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## Amines from vertebrates guide triatomine bugs to resources



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## ABSTRACT

Most triatomine bugs (Heteroptera: Reduviidae) are nest-living insects that require vertebrate blood or invertebrate haemolymph to complete their life cycle. Vertebrates accumulate excretory products in or near their nesting sites and we hypothesize that triatomines use emanations from such host wastes when searching for resources. Here we recount how triatomine bugs increase upwind locomotion on a servosphere in response to volatile amine constituents of vertebrate excretions. Fresh chicken faeces is strongly attractive to *Rhodnius prolixus* nymphs. Ammonia induces attraction and an increase in both speed and total path length by *R. prolixus* on the servosphere. Whereas ethylamine and dimethylamine attract *R. prolixus*, *Triatoma infestans* and *Panstrongylus geniculatus*, other amine constituents of vertebrate excretions such as isobutylamine and hexylamine induce *R. prolixus* nymphs to walk faster and for a longer period. These amines are derived from generally occurring metabolites of vertebrates and from gut flora metabolism. We conclude that amines and other products associated with nesting hosts serve as signals for foraging triatomines.

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## 1. Introduction

Most triatomine bugs (Heteroptera, Reduviidae) are nest-living insects (Usinger et al., 1966) that require vertebrate blood, though some can live off of invertebrate haemolymph (Sandoval et al., 2013; Lorosa et al., 2000) to complete their life cycle. Of the nearly 140 extant species of this subfamily a few can live in association with humans. These synanthropic species serve as epidemiologically important vectors of Chagas disease (Bastien, 1998; Dujardin et al., 2000). The other spp. are exclusively associated with wild hosts, i.e. sylvatic bugs, but an increasing number of triatomine spp. are in the process of colonizing human habitats (Rabinovich et al., 2011). The most epidemiologically important triatomines are represented in the genera *Triatoma* Laporte, 1832; *Rhodnius* Stål, 1859 and *Panstrongylus* Berg, 1879 (Gaunt and Miles, 2000); 80% of triatomine species belong to these three genera. *Rhodnius prolixus* Stål, 1859, *Triatoma infestans* Klug, 1834 and *Panstrongylus geniculatus* (Latreille, 1811) are important vectors of Chagas disease. *R. prolixus* and *T. infestans* are the most widespread vectors of this disease whereas sylvatic populations are associated with, among other hosts, arboreal vertebrates and

guinea pigs. Both species are also well adapted to exploit domestic and peridomestic habitats where they show varying degrees of host preferences (Gürtler et al., 2009). *P. geniculatus* has been recorded as exclusively sylvatic (Lent and Wygodzinsky, 1979), habitually living in humid forests in a diverse array of vertebrate nesting sites, including armadillo (Dasypodidae) burrows. However, there are increasing reports of this species invading domestic and peridomestic habitats (Rabinovich et al., 2011) over large areas of Latin America: Venezuela (Felicangeli et al., 2004), Colombia (Wolff and Castillo, 2000), Brazil (Valente, 1999), Perú (Cáceres et al., 2002), Ecuador (Aguilar et al., 1999), Bolivia (Depickère et al., 2011) and Argentina (Damborsky et al., 2001). Furthermore, Aldana et al. (2011) has found morphometric evidence of *P. geniculatus* domiciliation in houses in the city of Caracas, Venezuela.

In general, triatomines spend most of the daytime sheltering near their nidicolous hosts and frequently search for blood at night when hosts are at rest or off guard and when the air is less desiccating (Heger et al., 2006). Within nests (*sensu lato*), triatomines can readily find a blood meal because of host proximity using heat (Lazzari and Núñez, 1989; Schmitz et al., 2000; Ferreira et al., 2007; Milne et al., 2009; Catalá, 2011), carbon dioxide (Núñez, 1982; Taneja and Guerin, 1995), ammonia (Taneja and Guerin, 1997) as well as volatiles from different chemical classes, i.e. carboxylic acids, alcohols and aldehydes, emanating from the nearby host (Guerenstein and Guerin, 2001; Otálora-Luna et al., 2004; Barrozo and Lazzari, 2004a,b; Ortiz and Molina, 2010; Ortiz et al., 2011). As transmission of heat decreases abruptly with distance

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from the host (Dusenbery, 1992), emanations of chemical origin may play a special role in triatomine searching for food and host refugia.

Previous studies on triatomines have mainly focused on odours emanating from the host skin and breath (Otálora-Luna et al., 2004; Guerenstein and Lazzari, 2009; Ortiz et al., 2011) which are by-products of host-associated microflora and host metabolism (Guerin et al., 2000). To identify chemicals cues that could assist triatomines in seeking food and host refugia we examine here the behaviour of *R. prolixus*, *T. infestans* and *P. geniculatus* in response to the odour of vertebrate excretions, based on the assumption that chemicals abundant near nesting vertebrates could serve as cues for host orientation. Particular attention is paid to the propensity of triatomines to show oriented responses to volatile amine emanations from hosts as such molecules are prominent constituents of vertebrate wastes (Spoelstra, 1980; Mackie et al., 1998; Le et al., 2005) and since receptor cells in olfactory sensilla on the antennae of *T. infestans* have been characterized as responding to ammonia and amines (Taneja and Guerin, 1997; Diehl et al., 2003).

