

**THE EFFECTS OF STARVATION ON OLFACTORY RESPONSES OF THE
BLOOD-SUCKING TRIATOMINE, *RHODNIUS PROLIXUS***

By

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A Thesis Submitted to The Honors College

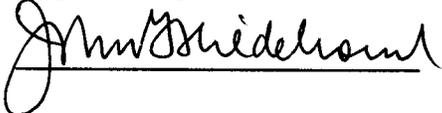
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Approved by:



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ABSTRACT:

The triatomine, *Rhodnius prolixus*, is a nocturnal, obligated blood-feeding insect that relies heavily on olfactory cues for finding mates, hosts, and refuges. Aggregation pheromones, present in conspecific feces and serve to mark refuge locations, elicit olfactory-driven behavior of the insect, which is modulated by temporal factors. However, it is not known if the physiological factor, hunger, also modulates the response to the pheromone. Behavioral experiments using nymphs of *R. prolixus* starved for three lengths of time post-ecdysis were conducted. Insects were individually tested during the three hours of scotophase in a dual choice, T-shaped olfactometer. Non-starved insects were found to distribute randomly in presence of two out of the four host-derived odors tested and aggregation pheromones. Starved nymphs significantly preferred the host-derived odors, but had reduced attraction to aggregation pheromones. Lastly, insects with prolonged starvation were significantly attracted to both aggregation pheromones and host-derived odors. Observed behaviors may present advantages for the insect in an ecological perspective.

INTRODUCTION:

Olfaction is a vital sensory modality for insects in a variety of context including foraging, mating, and searching of oviposition sites (Reisenman *et al.*, 2000). For nocturnal, hematophagous triatomines (Hemiptera: *Reduviidae*), commonly known as kissing bugs, cone-noses, or assassin bugs, olfactory cues are essential in searching for vertebrate hosts, which provide the nutritional blood meal required for both genders to grow at all life stages and in the case of adults, reproduce (Barrozo and Lazzari, 2004). Blood-feeding, however, is a dangerous task. A vertebrate host is not only a source of food but a potential predator as well for triatomines and all other hematophagous insects (Bodin *et al.*, 2009a, Bodin *et al.*, 2009b). Therefore, responses to olfactory stimuli should be modulated to reduce the risk of feeding.

Previously, studies have shown temporal and physiological factors (e.g. feeding status) modify the feeding behavior of triatomines in response to the same chemical or mechanical stimuli (Barrozo and Lazzari, 2004; Bodin *et al.*, 2009a; Bodin *et al.*, 2009b; Lazzari, 2009). A host-emitted chemical cue like carbon dioxide, for example, was highly attractive to the insects during the first few hours after dusk or early scotophase, when insects leave their shelter in search of food (Lazzari and Lorenzo, 2009). Yet, carbon dioxide becomes unattractive during the second period of highest activity, few hours before dawn or late scotophase, when insects return to shelter (Guerenstein and Lazzari, 2010; Lazzari, 2009). Moreover, the response of kissing bugs to host odors was also modulated by feeding status; insects that recently fed displayed repulsive behavior towards host-seeking odorants (Bodin *et al.*, 2009a, Bodin *et al.*, 2009b).

Kissing bugs also rely on chemical cues to find shelter or refuge. The feces that get deposited at refuge entrances contain aggregation pheromones that act as a chemical landmark to guide returning insects to shelter (Reisenman *et al.*, 2000; Vitta *et al.*, 2007). Likewise, the olfactory modulated response of triatomines to aggregation pheromones is temporally controlled. Highest attraction to aggregation pheromones was instead documented during late scotophase and reduced attraction to pheromones was observed during early scotophase (Bodin *et al.*, 2009a; Bodin *et al.*, 2009b; Reisenman *et al.*, 2000; Vitta *et al.*, 2007). Whether the olfactory-driven behavior towards aggregation pheromones is, nonetheless, modulated by the physiological condition of hunger is still unknown.

