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Spatio-temporal analysis of the role of faecal depositions in aggregation behaviour of the triatomine *Rhodnius prolixus*

MARIANEL L. FALVO, ALICIA N. LORENZO FIGUEIRAS and GABRIEL MANRIQUE

Laboratorio de Fisiología de Insectos, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, IBBEA, CONICET-UBA, Ciudad Autónoma de Buenos Aires, Argentina

Abstract. Several triatomine species aggregate over conspecific faeces. In some domestic species, this behaviour might help individuals to find their refuges because faeces are usually deposited around such places. In the present study, the defaecation behaviour of the triatomine bug *Rhodnius prolixus* Stål (Heteroptera: Reduviidae) is described in detail, including the temporal pattern of aggregation of nymphs with different nutritional status to faeces of different post-deposition times. First, using an experimental arena, the spatial distribution of faecal deposition spots released by nymphs, in relation to the position of a refuge, is recorded. The results show that a high density of faeces is deposited inside the refuge, and few and distant spots, in relation to the position of the refuge, are dropped outside. Second, whether the aggregation behaviour of nymphs is altered by the time elapsed from deposition of the faeces, and by the nutritional status of the insects, is determined. The post-deposition time of the faeces strongly modulates the aggregation response of the insects. Differences in aggregation between fed and unfed insects are only marginally significant; however, different temporal windows of aggregation according to the post-deposition times of the faeces are demonstrated between bugs of different nutritional status. The results are discussed with respect to the relevance of the aggregation signal in relation to the characteristics of the natural refuges used by *R. prolixus*.

Key words. Defaecation pattern, faeces, refuge marking, Triatominae.

Introduction

The haematophagous insect *Rhodnius prolixus* Stål (Heteroptera: Reduviidae: Triatominae) is one of the main vectors of *Trypanosoma cruzi*, the etiological agent of Chagas disease. This disease afflicts approximately six to seven million people worldwide, mostly in Latin America (WHO, 2015). These insects are adapted to live in domestic environments and, accordingly, are considered to be of major epidemiological importance (Monteiro *et al.*, 2003), although they also exist as a sylvatic species (Fitzpatrick *et al.*, 2008).

These blood-feeding nocturnal insects leave their refuges at dusk searching for their vertebrate hosts and return to their shelters before dawn, where they spend most of the daytime aggregated in akinesis. In several triatomine species, this aggregation behaviour is not only mediated by thigmotaxis, but also by chemical cues derived from the cuticle (Lorenzo Figueiras & Lazzari, 1998a; Vitta *et al.*, 2002; Lorenzo Figueiras *et al.*, 2009) and faeces (Schofield & Patterson, 1977; Ondarza *et al.*, 1986; Cruz-López *et al.*, 1993; Lorenzo Figueiras *et al.*, 1994; Lorenzo Figueiras & Lazzari, 1998b, 2002; Vitta *et al.*, 2007). The aggregation behaviour is well studied in *Triatoma infestans* (Lorenzo & Lazzari, 1996; Lorenzo Figueiras & Lazzari, 2000), which is primarily a domestic species and the most important vector of Chagas disease in the Southern Cone region of South America (Zeledón & Rabinovich, 1981).

Correspondence: Dr Gabriel Manrique, Laboratorio de Fisiología de Insectos, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Instituto de Biodiversidad y Biología Experimental y Aplicada – IBBEA, CONICET-UBA, Ciudad Universitaria, Pabellón II, C1428EHA Ciudad Autónoma de Buenos Aires, Argentina. Tel.: +54 11 4576 3300; e-mail: gabo@bg.fcen.uba.ar