## 2. Methods

### 2.1. Insects

*R. prolixus* and *T. infestans* colonies were fed once per month and the *P. geniculatus* colony two or three times per month on chickens, as the latter species has a lower tolerance to starvation, lower reproductive rate and a longer generation time (Cabello and Galindez, 1998). The three colonies were maintained in climate chambers: *R. prolixus* and *T. infestans* at  $80 \pm 10\%$  RH,  $22 \pm 1^\circ\text{C}$ , 12:12 LL:DD and *P. geniculatus* at  $90 \pm 10\%$  RH at the same temperature and light cycle. *R. prolixus* and *T. infestans* colonies are 20 years old (originating from colonies at the University of Cambridge, UK and subsequently maintained at the Swiss Tropical and Public Health Institute, Basel). The *P. geniculatus* colony originating from specimens collected in Montebello, Amalfi Municipality, Department of Antioquia, Colombia in May 2003 ( $6^\circ55'58''\text{N}$ ;  $75^\circ05'30''\text{W}$ ,  $18\text{--}24^\circ\text{C}$ ; annual rainfall 1000–2000 mm; Wolff et al., 2001) were reared in the laboratory since 2004. Fifth-instar *R. prolixus* and *T. infestans* nymphs were starved for 8–12 weeks and fifth-instar *P. geniculatus* nymphs, with a lower tolerance to starvation, for 4–6 weeks after moulting to induce high appetite levels for behavioural experiments. Fifth-instar nymphs were used as they were produced in higher numbers in the colonies than adults and, additionally, the latter risk flying from the servosphere (*vide infra*). Fifth-instars are suitable to study triatomine active dispersion as these nymphs can walk significant distances (29–60 m) from the nearest infested sites (Vázquez-Prokopec et al., 2004).

### 2.2. Behavioural recordings on a servosphere

The behavioural responses of walking triatomine nymphs were recorded using a servosphere (Kramer, 1976) along with two infrared-sensitive video cameras as already described (Taneja and Guerin, 1995; Otálora-Luna et al., 2004). The servosphere allows an untethered triatomine to walk unimpeded in all directions at the apex of a sphere (50 cm diam) in an experimental setup where sensory modalities other than the test chemostimuli presented in an air current are excluded. The sphere is mounted so that it can be rotated around two orthogonal axes in the horizontal plane by two low inertia servomotors. A beam of filtered incandescent light (filter cut-off 780 nm) from a position sensor fixed 26 cm directly above the apex of the sphere illuminates a circle 4.0 cm dia. A small square (ca. 2 mm square) of retroreflective material (3 M, No. 7610, Switzerland) glued to the pronotum of the bug reflects light back to

the sensor. The reflected light supplies the position sensor with information on the bug's movements and this is used to drive the servo-motors which compensates the sphere's position to maintain the insect at the apex. Movements of the sphere caused by the walking bug are recorded in a computer by pulse-generators mounted on the sphere equator thus permitting the reconstruction of the tracks described by the bugs. The  $x$ – $y$  coordinates of displacements made by the animal are recorded at 0.1 s intervals by two incremental pulse-generators mounted on the sphere's equator, both working at a resolution of 0.1 mm. This apparatus is an open loop set up, i.e. it records the behaviours a bug employs to approach a stimulus source but it does not allow the insect to reach it. In this manner locomotion of the insect does not change the concentration of the volatile stimulus it encounters in the air while walking, assuring almost constant stimulation over experimental time. A schematic representation of the servosphere is presented in McMahon and Guerin (2000). In addition, video records using a CCD camera were made of bug leg, antennal and body movements throughout each experiment. The insect was placed on the servosphere with forceps and the experiments started when the following behaviours were no longer observed: (a) appetite behaviours such as *reaching* in the air stream with the antennae or intense *tapping* on the antennae with the first legs (Otálora-Luna et al., 2004), (b) lowering of the body until it laid on the sphere (akinesis), or (c) no movement with the bug laying on one side (feigning to be dead). Records where such behaviours were noted were excluded to reduce the risk of recording behaviours associated with manipulation of the insect.

The responses of bugs to chemostimuli presented in the air stream (*vide infra*) were recorded in three consecutive 2-min periods: in the air stream alone (control), the air stream plus the chemostimulus (test) and the air stream after removal of the chemostimulus (end-control). Recordings of behaviours were made in the dark during the early and late scotophase as most triatomine species are more active during the night (Lent and Wygodzinsky, 1979; Barrozo et al., 2004). Most bugs walked downwind at the air speed employed (15 cm/s, *vide infra*). The few bugs that walked consistently upwind during the initial period in the odour-free air stream were discarded (between 1 and 2 each of 20 for *R. prolixus* and *T. infestans*, and between 1 and 2 of 10 for *P. geniculatus*).

### 2.3. Collection and preparation of biological extracts

Chicken faeces were obtained from one-year-old chickens reared in a cage with commercially available food and supplemented occasionally with vegetables. Human urine was obtained from an adult male who did not have a diet particularly rich in fish or other sea-foods (people who have a diet rich in sea-foods excrete more amines; Zhang et al., 1999). Human breath from the same adult was blown into a 1 l gas-wash bottle for 30 s and was left for at least 20 min before the start of a test to allow the temperature to drop to ambient levels.

To extract amines from faeces to water, chicken faeces (10 g) were acidified with a 20%  $\text{H}_2\text{SO}_4$  solution to reach pH 2, incubated for 10 min and centrifuged at 1000g for 10 min at  $-4^\circ\text{C}$ . To extract amines to dichloromethane (DCM, Merck, spectroscopy grade) the aqueous supernatant was mixed with DCM and brought to pH 10 by adding a 30% NaOH solution. The resultant basic fraction was washed with a saturated NaCl solution and conserved in a glass ampoule at  $-20^\circ\text{C}$ . Volatile components of fresh urine (200 ml) were entrained on to a porous polymer (500 mg of Soxhlet-extracted Porapak Q<sup>TM</sup>, Waters, 50–80 mesh, packed into a 6 cm long 6.23 mm i.d. glass tube and held with glass wool plugs). The polymer was preconditioned for 1.5 h with pure  $\text{N}_2$  at  $180^\circ\text{C}$ . Pure  $\text{N}_2$  was bubbled through the urine in a gas-wash bottle to convey volatiles to the porous polymer cartridge (150 ml/min) for 5 h.

