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Mating behavior of the solitary neotropical harvestman *Pachyloides thorellii* (Arachnida: Opiliones)

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Abstract. In order to study how sexual selection takes place during mating, it is necessary to have a clear knowledge of the interactions that occur throughout mating and which morphological and behavioral traits are involved. Available information about harvestman reproductive biology is mainly restricted to anecdotal field observations, most of them lacking a detailed description and quantification of mating behavior. In this paper, we study the reproductive behavior of the gonyleptid *Pachyloides thorellii* Holmberg, 1878 (Pachylinae) and provide quantitative and descriptive information about its sexual behavior. We observed 15 matings, measured females and males, and analysed our behavioral data in the context of individuals' sizes. We observed conspicuous pre-copulatory, copulatory and post-copulatory courtship. We also found that females have several strategies to reject males' mating attempts. Like most gonyleptids, males and females of *P. thorellii* mate in face-to-face position; however, we observed that both male and female clasp their chelicerae mutually. This behavior has not previously been reported for the suborder Laniatores. The information obtained through this study establishes the basis for further studies on this species' reproductive biology and supports the suitability of this species as a model to explore the importance of male copulatory courtship for female choice and sperm use.

Keywords: Sexual behavior, pre-copulatory courtship, chelicerae clasp, copulatory courtship

Opiliones is the third most diverse order within arachnids and it is divided into four suborders: Cyphophthalmi, Eupnoi, Dyspnoi, and Laniatores (Pinto-da-Rocha & Giribet 2007). In general, harvestmen are omnivorous, nocturnal creatures, showing high morphological and behavioral diversity (Savory 1938; Coddington et al. 1990; Adis & Harvey 2000). In Cyphophthalmi, reproduction can be achieved asexually through parthenogenesis or sexually through a spermatophore transference (Tsurusaki 1986; Machado & Macías-Ordóñez 2007). However, the most widespread sperm transfer mechanism in the order is direct copulation. Males possess an eversible penis that is introduced into the females' operculum to achieve sperm transfer (Machado & Macías-Ordóñez 2007).

Although studies regarding harvestman reproductive behavior have significantly increased in the last decade, there are still many gaps in our knowledge (Machado et al. 2015). Most studies on harvestman sexual behavior are field studies on Neotropical species of the suborder Laniatores, particularly of Gonyleptidae, with some kind of parental care. Sexual interactions in harvestmen mainly follow the scheme presented by Machado et al. (2015) where the mating process is divided into three stages: Pre-copulatory, Copulatory and Post-copulatory. In each stage, different sources of selection may shape both the morphology and the behaviors observed in different species (Fowler-Finn et al. 2014). Therefore, having a detailed description of the behaviors observed during these stages is the first step towards understanding sexual selection in each species.

During the Pre-copulatory stage, individuals generally evaluate their partner through courtship and decide whether to continue with further mating (Andersson 1994; Machado et al. 2015). In harvestmen, this stage is brief and courtship involves touching their partner's body (by males, females or both) using legs I and II. There is a small number of species for which courtship has been described; in *Chavesincola inexpectabilis* Soares & Soares, 1946 and *Pseudopucrolia* sp. (Gonyleptidae), the male taps the female's genital opening with legs II and gently touches her dorsum with legs I (Nazareth & Machado 2009, 2010), while in *Zygopachylus albomarginis* Chamberlin, 1925 (Manaosbiidae) it is the female that initially taps the male carapace and legs, and if the male returns the taps then copulation takes place (Mora 1990).

The Copulatory stage involves a closer evaluation of the partner, intromission, and sperm transfer. Stimulation through copulatory courtship is generally the most extended way in which such evaluation occurs (Eberhard 1996; Machado et al. 2015). Copulatory courtship is generally performed by males and consists of touching or grazing the legs and/or dorsum of the female. Males of *Discocyrtus pectinifemur* Mello-Leitão, 1937 and *C. inexpectabilis* (Gonyleptidae) tap females' bodies with legs II and I, respectively, during intromission. In other gonyleptid species such as *Acutisoma longipes* (Roewer, 1913), males intensively tap the dorsum and hind legs of females (Machado & Macías-Ordóñez 2007; Nazareth & Machado 2009).

