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Courtship behavior and potential indications for chemical communication in *Artemia franciscana* (Kellog 1906)

Conducta de cortejo y potenciales indicadores de comunicacion quimica en *Artemia franciscana* (Kellog 1906)

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ABSTRACT

Two behavioral experiments were performed to test that *A. franciscana* shows specific courtship patterns, which in part, would be governed by semiochemicals released by females. The courtship ethogram, based on 30 adult virgin pairs (1 female v/s 1 male) individually video recorded for 1 hour, revealed that males display four independent activity patterns: 1) approach and touch, 2) swim behind, 3) riding attempt, and 4) riding success. The analysis of the transitions among the observed patterns suggested two ways to achieve riding success, both requiring female touch. In one, males approach and touch females followed by riding attempt (27% probability of occurrence), but if the latter fails, males start over from approach and touch (23% probability of occurrence) to finally achieve riding success (19% probability of occurrence). The alternative route is approach and touch, swimming behind (7% probability of occurrence) and riding success (5% probability of occurrence). The role of semiochemicals released from females was assessed by using the polar solution (PS) extracted from water conditioned with 40 adult virgin females. Virgin males were soaked with PS for obtaining pseudo females. Afterwards, we confronted 1 pseudo female, 1 female, or 1 control male to either 1 male or 5 male. In all cases the behavior was recorded for 1 hour (N=10), and the frequencies of occurrence of each courtship patterns were analyzed. The most important results showed that: 1) virgin female and pseudo female elicited the same frequency of response in 1 male in all four courtship patterns ($P>0.05$); b) virgin female and pseudo female elicited the same response when tested against 5 males in approach and touch, swim behind and riding attempt ($P>0.05$). The fact that in all the cases control male always elicited the lowest frequency value in each courtship patterns, strongly suggests the possible role of sexual semiochemicals in the courtship behavior of *A. franciscana.* The combination of behavior and chemical cues suggests an elaborate pre-mating recognition.

Keywords: ethogram, courtship, *Artemia*, polar fraction, chemical cue

RESUMEN

Se realizaron dos experimentos conductuales para evaluar que *A. franciscana* muestra patrones de cortejo específicos, que en parte, serían gobernados por semioquímicos liberados por hembras. El etograma del cortejo, construido a partir de la grabación individual de 30 parejas adultas vírgenes (1 hembra v/s 1 macho) durante 1 hora, reveló que los machos mostraron cuatro patrones de actividad independientes: 1) acercamiento y toque, 2) nado en retroceso, 3) intento de monta, y 4) éxito de monta. El análisis de las transiciones entre los patrones observados sugirió dos maneras de conseguir una monta exitosa, en ambas el patrón acercamiento y toque fue fundamental. La primera transición consistió en que una vez que los machos se acercan y tocan a las hembras, intentan montar a las hembras (27% de probabilidad de ocurrencia), pero si esto no ocurre los machos comienzan nuevamente a acercarse y tocar a las hembra (23% de probabilidad de ocurrencia) para finalmente lograr una monta exitosa (19% de probabilidad de ocurrencia). La ruta alternativa es mediante la aproximación y toque para luego desarrollar el nado en retroceso (7% de probabilidad de ocurrencia) y montaje exitoso (5% de probabilidad de ocurrencia). El rol de los semioquímicos liberados por hembras fue evaluado mediante el uso de la solución polar (SP) obtenida desde agua acondicionada con 40 hembras vírgenes. Machos vírgenes fueron empapados con SP para obtener pseudo hembras. Luego, se enfrentaron 1 pseudo hembra, 1 hembra, 1 macho control a ya sea 1 o 5 machos.

En todos los casos, la conducta fue registrada durante 1 hora (n=10), y se analizaron las frecuencia de ocurrencia de cada uno de los patrones de cortejo. Los resultados más importantes mostraron que: 1) la hembra virgen y pseudo hembra elicitó la misma frecuencia de respuesta en 1 macho en los cuatro patrones de cortejo (P>0,05); b) hembras vírgenes y pseudo hembras obtuvieron la misma respuesta cuando se evaluaron contra 5 machos en acercamiento y toque, nado en retroceso e intento de monta (P>0,05). El hecho que en todo los casos el macho control siempre elicitó el menor valor de frecuencia en cada patrón de cortejo sugiere el posible rol de semioquímicos sexuales en la conducta de cortejo de *A. franciscana*. La combinación de señales químicas y conductuales sugieren un elaborado reconocimiento de pre-cortejo.