Entrained volatiles were eluted from the polymer with DCM and the first drops (400  $\mu$ l) were transferred to a glass ampoule and conserved at  $-20^{\circ}\text{C}$ . Neither ammonia nor carbon dioxide was detected from the chicken faeces and urine extracts (*vide infra* for detection instruments).

#### 2.4. Measurement of $\text{CO}_2$ and $\text{NH}_3$ levels from test samples

$\text{CO}_2$  released from fresh biological samples was measured in the air stream at the apex of the sphere with an infra-red gas analyzer (LI-COR LI-820, USA, flow 1.5 l/min, accuracy  $\pm 3\%$ , signal averaging 1 s, detection limit 5 ppm). Mean values over 10 s intervals for 30 min were compared with a control that consisted of a 1 l gas-wash bottle with 1 ml of nanopure water.  $\text{CO}_2$  released in human breath collected in the gas-wash bottle (mean increase 1130.10, SD 4.90 ppm) and in chicken faeces (mean increase 7.60, SD 3.25 ppm) was above local ambient levels (mean 401.03, SD 1.38 ppm,  $P < 0.01$ , *t*-test).  $\text{NH}_3$  released by fresh organic samples was measured in the air stream at the apex of the sphere with an electrochemical sensor (DrägerSensor Pac III S, Lübeck, Germany, accuracy  $\pm 1\%$ , flow 0.5 l/min). When the ammonia concentration was lower than 1 ppm (lowest sensitivity level of the sensor) the measurement was made at the exit of the gas-wash bottle in which the sample was held after allowing the ammonia level to accumulate. Subsequently this measured value was divided by 25, the dilution factor that arose from mixing the air from the gas-wash bottle in the humidified air stream flowing over the sphere apex. Mean values over 10 s intervals for 1 min were compared with controls. Mean concentrations of ammonia at the apex of the sphere were 123.11 ppb (SD 2.26) for diluted human breath and 95.01 ppb (SD 3.00) for chicken faeces. No ammonia was detected from controls.

#### 2.5. Chemostimuli and their delivery for behavioural recordings

After collection, biological samples were placed directly in gas-wash bottles using gloves or a spatula as necessary. Controls for biological samples consisted of gas-wash bottles with 2.5 g of cotton with 1 ml nanopure water. The following compounds and doses in the odour-delivery bottle (indicated in parenthesis) were tested separately on the servosphere: ethylamine (100  $\mu$ g), dimethylamine (100  $\mu$ g), methylamine (100  $\mu$ g), trimethylamine (100  $\mu$ g), isobutylamine (100  $\mu$ g), isopentylamine (100  $\mu$ g) and hexylamine (100  $\mu$ g). Two log-fold dilutions of dimethylamine (DMA) were also tested (10  $\mu$ g and 1  $\mu$ g). Ethylamine (EA), DMA and trimethylamine were prepared from 0.05% aqueous solutions with nanopure water, and the other chemicals tested (spectroscopy grade) were diluted in DCM. The concentration of  $\text{NH}_3$  tested was 200 ppb in the airstream at the apex of the sphere from 200  $\mu$ l of 0.25% aqueous  $\text{NH}_4\text{OH}$  placed on filter paper in the gas-wash bottle. These solutions were applied (200  $\mu$ l) to filter paper discs (120 mm diameter) placed at the bottom of a 1 l gas-wash bottle. DCM was left to evaporate for 6 s before placing the filter paper in the bottle. Controls consisted of bottles with the same amount of solvent on filter paper. Gas-wash bottles were then left for at least 10 min before presenting the chemostimuli to the bugs. The filter papers were replaced after 2–3 tests. Solvents and synthetic chemicals used in this investigation were  $\geq 97\%$  pure and acquired from Fluka, Merck and Aldrich (Switzerland). Nomenclature of chemicals follows the employed by NIST (<http://webbook.nist.gov/chemistry/>).

An automated stimulus delivery system, independent of the experimental room, was used as has been described previously (Taneja and Guerin 1995, 1997). Briefly, charcoal-filtered air passing through 1 l gas-wash bottles (160 ml/min) served to transport the vapours to a charcoal-filtered and humidified air stream

(15 cm/s, 4 l/min,  $23\text{--}25^{\circ}\text{C}$ , 40–60% RH) flowing over the bug at the apex of the sphere. The air from the gas-wash bottles was diluted 25-fold in the main air stream. Gas-wash bottles were washed with RBS 35-concentrate (Fluka, Switzerland) diluted in demineralized warm water ( $50^{\circ}\text{C}$ , 15 ml/l) for at least 3 h prior to use.

