UNIVERSIDAD DE LA FRONTERA

PROGRAMA DE DOCTORADO EN CIENCIAS DE RECURSOS NATURALES

BEHAVIORAL AND CHEMICAL ASPECTS INVOLVED IN THE MATE CHOICE OF THE BRINE SHRIMP *ARTEMIA FRANCISCANA*

DOCTORAL THESIS IN FULFILLMENT OF THE

REQUERIMENT FOR THE DEGREE

DOCTOR IN SCIENCE OF NATURAL RESOURCES

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TEMUCO-CHILE

2016

"BEHAVIORAL AND CHEMICAL ASPECTS INVOLVED IN THE MATE CHOICE OF THE BRINE SHRIMP *ARTEMIA FRANCISCANA*"

Esta tesis fue realizada bajo la supervisión del director de tesis, Dr. ANDRÉS QUIROZ CORTEZ, perteneciente al Departamento de Ciencias Químicas y Recursos Naturales de la Universidad de La Frontera, y es presentada para su revisión por la comisión examinadora.

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Acknowledgements

Summary

Mate choice is an important fitness trait as it impacts female reproductive output and offspring quality, contributing to maintaining reproductive isolation between different species and also between divergent or locally adapted populations. There are two important recognition processes through which an organism chooses a mating partner: a courtship behavior and chemical signaling. These processes act in combination in both terrestrial crustaceans and planktonic microcrustaceans, and inspired this thesis taking as study case the pelagic planktonic and extremophile brine shrimp *Artemia franciscana,* the sole macroscopic inhabitant of hypersaline lakes and lagoons. Under these extreme conditions, *Artemia* females evolved a life cycle combining two reproductive modes that are modulated by the environment: ovoviviparity (offspring in the form of free-swimming nauplii, when conditions are predictable) and oviparity (encysted offspring, under unpredictable conditions). Thus, the working hypothesis for this thesis is that a highly specialized mate choice occurs in the studied *Artemia* population, combining a courtship behavior and chemical cues. In a first step, a courtship ethogram, based on 30 adult pairs (1 female and 1 male) individually video recorded during 1 hour, was constructed. In a second step, the possible role of semiochemicals on the *Artemia* courtship was tested by isolating the polar chemical compounds released by females (N=40) (defined here as female essence) to the environment. The effect of female essence on the *Artemia* courtship behavior was evaluated using the ethogram results. For this process, one male was soaked with the female essence and was placed in the presence of 1) one non-soaked male or 2) five non-soaked males. The development of the different ethogram patterns was verified and recorded. Because the main courtship behavioral pattern obtained from the ethogram was "approach and touch";

the role of cuticular compounds was evaluated in a third experimental step. For this step, cuticular compounds extracted from males or females with a 2:1 chloroform:methanol mixture were used for soaking an artificial sponge that was exposed to one non-soaked male. The swimming speed generated by the male was recorded and analyzed. The ethogram revealed that males display four independent activity patterns, named as follows: 1) approach and touch, 2) swim behind, 3) riding attempt, and 4) riding success. These patterns suggest that an *Artemia* male exhibits an elaborate courtship in the presence of females, while the female response acts as a kind of selective pressure, reflected in the pattern "riding attempt", avoiding the male to engage in amplexus. Polar chemical compounds released by females elicited the same courtship behavior, determined in the ethogram, toward males. Specifically, one pseudo-female (male soaked with female essence) and one female elicited the same frequency of response from one male in all four courtship patterns ($P > 0.05$). Similar results were obtained when one pseudo-female and one female where placed in the presence of five males ($P > 0.05$). Cuticular compounds from either females or males increased the swimming speed of males by approximately 1.5 times in comparison with control treatments (salty water and solvents). Chemical characterization of the cuticular extracts showed the presence of four saturated fatty acids (myristic, palmitic, stearic, and arachidic acids) and five unsaturated fatty acids (oleic, linoleic, linolenic, gondoic, and erucic acids). Myristic acid was found only in female cuticular extract. These results show evidence that mate choice in *A. franciscana* involves a combination of a behavioral-courtship display and chemical signaling. The possible ecological implications of these aspects are discussed, and future directions are suggested in order to understand such a prominent trait as mate choice and its evolutionary effects.

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1.1 Introduction

1.1.1 Mate Choice

Mate choice or intersexual selection is an important fitness trait in organisms with sexual reproduction, as it affects female reproductive output and offspring quality (Eshel et al., 2000; Kokko, 2001; Anderson and Simmons, 2006; Cothran, 2008; Rode et al., 2011; Ahking and Gowaty, 2016). Likewise, mate choice plays an important role in maintaining reproductive isolation between different species (West and Kodric-Brown, 2015) and among geographically isolated and/or locally adapted populations within species which expres different morphs, physiological or behavioral characteristics (Zoppoth et al., 2013). At the species level, mate choice prevents interspecific hybridization and the breakdown of species genetic integrity (Laturney and Moehring, 2012), whereas in locally adapted populations, mate choice maintains population divergence, which is the first step toward speciation (Johannesson et al., 1995; Seehausen et al., 1997; Coyne and Orr, 2004; Bolnick and Kirkpatrick, 2012). Darwin, in his book "The Descent of Man and Selection in Relation to Sex" (1871) coined the term "sexual selection", highlighting the fact that mate choice is not a random process, as shown by the following statement: "*when we see many males pursuing the same female, we can hardly believe that the pairing is left to blind chance that the female exerts no choice, and is not influenced by the gorgeous colours or other ornaments with which the male alone is decorated*". He also suggested that females would play the choosy role, or the ability to select males with suitable traits (color or ornaments). Subsequently, other definitions reinforced the concept of mate choice as a non-random

process and also reinforced the important roles of the traits involved. For example, Halliday (1983) defined mate choice as *"any pattern of behavior, shown by a member of one sex, that leads to their being more likely to mate with certain members of the opposite sex than with others"*, and Heisler (1984) as *"the tendency of one sex, usually females, to mate non-randomly with respect to one or more phenotypic traits of the other sex"*. This definition and the previous ones highlight the importance of mate choice for sexual selection between individuals within a population that exhibits phenotypic variability in sexual traits or other sex-related characteristics.

The non-random mating emphasized by authors above, also called assortative mating in more recent literature (Jiang et al., 2013), entails a correlation (positive or negative) between male and female phenotypes (or genotypes) across mating pairs. Because the decision of which partner to select has both risks ("bad decisions") and benefits, sexual phenotypic traits of males (when females are choosy or selective) act as indicators of good health and vigor (Andersson, 1994). Thus, the most obvious benefit of selecting the right partner would be the maximization of the female's reproductive performance and the quality of the offspring (Jones and Ratterman, 2009; Rode et al., 2011), as well as maintaining the genetic integrity between species and/or between locally adapted populations. As such, mate choice plays a role as a pre-mating reproductive isolation mechanism (Andersson, 1994) in the sense that it prevents copulation. Instead, post-zygotic isolating mechanisms act after fertilization has occurred. This study focuses on pre-mating isolation, specifically on i) courtship and ii) chemical signaling.

1.1.2 How individuals select the most suitable partner?

Males or females, depending on which sex plays the choosy role (often females, for the purpose of this thesis), need to ensure a good reproductive output and well-adapted offspring, and this occurs if the right conspecific mate is chosen. A good decision has positive reproductive consequences (Rode et al., 2011) and evolutionary consequences such as maintaining population divergence, local adaptation and population persistence. Negative consequences for a locally adapted population could be an adaptive breakdown, in other words, an offspring unable to survive in its parental environment (Eshel et al., 2000; Kokko 2001; Cothran, 2008). Therefore, how does an individual choose the right conspecific partner in a phenotypically (and genetically) variable population? Mate choice frequently involves multimodal signals, through which an organism recognizes potential mating partners (Candolin, 2003). One form of mate choice is the ritual display of a series of behaviors to approach the female, which is known as courtship.

Courtship is closely related to male ornaments as Darwin described in "The Descent of Man and Selection in Relation to Sex" (1871), pointing out that males ostentatiously display their ornaments in front of females during the mating season. Courtship and "ornaments" are therefore important behavioral and sex traits upon which females can choose (Cratsley, 2004; Barske et al, 2011). Courtship has been defined as a series of stereotypical behaviors, shown by one sex in response to different signals shown by the opposite sex, in order to facilitate mating (Matthews, 1975; Ejima and Griffith, 2007). This mating behavior is displayed by most organisms, including insects (Phelan and Baker, 1990; Dukas et al., 2006), crustaceans (Sigvardt and Olesen, 2014), fishes (Roubertox,

1992; Kawase et al., 2013), birds (Fusani et al., 2007) and mammals (Morales-Piñeyrúa and Ungerfeld, 2012). Courtship has proved efficient to prevent inter-specific and inter-population mating (Arbuthnott, 2009; Yukilevich et al., 2016). Courtship specificity should increase with time since the population diverged (Arbuthnott et al., 2010; Polihronakis et al., 2012), and it is therefore expected to be optimized over the evolutionary history between the interacting sexes. In Darwinian terms, this means that both sexes have co-evolved traits to allow the recognition of suitable mates, expressed as increases in female reproductive output and offspring quality (Rode et al., 2011). Thus, how females select the fittest males is critical and has significant costs (energetic and reproductive) for them, but males also have a cost attached because the highly visible sexual traits or adornments make them more susceptible to predators (Zahavi, 1987).

Another common way to recognize a suitable mate from the same or a distinct population and/or species is through chemical signals (Smadja and Butlin, 2009), which are considered to be among the most highly specific forms of communication. A chemical cue is released by the emitter, and a receptor discriminates whether it comes from a conspecific individual or from another species (Goetze and Kiørboe, 2008). In the case of a conspecific potential mate, chemical cues can provide information on the reproductive status, i.e., virgin or non-virgin individuals (Ingvarsdottir, et al., 2002b), the premolt or postmolt stage (Bouchard, et al., 1996), or even on the parasite load of the prospective mate (Zala et al., 2004).

In crustaceans, the process of encounter, recognition, courtship and pairing commonly involves behavioral and chemical cues (specifically pheromones; see Table 1, and Dunham, 1978, 1988; Breithaupt and Thiel, 2011). For example, Van Leeuwen and Maly (1991)

showed that males of the copepod *Diaptomus leptopus* (Copepoda: Calanoida) exposed to water that previously contained conspecific gravid females (conditioned water) swam at significantly higher speeds than males exposed to water from non-gravid females or from conspecific males. Likewise, Ingvarsdottir et al. (2002b) reported that males of the caligid copepod *Lepeophtheirus salmonis* were significantly more attracted by conditioned water from pre-adult virgin females than by conditioned water from non-virgin females or conspecific males. Similarly, Pino-Marambio et al. (2007) reported that water from *Caligus rogercresseyi* females (Copepoda: Caligidae) elicited a significant attractant response to conspecific adult males. Obviously, in these experiments, there were no visual signals or physical contact between males and females. Females of *Tigriopus japonicus* (Copepoda: Harpacticoida) without cuticular proteins (extracted with trypsin) that are supposed to play a role in communication (Kelly and Snell, 1998), elicited a reduced mate guarding behavior on conspecific males than non-treated females (Ting et al., 2000). These evidences strongly suggest that there are chemicals substance mediating the relationship between male and female in aquatic microcrustaceans.

