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The role of silk in courtship and communication in mygalomorph spiders: Do males regulate their courtship in response to female mating status?

María Sofia Copperi[a,*], Nelson Ferretti[a], Alfredo V. Peretti[b]

[a] Instituto de Ciencias Biológicas y Biomédicas del Sur (INBIOSUR), Universidad Nacional del Sur, UNS-CONICET, San Juan 670, CP 800, Bahía Blanca, Argentina
[b] Laboratorio de Biología Reproductiva & Evolución, Instituto de Diversidad y Ecología Animal (IDEA), CONICET-UNC and Facultad de Ciencias Exactas Físicas y Naturales, Universidad Nacional de Córdoba, Av. Vélez Sarsfield 299, CP 5000, Córdoba, Argentina

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ABSTRACT

In spiders, pheromones are known to be responsible for attracting the opposite sex, eliciting male searching and courtship behaviors, as well as for synchronizing potential mates in space and time. Most spiders are cannibalistic and aggressive. Thus, early recognition of a female as a possible mate is essential for males, who may suffer high energetic or reproductive costs to the extreme of losing all fitness opportunities. In *Acanthogonatus centralis* Goloboff 1995, a mygalomorph spider, what female signs might be triggering male courtship behavior remain unknown, as well as whether males can discriminate between females. The aims of the present work were (1) establishing whether males can detect the presence of females using airborne and silk-borne signals and (2) determining whether males can discriminate the reproductive status and body condition of females. We found no evidence that airborne pheromones play a role in the sexual communication of *A. centralis*, but silk-bound contact signals function as a female advertisement. Also, this is the first study that demonstrates that male mygalomorph spiders can discriminate between different signals on silk through direct contact, showing a preference for unmated females.

1. Introduction

Animal communication takes place when at least one individual displays a behavior by sending a signal, and this affects the present or future behavior exhibited by another individual (Trabalon, 2013). Chemical communication, either *via* airborne or contact pheromones, is considered to be the most primitive kind of communication in animals (Pollard et al., 1987; Trabalon, 2013). The most crucial role of this communication, in many animal species, involves courtship when the partners are close to each other (Wyatt, 2014). Pheromones are chemical signals (olfactory and contact) that generally elicit mutually beneficial responses from intraspecific receivers (Gaskett, 2007; Karlson and Luscher, 1959; Wyatt, 2014). During the reproductive season, sex pheromones are responsible for attracting conspecifics of the opposite sex, eliciting their searching and courtship behaviors, and synchronizing potential mates (Karlson and Luscher, 1959; Wyatt, 2014).

The importance of sex pheromones for mate recognition has been documented for many species, and their role as signals and chemical indicators of mate choice is nowadays well established (Johansson and Jones, 2007; Steiger and Stökl, 2014; Wyatt, 2014). In most animals, females are the choosy sex due to their greater investment in the production of gametes (few, large and costly) (Andersson, 1994; Trivers, 1972; Williams, 1975). However, there may also be a male choice. Males tend to choose females with better body condition, a trait closely associated with higher fecundity (Bonduriansky, 2001; Edward and Chapman, 2011). Long courtships to unreceptive females may involve high energetic and/or reproductive costs for the male (Baruffaldi and Andrade, 2015; Byrne and Rice, 2006; Kelso and Verrell, 2002; Morse, 2010; Wedell et al., 2002).

Moreover, in polyandrous species, like most spiders, sperm competition may compromise the male´s reproductive success (Elgar, 1998). Therefore, being able to distinguish between females of different reproductive statuses or receptivity is advantageous for males since it would enable them to assess the risk of sperm competition before mating and to pay lower courtship cost by identifying unreceptive females (Assis et al., 2016; Guilford and Dawkins, 1991). Moreover, among spiders, if differences in the quality or quantity of chemical signals on silk or body cuticle were indicative of female size or reproductive status, having the capacity to detect them would be very advantageous for males (Assis et al., 2016; Harari and Steinitz, 2013).

Symonds and Elgar (2008) have estimated that about 80 percent of all pheromone research has been done on insects, whereas only about 1

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* Corresponding author.
E-mail addresses: sofia.copperisc@gmail.com, mscopperi@mendoza-conicet.gob.ar (M.S. Copperi).

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percent has focused on spiders. However, recognition of a conspecific sexual partner and knowledge of sexual receptivity may be critical for mating success in aggressive and potentially cannibalistic species such as spiders (Bukowski et al., 2001; Walker et al., 2003). Usually, spiders use airborne pheromones, which are emitted from the silk or body cuticle and which function as long-range signals, to attract males or elicit male searching behavior (Aisenberg et al., 2010; Gaskett, 2007; Schulz, 2004). Contact pheromones are usually associated with silk, draglines or substrate, and act as short-range attractants, triggering male courtship behavior and also providing information about the female's sexual status (Aisenberg et al., 2010; Gaskett, 2007; Schulz, 2004).

