

The Response of the Blood-sucking Bug *Triatoma infestans* to Carbon Dioxide and other Host Odours

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Abstract

Behavioural responses of *Triatoma infestans* larvae to carbon dioxide and other odours of vertebrate origin were investigated in a locomotion compensator. *T. infestans* oriented towards airstreams enriched with carbon dioxide exhibiting a threshold response between 300 and 400 p.p.m. above the ambient CO₂ background. The accuracy of the oriented response to carbon dioxide improved with stimulus intensity. Remarkably, insects did not show any change in their sensitivity threshold to carbon dioxide with the starvation time. The attractiveness to carbon dioxide depended on the time of the day, i.e. these nocturnal bugs only oriented towards carbon dioxide-loaded airstreams during the first hours of the scotophase. L-lactic acid did not evoke oriented responses when it was presented as a single stimulus in a wide range of intensities. However, a marked synergism was evident when L-lactic acid was combined with a sub-threshold concentration of carbon dioxide. Under this condition, the threshold response to carbon dioxide decreased to 75–150 p.p.m. above ambient CO₂ background. The isomer D-lactic acid evoked no response, either alone or in combination with carbon dioxide. When insects were stimulated with 1-octen-3-ol a significant positive orientation was found. This response was not modified by the addition of carbon dioxide.

Key words: behaviour, carbon dioxide, Chagas' disease, host location, semiochemicals, *Triatoma infestans*

Introduction

The haematophagous bug *Triatoma infestans* (Klug) (Hemiptera, Reduviidae: Triatominae) is the main vector of Chagas' disease in southern South America. At present this disease is one of the most important health problems in Latin America. According to the World Health Organization >18 million people are affected by this disease and ~90 millions are at risk of becoming infected. *T. infestans* is almost exclusively associated to human dwellings, with only one known wild population in Cochabamba, Bolivia (Dujardin *et al.*, 1987; Bermudez *et al.*, 1993). These bugs colonize human houses in a passive and/or active way, living in wall crevices and ceilings of rural houses and feeding mainly on humans, dogs and hens (Gürtler *et al.*, 1997). During hours of daylight they display little activity and are usually found in a quiescent state or 'akinesis', congregated inside refuges. During the night they display most of their activity, e.g. they search for food, mate, oviposition sites, etc. (Lazzari, 1992; Lorenzo and Lazzari, 1996, 1998). *T. infestans* exhibits a marked circadian organization of its

activities. Their locomotion is divided in two endogenously controlled temporal windows: one at dusk and another at dawn (Lazzari, 1992). These two peaks of activity appear to be associated with host seeking (dusk) and refuge search (dawn), respectively (Lazzari, 1992; Lorenzo and Lazzari, 1998).

In order to locate a food source, triatomines exploit different sensory cues released to the ambient by their vertebrate hosts. The heat emitted by warm-blooded animals is one of the most important cues guiding triatomines to their food source (Wigglesworth and Gillett, 1934; Lazzari and Núñez, 1989; Flores and Lazzari, 1996). Barrozo *et al.* (2003) showed that water vapour alone also represents a sufficient orientation cue for these bugs at short range and also enhances the response to heat in the long range. Several odours emitted by hosts are known to be involved in attractive responses in these bugs (Núñez, 1982; Taneja and Guerin, 1995, 1997; Guerenstein and Guerin, 2001), i.e. they activate and/or guide the insects to the proximity of the emitter. Among them, carbon dioxide, an activator and/or attractant for many other blood-sucking insects (Lehane,

1991), modulates the behaviour of triatomines such as *Rhodnius prolixus* and *T. infestans* (Wiesinger, 1956; Núñez, 1982; Taneja and Guerin, 1995). Wiesinger (1956) found that a warm (36–40°C) mixture of carbon dioxide and air was more attractive for *T. infestans* than warm air alone. According to Núñez (1982), CO₂ alone (0.1%, 1000 p.p.m.) would be enough to orient the bugs. Guerenstein *et al.* (1995) found attraction towards aerobic cultures of yeast (*Saccharomyces cerevisiae*) in *T. infestans* and provided evidence suggesting that the CO₂ emanating from these cultures would be mainly responsible for attracting these insects. Taneja and Guerin (1995) found oriented responses to 0.6% (6000 p.p.m.) of carbon dioxide in *T. infestans* and *R. prolixus* walking on a locomotion compensator. The same authors also observed that *T. infestans* showed a stronger attraction to mouse odour than to CO₂ alone.

Olfactory receptor cells on the triatomine antenna have shown to respond to carbon dioxide and carbon monoxide (Mayer, 1968). However, the response was not as strong as when human breath was blown over the antennae. Other volatile substances such as L-lactic acid, pyruvic acid, short-chain carboxylic acids, aldehydes, pyridine, furan, terpenes, alcohols and amines also evoked electrophysiological responses in antennal chemoreceptors of triatomines (Mayer, 1968; Bernard, 1974; Taneja and Guerin, 1995; Guerenstein and Guerin, 2001; Diehl *et al.*, 2003).

The aim of this work was to gain insights on the role of carbon dioxide in the host seeking behaviour of *T. infestans*. We analysed the orientation response evoked by airstreams artificially loaded with carbon dioxide. Additionally, we measured the threshold of sensitivity to carbon dioxide in behavioural assays, as well as its dependence on the nutritional state of the insects and on the daytime. Finally, we investigated the response to carbon dioxide when presented together with other volatiles associated to their warm-blooded hosts, to explore possible interactions between components of those odour blends on the behaviour of *T. infestans*.

Materials and methods

Animals

Third-instar larvae of *T. infestans* were provided by the Servicio Nacional de Chagas (Córdoba, Argentina) and maintained in our laboratory under a 12:12 h L:D illumination regime, at 28°C and 30–50% RH. The bugs were fed weekly on hens until moulting. Insects were starved for 20–30 days post-ecdysis before assays, except as otherwise stated (see below).

All the assays were conducted in a room maintained at 26 ± 1°C and under functional darkness (see below). Most of them were performed during the bugs' scotophase, except in those experiments that the daily variation on the behavioural response was evaluated. The assays along a day were divided in two moments, the photo- and the scotophase. The

photophase covered two intervals, from 0:00 to 5:00 h *Zeitgeber* time (*Zt*) and from 7:00 to 12:00 h (light on at 0:00 *Zt*), corresponding to the early and late day. The experiments performed during the scotophase also comprised two periods, from 12:00 to 17:00 h and from 19:00 to 0:00 h, conforming, respectively, to the early and late night.