#### 2.6. Behaviour analysis and statistics

The walking tracks of the triatomines were reconstructed by plotting the cumulative addition of consecutive positions. The *x*–*y* coordinates provided by the servosphere at intervals of 0.1 s were merged in step sizes of 3 units to efficiently summarize the tracks (Kitching and Zalucki, 1982). This merger provided step-size intervals of 0.3 s which allowed the insect to move at least 60% of its length before recording its next position, taking into account that a triatomine 5th. instar nymph's mean length is approximately 1.5 cm and average speed is approximately 3 cm/s. This step-size was long enough to reduce noise in the gait of the insect such as wobbling. Subsequently, a running mean was applied over five successive (0.3 s) intervals to smoothen track records in order to remove other noise related to the insect's gait that was detected by the servosphere. Additionally, 5 mm/s was considered the minimum speed the insect had to achieve to be considered walking; values below this speed were produced by other movements such as grooming the antennae or legs. Instantaneous displacement and direction were computed from the position changes within each interval. Other kinematic parameters were calculated from these instantaneous values, for control, test and end-control periods. Target vector (TV) and increased upwind locomotion (IUL) during the test period compared to the control were used as attraction indexes. Target vector was calculated by multiplying the cosine of the circular mean vector by its length (Jones, 1977; Batschelet, 1981; Bell, 1991). This term is equivalent to the "directionality" measure used by Jones (1977) and the "orientation index" of Barrozo and Lazzari (2004a,b). This variable incorporates both the mean direction of a path and the consistency with which a particular direction is maintained, i.e. path straightness, and comprises values between  $-1$  and  $+1$ . The increase in upwind locomotion was calculated as each insect's upwind walk during the test period minus the control period, where upwind locomotion was the walking distance in a cone  $60^{\circ}$  either side of due upwind. Speed, i.e. magnitude of velocity, and path length during the test period relative to the control period served as activation indexes, and were calculated as the difference between the median speed and median path length between control and test periods. The presence of several *fixed action patterns*, i.e. instinctive responses that occur reliably in the presence of identifiable stimuli, which are indivisible and run to completion (Eibl-Eibesfeldt, 1979; Lorenz, 1981), were identified but not quantified. Antennal tapping was observed each time the bug lifted one or both legs to tap the antennae one or more times and brought the leg or legs down. Cleaning behaviours were recorded when a bug cleaned antennae, tarsi, proboscis or anus. Increases in antennal reaching and antennal tapping as well as decreases in cleaning behaviours during the test compared to the control periods served as activation indicators.

Pairwise comparisons were done using Wilcoxon paired signed rank test. Multiple comparisons were done using this test with the Bonferroni correction to determine when post hoc tests were significant (Abdi, 2010). Video-recorded behaviours were compared using the two-tailed Wilcoxon paired signed rank test. Track analyses were performed using in-house developed software (gLocTrack) and statistical analyses were performed using R (version 2.2–1, Vienna, Austria; Ihaka and Gentleman, 1996) running on Linux. The interquartile range (IQR) is the difference between the 75th and 25th percentiles.

### 3. Results

#### 3.1. Behavioural responses to vertebrate waste products

The walking behaviour of *P. geniculatus* on the servosphere in the air stream alone was similar to that reported for *R. prolixus* and *T. infestans* nymphs (Taneja and Guerin, 1995, 1997). *P. geniculatus* walked in relatively straight bouts (median  $r$  0.83, IQR 0.022,  $n = 823$  bouts) punctuated by stops of variable duration (median 9.2 s, IQR 7.9) and performed similar antennal movements and cleaning behaviours as previously described for *R. prolixus* and *T. infestans*, i.e. scanning the air, touching the sphere, antennal cleaning, reaching and tapping (Otálora-Luna et al., 2004). The median length of walking bouts made by *P. geniculatus* was 85.70 mm (IQR 218.01). The median speed (9.81 mm/s, IQR 10.21,  $n = 31$  bugs) and maximum velocity (29.57 mm/s) reached by *P. geniculatus* was significantly lower than for *R. prolixus* (median 23.70 mm/s, IQR 22.12; maximum 43.71,  $n = 67$ ,  $P < 0.005$ ) but no different from *T. infestans* (median 11.13, IQR 18.63; maximum 29.60,  $n = 28$ ,  $P > 0.05$ ).

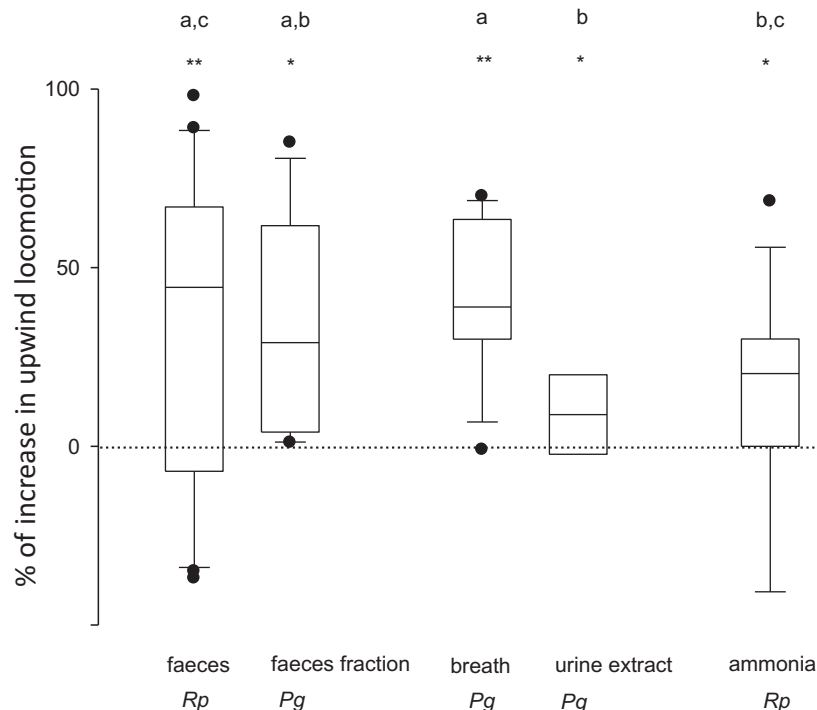
Activation behaviours and attraction were observed for triatomines when chicken faeces odour, human breath and human urine odour were presented to the bugs in the air stream (Fig. 1 and Table 1). Fresh chicken faeces (releasing 7.60 ppm CO<sub>2</sub> above ambient and 95.01 ppb NH<sub>3</sub>) proved strongly attractive to *R. prolixus* nymphs (Fig. 1). The median target vector (TV) shifted from  $-0.27$  during the control period to 0.56 during the 2-min stimulation period, and the median upwind locomotion increased by 44% during exposure to the chicken faeces odour compared to the control (IUL, Table 1). The volatiles emanating from chicken faeces also induced an increase of 5.30 mm/s in median speed and an increase of 23.92 cm in median path length compared to the air stream alone