Platnick (1971) classified the courtship of spiders according to three levels of the stimulus that triggers male courtship and most of the mygalomorph spiders were included under “level I”, meaning that the male begins to court after contact with the female. However from the 1980s onwards, and nowadays, most research on this group of spiders makes reference to a courtship that begins before direct contact between the spiders (Coyle and Shear, 1981), assuming the presence of signals deposited on the female silk (Costa et al., 2015; Costa and Pérez-Miles, 2002; Dor et al., 2008; Ferretti et al., 2013; Gaskett, 2007).

Knowledge about mygalomorph spiders has steadily grown over the last years, but comprehensive studies of communication in this group are still scarce. Ferretti et al. (2011) reported that the male *Acanthogonatus centralis* Goloboff 1995, a mygalomorph spider from central Argentina, performed intense tapping with its legs over the substrate and then over the female´s silk when courting. Although the authors did not specify what kind of signals triggered that behavior, they presumed the presence of airborne signals. *Acanthogonatus centralis* (Fig. 1) lives under stones, where females and juveniles construct their tunnel-webs connected to a short burrow (Ferretti et al., 2011) and shows an aggregate distribution (Pompozzi et al., 2019). Moreover, high individual motility was reported for both juveniles and adults (Ferretti et al., 2012), which increases the probability of mating encounters. Females continue to molt even in their adult stage. Therefore old spermathecae and remaining sperm are lost during molting, consequently female, from an operational and sperm storage point of view, which have mated previously become “virginal” again after each molt (Foelix, 2011; Pérez-Miles et al., 2007).

In this paper, the main objective was to assess the existence of both airborne and contact pheromones in *A. centralis* females, and determine whether males can detect the presence of females through airborne and/or silk-borne signals. Additionally, we examined the courtship and exploratory behaviors to discern whether males can discriminate the female's reproductive status and body condition. We predict that males use female signals to optimize the energetic cost involved in searching and courtship. We expect that silk-borne signals give males more information than do airborne signals, matching the behavior known for Mygalomorphae. This study promotes new research approaches on mygalomorph spiders regarding pheromone communication, structure, and function.

Fig. 1. *Acanthogonatus centralis* male.

2. Materials and methods

2.1. Collecting and rearing

We collected 30 adult *Acanthogonatus centralis* males from the locality of Sierra de la Ventana (38°04´21.3´´S, 62°03´02.6´´W), Buenos Aires Province, Argentina, during May-August 2014. Juveniles and possible adult female individuals were collected and reared under laboratory conditions. Individuals were considered sexually receptive females after molting in the lab and confirmed it by observation of well-developed spermathecae in the exuvia. Hence, we were sure that all the individuals used in the observations were females without sperm in their receptacles. These newly molted females were the group "unmated females" in the present study.

Specimens were kept in plastic Petri dishes, with soil as substratum with wet cotton wool (providing a water source). Individuals were fed once a week on *Gryllus assimilis* (Orthoptera, Gryllidae) or *Blatella germanica* (Blattodea, Blatellidae). We used an artificial 12 h light/dark cycle. The temperature during the experiments was 26.7 °C ± 1.52.

2.2. Experimental individuals

We used newly molted unmated females (N = 20) (individuals molted at least once in the laboratory after capture) and mated females (N = 10) (individuals molted at least once in the laboratory after capture and had copulated a week before experiments). The same 20 males were used for each trial. In the case of occasional deaths, a male was replaced by another individual from the initial stock (total deaths = 3) (no female died during the experiment). Pairs were not reused during different experiments. The period before reusing an individual was about 3 to 10 days. All the experiments were performed in the same order as we present them here. All spiders were weighed using an OHAUS PA313 Explorer Precision Balance and measured (carapace width) through digital photographs analyzed with ImageJ 1.45 (Schneider et al., 2012). Voucher specimens were deposited in the arachnological collection of the Laboratorio de Zoología de Invertebrados II, Universidad Nacional del Sur, Argentina.

2.3. Male behavioral assays

Behavioral trials were conducted matching the period of sexual activity of males. All experiments were recorded with a digital camera Panasonic SDR-S7 coupled to a fixed structure. Events were transcribed from digital videos using J Watcher 0.9 (Blumstein et al., 2000). All materials used in the test were washed with water and detergent, dried and sprayed with alcohol and finally dried again to remove possible remaining signals from previous experiments (Dodson et al., 2013; Gaskett, 2007; Walsh and Rayor, 2008).

2.3.1. Experiment 1: testing the occurrence of airborne female cuticular sex pheromones

For this experiment, we used an olfactometer consisting of a glass Y-shaped tube (Borosilicate glass 3.3 Pyrex CGW 7740). The device included an “introduction arm” with three defined regions and two “choice arms” (Walsh and Rayor, 2008) (Fig. 2). The olfactometer had three exits that were covered with a piece of voile, allowing airflow over the spider and preventing escapes. We placed fans at the end of each choice arm, which were on a different table in order to avoid transmission of seismic signals (Aisenberg et al., 2010; Quirici and Costa, 2005). The fans (8 cm in diameter) were used at 2292 rpm, producing a steady airflow. On the table with fans, a container of about 6.5 cm was placed between the choice arm and the fan (1 cm away). The container had its entrance sealed with a layer of voile. Females were introduced into the container, with the fan on, for 30 min before the assay, and kept in the same place until the end of the entire trial. The placement of containers was randomized with each trial.

