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Filling dynamics of the Brindley's glands in the blood-sucking bug *Triatoma infestans* (Hemiptera: Reduviidae)

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ABSTRACT

The filling dynamics of exocrine defensive glands is an important component of the defensive capacity of an insect in its natural environment. We studied the filling state and reloading rate of the Brindley's glands in the haematophagous Chagas disease vector *Triatoma infestans* (Hemiptera: Reduviidae). Quantitative analyses of isobutyric acid, the main secretion component, were carried out with glands dissected from adults under different scenarios of development, number of discharging events and feeding conditions. The alarm-pheromone function of the gland secretion was also assessed in bioassays with conspecific nymphs. Although pharate adults have their glands completely developed, these were not full until imaginal ecdysis. If kept undisturbed, the adults maintained a constant gland load, and discharged about 75% of the gland contents upon one disturbance event. While the glands can be discharged several times, full replenishing was not complete after one week, unless the insect had access to food. The escape behavior of nymphs in bioassays correlated with the chemical analyses, with nymphs showing significant avoidance only toward gland discharges from undisturbed or disturbed/fed adults. The results are discussed in reference to the feeding frequency and gregarious behavior of *T. infestans* under natural conditions, which suggest a relevant role of the filling dynamics of the Brindley's glands in the intraspecific communication of the insect.

1. Introduction

Exocrine glands are widespread among insects, and their products are used in different behavioral and ecological contexts, including sexual communication, defense, alarm and aggregation. Most Reduviidae have several exocrine glands in the thorax and abdomen, namely the Brindley's glands, metasternal glands, dermal glands, ventral glands and abdominal glands (Carayon et al., 1958; Staddon, 1983; Weirauch, 2006). In the subfamily Triatominae, ventral and abdominal glands are apparently absent, and only adult insects possess both the Brindley's and metasternal glands (Brindley, 1930; Schofield and Upton, 1978; Staddon, 1983).

The secretion from the metasternal glands contains several compounds, some of which are highly volatile aliphatic ketones, alcohols and acetals (Rossiter and Staddon, 1983; Manrique et al., 2006; Pontes et al., 2008; Vitta et al., 2009; Unelius et al., 2010; Bohman et al., 2011; Manrique and Lorenzo, 2012). It has been shown that these glands mediate sexual communication between adults of *Triatoma infestans*, *Rhodnius prolixus*, and *T. brasiliensis*, although there is no clear understanding of which compounds are responsible for such activity (Manrique et al., 2006; Crespo and Manrique, 2007; Pontes et al., 2008; Vitta et al., 2009; Zacharias et al., 2010). The Brindley's glands are located dorsally, extending into both sides of the second abdominal segment and opening into the metathoracic epimeron (Brindley, 1930; Kälin and Barrett, 1975; Staddon, 1983). Short chain acids, alcohols, esters, and a ketone have been reported as components of the Brindley's glands secretion of *T. infestans* males and females, with isobutyric acid as the main compound (*ca.* 35% of the blend, Manrique et al., 2006). The development of the Brindley's glands occurs during the fifth nymphal instar, and apparently ends just before adult ecdysis (Millen et al., 1979). These glands are discharged after mechanical disturbance, and their function is hence regarded as defensive, either as chemical defense, alarm pheromone, or both (Schofield, 1979; Ward, 1981; Cruz López et al., 1995; Rojas et al., 2002; Manrique et al., 2006). In *T. infestans*, for instance, adult insects emit volatile compounds from the Brindley's

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glands upon mechanical disturbance, and these volatiles trigger an escape response in the nymphs (Manrique et al., 2006).

