



## Do sexually dimorphic glands in the harvestman *Gryne perlata* (Arachnida: Opiliones) release contact pheromones during mating?

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**Abstract.** There are records of glands that produce sexual pheromones that are released into the environment or applied directly on sexual partners. Within Opiliones (Arachnida), several harvestmen in the suborder Laniatores have sexually dimorphic glands on legs I and IV, the mode of use of which is recorded only in two species but their function is unknown: while walking, males rub the glands against the substrate or against their body. Here we test an alternative and non-exclusive hypothesis that the glands present on the legs of male *Gryne perlata* (Cosmetidae) produce contact pheromones used in mating. We predicted that males would touch the females with the gland openings or with other male body parts previously rubbed by these glands. We also predicted that there are chemoreceptors on those parts of the females where males touch them. We analyzed 13 videos of *G. perlata* mating, a species in which the males have glands on legs I and IV of unknown function. We also analyzed 14 videos of *Discocyrtus pectinifemur* (Gonyleptidae) mating as a control, a species that lacks these glands. Finally, we looked for chemoreceptors on the legs of female *G. perlata* using a scanning electron microscope. During copulation, males of both species rubbed the legs of females with their first pair of legs, but not with the regions of these legs where the openings of the glands are. The fourth pair of legs were only used to support the body. Rubbing other body parts of the female by males with their glands was not observed during mating. Setae on the legs of the female did not have tip pores and therefore do not seem to be chemoreceptors. We therefore did not find any evidence that these sexually dimorphic glands in *G. perlata* release contact pheromones during mating.

### INTRODUCTION

Pheromones are chemical signals that can elicit responses in another individual of the same species (Symonds & Elgar, 2008). They can be used for long or close distance communication, or depend on contact to transfer information (Wyatt, 2003; Costa-Leonardo et al., 2009). Pheromones may be used in a variety of contexts including territory demarcation, foraging or reproduction among others (Agosta, 1992; Wyatt, 2003; Waldman & Bishop, 2004). Sexual pheromones are produced by glands that are usually sexually dimorphic in occurring in either sex. These glands occur in both vertebrates (Rasmussen & Krishnamurthy, 2000; Houck et al., 2007; Gonçalves & Brito-Gitirana, 2008) and invertebrates (Spiegel et al., 2002; Cuatianquiz & Cordero, 2006; Salerno et al., 2012).

Despite the great number of behavioural studies on sexual pheromones in arachnids, almost all of them were carried out on spiders (see references in Gaskett, 2007; Schulz, 2013) and a few on scorpions (Miller & Formanowicz, 2010; Taylor et al., 2012). Sexual pheromones in arachnids are typically released into the air (Uhl & Elias, 2011) or deposited on the ground (Steinmetz et al., 2004) and carry information about sex, age and virginity among others (Gaskett, 2007). Some male scorpions have a gland on their metasoma (“tail”) that they rub against the ventral region of the female. Females detect the secretions with chemoreceptors on the pectens, a sexually dimorphic pair of ventral appendages. If the glandular openings are covered with paraffin, male success is lower (Peretti, 1997; Olivero et al., 2015). Male secretions are not used for rec-

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ognition since the male has already been recognized and it is not a nuptial gift since there is no consumption of the secretions.

Harvestmen, arachnids belonging to the order Opiliones, include 6565 species (Kury, 2014) divided in four suborders: Cyphophthalmi, Dyspnoi, Eupnoi and Laniatores (Giribet & Kury, 2007). Sexual recognition seems to be mediated by contact with any part of their body (Willemart et al., 2009a) and possibly mediated by cuticular hydrocarbons, but this has never been tested. Some males of Cyphophthalmi have sexually dimorphic glands of unknown function on legs IV (Willemart & Giribet, 2010) and their opisthosoma (Sharma & Giribet, 2005). Some males in Eupnoi have glands on their genitalia (Macías-Ordóñez et al., 2010) and males in some Dyspnoi have glands on their chelicerae (Martens, 1969, 1973). In the latter two cases, females feed on secretions offered by males before mating. In the largest suborder Laniatores, males of several species have sexually dimorphic glands on legs I, III and IV, which typically consist of a swollen area on the leg where the glandular openings are located (Willemart et al., 2007; Willemart et al., 2010; Proud & Felgenhauer, 2011, 2013; Fernandes & Willemart, 2014). Although the mode of use of these glands has been studied (Willemart et al., 2007, 2009a, 2010: glandular regions are rubbed against the substrate), how these glands are used in the suborder have only recently been reported: males rub the glands either on the substrate or on their own body, probably spreading secretions (Fernandes & Willemart, 2014; Murayama & Willemart, 2015). Except for the case of secretions from the cheliceral glands on which females feed (Martens, 1969, 1973), there is no information on the function of these glands or why the males mark the substrate. The chemicals produced by these glands have not been identified.