Recording of walking paths

A locomotion compensator was used to analyse the orientation behaviour of the insects in an open-loop design for translation, but allowing free rotation (modified from Dahmen, 1980). It consisted of a hollow Styrofoam sphere (9.7 cm diameter, 2.5 g wt) suspended by a vertical airstream. A bug was tethered by its dorsal abdomen to a freely rotating stiff steel wire centred at the apex of the sphere, using double-sided sticky tape. When the tarsi contacted the surface of the sphere, the animal started to walk with a normal posture, displacing the sphere under its legs. Although an insect could walk and rotate freely changing its direction of locomotion, it was unable to change its distance to the airstream exit. The locomotion compensator included an optic sensor of a PC mouse separated by 1 mm from the inferior pole of the sphere, i.e. on the same axes of the insect position, but in the opposite side (Figure 1). The movements of the sphere induced by the insect were fed to a PC every 0.2 s (sampling interval) as *x* and *y* coordinates with the aid of software designed *ad hoc* (Diego Anfossi, unpublished). The walking paths of the bugs were reconstructed and analysed in their spatio-temporal components (see below).

The assays were monitored from the outside of the experimental room with the aid of an infrared-sensitive camera provided with an array of infrared LEDs (emission 900 nm). This light illuminated the scene without being perceived by the bugs (Reisenman *et al.*, 1998).

Stimulus delivery

A simultaneous-discrimination bioassay was developed, by confronting insects to two opposite airstreams (180°), both maintained clean (control versus control), or one bearing the test stimulus while the other was kept clean (control versus test). The rationale behind choosing two opposite air currents is related to the fact that this species exhibits spontaneous positive anemotaxis to odourless airstreams (Barrozo *et al.*, 2003). In this way, an insect exposed to two opposite clean airstreams (control versus control) could choose to walk towards any of the two streams, resulting in a non-oriented behaviour. This situation would be clearly distinguishable from the test assays that would provide an oriented direction towards an attractive odour source. On the other hand, the use of two clean currents settled at angles different from 180°, in a control condition, would result in an orientation towards a mean direction between both streams positions, i.e. different from a non-oriented behaviour. As a consequence, an attractive response of bugs to an

stream loading an stimulant odour should be tested against a similar positive response or average vector of the control condition, each sample carrying its own errors, such as dispersion and sinuosity of the paths. Therefore, our system allowed us to clearly differentiate between the two conditions (control or test) from a non-oriented behaviour or no mean direction, enhancing in addition the power of the statistical analysis.

The two opposite flows consisted of continuously delivered charcoal-filtered air, at $26 \pm 1^\circ\text{C}$ and $85 \pm 2\%$ RH, delivered tangentially to the apex of the sphere on the insect position (Figure 1). A pump forced the room air through the charcoal filter. Each airstream passed through two glass bottles arranged in series via silicone tubing (0.4 cm inner diameter), connecting ultimately with a glass tube (0.67 cm inner diameter, 5 cm length). Inside each glass bottle different odour sources were located. The glass-tube exits were placed 3 cm away from the insect location (Figure 1). The velocity of the air was set at 6 cm/s, measured at the exit of each glass tube, controlled by a needle valve and monitored by a flowmeter. In a normal position, the distance from tip to tip of the antennae is ~ 0.9 cm and a freely rotating bug scans a maximum span of 1.85 cm at the apex of the sphere (Barrozo *et al.*, 2003).

The first glass bottle (250 ml) loaded the passing air current with CO_2 , which was chemically generated inside this recipient through the following reaction: $\text{Na}_2\text{CO}_3 + \text{H}_2\text{SO}_4 \rightarrow \text{CO}_2 (\text{g}) + \text{H}_2\text{O} + \text{Na}_2\text{SO}_4$. Different solutions of Na_2CO_3 (0.01, 0.02, 0.04, 0.06, 0.1, 0.2, 0.3 M) were injected with a synchronic-motor-driven syringe at a constant flow (0.08 ml/min) over the recipient containing 100 ml of H_2SO_4 (0.01 or 1 M) to generate different amounts of CO_2 . The charcoal-filtered air passed at a flow rate of 90 ml/min over the surface of the reaction mixture, to achieve ~ 75 , 150, 300, 400, 800, 1500, 2300 p.p.m. of CO_2 above the ambient level (400 ± 20 p.p.m.). The reaction fluid was continuously stirred to ensure an appropriate homogenous mixture of the chemicals and stable CO_2 production. The CO_2 concentrations were verified between assays with a non-dispersive infrared sensor (model EGM-3, range 0–5000 p.p.m., accuracy 0.5% of the reading lecture; PP Systems). The control current (ambient CO_2 or 0 p.p.m. over the ambient) consisted of an airstream passed through an identical glass bottle, but only filled with 100 ml of H_2SO_4 (0.01 or 1 M). This allowed, in addition, maintenance of identical relative humidity in both streams.

The synthetic chemicals tested as stimuli were L-(+)-lactic acid (>99% purity), D-(–)-lactic acid ($\sim 95\%$ purity) and 1-octen-3-ol (>98% purity) (Fluka Chemie GmbH). Decadic solutions (1, 10, 100, 1000, 10 000 $\mu\text{g}/50\mu\text{l}$) of D/L-lactic acid and 1-octen-3-ol were prepared in distilled water or hexane, respectively. Fifty microlitres of the test solution and 50 μl of the corresponding solvent (control) were loaded onto different filter paper strips (2.5 cm^2) and each placed in the corresponding second glass bottle of the