While the chemistry and antipredatory function of exocrine defensive secretions have been thoroughly studied in countless insects, studies about the filling dynamics of tegumentary glands involved in chemical defense are rather scarce (Baldwin et al., 1990; Kearsley and Whitham, 1992; Whitman et al., 1992; Rossini et al., 1997). This, however, represents an important component of the defensive capacity of an insect in a natural environment and provides a broader picture of the adaptive value and cost of chemical defense in insects. Specifically in triatomines, Kälin and Barrett (1975) suggested that *R. prolixus* recover the capacity for a second scent release within 5 h after a prior disturbance. Moreover, even when they were disturbed on a daily basis, the insects were apparently able to release the Brindley's gland secretion. These results, however, were based on qualitative measures such as visual observation and smell detection of the secretion.

In this study, we report the filling dynamics of the Brindley's glands in *T. infestans*, both before and after the imaginal ecdysis, after single or multiple discharging events, and under different feeding conditions. Specifically, we quantified isobutyric acid, the main component of the Brindley's gland secretion, in glands from: (1) pharate adults inside fifth-instar nymphs, just before molting; (2) adults just emerged from imaginal ecdysis; (3) adults at different periods after a single discharge; (4) adults after several discharges; and (5) fed and unfed adults after a single discharge. In addition, we performed behavioral bioassays to evaluate the alarm response of nymphs when exposed to the secretion of adults after different feeding conditions.

2. Materials and methods

2.1. Insects

T. infestans were obtained from the Servicio Nacional de Chagas of Argentina and reared in the laboratory at 28 ± 1 °C, 30–60% RH, under a 12:12 h L/D photoperiod, with live hens as a blood meal source. Animals 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. All insects used for gland dissections were fed once a week until ecdysis of the fifth instar and were not fed as adults unless otherwise indicated. Fed adults were offered a single meal after their last experimental disturbance (see below).

To avoid eventual disturbances leading to gland discharges, all experimental insects were kept individually from their fifth nymphal instar in flasks (10 × 10 cm) lined at the bottom with a piece of filter paper (5 × 5 cm). Prior to dissecting the glands, the insects were chilled at 0 °C for 10 min in an ice-bath. For triggering a discharge, each leg was sequentially grabbed with forceps, completing 1 min of disturbance. Behavioral experiments were performed with fourth instar nymphs that had been starved for 10–25 days after molting. Insects were used once and then discarded.

2.2. Treatment groups

The contents of the Brindley's glands were compared among insects from different treatment groups as follows: (a) undisturbed pharate adults just before imaginal ecdysis ($N = 11$); (b) undisturbed newly (up to 3 h) molted adults before ($N = 10$) and after cuticle hardening ($N = 17$); (c) undisturbed 1-month old adults ($N = 7$); (d) unfed adults that discharged their glands once, dissected 3 h ($N = 14$), 24 h ($N = 9$) and 1 week ($N = 9$) after disturbance; (e) unfed adults that discharged their glands three ($N = 9$), four ($N = 9$) or five ($N = 6$) times on a weekly basis, all dissected one week after the last disturbance; (f) fed adults ($N = 9$) that discharged their glands weekly for three weeks, dissected 1 week after the last disturbance.

Pharate adults were identified as unsclerotized pink cuticle can be seen by transparency inside fifth instar nymph cuticle.

2.3. Brindley's gland dissection

The insects were chilled and affixed to dissecting dishes with modeling clay, leaving their thorax and abdomen exposed ventrally. This chilling process was previously used and showed to prevent the discharge of glands' content (Manrique et al., 2006; Vitta et al., 2009). Besides, the glands were dissected with microsurgical scissors under ice-cold Ringer's solution (Case, 1957) avoiding glands' release during the dissection procedure. Lateral incisions were performed from the 2nd abdominal sternite up to the thorax, and a median incision was cut along the abdomen to remove the ventral cuticle, thereby exposing the glands. These were then excised and placed individually into a glass vial (2 mL) with 0.5 mL of dichloromethane (Merck Química, Argentina). The vials with glands and solvent (gland extracts) were immediately stored at −20 °C until chemical analyses, which were conducted no later than 48 h after gland dissections.

