Sexual dialogue in Pachyloides thorellii (Opiliones: Gonyleptidae): a Neotropical harvestman with much to say

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Sexual dialogue in *Pachyloides thorellii* (Opiliones: Gonyleptidae): a Neotropical harvestman with much to say

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Abstract. Describing the signals involved in sexual interactions is crucial to understand how mating and fertilization success is achieved. We analyzed sexual interactions in the gonyleptid harvestman *Pachyloides thorellii* Holmberg, 1878 to test the possibility of associations between female and male behaviors. For that purpose, we recorded 21 sexual interactions of *P. thorellii* under laboratory conditions and performed fine scaled analyses of the videos. We found three female pre-copulatory behaviors ("Mouth parts protrusion," "Genital operculum opening," and "Ovipositor eversion") that seem to be related to sexual receptivity and cooperation with mating occurrence, and four copulatory behaviors ("Bucking," "Pulling," "Body lowering," and "Leg II movements") that could be indicating to the male that further stimulation is required or that mating is about to end. We also found that males use multimodal courtship displays that include the exchange of tactile and possibly chemical signals between sexes. This study shows that courtship and copulation in *P. thorellii* include intense information flow between sexes and female evaluation from the beginning until the end of the sexual interaction.

Keywords: Pre-copulatory courtship, female control, copulatory courtship, genital contact, copulatory communication

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Male-female sexual interactions in most animal species with internal fertilization can be divided into three main phases: pre-copulatory, copulatory, and post-copulatory. The pre-copulatory phase traditionally involves acoustic and/or visual displays, emission of chemical compounds, and tactile courtship that are usually performed by the males before intromission (examples in Andersson 1994). During the pre-copulatory phase, males and females can evaluate species identity, as well as estimate the quality of their potential mating partners (Jennions & Petri 1997). The copulatory phase starts with intromission and may also involve displays and tactile courtship performed by the male both on the external surface of the female body and inside her reproductive tract, using special morphological traits on his genitalia (Eberhard 1985, 1996; Aisenberg & Peretti 2015). Finally, the post-copulatory phase involves all sorts of interactions that occur after intromission is finished, including post-copulatory tactile courtship, mate-guarding, and cryptic female choice (Alcock 1994; Eberhard 1996). From the male perspective, the main role of the copulatory and post-copulatory interactions is to increase the probability of fertilizing the greatest number of eggs and maximizing progeny, whereas from the female perspective, the main role of these interactions is to ensure sperm transfer and gather information to select the best mating partners (Eberhard 2009). Natural and sexual selection may act on each of these three phases, shaping the evolution of morphological, physiological, and behavioral traits involved in male-female sexual interactions (Andersson & Simmons 2006). Therefore, detailed descriptions of sexual interactions can help enlighten the evolution of reproductive traits in both males and females.

The three phases of male-female sexual interactions can be easily observed and distinguished in species of the order Opiliones, commonly known as harvestmen or daddy long-legs (reviewed in Machado et al. 2015). Although the pre-copulatory phase is usually fast, involving mostly leg tapping and rubbing performed by the male on the female body (e.g., Nazareth & Machado 2009; Fowler-Finn et al. 2014), there are records of some species in which males can offer nuptial glandular secretions to females before intromission (Martens 1969). Copulatory interactions in harvestmen can also involve male leg tapping and rubbing on the female body, and complex genital interactions that may include tactile stimulation of the female genitalia and delivery of glandular secretions as nuptial gifts (e.g., Burns et al. 2013; Pérez-González & Werneck 2018). Post-copulatory tactile interactions are rarely reported in harvestmen, but in some species males exhibit mate-guarding, which may last as much as 48 hours (e.g., Macías-Ordóñez 2000; Buzatto & Machado 2008). Moreover, given that harvestmen are in general highly polygamic and females can store sperm from several different males, sperm competition and cryptic female choice are probably ubiquitous processes in species of the order (reviewed in Macías-Ordóñez et al. 2010 and Machado et al. 2015).

Stanley et al. (2016) described and quantified male-female sexual interactions of the Neotropical harvestman *Pachyloides thorellii* Holmberg, 1878 (Laniatores: Gonyleptidae). In this solitary species, the pre-copulatory phase starts when the male courts the female by touching her dorsum and legs with his first and second pair of legs. When the male touches the female, she can reject him by (i) quickly moving away from him, (ii) retracting her legs toward her body, and/or (iii) lowering her cephalothorax to the substrate, thus preventing intromission (Stanley et al. 2016). If the male is not rejected, he will continue to court, and the pair may adopt the typical mating position of Laniatores (see Machado et al. 2015). In this position, male and female stay face-to-face, with the male grabbing the female pedipalps with his own pedipalps, and both of them grabbing each other chelicerae. Once the couple adopts the mating position, through raising the anterior part of their bodies and forming a 90° angle between themselves, the male everts his penis and introduces it into the female genital operculum (Stanley et al. 2016). During intromission, the male continues to touch the female dorsum with his first pair of legs, initiating the copulatory phase. Females generally remain still, maintaining the mating position and slowly waving the second pair of legs in the air (Stanley et al. 2016). Intromission ends when the male retracts the penis from the female genital operculum. During the post-copulatory phase, the male remains near the female for a few minutes, touching her with his first and second pair of legs while she cleans her legs and genital operculum using her mouthparts (Stanley et al. 2016).

Similar to several insect species (summarized in Rodríguez 2015), males and females of *P. thorellii* could be exchanging information during all phases of the sexual interaction. This sexual dialogue implies that behaviors performed by individuals of one sex could be associated with predictable behavioral responses by individuals of the other sex. For instance, stimulatory behaviors performed by males, such as leg tapping, could increase the frequency of female behaviors related to mating receptiveness, such as adopting the face-to-face position or opening the genital operculum (Macías-Ordóñez et al. 2010). In the same way, some behaviors performed by females may communicate to males that further stimulation is required to continue the sexual interaction. Thus, typical rejection behaviors performed by harvestman females, such as lowering the cephalothorax, should be followed by an increase in the frequency of stimulatory behaviors performed by the males. On the other hand, the signals displayed could be messages of resistance from one sex to courtship and/ or copulatory behaviors performed by the other sex. In this context, fine-scaled observations have shown to be essential tools for testing these hypotheses in arthropods, and particularly, in spiders (Eberhard 1996; Peretti & Córdoba-Aguilar 2007; Peretti & Aisenberg 2011).

This study delves into the previous behavioral descriptions of male-female sexual interactions in *P. thorellii* (Stanley et al. 2016) with the aim of searching for correlations between sexes in courtship and copulatory behaviors performed during sexual interactions. We expect to identify female and male responses to pre-copulatory and copulatory behaviors, indicating female sexual receptivity and willingness to continue or resume the sexual encounter, and male behaviors to increase mating duration and fertilization success.