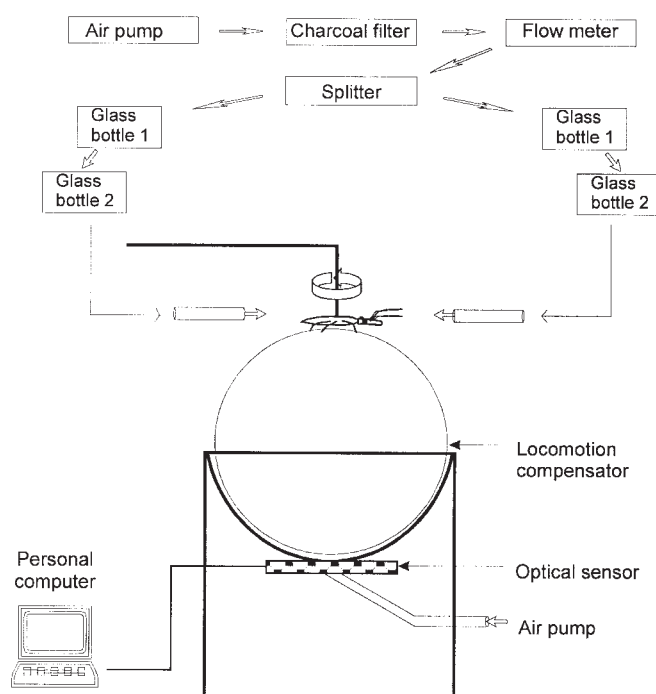


Figure 1 Experimental arrange for recording the oriented response of *T. infestans* to carbon dioxide and other chemicals under open-loop conditions. The locomotion compensator consisted of a hollow Styrofoam sphere suspended by a vertical airstream. The bug was fixed by its dorsal abdomen to a freely rotating wire centred at the apex of sphere. The insect could walk and rotate freely, but not change its distance to the air outlet. An optic sensor placed in the opposite pole of the sphere allowed registering the walking paths in a PC. A pump forced the room air through a charcoal filter and a flowmeter. The airstream was split in two identical streams that passed through the first series of glass bottles (glass bottle 1, 250 ml), inside one of which the CO_2 could be generated, whereas the other served as control. In the second couple of bottles (glass bottle 2, 20 ml) the chemical stimulus and the correspondent solvent could be loaded or kept empty.

series (vol. 20 ml, see Figure 1). Hexane was left to evaporate for 30 s before placing the filter paper in the glass bottle. The flasks were left to equilibrate for 10 min before presenting the evaporated vapours to the bugs. Filter papers for both control and test glass bottle were replaced after every test. The containers and the connecting tubing used to deliver the stimulus sources were replaced every time a different synthetic compound was tested, to prevent contamination.

In order to set up a condition of maximal stimulation for the insects, a live mouse was used as a stimulus source, i.e. as a positive control. The mouse was placed inside the 250 ml glass bottle of the arrangement.

To avoid eventual environmental biases, the sides of the test and control currents were changed between assays in a random fashion.

Before a test began, each insect was allowed 120 s to habituate to the experimental situation without stimulation (in still air). After this time, the two airstreams were switched on

and the walking pathway of the individual was recorded for 180 s.

Each individual insect was naive at the experimental situation, tested only once and discarded afterwards.

Data analysis

The experimental bugs were confronted with opposite airstreams carrying different combination of stimuli, i.e. control versus control, CO₂ versus control (100 ml H₂SO₄); L-lactic acid, D-lactic acid or 1-octen-3-ol versus control (corresponding solvent); CO₂ plus L-lactic acid, D-lactic acid or 1-octen-3-ol versus control (100 ml H₂SO₄ plus corresponding solvent); mouse versus control (empty bottle). The control versus control assays were carried out at the beginning of every experimental series. The pathways followed by the insects were analysed by means of circular statistics (Batschelet, 1965; Zar, 1984). The mean walking angle (α_i) displayed by each insect along the experimental time was computed and subsequently, for every experimental group a mean angle (α_m) and the length of the resultant mean vector (r) were calculated. The relative position of the stimulus-delivery current was conventionally designated as 0° and the control current as 180°. Whereas α extends from 0 to 360°, r varies between 0 and 1 (0 indicating a non-defined mean direction and 1 a straight path to a given direction). The statistical evidence of directness was tested following the Rayleigh test (Batschelet, 1965; Zar, 1984), being H_0 , the sampled population is uniformly distributed around a circle, versus H_a , the population does not follow a uniform circular distribution; circular uniformity implies no mean direction. When the data did deviate significantly from uniformity, the V -test (Zar, 1984) was carried out to assess whether the mean angle calculated from the sample was statistically distant from the stimulus direction (0°).

In order to compare angular dispersions between samples, angular distances of the individual data (α_i) from the expected mean angle or stimuli position (0°) for every experimental group of insects were computed. Differences were statistically evaluated through the Kruskal–Wallis test (critical value approximated to a χ^2 distribution) and followed by non-parametric Tukey-type multiple comparisons (Zar, 1984).

Additionally, for an easier visualization of the data, an orientation index was calculated, multiplying the cosine of the mean angle (α_m) by the length of the mean resultant vector (r), as $\cos(\alpha_m) \times r$. The orientation index varies between -1 and 1 (-1 indicates orientation away from the stimulus and 1 orientation towards the stimulus location).

From the individual pathways, the following parameters were also calculated: total distance walked (cm), total walking time (s) and mean walking velocity (cm/s). Besides, a linearity index was calculated by dividing the end-point distance of the pathway by the total distance walked, resulting in values between 0 and 1 (values around 0 indicate tortuous trajectories and 1 denote a perfect rectilinear paths). Linear data were analysed by means of one-way analysis of variance (ANOVA). In all cases, the data met the assumptions, i.e. normal distribution of residuals (Lilliefors test) and homogeneity of variance (Bartlett test) (Zar, 1984) and did not require transformation.

Results

The response of *T. infestans* to CO₂

When *T. infestans* larvae were stimulated with two opposite and identical odourless airstreams (control, 0 p.p.m. of CO₂ over the ambient) no oriented behaviour was observed (orientation index = -0.11; $n = 95$; Rayleigh test, not significant; Figures 2 and 3). This was also observed when insects

