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Interaction between two aggregation chemical signals in *Triatoma infestans* (Hemiptera: Reduviidae)

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ABSTRACT

The nymphs and adults of *Triatoma infestans* spend much of their time aggregated among themselves within narrow and dark shelters. The search for a suitable shelter depends in part on the recognition of chemical signals coming from the feces and the cuticle of the other individuals who use the refuge. The aim of this study was determine the possible interaction between the chemical signals associated to the feces and to the cuticle of *T. infestans*. The results showed that the insects remained significantly more time on the feces that had contact with legs and the feces plus footprints than feces or footprints alone, demonstrating the interaction between evaluated signals. These results demonstrates also that feces extracted a chemical stimulus from the legs. Understanding the interaction feces-legs as an interaction feces-cuticle of legs, the results suggest that the feces could extract some cuticular compound with activity on the behavior of the insects. This is the first report of the interaction between the two aggregation signals recognized in *T. infestans* and of the increase in the behavioral response of insects exposed to feces that had contact with a cuticular structure.

1. Introduction

Triatoma infestans (Hemiptera: Reduviidae) is a hematophagous insect of the subfamily Triatominae and is the main vector of Chagas's disease in the southern of Southern Cone of South America. Although some studies reported the existence of sylvatic populations (Noireau, 2009), the habitat of this species is mainly the human dwelling in rural zones of the endemic area (Lent and Wygodzinsky, 1979). In dwellings, nymphs and adults of *T. infestans* spend a lot of time aggregated among them within narrow and dark shelters. These refuges temper external climatic variations and protect against predators. In addition, considering the presence of conspecifics, the shelter also facilitate the transfer of symbionts and the meeting of couples (Lazzari et al., 2013). Domiciliary and peridomiciliary areas of the dwellings offer a plethora of possible shelters, but not all them present the optimal environmental characteristics nor are inhabited by conspecifics. The search for a suitable shelter depends in part on the recognition of chemical signals coming from the feces and the cuticle of the other individuals who use the refuge (Lazzari et al. 2013; Barrozo et al., 2017).

The feces of *T. infestans* release aggregation signals and their pattern of distribution around the shelters suggests a role as chemical landmarks that guide insects to their refuges (Lorenzo and Lazzari, 1996; Lorenzo-Figueiras et al., 1994). The aggregation in *T. infestans* also occurs on surfaces previously transited by other bugs who seem to have deposited cuticular compounds through physical contact of the legs or body with the substrate. This signal was described as footprints (Lorenzo-Figueiras and Lazzari, 1998). The two aggregation signals show chemical and physiological differences. The fecal pheromone can be extracted by polar solvents, seems be composed by highly volatile compounds and is detected as an olfactory stimulus (Lorenzo-Figueiras et al., 1994; Lazzari et al., 2013). On the other hand, the cuticular pheromone is extracted by non-polar solvents, seems be composed of epicuticular lipids and is detected by contact chemoreceptors (Lorenzo-Figueiras and Lazzari, 1998; Lorenzo-Figueiras et al., 2009). Both signals also differ in the mechanism of orientation induced: the fecal signal induces a taxis acting as a true attractant factor while the behavior elicited by the cuticular signal would be a kinesis acting as an arresting factor.

Although both pheromones are associated with shelters, both spatially and in their supposed biological meanings, the possible interaction between them has not been studied. A possible interaction scenario has been proposed considering the orientation mechanisms associated with each signal. The olfactory signal released from the feces would attract the insects to the shelter; when they reach at the refuge, the