A hypothesis that has not been tested for Laniatores is that the secretions of these sexually dimorphic glands are used as contact pheromones during mating. This would be similar to what has been described for scorpions, in which males bring sexually dimorphic glands into contact with chemoreceptors of the females during courtship (Peretti, 1997; Olivero et al., 2015). The prediction would be that the region with the glandular openings in the male would touch the body of the female harvestman. Alternatively, the male would transfer the secretions to another part of its own body that would then transfer the chemicals to the female. Such contact could occur either before the male inserts its penis into the female's genitalia (pre-copulatory courtship), during (copulatory courtship) or after the male removes its penis (post-copulatory courtship). Courtship may not only increase the time spent copulating and therefore increase the number of sperm transferred but also increase the chances of fertilization of the ovulae by the sperm by cryptic female choice after copulation (Eberhard, 1996). Males of some species of harvestmen such as *Gryne perlata* Mello-Leitão, 1936 (Cosmetidae) have glands opening on the retrolateral region of the metatarsi of legs I and on dorsal, lateral and ventral regions of the metatarsi of legs IV (Fernandes & Willemart, 2014). Al-

though nothing is known about their function, glands on legs IV are gently touched or rubbed against the substrate when the male is walking or rubbed against its own legs. Glands on legs I are only rubbed against other legs (Fernandes & Willemart, 2014). Mating in *Gryne perlata* has not been described but males of Laniatores use legs I to tap their own penis or the female's body, and legs IV do not contact the female (e.g. Willemart et al., 2009a; Requena & Machado, 2013). We therefore describe the copulatory behaviour of *G. perlata* in order to test the hypothesis that contact pheromones are released from these glands during mating. We predicted that (1) specific regions of the glands would directly or indirectly (via transfer to another body part of the male) touch some body part of the female and that, if (1) was confirmed, (2) we would find chemoreceptors on the females where the male contacts her with his glands. We used another harvestman species (*Discocyrtus pectinifemur* – Gonyleptidae), which lacks swollen areas on its legs (Mello-Leitão, 1937), as a control, predicting that males in this species would not touch females with the region of its legs where the glands are in *G. perlata*.

## MATERIAL AND METHODS

### Collection and maintenance in the laboratory

We collected the individuals of both species manually during the day. We collected *Gryne perlata* in a savannah area in the city of Irajuba, State of Bahia, northeast of Brazil, in December 2011. We collected *Discocyrtus pectinifemur* in a fragment of forest in the city of Campinas, State of São Paulo, southeast of Brazil, in January 2012. We maintained the animals individually in the laboratory in plastic containers (*G. perlata*: diameter 20 cm × height 8 cm; *D. pectinifemur*: 12 cm × 4 cm × height 8 cm), with humid soil on the bottom and containing a wet cotton ball. We fed the animals once a week on moistened dog food and the temperature in the laboratory followed that of the environment.

### Recordings

We recorded the mating behaviour at night under fluorescent light, using a Sony Handycam DCR-TRV361. To increase the chances of observing copulation, we introduced two males and a female into an arena (diameter 20 cm × height 8 cm) containing humid soil and pieces of cork. We analyzed recordings of 13 matings of *G. perlata* and 14 of *D. pectinifemur*.

