Female odours promote the activation of sheltered kissing bug *Rhodnius prolixus* males and modulate their orientation

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Abstract. The existence of a pheromone emitted during copulation has been reported for *Rhodnius prolixus* (Hemiptera: Reduviidae) Stål. Adults possess one pair of metasternal glands (MGs) from which female *R. prolixus* release volatiles mainly at night. We investigated whether these volatiles emitted by adult *R. prolixus* can modulate sexual-related behaviours of opposite and/or same sex individuals. We first used a shelter bioassay to test if adult activity patterns can be affected by chemical signals emitted by opposite sex conspecifics. We observed that males left the shelter more frequently in the presence of females, showing higher activity and an increase of intrasexual copulation attempts. Females showed no shelter-related activation in the presence of male odours. Second, we used a locomotion compensator device to investigate whether females or males show oriented responses to odours emitted by adults. We found that males oriented towards air currents carrying female odours but females did not orient towards odours emitted by adults. Finally, we observed that males oriented towards the female MG compounds. Thus, a volatile chemical signal emitted by females from their MGs promoted the activation of sheltered males and modulated orientation to air currents.

Key words. Chemical signals, locomotor activity, metasternal glands, sex pheromone, shelter.

Introduction

Rhodnius prolixus Stål is the main vector of Chagas disease in northern South America and parts of Central America (Schofield, 1994). This species is well adapted to live in human dwellings and is considered to have great epidemiological importance (Monteiro *et al.*, 2003). Recent data indicate that Chagas disease affects 15 million people and that another 90 million are exposed to risk of transmission in Latin America (Coura & Dias, 2009).

During daylight hours, triatomines are usually found in an inactive state or 'akinesis', aggregated inside shelters. Instead, they display most of their activity during the night, leaving the shelters to search for food (Lazzari, 1992; Lorenzo & Lazzari, 1996, 1998). *Triatoma infestans* Klug exhibits a marked circadian organization of its activities, and locomotion activity is divided in two endogenously controlled peaks, one expressed at dusk and another at dawn (Lazzari, 1992). The two peaks of activity appear to be associated with host seeking and shelter search, respectively (Lazzari, 1992; Lorenzo & Lazzari, 1998; Bodin *et al.*, 2008). There is no information available on whether mate search is also expressed at a particular temporal window. There is behavioural evidence that triatomines use pheromones for sexual communication, as it was reported

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that mating couples of *R. prolixus* emit volatiles that promote the aggregation of conspecific males. In addition, Manrique and Lazzari (1995) showed that mating *T. infestans* exhibit a similar behaviour. Triatomine adults have two pairs of exocrine glands, Brindley's (BGs) and metasternal glands (MGs), which emit secretions at two different sites on the surface of the thorax. The BG volatiles with isobutyric acid as the most abundant compound appear to have defensive purposes but also to act as an alarm pheromone (Ward, 1981; Manrique *et al.*, 2006). Furthermore, it has been suggested that the secretions of these glands mediate triatomine sexual communication (Fontán *et al.*, 2002; Rojas *et al.*, 2002; Guerenstein & Guerin, 2004). Nevertheless, several previous reports have recently indicated that MGs are the main source of sexual communication signals of these insects (Manrique *et al.*, 2006; Crespo & Manrique, 2007; Pontes *et al.*, 2008; Vitta *et al.*, 2009; Zacharias *et al.*, 2010; Manrique & Lorenzo, 2012; Pontes & Lorenzo, 2012). In particular, Crespo & Manrique (2007), Pontes *et al.* (2008) and Pontes & Lorenzo (2012) reported that the occlusion of MGs affected mating success and male aggregation around mating pairs in two different species, whereas the occlusion of BGs did not. The volatile compounds produced by the MGs of *T. infestans, Triatoma brasiliensis* Neiva, *R. prolixus* and *Triatoma dimidiata* Latreille have been identified (Manrique *et al.*, 2006; Pontes *et al.*, 2008; Vitta *et al.*, 2009; May-Concha *et al.*, 2013). Interestingly, Pontes *et al.* (2008) demonstrated that female *R. prolixus* emit these odours during the early scotophase and suggested that this would act as a form of calling behaviour.

