

## RESEARCH ARTICLE

# Impact of elevated CO<sub>2</sub> background levels on the host-seeking behaviour of *Aedes aegypti*

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**ABSTRACT**

Mosquitoes rely on carbon dioxide (CO<sub>2</sub>) to detect and orient towards their blood hosts. However, the variable and rapid fluctuations of atmospheric CO<sub>2</sub> concentrations may have an impact on the host-seeking behaviour of mosquitoes. In this study, we analysed the effect of transient elevated background levels of CO<sub>2</sub> on the host-seeking behaviour and the physiological characteristics of the CO<sub>2</sub>-sensitive olfactory receptor neurones (ORNs) in female yellow fever mosquitoes, *Aedes aegypti*. We show that the take-off and source contact behaviour of *A. aegypti* is impeded at elevated background levels of CO<sub>2</sub> as a result of masking of the stimulus signal. The mechanism underlying this masking during take-off behaviour is one of sensory constraint. We show that the net response of the CO<sub>2</sub>-ORNs regulates this CO<sub>2</sub>-related behaviour. Since these neurones themselves are not habituated or fatigued by the transient elevation of background CO<sub>2</sub>, we propose that habituation of second-order neurones in response to the elevated CO<sub>2</sub>-ORN activity could be one mechanism by which the net response is transduced by the olfactory system. The findings from this study may help to predict future shifts in mosquito–host interactions and consequently to predict vectorial capacity in the light of climate change.

**KEY WORDS:** Olfaction, Single sensillum recording, Wind tunnel, Climate change, Absolute detector

**INTRODUCTION**

Volatiles play an important role in the host-seeking behaviour of mosquitoes (Takken and Knols, 1999). Carbon dioxide (CO<sub>2</sub>) is one of the most important volatiles, a key kairomone emitted by vertebrates, which has been shown to be a reliable cue for mosquitoes to detect and orient towards a host species (Mboera and Takken, 1997). Such a role for CO<sub>2</sub> in mosquito foraging was recognised by Rudolfs as early as 1922 (Rudolfs, 1922). However, diel atmospheric CO<sub>2</sub> concentrations vary between 350 parts per million (ppm) and 500 ppm (up to 1000 ppm in dense vegetation), and rapid fluctuations are features of natural CO<sub>2</sub> sources (Gillies, 1980; Guerenstein and Hildebrand, 2008). Consequently, variations in ambient CO<sub>2</sub> levels could affect the ability of mosquitoes to process CO<sub>2</sub> fluctuations (Grant et al., 1995) and modulate their host-seeking behaviour. To date, there have been no published studies that have dealt with the effects of elevated ambient CO<sub>2</sub> levels on mosquito behaviour. Analysis of CO<sub>2</sub> processing in the context of host-seeking behaviour could help to identify the mechanisms underlying these effects, to predict shifts in

mosquito–host interactions and consequently to predict vectorial capacity.

Activation and source finding by host-seeking female mosquitoes occur when the fine-scale structure of the CO<sub>2</sub> plume is filamentous, i.e. when flying mosquitoes are exposed to intermittent increases in CO<sub>2</sub> concentration above background, as shown under controlled conditions (Geier et al., 1999; Dekker et al., 2001). Such a plume is encountered naturally by mosquitoes up to tens of metres from a host (Costantini et al., 1996; Zöllner et al., 2004). However, in a variable ambient CO<sub>2</sub> background, the effective range of attraction could decrease, due to limitations of the CO<sub>2</sub> chemosensory system.

Highly sensitive olfactory receptor neurones (ORNs), housed in the maxillary palp capitata peg sensilla of mosquitoes, detect CO<sub>2</sub> (Grant et al., 1995; Lu et al., 2007; Syed and Leal, 2007). Pulses of CO<sub>2</sub> elicit phasic–tonic responses in these ORNs, and the pulse duration is dependent on the concentration and length of the stimulus pulse (Grant et al., 1995). Grant et al. have shown that this response of the CO<sub>2</sub>-ORNs might be impeded in high ambient concentrations of CO<sub>2</sub>. However, we do not know how mosquitoes encode such an impediment and how it affects the behavioural response of mosquitoes to CO<sub>2</sub>.

In this study, we analyse the sensory mechanism underlying the behavioural response of the yellow fever mosquito, *Aedes aegypti*, to CO<sub>2</sub>. We show that female *A. aegypti* encode pulsed CO<sub>2</sub> stimuli, dependent on ambient CO<sub>2</sub> levels, as a net sensory response. Furthermore, we show that the net sensory response dictates behavioural activation across ambient CO<sub>2</sub> levels. The effect of ambient CO<sub>2</sub> levels on the host-seeking behaviour and vectorial capacity of mosquitoes is discussed.