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insects would decrease their locomotor activity by contacting cuticular signal present on the surface of other insects or deposited on the substrate as footprints (Lazzari et al., 2013). On the other hand, Galvez-Marroquin et al. (2017) identified the chemical composition of feces of *T. dimidiata* that promoted conspecific aggregation and detected that the volatile extract from feces present hydrocarbons that are part of the cuticle of this species as reported by Juárez et al. (2002). Moreover, one of these hydrocarbons (i.e. *n*-tricosane) elicited aggregation when evaluated individually. Galvez-Marroquin et al. (2017) briefly discusses the possible origin of the compounds present in the excreta and focuses mainly on the metabolic activity of symbiotic microorganisms. However, the fact that feces were collected on a filter paper placed under a vessel containing a group of insects (Galvez-Marroquin et al., 2017) allows to speculate that the feces might have acquired epicuticular hydrocarbons when they contacted the cuticle of other insects. In this context, other interaction scenario can be proposed in which the feces could acquire cuticular lipids when they contact the cuticular surface, and then both chemical signals would be deposited together. Once deposited, they could act sequentially as suggested in the first scenario.

The aim of this study was to determine the possible interaction between the chemical signals associated to the aggregation behavior in *T. infestans*. In particular, the hypothesis that feces acquire a greater effect on the behavior of insects when they contact the cuticle was evaluated. For this, the present research determined the behavioral response of *T. infestans* to feces, footprints, feces plus footprints and feces having had contact with cuticle.

2. Materials and methods

2.1. Insects

The insects used were laboratory-reared descendants from field insects collected from 25 de Mayo locality, department of Quitilipi, Chaco, Argentina. The insects were reared and maintained for two generations in the Centro de Referencia de Vectores (CeReVe) (Santa María de Punilla, Córdoba, Argentina) under controlled conditions (26 ± 1 °C, 60 ± 10% RH and 12:12 h L:D) and were fed fortnightly.

2.2. Experimental device

The behavioral assays were performed in a circular glass arena (15 cm diameter) with the floor covered with filter paper and divided in two equal sectors. A piece of filter paper (0.5 × 0.5 cm) impregnated with the stimulus to be tested (experimental paper) or clean (control paper) supported on a coverslip was placed in each sector. The position of the stimulus was reversed in every replicate to control biases due to external asymmetries. The arena was inside an experimental room (28 × 35 × 27 cm) at 23 ± 1 °C and 37 ± 10% RH and kept dark. The behavior of the insects was recorded by means of an infrared videocamera (AHD-318Q-T) connected to a monitor.

2.3. Evaluation of behavioral response

One fifth-instar nymph was gently located in the center of the arena and after 5 min of the acclimation the insect was liberated and could walk freely. The insect was observed for 10 min and the time spent in each zone was registered. Between replicates, the materials were carefully cleaned with ethanol (70%) and the filter papers were replaced to eliminate putative cues deposits by the insects. The assays were conducted using nymphs between 10 and 20 days old and fasted since last molt. Each insect was evaluated in their scotophase and was used only once.

2.4. Experimental series

Four one-way series (i.e. stimulus vs control) were performed: feces vs control, feces with legs vs control, footprints vs control, and feces with footprints vs control. Three two-way series (i.e. stimulus vs stimulus) were performed: feces vs feces with legs, feces vs feces with footprints, and feces with legs vs feces with footprints. A control series (i.e. control vs control) was performed to test whether the arena and surrounding environment were symmetrical. We performed 30 trials per series.

2.5. The stimuli

2.5.1. Feces

Fecal droplets were obtained of fifth-instar nymph by slight compression of the last section of the abdomen with a forceps. Feces from 15 insects were collected in a plastic tube (1.5 ml), maintained with the tube closed and used 24 hr later in a liquid state. This time interval guarantees their aggregating activity (Lorenzo-Figueiras et al., 1994). Any contact between the insect with the plastic tube or the fecal droplets with legs was avoided to exclude other potential cues. The insects were fed between 10 and 20 days after molt and two days before the experiment. The experimental filter papers were impregnated with 2 µl of the collected feces while control papers were impregnated with 2 µl of distilled water.