2.4. Gland extraction and analysis

The degree of filling of the Brindley's glands was assessed by measuring their isobutyric acid contents. Prior to the analysis, the gland extracts were sonicated (Branson 200, Taiwan) for 10 min, and with no further purification they were injected in a gas chromatograph (GC). The analyses were performed on a HP 5890 Series II Gas Chromatograph, equipped with a polar fused silica capillary column (DB-WAX, 30 m × 0.25 mm id, 0.25 μm film thickness, J & W Scientific, USA). Hydrogen was used as carrier gas (1 mL/min) and injections (1 μL) were made in the splitless mode. The injector and detector (FID) temperatures were 220 °C and 250 °C, respectively, and the column oven was heated from 40 °C (4 min) up to 250 °C at a rate of 7 °C/min. The injection procedure of gland extracts did not block the capillary column because we injected the solvent of the upper part of the vial assuming that the solid residues of the gland extracts remained at the bottom of the vial.

The isobutyric acid contents were determined from a calibration curve ($r^2 = 0.9992$) obtained from peak areas of standard solutions of isobutyric acid (Fluka, Germany) in dichloromethane, with concentrations of 4, 10, 20, 50 and 100 ppm. The standard solutions were injected under the same conditions described for the gland extracts. The identification of isobutyric acid in the gland extracts was confirmed by comparison of the retention time and mass spectrum of the chromatographic peak in the gland extract, with those of the synthetic standard [diagnostic ions (%): 88 (M^+, 10), 73 (42), 71 (3), 60 (5), 45 (14), 43 (100), 42 (14), 41 (52), 39 (16)].

2.5. Alarm behavior bioassays

When exposed to volatiles released by disturbed adults, nymphs show increased locomotion activity and escaping response (Manrique et al., 2006), indicating that these volatiles function as an alarm pheromone in *T. infestans*. The degree of filling of the Brindley's glands may have an effect on their biological function. Therefore, gland volatile emissions from adults with different treatments (disturbed/undisturbed; fed/unfed) were evaluated in regards to the alarm response they elicited on nymphs.

Adult odors were tested on fourth instar nymphs using an experimental arena without air current (Minoli et al., 2013). It consisted in a rectangular acrylic arena (15 × 10 × 4 cm) covered

by a transparent lid, with two holes on the floor (diam. 2.5 cm). This arena was attached to a lower chamber (15 × 10 × 1 cm) that was divided into two equal sections by an odor-impermeable transversal plate. Each section of the lower chamber was in turn communicated to a flask (10 mL) screwed through an opening in its floor, which contained the odor source.

The odor flasks contained one live *T. infestans* adult, and the dyads tested in the bioassays were as follows: (a) undisturbed adult placed in each flask (control experiment); (b) unfed adult disturbed for the first time just prior to the bioassay *vs.* an undisturbed adult; (c) unfed adult disturbed for the fourth time (weekly) just prior to the bioassay *vs.* an undisturbed adult; and (d) fed adult disturbed for the fourth time (weekly) just prior to the bioassay *vs.* an undisturbed adult. The treatment (a) (control experiment) was conducted to discard any spatial heterogeneity due to the experimental design used. As this control did not differ from a random distribution (see Results) the results obtained could be assigned to the effect of the tested odors.

The walking area in the arena was covered with filter paper cut with holes that matched those on the floor to allow diffusion of odors. The paper was exchanged after every replicate to avoid any chemical cue left by the tested nymphs. The bioassay was done with individual fourth instar nymphs, which were placed in the arena immediately after screwing the flasks with the volatile stimuli. The nymphs were placed in the middle of the arena and left covered with a flask for 1 min, after which it was released and its behavior registered during 4 min by means of a videocamera connected to a digital recorder.

