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RESEARCH ARTICLE

Chemically induced plasticity in early life history of *Palaemon argentinus*: are chemical alarm cues conserved within palaemonid shrimps?

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ABSTRACT

Most aquatic animals use infochemicals from both conspecifics and heterospecifics to assess local predation risks and enhance predator detection. Released substances from injured conspecifics and other species (chemical alarm cues) are reliable cues to indicate an imminent danger in a specific habitat and often mediate the development of inducible defenses. Amphibian and fish embryos have been shown to acquire this information while at the embryonic stage of development, in relation to the developing nervous system and sensory development. With the exception of *Daphnia*, there is no information on chemically mediated responses to alarm cues in embryos of any crustacean groups. Therefore, we tested whether embryo exposure to chemical cues simulating predation on conspecifics or heterospecifics (closely related, non-coexisting species), or a mixture of both, alters embryonic developmental time, size and morphology of the first larval instar in *Palaemon argentinus* (Crustacea: Decapoda). Embryonic exposure to chemical alarm cues from conspecifics shortened the embryonic developmental time and elicited larger larvae with a longer rostrum. Rostrum length of the first larval instar changed independently of their size, thus elongated rostra can be considered a defensive feature. Embryonic developmental time was not altered by chemical alarm cues from either heterospecifics or the mixed cues treatment; however, exposure to these cues resulted in larger larvae compared with the control group. Chemically induced morphological plasticity in larvae in response to alarm cues from con- and heterospecifics suggests that such cues are conserved in palaemonids shrimps, providing embryos with an innate recognition of heterospecific alarm cues as predicted by the phylogenetic relatedness hypothesis.

KEY WORDS: Chemoreception, Embryo, Hatching, Induced defenses, Macro-crustacean, Predation risk

INTRODUCTION

The ability to detect and respond to changes in surrounding medium enables organisms to cope with environmental challenges and an early response during ontogeny plays a major role driving developmental plasticity (Braendle and Flatt, 2006; Burggren and Duvansky, 2018; Fusco and Minelli, 2010; Gilbert and Epel, 2009; Gilbert et al., 2015; Moczek, 2007; Pigliucci et al., 2006; West-Eberhard, 2003, 2005). Phenotypic plasticity, i.e. the ability of many organisms to produce different phenotypes with a given genome in response to environmental stimuli (Pigliucci et al., 2006; West-Eberhard, 2003), can be described as a chain process where sensory systems are the first step by which environmental information is acquired by the organism, and the phenotypic modification is the final product (DeWitt and Scheiner, 2004; Gazzola et al., 2015). Predator-induced phenotypic modification of prey is a classic example of phenotypic plasticity, including the so-called inducible defenses that are thought to improve the fitness of organisms despite its inherent tradeoffs (e.g. Auld et al., 2010; DeWitt et al., 1998; Gazzola et al., 2015; Weiss and Tollrian, 2018). The expression of inducible defenses in prey organisms is chemically mediated, i.e. chemical compounds or 'infochemicals' activate their development (e.g. Gilbert, 2005; Harvell, 1990; Tollrian and Harvell, 1999; Tollrian et al., 2015; van Buskirk et al., 2011; Weiss and Tollrian, 2018). These infochemicals mediate intra- and interspecific interactions between aquatic organisms, explicitly affecting internal physiological processes, which leads to the development of effective inducible defenses such as morphological and life-history trait modifications that reduce the risk of predation (e.g. Ferrari et al., 2010b; Gazzola et al., 2015; Herzog et al., 2016; Ocasio-Torres et al., 2014; Selander et al., 2015; von Elert, 2012; Weiss et al., 2018).

Prey organisms can perceive infochemicals about the presence of their predators through either direct or indirect sources (Dicke and Grostal, 2001; Ferrari et al., 2010b; Wisenden, 2015). Cues from direct sources consist of chemicals that are produced or acquired (e.g. Ringelberg and van Gool, 1998) by the predator and then recognized by the prey (e.g. kairomones), while cues from indirect sources are not produced by the predator (Dicke and Grostal, 2001). Prey-released cues from injured or freshly killed prey (i.e. chemical alarm cues) are believed to warn conspecifics and heterospecifics about high-risk areas where predation has recently occurred (e.g. active predators) (e.g. Chivers and Smith, 1998; Chivers et al., 1996; Shabani et al., 2008; Wisenden, 2015). All major groups of aquatic organisms, from protists to vertebrates have shown antipredatory responses to chemical cues released from injured conspecifics (e.g. Tollrian and Harvell, 1999; Ferrari et al., 2010b; Hazlett and McLay, 2005; Laforsch et al., 2006; Schaum et al., 2013; Schoeppner and Relyea, 2009; Spivey et al., 2015; Wisenden, 2000; Wisenden and Millard, 2001). In turn, multiple taxa such as freshwater fishes, amphibians, mollusks and crustaceans also show antipredatory responses to chemical cues from heterospecifics (e.g. Chivers et al., 2002; Dalesman and Rundle, 2010; Hazlett and McLay, 2005; Mitchell et al., 2012; Sullivan et al., 2003; Shabani et al., 2008). Responses to heterospecific chemical cues may arise by one of two non-exclusive mechanisms (Mitchell et al., 2012; Schoeppner and Relyea, 2009): (1) individuals may possess an innate recognition of