### Scanning electron microscopy

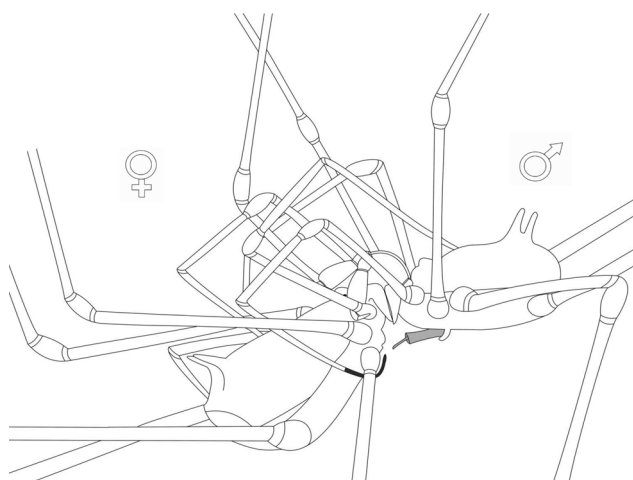
We micrographed the trochanter and femur of leg III of two males and two females of *G. perlata* preserved in alcohol 70%. These are the regions in the females that our study have shown to be touched by males during copulation. We cleaned the material with a brush, mounted it on an aluminum stub using double sided adhesive tape, dried it in an oven at 40°C for 24 h, sputter coated with gold (Sputter Coater Balzer SCD 50) and photographed with a Zeiss DSM 940 at the Bioscience Institute of the University of São Paulo.

## RESULTS

### Behaviour

#### *Gryne perlata*

We defined the beginning of mating as the moment when both male and female face each other and elevate the anterior regions of their bodies and the male uses its trochanter I and pedipalps to hold the female (Fig. 1). In this way,



**Fig. 1.** Mating posture of the harvestman *Gryne perlata* (Cosmetidae). Note the male's penis (grey) and the tarsi of its first pair of legs hooked round and rubbing the trochanters of the female's third pair of legs (black).

the male can insert its genitalia into the female's genitalia. The end of mating was defined as the moment when either the male or the female lowered the anterior region of their bodies. Pre and post mating are relative to these behaviours. The behavioural categories for *G. perlata* males are defined in Table 1, and the behavioural sequences are shown in Fig. 2.

#### Pre-mating

Males approach partner ( $n = 10$  – not all the videos show males approaching), solicit copulation and insert their penis into the female's genitalia (penis in) ( $n = 3$ ). Simultaneously with soliciting copulation, males display hook and rub trochanter III with tarsus I ( $n = 13$ ).

#### Mating and post mating

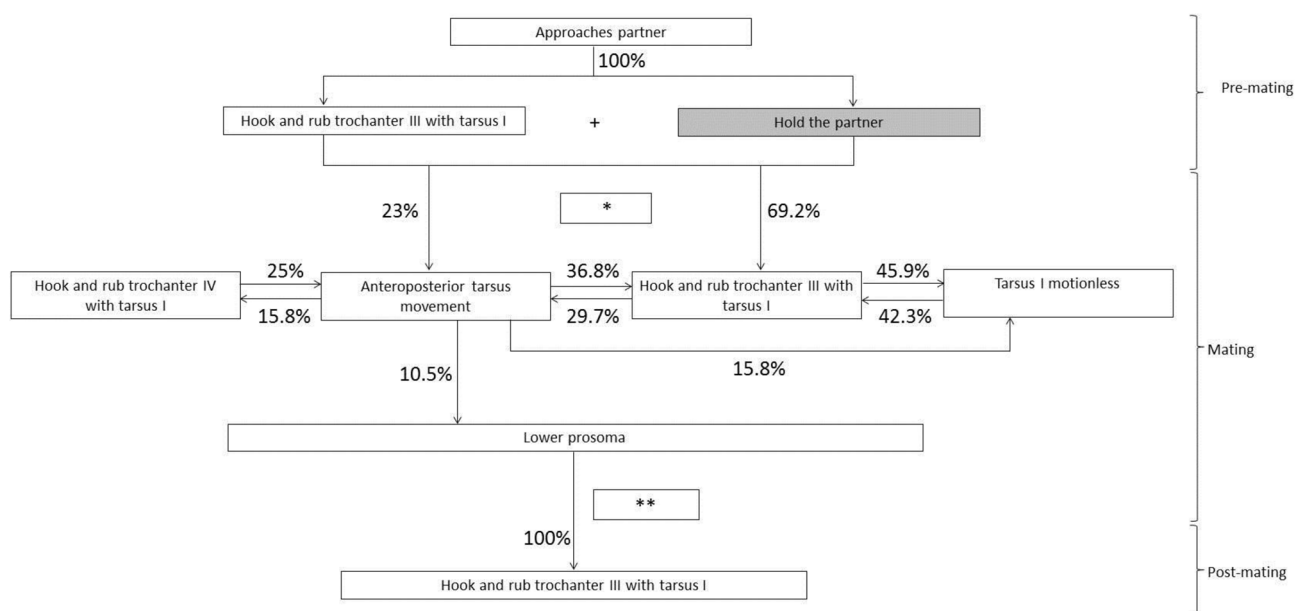
After starting copulation, males exhibited one or more than one of the following behaviours: hook and rub trochanter III with tarsus I ( $n = 12$ ); anteroposterior movement of tarsi I ( $n = 10$ ); hook and rub trochanter IV with tarsus I ( $n = 3$ ); tap female's dorsum ( $n = 3$ ) and tarsi I motionless ( $n = 10$ ). The behaviours that lasted longest during copulation were: hook and rub trochanter III with tarsus I for 45.3s (SD = 41.7s; min = 9s; max = 138s), which lasted for 47% of the time spent mating; and tarsi I motionless for 45.6s (SD = 30.2s; min = 18s; max = 109s), which lasted for 36.9% of the total time spent mating.