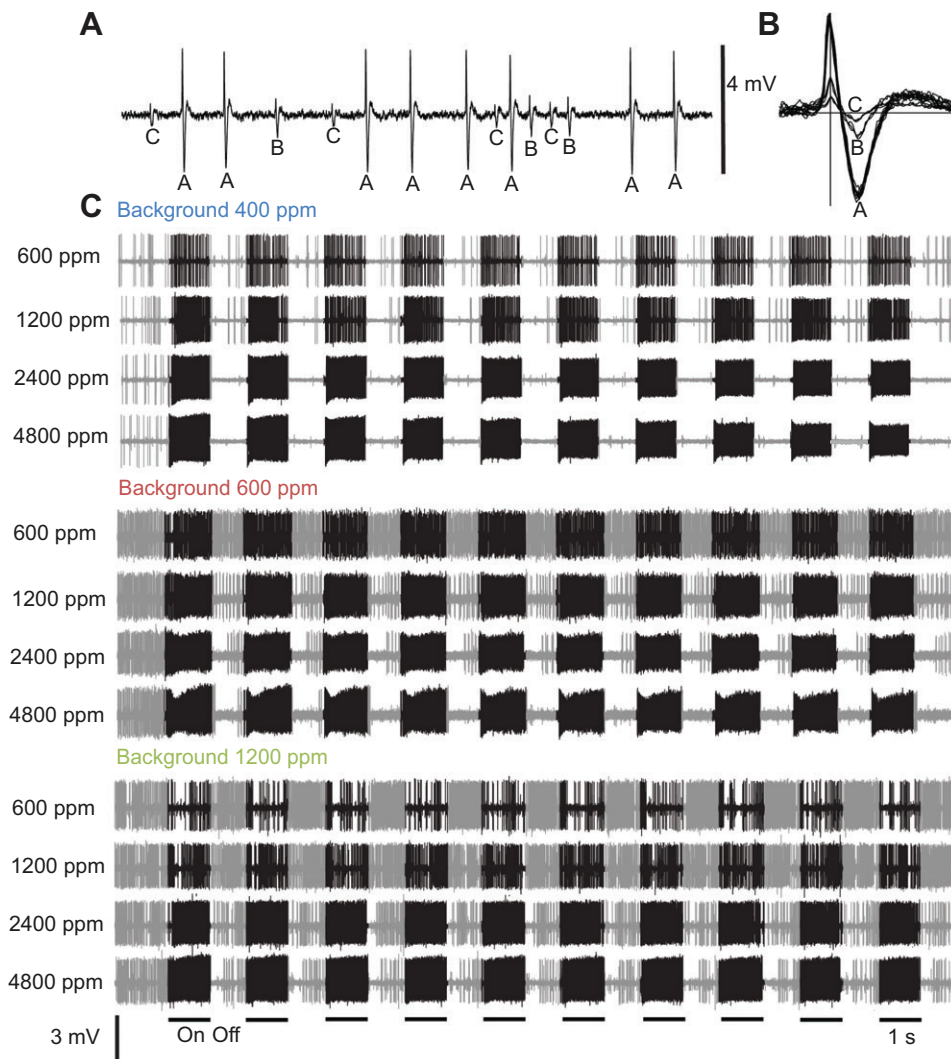
**RESULTS****CO<sub>2</sub>-ORN responses in different background levels of CO<sub>2</sub>**

Stimulation with increased concentrations of pulsed CO<sub>2</sub> elicited a significant increase in the A-cell response (background level 400 ppm:  $F=61.54$ , d.f.=3,  $P<0.001$ ; background level 600 ppm:  $F=480.81$ , d.f.=3,  $P<0.001$ ; background level 1200 ppm:  $F=379.54$ , d.f.=3,  $P<0.001$ ), as well as in the net response when the stimulus concentration exceeded that of the background level (background level 400 ppm:  $F=75.36$ , d.f.=3,  $P<0.001$ ; background level 600 ppm:  $F=425.76$ , d.f.=3,  $P<0.001$ ; background level 1200 ppm:  $F=403.47$ , d.f.=3,  $P<0.001$ ) (Fig. 1C; Fig. 2). Conversely, a significant decrease in the net response was observed when the stimulus concentration was lower than that of the background level ( $t=-17.82$ , d.f.=9,  $P<0.001$ ,  $t$ -test), i.e. when stimulating with 600 ppm CO<sub>2</sub> in a 1200 ppm CO<sub>2</sub> background (Fig. 2). Hence, the overall net response of the CO<sub>2</sub>-ORNs was significantly affected by the background level of CO<sub>2</sub> ( $F=4.0$ , d.f.=2,  $P=0.03$ ). The decrease in net response was contributed to by a significant increase in the interstimulus activity of the CO<sub>2</sub>-ORNs at 600 and 1200 ppm CO<sub>2</sub> background levels, compared with that at 400 ppm CO<sub>2</sub> ( $F=46.47$ , d.f.=2,  $P<0.001$ ) (Fig. 2).

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Received 18 June 2013; Accepted 14 October 2013



**Fig. 1. The temporal characteristics of CO<sub>2</sub> chemosensation in female *A. aegypti* in different background levels of CO<sub>2</sub> to four different concentrations of pulsed CO<sub>2</sub> stimuli.** (A) An extracellular recording from a capitata-peg sensillum showing the spontaneous activity of the large amplitude 'A', the intermediate 'B' and the small 'C' neurones, respectively. Vertical bar indicates the spike amplitude in millivolts (mV). (B) Waveform analysis of the extracellular recording in (A) shows the distinct waveforms of each neurone. (C) Detection of pulsed CO<sub>2</sub> by the CO<sub>2</sub>-ORNs, in different CO<sub>2</sub> backgrounds, over ascending concentrations. Black spikes represent the response by CO<sub>2</sub>-ORNs to a pulsed train of 1 s CO<sub>2</sub> stimuli and the grey spikes indicate the interstimulus activity of the CO<sub>2</sub>-ORNs. The vertical bar denotes the amplitude in millivolts. The horizontal bar represents the pulsed train of 1 s CO<sub>2</sub> stimuli.