Hence, the focus of this study was to examine the effects of hunger on the olfactory modulated behavior of triatomines in response to aggregation pheromones and additionally to other host-derived odorants and odorant mixtures. Since triatomines become increasingly vulnerable to host predation after blood-feeding and nymphs prepare to molt, it was hypothesized that for a period post-feeding insects would be attracted to aggregation pheromones regardless of the time of day. Conversely as the duration after feeding lengthens, insects were predicted to become more attracted to host odorants and less to aggregation pheromones.

The species of triatomines, *Rhodnius prolixus*, the main vector of Chagas Disease in Mexico, Central, and Northern South America (Coura and Borges-Pereira, 2010), was used to assess the presumptions. Findings of the biology and olfactory behavior of kissing bugs could contribute to useful tools in the monitor and control of these insects (e.g. odor-baited traps and detection devices) in endemic areas (Guerenstein and Lazzari, 2010; Guerenstein

et al., 1995), and offer invaluable insights on the regulation of the olfactory response in insects, overall.

MATERIALS AND METHODS:

Insect rearing and collection:

Laboratory colony of *R. prolixus* was kept in a temperature and humidity controlled setting, where feeding occurred weekly on artificial blood feeders filled with bovine blood (Núñez and Lazzari, 1990). Fourth (N4) and third (N4) nymphal stages were separately collected 4-5 days post-ecdysis and placed in a reverse 12-hour light/dark cycle (8:00PM/8:00PM). Nymphs were sorted into three experimental groups according to the period in which the insects would remain unfed: non-starved (14 ± 2 days [N4], 10 ± 2 days [N3]), starved (6-7 weeks [N4]; 5-6 weeks [N3]), and more starved (8-9 weeks [N4]).

Behavioral test:

The olfactory response of *R. prolixus* was tested during the first three hours of scotophase in a dual-choice, T-shape designed olfactometer, or the “T-maze” setup (Fig. 1). The main T-shaped apparatus was fashioned from tygon tubing and spare ones of equal dimensions were also constructed. The design allowed for one arm to deliver a constant, charcoaled-filtered airstream (0.5 LPM) of odor-laden air, while the opposing arm simultaneously delivered odorless air (control) with the same constant airstream. Mass flow controllers, linked to the setup, monitored and supplied the correct unit of air to each arm. The unidirectional flow of air in the olfactometer presented an enclosed environment

in which each insect being tested would only receive the mixture of the air sent by the arms without external olfactory inputs.

