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Under review

Orientation of *Belminus* triatomines to cockroaches and cockroaches' fecal volatiles: an ethological approach

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Abstract

Most triatomine bugs (Hemiptera: Reduviidae: Triatominae) are hematophagous, though *Belminus* species can live off of cockroach hemolymph to complete their life cycle. In this work we described the fixed action pattern (FAP) employed by *B. ferroae* to identify, approach and suck on a living cockroach. The FAP described here is composed of the following stereotyped behaviors: 1) visual and/or olfactory detection of the cockroach, 2) reaching, 3) cautious approach, 4) antennal exploration, 5) extension of the proboscis, 3) piercing to sedate, 5) walking away and waiting (post sedation behavior), 6) second cautious approach, 7) extension of the proboscis, 8) piercing to suck hemolymph. In order to identify chemicals cues that could elicit such FAP, we examined the behavior of *B. corredori*, *B. ferroae* and *B. herreri* in response to the cockroaches' odor, fresh cockroach feces and fresh rodent wastes. The last two sources were tested based on the assumption that abundant chemicals near host refuges could serve as cues for host orientation. We found the cockroach odor emanating from a box significantly attracted *B. herreri* in a still air olfactometer. The three *Belminus* species approached the captive cockroach after one hour, but avoided to climb the box. Odors emanating from the cockroach feces attracted *B. corredori* and *B. ferroae* in a Y-olfactometer. The FAP sequence observed suggests *Belminus* bugs are not predators like the rest of reduviids (assassin bugs) —but are kleptophagous ectoparasites, since they do not attack and kill a prey but rather steal hemolymph from its invertebrate host. Triatomines and their hosts have intimately shared the same refuge for millions of years. Similar odors occur across invertebrate and vertebrate refuges, and are recurrent in human abodes, thus plausibly explaining how these kleptophagous bugs can readily switch to the domestic habitat.

Keywords

Triatomines, excreta, olfaction, sensory ecology, proboscis extension

Introduction

The Triatominae (Hemiptera: Reduviidae) or kissing bugs are the insect vectors of American trypanosomiasis or Chagas disease. More than 18 million people are infected and approximately 120 million live at risk (Añez et al. 2004). However, according to an editorial published in The Lancet (Anonymous 2006), Chagas illness is the most neglected of neglected human diseases.

Triatomine bugs have a unique way of life, they are nest-living ectoparasites (Guerin et al. 2000) and vectors of *Trypanosoma cruzi* (Kinetoplastida, Trypanosomatidae) –the causative agent of Chagas disease. Triatomines usually live closely associated with their host, sharing the same refuge and, constantly feeding on the same source during the course of their life. In natural conditions most triatomine species bite humans and other vertebrate hosts during the night while they sleep. These blood-sucking bugs acquire the zoonosis by biting an infected vertebrate host (e.g, opossums, armadillos and bats, among others). In the bug the parasite goes into a specialized stage, the epimastigote, which moves onto the rectum where it becomes infectious (Schofield et al. 1987). Infectious *T. cruzi* are called metacyclic trypomastigotes. Triatomines defecate during feeding or shortly after feeding, thus vectoring the parasites by defecating on another host. The trypomastigotes in the feces are capable of swimming into the host's cells using flagella. They enter the human host through the bite wound or by crossing mucous membranes. The parasite *T. cruzi* is able to live in the gut of all triatomines, however, some bug species are more efficient vectors than others due to different factors such as, triatomine density, host preference (Gütler et al. 2009) and defecation index –i .e., time between feeding and defecation (Aldana and Lizano 2004).

Triatomines are a coherent taxonomic group among reduviids (Schaefer 2005). They are classified into 5 tribes and 15 genera including about 150 described species, most of which occur exclusively in America (Otálora-Luna et al. 2015). Triatomines exhibit behavioral and morphological features which distinguish them from most Reduviidae (or assassin bugs). Triatomines are unusual among Reduviidae because they are structurally adapted to hematophagy; although, not all are strict blood-sucking species, as we will show later. Reduviids are themselves an unusual family of the large order Hemiptera (or true bugs). Reduviids are unusual among the Hemiptera because almost all are terrestrial predators of other arthropods – most other predatory Hemiptera are aquatic. It is widely accepted that triatomines derived or evolved from primitive zoophagous reduviids (Cobben 1979). However, there is controversy over whether this occurred once (monophyletic hypothesis) or more times (polyphyletic hypothesis) during evolution (Otálora-Luna et al. 2015). Schaefer (2005) strongly suggested that we can learn about the subfamily Triatominae by knowing such phylogenetic relationships. According to this author there is enough consistence between behavioral, physiological and morphological features around triatomines' way of life to consider them a monophyletic or paraphyletic (i.e., a natural) group. However, taxonomists of Triatominae have drawn more attention to morphological traits than to behavioral patterns (Lent and Wygodzinsky 1979, Páez-Rondón et al. 2019). In some cases taxonomic groups are indistinguishable in terms of their morphological features (Hutchinson 1965), but even the most morphologically similar species have distinguishable differences in their habits (Imanishi 2011). According to Imanishi, ethological studies might serve to distinguish taxonomic groups. Although, behavior is not an ordinary phenotypic attribute as not all behavioral traits are heritable, an important portion of behavior, i.e. instincts, can be considered part of triatomine phenotype and can be accurately measured if observational conditions are controlled following ethological precepts. According to Lorenz (1966) "coordination of movements" or "phylogenetically adapted motor patterns" characterize species and the concept of homology can be applied to them just as well as morphological descriptions. The approach followed in this study has departed from the mentioned ethological premises. We aim to learn about the subfamily Triatominae by describing behavioral characters as indicators of phylogenetic relationships. The pursuit of a distinctive behavioral pattern that is unique to Triatominae, i.e an autapomorphy, guided our current experimental model. In this respect, we chose the genus *Belminus* as an experimental model to identify an ethological feature that reveals phylogenetic relationships.

The genus *Belminus* (Bolboderini) is among the least well-known groups of triatomines, and is composed of the species: *Belminus corredori* Galvão & Angulo, 2006; *Belminus costaricensis* Herrer, Lent & Wygodzinsky, 1954; *Belminus ferroae* Sandoval, Pabón, Jurberg & Galvão, 2007; *Belminus herreri* Lent & Wygodzinsky, 1979; *Belminus laportei* Lent, Jurberg & Carcavallo, 1995*; Belminus peruvianus* Herrer, Lent & Wygodzinsky; 1954, *Belminus pittieri* Osuna &Ayala, 1993; and *Belminus rugulosus* Stål, 1859. The geographical distribution of these relatively small (compared to other species of) triatomines is discontinuous and known only from a scarce number of specimens captured in Central America, Colombia, Peru, Venezuela, and northern Brazil. *B. peruvianus, B. herreri* and *B. ferroae* have been reported vectoring *T. cruzi* inside human dwellings of Peru and Colombia (Sandoval et al. 2010). Very little is known about the genus *Belminus*, among other reasons, because it is difficult to maintain these species in captivity. The feeding behavior of *Belminus* bugs is currently being unveiled, and the most controversial revelation is that these peculiar species live off invertebrate hemolymph to complete their life cycle. They are facultative bloodsuckers but can survive by exclusively feeding on fluids of other arthropods. Sandoval et al. (2004, 2010) studies on *B. herreri* and *B. ferroae* concluded that cockroaches (Blattodea: Blaberidae) are the principal hosts in human abodes, although adults occasionally feed on humans. These authors captured *B. herreri* and *B. ferroae* in houses where cockroaches were present; >86% of the intestinal contents tested reacted with *Periplaneta americana* antiserum, while only a small proportion reacted to human blood antisera. Sandoval et al. (2013) demonstrated that *B. ferroae* showed a higher fitness when feeding on *Blaberus* cockroaches compared with mice, i.e. a higher adaptation to the invertebrate host.

