# 5. The detection of carbon dioxide and its role in the orientation to hosts by haematophagous insects

*Alan J. Grant and Robert J. O'Connell*

## Abstract

Host orientation behaviours of mosquitoes and other blood-seeking insects are controlled to a large degree by signals released by the host, including heat, moisture and sound, as well as visual and olfactory cues. Clearly olfactory signals play a primary role in mediating such behaviours. Although other olfactory signals such as lactic acid, 1-octen-3-ol and ammonia have been implicated in orientation, perhaps the most important of these volatiles is carbon dioxide ($CO_2$). Carbon dioxide is a by-product of cellular respiration that is released in large amounts by potential hosts and has been associated with mosquito behaviour since the early 1920s. In this report we focus on aspects of carbon dioxide detection including the morphological and physiological characteristics of sensory structures located on the maxillary palps which contain receptor neurons responsive to $CO_2$. The relevant characteristics of these receptor neurons include the threshold and slope of the concentration response functions, the temporal pattern of the discharge, the effects of different background $CO_2$ concentrations and the physiological condition of the insect. Among these conditions are age, diapause status and whether the species is autogenous or anautogenous. The physiological characteristics of $CO_2$ detection in female mosquitoes are contrasted with similar characteristic in male mosquitoes as well as in other insects such as biting midges (Ceratopogonidae). The recent advances involving molecular research to further our understanding of the cellular processes of $CO_2$ detection and the role of deterrents and repellants on such processes are discussed. Finally, the uses of $CO_2$ as a tool in the design of control strategies including the development of traps for monitoring and potential control are discussed.

**Keywords**: carbon dioxide, mosquitoes, biting midges, behaviour, sensory physiology, blood feeding

## Introduction

Each year, there are approximately three hundred million new cases involving the major diseases transmitted by mosquitoes. One and a half million fatalities can be attributed to these diseases or their complications each year (Breman 2001, Mayor 2008). For decades, efforts have been made to better understand the mechanisms that underlie host attraction, feeding and mating of haematophagous insects (Friend and Smith 1977, Galun 1977). In particular, many have attempted to describe and synthesise the complex of signals which control host-seeking behaviour in mosquitoes (Davis and Bowen 1994, Daykin *et al.* 1965, Takken 1991, Zwiebel and Takken 2004). A number of factors including: moisture, heat, (Clements 1963, 1999); host volatiles such as: lactic acid (Acree, Jr. *et al.* 1968, Smith *et al.* 1969), carbon dioxide ($CO_2$) (Gillies 1980, Mayer and James 1969) and other compounds (Kline *et al.* 1990, 1991) appear to be important stimuli for mosquito orientation.

Many biting insects, including anautogenous mosquitoes, require a blood meal before oogenesis. For example, a female *Aedes aegypti* (L.) mates soon after emergence and sometime later she blood feeds. Within several days of blood feeding, she lays her eggs. The blood serves as a primary

source of energy for egg development. To acquire this blood meal, the female mosquito must locate a host for feeding. One of the sensory cues involved in this host-seeking behaviour is $CO_2$; a primary byproduct of cellular respiration, which is released in large quantities by all potential hosts. Carbon dioxide had been implicated in mosquito orientation as early as the 1920s (Rudolfs 1922, Crumb 1922) and continues to be seen as one of the important chemical signals utilised by mosquitoes and other haematophagous insects in their host-locating and feeding behaviours (Bowen 1991, Galun 1977, Gillies 1980). To serve that end, female mosquitoes, as well as many other biting insects, prove to be equipped with an array of sensors that are highly sensitive and specifically tuned to respond to behaviourally relevant doses of $CO_2$ (Grant and Kline 2003, Grant and O'Connell 1996a, Grant *et al*. 1995, Kellogg 1970). Carbon dioxide-sensitive receptor neurons have been identified and studied morphologically in mosquitoes (McIver 1972b), and their physiological properties (Grant *et al.* 1995, Grant and O'Connell 1996b) have been explored in several other arthropods, including Lepidoptera (Bogner 1990, Bogner *et al.* 1986), Hymenoptera (Lacher 1964, Stange and Diesendorf 1973), other Diptera (Bogner 1992, Grant and Kline 2003) and Arachnida (Steullet and Guerin 1992). It is clear from all these studies that attraction to hosts is a complex behaviour that can be modified by a large number of environmental effectors many of which may be unique to a particular species (Davis 1996).