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alarm cues common to closely related species (the 'phylogenetic relatedness hypothesis') and/or (2) individuals may acquire recognition of relevant alarm cues through experience (the 'ecological coexistence hypothesis'). The ecological coexistence hypothesis posits that responses to heterospecific alarm cues arise due to individuals co-existing with species that are part of the same prey guild (that may or may not be closely related) which may have a fitness advantage (Chivers and Smith, 1998; Mitchell et al., 2012; Schoeppner and Relyea, 2009). Several studies have supported this hypothesis by demonstrating that closely related, coexisting prey display similarly strong defenses (Schoeppner and Relyea, 2009). The phylogenetic relatedness hypothesis states that responses to heterospecific alarm chemicals are due to the homology of alarm cues among closely related species, i.e. alarm cues are conserved within taxonomic groups; thereby, the composition should be similar having been derived from a recent common ancestor (Mitchell et al., 2012). The composition of alarm cues is expected to involve a blend of alarm molecules, in which some could have the same conserved molecular structure as those from a recent ancestor, while others could be different agent(s) from closely related species (as recently demonstrated for other infochemicals called copepodamides that mediate interactions between marine zooplankton and their prey; Selander et al., 2015). Although identification of most infochemicals is still needed, exploring the specificity of the responses can shed some light on the degree of conservation of molecules acting as an alarm. Few recent studies have examined whether alarm cues from closely related, but non-coexisting species can induce antipredator responses too (Schoeppner and Relyea, 2009).

Being able to distinguish infochemicals indicative of a predation risk is fundamental for prey to avoid being eaten and early detection is the key to achieving such an advantage, which can translate into fitness benefits (Atherton and McCormick, 2015; Ferrari et al., 2010a,b; Lima and Dill, 1990; Mathis et al., 2008; Schoeppner and Relyea, 2009). Recent studies in fishes and amphibians have shown that the earliest time at which an individual can acquire this information is while the embryo is still in the egg when it is in contact with aquatic medium (e.g. Atherton and McCormick, 2015; Ferrari and Chivers, 2009; Ferrari et al., 2010a; García et al., 2017; Gazzola et al., 2015; Lehman and Campbell, 2007; Mandrillon and Saglio, 2007, 2008; Mathis et al., 2008; Nelson et al., 2013; Orizaola and Braña, 2004). Fish embryos have been shown to detect and react to chemical cues indicative of predation risk and they were able to use this information before they hatched (e.g. Atherton and McCormick, 2015; Nelson et al., 2013). Common frog embryos (*Rana temporaria*) are apparently unaffected by chemical alarm cues; however, embryonic exposure to such cues causes morphological plasticity of hatchlings (Mandrillon and Saglio, 2008). The ability of *R. temporaria* embryos to perceive a predation risk is related to the development of the nervous system and occurs after the completion of neurulation, i.e. there is a critical embryonic period conditioning the morphological responses of hatchlings (Mandrillon and Saglio, 2008). Nevertheless, embryos of the skipper frog *Euphlyctis cyanophlyctis* seem to recognise infochemicals, indicative of predation risk even before neurulation (Supekar and Gramapurohit, 2017). For most invertebrate taxa, however, information on whether embryonic responses to chemical predation cues occur at any point in development or if there are windows of sensitivity/responsiveness is still scarce. For instance, in the water flea *Daphnia pulex* there is a period during embryonic development with great sensitivity to kairomones from invertebrate predator (Imai et al., 2009; Laforsch and Tollrian, 2004; Miyawaka et al., 2015). As in *D. pulex*, in *Daphnia cucullata* predator kairomones induce morphological defenses that are already present in late stages of embryonic development (Laforsch and Tollrian, 2004). Whether the embryos of macro-crustaceans can use chemical predation risk cues as kairomones and/or alarm cues to alter hatching responses and/or hatchling traits has not been reported yet.

Here, we performed experiments in *Palaemon argentinus* to: (1) determine whether embryos can perceive chemical alarm cues; (2) assess potentially inducible traits; and (3) gather information about specificity of the responses in relation to the origin of the chemical alarm cues. Our laboratory experiment involved the direct exposure of female *P. argentinus* with newly spawned eggs to conspecific alarm cues and heterospecific alarm cues from a closely related but allopatric shrimp. We assume that if embryos can respond to conspecific alarm cues, then heterospecific cues from a closely related but allopatric species should induce similar responses. Our assumption is based upon the premise that closely related prey produces similar chemical alarm cues and, thereby, should induce similar behavioral and/or morphological defenses. We tested our assumptions by exposing females of *P. argentinus* with newly laid eggs to the following four treatments: (1) distilled water (control, C); (2) macerated adult conspecifics (crushed conspecifics, CC); (3) macerated adult heterospecifics (crushed heterospecifics, CH); or (4) a mixture of both macerated adult species (conspecifics and heterospecifics, CC+CH). By combining conspecific and heterospecific stimuli, we explored whether the alarm molecules in the mix would have the potential to induce defenses just as the individual application of CC and CH treatments.