In this study, departing from an ethological approach, we aim to characterize *Belminus* genus taxonomically by identifying and observing behavioral patterns elicited by sensory cues emanating from the cockroach host. Given that *Belminus* are synanthropes and are involved in maintaining the life cycle of *T. cruzi*, there is no doubt about their eco-epidemiological relevance. But, there are phylogenetic concerns about the fact that *Belminus*, as members of Triatominae, are not strict bloodsuckers. *Belminus* species feed on blood, however they can complete their life cycle by exclusively feeding on arthropod hemolymph. Indeed, hemolymph is the primary source of food of *Belminus* species when living in human adobes, as mentioned above. Thus, hemaotophagia is not an obvious autapomorphy within Triatominae, which serves as evidence against monophyly of this subfamily. The feeding behavior that distinguishes *Belminus* would be a primitive habit among Reduviidae, which suggests that *Belminus* are phylogenetically closer to their primitive reduviid cousins, the assassin bugs, and more distant from bloodsucking triatomines. However, this bit of evidence add to previous bits of evidence, which according to Schaefer (2005) “point in so many directions at once that, in fact, they point in none and, so far, prove nothing”. In this work, instead of focusing on the quality of triatomines’ meals (blood or hemolymph) we focused on the *natural history* of their feeding behavior. We looked for a behavioral pattern that can be recognized as an autopomorphy, i.e. shared by *Belminus* and all triatomine species.

When a kissing bug approaches to bite a host, it performs a highly stereotyped behavioral sequence called *fixed action pattern* (FAP) which is relatively invariant within Triatominae, and within Heteroptera. According to Páez-Rondón et al. (2018) different stimuli, such as temperature, chemical, visual and olfactory cues alone suffice to initiate the FAP associated with triatomine feeding. The behavioral sequence described by these authors starts with orientation to an object; subsequently the following behaviors are observed: visualization, exploring with the antennae, touching with the legs, extension of the proboscis and sucking of liquid. In this work, based on Páez-Rondón et al. (2018) discovery, we described the FAP employed by *B. ferroae* to identify, approach and suck a living cockroach. The “orientation” approach used by this author and in the current study, according to Roeder (1998), is somewhere between behaviorism (i.e., learning psychology) and ethology. Considering Crist (1998) criticism of the semantic style of the founders of ethology –and considering some stylistic elements used by the pioneers of the behavioral observation of animals (Darwin and Wallace 1858, Darwin 1983, Humboldt 1991)– we searched for the distinctive innate traits that might facilitate the phylogenetic placement of *Belminus* species. The primary question from an ethological perspective was: how do *Belminus* species approach their prey compared to the ancestral assassin bugs? Previous studies on triatomine orientation behavior have mainly focused on odors emanating from the host's skin and breath (Otálora-Luna et al. 2004, Guerenstein and Lazzari 2009, Aldana et al. 2008, Ortiz et al. 2011) which are by-products of the host-associated microflora and host metabolism of vertebrates. For many vertebrates, chemicals present in feces and urine constitute important signals that serve to mark their territory (Young and Henke 1999, Brennan 2001); sylvatic hosts such as opossums, armadillos and bats, as well as synantrophic host such as chickens dogs, cats and rodents cumulate excretory products in or near their nesting sites. Triatomines –as ectoparasites– use emanations from such host waste when searching for resources. Humans are not an exception; we have captured triatomine bugs in bathrooms, latrines, sewers and trashcans in rural and urban endemic areas (Aldana and Otálora-Luna 2019). Otálora-Luna and Guerin (2014) found that amines present in vertebrate waste (i.e. feces and urine) attract *Rhodnius prolixus*, *Triatoma infestans* and *Panstrongylus geniculatus*. To the best of our knowledge this is the first time host's feces were considered as a source of kairomones, indicating a food source for triatomine bugs. These authors designed their experiments and addressed their conclusions hypothesizing that triatomines are ectoparasites closely associated with their host; normally sharing the same refuge. Given our ethological focus, in this study we performed a selection of odor sources based on our previous *ectoparasite hypothesis* (Otálora-Luna and Guerin 2014) and our previous experimental model, i.e. observation of triatomine FAP (Páez-Rondón et al. 2018). In order to elucidate which chemical stimuli emanating from host excretions trigger the feeding instinct of *Belminus*, we assessed the orientation responses of *B. corredori*, *B. ferroae* and *B. herreri* to volatiles produced by cockroaches and host wastes, as well as rodent wastes.

Summarizing, this study aimed to describe a) the FAP associated with feeding in *Belminus*, b) determine if host volatiles alone elicit the FAP or chemotaxis in *Belminus*, and c) propose such FAP as a character that distinguish *Belminus* and all triatomines from other reduviids.

Materials and methods

Insects

The fifth-instar individuals of *Belminus species* used in this study originated from laboratory colonies containing insects that were collected from domiciles in different localities of Colombia (Sandoval et al. 2004, Galvão and Angulo 2006, Sandoval et al. 2010). Collected bugs were captured from the following Colombian locations: *B. ferroae* specimens were captured in Santa Catalina (07°07′26″N, 72°11′24″) and San Alberto (17°12′40″N, 72°19′12″) in the municipality of Toledo, Northern Santander; *B. herreri* specimens were captured around a house in the municipality of El Carmen, Santander (6°39′30″N, 73°38′56″) and in dwellings in the municipality of San Martin, Cesar (07°58′47″N, 73°32′26″); and *B. corredori* specimens were captured in a house in San Gil, Santander (6°33′18″N, 73°04′00″). Colonies started in the laboratory during the following years: *B. herreri* in 2000, *B. corredori* in 2002 and *B. ferroae* in 2005. Bugs were fed on cockroaches in laboratory conditions as described by Sandoval et al. (2013). *Belminus* colony has been at the premises of the Venezuelan Institute for Scientific Research (Mérida, Venezuela) for five years. Insects were kept at 23 ± 2°C, 82 ± 7% RH (relative humidity) in light dark cycle of 12/12 hours, and starved for 8–12 days from emergence before assays. They were fed 5th nymphs of *Blaberus giganteus* cockroaches (Blaberidae), which were fed food formulated for canines and corn seeds. *Belminus* bugs and cockroaches shared the same container since hatching for 5 months. Each bug was used only once to avoid bias from learning (Aldana et al. 2008).