### Consistency of pulsed stimuli of CO<sub>2</sub>

To investigate the correlation between CO<sub>2</sub>-ORN and behavioural responses, a similar stimulus protocol was adopted in all experiments. To ensure that the train of pulsed stimuli were neither molecularly nor turbulently diffused in the air stream of the wind tunnel, we designed a protocol to produce pulse trains consistent in shape and amplitude throughout the wind tunnel. To verify this consistency, a train of distinct pulsed stimuli of CO<sub>2</sub> was visualised by using acetone as tracer gas using a mini-photo ionisation detector (mini-PID) to track the ascending flow of known concentrations of acetone at different positions (centre and lateral sides) and distances (release chamber, halfway and source contact). This explicitly showed that the pulsed stimuli had a consistent shape and were clearly separated from one another throughout the wind tunnel (Fig. 3A, left inset,  $N=10$ ). The amplitude of each discrete pulse was shown by linear regression to be consistent in all positions in the wind tunnel assayed for each flow rate (Fig. 3A, right inset,  $N=10$ ).

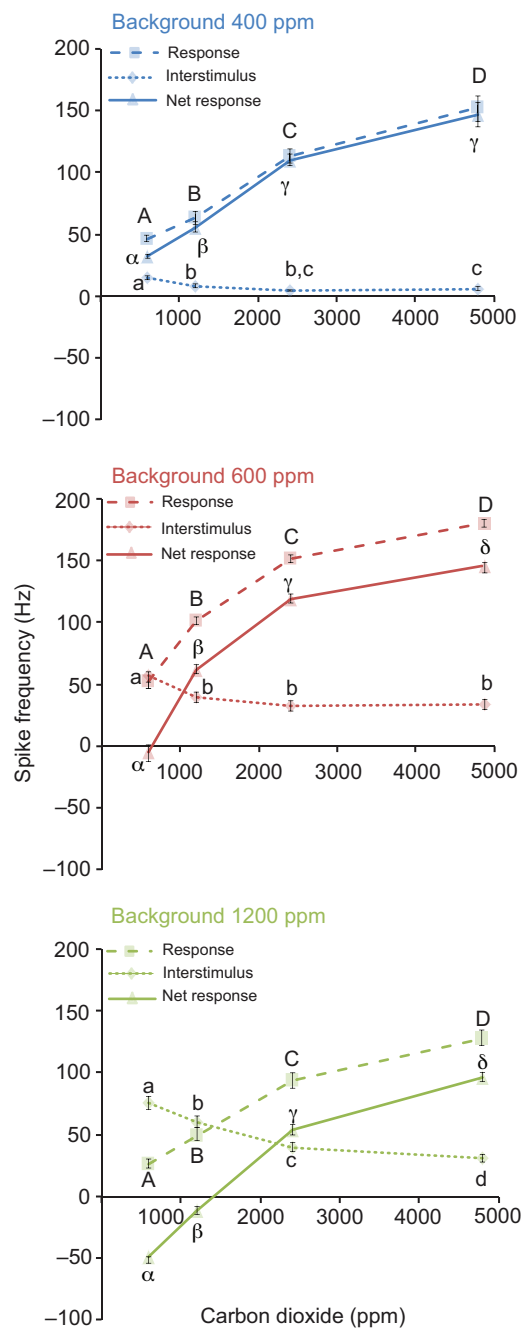
### Behavioural responses to CO<sub>2</sub> in different CO<sub>2</sub> backgrounds

The host-seeking behaviour of female *A. aegypti* was significantly affected by both the stimulus concentration and the background level of CO<sub>2</sub> (Fig. 3B). Time to take-off was significantly decreased when stimulus concentrations exceeded 1200 ppm CO<sub>2</sub>, at background levels of 400 and 600 ppm CO<sub>2</sub> (Fig. 3B). At a

background level of 1200 ppm CO<sub>2</sub>, however, time to take-off was not affected by stimulus concentration ( $F=3.6$ ,  $d.f.=29$ ,  $P=0.3$ ) (Fig. 3B). In addition, time to take-off at this background level was significantly increased compared with that observed at background levels of 400 and 600 ppm CO<sub>2</sub> (Fig. 3B). Once the mosquitoes had taken off, the time to reach the halfway mark in the wind tunnel was not significantly affected by either stimulus concentration or background level of CO<sub>2</sub> (data not shown). In contrast, the time to source contact was affected by both stimulus concentration and the background level of CO<sub>2</sub> (Fig. 3B). At a background level of 400 ppm CO<sub>2</sub>, the time to source contact was significantly decreased as stimulus concentration increased (Fig. 3B). However, at background levels of 600 and 1200 ppm CO<sub>2</sub>, mosquitoes did not take a significantly shorter time to reach the source at any stimulus concentration (Fig. 3B).

### Correlation between behaviour and physiology

The net sensory response over increasing stimulus concentrations among the different background levels was compared, following normalisation (Fig. 4). The slopes of the net response curves in 600 and 1200 ppm CO<sub>2</sub> background levels were significantly different from that of the 400 ppm CO<sub>2</sub> level ( $F=6.52$ ,  $d.f._n=76$ ,  $d.f._d=76$ ,  $P=0.013$ ), but not from each other ( $F=0.48$ ,  $d.f._n=1$ ,  $d.f._d=76$ ,  $P=0.49$ ). A net response threshold of  $\geq 100$  spikes s<sup>-1</sup> was found



**Fig. 2.** Averaged spike frequency ( $N=10$ ) of response, interstimulus activity and net response of the CO<sub>2</sub>-ORNs in different CO<sub>2</sub> backgrounds. Letters and symbols denote significant differences within the response, interstimulus activity and net response over increasing concentrations. Error bars represent the standard error of the mean. The spike frequency is represented in hertz (Hz).