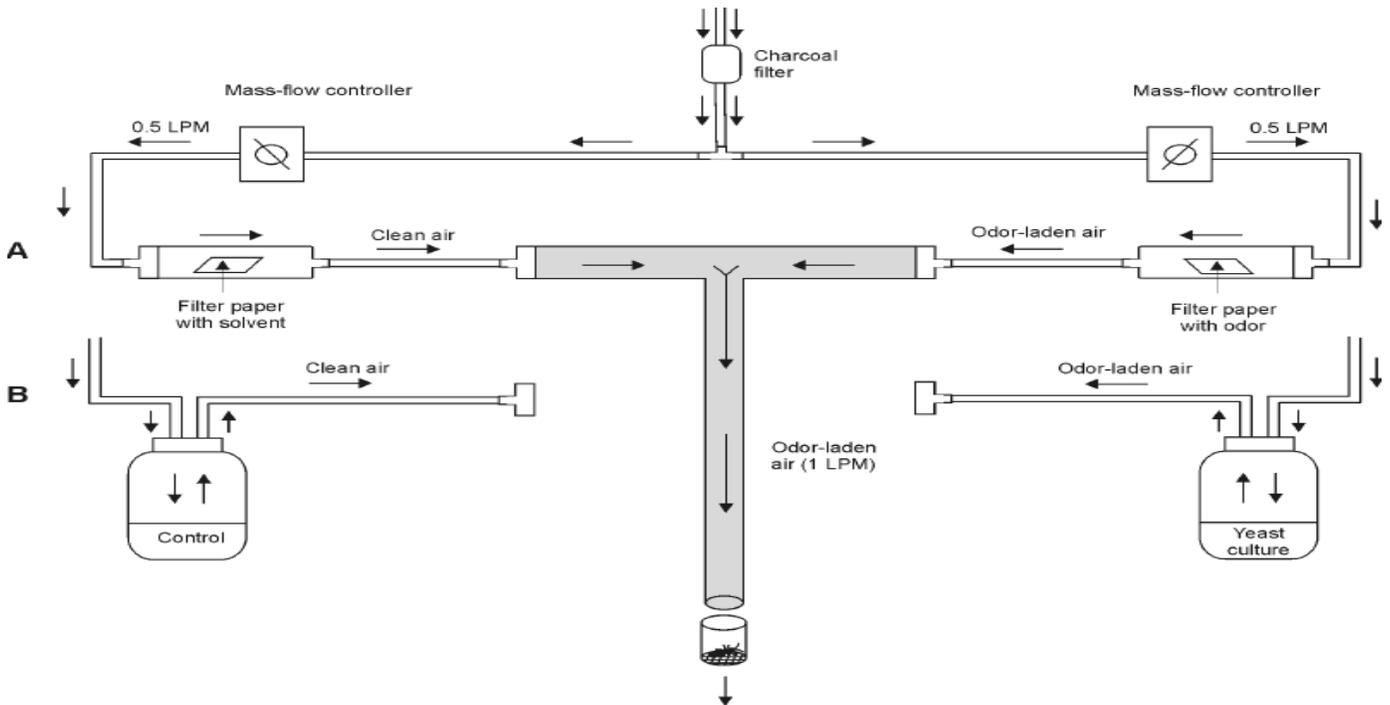


Figure 1: Setup of dual-choice, T-shaped olfactometer. A filtered airstream flowed through each arm at 0.5 LPM. 1A) Host-derived odor (50 μ L), feces, or solvent control (mineral oil) was loaded onto filter paper and placed in a glass syringe. 1B) Yeast was cultured in sealed glass jars and influx of air displaced yeast volatiles into the arm of the T-tube. Arrows indicate airflow direction.

Tested odors included single host-derived odors (0.05% limonene, 0.05% α -pinene, 0.006% nonanal), yeast cultures (contained a mixture of volatiles and a source of CO₂), and aggregation pheromones (feces). The concentration for each host-derived odor was made by dilution of the pure solute with mineral oil as solvent. Host-derived odors were chosen based on chemicals that were detected on external areas (e.g. skin) of common vertebrate hosts (Barrozo and Lazzari, 2004; Guerenstein and Lazzari, 2010; Lazzari, 2009; Ortiz and Molina, 2010). Triatomines have also been shown to exhibit a high attraction

towards yeast volatiles (Guerenstein *et al.*, 1995); therefore, the yeast volatiles acted as the positive control for this experiment.

When testing, an odorant of 50 μ L was impregnated on 1 x 1 mm pieces of filter paper and placed in a glass syringe that connected to the overall apparatus (Fig 1). Yeast cultures were cultivated in tightly sealed glass jars and connected to the T-maze setup. Feces, the source of aggregation pheromones, were freshly collected on 1 x 1 mm filter papers by gentle, manual depression of the abdomen of the insects, and then employed in experiments within 1-4 days to avoid the loss of effectiveness (Bodin *et al.*, 2009a; Bodin *et al.*, 2009b). The odorless arm contained clean air or filter paper loaded with mineral oil (50 μ L).

Once nymphs reached the desired starvation time, insects were individually transferred to a short release tube and left undisturbed for 5-10 minutes before testing. The release tubes had one end covered by mesh and a free end to slide into the opening of the longest arm of the T-maze. Ideally, the insect walked upstream when released into the T-maze, reached the T-intersection, and made a choice to continue into the arm with odor-laden air or odorless air. A choice was considered if an insect traveled into the arm halfway and made the decision within 8 minutes.