Palabras clave: Etograma, cortejo, *Artemia*, fracción polar, señal química

INTRODUCTION

Mate choice is a critical fitness parameter influencing offspring output and quality, and the persistence of a population in a given environment (Andersson & Simmons 2006; Jones & Ratterman 2009). Therefore, species have evolved suites of traits to optimize mate selection, such as an elaborate courtship behavior and/or chemical signals (Eisner & Meinwald 1995; Dicke & Takken 2006; Müller-Schwarze 2006; Breithaupt & Thiel 2011). Such specificity favors the recognition of suitable conspecific mates within a population, and prevents interspecific mating (Smadja & Butlin 2009). This study focuses on the brine shrimp *Artemia franciscana* (Crustacea: Anostraca), a paradigmatic planktonic microcrustacean that inhabit hypersaline environment (salty lakes) distributed in North, Central and South America (Triantaphyllidis et al. 1998; Van Stappen 2002). *A. franciscana* is the most studied and widely distributed of all *Artemia* species recognized by the criterion of reproductive isolation, among other traits (Gajardo et al. 2002; Gajardo & Beardmore 2012). Although, *Artemia* is a model organism for many basic disciplines, and an example of adaptation to extreme environmental conditions (Gajardo et al. 2002). In relation to how partners mate, Forbes et al. (1992) reported that large males had a pairing advantage over smaller males and that there are selective pairing by sized in laboratory conditions for *A. salina.*

Three reasons suggest the existence of pre-mating intra-specific recognition in *Artemia*. Firstly, the species has overlapping generations (Rode et al. 2011) due to the ability of females to switch offspring quality between free-swimming nauplii and resting eggs (embryo at gastrula stage), commonly referred as cyst (King et al. 2014), if environmental conditions are perceived as stable or stressful respectively (Van Stappen 2002; Gajardo et al. 2002). Second, while nauplii allow rapid population expansion in a short period of time, eggs account for overlapping generations due to their ability to resist several environmental stresses, thanks to a protective shell (chorion) and physiological mechanisms that allow them to remain viable for years almost dehydrated (Clegg & Gajardo 2009; Gajardo & Beardmore 1989). Thirdly, because they accumulate in lake shores and eventually hatch when suitable conditions resume (Gajardo & Beardmore 2012), females can be fertilized by males born from eggs released the same year in that was release the egg from which this female was born (pairs formed by female and male born from eggs produced the same year), or by males hatched from eggs that were produced in different years (past or future with respect to the egg from which this female was born) (Rode et al. 2011). On the other hand, a coevolutionary study using dormant cysts of *A. franciscana* collected from the same population over a 23-year period was carried out by Rodes et al. (2011) to investigate male-female coevolution in natural conditions over time. These authors developed a cross experiment showing that females had a better performance, specifically reproductive traits, when mated with their contemporary males than when they mated with males from the future or the past.

This behavior was observed previously by Zapata et al. (1990) in a multiple-choice experiment (males in excess). Those successful in getting females in amplexus, i.e. the stage prior to copula, were genetically different (more heterozygous for multiple alozyme loci) than those remaining without females (Zapata et al. 1990). Such success could be either attributed to choosy females, or to more active males that make better use of the available energy to remain attached to females for longer periods of time whilst females try to dislodge them by sudden movements.

Finally, *Artemia* populations tend to be locally adapted building local populations (Gajardo et al. 2002).

In Crustaceans, one of the more common recognition in pre-mating intra-specific is through chemical communication (Breithaupt & Thiel 2011). In Decapoda like *Carcinus maenas*, *Portunus sanguinolentus* and *Telmessus cheiragonus*, has been demonstrated the existence of certain precopulatory behavioral patterns mediated by seniochemicals (Ryan 1966; Bamber & Naylor 1996; Kamio et al. 2000). In microcrustaceans, the information is scarce, but is possible find antecedents related to the existence of chemical communication involved in intraspecific male-female relationships in copepods such as *Lepeophtheirus salmonis* (Ingvarsdóttir et al. 2002b) and *Caligus rogercresseyi* (Pino et al. 2007). In *Artemia*, there is no precedent, to our knowledge, related to the existence of behavioral patterns or chemical communication at precopulatory level. However, the fact that female has a better mate performance with contemporary males than those coming from the past or future, would suggest that this selection could be governed by contemporary physic and chemical factor. Semiochemicals, such as sex pheromones could have a relevant role in this system. Hence, we hypothesize that pre-mating recognition is mediated by behavior and chemical cues.