Since the early extirpation experiments of Roth and others (Jones and Madhukar 1976, Omer and Gillies 1971, Roth 1951), it has been clear that chemoreceptor neurons in sensilla located on the maxillary palps together with those on the antenna, play an important role in the detection and processing of the chemical stimuli implicated in initiating and modulating host location and feeding behaviours in insects (Bowen 1991, Gillies 1980, Kline *et al.* 1991, Takken and Kline 1989). Early physiological studies of the peripheral sensory system in mosquitoes had focused largely on the responses of olfactory receptor neurons in antennal sensilla. The antenna does contain a highly sensitive L-lactic acid receptor neuron, whose activity can be modulated by the behavioural repellent N,N-diethyl-m-toluamide (DEET) (Davis 1977, 1985, Davis and Sokolove 1976) The response characteristics of other antennal sensilla will be discussed by other author's in this volume and will not be addressed further in this review. A single early report (Kellogg 1970) described electrophysiological responses to $CO_2$ in neurons housed in sensilla basiconica on the maxillary palp of female mosquitoes. We now know that one of the three sensory neurons normally found in sensilla basiconica responds to small increments in $CO_2$ concentration and is sensitive to concentrations that are appropriate for orientation behaviour (Grant *et al.* 1995). In addition to the $CO_2$-sensitive neuron, the maxillary palp sensillum is innervated by two other spontaneously active receptor neurons. The neuron producing the smallest amplitude action potential is sensitive to stimulation with low concentrations of 1-octen-3-ol (octenol) (Grant and O'Connell 1996b), another mosquito attractant (Takken and Kline 1989). The neuron producing the intermediate-sized action potential is sensitive to yet another compound (unpublished data) and all three of these cells have axons that project directly to the brain. These three cells are developmentally related to each other so it is possible that they all could share similar functional purposes guiding host-locating behaviours.

## Morphology of the maxillary palp and sensilla

Like many female mosquitoes, *Ae. aegypti* have specialised sensilla on the ventral lateral surface of the fourth subsegment from the base of each maxillary palp (McIver 1972a, 1982). This overall morphology is shared by many species of mosquitoes. For illustrative purposes we show in Figure 1A-B scanning electron micrographs (SEMs) from a female *Culex pipiens* Linnaeus. Male mosquitoes also possess morphologically similar sensilla that are distributed along the distal half of the third

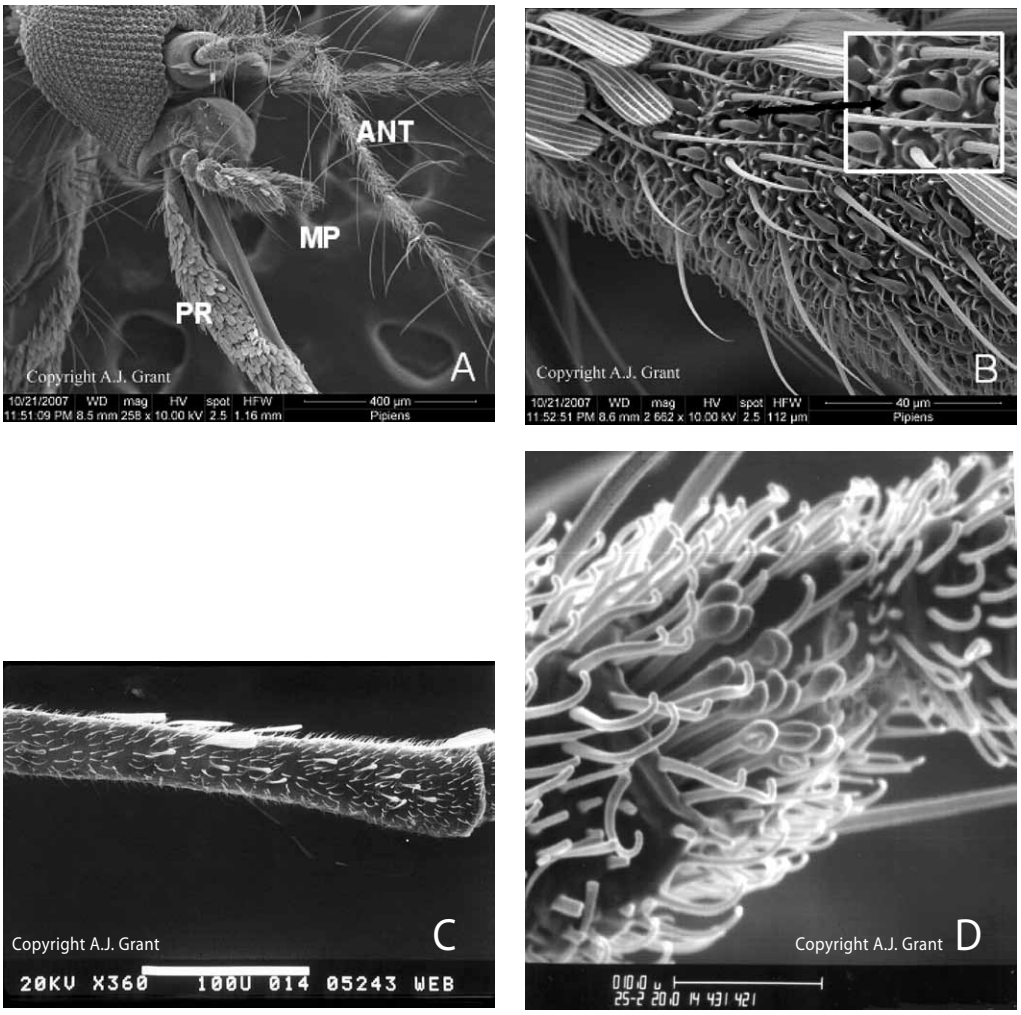


*Figure 1. Scanning electron micrographs (SEMs) of maxillary palps of mosquito species (A-C) and biting midges (D). (A) Portion of the head of a 21-22 day old female* Cx. pipiens *showing the maxillary palps (MP), antennae (ANT), and proboscis (PR). (B) Fourth subsegment of a maxillary palp of a 21-22 day old female* Cx. pipiens *showing individual sensilla. Inset: individual sensillum containing a neuron sensitive to $CO_2$. (C) Portion of the maxillary palp of a male* Ae. aegypti *depicting the basiconic sensilla containing neurons sensitive to $CO_2$. (D) Portion of the maxillary palp of a female* C. furens *(Poey) showing a cluster of $CO_2$-sensitive basiconic sensilla situated in an enlarged depression on the distal portion of the third subsegment from the base.*