Females would sometimes initiate but not conclude the interruption of mating by lowering prosoma ( $n = 4$ ). In the four matings where attempts to interrupt mating were not completed, lowering prosoma was observed once in two matings, 4 times during the same mating and twice in another mating, always with a different pair of animals. Before the females displayed lower prosoma, males had their tarsi I motionless for 9.2s ( $n = 2$ ) or anteroposterior tarsus movement for 5.7s ( $n = 2$ ). After females attempted to end mating, males exhibited hook and rub trochanter III with tarsus I of the females. In four cases, females resumed mating by elevating the prosoma again.

Males interrupted mating 5 and females 8 times by lowering the prosoma, which was always followed by penis withdrawn ( $n = 4$ ). Females also pulled back, causing the males to release the grip of their pedipalps. Five seconds prior to females ceasing to mate, males exhibited the following behaviour: hook and rub trochanter III with tarsus I ( $n = 1$ ); tarsus I motionless ( $n = 5$ ) and anteroposterior movement of tarsi I ( $n = 2$ ). After the females lowered their prosoma, males exhibited: hook and rub trochanter III with

**Table 1.** Definitions of the behaviour of the male harvestman of *Gryne perlata* recorded during mating. The numbers in parentheses indicate whether the behaviour was recorded before (1) or during/after mating (2).

Behavioural category	Description
Anteroposterior movement of tarsi I (2)	Rapid anteroposterior movements of the tarsi of legs I of males after they are held at an angle of approximately 90° with the floor, less than 1 mm from the lateral region of the female's body and between her III and IV pairs of legs.
Approaches partner (1)	The male orients toward the female and walks directly towards her (following Mora, 1990).
Holds partner	In the face-to-face position, the male uses its pedipalps to hold the female by her trochanter.
Hooks and rubs trochanter III with tarsus I (1) (2)	Male hooks its tarsus I and rubs its ventral region around the retrolateral region of the trochanter III of the female in a circular motion. The hook is more U-shaped in <i>G. perlata</i> and more L-shaped in <i>D. pectinifemur</i> .
Hook and rub trochanter IV with tarsus I (2)	Male hooks its tarsus I and rubs its ventral region around the retrolateral region of trochanter IV of the female in a circular motion. Alternatively, leg 1 of the male is straight and the retrolateral region of tarsus I is rubbed against the trochanter or femur IV of the female.
Lower prosoma (2)	Male and/or female direct the opisthosoma upwards, lowering the prosoma. Females can also pull back (Requena & Machado, 2014).
Penis inserted (2)	Male inserts its genitalia into those of the female.
Penis withdrawn (2)	Male removes genitalia from female's genital opening.
Solicit copulation (1)	The male grasps the female's trochanter I by means of its pedipalps and raises the anterior portion of the female's body, so that their ventral surfaces come into close contact (Requena & Machado, 2013).
Tap female's dorsum (2)	Male taps female's dorsum with the ventral region of legs I.
Tarsus I motionless (2)	Male holds its leg so that the tarsus forms an angle of 90° with the floor.
Whipping movements with legs II	Male whips the female with legs II by very rapidly articulating the trochanter/femur joint.