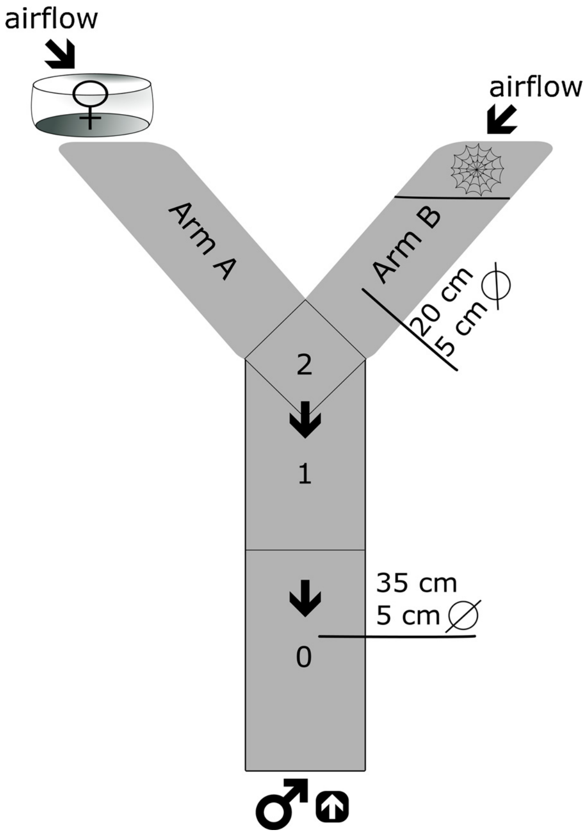

Fig. 2. Schematic draw of the glass Y-shaped tube olfactometer used to test the occurrence of airborne female cuticular sex pheromones (arm A) and silk-based pheromones (arm B). The male was placed into the introduction chamber (white arrow). Black arrows indicate directions of airflow.

For assessing the role of airborne pheromones in *A. centralis*, we released one male into the introduction arm. Then, we recorded the movement and behavior of each male over a 15-min period and registered the following parameters: i) the first arm visited, ii) total time spent inside each arm, iii) total time spent on each region, and iv) time spent by the male to make the first decision (choice latency). Female cuticular pheromone treatments consisted of males tested with Unmated vs. Control (N = 20 males), Mated vs. Control (N = 20 males), Unmated vs. Mated (N = 20 males). Moreover, ten males were exposed to two controls to prove the absence of behavioral asymmetries or laterality (Control vs. Control) (Ruhland et al., 2017). Control treatments comprised an empty container.

2.3.2. Experiment 2: testing the occurrence of airborne female silk sex pheromones

To assess silk-based signals, we used the same previously described maze devices and recorded, over a 15 min period: i) first arm visited, ii) total time spent inside each arm, iii) total time spent on each region, and iv) time spent by the male to make the first decision (choice latency). Each female was confined in a recipient for three days. All deposited silk was carefully removed and placed at the end of the choice arms. A piece of voile was laid 5 cm before the end of each choice arm, preventing the spider from contacting the silk. Silk-based pheromone treatments consisted of Unmated Silk vs. Control (N = 20 males), Mated Silk vs. Control (N = 20 males), Unmated Silk vs. Mated Silk (N = 20 males). Control treatments comprised an empty choice arm.

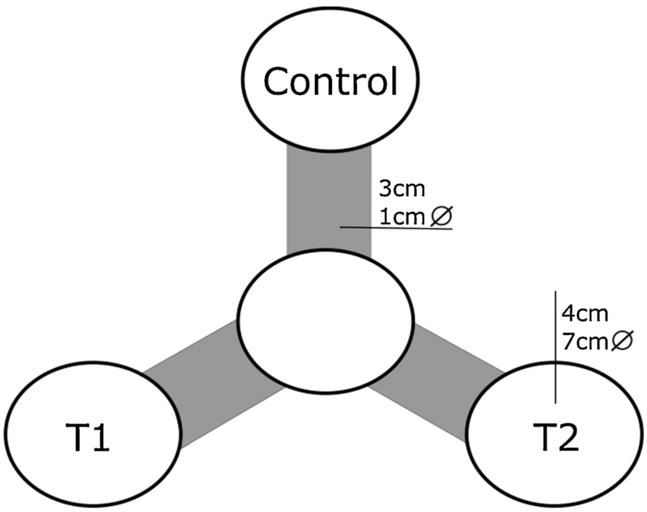

Fig. 3. Triple arena device used in female silk-based contact sex pheromones. The male was placed into the central chamber. T1: treatment one. T2: treatment 2.