MATERIALS AND METHODS

Animals collection and maintenance

During the middle of the breeding season (5 and 12 January 2018), we collected females with fully developed ovaries (almost ready to spawn) and adult males of the freshwater shrimp *Palaemon argentinus* Nobili 1901 (previously known as *Palaemonetes argentinus*; see DeGrave and Ashelby, 2013) from the littoral zone of Los Padres Lake, Buenos Aires Province, Argentina (Pampa Plain, 37°55′S, 57° 43′W). Los Padres Lake is a small, shallow and eutrophic lake (area, 2 km^2; mean depth, 1.8 m) with a polymictic thermal regime and alkaline water (pH=8.6) (González Sagrario et al., 2009). The transparency is variable with turbid and clear periods that varies from year to year (Allende et al., 2009; González Sagrario et al., 2009). Shrimp were collected using a hand net (45 cm width, 30 cm deep, 1 mm mesh size). Shrimp were kept for 1 week in a laboratory aquarium (30 cm×30 cm, 50 cm depth) filled with tap water with constant aeration and were fed daily with nauplii of *Artemia* spp. and fish food (TetraMin Pro; TetraGmbH, Melle, Germany). We examined the aquarium every morning to collect females with newly laid eggs.

The oriental shrimp *Palaemon macrodactylus* Rathbun 1902 was collected from Mar del Plata Harbor, Argentina (38°02′27″S, 57°32′ 18″W) using a hand net (300 µm mesh size). In 2006, this invasive shrimp was registered for the first time in Mar del Plata Harbor (Spivak et al., 2006), since then, several reproductive and developmental aspects have been studied in this established marine population (Vázquez et al., 2012). Although the oriental shrimp is a euryhaline species, it has not yet been found in shallow inland lakes from the Pampa plain, likely because it is unable to complete its embryonic development in fresh water (Vázquez et al., 2016). Therefore, the native freshwater shrimp and the invasive oriental shrimp do not cohabitate and are allopatric yet closely related species. Shrimps were kept in a laboratory aquarium (30 cm×30 cm, 20 cm depth) filled with filtered sea water, constant aeration and were fed daily with nauplii of *Artemia* spp. and fish food (TetraMin Pro) until use.

Stimulus preparation

Alarm cues were prepared fresh directly before being used in each trial by crushing adult shrimps with a pestle in distilled water, then filtering with 200 µm mesh size. To ensure similar concentrations of stimuli from macerated individuals of different sized species, live shrimps were gently dried with tissue paper and weighed to 0.0001 g. Each single batch of alarm cues was obtained from 250 to 350 mg of wet weight tissue, corresponding to about between 10 individuals of the smaller species *P. argentinus* and 2 individuals of the larger species *P. macrodactylus*. We prepared one single batch per each stimulus with an average concentration of 15.49±0.58 mg ml^{-1} (Gancedo and Ituarte, 2017). By combining similar aliquots (with a ratio 1:1) of each species-specific stimulus, we obtained the mixed treatment. Then, we added an aliquot (3 ml) of the corresponding stimuli to experimental animals. Control animals received 3 ml of distilled water to reproduce the slight mechanical disturbance caused by liquid introduction.

Experimental animals

Like many decapod crustaceans, development of shrimp embryos occurs externally on the pleopods of the female. Soon after a female with developed ovaries molts, one or more males may deposit spermatophores externally on her ventral thorax. Eggs are extruded within 2–3 h of mating, fertilized as they pass over the spermatophores and attached with adhesive material or ‘cement’ to pleopodal setae that form the incubation chamber (Bauer, 2004). During the embryonic development period (roughly 2 weeks during summer months, but dependent on temperature) the female may re-initiate oogenesis to provide new ova for extrusion at the next molt (Bauer, 2004). We were able to collect between 4 and 8 freshly ovigerous females per day from a single stock aquarium (see Animal collection and maintenance), then each mother shrimp was randomly assigned to one of the four treatments. We repeated this procedure until we reached a minimum of five ovigerous females (replicates) per each stimulus treatment and it was accomplished on 3 consecutive days.

Experiment started with the addition of the corresponding stimulus to the females of *P. argentinus* with freshly laid eggs, which were kept in individual plastic and opaque containers (1 liter) filled with 800 ml tap water (water depth 10 cm). An empty plastic tube (diameter 1.7 cm, length 5 cm) was offered as a refuge. Each ovigerous female was considered to be an experimental unit. Every morning, we fed shrimp with two flakes of fish food (TetraMin Pro). Approximately 1 h after feeding, the corresponding stimulus was carefully introduced with a syringe into the plastic containers. Experimental units were provided with constant aeration, temperature (23±2°C) and light (14 h:10 h light:dark cycle) to simulate summer conditions.

Since active substances that are presumably the source of chemical alarm cues in adult decapods have been shown to lose bioactivity after 24 h (Acquistapace et al., 2005; Shabani et al., 2008) and due to our interest in the sensitivity of embryos to chemical alarm cues that trigger a response rather than the detection of developmental windows during which responses can be possible, we used a repeated mode of treatment exposure (every day from the onset of spawning). Thus, water of the experimental units was completely replaced every other day. Non-conditioned water was also changed every other day to reproduce the slight mechanical disturbance caused by water management in all treatments. Water changes (when applicable) were done after shrimp were fed and before the introduction of water stimuli.