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As in *T. infestans* and other triatomines, nymphs of *R. prolixus* exhibit aggregation behaviour around their faeces (Lorenzo Figueiras & Lazzari, 2002). Orientation towards faeces occurs mostly during the early photophase and it is suggested that olfactory receptors would be more sensitive to this stimulus at this time because it coincides with the return to their refuges, after feeding and diuresis (Bodin *et al.*, 2008). In addition, feeding status can modulate the response of *R. prolixus* towards aggregation pheromones, such as those present in triatomine faeces (Reisenman *et al.*, 2013). In *T. infestans*, faeces that are freshly deposited do not evoke assembling behaviour until 3 h after deposition, from which time the faeces become attractive to the insects for approximately 10 days (Lorenzo Figueiras & Lazzari, 2000). However, in *R. prolixus*, the time during which the odours from the faeces of conspecifics remain active in promoting such aggregation behaviour is still uncertain. It is also unknown whether *R. prolixus* displays any particular defaecation behaviour related to the use of the refuge.

The present study investigates the role of faeces in the aggregation behaviour of *R. prolixus* and its relationship with the use of refuges. First, the spatial pattern of defaecation is analyzed (i.e. the distribution of faecal depositions spots related to the position of a refuge) and the defaecation behaviour is also described. Second, activation (i.e. the time that faeces become attractive) and persistence of the aggregation signal are each determined, as well as whether the aggregation behaviour is modified according to the post-deposition time of the faeces and the nutritional status of the insects.

Materials and methods

The insects were reared in the insectary under an LD 12 : 12 h illumination regime at 28 ± 2 °C and 60 ± 20% relative humidity. All instars were fed weekly on live hens that were handled according to the biosafety rules from the Servicio de Higiene y Seguridad of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. For the assays, fifth-instar nymphs of *R. prolixus* were used. They were fed according to the details of each experiment (see below). Insects were used only once and then discarded.

Experiment 1: Defaecation behaviour

The aim of these assays was to investigate the existence of a particular pattern of defaecation in *R. prolixus*. Initially, the spatial distribution of deposited faeces was analyzed in relation to the position of a refuge, and then the defaecation behaviour was described in detail.

The spatial distribution of faecal depositions in relation to a refuge's position

All experiments were performed in an experimental room with a controlled environment under an LD 12 : 12 h illumination regime at 25 ± 2 °C and 30–60% relative humidity. Assays were carried out by releasing 15 nymphs into a rectangular glass arena ($40 \times 32 \times 20\,cm^3$) lined with filter paper as a substrate that allowed the insects to walk freely for 60 days (Fig. 1a). In the middle of one wall of the arena, an aperture ($2 \times 1.5\,cm^2$) connected the arena to a corrugated cardboard box ($8 \times 8 \times 1.5\,cm^3$) that served as a refuge. This access allowed insects to move freely in or out of the refuge during the experimental time. This experiment was replicated 12 times.

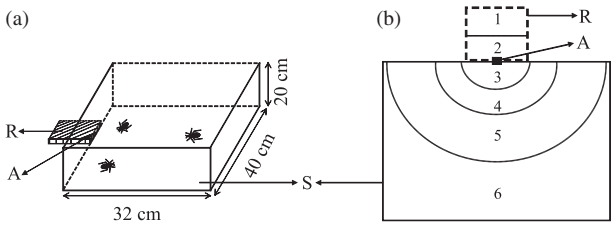

Fig. 1. Experimental device used to study the spatial pattern of defaecation of *Rhodnius prolixus*. (a) Lateral view. (b) upper view. R, refuge; A, access to refuge; S, substrate of filter paper. Different areas were delimited (1 and 2 inside; 3–6 outside the refuge) to evaluate the spatial distribution of faecal depositions by fifth-instar nymphs in relation to a refuge's position.

Before starting the assays, nymphs that were 7 days postecdysis were fed for 10 min to ensure survival during the 60-day experiments. Four hours later, the insects were gently released near the access of the refuge. At the end of each assay, insects were removed and the distribution of the faecal depositions in relation to the refuge was analyzed. Therefore, the experimental arena was divided into six different areas (Fig. 1b). The refuge floor was divided in two equal halves (each $4 \times 8\,cm^2$) corresponding to the areas 1 and 2, with the latter closer to the entrance than the former. Outside the refuge, the substrate was divided into four areas: areas 3–5 were delimited by a semi-circumference of radius 5, 10 and 20 cm, respectively; area 6 was delimited by the glass arena. The number of faecal spots in each area was counted.

