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Effect of the mating plug on female chemical attractiveness and mating acceptance in a scorpion

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Abstract

After mating, females may experience a decline in sexual receptivity and attractiveness that may be associated with changes in the production and emission of sex pheromones. In some cases, these changes are produced by chemical substances or structures (e.g., mating plugs) produced by males as a strategy to avoid or reduce sperm competition. In scorpions, sex pheromones may be involved in finding potential mates and starting courtship. Here, we tested the hypothesis that the males of *Urophonius brachycentrus*, a species that produces a mating plug, use chemical communication (sex pheromones) to detect, localize, and discriminate females according to their mating status (virgin or inseminated), aided by chemical signaling. We also explored the effect of extracting of the mating plug on chemical communication and mating acceptance. We used Y-maze olfactometers with different stimuli to analyze male choice and exploration time. To evaluate mating acceptance, we measured the attractiveness and receptivity of females of different mating status. We found that chemical communication occurs through volatile pheromones, but not contact pheromones. Males equally preferred sites with virgin or inseminated females with removed mating plug. In turn, females with these mating statuses were more attractive and receptive for males than inseminated females. This study suggests that the mating plug significantly affects female chemical attractiveness with an effect on volatile pheromones and decreasing sexual mating acceptance of females. The decline in the female's sexual receptivity is a complex process that may respond to several non-exclusive mechanisms imposed by males and strategically modulated by females.

KEYWORDS
genital plug, sex pheromones, sexual selection, sperm competition, *Urophonius brachycentrus*, virgin females

1 | INTRODUCTION

Sexual selection traditionally operates within two different processes, female mate choice and male–male competition (Andersson, 1994; Darwin, 1871; Hoquet, 2015). Both can continue after mating,

in the form of cryptic female choice or sperm competition, as non-exclusive processes of post-copulatory sexual selection (Birkhead & Pizzari, 2002; Eberhard, 1996; Parker, 1970; Wigby & Chapman, 2004). Sperm competition may favor different male adaptations at different levels (morphological, behavioral, and physiological traits) to increase their reproductive success (Parker, 1970; Simmons, 2014). For example, some males use chemical substances (pheromones

or antiaphrodisiacs) (Andersson, Borg-Karlson, & Wiklund, 2000; Malouines, 2017) to prevent or mitigate sperm competition by manipulating the female's behavior and physiology, aiming to decrease post-copulatory sexual receptivity (Simmons, 2014).

Pheromones are highly specific chemical compounds involved in a wide variety of behaviors (Shorey, 1973; Wyatt, 2003), and there are two main types: Volatile pheromones have a low molecular weight that allows them to cover great distances aided by wind or water, while contact pheromones have high molecular weights and thus remain in the substrate and require contact between the emitter and receiver (Wyatt, 2014). Many animal groups use chemical communication through sex pheromones as a signaling method during courtship and mating (Bradbury & Vehrencamp, 2000; Johansson & Jones, 2007; Wyatt, 2003). Because the males of some species can exercise mate choice (Bonduriansky, 2001), it has been observed that the males can sense the female's pheromones and get information about her reproductive status (Riechert & Singer, 1995; Thomas, 2011; Ting, Kelly, & Snell, 2000). This ability gives the males a selective advantage since they can find females with higher reproductive potential such as virgin females and detect lower levels of sperm competition (Bonduriansky, 2001; Thomas, 2011). Also, males may reduce potential costs of aggression coming from reluctant females (Arnqvist & Rowe, 2005; Huck & Banks, 1984).

Another mechanism to avoid or reduce the sperm competition risk is the blocking of the female genital opening with a mating plug (Birkhead & Møller, 1998; Simmons, 2001; Wigby & Chapman, 2004). The male production of mating plugs is a widespread phenomenon in the animal kingdom and has evolved independently many times (Shine, Olsson, & Mason, 2000). Mating plugs are diverse in structure and effectiveness, being generally formed by male substances, such as coagulation of ejaculate, or portions of genitalia (Peretti, 2010; Simmons, 2001; Uhl, Nessler, & Schneider, 2010; Wigby & Chapman, 2004). Sometimes, females may also have a role in mating plug formation (Aisenberg & Eberhard, 2009; Eberhard, 1996). Mating plugs have evolved under sexual selection to enhance male paternity and can have consequences on the female's physiology or behavior, such as causing a decrease in sexual receptivity to new males (Parker, 1970; Simmons, 2001, 2014).

In males, chemical communication and mating plug usage can be combined. For example, O'Donnell, Ford, Shine, and Mason (2004) showed that male garter snakes produce a mating plug with antiaphrodisiacs pheromones that inform new males of the female's reproductive status (i.e., has a repellent effect). In females, the mating plug can trigger a decrease in sexual receptivity to newcoming males by increasing her refractory period (Baer, Morgan, & Schmid-Hempel, 2001; Takami, Sasabe, Nagata, & Sota, 2008). This decline in sexual receptivity may be related to a decrease in the emission of female pheromones (i.e., pheromonostasis) or to a change in her pheromone profile (Thomas, 2011).