Table 1. Semiochemicals sorted according their function (Nordlund and Lewis, 1976)

SEMIOCHEMICALS OR CHEMICAL SIGNALS	Pheromones Ej: • Trail-marking • Alarm • Aggregation • Sexual	Intraspecific Function		Emitter (+) Receiver (+)
	Allelochemicals	Interspecific Function	Kairomones	Emitter (-) Receiver(+)
			Sinomones	Emitter (+) Receiver (+)
			Allomones	Emitter (+) Receiver (-)

1.1.3 Mate choice in an extreme environment: the case of the brine shrimp *Artemia*.

As small, blind, marine pelagic planktonic organisms living in a non-intuitive (at least from the human perspective) three-dimensional world, give the initial impression that they reproduce by random encounters (Titelman et al., 2007). However, they do select their mates, as shown in a recent study of the pelagic copepod *Acartia tonsa* (Ceballos and Kiørboe, 2010). This species has evolved the use of remote signals, and females attract

males by producing pheromones and hydromechanical cues. As reported by Ceballos and Kiørboe (see also Titelman et al., 2007), there are copepods exhibiting pre-mating behavior and sophisticated pre-nuptial dances that may be interpreted as courtship. Additionally, females show a resistance behavior, typically seen in the selective sex. Motivated by such findings, this thesis inquired whether the pelagic planktonic brine shrimp *Artemia*, the sole macroscopic species living in hypersaline lakes and lagoons (although depending on salinity, *Artemia* may co-exist with salt-tolerating copepods), could exhibit a courtship pattern and chemical cues to recognize conspecifics. There are many reasons to anticipate that mate choice would involve both types of sex recognition within *Artemia* populations, and these are essentially linked with the life history the organism evolved to cope with the harsh, extremely salty and variable conditions encounter in hypersaline lakes and lagoons (see review in Gajardo et al. 2002). The genus *Artemia* is composed of six sexual species (there are parthenogenetic types in Eurasia) with regional endemism: *Artemia salina* (Mediterranean region), *A. tibetiana* (Tibet), *A. sinica* (China), *A. urmiana* (Lake Urmia, Iran), *A. persimilis* (Chile, Argentina) and *A. franciscana* (North, Central and South America). The latter is the most widely distributed and best-studied species (its genome will be sequenced soon). This species has been selected for this intersexual study at the population level for various reasons (Table 2). Firstly, it is easily manipulated and raised under laboratory conditions and thus is amenable to experimental studies. Secondly, it is widely distributed in a north-south altitudinal gradient (Parraguez et al., 2009) in island-like salty lakes that are highly heterogeneous in the ionic composition of their water and are subject to specific local environmental pressures and demographic fluctuations (cycles of population extinction and re-colonization). Such conditions facilitate inter-population

differentiation and ecological isolation (Abreu-Grobois, 1987; Bowen et al., 1988; Van Stappen 2002). An example of this differentiation is represented by *A. monica* (Mono Lake, USA), an *A. franciscana* population that is ecologically isolated because the conditions of Mono Lake (predominance of carbonates) are intolerable both to other *A. franciscana* populations and to other *Artemia* species (Browne and Bowen, 1991). Thirdly, to cope with stressful conditions, the reproductive cycle allows overlapping generations. Females combine the production of free-swimming nauplii (ovoviviparous reproduction mode) and encysted offspring (oviparous mode) (Figure 1), that is, the production of cysts that are embryos in a diapause state covered by a resistant shell (chorion). Populations differ in their proportions of nauplii and cysts (Browne, 1980). The ability of females to switch between producing predominantly nauplii, under stable environmental conditions, and encysted progeny under stressful conditions, such as in lagoons that dry out seasonally, has adaptive significance for populations exposed to critical environmental conditions, as cysts can remain viable for years. Therefore, cysts accumulating along of lagoons or lake shores may have been produced by females of different times or generations, thus containing a memory of the past (Gajardo and Beardmore, 2012), When those cysts hatch, individuals from different generations can encounter and reproduce with varying quality of female reproductive output depending whether the female mates with a male from the same generation or from another (Rode et al., 2011). Rode et al. (2011) made an interesting series of experiments with cysts from a population of *A. franciscana* from the Great Salt Lake (Utah) collected in three different years: a) 1985, b) 1996, and c) 2007. They crossed individuals from different years and from the same year (Figure 2) and evaluated survival and reproductive output of females mated either with males from the same year

(contemporary males) or from two different years (males from the past and the future, respectively). These authors showed that females mated with contemporary males (Figure 3): i) survived best, ii) although tended to produce less offspring per week, and a lower egg-laying rate, the lifetime reproductive success did not differ between time-shifts, and iv) relative fitness increase. According the above, the mating process in *Artemia* is not random at least for females, as the reproductive output and offspring survival is affected by the mating partner.

Fourthly, there are indications of sexual "adornments" in *Artemia* males, such as the claspers that males use to grasp females in amplexus (or the "riding" position). Claspers play a critical role when males try to grasp females, as the females express a resistance behavior typically seen in the choosy sex, in order to test the condition or characteristics of the male. Once a male grasps a female, the two ride together in what is believed to be a sort of male guarding behavior through which the male prevents the female from being approached and, potentially, fertilized by other males. This is a typical behavior in crustaceans (Jormalainen, 1998). *Artemia* also exhibit subtle morphological structures of the "key-lock" variety (Rogers, 2002), as well as microscopic structures that may also play a role in amplexus, thus isolating species and populations (Mura and Gajardo, 2011). Last but not least, indirect evidence of females selecting males was provided by Zapata et al. (1990) in multiple-choice experiments (excess of males competing for females).
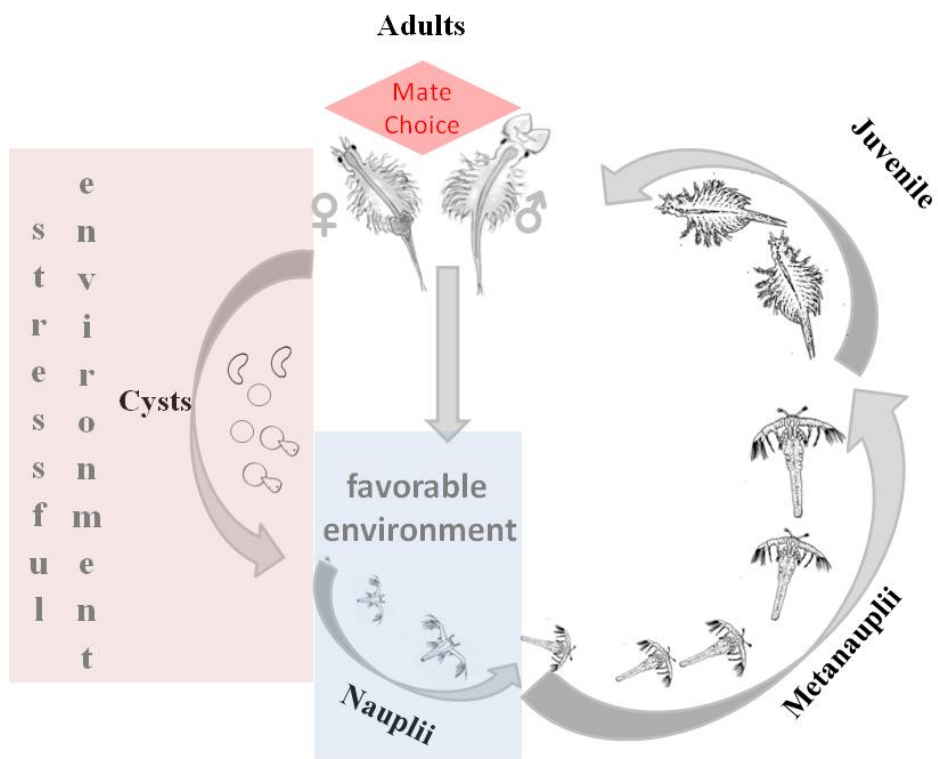
Figure 1. Main components of *Artemia* life cycle.
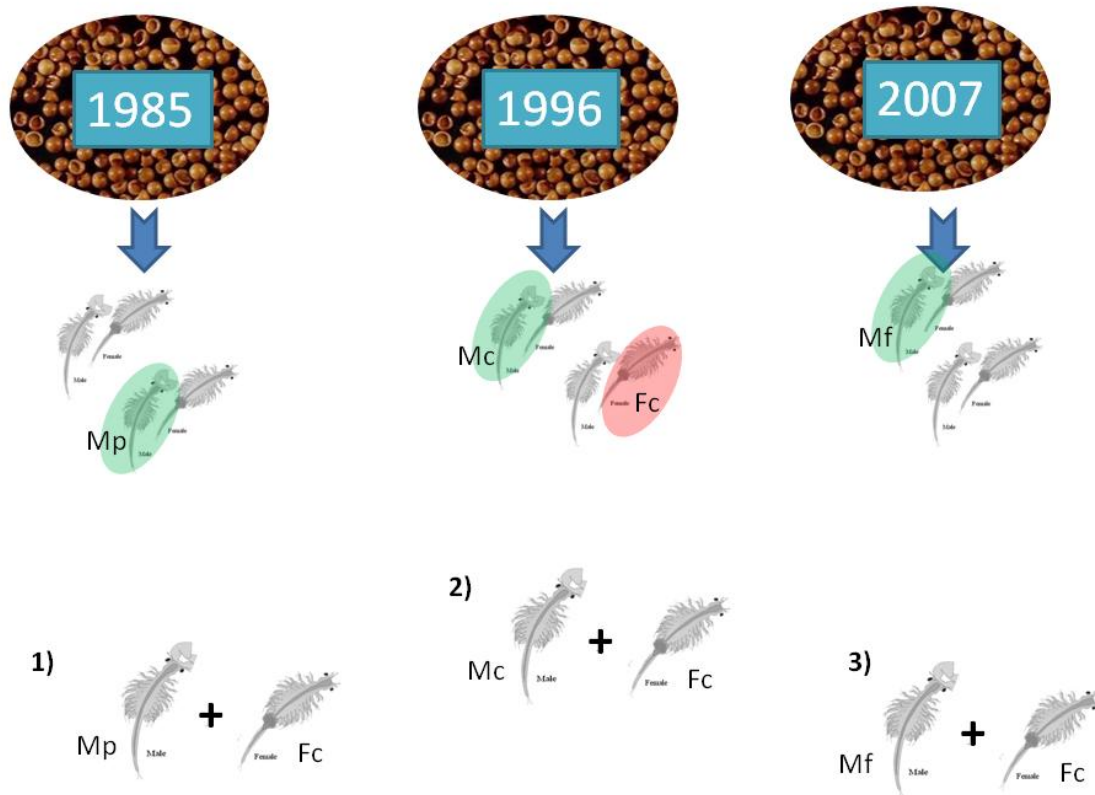
Figure 2. Experimental design developed by Rode et al. (2011). Fc: contemporay female, Mc: contemporary male, Mp: male from the past, Mf: male from the future. 1) cross experiment conformed by one sibling contemporary female (1996) and one male from the past (1985), 2) cross experiment conformed by one sibling contemporary female (1996) and one contemporary male (1996), 3) cross experiment conformed by one sibling contemporary female (1996) and one male from the future (2007).
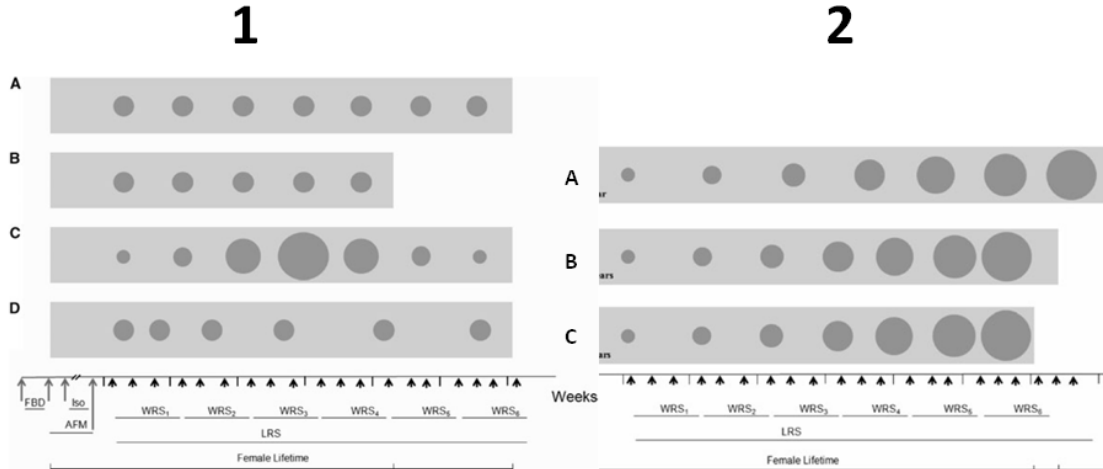
Figure 3. Schematic illustration of: (1) the survival and reproductive traits analyzed: Female lifetime reproductive success (LRS) of females B, C, and D differs from the reference female A (with constant brood size and interbrood interval) in one trait: lifespan (B), weekly reproductive success (WRS, C) and interbrood interval (reproductive rate, D). Tick marks on the horizontal axis represent the weeks after first reproduction. Vertical arrows are observations. Light gray rectangles represent female life span. Dark gray circles represent reproductive events and circle size represents brood/clutch size. WRS is the number of offspring produced over a 7-day period. FBD=time between the start of the experiment and female birth. AFM=female age when the first male was introduced. Iso=isolation time between brood individualization and first male introduction.

And (2) the results obtained: Females survived best when mated to contemporary males (rectangle length in A, B and C). Female lifetime reproductive success did not differ between time-shifts (same number of circles in A, B and C). Female interbrood interval decrease was shallower when females were mated to contemporary males (longer distances between the circles in A, compared to B and C). Schema and its components extracted from Rode et al. (2011).

Table 2. Factors supporting for a study of mate choice between heterogeneous populations of the brine shrimp *Artemia franciscana* (modified from Gajardo and Beardmore, 2012).