2.5.2. Feces with legs

Second and third pair of legs obtained from fifth-instar nymph died 24 hs before were immersed for 24 hs in feces obtained following the procedure described above (see Feces). Two microliters of these feces were used to impregnate the experimental paper while 2 µl of distilled water was used to impregnate the control papers.

2.5.3. Footprints

A group of 5 fifth-instar nymph with their anus completely occluded were allowed to walk freely on a piece of filter paper (9 cm diameter) placed inside of a glass container (9 cm diameter). After 24 h, these papers were examined to confirm the absence of fecal spots and were cut into pieces of 0.5 × 0.5 cm for the experiments. Clean filter paper was used as control. The occlusion of the anus was performed with water-based correction fluid (Aqua fluid, Berol, Newell Rubbermaid, Argentina S.A) and verified using a stereomiscroscope (Nikon SMZ745). The insects were between 10 and 20 days old and were fasted since molt.

2.5.4. Footprints with feces

Pieces of filter paper (0.5 x 0.5 cm) with footprints obtained following the procedure described above (see Footprints) were impregnated with 2 µl of feces obtained following the methodology described in the section Feces. The control papers were impregnated with 2 µl of distilled water.

2.6. Statistical analysis

The time that the insects remained in one zone was compared with the theoretical value assuming a random distribution (i.e. 5 min) using the one-sample Wilcoxon test. The differences were considered statistically significant if $P < 0.05$. A Wilcoxon two-sample test was used to compare the treatments that presented significant difference with their control. The Bonferroni correction was applied to adjust the significance level for each of the comparisons made, thus, the differences were considered statistically significant if $P < 0.017$ (overall α level = 0.05). The non-parametric test was used as the data did not follow a normal distribution according to the Shapiro-Wilk test (Sokal and Rohlf, 2009). The data were analyzed with the MINITAB Statistical Software, version 17 (Minitab Inc., PA, U.S.A.).