In spite of these facts, the processes involved in the activation of the sexual behaviour of *R. prolixus* males need to be characterized, as well as their orientation to females during locomotion. This information is needed to allow the development of behaviour manipulation techniques such as sexual confusion, mating disruption or the use of traps baited with sex pheromones. In the present study, we evaluated whether male or female odours are capable of inducing adult triatomines to increase sex-related activities, i.e. to cease akinesis, leave the shelters and engage in locomotion in search of mates. Moreover, we assessed whether odours from males and females added into airstreams odour promoted orientation behaviour in these insects. Finally, we evaluated whether female MG products are necessary for male orientation during locomotion.

Materials and methods

Insects

The insects were obtained from the *R. prolixus* colony at the Centro de Pesquisas René Rachou, which was established more than 20 years ago from a batch of domiciliary insects captured during field work in Honduras and donated by Dr Carlos Ponce (Ministerio de Salud Publica, Honduras). Groups of fifth instar larvae were sorted by sex and placed in separate flasks to keep them virgin. All insects were kept at $26 \pm 2\,°C$ and $60 \pm 10\%$ RH, under a LD 12:12 photoperiod for at least 3 days before the experiments. For shelter assays, insects

were fed at day 10 after ecdysis and used 20 days later. For locomotion compensator studies, insects were fed at day 10, and used 20 days after ecdysis. All assays were performed at $26 \pm 2\,°C$ and $60 \pm 10\%$ RH.

Experiment 1

We studied the modulation of activities that might lead to copulation when a chemical background containing intra- or cross-sex odours was added. Experiments were conducted in a glass arena of $40 \times 40 \times 20$ cm. In the centre of the arena an artificial shelter (10×10 cm, made of a piece of corrugated cardboard of 10×20 cm folded in the middle) with two lateral accesses was offered to the bugs (Lorenzo & Lazzari, 1998). The behaviour of a group of 20 males or females was recorded by means of an infrared sensitive video camera (HDL, CCD, HM 30/60), starting 2 h before the beginning of the scotophase and finishing 2 h after this phase, reaching a total recording period of 16 h. Insects were released at the centre of the arena and given 3 days to acclimatize to the environment. After this interval, a 2 day long assay took place. During the first night the spontaneous activity (control) of the insects was recorded; subsequently the same insects were exposed to odours of bugs of the opposite sex during the second night and their activity was recorded.

Stimuli were presented in flasks (height: 9.5 cm; diameter: 6.5 cm) hung 15 cm above the shelter. A nylon mesh was used to close the flask after introducing the bugs (a group of 10 male or female insects), therefore allowing odours eventually released by these insects to reach the arena. The stimulus source was presented 10 min before the scotophase started. In control assays, clean empty flasks were hung above the shelter at the same time and position. The following experimental conditions were tested separately: (a) 20 sheltered males exposed to an empty flask (control); (b) 20 sheltered males exposed to a flask containing 10 females; (c) 20 sheltered females exposed to an empty flask (control) and (d) 20 sheltered females exposed to a flask containing 10 males. For each treatment six replicates were performed.

Video recordings were analysed for the following parameters: (a) the number of insects outside the shelter; (b) the intensity of locomotor activity; and (c) the number of mating attempts performed between males. All parameters were analysed cumulatively (on an hourly basis) in order to describe any temporal variation in insect activity. Locomotor activity was measured by tracing two perpendicular axes forming a cross centred on the middle of the screen and counting each time a bug crossed any of these lines.