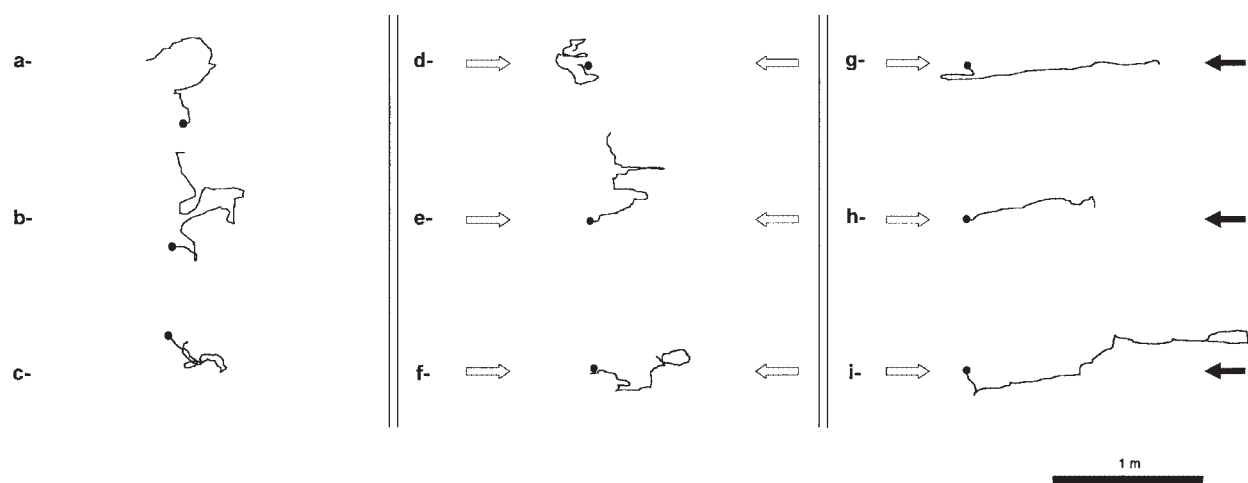


Figure 2 Sample records of pathways described by nine insects when confronted with: (a–c) still air conditions; (d–f) control versus control airstreams carrying ambient levels of carbon dioxide (400 ± 20 p.p.m.); and (g–i) control versus test airstream enriched with 1500 p.p.m. of CO₂ over the ambient levels. Dots denote the starting point of the walking bugs. Open arrows indicate the direction of the control air currents and filled arrows denote CO₂-enriched airstream direction.

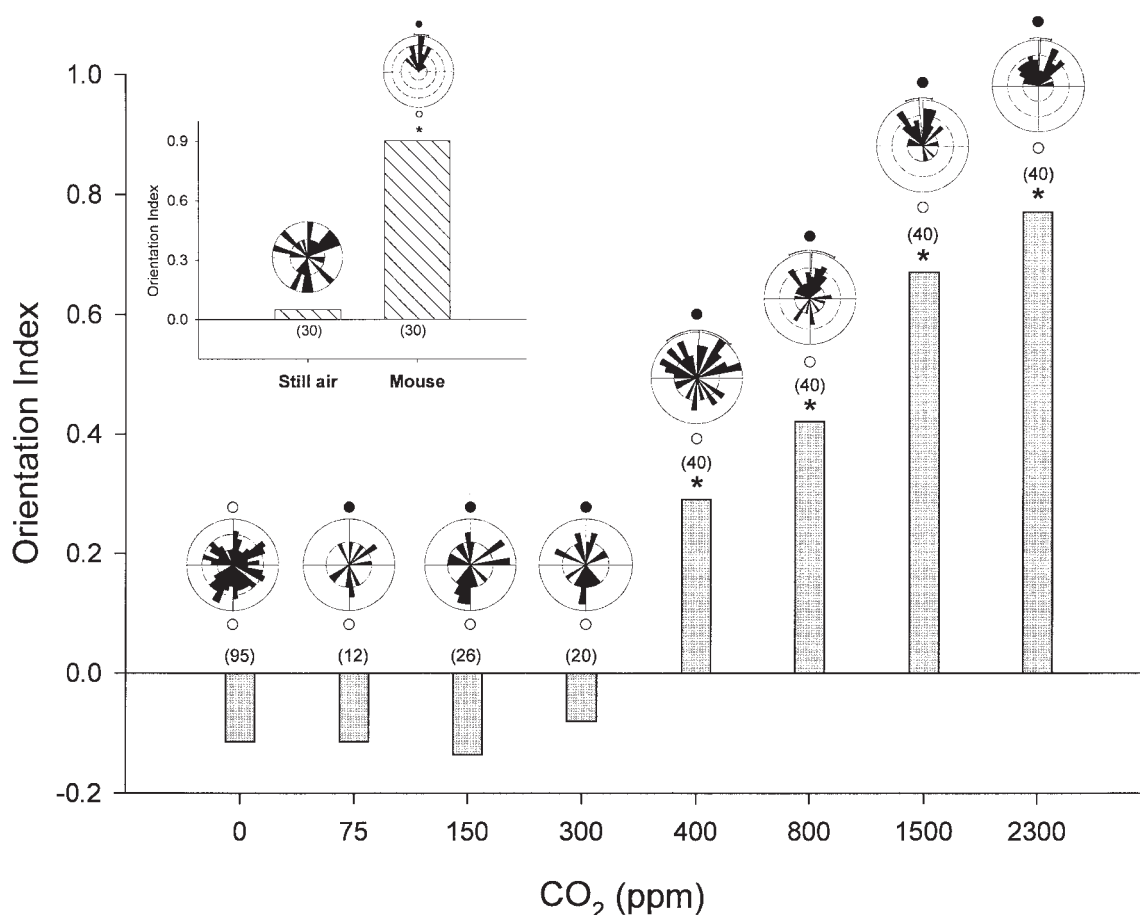


Figure 3 Orientation of *T. infestans* to airstreams carrying different levels of carbon dioxide. Results are expressed by means of an orientation index: 1 indicates orientation towards the stimulus and -1 orientation away from the stimulus. Asterisks denote the simultaneous occurrence of two conditions: statistically significant differences from a uniform distribution and a significant mean direction around the stimulus location (0°); $P < 0.05$ for both. The circular histograms (rose diagrams) represent the frequency of the angles displayed by the animals, which is proportional to the area of the wedge (bar width of 10°). The lines in diagrams depict the mean angle (α_m) and the 95% confidence. Open dots indicate control airstreams position (0 p.p.m. of CO_2 over the ambient) and filled dots denote test airstream location. The inset shows the orientation index and the rose diagrams of bugs under still air conditions or stimulated with a live host. Number of insects tested shown in brackets.