1. This species is easily cultured, maintained and manipulated under laboratory conditions, which facilitates experimental studies.

2. Populations of *A. franciscana* are widely distributed in scattered or isolated salty lakes (inland-like, resembling biogeographical islands).

3. Salty lakes are highly heterogeneous in water ionic composition (somewhat ecologically isolated). Both factors 2 and 3 suggest that there are locally adapted populations.

4. Female switch offspring type depending on whether environmental conditions are perceived as favorable (free-swimming nauplii; ovoviviparous mode of reproduction) or unfavorable (encysted embryos; oviparous mode).

5. Cysts are embryos covered by a thick shell that protects them from environmental aggression, and they remain viable even almost dehydrated for years.

6. Cysts released by females from different generations accumulate along lakeshores and can hatch at the same time. When this occurs, females face the challenge of mating with contemporary males or with males from the past or future (Rode et al., 2011).

7. There are evident sex-related morphological structures (claspers) and subtle, microscopic differences that might play a role in mate choice.

8. There is evidence of females of this species being choosy (Zapata et al 1990).

Based on the arguments given above, mate choice in *Artemia franciscana* is expected to be a highly specific process in which females appear to play the selective role, i.e., females are choosy. A combination of behavioral (courtship) and chemical cues is anticipated to be involved in *Artemia* mate choice. Such selection is not trivial, as males could have hatched from cysts released by females from a different generation or, alternatively, males and females from isolated salty lakes that may contain locally adapted populations (exhibiting subtle or evident phenotypic or behavioral differences) could come into contact. Due to the stringent environmental conditions of the salty lakes, the decision of mating with a given male will influence the type of offspring produced and their survival, and it will thus affect population continuity.

1.2 Hypothesis and research objectives

1.2.1 Hypothesis

Mate choice in *Artemia franciscana* is a critical process that affects female reproductive output, as well as offspring quality and fitness in a stressful environment. Hence, this thesis tested the following two hypotheses:

i) A combination of an elaborate courtship (a series of independent sequential steps) and chemical cues is expected in the mate choice between conspecifics of opposite sexes.

ii) Cuticular components found in males and females play a role as short-range chemical signals that help with finding and recognizing suitable mates by affecting the swimming speed of males.

1.2.2 Research objectives

1.2.2.1 General objectives

To determine the existence of behavioral patterns and the involvement of long- and short-range chemical signals in the courtship of *A. franciscana*.

1.2.2.2 Specific objectives

1. To determine the existence of a courtship behavioral pattern in the mating of *A. franciscana*.

2. To determine the presence of long- and short-range chemical signals involved in the courtship behavior of *A. franciscana*.

Chapter II

Mating behavior and chemical communication in Crustacea

2 Chapter II: Mating behavior and chemical communication in Crustacea

2.1 Introduction

Chemical communication is ubiquitous in nature (Thiel and Briethaupt, 2011; Nehring et al., 2013), has existed for a long time during evolution (Wyat, 2014) and is a stable type of signal that is not modified by changes in the abiotic conditions (Bargmann, 2006). It plays an important role in the mating behavior of organisms (Wyatt, 2003), such as in mate searching (Baruffaldi et al., 2010; Yen and Lasley, 2011) and mate recognition (Roberts and Uetz, 2005); thus, it helps with maintaining reproductive isolation between different species and with population divergence (Roberts and Uetz, 2004; Smadja and Butlin, 2009).

In Crustacea, Ryan (1966) showed for the first time the existence of chemical signals involved in mating behavior. He determined that *Portunus sanguinolentus* (Crustacea: Decapoda) males display a mating behavioral response in presence of a conspecific female in premolt stage, and a similar behavior is replicated by males when exposed to water containing chemical compounds from premolt females but not to water containing compounds from intermolt females. Other studies have also confirmed the importance of chemical communication in the mating behavior of large crustaceans and microcrustaceans (Atema and Engstrom, 1971; Katona, 1973; Dunham, 1978; Atema and Cowan, 1986; Kamio et al., 2000; Ingvarsdóttir et al., 2002b; Ting and Snell, 2003, Bailey et al., 2006, Zhang et al., 2011; Briethaupt and Thiel, 2011). In all these investigations, a previous indication of a courtship-like mating behavior led to the search for chemical signals (Kamio and Derby, 2011; Hardege and Terschak, 2011). This is consistent with the definition given

by Wyatt (2003), who defines chemical signals present in the mating process of organisms (pheromones) as *"molecules that are evolved signals, in defined ratios in the case of multiple component pheromones, which are emitted by an individual and received by a second individual of the same species, in which they cause a specific reaction, for example, a stereotyped behavior or a developmental process"*.

This chapter seeks to review recent precedents regarding mating behavior in crustaceans and is intended to discuss the most relevant research demonstrating the existence of chemical communication and its close relationship with mating behavior in macro- and microcrustaceans.

2.2   Crustacean mating behavior

Crustacean mating behavior commonly comprises several and diverse steps, in which males are usually the most active individuals (Table 3). According to Almerao et al. (2010), *Aegla platensis* (Crustacea: Decapoda) showed three phases of mating behavior:

1) Precopulatory phase: This phase begins with an agonist male behavior during which the male walks toward the female while raising and opening his pereiopods (chelipeds). Then, the male approaches the female, touching her with his pair of antennae and second, third, and fourth pairs of pereiopods. The female may allow the male to approach her or may walk or swim away from him, a behavior apparently dependent on the female's stage of gonadal development. Once the male's approach is successful, he begins courtship immediately, a behavior that includes lifting himself up on the tips of his pereiopods, with

chelipeds opened and raised. The male alternates between thrusting and striking his telson and uropods against his own thoracic sternites toward the female. When the female displays a submission posture (bends down on the substrate), the male touches her with pereiopods and antennae, climbs on her cephalothorax, and repeatedly vibrates his abdomen close to her.

2) Copulatory phase: During this phase, the male and female face each other and touching each other with their antennae. Then, the male grasps the female with his first pair of pereiopods, lifts the female from the substrate, and rotates her approximately 90º, positioning himself under the female. In this position, he holds the antennae of the female with the first pereiopods (chelipeds) and grasps the body of the female with his second, third, and fourth pairs of pereiopods. Still in this position, both the male and female slightly open their abdomens, and the female releases the oocytes through the genital pores inside her abdominal chamber. Copulation is completed when the female touches the mouthparts of the male (the male is still under the female), at which point the male releases the female.

3) Postcopulatory phase: During this phase, when the female is released, she moves away from the male and tightly closes her abdominal chamber. The male remains close to the female and sometimes climbs onto her and touches her body with his antennae and pereiopods (male guarding behavior). Then, the female begins grooming her abdominal chamber and eggs with her fifth pair of pereiopods.

Thiel et al. (2010) reported three main events involved in the mating behavior of *Rhynchocinetes brucei* (Crustacea: Decapoda): 1) Contact, through different postures by male over the female body (overlap, touching, and cage position), 2) spermatophore transfer event, and 3) mate guarding.

Spíndola and Feitosa (2012) described the mating behavior sequence of *Ucides cordatus* (Crustacea: Decapoda): courtship begins when a receptive female allows a male to approach (but can reject him by moving away); the male extends the chelipeds and the second pair of pereiopods laterally, forming an arch around the female, while intermittently opening and closing his abdomen. Standing on the third, fourth and fifth pair of pereiopods, the male then draws the female closer until she is firmly secured, and with the body raised, the male introduces the gonopod into the female's gonopore. When the copulation has finished, the male releases the female. In the decapod crustacean *Portunus pelagicus* (Soundarapandian et al., 2013), the mating ritual is divided into six phases, as follows: 1) pre-molt guarding, which starts when one male (with a hard shell) moves towards the female (at the verge of the premolt stage) and extends his large chelate in front of her. Then, the male catches the female with his chelae and embraces her for a short time while using his walking legs to hold her beneath him, forming a position called the cradle-carrying position (in this position, both animals face in the same direction). 2) Molting: when the female starts molting, the couple disengages from the cradle-carrying position. In the meantime, the male crab moves around the female, helping her to molt by removing the shell. 3) Precopulatory guarding: after molting has finished, the male and female rejoin in the cradle-carrying position, sometimes until the start of mating. 4) Copulation: this stage begins when the exoskeleton of the female is soft. The male crab rotates the female by using his walking legs and uses his chelate to turn her over. The female, positioning herself upside down beneath the male, extends her abdomen, exposing the gonophores, thus allowing the male to insert his paired gonopods into her genital pores. (By this time, the male and female crabs are facing in opposite directions.) The ventral surfaces of the male

and female are facing each other, and the abdomens of both are flung backwards. During copulation, the male often walks around with the female attached to its ventral surface, holding her with his third and fourth walking legs. 5) Postcopulatory guarding: after copulation, the male frees the female from the mating position and embraces her to form the cradle-carrying position, which continues for only a few hours. The female is inactive until she attains normal hardness of her exoskeleton. 6) Spawning: during mating, the male crabs deposit spermatophores in the spermatheca of the female, and they are stored until the female is ready for spawning. During spawning, the eggs are liberated from the ovaries and pass through the seminal receptacles containing the stored sperms, which are liberated from the spermatophores to fertilize the eggs. Then, fertilized eggs are extruded through the gonophores present in the sternites of the sixth thoracic segments of the third pair of legs, and these eggs become attached to the smooth setae present on the endopodites of the four pairs of pleopods in the abdominal flap of the female.

For the brine shrimp *Artemia* (Crustacea: Anostraca), the mating behavior has not been reported. However, there are antecedents for other members of Anostraca (Table 3). Moore and Ogren (1962) described the mating behavior of *Eubranchipus holmani* as follows: 1) The female adopts a stationary position with a body declination of approximately 45 degrees to water surface. 2) The male approaches under the body of the female and adopts a stationary position. Then, the male abruptly leaves the stationary position and either swims away or attempts to engage the female in amplexus. 3) After the male has grasped the female in amplexus, she briefly performs strong movements in a sort of vertical circle. If the male succeeds in keeping the female in amplexus, the female swims slowly and carries the male. A similar pattern was described by Wiman (1981) for *Streptocephalus mackini, S.*

dorothae, S. texanus, S. seali, and *S. bouvieri.* The mating behavior of these shrimps consists of a series of events that may differ in frequency or duration, but not in sequence, between species: 1) detection and orientation, 2) station-taking, 3) clasping, 4) intromission, and disengagement. When the male detects a conspecific female, he swims towards her with fast movements using the uropods, and he positions his head just above the female's posterior abdominal segments. At that stage, the male quickly moves his claspers to grasp the female in amplexus. Afterward, copula takes place using one of the two eversible penises. Finally, the animals disengage and continue with their normal swimming.

In summary, courtship in Decapoda follows a behavioral pattern with some similarities, as well as species-specific particularities. Among the specific differences, the mate-guarding behavior seems to exhibit more variability, as some species express the behavior before copula (precopulatory mate-guarding) and others after copula has occurred. In Anostraca, the behavioral pattern tends to be less variable (Moore and Ogre, 1962; Wiman, 1981). In all cases, observation of the courtship ritual leads to the expectation that the sending and recognition of chemical cues should also be involved, perhaps a type of sex pheromone (Johansson and Jones, 2007).

Table 3. Summary of mating patterns for different orders belonging to Crustacea.