Among arachnids, scorpions are a very good model organism to analyze chemical communication and mating plug production and their interaction. Pheromones have been proposed as a sensory channel for scorpions since their vision is generally considered very poor (Root, 1990). Scorpions have a pair of sexually dimorphic mechanosensory and chemosensory appendages, the pectines (Gaffin & Brownell, 1997; Kladt, Wolf, & Heinzel, 2007; Polis & Sissom, 1990; Stahnke, 1973), which seem to mediate chemically mediated orientation behaviors such as substrate scanning before and during mating (Gaffin & Brownell, 1992; Mineo & Del Claro, 2006; Tallarovic, Melville, & Brownell, 2000). Studies in several species of scorpions show that they can use chemical signaling to find potential mates and begin courtship (Gaffin & Brownell, 1992, 2001; Melville, Tallarovic, & Brownell, 2003; Steinmetz, Bost, & Gaffin, 2004; Taylor, Cosper, & Gaffin, 2012). In many scorpion species, males transfer a mating plug after sperm transfer and this plug may be of different types and effectiveness (Peretti, 2010). Although much effort has been put into analyzing pheromonal communication and mating plug morphology, the evaluation of the capability of males to detect the female's mating status, and the relationship between the presence of a mating plug and the female's pheromone emission has been neglected in scorpions.

Urophonius brachycentrus (Thorell, 1876) represents a good model organism for this analysis because the females present a mating plug after insemination (Carrera, 2008; Peretti, 2010). This mating plug is detached from the male spermatophore and cannot be expelled by the female or removed by other males. For this reason, females mate once during the reproductive season (Peretti, 2010; Vrech, Olivero, Mattoni, & Peretti, 2014), and the high effectiveness of this mating plug in limiting sperm competition is thought to be the result of strong sperm competition in the past that led to the forced monandry seen in the present (Peretti, 2010).

In the present analysis, we use the neotropical scorpion *U. brachycentrus*, as a model organism to: (1) examine what type of chemical communication is involved in female detection by the male; (2) determine whether the male discriminates the female's mating status by means of chemical detection; and (3) test the effect of the mating plug on mating acceptance. For this purpose, we focus first on the detection of female volatile and contact pheromones by males. Then, we analyze the chemical attractiveness of females with different mating statuses. Finally, we examine the effect of the mating plug on female sexual attractiveness and receptivity.

2 | MATERIALS AND METHODS

2.1 | Study species

Urophonius brachycentrus is a South American scorpion that belongs to the Bothriuridae family. Their surface activity (i.e., the time when they disperse, feed, and mate) occurs in autumn and winter of the Southern Hemisphere (between May and September) (Maury, 1969). During October, females remain in diapause inside underground chambers, and from November to January, they give birth (Peretti, 1997). As in other Bothriuridae, females are semelparous and die soon after the offspring become independent (Peretti, 1997). *U. brachycentrus* individuals are small to medium in size (females: 3.1–3.7 cm; males: 2.4–3.4 cm) (Ojanguren Affilastro, 2005).

We collected the specimens in Tanti, Department of Punilla, Córdoba, Argentina (31°22′57″S, 64°35′32″W AMSL), during May to June 2012, 2014, 2016. The collecting method consisted of turning rocks during the day. Each individual was identified as juvenile or adult, sexed (and assigned an individual collection number). We deposited voucher specimens at the Laboratorio de Biología Reproductiva y Evolución (LBRyE), Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Argentina. Individuals were kept inside plastic vials (7.2 cm × 5.5 cm) with a moistened cotton as water supply and were fed with one larva of *Tenebrio molitor* (Tenebrionidae) every 15 days. All animals were kept under an inverted 12:12-hr light:dark cycle.

2.2 | Identification of female mating status and removal of mating plugs

We classified the females according to their reproductive status by examining the genital opening. The presence of the mating plug allows precise identification of female mating status (virgin or inseminated) (Peretti, 2010; E. Romero-Lebron, M.A. Oviedo-Diego, personal observations). We placed each female inside a re-sealable zipper plastic storage bag to limit its movement. The bag was perforated to access the zone of the genital operculum, which was gently lifted using fine-tip tweezers. Observations were made under a stereoscopic dissection microscope (Nikon SMZ1500). The females were classified as "virgin" (VF) when the mating plug was absent and "inseminated" (IF) when the mating plug was visible. The plug of a group of females was manually removed using fine-tip tweezers, and these females were classified as "inseminated with the mating plug removed" (RF). The manipulated females rested for 72 hr before the beginning of the experiments. The group of females whose mating plugs were not removed was handled in a similar way to control the possible effect of manipulation on sexual behavior.