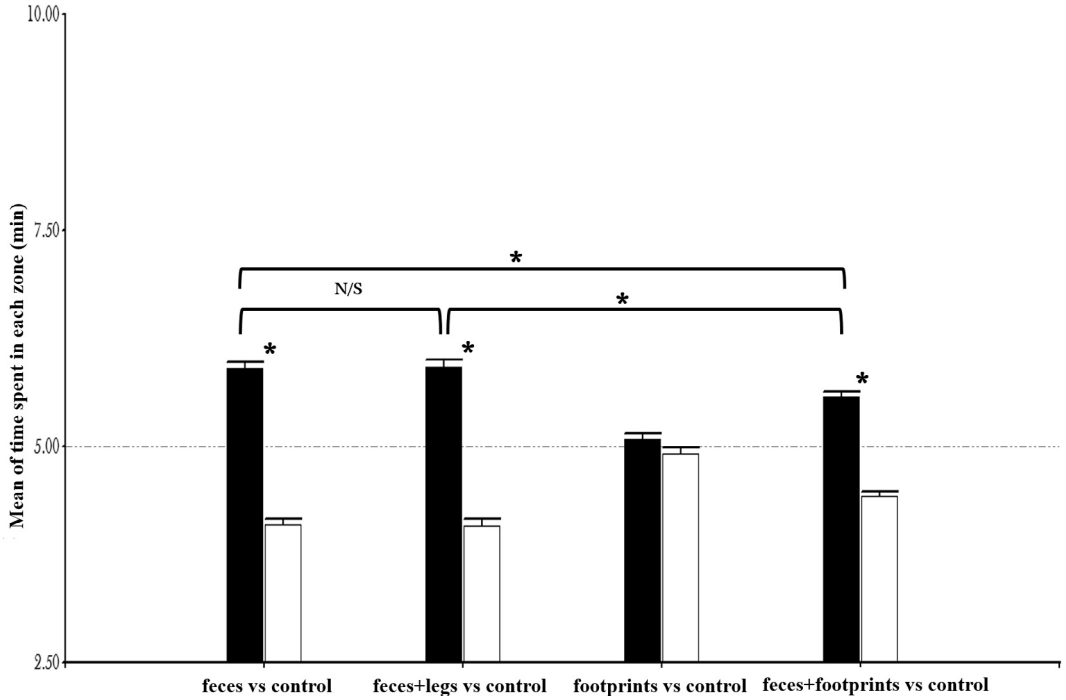

Fig. 1. Time spent by the fifth-instar nymphs of *Triatoma infestans* in each side of the one-way series (i.e. stimulus vs control). Asterisks represent significant differences within each series (Wilcoxon test) and between the treatments that presented significant difference with their control (Wilcoxon two-sample test). Dashed line indicates the expected value from a random distribution.

2.7. Ethics statement

The chicken were cared and handled in accordance with resolution 1047/2005 of the National Council of Scientific and Technical Research (CONICET) regarding the National Reference Ethical Framework for Biomedical Research with Laboratory, Farm, and Nature Collected Animals; and the National Law 14346 regarding Animal Welfare.

3. Results

The results obtained in control series demonstrated that experimental arena did not reveal spatial asymmetries ($W = 169$, $P = 0.299$). Fig. 1 shows the results obtained for the four one-way series (i.e. stimulus vs control). The results showed that insects remained significantly more time on the side that contained the feces ($W = 465$, $P < 0.0001$), feces with legs ($W = 465$, $P < 0.0001$) and feces with footprints ($W = 462$, $P < 0.0001$) than on the control side. In contrast, a random distribution was observed when the footprints was evaluated ($W = 298$, $P = 0.181$). In addition, the treatments that showed significant differences with their respective control were compared. These results showed that the feces with footprints evoked a significantly lower response than the feces ($W = 1142$, $P = 0.0008$) and the feces with legs ($W = 1139$, $P = 0.001$). In contrast, there was not significant difference between feces and feces with legs ($W = 915$, $P > 0.05$) (Fig. 1).

Fig. 2 shows the results obtained for the three two-way series (i.e. stimulus vs stimulus). The insects remained significantly more time on the side that contained the feces with legs ($W = 465$, $P < 0.0001$) or the feces with footprints ($W = 0\ 461$, $P < 0.0001$) than on the feces side. Finally, the insects spent significantly more time on the feces with legs than on the feces with footprints ($W = 121$, $P = 0.02$).

4. Discussion

The present study determined the behavioral response of fifth-instar nymphs of *Triatoma infestans* to the combination of two aggregation signals. The research showed that the feces that had contact with legs and the feces with footprints were able to promote greater behavioral response as compared to feces and footprints. This is the first work that reports the interaction between the two aggregation signals recognized in *T. infestans* and the increase of the behavioral effect of feces after contact with a cuticular structure.

The higher responses to feces with footprints and feces previously exposed to legs as compared to pure feces were results compatible with the joint exposure to two active stimuli. This was expectable for feces with footprints due that both stimuli are demonstrated aggregation signals for triatomines. The feces were reported in *T. infestans* and other triatomines as a signal with intra and interspecific activity (Schofield and Patterson, 1977; Cruz-López et al., 1993; Lorenzo-Figueiras and Lazzari, 1998, 2002; Lorenzo-Figueiras et al., 1994; Pires et al., 2002; Vitta et al., 2002, 2007). On the other hand, Lorenzo-Figueiras and Lazzari (1998) demonstrated that *T. infestans* aggregates on substrates transited by conspecifics and suggested their cuticular origin. Subsequent studies demonstrated the same behavior in other triatomines (Pires et al., 2002; Vitta et al., 2002). In contrast, although the result obtained with feces exposed to legs was the prediction derived from the hypothesis under evaluation (i.e. the feces acquire a greater effect on the behavior of the insects when they contacts the cuticle), it was not necessarily expected since the presence of a second stimulus was unknown. Thus, this result is the key observation of the present study because demonstrates that the feces enhanced their effect when contacted with legs. As the legs were removed before the behavioral assay, the result demonstrates also that the feces extracted a chemical compound from the legs, or the legs added a compound into the feces, that increase the behavioral response in the bugs. So, the insects were exposed to two signals: the feces and a substance coming from the legs. Finally, if the interaction feces-legs is understood as mainly an interaction feces-cuticle of legs, the results suggest a new hypothesis: the feces of *T. infestans* are able to extract cuticular compounds with behavioral effect.