(Table 1). A DCM extract containing basic and neutral volatile products from chicken faeces proved attractive to *P. geniculatus* nymphs (Fig. 1) as bugs walked more upwind during stimulus delivery (TV in test 0.68, IUL 29.46%) but did not walk faster or for a longer period than in the control period (Table 1). Diluted breath (releasing 1130.10 ppm CO<sub>2</sub> above ambient and 123.11 ppb NH<sub>3</sub>), used as a positive control, elicited activation and attraction of *P. geniculatus* nymphs that walked more upwind during stimulation than in the air stream alone (TV in test 0.75, IUL 39.37%, Fig. 1 and Table 1). Diluted breath induced an increase in median speed of 9.81 mm/s and in median path length of 68.51 cm in *P. geniculatus* compared to the control (Table 1). Increases in reaching and antennal tapping and decreases in cleaning behaviours were observed when fresh faeces odour was delivered to *R. prolixus* and diluted breath was delivered to *P. geniculatus*.

The extracted odour of human urine on a porous polymer proved attractive to triatomine nymphs (TV in test 0.57, IUL 12.75%) but less so compared to fresh faeces and diluted breath (Fig. 1). However, the urine extract elicited a higher level of activation than the faeces extract by causing an increase in the median speed by 8.66 mm/s (Table 1). In these tests, ammonia alone at 500 ppb attracted *R. prolixus* nymphs (TV in test 0.12, IUL 21.01%) and caused activation of the bugs by increasing the median speed by 7.05 mm/s and the median path length by 52.29 cm (Table 2), but proved less attractive than human breath.

#### 3.2. Behavioural responses to volatile amines

Volatile amines evoked the strongest behavioural responses of the compounds tested in this study. EA and DMA induced attraction in *R. prolixus*, *T. infestans* and *P. geniculatus* fifth-instar nymphs (Fig. 2) causing an increase in both target vector and in the relative



**Fig. 1.** Responses of triatomine fifth-instar nymphs to odours of fresh chicken faeces, a basic fraction of chicken faeces in dichloromethane, diluted human breath, a human urine odour extract and ammonia on a servosphere. The species tested are abbreviated as *P. geniculatus* (*Pg*) and *R. prolixus* (*Rp*). Responses are presented as the % increase in upwind locomotion in a cone 60° either side of due upwind in the test period relative to that in the preceding control period. Upwind locomotion increased significantly (Wilcoxon paired test, \* $P < 0.05$ , \*\* $P < 0.01$ ) during exposure of the bugs to all the biological samples tested and to ammonia (contained in both breath and faeces, but not in the faeces fraction). Treatments sharing the same letter are not significantly different (Wilcoxon non paired test,  $P > 0.05$ ). In the box plots, the lines within a box mark the median, the lower and upper boundary lines of a box indicate the 25th and 75th percentiles, the bars below and above indicate the 10th and 90th percentiles, respectively, and the points represent data beyond these limits.

**Table 1**

Activation and attraction responses of triatomine fifth-instar nymphs on a servosphere to odours of different biological substrates.

Biological substrate	N° of bugs tested	Sp.	Amount at source	Target vector (3)		Change from control to test period (3)		
				Control	Test	IUL (4) (%)	Speed (5) (mm/s)	Path length (5) (cm)
Chicken faeces	18	Rp (1)	2 g	-0.27	+0.56**	44.00**	5.30*	23.92*
Chicken faeces basic fraction	12	Pg (2)	200 µl	+0.24	+0.68**	29.46*	7.86	9.06
Urine porous polymer extract	10	Pg	200 µl	+0.01	+0.57*	12.75*	8.66*	8.13
Diluted breath	8	Pg	400 ml	+0.15	+0.75**	39.37**	9.81*	68.51**

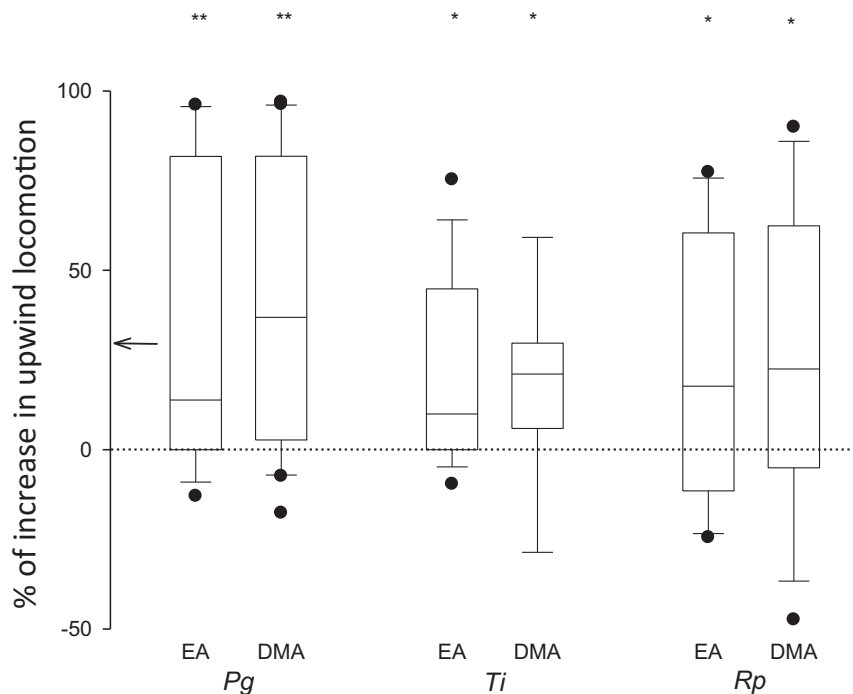
(1) Rp: *R. prolixus*, (2) Pg: *P. geniculatus*, (3) Median values are presented for activation and attraction responses of bugs (Wilcoxon paired test, \* $P < 0.05$ , \*\* $P < 0.01$ ), (4) IUL is the increase in upwind locomotion during the test period relative to the control period, (5) speed and path length are also presented as increases during the test period relative to the control period.

