
Chemosensory and Behavioural Adaptations for Haematophagy in Triatomine Bugs (Heteroptera:Reduviidae)

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Le doyen :

J.-P. Derendinger

*To Andrea,
who complements my life*

*and to my grandmother,
who suffers from Chagas disease*

From many directions workers are tunneling hopefully into the mountain, some with steam shovels and others with dental drills. Some travel blindly in a circle and come out close to their point of entrance; some connect, usually in a mismatched fashion, with the burrows of others. Some have chosen to disregard the random activities of their fellows and have worked out in a small region an elegant system of interconnecting tunnels of their own. Both the attraction and the confusion of this multitudinous excavation lie in the fact that none of the workers know precisely what they are looking for or what they are likely to find.

KENNETH D. ROEDER

Knowing the domiciliary habits of the insect, and its abundance in all the human habitations of the region, we immediately stayed on, interested in finding out the exact biology of the barbeiro, and the transmission of some parasite to man or to another vertebrate.

CARLOS CHAGAS

Life may be defined in many ways, but the most fundamental focus on information.

DAVID B. DUSENBERY

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Abstract

Triatomine adaptation for haematophagy (review)

All members of Triatominae (Heteroptera, Reduviidae) are well adapted to haematophagy. Species within this subfamily are associated with nidicolous hosts and are potential vectors of Chagas disease to humans. Through evolution these bugs have acquired special morphological, physiological and behavioural adaptations to become efficient bloodsuckers. Phylogeny suggests that triatomines evolved from reduviid predators which in turn descended from phytophagous hemipterans. Primitive traits first developed by hemipterans and subsequently inherited and optimized by the reduviid ancestors of triatomines, facilitated haematophagy in these insects. Piercing-sucking mouthparts that originally permitted phytophagous hemipterans to access rich juices of plants subsequently permitted predator reduviids to access haemolymph of arthropods, and later to access the blood vessels of vertebrates. The poisonous saliva of reduviid predators used to immobilize and digest captured prey changed to a more benign saliva used to leave the host unaware of the triatomine bite. There were other structural modifications that permitted efficient bloodsucking in triatomines but, the decisive step that allowed the shift from predation to haematophagy was a change of behaviour. The early association of a reduviid predator with a nesting vertebrate, an event that took place some 60 mya, permitted the shift from an arthropod prey to a vertebrate host. The ancestor or ancestors of triatomines were attracted by the abundant arthropod prey supply present in the vertebrates refugium. The prolonged close association with nidicolous mammals and birds eventually resulted in specialization for feeding directly and efficiently on these animals and minimized the time spent searching for food. First contact with modern humans by triatomines took place more recently when *Homo sapiens* reached America. The development of sedentary populations produced ecological disturbance and environmental pressures that facilitated the adaptation of sylvatic triatomine species to human dwellings some 10'000 years ago.

Ecology of sensing in triatomines (experimental research)

Through experimental research we have described the searching strategies used by *Rhodnius prolixus*, indicated as appetite behaviours, that are used prior to, during and after encounter with the host metabolites CO₂ and NH₃ in air. A combination of 1'000 ppm CO₂ plus 30–40 ppb NH₃ in an air stream induced *R. prolixus* nymphs walking on a servosphere to perform a series of appetite behaviours. Shortly after the onset of stimulation, the nymphs turned sharply upwind towards the source of the chemostimuli (within 13±9 s) from mostly downwind and crosswind walks in the air stream alone. The mean vector angles of these upwind tracks were concentrated in a cone 60° either side of due upwind. The upwind-walking bugs stopped more frequently but for a shorter duration and walked at a higher speed than before stimulation.

Other behavioural and electrophysiological experiments were performed in order to identify which compounds serve triatomines to localize their habitat. Electroantennogram recordings revealed ammonia to be the compound to which *Panstrogylus geniculatus* adults showed the highest response among the chemicals tested. *Triatoma infestans*, *R. prolixus* and *P. geniculatus* were attracted to volatiles from chicken faeces and human

urine samples. Ethylamine and dimethylamine delivered at ~100 ppb to the apex of a servosphere proved attractive to the three species, increasing the target vector and upwind displacement of fifth-instar nymphs. These behaviourally active amines are known to be present in vertebrate urine and faeces, and may play an important role in guiding triatomines to hosts and refugia.

Capturing devices were designed and tested with the three species. A simple bicylinder trap proved to be efficient alone (> 95 % of bugs released in an experimental arena were captured), i.e. without an odour dispenser. *R. rolixus*, *T. infestans* and *P. geniculatus* were more attracted to another (cylinder) trap baited with CO₂ and NH₃ than this trap alone: more than 73% of the total bugs were captured. These traps may be useful to study of the prevalence of triatomines in *Trypanosoma cruzi*-endemic areas.

Keywords: Sensory ecology, orientation behaviour, neurophysiology, odours from vertebrate wastes, triatomine evolution, haematophagy, electroantennogram, servosphere, gas chromatography, triatomine surveillance and control, traps, triatomine domiciliation, Latin America, Chagas disease, Reduviidae, Triatominae, *Rhodnius prolixus*, *Triatoma infestans*, *Pastrongylus geniculatus*.

Résumé

Adaptations olfactives et comportementales pour l'hématophagie chez les Triatominae (Heteroptera: Reduviidae)

Chapitre I

Les Triatominae (Hemiptera, Reduviidae) sont toutes bien adaptées à l'hématophagie. Au sein de cette sous-famille, certaines espèces sont des vecteurs pouvant potentiellement transmettre la maladie de Chagas à l'homme. Durant l'évolution, ces punaises sont devenues des « suceurs de sang » en développant une morphologie, une physiologie et des adaptations comportementales spécialisées. Les études phylogénétiques suggèrent que les triatomes ont évolué à partir de réduves prédatrices. Ces dernières descendraient d'hémiptères phytophages. Les premières adaptations primitives de certains hémiptères ont été héritées puis optimisées par les réduves. Cela a facilité à modeler l'hématophagie chez ces insectes. A l'origine, l'appareil buccal de type perceur-suceur des hémiptères phytophages leur permettait d'accéder à la riche sève des plantes. Ensuite, cet appareil buccal a permis aux réduves prédatrices d'accéder à l'hémolymphe des arthropodes et finalement, au sang des vertébrés. La salive toxique des réduves prédatrices utilisée initialement pour immobiliser et digérer les proies capturées, a évolué pour devenir finalement bénigne chez l'hôte victime d'une piqûre. Même si d'autres modifications ont eu lieu au niveau de la structure, l'étape décisive qui a permis aux triatomes de devenir d'efficaces suceurs de sang, est liée aux modifications comportementales. Les premières associations d'une réduve prédatrice avec un vertébré au sein d'un terrier ou d'un nid ont eu lieu il y a 60 millions d'années. L'ancêtre des triatomes était attiré par l'abondance d'arthropodes et donc de proies présentes dans ces lieux. Au fil du temps, ces réduves ont délaissé ce type de proies au profit de repas sanguins prélevés sur des vertébrés hôtes. C'est l'association étroite et prolongée de ces hémiptères avec des mammifères et des oiseaux qui ont poussé ces insectes à se nourrir directement et de manière efficace sur ces animaux. Ainsi, le temps de recherche de la nourriture a été minimisé. Les premiers contacts entre les triatomes et l'homme moderne ont eu lieu plus récemment, au moment où l'*Homo sapiens* a atteint l'Amérique latine. Les perturbations écologiques et les pressions environnementales engendrées par la sédentarisation des populations humaines ont poussé les triatomes sylvestres à s'adapter aux habitats humains.

Chapitre II

Les adaptations olfactives et comportementales acquises par les Triatominae pour la recherche d'une source de nourriture ou d'un lieu de refuge étaient fondamentales pour la survie de leurs membres. A travers cette recherche expérimentale, nous avons décrit les stratégies de recherche utilisées par *Rhodnius prolixus*. Ces stratégies sont appelées comportements d'appétence et sont utilisées avant, pendant et après la rencontre avec les métabolites de l'hôte (notamment le CO₂ et le NH₃) présents dans l'air environnant. Une combinaison de 1'000 ppm de CO₂ et 30-40 ppb de NH₃ dans un flux d'air suffisaient à induire chez les nymphes de *R. prolixus*, marchant à la surface d'une servosphère, une série de comportements d'appétence. Peu après le début de la stimulation, les nymphes se tournaient rapidement face au vent et en direction de la source du stimulus chimique (dans les 13±9 s) alors qu'elles se déplaçaient essentiellement dans le sens du flux d'air ou de manière perpendiculaire à celui-ci pendant l'absence de stimulus chimique. Les angles

vectoriels moyens des trajectoires en direction du vent étaient concentrés dans un cône de 60° de part et d'autre du flux d'air. Les punaises marchant en direction du vent s'arrêtaient plus fréquemment mais pour de plus courtes périodes. De plus, elles marchaient à une vitesse plus élevée qu'avant la stimulation. Durant les arrêts et en présence du stimulus chimique, les punaises corrigeaient fréquemment leurs angles de course. De plus, elles étendaient leurs pattes antérieures afin d'atteindre une hauteur d'air plus élevée avec leurs antennes. Dans le flux d'air dépourvu de stimulus chimique, les nymphes de *R. prolixus* échantillonnaient fréquemment la surface de la sphère avec leurs antennes et les nettoyaient avec leurs tarsi antérieurs. Cependant, les nymphes ne tapotaient que brièvement le flagellum de l'antenne gauche ou droite sur le premier tarse correspondant, en présence du stimulus chimique, et elles ne touchaient jamais la surface de la servosphère. Après la fin de la stimulation, les punaises continuaient à répondre avec les mêmes comportements d'appétence mais elles marchaient perpendiculairement au flux d'air de façon plus tortueuse en essayant de regagner le contact avec le stimulus chimique.

Chapitre III

Les Triatominae sont des Reduviidae vivant en groupe. Elles ont besoin de sang de vertébrés pour accomplir leur cycle de vie. Pour y parvenir, elles doivent être capables de détecter des signaux olfactifs provenant directement de leur hôte et du refuge de ce dernier. En occupant des nids, des terriers ou des abris, les vertébrés accumulent beaucoup de déchets à l'intérieur ou près de ceux-ci. Les triatomines seraient capables d'utiliser cette riche source d'odeurs pour trouver ce genre de refuges. Dans ce chapitre, nous décrivons comment deux espèces de triatomines domestiques *Triatoma infestans* et *Rhodnius prolixus* ainsi qu'une espèce sylvestre *Panstrongylus geniculatus* sont attirées par des substances volatiles provenant d'échantillons de fèces de poule et d'urine humaine. Ces odeurs pourraient être impliquées dans la signalisation de la présence d'un hôte vertébré dans un refuge. Parmi tous les produits chimiques testés, les électroantennogrammes ont révélé que l'ammoniac est le composé auquel les *P. geniculatus* adultes montrent la plus forte réponse. Avec la servosphère, l'émission d'éthylamine et de diméthylamine à une concentration d'environ 100 ppb s'est révélée fortement attirante pour ces trois espèces. Chez les nymphes du cinquième stade, ces composés ont provoqué l'augmentation du déplacement face au vent. Des comportements d'activation ont aussi été observés : des nymphes de *P. geniculatus* ont marché plus vite et plus loin quand ces deux amines à courte chaîne étaient présentes dans le courant d'air. L'isobutylamine et l'hexylamine n'ont pas provoqué d'attraction mais ont induit l'activation de nymphes *R. prolixus* : une augmentation médiane de la vitesse de 16,56 mm/s et une augmentation médiane de déplacement de 43,29 cm a été provoquée par l'isobutylamine. Toutes ces amines agissant sur le comportement sont connues pour être présentes dans l'urine et les fèces des vertébrés. Notre conclusion est que l'attraction envers ces composés ainsi que d'autres composés présents près du refuge de l'hôte représentent une adaptation fondamentale pour le guidage de ces punaises vers les ressources. Cette adaptation des Triatominae auraient probablement été héritée de Reduviidae ancestrales qui utilisaient les refuges de vertébrés pour leurs source de proies d'arthropodes et comme abri.

Chapitre IV

Le développement d'un outil permettant de contrôler et surveiller les triatomines est fondamental dans la lutte contre la maladie de Chagas. Un tel outil permettrait à l'administration régionale de la santé publique à déployer des ressources nécessaires en

fonction du degré de l'infestation. Nous avons développé un système de contrôle efficace, c'est-à-dire un dispositif de capture permettant de détecter dans des zones endémiques la présence des vecteurs de la maladie de Chagas. Nos études se sont basées sur des enregistrements électrophysiologiques et des découvertes effectuées avec le compensateur de locomotion (servosphère). Quand 60 insectes ont été libérés dans une arène expérimentale, le piège (bi-cylindrique) s'est avéré très efficace (> 95 % des insectes ont été capturés), même sans le distributeur d'odeur. Un autre dispositif (piège cylindrique) s'est montré moins efficace pour la capture de triatomes mais plus approprié pour évaluer les effets d'odeurs. *R. prolixus*, *T. infestans* et *P. geniculatus* étaient plus attirés par le piège cylindrique amorcé avec du CO₂ et du NH₃ que par la seule présence du piège: plus de 73 % des insectes totaux ont été capturés dans le piège d'essai. Le piège bi-cylindrique amorcé avec du CO₂ et du NH₃ est utile pour l'étude de la fréquence de triatomes dans des secteurs endémiques de *Trypanosoma-cruzi*.

Mots clés: Écologie sensorielle, comportement d'orientation, neurophysiologie, odeur de déchets des vertébrés, évolution des triatomes, hématophagie, électroantennogramme, servosphère, chromatographie en phase gazeuse, contrôle et surveillance de triatomes, pièges, domiciliation des triatomes, Amérique Latine, Maladie de Chagas, Reduviidae, Triatominae, *Rhodnius prolixus*, *Triatoma infestans*, *Panstrongylus geniculatus*.

Resumen

Adaptaciones olfativas y de comportamiento para la hematofagia en los triatominos (Heteroptera, Reduviidae)

Capítulo I

Todos los miembros de la subfamilia Triatominae (Heteroptera, Reduviidae), vectores potenciales de la enfermedad de Chagas, están bien adaptados a la hematofagia. A través de la evolución han adquirido las adaptaciones morfológicas, fisiológicas y de comportamiento necesarias para convertirse en insectos chupadores de sangre. La filogenética sugiere que los triatominos evolucionaron de reduvidos depredadores que a su vez descendieron de hemípteros fitófagos. Ciertas características primitivas, que fueron primero desarrolladas por los hemípteros y subsiguientemente heredadas y optimizadas por los reduvidos ancestros de los triatominos, facilitaron y moldearon la hematofagia en estos insectos. Las piezas bucales picadoras y chupadoras que originalmente les permitieron a los hemípteros fitófagos acceder a los ricos jugos de las plantas, luego les permitieron a los depredadores reduvidos acceder a la hemolinfa de los artrópodos, y finalmente permitieron que los triatominos accedieran a los vasos sanguíneos de los vertebrados. La saliva venenosa que los depredadores reduvidos utilizaban para inmovilizar y digerir la presa capturada se transformó a una saliva más benigna capaz de dejar a los huéspedes inadvertidos de las picaduras. Al igual que éstas, hubo otras modificaciones estructurales que les permitieron a los triatominos chupar sangre eficientemente. Sin embargo, el evento decisivo que permitió el salto de la predación hacia la hematofagia fue un cambio de comportamiento. La asociación temprana de un reduvidos depredador con un vertebrado nidícola, incidente que ocurrió hace unos 60 millones de años, permitió el cambio de una presa artrópoda por un huésped vertebrado. El ancestro de los triatominos fue atraído al refugio del vertebrado por la abundante reserva de presas artrópodas presentes en este. La asociación íntima y prolongada con aves y mamíferos nidícolas resultó eventualmente en la especialización para alimentarse directa y eficientemente de estos animales y minimizó el tiempo invertido en la búsqueda de alimento. El primer contacto que los triatominos realizaron con seres humanos ocurrió más recientemente cuando el *Homo sapiens* llegó a América. El desarrollo de poblaciones sedentarias produjo daños ecológicos y presiones ambientales que forzaron la adaptación de especies silvestres al domicilio humano hace unos 10'000 años atrás.

Capítulo II

Los triatominos adquirieron adaptaciones olfativas y de comportamiento para buscar rápida y eficientemente comida y refugio. A través de experimentos hemos descrito las estrategias, llamadas comportamientos de apetito, que son utilizadas por *Rhodnius prolixus* antes, durante y después de encontrar en el aire los compuestos metabólicos CO₂ y NH₃ cuando caminan en una servósfera. Una combinación de 1'000 ppm de CO₂ y 30–40 ppb de NH₃ en una corriente de aire indujo a las ninfas de *R. prolixus* a llevar a cabo una serie de comportamientos que pusieron en evidencia su interés en el estímulo. Poco antes de aparecer el olor en la corriente de aire las ninfas giraron precipitadamente (en 13±9 s) para luego caminar viento arriba en dirección del estímulo, cuando antes, en el control, caminaban principalmente a través del viento o viento abajo. Los ángulos de los vectores promedio que describen estas caminatas apuntan en dirección del viento, concentrados en un cono de 120°. Los triatominos que caminan en dirección del viento se detienen con

mayor frecuencia pero por menos tiempo, además caminan más rápido que antes de la estimulación. Durante las paradas, los triatominos giraron y cambiaron de curso, elevaron la parte anterior del cuerpo extendiendo las piernas delanteras y batiendo las antenas en lo alto. Cuando la corriente de aire no contenía los olores, las ninfas tocaban la superficie de la esfera o limpiaban cuidadosamente sus antenas. Sin embargo, en presencia del estímulo químico, estos insectos sólo golpeaban breve y rápidamente el flagelo de la antena izquierda o derecha con el correspondiente tarso del primer par de patas y nunca tocaban la superficie de la esfera. Luego de la estimulación, los triatominos continuaron respondiendo con los mismos comportamientos de apetito, pero caminaron más tortuosamente y en dirección perpendicular al viento en un esfuerzo por recontactar el estímulo.

Capítulo III

Los triatominos son reduvídeos nidícolas que necesitan sangre de un vertebrado para completar su ciclo de vida. Para tal fin, deben ser capaces de detectar las señales olfativas que emanan del huésped y del refugio en el que éste habita. Los vertebrados que habitan en el refugio acumulan, dentro o alrededor del mismo, grandes cantidades de productos de desecho. Es probable que los triatominos sean capaces de utilizar esta fuente rica en olores para localizar dicho refugio. En este trabajo exponemos cómo las especies domésticas *Triatoma infestans* y *R. prolixus*, y la especie selvática *Panstrongylus geniculatus* son atraídas por volátiles provenientes de muestras de heces de gallina y de orina humana. Estos olores están asociados a la presencia de un huésped vertebrado en un refugio. A través de electroantenogramas se ha podido detectar que el amoníaco es el compuesto al cual adultos de *P. geniculatus* muestra respuestas de mayor magnitud en comparación con otros compuestos químicos que también fueron ensayados. La etilamina y la dimetilamina suministradas a ~100 ppb en una corriente de aire resultaron altamente atractivas para las tres especies, incrementando el desplazamiento viento arriba de las ninfas de quinto estadio en una servósfera. También se observaron comportamientos de activación, i.e. las ninfas de *P. geniculatus* caminaron más rápidamente y mayores distancias cuando estas dos aminas de cadenas cortas estaban presentes en la corriente de aire, que cuando la corriente de aire no contenía estas moléculas. La isobutilamina y la hexilamina no produjeron atracción, pero indujeron la activación de las ninfas de *R. prolixus*: la isobutilamina produjo un incremento medio de la velocidad de 16.56 mm/s y un incremento medio del desplazamiento de 17.33 cm, y la hexilamina produjo un incremento de velocidad de 13.93 mm/s y un incremento en el desplazamiento de 43.29 cm. Todas estas aminas que activan el comportamiento se encuentran presentes en la orina y heces de los vertebrados. Por lo tanto, podemos concluir que estos y otros compuestos que se encuentran cerca del refugio del huésped representan un recurso fundamental que ayuda a los triatominos a localizar dicho habitat. Probablemente esta característica fue heredada de sus ancestros depredadores reduvídeos que también utilizaban la vivienda del vertebrado como una fuente de alimento y como refugio.

Capítulo IV

Trabajar en el desarrollo de una herramienta de vigilancia y control de los triatominos es fundamental para combatir la enfermedad de Chagas. Esta herramienta puede servir de ayuda a las autoridades sanitarias para estimar los recursos necesarios que han de invertirse para controlar un brote según sea su escala de infección. Hemos desarrollado un sistema de

monitoreo, i. e. dispositivo de captura, para detectar la presencia de triatominos vectores de la enfermedad de Chagas en áreas endémicas. Nuestros estudios aprovecharon mediadas electrofisiológicas y hallazgos obtenidos en un compensador de movimiento (servósfera). La trampa (bicilíndrica) demostró ser efectiva por sí sola (>95% de los insectos fueron capturados), i.e. sin dispensador de olor, cuando 60 insectos fueron liberados en una arena experimental. También fue probado otro dispositivo (trampa cilíndrica) que resultó ser menos efectivo para capturar los insectos, pero más apropiado para probar el efecto de los olores en la captura de triatominos. *R. prolixus*, *T. infestans* y *P. geniculatus* fueron más atraídos hacia la trampa cilíndrica cebada con CO₂ y NH₃ que la trampa por sí sola: más del 73% del total de los insectos fueron capturados en esta trampa piloto. La trampa bicilíndrica cebada con CO₂ y NH₃ es útil par estudiar la prevalencia de triatominos en zonas endémicas de *Trypanosoma cruzi*.

Palabras clave: Ecología sensorial, comportamiento de orientación, neurofisiología, olores de desechos de vertebrados, evolución de los triatominos, hematofagia, electroantenograma, servósfera, cromatografía de gases, vigilancia y control de triatominos, trampas, domiciliación de los triatominos, América Latina, enfermedad de Chagas, Reduviidae, Triatominae, *Rhodnius prolixus*, *Triatoma infestans*, *Pastrongylus geniculatus*.

Preface

The study of triatomine bugs is of ecological, evolutionary, physiological and medical interest. Ecologically and evolutionary, because these bugs have radiated throughout the American, Asian, Australian and African continents giving rise to one of the few major haematophagous groups within the Heteroptera with more than 130 species adapted to diverse habitats at different latitudes and altitudes. Physiologically, because they have been easy to maintain and manipulate under laboratory conditions, being used as a practical model to study insect physiology for over one hundred years. The medical interest lies on the fact that these bloodsuckers are vectors of *Trypanosoma cruzi*, the causative agent of Chagas disease or American trypanosomiasis, an illness that infects nearly 18 million people and causes some 21'000 deaths per year in Latin America.

For a better understanding of these disease vectors we have decided to consider the ways (how) and motives (why) by which they search for resources in the environment. We know they move about for many reasons: to search for food, to find a potential mate, to find shelter or a suitable nesting site, to escape predators and explore new territories. The most important mode of locomotion in triatomines is by walking, but adults occasionally fly short distances. With some probability, a bug may encounter a blood meal, shelter or a nesting site even if just transported passively or if it searches at random. For example, *Rhodnius prolixus* Stål, 1859 can arrive in the domestic habitat by being carried on palm tree leaves used for constructing rural houses, and *Panstrongylus geniculatus* (Latreille, 1811) can enter houses by flying towards artificial “hypnotizing” light during the dark humid night of the tropics. However, the probability of arriving at a particular site or of finding a relevant target will be much higher if the insect uses environmental information that may reliably guide it to its goal.

Visual, chemical, acoustic, vibratory and tactile stimuli are all relevant cues for orientation by insects. Volatile compounds are particularly important to triatomines for two reasons, a) most triatomine species search for hosts during the night when light is almost absent and b) information present in odours can travel further away, often carried by flows of air, overcoming obstacles that other stimuli cannot bypass. Triatomine antennae apart from serving for touch and taste are the organs that perceive odours. Each antenna bears hundreds of different odour receptor cells but we only know a few particular types in Triatominae. In addition, we do not know much about how and why triatomines respond to this multidimensional source of volatile chemical information.

Our research employs an *ecology of sensing** approach, so in our hypotheses two main lines of query are implicit. Which are the chemical cues that a triatomine can find in its environment and how does it use these cues to survive and eventually increase its fitness? In order to answer these questions, we explored current knowledge on the chemical ecology of triatomines and that of other blood-sucking arthropods and their hosts. The use of this comparative approach is based on the assumption that there must be considerable analogy between arthropods that developed similar ways of life, i.e. haematophagy, regardless of their evolutionary origins. This allowed us to develop a criterion which guided our selection of key products and metabolites that we expect evoke some physiological and/or behavioural responses in triatomines. Questions regarding to the

* The general approach taken here is more in the domain of *ecology of sensing* than of *sensory ecology*, because the latter deals with *how* organism acquire and use sensory information to survive and the former takes a broader perspective and also deals with *why* organisms use this kind of information leaving a place for evolutionary questions.

orientation behaviour of triatomines *vis a vis* key volatile stimuli are studied and discussed in the context of triatomine sensory ecology and natural history. Finally, we propose that by answering such general questions we can develop tools for triatomine control.

In the first chapter of this thesis a synthesis of our current knowledge on triatomine evolution is presented. The important physiological and behavioural adaptations that these insects have acquired through evolution to become haematophagous and be associated with humans are underlined. The second chapter describes searching strategies by *R. prolixus* indicated as appetite behaviours that are used prior to, during and after encounter with the host metabolites CO₂ and NH₃ in air. The third chapter focuses on research for other chemical cues used by *R. prolixus*, *Triatoma infestans* (Klug, 1834) and *P. geniculatus* to find resources based on our knowledge of the ecology of these bugs. The fourth chapter proposes a capturing device incorporating relevant host metabolites to be used for the surveillance of these vectors.

This study improves our understanding of the Triatominae subfamily by exploring some behavioural and physiological characteristics necessary for the survival of these insects and very probably, of many other haematophagous and non-haematophagous arthropods. Through our findings, we have been able to find possible explanations for some triatomine behaviours, predict possible scenarios related to sylvatic triatomine domiciliation and propose a tool that may help in monitoring populations of these vectors of disease.

Chapter I

Introduction

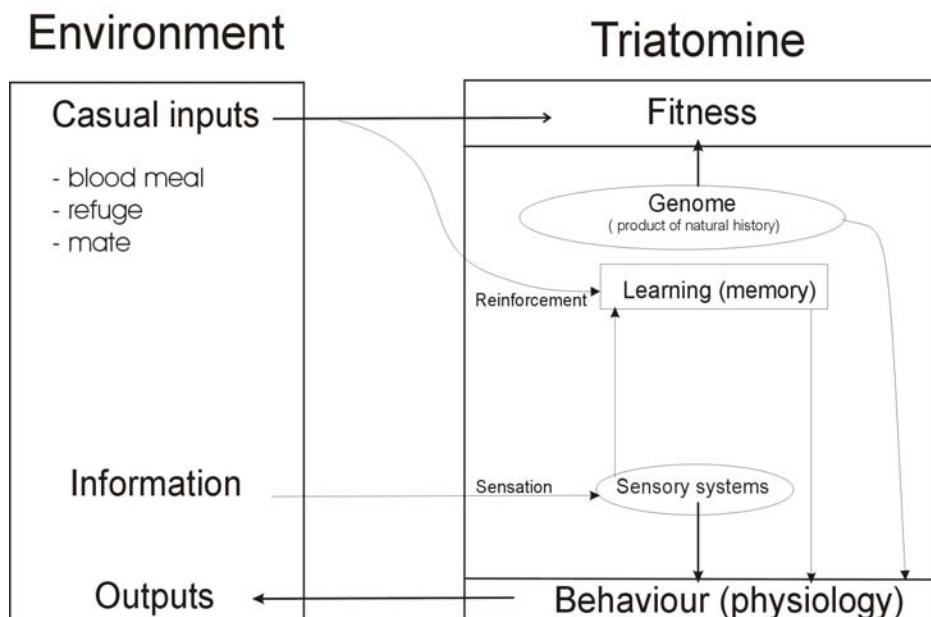
“Die ewige Wiederkehr des Gleichen.”
- Friedrich Nietzsche

“The only stable thing is change.”
- Heraclitus

Introduction: Evolution of the blood-sucking habit in Triatominae (Heteroptera: Reduviidae)

How triatomines respond to their environment depends on the (sensory) information these bugs acquire from their surroundings and the (genetic) information they have inherited from their ancestors (Fig 1). The genetic influence is a consequence of what triatomine ancestors dealt with in the past. Certainly, the laws of physics and chemistry have remained unchanged since the appearance of the first triatomines on earth, so the evolution of these insects' sensory systems has been constrained over time by common principles. But, the driving force of triatomine evolution is constituted by the geographical and climate changes that the planet has experienced through millions of years until present. This means that it is very relevant to consider unchanging laws and also the dynamics of triatomine natural history in order to understand morphological, physiological and behavioural adaptations in these bugs. Here we present a brief introduction to triatomine phylogeny and show how these insects have overcome (or have taken advantage of) ancestral traits to adapt to haematophagy and to human habitats.