A generalized linear model analysis (Crawley, 2007) with negative binomial error distribution and log-link function was used to fit the number of faecal spots against the different areas of the arena. This statistical analysis was carried out using R, version 2.15.1 (R Development Core Team, 2013).

Description of the defaecation behaviour of R. prolixus

To analyze and describe the defaecation behaviour of *R. prolixus*, three fifth-instar nymphs were gently released into a glass experimental arena ($20 \times 20 \times 11\,cm^3$) and left for 4 days. These insects were 7 days postecdysis, and were fed 2 days before the experiment for 10 min. The arena was lined with filter paper as a substrate, and contained a refuge similar to the one described above, although, in this case, it was covered by a transparent film that allowed direct viewing of the insects' behaviour inside the refuge. A semicircular Teflon tape surrounded the refuge and delimited an area of $52\,cm^2$ where insects could freely move once outside the refuge. The

assays were recorded using a camera (KPC133ZDP/F36; RoHS, Taiwan) that emitted light in the infrared spectrum, which would not be perceived by the insects (Reisenman *et al.*, 1998) and allowed filming in darkness. The camera was connected to a digital video recorder (RoHS, KPD674ZB (EU)-ARG-B, Taiwan), and this in turn was connected to a monitor (Vi.Cam System, China). The stored videos were observed and the defaecation behaviour was described. Twelve replicates were performed for this experiment. The environmental conditions of the experimental room remained under an LD 12 : 12 h illumination regime at 27 ± 1 °C and 30–60% relative humidity.

Experiment 2: Aggregation of nymphs of different nutritional status to faeces of different post-deposition times

The aim of this second experiment was to investigate whether the aggregation signal present in the faeces of *R. prolixus* is activated in a time-dependent form. In parallel, it was also determined whether the nutritional status of nymphs could modulate this aggregation response. Hence, the aggregation response of fed or unfed nymphs towards faeces deposited at different times prior to the start of the experiments was studied.

Experimental set-up for the time-based studies

All experiments were performed in an experimental room with a controlled environment at 25 ± 2 °C, 30–60% relative humidity and functional darkness (i.e. in absence of light to avoid the possible utilization of visual cues by nymphs). Experiments were performed according to the circadian rhythm of the insects, between the late scotophase and early photophase, a period in which they would be returning to the refuge. Each assay was conducted in a circular glass arena (diameter 15 cm), lined with filter paper on the bottom, divided into two equal semicircular sectors. The sections were divided virtually because the division was added digitally during later analysis. In each sector, a half-folded square piece of filter paper (3.5 cm) was placed to provide a refuge. One was covered with faeces (see below) and the other remained clean as a control. The position of the papers was rotated 90° clockwise between assays to control for any spatial bias. The treated and control papers were manipulated with different tweezers to prevent cross-contamination. The arena and tweezers were cleaned with ethanol (96%) between assays.

Collection of faeces

Faeces used as stimuli were deposited by fifth-instar nymphs of 2–3 weeks postecdysis. The insects were fed for 30 min and then left to defecate on a filter paper (diameter 8 cm) placed on the bottom of the feeding flask for approximately 4 h. The faeces covered filter paper was removed from the flask and cut in squares of 3.5 cm that were stored in a Petri dish until their utilization (i.e. different times according to the series).

Experimental assays using faeces of different post-deposition times

Assays were performed using faeces of different post-deposition times as a stimulus: 0 (i.e. time elapsed from up to 5 h after deposition), and 1, 3, 7, 10, 14 or 21 days old. Furthermore, to analyze the effect of the nutritional status of nymphs in the aggregation response, 7–12-day-old postecdysis insects were used, either unfed or fed (i.e. which ingested blood 4 h before the assay).