All choices were recorded by observation, and insects visibly startled were noted but not counted in the final results. Insects were used only once and tested based on availability. To avoid bias from scent tracts left by previously tested individuals (Vitta *et al.*, 2007), the arms of the T-shaped were switched after every test and the entire T-maze tube was replaced every three trials. Odorants were either reloaded every 15-20 minutes

to control for volatile loss. In addition, elimination of visual factors was executed by framing the experimental setup with a symmetrical folder barrier. Experiments were conducted in the dark with a red incandescent lamp as the sole light source. A control series with clean air present in both arm of the T-maze was ran to ensure the airstream was free of odor contaminants and corrected for appositional issues that might affect the animals' response.

T-maze components were thoroughly cleaned with 70% ethanol spray and given time to air dry before the next use. After six to seven experiments, all parts were entirely submerged in pure ethanol to remove any volatile residues.

Statistical analysis:

Results were analyzed using the binomial probability distribution test. Statistical significance was based on the expected 50% random distribution of insects to the odor-laden air and clean air for each series.

RESULTS:

The olfactory modulated response of the insects (N = 329) to the tested odors varied among the three experimental groups. In the control series, animals (tested only from the starved group) displayed random distribution between the two arms of the olfactometer in absence of any odorants ($p > 0.05$). The group of non-starved insects randomly distributed in the presence of yeast volatiles, nonanal, and aggregation pheromones ($p > 0.05$); however, the same group was repelled by (-)-limonene ($p = 0.046$) and significantly preferred α -

pinene ($p=0.004$). Nymphs in the starved group were attracted to all host-derived odorants and preferred the odorless air when tested with aggregation pheromones ($p<0.05$ in all cases). On the contrary, the more starved insects were significantly attracted to (-)-limonene ($p=0.032$) as well as to aggregation pheromones ($p=0.032$). Proportions of olfactory response of insects to each odor series are graphically represented in *Fig. 2*, and the *Appendix* can be referenced for numerical values.