Behavioral observations

Three different arenas were used to study the behavior of *Belminus* species. Bioassays were conducted at 24 °C and 68% RH, and were illuminated by fluorescent ceiling lights (brightness 2000 lux, color temperature 4000 K), unless otherwise specified. All experiments were performed in Merida, Venezuela, between 1700 and 1860 meters above the sea level.

Arena 1: Ethological responses of *B. ferroae* were examined in a 10 cm Petri dish made of glass, where a 3-cm living *B. giganteus* nymph was enclosed with a *B. ferroae* bug (Fig 1Ai). *Phase I*: In the first part of the experiment the cockroach was confined in a 5.8 cm plate tight to the center of the arena with a low odor thermoplastic adhesive (tec bond 341, HotMelt), to avoid odor escaping from around the lid. Thus, only visual contact was allowed between the cockroach and the bug. The location and behavior of the cockroach and the triatomine were recorded for 10 minutes. *Phase II*: The cockroach was released, so both individuals could physically interact for 10 additional minutes. *Controls*: These consisted of a second series of two bioassays in which either the cockroach or the bug were absent. Each experiment was repeated 10 times.

Arena 2: Orientation responses of *B. corredori*, *B. ferroae* and *B. herreri* to an odorous still air were tested in a square glass arena (29 x 29 cm). A living fifth instar *B. giganteus* was confined in an opaque-black cardboard box (length 6.5, wide 8 cm, height 2.5), placed in the center of a side (Fig 1B). The side of the box facing the center of the arena had 12 holes (dia. 2 mm). A similar empty box was placed as a control at the other end. Each bug was placed in a central 3 cm circle. The bug, initially covered with a circular Petri dish (2.8 cm), was released after 2 min of adaptation. We noted which half of the arena the insect preferred during a 5 minute period. A bug was considered attracted to cockroach volatiles if it preferred the half of the arena where the cockroach was present. In a secondary phase of the bioassay, the position of the bug was noted after one hour of starting each experiment. A bug was considered attracted if it ended next to the box where the cockroach was present, i.e. no more than 3 cm far from the box. As some *Belminus* individuals may be reluctant to move for long periods (sometimes hours) after being manipulated, insects that did not leave the circle after five minutes were discarded (23%). Stimuli positions were interchanged every 2 insects; the arena was cleaned before making such changes. In order to test for asymmetries in the experimental setup *Belminus* bugs were observed in an odorless control trial, where two empty boxes were placed in the arena. Each experiment was repeated 20 times.

Arena 3: Triatomine responses to an odorous air current were tested in a Y-tube dual choice olfactometer made of glass (Fig 1C). The three *Belminus* species were tested separately against volatiles emanating from 1) living cockroaches, 2) cockroach feces, and 3) mouse feces. One triatomine bug was allowed to a) walk upwind in the stem (length 22 cm, i.d. 2.370 cm, e.d 2.804 cm), and once it reached the junction, b) made a choice between the two arms (length 13 cm) of the Y-olfactometer. Orientation was quantified by recording triatomines’ choice. All individuals walked from the base of the Y olfactometer and made a decision. A preliminary spatial control experiment with two clean arms was performed to test asymmetries, and no significant differences were found. A pump was used to draw charcoal-filtered air (stimulus controller, CS-55, Syntech) from the two arms (10 ± 2 cm/s airspeed as measured by a hot-wire anemometer, 2440, Kurtz, accuracy ± 0.01 m/s), one of which was connected to a 500 ml glass gas-wash bottle containing the odor source; the other one held a similar clean (control) bottle. Both air-streams reached the ‘‘Y’’ stem (23± 2 °C and 70 % RH as measured by a thermo-hygrometer, EA80, Extech Instruments, accuracy ± 3% RH and ± 1 °C). Tubes, fittings and valves were made of polytetrafluoroethylene (PTFE, International Polymer Solutions Inc). Headspace odors –i.e. volatiles emanating from the vapor phase above the sample in a vial– were tested by placing the odorous samples (see next section) in 500 ml glass gas-wash bottles. Bottles were left to equilibrate for at least 10 min before blowing volatiles, and an additional 15 minutes elapsed between experiments so cockroaches and wastes had time to emit more volatiles. Air blown over filter paper and rice hulls dampened with 5 ml of water served as controls for cockroach feces and mouse wastes respectively. The control for cockroaches consisted of a clean gas-wash bottle with a similar wet filter paper. Water served to add humidity to match the humidity produced by organic samples (Otálora-Luna and Guerin 2014). The air-stream passing through the bottles was injected at 150 ml/min. Bugs walking on the Y-olfactometer were stimulated until a response was observed, < 30 min. Flask positions were interchanged for every insect. The Y-olfactomenter was cleaned before changing positions. In order to test for asymmetries in the experimental setup *Belminus* bugs were observed in an odorless control trial, where two empty bottles were tested. In order to remove odors from the laboratory the experimental setup was positioned near an exhaust hood, which was able to exchange the air in the room 15 times per hour. Mouse waste was not tested on *B. herreri* as this colony was not large enough to provide an adequate number of naïve individuals.

Odor collection for arena 3

Cockroach odors emanated from three living *B. giganteus* 5th nymphs. Cockroach feces were collected (20 g) and separated from extraneous material (*e.g.*, dead insects, exuviae, oothecae and food waste) from the bottom of the containers used for mass-rearing of *B. giganteus*. Mouse wastes (30 g) consisted of rice hulls impregnated with feces and urine of these rodents (albino mice, strain NMRI). All samples were placed separately in 500 ml glass gas-wash bottles.

Visual recording of arenas

To analyze behavioral details of Arenas 1-3, frontal and top views of the experimental arenas were filmed full-screen using a video CMOS camera (EOS Rebel T7i, Canon) equipped with a zoom lens (EF-S 15-55 mm f, IS, Canon) coupled to a macro lens (close-up +10, 58 mm, Commander) to obtain a field view of 40 mm (in the diagonal) –a pivoting base allowed to visually cover the entire arena. Experiments were conducted in a darkened room. The source of light for arenas 1 and 2 consisted of a LED lamp (2850K, 570 lumens, Reveal, General Electric). The source of light for arena 3 consisted of a LED infrared lamp (840 nm, Cmvision) –the infrared sensitivity of the camera allowed to observe the experiments in the dark. Recordings were analyzed by a computer video software (version 9.1.2.7, Wondershare Filmora9) for playback, tracking and editing. The ethological language used to describe triatomine behaviors followed suggestions of Kogon (1941) and Crist (1998).

Statistics

Comparisons of behavioral responses observed in the arena 1 were made using one-tailed binomial test. Comparison of behavioral responses for each species in the arenas 2 and 3 were made using two-tailed binomial test. A chi-square test of independence was performed to examine the relation between the *Belminus* species and orientation responses in arenas 2 and 3. When dependence resulted insignificant in the previous test, *Belminus* species were treated as a single group –as a genus– for a two-tailed binomial test. When dependence resulted significant, multiple chi-square pairwise comparisons were performed among species, after post hoc Holm-Bonferroni correction (Ruxton and Beauchamp 2008). Statistical analyses were analyzed using R (version 3.5.3, Vienna, Austria; Ihaka and Gentleman 1996).