Experiment 2

We studied the orientation of individuals to air-current bearing intra- or cross-sex odours. A locomotion compensator was used to analyse the orientation of each insect in an open-loop design for translation that allowed free rotation of the bug, as described elsewhere (Barrozo *et al.*, 2003). It consisted of a hollow Styrofoam® sphere (9.7 cm diameter, 2.5 g wt) floating

on a vertical airstream. Individual insects were tethered by the dorsal thorax to a freely rotating metal rod centred at the apex of the sphere. After tarsal contact was allowed on the surface of the sphere, animals immediately started to walk, displacing the sphere under their legs. Although insects could walk and rotate freely, thus changing the direction of locomotion, they were unable to change the distance to the sources of stimuli. The locomotion compensator presented a PC mouse optic sensor 1 mm below the inferior pole of the sphere, i.e. on the opposite side of the position of the insect. The displacements of the sphere induced by the insect were detected by this sensor, sampled every 0.3 s, transformed into x-y coordinates with the aid of software (Barrozo *et al.*, 2003) and stored by a computer. The walking paths of the bugs were reconstructed using these data and spatio-temporal components analysed.

Stimulus delivery

A simultaneous discrimination bioassay was used to expose insects to odourant stimuli. In control trials, two opposed (180°) clean airstreams, i.e. control vs. control, were presented to insects simultaneously. In test trials, one of the airstreams bore the odour whereas the other was kept clean (test vs. control). The rationale for using two opposite air currents is discussed elsewhere (Barrozo & Lazzari, 2004a).

The two opposite streams were driven by a pump that used air from the experimental room. The flows were delivered tangentially at the apex of the sphere to reach the position of the insects (Barrozo *et al.*, 2003). These airstreams previously passed through glass bottles (125 mL) containing the stimuli source and were transported by silicone tubing (0.4 cm inner diameter) and ultimately delivered by a glass tube (0.67 cm inner diameter, 5 cm length). The outlets of the glass tubes were placed 5 cm away from the apex of the sphere (Barrozo *et al.*, 2003). Air velocity was set at 8 cm/s at each tube outlet with the aid of an anemometer (Testo 405-V1; Testo Inc., Lenzkirch, Germany). In each test assay, the air flowing through one bottle was kept clean whereas that flowing through the remaining bottle carried the odours emitted by two adult *R. prolixus* of the same sex that were kept inside.

Before each test, insects were allowed to acclimatize in still air for 120 s. Subsequently, the airstreams were turned on and the trajectories of the insects were recorded for 900 s. All insects were tested once and discarded afterwards. Thirty insects were tested for each of the stimuli evaluated. To investigate whether females or males show oriented responses to odours from adults the following treatments were tested: (a) two clean airstreams (control), (b) one clean airstream vs. one airstream with male odour added and, (c) one clean airstream vs. one airstream with female odour added. Additionally, we tested if female MG volatiles modulate male orientation responses. Thus, males were exposed to (d) a clean airstream vs. one airstream carrying the odours emitted by two females with occluded MGs (occlusion procedure explained below) and to (e) one clean airstream vs. one airstream carrying the odours emitted by two control treated females (paraffin applied, MGs not occluded). Females were exposed to (f) one clean airstream vs. one airstream carrying the odour emitted by a mouse. This treatment acted as a positive control ($n = 25$) to set up a condition of maximal responsiveness for females. Treatment (days) was developed to analyse whether the odours produced by female MGs were responsible for promoting the orientation presented by walking males. A wax-pen (HOT PEN #2; Max Wax, Washington, USA) was used to melt paraffin wax (melting point: 56–58 °C; Sigma-Aldrich, St. Louis, MO, U.S.A.) for selectively occluding female MG orifices. The occlusion of gland orifices was carried out 24 h before assays were performed (Crespo & Manrique, 2007). Groups of no more than 30 assays per day, corresponding to an experimental series (a) (10 assays), (b) (10 assays) and (c) (10 assays), were conducted on the same day in order to grant a block design for them. Based on the results from these series, orientation of males was subsequently evaluated in series (d) and (e), also using a block design. As females showed no orientation to conspecifics, series (f) was performed as a positive control for them.

Statistical analysis

Student's *t*-test for independent samples was used to compare results of control shelter assays between sexes (Experiment 1). Student's *t*-test for paired samples was used for comparing results from control and experimental assays with insects of the same sex. The discriminatory limit used in these comparisons was $P < 0.05$. An activity index was calculated to compare the level of activity presented independently of the number of active insects. For this, the amount of locomotor activity was divided by the number of insects found outside the shelter.