Table 1. Embryonic developmental time and morphological parameters measured in the first larval instar of *Palaemon argentinus*

Treatment	Female	Embryos Developmental time (days)	First larval instar (zoea I)							
			N	*n*	CL (mm)	RL (mm)	TW (mm)	TL (mm)	EL (mm)	EW (mm)
C	A	17	53	10	0.603±0.021	0.566±0.023	0.666±0.025	0.986±0.039	0.206±0.009	0.141±0.015
	B	18	60	10	0.614±0.018	0.551±0.016	0.637±0.018	1.000±0.012	0.199±0.010	0.150±0.009
	C	17	72	8	0.664±0.053	0.544±0.022	0.687±0.027	0.998±0.019	0.199±0.011	0.148±0.012
	D	15	15	10	0.623±0.013	0.565±0.020	0.643±0.015	0.934±0.021	0.202±0.014	0.148±0.014
	E	14	20	9	0.626±0.021	0.569±0.022	0.640±0.029	0.933±0.026	0.191±0.012	0.149±0.012
	F	16.5*	WLR		–	–	–	–	–	–
CC	G	11	67	10	0.643±0.012	0.635±0.009	0.687±0.029	1.004±0.052	0.222±0.012	0.156±0.025
	H	15	67	7	0.619±0.040	0.593±0.029	0.629±0.022	0.964±0.042	0.210±0.008	0.161±0.013
	I	11.5*	105	10	0.705±0.036	0.592±0.020	0.666±0.013	0.993±0.020	0.211±0.013	0.159±0.012
	J	16	49	10	0.741±0.027	0.576±0.021	0.689±0.014	1.031±0.029	0.215±0.008	0.152±0.009
	K	12	81	10	0.727±0.030	0.576±0.017	0.687±0.018	0.983±0.027	0.209±0.011	0.154±0.009
	L	12.5*	33	10	0.714±0.015	0.574±0.020	0.664±0.015	1.004±0.026	0.197±0.015	0.147±0.013
	M	13.5*	WLR		–	–	–	–	–	–
CH	N	12	57	10	0.719±0.036	0.512±0.024	0.657±0.034	0.977±0.035	0.204±0.012	0.147±0.016
	O	12	7	7	0.737±0.035	0.534±0.026	0.665±0.027	0.967±0.025	0.197±0.013	0.145±0.021
	P	12	34	7	0.692±0.024	0.546±0.019	0.689±0.008	1.020±0.016	0.201±0.009	0.161±0.008
	Q	16	29	10	0.727±0.023	0.546±0.027	0.675±0.011	1.015±0.028	0.204±0.018	0.165±0.013
	R	17	9	8	0.711±0.031	0.544±0.015	0.667±0.017	1.009±0.028	0.199±0.006	0.152±0.013
CC+CH	S	13	27	10	0.718±0.017	0.499±0.020	0.660±0.012	1.000±0.023	0.200±0.007	0.152±0.012
	T	15	28	10	0.705±0.021	0.517±0.025	0.648±0.016	0.993±0.018	0.220±0.011	0.166±0.014
	U	16	67	10	0.716±0.026	0.539±0.029	0.680±0.021	1.008±0.020	0.208±0.009	0.159±0.012
	V	16	37	9	0.738±0.025	0.551±0.018	0.714±0.006	1.068±0.022	0.215±0.008	0.165±0.011
	W	16	19	9	0.664±0.033	0.523±0.036	0.637±0.029	0.916±0.110	0.191±0.016	0.145±0.015

Effects of embryonic exposure to distilled water control (C), conspecific alarm cues from crushed adult shrimps (CC), heterospecific alarm cues from crushed adult shrimps (CH), a mixing of both crushed adult species (conspecifics and heterospecifics, CC+CH). Embryonic developmental time: from spawning of eggs to onset of larval hatching; *Larvae from the same clutch that hatched on two consecutive days (the average of these days was considered as the completion of hatching); *N*=total number of larvae recovered per female (clutch); *n*=number of larvae used for morphological measurements; WLR, without larval recovery; CL, carapace length; EL, largest axis of the pigmented surface of the eye; EW, the smallest axis of the pigmented surface of the eye; RL, rostrum length; TL, telson length; TW, telson width. Values are means±s.d. Embryonic exposure to chemical treatments induced changes in two (carapace and rostrum lengths) of the six morphological parameters measured in larvae. In gray, embryonic and larval traits induced by chemical alarm cues that differed from those of control animals.