To asses differences between experimental dyads, the time spent in each side of the arena was registered, and a preference index (PI) ranging from −1 to 1 was calculated as PI = (*T* − 120)/120, where *T* is the time in seconds spent in the side of the arena opposite to the undisturbed adult, which served as control. Therefore, PIs near −1, 0 or 1 indicate repellence, no effect, or attraction to the tested stimulus, respectively. The assignment of the odor source to each flask was reversed after every replicate to eliminate directional bias. The experiments were conducted under 50 ± 10% RH, 25 ± 1 °C, during the first 5 h of the scotophase, when *T. infestans* show maximal activity (Lazzari, 1992).

2.6. Statistical analysis

The amount of isobutyric acid present in the Brindley's glands of insects from different treatment groups were registered and expressed in relation to the amount found in the Brindley's glands of undisturbed, newly-molted adults after cuticle hardening. This amount (henceforth referred to 1 adult-equivalent or 1 AEq) was regarded as the average value representing full adult glands. Differences between the AEq of each treatment group and the content of full glands (i.e., AEq = 1) were analyzed by One-Sample *t*-tests. Differences between two or more treatments were assessed by comparing the absolute value of isobutyric acid present in the glands by means of *t*-tests or One-way ANOVA followed by Fisher's multiple comparisons. The orientation responses in the behavioral bioassays (PIs) were assessed by means of One-Sample *t*-tests (Ho: PI = 0) (Zar, 1996).

3. Results

3.1. Brindley's gland contents in undisturbed adults and the relationship with adult development

The isobutyric acid contents of the Brindley's glands of undisturbed adults just after cuticle hardening was 5.7 ± 0.7 μg/gland (mean ± SE, *N* = 17). Taking into account that each adult presents a pair of Brindley's glands, we estimated 11.4 μg as the value of one adult-equivalent (1 AEq, shown in Figs. 1–4 with a dotted line).

The gland contents of pharates, newly-molted adults with soft cuticle, and one-month old adults, are shown in Fig. 1. When compared to 1 AEq, glands from pharates contained significantly lower isobutyric acid (0.2 AEq, $P < 0.05$, *t*-test), while no differences from 1 AEq were found for newly-molted unsclerotized adults or one-month old adults ($P > 0.05$, *t*-test). The isobutyric acid contents were also different among treatment groups (One-way ANOVA, $P < 0.05$), with glands from pharates containing significantly lower isobutyric acid than newly-molted adults with soft cuticle ($P = 0.031$, Fisher's test). Gland contents from undisturbed one-month old adults were intermediate, and did not show significant differences with either group ($P > 0.05$, Fisher's test).

3.2. Gland reloading after a single disturbance

Gland discharge significantly affected the isobutyric acid available in the glands for future discharges (One-way ANOVA, $P < 0.05$, Fig. 2). A single discharge decreased on average 75% of the gland contents, from 5.7 ± 0.7 μg/gland found in undisturbed adults (1 AEq), to 1.5 ± 0.2 μg/gland (mean ± SE, *N* = 14) found 3 h after

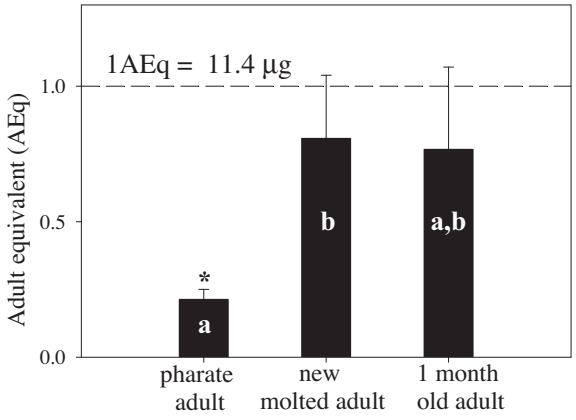

Fig. 1. Isobutyric acid contents (relative to 1 AEq) in glands from undisturbed adults at different developmental stages. Error bars indicate SE, asterisks indicate a significant difference with 1 AEq (*t*-test, $P < 0.05$). Different letters within bars represent significant differences among groups (One-way ANOVA followed by Fisher's Test, $P < 0.05$).