subsegment (Figure 1C) (McIver 1972a, 1982). The presence of these sensilla is prevalent among mosquitoes, but their numbers and distribution varies from species to species (Braverman and Hulley 1979). Female *Ae. aegypti* possess approximately 25 sensilla on each palp. The sensilla basiconica in both *Ae. aegypti* and *Cx. quinquefasciatus* Say are restricted to the fourth subsegment from the head, whereas *Anopheles stephensi* Liston have sensilla basiconica distributed along the terminal three subsegments of the palp. It has been suggested that differences in the number

and distribution of particular chemoreceptors on the antennae and maxillary palps of various species of mosquito are related to differences in the insect's relative preferences for particular hosts (Braverman and Hulley 1979) or in their landing and probing responses to them. In addition to the variation in distribution of sensilla, the overall length of the maxillary palps varies. The palps of *An. stephensi* are significantly longer (about 1,450 µm) than the palps of either *Ae. aegypti* (about 350 µm) or *Cx. quinquefasciatus* (about 300 µm). Although there is considerable variation in the external morphology of the palps and the distribution of sensilla along them the physiological characteristics of the neurons that innervate them show strong similarities (Figure 2); particularly with regards to the response of the neuron producing the largest amplitude action potential (up to 300 µV peak to peak), which is reliably sensitive to stimulation with $CO_2$ (Grant *et al.* 1995, Kellogg 1970). However, our recent unpublished studies suggest that several Anopheline species have reduced sensitivity. Lu *et al.* reported that *An. gambiae* Giles showed an elevated threshold and a diminished dose response functions (Lu *et al.* 2007).

The presence of these $CO_2$-sensitive sensilla is often correlated with blood feeding behaviour. In other arthropods, sensilla with similar morphological features are found to contain neurons which also respond to $CO_2$. For example, biting midges possess similar sensilla with neurons that respond to $CO_2$. Although the Phantom Midge (Family *Choaboridae*) is taxonomically related to the mosquito, it had long been thought to be a non-biting insect. Subsequent investigations revealed the presence of blood in the digestive tract and an anatomical study revealed that these animals have mouthparts appropriate for biting and that they also possess basiconic sensilla on the palps which are morphologically similar to those examined here (McKeever and Pound 1979). To date, this correlation only holds for females of the species.

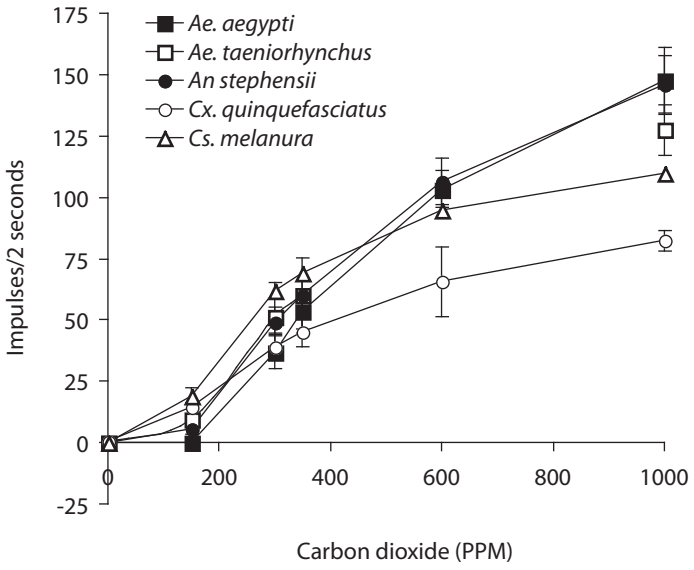


*Figure 2. Mean±SE number of action potentials during 2 s stimulus pulses of the indicated concentrations of $CO_2$ from s. basiconica on the maxillary palps of individuals from five different species of female mosquito (*Ae. aegypti, An. stephensi, Cx. quinquefasciatus, Cs. melanura *(Coquillett), and* Ae. taeniorhynchus *(Wiedemann)). All of these functions were established in a background environment containing 0 ppm $CO_2$.*

## Methodology in electrophysiological studies

Standard electrophysiological techniques can be used to record extracellular responses from the receptor neurons located in the sensilla found on insects. For $CO_2$-sensitive neurons in biting insects such as mosquitoes and biting midges, details of the recording methodology have been described fully in previous reports (Grant and O'Connell 1986, O'Connell 1972) and are summarised below. To insure stable recordings, the insect must be carefully immobilised with the target structure positioned in a manner that allows unobstructed access of the recording microelectrode to the selected sensillum. Whole adults are secured on a glass plate with minute strips of sticky tape or fibre threads to secure the thorax, abdomen, legs and wings. Once the insect is secured, the preparation is positioned under the objectives of a compound light microscope with an effective magnification of approximately 750X.