METHODS

Breeding and maintenance.—We raised individuals of *P. thorellii* in the Departamento de Ecología y Biología Evolutiva, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay. The breeding of this species started in 2008 from adult individuals collected in the locality of Marindia (34°46′S, 55°49′W), Canelones, Uruguay. From that year on, several controlled crossings were performed under laboratory conditions that allowed us to rear juveniles until obtaining virgin adults. After hatching, the nymphs were maintained in their mothers' container until they reached the subadult instar (approximately two months after hatching; Stanley 2011). After this, they were isolated in cylindrical plastic airtight containers (10 cm diameter, 8 cm high) with sand as substrate, wood pieces and rocks as shelter, and a wet cotton wool as water supply. Individuals were fed weekly with pieces of *Zophobas morio* Fabricus, 1776, and *Tenebrio molitor* Linnaeus, 1758 (Coleoptera) larvae, cucumber, apple and cat food (FirstClass®), according to previous studies on this species (Stanley et al. 2016). The average temperature ± standard deviation (SD) during the breeding period was 22.7 ± 3.2°C in 2015 and 22.5 ± 3.3°C in 2016. All individuals used in the trials were virgin, unrelated, and with one to six months of adult age.

Behavioral observations.—We carried the observations during April and May in 2015, and during May and June in 2016, at an average temperature of 21.6 ± 1.7°C. We placed each female in a glass container of 15 × 15 cm wide and 4 cm tall, with sand as substrate, a piece of tree bark as potential refuge, and a wet cotton wool as water supply. We placed each male in the container immediately before the beginning of each encounter, 24 h after we placed the female in the container. We carefully picked up the males to prevent the release of defensive chemical secretions and placed them approximately 10 cm away from females. If within the first 15 min after the beginning of the trial the male did not court the female, the trial was stopped, and we retried with the same couple 24 h later. Instead, if the male courted the female, we continuously monitored them for up to 30 min (in the case of successive rejections by the female), or until the end of mating interactions (in the case of successful copulations). We conducted the behavioral observations under red light, following previous studies on this species (Stanley et al. 2016). Instead of placing the glass container on a fixed surface we placed it on a stool to be able to rotate the container 360° while filming, and placed a 2 cm thick foam pad between the stool and the container to minimize vibrations while steering. We recorded each encounter with a Sony video camera (DCR-SR87) fixed to a tripod at the same height as the container. We recorded female body measures before placing them in the glass container, whereas the male body measures were recorded after the trials. We weighed all individuals using a digital balance (Sartorius Weighting Technology Germany AZ214; precision 0.1 g) and recorded dorsal scute length and width under a stereoscopic microscope (OLYMPUS SZ-61; precision 0.01 mm), following Willemart et al. (2008).

We analyzed the videos in detail to determine the frequency and duration of female and male sexual behaviors. We considered that the pre-copulatory phase initiated when the male performed the behavior "Touch with legs I–II" and finished when the penis was inserted into the female's genital operculum (i.e., "Penis insertion," see Table 1). The copulatory phase lasted from "Penis insertion" until "Penis retraction," when the male withdrew the penis from the female's genital operculum (see Table 1). Finally, we considered that the post-copulatory phase lasted from "Penis retraction" until "Separation" of the couple (see Table 1). The duration of behaviors "Mouthparts protrusion" and "Opening of genital operculum" refers to the time these behaviors initiated until "Penis insertion" occurred or the behaviors ended.

Table 1.—Behaviors observed during male-female sexual interactions in the harvestman *Pachyoides thorelli*. The list is based on the original description provided by Stanley et al. (2016), but it also includes novel behaviors described in the current study (marked with an asterisk) and modified definitions. The phase of the male-female sexual interactions in which each behavior was observed is indicated in the third column.

Behavior	Description	Phase
Touch with legs II	Mutual touches with the tarsus of the second pair of legs. The individual stands still on the substrate touching the dorsal scute and/or the first three pairs of legs of the mating partner. It can be performed either by males or females.	Pre-copulatory
Touch with legs I-II	Male intensively taps female's dorsal scute with the tarsus of the first pair of legs, while "Touch with legs II" continues.	Pre-copulatory
Rushing	Male extends his pedipalps and quickly approaches the female (frontally, laterally, or from behind).	Pre-copulatory
Male over female	Male climbs over female dorsal scute and slides over it while he extends his pedipalps and rubs female dorsum. "Touch with legs I-II" continues and "Male over female" ends when the male is located in front of the female in a face-to-face position.	Pre-copulatory
Mouthparts protrusion*	Female protrudes her mouthparts (Fig. 5A).	Pre-copulatory
Genital operculum opening*	Female opens the genital operculum (Fig. 5A).	Pre-copulatory
Ovipositor eversion*	The female everts the ovipositor after opening the genital operculum (Fig. 5C).	Pre-copulatory
Grabbing	Once in the face-to-face position, the male uses the claw of his pedipalps to grab the coxae of the female first pair of legs. Both male and female grab each other chelicerae. Male continues with the behavior "Touch with legs I-II".	Pre-copulatory
Elevation	Using his fourth pair of legs as support, the male elevates the anterior part of his body together with the anterior part of the female body forming a 90° angle between them.	Pre-copulatory
Bucking*	Female rapidly extends her fourth pair of legs elevating the posterior and lowering the anterior part of the body.	Pre-copulatory and Copulatoty
Insertion attempt*	While grabbing the female, the male everts his penis toward the female genital operculum and moves the posterior part of his body like a pendule. First, he moves his abdomen up and backwards, and then down and forward, rubbing the female ventral zone with the glans. Immediately, he retracts the penis when he does not manage to insert the glans into the female genital operculum (Fig. 5B).	Pre-copulatory
Failed insertion*	Identical to "Insertion attempt," but the male does not direct the penis toward the genital operculum but to one side of the female body (Fig. 5C).	Pre-copulatory
Penis insertion	Male introduces the glans into the female genital operculum.	Copulatory
Leg tapping	Male places the tarsi of the first pair of legs on the dorsal scute of the female and slides them toward the sides of her body. He maintains his second pair of legs in the air, alternating between the right and left and touching the female on the sides and dorsum. Description modified from the behavior "Copulatory courtship" in Stanley et al. (2016).	Copulatory
Penis vibration*	Male rapidly moves the penis up and down after insertion (Fig. 6A).	Copulatory
Mouthparts-genitalia contact*	The distal end of the penis shaft contacts the female mouthparts, which are protruded (Fig. 6B).	Copulatory
Insertion movements*	Maintaining the glans in the female genital operculum, the male performs pendular movements (idem to "Insertion attempt"), moving his abdomen up and backwards, and then down and forward.	Copulatory
Insertion only*	Male maintains the insertion without performing any other behavior.	Copulatory
Pulling	Female pulls backwards from the male using her third and fourth pair of legs as a support.	Pre-copulatory and Copulatoty
Legs II movements	Female moves the second pair of legs slowly.	Copulatory
Body lowering	Female bends her legs lowering her body.	Pre-copulatory and Copulatoty
Penis retraction	Male withdraws the penis from female genital operculum and retracts it into his body.	Copulatoty
Separation	Male releases female pedipalp and chelicerae, as the female releases male chelicerae. Description modified from the behavior "Separation" in Stanley et al. (2016).	Post-copulatory
Ovipositor cleaning	Female everts ovipositor until its distal extreme contacts her mouthparts, scrapes the ovipositor with the claws of her pedipalps, and takes them to her mouthparts. She repeats this behavior several times (Fig. 7). Name and description modified from the behavior "Operculum cleaning" in Stanley et al. (2016).	Post-copulatory
Moving away	One individual (male or female) moves apart from the other. Description modified from the behavior "End" in Stanley et al. (2016).	Post-copulatory
Remaining still	One individual (male or female) remains still. Description modified from the behavior "End" in Stanley et al. (2016).	Post-copulatory
Ending	One or both individuals move far away from each other, reaching a distance greater than the length of their second pair of legs. Description modified from the behavior "End" in Stanley et al. (2016).	Post-copulatory