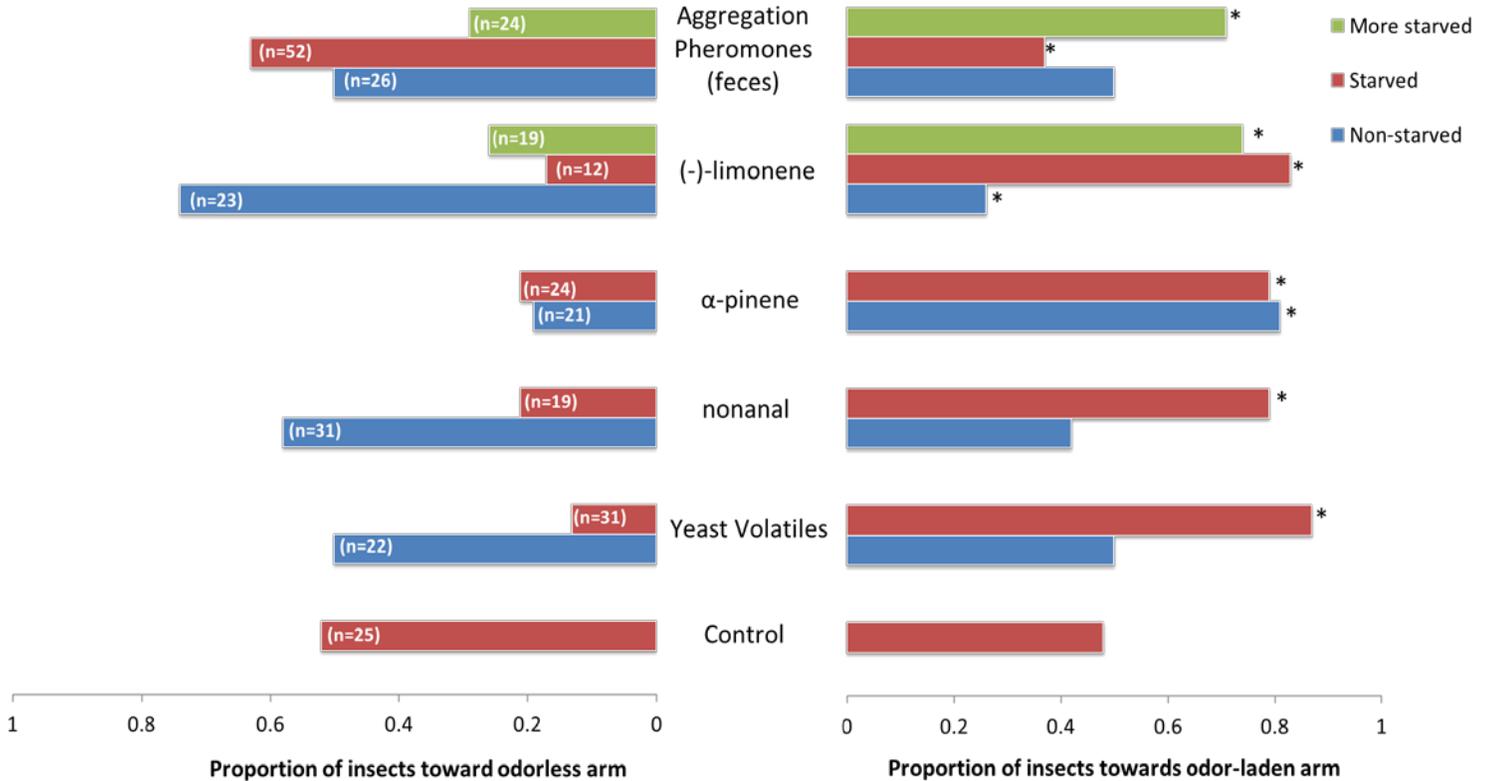


Figure 2: Olfactory response of insects at different starvation stages with respect to host-derived odors or conspecific aggregation pheromones. Data indicates the proportion of insects selecting the odor-laden arm of the maze (right) or odorless arm (left). Asterisks indicate statistical significance (binomial tests, $p<0.05$; $N=329$) for each test group and odor. White numbers listed in bars represent the sample size per series.

To further investigate the observed behavior of insects with prolonged starvation, this group of insects was tested in a competence series: odorless air was removed and both aggregation pheromones and (-)-limonene were simultaneously presented in the T-maze apparatus. As shown in *Fig. 3*, the insects preferred the host-derived odor over the aggregation pheromone, but the proportion of insects was not significantly different ($p=0.191$; $N=47$).

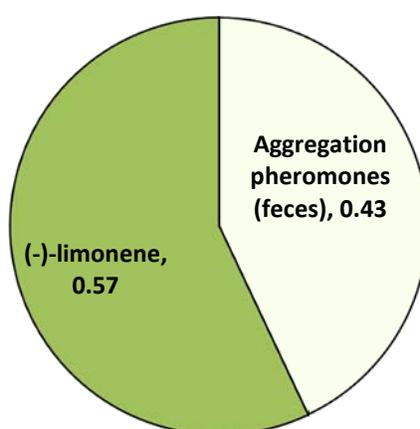


Figure 3: Proportion of more starved insect group response to simultaneous presentation of host-derived odor ((-)-limonene) and aggregation pheromones.

DISCUSSION:

Results indicate the olfactory modulated behavior of *R. prolixus* to certain host odorants and aggregation pheromones is affected by hunger status. The starved group performed as predicted. There was a high attraction towards host-derived odors and reduced attraction to aggregation pheromones. For the non-starved group, the insects were mainly indifferent towards the odors except for (-)-limonene and α -pinene, which were significantly repelled and preferred respectively. Because this group experienced the

shortest period of starvation post-ecdysis, the high attraction to the host-odorant, α -pinene, was against predictions. The single odorant, α -pinene, has been isolated from vertebrate host of multiple hematophagous insects and was found to initiate neuronal excitation in kissing bugs (Guerenstein, 1999; Guerenstein and Lazzari, 2009). Despite these facts, there is no support of α -pinene regulating the host-feeding response in blood-sucking insects and therefore, the odorant could, otherwise, play an alternative physiological role in these insects.