The recording microelectrodes are hand-made from 125-µm diameter straightened tungsten wire. They are electrolytically sharpened to a tip diameter less than 1 µm in a 10% electrolyte solution (Galbreath and Galbreath 1977, Hubel 1957). An indifferent electrode of similar design is inserted into the eye of the insect and the recording electrode is inserted through the cuticle at the base of an individual sensillum. The microelectrodes are mounted in micromanipulators for positioning. Following placement of the recording electrode in the fluid space of the sensillum the electrical signals obtained from the neurons within the sensillum are band-passed filtered, amplified and sent in parallel to an audio monitor and a computer for subsequent data acquisition, action potential discrimination, analysis and storage. The basic work station, including the microscope, manipulators, preparation stage and amplifier are all mounted on a vibration isolation table in a Faraday cage. The electrical circuits required for this apparatus are isolated and independently earthed through a dedicated ground.

Data acquisition and analysis are accomplished using a software programme Autospike, developed by the late Dr. Jan N.C. van der Pers (www.syntech.nl/s). This programme is similar in principle to one used earlier (O'Connell *et al.* 1973) and is designed to record and analyse electrophysiological information from insect chemosensilla. This programme runs on a Windows platform with an analog-to-digital/digital-to-analog (AD/DA) interface via an IDAC controller. Discrimination and sorting of action potentials arising from the several neurons usually found in a single sensillum are easily accomplished with Autospike. Electrophysiological preparations from mosquitoes and other insects typically last for several hours before there are detectable changes in their response properties.

Chemical stimulation of olfactory receptor neurons in insects is usually accomplished with two opposing gas streams directed towards the sensory structure (Borroni and O'Connell 1992). One of these is normally on and carries the desired background stream (550 mls/min). The second stream is normally off and carries the desired stimulus (440 mls/min) stream. An independent controller activates the valves controlling these two streams and also initiates data collection by the software. To reliably manipulate $CO_2$ stimulus concentration the flow dilution olfactometer design (O'Connell and Mozell 1969) normally used in olfactory studies should not be used. In this basic design a stock concentration of $CO_2$ is diluted to the desired stimulus concentration by adding diluent flow from a pure air stream and adjusting the ratio of flow in the two gas streams to produce the final desired concentration. Although the bulk concentration of $CO_2$ can be adjusted in this fashion, its use reveals a considerable variation in neuronal response. This appears to be due to inconsistent mixing within the stimulus stream. As we shall discuss later, $CO_2$ receptor neurons appear to have an enhanced ability to follow rapid changes in concentration

especially when compared to the more tonic pheromone receptor neurons normally found in insect antennae (Borroni and O'Connell 1992). Because of this factor, reliable $CO_2$ stimuli should be delivered without dilution from certified formulated gas cylinders, each containing a calibrated concentration of $CO_2$. For our studies we chose 0, 150, 300, 350, 600 and 1000 ppm $CO_2$, each containing 20% purified oxygen, and the remainder made up with purified nitrogen. Between $CO_2$ stimulations, the preparation is bathed in a stream of carbon dioxide-free synthetic air (0 ppm $CO_2$). For each preparation 2 s pulses of $CO_2$ are sequentially presented at each of the certified concentrations. The interval between individual stimuli is normally 10-20 s. Following a brief rest period of approximately one to five minutes the whole protocol is repeated. Reproducibility of the response to a particular stimulus concentration is very high and individual responses at a particular concentration rarely differ from each other by more than a few impulses. Three to five repetitions of this protocol are made for each preparation to form the average. T-tests are then performed for the grand average response across all preparations at each dose to determine if there are any statistical differences between treatments.

## Temporal pattern of response

The maxillary palp sensilla of both mosquitoes and biting midges (Diptera: Ceratopogonidae) contain neurons that are responsive to stimulation with low concentrations of $CO_2$. Stimulation of these neurons with pulses of $CO_2$ elicits typical biphasic action potentials with peak-to peak amplitudes of several 100 µVs (Figure 3). This neural signal is sent to the insect brain where