The paths followed by the insects in the locomotion compensator (Experiment 2) were analysed by means of circular statistics (Batschelet, 1965; Zar, 1984). The mean angle of the trajectory (i) displayed by an insect throughout the experimental time was computed and, for every experimental group, a mean angle (μ) and the length of the resultant mean vector (r) were subsequently calculated. The relative position of the odour-associated airstream was conventionally designated as 0° and the control one as 180°. Whereas μ extends from 0 to 360°, r varies between 0 and 1 (0, indicating a non-defined mean direction and 1, a straight path to a given direction). The statistical evidence of directness was tested according to the Rayleigh test (Batschelet, 1965; Zar, 1984). *Ho*, considered that the sample was uniformly distributed around a circle vs. *Ha*, indicating that the sample did not follow a uniform circular distribution. In this conception circular uniformity implies no defined mean direction. When data did deviate significantly from uniformity, a V-test (Zar, 1984) was carried out to assess whether the mean angle calculated from the sample was statistically different from the stimulus direction (0°).

Additionally, an orientation index (OI) was calculated as the product of the cosine of the mean angle μ multiplied by the length of the mean resultant vector r, as $\cos(\mu)\, r$. The OI varied between −1 and 1 (−1, indicates orientation away from the stimulus and 1, orientation towards the stimulus location).
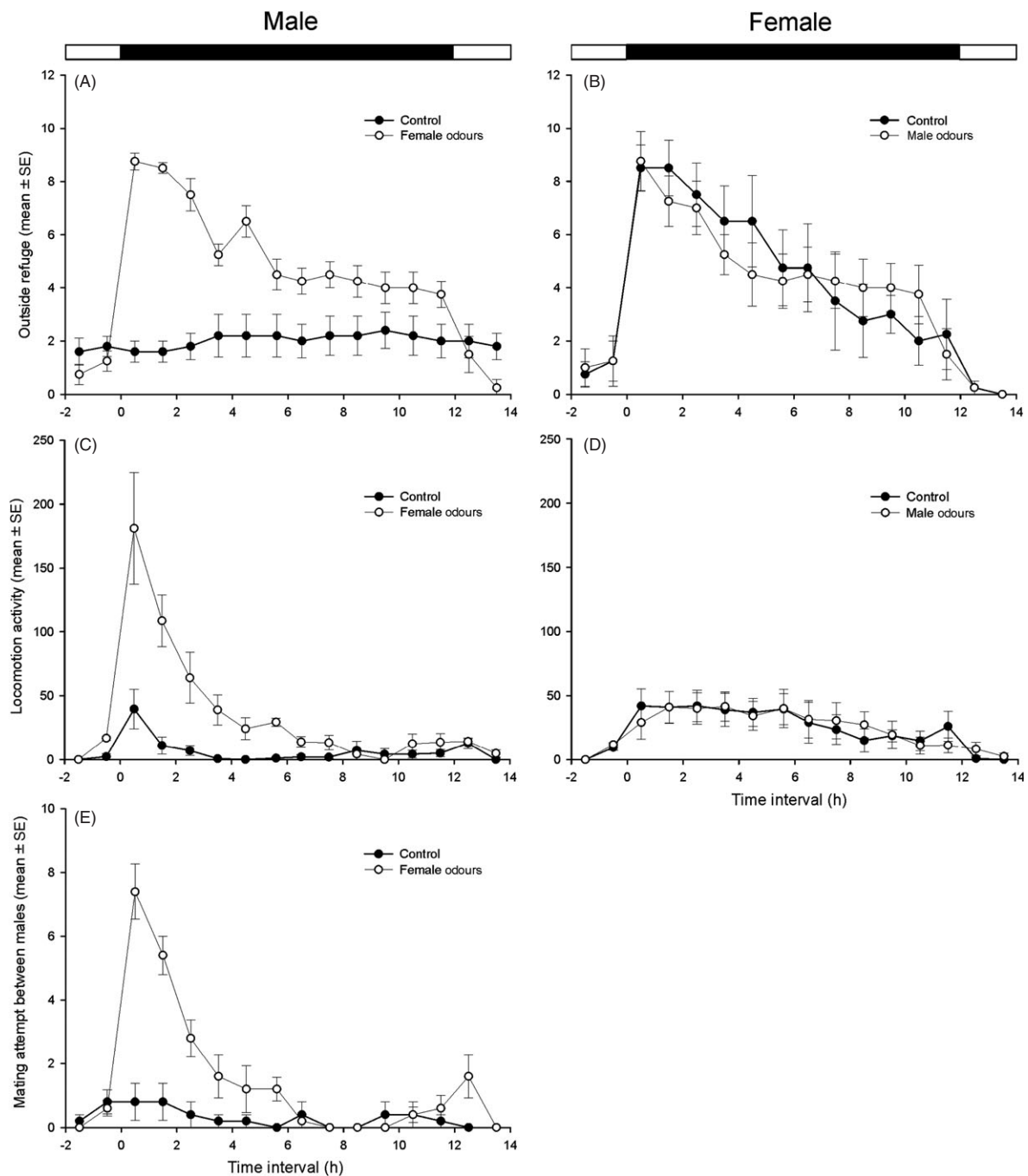
Fig. 1. Activity of insects associated with shelters. Variation in the number of males (A) and females (B) that left the shelters, temporal pattern of locomotor activity of males (C) and females (D) outside shelters, and temporal variation of the frequency of mating attempts between *R. prolixus* males (E). (●) Control: spontaneous activity. (○) Test: presence of opposite sex individuals. The top horizontal bars represent the cycle of illumination: white/photophase; black/scotophase. The values represent the average of six replicates (per hour)/series.