MATERIALS AND METHODS

Sample Origin

Artemia franciscana individuals were collected in the Cejar Lagoon (3 ha in extent and an average water depth 10 m) (Gajardo & Beardmore 1993) in northern Chile (23°02'S – 68°13'W), and upon arrival at the Laboratorio de Química Ecológica of Universidad de La Frontera, they were placed in 5 L aquaria with water brought from the sample site, which was slowly replaced with artificial seawater (35 ppt). Meta-nauplii (2.2 - 2.6 mm in length) obtained from *Artemia* cultures were individually placed in Falcon tubes with 40 mL of artificial seawater (35 ppt), to keep (approximately 20 days) them virgin. Artemias were fed with *Dunaliella tertiolecta* (1.2×10^6 cells mL^{-1} per individual) every two days and constant aeration was provided by an aquarium pump (BOYU SC-7500) according to Gajardo & Beardmore (1993).

Ethogram

Pairs formed (30) of virgin male and female individuals showing evident claspers and ovisacs respectively (approximately 20 days of age). Each pair was placed in a plastic jars with 500 mL of artificial seawater (35 ppt) illuminated with natural light (16-h light/8-h dark) to record their behavior for 60 min using Ethovision 3.1, Noldus Technologies (PANASONIC WV-BP330/GE video camera) (Mutis et al. 2009). The effect of the size of the individuals was minimized by selecting by eye individual pairs of similar size. The courtship pattern emerged were established as in statistical analysis below.

Conditioned Water (CW)

Once the different courtship patterns developed by *A. franciscana* were identified, the possible role of chemical cues released from virgin female was evaluated. Consequently, an experiment was designed for eliciting the production of potential semiochemicals. Specifically, 40 virgin *A. franciscana* females were placed in a beaker with artificial seawater (5 L; 35 ppt), prepared according to the methodology described by Pino-Marambio et al. (2007) during 48 h for obtaining "conditioned water" (CW). To prevent the interference of microalgae in the chemical isolation process, virgin individuals were not fed during water conditioning. Artificial seawater without females was used as a control, hereafter referred as "control conditioned water" (CCW).

Solid Phase Extraction (Spe) And Vacuum Distillation

SPE was performed for the adsorption of the semiochemicals present in CW and CCW following the protocols reported by Ingvarsdottir et al. (2002a, 2002b), Bailey et al. (2006) and Pino-Marambio et al. (2007). Trapping columns (Biotage, Uppsala - Sweden) consisted of 6 mL glass cartridges containing two layers (C2 (500 mg) over ENV + (200 mg)). The extraction was performed using a vacuum manifold (VacMaster-10 SPE (Biotage). Ten SPE cartridges were preconditioned with 2 mL methanol (HPLC grade, Darmstadt - Germany), which was subsequently removed with 2 mL water (HPLC grade, Darmstadt - Germany). After the extraction, interfering compounds were removed with 2 mL water (HPLC grade, Darmstadt - Germany). Chemical compounds contained in both adsorbents were eluted with 2 mL ethanol (HPLC grade, Darmstadt - Germany), yielding a volume of 10 mL of ethanol extract.

SPE extracts were separated into two fractions by vacuum distillation (under 0.04 torr) over 24 h at 25°C, as described previously by Pino-Marambio et al. (2007). This technique allowed the separation of the non-polar and polar fractions. This process yielded an ethanolic distillate containing non-polar chemical compounds and a residue constituted mainly of polar components (Pickett & Griffiths 1980; Ingvarsdottir et al. 2002a, 2002b; Bailey et al. 2006). The residue was then dissolved with 1 mL of ethanol (HPLC grade, Darmstadt - Germany) and 1 mL of water (HPLC grade, Darmstadt - Germany). This polar solution (PS) was used in the behavioral bioassays described below.