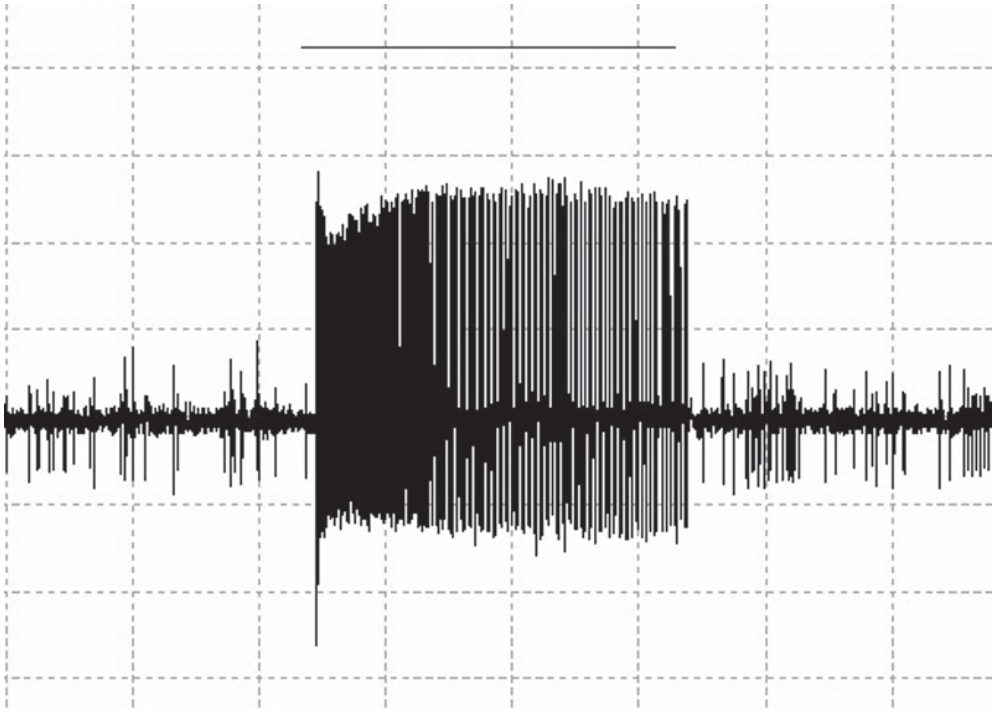

*Figure 3. Typical electrophysiological response from a female* Cx. pipiens *in response to a 2 s stimulation of 600 ppm $CO_2$ in a background of 0 ppm $CO_2$.*

environmental $CO_2$ information is processed. Processing in the central nervous system probably is dependent not only on the presence or absence of impulse activity in particular afferents, but also on the pattern of action potentials generated in them during the odour stimulus. Generally these neurons do not produce nerve impulses in environments lacking $CO_2$. To evaluate the temporal pattern of activity for the $CO_2$-sensitive neurons in the maxillary palp sensilla, we can evaluate the instantaneous frequency of action potentials generated by these neurons during the stimulus pulse (Figure 4). Both mosquito and biting midge $CO_2$-sensitive cells respond with a sharp 'phasic' burst of activity, lasting 100-200 ms, followed by a lower tonic level of response whose duration and magnitude is dependent on stimulus concentration and the length of the stimulus pulse. Step increases in stimulus concentration elicit phasic increases in activity in both mosquito and biting midge receptor neurons, step decreases in concentration elicit reciprocal phasic reductions in activity followed by a slower increase in activity as the response returns to a level proportional to the new background concentration of $CO_2$. This latter pattern can only be observed when steps in stimulus concentration are given from a background level of $CO_2$. This pattern of discharge is widely believed to provide a sharpening mechanism (Dionne 1992) that allows the nervous system to detect the small changes in concentration that likely signals the presence of potential hosts (Grant and O'Connell 1996a).

The difference in the temporal pattern of discharge may also reflect intrinsic differences in the way in which the respective chemical signals in the environment are processed. The response properties of $CO_2$-sensitive receptor neurons to step changes in concentration suggest the presence of at least two different mechanisms. On one hand, there appears to be a tonic response mechanism that simply reads out a response rate proportional to the steady state concentration of $CO_2$ with relatively little desensitisation over time. On the other hand, there is also a rate-sensitive mechanism whose output rapidly follows both the direction and the rate of change of $CO_2$ concentration. As is the case with cold receptor neurons in vertebrates (Darian-Smith *et al.* 1973) and invertebrates

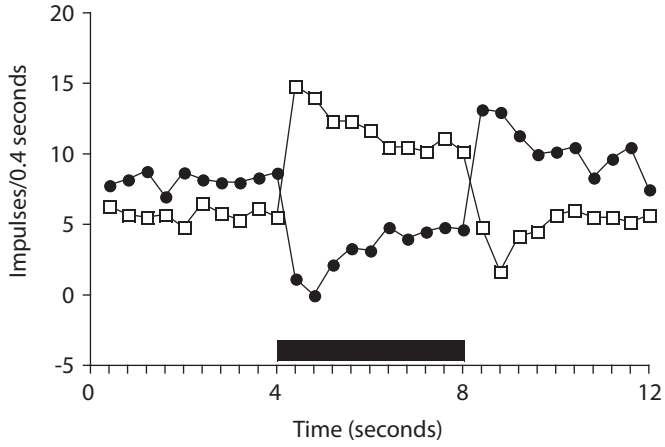


*Figure 4. The temporal pattern of response from a $CO_2$-sensitive receptor neuron in a female* Ae. aegypti. *The open symbols represent the average response to a 4 s stimulus pulse stepping up to 350 ppm $CO_2$ from a background concentration of 300 ppm. The solid symbols represent the average response to a 4 s stimulus pulse stepping down to 300 ppm from a level of 350 ppm $CO_2$. In both cases the number of action potentials was averaged in 400 ms time bins and represents the mean from a single sensillum stimulated 10 times with each pulse protocol.*