2.3 | Devices and protocols for pheromone and male detection trials

2.3.1 | Volatile pheromones

To analyze the male detection and orientation toward emission sites of female volatile pheromones (localization), we used a Y-maze olfactometer (modified from Melville et al., 2003) (Figure 1a). This maze made of round Pyrex glass had three branches and was placed on an expanded polystyrene platform (EPS) (1.5 cm height) to reduce
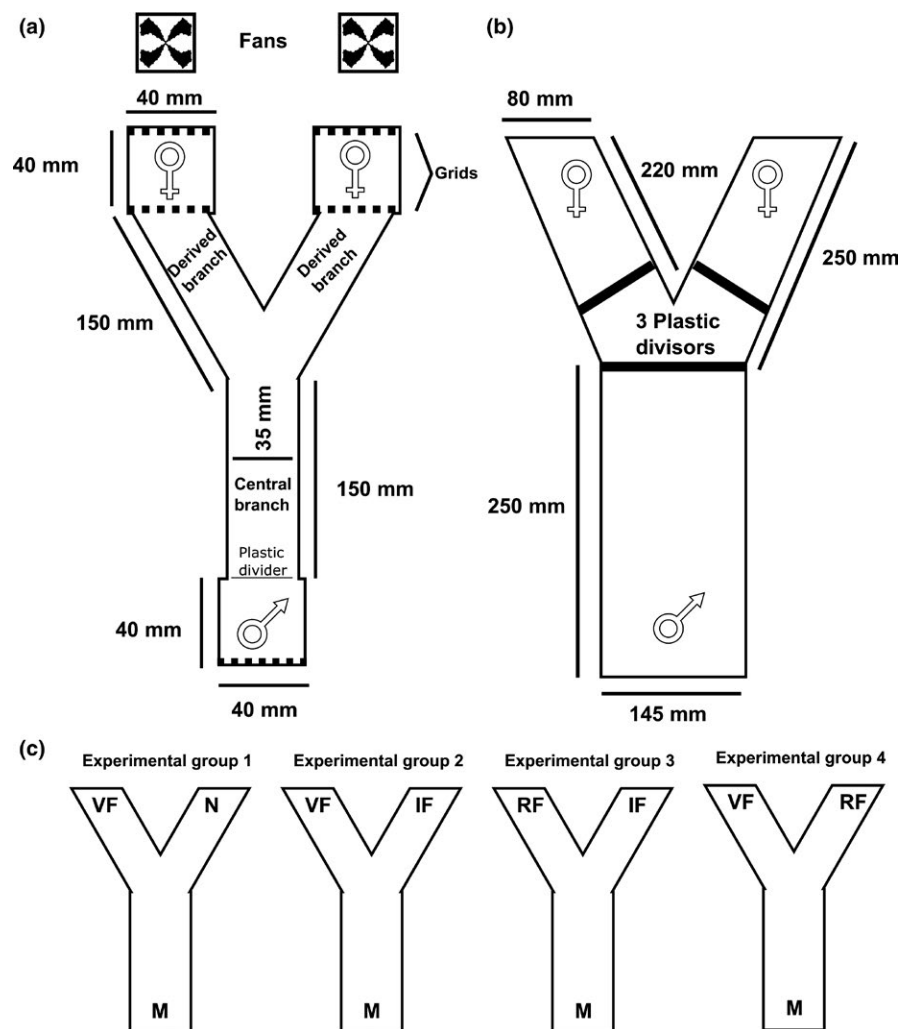

FIGURE 1 Devices and protocols for trials of pheromones male detection. (a) Y-maze olfactometer for volatile pheromone trials. (b) Y-maze olfactometer for contact pheromone trials. (c) Experimental groups of pheromone trials. IF, inseminated female; M, male; N, nothing (without stimulus); RF, inseminated female with mating plug removed; VF, virgin female; ♂, male; ♀, female

external vibrations. We placed an individual plastic chamber (4 cm length × 4 cm diameter) at the end of each of the three branches. Later, we put a male in the individual chamber at the end of the central branch (15 cm length × 3.5 cm diameter), and the chambers at the end of the two lateral branches (15 cm length × 3.5 cm diameter) were empty or occupied by stimuli-branches. All individuals were placed in their respective chambers 30 min before the trials for acclimatization (Koh, Seah, Yap, & Li, 2009). We chose the branches randomly where stimuli-branches and control (i.e., without stimulus) were placed. During this period, and to avoid early stimulation of the male, we separated the male's chamber from the central branch using a plastic divider, which we removed when the trial began. A mild stream of air passed through the maze from the lateral branches to the central branch, generated using a computer fan at the end of each lateral branch (Gaskett, 2007). We separated chambers with females from lateral branches with a fine tulle mesh to prevent physical contact between females and males during the trial but allow continuous air streams. The chambers containing females were separated from the lateral branch (1 cm separation) to avoid the vibration communication that is common in scorpions (Briceño & Bonilla, 2009). The observations were made with red light illumination homogeneously distributed over the olfactometer (Machan, 1968; following Gaffin, 2011). Each observation lasted 30 min and was digitally recorded with an HD camera (Sony DSC-DCR-SR65E) with night shot function. After each trial, the Y-maze and the three experimental chambers were washed using ethanol 70% to eliminate any possible chemical trace (Koh et al., 2009) and then with distilled water to eliminate any remaining trace of ethanol.