For each assay, six insects were placed in the middle of the experimental arena by inverting a cylinder flask ($5 \times 3\,cm^2$) containing them. After 3 min of familiarization, the flask was removed and the nymphs were then free to move. The behaviour of the insects was recorded from the outside of the room by a videocamera (RoHS conform, KPC133ZDP/F36, Taiwan) connected to a monitor (Vi.Cam System, China). After 1 h, the number of insects in each half of the arena was recorded from the obtained video. An aggregation index (Cruz-López *et al.*, 1993) was calculated as the difference between the number of insects in the sector containing the faeces and the ones found in the clean sector, divided by the total number of insects released in each assay. The index ranged between −1 (no aggregation to faeces) and 1 (aggregation to faeces), with 0 corresponding to a random response. Twenty replicates were performed for each experimental group. The attraction of triatomines to faeces is expected to be independent of the presence of other individuals. Assays in which insects were still walking after a period of 1 h were discarded (3.5% of the total assays).

The data were analyzed by means of parametric statistics after verification of the assumptions [i.e. normal distribution of residuals (Schapiro-Wilks test) and homogeneity of variance (Levene's test)]. Differences between the aggregation index were assessed by means of two-way analysis of variance (factors: post-deposition time and nutritional status) followed by Tukey's post-hoc means comparisons (Zar, 1984). Principal effects were analyzed because not significant interaction was found between the two factors ($F = 0.4859$, d.f. = 6, $P = 0.8186$).

To assess the temporal windows of the post-deposition time of faeces in which fed and unfed insects aggregated, a one-tailed one sample *t*-test was applied comparing whether each point was significantly different from 0. INFOSTAT (http://www.infostat.com.ar) was used for all calculations.

Results

Defaecation behaviour

Nymphs released in the experimental arena exhibited a particular spatial pattern of defaecation in which far more faecal spots were deposited inside the refuge than elsewhere (Fig. 2). No significant differences between the area categories 'Area 1' and 'Area 2' were found, showing that there was no tendency to mark the access from the inside of the shelter ($P = 0.35$). Outside the refuge, few and distant spots were found. All areas outside the refuge had significant less spots than 'Area 2' ($P < 0.0001$).

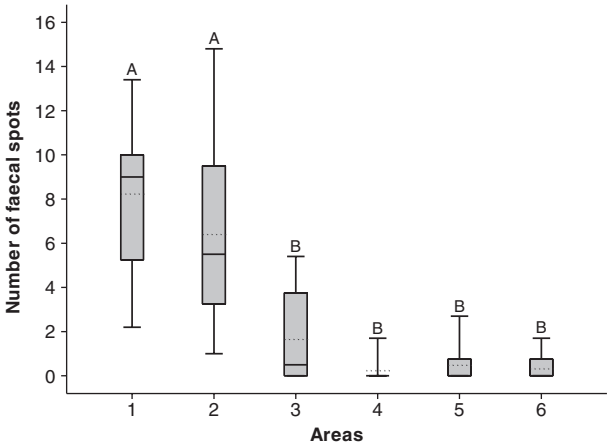

Fig. 2. Spatial distribution of faecal depositions by fifth-instar nymphs of *Rhodnius prolixus* in relation to a refuge. Boxes represent the first and third quartiles of the number of faecal spots in each area of the experimental arena. For each one, the median (solid line) and the mean (dotted line) are shown ($n = 12$). The highest numbers of faeces were found in areas 1 and 2 corresponding to the inside of the refuge. Areas 3–6 were outside the refuge, with increasing distance from the refuge. Different uppercase letters show significant differences between the numbers of faecal spots ($P < 0.001$). Results obtained from the generalized linear model (Theta: 3.38, SE = 1.69).