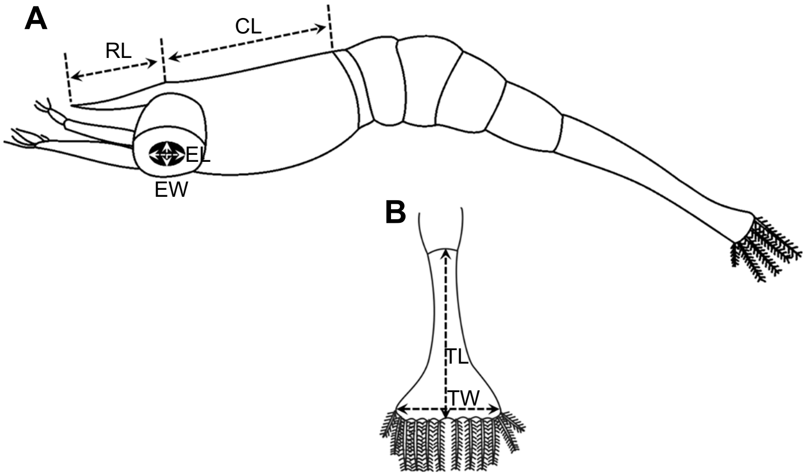

Fig. 1. Morphometric measurements made on first-instar *Palaemon argentinus* larvae. (A) Lateral view of the zoea I showing the linear measures used in morphological analysis. (B) Dorsal view of telson. The first larval instar is drawn without pereiopods and modified from Menú-Marque (1973). CL, carapace length; EL, largest axis of the pigmented surface of the eye; EW, the smallest axis of the pigmented surface of the eye; RL, rostrum length; TL, telson length; TW, telson width.

Response variables

Since hatching usually takes place at night, we checked the experimental units every morning in order to recover larvae soon after hatching. We considered the embryonic phase as the period between egg laying and the onset of larval hatching; when larvae hatched on two consecutive days, we considered the average of these days as the completion of hatching (Table 1). Immediately after larval release, we removed the mother shrimp from the plastic containers and carefully collected the first-instar *P. argentinus* zoeae using a plastic pipette. We measured larval carapace lengths with an Olympus SZX7 stereomicroscope (Olympus, Tokyo, Japan) from the posterior orbital margin to the dorso-posterior edge (Fig. 1) to the nearest 0.01 mm. In addition to carapace length of the zoea I larvae (194 nm; Table 1), we also measured rostrum length (from the rostrum tip to the posterior orbital margin), telson width, telson length, the largest and the smallest axis of the pigmented surface of the eye (Fig. 1). We determined all the measurements in subsets of at least 7 larvae per clutch, usually 10 (Table 1). Zoeae were obtained from 21 out of 23 clutches of eggs (Table 1). Two units, one from the control and one from the conspecifics were accidentally lost during analysis.

Data analysis

We assessed whether embryo exposure to chemical stimuli alters embryonic developmental time using a one-way ANOVA with chemical stimulus (C, CC, CH and CC+CH) as factors. To evaluate the effect of embryo exposure to chemical stimuli on larval traits, we used linear mixed models (LMMs). LMMs are particularly useful when there are several measurements that come from sets of larvae belonging to a single female (clutch). Since female identity provided variability to each one of the morphological response variables (all $P<0.001$), we kept female identity as a random factor in our models. We analyzed the effects of embryo exposure to chemical stimuli on the following larval traits: carapace and rostrum length, telson width, telson length, the largest and the smallest axis of the pigmented surface of the eye using LMMs with chemical stimulus as fixed factor and female identity as random factor. As carapace and rostrum lengths differed between chemical treatments, we assessed whether the elongation of the rostrum was related to body size using a LMM with chemical stimuli as fixed factor, carapace length of larvae as covariate and female identity as a random factor. We used the function Lme in the package ‘nlme’ (https://cran.r-project.org/web/packages/nlme) and the multcompView package (https://cran.r-project.org/web/packages/multcompView) for *post hoc* comparisons (Tukey test). All statistical analyses were performed using R Software (v3.5) (https://www.r-project.org/).

We explored whether LR depends on LC with a linear mixed model, with water stimuli (C, CC, CH, CC+CH) as fixed factors, LC as covariate and female identity as random factor in the models. We used the function Lme in the package ‘lme4’ (https://cran.r-project.org/web/packages/lme4). We explored if LR depends on LC with a linear mixed model, with water stimuli (C, CC, CH, CC+CH) as fixed factor, LC as covariate and female identity as random factor in the models with the function Lme in lme4.

RESULTS

Exposure to chemical alarm cues throughout embryonic development elicited changes in embryonic developmental time and morphology of the first larval instar of *P. argentinus*. The embryonic developmental time differed among treatments ($F_{3,19}$=3.76; P=0.028) since chemical alarm cues from conspecifics (CC) shortened the time until hatching (Tukey test, $P<0.05$) compared with the other three treatments (Fig. 2). Size at hatching was affected

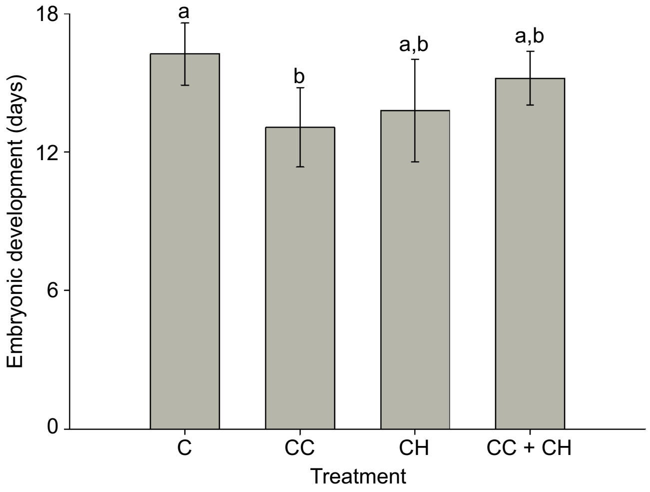

Fig. 2. Time between egg laying and larval hatching. Effects of embryonic exposure to distilled water control (C), *n*=6; conspecific alarm cues from crushed adult shrimps (CC), *n*=7; heterospecific alarm cues from crushed adult shrimps (CH), *n*=5; a mix of both crushed adult species (conspecifics and heterospecifics, CC+CH), *n*=5. Values are overall means±s.d.; for further details, see Table 1. Different letters indicate significant differences among treatments.