Fig. 1 Input-output relationships of a triatomine and its environment based on Dusenbery (2001).



Triatomine capability to interact with its habitat is defined in its inherited **genome**. Each bug reacts to the environment according to its physiological needs and environmental information received. These behavioural **outputs** modulate the casual **inputs** from the environment. If the inputs are favourable, the individual increases its **fitness** and the probability that its genes can be passed to its descendants.

1. Brief phylogeny of Triatominae

The origin of Hemiptera probably dates back to the late Carboniferous¹, about 300 mya, with fossils found dating from the Permian² (Wootton 1981, Burmester 2001; see Table 1).

Approximately 80'000 species of this monophyletic order have been described and it is by far the largest and most diverse group of hemimetabolous insects. The success of Hemiptera is based on the following (Dolling 1991):

1. Their bodies are compactly flat, a feature that allows them to easily find refuge and sometimes food³.
2. Many species are capable of flying during the adult stage, an important adaptation to exploit new and better resources.
3. As in the adults of most successful holometabolous insects their central-nervous system is greatly concentrated, permitting a common rapidity and diversity in behavioural responses and a great capacity to learn.
4. Their piercing-sucking mouthparts permit access to rich juices of plant and animal tissues. It is believed that the first Hemiptera were phytophagous and that entomophagy and haematophagy evolved from these sap and phloem suckers.

Traditionally the Hemiptera is divided into two groups, Homoptera (Sternorrhyncha and Auchenorrhyncha) and Heteroptera, the latter includes Triatominae. With more than 38'000 known species, the Heteroptera is now accepted as an order in its own right and as a monophyletic taxa (Dolling 1991). This group possesses characteristic scent glands with conspicuous external openings, although absent in some aquatic forms. Only Heteroptera possess a gula - an area of cuticle closing the head capsule ventrally behind the mouthparts - a specialized morphological adaptation for sucking liquids. The result is the capacity to bring the rostrum forwards, allowing greater freedom of movement than is possible in Sternorrhyncha and Auchenorrhyncha. This versatile proboscis enabled heteropterans to exploit a greater variety of foodstuff, including animal tissue. Indeed, predation and haematophagy is confined to this group and many of their families consist entirely of predators (assassin bugs) which occupy a wider range of habitats than do the plant-feeding members of the suborder. A few families such as Cimicidae, Polyctenidae and Reduviidae have developed the capacity to feed on vertebrate blood even though it is a very unusual habit in Heteroptera. Cimicidae (bed bugs) feed only on the blood of vertebrates, usually

¹ The Carboniferous Period occurred from about 354 to 290 million years ago during the late Paleozoic Era. Pangea was assembled by the collisions of three main blocks, Gondwana, Euramerica and Siberia, during the late Carboniferous and early Permian periods. One of the greatest evolutionary innovations of the Carboniferous was the amniote egg, which allowed further exploitation of land by tetrapods (reptiles, birds and mammals). Many kinds of arthropods evolved encouraged by the oxygen-rich atmosphere, the abundance of food in the decaying forest leaf-litter, and the absence of large terrestrial vertebrates.

² The Permian is so named in recognition of the largest mass extinction recorded in the history of life on Earth. It lasted from 248-290 mya and it was the last period of the Paleozoic Era. Pangea was present but Asia was broken up at the time. Some groups of animals survived the Permian mass extinction in greatly diminished numbers but they never again reached the ecological dominance they once had clearing the way for another group like as dinosaurs. During this period the great forests of fern-like plants shifted to gymnosperms including modern conifers.

³ For example, the small size and flattened shape of many members of the predator family Anthocoridae enhance the opportunities of both adults and nymphs to enter cracks and crevices and to get under the barks of trees (Lattin 1999) where they have specialized in feeding on the arthropods living there.

birds, bats and humans (Lehane 2005). All members of the small family of Polyctenidae (e.g. *Eoctenes spasmae*) feed on microchiropteran bats of both the New and Old World (Kim 1985). Members of Triatominae (from the Reduviidae family, see below) feed exclusively on blood of diverse nesting vertebrates. Probably, these haematophagous heteropterans (i.e. Cimicidae, Polyctenidae and Reduviidae) all descended from early predator forms.

Table 1. Geological time scale

Eon	Era	Period	Epoch	Dates (mya)	Originated group (mya)		
Phanerozoic	Cenozoic	Quaternary	Neogene	Holocene	10,000 years ago to the present	Triatomine domestication	
					Pleistocene	0.01	
		Tertiary	Paleogene		Pliocene	2	↑ (nesting vertebrates diversification)
					Miocene	13	
					Oligocene	25	
					Eocene	36	
			Paleocene	58			
		Mesozoic	Cretaceous			65	Triatominae (~60-90)
			Jurassic			136	Birds (~150 mya)
			Triassic			190	Mammalia (~200 mya)
	Paleozoic	Permian			225	Reduviidae (~230)	
		Carvoniferous			280	Hemiptera (~300 mya)	
		Devonian			345		
		Silurian			405		
		Ordovician			425		
	Cambrian			500			

Records of haematophagy in other heteropteran families are anecdotal or just show facultative haematophagy. Some Lygaeidae (seedbugs) of the tribe Cleradini are predators that descended from early phytophagous seed-eating forms and that normally feed on invertebrates living in bird and rodent nests. But seedbugs can also, as is the case of more developed nymphs and adult forms of *Clerada apicicornis*, steal blood from recently fed triatomines or feed directly on nestling vertebrates under laboratory conditions (Torres *et al.* 2000). Apparently, some Anthocoridae (flower bugs), notably *Anthocoris pilosus* and *Lyctocoris campestris*, are facultative blood-suckers, and a few Miridae (leaf bugs) will gorge on vertebrate blood if allowed to do so (Carcavallo *et al.* 2000). In addition, at least one member of the Physoderinae (*Cryptophysoderes faichildi*, Carcavallo *et al.* 2000), another subfamily of Reduviidae, may also feed on vertebrate blood. Nevertheless, most bites of these families are probably exploratory and do not lead to prolonged feeding.

The Reduviidae represents an ancient family with 32 subfamilies and 6'601 described *spp.* (Maldonado Capriles 1990) with fossil records suggesting that the earliest predatory forms arose from phytophagous hemipterans during the Permian/Triassic periods some 230 mya (Table I). The family is seen largely as a tropical one, nevertheless there are reduviids on all five continents. There are morphological characteristics that allow one to place heteropterans in the Reduviidae family (Dolling 1991). The transverse constriction of the head between the eyes is encountered elsewhere only in Berytidae. The reduviid (and phymatid) sedentary mode of capturing prey and use of the proboscide for stinging and defence seem to have contributed to the reduction of the *methatoracic* and *dorsal abdominal* scent glands and to the evolution of new exocrine glands according to Aldrich (1988). Thus, two pairs of sac like glands have risen: *Brindleys'* glands located on the lateral edge of the metathorax and *ventral* scent glands. Secretion of isobutyric acid is always present in triatomine Brindleys' glands, except for *Dipetalogaster maximus* that does not bear such glands (Guerenstein & Guerin 2004). The presence of a mostly very short and robust rostrum (labium) conspicuously arcuate (removed from the body) that does not extend beyond the prosternum is a characteristic unique to the group. These mouth features are secondarily lost in Triatominae and in some harpactorines which have a more elongated and flexible labium. Apart from a handful of species, all reduviids are able to stridulate by rubbing the apex of the rostrum in a transversely striated groove at the front of the prosternum and have a lateral insertion of the four-segmented antennae. In relation to their behaviour, reduviids are considered an important group of aggressive predators, most species being raptorial entomophagous pouncing on their victims and holding them with their front legs variously modified for the purpose (Fig. 2). Certainly, the morphological and behavioural adaptations developed by Reduviidae to predation opened the way for the radiation of the Triatominae subfamily.

**Fig. 2 Wheelbug *Arilus cristatus* (Reduviidae: Harpactorinae)
on the underside of Purple Coneflower in July**

Photo: Tim Nichols



Triatominae comprises 137 known species grouped in 19 genera in 6 tribes (Galvão *et al.* 2003), see Table 2. Most species are confined to America and a few, representing two genera, inhabit continental India (6 spp. of *Linshcosteus*) and south and south-east Asia to New Guinea and northern Australia (8 spp. of the *T. rubrofasciata* complex); no autochthonous endemic species are found in Africa. Lent & Wygodzinsky (1979) supported a monophyletic origin for triatomines based on three autapomorphies:⁴ a) the hematophagous feeding habit, b) elongated and nearly straight labium with flexible membranous connections between segments 3 and 4, allowing an upwardly pointed distal rostral segment when the rostrum is in the feeding position, c) loss of dorsal abdominal scent glands in nymphs. But recently the monophyletic origin of the subfamily has been put in doubt (Schofield 2000a, 2000b) and there is controversy concerning the origin of triatomines with considerable difference of opinion. It is not clear if this subfamily descended from one ancestor (monophyletic), from two (diphyletic) or more (polyphyletic); Schaefer (2003, 2005) has provided a critical discussion on this matter. In order to elucidate the dispute this author proposes to study the reduviid subfamily Physoderinae or other hypothetical sister groups of triatomines. It is important to note that with the exception of Triatominae little research has been carried out on other reduviid subfamilies. Fortunately, there is an increasing number of genetic studies which serve to clarify the taxonomic relationships between triatomine species (Hypša *et al.* 2002, Sainz *et al.* 2004, Abad-Franch & Monteiro 2005). Integration of this phylogenetic knowledge with ecological studies will shed light on the natural history of Triatominae.

⁴ An autapomorphy is a derived trait unique to any given taxon, i.e. shared by the ingroup taxa, but excluded from its outgroup taxa.

Table 2. Classification of the subfamily Triatominae (after Galvão *et al.* 2003)

Tribe:	Alberproseniini	Bolboderini	Cavernicolini	Linschosteini ^b	Rhodniini	Triatomini
Genus	<i>Alberprosenia</i> (2 spp.)	<i>Belminus</i> (6 spp.) <i>Bolboderia</i> (1 sp.) <i>Microtriatoma</i> (2spp.) <i>Parabelminus</i> (2 spp.)	<i>Cavernicola</i> (2 spp.) <i>Torrealbaia</i> (1 sp.) ^a	<i>Linschosteus</i> (6 spp.)	<i>Psammolestes</i> (3 spp.) <i>Rhodnius</i> (16 spp.)	<i>Dipetalogaster</i> (1 sp.) <i>Eratyrus</i> (2 spp.) <i>Hermanlenticia</i> (1 sp.) ^c <i>Meccus</i> (6 spp.) ^d <i>Mepraia</i> (2 spp.) ^e <i>Nesotriatoma</i> (3 spp.) ^f <i>Paratriatoma</i> (1 sp.) <i>Panstrongylus</i> (13 spp.) <i>Triatoma</i> (67 spp.)

a *Torrealbaia martinezi* is a new species of Cavernicolini.

b Linschosteini is a new tribe

c *T. matsunoi* is placed in the genus *Hermanlenticia*.

d *Meccus* is a revalidated genus.

e *Mepraia spinolai* was considered previously as member of the genus *Triatoma*.

f *Nesotriatoma* is a new genus.

Triatomines, unlike other hematophagous insects such as mosquitoes or tsetse flies, are nest-living bloodsuckers⁵ occupying the host's abode and feeding during the night when the host sleeps. Tree-nesting vertebrates such as birds, reptiles, sloths and opossums and burrow-inhabiting vertebrates such as armadillos, guinea pigs, rabbits, rodents and bats are their usual hosts. Underground enclosures, nests in hollow trees, bird nests, fallen logs, fronds of palm trees, bromelias, the underside of loose bark and similar locations normally harbour these insects.

2. Requirements for haematophagy

The primitive morphological, physiological and behavioural adaptations developed by the first predatory hemipterans and inherited and subsequently optimized by the reduviid ancestors of triatomines facilitated and moulded haematophagy in this subfamily. The reduviid predators had already developed piercing mouthparts, protein digestive enzymes, aggressive and hunting behaviours on animal targets, as well as associations with nesting vertebrates. The path to haematophagy was delineated and the prize, nutritious blood, was a driving force. But other inherited characteristics such as the predecessors' poisonous

⁵ Other examples of arthropods that have exploited the nidicolous habitat include most Argasidae, Phlebotominae and Cimicidae, many Gamasina and some Siphonaptera. Balashov (1984) calls all of these arthropods «nest-burrow bloodsuckers»

saliva and the poor vitamin pool contained in the new food source represented some obstacles that needed to be overcome.

Fig. 3 *R. prolixus* nymph

Photo: Michele Vlimant



One of the most important morphological characteristics that facilitated the evolution of haematophagy in Heteroptera was the pre-adaptation for piercing which opened a route for the evolution of the blood-sucking habit in at least 3 families (see above). Nevertheless, some improvements in the reduviid predatory ancestor's mouth parts were necessary for efficient sucking of blood in Triatominae. When comparing the current predatory reduviids with haematophagous triatomines some rostrum differences in structure and function are observed. The mouthparts of predators which are generally short, robust (strongly chitinized) and often curved, permit an efficient perforation of the prey's hard exoskeleton. Instead, triatomines developed a more elongated, slim and slender proboscis (Fig. 3) able to pierce the host's soft integument in search of blood vessels after penetration (Carcavallo *et al.* 2000, Lehane 2005).

Another obstacle that triatomines needed to overcome, in order to facilitate regular blood intake from host, was the poisonous saliva of their reduviid ancestor. Most bites of predatory reduviids are very painful to vertebrates because of their marked toxic and proteolytic saliva, necessary to immobilize and digest their invertebrate prey. This saliva can also be used as a defence against vertebrates. In triatomines this is not the case, few digestive enzymes have been found in their saliva and those that are present exist at very low concentrations making it unlikely that kissing bugs' saliva have important factors for blood digestion (Lehane 2005). In order to avoid the defensive reactions on the sleeping vertebrate these rapid-feeding bloodsuckers developed a more benign saliva. This saliva has anti-haemostatic and anti-pain proteins and some other small molecules (Andersen *et al.* 2005) that inhibit the normal blood clotting process, facilitate penetration of the skin and neutralize the inflammatory response. Such adaptation served to increase triatomine chances of surviving the most risky episode of their lives, i.e. piercing the host's skin.

Having located and transferred the blood from the host to the midgut, these insects needed to deal with this rich protein meal. The Triatominae subfamily preserves some digestive characteristics of its entomophagous ancestors, having inherited a particular set of digestive proteins for lysis of the blood cells (cathepsins) from reduviid predators. The phytophagous ancestors of early reduviids lost their capacity to use trypsin, the usual

digestive protease, because plant sap has virtually no protein and plant seeds have potent antitrypsins. Thus, reduviid predators and haematophagous triatomines developed the capacity to secrete cathepsins in the gut for the digestion of their respective protein-rich meals (Billingsley 1990, Lehane & Billingsley 1996). Cathepsins⁶ are a family of primitive proteases normally present in the intracellular lysosomes. They are generally active at low pH (optima 5.5 - 6), so kissing bugs developed a system to acidify the lightly alkaline blood (pH~7.4, Terra 1988).

Triatomines also conserve some behavioural characteristics of their ancestors. Some triatomines currently feed on invertebrates during their early stages in natural and laboratory conditions: young instars of *Eratyrys mucronatus* can feed on the haemolymph of large arachnids (*Amblypygi*) which inhabit hollow trees (Gaunt & Miles 2000) and *Belminus herreri* and *B. peruvianus* are unable to take bloodmeals directly from birds and mammals during their early instars but can do so from gorged bugs by cleptohaematophagy or cannibalism in the laboratory (Sandoval *et al.* 2000). Furthermore, *B. herreri* has been reported to feed on Blattidae in rural dwellings in Colombia (Sandoval *et al.* 2004). Although Lorosa *et al.* (2000) succeeded in getting all nymphal stages of *T. circummaculata* and *T. rubrovaria* to feed exclusively on Blattidae lymph under laboratory conditions, these authors apparently failed in completing the entire life cycle, i.e. in order to succeed into a second generation these species needed vertebrate blood.

The blood meal, although very rich in proteins, does not have all the nutrients that triatomines required to survive, so these bugs as obligate blood-sucking arthropods needed to evolve a mutualism with micro-organisms that produce B vitamins (thiamine, pantothenic acid, pyridoxine, folic acid and biotin) that are deficient in vertebrate blood (Douglas & Beard 1996). These symbionts are so important that most blood-suckers conserve them within mycetocytes, i.e. cells lining the ventriculus of the midgut (e.g. in the tsetse fly) or in a specialized organ known as mycetome (e.g. in the leech). It is well known that plant-feeding heteropterans frequently harbour symbionts whose primary function is the synthesis of similar essential nutrients in specialized crypts or caeca of the midgut or in mycetocytes. But gut symbionts are lacking in the wholly predaceous families of Heteroptera (Dolling 1991). Triatominae could therefore not inherit specialized cells or organs to contain these important symbionts from their hypothetical predaceous ancestor and evolved a more primitive way to conserve these bacteria. Indeed, triatomine symbionts live freely in the lumen of the midgut and are acquired by the ingestion of conspecific faeces and cannibalism (Douglas & Beard 1996, Eichler & Schaub 1998, 2002). Much emphasis has been given to an actinomycete *Rhodococcus* (= *Nocardia*) *rodinii*, however other bacteria, including *Streptococcus*, *Staphylococcus*, *Corynebacterium*, *Mycobacterium*, *Pseudomonas* and *E. coli* have been isolated from the midgut of *R. prolixus* (Douglas & Beard 1996). Rapid turnover of the enormous volume of ingested blood through the excretion of large amounts of water and salts (Kollien *et al.* 2001) and triatomine aggregation behaviours (Figueiras & Lazzari 2000) facilitate symbiont transmission between conspecifics.

Reduviid forelegs underwent interesting morphological adaptations from the predator form to the blood-sucker form related to their different ways of life. On the former, forelegs are usually longer and adapted with sticky organs on the fore tibia (i.e. fossulae spongiosae) to grasp and manipulate the invertebrate prey (Gorb & Beutel 2001). The presence of

⁶ Similar cysteine proteinases were found in some Coleoptera families (supraorder Cucujiformia), thus Terra *et al.* (1996) have suggested that the ancestor of this group was adapted to eat seeds rich in trypsin inhibitors.

fossulae spongiosae is plesiomorphic⁷ within Reduviidae (Weirauch 2005). Although forelegs are more simplified on triatomines, some species retain these adhesive pads for assisting adults in climbing smooth surfaces, a useful adaptation for bugs that fly and live in trees as most *Rhodnius* adults do (Beutel & Gorb 2001).

Aside from the above mentioned structural adaptations there were also behavioural ones. Probably, the decisive step that allowed the shift from predation to haematophagy was, in fact, a change of behaviour. Other examples have proven that behaviours can initiate evolutionary adaptations to new ecological niches (Futuyma 1998). As Mayr (1963) has explained: “A shift into a new niche or adaptative zone is, almost without exception initiated by a change of behavior. The other adaptations to the new niche, particularly the structural ones, are acquired secondarily. With habitat and food selection – behavioral phenomena - playing a major role into a new adaptative zone, the importance of behavior in initiating new evolutionary events is self-evident”. Most researchers agree that the change of “prey” that took place in Triatominae developed from the association of a reduviid predator with a vertebrate (Schofield 2000b, Jurberg 2003). This conclusion is based on the knowledge of phylogeny and behavioural adaptations of current reduviid predators and triatomines. At present, most reduviid predators are normally free living and active hunters that seek out and attack prey,⁸ whereas others adopt an ambush strategy waiting for their prey to pass nearby and attack by surprise,⁹ and many have been found living in vertebrate nests and burrows (sometimes feeding on triatomines; Carpintero 1981). Some of these could be transitional forms between a predatory and a blood-sucking reduviid, i.e. sister groups of triatomines.

Members of the following reduviid subfamilies have been very tentatively suggested to be close relatives of Triatominae by many authors:

- Physoderinae (Lent and Wygodzinsky 1979, Carcavallo *et al.* 2000)
- Reduviinae (Carcavallo *et al.* 1999, 2000)
- Harpactocorinae (Carcavallo *et al.* 1999, 2000)
- Emesinae (Schofield 2000a)
- Peiratinae (Schofield 2000a)

Some species of these predator subfamilies show morphological similarities to triatomines and many can apparently feed on blood if allowed to do so, but knowledge of their biology is very poor. Do any species among these candidates share some morphological, physiological and behavioural features with triatomines? It would be very interesting to know if these reduviid predators aggregate in refugia, are night active, prefer to search for food near the refuge of a vertebrate, are attracted to vertebrate odours or share any other behavioural characteristic with triatomines.

⁷ An evolutionary primitive trait that is homologous within a particular group of organisms but is not unique to members of that group and therefore cannot be used as a diagnostic or defining character for the group (Martin & Hine 2000).

⁸ For example, the reduviid subfamily, Peiratinae actively pursues its prey along the ground or on vegetation, grabbing the victim with the forelegs before immobilising it (Dolling 1991).

⁹ Members of the reduviid subfamily Emesinae live in spider webs and steal the trapped prey of the spider (Dolling 1991).

3. Evolutionary succession

Certainly, there was an evolutionary succession of steps from the predator assassin bug to the haematophagous kissing bug. Early reduviid contacts with vertebrates defined many of these evolutionary steps. Primitive birds and mammals that appeared in the Mesozoic era (Table I) were not dominant until dinosaurs became extinct about 65 mya. But their great radiation between the late Cretaceous and early Tertiary (Delsuc *et al.* 2004, Ericson *et al.* 2003) must have provided an ample supply of new niches for opportunistic species of various arthropod groups (Kim 1985). The new vertebrate species occupied more ecological niches and reached a greater diversity of life-styles than did early reptiles. Many of them were relatively small and lived in nest type dwellings on trees or in subsoil burrows. It is reasonable to assume that the nests of such birds and mammals were continuously invaded and colonized by many different opportunistic arthropods, which were likely scavengers and saprophagous feeding on organic remains and profiting from the vertebrate refuge. The ancestral reduviid predator was attracted by this abundant arthropod prey supply. Presumably, these early triatomines first fed on soft invertebrates abundant in nests (e.g. caterpillars and larvae of other insects) and later attempted to pierce the skin of newborn (and thus defenceless) vertebrates. It is likely that these bites were initially exploratory and occurred just occasionally. The prolonged close association with nidicolous mammals and birds eventually resulted in specialization to feeding directly and efficiently from the vertebrate itself and minimized the time spent searching for food. Aside from the abundant supply of food and protection from diverse enemies, the nest also provided favourable and constant microclimatic conditions (Heger *et al.* 2006). The protection from climatic extremes entailed a reproduction less dependent on seasonal changes and an increase in population density of early triatomines. Furthermore, aggregation in a closed environment near the host afforded many other advantages. It facilitated sexual encounters and symbiont acquisition via conspecific faeces ingestion and cannibalism. It permitted the development of an alarm system based on intercommunication between conspecifics -valuable to deal with predators. It also laid the way for the production of high quantities of faeces with chemical cues useful for triatomines to return to their refuge after feeding.

4. Radiation

The absence of triatomine endemic forms in Africa, complete absence in Europe and existence of very few species in Asia (i.e. pre-Gondwanian distribution), indicate that these bugs arose after continent separation began, probably in the mid-Cretaceous (Table I). It is likely that the subfamily evolved in North or South America where most species are present (Hypša *et al.* 2002). Poinar (2005) reported a triatomine (*T. dominicana*) infected with trypanosomes (*T. antiquus*) as well as bat hairs fossilised in Dominican amber that indicates the existence of triatomine association with trypanosomatids and nesting vertebrates in the mid-Tertiary period (at least 15 mya according to the amber dating). The particularly high degree of speciation reached by birds and nesting mammals in the tropical zone of America, as a consequence of several factors operating during the Cenozoic in the Amazon region (Nores 1999), provided a high diversity of “vacant” nest-type habitats that made the diversification of triatomines in this region and probably the radiation of *Lishcosteous* in tropical Asia possible. In relation to *Lishcosteous* it is not clear if these species are autochthonous to Asia and they developed the haematophagous habit there, or

radiated from America to the Old World in association with their hosts several mya (Schaefer & Coscaron 2001). Radiation of the Old World species of *Triatoma* probably occurred not more than 350 years ago just as *T. rubrofasciata*, associated with domestic rats, was disseminated by shipping during human exploitation of commercial sea routes (Patterson *et al.* 2001)

5. *Triatomine associations with humans*

All triatomine species are potential vectors of *T. cruzi* to humans, but the majority of these are inhabitants of wild ecotopes and have, until the present, never been reported invading houses (Silveira 2000, Gaunt & Miles 2000). Only a small fraction (about 5%) are of medical and epidemiological importance, as is the case of *T. infestans* (Fig. 4), *R. prolixus*, *T. dimidiata*, *P. megistus*, *T. sordida*, *T. brasiliensis* and *R. pallens* which are responsible for most Chagas disease cases, mainly because of their wide geographical distribution and capacity to colonize the houses of impoverished human populations. Between the two extreme categories of wild and domiciliary triatomines there is an important and increasing number of species that even though they live in sylvatic habitats have been reported invading the domestic and peridomestic habitat where they can transmit the Chagas pathogen. The emergence of these new domestic species is facilitated where human activities impact natural habitats.

Fig. 4 *T. infestans* (Reduviidae: Triatominae) on the skin of a human host.

Photo: Pablo Guerenstein



Triatomines first contact with modern humans took place recently when *Homo sapiens* reached America from Siberia probably 15'000 years ago (Bortolini *et al.* 2003, Dillehay 2003, González-José *et al.* 2005). Although no one is certain when the first American settlers lodged triatomines in their dwellings, it is quite clear that the association was well established during precolumbian times. There is a 9'000-year record that indicates a high prevalence of Chagas disease in Indians living along South America's coastal area of the Atacama Desert (Aufderheide *et al.* 2004). Arguably, domiciliary triatomines were the vectors responsible for such Chagas disease cases at that time.

It is interesting to note that there has been a clear-cut difference between *highland* and *lowland* Amerindian populations in their association with triatomines (Coimbra 1988). The former populations clearly have been associated with triatomines and suffered from

Chagas disease long before European contact. On the other hand, there have been no reports of *T. cruzi* among lowland tribes or of bug vectors in their traditional houses¹⁰ despite thousands of years of occupation by these tribes of the Amazonia Basin.