2.3.3. Experiment 3: testing if body condition was encoded in the contact sex pheromones on silk

We used the male behavioral response to chemical and mechanical stimuli as evidence of female contact sex pheromones and male discrimination of female mating status and body condition. We used a triple arena device (Fig. 3) (used in Papke et al., 2001; Riechert and Singer, 1995), which consisted of three interchangeable plastic recipients located around a central container of the same size and connected to it by plastic tubes. A filter paper disc was placed into each of the three choice recipients. A female was placed inside a choice recipient and allowed to move and deposit silk freely over the filter paper surface for 24 h. Before the beginning of the trial, we removed the females from their recipient and reconnected these to the arena device. We released the focal male into the central container and observed its behavior towards the three surrounding treatment containers over a 20 min period. The placement of treatments in the arena device was randomized with each trial (Riechert and Singer, 1995).

Data recorded consisted of: i) latency (time from introduction of the male in the choice container until the beginning of courtship); ii) total time spent on each container; iii) frequency of scratching behavior, that is, male extending its first two pairs of legs forward, touching the substrate, then moving the legs backward.

We tested if body condition was encoded in the pheromone production, with treatments including males faced with Silk of Large Unmated females vs. Silk of Small Unmated females vs. Control (N = 20 males); Silk of Large Mated females vs. Silk of Small Mated females vs. Control (N = 20 males).

The control treatment was Control vs. Control vs. Control (N = 10 males) to prove the absence of behavioral asymmetries or laterality (Ruhland et al., 2017). This treatment comprised the triple arena device with three containers, each with a filter paper without silk at the base.

2.3.4. Experiment 4: testing if mating status was encoded in the contact sex pheromones on silk

We used the male behavioral response to chemical and mechanical stimuli as evidence of female contact sex pheromones and male discrimination of female mating status. We used the same triple arena device and released one male into the central chamber. Then, we recorded the movement and behavior of each male over a 15-min period and recorded: i) latency (time from introduction of the male in the choice container until the beginning of courtship); ii) total time spent on each container; iii) frequency of scratching behavior, that is, male extending its first two pairs of legs forward, touching the substrate, then moving the legs backward.

Here we tested whether information on the mating status of females was available to males through their pheromones and the treatments

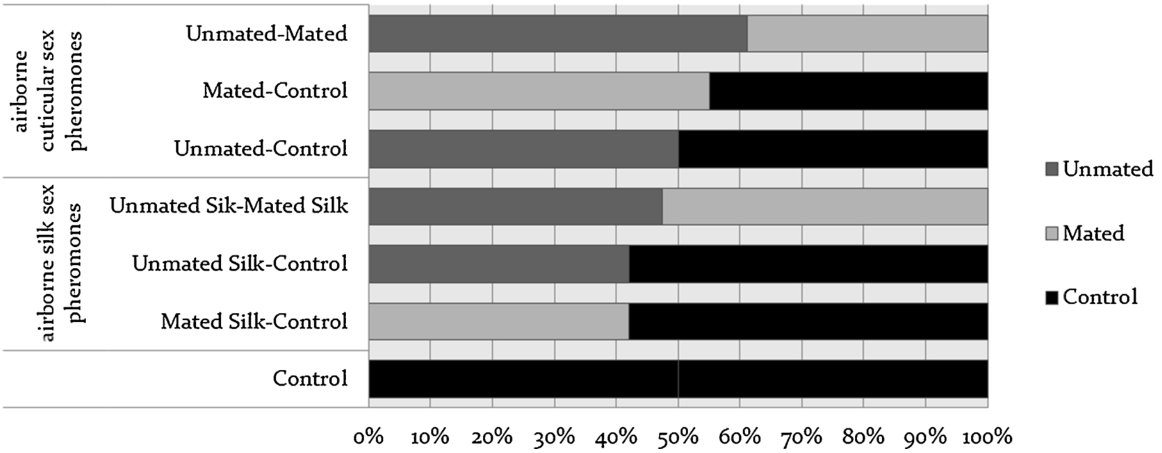

Fig. 4. First arm chosen by *A. centralis* males in experiments of airborne female cuticular and silk-based sex pheromones (expressed in mean percentage values).

were Silk of Unmated females vs. Silk of Mated females vs. Control (N = 20 males).

2.4. Statistical analysis

Data were analyzed using the statistical package PAST v.3.02 (Hammer et al., 2001). We checked the normality of data distribution using the Shapiro–Wilk test and variance homogeneity with the Levene test. Non-normal data were analyzed using nonparametric tests. Specific tests employed are given in the results section.