**Table 2**

Activation and attraction responses of triatomine fifth-instar nymphs on a servosphere to ammonia and amines.

Compound	N° of bugs tested	Sp.	Amount at source	Target vector (4)		Change from control to test period (4)		
				Control	Test	IUL (5) (%)	Speed (6) (mm/s)	Path length (6) (cm)
NH <sub>3</sub>	14	Rp (1)	500 µg	-0.34	+0.12*	21.01*	7.05*	52.29*
Ethylamine	13	Rp	100 µg	-0.22	+0.21*	17.68*	0.98	42.13*
Dimethylamine	12	Rp	100 µg	-0.44	+0.25*	26.66*	6.50*	23.72*
	14	Rp	10 µg	-0.27	-0.41	6.23	9.12	0.85
	13	Rp	1 µg	-0.19	-0.13	0.00	-0.13	-15.00
Methylamine	12	Rp	100 µg	-0.32	+0.15	7.25	1.33	-9.35
Trimethylamine	12	Rp	100 µg	+0.07	-0.13	8.26	-5.68	-3.28
Isobutylamine	11	Rp	100 µg	+0.09	+0.16	12.02	16.56*	17.33*
Isopentylamine	10	Rp	100 µg	+0.09	+0.15	11.21	-11.26*	-7.14
Hexylamine	10	Rp	100 µg	+0.08	-0.14	0.21	13.93*	43.29*
Ethylamine	14	Ti (2)	100 µg	+0.14	+0.32*	16.02*	0.14	104.54*
Dimethylamine	15	Ti	100 µg	+0.16	+0.29*	22.25*	2.52	56.94*
Ethylamine	12	Pg (3)	100 µg	+0.15	+0.30**	41.57**	7.86*	52.66*
Dimethylamine	18	Pg	100 µg	-0.05	+0.36*	32.11**	5.77*	48.55*

(1) Rp: *R. prolixus*, (2) Ti: *T. infestans*, (3) Pg: *P. geniculatus*, (4) Median values are presented for activation and attraction responses of bugs (Wilcoxon paired test, \* $P < 0.05$ , \*\* $P < 0.01$ ), (5) IUL is the increase in upwind locomotion during the test period relative to the control period, (6) Speed and path length are also presented as increases during the test period relative to the control period.



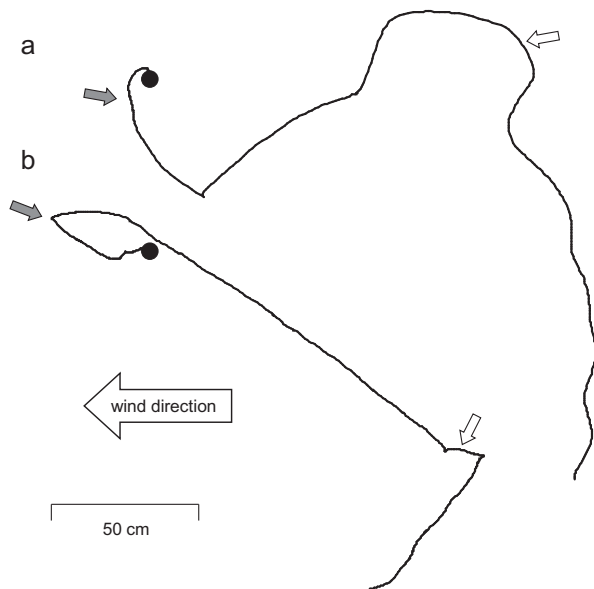
**Fig. 2.** Responses of fifth-instar *P. geniculatus* (Pg), *T. infestans* (Ti) and *R. prolixus* (Rp) nymphs to ethylamine (EA) and dimethylamine (DMA) on a servosphere. Responses are presented as the % increase in upwind locomotion in a cone 60° either side of due upwind in the test period relative to that in the previous control period. Upwind locomotion increased during exposure of all three triatomine spp. to ethylamine and dimethylamine at a source dose of 100 µg (Wilcoxon paired test, \* $P < 0.05$ , \*\* $P < 0.01$ ). The arrow on the ordinate indicates the median level of attraction for *P. geniculatus* elicited by the basic fraction of chicken faeces that contains these amines. There is no significant difference between treatments (Wilcoxon non paired test,  $P > 0.05$ ). For explanation of boxplots see legend to Fig. 1.

upwind locomotion by the three species (Table 2). *P. geniculatus* nymphs walked faster and for a longer period with either of these two amines present in the air stream compared to the air stream alone, and both *R. prolixus* and *T. infestans* also walked more in the presence of EA or DMA (Fig. 3). Lower concentrations of DMA in the air stream did not elicit oriented responses from *R. prolixus* except for an increase in antennal reaching and tapping behaviours recorded during stimulation.

Methylamine, trimethylamine, isobutylamine, isopentylamine and hexylamine failed to elicit attraction in *R. prolixus* nymphs (Table 2). Nevertheless, isobutylamine and hexylamine did activate this species: isobutylamine induced increases of 16.56 mm/s in speed and of 17.33 cm in path length, and hexylamine induced increases of 13.93 mm/s in speed and of 43.29 cm in path length relative to the control. Increases in antennal reaching and tapping were also recorded in the presence of isobutylamine and hexylamine, as well as in the presence of methylamine and trimethylamine. Isopentylamine induced a decrease of –11.26 mm/s in the walking speed of *R. prolixus* but, no increases in antennal reaching and tapping were observed.