Results

Arena 1

Phase I: *B. ferroae* nymphs walked along the border (sidewall) of the 10-cm dish; showing positive thigmotaxis (Fig 1A). The bugs hardly ever edged the 5-cm dish, which contained the cockroach (two bugs, each on one occasion). Most bugs depicted a swinging walk along the perimeter of the former dish, alternating between clockwise and counterclockwise directions. This to-and-fro circuit approximated and rarely exceeded 180°. Triatomine walking bouts were not continuous but were punctuated by stops, which comprised 20% of the time. The cockroach showed several attempts to escape by moving into the confined space, occasionally ramming against the sidewall of the dish. There were no obvious sings of interaction between the triatomine and the cockroach despite their proximity.

Phase II: Shortly after being released the cockroach moved profusely and showed positive thigmotaxis; although walking tracks were discontinuous —punctuated by stops. The bug interrupted the positive thigmotactic behavior and remained motionless for 5 seconds. Then the bug faced the cockroach and lifted the anterior part of its body (~40°) through an extension of its forelegs in order to reach higher with its antennae held up (~60° with respect to ground), a behavior referred to here as *reaching*. The bug continuously waved its antennae (i.e., semaphore movement, ~30°). Eventually, when the cockroach came to a halt, the bug stopped reaching and walked very cautiously to the cockroach (Fig 1B) by bending down the anterior part of its body through flexing of their forelegs (i.e., stalking). Thus, the triatomine walking velocity decreased substantially compared to the previous phase. The bug approached the abdomen of the cockroach and touched it with its antennas (i.e., antennal exploration). Then the bug extended the proboscis, pierced the cockroach abdomen briefly (~2 seconds), and finally walked away.

Proboscis flexibility allowed the bug to pierce the cockroach from below (Fig 1Ca). After 10 seconds of being pierced, the cockroach experienced alternating spasms with slow, clumsy and erratic movements. The cockroach displayed obvious sings of disorientation. In such condition, the cockroach did not show positive thigmotaxis but rather slow wide-ranging movements. The occasion was seized to approach the cockroach in the same manner as before –the triatomine bug walked cautiously and eventually inserted the stylets. This time the bug sucked cockroach hemolymph for 2 minutes; repeating this kleptophagic behavior on two or more occasions. The triatomine abdomen swelled prominently. Summarizing the description of *Belminus*' FAP we categorized the following stereotyped behaviors: 1) visual and/or olfactory detection of the cockroach, 2) reaching, 3) cautious approach, 4) antennal exploration, 5) extension of the proboscis, 3) piercing to sedate, 5) walking away and waiting (post sedation behavior), 6) second cautious approach, 7) extension of the proboscis, 8) piercing to suck hemolymph. The sequences 6-8 were repeated more than once. The cockroach did not die.

Controls: During the control experiments, where only one of the two insects was present, the triatomine bug and the cockroach showed similar behaviors to those observed in the phase I.

The FAP described in the phase II was not observed during the phase I or during the two controls (P<0.001).

Arena 2

During the first 5 min *B. herreri* orientated their walks toward the cockroach (78%, P=0.012), however *B. corredori* (65%, P=0.169) and *B. ferroae* (58%, P=0.193) orientation did not significantly differ from random (Fig 2). The chi-square test of independence showed that the three species did not behave differently (P=0.501). Thus, when we tested the three species a as group (n=56); *Belminus* genus oriented its walk towards the cockroach (68%, P=0.0029).

Eventually, after 1 hour, all individuals from the three species ended up approaching the box (100%, P<0.001), and remained 0.5-3 cm close, but the odors or vibrations induced by the captive cockroach prevented the bugs from contacting the box. Bugs made explorative approaches to the box, by touching it with antennae and brief walks, but eventually desisted from climbing. The FAP or proboscis extension were not observed in this arena, only chemotaxis was observed.

Controls: During the odorless control experiments, where two empty boxes were tested on *B. corredori* (n=20), orientation did not significantly differ from random (left: 9, right: 11, P=0.160).

Arena 3

The FAP or proboscis extension were not observed in the Y-olfactometer, only chemotaxis was observed in certain cases. When one arm of the Y-olfactometer was provided with the headscape of living cockroaches and the other with clean air, *B. corredori* (P=0.005), *B. ferroae* (P=0.04) *and B. herreri* (P=0.014) avoided the odorous air current (Fig 3). The chi-square test of independence showed that there was no significant relation between species and behavioral responses (P=0.347); *Belminus* genus (n=52) avoided volatiles emanating from cockroaches (79%, P=0.000013).

Similarly, *B. corredori* (P=0.001) and *B. ferroae* (P=0.008) avoided the extreme of the Y-olfactometer when provided with the headscape of mouse wastes (Fig 3). The chi-square test of independence showed that there was no significant relation between species and behavioral responses (P=0.477); *Belminus* genus (n=48) avoided volatiles emanating from mouse wastes (79%, P=0.000023).

However, volatiles emanating from cockroach feces attracted *B. corredori* (P=0.019) and *B. ferroae* (P=0.003), while *B. herreri* did not show attraction or avoidance behaviors (P=0.0541). The chi-square test of independence showed that there was a significant relation between species and behavioral responses (P=0.0208). When performing multiple pairwise comparisons, chi-square tests showed that the behavioral responses of *B. corredori* and *B. ferroae* were not different (P=0.766), the behavioral responses of *B. corredori* and *B. herreri* were not different (P=0.0683), and the behavioral responses of *B. ferroae* and *B. herreri* were different (P=0.0392) (Fig 3).

Controls: During the odorless control experiments, where two empty flasks were tested on *B. corredori* (n=22), orientation did not significantly differ from random (left: 12, right: 10, P=0.154).

Discussion

Páez-Rondón et al. (2018) exposed *R. prolixus, T. infestans* and *P. geniculatus* to a drop of water, a plastic dummy and a piece of guava fruit, and discovered that all of these objects elicit the FAP associated with feeding. In this study, we report for the first time such behavioral pattern in *Belminus* –from an ethological point of view– which reveals the novelty of our work.

Furthermore, according to Tinbergen (1963) an ethologist will attempt to recognize elements of animal's *own* behavior; we showed that kleptophagy is a *phenomena proper* (sensu Portmann 1967, Kleisner 2008, Kleisner 2015, Aldana and Otálora-Luna 2019) of *Belminus* genus and the rest of triatomines. The manner in which *Belminus* bugs approached the cockroach, performing a highly stereotyped sequence that ended in proboscis lifting and sucking, and that did not result in the immediate death of the host, defines its phyloegentic relationship within Triatominae.