(Fig. 4), which correlated with a significant decrease in time to take-off flight (Fig. 3B) regardless of background CO<sub>2</sub> level (Figs 4, 5). At no tested CO<sub>2</sub> concentration, in a background level of 1200 ppm, did the mosquitoes decrease their time to take-off (Fig. 4), which correlated with the maximum net response generated of  $92.5 \pm 4.1$  spikes s<sup>-1</sup> (Figs 4, 5).

A correlation was observed between net sensory response of the CO<sub>2</sub>-ORNs and the behavioural response to pulsed stimuli of CO<sub>2</sub> in elevated background of CO<sub>2</sub> levels (Fig. 5). The time to take-off

flight was significantly decreased as the stimulus concentration of CO<sub>2</sub> exceeded 1200 ppm at the CO<sub>2</sub> background of 400 and 600 ppm, which was significantly correlated with the net response threshold of  $\geq 100$  spikes s<sup>-1</sup> (400 ppm at  $r=0.9764$ , 95% confidence interval: 0.2489 to 0.9995,  $P=0.0236$ ; 600 ppm at  $r=0.9830$ , 95% confidence interval: 0.3970 to 0.9997,  $P=0.0170$ ; Figs 4, 5). In addition, the upwind flight towards source contact at CO<sub>2</sub> background of 400 ppm was correlated with the net response ( $r=0.9644$ , 95% confidence interval: 0.04438 to 0.9993,  $P=0.0356$ ; Fig. 5). However, at the higher background levels of CO<sub>2</sub>, the stimulus concentration had no effect on upwind flight (Fig. 5).

## DISCUSSION

In this study, we have analysed the sensory mechanism that is involved in constraining the behavioural response of *A. aegypti* to CO<sub>2</sub> at elevated background CO<sub>2</sub> levels. Knowledge gained through this study sheds new light on the effects of varying CO<sub>2</sub> environments on the interactions between insects and hosts, in general, and between disease-vector mosquitoes and their blood hosts specifically. The study also improves our ability to predict shifts in vectorial capacity and other community interactions in future environments.

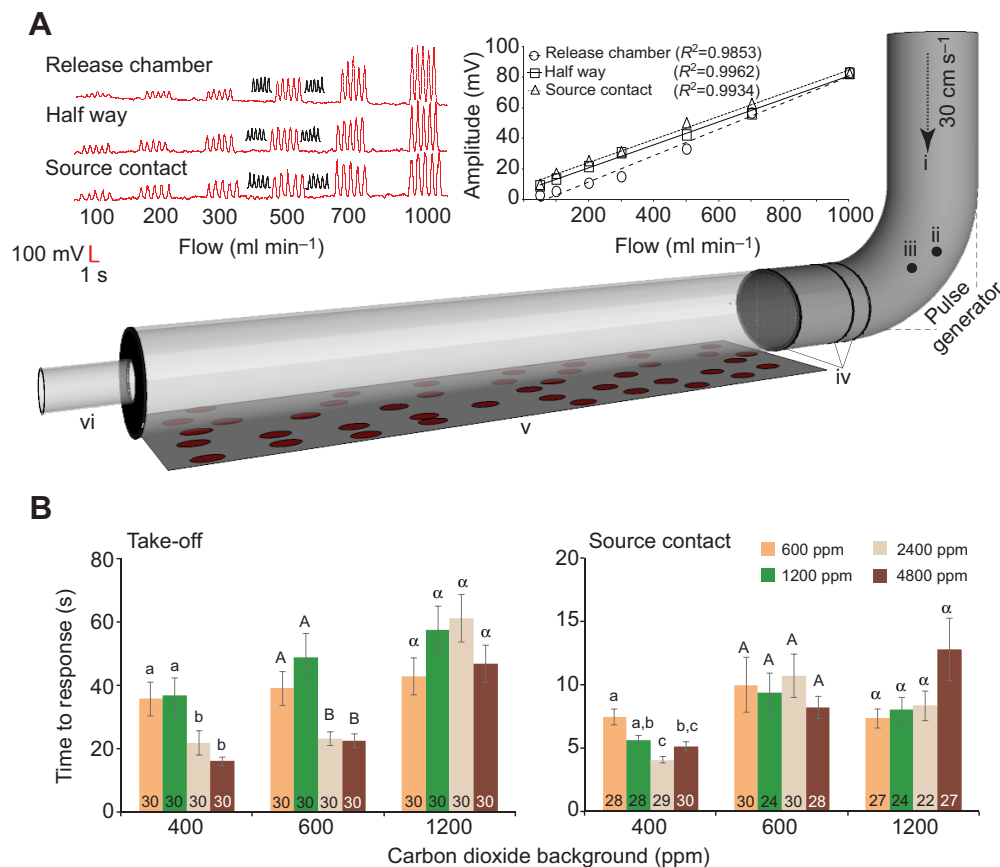
### Impact of elevated CO<sub>2</sub> levels on mosquito behaviour

Carbon dioxide activates and modulates the host-seeking behaviour of insects, including mosquitoes, in a concentration-dependent manner (Dekker et al., 2001; Guerenstein and Hildebrand, 2008; Dekker and Cardé, 2011). We have shown that a transient elevation of the background level of CO<sub>2</sub> significantly affects the behavioural response of the mosquitoes. Specifically, our behavioural data suggest that an elevation of the background level of CO<sub>2</sub> adds a masking effect that reduces the detection of the CO<sub>2</sub> stimulus, affecting both activation and source finding. A similar mechanism has been reported to affect oviposition by the pyralid moth *Cactoblastis cactorum*, and its peripheral reception (see below) (Stange, 1997). This mechanism is closely analogous to the attraction of male moths towards the pheromone of calling females, which decreases with an increase in the background concentration of pheromone (Sanders, 1982; Sanders and Lucuik, 1996; Schofield et al., 2003).