In order to compare the OIs calculated for the different experimental female groups, differences were statistically evaluated through an ANOVA test and followed by Tukey's multiple comparisons (Zar, 1984). A Kruskall–Wallis test followed by Dunn's multiple comparisons was used to analyse the significance of results obtained with males.

Results

Experiment 1

Males of *R. prolixus* left the shelters more frequently in the presence of females (Fig. 1A, Student's *t*-test for paired
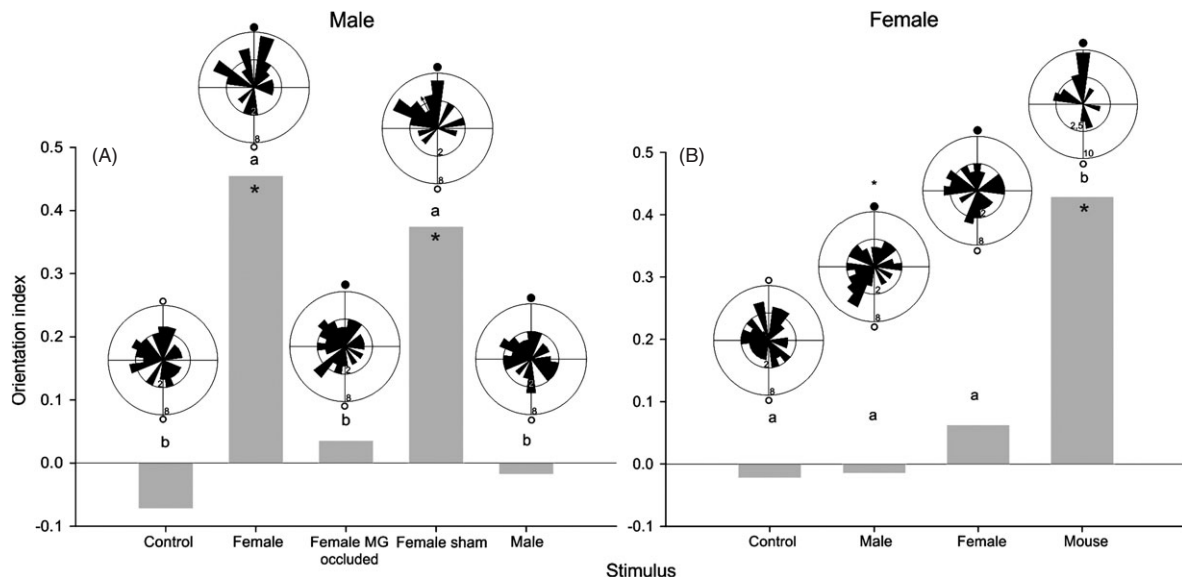
Fig. 2. Orientation activity. Orientation and activation of male (A) and female (B) *Rhodnius prolixus* to airstreams carrying odours from conespecifics odour. Results are expressed by means of an orientation index (OI): values near 1 indicate orientation towards the odour associated airstream and near −1 orientation away from it. Different letters denote statistically significant differences between groups. (a) Kruskall–Wallis $H = 50.4$, d.f. $= 4$, $P < 0.001$; followed by Dunn's multiple comparisons test; (b) ANOVA $F = 10.4$, d.f. $= 3$, $P < 0.001$ followed by Tukey's test. The circular histograms (rose diagrams) represent the frequency of the path angles displayed by the animals, which is proportional to the area of the wedge (bar width of 15°). Asterisks denote the simultaneous occurrence of two conditions: statistically significant differences from a uniform distribution and a significant mean direction to the stimulus location (0°); $P < 0.05$ for both. White dots indicate the position of the control airstream and black dots denote the test airstream location.