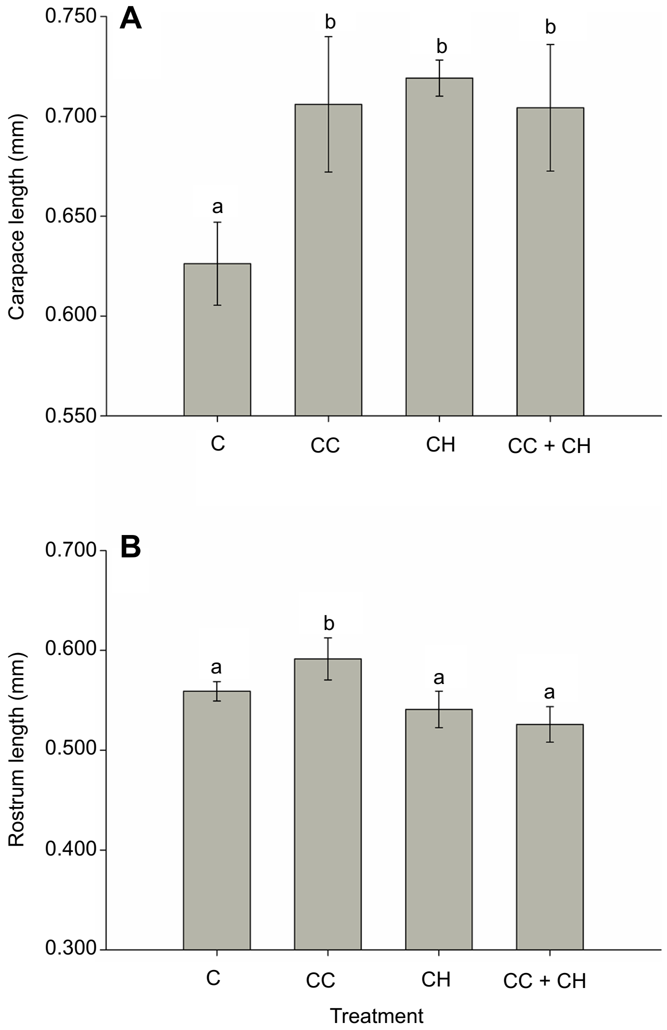

Fig. 3. Morphometric parameters of first-instar *P. argentinus* zoeae. Effects of embryonic exposure to alarm cues on (A) carapace length and (B) rostrum length of the zoea I. Distilled water control (C), *n*=47; conspecific alarm cues from crushed adult shrimps (CC), *n*=57; heterospecific alarm cues from crushed adult shrimps (CH), *n*=42; a mixing of both crushed adult species (conspecifics and heterospecifics, CC+CH), *n*=48. Values are overall means ±s.d.; for further details, see Table 1. Different letters indicate significant differences among treatments.

by embryonic exposure to chemical alarm cues ($F_{3,17}$=6.54; P=0.004). All embryonic treatments resulted in larger larvae compared with those in the distilled water control (Tukey test, all P<0.05; Fig. 3A). In addition, rostrum length of larvae was also affected by embryonic exposure to chemical alarm cues ($F_{3,17}$=9.84; P=0.0005), it did not depend on carapace length of larvae ($F_{1,168}$=0.91; P=0.34) and there was no interaction between carapace length and treatments ($F_{3,168}$=0.84; P=0.47). Chemical alarm cues from conspecifics (CC) produced larvae with longer rostra compared with the distilled water control (P<0.05; Fig. 3B). Embryo treatments did not affect other morphological traits measured in larvae: telson length ($F_{1,3}$=1.08; P=0.38), telson width ($F_{1,3}$=0.54; P=0.66), length of the eye ($F_{1,3}$=2.17; P=0.13); width of the eye ($F_{1,3}$=1.78; P=0.19).

DISCUSSION

The results of the present study indicated that shrimp embryos use infochemicals from macerated con- and heterospecific adults to alter hatching time and first larval instar morphology. As far as we know, this is the first report on chemically mediated responses to alarm cues for embryos of a decapod crustacean. We found that embryos of *P. argentinus* hatched in anticipation and produced larger larvae with longer rostra in response to chemical cues released by adult conspecifics. By contrast, exposure to heterospecific cues and to a mix of con- and heterospecific cues did not alter the time to hatch, whereas both cues led to larger larvae. The absence of phenotypic modification in the timing of hatching is not always indicative of no effect (e.g. Ituarte et al., 2014; Mandrillon and Saglio, 2008; Mueller, 2018). The larger size at hatching in response to conspecific and heterospecific alarm cues indicates that the embryos of *P. argentinus* could have recognized cues that are common to palaemonid shrimps.