Our behavioural data suggest that mosquitoes are able to cope with the present natural diurnal and seasonal changes in atmospheric CO<sub>2</sub> concentrations, i.e. 350–500 ppm (up to 1000 ppm in dense vegetation) (Gillies, 1980; Guerenstein and Hildebrand, 2008). However, it is unclear if they will be able to do so following the predicted ongoing increase in atmospheric CO<sub>2</sub>, 550–1000 ppm by the turn of the next century (Guerenstein and Hildebrand, 2008). This ongoing increase in ambient CO<sub>2</sub> level may reduce the vectorial capacity of mosquitoes by limiting the effective range of attraction to their blood hosts (Zöllner et al., 2004). Additional studies, however, are required to investigate the ability of mosquitoes to adapt to ongoing increases in atmospheric CO<sub>2</sub> levels.

### Constraint of the CO<sub>2</sub>-ORNs

Limitations of the CO<sub>2</sub>-induced behavioural response are dependent on the physiological response of their CO<sub>2</sub>-ORNs. In *A. aegypti*, CO<sub>2</sub>-ORNs respond in a concentration-dependent manner at all background levels of CO<sub>2</sub>. A dampening of the signal resolution occurs between 2400 and 4800 ppm, which corresponds to a previously observed upper behavioural response threshold (Costantini et al., 1996). A plausible mechanism that the olfactory system of mosquitoes uses to minimise this loss of signal resolution is to subtract the spontaneous (interstimulus) activity of the ORNs



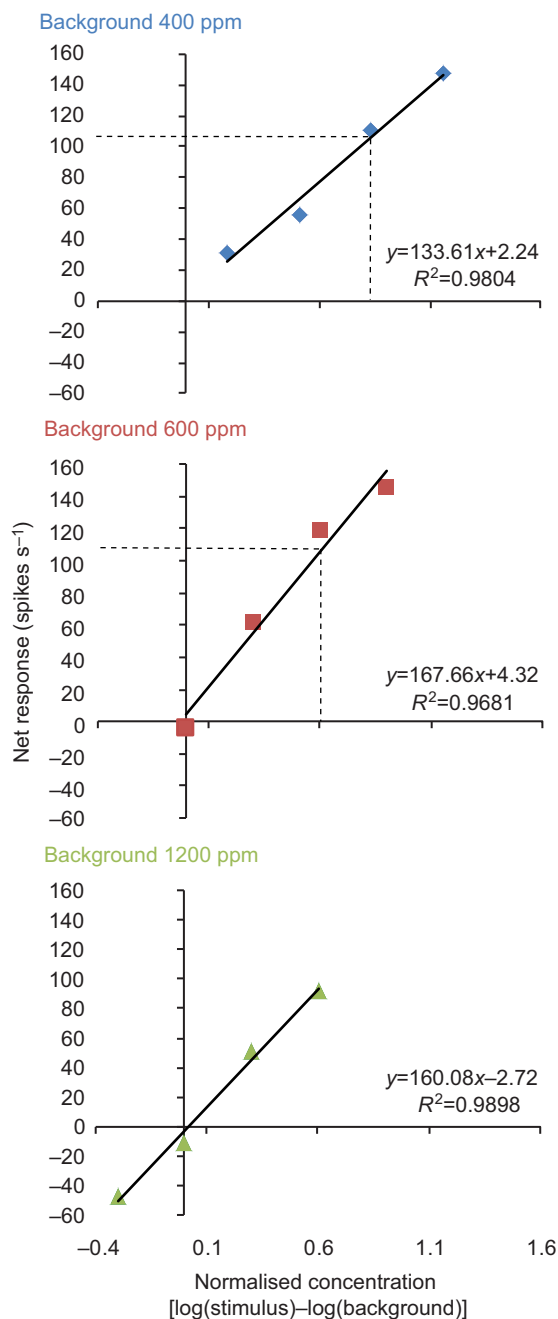
**Fig. 3. Behavioural responses of female *A. aegypti* in different background levels of CO<sub>2</sub>.** (A) Behaviour was assessed in a wind tunnel assay, as shown in the schematic diagram in the bottom panel: (i) charcoal-filtered and humidified air, (ii) pressurised air inlet, (iii) stimulus inlet into which CO<sub>2</sub> was injected, (iv) stainless-steel mesh plume diffusers, (v) glass flight tunnel and (vi) release chamber. The upper panels demonstrate that the pulsed stimuli (here shown as five cycles of 1 s on and 1 s off) maintain their amplitude and shape throughout the wind tunnel and at all tested flow rates. The upper left panel shows the consistent and distinct pulsed stimuli at ascending flow rates of known concentration of acetone in the wind tunnel. Discrete pulsed stimuli were measured in the centre (in red) and at the lateral sides (in black) of the release chamber, at halfway and at the source. The upper right panel presents a graphical representation of the distinct pulsed stimuli, which shows the average amplitude of each of the five distinct pulses ( $N=10$ ) at different positions and the regression correlation coefficients ( $R^2$ ) that demonstrate the consistency of the stimulus amplitude at the different positions within the wind tunnel with increasing flow rates. (B) Female mosquito attraction towards pulsed stimuli of four concentrations of CO<sub>2</sub> in the wind tunnel at different CO<sub>2</sub> background levels is shown ( $N=30$  for each background level). Numbers inside the bars represent the number of tested individuals that took off (left panel) or made source contact (right panel). Letters indicate the significant differences within the background levels over increasing concentrations.