Highland Indians formed complex societies that included a stratified organization and highly populated cities. The intensive agricultural practices (e.g. potato and maize) that these civilizations developed more than 4'000 years ago (Perry *et al.* 2006) surely produced a strong impact on the triatomine environment and promoted its domiciliation. Additionally, early domestication of vertebrates by highland civilizations facilitated triatomine colonization of the peridomestic habitat. *T. infestans* invasion of sedentary human populations probably started by its association with guinea pigs (*Cavia porcellus*), a natural host of this bug (Panzera *et al.* 2004). In deed, when Spanish explorers first encountered the Incas, the guinea pig and other wild animals were already semidomesticated as a source of food in the southern region (Spotorno *et al.* 2004).

Fig. 5 Amazon Yanomami Idians

Photo: Anonymous



On the other hand, Lowland Indians were organized in less complex societies and were highly nomad as they still are in present-day Amazonia (Fig 5). It has been reported that these tribes present low indexes of Trypanosomiasis and Leishmaniasis, another zoonosis transmitted by a nesting bloodsucker insect (sandflies, Phlebotominae). According to Coimbra (1988) small settlements that rarely exceeded 500 people and high village mobility explain the Amazon Indians' "immunity" to these local diseases. Mobility is an efficient adaptative response by Amazonian populations to many environmental constrains, this cultural practice avoids declining soil fertility and weed infestation of gardens after repeated cropping, and protein (animal prey) scarcity due to intensive hunting.¹¹ Another relevant element that serves to protect Lowland Indians from vector-borne diseases is the rare practice of animal domestication. Although some people (usually children) keep immature individuals found in the forest as pets, these seldom breed in captivity, so the

¹⁰ Ancient Native Americans, as modern natives still do, built houses of wattle and thatch that provided ideal refuge opportunities and facilitated the entrance of arboreal bugs carried in tree construction material. Triatomines like *R. prolixus* are capable of sticking their eggs on palm trees which have been used by Amerindian and non-Indian people to construct house roofs, especially in the tropics where palm roofs decrease house air temperature. But these facts have not been demonstrated as being sufficient for triatomine domiciliation to take place. Indeed, some authors propose that the characteristic design of certain lowland Indian houses could provide barriers against triatomine domiciliation (Coimbra 1988).

¹¹ There are other factors that promote mobility such as avoidance of too many graves in the traditional house, the death of the tribe leader, proliferation of cockroaches in the houses or anthills in the village surroundings. Village fissioning also occurs as a means of alleviating social tensions when the population reaches a certain size (Coimbra 1988).

number of animals in houses is low and unstable. All these factors undoubtedly have contributed to obviate triatomines domiciliation among lowland Amazonian tribes.

Modern American societies have been experiencing the same cultural practices that led triatomine domiciliation of highland populations thousands of years ago. These practices produce ecological disturbance and environmental pressures that facilitate the adaptation of sylvatic triatomine species to human houses. The existence of highly endemic areas of Chagas disease in Latin America strongly depends, as probably was the case in ancient Indian civilizations, on differential political and socio-economic development. Recently reported Chagas disease cases in the Brazilian states of Pará and Amazonas are in non-Indian peoples and inhabitants of periurban or rural areas (Coimbra 1988). Currently, there is a great and increasing potential of Chagas disease endemization in Amazonia due to recent human migrations and the environmental degradation inflicted on the region.

6. Epilogue

Triatomine evolution is a dynamic process where domiciliation is one of the fastest changing dimensions. We may presently be observing over a very short time scale the adaptation of many sylvatic species to the human domicile. This major change of hosts can occur very rapidly as a consequence of the way humans influence their surroundings as mentioned above. Regrettably, there is a lack of research on the way cultural heritage has influenced Chagas disease epidemiology (Coimbra 1988).

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Chapter II

Appetence behaviours of the triatomine bug *Rhodnius prolixus* on a servosphere in response to the host metabolites carbon dioxide and ammonia

“The information used by the animal for the guidance of its behaviour has often proved to be simple.”

- Friedrich G. Barth & Axel Schmid

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Appetence behaviours of the triatomine bug *Rhodnius prolixus* on a servosphere in response to the host metabolites carbon dioxide and ammonia

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Abstract A combination of 1,000 ppm CO₂ plus 30–40 ppb NH₃ in an air stream induced *Rhodnius prolixus* nymphs walking on a servosphere to perform a series of appetence behaviours. Shortly after the onset of stimulation the nymphs turned sharply upwind towards the source of the chemostimuli (within 13 ± 9 s) from mostly downwind and crosswind walks in the air stream alone. The mean vector angles of these upwind tracks were concentrated in a cone 60° either side of due upwind. The upwind walking bugs stopped more frequently but for a shorter duration and walked at a higher speed than before stimulation. During stops in the presence of the chemostimuli the bugs frequently corrected their course angles and extended their forelegs to reach higher with their antennae in the air. In the air stream alone, *R. prolixus* nymphs frequently sampled the sphere surface with the antennae and cleaned their antennae with the foreleg tarsi. However, the nymphs only briefly tapped the left or right antennal flagellum on the corresponding first leg tarsus and never touched the servosphere surface in the presence of the chemostimuli. After chemostimulus removal from the air stream the bugs continued to respond with the same appetence responses as during stimulation, but walked more tortuously in a crosswind direction in an effort to regain contact with the chemostimuli.

Keywords *Rhodnius prolixus* · Triatomine bug · Blood-sucking · Behaviour · Servosphere

Introduction

The triatomine bug *Rhodnius prolixus* Stål, 1859 (Hemiptera: Reduviidae), referred to hereafter as *Rhod-*

nus, is an important vector of *Trypanosoma cruzi*, the causative agent of Chagas' disease in Central and South America (Lent and Wygodzinsky 1979). This species, as virtually all members of the tribe Rhodniini, is primarily associated with palm tree habitats and has a wide range of hosts including birds, rodents, marsupials, sloths and reptiles (Lent and Wygodzinsky 1979). *Rhodnius* colonized human dwellings due to the use of palm trees in the construction of houses in rural areas, providing the bugs with a stable niche with a constant food supply. Furthermore, this particular species has been used to study insect orientation behaviours since the beginning of the last century (Wigglesworth and Gillett 1934) and has long since served as a model species in insect physiology (Wigglesworth 1984).

There seems to be an olfactory component to host finding in virtually all blood-sucking insects (Lehane 1991; Guerin et al. 2000). Triatomines use different sensory cues to locate hosts and refugia, but volatile chemostimuli alone can induce appetence behaviours (Taneja and Guerin 1995). CO₂, with a very constant concentration in mammalian breath (4.5%; Lindstedt and Thomas 1994) is generally accepted to be a dominant cue that serves as both an activation and orientation cue for all blood-sucking insects including triatomines (Wiesinger 1956; Mayer 1968; Nuñez 1987; Taneja and Guerin 1995). NH₃ is present in triatomine (Harrington 1961; Taneja and Guerin 1997) and host animal excretions (Lentner 1981; Albone 1984) and as such occurs in both refugia used by triatomines as well as in host dwellings. Furthermore, NH₃-sensitive receptor cells occur in basiconic grooved-peg sensilla of *Triatoma infestans* (Diehl et al. 2003) that respond to levels as low as 2 ppb (Taneja and Guerin 1997). The NH₃ concentration in human breath varies between 120 to 1,280 ppb (Norwood et al. 1992). As both CO₂ and NH₃ alone attract *Rhodnius* and *T. infestans* on a servosphere (Taneja and Guerin 1995, 1997; Guerin et al. 2000), we use here a combination of these products to study the appetence responses of *Rhodnius* on a servosphere, we describe the behaviours contributing to such responses and the strategies used by these bugs to try to

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recontact the chemostimuli after their removal from an air stream.

Materials and methods

Insects

A colony of *Rhodnius* is maintained at 28°C, 80% RH and 12:12 h L:D cycle in a climate chamber. The bugs are fed on chickens once a month. Experiments were made with fifth-instar nymphs starved for 7–8 weeks after moulting.

Behavioural recordings

A servosphere (Kramer 1976) was used to record the walks of *Rhodnius* nymphs. The servosphere is an open loop set up, i.e. it records behaviours the bugs employ to approach a goal but without reaching it. This design satisfies the minimum of experimental conditions required to study a walking arthropod's behaviour (Kennedy 1977). The apparatus allows a walking untethered triatomine liberty to move unimpeded in all directions at the apex of a sphere (50 cm diameter) in an experimental set up where sensory modalities other than chemostimuli are excluded (Taneja and Guerin 1995). The co-ordinates of displacements of the bug on the servosphere are supplied to a computer (SAMII 68 K; WWS, Ettlingen, Germany) by two incremental pulse-generators mounted on the sphere's equator, both working at a resolution of 0.1 mm and recording at 0.1-s intervals. Subsequent analysis of these records was performed using in-house developed track analysis software (sLocTrack) running under Linux. Each bug was allowed a 2-min period of acclimatisation before recording started. The responses of 24 bugs were recorded in three consecutive 2-min periods: in the air stream alone (C1), the air stream plus chemostimuli (T) and the air stream after removal of the chemostimuli (C2). To assess the effect of the air stream alone on the behaviour of the triatomines, the behaviours of 21 other bugs were observed for 2 min each in still air.

All behaviours on the servosphere were also recorded on video tape (Panasonic VCR NV-180) using two infrared-sensitive video cameras (Canon Ci 20PR, Japan and PCO Computer Optics 77CE, Germany), one positioned at 70° to the apex of the sphere over the air delivery tube and the second placed tangentially to the apex of the sphere from the downwind position. Both views were observed simultaneously using a screen splitter (Panasonic production mixer, WJ-MX12, Japan).

Chemostimuli and their delivery

The stimulus delivery system was as described in Taneja and Guerin (1995). This consisted of a charcoal-filtered air-stream (15 cm s⁻¹ as measured by a hot-wire ane-

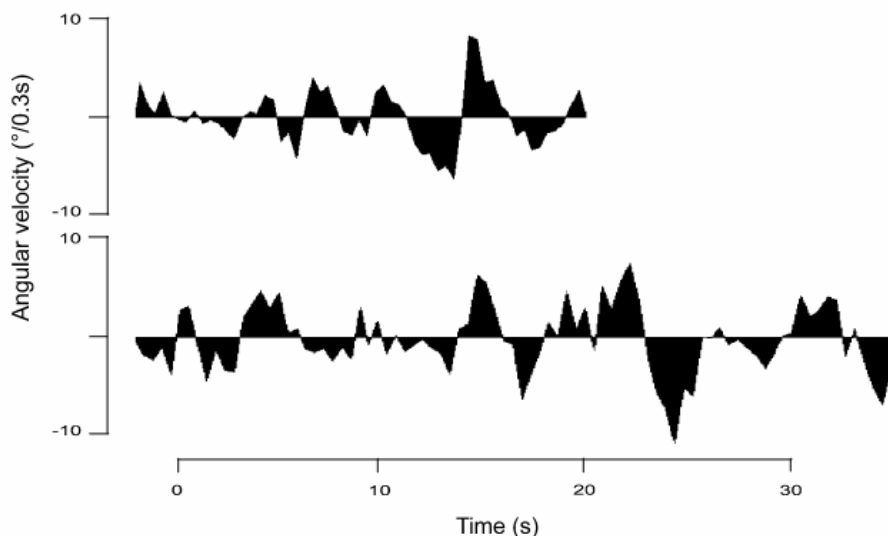
nometer, ThermoAir2, Schiltknecht Messtechnik, Switzerland, accuracy ±0.01 m s⁻¹, rise time <1 s) maintained at 24°C and 80% RH (as measured by a dew point thermohygrometer, MTR 5.3, DMP, Switzerland, accuracy ±0.5 K, rise time <2 min) directed to the apex of the servosphere into which a mixture of 1,000 ppm CO₂ and 30–40 ppb NH₃ were injected as chemostimuli (Taneja and Guerin 1995, 1997). The onset and cessation of stimulus delivery to the bug was controlled automatically (Taneja and Guerin 1995). Carbon dioxide came from a pressured cylinder containing 2% CO₂ in O₂ injected at 50 ml min⁻¹ into the air stream. Ammonia came from a gas-wash bottle containing a filter paper disk (12.5 cm diameter) wetted with 0.2 ml of diluted NH₄OH (Fluka, Switzerland) in nanopure water (71.43 mmol⁻¹). The air passing over this filter paper disk was injected at 160 ml min⁻¹ into the air stream. The filter paper was treated once for tests with 4–5 bugs. The controls consisted of 50 ml/min charcoal-filtered air alone for CO₂ and 160 ml min⁻¹ charcoal-filtered air passing through a gas-wash bottle with 0.2 ml water on filter paper for NH₃. The concentrations of CO₂ in the air at the apex of the sphere was measured with an infrared gas analyser (LI-COR LI-820, Nebraska, USA, accuracy ±3%, rise time 5 ppm in ≤20 s) and the concentration of NH₃ was measured with an electrochemical sensor (Dräger Sensor Pac III S, Lübeck, Germany, accuracy ±1%, rise time 1 ppm in ≤20 s).

Behaviour analysis

As the mean length of a *Rhodnius* fifth-instar nymph is 1.5 cm and its average walking speed is approximately 30 mm s⁻¹, the basic track recording intervals of 0.1 s were merged to provide intervals of 0.3 s, i.e. allowing the bug to move at least 60% of its length before recording its next position. Subsequently, a running mean was calculated over five successive (0.3 s) intervals to smoothen the data in order to remove some noise related to the insect's gait that was picked up by the servosphere. To identify stops, 3 mm/s was considered the minimum speed the animal had to achieve to be considered walking; all displacements slower than this were discarded.

From these modified track recordings the following instantaneous statistics were calculated; direction (ϕ) with respect to wind (0°), instantaneous speed (v) and angular velocity (ω). The instantaneous walking speeds of the bugs (v) in each experimental period were compared using Student's *t*-test; compliance to normal distribution (Shapiro-Wilk test for normality and QQ normal plot method) and equality of variances (Leven's test) were tested. Parameters, termed summary statistics, were calculated for each period of each track (C1, T and C2). These summary statistics were overall displacement (D), number of stops (NS), stop duration (SD), mean direction of the track (Φ) and path straightness r (mean

Fig. 1 Angular velocity in degree per 0.3 s made up by *Rhodnius* nymphs walking on the servosphere. By designating left turning angles as (-) and right turning angles as (+) and applying a running mean over five consecutive 0.3-s intervals, regular oscillations left and right of a relatively straight course are apparent



vector length, Batschelet 1981), relative upwind displacement (RUD, i.e. walking distance in a cone 60° either side due upwind) and target vector (TV, calculated by multiplying the cosine of the mean direction of the track Φ by the path straightness r , Jones 1977). All summary statistics except Φ were compared using the two-tailed Wilcoxon paired signed rank test. Rao's spacing test (Batschelet 1981) was applied to determine whether the distributions of Φ in a given period differed from uniformity. In periods where a particular orientation angle was expected, the data were subjected to a modified Rayleigh V -test (Batschelet 1981). Since the mean vector angles of the tracks were mostly distributed across the air stream after removal of the chemostimuli, the corresponding mean angles were doubled (Batschelet 1981) before applying the modified Rayleigh V -test. The dispersion, in degrees, of the mean track directions (Φ) for all 24 bugs was estimated as the mean angular deviation s (Batschelet 1981). In addition, major changes in walking direction that occur after onset of chemostimulation were analysed from plots of the tracks and by replaying the video recordings at a resolution of 1 frame/12.5 s (Panasonic VCR AG-7350, Japan).

As bugs stop frequently during walks, criteria for automatically identifying a walking bout were established in our software in order to examine if the mean direction of such walking bouts was modified in response to exposure of the bugs to the chemostimuli. This was done as follows: if the bug's speed increases above the minimum speed, start a new walking bout and if it drops below the minimum speed interrupt the walking bout; if the walking bout duration is < 0.5 s (minimum walking bout duration) then discard the bout; if a walking bout starts during one experimental period and finishes during the following one, it is divided into two walking bouts in the corresponding periods. Summary statistics for walking bouts were calculated and analysed as described above. All statistical analyses were per-

formed using R for Linux version 1.6–24, Vienna, Austria (Ihaka and Gentleman 1996).

Results

Rhodnius nymph behaviours in still air

Triatomine behaviour in still air is firstly described here as an air stream was used to deliver the chemostimuli. *Rhodnius* nymphs walked in straight bouts (median $r=0.996$, IQR¹ 0.004; median length of a bout 85.07 mm, IQR 162.71, $n=321$) punctuated by stops (median 3.2 s, IQR 4.1 s, $n=335$ stops). The median distance walked during the 2-min recording was 1,924 mm (IQR 1,498 mm, $n=21$) and the median number of stops/bug was 19 (IQR 25). The bugs began a walking bout at a high speed (mean 45 mm s^{-1}) that gradually decreased by some 20% to end in an abrupt stop. The mean instantaneous velocity during these walks was 27.89 mm s^{-1} . These walking bouts also included intervals (< 1 s) at relatively low speed (mean 11 mm s^{-1}). The bugs walked by turning successively right and left of course in a zigzag path (Fig. 1). The median angular velocity was $8.8^\circ/\text{s}$ (IQR $11.6^\circ \text{ s}^{-1}$). No bug turned at an angular velocity higher than $61.2^\circ \text{ s}^{-1}$ (that occurred only at low walking speeds of $< 10 \text{ mm s}^{-1}$); at peak walking speeds ($> 40 \text{ mm s}^{-1}$) angular velocities only reached $14.3^\circ \text{ s}^{-1}$.

During walking bouts the bug periodically scanned the air by moving their antennae up and down either simultaneously or alternatively with the left or right antenna ($\pm 30^\circ$ with respect to the body long axis) ca. every 2 s, irregularly touched the sphere surface with their antennae and eventually undertook a change in

¹IQR: interquartile range, i.e. the difference between the 75th and 25th percentiles.

direction. During stops, the bugs either changed direction by rotating the body and/or scanned the air by moving the antennae (maintaining the thorax–abdomen axis parallel to the ground), or undertook cleaning of the antennae (14 out of 21 nymphs did so). Antennal scanning of the air during stops was performed by moving both antennae simultaneously or alternatively up and down, and laterally. Antennal cleaning is a stereotyped behaviour: the bugs lift and join both first leg tarsi over one antenna and run them distally along the flagellum (through a small comb on the inner side of the fore tibia as described by Campanucci et al. 1998) while slightly lifting the head in the opposite direction. This is followed by rubbing the tarsi against one another and subsequent preening of the second antenna. The entire process lasts approximately 1 min 20 s if uninterrupted.

Of the 21 insects recorded while walking in still air, 8 made consecutive clockwise or counter-clockwise circles while walking. Circling was not continuous but was interrupted by stops. The fraction of the track walked in circles was very variable; seven bugs made from 2 to 5 circles of variable size, shape and sense of rotation, accounting for 22.2% of the track, and one individual made 32 consecutive very regular clockwise circles (ca. 12 cm diameter) throughout its walk. The tracks of nymphs that did not circle were not very straight (median $r=0.36$, IQR 0.28, $n=13$), nevertheless the mean vector angles describing these walks showed no bias for a given direction in still air ($p > 0.8$ Rao's spacing test).

Behaviours in the air stream alone

In contrast to the situation in still air, nymphs in the air stream without the chemostimuli walked relatively straight (median $r=0.75$, IQR 0.39, $n=24$) but no bugs circled. The mean vector angles of the tracks were either downwind or crosswind ($P < 0.005$ Rao's spacing test, $s=59^\circ$, Fig. 3a). In the air stream, the nymphs walked less (median 936 mm, IQR 823 mm, $n=24$, $P < 0.001$), stopped less (median 7, IQR 11, $P < 0.01$) but for a longer duration (median 11.14 s, IQR 6 s, $P < 0.001$) than in still air. The bugs' mean instantaneous walking velocity in the air stream (24.06 mm s^{-1}) was lower compared to still air ($P < 0.0001$). Fifteen of the 24 nymphs cleaned their antennae during stops (3 only cleaned one antenna).

Responses to CO_2 plus NH_3 in the air stream

The addition of CO_2 and NH_3 to the air stream induced 22 of the 24 nymphs tested to turn sharply upwind (median of these upwind turns 112° , IQR 43°) shortly after the onset of stimulation (median time to response 13 s, IQR 9 s, maximum 34 s, Fig. 2). The straightness of the walks (median $r=0.57$, IQR 0.37) did not diminish significantly ($p > 0.152$) compared to walks made in the air stream alone, but the mean vector angles

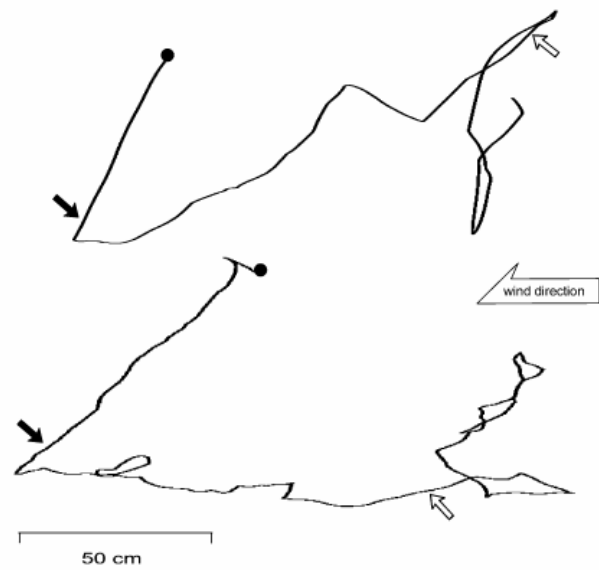


Fig. 2 Tracks made by *Rhodnius* nymphs on the servosphere depicting responses to 1,000 ppm CO_2 plus 30–40 ppb NH_3 in the air stream (arrow). The tracks started (filled circle) with the bugs walking crosswind or downwind in the air stream alone (15 cm s^{-1}). After the chemostimuli were added to the air stream (bold arrows on the tracks), the bugs turned to walk upwind. After removal of the chemostimuli from the air stream (empty arrows) the bugs at first continued to walk upwind but thereafter walked mainly crosswind

of the tracks were concentrated in a cone 60° either side of due upwind ($P < 10^{-7}$ modified Rayleigh V -test; angular deviation 30° , Fig. 3b). The bugs still continued to walk in bouts but the distribution of these walking bouts shifted from downwind and crosswind runs in the air stream alone to upwind runs following stimulus delivery (modified Rayleigh V -test, $P < 0.001$, Fig. 4). There is, however, a deviation of the running bouts to one side of upwind due to a slight asymmetry in the airflow, detected after the experiments were completed. The median proportion of the distance walked upwind during the 2-min stimulation period reached 63% as compared to 7% in the air stream alone (Fig. 5a). The target vector values increased to 0.48 (IQR 0.27) indicating the efficiency with which the triatomines responded to the chemostimuli (Fig. 5b).

Although *Rhodnius* nymphs stopped more frequently during stimulation (median 15, IQR 12) they walked farther (median 1,614 mm, IQR 781 mm) than in the air stream alone (Fig. 6). This was because the median duration of stops was shorter (3.95 s, IQR 3.2 s, $P < 0.0005$) during exposure to chemostimuli and that the bugs' mean instantaneous velocity (24.77 mm s^{-1}) increased compared to that in the air stream alone ($p < 0.05$). During stops, the bugs frequently lifted the anterior part of their body ($\sim 40^\circ$) through extension of their fore legs in order to reach higher with their antennae held up (ca. 60° with respect to ground), a behaviour not seen in still air or in the air stream alone and referred to here as antennal

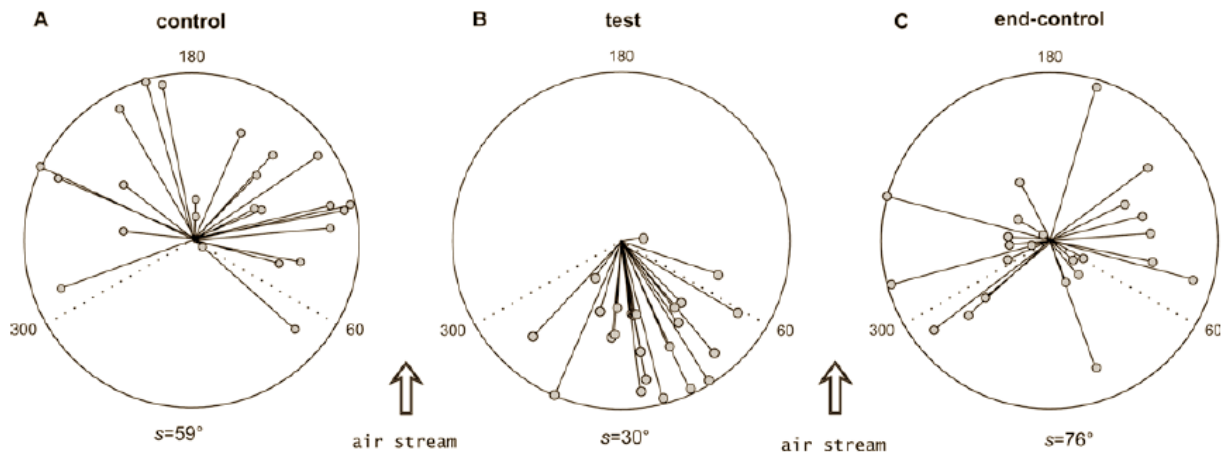
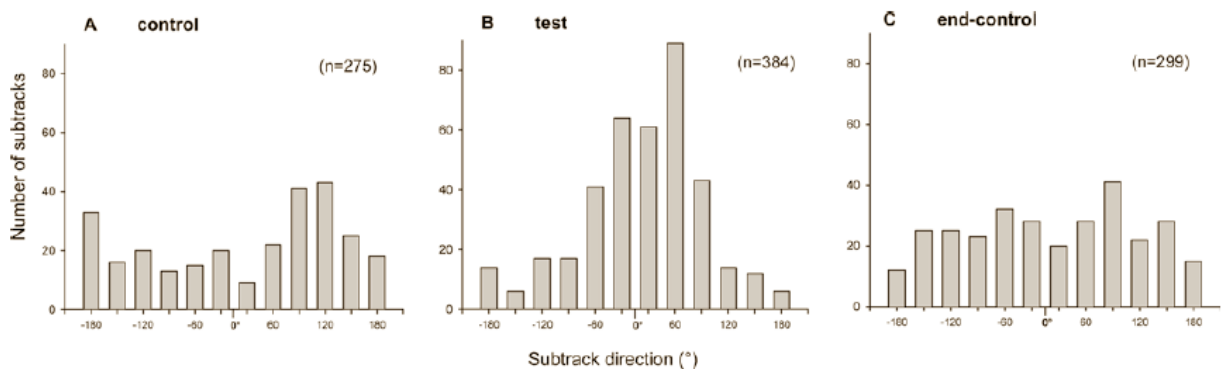


Fig. 3a-c Polar plots of the distribution of the mean vector angles of tracks described by *Rhodnius* fifth instar nymphs ($n = 24$) on the servosphere during consecutive 2-min periods: in the air stream alone (a), air stream with 1,000 ppm CO_2 plus 30–40 ppb NH_3 (b) and the air stream alone again (c). The mean vectors of the tracks shifted from crosswind and downwind in the initial control period to the upwind cone (60° either side of due upwind) during exposure to CO_2 plus NH_3 (test), and to crosswind after the chemostimuli were removed from the air stream (end-control). The nearer the vector weighting to the perimeter the straighter the walk in that direction. The mean angular deviation (s) below each plot is a measure of the dispersion of the directions (Batschelet 1981) taken by bugs in each period. The bug walked more crosswind but in a more tortuous manner after treatment removal from the air stream (end-control)

reaching. During exposure to the chemostimuli the bugs did not touch the sphere surface with their antennae. Rather than cleaning the antennae with the foreleg tarsi,

Fig. 4 Histograms of the mean vector angles of subtracks made by 24 *Rhodnius* nymphs during successive 2-min periods in an air stream (upwind 0° ; a), in an air stream with a mixture of 1,000 ppm CO_2 plus 30–40 ppb NH_3 added (b) and in the air stream alone again (c). A subtrack is a walking bout of 0.5 s or more and with a minimum speed of 3 mm s^{-1} , and the subtrack vector angle is the circular mean (Batschelet 1981) of the instantaneous course angles recorded for each walking bout. The bugs walked essentially downwind and crosswind during the initial period in the air stream alone (control). There was a shift ($P < 10^{-16}$; Wilcoxon unpaired test) to a higher proportion of subtracks in the upwind direction during exposure to CO_2 plus NH_3 (test) but this disappeared after removal of the chemostimuli (end-control)



as in still air and in the air stream alone, the bugs exposed to the chemostimuli briefly tapped ($< 1 \text{ s}$) the left or right flagellum on the corresponding first leg tarsus, a behaviour referred to hereafter as antennal tapping. Of the 24 bugs tested, 11 exhibited this behaviour while walking or during brief stops.