The prerequisites for evolution of inducible defenses include fluctuating predator conditions, the existence of a cue that reliably indicates the presence of the predator, the effectiveness of the defense, and finally, defense-associated costs that exceed the benefits in the absence of a threat (Tollrian and Harvell, 1999; Herzog et al., 2016). The longer rostrum and carapace length of larvae of *P. argentinus*, as observed in zoeae I from embryos exposed to conspecific alarm cues, were also seen in an experiment in which embryos developed in the presence of a predatory fish feeding on conspecific ovigerous females (Ituarte et al., 2014). That experiment, however, did not allow for discrimination between chemical and/or visual stimulus that elicited the observed morphological changes in the first-instar *P. argentinus* zoeae. In juveniles of the shrimp *Xiphocaris elongata*, rostrum growth was induced by infochemicals from a predatory fish, although it was not possible to distinguish whether chemical alarm cues can also induce such a response on their own (Ocasio-Torres et al., 2014). Our present results demonstrate that conspecific alarm cues from adult individuals provided reliable information to embryos about a potential hazard, being the first experimental evidence that chemical alarm cues induce an elongation of the rostrum in *P. argentines*, as well as a larger size at hatching. As conspecific alarm cues elicited larger larvae with longer rostra compared with the control animals, and rostrum length changed independently of their body size, elongated rostra in the first zoeal instar can be considered as a defensive inducible response.

In shallow lakes from the Pampean region, benthic adult and juvenile *P. argentinus* along with other macroinvertebrates and small littoral fish species (12.5–16 mm body length) show a distribution completely associated with littoral macrophytes (González Sagrario and Balseiro, 2010). In turn, small littoral fishes prey often on benthic stages of *P. argentinus* (Rosso, 2006). Water transparency, along with changes in macrophyte abundance, has been shown to affect trophic interactions in these shallow lakes (e.g. Colautti et al., 2014; González Sagrario et al., 2009; Sosnovsky et al., 2010). Fish diet analyses support the relevance of the littoral zone as a foraging area with an estimated contribution of littoral carbon accounting over the 80% of the biomass ingested by pelagic fish species (e.g. *Odontesthes bonariensis*, *Oligosarcus jenynsii* and *Rhamdia quelem*) (González Sagrario et al., 2018). Such strong linkage between the littoral and the pelagic occurs through the consumption of littoral macroinvertebrates (e.g. *P. argentinus*) and small littoral fish species (e.g. *Cnesterodon decemmaculatus*, *Jenynsia multidentata*, *Astyanax* sp.) (González Sagrario et al., 2018). Accordingly, the benthic adult and juvenile stages as well as the first larval instar of *P. argentinus* (just before reaching the plankton) must face a high predation risk imposed by a rich littoral predator assemblage composed by small gape-limited fish as well as by large omnivorous and voracious macroinvertebrates (Gonzalez

Sagrario et al., 2009). The zooplankton, in turn, must face planktivorous fishes and predacious invertebrates in the pelagic zone (González Sagrario et al., 2009). Even though zooplankton do not undergo diel horizontal migration from the open water to the littoral of shallow lakes to avoid pelagic predators, littoral predators turn the macrophytes into highly risky areas (González Sagrario et al., 2009; González Sagrario and Balseiro, 2010). The planktonic phase of *P. argentinus* includes at least seven larval instars (Menú-Marque, 1973) before reaching the juvenile phase that enters the benthic adult habitat. Although not reported for the planktonic larval stages of *P. argentinus*, other larger- and medium-sized (0.9–0.7 mm length) zooplankters showed an avoidance of the littoral zone during the daytime and at night, selecting open water, despite the associated predation pressure in the pelagic area (González Sagrario and Balseiro, 2010). A large size in combination with an armor exoskeleton and long rostrums could make shrimp zoeae less vulnerable to gape-limited fish predators (Morgan, 1987, 1989). Moreover, grass shrimp zoeae are very effective at escaping from fish due to their post-contact behavioral response, which enhances the effectiveness of their morphological defenses (Morgan, 1987). Predatory fish and macroinvertebrates, however, are expected to show different feeding strategies and/or different prey selectivity. Therefore, further research should address how specific the reported inducible responses are, i.e. whether or not different predatory fish and/or macroinvertebrates also trigger morphological change.

Spines of decapod larvae are known to limit the size at which planktivorous and opportunistic predatory fish begin to prey on zoeae (Morgan, 1989). Once detected, spines are more effective than a large body at deterring predation by gape-limited fishes, because they not only increase the size of the zoeae but are noxious (Morgan, 1987). Spines enhancing survival of attacked zoeae, regenerate quickly, are effective while partially regenerated, cost relatively little to produce (i.e. they are composed only of epidermis, hemolymph, and cuticle) and do not greatly increase the visibility of zoeae (Morgan, 1987). Since zoeae do not evade attacks by fishes but rely on their spines and armor to survive initial attacks, induced rostrum growth may allow larvae to respond relatively quickly to the presence/absence of predators. Although physiologically different, inducible changes in life history (such as the time until hatching) are functionally related to inducible morphological changes, as both anti-predator responses provide an adaptive response to a size-selective predator (von Elert, 2012). Larval survival of *P. argentinus* could be increased by anticipating the time to hatch, i.e. leaving soon the littoral zone of shallow lakes where females live and the risk of predation is higher.