Furthermore, the group with the longest period of starvation also behaved in disagreement with what was expected. When presented with aggregation pheromones, a proportion of 0.71 insects preferred the odor, which was near equivalent to the proportion (0.74) of more starved animals that preferred the odor-laden arm in presence of the host-derived odor tested, (-)-limonene. We suspected the prolonged nutritional deprivation led to an extremely weakened state in which the animals would remain aggregated, unless a clear olfactory cue signaling food was detected. Results from the competence series suggested otherwise (Fig. 3). There was a tendency for the insects to orient toward the odor, but the proportion was statistically insignificant. However, the data does confirm observations made by Takano-Lee and Edman (2001), who documented a tendency for *R. prolixus* to seek for refuge more frequently when fasted than blood fed in a stimulated house environment.

The olfactory response of these insects show high integrity given that our results generally paralleled with previous behavioral assessments of triatomines using Dahmen's (1980) design to measure orientation (Barrazo and Lazzari, 2004; Guerenstein and Lazzari,

2009; Bodin *et al.*, 2009a). Thus, olfactory sensory modulations can certainly be viewed as an adaptation of hematophagy that dictates the survival of blood-sucking triatomines.

Future directions are to study other levels of modulation and the mechanisms involved with the olfactory response of *R. prolixus* induced by physiological factors. Electrophysiological techniques such as electroantennogram recordings (EAGs), completed in the malaria vector, *Anopheles gambiae*, can be used to provide a crude analysis of the firing patterns of the odor receptors in the antennae to different odors (Costanini *et al.*, 2001). Evidence from other studies also suggest neurohormones of both kinin-like and CRF-like peptides, released into the hemolymph of *R. prolixus* post-feeding, could be another factor responsible for regulation of olfactory responses (Te Brugge and Orchard, 2002; Bodin *et al.*, 2009a). Detailed examination of the hemolymph factors is required to determine any link to mediation of olfaction.

In conclusion, the research has found that the nutritional state of *R. prolixus* does impact the olfactory behavior towards odors with varying biological meanings. By continuing to study kissing bugs, we can increase our understanding on the physiological and biological adaptations that allow hematophagous insects to exploit vertebrate blood as a food source, and link such discoveries to assist in the control of vector-borne diseases.

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APPENDIX:

Toward Odor	Control	Yeast Volatiles	Nonanal	alpha-pinene	(-)-Limonene	Aggregation Pheromones (Feces)
Non-starved	n/a	0.5	0.42	0.81	0.26	0.5
Starved	0.48	0.87	0.79	0.79	0.83	0.37
More Starved	n/a	n/a	n/a	n/a	0.74	0.71
Away from Odor	Control	Yeast Volatiles	Nonanal	alpha-pinene	(-)-Limonene	Aggregation Pheromones (Feces)
Non-starved	n/a	0.5	0.58	0.19	0.74	0.5
Starved	0.52	0.13	0.21	0.21	0.17	0.63
More Starved	n/a	n/a	n/a	n/a	0.26	0.29

Table 1: Orientation of insects to different odors shown in proportions. Odorants were presented in the T-shaped olfactometer.

	Non-starved	Starved	More Starved
Yeast Volatiles	0.058	0.000	n/a
(-)-limonene	0.019	0.047	0.032
alpha-pinene	0.004	0.000	n/a
nonanal	0.093	0.010	n/a
Aggregation pheromones (feces)	0.577	0.035	0.032
Competence Test	n/a	n/a	0.191

Table 2: P-values obtained from Binomial Probability Distribution Test for each odorant series.