The possible ability of the feces of *T. infestans* to extract cuticular compounds increasing their behavioral effect is a hypothesis that is

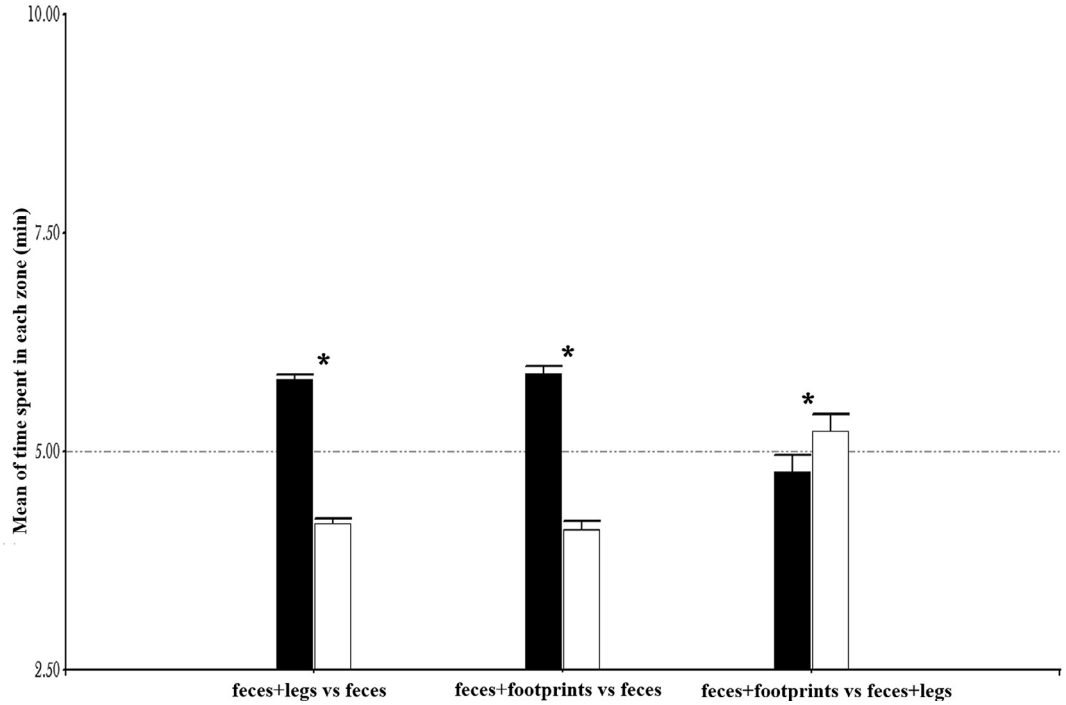

Fig. 2. Time spent by the fifth-instar nymphs of *Triatoma infestans* in each side of the two-way series (i.e. stimulus vs stimulus). Asterisks represent significant differences within each series (Wilcoxon test). Dashed line indicates the expected value from a random distribution.