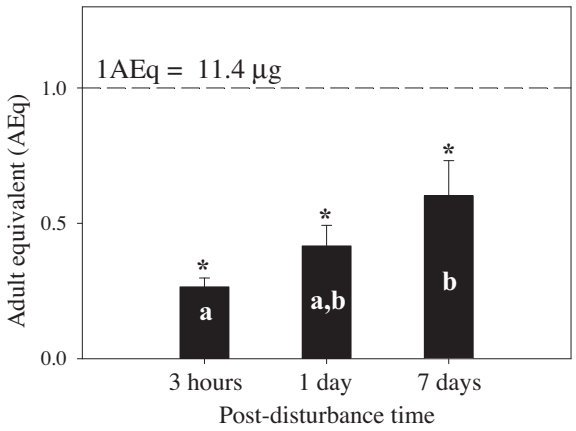

Fig. 2. Isobutyric acid contents (relative to 1 AEq) in glands from once-disturbed adults, dissected 3 h, 1 day or 7 days after the disturbance event. Error bars indicate SE, asterisks indicate a significant difference with 1 AEq (*t*-test, $P < 0.05$). Different letters within bars represent significant differences among groups (One-way ANOVA followed by Fisher's Test, $P < 0.05$).

the discharge ($P < 0.05$, t-test, Fig. 2). While one week was not enough to recover the initial (1 AEq) isobutyric acid contents ($P < 0.015$, t-test, Fig. 2), the gland contents one week after a single discharge were significantly higher than 3 h after disturbance (Fisher's test, $P < 0.01$). The gland contents one day after the discharge were intermediate, indicating that gland reloading occurs gradually.

3.3. Brindley's glands contents after several disturbances

Multiple gland discharges significantly reduced the contents of the Brindley's glands (One-way ANOVA, $P = 0.022$, Fig. 3). Glands that were discharged three or more times presented lower amounts of isobutyric acid than those discharged only once, even considering that one week was allowed for gland recovery after the last disturbance. Indeed, when comparing gland contents in adults disturbed one, three, four or five times (weekly), a significant progressive decrease in isobutyric acid can be observed (Fisher's test, $P = 0.002$, Fig. 3).

3.4. Nutritional status and gland reloading

Feeding had a significant effect in the insect's capacity to reload the Brindley's glands (t-test, $P = 0.001$, see different letters in Fig. 4). While the isobutyric acid contents of unfed adults that discharged their glands three times were significantly lower than 1 AEq (Fig. 4), fed adults under the same conditions (fed after the last disturbance event) fully recovered the original (1 AEq) isobutyric acid contents (t-test, $P > 0.05$).

3.5. Alarm behavior bioassays

The behavioral bioassays showed that nymphs respond differently when exposed to volatiles from adults with different histories of disturbance and feeding (Fig. 5). The preference index (PI) of nymphs, which expresses the relative time spent in each side of the arena, was not significantly different from zero when the nymphs were exposed to odors from two undisturbed adults (control experiment, treatment *a* in Section 2.5, Fig. 5). The nymphs significantly avoided the side of the arena containing volatiles from an unfed when it was disturbed for the first time (treatment *b*), but such response was not observed when the adults had been disturbed weekly for four weeks, and remained unfed during that period (treatment *c*). Finally, volatiles from adults that had been