samples, $P < 0.01$). By contrast, females were not affected by the presence of males in the experimental room (Fig. 1B, Student's *t*-test for paired samples, NS). Females left the shelters more frequently than males only in the control assays (Student's *t*-test for independent samples, $P < 0.002$). However, no significant difference in spontaneous locomotor activity was observed between males and females (Fig. 1C, D, Student's *t*-test for independent samples, NS).

The locomotor activity of males was significantly increased in the presence of females (Fig. 1C, Student's *t*-test for paired samples, $P < 0.02$). However, when females were stimulated with the presence of males, no evident changes were observed in the locomotor activity (Fig. 1D, Student's *t*-test for paired samples, NS).

The frequency of copulatory attempts between males was also evaluated in this experiment, given that this behaviour is commonly observed among males of this species. We worked under the assumption that males could be aroused by the presence of volatile signals emitted by females inducing an increased number of mating attempts. In fact, an increase in the number of mating attempts performed by males was observed in the presence of females (Fig. 1E, Student's *t*-test for paired samples, $P < 0.001$).

Experiment 2

The addition of female odours to one of the airstreams induced a change in the behaviour of *R. prolixus* males

(Kruskall–Wallis $P < 0.001$). The OIs recorded with males stimulated with odours from intact females or *sham*-treated females were significantly higher (Dunn's test, $P < 0.001$ for both) than those observed in the presence of male odours and clean airstreams (Dunn's test, NS).

Males oriented towards the airstream loaded with female odour (Fig. 2A, OI $= 0.46$; Rayleigh test, $P = 0.04$; V-test, $P = 0.008$). The analysis of data evidenced a non-oriented locomotion activity when males were presented with two opposite and identical clean airstreams (Fig. 2A, OI $= −0.07$; Rayleigh test $P = 0.17$). In addition, when males were presented with one of the airstreams associated with male odour, non-oriented trajectories were exhibited (Fig. 2A, OI $= −0.017$; Rayleigh test $P = 0.7$).

Males exhibited non-oriented trajectories when one of the airstreams carried the odour of females that had the MGs occluded (Fig. 2A, OI $= 0.035$; Rayleigh test $P = 0.13$). Nevertheless, males oriented to airstreams loaded with odours from *sham*-treated females (Fig. 2A, OI $= 0.37$; Rayleigh test $P < 0.001$; V-test $P < 0.001$).

The OI of females stimulated with mouse odours were significantly higher than those of all other female groups tested (Fig. 2B, Tukey's $P < 0.001$) and females oriented significantly towards an airstream enriched with host-emitted signals (Fig. 2B, OI $= 0.43$; Rayleigh test $P = 0.002$; V-test $P < 0.001$). Females stimulated with female odours (OI $= 0.06$; Rayleigh test $P = 0.7$), male odours (OI $= −0.014$; Rayleigh test $P = 0.2$) or clean airstreams (OI $= −0.02$; Rayleigh test $P = 0.32$) showed no oriented responses (Fig. 2B).