The response variable for the trials with volatile pheromones was "first lateral branch chosen by the male" and involved three options: 1. Lateral branch A; 2. Lateral branch B, and 3. No branch choice. It was considered "choice" when the male entered one of the lateral branches (more precisely when the anterior prosoma portion of the male crossed the maze junction). Lack of choice (3. No branch choice) was when the male did not leave the central branch or the chamber at the end of the central branch.

2.3.2 | Contact pheromones

A rectangular PET plastic Y-maze olfactometer was used (following Miller & Formanowicz, 2011) (Figure 1b). The Y-maze had three branches, a central branch (25 cm length × 14.5 cm width) and two lateral branches (25 cm length × 8 cm width). The maze was also laid on polystyrene sheet (EPS) (1.5 cm height) to reduce external vibrations. Males and stimuli-branches were placed inside the maze 24 hr before the beginning of the trial, which was enough time for the potential contact female pheromones to impregnate a filter paper covering the base of the Y-maze (Koh et al., 2009). We made one division of PET plastic at 22 cm from each of the three branches forming three rectangles that prevented physical contact between individuals. We placed the male in the rectangle of the central branch and stimuli-branches in the polygons formed inside the lateral branches (Figure 1b). After 24 hr, stimuli-branches and plastic dividers were

removed, and the male was able to freely access all branches for 2 hr (Miller & Formanowicz, 2011). During this time, the male's activity was digitally recorded with an HD camera with night shot function, and the trials were made under red light illumination homogeneously distributed over the Y-maze (Machan, 1968; following Gaffin, 2011). At the end of the essay, the filter paper was discarded, and the Y-maze was washed with ethanol 70% to eliminate possible remains of chemical substances from previous groups (Koh et al., 2009) and distilled water to remove any trace of ethanol.

The response variable for trials involving contact pheromones was the "exploration time on each branch" (seconds) and was quantified from the recorded videos as the time the male spent in each branch. In this analysis, we did not consider the lack of choice (i.e., 3. No branch choice) because the male started in the central branch and passed through it every time he changed stimuli-branches, resulting in an artificial overestimation of time spent in the central branch.

2.3.3 | Experimental groups for both pheromonal trials

We tested four experimental groups for both volatile and contact pheromone trials (Figure 1c). Males (n = 20 per trial) were faced with different combinations of stimuli in the lateral branches of the Y-mazes. These males were reused in all experiments in a random order (except in trials of 2016). In the first trial, we placed a virgin female in one lateral branch and no female in the other lateral branch (VF vs. None). In the second trial, we exposed the males to females of different reproductive status: We placed a virgin female in one lateral branch and an inseminated female in the other lateral branch (VF vs. IF). The following two experimental groups gave information about the effect of mating plug removal on the male's orientation and preference: The third trial consisted of males exposed to an inseminated female with the mating plug removed in one lateral branch, and an inseminated female with mating plug in the other lateral branch (RF vs. IF). In the last trial, we exposed the males to an inseminated female with the plug removed in one lateral branch and a virgin female in the other lateral branch (RF vs. VF).

2.4 | Testing female receptivity and attractiveness

We carried out sexual encounters inside rectangular mating arenas made of glass (10.5 cm × 7 cm × 14.5 cm) with soil and stones as substrate. First, we placed the female on one side of the mating arena and then placed the male on the opposite side. We digitally videotaped the sexual encounters with an HD camera with night shot function. Individuals were randomly selected for three trials: (1) males versus virgin females (VF) (n = 30); (2) males versus inseminated females with mating plug (IF) (n = 30); and (3) males versus inseminated females with the mating plug removed (RF) (n = 30). Males were reused in all experiments in random order. To determine the female's sexual attractiveness, we analyzed the mating behaviors of males. Assuming the female was attractive, the male

would try to grip the female's pedipalps with its own and perform juddering (i.e., the male jerks its whole body back and forth quite rapidly) (Olivero, Mattoni, & Peretti, 2017; Polis & Sissom, 1990). These behaviors are the positive sexual displays toward attractive females and a necessary component of the initial phase of the courtship (Peretti & Carrera, 2005). Sexual encounters were evaluated for a maximum of 30 min, and a trial was considered unsuccessful if the male did not grasp the female's pedipalps or perform juddering.