Behavioral Bioassays

For bioassays (all in 35 ppt artificial seawater) 1 virgin male was soaked with 70 µL of the polar solution (PS, residue with polar component from CW, diluted as indicated above) for 1 min. Such male, hereafter referred as a "pseudo female" was confronted to 1 virgin male and to 5 virgin males. In both cases the frequencies of the behavioral patterns previously identified in the ethogram were recorded for 1 hour using behavioral tracking software (Ethovision 3.1, Noldus Technologies). As a positive control, the behavior of 1 virgin female tested against either 1 virgin male (experiment 1) or 5 virgin males (experiment 2) was also recorded for 1 hour. As a negative control, 1 virgin male soaked in PS obtained from CCW (control male) was tested against either 1 or 5 virgin males. Treatments and controls were replicated 10 times.

Statistical Analysis

The resulting ethogram based on the analysis of 30 individual pairs describes the courtship patterns on a first order Markov table constructed to analyze the frequency of transitions from one behavior to all other possible behaviors. The independence of courtship patterns was estimated following the methodology described by Fagen & Young (1978), with expected values calculated using a Chi-square test (X^2) according to Martin & Bateson (2007). For the behavioral bioassays, differences between controls and experimental groups were tested with the non-parametric Kruskal-Wallis test ($P<0.05$) followed by Conover-Inman's test, using StatsDirect V.2.2 software (StatsDirect. Ltd., UK).

RESULTS

Ethogram

The ethogram of *A. franciscana* males (FIGURE 1) revealed four independent behaviors ($X^2=46.77$; $P<0.05$) named as follows: 1) approach and touch: after moving around exploring for females, a male approaches and touches the female's body with his clasper; 2) swim behind: a male swims behind the female in a sort of specific orientation without touching her; 3) riding attempt: a male attempt to grasp female in amplexus (also called riding position) but fails due to her sudden movements clearly intended to dislodge him; and 4) riding success: a male grasps the female in amplexus, and the couple rides together for 5 min. FIGURE 1 shows the most frequent transitions among the four behaviors, suggesting two main ways to achieve riding success. Both require that males approaching or touching females, but the most frequent one follows riding attempt afterwards (27% probability of occurrence). If riding attempt fails male returns to approach and touch (23%) to finally achieve riding success (19%). The less frequent pattern starts with approach and touch and then swimming behind (7%) to end-up in riding success (5%) (Table 1).

Behavioral Bioassays With Ps

Figures 2 and 3 show the number of courtship patterns carried out by a male of *A. franciscana* in the two experimental conditions described in the methods section (behavioral bioassay). All pairs (1 female/1 male; 1 pseudo female/1 male; 1 control male/1 male; 1 female/5 males; 1 pseudo female/5 males; 1 control male/5 males) developed all four courtship patterns, but only two pairs, 1 female/1 male and