Finally, mating is followed by a mate guarding period (Post-copulatory stage). In this stage, the male remains with the female and continues to court and/or stimulate her in order to reduce sperm competition and increase reproductive success (Simmons 2001; Machado et al. 2015). In some harvestman species, males remain with the female, touching her from time to time with legs I and II, until she lays one or more eggs. This period can range from a few minutes (*Z. albomarginis* (Mora 1990); *Iporongaia pustulosa* Mello-Leitão, 1935 (Requena & Machado 2014)) to several days (*A. longipes* (Machado & Olivera 1998); *C. inexpectabilis* (Nazareth & Machado 2009)).

The goal of the present study was to describe the mating behavior of the gonyleptid *Pachyloides thorellii* Holmberg, 1878. This is the first mating description for a solitary species lacking any kind of post-oviposition parental care. We first provide information about the behaviors that conform to the Pre-copulatory, Copulatory and Post-copulatory stages with a

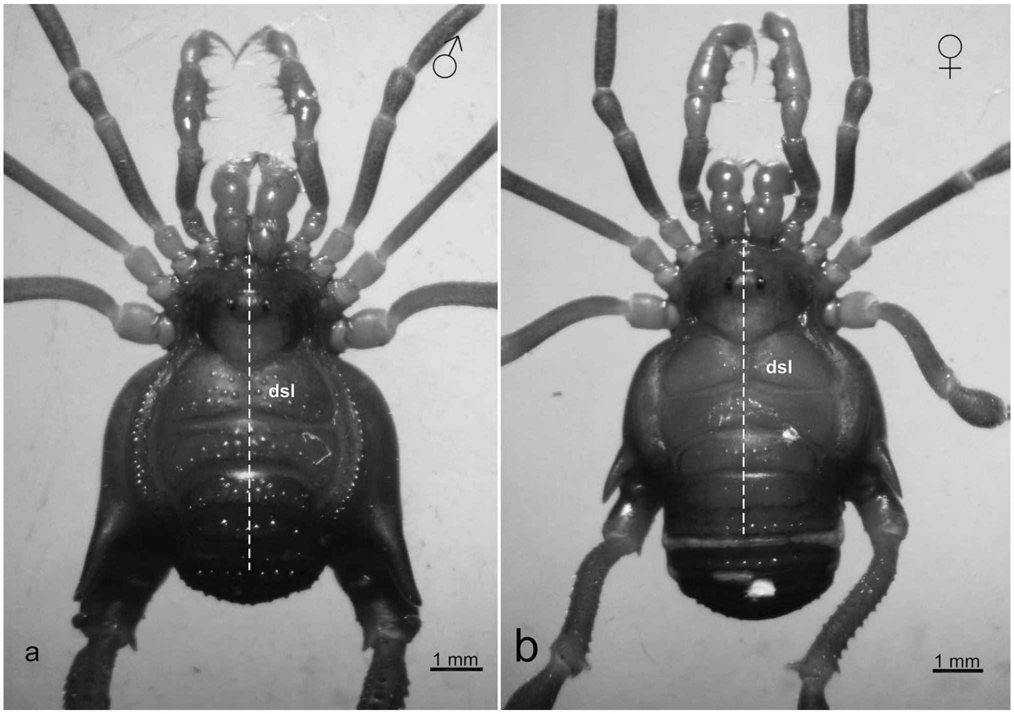

Figure 1.—Measure taken for males (a) and females (b) of *P. thorellii*. dsl: dorsal scute length.

flow diagram. Second, we examine relative sizes of the sexes in the context of the observed behaviors to evaluate whether body size affects mating duration. And finally we provide, for the first time, detailed photographs of the genitalia of this species.

METHODS

Study organisms.—*Pachyloides thorellii* inhabits cryptozoic environments in southern Uruguay, characterized by the presence of leaf litter and pieces of tree bark. Males and females have similar sizes and, contrary to what is observed in other species of the family, they do not seem to be sexually dimorphic (Pinto-da-Rocha & Giribet 2007; Giuliani 2008; Willemart et al. 2008). Females start ovipositing approximately a month after copulation and perform several ovipositions in which each egg is placed in isolation under a rock or inside a tree bark fissure (Stanley 2011).

Collection and maintenance in captivity.—We collected adult individuals of *P. thorellii* at Marindia (Canelones, Uruguay; 34°46′S, 55°49′W), during January 2008 and 2009. The individuals were taken to the Laboratorio de Etología, Ecología y Evolución (I.I.B.C.E., Montevideo, Uruguay) and held individually in Petri dishes of 9 cm diameter and 1.5 cm height, with sand as substrate and wet cotton wool as a water supply. They were fed *ad libitum* once a week with apple and cucumber pieces, cat food, and pieces of *Tenebrio molitor* (Coleoptera) larvae. We maintained individuals under natural photoperiod. The average temperature during breeding was 26.3 °C (± 1.8 SD, range = 17.5–37).