(Altner and Loftus 1985) these phasic properties provide a sharpening mechanism that greatly exaggerates the response output of the receptor neuron as stimulus intensity changes rapidly. For example, the average tonic levels of activity elicited by $CO_2$ backgrounds of 300 and 350 ppm differ from each other by only about 6 impulses/s (Figure 4). However, a rapid step from 300 to 350 and back to 300 ppm elicits peak phasic responses that differ from each other by approximately 38 impulses/s. Although we do not yet know the 'following' capabilities of the $CO_2$ receptor, the ability of the neuron to produce phasic bursts whose magnitude and sign are a function of the magnitude and sign of the change in $CO_2$ concentration should have the effect of enhancing the sensory signal elicited and transmitted to the central nervous system by rapid changes in concentration. It is likely that this ability of the system to follow rapid changes in concentration accounts for the difficulty associated with standard dilution techniques of stimulation referenced earlier.

Depending on the nature of the central processing associated with these temporal inputs, the ability of the sensory system to follow rapid changes in $CO_2$ concentration might be enhanced by these characteristics. This pattern of response would also be particularly appropriate if mosquitoes oriented to hosts by following the rapidly changing increments in $CO_2$ concentration that are likely to be encountered in nature (Elkinton *et al.* 1984), as opposed to simply flying upwind toward a source in a steadily increasing gradient as might be imagined from a point source release of an odorant. It should be clear then that receptor neurons in insect olfactory sense organs can vary in both their chemical sensitivity and in their temporal characteristics (see Chapter 4, this volume).

## Responses in different background concentrations of $CO_2$

For ease of comparison, most electrophysiological tests reported here were to step increases in $CO_2$ concentration from a background environment lacking $CO_2$ (0 ppm $CO_2$). The resulting response magnitude fits a log function dependent simply on $CO_2$ concentration. However, biting insects in nature never encounter environments without $CO_2$. Therefore, when we tested the same set of $CO_2$ concentrations applied from different background levels of $CO_2$ (Figure 5), we obtain some useful additional information about the sensory neuron. Here too, we see the same basic pattern of response to stimulation in different background concentrations of $CO_2$ in mosquitoes and biting midges. The average numbers of impulses generated in different background concentrations during a 2 s stimulus containing a given $CO_2$ concentration are similar or slightly less as long as the background concentration is lower than the stimulus concentration. However, if the background concentration is higher than the stimulus concentration, the average number of impulses generated to the stimulus step is greatly diminished. For example, in backgrounds containing 0, 150 or 300 ppm $CO_2$ the magnitude of responses elicited by a stimulus containing 600 ppm $CO_2$, are comparable to each other. However, in a background concentration of 1000 ppm $CO_2$, the magnitude of response to a similar stimulus containing 600 ppm $CO_2$ is greatly reduced.

One implication of this observation is straightforward. One can imagine that the reduced sensory signal may impede the animal's ability to find hosts in high backgrounds. Conversely, the animal is more likely to only find hosts that emit $CO_2$ concentrations higher than the background. From this we can surmise that continued increases in the environmental concentrations of $CO_2$ might result in large shifts in host-seeking behaviour. However, without additional information on the response of hosts to these changes it is impossible to predict how these shifts could alter host detection and subsequent disease transmission.

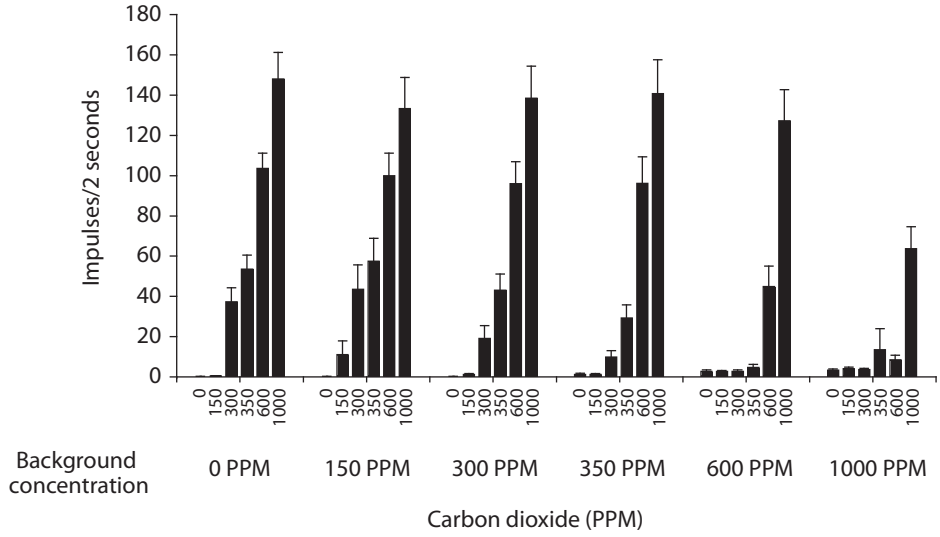


*Figure 5. Mean + SE responses of $CO_2$-sensitive receptor neurons in female* Ae. aegypti. *The stimuli consisted of 2 s pulses of different concentrations of $CO_2$ delivered from each of the indicated background levels of $CO_2$.*