#### 4. Discussion

In the present study, triatomine bugs are attracted by the faeces of a chicken, the breath and urine of an experimenter, as well as DMA and EA. Behavioural responses to volatile constituents of such vertebrate wastes and to volatile amines recorded on a servosphere demonstrate that these compounds play a role in the sensory ecology of triatomines. DMA and EA are attractive on their own and cause an increase in path length by *R. prolixus*, *T. infestans* and *P. geniculatus*. These behavioural responses to amines by three triatomine spp. complement earlier findings showing how DMA and EA evoke electrophysiological responses in olfactory receptor cells in



**Fig. 3.** Tracks made by *R. prolixus* (a) and *P. geniculatus* (b) nymphs on the servosphere in response to, respectively, ethylamine and dimethylamine. Each amine was released into the airstream from a source dose of 100  $\mu$ g. Tests were divided into three consecutive 2 min recording periods, i.e. air stream alone, the air stream plus the test stimulus and the air stream alone following removal of the test stimulus. The tracks started (filled circle on the tracks) with the bugs walking crosswind or downwind in the air stream alone (15 cm/s). On addition of amines to the air stream (bold arrows on the tracks) the bugs turned to walk upwind, and on removal of the amines from the air stream (empty arrows on the tracks) the bugs changed direction to a mainly cross-wind course. Note how the bugs walked for longer distances when exposed to the amines.

grooved-peg sensilla on the antenna of *T. infestans* (Taneja and Guerin, 1997; Diehl et al., 2003). Except for dimethylamine that attracts the Mexican fruit fly *Anastrepha ludens* (Robacker et al., 2000), to our knowledge behavioural responses to ethylamine and dimethylamine have not been reported earlier in an insect. In the present study ammonia induces attraction and an increase in both speed and path length by *R. prolixus*. Previous studies show that ammonia has similar effects on the behaviour of *T. infestans* (Taneja and Guerin, 1997; Guerin et al., 2000) and also attracts *R. prolixus* when combined with CO<sub>2</sub> (Otálora-Luna et al., 2004). Ammonia affects the behaviour of other haematophagous insects, contributing to the attractive effect of host odours on the mosquito *Aedes aegypti* in an olfactometer (Geier et al., 1999), and affects the response of *Anopheles gambiae* to a laboratory trap (Njiru et al., 2006) as that of the sandflies *Lutzomyia longipalpis* and *Lutzomyia intermedia* to field traps (Andrade et al., 2008).

Isobutylamine and hexylamine also affect *R. prolixus* behaviours in this study by causing an increase in speed, overall path length, antennal reaching and tapping, i.e. behavioural responses similar to those recorded when fresh chicken faeces and human breath are presented. A previous study in this laboratory has shown that isobutylamine evokes the strongest response among a range of host volatiles from an olfactory receptor cell in grooved-peg sensilla on the antenna of *T. infestans* (Diehl et al., 2003). This grooved-peg sensillum type also occurs on culicid antennae (McIver, 1982) where receptor cells sensitive to ammonia and amines occur (Pappenberger et al., 1996; Meijerink et al., 2001). In addition to ammonia- and amine-sensitive receptor cells, the *T. infestans* and *R. prolixus* grooved-peg sensilla house carboxylic acid-sensitive receptor cells (Guerenstein and Guerin, 2001; Diehl et al., 2003). Since these grooved-peg sensillar structures are also reported from other triatomine genera (*Panstrongylus*, *Dipetalogaster*, *Cavernicola*; Catalá, 1997), sensitivity to ammonia, volatile amines and carboxylic acids is probably widespread across the Triatominae. Since these triatomine bugs share a similar way of life, antennal receptor cell responses to these products may represent a common chemosensory feature among triatomines that facilitates perception to products originating from hosts or their waste products.

For many vertebrates, chemicals present in skin secretions, sex gland secretions, saliva, faeces and urine constitute important signals that serve to mark their territory (Young and Henke, 1999; Brennan, 2001). Volatile amines reported as eliciting behavioural responses from triatomines in this study are end-products of primary vertebrate catabolic pathways. Vertebrate faeces and urine contain volatile amines including DMA, EA, isobutylamine and hexylamine (Hartung and Rokicki, 1984; Sato et al., 2001; Cai et al., 2003). Fresh chicken faeces that is shown to attract *R. prolixus* in this study liberates ammonia, volatile amines and other compounds including carboxylic acids, aromatics, alcohols, and CO<sub>2</sub> from the activity of bacteria originating in the vertebrate gut (Spoelstra, 1980). Part of the ammonia produced by deamination of amino acids in the gastrointestinal tract is excreted in vertebrate faeces, but most of it is transformed in the liver to urea or uric acid to provide substrates that serve as precursors for amines. Ammonia is liberated from urea and uric acid shortly after waste excretion by the action of bacteria (Mackie et al., 1998). DMA and EA have diverse metabolic precursors in vertebrates with trimethylamine N-oxide degraded by gut flora as the major dietary source of DMA (Zhang et al., 1998, Fig. 4). There is also an unidentified pathway in mammals that leads to the endogenous formation of DMA (Zeisel et al., 1985). Choline, an essential nutrient needed for diverse processes in vertebrates, is a precursor of both DMA and EA as can be degraded by the activity of gut flora to either EA plus ethanolamine or to trimethylamine which is readily demethylated to DMA (Le et al., 2005). EA can also be obtained by decarboxylation of alanine or amination of ethanal by anaerobic