were kept under still air conditions on the locomotion compensator (orientation index = 0.05; $n = 30$; Rayleigh test, not significant; Figures 2 and 3). Nevertheless, larvae were able to detect and orient towards air currents enriched with carbon dioxide above the ambient levels. As an example, Figure 2 shows how the presence of carbon dioxide modified the walking behaviour of *T. infestans*. The insects walked significantly towards the carbon-dioxide-loaded airstream when they were stimulated with levels of at least 400 p.p.m. above the ambient (Figure 3; V -test, for 400 p.p.m.: $u_{(40)} = 2.6$, $P < 0.005$; for 800 p.p.m.: $u_{(40)} = 3.8$, $P < 0.0005$; for 1500 p.p.m.: $u_{(40)} = 6$, $P < 0.0005$; for 2300 p.p.m. $u_{(40)} = 6.9$, $P < 0.0005$). Below this level of carbon dioxide the insects exhibited a non-oriented behaviour on the sphere (Rayleigh test, not significant), indicating a threshold response to carbon dioxide lying between 300 and 400 p.p.m. over the atmospheric background (Figure 3). Above the threshold level, the insects usually maintained a rather straight course; however, their walking mean

direction was not right away to the test airstream outlet (0°), but usually biased some degrees to the right or to the left (Figure 3). The statistical analysis of the data displayed in Figure 3 (see rose diagrams), evinced a significant reduction of the angular dispersion with increasing levels of CO_2 above the threshold, from 400 to 2300 p.p.m. (Kruskal–Wallis test, $H_{(df,3)} = 14.2$, $P < 0.003$). The angular dispersion was 70.3° for 400 p.p.m., 59.2° for 800 p.p.m., 40.5° for 1500 p.p.m. and 35° for 2300 p.p.m. Animals exposed to 400 p.p.m. showed a significantly higher deviation from the stimulus direction than insects stimulated with 1500 and 2300 p.p.m. of CO_2 over the ambient levels (non-parametric Tukey-type comparisons, $Q_{(\infty,4)} = 4.8$ and $Q_{(\infty,4)} = 4.4$, respectively; $P < 0.05$ for both cases; Figure 3).

Besides, the behaviour of *T. infestans* towards a live host is shown in Figure 3, as a reference of maximal orientation response. It is worth mentioning that a live mouse constitutes a complex source of sensory stimuli, including heat, water vapour and a wide diversity of chemical cues. Bugs

oriented significantly towards an airstream enriched with host-emitted cues (orientation index = 0.9; V -test, $u_{(30)} = 5$, $P < 0.0005$).

We found no differences either in the total distance walked (range: 1.6–2.9 m), total walking time (106–130 s), mean walking velocity (1.0–2.5 cm/s) or linearity index (0.48–0.61) between insects exposed to still air or control conditions and those confronted to different levels of carbon dioxide over the ambient, including levels above the threshold concentration (ANOVA, not significant).

The effect of starvation

We tested the response of insects previously submitted to a prolonged starvation period (60 days) to evaluate any potential shift in the threshold level of the response to carbon dioxide. The starved animals stimulated with 300 p.p.m. of CO_2 above the background level showed a non-oriented behaviour on the sphere, while when exposed to 400 p.p.m. they exhibited a significant attraction to carbon dioxide, as stated for the previously tested bugs. As a consequence, they did not display any variation in the threshold sensitivity to carbon dioxide with starvation in the range 20–60 days post-ecdysis (orientation index for 300 p.p.m. = 0.12, $n = 20$, Rayleigh test, not significant; orientation index for 400 p.p.m. = 0.42, V -test, $u_{(20)} = 2.65$, $P < 0.005$).

The temporal modulation of the response

Taking into account the marked temporal organization of the behaviour of *T. infestans*, which exhibits a bimodal pattern of spontaneous locomotor activity with peaks at the early and the late scotophase (Lazzari, 1992), a temporal modulation of the response to carbon dioxide was tested. The experiments were conducted during different times of the day, during both the photophase and the scotophase, stimulating bugs with airstreams carrying CO_2 levels above the threshold of responsiveness (Figure 4). During the two periods of the photophase tested, the insects exhibited a non-oriented behaviour on the sphere for all concentrations analysed (Rayleigh test, not significant). The same behaviour was observed in the assays conducted during the late scotophase (Rayleigh test, not significant). By contrast, a significant oriented response towards the stimulus position was observed at the early night for all the CO_2 concentrations tested (V -test, for 400 p.p.m.: $u_{(20)} = 2.3$, $P < 0.02$; for 1500 p.p.m., $u_{(20)} = 4.3$, $P < 0.0005$; for 2300 p.p.m., $u_{(20)} = 4.6$, $P < 0.0005$). It is worth emphasizing here that, as mentioned in the Material and methods section, all assays described in the preceding sections were conducted during the initial hours of the scotophase.

Responses to CO_2 in combination with other chemicals

Behavioural responses to other attractants for blood-sucking insects, such as lactic acid and 1-octen-3-ol were tested, and the effects of combining them with carbon dioxide analysed. When offered alone upon an air current,

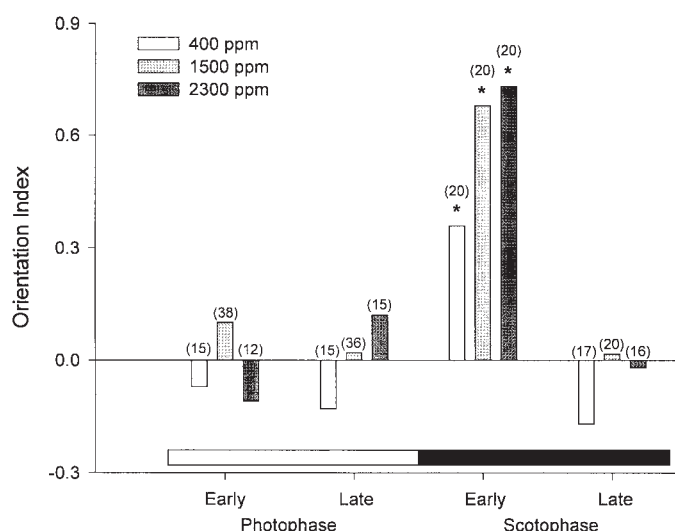


Figure 4 Orientation response of *T. infestans* to different concentrations of carbon dioxide (400, 1500 and 2300 p.p.m.) above the background, at different moments of the day, i.e. early and late photophase and early and late scotophase. Asterisks denote statistically significant differences from a uniform distribution and a significant mean direction around stimulus location (0°), both conditions met simultaneously at $P < 0.05$. Number of insects tested shown in brackets.