Response to removal of the chemostimuli from the air stream

The bugs continued to respond with an upwind walk for some time after removal of the chemostimuli (median 16 s, IQR 9 s, $n = 24$). Thereafter, they walked more crosswind ($p < 10^{-8}$ modified Rayleigh V -test) as indicated by the mean direction (Φ) of the tracks (Fig. 3c), and significantly less straight (median $r = 0.47$, IQR 0.48, $P < 0.05$, $n = 24$) compared to the initial clean air stream situation. The bugs' instantaneous velocity increased after stimulus removal (mean 26.26 mm s^{-1} , $P < 0.0001$) but the frequency of stops and the distance walked remained the same (Fig. 6). The triatomines continued to scan the air with their antennae by lifting the thorax during stops and did not touch the ground with the antennae. They did not clean the antennae with the forelegs but antennal tapping was observed in 13 of the 24 records after stimulus removal.

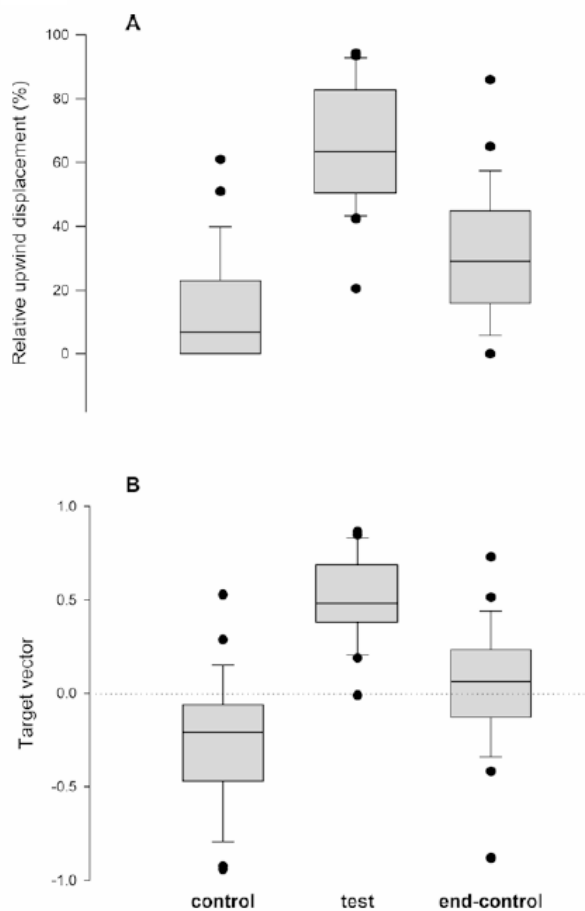


Fig. 5 Responses elicited by a mixture of 1,000 ppm CO₂ plus 30–40 ppb NH₃ in fifth instar *Rhodnius* nymphs on the servosphere were assessed using upwind displacement, i.e. the relative distance walked in a cone 60° either side of due upwind (a), and target vector (b; Jones 1977, see Materials and methods). a The relative upwind displacement during the initial period in the air stream alone (6.8%; control) increased significantly during exposure to CO₂ plus NH₃ (63%, $P < 10^{-7}$; test) and decreased again after removal of the chemostimuli (29%, $P < 10^{-5}$; end-control) but to a level higher than in the initial control period ($P < 0.01$). b Target vector was highest during exposure to the chemostimuli ($P < 10^{-5}$; Wilcoxon paired test). 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, bars below and above indicate the 10th and 90th percentiles, respectively, and the points represent data beyond these limits

Discussion

Mechanisms of orientation without chemostimuli in the air stream

In still air the starved triatomines, driven either by hunger or need for a shelter, indulge in non-oriented behaviour or ranging (Dusenbery 1992) likely to bring them into contact with a stimulus. Some individuals deprived of chemical, auditory, visual or mechanore-

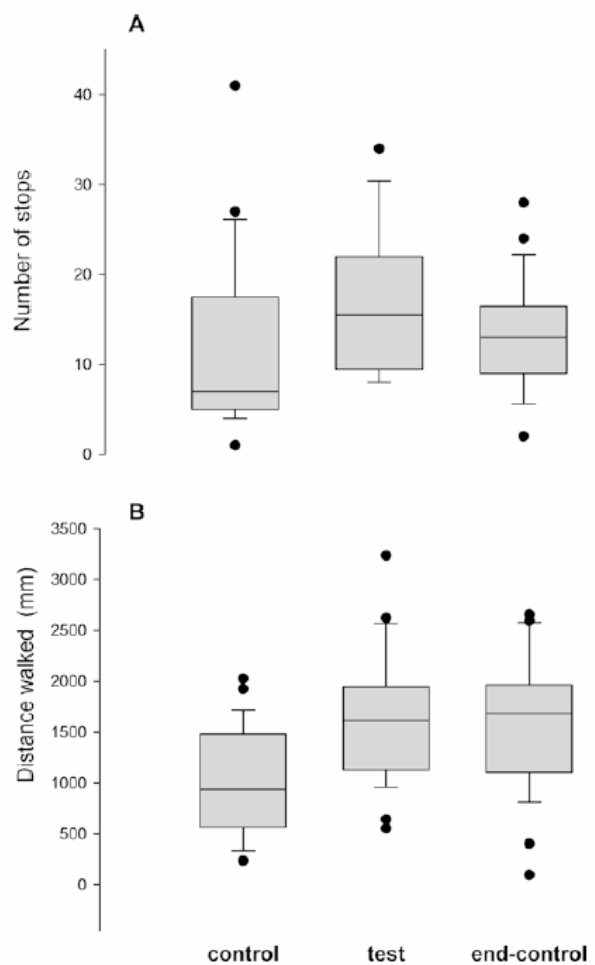


Fig. 6 *Rhodnius* fifth instar nymphs exposed to a mixture of 1,000 ppm CO₂ plus 30–40 ppb NH₃ on the servosphere stopped more frequently (a) but walked more (b) than in the air stream alone. a The number of stops increased on exposure to the treatment ($P < 0.05$; test) and remained higher after removal of CO₂ plus NH₃ from the air stream (end-control) compared to the initial period ($P < 0.01$; control). b The distance walked also increased during the test period compared to the initial control period ($P < 10^{-4}$) and remained higher than for this initial period even after removal of the test molecules from the air stream ($P < 0.005$). For boxplot explanations and statistics see legend to Fig. 5

ceptive information from an air stream make irregular paths containing many circular elements as has been reported for other arthropods (Bell and Kramer 1979; Heinzel and Böhm 1989; Lönnendonker and Scharstein 1991; McMahon and Guerin 2000). This may be due to the incapacity of the bugs to maintain a straight course in the absence of a ‘collimating’ stimulus, i.e. one unrelated to a particular goal that serves the purpose of maintaining a straight course (Dusenbery 1992). On the other hand, *Rhodnius* in an air current is provided with such a collimating stimulus inducing the bug to walk relatively straight downwind or crosswind.

Mechanism of orientation to the chemostimuli

The combination of CO₂ and NH₃, two small molecules, induces goal oriented behaviours by *Rhodnius* on the servosphere. The triatomines switch to a series of endogenously driven behaviour patterns that reflects their appetite (*sensu lato*). Two different goal oriented behaviours can be distinguished as in other species: guiding (Dusenbery 1992) and casting (Kennedy and Marsh 1974; Dusenbery 1992). Guiding behaviours include the mechanisms the bugs employ to approach the resource of the chemostimuli in the air stream, and casting behaviours (treated below), are those undertaken to regain contact with the chemostimuli after loss (David et al. 1983).

Firstly, upon delivery of CO₂ and NH₃ into the air stream the triatomines turn to walk upwind, i.e. chemoanemotaxis. This certainly entails some mechanosensory input in response to the air current direction. The bugs walk in the direction of the source of the chemostimuli while stimulation persists, making sequential corrections in the course angle during stops and walking bouts vis-à-vis the air stream. The increase in walking speed, upwind displacement, increased number of stops of shorter duration and antennal reaching observed during exposure to the chemostimuli all demonstrate the appetite of the bugs. Antennal reaching in the air stream during stops may, under the open loop conditions on the servosphere, represent yet another behavioural adaptation: as foreleg extension accompanies antennal reaching this could also be interpreted as an attempt by the bug to reach on to a close by host. Antennal tapping in the presence of the chemostimuli is a less time consuming alternative to the antennal cleaning behaviour observed in the air stream alone, thus improving the efficiency of the bug's guiding response. Furthermore, alternation between walks and pauses where antennal scanning occurs has been referred to as saltatory search (O'Brien et al. 1990), i.e. a searching cycle that functions to improve efficiency of the foraging tactic of a species like a triatomine that requires an isolated resource. Bilateral antennal sensing of the air current containing the chemostimuli during each stop plays a crucial role in the directed walk towards the stimulus source.

Behaviours in the air stream after stimulus removal

The more crosswind and tortuous walks performed by the bugs after treatment removal from the air stream represent an effort by the bugs to regain contact with the chemostimuli. Crosswind displacement on loss of the chemostimuli has already been observed for triatomines (Taneja and Guerin 1995) and for flying moths (Kennedy and Marsh 1974; David et al. 1983). Geometrical analysis of casting behaviours (Dusenbery 1992) has shown that crosswind walking increases the probability of encounter with a chemostimulus over upwind,

downwind or a random search strategy. In addition, the relative high speed, more frequent and shorter stops and antennal reaching and tapping observed in the presence of the chemostimuli persist after cessation of stimulation.

The observed appetite behaviour of *Rhodnius* in the presence of NH₃ plus CO₂, two simple and ubiquitous molecules, is interesting from a biological and epidemiological point of view. Rose (1998) working under semi-natural conditions has shown that ammonia alone can activate *T. infestans* (i.e. induce exit from a refuge) but is not enough to recruit the bugs to the source. Furthermore, this author found that even though CO₂ alone under the same conditions could attract *T. infestans* to the source, addition of NH₃ in the air produces an effect greater than the sum of the individual effects of these two molecules in terms of activation and recruitment. The behavioural effect of these molecules on triatomines is not surprising as both are central catabolic products associated with respiration and excretion in animals. As such, the mixture of CO₂ and NH₃ may prove practical in developing a lure to monitor presence of these disease vectors. Both to the experiments of Rose (1998) and those detailed here lend us to conclude that *Rhodnius* may use the combination of CO₂ plus NH₃ to find either a host or a refuge.

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Chapter III

Volatiles Present in Vertebrate Wastes Attract Triatomines: An Evolutionary Explanation

“Much contemporary study of behaviour consists of developing and testing predictive hypothesis about the adaptive value of behaviours in order to understand how they are selectively advantageous.”

- Douglas J. Futuyma

Abstract

Triatomines are nest-living reduviids that require vertebrate blood to complete their life cycle. Nesting vertebrates accumulate plenty of waste products in or near their dwellings and triatomines might be able to use this rich source of odours to find such refuge. Here we recount how the domestic triatomine species *Triatoma infestans* and *Rhodnius prolixus* and the sylvatic *Panstrongylus geniculatus* respond to volatiles from chicken faeces and human urine samples, odours that may be implicated in marking the presence of a vertebrate host occupying a nest. Electroantennogramms revealed ammonia to be the compound to which *P. geniculatus* adults showed the highest response among the chemicals tested. Ethylamine and dimethylamine delivered at ~100 ppb to the apex of a servosphere proved highly attractive to the three species, increasing the target vector and the upwind displacement of the fifth-instar nymphs. Activation behaviours were also observed, i.e. *P. geniculatus* nymphs walked faster and farther when these two short chain amines were present in the air stream compared to the air stream alone. Isobutylamine and hexylamine failed to elicit attraction but induced the activation of *R. prolixus* nymphs: isobutylamine induced a median increase of 16.56 mm/s in speed and a median increase of 17.33 cm in displacement, and hexylamine induced an increase of 13.93 mm/s in speed and an increase of 43.29 cm in displacement. All of these behaviourally active amines are known to be present in vertebrate urine and faeces. We conclude that these and other compounds present near the host nest represent a fundamental resource that serves triatomines to localize such habitat. We believe this adaptation was probably inherited from their predator reduviid ancestors who also used the vertebrate dwelling as a source of arthropod prey and refuge.

Keywords: Behaviour, sensory ecology, faeces, urine, wastes, malodorous, dimethylamine, ethylamine, scent marking, Triatominae, *Panstrongylus geniculatus*.

INTRODUCTION

It is well accepted that all Triatominae species (Heteroptera, Reduviidae) are associated with nidicolous hosts (see Chapter I) from which they take blood to complete their life cycle. Of the nearly 130 extant species of this subfamily very few (only 5%, Dujardin *et al.* 2000, Silveira 2000) are associated with humans (i.e. domestic species), a prerequisite to being vectors of Chagas disease. The rest are associated with wild hosts and are termed sylvatic species. The three genera of greatest epidemiological significance are *Triatoma*, *Rhodnius* and *Panstrongylus* (Bastien 1998). *R. prolixus* and *T. infestans* are well adapted to live in houses and have a wide distribution; when sylvatic, these species are associated with arboreal vertebrates and guinea pigs, respectively (Carcavallo *et al.* 2000). *P. geniculatus* has previously been noted as exclusively sylvatic with little importance in transmitting Chagas disease, usually living in humid forests in diverse vertebrate nesting places and especially in armadillo (Dasypodidae) burrows (Lent & Wigodzinisky 1979). Currently there are increasing reports of this species invading domestic and peridomestic habitats over a vast area: Venezuela (Pifano 1986, Reyes-Lugo & Rodriguez-Acosta 2000, Feliciangeli *et al.* 2004, Carrasco *et al.* 2005), Colombia (Wolff & Castillo 2000), Brazil (Valente *et al.* 1998, Valente 1999, Luitgards-Moura *et al.* 2005), Perú (Cáceres *et al.* 2002, Cuba *et al.* 2002), Ecuador (Amunarriz *et al.* 1991, Aguilar *et al.* 1999) and Argentina (Damborsky *et al.* 2001).

Domestic and sylvatic triatomines spend most of the photophase hiding near a vertebrate's resting place and often search for blood at night when the host is immobile or off guard and when environmental conditions are most favorable (Heger *et al.* 2006). In nests (*sensu lato*) triatomines can easily find their host because of its proximity. As orientation cues they can use the high quantities of heat and carbon dioxide that emanate from the nearby vertebrate (Schmitz *et al.* 2000, Taneja & Guerin 1995). Clearly, vertebrate heat transmission that decreases abruptly with distance (Dusenbery 1992) and carbon dioxide that has little specificity -produced by animals, plants and microorganisms - are not useful on their own for bugs to search for a distant inhabited nest. When for any reason the host is not available nearby or the colony size exceeds the resources available, the bugs are forced to find a way to new sources of food and refuge, i.e. active dispersion (Dujardin *et al.* 2000). It is known that these insects will disperse actively by walking or flying considerable distances (Cecere *et al.* 2004) when climatic conditions are favorable and will do so primarily motivated by hunger (Gomez-Nuñez 1969, Galvão *et al.* 2001, Vázquez-Prokopec *et al.* 2004). The role that volatile chemical cues could play in triatomine invasion of sylvatic vertebrate dwellings and human abodes is not completely understood.

In order to establish which odours can assist *R. prolixus*, *T. infestans* and *P. geniculatus* in seeking a stable niche, here we explore current knowledge of the chemical and evolutionary ecology of these bugs and their vertebrate hosts. Based on the presumption that odours present near nesting vertebrate refugia play a general role in triatomine localization of such resources, behavioural and electrophysiological bug responses to odours present in vertebrate wastes (Hobbs *et al.* 1995, Mackie *et al.* 1998, Wiles *et al.* 2001, Le *et al.* 2005), scent gland secretions (e.g. axilla sweat, Natsch *et al.* 2003) and other materials abundant in nests (e.g. feathers, Allan *et al.* 2006), were tested on a servosphere and by electroantennogram recordings. As terpenes are also occasionally found in herbivore wastes (Sato *et al.* 2001), their role was also investigated. In this chapter we concentrated our study on amines which, among the range of compounds

tested, elicited the strongest behavioural responses and have previously been reported to evoke responses from olfactory receptor cells in the grooved peg sensillum on triatomine antennae (Taneja & Guerin 1997, Diehl *et al.* 2003).

METHODS

Insects

R. prolixus and *T. infestans* colonies were fed once per month and *P. geniculatus* colony two or three times per month on chickens, as the latter species can only starve for a shorter time than the other two (Cabello & Galindez 1998). The three colonies were maintained in climate chambers: *R. prolixus* and *T. infestans* at 80 % RH, 22°C, 12:12 LL:DD and *P. geniculatus* at 90 % RH at the same temperature and light cycle. *R. prolixus* and *T. infestans* colonies are 20 years old (originating from the Swiss Tropical Institute, Basel). The *P. geniculatus* colony originated from specimens collected in Montebello (Wolff *et al.* 2001), Amalfi Municipality, Department of Antioquia, Colombia (6°55'58''N; 75°05'30'' W, 18-24 °C; annual rain 1000-2000 mm) in May 2003 and reared at the University of Neuchâtel laboratory since 2004. Fifth-instar *R. prolixus* and *T. infestans* nymphs were starved for 8-12 weeks and fifth-instar *P. geniculatus* nymphs were starved for 4-6 weeks after moulting to standardize hunger level for behaviour experiments. *P. geniculatus* adults were starved for less than one week and kept near 99 % RH at least three days before electrophysiology experiments.

Behavioural recordings

The method of recording the behavioural responses of walking triatomine nymphs using a servosphere (Kramer 1976) and two infrared-sensitive video cameras has been described earlier (Taneja & Guerin 1995, Otálora-Luna *et al.* 2004). The servosphere allows a walking untethered triatomine nymph to walk unimpeded in all directions at the apex of a sphere (50 cm diam) in an experimental set up where sensory modalities other than the test chemostimuli in an air current are excluded. Displacements of the sphere caused by the moving animal are supplied to a computer by pulse-generators mounted on the sphere equator allowing the reconstruction of the tracks described by the bugs. This apparatus is an open loop set up, i.e. it records the behaviours a bug employs to approach a goal but without reaching it. A schematic representation of the servosphere is presented by McMahon & Guerin (2000) and Guerin *et al.* (2000). In addition, video records are made of the bugs' legs, antennae and general body movements throughout each experiment and recorded on video cassettes (Panasonic VCR AG-7350, Japan). The bug was placed on the servosphere with forceps. Experiments started when the following behaviours were no longer observed: a) the bug performs appetite behaviours such as *reaching* up in the air stream or intense *tapping* (see below), b) the bug lowered its body until it laid on the sphere (akinesis) or c) the bug did not move with its body laying on one side (pretending to be dead). This assured the animal was not influenced by the recent manipulation or the experimenter's recent proximity to the insect. The responses of bugs were recorded in three consecutive 2-min periods: in the air stream alone (C1), the air stream plus the chemostimulus (T) and the air stream after removal of the chemostimulus (C2). Behavioural recordings were performed in the dark during the early and late scotophase as

most triatomine species are more active during the night. Bugs that walked consistently upwind during the initial period in the air stream were discarded.

Behaviour analysis

Criteria used to smoothen track recordings and for automatically identifying when the bug stops walking is described in [Otálora-Luna *et al.* \(2004\)](#). Several parameters were calculated for each period of each track (C1, T and C2); mean direction of the track (Φ), path straightness r (mean vector length, [Batschelet 1981](#)), target vector¹² (TV, calculated by multiplying the cosine of the mean direction of the track Φ by the path straightness r , [Jones 1977](#)), upwind displacement (UD, i.e. walking distance in a cone 60° either side of due upwind), median speed (V), overall displacement (D), number of stops (NS) and median turning velocity (TU, [Bell 1991](#)). All parameters except Φ were compared using the two-tailed Wilcoxon paired signed rank test. Comparisons between treatments were made using the two-tailed Wilcoxon non-paired signed rank test. Rao's spacing test and V-test ([Batschelet 1981](#)) were applied to determine whether the distributions of Φ in a given period differed from uniformity. In periods where a particular orientation angle was expected, the data was subjected to a Rayleigh test with specified mean direction ([Batschelet 1981](#)).

Other behavioural observations described previously ([Otálora-Luna *et al.* 2004](#)) were catalogued as follows: *antennal reaching* was noted when a bug lifted the anterior part of its body (ca. 40°, with respect to the ground) through extension of their forelegs in order to reach higher with the antennae held up (ca. 60°, with respect to the ground) and flicked them to scan the air, and *antennal tapping* was noted when a bug tapped the right or left antennal flagellum with the corresponding foreleg. Antennal tapping was counted 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 noted when a bug cleaned antennae, tarsi or proboscis. Increased target vector and increased upwind displacement (IUD) during the test compared to control period (C1) were used as *attraction indexes*. Increases in speed, walking distance, number of stops, antennal reaching and antennal tapping, and a decrease in cleaning behaviours compared to C1 were catalogued as *activation indexes*. Video-recorded behaviours were compared using the two-tailed Wilcoxon paired signed rank test. Track analyses were performed using in-house developed software (sLocTrack) and statistical analysis were performed using R (version 2.2–1, Vienna, Austria; [Ihaka & Gentleman 1996](#)) running under Linux.

Biological samples collection and extractions

Chicken faeces and feathers were obtained from chickens fed on commercially available food and supplemented occasionally with vegetables. Feathers were pulled with latex gloves from two one-year-old chickens and faeces were collected from a recently cleaned chicken cage. Urine and axilla sweat were obtained from an adult male who did not have a diet particularly rich in fish or other sea-products (people who have a diet rich in sea-products excrete more amines than normal, [Zhang *et al.* 1999](#)). Fresh axilla sweat was collected on cotton held by surgical tape on an adult male armpit for 1 hour during

¹² This variable incorporates both the mean direction of a path (Φ) and the consistency with which a particular direction is maintained (r).

exercise. Human breath from an adult male was blown into a 1 l gas-wash bottle for 30 s and the bottle was left to equilibrate for at least 20 min before the start of a test to avoid the influence of heat. *Pinus sylvestris* essential was obtained from Aromax[®], Budapest.

CO₂ released by fresh biological samples was measured at the apex of the sphere by an electronic infra-red gas analyzer (LI-COR LI-820, USA, accuracy ±3%, signal averaging 1s, flow 1.5 l/min) connected to a computer. Mean values over 10 s intervals for 30 min were compared with a control that consisted of a gas-wash bottle with 1 ml of nanopure water. CO₂ released above ambient levels (mean 401.03, SD 1,38 ppm) by human breath (mean increase 1130.10, SD 4.90 ppm) and chicken faeces (mean increase 7.60, SD 3.25 ppm) was significantly higher compared to ambient (p<0.01). The CO₂ measured on the apex of the sphere produced by axilla sweat (mean increase 2.98, SD 2.20 ppm) and feathers (mean increase 0.11, SD 3.17 ppm) was not above ambient levels (p>0.05, *t*-test). NH₃ released by fresh organic samples was measured at the apex of the sphere by an electrochemical sensor (DrägerSensor Pac III S, Lübeck, Germany, accuracy ± 1%, flow 0.5 l/min). When the ammonia concentration was lower than 1 ppm (lowest sensitivity level of the apparatus) the measurement was made at the exit of the gas-wash bottle in which the sample had been held to allow the ammonia level to accumulate. Subsequently the measured value was divided by 25, the dilution factor that arose from mixing the air transporting the vapours from the gas-wash bottle into the humidified air stream flowing over the sphere apex (see below). Mean values over 10 s intervals for 1 min were compared with controls. Mean concentrations of ammonia on the apex of the sphere were 123.11 ppb (SD 2.26) for diluted human breath, 95.01 ppb (SD 3.00) for chicken faeces, 81.77 ppb (SD 5.21) for axilla sweat. No ammonia was detected from feathers and controls.

To avoid the confounding effect of having CO₂ released from the biological samples, extractions of chicken faeces and of human urine and axilla sweat were performed. Chicken faeces (10 g) were acidified with a H₂SO₄ solution (20%) to reach pH 2, incubated for 10 minutes and centrifuged at 1'000 g for 10 min at -4° C. The supernatant was brought to pH 10 by addition of a NaOH (30%) solution and mixed with dichloromethane (DCM). Amines and neutral molecules were extracted in the DCM fraction, washed with a saturated NaCl solution and conserved in a glass ampoule at -20° C. Volatile components of urine (200 ml) were entrained immediately after collection on to a porous polymer (500 mg of Soxhlet extracted Porapak QTM, Waters, 50-80 mesh, packed into a 6 cm long, 6.23 diam glass tube and stoppered with glass wool plugs). The polymer was preconditioned for 1.5 h with pure N₂ at 180° C. Pure N₂ was bubbled through the urine contained in a gas-wash bottle to the porous polymer cartridge (150 ml/ min) for 5 hours. The entrained volatiles were eluted from the polymer with DCM and the first drops (400 µl) were transferred to a glass ampoule and conserved at -20°C. Human axilla sweat (5 g) was absorbed on cotton (2.5 g) as described above. The wet cotton was packed into an 11 cm long and 2.5 cm diam glass tube. The entrained volatiles were eluted with DCM (1 ml) and by compressing the cotton with a glass rod. The first few drops (200 µl) were transferred to a glass ampoule and conserved at -20° C. Neither ammonia nor carbon dioxide was detected in chicken faeces and urine extracts.

Chemostimuli and their delivery for behavioural recordings

An automated stimulus delivery system was used as has been described previously (Taneja & Guerin 1995, 1997). Briefly, clean air passing through 1 l gas-wash bottles (160 ml/min) served to transport the vapours to a humidified clean air stream (15 cm/s, 4 l/min, 23-25° C, 40 and 60% RH) flowing over the bug on the apex of the sphere. The air injected from the gas-wash bottles was diluted 25-fold in the main air stream. Gas-wash bottles were washed with RBS (35-concentrate, Fluka) diluted in demineralized warm water (50°C, 15 ml/l) for at least 3 h prior to use.

After collection, biological samples were placed directly in gas-wash bottles using gloves or spatula as necessary. Controls consisted of bottles with 2.5 g of cotton wetted with 1 ml of nanopure water. Dilutions of the synthetic compounds tested were made in DCM, paraffin oil (Merck, spectroscopy grade) or nanopure water; extractions of biological samples were not diluted. These solutions were applied (200 µl) to filter paper discs (120 mm diameter) and placed at the bottom of a gas-wash bottle. The DCM was left to evaporate for 6 s before placing the filter paper in the bottle. Controls consisted of bottles loaded with the same amount of solvent on filter paper. Gas-wash bottles were then left to equilibrate for at least 10 min before presenting the chemostimuli to the bugs. The filter papers were replaced after 2-3 tests.