Species	Order	Habitat	Mating Patterns	Authors
Eubranchipus holmani	Anostraca	Temporary Freshwater Environments	Sstation taking, amplexus	Moore and Ogren (1962)
Gonodactylus bredini	Stomatopoda	Rocks and rubble in littoral and sub-littoral zones of Atlantic.	Male start the approach, swim over the female, palpates her body with his antennules and grasps her at the telson. Or, Female start the approach from the front or side of the male and both oppose their genital openings for the copula.	Dingle and Caldwell (1972)
Streptocephalus seali, Streptocephalus mackini, Streptocephalus dorothae, Streptocephalus texanus,	Anostraca	Temporary Ponds	Detection and orientation, station taking, clasping, intromission, and disengagement.	Wiman (1981)

and *Streptocephalus bouvieri*.				
Cyzicus grubei	Diplostraca	Temporary Freshwater Environments	Capture, Positioning, and Copulation.	Pérez-Bote (2010)
Lynceus brachyurus	Laevicaudata	Temporary Ponds	Male approaching to female, searching behavior (male swim around to female), male attempts to clasp the female (using his claspers), male hold on the edge of one of female valves, Bending of hind body and brushing behavior of Male (male attempts open the female valve and copulate with female).	Sigvardt and Olesen (2014)

2.3 Chemical communication in Crustacea

2.3.1 Chemical signals and mating behavior

The study of semiochemicals involved in the mating behavior of Crustacea began in the 1960s, and the list has increased considerably to the present day. The largest number of studies have been focused on decapod species, for which evidence of chemical communication has been demonstrated (Table 4); in a few cases, general chemical information has been reported about the putative pheromones, but only for the crab *Erimacrus isenbeckii* have pheromones been partially identified (Table 5). Compared to decapods, considerably less information is available on semiochemicals involved in the mating behavior of aquatic microcrustaceans. In the marine harpactocoid copepod *Tigriopus japonicus*, males exposed to females previously washed with trypsin or to females not treated with this peptidase allowed the inference that males were able to distinguish glycoproteins present on the cuticular body surface of females because females treated with trypsin were significantly less attractive to males than untreated females. In parallel, a monoclonal antibody that was synthesized using a trypsin-released protein fragment was able to recognize the surface protein of *T. japonicus* females (Ting et al., 2000). Later, Ting and Snell (2003), using the antibody developed previously, identified two proteins (70,000 Da and 36,000 Da) from the cuticular body surface of *T. japonicus* females. The authors reported that these proteins are similar to α-2-macroglobulin, a protease inhibitor, and postulated an interesting model of detection of the female pheromone by the male. Other examples include the ectoparasite *Lepeophtheirus salmonis* (Copepoda: Caligidae), which uses chemical signals for locating fish hosts (Ingvarsdottir et

al., 2002a), and mates (Ingvarsdottir et al., 2002b). The intraspecific study made it clear that males were significantly attracted to: a) preadult II virgin females, b) total extract from water conditioned with preadult II virgin females, and c) vacuum-distilled nonpolar fraction from water conditioned with preadult II virgin females. A similar study established that young and adult females of the ectoparasite *Caligus rogercresseyi* (Copepoda: Caligidae) elicited an attractant response from conspecific adult males (Pino-Marambio et al., 2007). These authors suggested that *C. rogercresseyi* females release a sexual attractant, but they did not identify the compounds involved in this behavior.

2.3.2 Chemical signals and swim behavior

Swimming is an important behavior closely related to the mating process in microcrustaceans and reported to be strongly influenced by semiochemicals. One example is the change in swimming speed of the small cyclopoid copepod *Oithona davisae* (0.3 mm in body length). Males showed significantly (P<0.0001) greater swimming activity and speed (>40 mm s^{-1} or >100 body lengths s^{-1}) in the presence of virgin females compared to the swimming activity and speed in absence of females (15 mm s^{-1}) (Kiørboe, 2007). According to Heuschele and Kiørboe (2012), *O. davisae* males placed into a bottle containing water previously conditioned with conspecific virgin females exhibited significantly (P<0.00031) greater swimming activity and speed compared to their activity and speed when the water used was previously conditioned with conspecific mated females (7.64 mm s^{-1}), or with virgin males (4.15 mm s^{-1}). Likewise, Van Leeuwen and Maly

(1991) showed that there was a significant increase in the swimming speed of *Diaptomus leptopus* males (Copepoda: Calanoida) in the presence of gravid females (P<0.025) but not in the presence of males (P>0.25) or nongravid females (P<0.10). Seuront (2013) suggested that *Eurytemora affinis* (Copepoda: Calanoida) males are able to detect pheromones emitted by non-ovigerous or ovigerous females. Males spent significantly more time following an artificial trail constituted of water conditioned using non-ovigerous or ovigerous females (84.6% and 81.3% of the time, respectively) than when exposed to a trail made with male-conditioned water or to an unscented control trail (9.3 and 9.4% of the time, respectively). In addition, significant differences were found between the swimming speed of males following a trail made with water conditioned with non-ovigerous (3.75 mm s^{-1}) or ovigerous females (3.22 mm s^{-1}) with respect to the swimming speed developed when males followed a trail made with water conditioned using males (2.53 mm s^{-1}) or an unscented control trail (2.49 mm s^{-1}). Meanwhile, Tsuda and Miller (1998) reported that the calanoid copepod *Calanus marshallae* followed a series of swimming patterns (hop and sink, search, chase, escape, dance). "Search" and "dance" were performed by males only in the presence of newly molted females. Both patterns imply an increase in the swimming speed to 10 times higher than the other swim patterns, which did not require the presence of newly molted females. At the same time, Doall et al. (1998) reported that males of the calanoid copepod *Temora longicornis* (1 mm in body length) developed a swimming speed (9.73±3.18 mm s^{-1}) greater than the swim speed shown by females (5.91±2.28 mm s^{-1}) and that males increased their swimming speed in the presence of females (24.96±9.39 mm s^{-1} or >20 body lengths s^{-1}). For the calanoid copepod *Hesperodiaptomus shoshone*, Yen et al. (2011) reported that the swimming speed of males (1.25±0.14 mm s^{-1}) was 1.75 times

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higher after detecting the trail mimic using female-conditioned water (1.67±0.21 mm s^{-1}) (P=0.0007). Another example of the possible chemical communication involved in the swimming patterns was reported by Nihongi et al. (2004) for the calanoid copepod *Leptodiaptomus ashlandi*. The male swimming speed (1.36 mm s^{-1}) in the presence of females was 1.75 times higher than the swimming speed in absence of females (P< 0.01). These results indicated that the conditioned water contained semiochemicals used as chemical cues for recognizing potential mate females by conspecific males of *O. davisae, E. affinis,* and *H. shoshone*.

In the case of the brine shrimp *Artemia*, the information about the chemical communication is nonexistent. However, there is indirect information about the ability to detect chemical signals. Tyson and Sullivan (1979) found and described, by means of scanning and transmission electron microscopy, two types of cuticular sensilla (named type 1 and type 2 sensilla) on the antennules of adult *Artemia*. Both types of sensilla were subjected to dye-penetration experiments using a solution of crystal violet, a common technique used for identifying chemoreceptors in insects (Slifer, 1970). Light microscopic examination of these two types of sensilla indicated that type 2 sensilla were permeable to the dye, and consequently may have chemoreception function, whereas type 1 sensilla were not.

Table 4. Sex pheromone evidence in decapod crustaceans

Species	Experimental Antecedents	Main Results	Reference
Portunus sanguinolentus	Behavioral observation of the male behavior in presence of females in different stage of molt	Male exhibit the same mating behavior when are exposed to both premolt female and conditioned water with premolt females	Ryan (1966)
Chionoecetes opilio	Observation of male behavior exposed to female conditioned water.	Males exhibited significantly increased activity exposed to water from a source aquarium containing moulted pubescent females, egg-stripped multiparous females and moulted immature females than exposed to water from an aquarium containing berried multiparous females, eggs alone, adolescent males or an empty aquarium.	Bouchard et al. (1996)

Telmessus cheiragonus	Behavioral observation of males exposed to sponges dipped in premolt (1 min or 20 h) and postmolt (1 min or 20h).	Males showed copulation behavior in presence of sponge previously dipped, in a common aquarium, with postmolt females.	Kamio et al. (2002)
Palaemonetes pugio	Behavioral observation of males in presence of females in different stages of life.	Males that make physical contact with a postmolt parturial (with eggs) female react with copulatory behavior (grasping and/or mounting).	Caskey and Bauer (2005)
Portunus pelagicus	Sponges injected with urine of: (a) male crab, (b) premoult and (c) postmoult female crabs.	Sponge containing premoult and postmoult female urine was attractive to sexually receptive males.	Vasudevan and Mahin (2013)

Table 5. Chemical traits of the putative sex pheromones in Crustacea.

Species	Compound	Emitter	Receiver	Author (s)
Erimacrus isenbeckii	Ceramide	Females	Males	Asai et al. (2000)
Telmessus cheiragonus	> 1000 Da*	Females	Males	Kamio et al. (2002)
Carcinus maenas	>1000 Da*	Females	Males	Hardege et al. (2002)
Tigriopus japonicus	70000 Da*	Females	Males	Ting and Snell (2003)
Tigriopus japonicus	36000 Da*	Females	Males	Ting and Snell (2003)
Carcinus maenas, Chionoecetes opilio, Stenorhynchus seticornis	Uridine diphosphate	Females	Males	Bublitz et al. (2008)
Carcinus maenas	Uridine diphosphate	Females	Males	Fletcher and Hardege (2009)

*Da = mass unit which defines the size of a protein

2.4 Conclusions

1. Mating behavior in crustaceans has significant impact on species integrity and population divergence, so this topic has been experimentally studied from behavioral (courtship) and chemical signaling perspectives, with the former often leading to the latter.

2. Mating behavior in large crustaceans, such as decapods, includes an elaborate combination of a behavior ritual such as the courtship, as well as chemical signals that are commonly species-specific.

3. Mate choice in small freshwater (*Diaptomus leptopus, Hesperodiaptomus shoshone*.), euryhaline (*Eurytemora affinis)*, and marine planktonic individuals (*Oithona davisae, Calanus marshallae, Tigriopus japonicus*), as well as freshwater Anostracans (*Artemia* relatives), also involves a courtship-like behavior and chemical signaling, but these processes have been less studied.

4. Bio-directed behavioral experiments using conditioned water has been shown, in most cases, to be the most widely used and suitable method to establish the existence of chemical communication in Crustacea.

5. The possible involvement of sex pheromones remains poorly understood for the order Anostraca. In the case of *Artemia*, although there are indirect antecedents indicating the morphological and physiological capacity of *Artemia*'s antenna for detecting chemical cues, there have been no reports regarding chemical communication in these species.

Chapter III

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

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3 Courtship behavior and potential indications for chemical communication in

***Artemia franciscana* (Kellog, 1906)**

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3.1   Introduction

Mate choice is a critical fitness parameter influencing offspring output and quality, and the persistence of a population in a given environment (Andersson and Simmons, 2006; Jones and Ratterman, 2009). Therefore, species have evolved suites of traits to optimize mate selection, such as an elaborate courtship behavior and/or chemical signals (Eisner and Meinwald, 1995; Dicke and Takken, 2006; Müller-Schwarze, 2006; Breithaupt and Thiel, 2011). Such specificity favors the recognition of suitable conspecific mates within a population, and prevents interspecific mating (Smadja and 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 and 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 is 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 and Gajardo, 2009; Gajardo and Beardmore, 1989). Thirdly, because they accumulate in lake shores and eventually hatch when suitable conditions resume (Gajardo and 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 and Thiel, 2011). In Decapoda like *Carcinus maenas*, *Portunus sanguinolentus* and *Telmessus cheiragonus*, has been demonstrated the existence of certain precopulatory behavioral patterns mediated by semiochemicals (Ryan, 1966; Bamber and 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 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.

3.2   Materials and methods

3.2.1   Sample origin

Artemia franciscana individuals were collected in the Cejar Lagoon (3 ha in extent and an average water depth 10 m) (Gajardo and 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 x 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 and Beardmore (1993).

3.2.2   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.

3.2.3 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).

3.2.4 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 and 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.

3.2.5   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.

3.2.6   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 and Young (1978), with expected values calculated using a Chi-square test (X^2) according to Martin and 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).