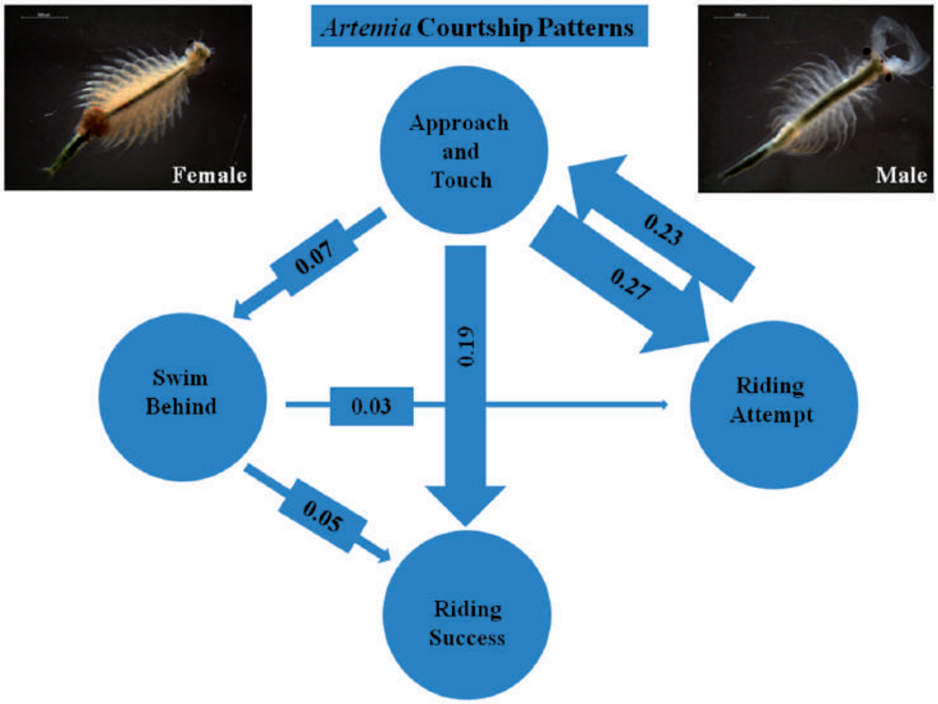

Figure 1. The components of the courtship ethogram of *A. franciscana* (N = 30 couples, P = 0.05). The values in arrows indicate the probabilities of transitions from one behavior to other. Each percentage indicates the probability that this step occurs, the complement to 100% is the probability that this step does not occur. Transitions are not indicated in the diagram were not significant (P>0.05).

Figura 1.Componentes del etograma cortejo de *A. franciscana* (N = 30 parejas, P = 0,05). Los valores de las flechas indican las probabilidades de transición de un comportamiento a otro. Cada porcentaje indica la probabilidad de que este paso ocurra, el complemento al 100% es la probabilidad de que no se produzca este paso. Las transiciones no indicadas en el diagrama no fueron significativas (P>0,05).

1 pseudo female/1 male, showed the same frequencies in all four behaviors (P>0.05), whilst 1 control male/1 male exhibited lower frequency of approach and touch and riding attempt (P<0.05). When 1 female, or 1 pseudo female, were independently confronted to 5 males, the frequencies of approach and touch, swim behind and riding attempt were the same (P>0.05). However, the frequency of riding success was higher in the pair 1 female/5 males than 1 pseudo female/5 males (P<0.05), and this one was higher than the control pair (1 control male/5 males) (P<0.05). Noteworthy, control pairs (1 control male/1 male; 1 control male/5 males) always showed the lowest frequency in every behavior.

Table 1. Comparative matrix of the behavioral transitions identified for *Artemia franciscana*. The interactions are unidirectional; the top row contains the behaviors that move to the conduct contained in the left column. Each percentage indicates the probability that this step occurs, the complement to 100% is the probability that this step does not occur.

Tabla 1. Matriz comparativa de las transiciones de conducta identificadas para *Artemia franciscana*. Las interacciones son unidireccionales; la fila superior contiene los comportamientos que se mueven a la conducta contenidas en la columna de la izquierda. Cada porcentaje indica la probabilidad de que esa etapa ocurra, el complemento a 100% es la probabilidad de que no se produzca esa etapa.

	Approach and Touch	Swim Behind	Riding Attempt	Riding Success
Approach and Touch	-	ns	23%	ne
Swim Behind	7%	-	ns	ne
Riding Attempt	27%	3%	-	ne
Riding Success	19%	5%	ns	ne