L-lactic acid was not able to evoke any attractive response of the bugs over a wide range of intensities (in all cases, Rayleigh test, not significant; Figure 5). However, the addition of 300 p.p.m. of CO_2 above the ambient level (a concentration below the threshold response), evoked a significant orientation of the bugs towards the stimuli direction, with 100 μg of L-lactic acid as the only effective stimulus dose observed under our experimental conditions (V -test, $u_{(20)} = 2.8$, $P < 0.002$). Hence, neither compound was attractive when tested alone, but highly effective in evoking orientation when they were presented together (i.e. synergism) in an adequate proportion and concentration.

In contrast to the synergism found between L-lactic acid and carbon dioxide, no effect was observed when D-lactic acid was combined at the same dose (100 μg) with 300 p.p.m. of carbon dioxide above the ambient levels (orientation index = 0.002, $n = 20$, Rayleigh test, not significant) nor when D-lactic acid was presented alone (orientation index = 0.14, $n = 20$, Rayleigh test, not significant).

Moreover, we evaluated to what extent the presence of L-lactic acid could increase the sensitivity of the insects to carbon dioxide. Figure 6 shows that the bugs were able to discriminate carbon dioxide levels of 150 p.p.m. above the ambient level in the presence of 100 μg of L-lactic acid (V -test, 150 p.p.m. plus L-lactic acid: $u_{(25)} = 3.6$, $P < 0.0005$; 300 p.p.m. plus L-lactic acid: $u_{(15)} = 2.7$, $P < 0.005$), i.e. the threshold response to carbon dioxide decreased from between 300 and 400 p.p.m. when presented alone to values between 75 and 150 p.p.m. when combined with L-lactic acid.

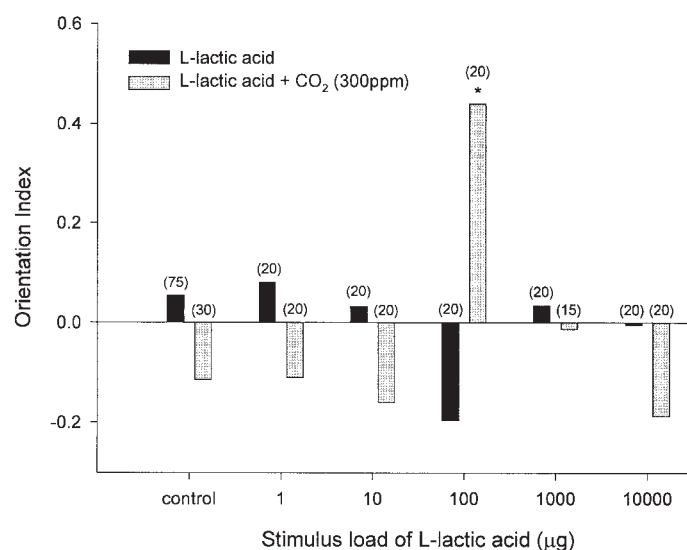


Figure 5 Effect of L-lactic acid added at different intensities, either alone or in combination with carbon dioxide. The black bars indicate the orientation index of insects stimulated only with L-lactic acid. Distilled water (solvent) served as control. The grey bars denote the stimulation with L-lactic acid and carbon dioxide at a sub-threshold concentration (300 p.p.m. over the ambient level) (see Figure 3). The control condition for testing the mixture consisted on the combination of distilled water and an airstream with 300 p.p.m. of CO₂ above the environment. The asterisk denotes both, the statistically significant differences ($P < 0.05$, see text for more statistical details) from a uniform distribution and a significant mean direction around stimulus location (0°). Number of insects tested shown in brackets.

1-Octen-3-ol represented an orienting cue by itself for *T. infestans*, since the insects were attracted to air currents loaded with 100 µg of this compound (V -test, $u_{(36)} = 4.7$, $P < 0.0005$; Figure 7). The addition of carbon dioxide (300 p.p.m.) did not modify the response of the bugs to this chemical.

In accordance with our assays involving CO₂ as a single stimulus (see above), under our experimental conditions, the general locomotor activity of bugs (i.e. total distance walked, total walking time, mean walking velocity, linearity index) stimulated with L- or D-lactic acid or 1-octen-3-ol alone or in combination with carbon dioxide above the ambient levels, was not statistically different from the respective controls (ANOVA, not significant).

Discussion

Triatoma infestans, as other haematophagous insects, responded to airstreams loaded with amounts of carbon dioxide above the ambient level. We have analysed different aspects of this response, using an open-loop bioassay based on the simultaneous discrimination of two air currents. Here, we showed that the simultaneous exposition to two opposite odourless currents leads the bugs to a non-oriented behaviour (as in still air) and to display a preference for one of the streams only when an attractive stimulus is presented.

We have established that *T. infestans* is attracted to carbon dioxide when it is present at concentrations >300 p.p.m.

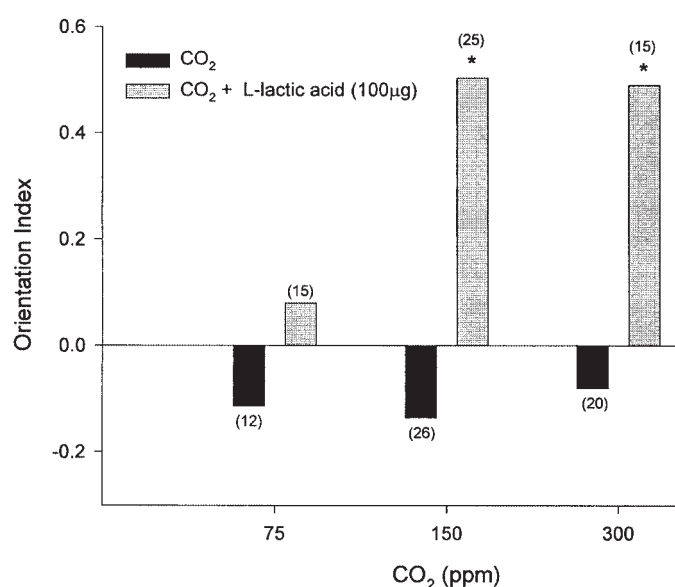


Figure 6 Effect of L-lactic acid over the sensitivity to carbon dioxide. The black bars represent the response to different sub-threshold levels of carbon dioxide (data from Figure 3). The grey bars show the combination of the different concentrations of carbon dioxide and L-lactic acid added at a single dose (100 µg; see Figure 5). Asterisks denote both, statistically significant differences ($P < 0.05$, see text for further statistical details) from a uniform distribution and a significant mean direction around stimulus location (0°). Number of insects tested shown in brackets.