3.3 Results

3.3.1 Ethogram

The ethogram of *A. franciscana* males (Figure 4; Table 6) 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 4 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%).
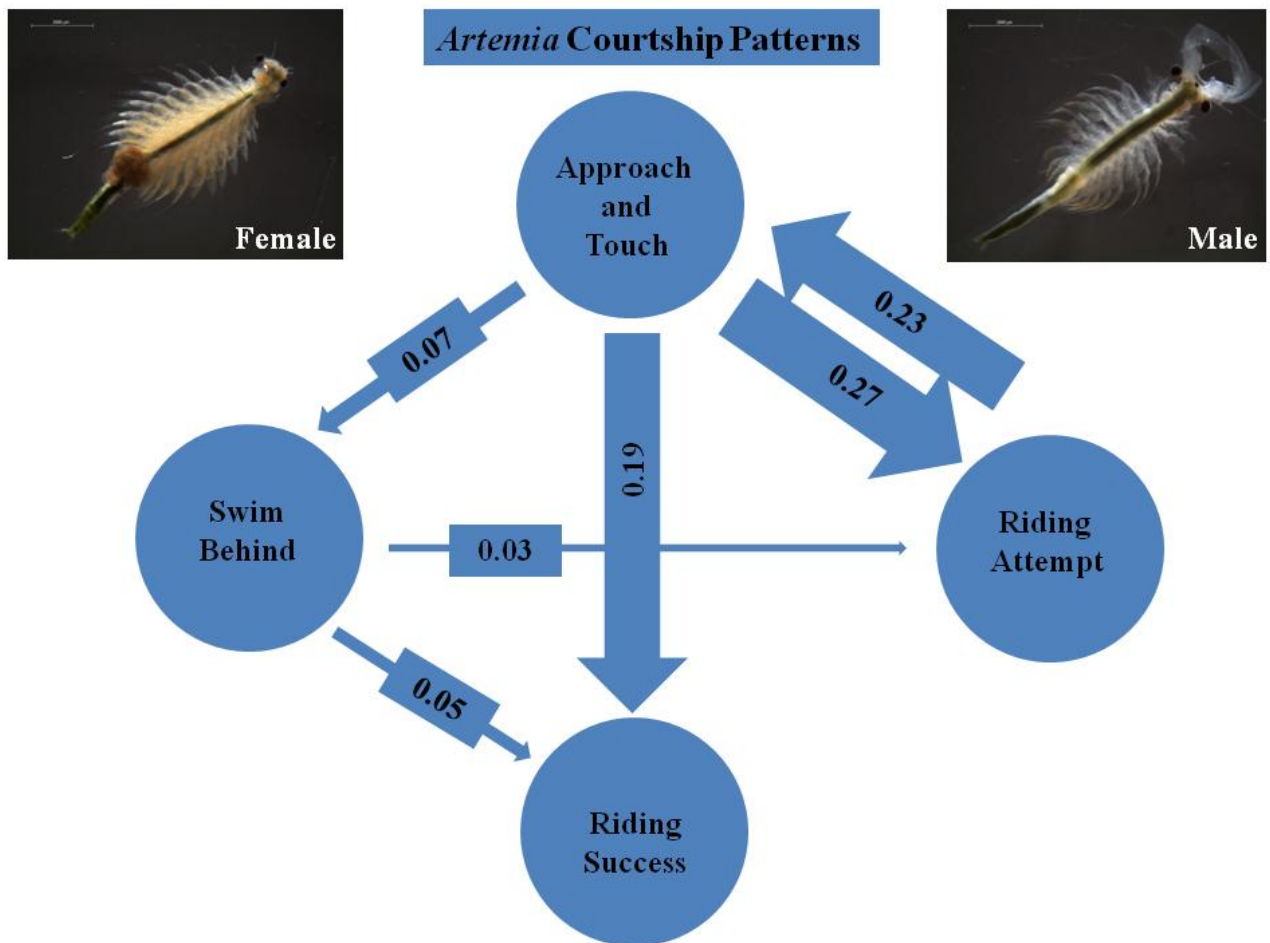
Figure 4. The components of the courtship ethogram of *A. franciscana* (N=30 couples, P = 0.05). The values in arrows indicate the probabilities of transitions from one behavior to other. Each percentage indicates the probability that this step occurs, the complement to 100% is the probability that this step does not occur. Transitions are not indicated in the diagram were not significant (P>0.05).

Table 6. 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.

	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

3.3.2   Behavioral bioassays with PS

Figures 5 and 6 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 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.
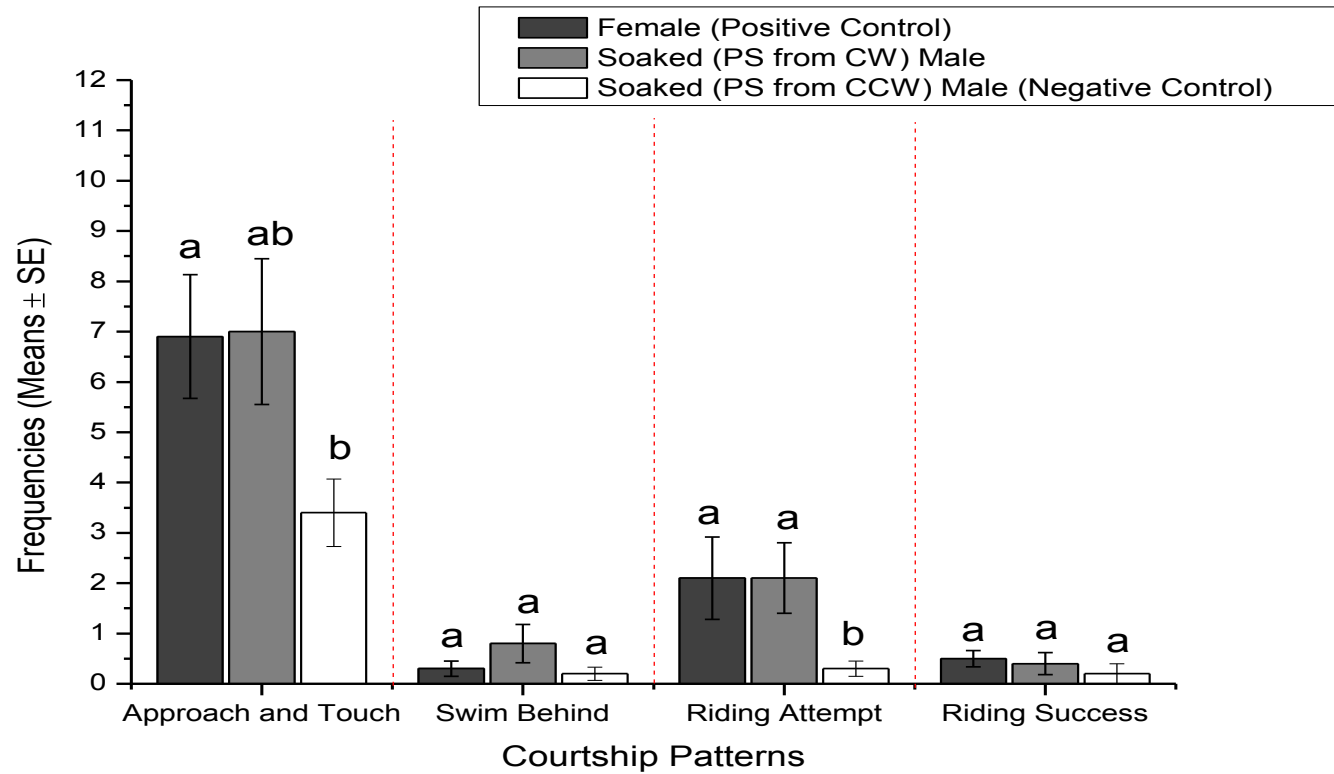
Figure 5. Behavioral experiment with 1:1 ratio. Response elicited by one female, one soaked male with PS from CCW, or one soaked male with PS from CW on one conspecific non-soaked male, respectively (N=10). Different letters indicate that there are significant differences based on the Kruskal-Wallis test followed by Conover-Inman's test (P<0.05).
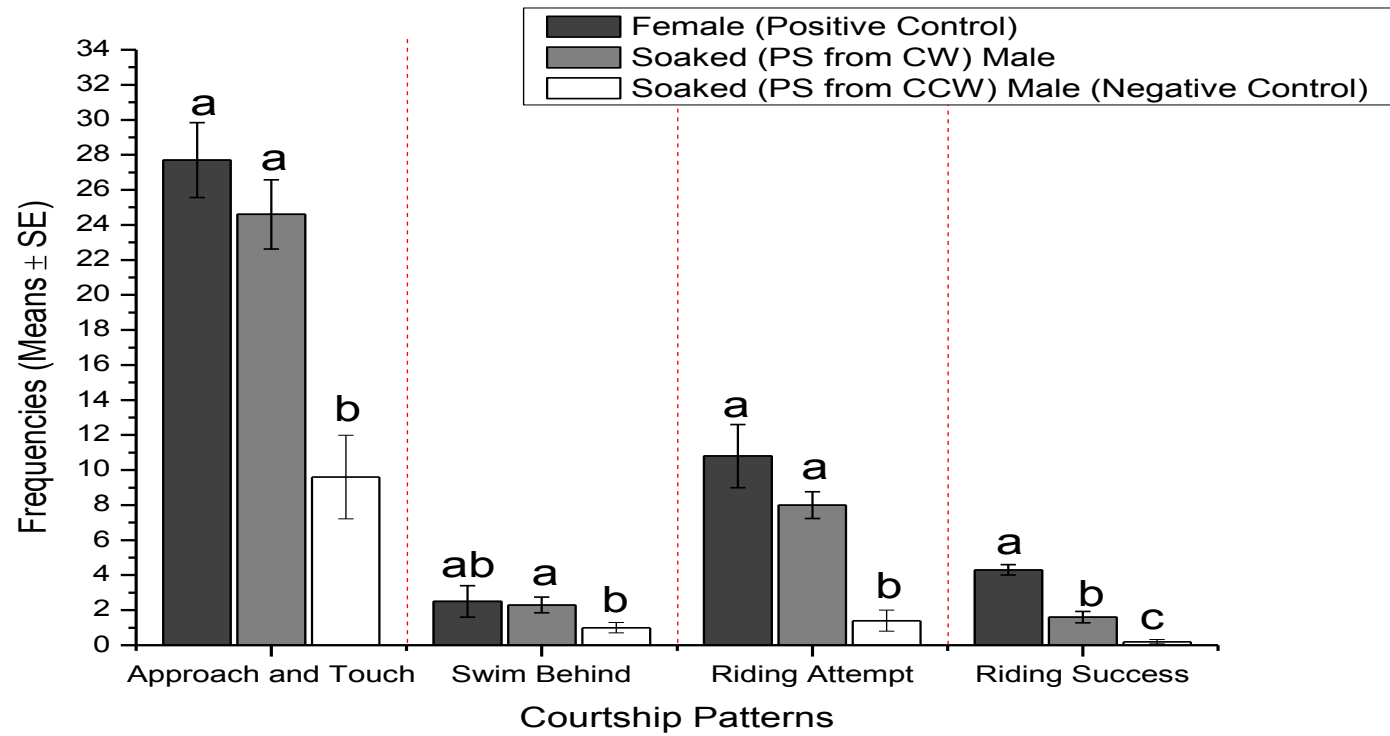
Figure 6. Behavioral experiment with 1:5 ratio. Response elicited by one female, one soaked (PS from CCW) male, or one soaked (with PS from CW) male on five conspecific non-soaked males, respectively (N=10). Different letters indicate that there are significant differences based on the Kruskal-Wallis test followed by Conover-Inman's test ($P<0.05$).

3.4 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 and 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.

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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 and 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 and 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 and 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 and 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; Vasudevan, 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 and Gherardi, 2010; Okamura and Goshima, 2010).

The observation of specific courtship 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 and Gajardo, 2011).

3.5   Conclusions

Brine shrimp *Artemia franciscana* showed four patterns in courtship behavior. These behavioral patterns were similar to the patterns previous to copula described for other Anostrocan (*Streptocephalus* sp., and *Eubranchipus holmani*). Ethogram constructed indicates that females showed strength against the male riding attempt, which might be, in

some way, an indicative of selective pressure by females towards males, and allows speculate about the existence of mate choice. Furthermore, for the first time is show behavioral evidence of the probable existence of a sex pheromone involved in the courtship behavior of *Artemia*. The putative pheromone have polar traits, because in the bioassays was used the polar fraction obtained from conditioned water of *A. franciscana* female. The evolutionary relevance of this finding in relation to the ecological divergence of *Artemia* habitats should be discussed.

Chapter IV

Cuticular compounds of *Artemia franciscana* (Kellog, 1906) (Crustacea: Anostraca) increase the swim speed of conspecific males

4 Cuticular compounds of *Artemia franciscana* (Kellog, 1906) (Crustacea: Anostraca) increase the swim speed of conspecific males

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4.1 Introduction

The brine shrimp *Artemia franciscana* is the most widespread member of the genus *Artemia* (Crustacea: Anostraca) and is the unique microcrustacean that inhabits hypersaline environments (Persoone and Sorgeloos, 1980; Van Stappen, 2002). *Artemia* is an excellent extremophile model for understanding the adaptation process both in nature and under laboratory conditions, due to the extreme conditions where these organisms develop (Gajardo et al., 2002; Gajardo and Beardmore, 2012). It is also an excellent bioindicator of xenobiotic and toxic compounds in the environment and is a good resource for marine aquaculture (Bengtson et al., 1991; Sorgeloos et al., 2001). *A. franciscana* have a sexual reproduction mode, and, similar to other microcrustaceans inhabiting large water spaces in relation to their size, the maintenance of the species depends on the probability of contact between males and females and of the subsequent effective mate recognition (Briethaupt and Thiel, 2011). This probability of encounter may be influenced by chemical factors (Briethaupt and Thiel, 2011; Ceballos and Kiørboe, 2010) that directly influence the activity of both sexes (in the case of *Artemia*, males that devote energy to searching for females), specifically swimming speed (Seuront, 2013). The faster the animal moves, the larger the area covered in less time (Gerritsen, 1980; van Duren et al., 1998); thus, the likelihood of finding a conspecific female increases (Kiørboe, 2008).