Behavioral observations.—The experiments were performed in March 2008 and 2009, with a room temperature of 24 °C (± 1.2 SD, range = 20–30). Females were placed in Petri dishes of 14.5 cm diameter and 2.5 cm height (encounter arena), with sand as substrate and wet cotton wool to maintain humidity, 24–48 h before the experiments, for acclimation and stress reduction. Males were placed inside the arena immediately before the beginning of each encounter. Males were carefully picked up with forceps by one of their legs IV, to prevent the release of chemical substances that could affect behavior. Then they were gently placed approximately at 10 cm from the female. Each trial lasted 30 minutes after the introduction of the male or until the end of mating. If mating was not observed, the same couple was tested again 24 h later.

All the observations took place under red light (placed 50 cm from the arena). We recorded each encounter with a Sony Handycam video camera (DCR-SR40 Nightshot; Sony Corp., Tokyo, Japan) and took notes of all interactions. We analyzed the video recordings with JWatcher computer program (Version 0.9, Blumstein et al. 2000), to determine the frequency and duration of each behavior. We used the frequency of transition from one behavior to the other and expressed them in percentages to construct the flow diagram.

Morphological features.—After the trials, individuals were fixed and preserved in ethanol 95%. Both males and females were photographed with a digital camera (Nikon Coolpix 5100) mounted on a stereoscopic microscope (Nikon SMZ-10; Nikon Corp., Tokyo, Japan). We took three separate pictures per individual and using ImageTool software (Version 3.0; Wilcox et al. 1995) software, we measured the length of dorsal scute (Fig. 1). We analyzed the average of the measures taken from the three pictures. Following Willemart et al. (2008), we used dorsal scute length as a size reference to calculate an index of size difference for each couple (dorsal scute length of male divided by dorsal scute length of female). This index was related to mating duration in a linear regression, transforming

Table 1.—Description of *P. thorellii* mating behavior units observed. Rejection units were only performed by females.

Behavior	Category	Description
Touch with leg II	Pre-copulatory	Mutual touches with the tarsus of the second pair of legs. The individuals stand still on the substrate touching dorsum, sides and/or first three pair of legs of the partner.
Touch with leg I and II	Pre-copulatory	Male intensively taps female's dorsum with the tarsus of the first pair of legs, while Touches with leg II continues.
Rush	Pre-copulatory	Male extends its pedipalps and quickly approaches the female.
Male over female	Copulatory	Male climbs over female's dorsum and slides over it while he extends its pedipalps and grazes female's dorsum. Touch with leg I and II continues and this behavior ends when the male locates himself in front of her in a face-to-face position.
Grabbing	Copulatory	Once in face-to-face position the male uses the claw of his pedipalps to grab the coxae of the female's first pair of legs. Both grab each other's chelicerae (Fig. 5). Male continues Touch with leg I and II.
Elevation	Copulatory	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's body forming a 90° angle between them.
Copulatory courtship	Copulatory	Male puts the tarsus of both legs I in the dorsum of the female and slides them towards the sides of her body. He maintains his second pair of legs in the air alternating between right and left to touch the female on the sides and dorsum.
Pulls	Copulatory	Female pulls backwards from the male using her third and fourth pair of legs as support.
Leg II movements	Copulatory	Female moves the second pair of legs slowly.
Lowers body	Copulatory	Female bends her legs lowering her body.
Separation	Post-copulatory	Male retracts penis and releases female's pedipalp and chelicerae as the female releases male's chelicerae.
Operculum Cleaning	Post-copulatory	Female scraps the operculum with the claws of her pedipalps and takes them to her mouth. She repeats this several times.
Leg Cleaning	Post-copulatory	Individuals slide their legs through their chelicerae.
End	Post-copulatory	One or both individuals move far away from the other.
		Rejection units
Run away	Pre-copulatory	Female quickly moves away from the male when he touches her.
Bending	Pre-copulatory	Female retracts legs I, II and pedipalps towards her body while elevating the abdomen and lowering the cephalothorax to the substrate.
Kicking	Pre-copulatory	The female rapidly extends leg IV when male approaches.

each variable into logarithmic values. Voucher specimens were deposited in the Colección Entomológica de la Facultad de Ciencias, Montevideo, Uruguay.