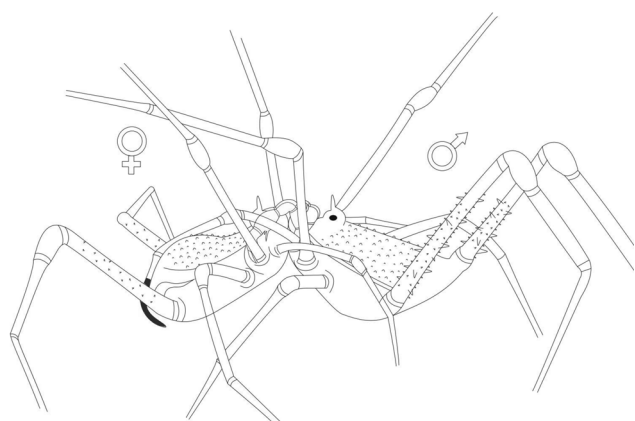


**Fig. 2.** Flow chart of the mating behaviour of males of the harvestman *Gryne perlata* (n = 13). Only behaviours having an incidence of more than 15% are shown except for the value 10.5%. “Hold the partner”, in grey, which lasts until the end of mating. The behaviors Hook and rub trochanter III with tarsus I, hold partner and Tap female’s dorsum occur consecutively in this order. The behavior Hook and rub trochanter III with tarsus I is shown three times for the sake of clarity. The penis was not always visible because of the angle of recording, but the other behaviors reliably allow us to infer when the male inserted and withdrew his penis. \* Penis inserted (based on observations when penis was visible, n = 3). \*\* Penis withdrawn (based on observations when penis was visible n = 5).

tarsus I (n = 4); tarsus I motionless (n = 2) and anteroposterior movement of tarsi I (n = 2).

Five seconds prior to males ceasing mating, they exhibited: hook and rub trochanter III with tarsus I (n = 2) and tarsus I motionless (n = 3). After lowering the prosoma, males exhibited: hook and rub trochanter III with tarsus I (n = 3) and kept tarsus I motionless (n = 2). In all cases when males ceased mating, females slowly moved their second pair of legs anteroposteriorly.

Two females mated more than once, with one female mating twice with the same male and the other mating with two different males. Two males interrupted mating by biting the females with their chelicerae, while vigorously tapping them with their first pair of legs and pedipalps. One of these females also bit the legs of the mating male. In both



**Fig. 3.** Mating posture of the harvestman *Discocyrtus pectinifemur* (Gonyleptidae). The male keeps its tarsus I (black) motionless on the female’s free tergites and anal operculum.

cases, the mating male ceased mating. In one of the cases, the intruder started copulating with the female.

Males did not touch or rub the sexually dimorphic glands on legs IV on any part of the females’ body, its own body or on the substrate during pre-mating, mating or post mating. The fourth pair of legs were only used to support the body.