## Detection of $CO_2$ by male mosquitoes

Male mosquitoes possess morphologically similar sensilla to those structures found on the maxillary palps of females (McIver 1972a, 1980). They prove to contain $CO_2$-sensitive receptor neurons as well (Grant *et al.* 1995). The spatial distribution of these *sensilla basiconica* on the male palp is not restricted to the fourth sub-segment from the head, as in the female of many species. Instead, the distribution includes the distal half of the third subsegment from the head (Figure 1C). Neurons that innervate these male sensilla have thresholds and sensitivities to $CO_2$ that are similar to those in females (Figure 6, Grant and O'Connell 2007).

The apparent ability of males to detect $CO_2$ raises questions about the potential function of this chemical stimulus in their biology. Male mosquitoes are not known to blood feed, as their mouthparts are not adapted for piercing (Matheson 1945). But males do feed on plant nectars. Perhaps their ability to detect $CO_2$ is utilised in their search for these food sources. Depending on when during the day they are active this input may be useful in detecting either sources or sinks of $CO_2$ involved with the photosynthetic activity of plants. Alternatively, male *Ae. aegypti* have been observed near human hosts where mating occurs as the females seek a blood meal (Hartberg 1971). This suggests that males of some species may use $CO_2$ as a secondary cue to orient to places where females are likely to be more abundant and available for mating.

## Effect of age on $CO_2$ responses

Following emergence, female mosquitoes typically perform a series of behaviours which includes mating, feeding and oviposition. For an anautogenous mosquito such as *Ae. aegypti,* a blood meal is required to obtain the protein needed for egg production. To acquire this blood, the female engages in periods of host-seeking. Host seeking is governed by sensory cues associated

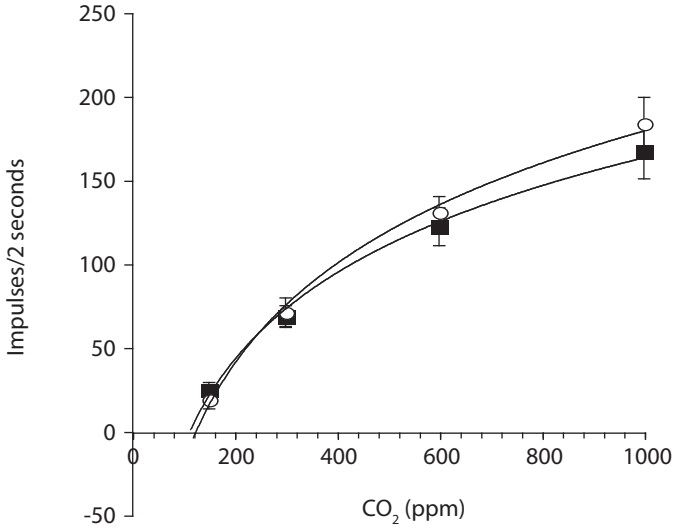


*Figure 6. Mean±SE concentration-response function from male (open circles; n=20) and female (solid square; n=30). $CO_2$-sensitive receptor neurons in female* Ae. aegypti *in response to a 2 s stimulation of the indicated concentration of $CO_2$. Stimulation with the control concentration of 0 ppm $CO_2$ elicited no response in any mosquito. Male responses were not statistically different from female responses at any concentration tested.*

with host-produced odours (Takken 1991) including $CO_2$. Mating generally occurs within the first few days following emergence (Christopher 1960, Clements 1999). Blood feeding is not required for copulation to occur. Numerous behavioural reports suggest that for most anautogenous mosquitoes relatively little feeding activity occurs during the first few days of adult life and host-seeking and feeding activities increase dramatically from then to 96 h after emergence (Christopher 1960, Davis 1984a, Seaton and Lumsden 1941). This enhanced host-seeking level is maintained for several weeks. Since host-seeking behaviour changes as the insect ages, it is interesting to evaluate the dynamic sensitivity of the $CO_2$ receptor neurons across this timeframe. To that end, we recorded electrophysiological responses from sensory neurons to stimulation with various concentrations of $CO_2$ in newly emerged adults (0-2 d old) when host orientation is reduced, and in older insects (4-7 d old) when host orientation reaches a maximum. Female *Ae. aegypti* show pronounced differences in the sensitivity of their $CO_2$ receptor neurons across these young and older age classes (Figure 7, Grant and O'Connell 2007). Young females have a significantly lower overall response rate to stimulation with 300, 600 and 1000 ppm $CO_2$ when compared with the same stimuli in older females. Recording from male *Ae. aegypti* did not show a comparable age dependent difference for any of the $CO_2$ concentrations tested (Grant and O'Connell 2007).This difference in sensitivity as a function of age was also observed for female *Cx. pipiens* (unpublished results). Similar differences in the rates of development of sensory systems have been studied electrophysiologically and reported in other insects as well (Blaney *et al.* 1986, Crnjar *et al.* 1990, Davis 1984b, Schweitzer *et al.* 1976).