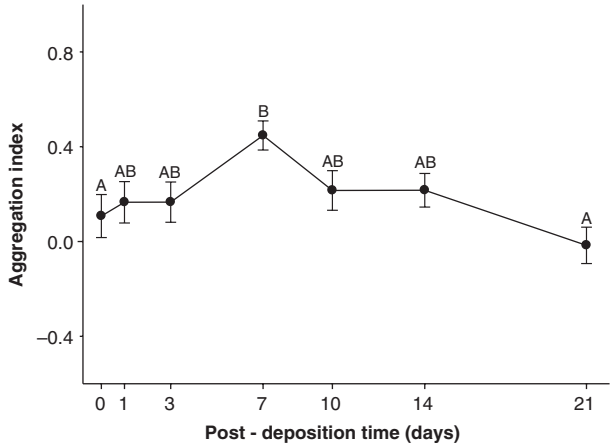

Fig. 3. Effect of post-deposition time of faeces on the aggregation response of *Rhodnius prolixus*. The aggregation behaviour varied significantly with the post-deposition time of the faeces. Each dot represents the mean ± SE aggregation index ($n = 40$). Different uppercase letters show significant differences within the factor post-deposition time of the faeces (*a posteriori* Tukey's comparisons).

These results provide evidence that nymphs of *R. prolixus* deposit their faeces mainly inside the refuges.

Regarding the behaviour of defaecation, 12 defecating events were observed in the video records. In all of them, *R. prolixus* nymphs carried out a sequence of stereotypical steps. First, they remained immobile for a few seconds. Then, they walked backwards between 2 and 4 cm and defecated at the new location. Finally, insects walked the same distance forward, returning to the initial position to stay there or keep walking.

Aggregation of nymphs of different nutritional status to faeces of different post-deposition times

When released on an experimental arena with faeces added at one sector, insects presented a change in aggregation behaviour related to the post-deposition time of the faeces but not with the nutritional status of nymphs. The aggregation behaviour of these insects varied significantly with the post-deposition time of the faeces ($F = 3.0713$, d.f. = 6, $P = 0.0064$) but not with the nutritional status of nymphs ($F = 3.4226$, d.f. = 1, $P = 0.0654$). Post-hoc comparisons revealed that 7-day-old faeces were significantly more attractive than either newly-deposited faeces (0 day) or 21-day-old faeces ($P < 0.05$ in both cases) (Fig. 3).

Although there was no significant effect of nutritional status on aggregation behaviour, there was evidence of a difference between the behaviour of fed and unfed insects. To investigate this trend further (i.e. an aggregation index significantly different from 0), the temporal window of the post-deposition time of faeces that provoked an aggregation response (one tailed *t*-test; asterisks in Fig. 4) was determined for both fed and unfed larvae. Insects of the unfed group began to aggregate at faeces 24 h after deposition. This effect was maintained with faeces up to 14 days old, and was lost with 21-day-old faeces (0 day, $P = 0.2336$; 1 day, $P = 0.0499$; 3 days, $P = 0.0266$; 7 days, $P < 0.0001$; 10 days, $P = 0.0285$; 14 days, $P = 0.0303$; 21 days, $P = 0.4008$). When animals were fed before the assays, the aggregation response was significant with faeces of 7 and 14 days of deposition, with a tendency to aggregate to 10-day-old faeces (0 day, $P = 0.1844$; 1 day, $P = 0.1977$; 3 days, $P = 0.2763$; 7 days, $P = 0.0001$; 10 days, $P = 0.0667$; 14 days, $P = 0.0167$; 21 days, $P = 0.7859$). The effect of the aggregation signal, as in the unfed group, was lost at 21 days post-deposition. These results show a clear difference in the aggregation behaviour of fed and unfed nymphs.

Discussion

The present study describes the defaecation behaviour and the spatial pattern of faeces distribution in relation to the refuge in *R. prolixus*, a species that presents sylvatic and domiciliary habits. Additionally, the aggregation behaviour mediated by faeces is studied to determine the activation and persistence of the aggregation signal, and to analyse whether the aggregation behaviour is modified according to the deposition time of the faeces, as well as the nutritional status of the insects. It is demonstrated that *R. prolixus* deposits its faeces mainly inside the refuge. In addition, the results show that the post-deposition time of faeces has an effect on the aggregation response and that fed and unfed insects have different temporal windows of aggregation.