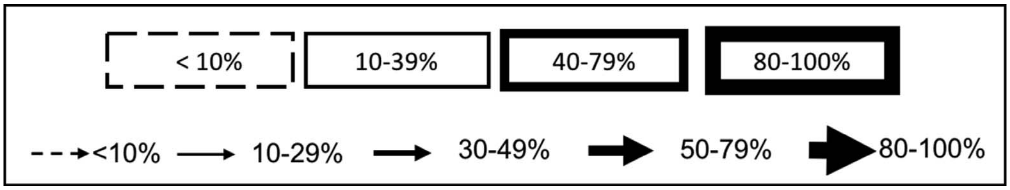

Figure 1.—Key to flow diagrams in figures 2–4, which illustrate male-female sexual interactions in the three phases of courtship and mating in the harvestman *Pachyoides thorellii* Pre-copulatory phase (Fig. 2), Copulatory phase (Fig. 3) and Post-copulatory phase (Fig. 4). In all, grey squares contain behaviors that were performed only by males, white squares contain behaviors that were performed only by females, and dotted squares contain behaviors that were performed by both sexes. Arrow thickness represents frequencies of transition between behaviors, with corresponding values expressed as percentages. Square thickness represents the frequencies of occurrence of each behavior and their values are expressed in percentages. (*) Frequencies of transition significantly higher than expected by chance; (-) Frequencies of transition significantly lower than expected by chance; LT: Leg tapping; PV: Penis vibration; MGC: Mouthparts-genitalia contact; LIIM: Legs II movements.

Statistical analyses.—We quantified frequencies and durations of each behavior and constructed a flow diagram for each phase (i.e., pre-copulatory, copulatory, and post-copulatory). The statistical analyses were performed only with the trials that resulted in copulation and between those behavioral transitions that were from one sex to the other. Behavioral transitions from the same sex were removed from the analysis. We used Chi-square tests to determine whether each transition between male and female behaviors occurred more frequently than expected by chance and used a *P*-value of 0.05 as the limit of statistical significance. All statistical analyses were performed in the software PAST version 3.0 (Hammer et al. 2003).

RESULTS

We obtained 21 successful copulations ($n = 80$ trials) that showed a mean ± SD duration of 764.2 ± 339.8 s (range = 103–1340 s). A complete list of the behaviors recorded in male-female interactions is presented in Table 1. Figure 1 gives a key to the behavioral flowcharts depicted in Figs. (old 1-3)>2–4.

Pre-copulatory phase.—The pre-copulatory phase (Fig. 2) had a mean ± SD duration of 48.7 ± 43.3 s (range = 17–210 s). This phase started with "Touch with legs I–II" (Table 1). During this behavior, the male courted the female by touching her dorsum and legs with his first and second pair of legs. If the female did not move away from him, the male continued touching her while he adopted the "Male over female" position (Table 1). We observed that during the "Male over female" position the female usually performed "Mouthparts protrusion" ($X^2 = 31.35$, 1 df, $P < 0.001$; Fig. 5a). Then, once in the face-to-face position, "Grabbing" and "Elevation" occurred. Next, the female performed "Genital operculum opening" ($X^2 = 6.63$, 1 df, $P = 0.01$; Fig. 5a,b), a behavior that was followed by "Penis insertion" ($X^2 = 112.89$, 1 df, $P <$ 0.001). In one case, we observed the male everting the penis and trying to insert it between the coxae of female legs II and III ("Failed insertion"). After a couple of attempts, the female performed "Ovipositor eversion" until the male redirected his penis and accomplished the insertion (Fig. 5c).

Copulatory phase.—The copulatory phase began with "Penis insertion" (Fig. 3). This phase had a mean ± SD duration of 704.3 ± 342.9 s (range = 58–1300 s). During this phase, males were observed performing three behaviors either simultaneously or at different moments: (1) "Leg tapping": the male performed delicate touches with the first pair of legs on the female dorsum and legs (Table 1); (2) "Penis vibration": during insertion, the male moved the penis rapidly up and down (Fig. 6a; Table 1); (3) "Mouthparts-genitalia contact": the penis shaft was contacted by female mouthparts while she maintained "Mouthparts protrusion" (Fig. 6b; Table 1). This contact between the female mouthparts and the penis shaft was maintained during the duration of penis intromission. In some cases, we observed the female moving the mouthparts when the penis shaft was not in contact with it.

As the flow diagram shows (Fig. 3), males performed the three behaviors mentioned above in any combination. During the copulatory phase, we also observed males performing "Insertion movements," in which they elevated their abdomen, partially retracting the penis without interrupting intromission, and immediately lowering their abdomen, resuming full insertion into the female reproductive tract (Table 1). The "Insertion movements" seemed to occur periodically at mean ±SD intervals of 13 ± 5.6 s. Once the male started "Insertion movements," he generally maintained them while "Leg tapping," "Penis vibration," and "Mouthparts-genitalia contact" occurred.