Our data indicate that embryos of *P. argentinus* were able to recognize infochemicals from closely related and non-coexisting adult heterospecifics, i.e. without any prior experience with such cues. The phylogenetic-relatedness hypothesis predicts that prey should respond innately to cues from heterospecifics and any cue that is structurally similar to the conspecific alarm cue (Schoeppner and Relyea, 2009). Our data support the phylogenetic-relatedness hypothesis; otherwise embryo exposure to alarm cues from an allopatric species should not have induced larger body size in the first larval instar. In turn, the intensity of the induced responses have shown to be stronger to conspecifics than to heterospecifics, indicating that the target species could distinguish between those chemical alarm cues (Mirza and Chivers, 2001; Shabani et al., 2008; Tran, 2014). Embryo exposure to conspecific alarm cues induced stronger plastic responses (faster embryonic development and larger larvae with longer rostrum) than to heterospecifics, which suggest that *P. argentinus* embryos distinguished between con- and heterospecific alarm cues. Since most of the inducible defenses are not simply on–off responses, the level of response often increases with the level of stimulus beyond an initial threshold (Harvell, 1990), whereby the development of larger larvae (but without modification in rostrum length) in response to the mixture of con- and heterospecifics indicates that alarm cues from conspecifics into the mix were insufficient to induce full larval defenses. From these results, it is reasonable to assume that the faster embryonic development, larger size and longer rostrum could have been triggered by more than one molecule involved in a blend of conspecific alarm molecules. Some of these alarm molecules present in the heterospecifics could conserve a molecular structure capable of inducing a response in *P. argentinus* (size), while others do not (those involved in embryonic developmental time and rostrum length of larvae). This could explain why the development of larger larvae occurred even with half the concentration of the species-specific alarm molecules, but such a concentration was insufficient to elicit a modification in rostrum length. Therefore, our results suggest that some alarm molecules are conserved within palaemonid shrimp, as also proposed for other taxonomic groups (e.g. Hazlett and McLay, 2005; Mirza and Chivers, 2001; Mitchell et al., 2012).

In general, chemical cues bind to chemoreceptors that are located on some kind of chemoreceptive organ (Weiss and Tollrian, 2018; Weiss, 2019). For example, the receptors for the detection of predator cues released by the backswimmer *Notonecta glauca* were recently shown to be located on the first antennae of *Daphnia longicephala* (Weiss et al., 2015, 2016). In *D. pulex*, neckteeth can be induced as early as the embryonic stages (Imai et al., 2009; Laforsch and Tollrian, 2004; Naraki et al., 2013). Exposure of just several hours to the kairomone at the end of embryonic stage is enough to induce neckteeth formation (Miyakawa et al., 2015). This kairomone sensitivity is reported to start with the shedding of the third egg membrane, which liberates the chemosensilla of the first antennae (Laforsch and Tollrian, 2004; Weiss et al., 2016). In malacostracan adult crustaceans, the chemoreceptors for olfaction are centralized on both antennae but not limited to these (Shabani et al., 2008); thus, critical periods for cue sensing during embryonic development can likely be linked to developing chemoreceptive structures (tissues/organs) and/or differential expression of genes coding for chemoreceptor proteins (e.g. Leal et al., 2013). Moreover, whether the recognition of infochemicals is limited or not to certain developmental time windows, as well as the time course of exposure that is necessary to activate the response, remain largely unknown for malacostracans and most aquatic animals (Mueller, 2018).

Embryos cannot escape from their eggs, thus it is important for them to sort out relevant stimuli. In fact, acquiring information about predators even before being directly exposed to them would provide prey with a great advantage (Ferrari and Chivers, 2009). This research contributes to our knowledge of an innate recognition of chemical alarm cues while at the embryonic stage of development in a shrimp species and opens up the question about whether invertebrate embryos could also acquire information about predation by learning. For instance, many organisms could respond innately to some cues (i.e. cues from conspecifics and heterospecifics that are structurally similar) while they could also learn to respond to other cues (cues from heterospecifics that are also consumed by a common predator), and both types of responses are important in producing effective anti-predator defenses (e.g. Atherton and McCormick, 2015; Ferrari and Chivers, 2009; Nelson et al., 2013; Schoeppner and Relyea, 2009). Amphibians and fish can learn while at the embryonic stage of development about the dangerousness of a novel predator, i.e. embryos learn to recognize unknown heterospecific cues (e.g. Ferrari and Chivers, 2009; Ferrari et al., 2010a; Nelson et al., 2013). Embryonic learning of predators is an exciting new area of research, whereby prenatal learning in invertebrates has been barely shown and is still debated (Darmaillacq et al., 2008; Isingrini et al., 1985; Romagny et al., 2012). In such a sense, invertebrate groups with externally brooded embryos may offer a powerful model system to test learning and generalization abilities.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: R.B.I., C.C.B.; Methodology: R.B.I.; Software: M.G.V.; Formal analysis: R.B.I., M.G.V., C.C.B.; Investigation: R.B.I., M.G.V., C.C.B.; Writing - original draft: R.B.I.; Writing - review & editing: C.C.B.; Funding acquisition: C.C.B.

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