The following compounds¹³ were delivered separately: dimethyltrisulphide (DMTS), acetone, 1-octen-3-ol, myrcene, linalool, 4-methyl pentanoic acid (4MPA), ammonia, methylamine, ethylamine, dimethylamine, trimethylamine, isobutylamine, isopentylamine, hexylamine, skatole, phenol, p-cresol and indole. The following mixtures were also tested: p-cresol + phenol, p-cresol + skatole, p-cresol + skatole + phenol, 1-octen-3-ol + isobutyric acid, 4MPA + DMTS, 4MPA + nonanal, 4MPA + nonanal + β -caryophyllene + skatole and 4MPA + butyric acid + β -caryophyllene + skatole. A dispenser used in a mosquito lure (BG-Sentinel trap, Biogents, University of Regensburg, Germany) that releases ammonia from ammonium bicarbonate in a plastic matrix (at 5 ppm on the apex of the sphere) and hexanoic acid from a polyethylene tube (3 mm diam, 100 mm long) + L-lactic acid from a separate silicone tube (3 mm diam, 1000 mm long), the same dispenser without the ammonia, and a dispenser used as a tsetse fly lure (POCA dispenser, Chemical Research Center, Hungarian Academy of Science, Hungary) that diffuses 3-n-propylphenol, 1-octen-3-ol and p-cresol were also tested (IAEA-TECDOC 2003). Acetone was added to the POCA dispenser by placing 1 µg diluted in 200 µl of paraffin oil on filter paper. The highly volatile compounds DMTS and acetone were diluted in paraffin oil because this solvent prolongs the release over time by roughly 5-fold compared with DCM. NH₃ (NH₄OH), methylamine, ethylamine (EA), dimethylamine (DMA) and trimethylamine were diluted in water until reaching 150 mM. Log solutions of DMA were tested too. The concentration of NH₃, measured as mentioned above, was 200 ppb for 200 µl of diluted NH₄OH (150mM) placed on filter paper and 5000 ppb for the BG-Sentinel trap dispenser on the apex of the sphere. The rest of chemicals tested, including all mixtures, were diluted in DCM. Most solvents and synthetic chemicals used in this investigation were all at least 97% pure. NH₄OH, methylamine, dimethylamine, ethylamine and trimethylamine were 25%, 40%, 40%, 70% and 45% solutions respectively.

¹³ Nomenclature of chemicals in this thesis follows the employed by NIST (<http://webbook.nist.gov/chemistry/>).

Electroantennograms

Electroantennograms from *P. geniculatus* adults were recorded as described in [Taneja & Guerin \(1997\)](#). After anaesthetising the bug with CO₂ it was mounted dorsally on a glass slide. The head and antennae were laid out on a second thin (1 mm) glass coverslip with double-side sticky tape. It was necessary to use a black background (insulating tape) as the antennal flagellar segments are transparent under the incident cold-light source employed. Both antennae were carefully embedded in the tape glue using a human eye lash held in a wooden toothpick, except for the tip of the distal flagellum of one antenna that was elevated at roughly 60° and held up by a piece of plasticine in the direction to the recording electrode (see below). The slide with the bug was mounted under a microscope (maximum 800 times amplification, WILD M3Z, Heerbrug Switzerland) in such a way that the antenna was bathed in a charcoal filtered air-stream (90-100% RH, 23±2° C) delivered at 1 m/s via a glass water-jacketed tube (7 mm i.d.) whose outlet was 1 cm from the preparation. A chloridised silver electrode in a drawn-out glass capillary (tip diam. ca. 2 µm) filled with KCL (50 mM) solution was inserted between the scape and the second proximal segment as reference electrode. A second silver electrode in another drawn-out glass capillary (tip diam. ca 20 µm) covered roughly 1 mm of the tip of the elevated flagellum and was used for recordings. Instruments used for recording (Syntech, The Netherlands) are described by [Taneja & Guerin \(1997\)](#). Micromanipulators (Leitz, Switzerland) permitted accurate positioning of the electrodes.

Chemostimuli and stimulus delivery for electrophysiological recordings

The stimulation system is described by [Taneja & Guerin \(1997\)](#) and [Guerenstein & Guerin \(2001\)](#). Different doses of the following synthetic chemicals (Merck and Sigma, analytical grade) were tested to study the response profiles of receptor cells located on the antennae: DMTS, skatole, indole, phenol, p-cresol, isobutyric acid, heptanoic acid, 4-methylpentanoic acid, α-pinene, linalool, β-caryophyllene, isoprene, nonanal, trans-2-hexenal, 1-octen-3-ol and ammonia. All compounds were diluted in DCM except for ammonia that was diluted in water from a 25% solution. DCM and water served as solvent blanks. 10 µl of each solution were deposited on a small filter paper strip (0.8 x 3 cm) that was inserted into the stimulus cartridge syringe after solvent evaporation. Taking into account the amount delivered from the stimulus syringe, the dilution in the humidified air stream and the surface area of the antenna, the relative amount of synthetic reaching the antenna has been estimated and corresponds approximately to 1/10000 of the amount placed on the filter paper. The following solutions of NH₄OH were made: 15 mM, 150 mM, 1.5 M, 15 M. The ammonia concentration passing over the antennae was calculated using the calibration curve of [Taneja & Guerin \(1997\)](#).

Gas chromatography coupled mass spectrometry (GC-MS)

Constituents of chicken feathers, chicken faeces and chicken faeces basic fraction were identified by gas chromatography (HP 5890 Series II) linked mass spectrometry (HP 5971 A) with He at 30 cm sec⁻¹ as carrier gas ([Steullet & Guerin 1994](#)). The GC was equipped with a thermal desorption unit (TDS 2, Gerstel[®], Germany) and a cooled split/splitless injection system (CIS 3, Gerstel[®], Germany) installed on the head of a high-resolution capillary column.

Fresh feathers, guinea pig and chicken faeces volatiles initially trapped on a Tenax™ GR cartridge were thermally desorbed in splitless mode via the TDS 2 unit programmed to increase from 30°C to 220°C at a rate of 30°C/min. A heated (240°C) transfer line evacuated the volatiles to the CIS 3 cooled to -80°C with liquid nitrogen where volatiles were cryotrapped. At the end of the TDS 2 transfer the CIS was subjected to ballistic increase (12°C sec⁻¹) in temperature to reach 240°C to transfer the volatiles onto the high resolution capillary column. The dichloromethane extract (1 µl, see above) of the basic fraction of the chicken faeces was injected via the CIS 3 unit in splitless mode programmed to increase from 30° to 210° at a rate of 12°C/s. Chemical identity of biologically separated compounds was determined using mass spectrum library matches (Wiley 7th Nist 98).

Two types of columns were used: FFAP, L 30 m, ID 0.25 mm, film thickness 0.25 µm (BGB Analytik, Switzerland) for the analyses of fresh feathers and faeces volatiles and OPTIMA-5-AMINE, L 30 m, ID 0.25 mm, film thickness 1 µm (Macherey-Nagel, Switzerland) for the analysis of the faeces basic fraction. The oven temperature programmes were set at 40°C for 5 min with a linear increase to 230°C at 5°C/min for the polar FFAP column, and 40°C for 5 min with a linear increase to 280°C at 5°C/min, for the OPTIMA-5-AMINE column.

RESULTS

Behaviours of the three triatomine species in clean air

P. geniculatus walking behaviours on the servosphere did not differ greatly from *R. prolixus* and *T. infestans* nymphs. The former walked in relatively straight bouts (median 0.83, IQR* 0.022, n=823 bouts) punctuated by stops of variable duration (median 9.2 s, IQR 7.9) as has been reported before for the other two species (Taneja & Guerin 1995, 1997, Otálora-Luna *et al.* 2004). The median length of a walking bout was 85.70 mm (IQR 218.01). The median instantaneous velocity (9.81 mm/s, IQR 10.21, n=31 bugs) and maximum velocity (29.57 mm/s) during walking 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 did not differ much from *T. infestans* (median 11.13, IQR 18.63; maximum 29.60, IQR, n=28, p>0.05). Triatomines walked more crosswind and downwind in the clean air as was evidenced by their mean angles (p<0.01 Rao's spacing test).

P. geniculatus and *T. infestans* performed the same antennal behaviours as have been described for *R. prolixus* (Otálora-Luna *et al.* 2004). Apart of antenna cleaning, the following cleaning behaviours, performed with less frequency (<0.02%, n= 60), were also observed: cleaning of the proboscis, cleaning of the anus and cleaning of a left or right foreleg tarsus. Cleaning of the proboscis was performed only rarely and after the bug had cleaned antennae and as in antennal cleaning the bug lifts and joins both foreleg tarsi over the extended proboscis to run them distally along it. Cleaning the anus was performed by dragging it along the sphere surface in semicircular movements. Foreleg tarsus cleaning was performed by rubbing it with the second leg ipsilateral tarsus. *P. geniculatus* was observed to irregularly perform a behaviour never observed in either *R. prolixus* and *T. infestans*: during stops one of each of ten bugs extended one or both hind legs

* IQR: interquartile range, i.e. the difference between the 75th and 25th percentiles.

simultaneously and subsequently bended them within after rubbing the sphere with tarsi, a behaviour referred here as *hind leg flexing*.

Behavioural responses to vertebrate odours

Appetence behaviours, including attraction and activation, similar to those elicited by host metabolites on the servosphere (Taneja & Guerin 1995, 1997, Guerenstein & Guerin 2001, Otálora-Luna et al 2004) were observed in this study when chicken faeces, urine and other biological substrates were tested in the same apparatus (Table I).

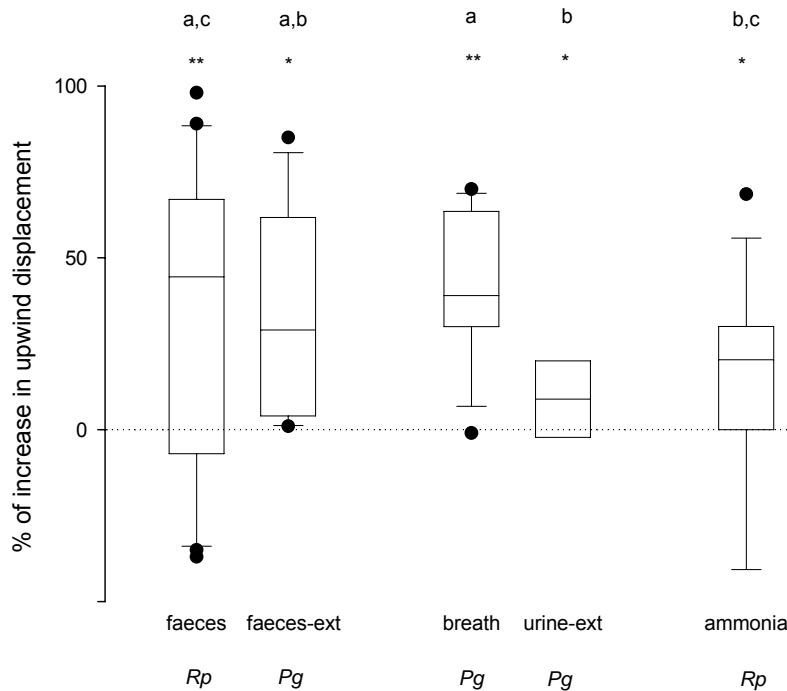


Fig 1. Responses of *P. geniculatus* (*Pg*) and *R. prolixus* (*Rp*) fifth-instar nymphs to fresh chicken faeces (*Rp*), a basic chicken faeces extract (*Pg*), diluted human breath (*Pg*), a human urine extract (*Pg*) and ammonia (*Rp*) on a servosphere. Responses are presented as the % increase in upwind displacement in a cone 60° either side of due upwind in the test period compared to that in the previous control period. Upwind displacement 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 the faeces fraction). Treatments sharing the same letter are not significantly different (Wilcoxon no 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.

Fresh chicken faeces, that produced CO₂ and NH₃ in low concentrations (7.60 ppm CO₂ above ambient and 95.01 ppb NH₃)* proved highly attractive to *R. prolixus* nymphs. The median target vector (TV) shifted from -0.27 during the control period to 0.56 during the 2-min stimulation period (Table I), and the median upwind displacement increased to 44 % during the test period compared to the control period (IUD, Table I, Fig 1). Volatiles emanating from chicken faeces also induced an increase of 5.30 mm/s in the median speed and an increase of 23.92 cm in the median displacement (Table I) compared to the air stream alone. A selective extraction of basic and neutral products from chicken faeces in dichloromethane also proved attractive to *P. geniculatus* nymphs (Fig 1). In this case, the bugs walked more upwind during the stimulus delivery (TV in test 0.68, IUD 29.46 %) but they did not walk faster or farther than in the control period (Table I).

* over the apex of the sphere

Diluted breath (with 1130.10 ppm CO₂ above ambient and 123.11 ppb NH₃), used as a positive control, elicited attraction and activation behaviours from *P. geniculatus* nymphs as strong as those elicited by fresh chicken faeces from *R. prolixus* nymphs (Fig 1, Table I). *P. geniculatus* nymphs walked more upwind during stimulation with diluted breath than in the air stream alone (TV in test 0.75, IUD 39.37%). Diluted breath also induced an increase of the median speed by 9.81 mm/s and the median displacement by 68.51 cm (Table I) compared to the control period.

Table I. Responses of *R. prolixus* (*Rp*) and *P. geniculatus* (*Pg*) fifth-instar nymphs on the servosphere to different biological substrates

Compound	sp.	Bugs tested	Amount at source	Target vector		Change from control to test period		
				control	test	IUD (%)	speed (mm/s)	displacement (cm)
chicken faeces	<i>Rp</i>	18	2 g	-0.27	+ 0.56 **	44.00 **	5.30 *	23.92 *
	<i>Pg</i>	12	200 µl	+ 0.24	+ 0.68 **	29.46 *	2.86	9.06
diluted breath	<i>Pg</i>	8	400 ml	+ 0.15	+ 0.75 **	39.37 **	9.81 *	68.51 **
	<i>Pg</i>	10	200 µl	+ 0.01	+ 0.57 *	12.75 *	8.66 *	8.13
feathers	<i>Rp</i>	17	5 g	- 0.57	+ 0.20 *	14.08 *	3.07	44.47 *
	<i>Rp</i>	25	2 g	- 0.12	+ 0.42 *	15.19 *	12.55 *	59.96 *
axilla sweat	<i>Rp</i>	13	200 µl	- 0.35	+ 0.52 *	13.25 *	5.72 *	95.72 *
	<i>Pinus sylvestris</i> essential oil	<i>Rp</i>	10	200µl	- 0.18	-0.06	0.21	- 13.34 **

Attraction and activation are represented by median values. IUD is the increase in upwind displacement; Wilcoxon paired test, * P<0.005, ** P<0.01.

Table II. Responses of *R. prolixus* (*Rp*), *T. infestans* (*Ti*) and *P. geniculatus* (*Pg*) fifth-instar nymphs on the servosphere to ammonia and aliphatic amines

Compound	sp.	Bugs tested	Amount at source	Target vector		Change from control to test period		
				control	test	IUD (%)	speed (mm/s)	displacement (cm)
NH ₃	<i>Rp</i>	14	500 µg	-0.34	+ 0.12 *	21.01 *	7.05 *	52.29 *
ethylamine	<i>Rp</i>	13	100 µg	-0.22	+ 0.21 *	17.68 *	0.98	42.13 *
dimethylamine	<i>Rp</i>	12	100 µg	-0.44	+ 0.25 *	26.66 *	6.50 *	23.72 *
	<i>Rp</i>	14	10 µg	-0.27	-0.41	6.23	9.12	0.85
	<i>Rp</i>	13	1 µg	-0.19	-0.13	0.00	-0.13	-15.00
methylamine	<i>Rp</i>	12	100 µg	-0.32	+ 0.15	7.25	1.33	-9.35
trimethylamine	<i>Rp</i>	12	100 µg	+ 0.07	-0.13	8.26	-5.68	-3.28
isobutylamine	<i>Rp</i>	11	100 µg	+ 0.09	+ 0.16	12.02	16.56 *	17.33 *
isopentylamine	<i>Rp</i>	10	100 µg	+ 0.09	+ 0.15	11.21	-11.26 *	-7.14
hexylamine	<i>Rp</i>	10	100 µg	+ 0.08	-0.14	0.21	13.93 *	43.29 *
ethylamine	<i>Ti</i>	14	100 µg	+ 0.14	+ 0.32 *	16.02 *	0.14	104.54 *
dimethylamine	<i>Ti</i>	15	100 µg	+ 0.16	+ 0.29 *	22.25 *	2.52	56.94 *
ethylamine	<i>Pg</i>	12	100 µg	+ 0.15	+ 0.30 **	41.57 **	7.86 *	52.66 *
dimethylamine	<i>Pg</i>	18	100 µg	-0.05	+ 0.36 *	32.11 **	5.77 *	48.55 *

Attraction and activation are represented by median values. IUD is the increase in upwind displacement; Wilcoxon paired test, * P<0.005, ** P<0.01.

The urine extract proved less attractive to *P. geniculatus* nymphs (TV in test 0.57, IUD 12.75%) compared to fresh faeces and diluted breath (Fig 1). On the other hand, the urine extract elicited a higher level of activation than the faeces extract, the former producing an increase of 8.66 mm/s in median speed whereas the latter did not produce an increase in speed (Table I). Ammonia alone at 500 ppb proved less attractive (TV in test 0.12, IUD 21.01%) than breath when tested on *R. prolixus* nymphs (Fig 1), but still elicited activation of the bugs increasing the median speed by 7.05 mm/s and the median displacement by 52.29 cm (Table II).

Volatiles present in chicken feathers, axilla sweat and the axilla sweat extract in dichloromethane, also evoked significant attraction and activation responses by *R. prolixus* during the 2-min stimulation (for details see Table I). On the other hand, essential oil of *P. sylvestris* did not elicit attraction or activation behaviours on *R. prolixus* nymphs. This essential decreased walking speed by -13.34 mm/s and displacement by -37.20 cm during the test period compared to the control period. *P. sylvestris* essential did not increase antennal reaching and antennal tapping during the test compared to the previous control and did not increase the distance walked in a downwind cone 60° either side of due downwind (TV in test 0.08, IUD 15.01%, $p>0.05$) compared to control.

Behavioural responses to aliphatic amines

Among the seven amines tested, ethylamine (EA) and dimethylamine (DMA) caused the strongest responses on *R. prolixus*, *T. infestans* and *P. geniculatus* fifth-instar nymphs (Table II). EA and DMA proved highly attractive, increasing the target vector and the relative upwind displacement of the three species (Fig. 2). *P. geniculatus* nymphs walked faster and farther when these two amines were present in the air stream compared to the air stream alone (Table II) and this was confirmed with *R. prolixus* and *T. infestans*, except for *R. prolixus* that did not walk faster in presence of EA and *T. infestans* that did not walk faster in presence of EA and DMA. Lower concentrations of DMA did not elicit responses from *R. prolixus* (Table II) except for an increase in reaching and tapping behaviours during stimulation.

Methylamine, trimethylamine, isobutylamine, isopentylamine and hexylamine failed to elicit attraction on *R. prolixus* nymphs (Table II). Nevertheless, isobutylamine and hexylamine induced activation on this species: isobutylamine induced an increase of 16.56 mm/s in speed and an increase of 17.33 cm in displacement, and hexylamine induced an increase of 13.93 mm/s in speed and an increase of 43.29 cm in displacement relative to control. Increase in reaching and tapping and decrease in cleaning behaviours were also observed for both amines. On the other hand, isopentylamine induced a decrease of -11.26 mm/s in speed on *R. prolixus* (Table II).

Behavioural response to other compounds

None of the aromatic compounds tested elicited attraction responses in triatomines but skatole at 100 ng and phenol at 100 µg (source dose) induced a decrease in displacement (Table III). 4MPA at 1 mg – 10 mg increased the target vector and upwind displacement as well as the speed and displacement of *R. prolixus* (Table IV). DMTS at 1 µg increased the upwind displacement, but failed to increase the target vector, speed or displacement of

R. prolixus. The blend of 4MPA + nonanal + β -caryophyllene + skatole (1 μ g:10ng:10ng:10pg) increased target vector but did not increase other attraction or activation parameters (Table V) except for some tapping of antennae. The blend of 4MPA + DMTS (100 μ g:100ng) increased speed. The blend of 4MPA + butanoic acid + β -caryophyllene + skatole (1 μ g:1 μ g:1ng:100pg) also increased speed as well as reaching and tapping. On the other hand the blend of 4MPA + nonanal (100 μ g:10 μ g) decreased displacement on *R. prolixus* and did not affect other behaviours.

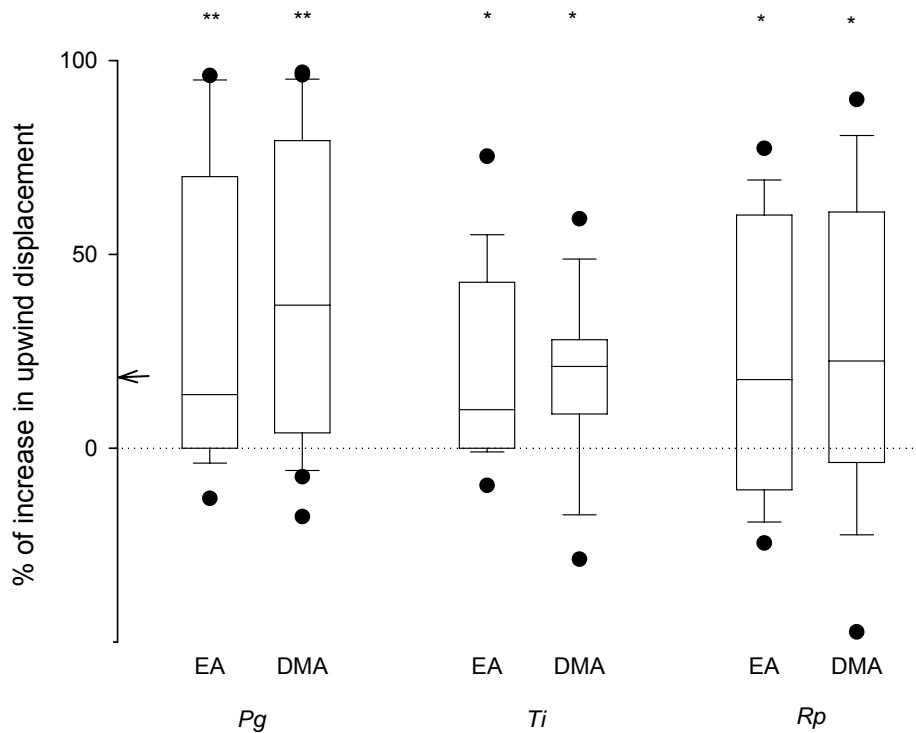


Fig 2. Responses of fifth-instar *P. geniculatus* (*Pg*), *T. infestans* (*Ti*) and *R. prolixus* (*Rp*) nymphs to ethylamine (EA) and dimethylamine (DMA) on a servosphere. Responses are presented as the % increase in upwind displacement in a cone 60° either side of due upwind in the test period compared to that in the previous control period. Upwind displacement increased during exposure of all three triatomine *spp.* to ethylamine and dimethylamine at 100 μ g source dose (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 chicken faeces basic fraction that contains these amines. There was no significant difference between treatments (Wilcoxon no paired test, $P > 0.05$). For explanation of boxplots see legend for Fig. 1

The blend of hexanoic acid, L-lactic acid and ammonia diffusing from a dispenser (see Methods) activated and attracted *R. prolixus* nymphs (Table V). But, when the ammonia dispenser that released 5 ppm ammonia to the apex of the sphere was taken away, no attraction was observed, on the contrary, bugs walked less than in control (Table V).

Electrophysiology

The electroantennogram dose response curves recorded to increasing doses of ammonia, isobutyric acid, 4-methyl pentanoic acid and skatole, reported to be present in vertebrate wastes and nonanal found in feathers (Fig 3) revealed ammonia to be the compound to which *P. geniculatus* showed the highest response (Fig 4). EAG responses to ammonia were discernible down to 20 ppb (see Methods). The antenna responses to isobutyric acid and nonanal started at 10 ng (source dose) whereas the EAG responses recorded for 4-methyl pentanoic acid and skatole started at source doses of 100 ng - 100 μ g.

Table III. Responses of *R. prolixus* fifth-instar nymphs on the servosphere to aromatic products

Compound	Bugs tested	Amount at source	Target vector		Change from control to test period		
			control	test	IUD (%)	speed (mm/s)	displacement (cm)
skatole	10	1 ng	-0.08	-0.32	-2.00	1.12	0.48
	21	10 ng	-0.27	-0.21	0.01	-1.31	-9.01
	10	100 ng	+0.03	-0.27	-0.04	-6.14	-38.36 *
	11	1 µg	-0.45	-0.01	-0.07	-3.25	-0.21
phenol	8	100 ng	-0.16	-0.12	9.05	-4.05	-18.00
	7	100 µg	-0.47	-0.08	-23.20	-18.01 *	-21.5 *
p-cresol	9	10 µg	-0.01	-0.03	12.00	2.03	13.04
indole	8	100 ng	-0.31	-0.57	-19.21	-12.24	-11.37
	8	1 µg	+0.02	+0.01	4.99	-6.09	-12.15
	10	10 µg	-0.09	0.02	6.12	5.77	25.94

Attraction and activation are represented by median values. IUD is the increase in upwind displacement; Wilcoxon paired test, * P<0.005.

Table IV. Responses of *R. prolixus* fifth-instar nymphs on the servosphere to natural products

Compound	Bugs tested	Amount at source	Target vector		Change from control to test period		
			control	test	IUD (%)	speed (mm/s)	displacement (cm)
dimethyl trisuphide	17	1 µg	-0.37	+ 0.18	17.75 *	4.57	5.26
	16	100 µg	-0.32	- 0.02	2.21	- 3.15	0.36
	18	1 mg	-0.15	- 0.06	7.02	- 3.67	4.36
acetone	16	10 µg	-0.20	+ 0.04	0.00	- 2.99	38.40 *
1-octen-3-ol	15	1 µg	-0.48	- 0.41	0.09	3.05	12.70
	14	10 µg	-0.30	0.01	11.08	1.04	8.21
myrcene	8	100 ng	-0.08	- 0.12	0.11	- 2.17	- 9.25
	7	10 µg	-0.12	- 0.23	0.00	- 5.20	- 11.46
	8	1 mg	-0.11	- 0.25	-12.00	- 1.21	- 6.44
linalool	15	100 ng	-0.44	- 0.51	0.00	- 0.38	0.74
4-methyl pentanoic acid	10	100 ng	-0.32	- 0.22	8.02	4.19	1.99
	14	100 µg	-0.36	- 0.41	0.00	- 3.57	8.36
	15	1 mg	-0.80	+ 0.34 *	15.47 *	10.47	27.79 *
	14	10 mg	-0.43	+ 0.27 *	24.65 *	18.09 **	92.57 **

Attraction and activation are represented by median values. IUD is the increase in upwind displacement; Wilcoxon paired test, * P<0.005, ** P<0.01.