Belminus bite was not toxic to the cockroach but rather analgesic. Furthermore, when rearing *Belminus* triatomines in our laboratory we have observed that a single cockroach is able to survive after being bitten by a dozens of these bugs for many months (data not shown). Most predator reduviids have highly toxic venoms which kill the prey immediately (Walker et al. 2018). Triatomine saliva is not that toxic but is able to inhibit voltage-gated sodium channels which may account for the anesthetic effect (Walker et al. 2016). From these physiological studies and our ethological observations we might conclude that *Belminus* are not able to *kill* its *prey* but to *parasite* its *host*. The use of the terms parasite and host could be debated since triatomines do not live directly on the host –as ticks, lice or fleas. However, triatomines closely share their lives with their hosts, in the same refuge, and that is why Guerin et al. (2000) called them ectoparasites. Alternatively, we could also call triatomines carnivores (Cobben 1979, Livingstone et al. 1998), zoophagous (Boyd et al. 2002) or micropredators (Poulin and Randhawa 2013, Wilson et al. 2017). But these categories are less concrete; for example, contrary to mosquitos, which have been catalogued as micropredators, triatomines live closely associated with their host. In any case, the purpose that prevails here is to behaviorally and phylogenetically distinguish triatomines from the rest of reduviids. The parasitic strategy (kleptophagy) described in this study is found only in one taxon (Triatominae), but not found in any other outgroup reduviid taxa (Reduviidae subfamilies).

Another aspect worth considering is the form of the proboscis of triatomines which is very different from the rest of reduviids (Lent and Wygodzinsky 1979). Reduviids have a hook-shaped proboscis that when extended points downwards. However, the triatomine proboscis is straight and possesses a membranous joints, a treat used by Lent and Wygodzinsky (1979) to distinguish the Triatominae from other reduviid subfamilies. This morphological feature is associated with the hematophagous –and kleptophagous– habit that distinguish triatomines. *Belminus* bugs used such proboscis flexibility to pierce the cockroach from below in arena 1. The proboscis behavior performed by *Belminus* and all triatomines is not possible within predator reduviids which have a hook-shaped proboscis solely capable of piercing its prey from above. Cobben (1979) regards triatomines as an “advanced” subfamily within Reduviidae according to this mouth-part structure and function. Such morphologic-behavioral *coupling* (sensu Varela 1988) illustrates a *structural adaptation* (sensu Croizat 1962, Greham 1984, Otálora-Luna et al. 2017), and deserves special consideration as it might be tracing the course of evolution of kleptophagy in these insects (Aldana et al. 2019).

Belminus is able to detect thermal cues as they feed on humans and other homoeothermic vertebrates. But, they prefer poikilothermic arthropods as hosts (Sandoval *et al*. 2013), whose temperature rarely exceeds that of warm-blood vertebrates. Wigglesworth and Gillett (1934 a,b) suggested that triatomines are attracted to their host mainly by warmth. Vinauger et al. (2013) stated that "heat constitutes the only necessary and sufficient signal to trigger the PER" or proboscis extension response (Flores and Lazzari 1996). Haridass and Ananthakrishnan (1980) stated that "unlike predatory reduviids, in the hematophagous *T. rubrofasciata*, feeding behavior is elicited only by a temperature gradient arising from their vertebrate hosts, as is also the case with other blood feeding insects". In disagreement with these statements we suggest that feeding behavior in all Triatomine subspecies is not solely driven by heat. In line with previous studies, visual, olfactory and tactile stimuli can also trigger the FAP (Páez-Rondón et al. 2018). In this study we confirmed that non-thermal stimuli from poikilothermic insects elicit positive taxis and the FAP in *Belminus* species. Such elicitation flexibility of stimuli for triggering orientation behaviors and the FAP was decisive in the change of behavior that permitted the primitive predateous reduviids to become kleptophagous, driving the evolution of Triatominae. Presumably, early triatomines fed on abundant soft invertebrates in the nests and burrows of vertebrates, then later encountered their defenseless newly born offspring, tasted them, extended the proboscis, punctured and penetrated their skin (Otálora-Luna et al. 2015). The peculiar form and flexibility of triatomine proboscis appeared, the quality of their saliva became less toxic and more analgesic, and their FAP acquired a kleptopagous *aspect* (sensu Portmann 1967), thus triatomines became an advanced group among Reduviidae. Eventually, this elicitation flexibility also facilitated the process of domestication of several sylvatic species, like *Belminus sp*.

The feeding FAP observed here is derived from an ancestral pattern of hemipterans. Hatfield et al. (1983) described similar sequential behaviors associated with feeding in the phytophagous bug *Lygus lineolaris* (Hemiptera: Miridae). Haridass et al. (1987) distinguish a series of categories where reduviids can be grouped based on their approaching behaviors during feeding: (i) 'blood feeding' type, (ii) 'sticky-trap' type, (iii) 'raptorial' type, (iv) 'wait and grab' type, (v) 'pin and jab' type and (vi) 'chase and pounce' type. Reduviid predators exhibit an “assessing” and “ambush” strategy (Edwards 1962, Ables 1978), which is different from the FAP described for *Belminus vis à vis* the way the victim is handled. A typical member of the Harpactocorinae sits "pinned” and waits for a prey to arrive on a flower to “jab” it (Haridass et al. 1988, Ambrose 1999). Edwards (1962) described the feeding FAP for the harpactocorine *Rhinocoris carmelita* and the reduviine *Platymeris rhadamanthus* as follow: arousal, orientation, fixation, approach, (facultative) pounce, extension of rostrum, grasping, apprehension, insertion of styletes and injection of saliva. Srikumar et al. (2014) described the feeding FAP for harpactorine reduviid predators as follow: arousal, approach, capture, rostral thrust, paralyzing, sucking, and post-predatory behaviors. Other authors have described similar behavioral sequences in Reduviidae (Ables 1978, Haridass and Ananthakrishnan 1980). Ables (1978) observed that “smaller, more mobile prey are usually ‘ambushed’, whereas larger, less mobile prey are ‘stalked’” and “when prey were detected the predator became motionless with the fore tibiae raised and the antennae extended.” This author added that “initial contact with prey usually consisted of antennation and then insertion of the predator's proboscis”. According to Srikumar et al. (2014) “after prey were pinned and jabbed, the reduviids paralyzed them by injecting toxic saliva". Some reduviid species transport the prey beneath leaves and twigs to a safe and secluded place before sucking out its body fluids (Srikumar et al. 2014). Sometimes they grip it with the fore and mid-legs until the victim succumbs (Edwards 1962).

Such ambushing, grasping, dragging, apprehension, paralyzing and similar murderous behaviors were not observed in *Belminus* bugs. The paralyzing (lethal) effect elicited by assassin bugs differs from the sedation effect caused by *Belminus*, as the latter do not pursue to *kill* but to *ectoparasite*. Triatomines have adapted to live together with their hosts. It should be stressed that cockroaches eventually recover from lethargy after being sucked, and shall survive for upcoming bites. Thus, *Belminus* species are not predators like the rest of reduviids. *Belminus* are not assassin bugs. As with other triatomine species, *Belminus* are not real *hunters* but *thieves*, i.e., ectoparasites that take advantage of the least carelessness of the host to steal its fluids.