Certain female's behaviors in response to attracted males were indicators of female sexual receptivity. If a female allowed the male to grasp the pedipalps, this female was considered as sexually receptive. Receptiveness was only evaluated over attractive females. The female that is attractive to the male (i.e., male exhibited juddering and tried to grip the female's pedipalps) is receptive if it allows the grip, otherwise it will reject the male (i.e., not receptive).

2.5 | Statistical analysis

In trials of volatile pheromones, the response variables involved three possible options (1. Lateral branch A; 2. Lateral branch B; and 3. None). A multiple-proportion test (chi-square goodness-of-fit tests) was used (Siegel & Castellan, 1988; Zar, 1999) to compare the observed distribution to an expected distribution with a Bonferroni correction ($\alpha = 0.0167$) since we considered important to include the lack of choice (3. None) as a possible answer in these cases (Abdi, 2007). In contact pheromones trials, the variable "exploration time

on each branch" was modeled according to its best distribution. To find the best relative distribution for the data, we used the package fitdistrplus (Delignette-Muller & Dutang, 2015) in R v. 3.3.3 64 bit (R Core Team, 2017). Gamma was the best distribution for the data and was modeled with the package glmmADMB for generalized linear mixed models (GLMMs) (Fournier et al., 2012). We modeled each experimental group independently (1. males vs. VF and empty [absence of a female]; 2. males vs. VF and IF; 3. males vs. RF and IF; 4. males vs. VF and RF) since the levels of the factor with fixed effect "branch explored" were different for each group. Also, the random effect variable "male identity" was added to account for multiple measurements on the same males. The validation of the fitted models was assessed graphically with residual analysis. Also, a multiple-proportion test was used to observe if there were statistically significant differences between the attractiveness proportions of females of different mating status (1. VF, 2. IF, 3. RF). Finally, considering only the attractive females, we compared the proportions of sexual receptivity (i.e., females allowing pedipalp grasping) according to the mating status.

3 | RESULTS

3.1 | Volatile pheromones

In the first experiment (VF, None), males chose in greater proportion the branch containing the virgin female compared to the empty

TABLE 1 Male branch choice in volatile (proportion of choice) and contact (average branch exploration time ± standard error of the mean) pheromone trials in different experimental groups

Experimental group	Male branch choice	Volatile pheromones		Contact pheromones	
		Proportion of choice	$\alpha = 0.0167$	Average branch exploration time (s)	$\alpha = 0.05$
1	VF	0.65 (13/20)	$X^2 = 9.70$	2,855 ± 481	$X^2 = 3.519$
	Nothing	0.25 (05/20)	$p = 0.008***$	1,521 ± 301	$p = 0.061$
	None/Central	0.10 (02/20)	$df = 2$ ($n = 20$)		$df = 1$ ($n = 17$)
2	VF	0.65 (13/20)	$X^2 = 9.10$	2,778 ± 513	$X^2 = 0.188$
	IF	0.15 (03/20)	$p = 0.011**$	2,507 ± 388	$p = 0.668$
	None/Central	0.20 (04/20)	$df = 2$ ($n = 20$)		$df = 1$ ($n = 18$)
3	RF	0.70 (14/20)	$X^2 = 12.1$	3,045 ± 564	$X^2 = 3.229$
	IF	0.15 (03/20)	$p = 0.002***$	1,364 ± 354	$p = 0.072$
	None/central	0.15 (03/20)	$df = 2$ ($n = 20$)		$df = 1$ ($n = 18$)
4	RF	0.50 (10/20)	$X^2 = 2.80$	1,968 ± 500	$X^2 = 0.066$
	VF	0.30 (06/20)	$p = 0.247$	2,213 ± 497	$p = 0.797$
	None/central	0.20 (04/20)	$df = 2$ ($n = 20$)		$df = 1$ ($n = 17$)