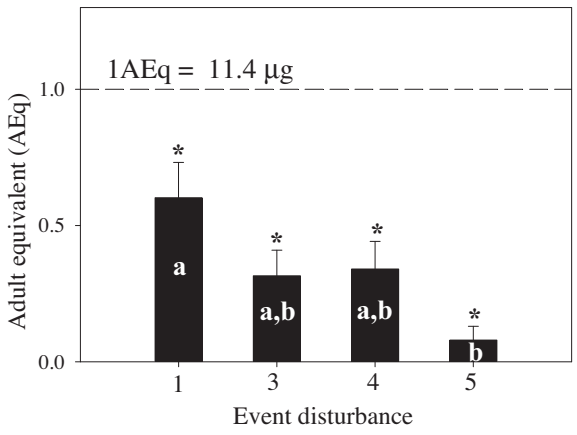

Fig. 3. Isobutyric acid contents (relative to 1 AEq) in glands from adults disturbed one, three, four or five times, on a weekly basis. Error bars indicate SE, asterisks indicate a significant difference with 1 AEq (t-test, $P < 0.05$). Different letters within bars represent significant differences among groups (One-way ANOVA followed by Fisher's Test, $P < 0.05$).

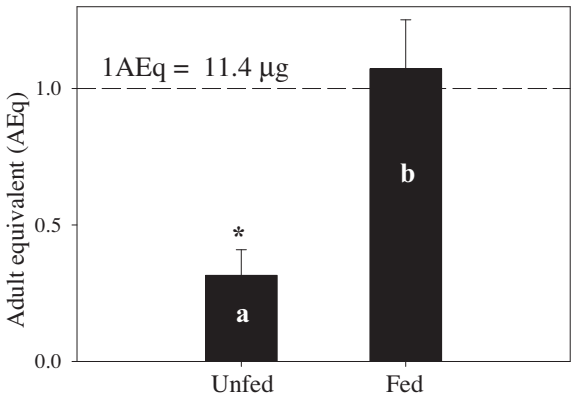

Fig. 4. Isobutyric acid contents (relative to 1 AEq) in glands from adults disturbed three times, according to their feeding status. Error bars indicate SE, asterisks indicate a significant difference from 1 AEq (t-test, $P < 0.05$). Different letters within bars represent significant differences among groups (One-way ANOVA followed by Fisher's Test, $P < 0.05$).

similarly disturbed, but had access to food during the four-week period, caused an escape response on the nymphs (treatment *d*, Fig. 5).

4. Discussion

Our results describe the filling state and reloading dynamics of the Brindley's glands in *T. infestans* under different scenarios of development, number of discharging events and feeding conditions. The development of the Brindley's glands occurs during the fifth nymphal instar, and ends just before imaginal ecdysis (Millen et al., 1979). We found that the glands are not fully loaded at the pharate stage, but as soon as the insect molts into an adult, even before cuticle hardening, the gland contents reach a maximum and remain unchanged if no discharges are triggered. Since the fifth-instar cuticle covers the pharate adult, and there are no gland openings at this stage, it can be expected that the Brindley's glands remain non-functional, explaining the low contents found in this study. After ecdysis, however, the glands must become quickly loaded and functional for the new adult to be defended, and this also correlates with our findings.

When disturbed, the insect discharges most of its gland contents, but about 25% of the original secretion remains available for further discharges. The glands are then gradually reloaded, even if the insect has no access to food. Although not fully recovered within one week, the glands can be discharged several times on a weekly basis, resulting in a progressive decrease in the remaining gland contents. As it may be expected, our results show that the gland contents recover remarkably to their original levels when blood meals are available to the insects.

Chemical analyses of gland contents also correlated with our behavioral bioassays for testing alarm-pheromone activity. While the first adult discharge elicited an escape response in conspecific nymphs, gland discharges from adults that had been disturbed several times did not, unless the insect had access to blood meals prior to the test. This indicates that the discharge of the Brindely's glands bears a physiological and ecological cost to the insect. Inasmuch as feeding is required for gland reloading, it seems clear that resource allocation must be directed towards this process. Moreover, at least regarding their alarm function, the glands become less efficient after several discharges, and one may presume that their defensive role also becomes compromised.