This suggest that kleptophagy is an ethological (i.e. taxonomic, phylogenetic and evolutionary) characteristic of *Belminus* as well as of Triatominae. Since, *Belminus* are not strict hematophagous, this trait might deserve taxonomic revision. We propose to consider kleptophagy as an attribute that groups triatomines. Kleptophagy is a non-aggressive behavior which contrast with the violent predatory behaviors that characterize ancestral reduviids.

Immelmann (1983) underline that while appetitive behavior might contain taxis components as well as FAP, not all taxis derive in FAP. Clearly, the FAP requires an external stimulus to be triggered but different modalities alone could be sufficient. In arena 1, phase 1, visual cues –if apparent to *Belminus* bugs– were not sufficient to trigger the FAP or host mediated taxis. Probably, the bug was not able to see the cockroach through the glass. However, we have previously observed that a visual cue –a plastic dummy resembling a drop of water– is sufficient to elicit the FAP (Páez-Rondón et al. 2018). It is difficult to stablish which cues elicited the FAP when the glass barrier was removed (phase 2) but, certainly the FAP was triggered until completion by the presence of the host, and probably visual cues played an important role in this opportunity. Extension of the fixed bug's proboscis to the cockroach and sucking observed in arena 1 may be catalogued as a *reflexes* (Páez-Rondón et al. 2018). Approach to the cockroach (arena 1), to the black box containing the cockroach (arena 2) and positive decisions made in the Y-tube (arena 3) may be catalogued as taxis. Taxis, proboscis extension and sucking are clearly part of the FAP, and at some point there is a superposition of all of them (Tinbergen 1951, Lorenz 1977, Eibl-Eibesfeldt I 1979). While, olfaction was required in arenas 2 and 3 to elicit taxis behavior, it was not sufficient to elicit the FAP.

For future studies, we suggest to test olfaction and visual cues alone, using a cockroach dummy or providing multimodal stimuli on the servosphere –to see whether olfaction is sufficient –or only required– to elicit FAP. We also suggest to combine different stimuli to clarify how convergence between visual (different light treatments) and olfactory cues works (Reisenman et al. 2000, Otálora-Luna and Dickens 2011).

Only *B. herreri* showed significant attraction to the black box where the cockroach was confined, during the first 5-min period in the still-air olfactometer. Thus, this species was able to detect the cockroach in still air conditions at 10 cm (78%, arena 2). Interestingly, the three species were attracted to the cockroach odors after one hour. Probably, *B. corredori* and *B. ferroae* reached the box containing the cockroach by *chance* after one hour of trial-and-error, but it is worth to note that all tested bugs continued walking close to this box after that hour, thus a short-range attraction effect can be inferred from this experiment; although proboscis extension was never observed. They made attempts to climb the box, but cockroach's movements, and probably pungent odors, caused the triatomine bugs to keep a distance. Such *Belminus*' avoidance reaction merits further ethological and physiological consideration. The difference between the initial attraction and the attraction at the end of an hour could also indicate that the cockroaches were not the preferred host, suggesting that there is a greater latency to react to cues emitted by cockroaches. Probably, these *Belminus* species have different hosts in nature. Our knowledge of *Belminus*' sylvatic life is very limited. For future studies, we suggest improvement of these arenas in order to test olfaction alone, without any visual cues, to determine whether olfaction is sufficient (or only required) to elicit the FAP. We also recommend sampling and observing *Belminus* bugs living in natural conditions, a real challenge!

Belminus species were not attracted to volatiles emitted by cockroaches in the Y-olfactometer, and indeed the three species *B. corredori, B. ferroae* and *B. herreri* avoided this odor. This result was surprising as the three *Belminus* bugs tested in this study have lived exclusively off cockroach hemolymph. The cockroaches acted stressed, i.e. exhibiting compulsive movements, in the olfactometer flask probably due to the negative pressure produced by the air flow.

Possibly, they were producing allomones (*i.e.*, defensive volatile secretions) during the experiments (Schal 1992), which might explain the avoidance behaviors observed here. Mouse's wastes were also avoided by *B. corredori* and *B. ferroae*. Avoidance of mice feces might be due to potential predation by this vertebrate. However, both species were attracted to volatiles emanating from cockroach feces. Sandoval et al. (2004, 2010, 2013) demonstrated that *Belminus* species are much better adapted to cockroaches than to mice. These authors found domestic *Belminus* species closely living in, and feeding on, cockroaches in human houses. In nature, sylvatic *Belminus* are probably associated with insect-hosts living under rocks or in tree barks, or are associated with invertebrates living inside vertebrate refuges such as caves, borrows, nests, etc. Our result confirmed the hypothesis proposed previously by Otálora-Luna and Guerin (2014) –volatiles from host wastes (i.e. cockroach feces) emanating from host refuges stimulate triatomines searching behavior.

But, which volatiles are emitted by cockroach feces? Besides specific pheromones (Schal 1992), nitrogenous compounds such as ammonia, methylamine, dimethylamine and trimethylamine (Sakuma and Fukami 1990) as well as carboxylic acids such as acetic, propionic, isobutyric, butyric, isovaleric and valeric acid (McFarlane and Alli 1985) are major components of the excreta of cockroaches, many of which elicit searching behaviors in other triatomine species (Guerenstein and Lazzari 2009, Ortiz et al. 2011, Otálora-Luna and Guerin 2014). Ammonia attracts triatomines and emanates in large amounts from both vertebrate feces (Otálora-Luna and Guerin 2014) as well as from cockroaches' feces (Mullins and Cochrand 1972). *Belminus* avoidance responses to mice wastes and attraction responses to cockroach feces suggest that *Belminus* species might recognize unknown specific as well as known general volatiles (e.g.

ammonia, CO_2, carboxylic acids, aromatics, etc.) emitted by excretory products of their host. Odors from vertebrate and invertebrate hosts, while sharing several molecules, produce ambivalent effects depending on the dose, combination with other molecules, combination with other modalities (e.g. visual cues), physiological state of the insect, among other factors. For example, we know that isobutyric acid and ammonia have different behavioral effects on triatomines depending on the dose. They evoke repellency and scape responses when released " suddenly" at high doses but are attractive at low doses (Schofield 1975, Guerenstein and Guerin 2001, Otálora-Luna and Guerin 2014). Such parsimony or use of the same chemical as a releaser of different behaviors is common in arthropods (Blum 1996). This might explain how the cockroach in arena 1 elicited the FAP while the cockroach in the box (arena 2) or in the bottle (arena 3) did not elicited the FAP. Probably, the visual and/or physical (tactile) presence of the cockroach was *required* as volatiles were not *sufficient*.