During the copulatory phase, we observed that when "Leg tapping" stopped, females tended to perform behaviors such as "Bucking," "Pulling," "Body lowering," and "Legs II movements" (Table 1). Moreover, we detected the following significant transitions: (1) "Mouthparts-genitalia contact" and "Penis vibration" followed by "Bucking" ($X^2 = 20.66$, 1 df, $P < 0.001$); (2) "Mouthparts-genitalia contact" and "Penis vibration" followed by female "Pulling" ($X^2 = 21.36$, 1 df, $P < 0.001$); (3) "Penis vibration" followed by female "Bucking" ($X^2 = 37.07$, 1 df, $P < 0.001$); (4) "Mouthparts-genitalia contact" followed by female "Pulls" ($X^2 = 8.38$, 1 df, $P =$ 0.0038); and (5) "Insertion" followed by female "Body lowering" ($X^2 = 11.88$, 1 df, $P < 0.001$). We also observed that some female behaviors were significantly followed by male behaviors: (1) "Bucking" was followed by "Leg tapping" and "Mouthparts-genitalia contact" ($X^2 = 82.92$, 1 df, $P <$ 0.001); (2) "Body lowering" was followed by "Leg tapping," "Mouthparts-genitalia contact," and "Penis vibration" ($X^2 =$ 12.25, 1 df, $P < 0.001$); and (3) female "Leg II movements" were followed by "Leg tapping" and "Mouthparts-genitalia contact" ($X^2 = 9.61$, 1 df, $P = 0.0019$). Some female copulatory behaviors occurred less frequently after male "Insertion movements" ("Bucking": $X^2 = 125.52$, 1 df, $P < 0.001$; "Pulling": $X^2 = 56.39$, 1 df, $P < 0.001$; "Leg II movements":

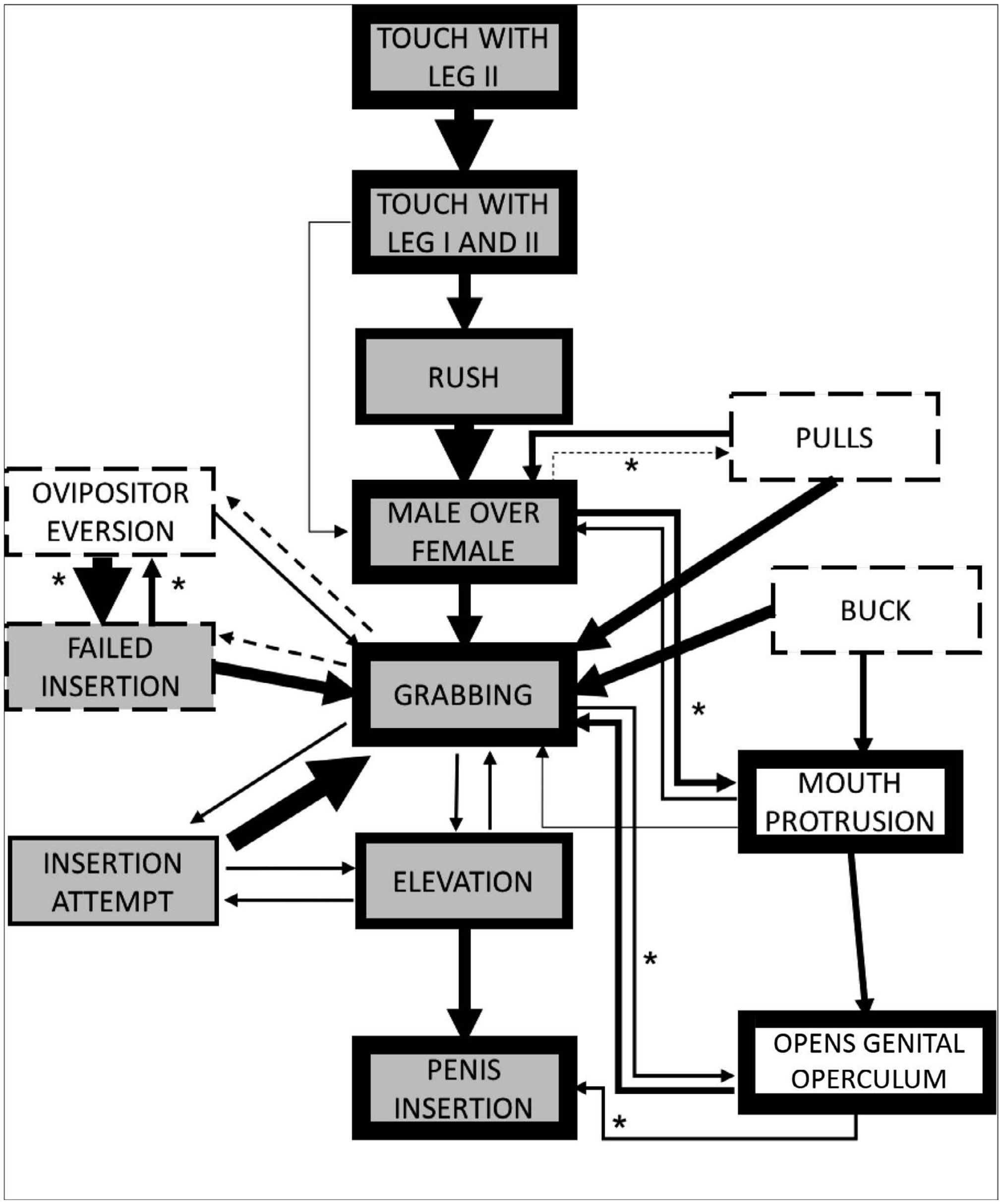

Figure 2.—Flow diagram of male-female sexual interactions in the Pre-copulatory phase of the harvestman *Pachyoides thorellii*. See Figure 1.

$X^2 = 7.97$, 1 df, $P = 0.005$). Finally, "Bucking" ($X^2 = 52.42$, 1 df, $P < 0.001$), "Pulling" ($X^2 = 1427.50$, 1 df, $P < 0.001$), and "Body lowering" ($X^2 = 38.03$, 1 df, $P < 0.001$) were significantly followed by "Penis retraction" and therefore the end of sperm transfer (Fig. 3). We found that in 81% ($n = 17$) of the successful copulations "Penis retraction" was preceded by one of these three female behaviors.

Post-copulatory phase.—"Penis retraction" indicated the end of the copulatory phase and beginning of the post-copulatory phase (Fig.4). This phase had mean ± SD duration of 11.3 ± 15.2 s (range = 1–70 s). After "Penis retraction" the couple maintained the chelicerae embraced for a mean ± SD of 2.7 ± 2.1 s (range = 1–7s) and the male continued the behavior "Touch with legs I and II" during the whole phase. We observed that after "Penis retraction" females could perform "Pulling," "Bucking," and/or "Body lowering," and all those behaviors lead to the "Separation" of the couple (Fig. 4). However, the transition frequency between "Penis retraction" and any of these female behaviors was not significantly different than expected by chance. We detected that when the female performed "Pulling," the male generally responded with "Touches with legs I and II," and the other way round (female "Pulling" followed by male "Touches with leg I and II": $X^2 = 4.83$, 1 df, $P = 0.028$; male "Touches with leg I and II" followed by female "Pulling": $X^2 = 4.55$, 1 df, $P = 0.033$). We also observed that male "Touches with legs I and II" could continue even after "Separation." After "Separation" females performed "Ovipositor cleaning" in 76% of the cases ($n = 16$) (Table 1) (Fig. 7). "Ovipositor cleaning" had a mean (± SD) duration of 41.2 ± 37.3s (range = 10–145 s) and in one-third of the cases (31%) females did it more than once (maximum = 3).