* ns = Transitions not significant (P>0.05).
**ne = Transition not evaluated

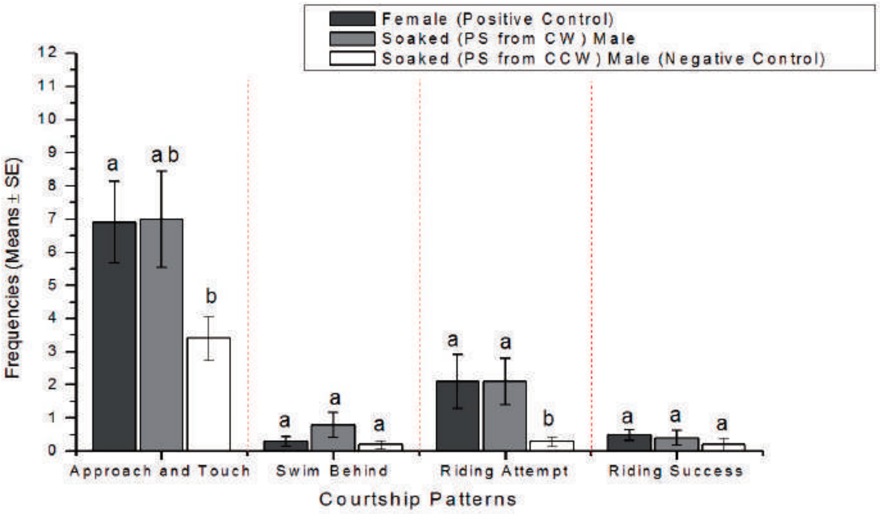

Figure 2. Behavioral experiment with a 1:1 ratio. Response elicited by one female, one soaked male with PS from CCW, or one soaked male with PS from CW on one conspecific non-soaked male, respectively (N=10). Different letters indicate that there are significant differences based on the Kruskal-Wallis test followed by Conover-Inman's test (P<0.05).

Figura 2. Experimento conductual en una proporción 1:1. Respuesta provocada por una hembra, un macho empapado con PS de CCW, o un macho empapado con PS de CW sobre un macho conespecifico no empapado, respectivamente (N=10). Letras diferentes indican que existen diferencias significativas basadas en el test de Kruskal-Wallis seguido por el test de Conover-Inman (P<0,05).

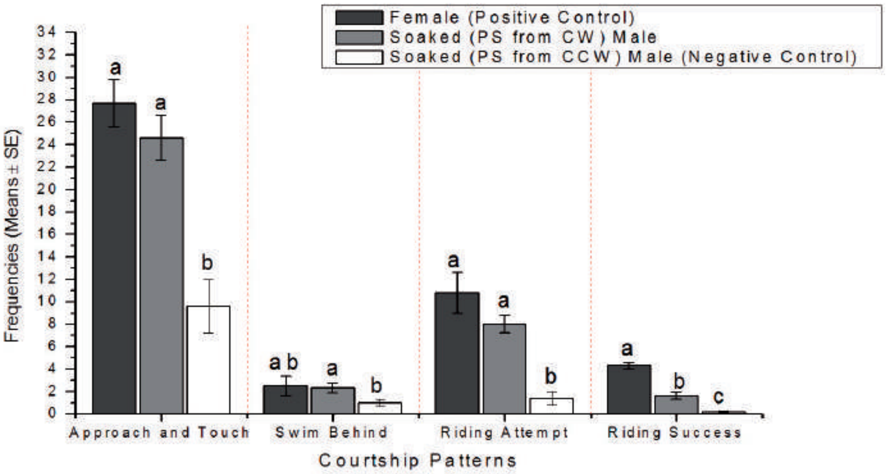

Figure 3. Behavioral experiment with a 1:5 ratio. Response elicited by one female, one soaked (PS from CCW) male, or one soaked (with PS from CW) male on five conspecific non-soaked males, respectively (N=10). Different letters indicate that there are significant differences based on the Kruskal-Wallis test followed by Conover-Inman's test (P<0.05).

Figura 3. Experimento conductual en una proporción 1:5. Respuesta provocada por una hembra, un macho empapado con PS de CCW, o un macho empapado con PS de CW sobre cinco machos conespecificos no empapados, respectivamente (N=10). Letras diferentes indican que existen diferencias significativas basadas en el test de Kruskal-Wallis seguido por el test de Conover-Inman (P<0,05).

DISCUSSION

This article provides the first courtship ethogram of *Artemia franciscana*, a sexual crustacean inhabiting hypersaline lakes in North, Central and South America (Gajardo et al. 2002). So far, only there are antecedents related to mating patterns described for anostracan freshwater relatives of *Artemia* (*Streptocephalu mackini* and *Eubranchipus holmani*). According to Moore & Ogren (1962), Wiman (1981) and Belk (1991), anostracan mating behavior follows a set pattern: detection, orientation, station taking, amplexus, intromission, copulation and disengagement.

A. franciscana males displayed four independent behavioral patterns to get females in amplexus, or riding position, the stage at which copula takes place: approach and touch, swim behind, riding attempt and riding success. These behavioral patterns consistently appeared in the behavioral bioassays in which one female and the so-called pseudo female were confronted to males in two ratios (1:1and 1: 5). Pseudo female correspond to one virgin male soaked in the polar solution (PS) extracted from conditioned water (CW) with conspecific females, which induced males to respond as if they were in front of females.