3. Results

3.1. Experiment 1: testing the occurrence of airborne female cuticular sex pheromones

No preference was observed in the first choice arm when comparing Control vs. Control (10/10) (binomial test: p = 1; N = 10), Unmated vs. Control (10/10) (binomial test: p = 1; N = 20), Control vs. Mated (9/11) (binomial test: p = 0.824; N = 20) or Unmated vs. Mated treatments (11/7) (binomial test: p = 0.481; N = 20) (Fig. 4). As predicted, we observed no males showing courtship behavior in any of the treatments (N = 60). However, the relative time spent in each arm was different in the Unmated vs. Control treatment. In this case, males spent more time in the control arm (Mann-Whitney U-test = 93, $p < 0,005$). Males showed no significant differences among the remaining treatments: Control vs. Control (Mann-Whitney U-test = 49, p = 0.969), Mated vs. Control (Mann-Whitney U-test = 192, p = 0.839), and Unmated vs. Mated (Mann-Whitney U-test = 163, p = 0.321) (Fig. 5). Concerning choice latency, no significant difference was observed among treatments (Kruskal–Wallis ANOVA, $\chi 2 = 1.473$, p = 0.479). Latency means were: Unmated vs. Control 58.45 ± 68.94 s SD (range: 2.86–241.44), Mated vs. Control 58.05 ± 82.77 s SD (range: 2.30–280.40) and Unmated vs. Mated 89.14 ± 127.59 s SD (range: 2.02–471.76). The latency of choice did not decrease throughout the days of the experiments ($r_s = 0.224$, p = 0.533).

3.2. Experiment 2: testing the occurrence of airborne female silk sex pheromones

Males showed no significant differences in the first arm choice among the different treatments: Unmated Silk vs. Control (8/11) (binomial test: p = 0.648; N = 20), Mated Silk vs. Control (8/11) (binomial test: p = 0.648; N = 20) and Unmated Silk vs. Mated Silk (9/10) (binomial test; p = 1; N = 20) (Fig. 4). Males did not court or display searching behavior in any of the 60 tests when exposed to experiments with silk-based airborne signals. No significant differences were found when comparing time spent inside each arm in the treatments Unmated Silk vs. Control (Mann-Whitney U-test = 148, p = 0.163) or Unmated Silk vs. Mated Silk (Mann-Whitney U-test = 156.5, p = 0.244). However, males spent more time in the arm in the Mated Silk vs. Control treatment (Mann-Whitney U-test = 101.5, $p < 0.05$) (Fig. 5). Regarding choice latency, males showed no differences in time spent choosing an arm (Kruskal–Wallis ANOVA, $\chi 2 = 0.7211$, p = 0.697). Latency means were: Unmated Silk vs. Control 58.40 ± 109.53 s SD (range: 1.78–402.38), Mated Silk vs. Control 105.91 ± 225.80 s SD (range: 1.49–899.59) and Unmated Silk vs. Mated Silk 44.91 ± 84.67 s SD (range: 1.22–319.76). The latency of choice did not decrease during the experimental days ($r_s = 0.2619$, p = 0.536).

3.3. Experiment 3: testing if body condition was encoded in the contact sex pheromones on silk

Males courted in 92.5 percent of experiments (N = 40) with filter paper having silk of both unmated and mated females but never courted on filter paper without silk (control). The mean time males spent on each paper disc did not differ significantly in the Control vs. Control vs. Control treatment (Kruskal–Wallis ANOVA, $\chi 2 = 0.1376$, p = 0.9335). When we tested if body condition was encoded in the pheromone production, the mean values of the time males spent on each paper disc differed significantly in the treatments Large Unmated Silk vs. Small Unmated Silk vs. Control (Kruskal–Wallis ANOVA, $\chi 2 = 7.22$, $p < 0.05$) and Large Mated Silk vs. Small Mated Silk vs. Control (Kruskal–Wallis ANOVA, $\chi 2 = 8.989$, $p < 0.01$). Males spent more time in both groups of females than in the control. In the combination Large Unmated Silk vs. Control (Wilcoxon signed rank test = 153, $p < 0.005$) males showed differences in permanence, preferring the silk of unmated females, but we did not find any effect of time spent in the treatments Large Unmated Silk vs. Small Unmated Silk (Wilcoxon signed rank test = 121.5, p = 0.28) or Small Unmated Silk vs. Control (Wilcoxon signed rank test = 114, p = 0.21). In both combinations, Small Mated Silk vs. Control (Wilcoxon signed rank test = 104, $p < 0.005$) and Large Mated Silk vs. Control (Wilcoxon signed rank test = 126, $p < 0.005$), males showed differences in permanence, preferring the silk of mated females, but we found no effect of time spent in the treatments Large Mated Silk vs. Small Mated Silk (Wilcoxon signed rank test = 113, p = 0.7618). We observed differences in courtship occurrence among treatments (Fig. 6), but males showed no differences in courtship frequency in Large Unmated Silk vs. Small Unmated Silk (15/12) (McNemar test: p = 0.508; N = 20) or Large Mated Silk vs. Small Mated Silk (13/12) (McNemar test: p = 1; N = 20). The groups of small and large females showed significant differences in weight (Weight: Unmated: 0.579 ± 0.022 SE vs. 0.712 ± 0.01 SE, t=-7.1691, $p < 0.001$; Mated: 0.618 ± 0.013 SE