Several authors have established that one of the most effective ways for male crustaceans to find and recognize an opposite sex conspecific is through sexual pheromones emitted by females (Briethaupt and Thiel, 2011; Ceballos and Kiørboe, 2010). Such signals have

proved to affect swimming speed in crustacean males, as shown by experiments in which males are exposed to 1) live females (van Leeuwen and Maly, 1991; Tsuda and Miller, 1998; Nihongi et al., 2004; Heuschele and Kiørboe, 2012) or 2) conditioned water from live females (Yen et al., 2011; Seuront, 2013). Tsuda and Miller (1998) showed that males of the marine calanoid copepod *Calanus marshallae* developed specific swimming patterns, such as search and dance, in the presence of newly molted females. Both behaviors increased male swimming speed compared to males not confronted with newly molted females, which is interpreted as the males being subjected to a stimulus, most likely a sex pheromone released by females.

Recently, Seuront (2013) reported the swimming speeds of males of the euryhaline calanoid copepod, *Eurytemora affinis,* when confronted with estuarine water, male-conditioned water, non-ovigerous female-conditioned water, and ovigerous female-conditioned water. Males significantly increased their swimming speed in the presence of non ovigerous and ovigerous females in contrast to their speeds when confronted with estuarine water or male-conditioned water. The explanation proposed by Seuront (2013) was that the presence of a "diffuse background pheromone concentration" produced by females would stimulate the swimming behavior of males.

Studies have shown the existence of lipophilic compounds (glycoproteins, hydrocarbons and fatty acid) on the body surfaces of decapod crustaceans (Caskey et al., 2009) and of microcrustaceans (Snell and Morris, 1993) and have advanced an explanation regarding how these compounds affect the mating behaviors of these organisms (Caskey and Bauer,

2005; Zhang et al., 2011; Kelly and Snell, 1998; Ting et al., 2000; Ting and Snell, 2003). However, the understanding of how these compounds affect the swimming speed in crustaceans has not yet been clarified.

This study tested the hypothesis that cuticular compounds present on the body surface of *A. franciscana* females play a role in the swim speed of conspecific males. To this end, cuticular compounds extracted from males or females using a 2:1 chloroform:methanol solution were used for soaking artificial sponges to which individual non-soaked conspecific males were exposed. The swimming speed developed by each male was recorded and analyzed.

4.2 Material and methods

4.2.1 Sample origin

A. franciscana individuals were collected in the Cejar Lagoon (a 3 ha salty lagoon with relatively shallow depth, 10 m) located in the Atacama desert 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 saltwater (35 ppt). The juvenile instar meta-nauplii (2.2-2.6 mm in length) obtained from *Artemia* cultures were individually placed in Falcon tubes with 40 mL of artificial salty water (35 ppt) for approximately 20 days, to keep them virgin. *Artemia* individuals were fed *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 and Beardmore (1993).

4.2.2   Cuticular extract solution

Virgin females and males (approximately 20 days old) were grouped by sex to proceed to cuticular extraction from 40 virgin males (MCE) and 40 virgin females (FCE). This was performed according to the methodology of Caskey et al. (2009). Briefly, individuals were placed in a beaker with 5 mL of deionized water for 30 seconds in order to remove salt and other elements from the exoskeleton. Then, individuals were placed in a beaker with 5 mL of methanol (HPLC grade, Darmstadt-Germany) to make the surface miscible with the extraction solvent and, finally, placed in a beaker with 12 mL of a 2:1 mixture of chloroform and methanol (HPLC grade, Darmstadt-Germany) for 1 min to extract compounds from the surface. The extracts were filtered through a syringe filter (Millipore, Durapore 0.22 µm) with a syringe (BD Plastipak^{TM} 0.50 x 15 mm) and stored at 4°C until bioassays and esterification.

4.2.3   Bioassay

To determine the probable effect of chemical stimuli on the swimming speed of *A. franciscana* males, artificial sponges (3 x 5 mm) were soaked with cuticular extracts

obtained from *A. franciscana* males or females. The sponges were pre-washed with dichloromethane (HPLC grade, Darmstadt-Germany) in a Soxhlet system and then rinsed with artificial salty water, in order to eliminate possible colorants and other compounds. Once dry, the sponges were soaked with the cuticular extract solution obtained as described above.

The experiment consisted of placing one sponge in the center of a Petri dish (90 x 20 mm) on a flat base, above which a digital camera was placed for tracking and recording the animal's activity (swimming speed) (Figure 7). For 30 min, 1 male was confronted with a sponge soaked with 450 μL (equivalent to 1.5 females based on the volume (12 mL) and number (40) of females used in the extraction process) of FCE or MCE. Sponges soaked with artificial salty water (SW) or a 2:1 mixture of chloroform and methanol (CM) were used as controls. The entire video tracks were analyzed using behavior-tracking software (Ethovision 3.1, Noldus Technologies). All bioassays were replicated 10 times. Differences between groups were tested using the non-parametric Kruskal-Wallis test (P<0.05) with StatsDirect V.2.2 software (StatsDirect, Ltd., UK), followed by Conover-Inman's test for separation of groups (P<0.05) (Conover, 1999).
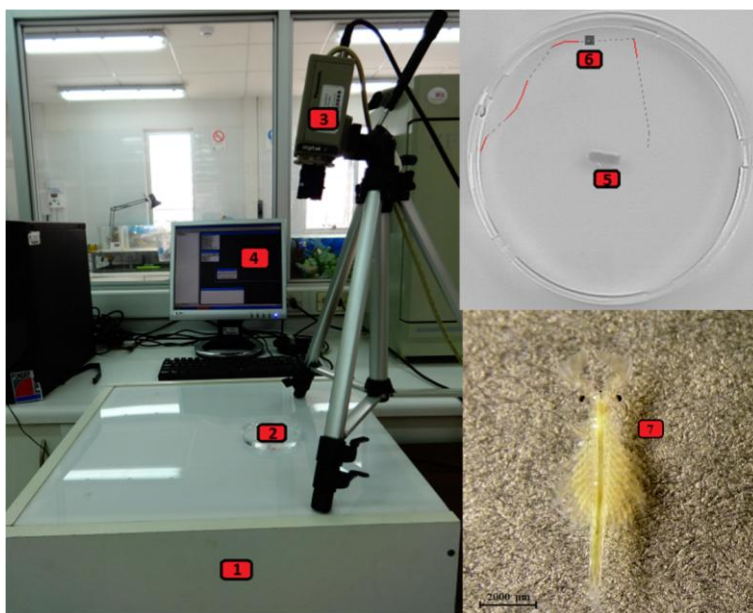
Figure 7. Bioassay set-up: (1) flat base, (2) Petri dish, (3) digital camera, (4) computer with behavioral tracking software (Ethovision 3.1, Noldus Technologies), (5) Sponge (soaked with various extracts, as indicated), (6) *Artemia* movement tracked during bioassays, and (7) *Artemia franciscana* male.

4.2.4   Preparation of fatty acid methyl esters

Fatty acid methyl esters (FAMEs) were obtained by adding 500 µL of an esterification reaction mix (methanol:hydrochloric acid:chloroform, 10:1:1) (HPLC grade, Darmstadt-Germany) to 3 mL of cuticular extract. The mixture was stirred in a vortexer for 20 s at 120 rpm and was immediately incubated at 90 ℃ for 45 min to complete the esterification reaction. The reaction tubes were left to cool at room temperature. Subsequently, 400 µL of distilled water was added to each tube, and the mix was stirred. Then, FAMEs were extracted using hexane/chloroform (4:1 v/v, 3 x 400 µL). Samples were centrifuged at

3,000 rpm for 15 min. The organic fractions were pooled, and hexane (HPLC grade, Darmstadt-Germany) was added to obtain a final volume of 1.0 mL (Lewis et al., 2000).

4.2.5 Analysis and quantification of the fatty acid methyl esters by GC-FID

Fatty acids were identified as methyl esters using a gas chromatography-flame ionization detector (GC-FID) (Fison GC 8000 series model, Italy) equipped with a BPX70 capillary column (30 m x 0.22 mm x 0.25 μm film thickness). A 1 μL sample, in splitless mode, was injected for each sample, with the injector temperature at 250 ℃. The initial column temperature was 100 ℃ for 1 min, increasing 6 ℃/min until reaching 250 ℃, and this temperature was maintained for 10 min. A commercial fatty acid methyl ester mix (Sigma Aldrich) was used for identification and quantification.

4.2.6 Analysis by GC-MS

FAME identification was corroborated using a gas chromatograph (Model Focus; Thermo Electron, Waltham, MA) coupled to a mass spectrometer (model DSQ; Thermo Electron) (GC-MS) equipped with a DBP-5 capillary column (30 m x 0.22 mm x 0.25 μm film thickness). Helium was used as gas carrier at a flow rate of 1.5 mL/min. Ionization was performed by electron impact at 70 eV and 250 °C. The GC oven was programmed to remain at 40 °C for 1 min and then increased 5 °C/min to 250 °C. The identification was

corroborated via comparisons between experimental mass spectra and those in the NIST mass spectral database (NIST ver. 2.0; Thermo).

4.3   Results

4.3.1   Swim speed

Swimming speed of *A. franciscana* males was significantly affected depending on the chemical stimulus provided in the artificial sponge (Figure 8). Sponges soaked with FCE elicited a faster swimming speed in males (2.897 cm/s±0.26) than sponges soaked with the controls SW (1.969 cm/s±0.24) (P=0.0394) and CM (1.868±0.30) (P=0.0176). Meanwhile, males exposed to sponges soaked with MCE showed no significant differences in swimming speed compared with males exposed to sponges soaked with FCE (P=0.743) or SW (P=0.0791). There was no a significant difference between the swim speed developed by the male when are exposed to the two controls sponge (soaked with SW or CM) (P>0.05).
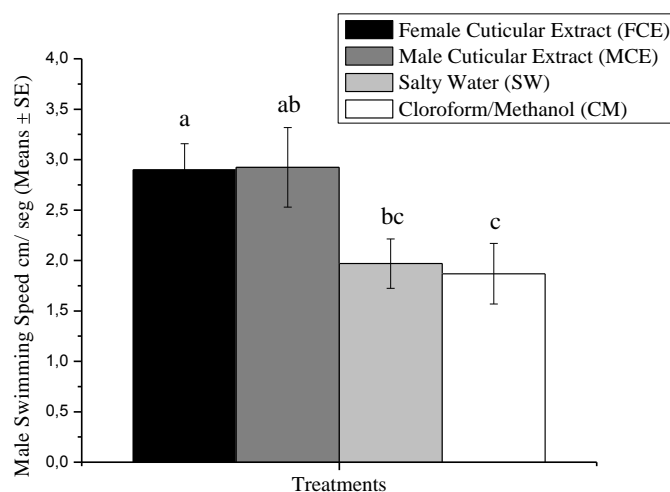
Figure 8. Swimming speed elicited in *Artemia franciscana* males by a sponge soaked with female cuticular extract (FCE), male cuticular extract (MCE), salty water (SW), or a 2:1 chloroform:methanol mixture (CM) (N=10). Different letters indicate significant differences based on the Kruskal-Wallis test followed by Conover-Inman's test (P <0.05).

4.3.2 Chemical analysis of cuticular extract

Qualitative differences were observed between the fatty acid profiles of FCE and MCE from *A. franciscana* (Table 7). Four saturated fatty acids (myristic, palmitic, stearic, and arachidic acids) and five unsaturated fatty acids (oleic, linoleic, linolenic, gondoic, and erucic acids) were identified using GC-MS analysis. The FCE and MCE fatty acid profiles shared the same compounds, with the exception of myristic acid, which was only present in FCE (4.6 μg/individual). Palmitic acid was the most abundant fatty acid found in both FCE (50.5 μg/individual) and MCE (57.0 μg/individual). By contrast, erucic acid was found in small amounts in both FCE (1.3 μg/individual) and MCE (1.1 μg/individual).

Table 7. Identification by GC-MS of fatty acids from female cuticular extracts and male cuticular extracts of *Artemia franciscana.*

RT	Fatty acids	Area (%)		Individuals equivalent (µg/individuals)	
		Female	Male	Female	Male
12.30	Myristic	12.3	nf*	4.6	nf*
14.98	Palmitic	41.1	42.9	50.5	57.0
17.48	Estearic	19.6	22.7	32.3	40.4
17.93	Oleic	11.7	10.2	31.5	37.7
18.74	Linoleic	1.9	1.3	6.0	5.4
19.66	Linolenic	6.0	3.3	18.2	24.5
19.81	Arachidic	0.7	2.3	11.7	2.8
20.22	Gondoic	0.5	0.3	1.6	4.0
22.33	Erucic	0.2	0.2	1.3	1.1

*nf: not found

4.4   Discussion

Previous reports had suggested that the swimming speed of several microcrustaceans is affected by chemical compounds emitted by conspecifics (van Leeuwen and Maly, 1991; Tsuda and Miller, 1998; Nihongi et al., 2004; Yen et al., 2011; Heuschele and Kiørboe, 2012; Seuront, 2013). Our results showed that, indeed, males increase their swimming speed in the presence of both FCE and MCE, suggesting at least two possibilities: 1) assuming that males tend to compete for females when the latter are scarce (Zapata et al., 1990), males that are more active or that swim faster would have an advantage in mate choice compared with less-active males; and 2) similar cuticular compounds may be

produced by both males and females. The first possibility is relatively common in crustaceans. Sneddon et al. (1997) and Okaruma and Goshima (2010) reported in the shore crab *Carcinus maenas* and the hermit crab *Pagurus filholi* that males expressed aggressive behavior, attacking the opponents using their chelipeds, when confronted with conspecific males. Similarly, Sneddon et al. (2003) reported that such aggressive behavior of *C. maenas* males increased when exposed to conditioned water from conspecific females.