Note. "None" refers to the case the male stays in the central branch and does not choose any derived branch. A significance level α of 0.0167 was considered (Bonferroni correction) for volatile pheromone trials, and a significance level of 0.05 for contact pheromone trials.
IF: inseminated female; RF: inseminated female with mating plug removed; VF: virgin female.
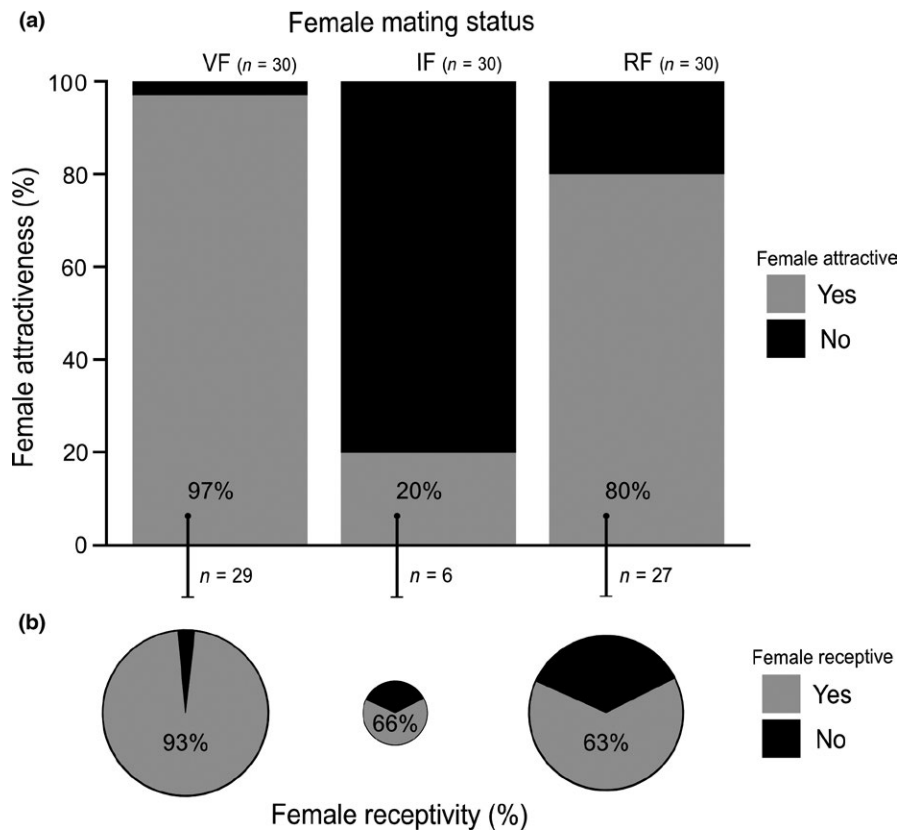The significance of **$p < 0.01$ and ***$p < 0.001$.

FIGURE 2 Sexual attractiveness and receptivity trials. (a) Sexual attractiveness (%) of females with different mating status. (b) Sexual receptivity (%) of females with different mating status that were attractive to males (refer to text for precise explanation). Different diameters refer to the sample size used. IF, inseminated female; RF, inseminated female with mating plug removed; VF, virgin female

branch and the lack of choice (Table 1). In the trial with virgin and inseminated females (VF, IF), males chose the lateral branch with the virgin female in greater proportion compared to the proportion of males that chose inseminated females or did not choose at all (Table 1). In the third experiment (RF, IF), males chose significantly more often the branch containing inseminated females with removed plug over the other branch with inseminated females and the lack of choice (Table 1). In the last test (VF, RF), we did not find statistically different the proportions of choice.

3.2 | Contact pheromones

There was no preference of males for any of the stimulus of the Y-maze (Table 1). We did not find statistically significant differences in branch exploration time between virgin (VF) and inseminated (IF) females, nor between inseminated females with mating plugs removed (RF) and virgin females (VF) (Table 1). We found low variability of the random effect in all the models (variance <0.00005, SD < 0.0005).

3.3 | Attractiveness and receptivity of females with different mating status

Males grasped female's pedipalps and performed juddering more frequently to virgin females and inseminated females with removed mating plugs compared to inseminated females (Figure 2a). Statistical analyses showed a difference between virgin females and inseminated females (X^2 = 15.114, p = 0.0001, df = 1), and between

inseminated females with removed plug and inseminated females (X^2 = 13.364, p = 0.0003, df = 1). No statistically significant differences were found when comparing the proportions of males attracted to virgin females versus inseminated females with a removed mating plug (X^2 = 0.071, p = 0.789, df = 1) (Figure 2a).

Concerning the female's sexual receptivity, we only evaluated those trials where the males were attracted to females (1. VF n = 29, 2. IF n = 6, 3. RF n = 27) (see diameters in Figure 2b). Sexual receptivity did not differ between virgin females and females with removed mating plug (X^2 = 0.058, p = 0.810, df = 1) (Figure 2b). Six inseminated females that were attractive to males were also receptive to them (66%). We did not find statistically significant differences of this proportion with the proportion of receptivity of inseminated females with removed plugs (RF) (X^2 = 0.001, p = 0.979, df = 1), nor with the proportion of virgin females (X^2 = 0.046, p = 0.831, df = 1) (Figure 2c).

4 | DISCUSSION

Our results show that males of *U. brachycentrus* can detect and locate females using volatile pheromones. We found that males can discriminate between the trails of mated and inseminated females. The mating plug may be related to female pheromone production, as the removal of the plug altered the male ability to localize the females. The presence of the mating plug produces a decline in female sexual and chemical attractiveness. Both sexual attractivity

and receptivity of females seem to be regained after mating plug removal.