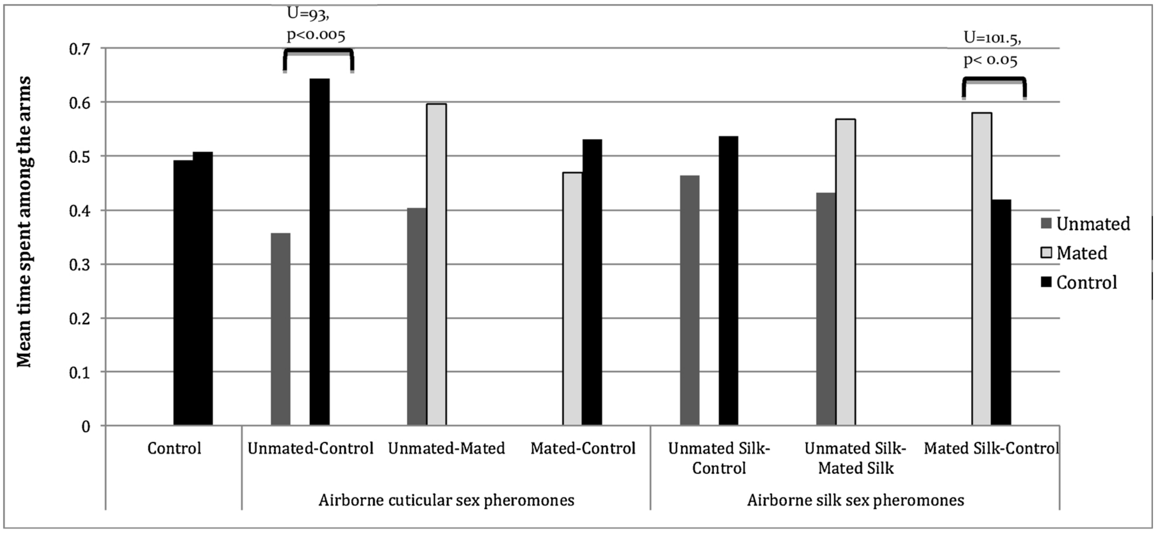

Fig. 5. Time spent between arms by *A. centralis* males in experiments of airborne female cuticular and silk-based sex pheromones (expressed as mean time rate).

vs. 0.764 ± 0.015 SE, $t = -3.73$, $p < 0.005$).

The number of “scratches” in the body condition treatment did not differ between small-large female combinations (Unmated: Wilcoxon signed rank test = 118, $p = 0.1569$; $N = 20$; Mated: Wilcoxon signed rank test = 106, $p = 0.6580$; $N = 20$). We found no relationship of female weight or total length with number of male scratches among treatments (weight: Unmated: $r_s = -0.22$, $p = 0.915$; Mated: $r_s = 0.095$, $p = 0.638$; total length: Unmated: $r_s = -0.062$, $p = 0.782$; Mated: $r_s = 0.050$, $p = 0.837$). Nor was male weight related to the number of scratches, total time spent on each container or courtship latency in any of the three treatments. These relationships are presented in Supporting Information (Table S1).

3.4. Experiment 4: testing if mating status was encoded in the contact sex pheromones on silk

Males courted in 95 percent of experiments ($N = 20$) with filter paper having silk of both unmated and mated females but never courted

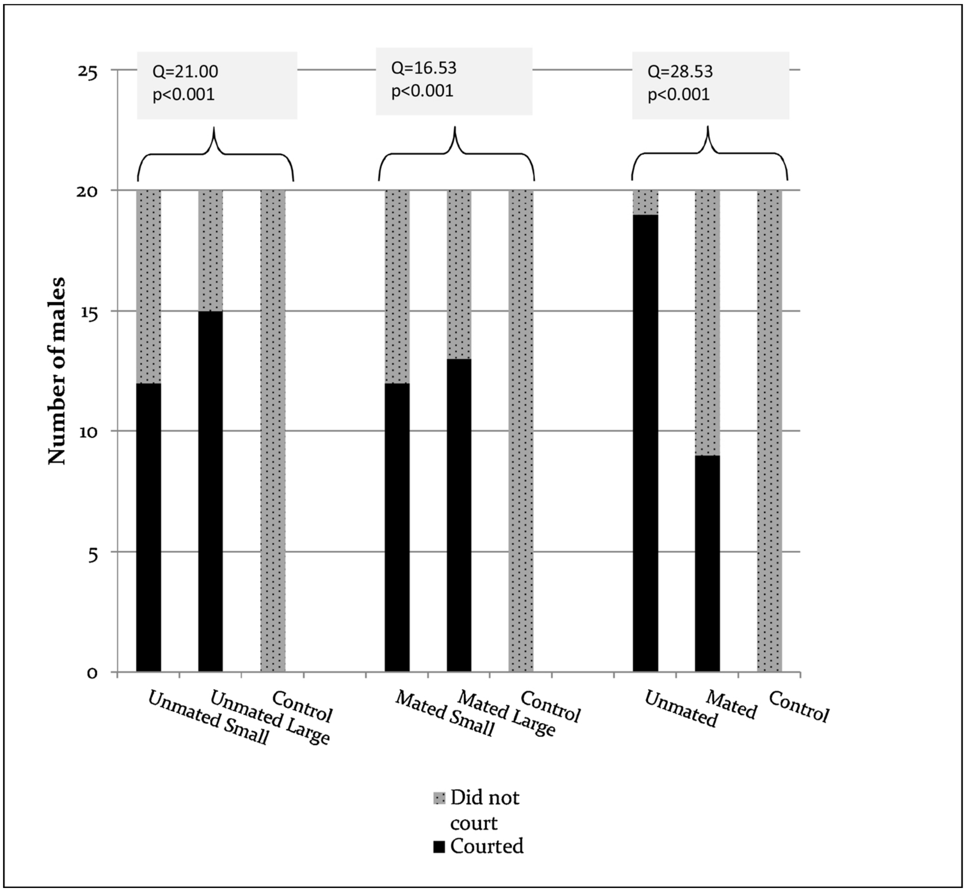

Fig. 6. Frequency of courtship occurrence for *A. centralis* males in each treatment of female contact sex pheromones on silk.