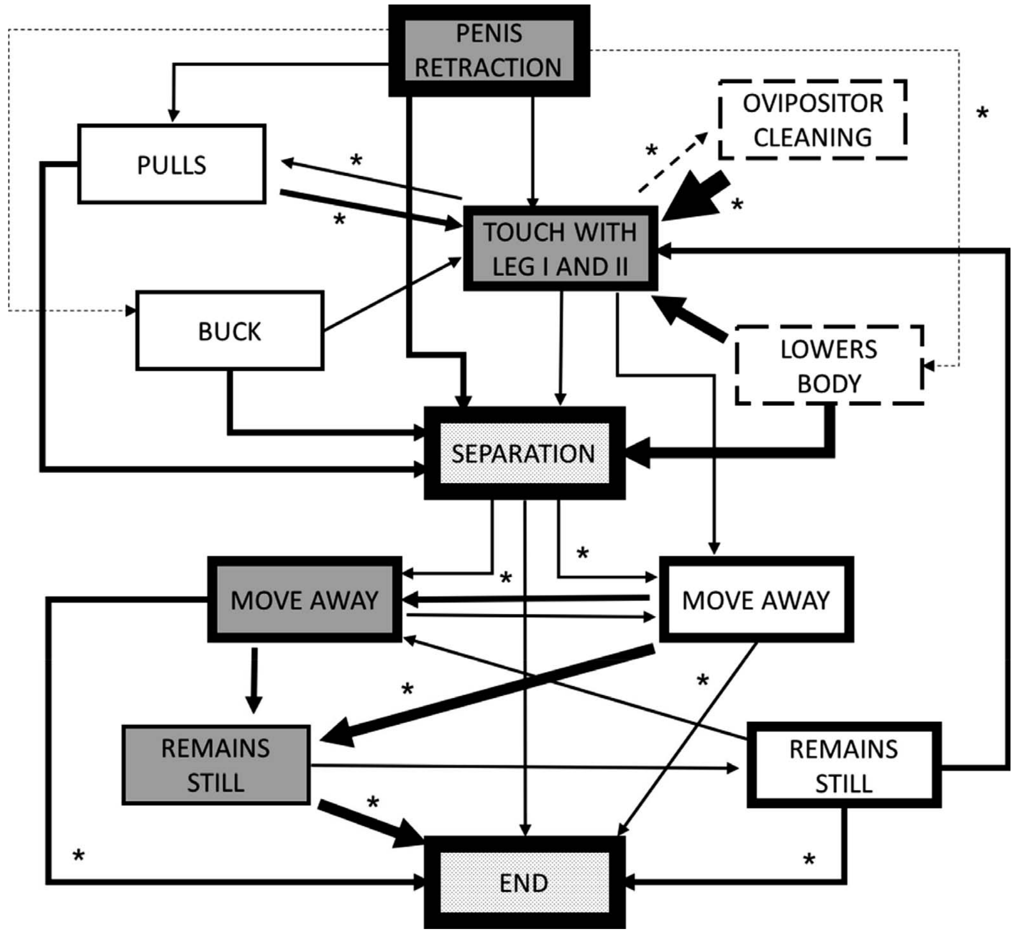

Figure 3.—Flow diagram of male-female sexual interactions in the Copulatory phase of the harvestman *Pachyoides thorellii*. See Figure 1.

The end of the post-copulatory phase occurred when one or both individuals moved away from each other at a distance greater than the length of their legs II ("Ending"; see Table 1). After "Separation," the female was generally the one "Moving away" ($X^2 = 6.17$, 1 df, $P = 0.013$), which could lead to "Moving away" of the male, too ($X^2 = 12.47$, 1 df, $P < 0.001$), or "Remaining still" ($X^2 = 155.27$, 1 df, $P < 0.001$). All of the last three behaviors could lead to "Ending" (female "Moving away" followed by "Ending": $X^2 = 12.24$, 1 df, $P < 0.001$; male "Moving away" followed by "Ending": $X^2 = 218.98$, 1 df, $P = 0.001$; male "Remaining still": $X^2 = 47.24$, 1 df, $P < 0.001$).

DISCUSSION

In this study, we quantified male-female sexual interaction in the harvestman *P. thorellii* in order to search for correlations between sexes in courtship and copulatory behaviors. Positioning the camera at the same height of the couple to record from the sides and a more exhaustive analysis of each encounter compared to Stanley et al. (2016), enabled us to record new behaviors that support the hypothesis of female control and couple cooperation instead of coercion in this species. We found that during the pre-copulatory phase of sexual interactions females can either reject the males or protrude their mouthparts and open the genital operculum in response to male courtship (i.e., "Leg tapping"). Given that "Mouthparts protrusion" always occurred before "Genital operculum opening" and preceded "Penis insertion," the former female behavior can be interpreted as an indication of sexual receptivity. During "Mouthparts protrusion" females contacted the penis, and it is possible that they feed on glandular secretions produced by the males. The presence of glandular secretions on the penis has already been reported for species belonging to the genus *Leiobunum* (Eupnoi: Sclerosomatidae), in which the delivery of nuptial gifts is a necessary step in the copulation process (Burns et al. 2012, 2013; Fowler-Finn et al. 2014). The glandular secretions may function in *P. thorellii* as an estimator of male quality, to help the female decide whether the pre-copulatory phase will proceed to the copulatory phase (Eberhard 1996). To our knowledge, this study is the first report of possible transference of nuptial gifts through the penis in a species of the suborder Laniatores. Future morphological studies will analyze the male genitalia looking for accessory glands and/or other structures from which the females could obtain any substance before or during intromission in this species.