Table V. Responses of *R. prolixus* fifth-instar nymphs on the servosphere to mixtures of natural products

Compound	Bugs tested	Amount at source	Target vector		Change from control to test period		
			control	test	IUD (%)	speed (mm/s)	displacement (cm)
p-cresol + phenol	10	10:10 µg	-0.48	-0.01	6.00	2.74	0.00
p-cresol + skatole	10	10:10 µg	-0.58	-0.71	-0.01	1.19	14.17
p-cresol + skatole + phenol	10	10:10:10 µg	-0.43	-0.56	-0.03	9.01	3.99
1-octen-3-ol + butanoic acid	9	1:1 µg	0.11	0.29	12.52	-3.19	-21.14
4MPA + DMTS	11	100 mg:100 ng	-0.15	-0.28	11.00	13.85 *	2.49
4MPA + nonanal	10	100:10 µg	-0.14	-0.29	-7.49	-11.13	-43.74 *
4MPA + nonanal + β-caryophyllene+ skatole	9	1 µg:10 ng:10 ng:10 pg	0.00	0.36 *	16.63	-5.02	11.29
4MPA + butanoic acid + β-caryophyllene+ skatole	12	1 µg:1 µg:1 ng:100 pg	0.13	0.21	0.00	22.22 *	-3.12
NH ₃ + lactic acid+ hexanoic acid ^a	14	dispenser	+ 0.02	+ 0.56 *	45.88 *	5.27	21.33 *
lactic acid+ hexanoic acid ^a	8	dispenser	-0.84	-0.31	0.00	-6.89	-117.38 *
POCA ^b	11	dispenser	-0.57	-0.52	0.00	5.19	7.08

a. BG-Sentinel trap dispenser.

b. 3-n-propylphenol, 1-octen-3-ol, p-cresol, acetone. For dispenser descriptions see Methods.

Attraction and activation are represented by median values. IUD is the increase in upwind displacement; Wilcoxon paired test, * P<0.005, ** P<0.01.

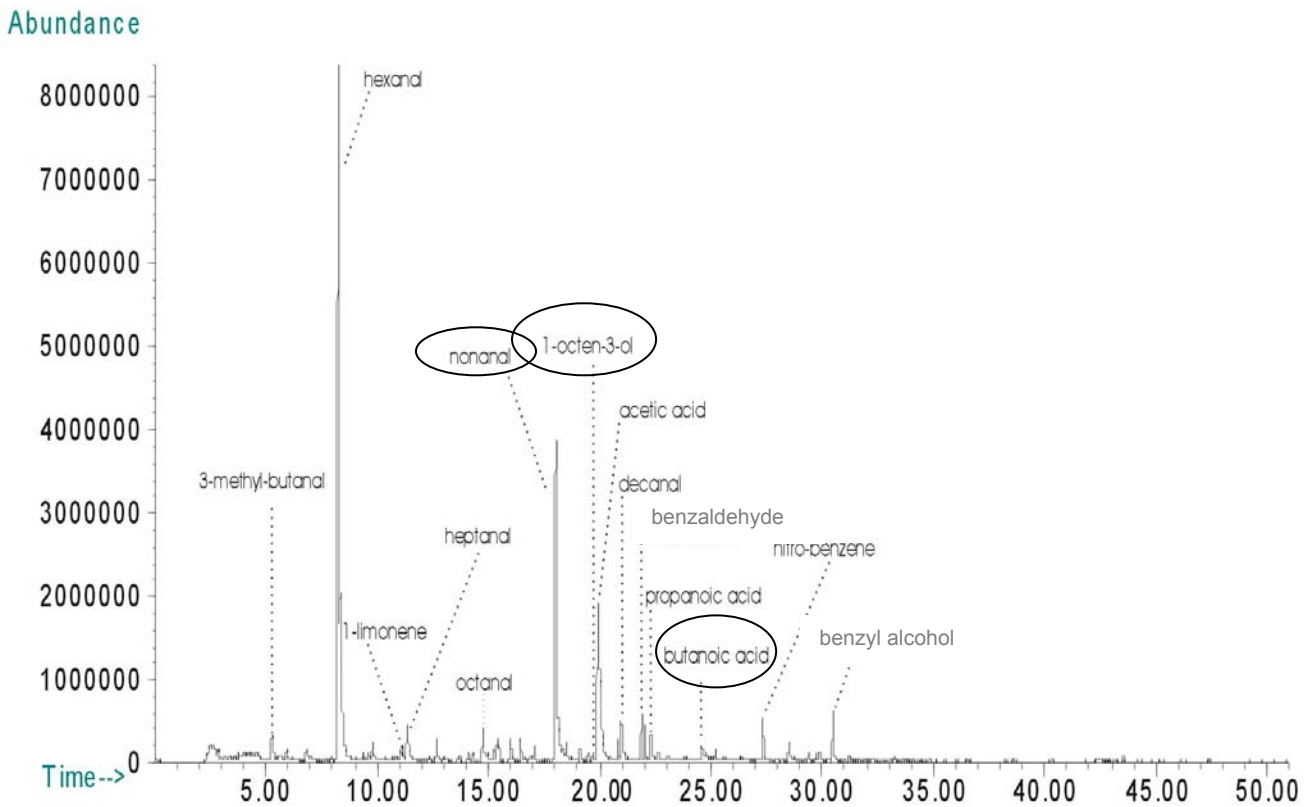


Fig. 3 Identification of compounds present in chicken feather headspace volatiles collected on a porous polymer and analyzed by GC-MS. Aldehydes, short chain carboxylic acids, alcohols and terpenes were found in the feather odour. Nonanal, 1-octen-3-ol and butanoic acid (circled) were tested in behavioural experiments (see Table IV, V).

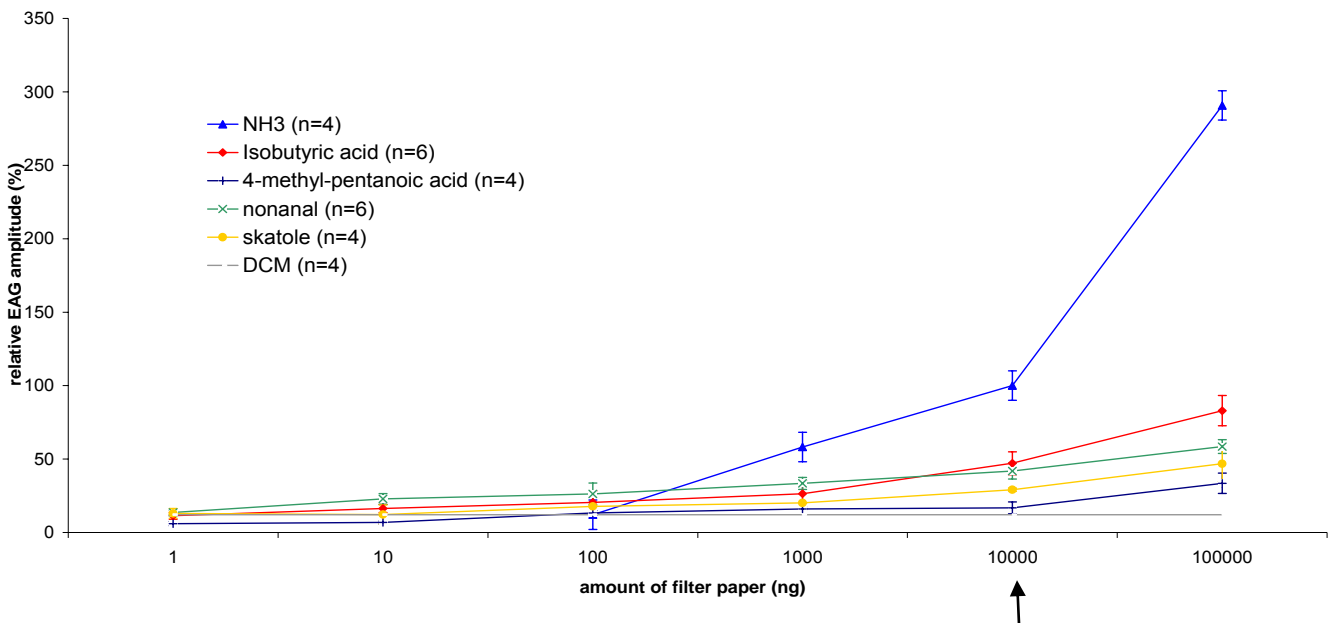


Fig. 4 EAG responses of *P. geniculatus* to five chemostimuli found in vertebrate wastes and feathers. Responses are normalized to 10 μ l of NH_4OH (150mM, indicated by an arrow) on filter paper as reference (representing 100%) that corresponds approximately to 20 ppb of ammonia in air.

DISCUSSION

Behaviours of triatomine spp. on the servosphere

R. prolixus, *T. infestans* and *P. geniculatus* fifth instar nymphs behaviours did not differ greatly on the servosphere in the absence of chemostimuli in a clean air stream. The three species walked in relatively straight bouts punctuated by stops and performed similar antennal movements and cleaning behaviours as previously described for *R. prolixus* by Otálora-Luna *et al.* (2004). Additionally, cleaning of the extended proboscis, a behaviour rarely observed, is reported here for first time for the three species. This behaviour is of particular significance as triatomines approaching a host will need to keep their proboscis gustatory sensilla (Catala 1996) clean of dust particles in order to be able to recognize the vertebrate skin and blood (Smith & Friend 1982) when the occasion arises. Hind leg flexing was a behaviour exclusively performed by *P. geniculatus* nymphs on the sphere. They lifted their hind legs over the abdomen after scraping the sphere surface with tarsi, in an attempt to move particles from the ground to the abdomen surface. This may have a role in camouflage of this species. All nymph states of *P. geniculatus* have been observed in the laboratory to cover their abdomens with available particles, by performing a similar behaviour but this has never been observed on *R. prolixus* and *T. infestans* nymphs. Such camouflage behaviour by *P. geniculatus* nymphs has also been observed in *T. dimidiata*, *T. phyllosoma*, *P. megistus* and *P. herreri* (Zeledón 1981) and in *T. nigromaculata* (Heger 2006, personal communication). The camouflage behaviour is useful for species that live in burrows where dust particles are abundant as is the case for *P. geniculatus*, but less useful for other species like *R. prolixus* that live naturally in trees and *T. infestans* that live in rocky habitats. Additionally, the fact that *P. geniculatus* nymphs continued walking after performing hind leg flexing suggests that this is a tactic that forms part of foraging strategy. Nymphs covered by ground particles can be less visible to predators and for hosts while exploring open spaces.

Attraction to vertebrate wastes

Chicken faeces caused a similar response as diluted breath on *R. prolixus* (Table I) even though the former sample produced lower concentrations of CO₂ and NH₃. Furthermore, the chicken faeces fraction with amines (Appendix 1) and neutral compounds still proved attractive for *P. geniculatus*. A similar result was obtained when the urine odour extract was tested on this species. This demonstrates that bird faeces and mammalian urine odour constituents are responsible on their own of triatomine attraction. In addition, the chemotactic response to chicken faeces basic fraction suggests a role for aliphatic amines in such attraction behaviour.

Ammonia elicited attraction and activation behaviours when tested on *T. infestans* (Taneja & Guerin 1997) and *R. prolixus* (Fig 1, Table I) and elicited the highest EAG response among the products tested on *P. geniculatus* (Fig 4). Taneja & Guerin (1997) previous electrophysiological studies demonstrated the presence of olfactory receptor cells in the grooved peg sensillum on triatomine antennae for methylamine, dimethylamine, ethylamine and diethylamine at a threshold some 10 times higher than that of NH₃. Diehl *et al.* (2003) found that isobutylamine and 2-butylamine were the best stimulants for receptors within the grooved peg sensillum among different short-chain aliphatic amines tested. These authors also reported that receptor cells within the grooved peg sensillum

also responded to methylamine, dimethylamine, trimethylamine, ethylamine and diethylamine evoking moderate to weak responses.

Based on these facts, we chose a series of aliphatic components containing nitrogen in their structure (i.e. amines). Among the aliphatic amines tested, dimethylamine and ethylamine on their own proved attractive for all three triatomines *spp.* tested. As DMA and EA are both excreted in considerable quantities by birds and mammals (Hartung & Rokicki 1984, Robacker *et al.* 2000, Le *et al.* 2005), our results suggest that these amines, among other compounds present in host wastes, act as information cues signaling resources for triatomines. In addition the role of other amines such as isobutylamine and hexylamine should not be underestimated as these two molecules also affected triatomine behaviours, i.e. increase in speed and displacement by the bugs tested (Table II).

The major amine constituents of animal wastes are end-products derived from general vertebrate and gut flora metabolism and as such do not vary greatly between vertebrate species (Mackie *et al.* 1998). In mammals, excess of DMA, EA and other amines such as methylamine, trimethylamine, propylamine, isopropylamine are absorbed in the gut and excreted in the urine (300-700 µg/l, Cai *et al.* 2003) and lower concentrations are also found in faeces. In fact, DMA is the most abundant short-chain aliphatic amine present in human urine (roughly 0.25 mg/Kg/24h) and rat urine (roughly 2 mg/Kg/24h) and it is also present in other vertebrate body fluids such as saliva, gastric juice, blood and vaginal secretions (Zhang *et al.* 1993, Zhang *et al.* 1995). Vertebrates must get rid of DMA since this compound is highly toxic and it is an immediate precursor of dimethylnitrosamine¹⁴, a recognized carcinogen for a wide variety of animal species. Trimethylamine N-oxide degraded by the gut flora is the major dietary source of DMA (Zhang *et al.* 1998, Fig 5).

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 physiological purposes, is a precursor of both excreted DMA and EA. Choline is degraded by the activity of gut flora to either EA plus ethanolamine or to trimethylamine which is easily demethylated to DMA (Le *et al.* 2005, Fig 1). EA can also be obtained by decarboxilation of alanine or amination of ethanal by anaerobic bacteria present in vertebrate faeces (Spoelstra 1980).

Evolutionary and ecological implications

Triatomine *spp.* radiated early (60 mya, see Chapter I) to occupy different ecotopes but still share the same adaptative zones today (haematophagy and association with nidicolous hosts). These bugs have common morphological sensory adaptations, i.e. similar olfactory sensilla types (Carbajal de la Fuente & Catalá 2002, Diehl *et al.* 2003). In addition, similar chemotactic responses are recorded here for three different triatomine species to volatiles originating from host wastes. We propose that these chemosensory and behavioural adaptations, probably inherited from their reduviid predator predecessors, permitted all species of the Triatominae to adapt to haematophagy. Major radiations of modern birds and mammals between the late Cretaceous and early Tertiary (Ericson *et al.* 2003, Delsuc

¹⁴DMA undergoes nitrosation under weak acid conditions to give dimethylnitrosamine. This animal carcinogen has been detected and quantified in human urine samples and it may also arise from nitrosation of DMA by nitrogen oxides present in acid rain in highly industrialized countries (Zhang *et al.* 1998).

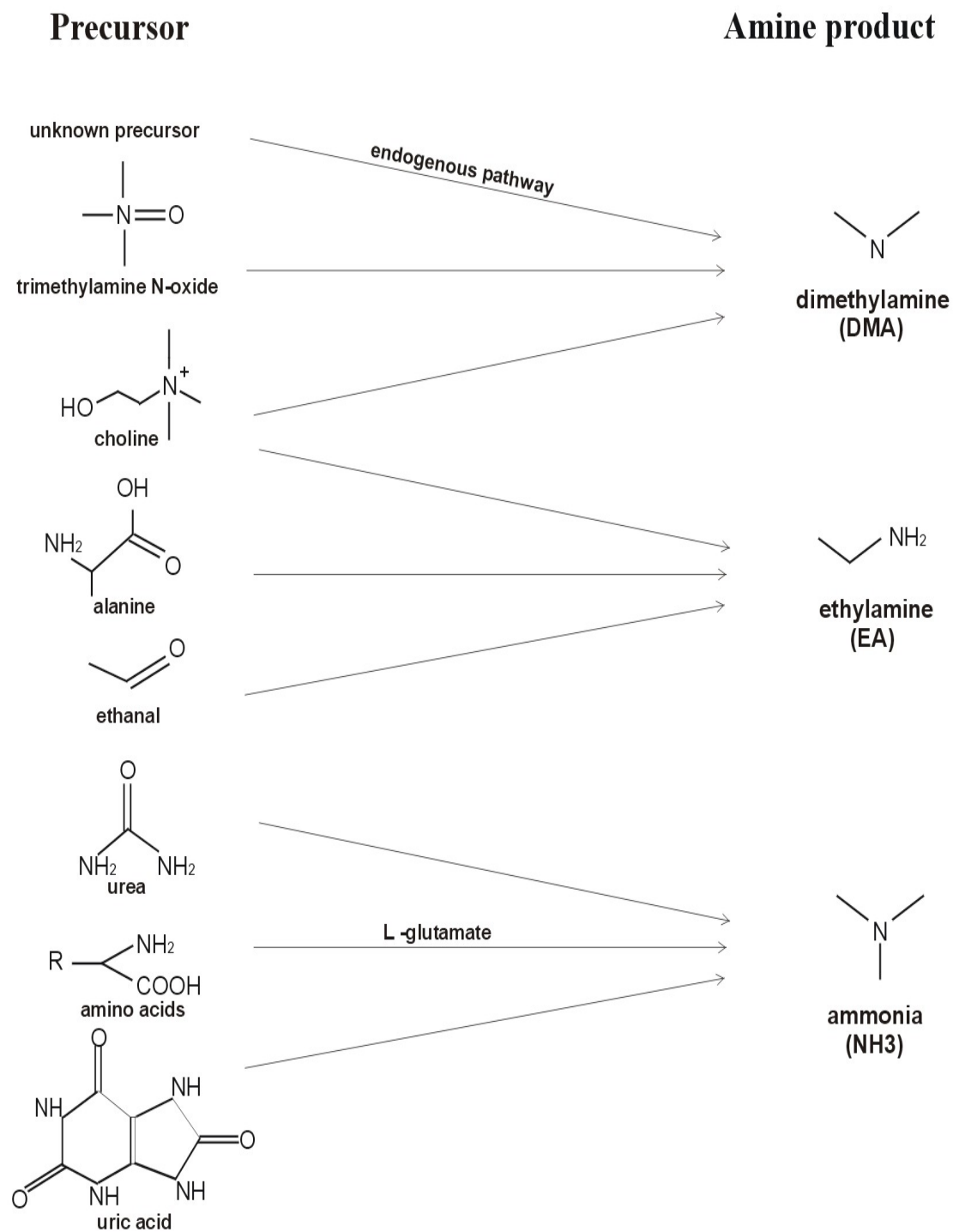


Fig 5. Origin of volatile amines excreted in faeces and urine that elicit attraction on triatomines

et al. 2004) must have provided ample new niches for opportunistic species of various arthropod groups (Kim 1985). Presumably, during the early Mesozoic, the ancestral reduviid predators were already well adapted to invade and inhabit nidicolous vertebrate refugia attracted by an abundant supply of arthropod prey and stable microclimatic conditions (Heger *et al.* 2006). The prolonged triatomine association with birds and nesting mammals resulted in specialization to feed directly on vertebrate blood (Chapter I). As many early and extant nesting vertebrates use their excretions to mark the nest or burrow entrance (Young & Henke 1999, Brennan 2001), triatomine ancestors probably developed the capacity to identify such rich sources of odours in order to find vertebrate dwellings. This provided a fundamental step in the reduviid colonization of bird nests, mammalian burrows, tree holes and other vertebrate refugia and eventually human dwellings.

Based on our findings we propose that *P. geniculatus* and other sylvatic triatomine *spp.* could use the same chemical cues that they use to locate their wild hosts, to exploit domestic ones. This shift would be most likely to occur when environmental disturbances of wild habitats by human activities occur. Amines present in human and domestic animal excretions could play an important role in localization of houses by domestic and sylvatic triatomine species. As *P. geniculatus* adapted to the domestic niche, i.e. specialized to live at the expense of humans, it could eventually develop a habit¹⁵ to search for human dwellings as other domestic species do. On the other hand, triatomine species that are highly specialized to a particular niche risk extinction if similar damage of their environment occurs.

There are other cases of adaptation to the domestic environment in Insecta which lead to the successful transmission of a pathogen. Sandflies (Psychodidae: Phlebotominae), vectors of Leishmaniasis over a wide area of the planet, are often restricted to habitats such as reptile, mammal and bird nesting sites as well as earthen floor of human domiciles (Felicciangeli 2004). Schlein *et al.* (1989, 1990) found that the presence of cow manure serves as both oviposition attractant and stimulant in the sandfly *Phlebotomus papatasi* under field and laboratory conditions. Additionally, it has been found that extracts of rabbit and chicken faeces promote egg laying in the sandfly *Lutzomyia longipalpis* (Elnaïem & Ward 1992, Dougherty *et al.* 1993, 1995) in the laboratory. Clearly, it has selective advantages for sandfly females to find oviposition sites in animal shelters rich in organic debris that serve as food for larvae¹⁶. Additionally, the presence of vertebrate animal wastes may indicate the availability of host for females (as must be the case for triatomines) and induce the formation of leks of males (Spiegel *et al.* 2005). In general, from these observations and results presented in this thesis we can conclude that odours from vertebrate wastes are highly convenient cues that render a suitable habitat apparent for nest-living haematophagous arthropods.

Amines have also elicit behavioural responses from non-haematophagous arthropods. Various species of tephritid fruit flies (Tephritidae) feed on bird faeces droppings to complement their carbohydrate diet that is poor in nitrogen (Fagan *et al.* 2002). To do so these frugivorous dipterans use amines emanating from bird faeces to locate such rich nitrogen resources. Behavioural experiments on the Mexican fruit fly (*Anastrepha ludens*)

¹⁵ Triatomines' capacity to learn has recently been demonstrated (Abramson *et al.* 2005).

¹⁶ Immature stages of some sandflies have also been found in buttress roots of trees in Panama (*Lutzomyia ovallesi*) and in termite hills in Kenya (*Phlebotomus celiae*) (Felicciangeli 2004).

have demonstrated that DMA (1-100 µg) and 1-pyrroline¹⁷ (100 ng -100 µg, source dose) alone, both identified in duck faeces, attract sugar-fed and sugar-starved adults (Robacker *et al.* 2000). Blowflies (Calliphoridae) are also attracted by amines (Ashworth & Wall 1994). These insects feed on protein rich material in decomposition. Several calliphorid species use carrion as an energy resource for larvae; laying eggs on live hosts, animal excrement and bird nests. Sheep blowflies *Lucilia sericata* and *L. cuprina* oviposition is elicited by the presence of nitrogen-rich compounds (Ashworth & Wall 1994).

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¹⁷ 1-pyrroline was found here in chicken faeces basic fraction (Appendix 1)

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Chapter IV

A Device for Trapping Domestic and Sylvatic Triatomines

“In addition to being motivated by their basic curiosity about how the world works, humans have practical reasons for maintaining an interest in how organisms obtain and use information about their environment. ... Our ancestors could hunt and fish more successfully if they understood the sensory abilities of their prey so as to avoid alerting them while stalking or trying to attract them with lures.”

-David. B. Dusenbery

Abstract

Work on the development of a tool for surveillance of triatomine bugs is fundamental in the fight against Chagas disease. Such tool can help health authorities to efficiently deploy the necessary resources to be invested in the control of an outbreak according to the scale of the infestation. We developed an effective monitoring system, i.e. a capturing device, to detect the presence of triatomine vectors of Chagas disease in endemic areas. Our studies took advantage of previous electrophysiological recordings and locomotion compensator (servosphere) findings. The (bicylinder) trap proved to be efficient alone (> 95 % of bugs were captured), i.e. without an odour dispenser, when 60 bugs were liberated in an experimental arena. Another device (cylinder trap) that resulted less effective in capturing bugs than the bicylinder trap, but more appropriate to test the effects of odours on triatomine capture, was also tested. *Rhodnius prolixus*, *Triatoma infestans* and *Panstrongylus geniculatus* were more attracted to the cylinder trap baited with CO₂ and NH₃ than this trap alone: more than 73% of the total bugs were captured in the test trap. The bicylinder trap baited with CO₂ and NH₃ may be useful to study the prevalence of triatomines in *Trypanosoma cruzi*-endemic areas.

Keywords: triatomine behaviour, triatomine surveillance and control, traps, experimental arena, triatomine domiciliation, Latin America, Chagas disease, Reduviidae, Triatominae, *Rhodnius prolixus*, *Triatoma infestans*, *Pastrongylus geniculatus*.

INTRODUCTION

The development of precise and reliable methods for the early detection of triatomine household infestations is necessary for effective Chagas disease prevention through vector control (Gürtler *et al.* 2001). This can permit local health authorities to monitor the effects of control campaigns and establish the need for additional treatment according to the scale of the infestation.

Currently, there are three general methods to quantify bug occurrence and density in domestic and peridomestic sites: the use of human collectors, sampling devices and house demolition. Human collection is a commonly used method, but since triatomines are nocturnal and hide in cracks and crevices, it is difficult for trained personnel, much less homeowners, to be aware of their presence. A more standardized method is timed manual collection using an irritant spray (i.e. flushing out method, Pinchin *et al.* 1980, 1981) but this is costly, requires skilled staff, and lacks sensitivity and precision (Gürtler *et al.* 1999). House demolition has proved to be the most effective method of quantifying bug infestation, but is obviously too expensive and very impractical (Rabinovich *et al.* 1995). The use of sampling devices proves to be the most sensitive and cost-effective method according to Gürtler *et al.* (1995). These devices involve Gómez-Núñez boxes (Gómez-Núñez 1965), fiberpot refugia (Takano-Lee & Edman 2001) and sensor boxes (Vázquez-Prokopec *et al.* 2002). All these devices are attractive to triatomines, through thigmotaxis and negative phototaxis, but the insects are not captured as they can freely enter and leave an enclosed space that just provides a refuge. The presence of alive or dead nymphs and adult forms as well as exuviae, faeces and eggs in sampling devices serves to detect bugs. Sensor sheets treated with phenolphthalin facilitate the visibility of triatomine faecal streaks (Gürtler *et al.* 2001). One of the drawbacks of sampling devices is that they reveal a rising trend of infestation over time due to the increasing faeces deposition by arriving triatomines, which results in the progressive attraction of more bugs to the refuge because of their own faeces (Taneja & Guerin 1997, Lorenzo & Lazzari 1998). Furthermore, this increasing sensitivity over time requires waiting for a period after the device has been installed (from weeks to months) until it effectively becomes a triatomine refuge and one can provide a measure of bug infestation.

Other sampling devices with odour dispensers have been designed to capture triatomines. The effectiveness of traps baited with CO₂ has already been proven using yeast (Pires *et al.* 2000) and dry ice (Botto-Mahan *et al.* 2002). The functioning of these capturing devices is based on the fact that CO₂ attracts triatomines, but few other sensory cues have been tested or considered in order to improve the efficacy of such traps. Furthermore, yeast is cheaply available, but production of CO₂ from yeast is not constant over time. Dry ice is relatively expensive and impractical for transporting, especially in rural areas. Another trap has been tested using a living mouse inside, i.e. a live-bait trap (Noireau *et al.* 2002). The use of a host as a bait can be very effective, but is impractical due to the transport and care of a living animal and the host's risk of losing its life *vis à vis* predators that occur in the sylvatic and peridomestic environment. Light has also been used to trap dispersing nymphs and adult bugs at night (Vázquez-Prokopec *et al.* 2004), but this method is impractical to be used indoors. Clearly, there is room to develop more precise and reliable methods to diagnose triatomine house infestation levels that can be of assistance to local health authorities in designing vector control programmes.

Recent research on the thermo-, hydro-, chemosensory and visual adaptations of triatomines and studies on their sensory ecology has provided knowledge that can be employed for development of simple trapping devices. Data is presented here on the development of a trap for these bugs using a behavioural approach. Experiments by both Rose (1998), working in semi-natural conditions, and our own (Otálora-Luna & Guerin 2004), working under controlled conditions on the locomotion compensator, demonstrated that a combination of CO₂ plus NH₃ attracts *T. infestans* and *R. prolixus*. As such, a CO₂ and NH₃ combination has proved practical in the development of a lure.