above the ambient. This value refers to the response to the sole addition of carbon dioxide into an airstream, but the sensitivity of these bugs is not invariable, being modulated by other factors, as we detail below. The behavioural sensitivity of *T. infestans* to CO₂ alone can be compared with that of other blood-sucking arthropods. *Stomoxys calcitrans* shows upwind anemotaxis behaviour and an increase in flight activity when stimulated with 60–100 p.p.m. of CO₂ above the ambient (Warnes and Finlayson, 1985; Schofield *et al.*, 1997). *Aedes aegypti* responds with upwind flight to odour plumes loaded with 500 p.p.m. above the background (Geier *et al.*, 1999). The tick, *Amblyomma variegatum*, is activated from the resting state by 1500 p.p.m. of CO₂ and attracted by a carbon dioxide source of 4500 p.p.m. in wind-tunnel experiments (Steullet and Guerin, 1992).

The concentration of carbon dioxide in atmospheric air is ~300–400 p.p.m., whilst a normal human exhalation reaches values of ~45 000 p.p.m. Excretion of carbon dioxide through the skin of the human body is relatively low, being only ~0.25% of that from the lungs (Frame *et al.*, 1972). Under natural conditions, local atmospheric CO₂ levels can vary considerably, depending on the time of the day and the density of vegetation (see Gillies, 1980). Triatomine bugs inhabit relatively closed environments that are shared with their hosts. Usual habitats include human dwellings, enclosures for domestic animals, bird nests, mammal burrows, etc. As a consequence, triatomines travel shorter distances searching for food than insects living in open areas. To get

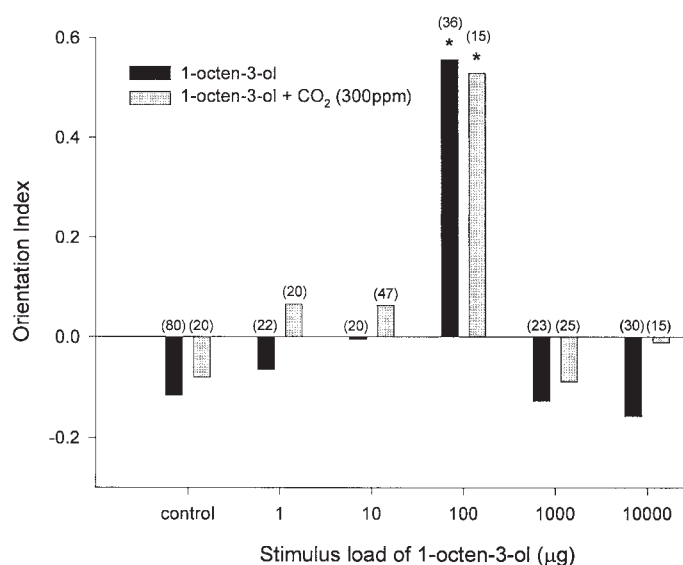


Figure 7 Effect of 1-octen-3-ol added at different intensities, either alone or in combination with carbon dioxide. The black bars represent the orientation index of insects stimulated only with 1-octen-3-ol. Hexane (solvent) served as control. The grey bars represent the stimulation with 1-octen-3-ol and carbon dioxide at a sub-threshold concentration (300 p.p.m.; see Figure 3). The control consisted on the combination of hexane and an airstream loaded with 300 p.p.m. of CO₂ over the ambient level. Asterisks denote both, statistically significant differences ($P < 0.05$, see text for further statistical details) from a uniform distribution and a significant mean direction around stimulus location (0°). Number of insects tested shown in brackets.

an approximate idea at which maximum distance *T. infestans* could detect levels of carbon dioxide similar to those released by hosts, we measured the CO₂ around a human being of 70 kg at different distances from the mouth, in a wide but closed space, over a background concentration of 440 ± 10 p.p.m. of CO₂. The concentration of carbon dioxide decreased exponentially with the distance to the human source and dropped below the bugs' threshold at ~ 0.80 m; the concentration of CO₂ could not be distinguished from background level at ~ 1 m from the source. Nevertheless, as suggested by Gillies (1980) and as we have demonstrated, the attractant effect of CO₂ can be greatly enhanced by the presence of one or several volatile chemical substances (see below).

Our bioassays allowed us to investigate different parameters of the response of *T. infestans*, when confronted to a carbon dioxide enriched airstream. On average, the bugs rarely maintained a straight path in direction to the stimulus, i.e. keeping an angle of 0° relative to the stimulus, but exhibited an angular dispersion whose magnitude depended on the concentration of the stimulus (Figure 3, rose diagrams). In that sense, the larvae showed a higher accuracy in their trajectories towards the stimulus position with increasing CO₂ concentrations and exhibiting a significantly smaller angular deviation. However, when the linearity index was computed from individual paths, it did not change significantly with CO₂ concentration. This fact

suggests to us that the angular dispersion of each experimental group could be more related to differences in the approaching angle of individuals, rather than in the sinusosity or tortuosity of the path followed by each insect.

Apart from orientation, no general increase in the mean walking velocity, nor in the total walking time or in the total walked distance, could be observed when carbon dioxide was added to an airstream. These results agree with previous observations by Núñez (1982), who using actographs did not find any stimulatory effect of carbon dioxide alone on the related species *Rhodnius prolixus*. In *T. infestans*, Taneja and Guerin (1995) observed that mouse odour, but not carbon dioxide evoked an increase in walking speed on a servosphere. However, Guerenstein and Guerin (2001) reported that these bugs walked faster on a locomotion compensator when exposed to an air current loaded with 1000 p.p.m. of carbon dioxide. Considering the discordant results obtained by different authors, the possible effect of CO₂ as a general locomotion activator in triatomines needs further investigation. From our experimental conditions, we cannot exclude the possibility that the contact of animals with the suspended sphere could have evoked an increase in locomotor activity. Thus, the addition of an orienting stimulus would just modify the direction, but not the speed of displacement.