Male harvestmen cannot force the opening of female operculum, so copulation cannot occur without female cooperation (Macías-Ordóñez et al. 2010; see also Burns et

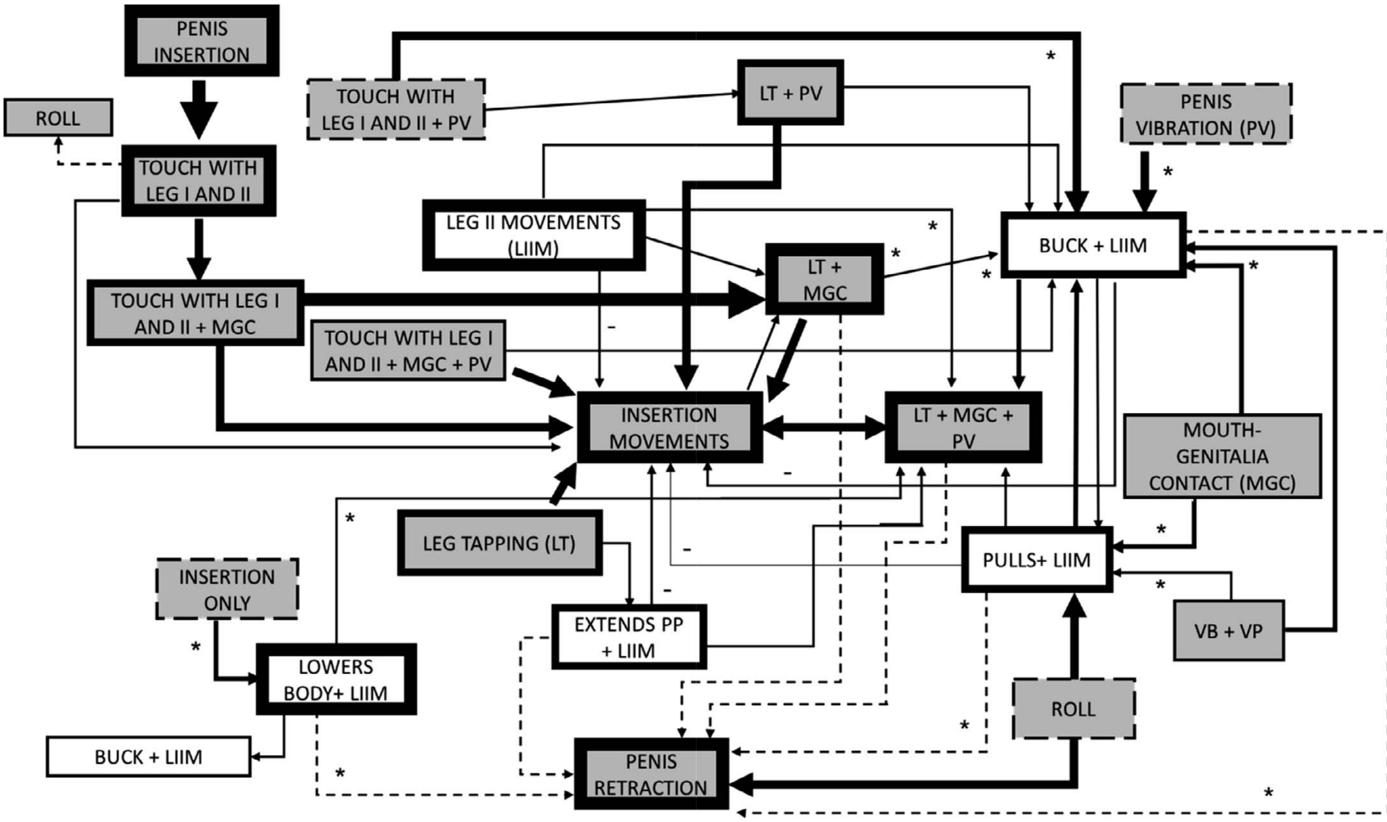

Figure 4.— Flow diagram of male-female sexual interactions in the Post-copulatory phase of the harvestman *Pachyoides thorellii*. See Figure 1.

al. 2013). We observed that sexually receptive females of *P. thorellii* indeed open their genital operculum voluntarily seconds before intromission occurs. Moreover, we observed a case in which the female everted the ovipositor when the male did not direct the penis in the operculum direction (see "Failed insertion" in Table 1). Ovipositor eversion clearly allowed intromission, reinforcing the notion that females cooperate with males during sexual interactions. We also observed that female pedipalps remained still and close to the substrate before and during intromission, and that the penis slid through the female mouthparts and pedipalps when the male tried inserting his penis. We suggest that all these female behaviors have the function of guiding the penis toward the genital operculum and placing the putative glands on the penis close to the female mouthparts during intromission. Once a male is accepted in the pre-copulatory phase, the female clearly cooperates, and the mating pair proceeds to the copulatory phase.

In this study, we report behaviors that seem to be related to female estimation and choice on male copulatory courtship. As we already stated, it is possible that glandular secretions are targets of female choice and estimators of male quality, similar to reports in other arthropod species (Eberhard 1996, 2010; Machado et al. 2015). Furthermore, penis vibration performed during intromission could also be associated with genital stimulation in *P. thorellii*. The tip of the ovipositor in Laniatores has a series of sensilla that could be stimulated during male genitalic movements with structures located on the distal part of the penis (Macías-Ordóñez et al. 2010). We also observed male body movements during sperm transfer, a behavior we called "Insertion movements." Males, without withdrawing the penis, move their abdomen back and forward in a pendulum like manner during insertion. In Eupnoi, this type of behavior is related to the insertion of the stylus in the female seminal receptacles (Macías-Ordóñez et al. 2010). However, this type of insertion would not be expected in Laniatores as the sperm is probably deposited at the lumen of the ovipositor (Macías-Ordóñez et al. 2010). Therefore, genital and body movements in Laniatores and Eupnoi males possibly have different functions. In *P. thorellii*, these movements may be involved in transporting the sperm to the seminal receptacles or removing sperm of previous males, similar to reports for other arachnid and insect species (see examples in: Eberhard 1996, 2011; Macías-Ordóñez et al. 2010; Briceño & Eberhard 2015; Machado et al. 2015). Finally, we cannot rule out the possibility that insertion movements could also be byproducts of ejaculations. Seminal products in Laniatores are pushed through the sperm duct by a muscular organ located at the base of the penis (Macías-Ordóñez et al. 2010). Thus, insertion movements may occur when males press the muscle associated with the penis and the seminal vesicle to promote sperm transfer.