In *Artemia* males, the ability to move faster has important benefits. For example, males forage for microalgae or food particles; thus, males that are more active are likely to obtain more food in a given period of time than less-active males. Similarly, males that are more active or energetic should have advantages both in seeking mates and in keeping females in amplexus for a longer time because they better resist the resistance behavior by which females actively take part in selecting qualified males (see chapter III) (Mura and Gajardo, 2011). Once males grasp females in amplexus by means of their modified antennae, the females display energetic movements to dislodge males (Zapata et al., 1990). Keeping females for themselves for longer periods (mate-guarding behavior, Jormalainen, 1998) ensures the repeated fertilization of the same female and prevents fertilization by other, less-active males.

Our results indicate that the two sexes produce similar cuticular compounds (Table 7), which may have an evolutionary role in mate finding and recognition because it contributes to the aggregation between organisms from the same species or population (Beauché and Richard, 2013). Fatty acids are commonly present in the cuticular extracts of insects

(Blomquist et al., 1987) but are less common in crustaceans (Caskey and Bauer, 2005; Zhang et al., 2011). Caskey et al. (2009) reported the presence of three saturated fatty acids (palmitic acid, stearic acid, and arachidic acid) and two polyunsaturated fatty acids (eicosatetraenoic acid and eicosapentaenoic acid) in cuticular extracts of the caridean shrimp *Palaemonetes pugio*. Palmitic, stearic and eicosapentaenoic acids were present in all developmental stages of *P. pugio* (postmolt parturial females, postmolt nonparturial females, postmolt males and intermolt females). However, Eicosatetraenoic acid was found only in postmolt parturial females and postmolt nonparturial females. Similarly, in the moth *Adoxophyes orana* (Lepidoptera: Tortricidae), whole-body extracts obtained from males and females showed that myristic and palmitic acid are male-specific and may act as a key component of a male pheromone that inhibits the mating behavior of other conspecific males (Otter et al., 1989). This is concordant with the differentiation observed in the present study for the fatty acids present in FCE and MCE. Myristic acid was only found in FCE, indicating that this fatty acid may have a sex-specific function. Admitting the complexity of the cues involved in mate recognition in a biological system (Dicke and Takken, 2006), the results of this work allow us to suggest that overlap of cuticular compounds between *A. franciscana* males and females might 1) help in reducing male-male aggression, which is indeed an uncommon behavior in *Artemia* and 2) contribute to the mate-finding and recognition between organisms from the same species or population.

We interpreted the increased swimming speed of males in the presence of FCE as the probable existence of a short-range or contact pheromone, consistent with other similar findings (van Leeuwen and Maly, 1991; Nihongi et al. 2004). The role of these secondary

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metabolites requires further investigation in *A. franciscana*, one of the most widely distributed and highly plastic *Artemia* species, which is also, found invading non-native habitats in the Mediterranean (Amat et al., 2005) and thus can be found coexisting with other species. Under such conditions, mate choice becomes especially relevant. Besides, mate choice is also relevant due to the highly heterogeneous population structure of this species (locally adapted populations). Future work should place attention to myristic acid, which is only present in female cuticular extracts, providing a basis for speculation on a sex-specific role in *A. franciscana* mating. Similarly, a more systematic testing of different fatty acids, and their respective proportions, should be the starting point for a more in depth intra-specific study.

4.5   Conclusions

1. Swimming behavior in *Artemia franciscana* males is an interesting trait for monitoring in mate-choice bioassays (using living individuals, conditioned water from these organisms or other biological fractions such as cuticular extracts) because it is affected by the presence of conspecific males or females: males react to the stimulus by moving at higher speed.

2. The swimming speed of *A. franciscana* males nearly doubled when they were confronted with cuticular extracts of conspecific males or females compared to the swimming speed in the absence of conspecifics (with salty water or a chloroform-methanol mixture (2:1)). This provides indirect evidence of chemical communication.

3. The identification of similar cuticular compounds in males and females, such as fatty acids, is more direct evidence that some type of chemical signal may be associated with these compounds, perhaps a short-range pheromone. Future behavioral bioassays using the various components (individually or in different blends) of the extracts obtained remain to be performed.

Chapter V

General discussion, concluding remarks and

future directions

5 General discussion, concluding remarks and future directions

5.1 General discussion

This research demonstrates that mate choice in a population of *Artemia franciscana* from Cejar Lagoon in northern Chile (23°02'S–68°13'W) is a specialized interaction between conspecific individuals that involves the combination of at least two important elements: a courtship behavior, complemented by the associated resistance behavior of females by which they actively participate in selecting prospective mates, a trait also seen in other crustaceans, and chemical cues. The two sets of pre-mating isolating traits seem to act jointly, with males displaying their "attributes" through a courtship ritual and females (the choosy sex) actively participating in selecting the proper mate. Likewise, both sexes are, apparently, able to provide some information to their conspecifics through chemical compounds located in the cuticle. The obvious benefit of such specialized mate choice is improved female reproductive output and offspring quality.

Although marine plankton (Ceballos and Kiørboe, 2010) and other related marine or freshwater crustaceans (Moore and Ogre, 1962; Wiman, 1981; Tsuda and Miller, 1998) do exhibit ritual courtship behavior, mate choice in the marine realm is often driven by chemical signals. In contrast to marine planktonic animals, *Artemia* exhibits some peculiarities associated with living in an extremely salty (hypersaline) environment, in which a great deal of energy is allocated to osmoregulation, survival, swimming for foraging food, and reproduction. Under such conditions, both the amount and type or quality of offspring become relevant; for example, in the oviparity reproductive mode,

females release encysted offspring that can cope with stressful and unpredictable environmental conditions because cysts are highly resistant and can remain viable for years while almost desiccated. In contrast, in the ovoviviparity mode, females produce free-swimming nauplii that are ready to bloom and cope with a new habitat, for example when a dried out shallow lagoon is flooded again. Thus, the environment exerts pressure on the female's reproductive output and offspring quality and, consequently, on the mating couple, as it is not irrelevant which male fertilizes the female (Rode et al., 2011). It is known that populations differ in the proportions of cysts and nauplii produced by females, as well as in their total reproductive output (Browne, 1980). In addition, because *Artemia* populations are locally adapted, offspring must be synchronized to such conditions; thus, it is expected that a mating pair from the same population produces better reproductive output than a combination of parents from different environments. Finally, the occurrence of overlapping generations (see Table 2; Chapter I) also affects the type of mates coming into contact. Giving the importance of mate choice in *Artemia*, it is understandable that females (the choosy sex) play an active role through resistance behavior (see chapter III), in addition to the role of males during courtship.

5.1.1   Courtship ritual in *Artemia* and related crustaceans.

A. franciscana males showed four behavioral patterns defined as follows (Tapia et al., 2015): 1) approach and touch, 2) swim behind 3) riding attempt, and 4) riding success. Patterns 1 and 2 are related to exploring to find females and then attracting their attention.

The third is when males attempt to get females in amplexus, a highly interactive stage, as males need to be sufficiently active and firm (the size, and perhaps shape, of the body and claspers being critical traits). The female's resistance behavior is also relevant because the females are the choosy sex. The last stage, when the couple rides in amplexus, is critical because it often leads to copula and also prevents other males from fertilizing that female (mate-guarding behavior). A relatively similar pattern has been described for anostracan crustaceans, which are close relatives of *Artemia*, for example, in species such as *Eubranchipus holmani, Streptocephalus mackini, S. dorothae, S. texanus, S. seali,* and *S. bouvieri* (Moore and Ogren, 1962, Wiman 1981, Belk 1991). In these species, males follow females and fertilize them in a process involving the following steps (see details in chapter II): detection and orientation, station taking, clasping, intromission, and disengagement. As observed in this study, *A. franciscana* males have three major paths to achieve riding success (or amplexus, the stage prior to copula), all of which require touching the females. In one path, males "approach and touch" females, soon thereafter achieving riding success (19% probability of occurrence). The second path involves the male "approaching and touching" the female but failing to clasp female in amplexus for a while (27%), after which they return to the first step and eventually succeed (achieving amplexus, 23%). The last and most indirect alternative route includes the male "approaching and touching" the female and then "swimming behind" her (7%), finally achieving "riding success" with the female (5%) (Figure 4; Table 6; Chapter III). Via resistance behavior, females also take an active role in selecting males.

Upon being touched, females tend to move or escape, a behavior also described in calanoid copepods such as *Temora longicornis*, *Calanus marshallae*, *Acartia tonsa* and *Eurytemora affinis*. Female in these species react with sudden and active movements when touched by males, thus preventing being grasped by males in amplexus and thus avoiding copulation and fertilization (Doall et al, 1998; Tsuda and Miller, 1998; Ceballos and Kiørboe, 2010; Seuront, 2013). Wiman (1981) had previously described this avoidance behavior in females of *Streptocephalus* (Anostraca) but did not go any further in clarifying the role that such energetic movements could play in mate choice. Such resistance behavior in *Artemia* would be the way by which females choose conspecific males with favorable characteristics, perhaps strong, larger or very active males, but also those with larger claspers that act as powerful tools to keep females for themselves in amplexus for a long time, thus preventing females copulating with other males, in what should be interpreted as a sort of guarding behavior. Such resistance behavior of *Artemia* females is consistent with the concept of "self-referent" by which females take an active role in evaluating male quality. Dechaume-Moncharmont et al. (2013) showed that females of the fish *Amatitlania nigrofasciata* (convict cichlids) prefer conspecific males approximately one-third larger than their own size, evidence that females discriminate male size as a function of their own size. Forbes et al. (1992) used the same argument when indicating that couples in pre-copula kept under laboratory conditions tended to be similar in size. Further evidence of females playing an active role in selecting males comes from the observation that females can keep their gonopores shut even when riding in amplexus with males in a sort of after-amplexus barrier (Rogers, 2002). Although in this thesis, individual size and clasper size and shape were not

evaluated in males, it is easy to predict that the resistance behavior could be neutralized by very active and stronger (grip strength) males or by those with greater clasper size. These animals are likely to be genetically different, as Zapata et al (1990) noted in *Artemia franciscana*. For example, males taking females in amplexus were more genetically variable than those unable to clasp females in a multiple-choice experiment designed to promote male competition for females.

As mentioned above, touching females seems to be an obligatory path to achieve riding success, and this is likely to be related to the existence of "odor" molecules acting as signals as observed in microcrustaceans such as copepods. Frey et al. (1998) and Ting et al. (2000) suggested that female surface glycoproteins serve as mate-recognition cues in harpacticoid copepods such as *Coullana canadensis* and *Tigriopus japonicus*. Glycoproteins are among the main constituents of the epicuticle (outermost portion of the cuticle) of arthropods, along with proteins, hydrocarbons and fatty acids (Nation, 2002; Ye et al., 2007). Cuticular compounds have been shown to play an important role in the mate recognition for arthropods. For example, two sympatric elm leaf beetles, *Pyrrhalta maculicollis* and *P. aenescens*, showed very different profiles of cuticular hydrocarbons (CHCs). In addition, males significantly preferred to mate with conspecific females instead of heterospecific females. However, males attempted to mate with heterospecific females previously soaked with CHCs from their conspecific females (Zhang et al., 2014).

Regarding the behavioral bioassays using the polar fraction from conditioned water with females, it important to note that males performed all behavioral patterns previously described in the basic ethogram (male and female confronted) independent of the stimulus

to which they were exposed (female, pseudo-female or other male). However, differences were observed in the number of times each pattern was performed. Females and pseudo-females (males previously soaked with the polar fraction from water conditioned with conspecific females) elicited the same frequency of response from one male in all four courtship patterns (P>0.05), and virgin females and pseudo-females elicited the same responses when tested against five males in approach and touch, swim behind and riding attempt (P>0.05). In all cases, the control male always elicited the lowest frequency value for each courtship pattern. Similar methodology and results were presented by Harderge et al. (2002) for *Carcinus maenas* (Crustacea: Decapoda). Males try to mate with conspecific males when the latter have been previously dipped into a tank containing water conditioned with conspecific females. Meanwhile, males in the presence of control males (dipped in a tank containing sterile-filtered seawater) show an agonistic behavior. Males of *C. maenas* attempt to pair with objects like stones if such objects are dipped in water conditioned with females in what is recognized as a primary indication of some chemical compound released by females into the water.