It is widely known that the nutritional state of animals modifies their motivation to feed. This accepted fact could eventually be expressed as a decrease in the threshold response to host-associated stimuli (e.g. CO₂), i.e. at a behavioural level. This was not the case for *T. infestans*, since bugs did not express a change in their sensitivity to carbon dioxide with increasing starvation levels. Although few data are available in this respect on other haematophagous insects, in at least one species the sensitivity increases with the starvation time at the sensory level. The antennal receptors of *S. calcitrans* usually respond to an increase in carbon dioxide levels of 230 p.p.m., but starvation reduces this threshold of responsiveness (Warnes and Finlayson, 1986).

Many morphological, physiological and behavioural processes in triatomine bugs have been shown to be temporally modulated, e.g. locomotor activity, egg hatching, oviposition, ecdysis, dispersion, thermopreference, light/dark adaptation of compound eyes, refuge incoming/outgoing and feeding motivation (Ampleford and Steel, 1982; Constantinou, 1984; Ampleford and Davey, 1989; Lazzari, 1991, 1992; Lorenzo and Lazzari, 1998; Reisenman *et al.*, 1998, 2002; Minoli and Lazzari, 2003). Furthermore, we verified here that the sensitivity of *T. infestans* to carbon dioxide also varied with the time of the day. We have tested the response to carbon dioxide during the photophase and the scotophase of these nocturnal bugs, including the two main periods of spontaneous activity, i.e. at the beginning and at the end of the night (Lazzari, 1992). Attraction to carbon dioxide in *T. infestans* only occurred during the

initial hours of the night. This finding agrees with previous evidences on the use of temporal windows by these insects: during the early night their activity seems to be associated to host seeking and at the end of the night to refuge search (Lazzari, 1992; Lorenzo and Lazzari, 1998). As carbon dioxide is a host-related cue, a maximal sensitivity during the time the bugs search for food reinforces this assumption. It would be of interest to analyse in the future whether this modulation occurs at the sensory level, as occurs in some blood-sucking insects with other host-related cues (Davis, 1984) or at the central level, as is the case of the phototactic response of *T. infestans* (Reisenman *et al.*, 1998, 2002).

Many data on the daily modulation in the response to pheromones are available in the literature (e.g. Baker and Cardé, 1979; Zhukovskaya, 1995; Linn *et al.*, 1996), but there is little information concerning the temporal modulation of behavioural responses to host-related cues in haematophagous insects (Brady, 1975).

The behavioural responses of *T. infestans* to carbon dioxide in combination with other chemicals revealed different effects, rather than general ones. When offered alone and in a wide range of stimulus doses, L-lactic acid did not evoke any oriented response in the bugs. However, when this chemical, at a particular dose, was combined with a sub-threshold amount of carbon dioxide, *T. infestans* expressed a positive oriented response towards the mixture. This effect reveals a truly synergism, since bugs significantly oriented to the combination of both stimuli, that, when offered singly, were not able to evoke a response. Moreover, the sensitivity threshold to carbon dioxide decreased to a level between 75 and 150 p.p.m. when L-lactic acid was also present. L-lactic acid has been detected on human skin (Acree *et al.*, 1968; Bernier *et al.*, 2000). It was found to be the major component of human sweat produced by eccrine glands (see Braks *et al.*, 1999), with concentrations ranging from 0.5 to 5 mg/ml (Eiras and Jepson, 1991; Cork and Park, 1996; Geier *et al.*, 1996). The concentration revealing synergism with CO₂ in our assays was equivalent to 2 mg/ml, i.e. lying at levels present in human hosts.

In other haematophagous insects, such as mosquito species, L-lactic acid presented as a single stimulus, is only slightly or non attractive (Geier *et al.*, 1996; Braks *et al.*, 2001), but in combination with carbon dioxide it acts as a synergist by increasing the attractiveness to this gas in behavioural assays (Eiras and Jepson, 1991; Geier *et al.*, 1999; Dekker *et al.*, 2002). Some authors pointed out the importance of lactic acid as a guiding cue, relating the olfactory-based host preference to differences in the amount of lactic acid in the odour samples (Steib *et al.*, 2001; Dekker *et al.*, 2002).

It is also worth mentioning that we found a high specificity in the response to the L-isomer of lactic acid. This result is in accordance with a previous report by Bernard (1974), which recorded single cell responses from the thick-walled basi-

conic sensilla of *T. infestans* to L-lactic acid. Although, he did not observe any response to D-lactic acid on this olfactory hair.

1-Octen-3-ol was first isolated from cattle odours (Hall *et al.*, 1984) and is also present in small amounts in human sweat (Cork and Park, 1996). This compound was identified as a potent olfactory attractant mainly when combined with carbon dioxide for some mosquitoes species and tsetse flies (Vale and Hall, 1985; Takken and Kline, 1989; Van Essen *et al.*, 1994). In the latter group, the 1-octen-3-ol offered alone also significantly increases fly captures in field studies (Hall *et al.*, 1984; Dransfield *et al.*, 1986). In the present work, it was demonstrated that 1-octen-3-ol appeared as effective in evoking positive orientation for *T. infestans* when tested as a single compound, but neither a synergistic effect or a summed response was observed when presented together with CO₂. In general, blood-sucking insects seem to be guided by the same group of host-emitted cues. Nevertheless, further investigations are required to clarify the underlying mechanisms and to understand the differences observed.

It is worth mentioning that this work demonstrates, for the first time, the attractant effect of L-lactic acid (+CO₂) and 1-octen-3-ol in a triatomine species. Remarkably, *T. infestans* larvae showed an oriented response to a single stimulus load under both chemical stimulations. Therefore, it would be important to stress here that in this work, a wide range of intensities of both odours were tested, probably excluding several other putative attractive intensities. So, the behavioural response of *T. infestans* might not be just restricted to only one intensity. The wide interval among the tested doses does not allow us to establish the form of the dose-response curves. As a consequence, the stimulus load capable to induce an attractive response should be only taken as approximate, although capable to reveal the ability of the bugs to perceive L-lactic acid and 1-octen-3-ol.

In summary, we have described aspects of the response of *T. infestans* to carbon dioxide, such as sensitivity threshold, approaching strategies, temporal modulation, dependence on nutritional status and responses, together with other chemical cues. Many of these aspects appear as novel for blood-sucking insects and suggest that these walking insects could be an excellent model system to study the sensory ecology of the haemathophagous life.

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