at a given CO<sub>2</sub> background level from the stimulus response. This generates a net response that remains linear throughout the ecologically relevant range of CO<sub>2</sub> stimuli. This strategy, while maintaining signal resolution to elevated CO<sub>2</sub> stimuli, requires stronger stimuli in increased CO<sub>2</sub> background levels to produce an equivalent signal to that generated in a lower CO<sub>2</sub> background. The requirement for a stronger stimulus in the elevated backgrounds is likely to be mitigated by the reduction of the membrane potential needed to achieve the threshold for firing an action potential. This is a result of the increased baseline membrane potential predicted during the interstimulus periods in elevated background CO<sub>2</sub>. Thus mosquitoes are able to accurately detect the level of CO<sub>2</sub> stimulus above background CO<sub>2</sub> levels over a broad range of concentrations. This sensitivity ensures that a mosquito is able to respond to a plume of host-emitted CO<sub>2</sub> in which distance from the source becomes the limiting factor in elevated backgrounds of CO<sub>2</sub>.

Carbon dioxide-ORNs have been described as absolute concentration detectors, i.e. at or below the background CO<sub>2</sub> level, the sensory response to CO<sub>2</sub> remains linear (Syed and Leal, 2007), and background CO<sub>2</sub> concentration has little to no effect on the

stimulus response (Grant et al., 1995). Our electrophysiological analyses revealed that the CO<sub>2</sub>-ORNs meet the first criterion, but not the second. Mosquito CO<sub>2</sub>-ORNs exhibit compressive non-linearity (Stevens, 1971). The response to CO<sub>2</sub>, as a function of the stimulus (spikes s<sup>-1</sup> ppm<sup>-1</sup>), was significantly reduced when the background level increased. Similar observations have been made for CO<sub>2</sub> detection in the moth *C. cactorum* (Stange and Wong, 1993; Stange, 1997), for 1-octen-3-ol detection in the fly *Musca domestica* (Kelling et al., 2002), and for pheromone detection in a wide variety of moths (Willis and Baker, 1984; Mafra-Neto and Baker, 1996; Evenden et al., 2000). The compressive non-linearity suggests that insects experiencing elevated background concentrations of stimuli either are unable to perceive the stimulus or are habituated, which consequently affects their behavioural performance.

We found no indication of sensory neurone habituation or fatigue during the electrophysiological experiments (<5 min exposure to the elevated CO<sub>2</sub> background), as is demonstrated by there being no difference in CO<sub>2</sub>-ORN response to stimulus or interstimulus during the first, fifth or tenth stimulation. The duration of the exposure of the mosquitoes to the background CO<sub>2</sub> during the behavioural



**Fig. 4. Take-off flight behaviour is correlated with the net physiological response of the CO<sub>2</sub>-ORNs to normalised CO<sub>2</sub> concentrations in *A. aegypti* females.** The dotted lines indicate the behavioural threshold in net physiological response, i.e. at which time to take-off flight was significantly reduced.

assays (<3 min) was less than that used for the physiological assays. Yet, with no evidence of ORN habituation or fatigue, the modulation behaviour is still evident within this time period. We argue that this is due to the sensory information included both in the stimulus response and the interstimulus response: what we have termed the net response. Although we have not investigated the response profile of second-order neurones in the antennal lobe in this study, we propose that second-order neurones may become habituated to the background firing of the CO<sub>2</sub>-ORNs. In fact, this may be one mechanism by which the difference between the background firing

of the ORN could be subtracted from the stimulus, resulting in the transfer of the net response to the higher brain centres. In this model, the absolute sensory constraint on the activation behaviour is the difference between the background and maximum firing rate of the CO<sub>2</sub>-ORNs. If the difference is less than the physiological threshold then no decrease in time to activation behaviour can occur. This system provides signal resolution in a sea of noise, which would otherwise result in the activation of host-seeking behaviour in elevated background CO<sub>2</sub> concentrations in the absence of a host.

### Future perspectives

Considering the reliance on CO<sub>2</sub> sources, the CO<sub>2</sub>-sensory system of mosquitoes is an ideal target to disrupt their host-seeking behaviour. In this study, we showed that an elevation of ambient CO<sub>2</sub>, of up to three times that of normal concentration, adds a masking effect that significantly reduces the detection of this key host kairomone. In line with our finding, Turner et al. (Turner et al., 2011) recently showed that host-seeking mosquitoes become disoriented by artificially prolonged activation of CO<sub>2</sub>-ORNs, which masks the ability of the mosquitoes to detect changes in the concentration of CO<sub>2</sub> in the environment. We believe that our study will provide a better understanding of the natural mechanisms involved in this masking process.