METHODS

Animals

R. prolixus, *T. infestans* and *P. geniculatus* colonies were maintained as in Chapter III.

Traps

Three types of traps were used in the experimental arena: *bicylinder trap*, *cylinder trap* and *box trap*.

The bicylinder trap (Fig. 1, A) resembles an upturned milk pail. It was made of two black cardboard cylinders. One upturned cylinder (*roof*, 17.5 cm diam, 10.5 cm high) covers another cylinder container (*base cylinder*, 13.8 cm diam, 10.5 cm high). An adhesive tape (1 cm wide) was placed around the inner upper border of the base cylinder to stop bugs from escaping. The surface of the adhesive tape was smooth, so it was not possible for bugs to crawl out of the trap. Both cylinders were fixed to each other by a cardboard cylindrical pillar (4 cm diam, 15.5 cm high) which served as a column to bear the roof cylinder over the base cylinder. This column contained the odour dispenser(s) and was surrounded by 5 holes (0.5 cm diam) located 2 cm above its base permitting diffusion of the vapours into the bicylinder trap. The bottom of the roof cylinder was 6 cm above the ground in order to let the CO₂ and NH₃ diffuse out over the ground (Fig. 1, A).

For one series of experiments double adhesive white tape (Tesa™, 5 cm wide) was placed on the outside of the base cylinder of the bicylinder trap baited with the CO₂ and NH₃ to capture the bugs trying to climb the sticky surface. The control did not have the double adhesive tape.

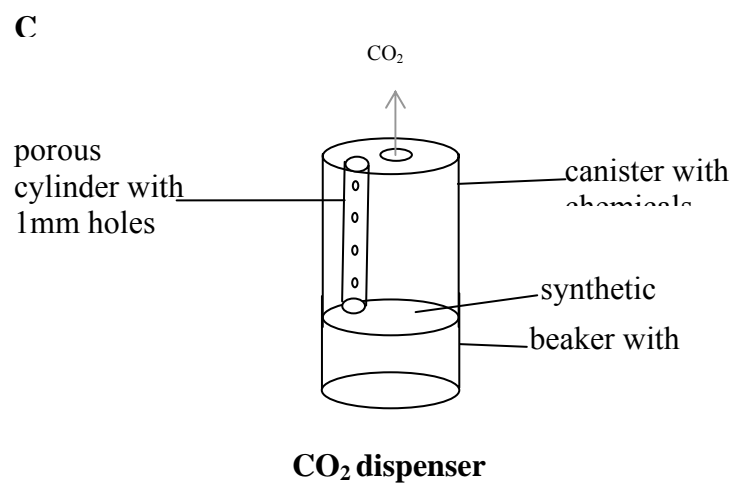
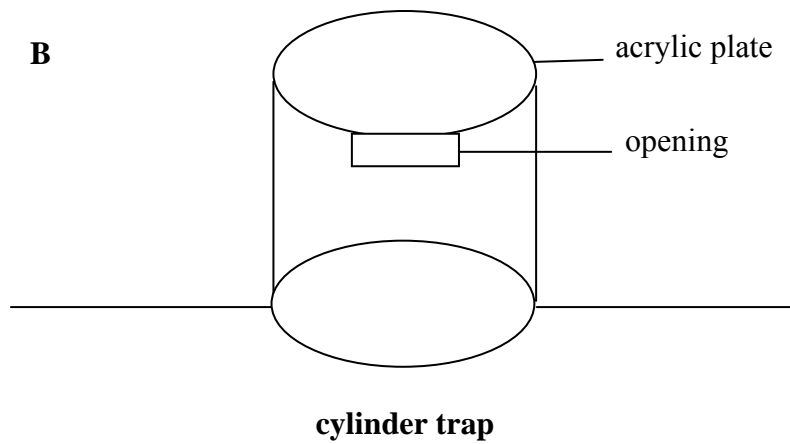
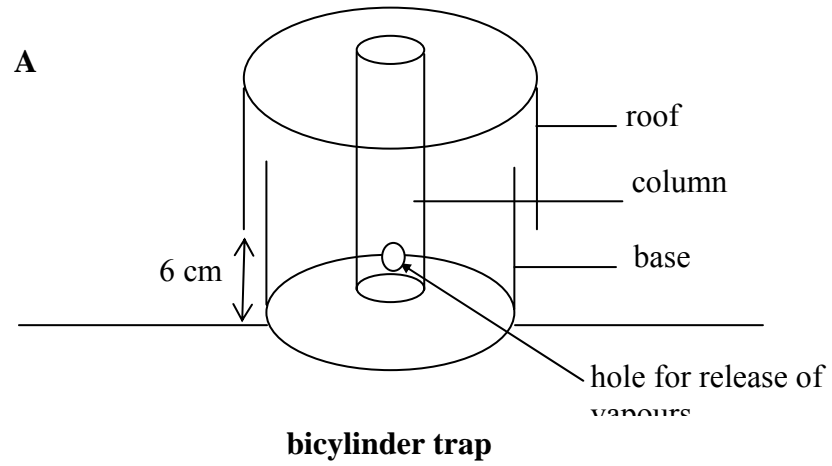


Fig 1. Bicylinder trap (A) and cylinder trap (B) used in experimental arena, and CO₂ dispenser (C) used in the cylinder and bicylinder trap.

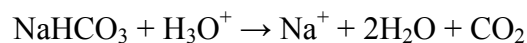
The cylinder trap (Fig 1, B) was of a simpler design as the bicylinder trap. It consisted of a cylinder made of black cardboard (13.8 cm wide, 10.5 cm high) covered with a transparent acrylic plate, with a rectangular slit (9.5 cm wide, 1 cm high) near the top (9.5 cm up). This slit allowed triatomines to enter the trap. As in the bicylinder trap, an adhesive tape (1 cm wide) was placed around the inside upper border of the cardboard container to stop bugs from escaping through the opening.

The box trap was constructed as Vázquez-Prokopec *et al.* (2002) with few modifications. It consisted of a one-liter recycled Tetra Brik™ (Tetra Pak Corp.) milk carton measuring 16 x 9.5 x 6 cm (long, wide, deep). All boxes were washed with RBS (35-concentrate, Fluka) diluted in demineralized warm water (approx. 50°C, 15 ml/l) for 3 h before use. Internally, the boxes contained a central column (20 cm x 4.5 cm) of accordion-folded black corrugated cardboard paper. The box was placed vertically on one of its 9.5 x 6 cm sides. The entrance measuring 14 x 1 cm (wide, high) was located at ground level and facing the bugs leaving the acrylic release box in the test arena (see below). The entrance had a 14 x 10 cm cardboard base, instead of leather as in the Vázquez-Prokopec *et al.* (2002) model.

CO₂ dispenser

Chemical principle

On the principle of an acid-base reaction to produce CO₂, two salts were mixed in a 2:1 ratio: sodium bicarbonate (12g, purity 99.5 %, Merck) and anhydrous citric acid (6g, purity 99.5 %, Fluka) in the presence of high humidity (95% RH). In water the sodium bicarbonate decomposes into Na⁺ and HCO₃⁻ and the acid decomposes into H⁺ and acid⁻. The HCO₃⁻ reacts with H⁺ to form H₂O and CO₂.



Dispenser

The dispenser (Fig 1,C) consisted of a 35 mm polyethylene film canister (Kodak, 3.3 cm diam, 5 cm high) and lid. The grey lid had a hole in the center (7 mm diam to allow the release of CO₂). The base of the black container was cut and replaced by synthetic porous (0.04 mm wide) gauze. The sealed ends of four small polyethylene syringe needle sleeves (BD Microlance, 5 mm diam, 4.5 mm high) were cut to make small cylinders. Four lines of fifteen holes each (1 mm diam) were made on one side of each cylinder and placed vertically inside the canister to improve water vapour contact with the salts. A beaker (3.3 cm diam, 3.5 cm high) was connected to the base of the film canister to provide water vapour to the sodium bicarbonate/citric acid powder mixture. The bicarbonate and citric acid were mixed and placed in the film canister, the lid closed and it was connected to the beaker containing 10 ml of water just before an experiment (~ 1 min). The salts held by the synthetic gauze in the canister were separated by 5 mm from the water surface to optimise a slow release of CO₂ (otherwise the reaction ran out in few seconds). After connecting the water beaker to the canister, the dispenser was immediately placed for an experiment inside the trap or was used for measuring the CO₂ release rate (see below).

Release over time

In order to estimate the release of CO₂, the air across the headspace of the dispenser was pumped at 1.5 l/min into an electronic infrared gas analyzer (LI-COR LI-820, USA, range 0-2000 ppm, accuracy ±3%, signal averaging 20 s) in a climate chamber at 23°C and 80%RH. The analyzer hose was placed 5 mm above the hole in the lid of the container. The control consisted of a dispenser with 10 ml of water and without any chemicals. A fan continuously extracted air from the climate chamber to avoid an increase of the background CO₂ level. Data measured by the LI-COR detector was directly recorded into a computer operated from outside the climate chamber (Fig 2).

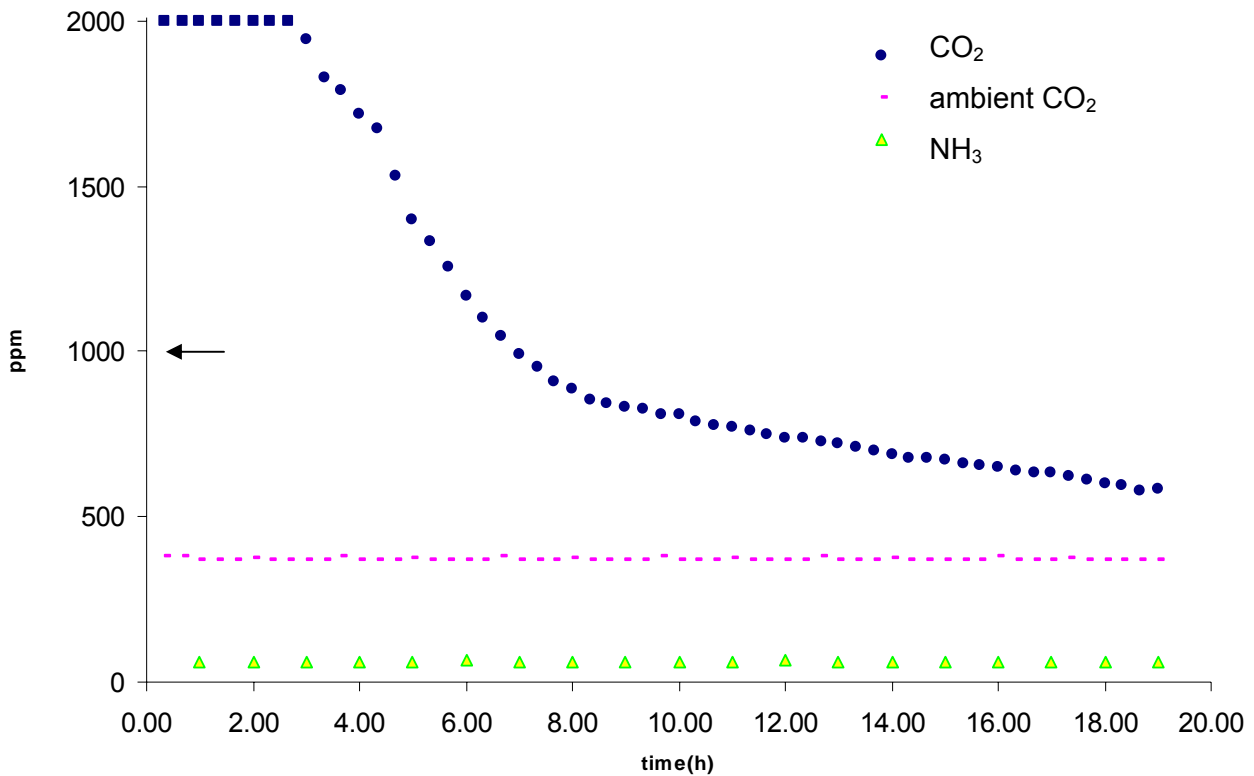


Fig 2. The concentration of CO₂ (every 20 min) and NH₃ (every 60 min) measured over dispensers for each of these molecules (see Methods) over 19 hours. The concentration of CO₂ was relatively high during the first 3 hours (squares correspond to values that surpass the 2000 ppm limit of the sensor) and then decreased regularly to approach 200 ppm above ambient (mean 370.85, SD 3.80) indicated by the dashed lines. The arrow indicates the CO₂ concentration used in test with *R. prolixus* nymphs, 1000 ppm (see Chapter II). The NH₃ concentration released from the dispenser was constant over 19 h (mean 61.13; SD 1.80).

NH₃ dispenser

Chemical principle

Ammonium carbonate¹⁸ (NH₄)₂CO₃ (3 g, 95.5% pure, Merck) was used as the NH₃ source. This salt is a 1:1 mixture of ammonium bicarbonate (NH₄HCO₃) and ammonium carbamate (NH₂COONH₄) which constantly releases NH₃ at room temperature.



Dispenser

The ammonia dispenser consisted of a glass beaker (1.9 cm diam, 3.9 cm high) covered with a plastic lid with a hole in its centre (5 mm diam) containing the ammonium carbonate powder. The ammonium carbonate powder was taken from a stock at -21°C, weighed and placed in the beaker 10 min before an experiment.

NH₃ release over time

In order to estimate the release of NH₃ over time, the air across the headspace of the NH₃ dispenser was pumped at 0.5 l/min into an electrochemical analyzer (Dräger Sensor Pac III S, Germany, range 0-200 ppm, accuracy ± 1%) in a climate chamber at 23°C and 80% RH. The analyzer hose was placed 5 mm above the hole in the lid of the canister. The control consisted of an empty beaker under the same conditions as above. Data measured by the electrochemical analyzer was manually noted every hour over 19 h from outside of climate chamber and then every 6h over 3 days. The ammonia mean concentration, 61.13 ppm, SD=21.80 ppm, was constant over 19h (Fig 2) and did not decrease over 3 days. The analyzer did not register any NH₃ over the control.

In order to estimate the release of NH₃ coming through the opening of the cylinder trap, the Dräger hose was placed 1cm from it. Measured data was manually noted every 10 min over 1 h, as before. The mean ammonia concentration was 15 ppm, SD=11.03 ppm.

In order to estimate the release of CO₂ from the ammonia dispenser, the LI-COR CO₂ analyzer was used as described above for the CO₂ dispenser, but measurements were made every 10 s over 30 min. An empty beaker was used as control. The mean CO₂ concentration was 81 ppm (SD 27 ppm) above ambient.

Mosquito lure dispensers

A dispenser (B-dispenser) used in a mosquito lure (BG-Sentinel trap, BioGents, University of Regensburg, Germany) that releases ammonia, hexanoic acid and L-lactic acid was used to bait the cylinder trap in a series of experiments.

¹⁸ This salt decomposes into ammonia, carbon dioxide and water at temperatures over 59 °C. It tends to cake over time, is very toxic and if it contacts nitrites (present in fertilizers) it can explode. It must be kept in tightly closed containers in a cool and dry place otherwise it loses its free flowing properties within a few days.

Test arena

Bicylinder and cylinder traps were tested in an experimental arena (Fig 3) in a climate chamber at 80 % RH, 22°C, 12:12 LL:DD. Light increased from 5:30h to 7:00h (artificial sunrise) to reach a maximum of 1400 lux and decreased from 5:30 pm to 7:00 pm (artificial dusk) to reach 0 lux. The circular arena (1.20 m diam) was limited by a metallic wall (45 cm high) painted grey with metallic paint that provided a smooth surface that the triatomine nymphs could not climb. The arena floor was made of aluminium. Test and control traps were placed in the middle of the floor 10 cm distant to each other. An acrylic refuge (7 cm wide, 3.5 cm high, 8 cm deep) for release of triatomines was placed on one side of the arena. The acrylic cage had a door (PlexiglasTM, 7 cm wide, 3.5 cm high) that faced the traps and that could be opened by pulling a cord to release the bugs. Both traps were equidistant to the refuge. Positions of traps were exchanged in each series of experiments to compensate for any asymmetry in the arena.

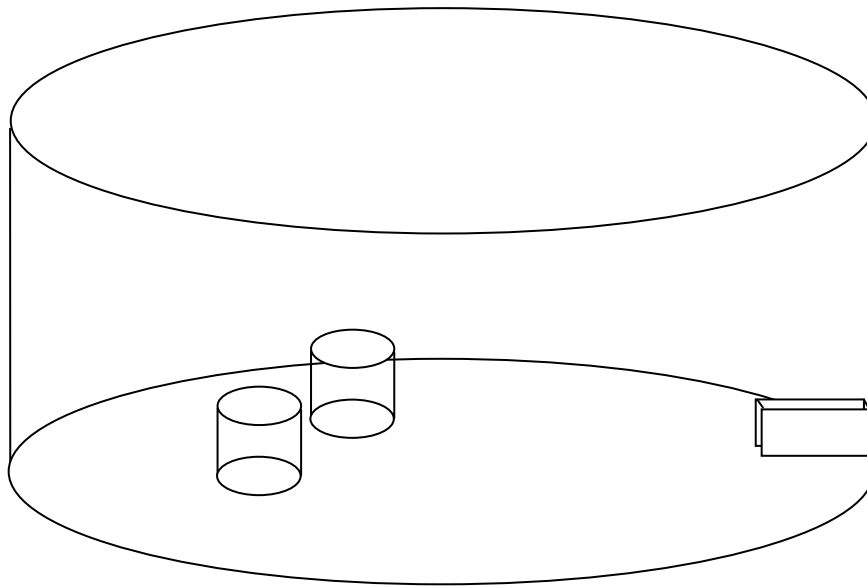


Fig 3. Test arena with test and control bicylinder or cylinder traps at the left, and the acrylic box from where the bugs were released at the right

Behavioural testing

Observations of the triatomine entry to the traps were observed using video. Triatomines were placed in the acrylic box for a 10 min acclimatisation period prior to their liberation. The traps were put in place 2 min before starting an experiment. Each experiment started 15 min after dawn (at 7:15h) and finished 15 min after sunset (19:15h). Trials were performed in darkness and in the scotophase to avoid providing visual stimuli to the bugs and because field and laboratory observations indicate peaks in foraging activity during this phase (Barrozo *et al.* 2004). Trapped bugs were counted at the end of each experiment. Traps were manipulated with gloves. The arena and the acrylic box were cleaned with ethanol (40%) at the end of each experiment.

Statistics

The distribution of insects in the arena was tested against a random distribution (i.e. one half of the bugs in each trap) by means of the log-likelihood ratio test (*G*-test with Williams correction, Sokal & Rohlf 1981), which is a *goodness of fit test*. In order to compare the results obtained in different sets of experiments a *test of independence* using the *G*-test was employed. All statistical analysis was performed using R version 2.2–1 for Linux, Vienna, Austria (Ihaka & Gentleman 1996).

RESULTS

Video recordings in the arena indicated that triatomines walked in bouts punctuated by stops. The stops were longer when the bugs reached the border of the arena or the border of a trap. After 2 hours of being released, 40% of bugs were inside or near the control and test bicylinder and cylinder traps and 60% of the bugs were inside or near the traps after 6 hours.

The bicylinder trap baited with a CO₂ plus NH₃ mixture was tested for its capacity to capture fifth-instar *R. prolixus* and *T. infestans* nymphs in the arena (see Table I). These vapours did not increase the number of bugs captured in the baited traps compared to controls ($p > 0.05$, *G*-test). Similar results were obtained for other odours and species tested (Appendix 3). Nevertheless, the median proportion of bugs captured by test and control bicylinder traps

Table I. Triatomine bugs captured in bicylinder traps in the experimental arena

Experiment	Species	No. of tests (k)	Mean N° of bugs captured by the test trap	% of bugs captured by test and control traps	Bugs captured in the test as % of the total bugs captured	G-test p value (test vs. control)
CO ₂ + NH ₃	<i>R. prolixus</i>	4	33.03	95.42	69.43	n.s
CO ₂ + NH ₃	<i>T. infestans</i>	3	10.00	100.00	50.00	n.s*

* n.s. not significant. Sixty bugs were tested in each experiment. There were no differences between the different sets of experiments ($p > 0.1$, *G*-test; see Methods).

(97, IQR 6.8 %, $n=60$, $k=12$)¹⁹ was always higher compared to the median proportion of bugs captured by test and control cylinder traps (61.6, IQR 8.6 %; $p < 0.01$) in different experiments (Table II). Furthermore, when a non-baited bicylinder trap and a box trap were placed together in the arena, the former captured more *R. prolixus* and *T. infestans* bugs than the box trap (Fig 4).

¹⁹ IQR means interquartile range, “n” is the number of bugs tested in each experiment and “k” is the number of times the experiment was repeated.

Table II. Triatomine bugs captured bugs in cylinder traps in the experimental arena

Experiment	Species	No. of tests (k)	Mean N° of bugs captured by the test trap	% of bugs captured by test and control traps	Bugs captured in the test as % of the total bugs captured	G-test p value (test vs. control)
CO ₂ + NH ₃	<i>R. prolixus</i>	4	28.60	65.33	72.96	0.005
CO ₂ + NH ₃	<i>P. geniculatus</i>	4	26.00	56.25	77.04	0.001
CO ₂ + NH ₃	<i>T. infestans</i>	4	25.25	56.67	74.26	0.01
NH ₃	<i>P. geniculatus</i>	4	23.25	68.33	56.71	n.s.
B-dispenser	<i>P. geniculatus</i>	6	25.00	60.00	69.44	0.05
B-dispenser	<i>R. prolixus</i>	4	16.75	59.58	46.85	n.s.

NH₃ was released from the salt mixture except in the case of test with the B-dispenser. There were no differences between the different sets of experiments ($p > 0.1$, G-test; see Methods).

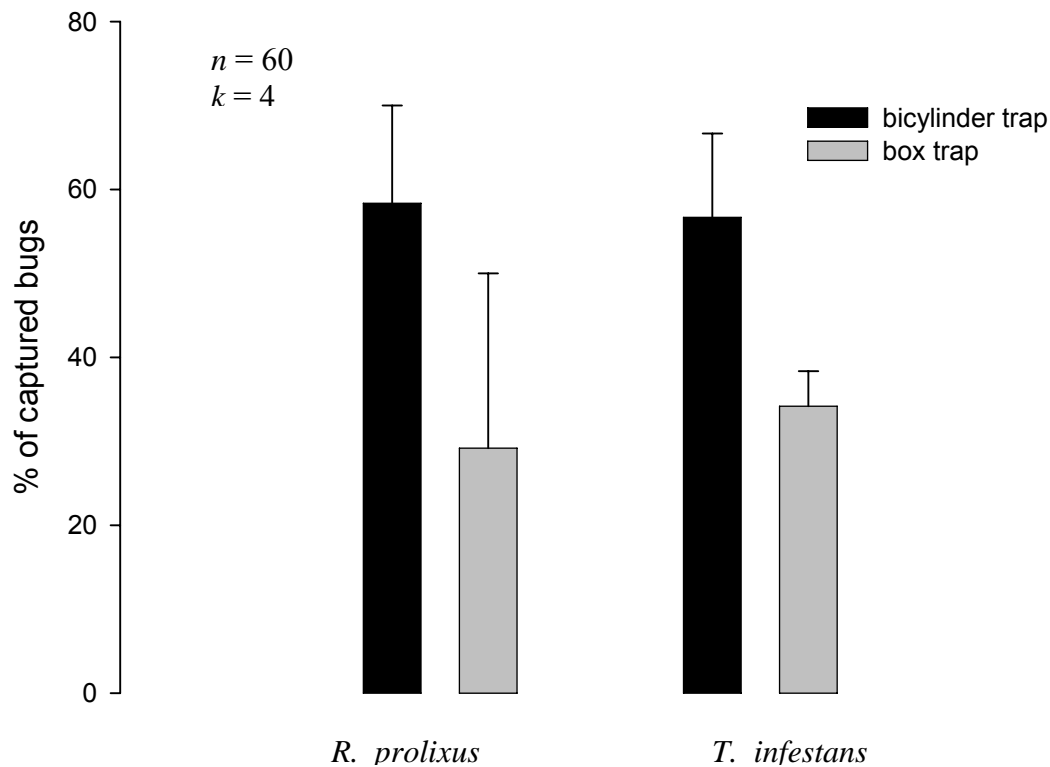


Fig 4. Proportion of *R. prolixus* and *T. infestans* captured when the bicylinder trap and the box trap were compared in the experimental arena (see Methods). The number of bugs captured in the bicylinder trap was higher than in the box trap ($p < 0.05$; G-test; n is the number of bugs and k the number of repetitions). The *upper boundary lines* indicate the median and the *bars above* represent the maximum values. The median proportions of uncaptured bugs over 12 hours, i.e. outside the test and control traps, were 12.5% for *R. prolixus* and 10.8 % for *T. infestans*.

Even though, the cylinder trap proved less effective in capturing bugs than the bicylinder trap, the cylinder trap was more appropriate to test the effects of different odours and dispensers on triatomine capture. The number of *R. prolixus*, *T. infestans* and *P. geniculatus* captured using a CO₂ + NH₃ dispenser in test cylinder traps was significantly higher than in control traps for all three species tested (Fig 5). But, ammonia alone was not sufficient to attract *P. geniculatus* to the test trap (Table II). A dispenser that is used in a trap for mosquitoes (see Methods), allowed more captures of *P. geniculatus* in the test trap compared to the control. However, the same dispenser did not elicit such an effect on *R. prolixus* nymphs (Table II).

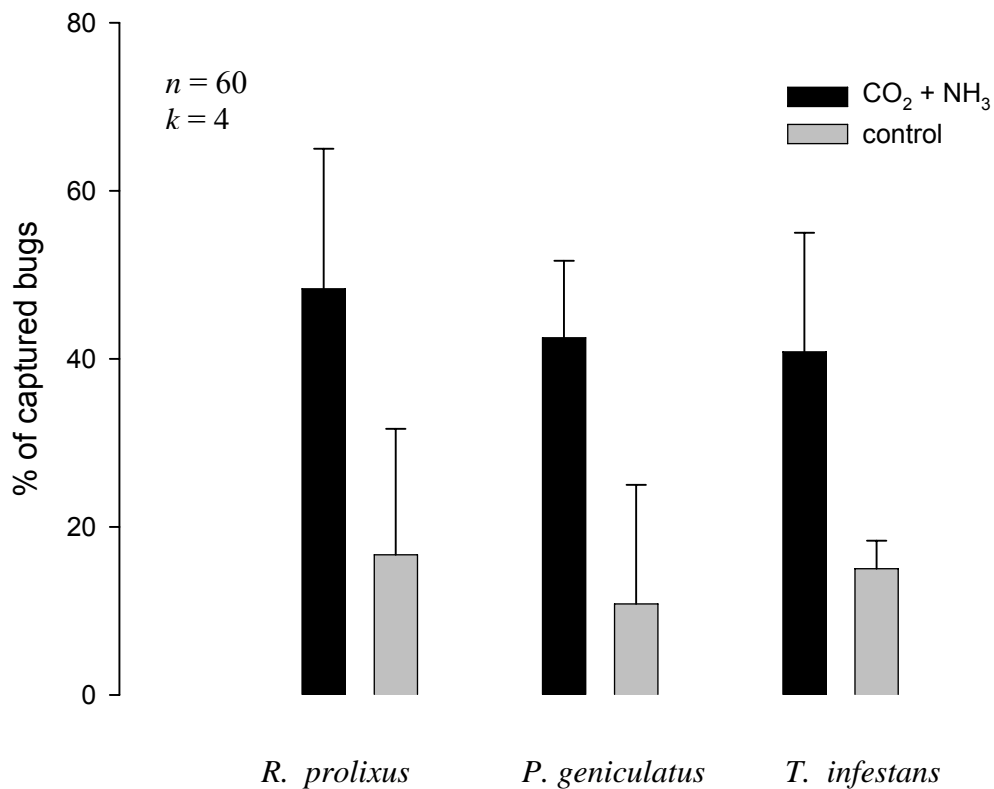


Fig 5. Proportion of triatomines captured in test (dispensing CO₂ and NH₃) and control traps using cylinder traps in the laboratory arena. Three different species were tested; *R. prolixus*, *P. geniculatus* and *T. infestans* following overnight experiments (see Methods). The proportion of bugs found in the test trap with the CO₂ plus NH₃ dispenser is higher compared to the control trap for all three species ($p < 0.005$, $p < 0.001$, $p < 0.01$ respectively for each species; G-test; n is the number of bugs and k experiment repetitions). There were no differences between species ($p > 0.5$; G-test). The upper boundary lines indicate the median and the error bars above represent the maximum values. The median proportions of uncaptured bugs, i.e. outside the test and control traps, were 36.7, 43.3, 42.5%, respectively, for *R. prolixus*, *P. geniculatus*, and *T. infestans*.