In recent decades, aggregation behaviour towards faeces and the different factors involved in this process have been studied in diverse triatomine species. For example, a predominantly domestic species such as *T. infestans* deposits its faeces mainly

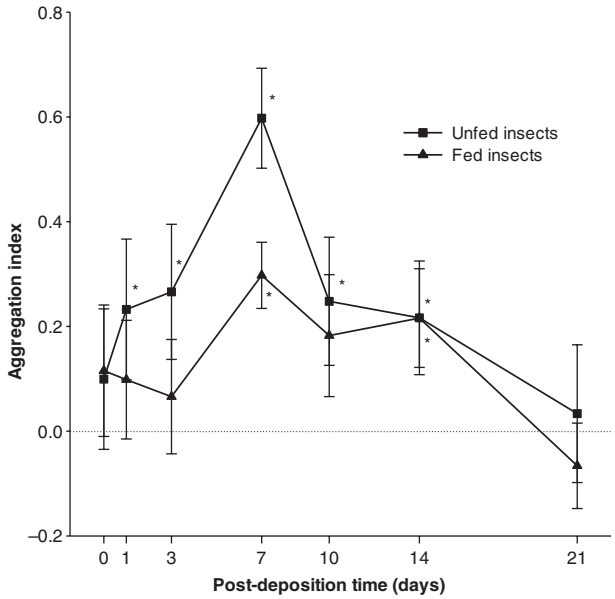

Fig. 4. Temporal window of post-deposition time of faeces that promoted an aggregation response in fed and unfed *Rhodnius prolixus* nymphs. Aggregation of fed (triangles) and unfed (squares) nymphs did not differ statistically. However, the temporal window of the post-deposition time of faeces to which unfed insects aggregated was wider than that for fed insects. Each dot represents the mean ± SE aggregation index ($n = 20$). Asterisks indicate an aggregation index significantly higher than 0 ($P < 0.05$, one-tailed *t*-test). The dotted line represents a random response (aggregation index = 0).

outside the refuge, near its access (Lorenzo & Lazzari, 1996). It is suggested that chemicals released by faeces might serve as a signal that guides the insects to the entrance of refuges after feeding. In a domestic environment, this could be essential for survival because the hosts are relatively far from the refuge; hence, the period of time between blood intake and the return to the refuge should be as short as possible to minimize the probabilities of being eaten by a predator. In addition to faeces, a chemical landmark left behind by the bugs on substrates that have been walked-over also promotes aggregation in *T. infestans* (Lorenzo Figueiras & Lazzari, 1998a). These species have cuticular compounds (Lorenzo Figueiras *et al.*, 2009) that mark the surfaces of the shelter and promote the arrestment of the insects within them. Therefore, for this domestic species, both olfactory and contact chemoreception signals might be involved in orientation, detection and aggregation behaviour inside a shelter.

When comparing previously published literature with the aggregation and defaecation behaviour as shown in *R. prolixus*, even though this species can colonize domestic environments, it appears that chemical signals may not have the same meaning or importance as in *T. infestans*. No aggregation response to cuticular chemical factors is found in *R. prolixus* (Lorenzo Figueiras & Lazzari, 2002) and faeces are deposited mainly inside the refuge, not marking its entrance (Fig. 2). Vitta *et al.* (2007) report a study of defaecation behaviour in *Triatoma brasiliensis*, and their results are consistent with those of the present study because this species also deposited a higher number of faecal marks inside the shelter rather than in the surrounding area. Vitta *et al.* (2007) suggest that defecating inside the refuge might avoid the detection of the faecal signal by other species or by predators. In the sylvatic environment, *R. prolixus* is found in palm trees, which are also inhabited by vertebrates. The refuges consist of bird's nests comprising materials such as twigs, leaves or other organic objects. In addition, eggs of *R. prolixus* are also found attached to pigeons (Gamboa, 1963). The first constraint to leaving marks or signals of faeces near the access of shelters is that, because of the structure of the shelters in the sylvatic environment, there is no definite access. In addition, there is the fact that *R. prolixus* in the sylvatic environment use refuges in which they are in direct contact with their hosts, and so they do not need to move very far from these refuges to obtain their food. Even if the host is absent for some time, *R. prolixus* can survive long periods of starvation; therefore, they can wait inside a refuge until, eventually, a new host is located nearby (Gómez-Núñez, 1964).