The use of waste odors to find resources is a structural adaptation widespread in triatomines, given that the associated volatile organic compounds are end-products derived from generally occurring metabolites of vertebrate and invertebrate gut flora metabolism and, as such, do not vary greatly between hosts (Otálora-Luna and Guerin 2014). These compounds could play an important role in house invasion by triatomine, as has been mentioned elsewhere (Otálora-Luna and Guerin 2014). *Belminus* species could well make use of the same general chemical cues they use to locate wild hosts for the exploitation of synanthropic invertebrates (*e.g.*, cockroaches) since similar odors occur across invertebrates and vertebrates, thus plausibly explaining how these small triatomines can readily switch to the domestic habitat (Otálora-Luna and Guerin 2014, Otálora-Luna et al. 2016).

Strict blood-feeding has been considered a behavioral feature that group Triatominae (Lent and Wygodzinsky 1979). However, as *Belminus* does not strictly depend on blood, such taxonomic character must be revised. The inconsistency does not necessarily compromises the systematic identification of the group. A proper behavioral category shall comprise *Belminus* as a triatomine. As kleptophagy is hitherto an advanced and unusual feature in Hemiptera and in Reduviidae itself, we believe Triatominae is a single evolutionary unit that has evolved only once –i.e. Triatominae is monophyletic (sensu Schaefer 2005). During the course of evolution *Belminus* bugs have selected and adapted those behavioral sequences that fit better to a) their ancestral constraints (Triatominae), b) physiological needs (carnivorous) and c) ecological scenarios (ectoparasitism). One surmises that kleptophagic behaviors of *Belminus* species detailed here have contributed to their match to the synanthropic niche. The presence of cockroaches in human houses facilitates *Belminus* species adaptation to the human habitat, where both cockroaches and humans are potential targets.

A better ethological –behavioral, systematic, evolutionary, etc.– understanding of the Triatominae is essential for the development of novel control programs. In particular, the study of instinctive behaviors of *Belminus* and other triatomine species is critical in the design of experiments aimed at improving control technologies.

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Conflict of interest

Fernando Otálora-Luna et al. declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and institutional guidelines for the care and use for animals were followed.

Research involving human participants and/or animals

This article does not contain any studies with human participants performed by any of the authors.

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Figure legend

Fig 1. Experimental arenas (1-3). Chemical and tactile interactions between the bug and the cockroach were impeded in arena 1 by a transparent barrier (Aia). Contact interactions were allowed by removing the smaller petri dish (Aib). All *Belminus* bugs ended piercing the anesthetized cockroach (Aiia), and eventually left gorged after sucking enough hemolymph (1Aiib). Arenas 2 and 3 are illustrated in B and C respectively. Relative size of the insect has been exaggerated for clarity.

Fig 2. Behavioral responses of *Belminus corredori* (*Bc*), *B. ferroae* (*Bf*) and *B. herreri* (*Bh*) to a cockroach captive in cardboard box. Gray bars = % of bugs that walked to the empty box (repelled), black bars = % of bugs that walked to the cockroach (attracted), n = number of tested bugs, * indicate P < 0.05 for the binomial test. The white arrow indicates the total percentage of *Belminus* species attracted to the cockroach (68%, P < 0.005). The black arrow indicates that the three species ended up close to the box containing the cockroach after one hour (P<0.001). A schematic representation of the arena is placed at the right top of the graph. Relative size of the insect has been exaggerated for clarity.

Fig 3. Behavioral responses of *Belminus corredori* (*Bc*), *B. ferroae* (*Bf*) and *B. herreri* (*Bh*) exposed to the headspace of cockroaches, cockroach feces and mouse waste. Gray bars = % of bugs that walked to the clean air (repelled), black bars = % of bugs that walked to the odor (attracted), n = number of tested bugs, * indicate P < 0.05 and ** indicate P < 0.01 for the binomial test. Different letters indicate P<0.05 for multiple chi-square tests after Holm-Bonferroni correction. A schematic representation of the Y-tube olfactometer is placed at the right top of the graph. Relative size of the insect has been exaggerated for clarity.