Discussion

This work reports that odours associated with adult females act as activation and orientation signals modulating the sexual behaviour of *R. prolixus* males. Moreover, it demonstrates that the secretions from female MGs are necessary for male orientation to be expressed.

Rhodnius prolixus females showed a clear spontaneous increase in leaving the shelter during the early hours of the scotophase. By contrast, male *R. prolixus* did not show any relevant disposition to leave shelters in the absence of female signals. Nevertheless, they showed an increase in the propensity to come out of the shelters when female signals were present in the experimental room. The peak activation of males was expressed during the same time interval shown spontaneously by females. In addition, during this period of time males also showed higher locomotor activity and a larger number of intrasexual mating attempts in the presence of female odour. Thus, we suggest that female odours promote an arousal in shelter-associated males, inducing them to leave the shelters. Meanwhile, male odours did not promote significant changes in the tendency of females to leave shelters or engage in locomotor activity. As females spontaneously left the shelters more often than males, we suggest that fasting induced females to leave the shelters in search of a host more intensely than males, as requirements for food are more pronounced in females owing to reproductive activity (Schilman & Lazzari, 2004). In other species of triatomines, such as *T. brasiliensis*, females are less resistant to fasting than males supporting the idea of females leaving the shelters more often than males (Costa & Perondini, 1973). Females of other groups of haematophagous insects, e.g., the tsetse fly *Glossina austeni* Newstead, present higher energy demands than males as the former need to take larger bloodmeals after ovulation (Davey, 1974).

The results of shelter assays showed that the hours early in the scotophase apparently represent the interval in which *R. prolixus* adults leave the shelter. In the case of males, this can almost exclusively be observed in the presence of female odours. Therefore, we suggest that males of this species might restrict most of their sexual activity to a relatively short temporal window related to the start of the scotophase. This interval overlaps with those of other known rhythmic activities described for triatomines (Lazzari, 1991, 1992; Barrozo & Lazzari, 2004b).

We have analysed different aspects of adult *R. prolixus* orientation using an open-loop bioassay based on the simultaneous discrimination of two air currents. The results obtained demonstrated that males and females exposed to two opposite clean air currents showed a non-oriented behaviour. These results are in agreement with those described for walking fifth instar *T. infestans* (Barrozo et al., 2003).

Previous works performed in a locomotor compensator have studied triatomine orientation responses using olfactory signals such as carbon dioxide, lactic acid, short-chain fatty acids and other host odours (Barrozo & Lazzari, 2004a, b). For the first time this design was used to demonstrate that *R. prolixus* males clearly orientate towards airstreams carrying female odours (Fig. 2). Interestingly, *R. prolixus* males only presented oriented responses when stimulated with airstreams carrying female odours. Females of this species spontaneously emit odours from the MGs (Pontes et al., 2008) and this fact might be the basis of the increase in male activity described here.

It has been shown that female MGs are the sources of volatiles that elicit male flight initiation (Zacharias et al., 2010). This conclusion was reached after occluding female MGs with paraffin wax and observing that this impairs male oriented take-off in response to female odour laden airstreams. Our results are in agreement with this, suggesting that males of this species can initiate flight or engage in oriented walking as a response to female odour laden airstreams. Whether one or the other behaviour is triggered may depend on other contextual signals. These sex-specific responses suggest that orientation by *R. prolixus* males is part of a mate location mechanism involving a sex pheromone emitted by females (Pontes et al., 2008). It is proposed that these signals would promote the activation and subsequent approximation of males to females from relatively long distances. Interestingly, the odours from female MGs would later mediate male aggregation, allowing females to have access to diverse males with which they may copulate (Baldwin et al., 1971; Crespo & Manrique, 2007; Pontes & Lorenzo, 2012). Whether these male aggregations observed in response to mating couples in *R. prolixus* are related to female choice processes needs to be clarified.

As it has been established that female *R. prolixus* produce a pheromone that elicits activation, orientation and male aggregation, the identification of key compounds should now proceed. These compounds may represent new avenues for developing control tools for eliminating domiciliary populations of these relevant Chagas disease vectors.

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