**Mating in *Discocyrtus pectinifemur***

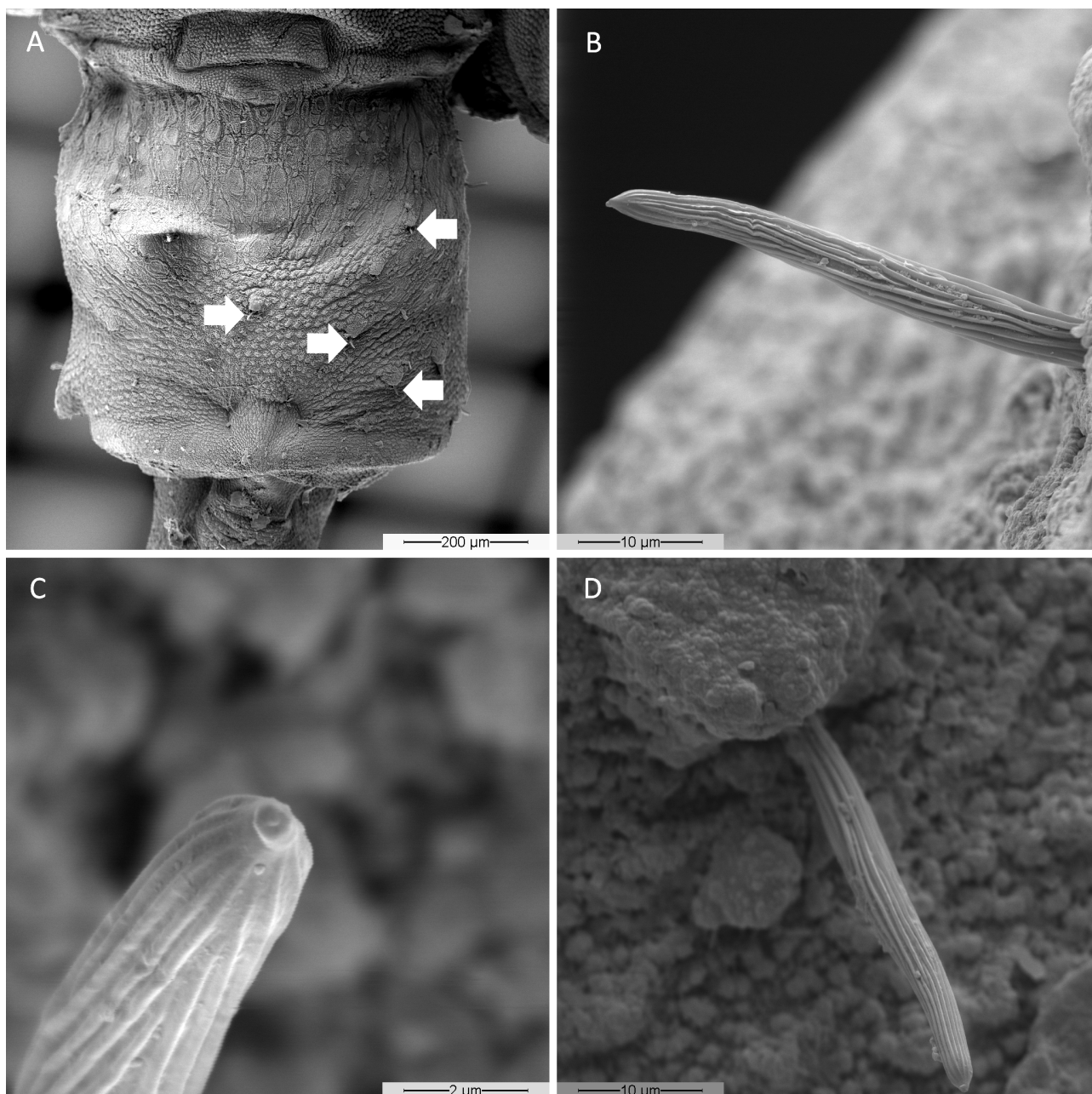
Because these videos were recorded for another purpose, they recorded only the mating described below (and not pre or post mating ). The mating posture is similar to that of *G. perlata*, but the males held the females with their pedipalps and by the trochanters of their second pair of legs. Males spent 38.7s (SD = 30.3s; max = 140s; min = 5s; n = 13) with their tarsi I motionless on the female’s free tergites and anal operculum and hook and rub femur III with tarsus I for 14.7s (SD = 10.2s; max = 34s; min = 1s; n = 7). The tarsi I in this species formed a more L-shaped hook than the more U-shaped hook in *G. perlata*. Hook and rub femur III with tarsus I was often intercalated with: touch female’s dorsum (n = 13); tarsus I motionless (n = 2), whipping movements with legs II (n = 4) and anteroposterior movement of tarsi I (n = 3).

**Scanning electron microscopy**

We did not find chemoreceptive sensilla on the trochanter and femur of the third pair of legs of males or females of *G. perlata*. However, we found sensilla chaetica without tip-pores and rough pit glands (Fig. 4) (see Willemart et al., 2007, 2009a), with the latter on the trochanter.

**DISCUSSION**

We did not find any evidence that males of *G. perlata* touch the female’s body with the sexually dimorphic



**Fig. 4.** A – trochanter of leg III of the females of the harvestman *Gryne perlata*, with white arrows indicating position of the setae depicted in B, C and D. Setae on the femur (B and C) and trochanter III (D) of leg III, lack wall pores or a tip pore.

glands on their legs I and IV during pre-mating, mating or after mating. The behaviour hook and rub trochanter III with tarsus I, could indicate they stimulate the female by means of secretions from the glands on metatarsus I, but *D. pectinifemur* exhibits the same behaviour and lacks these glands (see Willemart et al., 2010). Moreover, we found no evidence of chemoreceptors where the male's legs frequently touched the female. Our results therefore suggest that these glands in *G. perlata* are not involved in the release of contact pheromones during mating.