Fig 1

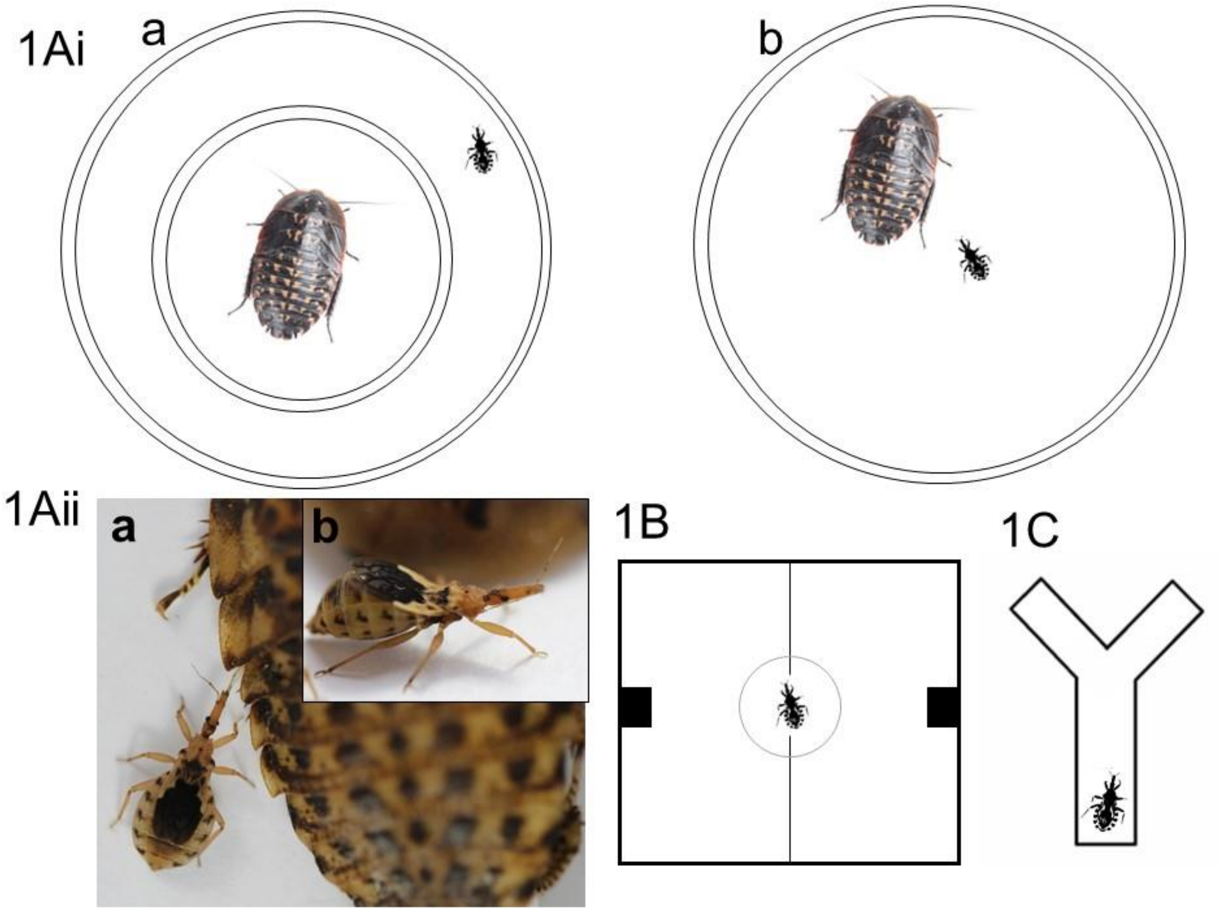


Fig 2

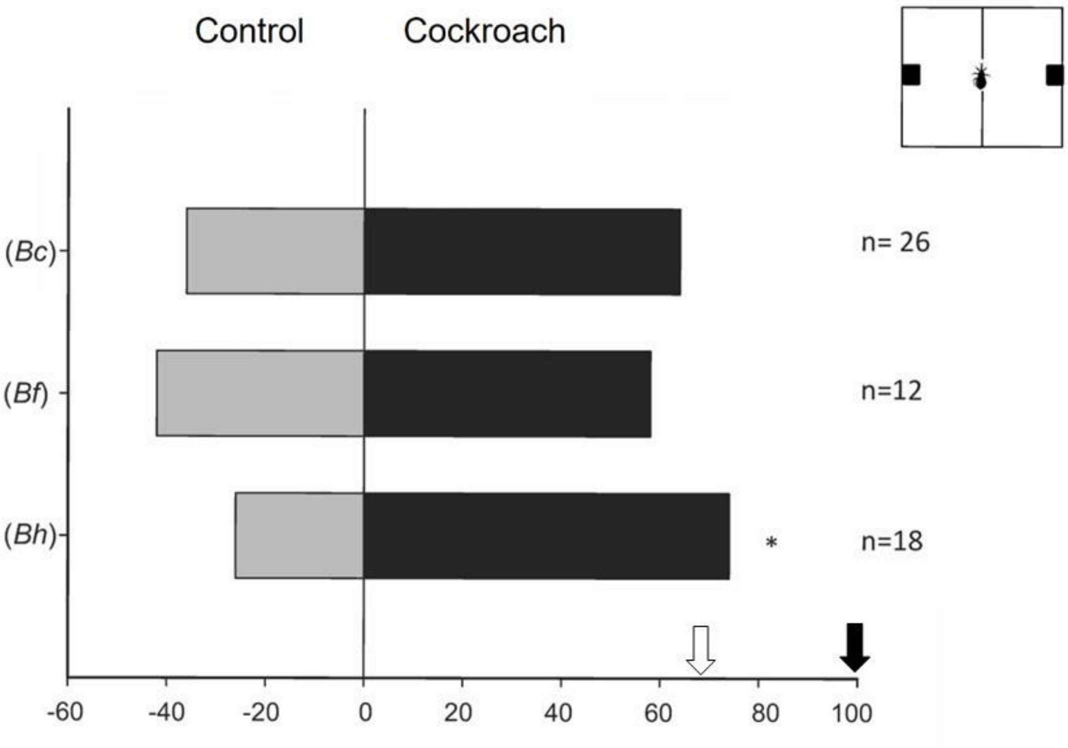
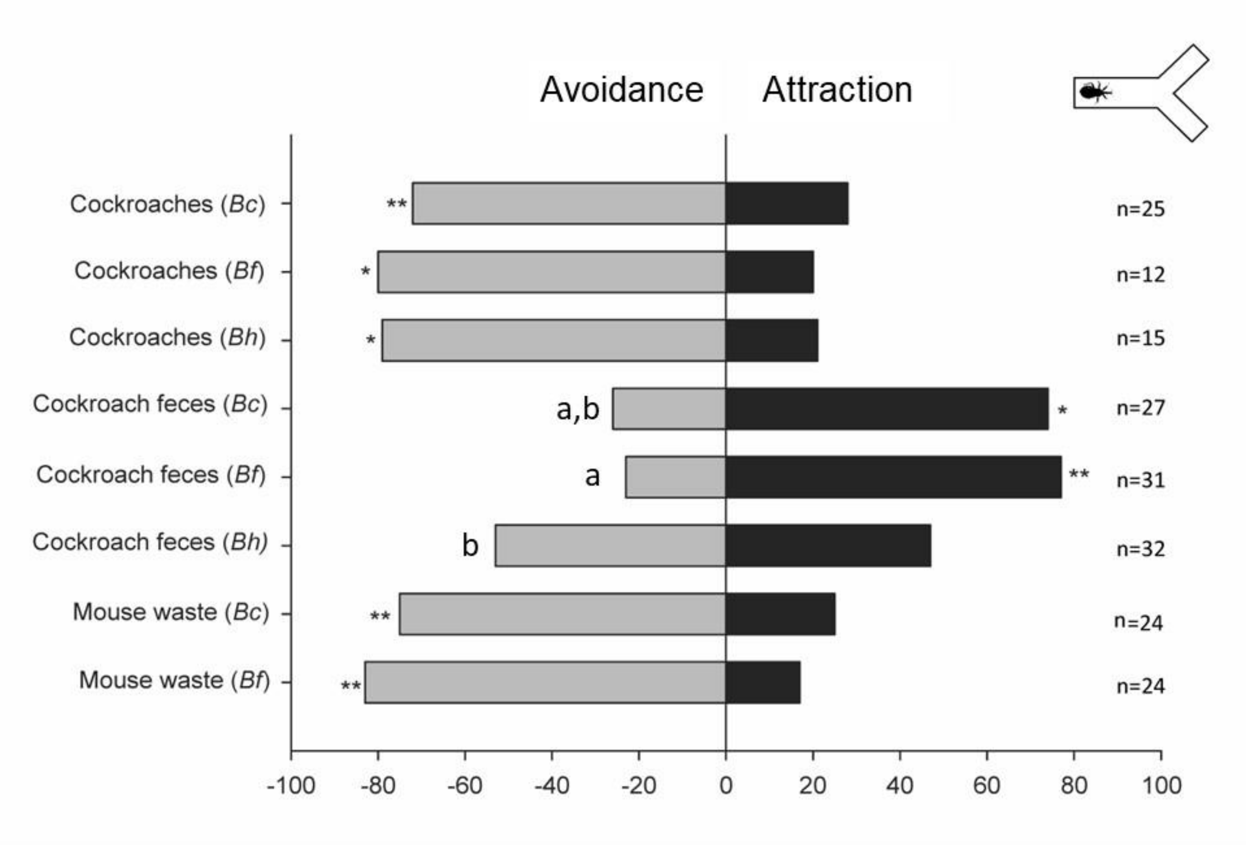


Fig 3


Avoidance
Attraction
Cockroaches (*Bc*)
Cockroaches (*Bf*)
Cockroaches (*Bh*)
Cockroach feces (*Bc*)
Cockroach feces (*Bf*)
Cockroach feces (*Bh*)
Mouse waste (*Bc*)
Mouse waste (*Bf*)
**
*
*
a,b
*
a
**
b
**
**
n=25
n=12
n=15
n=27
n=31
n=32
n=24
n=24
-100
-80
-60
-40
-20
0
20
40
60
80
100