### MATERIALS AND METHODS

#### Insects

*Aedes aegypti* (Rockefeller strain) were kept at 27°C, 65±5% relative humidity (RH) and at a 12:12 h light:dark period, as previously described (Cook et al., 2011). The ambient concentration of CO<sub>2</sub> during rearing and experiments was 400±5 ppm sugar-fed, 4- to 7-days post-emergence, female mosquitoes were used in this study.

#### Single sensillum recordings

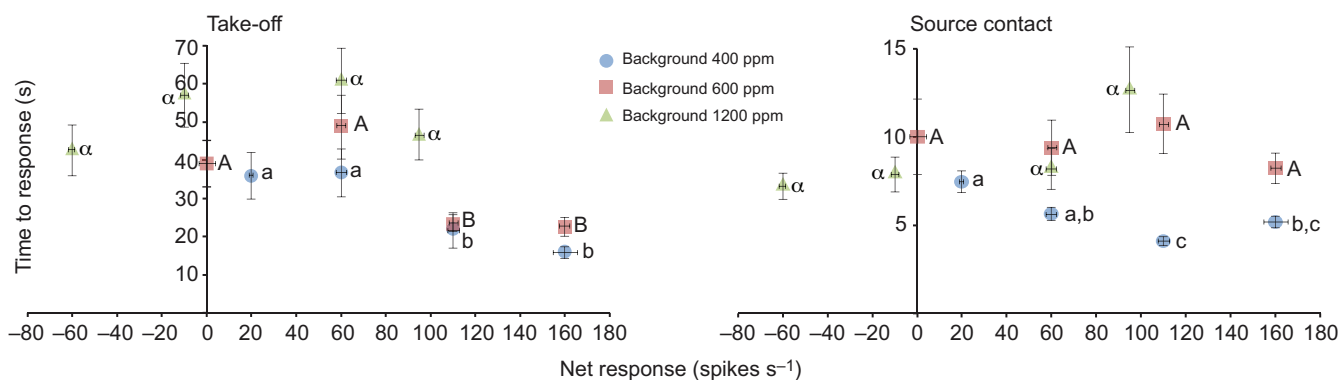
Capitate peg sensilla are found on the fourth segment of the maxillary palps of female *A. aegypti* (McIver, 1972). Each sensillum houses three ORNs, distinguishable by spike amplitude (Fig. 1A,B) (Cook et al., 2011). The ORN with the largest amplitude is, by convention, referred to as the A-cell and has previously been shown to respond to CO<sub>2</sub> (Grant et al., 1995). Single sensillum recordings from this cell were performed as previously described (Cook et al., 2011). A single recording was taken from each of 10 preparations at each background concentration. In total, recordings were made from 30 mosquitoes.

#### CO<sub>2</sub> stimulation

A continuous humidified airstream containing a background of either ambient (400), 600 or 1200 ppm CO<sub>2</sub> was delivered at 2 l min<sup>-1</sup> via a glass tube (7 mm i.d.). The elevated CO<sub>2</sub> backgrounds were obtained by diluting pure CO<sub>2</sub> (Strandmöllen, Ljungby, Sweden) directly into the airstream. The outlet of the tube was placed approximately 10 mm from the maxillary palps. An IDAC-4 (Syntech, Kirchzarten, Germany) was used to activate two-way Teflon solenoid valves (Teddington, Lanna, Sweden) that controlled the delivery of an embedded CO<sub>2</sub> stimulus into the glass tube through a separate CO<sub>2</sub> line. The stimulus was embedded into the airstream through a hole (2 mm i.d.) in the glass tube, 11 cm upstream of the maxillary palps, and the pulses were verified by using a CO<sub>2</sub> analyser (LI-820, LI-COR Biosciences, Lincoln, NE, USA). The solenoid valves were connected to separate gas cylinders containing metered amounts of CO<sub>2</sub> (600, 1200, 2400 and 4800 ppm) and oxygen (20%), balanced by nitrogen (Strandmöllen). A pulsed stimulus train of CO<sub>2</sub> was used, with stimulation for 1 s and an interstimulus interval of 1 s.

#### Wind tunnel bioassay

Behavioural experiments with pulsed CO<sub>2</sub> stimuli were performed in a glass wind tunnel (80×9.5 cm i.d.) (Fig. 3A), illuminated from above at 280 lx. A charcoal-filtered and humidified air stream (25±2°C, RH 65±2%) flowed



**Fig. 5. The behavioural response of female *A. aegypti* is correlated with the net physiological response of the CO<sub>2</sub>-ORNs.** Letters and symbols denote significant differences among the behavioural responses to CO<sub>2</sub> stimuli within each CO<sub>2</sub> background. Vertical and horizontal error bars represent the standard error of the means of time to take-off and net response, respectively.

through the wind tunnel at 30 cm s<sup>-1</sup>. The air was passed through a series of stainless-steel mesh screens to generate a laminar flow and a homogenous plume structure.