Polis and Sissom (1990) suggested that the use of pheromones in the recognition and detection of potential partners is crucial for the mating success of many scorpion species. Numerous works have tested the detection of contact pheromones in scorpions (e.g., Benton, 1992; Gaffin & Brownell, 1992, 2001; Melville et al., 2003; Miller & Formanowicz, 2011; Steinmetz et al., 2004; Taylor et al., 2012). Conversely, only a few studies have analyzed the use of volatile chemical signals in scorpions. For example, Nisani, Honaker, Jenne, Loya, and Moon (2018) showed that the scorpion *Hadrurus arizonensis* (Vaejovidae) could detect and avoid his natural scorpion predator using volatile chemical signals (kairomone). However, until now, there was no evidence of volatile chemical signals used in reproduction (i.e., sex pheromones). The present work provides the first evidence of the presence of volatile sex pheromones in scorpions.

We found that males detected and localized virgin females more frequently than empty control or inseminated females (Figure 3). This suggests the male's ability to discriminate the pheromone trails of females with different mating status. There are several examples in diverse taxa of males discriminating between inseminated and virgin females by means of the two types of pheromones (O'Donnell et al., 2004; Roberts & Uetz, 2005; Thomas, 2011). Discrimination of the mates' quality is an evolutionary advantage because males can direct their mating efforts toward females that are sexually receptive or more fecund (Bonduriansky, 2001; Dewsbury, 1982; Wedell, Gage, & Parker, 2002). In many taxa, males prefer virgin females, where sperm has less chance of competing with that of rival males (Engqvist & Reinhold, 2006; Kelly & Jennions, 2011), ensuring a greater reproductive success (Bonduriansky, 2001). In *U. brachycentrus*, virgin females may provide the highest reproductive value, as females mate only once due to the presence of the mating plug. The reduction of female attractiveness and receptivity after mating is frequent in many scorpion groups (Polis & Sissom, 1990). Also, virgin females have a lower rate of cannibalistic attacks against males (Polis & Farley, 1979). For example, in *Zabius fuscus* (Buthidae), females inseminated are less receptive and more aggressive to males (Peretti & Carrera, 2005). In *Mesomexovis punctatus* (Vaejovidae), the mating plug causes a decline in female's sexual receptivity, even though they remain attractive to males (Contreras-Garduño, Peretti, & Córdoba-Aguilar, 2006). In this situation, females are so reluctant to second matings that they could cannibalize the new male (Contreras-Garduño et al., 2006).

Regarding contact pheromone trials, males showed no preference for exploring sites that contained a female, or sites that contained females of any particular reproductive status (Figure 3). This would suggest that the males do not detect the presence of the female or her mating status by using contact pheromones. This contrasts to data in other scorpion species in which contact pheromones are considered the main form of chemical communication (Gaffin & Brownell, 1992, 2001; Melville et al., 2003; Tallarovic et al., 2000; Taylor et al., 2012). Contact sex pheromones have been

Effects of mating plug		Female mating status		
		VF	IF	RF
Male's chemical preference	Volatile pheromones	+	−	+
	Contact pheromones	−	−	−
Mating acceptance	Female attractiveness	+	−	+
	Female receptivity	+	−	+

FIGURE 3 Effect of the mating plug on sexual interactions. Males in volatile pheromones trials prefer virgin females (VF); these females are attractive for males and receptive for mating. Males do not prefer inseminated females (IF) in volatile pheromone trials and in sexual encounters (these females are not sexually attractive and receptive). Males in volatile pheromone trials prefer inseminated females with removed mating plug (RF); these females are sexually attractive and receptive. In contact pheromones trials, there was no evidence of sexual recognition to any female mating status. References "+" Positive, "−" Negative. IF, inseminated female; RF, inseminated female with mating plug removed; VF, virgin female

postulated more frequently for short-range interactions (Gaskett, 2007), while volatile pheromones are expected for mate encounter and the discernment of reproductive status (De Freitas, De Lima Mendonça, Nascimento, & Sant'Ana, 2004; Hammack, 1986; Hegdekar & Dondale, 1969). The use of contact pheromones may not be appropriate for *U. brachycentrus*, because this species has surface activity during winter (Maury, 1977) and lives in high mountain environments (Acosta, 1988). Under these conditions, contact pheromones would be easily washed out of the substrate by water or snow (Dondale & Hegdekar, 1973), favoring chemical communication by volatile pheromones since these would not be eliminated in this environment (Wyatt, 2003). Other methodologies should be considered for the future study of sex pheromones, such as the evaluation of cuticular hydrocarbons (CHCs), chemicals in the body surface, which have been studied extensively in insects (Ferveur, 2005; Howard, 1993). In spiders, CHCs have been proposed to act as pheromones and differences were observed in the blend produced between sexes (Trabalon & Bagnères, 2010). In scorpions, some studies have identified CHCs (Gefen, Talal, Brendzel, Dror, & Fishman, 2015; Hadley & Jackson, 1977), but there are still no studies on their possible role in intersexual communication in the context of mating. It may also be of interest to include the study of other types of sexual communication of scorpions, such as vibrations. Scorpions detect their prey by vibrations (Polis & Farley, 1979), and it has been suggested that these may be important in the sexual context (Briceño & Bonilla, 2009). Our data indicate a phase of detection and location where vibrations would not be involved. However, this could not be measured during sexual encounters where they may be playing an important role contributing to the mating acceptance observed in females.