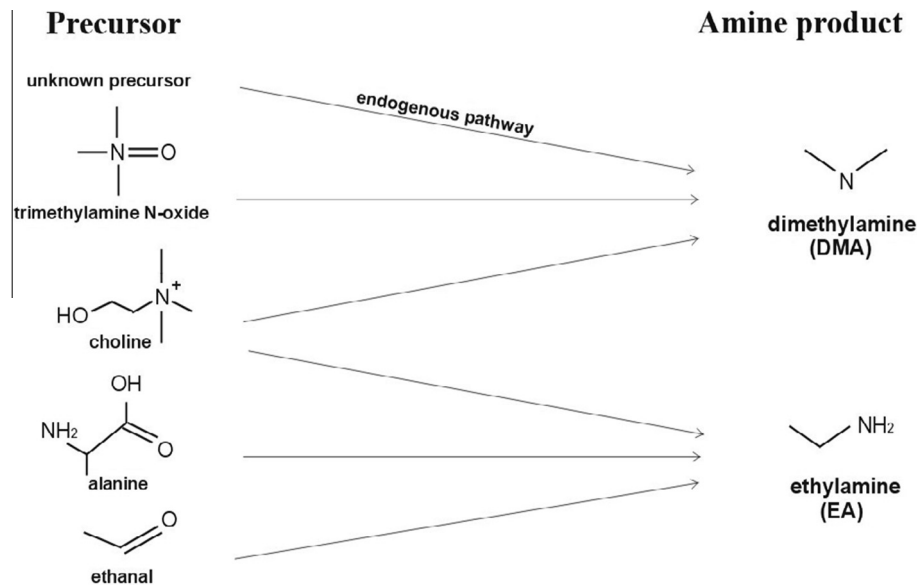


Fig. 4. Origin of volatile amines excreted in vertebrate wastes that elicit appetite responses in triatomine bugs.

bacteria present in the vertebrate gut (Spoelstra, 1980). In mammals, excess DMA, EA and other amines such as methylamine, trimethylamine, isobutylamine, isopentylamine and hexylamine are absorbed from the gut and excreted in urine but lower levels are also found in faeces (Spoelstra, 1980; Zhang et al., 1999; Mitchell and Zhang, 2001). Studies by Robacker et al. (2000) confirm the presence of ammonia, DMA and other volatile amines in bird faeces. Vertebrates must rid themselves of DMA since it is highly toxic and is a precursor of dimethylnitrosamine, a recognized carcinogen for a wide variety of animal species. DMA is the most abundant short-chain amine present in human urine (roughly 0.25 mg/kg/24 h; Zhang et al., 1995) and rat urine (roughly 2 mg/kg/24 h; Zhang et al., 1998), and as such, this may account for the behavioural responses that the extract of human urine induces in *P. geniculatus*. DMA is also present in other vertebrate body fluids such as saliva, gastric juice, blood and vaginal secretions (Zhang et al., 1993). The present findings underline how volatile amines from waste products of vertebrates may serve in signalling the presence of potential hosts to triatomines.

As the volatile amines that are the subject of this study could also be present in invertebrate excretions, their behavioural significance for triatomines can also be considered in the context of recognizing their own waste products in refugia (Taneja and Guerin, 1997; Lorenzo Figueiras and Lazzari, 1998, 2000) and for the recognition of invertebrate hosts and their refugia (Sandoval et al., 2013). We have established, for example, that the entomophagous triatomines *Belminus ferroae* and *Belminus corredori* are attracted by faeces of their cockroach host *Blaberus giganteus* (unpublished data).

This is not the only case among haematophagous insects where volatiles emanating from vertebrate wastes contribute to the survival of a vector of disease. Sand flies (Psychodidae: Phlebotominae), vectors of Leishmaniasis over a wide area of the planet, are often restricted to habitats such as termite, reptile, mammalian and bird nesting sites as well as to earthen floors of human dwellings (Felicangeli, 2004). Schlein et al. (1989, 1990) found that the presence of cow manure serves as an oviposition substrate for the sand fly *Phlebotomus papatasi* under field and laboratory conditions. Additionally, odour extracts of rabbit and chicken faeces promote egg laying in the sandfly *L. longipalpis* in the laboratory (El-naïem and Ward, 1992; Dougherty et al., 1993, 1995). Clearly, there is an advantage for sand fly females to exploit oviposition

sites near vertebrate shelters that are rich in organic debris and, as such, serve as food for larvae; and for sand fly males that form leks near these host resources to indicate its availability to blood-sucking females (Spiegel et al., 2005). As such, emanations from vertebrate wastes serve as cues that render a suitable resource more apparent to nest-living triatomines and sand flies.

Several studies indicate that triatomine host preferences shift with the availability of hosts and niche (Gürtler et al., 1997; Gürtler et al., 2009; Rabinovich et al., 2011). The use of amines to find resources may be widespread in triatomines, that feed on different hosts, given that these molecules are end-products derived from generally occurring metabolites of vertebrate and from gut flora metabolism and, as such, do not vary greatly between vertebrate species (Mackie et al., 1998).

Attraction to volatile amines could be an adaptation or *exaptation* (sensu Gould, 1982, 1991) that contributes to active dispersion of triatomines. The amine products tested here along with other triatomine chemostimuli identified elsewhere (Otálora-Luna et al., 2004; Guerenstein and Lazzari, 2009; Ortiz et al., 2011) could play an important role in house invasion by triatomine life stages that walk and fly (Gómez-Núñez, 1969; Vázquez-Prokopec et al., 2004). Triatomines could well make use of the same chemical cues they use to locate wild hosts for the exploitation of humans and domesticated animals since the same waste product occur across vertebrates, thus explaining how sylvatic spp. such as *P. geniculatus* can readily switch to the peridomestic habitat (Coimbra, 1988).

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jinsphys.2014.09.007>.

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