on filter paper without silk (control). When we tested if female mating status was advertised by pheromones (Unmated Silk vs. Mated Silk vs. Control), males spent more time in both groups of females than in the control (Kruskal–Wallis ANOVA, $\chi2 = 23.71$ $p < 0.001$), moreover they spent more time on the paper disc with silk of unmated than mated females ($W = 171$, $p < 0.005$). Courtship occurrence in the Unmated Silk vs. Mated Silk combination (without the control) was higher for unmated than mated females (19/9) (McNemar test: $p < 0.05$; $N = 20$) (Fig. 6). In this treatment, females had similar weight values (weight: Unmated 0.67 ± 0.03 SE vs. Mated 0.57 ± 0.038 SE, $t = 1.9075$, $p = 0.075$). In the female mating status treatment, males performed more scratches on the silk of unmated females (Unmated Silk vs. Mated Silk vs. Control: Wilcoxon signed rank test = 166.5, $p < 0.005$; $N = 20$). However, in the same treatment, we found no relationship between female weight or total length and the number of male scratches (weight: $r_s = -0.291$, $p = 0.133$; length: $r_s = -0.235$, $p = 0.258$). Nor was male weight related to the number of scratches, total time spent on each container or courtship latency. These relationships are presented in Supporting Information (Table S1). The latency to start courtship did not show significant differences among experiments 3 and 4 (Kruskal–Wallis ANOVA, $\chi2 = 9.471$, $p = 0.0916$).

4. Discussion

Acanthogonatus centralis relies on silk-borne pheromones to find and start courting a mate, and males can discriminate between unmated and mated females based on silk signals, a capacity not described for this group of plesiomorphic mygalomorph spiders.

First, our study indicates the absence of airborne chemical signals as part of the communication between *A. centralis* males and females. We did not record male preference for any of the stimuli we offered during the experiments. Males did not show a first choice dependent on female or silk presence. This result may indicate a lack of communication due to an absence of airborne chemical signals in females and their silk. *Acanthogonatus centralis* is a tube mygalomorph spider. Thus, its lifestyle may not favor the use of long-range airborne silk signals bound to silk, unlike most web-building species whose large webs are efficient for signaling (Kasumovic and Andrade, 2004; Xiao et al., 2009). This species has an aggregate distribution (Pompozzi et al., 2019) and males are abundant and active during the reproductive season (Ferretti et al., 2012); therefore, the likelihood of wandering males finding silk-bound sex pheromones is relatively high.

Second, regarding the time spent inside each arm in experiments of silk-based airborne signals, males spent more time in silk of mated females, and also in the control arm (Fig. 5). In the absence of male searching or courtship behavior, however, we assume that these results could indicate a lack of real preference for these stimuli.

In female spiders, sexual pheromones could be produced in silk glands (Schulz, 2004) or in the cuticle and be transported during silk production (Trabalon et al., 2005). In araneomorphs, airborne female cuticular pheromones have been identified and tested in several species (ex.: *Agelenopsis aperta* (Papke et al., 2001), *Argiope bruennichi* (Chinta et al., 2010), *Pholcus beijingensis* (Xiao et al., 2009)). In contrast, there is only one study on Mygalomorphae (*Brachypelma albopilosum*, Theraphosidae) that observed the existence of cuticular chemical compounds mediating communication between conspecifics. However, no mention was made of the properties of these volatile compounds (Trabalon, 2011).

As predicted, our study corroborated previous reports on Mygalomorphae families, showing that communication starts with female silk-based contact signals and is then mediated by other channels such as the mechanosensory mechanism (seismic and vibrational signaling) (Costa et al., 2013; Ferretti et al., 2013; Gaskett, 2007). For example, the male *Mecicobothrium thorelli* (Mecicobothriidae) communicates with the female through silk vibrations. Similarly, male individuals of *Eupalaestrus weijenberghi* (Theraphosidae) and *Acanthoscurria suina* (Theraphosidae) start courtship after contact with the silk at the entrance of the female's burrow, and then the couple uses seismic communication (Costa et al., 2013).