We found that males were chemically and sexually attracted in major proportion to females with the mating plug removed than to inseminated females (Figure 3). This suggests that the mating plug is related to the discernment mechanism of female mating status and that its presence may produce negative sexual recognition by the male. We obtained evidence that females partially regain sexual attractiveness after mating plug removal and can accept mating attempts from males. Also, the removal of the mating plug seems to produce a return of volatile pheromone emission in inseminated females. This indicates that it is not the "inseminated" condition that produces changes in chemical and sexual attractiveness, but the presence of the plug per se. Pheromone emission seems to be similar in virgin females and females with the mating plug removed, resulting in an equal proportion of male sexual attraction and male localization of these females. In contrast, inseminated females with a mating plug removed in *M. punctatus* and *Euscorpius italicus* were not receptive to new mating (Althaus et al., 2010; Contreras-Garduño et al., 2006). These studies in scorpions investigated the variation in female receptivity according to the moment of the plug extraction (and associated with changes in mating plug morphology). This approach must be included in future works in *U. brachycentrus* as the mating plug changes over time (M. Oviedo-diego, C. I. Mattoni, & A. V. Peretti, in prep). Differences in the composition and morphology of the mating plug could have specific mechanical, physiological, or both effects in the female and produce different receptivity responses when removed.

There may be a "male control" mechanism that could explain the influence of the mating plug on the change in female chemical and sexual attractiveness. The male produces a mating plug that triggers a decrease in female receptivity to new mating, by either mechanical or chemical effects, or both (Parker, 1970; Simmons, 2001; Wigby & Chapman, 2004). The mating plug may negatively affect the quantity or quality of the volatile pheromones emitted by the females to prevent female remating and enhance the paternity of the offspring (Gillott, 2003; Parker, 1984; Simmons, 2001). Besides, males may add antiaphrodisiac substances to the mating plug that may decrease the female's attraction to subsequent males mainly by a repellent effect (Malouines, 2017; O'Donnell et al., 2004). Consequently, the removal of the mating plug could explain the return of the attractiveness of females if the effect of the antiaphrodisiac is eliminated (O'Donnell et al., 2004). Our data confirm the detection and male chemical attraction by volatile pheromones toward virgin females and inseminated females with the plug removed, though we cannot rule out that, together with the plug, the male may transfer some antiaphrodisiac substances that mask or alter the female's pheromone emission.

From this perspective, there would be a sexual conflict since females would be deprived of the benefits of polyandry (Arnqvist & Nilsson, 2000; Arnqvist & Rowe, 2005; Jennions & Petrie, 2000). Alternatively, the female could strategically modulate the pheromone titer to control the timing and frequency of mating (Gaskett, 2007). This would be likely in cases where the female's costs of remating (e.g., loss of time and energy, exposure to parasites or predators, male harassment) outweigh the benefits of polyandry (Herberstein, Schneider, & Elgar, 2002; Kuntner, Gregorič, Zhang, Kralj-Fišer, & Li, 2012; Maklakov, Bilde, & Lubin, 2005; Maklakov & Lubin, 2004; Schneider & Lubin, 1998). The production of seminal substances (with "antiaphrodisiacs," or to form "mating plugs") could imply significant costs for the males (Dewsbury, 1982; Perry, Sirot, & Wigby, 2013; Wedell et al., 2002). Therefore, the characteristics and amount of seminal substances may reflect the quality of the male, and based on this, the female could exercise cryptic female choice (Eberhard & Cordero, 1995) and consequently modulate the emission of pheromones. In support of this, in our study, there were a few females that were receptive although they were inseminated. Future studies will evaluate the possibility that females can modulate the quantity or quality of pheromones as a form of cryptic female choice.

5 | CONCLUDING REMARKS

Although sex pheromone production, utilization, and detection have been widely studied in invertebrates, few investigations have shown the relationship between chemical communication and mating plugs and its consequences in the signaling of the female's mating status. Our study offers new data from scorpions, a neglected group of arthropods in these topics. This contribution demonstrates for the first time in scorpions that males can detect and localize females through volatile pheromones and that they are capable of discriminating female's mating status. The presence of the mating plug significantly affects female chemical attractiveness and produces a decrease in female mating acceptance (which can be reversed if the mating plug is artificially removed). Further studies may examine the proximal cause of the association between the mating plug and variation in chemical attractiveness. Complementary investigations could also focus on comparative analyses of the structure, function, and effectiveness of plugs among species of scorpions, as promising models of animals with spermatophore-mediated sperm transfer.

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