The defaecation behaviour of *R. prolixus* described in the present study is similar to that shown for *T. infestans* (Lorenzo & Lazzari, 1996). Both species have the stereotypical movement of walking backwards before defaecation and then walking forwards afterwards.

The spatial pattern of defaecation of *R. prolixus* (Fig. 2) probably establishes a gradient from a high to a low concentration of aggregation signal from the refuge. This gradient would guide the insects towards the proximity of the refuge; once inside, the high concentration would indicate the presence of a refuge being used by conspecifics. In a wild habitat, entering into a wrong refuge or a refuge exploited by other species leads to a longer period of time remaining outside the shelter, increasing the risk of predation. This is consistent with the fact that *R. prolixus* does not have an interspecific aggregation response to faeces from other species such as *T. infestans* (Lorenzo Figueiras & Lazzari, 2002). It is demonstrated in the present study that *R. prolixus* responds differently to the aggregation signal of faeces of distinct post-deposition times (Fig. 4). These findings are consistent with studies in *T. infestans* (Lorenzo Figueiras & Lazzari, 2000) showing that, for this largely domestic species, faeces aged between 3 h and 10 days old are attractive for conspecifics. The activation, persistence and deactivation of the aggregation signal present in faeces of *T. infestans* might be explained by the fact that chemical composition of faeces changes with time (Mota *et al.*, 2014). At least five compounds can be identified in the faeces of *T. infestans*, *Panstrongylus megistus* and *T. brasiliensis*, with highly variable abundances throughout the period sampled (Mota *et al.*, 2014). The chemical composition of *R. prolixus* faeces remains to be determined. However, the existence of interspecific interactions (i.e. faeces of *R. prolixus* that promote aggregation of other species of triatomines) (Cruz-López *et al.*, 1993; Lorenzo Figueiras & Lazzari, 2002) suggests that at least some of the chemical compounds may be the same between species, and would have a common evolutionary origin. In accordance with the results of the present study on the temporal dynamics of the aggregation response to faeces in *R. prolixus*, an interesting hypothesis to test using chemical analysis and further behavioural experiments is whether there

is a compound that masks the active compound in the initial hours, which begins to evaporate gradually at 24 h, and then completely within 1 week (day 7 post-deposition). In this way, the possible compounds responsible for aggregation remain uncovered for at least 1 week, before losing their biological activity.

Regarding nutritional status, the borderline significant ($P = 0.0654$) statistical value obtained for the comparison of aggregation behaviour of fed and unfed nymphs suggests that there might be an effect of the nutritional status on the aggregation behaviour of *R. prolixus*. In a deeper analysis, when the degree of aggregation is analysed for each post-deposition time of faeces, the temporal window of aggregation is from 1 to 14 days for unfed insects and from 7 to 14 days for fed insects. Accordingly, the nutritional status of insects can produce changes in the detection threshold of the olfactory receptors or modify the motivational states affecting the responses of *R. prolixus*, including the degree of aggregation. The different tendencies to aggregate between fed and unfed insects might be a result of insects, with minor resources, having a strong tendency to remain aggregated in refuges in the absence of a host. On the other hand, fed insects appear to show weaker aggregation responses, which is curious at the very least considering that they would only need to wait in a quiet hiding place until their next moult. It cannot be excluded that different results would be obtained if a larger refuge was offered for the insects in the assays employed in the present study.

Finally, the results reported in the present study might be helpful for improving methods related to the detection and control of *R. prolixus*. Faecal spots over walls and crevices are often used to detect a domiciliary triatomine infestation. However, pest control operators should be careful when indicating the absence of a colony of *R. prolixus* from a house or other residence simply as a result of no faeces being observed because these marks may be inside refuges, and therefore not visible.

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