constructed from following information: 1) The cuticular lipids are the suggested active components of the footprints of *T. infestans*. Lorenzo-Figueiras et al. (2009) showed that the free fatty acids fraction from epicuticular lipid extract, and two components of this fraction (i.e. octadecanoic acid and hexacosanoic acid), promoted aggregation in *T. infestans* suggesting the possible chemical base of the footprints landmarks. 2) Hydrocarbons promoting aggregation are present in feces and cuticle of *T. dimidiata*. Galvez-Marroquin et al. (2017) detected hydrocarbons in the feces of *T. dimidiata* and showed that three of them (i.e. *n*-octadecane, *n*-nonadecane and *n*-tricosane) elicited aggregation when evaluated individually. Interestingly, some of these hydrocarbons, including *n*-tricosane, were also detected in the epicuticle of *T. dimidiata* (Juárez et al., 2002). 3) The feces of the triatomines could extract lipids compounds. The feces of the triatomines are composed of urine and remnants of digested/undigested blood (Lehane, 2005). As blood of birds (i.e. the food provided in this study) and its remnants in the intestine of the triatomine have lipid compounds (Wainszelbaum et al., 2003; Lehane, 2005), then it is possible to speculate that this non-polar fraction of feces could extract hydrocarbons from the cuticle. 4) Finally, the results of the present study showed that the legs, a mainly cuticular structure, added a factor to the feces that increased their activity. Thus, taking into account the recently reviewed information and the results of this study, the possible extraction of attractant cuticular lipids by the feces is quickly deduced.

It is important to consider that if the feces are able to obtain lipids from the cuticle, this could occur within the proctodeum as it is lined by cuticle. In this way, two theoretical scenarios would be compatible with the possibility that the cuticle of the legs, or other external structure, increases the activity of the feces: the cuticle of the legs increases the amount of compounds provided by the proctodeal cuticle or the cuticle of the legs is different from that of the rectum and adds a compound that does not provide the rectum. It is not difficult to speculate with differences in the cuticles that interact with different external media. In this sense, Schmidt et al. (1998) did not mention the cement layer in the rectal cuticle of *Triatoma infestans* but this layer is present in the cuticle that covers the external structures of the triatomines (Juárez and Fernández, 2007). Therefore, both cuticles appear to be different and each one could contribute different components to the feces. Finally, we do not rule out the possibility that the factor contributed by the legs comes from a different source to the cuticle, e.g. rest of hemolymphatic material.

The previous studies demonstrated that the two aggregation signals in triatomines are detected by different sensory modality and promote different mechanism of orientation. The fecal pheromone acts as olfactory stimulus and induces a taxis (Lorenzo-Figueiras et al., 1994; Lazzari et al., 2013) while the cuticular pheromone is detected by contact chemoreceptors and the behavior induced would be a kinesis (Lorenzo-Figueiras and Lazzari, 1998; Lorenzo Figueiras et al., 2009). Thus, the feces act as an attractant factor while the cuticular signal acts as an arresting factor (Lorenzo-Figueiras et al., 1994; Lorenzo-Figueiras and Lazzari, 1998). Considering these differences, the following sequence could describe the behavior of the insects in the experimental series where both signals were together as a treatment: the insects are attracted by the feces towards the zone where both signals are deposited, when the insects arrive at that zone they are arrested by the contact with the cuticular signal. This sequence could occur when the two pheromones were deposited in the same area but independently (i.e. the treatment “feces with footprints”) and when they were possibly deposited together as part of the feces (i.e. the treatment “feces with legs”). In the nature, this situation could occur in the context of seeking refuge (Lazzari et al., 2013). The olfactory fecal signal would guide the insects towards the shelter; once around or inside the refuge, the insects would decrease their locomotor activity by the contact with the cuticular signal present in the integument of other insects, deposited on the substrate as footprints or extracted by feces and deposited together.