We found that when the female pulled, lowered her body, or bucked—behaviors that are considered rejection behaviors in this species (Stanley et al. 2016)—the male does not respond with insertion movements, but he rather intensifies courtship

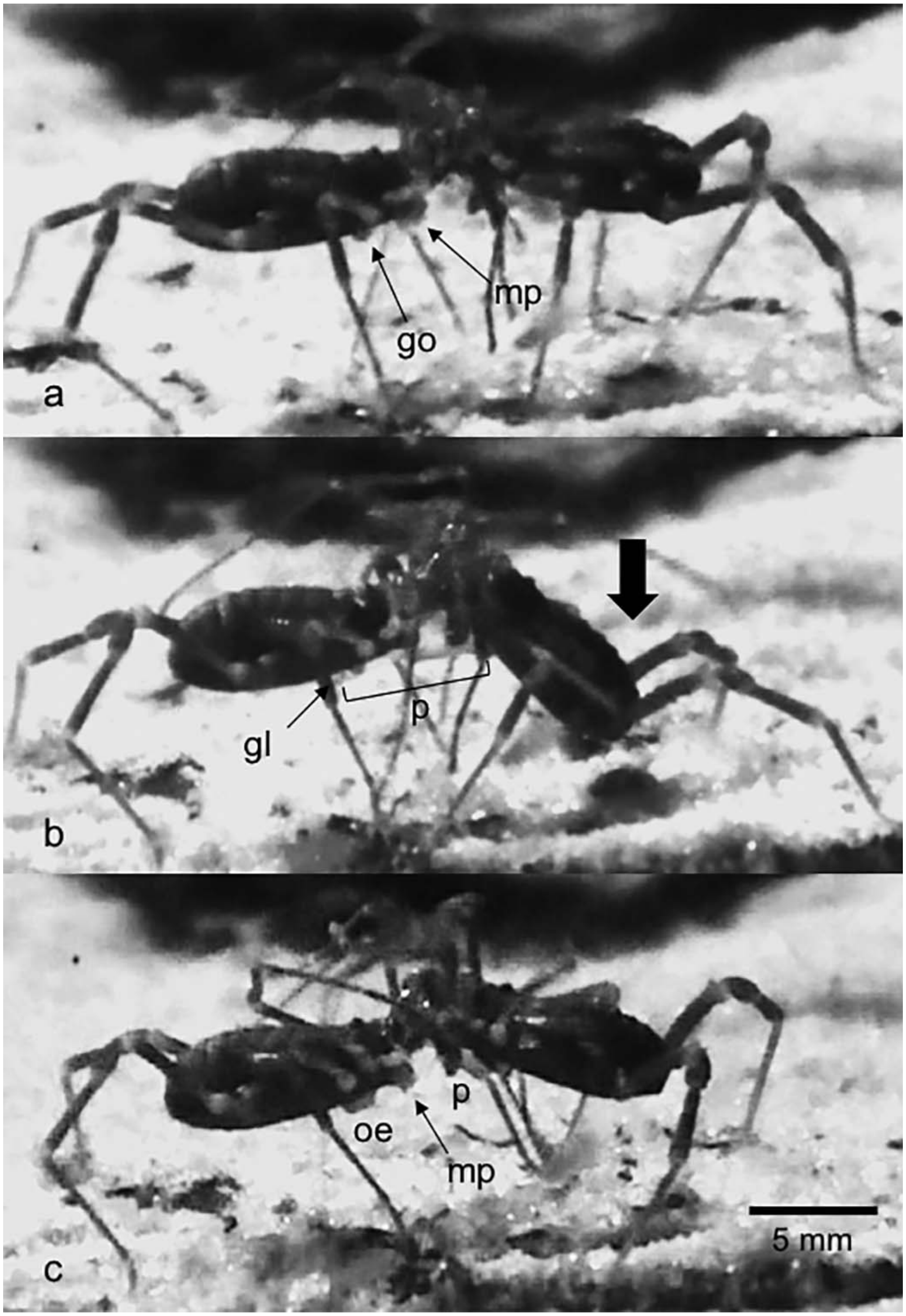

Figure 5.—Male (right) and female (left) behaviors during the pre-copulatory phase in the harvestman *Pachyloides thorellii*. (a) Face-to-face position and grabbing. (b) Insertion attempt. Black arrow shows the direction of male movements. The female maintains mouthparts protrusion and her genital operculum open while the male pushes the abdomen down and everts the penis. The attempt was unsuccessful as the glans of the penis is still visible and beyond the female genital operculum. (c) Failed insertion and ovipositor eversion. The male everts the penis between female legs, while the female maintains mouthparts protrusion and everts the ovipositor. mp: mouthparts protrusion; go: genital operculum; p: penis; gl: glans; oe: ovipositor eversion.

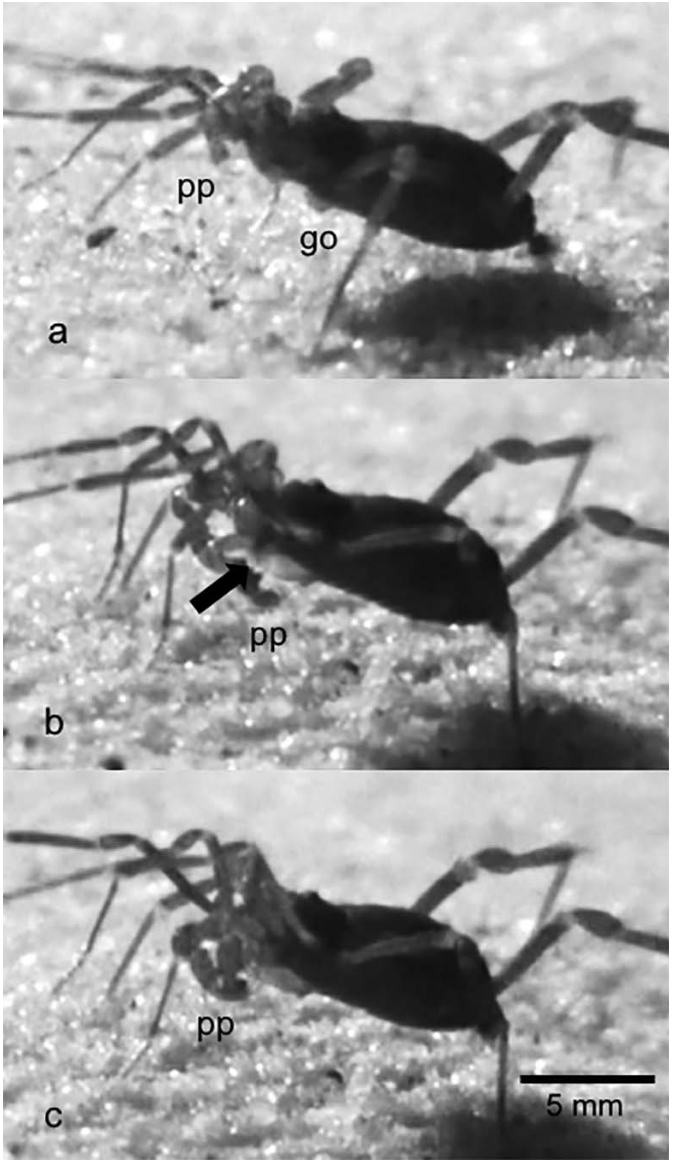

Figure 6.—Novel male behaviors described in the current study performed by *Pachyloides thorellii* during the copulatory phase. (a) Penis vibration. Arrow and dotted lines indicate the penis movement. (b) Mouthparts-genitalia contact. p: penis; s: penis sack; m: mouth; go: genital operculum.

by performing "Leg tapping." Therefore, it is possible that those female behaviors are signaling a negative sexual response to which males respond by intensifying courtship. Under this scenario, there would be a copulatory dialogue between the sexes in which each member of the couple would modify its behavior according to the signals emitted by its partner. In fact, this type of male-female interaction is expected to occur during mating when individuals are in direct physical contact, allowing them to detect subtle movements and/or behavioral and physiological changes of the partner, and quickly respond in order to obtain greater mating success (Rodríguez 2015). Reports about male-female dialogues during mating in arthropods are still scarce (for a review see Rodriguez 2015), and our study is probably the first in harvestmen.