Harvestmen have contact chemoreceptors on the distal parts of their legs, which are used in finding shelter (Teng et al., 2012; Santos et al., 2013), food and detecting predators (Willemart & Chelini, 2007; Chelini et al., 2009; Costa & Willemart, 2013), detecting alarm pheromones (Machado et al., 2002) and conspecific recognition (Willemart & Hebets, 2012). Setae that are contact chemoreceptors have a single pore at their tips, which was not the case for the setae that are on the body parts of males that rub against the females. These setae may therefore be contact mechanoreceptors.

Mating posture is similar in all laniatorids studied so far, with both sexes face-to-face and raising the anterior parts of their bodies (Mora, 1990; Machado & Oliveira, 1998; Nazareth & Machado, 2009; Requena & Machado, 2013). However, males of the gonyleptids *Acutisoma longipes* Roewer, 1913, *Chavesincola inexpectabilis* Soares & Soares, 1946, and *Iporangaia pustulosa* Mello-Leitao, 1935 hold the female's pedipalps with their pedipalps (Machado & Oliveira, 1998; Nazareth & Machado, 2009;

Requena & Machado, 2013), whereas the males of the cosmetid *G. perlata* hold the trochanter of the first pair of legs of the females with their pedipalps and those of gonyleptid *D. pectinifemur* hold females by their trochanter II (Table 1). It is unknown whether the typical spoon shaped pedipalps of cosmetids (unlike the cylindrical pedipalps of other species) are involved in grasping the opposite sex.

The behaviour hook and rub trochanter III with tarsus I starts before mating and lasts for about 50% of time spent mating in *G. perlata*. Considering that this behavior is not involved in pheromone releasing, this behaviour may be tactile in both of these species. Repeated touching of particular parts of the female body with legs or pedipalps during mating is common in harvestmen (Mora, 1990; Willemart et al., 2006, 2009b; Nazareth & Machado, 2009; Requena & Machado, 2013; this study, both species), but this is the first time that rubbing is reported. Tapping or rubbing different parts of the body of females may increase the chances of mating success for males at least in some spiders and scorpions (Eberhard, 1996; Huber & Eberhard, 1997; Peretti & Carrera, 2005). That may also be true for *G. perlata*.

In some cases, males of *G. perlata* keep the tarsi I motionless, which is followed by the females attempting to interrupt mating by lowering the anterior portion of the prosoma. Males would then start hook and rub trochanter III with tarsus I. In half of the cases when this happened ( $n = 8$ ), females resumed mating by raising the prosoma again. This suggests that males and females may be communicating during mating. In pholcid spiders, females respond to male pressure on her genitalia by stridulating, after which the males relax the pressure (Peretti et al., 2006). In those cases when female harvestmen resumed mating and the males responded, they increased the time spent mating, which may lead to more sperm being transferred to the females (Enders, 1993; Cuatianquiz & Cordero, 2006; Machado & Macías-Ordóñez, 2007; Pérez-Staples et al., 2010).

Lowering the prosoma is a typical posture of harvestmen at the end of mating (Machado & Macías-Ordóñez, 2007). However, in three cases, males hook and rub trochanter III with tarsus I after they lowered their prosoma, suggesting a post-copulatory courtship. In *C. inexpectabilis* and *I. pustulosa*, males also “tapped”/“gently touched” females after mating (Nazareth & Machado, 2009; Requena & Machado, 2013). Such behaviour could be related to cryptic female choice, which increases the certainty of the paternity of the male (Eberhard, 1996). Finally, unlike in *I. pustulosa* and *C. inexpectabilis*, in which females terminated mating (Nazareth & Machado, 2009; Requena & Machado, 2013), both males and females of *G. perlata* may terminate mating by lowering the prosoma and pulling back.

Our observations suggest that the sexually dimorphic glands of *G. perlata* do not release contact pheromones during mating. Therefore, the use of these glands in *G. perlata* may be restricted to spreading chemicals in the environment (Fernandes & Willemart, 2014).

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