In triatomines, only one study investigated how the aggregation behavior of *T. infestans* is modulated by the interaction between chemical signals present in its feces and another stimulus detected by a different sensory modality, in this case visual signal coming from spectral lights (Reisenman et al., 2000). The authors showed that the behavioral response of insects depended on specific combinations of the signals evaluated. On the other side, analysis of hydrocarbons from anal fluids involved in nest marking by the ant *Messor capitatus* (Grasso et al., 2005) suggests that territory marking using secretions issued from the

rectal sac may be another labeling function by the way of abdominal secretions. The same authors reported that the hydrocarbons in the feces were similar to those hydrocarbons on the cuticle of workers. In general, the primary function of the hydrocarbons is to protect the insect from desiccation. Secondarily, they play an important role when interacting with other cues in many species for intra and interspecific communication such as species and gender recognition, fertility signaling, chemical mimicry and aggregation (Breed, 1998; Howard and Blomquist, 2005; Vaidyanathan and Feldlaufer, 2013).

The sensory modalities involved in the reception of fecal and cuticular signals in conjunction with the experimental conditions would explain the random distribution in the series "footprints vs control" in comparison with the series "feces vs control" and "footprints plus feces vs feces". The feces act at a distance and the concentration would be the main determinant of its behavioral action. The distribution of time spent by insects in each area of the "feces vs control" series (i.e. different from chance) shows that the concentration of the fecal signal was enough to attract the insects. In addition to the concentration, the footprints must be contacted by the insects to exert its action, and this contact depends on chance if other factor is not present. The probability of the contact insect-footprints decrease when the area where footprints are deposited also decrease. Thus, when this area is below a threshold, the insect should show a random distribution. So, the random distribution in the "footprints vs control" series could be due to the fact that the concentration was very low or that the area with the cuticular signal was very small. However, the results obtained in the "feces plus footprints vs feces" series (i.e. greater behavioral effect of the combined treatment than feces) demonstrated that the concentration of footprints was sufficient to exert action. In this series, when the contact signal was deposited together with the attractant odor, the contact of the insect with the first signal no depended on chance but was favored by the presence of the odor that acted as a guide. Once contacted, the footprints arrested the insects. Therefore, the non-effect of the footprints was probably because the area where were deposited was very small. This could also explain the differences between "footprints vs control" series and the previous studies that demonstrated the aggregation effect of the footprints in *T. infestans*. Lorenzo-Figueiras and Lazzari (1998) used pieces of filter paper with footprints of 3 x 3 cm in an experimental arena of 13 cm diameter, while Lorenzo-Figueiras et al. (2009) used pieces of filter paper of 2 × 3 cm in an experimental arena of 12 cm diameter. In the present study, the area with footprints was smaller (0.5 × 0.5 cm) and occupied a considerably smaller portion of the experimental arena (15 cm diameter) than in the previous studies, suggesting that the dimensions of the experimental arenas explain the distribution pattern of the insects (i.e. at random or not).

A previous study demonstrated that the attractant activity of the feces depends on the time elapsed since its deposition, i.e. of the ageing of the feces (Lorenzo Figueiras and Lazzari, 2000). The authors showed that fresh feces (i.e. recently deposited) did not promote aggregation in *T. infestans* while dry feces (i.e. between 3 h and 10 days after deposition) induced the aggregation behavior. In this context, it is possible to ask if the acquisition of attraction may be due to the drying/evaporation that occurs naturally while the time passes or to some other process associated with time but independent of drying/evaporation. Although the present work did not aim to answer this question, the results showed that the feces were attractive 24 h after deposition (i.e. aged), in a liquid state (i.e. not dried) and kept in airtight tubes (i.e. with minimal evaporation), which suggests that the attractiveness of feces depends on some process associated with aging but different from drying/evaporation

5. Conclusion

The behavior of *T. infestans* against feces in contact with legs or with footprints described in this study shows the interaction of the two aggregation signals recognized in this species and suggest a possible role in the natural context (e.g. search for refuge). In addition, the present research showed that the feces that had contact with legs promoted a greater behavioral response than the feces alone demonstrating that the feces extracted an active compound from legs, presumably a cuticular component. However, further research is needed to answer whether the feces actually extract cuticular lipids and to determine the compounds present in feces in contact with legs that promote aggregation in *T. infestans*.

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Declarations of interest

None.

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