To investigate the direct correlation between sensory input and behaviour, we created distinct pulsed stimuli of 1 s on and 1 s off, embedded on the CO<sub>2</sub> background of 400, 600 and 1200 ppm, in a wind tunnel (Fig. 3A, inset). The transient elevated backgrounds were obtained by diluting pure CO<sub>2</sub> in the main airstream. The pulsatile plume structure was designed to be consistent in amplitude and structure throughout the length and breadth of the wind tunnel. For this, homogenous discrete pulse stimuli were created by pushing pure CO<sub>2</sub>-laden air into a pulse generator placed behind the stainless-steel mesh screens through a stimulus controller (SEC-2/b, Syntech) (Fig. 3A). Desired concentrations (600, 1200, 2400 and 4800 ppm) of pulsed CO<sub>2</sub> were obtained by regulating the CO<sub>2</sub> flow to the stimulus controller. To ensure distinct pulses of stimuli, pressurised air at 4.5 l min<sup>-1</sup> was introduced into the pulse generator just downstream of the point where the CO<sub>2</sub> was introduced (Fig. 3A). Various flow rates of known amounts of acetone as a tracer gas (99.9%, Chromasolv, Sigma-Aldrich, Stockholm, Sweden) were used to investigate the consistency of distinct pulse stimuli throughout the wind tunnel and measured at different positions (centre and lateral sides) and distances (40 cm 'source contact', 80 cm 'halfway' and 120 cm 'release chamber') from the pulse generator (Fig. 3A, inset). Five cycles of 1 s on and 1 s off were tested at each flow rate. The consistency in amplitude and the structure of the pulsed stimuli was visualised using a mini-PID (Aurora Scientific, Aurora, Ontario, Canada). The corresponding concentrations of CO<sub>2</sub> were calculated backwards from mini-PID responses to the known concentration of acetone with different flow rates (Fig. 3A, inset). The concentration of CO<sub>2</sub> was measured at the downwind and upwind end of the wind tunnel via the CO<sub>2</sub> analyser.

Female mosquitoes were kept individually in 7×2.6 cm i.d. glass release chambers in the wind tunnel room for 24 h before the experiments. Mosquitoes were provided with water through a moistened filter paper placed against the stainless-steel mesh, which covered one end of the chamber. The release chambers were placed in the centre of the downwind end of the wind tunnel. Thereafter, the following behavioural steps were observed: time to take-off flight, halfway and source contact, for a maximum of 120 s. Equal numbers of experimental and control flights were performed each day.

### Statistical analysis

Physiological activity was analysed (AUTOSPIKE, Syntech) as the number of spikes during interstimulus interval (1 s) subtracted from the number of spikes during the stimulus response (1 s), resulting in the net response (spikes s<sup>-1</sup>; 10 replicates per elevated CO<sub>2</sub> background level). The response, interstimulus activity and net response resulting from the first, fifth and tenth stimulus pulses were first assessed for normal distribution using the D'Agostino omnibus K<sup>2</sup> test (Prism version 5.01, GraphPad Software, La Jolla, CA, USA) and then compared by 2-way repeated measures analysis of variance (ANOVA) followed by Bonferroni *post hoc* test (Prism version 5.01).

Data resulting from the fifth stimulus pulse was then used for all subsequent analyses, as there were no significant differences between factors among the stimulus pulses. Power analysis was performed on net physiological response to determine the sample size. Furthermore, we determined the significant effect of stimulus concentration on each variable by a general linear model (GLM) 2-way ANOVA followed by Tukey's *post hoc* test (Minitab version 16.1.0). A one-sample *t*-test was performed to analyse the significance level of response to the stimulus of 600 ppm CO<sub>2</sub> in the background of 1200 ppm CO<sub>2</sub> compared with zero (Minitab version 16.1.0).

To define the relationship between CO<sub>2</sub>-ORN response and CO<sub>2</sub> background level, a regression analysis was performed separately for each insect tested. The slopes were used to analyse the statistical variation, with respect to background level of CO<sub>2</sub>, for net response (Prism version 5.01). Prior to linear regression analyses, we normalised the concentration regime by subtracting the logarithm of the stimulus concentration from the logarithm of the background level.

To describe the correlation between CO<sub>2</sub>-ORN net response (10 replicates per elevated CO<sub>2</sub> background level) and behavioural observation (30 replicates per elevated CO<sub>2</sub> background level), Pearson's correlation was used. Based on the prior behavioural (Figs 3, 5) and physiological (Figs 2, 5) analyses, thresholds for a significant reduction in time to take off ( $\leq 30$  s;  $\geq 100$  spikes s<sup>-1</sup>) and source contact ( $< 6$  s;  $\geq 65$  spikes s<sup>-1</sup>) were identified. The number of observations that matched these criteria for each dose in each background condition were counted and recorded as a ratio of the total number of observations. Pearson's correlation coefficients were calculated comparing the ratios of significant behavioural and physiological responses for each background condition.

### Acknowledgements

We would like to thank Professor Peter Anderson at the Unit of Chemical Ecology and Dr Richard Hopkins at the Department of Ecology, Swedish University of Agricultural Sciences, Ultuna for providing comments on a previous version of the manuscript. In addition, we thank Dr Jan-Eric Englund, Professor Fredrik Schlyter, Dr Eduardo Hatano and Dr Teun Dekker for advice on statistical analysis and experimental design.

### Competing interests

The authors declare no competing financial interests.

### Author contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: R.I., S.R.H. and S.M. Acquisition of data: S.M. Analysis and interpretation of data: R.I., S.R.H. and S.M. Drafting of the article: R.I., S.R.H. and S.M. Critical revision of the article for important intellectual content: R.I. and S.R.H. Obtained funding: R.I.

### Funding

This study was supported by the Linnaeus initiative 'Insect Chemical Ecology, Ethology and Evolution' IC-E3 (Formas, Swedish University of Agricultural

Sciences), and a PhD scholarship to S.M. from the Higher Education Commission (HEC) of Pakistan.

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