According to the observed in the ethogram, the most commonly displayed transition among the four behavioral patterns was "approach and touch" to "riding attempt". This means that males approaching and touching females had higher probability to reach riding success, the stage prior to copula in which males grasp females in amplexus. If such initial step is missed-out, males will face rejection (females move energetically to get rid of every male) with high probability, unless they go back to this step, as it was observed in the ethogram. Along the same line of reasoning, the "swim behind" pattern brings to mind the presence of a long-range signal, as reported in other crustaceans (Breithaupt & Thiel 2011). Such signals may be transported by various mechanisms (diffusion, laminar advection, and turbulent advection) before reaching the recipient, who thus needs to be properly positioned in relation to the emitter to receive the signal (see Webster & Weissburg 2009). Approach and touch females also evokes the probable involvement of short-range chemical signals. Although information on chemical signaling is almost inexistent in *Artemia* and, generally, chemoreceptors are poorly known in aquatic organisms (Peñalva-Arana et al. 2009), some chemoreceptors are reported to be located in the antenna sensorial system of *Artemia* (Tyson & Sullivan 1979). In *Daphnia pulex* (waterflea) a well-known aquatic crustacean and a freshwater *Artemia* relative, about 58 chemoreceptor genes and their products has been recently described and these, presumably, mediates many chemoperception abilities of waterfleas, such as gustatory abilities (gustatory receptors are similar to those in insects) (Peñalva-Arana et al. 2009). In the copepod *Tigriopus japonicus,* females exhibit a surface protein that may act as a contact signal capable of delivering information to conspecific males (Ting et al. 2000). Ting & Snell

(2003) purified and identified two proteins similar to α-2-macroglobulin, a protease inhibitor, from the surface of *T. japonicus*. The "riding attempt" behavior is a crucial previous step leading to the fertilization of *Artemia* females during amplexus (riding position). For this, male claspers are relevant tools for holding females tightly. The energetic female movements observed are clearly aimed to dislodge males, suggesting choosy females, a possibility previously suggested by Zapata et al. (1990). This trait could be indicating of male-female coevolution as female's ability to dislodge males by their sudden movements correlates with male's ability to maintain females grasped. Such behavior ensures the ability to hold females for reproduction under limiting conditions, allowing the population to persist under such conditions (Christy 1987; Wada et al. 1999; Sugumar 2010).

Because "riding attempt" behavior was observed in the behavioral bioassays in both positive and negative controls, we assume that the chemicals present in the PS from CW elicit a sexual response capable of confounding males in their ability to recognize the dimorphic female phenotype, revealing the importance of such signal. This would explain why *Artemia* males often tend to grasp males under a scenario of female shortage, as sometimes is common in laboratory cultures. Pseudo female (male soaked in PS) elicited such a response. In insects, cuticular compounds identified as contact sex pheromones can explain the homosexual behavior displayed by males (Peschke 1987), and the absence of male sex pheromones would also support such behavior (Billeter et al. 2009; Dukas 2010). However, typical compounds of *Artemia* males, if any exists, did not confound our results, as we found significant differences when males were soaked with PS from CW (treatment, conditioned water with females) in comparison with males soaked with PS from CCW (control conditioned water = without females). A study performed by Ibeas et al. (2009) identified cuticular compounds acting as contact sex pheromones in both male and female beetles (Coleoptera: Cerambycidae), with overlapping effects. Nevertheless, males recognize females because these compounds are present in different ratio.

Behavioral bioassays performed in this work using different ratios of mates (1:1 and 1:5) made clear males compete when females are limiting, a behavior common in the phylum Crustacea. Such competitive and even aggressive behavior has also been linked to the presence of chemical signals (Sneddon et al. 2003, Aquiloni & Gherardi 2010, Okamura & Goshima 2010).

The observation of specific courtship-like behavior patterns in *Artemia,* and the probable involvement of chemical cues suggest that mate choice is a complex phenotype in *A. franciscana*. One of the lines to follow-up refers to how general is the ethogram pattern, in other words, does the pattern repeats when locally adapted *A. franciscana* populations are compared? Does the pattern repeat among species? On the other hand, the identification of the chemical present in the polar extract isolated from conditioned water (CW), and the evaluation of structures involved in the emission and reception of chemical signals speak out of a sort of complementary male-female (co) evolution of these traits, the sort of key-lock or complementary structures referred elsewhere (Mura & Gajardo 2011).

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