Scanning electron microscope (Jeol JSM 5900LV) images were used to visualize the structures present on the penis and the ovipositor of *P. thorellii* individuals. Samples were critical point dried and sputter coated with gold, and scanned at the Servicio de Microscopía Electrónica de Barrido y Microanálisis, Facultad de Ciencias, Montevideo, Uruguay.

Statistical analysis.—All statistical analysis was performed using PAST (Version 1.18, Hammer et al. 2003).We selected $P = 0.05$ as the limit for statistical significance. We tested the behavioral and morphological data for normality and homogeneity of variances using a Shapiro-Wilk test and Levene test, respectively. If variables showed normality and homogeneity of variances, we used the parametric Student's *t*-test; if any of these conditions was not met we used the non-parametric Mann-Whitney *U*-test. We compared dorsal scute length between males and females to determine whether there was any size difference. Then we performed a multiple regression test using size differences within couples as the independent variable and the duration of the different stages defined during mating as the dependent variable. Finally, we performed a multiple logistic regression using presence and absence of rejection as the categorical variable and mating duration and size differences within couples as continuous variables.

RESULTS

Sexual behavior.—The average duration of the analyzed mating sequences was 690 seconds (± 198 SD, range = 486–1182 s, $n = 15$). We defined 14 behaviors (see Table 1 for description) and displayed the transitions from one behavior to the other in a flow diagram (Fig. 2). The mating process was divided into the three stages proposed by Machado et al. (2015): Pre-copulatory, Copulatory and Post-copulatory.

Pre-copulatory behavior.—Interactions between male and female began when one or both individuals waved their second pair of legs simultaneously or alternately while they remained still or walked around the arena. Contact was initiated by the male in 87% ($n = 13$) of the cases, by directing his second pair of legs and walking towards the female. In the remaining cases (13%, $n = 2$), females initiated contact in a similar way as the majority of males had. When they were close to each other, both male and female touched each other's dorsum and legs with the tarsi of their first and second pair of legs. The Pre-copulatory Behavior stage showed a mean duration of 18 s (± 12 SD, range = 2–48 s). This stage began with the behavior *Touch with leg I and II* and ended with the behavior *Grabbing*. The male touched the female with leg I and rapidly climbed over her dorsum (*Rush*). Once over the female, the male touched the female dorsum both with legs I and pedipalps and the touches with leg II accelerated. He immediately slid over the female until reaching a face-to-face position (*Male over