A test was made to evaluate the efficiency of double adhesive tape in capturing *R. prolixus* nymphs that tried to enter the bicylinder trap baited with CO₂ plus NH₃ (see Methods). The trap with the outer sticky surface only captured a median of 0.84 (IQR 1.67 %) bugs compared to the same cylinder trap without the sticky surface that captured a median of 95.83 % bugs (IQR 1.70 %; $n=60$, $K=2$; $p < 1 \times 10^{-15}$, G-test).

DISCUSSION

Using our previous knowledge on the sensory ecology of triatomines (Taneja & Guerin 1995, 1997, Guerin *et al.* 2000, Guerenstein & Guerin 2001, Diehl *et al.* 2003, Otálora-Luna *et al.* 2004) and behavioural studies presented here, we have developed a device that has proved to be effective in the laboratory for capturing triatomines. The bicylinder trap design in itself is so efficient at capturing triatomines that it is inappropriate for testing the effects of any added chemicals on trap efficacy. This could also be related to the fact that vapours emanating from the baited bicylinder trap increased the captures by the control trap in our test arena (Fig 1). Despite the fact that triatomines entering the bicylinder trap must climb a vertical surface and do not need to do so when entering the box trap, the insects entered the former trap in higher numbers than in the box trap (Fig 3). This could be related to the fact that the bicylinder trap provides its entire external perimeter for the bugs to enter at ground level (Fig 1), while the box trap only provides a rectangular slit entrance to access its interior on one side at ground level.

The cylinder trap with a slit on one side at 9.5 cm from the ground makes it more difficult for bugs to enter, but it proved practical for testing the effects of chemicals on bug capture. As suggested in Chapter II, the CO₂ plus NH₃ mixture is capable of attracting triatomines of the three different species *R. prolixus*, *P. geniculatus*, *T. infestans* in a trap. The response evoked by these two simple and ubiquitous molecules is not surprising as both have proved capable of triggering strong appetite behaviours by *R. prolixus* in a servosphere (Otálora-Luna *et al.* 2004), and both are present in host breath and wastes, as well as in triatomine faeces. Ammonia, also present in triatomine faeces and host wastes, has proved to attract *T. infestans* (Taneja & Guerin 1997) and *R. prolixus* (see Chapter III) in a servosphere, but it did not induce more significant captures of *P. geniculatus* nymphs in the cylinder trap. Rose (1998) found a similar result in semi-natural conditions, i.e. ammonia did not capture more bugs alone than when mixed with CO₂. The ammonia produced by our dispenser seems to be sufficiently stable over time and cheap enough for practical applications (Fig 5), but the CO₂ produced by the salts drops just after a few hours to 200 ppm above ambient.

Furthermore, when volatiles emanating from the mosquito lure containing aliphatic acids and NH₃ were tested in the cylinder trap, this resulted in an increase in the number of *P. geniculatus* captured. As such effect was not observed when the mosquito lure was tested in *R. prolixus*, the effectiveness of this lure in bugs capture is not conclusive.

The bicylinder trap with the double adhesive tape attached as a 5 cm band around the outside of the base cylinder to catch bugs trying to climb into the device baited with CO₂ and NH₃, captured very few bugs compared to the same trap without the sticky surface. It seems that bugs avoided the sticky surface. Double adhesive tape attached in a similar manner has been used in a trap baited with a live mouse in field (Noireau *et al.* 2002). In this case, cues emanating directly from the host were strong enough to catch bugs on the sticky surface. The capture method of the bicylinder trap seems more efficient without the double adhesive tape. As triatomine nymphs can easily climb the vertical base cylinder surface and are effectively captured once inside, the accessory sticky surface is not necessary. Nymphs and adults of all tested species, except for *R. prolixus* adults, are incapable of climbing smooth surfaces. Nevertheless, *R. prolixus* and other species that bear fossulae spongiosae on the tarsus may be capable of escaping from our bicylinder

trap. Since the triatomines enter the bicylinder trap so readily, it needs to be tested in the field or at least in semi-natural controlled conditions to evaluate its efficacy.

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Chapter V

General Discussions and Conclusions

Adaptation to haematophagy

All triatomine species live by sucking the blood of vertebrates regardless of the fact that most reduviids subfamilies are predaceous, i.e. live by sucking the haemolymph of invertebrates (Dolling 1991). Piercing mouth parts present ancestrally in Heteroptera facilitated the shift to the new host. Other physiological features needed by these bugs to be efficient haematophagous *spp.* were meliorated through the natural selection. Very probably two changes of behaviour initiated the blood sucking habit within the Reduviidae: a) the association of a triatomine ancestor with nesting mammals and b) the subsequent attempt to pierce vertebrate skin. The subsequent development of sensory and behavioural adaptations to search for *host nests* was essential for the evolutionary success of the subfamily Triatominae and olfaction was particularly important as most of its members are nocturnal.

Searching strategies

Triatomines have developed different strategies to locate food and refuge using chemical stimuli, the most ancient and universal source of information. These strategies can be classified according to the problems bugs need to solve (Weissburg & Dusenbery 2002): a) a search problem, to make contact with the odour (ranging) -which provides a larger target than the host itself-; b) an approach problem, of using the odour to move towards the source (guiding); c) a lost stimuli problem, of recovering contact with the odour plume and d) a termination problem, of determining that the source has been reached.

In still air *R. prolixus* indulge in random highly tortuous walking as has been reported previously for the tick *Amblyomma variegatum* on the servosphere (McMahon & Guerin 2000). This behaviour is probably due to the incapacity of both arthropods to maintain a straight course in the absence of a “collimating” stimulus. But, in the presence of an air current triatomines walk relatively straight downwind or crosswind. The air stream could be inducing bugs to run away from the desiccating air stream or to find a more sheltered place but it is also possible that crosswind walking could be part of ranging behaviour. As chemical stimuli are not available in the air, the bug must adopt an optimal pattern of locomotion to gain contact with such stimuli. The animal has no information about the location of a specific goal however, it perceives an air current that could eventually carry chemicals originating from an interesting source. According to the theory of optimal search (Dusenbery 1992) if an animal that moves in two dimensions is searching for a relevant stimulus plume in an air stream, cross-current searching will be 50% more efficient than parallel-current searching. The basic argument that sustains this geometrical estimate is simply that the plume presents a potentially larger target size for a bug that is walking across the current than for a bug that is walking parallel to the current.

After the bug has detected a stimulus that can be employed to move closer to a specific goal, the approach phase begins. The combination of CO₂ and NH₃ in an air stream has allowed us to characterize the guiding behaviours performed by starved *R. prolixus* nymphs on the servosphere. Other attractants of natural and synthetic origin tested on *T. infestans* and *P. geniculatus* nymphs evoked similar goal oriented behaviours. The increase in walking speed, upwind displacement, stops with antennal reaching, and the decrease in cleaning behaviours, all serve to increment the efficiency of the guiding behaviour. Saltatory search (O'Brien *et al.* 1990), a foraging tactic that consists in the

alternation between walks and pauses where antennal scanning occurs, was observed on the three species tested. This behaviour must also play a crucial role in the directed walk towards the stimulus source. Pauses permit bugs to check stimulus direction and could also be useful for the animal to rest imperceptible for a short period before the next movement, as continuous movement of the insect would make it more apparent for potential enemies and for the nearby host. *P. geniculatus* places dust particles on its abdomen by flexing its hind legs during short pauses. This illustrates the necessity of these animals to remain unapparent while walking.

Those behaviours undertaken by triatomines to regain contact with the chemostimulus (casting) after it has been removed from the air stream consist of continuing the upwind walk for some seconds at relative high speed. Then, a shift to crosswind displacement occurs and predominates over downwind displacement, so the insect returns to a ranging strategy as above. Similar strategies of casting have been reported in flying moths when these lose the odour plume (Kaisling 1997). Additionally, antennal reaching and tapping, performed in the presence of the stimuli, persists after cessation of the stimulation.

The tactics used by triatomines to solve the problem of “determining that the source has been reached” has not been treated in detail here because the sphere is an open loop setup. Even so, the reaching behaviour observed during stimulation could be an attempt by the bug to reach on to a nearby host. In our experiments the bugs were subjected to constant concentrations of chemostimuli on the servosphere. It would be interesting to run experiments where the concentration of the stimulus increased while the bug walks upwind and decreased when it walks downwind and ceased when it walked a certain distance crosswind. With such experiment we would be placing the bug in a virtual plume. With a narrowing plume as one approaches the source it would be possible to test if the bug is capable to measure distance between the limits of the cone to calculate the distance to the source. If this capability exists one may predict that when the bug arrives at the source it will perform more reaching than before.

Wastes and other materials abundant in vertebrate nests as sources of information

We have already discussed how volatile stimuli are searched for and how such stimuli are used by walking bugs to find resources (arguably flying adults could also use odour plumes to find resources). Another basic question that has also gained our interest is the nature of the products that induce such guiding behaviours in triatomines. Taking advantage of knowledge of the ecology and evolutionary origin of triatomines, we have found effective attractants for these insects in those materials that may be abundant in a vertebrate nests, e.g. faeces, urine, exocrine gland secretions and feathers. Activation and attraction behaviours are elicited by extracts of bird and mammal excretions. From the diverse volatiles compounds found in vertebrate wastes (Appendix 2, 3), amines (Chapter III) elicited the best behavioural response and dimethylamine and ethylamine were the most effective attractants among this chemical group. Aside from these short chain amines there are certainly other components that contribute to triatomines recognition of resources. Evidence for this is that other compounds such as hexylamine and isobutylamine, that did not prove attractive to these bugs, elicited activation behaviours. Furthermore, the short chain carboxylic acid 4-methylpentanoic acid, also present in vertebrate wastes, proved attractive to *R. prolixus* albeit the high concentration tested (1-10 mg source dose).

Chemotactic responses to dimethylamine and ethylamine have been recorded here for three triatomine species that occupy different ecotopes and are phylogenetically distant. Species within the *Triatoma* and *Panstrongylus* genera are considered to be closely related belonging to the Triatomini tribe, but more distant from *Rhodnius* which belongs to the Rhodniini tribe (Gaunt & Miles 2000). The Triatomini and Rhodniini diverged between 48.9 and 64.4 mya during the lower Tertiary period, estimated on a nuclear rDNA-based molecular clock (Bargues *et al.* 2000). Nevertheless, the fact *R. prolixus*, *T. infestans* and *P. geniculatus* species share a common way of life, being associated with nesting vertebrates, explains the common behavioural feature of attraction to vertebrate wastes.

As attraction to vertebrate wastes must be fundamental for haematophagous insects that are associated with nesting vertebrates, we could expect to find examples of similar chemotactic behaviors in other arthropod groups. Sandflies attraction to vertebrate faeces volatiles (Dougherty *et al.* 1995) confirms this and the use of dimethylamine and 1-pyrroline by tephritid fruit flies to localize duck faeces as a source of nitrogen (Robacker *et al.* 2000) demonstrates the importance of amines as chemical source of information.

Implications for triatomine domiciliation

Even though all the steps of triatomine adaptation to human dwellings are not fully understood, it is generally accepted that they do not occur at once but in a succession of steps (Coimbra 1988). *T. infestans* and *R. prolixus* show domiciliary habits, but originally they were both exclusively wild as most triatomines still are. Sedentary practices and domestication of vertebrates by highland precolumbian civilizations facilitated triatomine colonization of the peridomestic and domestic habitat thousands of years ago. Similar scenarios are currently generating domiciliation by sylvatic triatomine species in regions which formerly were not endemic for Chagas disease. This comes about when wild habitats harbouring bugs are damaged by human activities such as population displacements due to war, burning and devastation of primary forests, over-hunting of natural hosts and introduction of peridomestic farm animals and other agricultural practices in natural reserves, facts related to increased poverty in Latin America, but also to government programs of urbanization and agriculture (Walter 2003, Walter *et al.* 2005, Barata *et al.* 2005). Recent invasion of human dwellings by *P. geniculatus* carried in tree branches, by its own means of locomotion (Valente 1999, Wolff & Castillo 2000) and through observed association with pigs (Valente *et al.* 1998) and rats (Reyes-Lugo & Rodriguez-Acosta 2000) in forested regions altered by man are clearly preliminary steps of a domiciliation process by this species. Other sylvatic species of triatomines forced by human alteration of their environment are following the same pattern of domiciliation as *P. geniculatus*, i.e. a search for new sources of food and refuge in human dwellings.

Our results suggest that odours emanating from human settlements may serve sylvatic and domestic triatomines, just as for sandflies, as a source of information that guides invasion of houses. The presence of latrines and domestic animals (and their wastes) around the houses increase the production of volatiles that attract these insect vectors of disease to the human habitat. This in part explains why lowland Indians living in the Amazon basin have seldom been reported suffering from Chagas disease or Leishmaniasis, common diseases among non-Indian farmers in the same region (Coimbra 1988). Amazon Indians do not domesticate animals and do not accumulate high quantities of wastes around settlements because of their nomad culture. And more important, they do not inflict ecological

interference on triatomine and sandfly habitats as the newer settlers do through the practice of intensive agriculture in the Amazon region.

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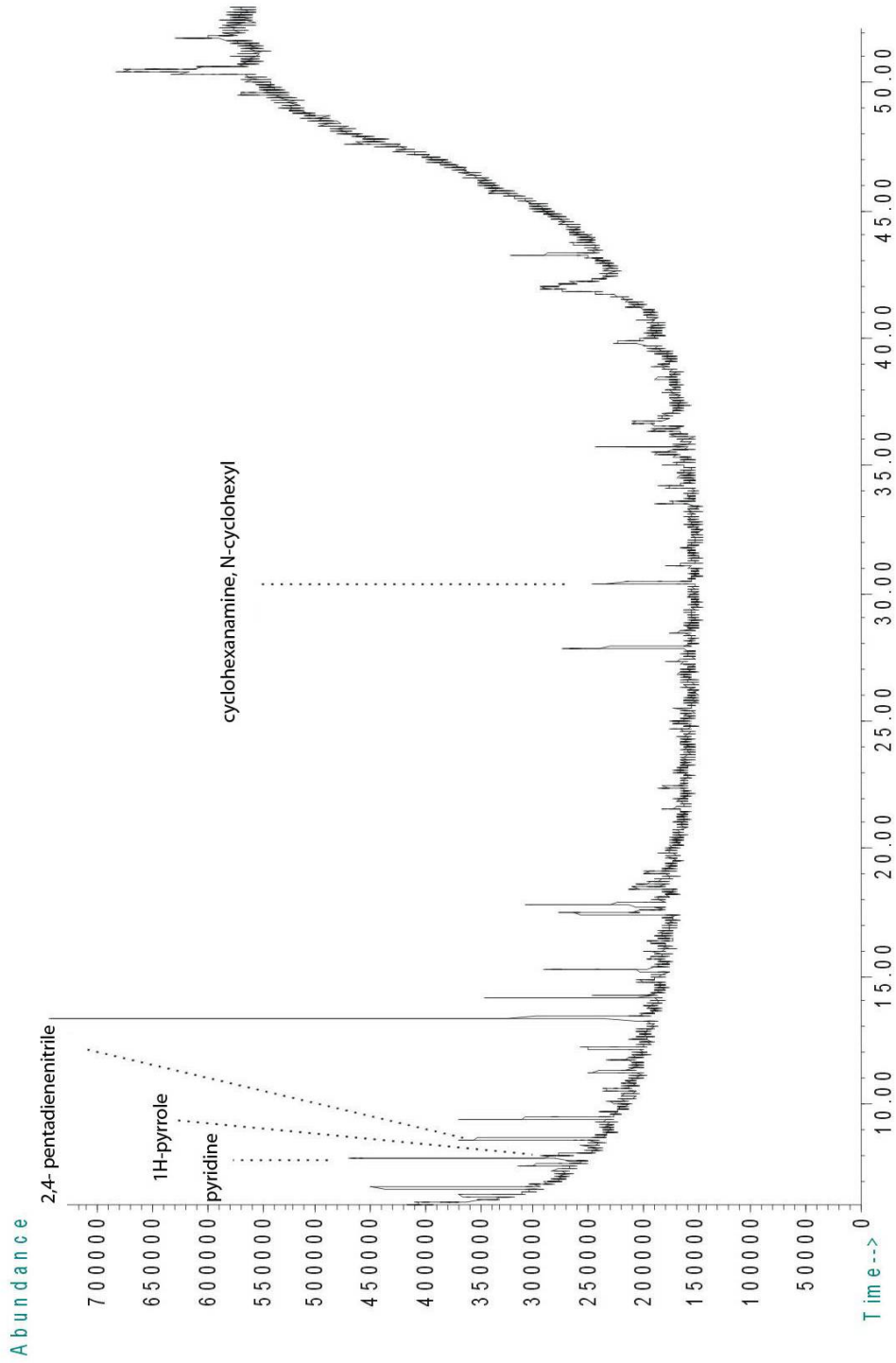
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Appendices

Appendix 1



Identification of compounds present in chicken faeces. Amines and nonpolar volatiles were collected in dichloromethane from a basic and neutral fraction and analyzed by GC-MS. Compounds that contain nitrogen were identified in the extract.

Appendix 2

Effects of different lures on capture of triatomine bugs in test versus control bicylinder traps.

Experiment	Species	N° of tests (k)	Mean N° of bugs captured by a test trap	% of bugs captured by test and control traps	Bugs captured in test as % of the total bugs captured	G-test p value (test versus control)
NH ₃	<i>T. infestans</i>	2	13.42	100.00	49.17	n.s ⁵
CO ₂ + NH ₃ ¹	<i>R. prolixus</i>	4	33.03	95.42	69.43	n.s
CO ₂ + NH ₃	<i>T. infestans</i>	3	10.00	100.00	50.00	n.s
4MPA + NH ₃ ²	<i>R. prolixus</i>	5	7.67	92.50	48.65	n.s
yeast ³	<i>T. infestans</i>	4	25.83	100.00	62.50	n.s
urine ⁴	<i>T. infestans</i>	4	30.06	100.00	65.83	n.s

(1) CO₂ and NH₃ dispensers are described in Materials and Methods, Chapter IV.

(2) 4MPA was used at 10 mg diluted in dichloromethane on a filter paper and placed in the trap in a glass vial, Materials and Methods, Chapter III.

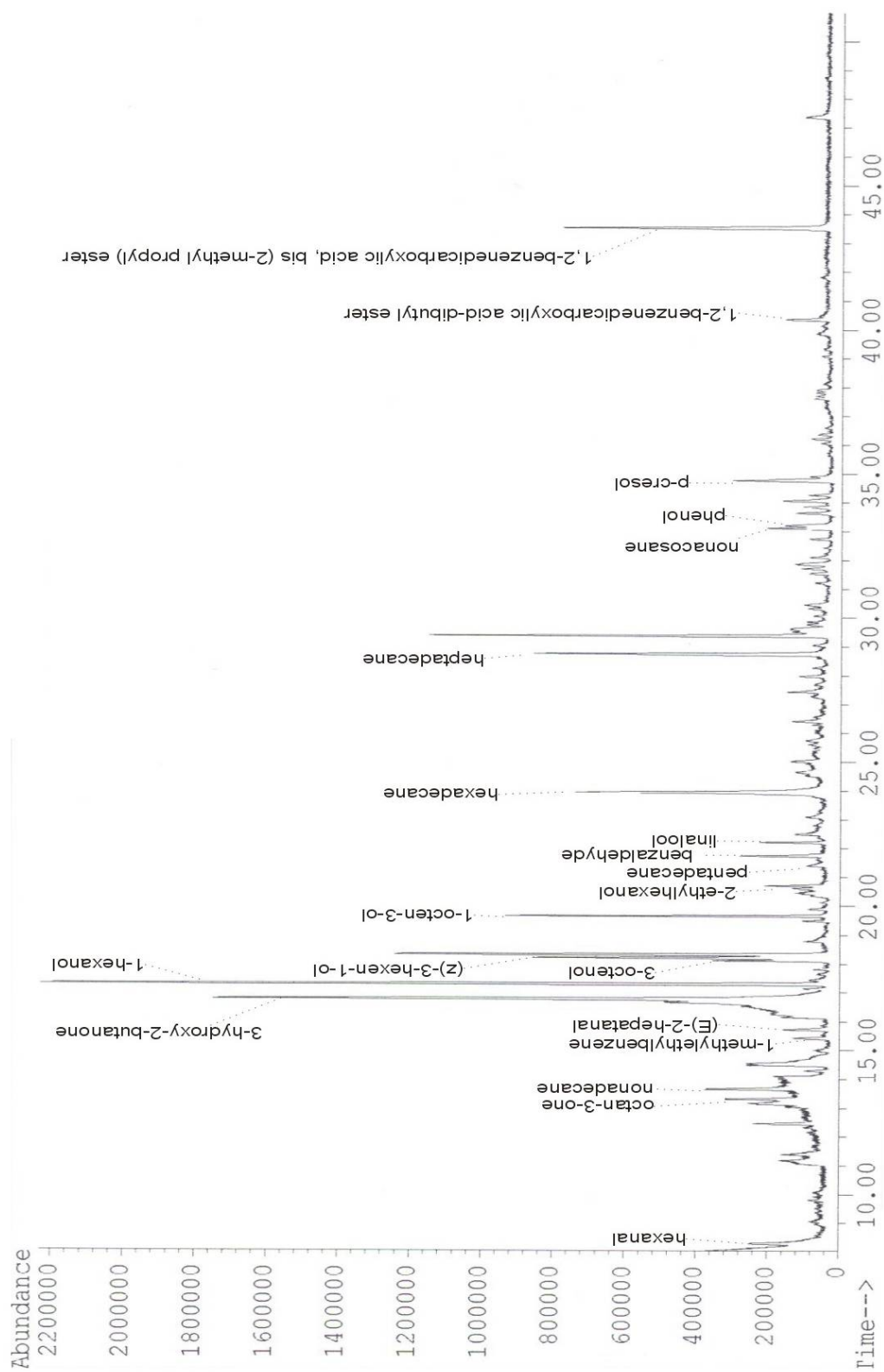
(3) 6 g of yeast and 4 g of sugar in 30 ml of water in a glass vial were used as lure.

(4) 30 ml of male human urine in a glass vial was used as lure

(5) n.s. not significant†

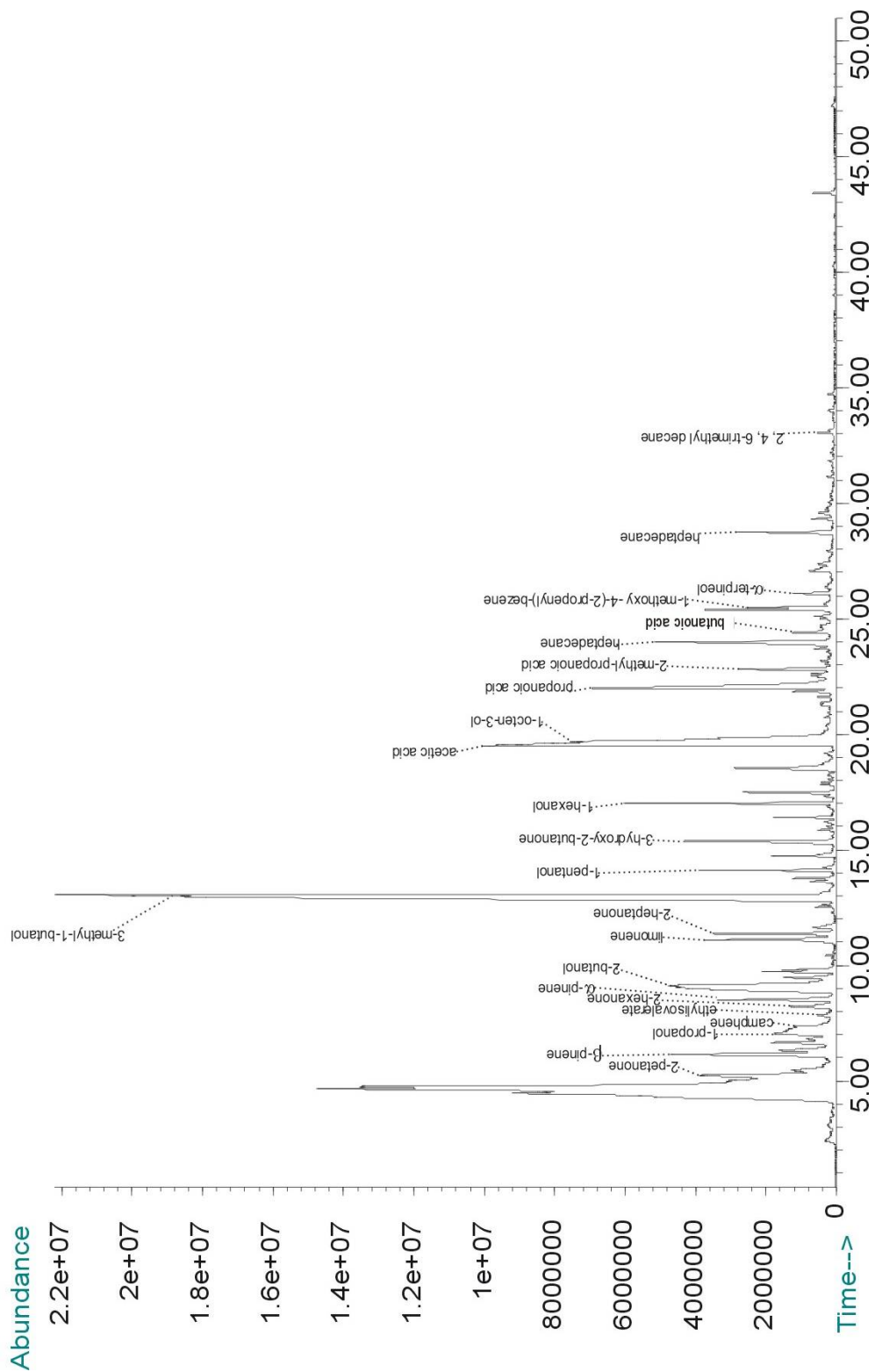
There were no differences between the different sets of experiments.

Appendix 3



Identification of compounds present in the headspace of chicken faeces. Volatiles were trapped on a porous polymer and analyzed by GC-MS (see Methods of Chapter III).

Appendix 4



Identification of compounds present in the headspace of guinea pig faeces. Volatiles were trapped on a porous polymer and analyzed by GC-MS (see Methods of Chapter III).