Feeding frequency in *T. infestans* has been studied under different conditions, and this information provides a relevant context to our study. Factors such as host availability and defensive behavior,

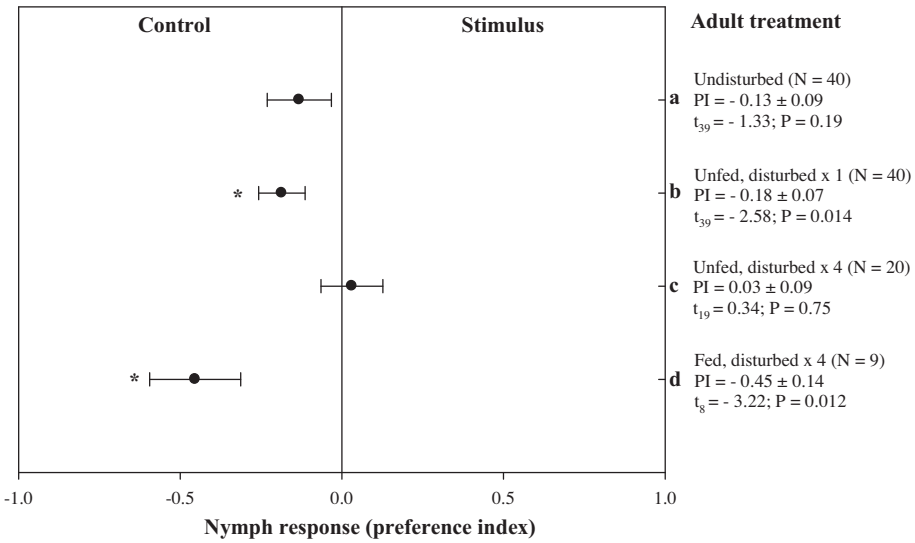

Fig. 5. Behavioral responses of nymphs (represented as preference index, PI) in the experimental arena containing adult volatiles. The different adult treatments were tested in dyads against undisturbed adults, which served as controls. The number of weekly disturbance events is indicated as x1 or x4, with the last disturbance performed just prior to the test. Error bars represent SE, asterisks indicate a significant preference toward the control side (PI different from zero, *t*-test, $P < 0.05$).

the degree of domestication of the insects, as well as seasonal environmental conditions, are all important variables affecting the feeding rate of *T. infestans* (Catalá, 1991; Lehane, 1991; López et al., 1999; Ceballos et al., 2005). In peridomestic populations, for instance, adults fed every 2.9, 4.3, 7 and 5.6 days in spring, summer, fall and winter, respectively (Ceballos et al., 2005). Domestic populations, however, predictably show more stable feeding rates of 4 days between meals (Gürtler et al., 2014). As a whole, feeding rates in *T. infestans* are high, and according to our results, this would imply that the Brindley's glands are probably full most of the time.

Of course, the natural frequency of gland discharges should also be taken into account, but no specific studies are available on this subject. Triatomine bugs avoid light, and during daytime they usually remain relatively inactive, aggregated in protected sites. At night, they must leave their shelters in order to feed, becoming exposed to predation, either by the hosts themselves or during host location. While the frequency of such hazardous encounters is unknown under natural conditions, the gregarious nature of *T. infestans* probably assures that even if one individual has discharged its glands before, others may compensate the resulting loss for alarm signaling. Such gregarious behavior, in which juvenile and adults coexist, emphasizes the relevance of alarm pheromones that function across life stages. Although in a natural scenario a prolonged and complex disturbance procedure such as the one used for us probably never occurs, we decided to standardize the disturbance procedure to assure the release of the alarm pheromone in order the treatments to be comparable. It is possible that our prolonged disturbance procedure evoked a higher release of alarm pheromone than that evoked by natural disturbance stimuli. Therefore, the amount of isobutyric acid found to be released in this work could be overestimated and the refilling times in a natural situation could be shorter.

In short, when taking into account the natural history of *T. infestans*, our results strongly suggest that the filling dynamics of Brindley's glands play a significant role in the intraspecific communication of this Chagas disease vector.

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