exposed than in control individuals (Cold and Forbes 2004; Pedersen et al. 2013). Chlorpyrifos is known to affect animals by the inhibition of acetylcholinesterases, causing hyper stimulation and erratic movements at lower doses and/or initial time (Montagna and Collins 2008; Gutierrez and Negro 2014). We hypothesized that the extended precopulatory guardian behavior might be related with the difficulties in mating caused by the uncoordinated movements. However, more studies are needed.

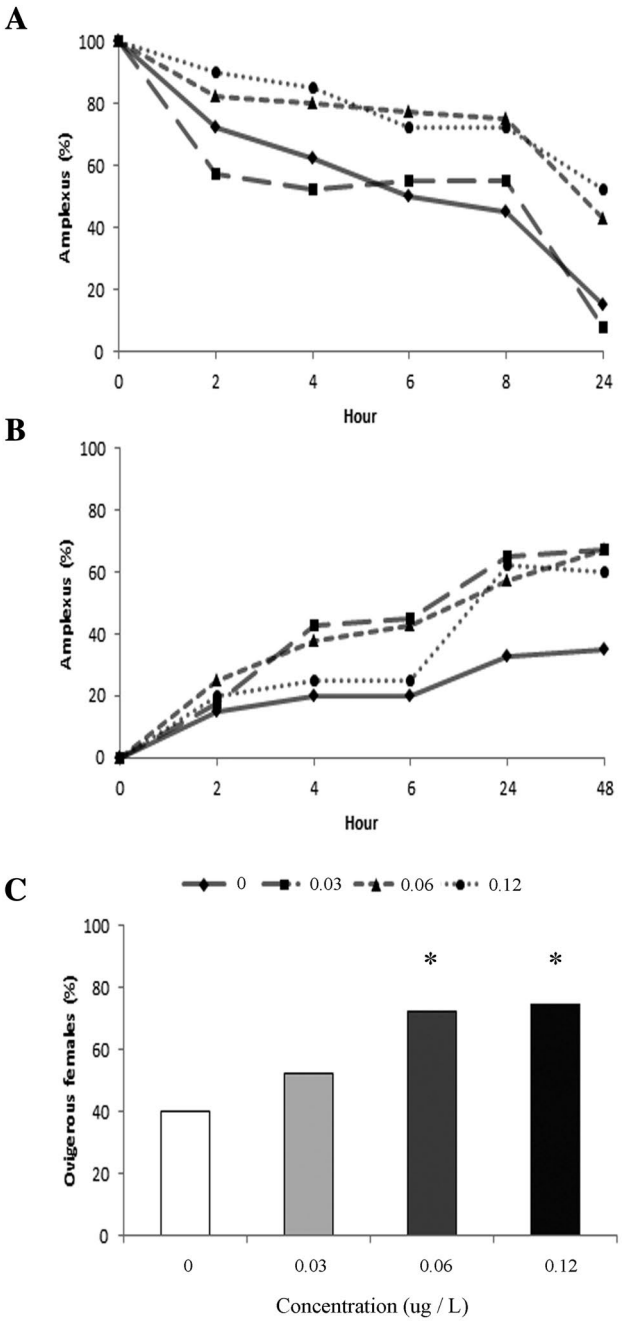

Fig. 2 a Amplexus decay (percentage) of *Hyalella curvispina* at different times and different chlorpyrifos concentrations. **b** Amplexus reform (percentage) of *Hyalella curvispina* at different times. **c** Ovigerous females (percentage) of *Hyalella curvispina* exposed to different chlorpyrifos concentrations. *significant differences ($p < 0.05$) in the proportion of ovigerous females regarding C0 (Chi-square test)

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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