Regarding contact pheromone experiments, we found that *A. centralis* males more often chose the containers with filter paper containing female´s silk than those with filter paper without silk. This observation supports the idea of using tactile-chemical signals on silk, which enable the communication involved in searching for a mate (Beyer et al., 2018; Wyatt, 2014). Moreover, we did not record any male behavior involving courtship or searching in control experiments. Indeed, these individuals spent less time in the control treatment than in the silk treatment. Using silk-based chemical signals allows females to continually release signals without the energetic costs of active pheromone release from glands (Gaskett, 2007). If females should actively emit the pheromones would lose valuable time that would delay other activities such as the capture of prey (Gaskett, 2007; Schulz, 1997)

A. centralis males do not show any preference for the silk of females in relation to their weight. This result contrasts with several types of research that show a correlation between female weight and fecundity and the male's choice regarding this feature (reviewed in Bonduriansky, 2001 and Huber, 2005). In the Large Unmated Silk vs. Small Unmated Silk vs. Control treatment, males spent less time on the silk of small unmated females than in the control group, but their courtship effort was equivalent to the effort they invested in the silk of large unmated females. Therefore, we considered the courtship effort to be more relevant than the time males spent randomly walking the different parts of the device. In small-large female combinations males no show differences in the number of "scratches". Hence, large and therefore older females, regardless of their reproductive background, would not be producing differences in courtship effort of males.

When we tested whether pheromones advertised female mating status, *A. centralis* males showed different frequencies of courtship behavior. Scratching behavior was more frequent in the treatment involving silk of unmated females than the silk of mated females. Also, courtship occurrence and time recorded were the highest during that treatment (Fig. 6). Therefore, mygalomorph males can discriminate between different signals on silk through direct contact, showing a preference for unmated females. In this scenario, males can explore tactile-chemical signals to gather information about female sexual receptivity. Similarly, in some araneomorphs, males show preference for courting unmated females over mated females (Baruffaldi and Costa, 2010; Riechert and Singer, 1995; Scott et al., 2018; Stoltz et al., 2007; Tuni and Berger-Tal, 2012) and use silk-based signals to discriminate their reproductive status (Baruffaldi and Costa, 2010; Riechert and Singer, 1995; Stoltz et al., 2007; Tuni and Berger-Tal, 2012). Preference for unmated females implies important benefits for males, such as lower rejection rates, less sperm competition and increased paternity success (Aisenberg and Costa, 2005; Baruffaldi and Costa, 2014; Bonduriansky, 2001; Pérez-Miles et al., 2007; Wedell et al., 2002).

Nevertheless, during the experiments, males also courted mated females, probably for maximizing reproductive success and for strategically varying their allocation of sperm (Andersson, 1994; Wedell et al., 2002). The persistence of signals on silk after mating also allows females to re-copulate with another male, thus bringing the benefits of polyandry, in that case, there would be sperm competition, inbreeding avoidance and an increase in offspring genetic diversity (Hosken and Stockley, 2003). Even though the preference for unmated females was observed in *A. centralis* males (possibly due to the high female receptivity), we expected that they would try to copulate with many females since the simple shape of the spermathecae (blind receptacle) may result in last-male sperm priority or sperm mixing (Uhl, 2000).

Hence, through this study, we can infer that a female's silk has information about her species and status, making communication possible between conspecific individuals. However, we neither isolated nor analyzed the chemical compounds present on silk of *A. centralis* females. Despite this fact, throughout all these years of research on different spider groups, it becomes clear that the chemical compounds used in communication are typically present in spiders' silk (Gaskett, 2007; Schulz, 2013). Two contact pheromones were previously reported for tarantulas (Theraphosidae) (Fukushima et al., 2003; Costa et al., 2015). In *Acanthoscurria gomesiana* there is a polar acetonitrile-soluble substance, which triggers male courtship, and in *Eupalaestrus weijenberghi,* a water-resistant pheromone. Although no detailed studies have been carried out involving isolation and effects of these compounds, the existence of a contact pheromone on silk of *A. centralis* females would be possible. However, *Misumena vatia* males (Thomisidae) can distinguish differences in sex and reproductive status based on the properties of silk and not on the detection of pheromones (Anderson and Morse, 2001; Leonard and Morse, 2006).

Like other mygalomorph spiders (Costa and Pérez-Miles, 2002; Dor et al., 2008; Gaskett, 2007), *A. centralis* females may deposit pheromones in the silk tube or at the entrance of the burrow, so males would be able to recognize the presence of a potential mate through silk traces. As cannibalism is likely to occur in this species (Pompozzi and Copperi, 2018), having the opportunity to recognize a possible mate before starting courtship is advantageous for males.

The present study suggests that, although the female's vibratory signals are used in early stages of courtship in several mygalomorphs (Ferretti et al., 2013); silk-bound sex pheromones of *A. centralis* females convey information about their reproductive status and trigger male courtship. In conclusion, after trying all possible combinations, as predicted, airborne pheromones do not appear essential for sexual communication in *A. centralis*, but silk-based contact signals may function as a female advertisement. *Acanthogonatus centralis* males invest higher courtship effort on silk from unmated than mated females, no matter the age.

In order to enhance our understanding of spider sex pheromones, and determine the structure and function of their chemical compounds, future studies will be expanded to a wide range of mygalomorph taxa.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.beproc.2019.103939.

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