Contrary to gonyleptid species with maternal or paternal care in which male mate guarding and oviposition occur immediately after copulation (Machado & Macías Ordóñez 2007), females of *P. thorellii* move away from males after copulation. In species of Mitobatinae (Gonyleptidae), in which there is also no parental care, there is no mate guarding and females also move away from males after copulation (Machado & Macías-Ordóñez 2007; Zatz et al. 2011; G. Machado pers. obs.). It seems, therefore, that parental care is associated with greater investment by males in securing fertilizations. In species without parental care, eggs are laid individually and the benefits of guarding a female should be lower. After mating, we observed females cleaning their operculum. We modified the description of the behavior "operculum cleaning" reported by Stanley et al. (2016), as we observed that the distal part of the ovipositor contacts the female mouth while she uses her pedipalps to grasp its proximal part (see Table 1). Even though it was not reported in all trials, the observations show that the "cleaning" behavior is frequent in the species. However, the function of this "cleaning" behavior is unclear yet, and it could be another stage of male evaluation. As an example, females could be dumping sperm similarly as has been reported for the pholcid spider *Physocyclus globosus* (Taczanowski, 1874) (Peretti & Eberhard 2009; Calbacho-Rosa & Peretti 2015). Therefore, future studies should focus on evaluating if the female is removing sperm from the ovipositor, and if this behavior has a significant effect on male reproductive success.

In this study, we observed that mating in *P. thorellii* is more complex than previously described, showing characteristics that suggest a dialogue between sexes from the beginning until the end of the sexual interaction. Moreover, we described female pre-copulatory behaviors that could be related to (i) sexual receptivity and cooperation with mating occurrence, and also (ii) regulation of male copulatory courtship. Finally, *P. thorelli* appears as an interesting model to test hypotheses related to copulatory dialogue and its implications for male and female reproductive success in harvestmen.

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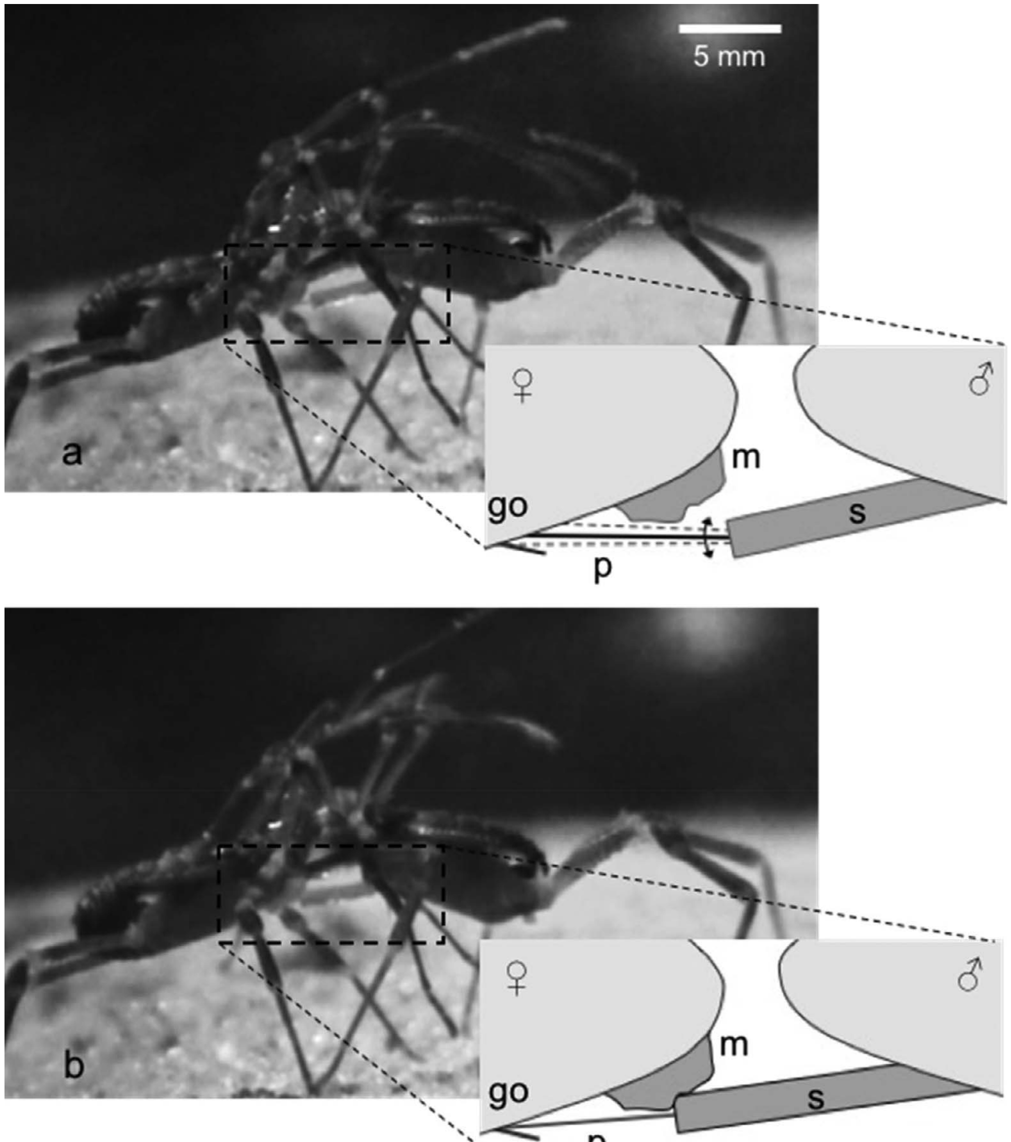

Figure 7.—Detailed observation of *Pachyloides thorellii* ovipositor cleaning behavior. (a) Female with open genital operculum. (b) Fully everted ovipositor. Black arrow indicates the contact between distal extreme of the ovipositor and mouthparts. (c) Ovipositor scraping. Female scrapes the ovipositor with her pedipalp claws from the base to the tip and then takes the claws to her mouth. pp: pedipalps; go: genital operculum.

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