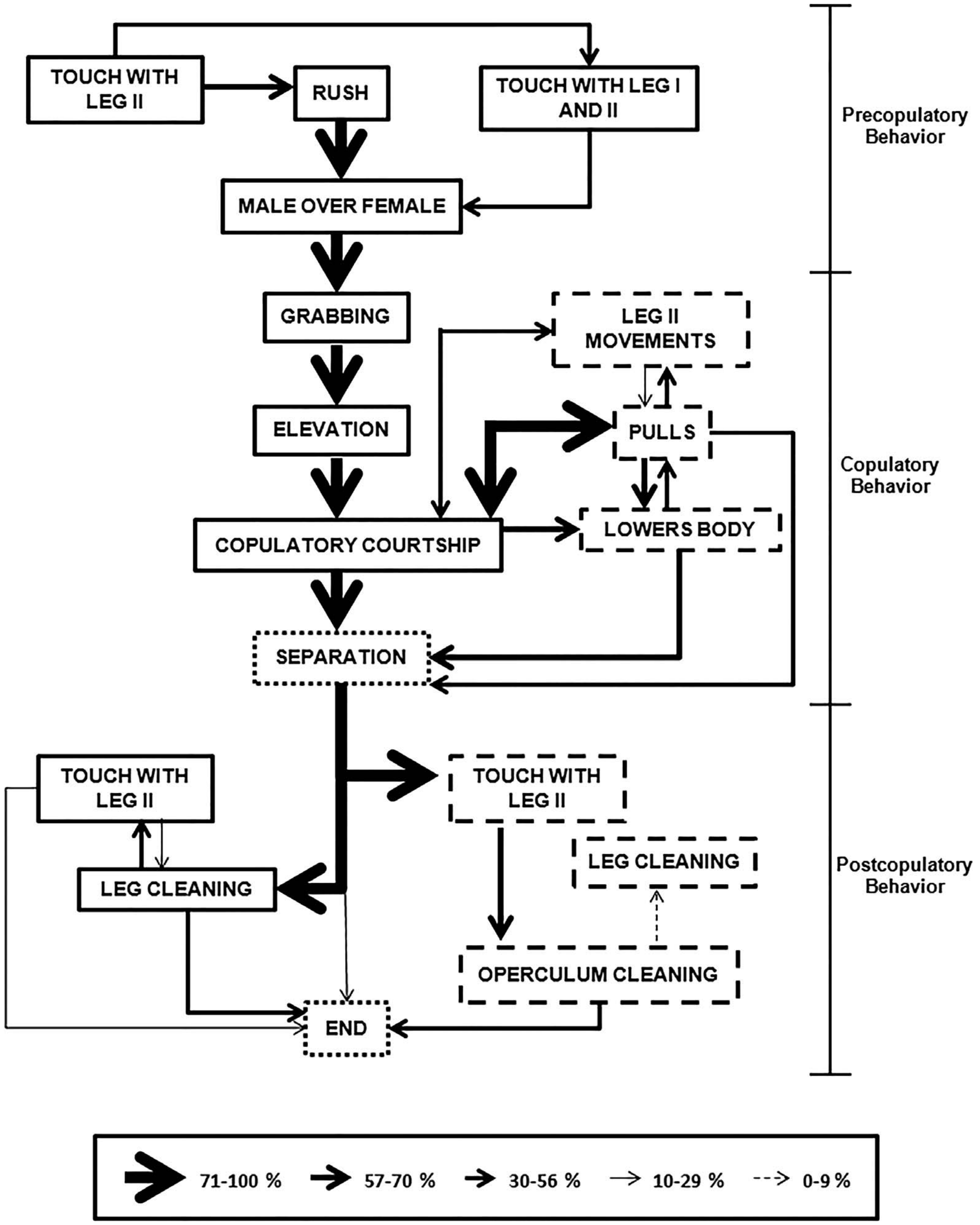

Figure 2.—Flow diagram of *P. thorellii*'s mating. Squares with continuous lines contain behavioral units that were performed only by males. Squares with dashed lines contain behavioral units that were performed only by females, and squares with dotted lines represent behavioral units that were performed by both individuals. Arrow thickness represents different frequencies of transition between units and their value is expressed in percentages.

female). During this behavior, the female was able to reject or offer certain resistance to the male's grabbing attempts. Finally, once in front of the female, the male grabbed the base of her first pair of legs with the claw of his pedipalp and then they both grabbed each other's chelicerae (*Grabbing*) (Fig. 3).

Thirty-three percent ($n = 5$) of the females accepted male courtship and mated without resistance; of the remaining 67% ($n = 10$), 60% ($n = 6$) resisted male attempts to mate but accepted later in the same trial, and 40% ($n = 4$) rejected males but accepted them 24 h later without resistance. The behaviors observed during female resistance and rejections were *Run away*, *Bending* and *Kicking* (see definitions in Table 1). Neither size difference nor mating duration were correlated with the presence and absence of rejection (logistic regression: Size difference: $\chi^2 = 2.1$, $P = 0.15$; Mating duration: $\chi^2 = 0.67$, $P = 0.46$).

Copulatory behavior.—The Copulatory Behavior stage had a mean duration of 654 s (± 152 SD, range = 403–954 s), and started with the behavior *Elevation*. After grabbing the female, the male elevated the front part of his body together with the female, reaching copulatory position (*Elevation;* see Table 1