In this work, the polar fraction obtained from conditioned water of *A. franciscana* females showed biological activity. This fraction has been previously postulated as responsible for the mating behavior developed by decapod crustaceans such as *C. maenas* and *Callinectes sapidus* (Hardege and Terschak, 2011; Kamio and Derby, 2011).

One important challenge for crustaceans prior to mate recognition is finding potential mates (Harderge, 2002). This is not an easy task in the tridimensional (volumetric) aquatic world or for individuals lacking visual abilities. Nevertheless, reports suggest that aquatic

copepods such as *Calanus marshallae* and *Eurytemora affinis* increase their swimming activity or speed in the presence of conspecifics of the opposite sex (Tsuda and Miller, 1998: Seuront, 2013), which allows them to scan the largest area possible, increasing their chances of finding conspecific mates (Gerritsen and Strickler, 1977). In this study, it was possible to determine that the swimming speed of *A. franciscana* males increased by approximately 1.5 times upon exposure to a sponge soaked with cuticular extract of conspecific males or females, whereas the swimming speed of males exposed to sponges soaked with artificial water or to the solvent used for extracting the polar fraction (chloroform:methanol) remained unchanged. Contrary to expectations, such an increase in activity occurred when *Artemia* males were confronted with extracts from either male or female conspecifics. This behavior may have different reasons; for example, both males and females may use signals to facilitate the aggregation of conspecifics belonging to the same species or population. This also occurs in the terrestrial crustacean *Armadillidium vulgare* (Isopoda: Oniscidea), in which different pheromones are released to facilitate mate finding (Beauché and Richard, 2013). Furthermore, Doall et al. (1998) mentioned that *Temora longicornis* (Copepoda: Calanoida) males are able to follow (track) signals of conspecific females, and, once they lose the signal, perform a "back-tracking behavior", i.e., return to a previous point of the female signal, the male's own signal, or a combination of the two. The authors mentioned that this behavior is probably used as a way of repositioning to return to the search for a mate. The increase in the swimming speed in *A. franciscana* males in the presence of conspecific females may be due to competition for

females when females are scarce. Hence, a more active individual, with a higher exploration capacity, has a greater likelihood of mating than another, less-active individual.

In summary, the courtship behavior in *Artemia*, combined with chemical signals, indicates the evolution of a highly specific mate choice whose benefits are understandable in the context of the life cycle of a species exposed to a harsh (extremely salty) environment. Table 2 (Chapter I) listed the natural conditions that have required the evolution of such specific mate choice. Two possible situations are highlighted in this discussion. First, locally adapted or ecologically isolated populations may come into contact when cysts from a given population are translocated by water birds such as flamingos or by wind (natural dispersers of cysts) or when cysts released by females from different generations accumulate along the shores of salty lakes and hatch simultaneously. Rode et al. (2011) showed that females choosing contemporary males (cysts produced during the same year as the female) performed better in some reproductive traits (inter-brood number) in comparison to females choosing males hatched from cysts released by parents in other generations.

The finding of a courtship pattern coupled to the release of chemical signals is novel for *Artemia*, and the specificity seen at the population level, often seen between species, poses new questions regarding the importance of mate choice in this unique extremophile.

5.2   Concluding remarks

1. This work provides evidence of a highly specific mate choice between *Artemia franciscana* individuals collected from Cejar Lagoon in northern Chile (23°02'S– 68°13'W): a courtship behavior and chemical cues.

2. The ethogram showed males displaying four independent behaviors after locating a female, all of which were expressed in all bioassays performed, though their frequency changed under certain circumstances.

3. Females of *Artemia* are choosy and actively participate in selecting a mate. This is inferred from a type of resistance behavior displayed when a male attempts to achieve amplexus. Females exert sudden movements to dislodge males in a kind of testing exercise of male abilities. This leaves open the question of what male abilities would be relevant in this process.

4. Bioassays using the polar chemical compounds released by females and using cuticular extracts from males and females provides evidence of the use of chemical cues in *A. franciscana.*

5.3 Future directions

The execution of this thesis has generated several questions that remain to be investigated, including the following:

1. A critical testing of the importance of what is considered a highly specialized mate choice would require a follow-up of the offspring of fertilized females, comparing, for example, intrapopulation and inter-population mating.

2. It is important to verify whether the four behavioral patterns revealed by the ethogram are the same in different populations and in different species.

3. The choosy role of females requires further investigation. Aspects to be researched include the following: a) what male traits are relevant to finding and then copulating with females? Examples include size, clasper size and shape, and/or ability to move. b) More realistic mate-choice bioassays, such as multiple-choice assays, should be planned to permit females to choose males of different quality. c) The genetic structure of critical phenotypic traits involved in mate choice (body size, clasper size, activity) also requires research.

4. The critical components of the polar fraction of the female-conditioned water should be identified, as should those present in the cuticles of males and females.

5. Long-range or short-range pheromones should be identified through bio-directed behavioral experiments. Future work should evaluate (individually) the activity of each of

the identified fatty acids and assess the proportions in which they are blended, as well as considering other components of the arthropod cuticle, such as hydrocarbons.

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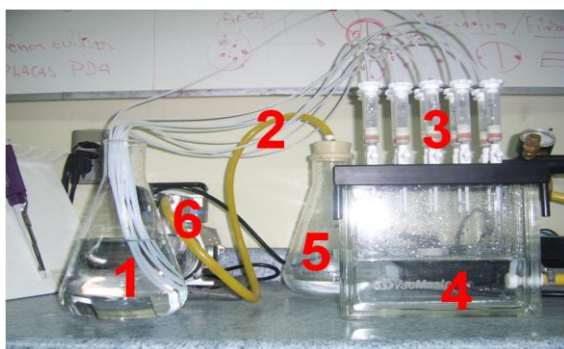
Figure 9. *Artemia franciscana* male (left) shows two modified antennas known as forceps (1 and 2) and female (right) shows an broadened uterus (3).


Figure 10. Culture of *Dunaliella tertiolecta*.

Figure 11. Solid Phase Extraction. 1) Conditioned water, 2) acetate hoses, through which flows the conditioned water, connecting with, 3) trapping columns, 4, 5, and 6) vacuum manifold system.


Figure 12. Vacuum Distillation. 1) Ultra vacuum pump, 2 and 3) gas traps immersed in liquid nitrogen (into the gas trap number 3 is positioned the recipient containing the SPE product (previously extracted from conditioned water), 4) lineal vacuum system, and 5) recipient containing residue polar compounds.
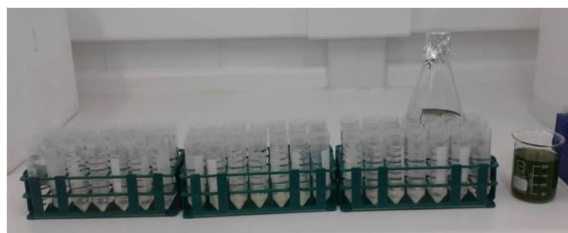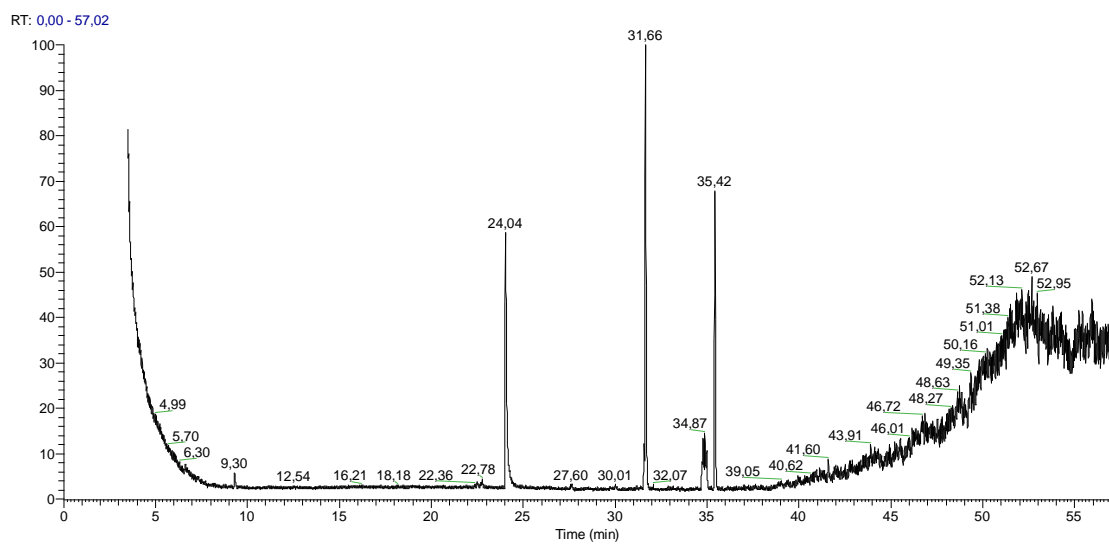
Figure 13. Falcon tube (50 mL) used for individual maintenance of *Artemia franciscana* in order to keep virgin individuals before bioassays.
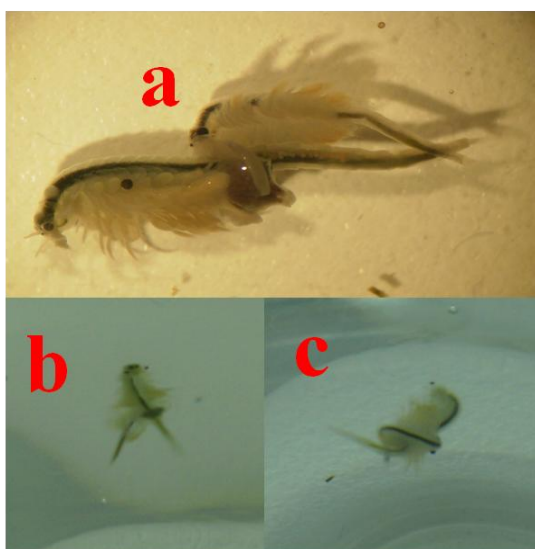
Figure 14. *Artemia franciscana* male and female in riding position (a). Male grasping at the female uterus (b), and male attempt to mate with female.
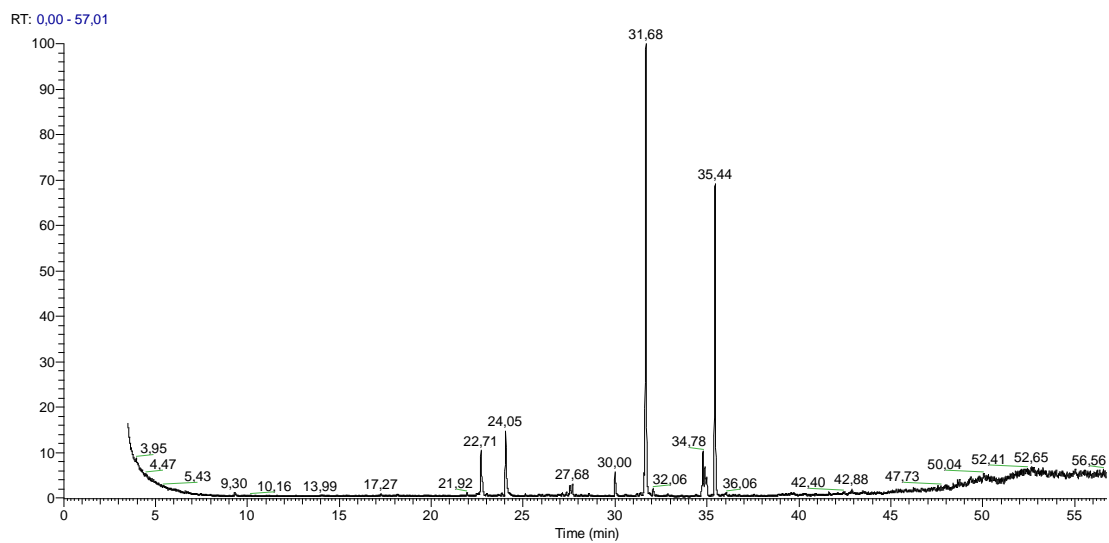
Figure 15. Total ion chromatograma of female esterified cuticular extract.

Figure 16. Total ion chromatograma of male esterified cuticular extract.