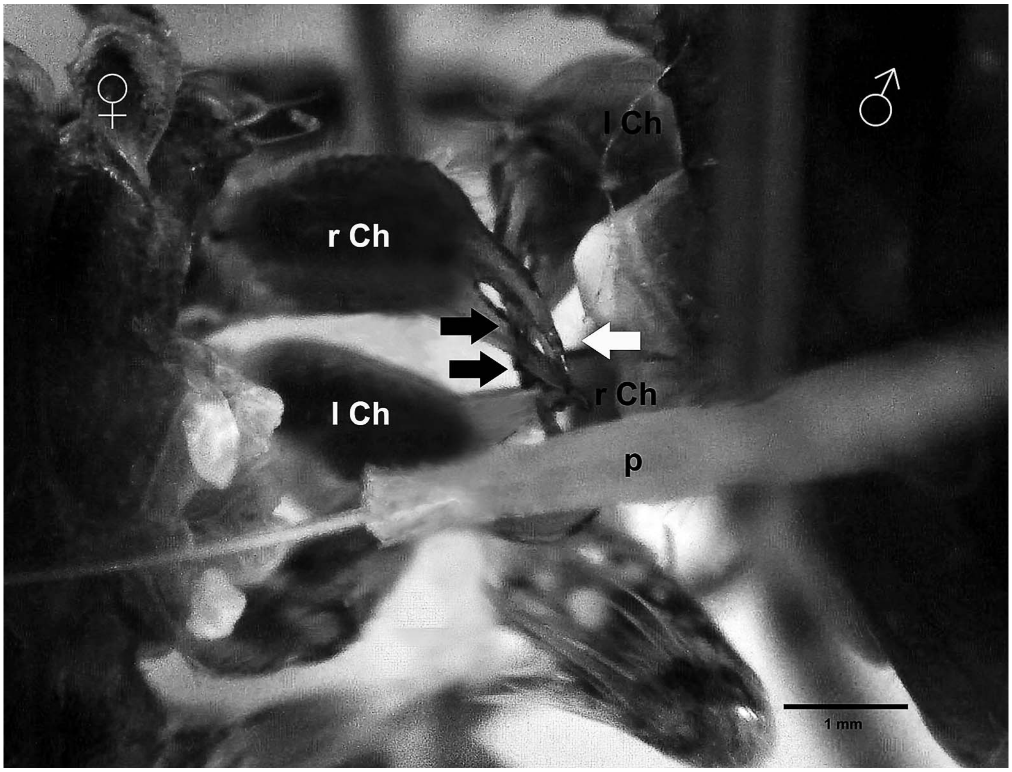

Figure 3.—Ventral detail of mutual cheliceral grabbing during copulation in *P. thorellii*. Black arrows show the sites where male chelicerae grabbed female chelicerae and the white arrow shows female site of grabbing. r Ch: Right chelicerae; l Ch: Left chelicerae; p: penis.

for further detail). Simultaneously, the male raised his third pair of legs until he got them on top of the female's second pair of legs. In this position, while performing *Copulatory courtship*, the male inserted his penis into the female's gonopore and did not withdraw it until the mating ended. At this point, we observed a decrease in the intensity of the male touches with legs I, which then remained constant until the couple separated. Females remained almost immobile during most of this stage, except at the beginning and near the end of the stage when they tried to pull away from the males' grasp. Males maintained their grasp and continued the copulatory courtship. Before *Separation*, females slowly started moving legs II (*Leg II movements*) and lowered their bodies by flexing their fourth pair of legs, which obliged males to withdraw the penis and release the female chelicerae and legs ($n = 11$). Males sometimes finished mating by freeing the female in absence of any of the mentioned female displays ($n = 4$).

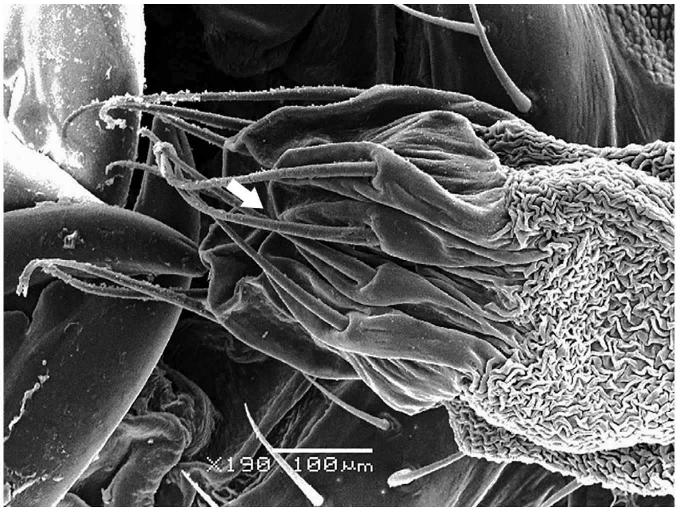

Figure 4.—SEM image of female ovipositor of *P. thorellii*. White arrow shows ovipositor opening.

Post-copulatory behavior.—The Post-copulatory Behavior stage started immediately after the couple separated and had a mean duration of 63 s (± 75 SD, range = 12–258 s). During that stage, both male and female stayed close to each other (approximately 1 cm away), touching each other's dorsum and legs with legs II. At the same time, each of them performed *Leg cleaning,* and during this stage all females were observed carrying out *Operculum cleaning*.

We did not observe a statistically significant relationship between the size ratio of the members of each couple and mating duration or duration of any of the stages (multiple regression: $r = 0.63$, $P = 0.23$).

Genital apparatus.—The female's ovipositor has four lobes, each carrying three long setae that point towards the center of the ovipositor, covering the entrance (Fig. 4). The male's penis has several ornamentations on its *pars distalis* (Fig. 5). We observed on both sides, two groups of three sensilla, one at the distal end and the other on the base (Figure 5a). Between those groups of sensilla, there is a spiny area that covers the edge of the distal part of the penis (Figure 5b). In the *glans*, we observe both the *ventral process* and the *stylus* (Figure 5). In a closer

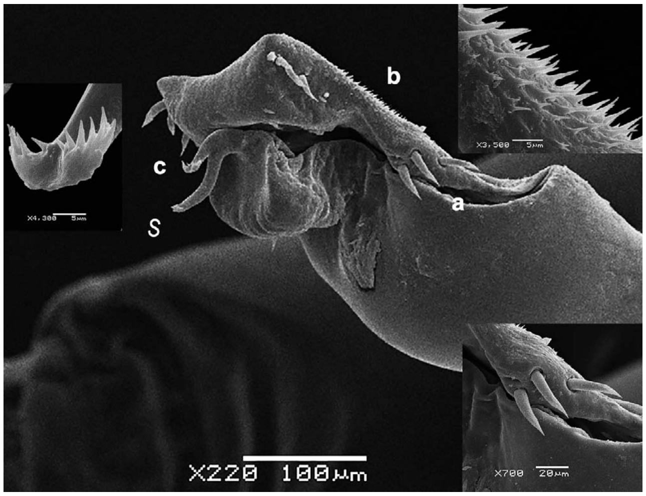

Figure 5.—SEM image of male penis of *P. thorellii*. a, b: detail of sensilla and spines in the *pars distalis*; c: ventral process; *S*: Stylus.

look, the ventral process shows several processes on its sides (Figure 5c).

DISCUSSION

We found that *P. thorellii* mating behavior is one of the longest found so far in the suborder Laniatores, clearly showing the three stages—Pre-copulatory, Copulatory and Post-copulatory—proposed by Machado et al. (2015). Males touched females with legs I and II from the beginning to the end of the interactions and females were able to resist and/or reject males. We observed that females cooperate with the male during copulation, through cheliceral holding and are able to end mating by lowering their bodies, forcing males to withdraw the penis and release their chelicerae. We found no relationship between the size ratio of each couple and either the probability of rejection or the duration of any stage or the whole mating.

As observed in other harvestman species, individuals of *P. thorellii* seem to identify conspecifics and differentiate males from females after touching them (Willemart et al. 2006; Fowler-Finn et al. 2014). Normally, individuals use their second pair of legs to orient themselves to nearby objects; these legs are not used for locomotion and they are constantly moving in a similar way to insect antennae (Machado et al. 2007). As it was observed, prior to contact, individuals direct their second pair of legs towards their conspecific, approach them, and finally contact takes place. Pre-copulatory courtship in *P. thorellii* is similar to that reported for other Laniatores (see Table 12.1 in Machado & Macías-Ordoñez 2007). Particularly in Gonyleptidae, courtship is short and initiates when one individual touches the other. Once the male detects the female, he tries to grab her and mate. However, females can accept mating or resist it. Resistance behaviors in *P. thorellii* resemble those observed in other members of the family (Gnaspini 1995; Elpino-Campos et al. 2001; Machado & Macías-Ordoñez 2007; Willemart et al. 2008; Nazareth & Machado 2009, 2010; Requena & Machado 2014). There was no relationship between rejected males and size ratio within couple members, and rejected males reinitiated courtship several times by touching the female with legs I and II; this behavior may have the function of increasing the probability of female mating acceptance as suggested by Willemart et al. (2006). These facts together with the pre-copulatory behaviors reported by Machado & Macías-Ordóñez (2007) in other species, suggest that Laniatores may rely more on courtship than on coercive behaviors as observed in Eupnoi (Machado & Macías-Ordoñez 2007). The time individuals remain in pre-copulatory courtship represents a window for evaluation of the potential partner and the length of this stage may be correlated with the capacity of the female to control sperm afterwards. It would be necessary to compare courtship duration and the frequency and duration of the behaviors observed during courtship with the number of offspring obtained from virgin females to assess the function of such behaviors.

As mentioned before, the copulatory behavior in *P. thorellii* is one of the longest found so far for the suborder (Matthiesen 1983; Gnaspini 1995; Machado & Oliveira 1998; Elpino-Campos et al. 2001; Machado & Macías-Ordóñez 2007; Nazareth & Machado 2009, 2010; Buzatto et al. 2011) and within species of other suborders (Eupnoi: Macías-Ordóñez 1997, 2000; Willemart et al. 2006; Dyspnoi: Pabst 1953; Martens 1969), and was characterized by tactile courtship (touches with legs I and II) like many other harvestmen (Machado & Macías-Ordóñez 2007). Copulatory position (face-to-face and forming a 90°angle) is similar to what is observed in other harvestmen; the mutual chelicerae holding has not been reported for the suborder Laniatores. Until now it has only been mentioned that females of *Zygopachylus albomarginis* extend their chelicerae and pedipalps and grab males by their cephalothorax to bring them closer, but there has been no mention of male cheliceral holding (Mora 1990). Male cheliceral holding was reported for two species of *Trogulus* Latreille, 1802 (Dyspnoi), in which a male grabs a female's body with legs I and II and her chelicerae with his chelicerae (Pabst 1953). In females, cheliceral holding was observed in a few species of the genus *Ischyropsalis* C.L. Koch, 1839 (Dyspnoi) (see Table 12.1 in Machado & Macías-Ordóñez 2007). Females grab the base of male's chelicerae with her chelicerae, bringing them close to her mouth and maintaining that position until mating ends (Martens 1969). The fact that females actively participate in holding and maintaining mating position suggests they have a greater control of mating duration. In fact, it was observed in these species and both *P. thorellii* and other gonyleptids that females are able to end mating (Pabst 1953; Martens 1969; Nazareth & Machado 2009). *P. thorellii* females lower their bodies, forcing males to withdraw the penis and release the chelicerae. The fact that the male and female hold each other's chelicerae could enable a more firm and stable position during mating and such stability could explain the longer matings observed. Males could use part of the mating time for several purposes: to remove sperm from previous matings (Thomas & Zeh 1984; Eberhard 1996; Birkhead & Møller 1998), to transfer accessory substances (nutritious or inhibitory of future matings) (Parker 1970; Simmons 2001) and/or to stimulate females for longer periods (Eberhard 1998). We found that the penis in *P. thorellii* has spines, sensilla and other projections, such as the ventral process, that could promote penetration of the penis, remove sperm, and/or stimulate the ovipositor during mating (Macías-Ordóñez et al. 2010). However, the function of these ornaments in this and other harvestman species is still unknown.

After mating, males remain near the female touching her with legs I and II; this fact could indicate the presence of Post-copulatory courtship. A similar behavior was observed in other Gonyleptidae species: in *Chavesincola inexpectabilis*, females oviposit immediately after mating (Nazareth & Machado 2009) and in *Goniosoma spelaeum* (Mello-Leitão, 1933) and *A. longipes*, males stay close and approach to reinseminate the female (Gnaspini 1995; Machado & Olivera 1998). In *P. thorellii*, females oviposit between 30 and 40 days after mating (Stanley & Toscano-Gadea, unpublished data). Males could stay with them for long periods during which they could reinseminate them and protect the female from other males. Even though in this study we separated the couple after they moved away from each other, Stanley (2012) observed that the same couple was able to mate up to six times with a separation of 24–48 h between matings. She also observed that males may fight with one another immediately after matings. These facts could imply that both post-copulatory courtship and mate guarding could be occurring in *P. thorellii*.

Females perform *Operculum cleaning* during most of the Post-copulatory stage. Due to the fact that this behavior is observed immediately after mating it is possible that females are removing and eating sperm (Pinto-da-Rocha & Giribet 2007; Macías-Ordóñez et al. 2010). Females of the fly *Prochyliza xanthosoma* prefer males that transfer great amount of sperm, because after mating they expel part of the ejaculate and feed from it (Bonduriansky & Rowe 2003; Bonduriansky et al. 2005). If females of *P. thorellii* are in fact expelling sperm, this would be one more feature in favor of female control over sperm in this species. Future studies should identify and quantify the substance that the female takes to her mouth and determine if the observed behavior is related with sperm dumping or with other substances with nutritional value being transferred to females as nuptial gifts (Eberhard 1998; Arqnvist & Nilsson 2000; Bonduriansky & Rowe 2003; Elgar et al. 2003; Bonduriansky et al. 2005, Peretti & Eberhard 2009).

P. thorellii seems to be a promising model in which to study the mechanisms responsible for sexual selection. This work provides the framework required for future sexual behavior research in the species. Studies involving courtship influence on mating duration and sperm use in female reproductive tract, as well as the causes promoting male fights, are already taking place.

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