In addition to the variation in overall sensitivity between young and older female mosquitoes, the temporal pattern of discharge to a continuous stimulus (1000 ppm $CO_2$) also differed between the two ages (Grant and O'Connell 2007). As we have argued before (Grant and O'Connell 1996b)

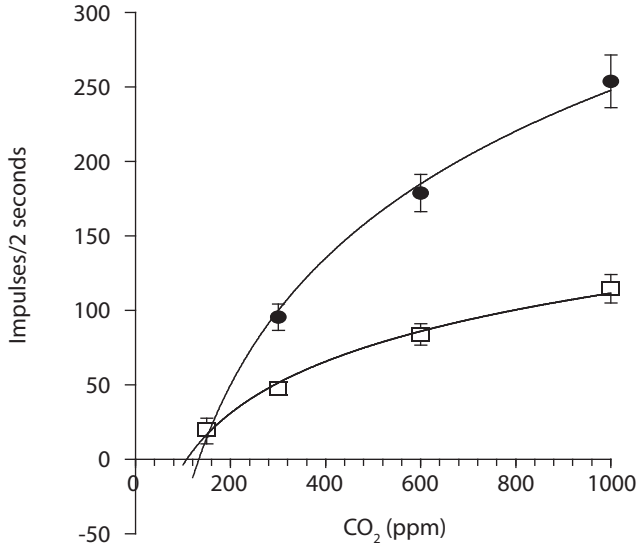


*Figure 7. Mean±SE concentration-response functions from young (open square; n=15) and older (solid circles; n=15) $CO_2$-sensitive receptor neurons in female* Ae. aegypti *in response to a 2 second stimulation of the indicated concentration of $CO_2$. Stimulation with the control concentration of 0 ppm $CO_2$ elicited no response in any mosquito. Responses were statistically different from each other at concentrations of 300, 600 and 1000 ppm. The solid circles and open squares represent data points and the solid lines represent functions fitted to these points.*

the temporal discharge pattern may enhance the neuron's ability to discriminate rapid changes in $CO_2$ concentration that are presumed to occur in the downwind stimulus plume emanating from a potential host. Thus, enhancing the capability of the peripheral sensory system to detect $CO_2$ changes as the female mosquito matures should enhance host-seeking in older females. On the other hand, these changes in sensitivity may be accidental and simply reflect the normal physiological maturation of the sensory system or the changes in the periphery may be timed by other more central processes in the mosquito which are designed to optimise the sensory capabilities of the system when it is required to mediate host-seeking behaviour. It is not possible to state the causal relationships between these host-related sensory capabilities and the development of host-seeking behaviours. However, one is tempted to speculate that the maturation we have demonstrated in the sensory system is responsible for the age-related shift in host-seeking behaviour.

## $CO_2$ detection in biting midges

In addition to mosquitoes, other biting insects detect $CO_2$ including but not limited to Tabanidae, Simuliidae, some Muscidae and Ceratopogonidae. Among the Ceratopogonids or biting midges, *Culicoides* is the most important genus with respect to the health and comfort of humans and animals (Kettle 1965, Linley *et al.* 1983). Annoyance from adult female biting midges often may become serious enough to affect outdoor recreational and work-related activities. Their presence also has an adverse effect on tourism and land development, especially in coastal areas of the United States (Linley and Davies 1971) and in parts of Europe. Animal and human health

are affected by disease organisms transmitted by adult female biting midges, which include protozoans, filarial worms and viruses (Blanton and Wirth 1979, Kettle 1965, Linley *et al.* 1983).

*Sensilla basiconica* on the maxillary palps of female *C. furens* (Poey), *C. stellifer* (Coquillett) and *C. mississippiensis* (Hoffman) all contain a receptor neuron which responds to stimulation with low levels of $CO_2$ (Figure 1D). These neurons produce typical biphasic action potentials with peak-to-peak amplitudes of up to 350 μV. As in our mosquito work, we recorded responses to stimulation with 0, 150, 300, 600 and 1000 ppm $CO_2$. The *Culicoides* $CO_2$-sensitive neurons have basic physiological characteristics that are similar to those in the mosquito $CO_2$-sensitive neurons (Grant and O'Connell 1996b, 2007, Grant *et al.* 1995). Both mosquito and biting midge receptor neurons respond with a phasic-tonic discharge pattern to increases or decreases in $CO_2$ concentration. The neurons of both are silent in environments without $CO_2$. However, there are several interesting differences between biting midges and mosquito response properties. First, in most cases, their response thresholds appear different. Stimulation with 150 ppm $CO_2$ elicited a response in *C. furens,* which is statistically larger than the activity seen in response to stimulation with 0 ppm $CO_2$ (Figure 8). *Aedes aegypti*, on the other hand, often require higher concentrations to elicit a significant increase in response (Grant *et al.* 1995). This indicates that the threshold of detection is lower for *C. furens* and as a consequence their neurons are more likely to be active at ambient levels. Second, the slopes of the concentration response functions are considerably different. The response function for *Ae. aegypti* is steeper than for *Culicoides.* This indicates that a small change in stimulus concentration will elicit a larger change in impulse activity in *Aedes* as

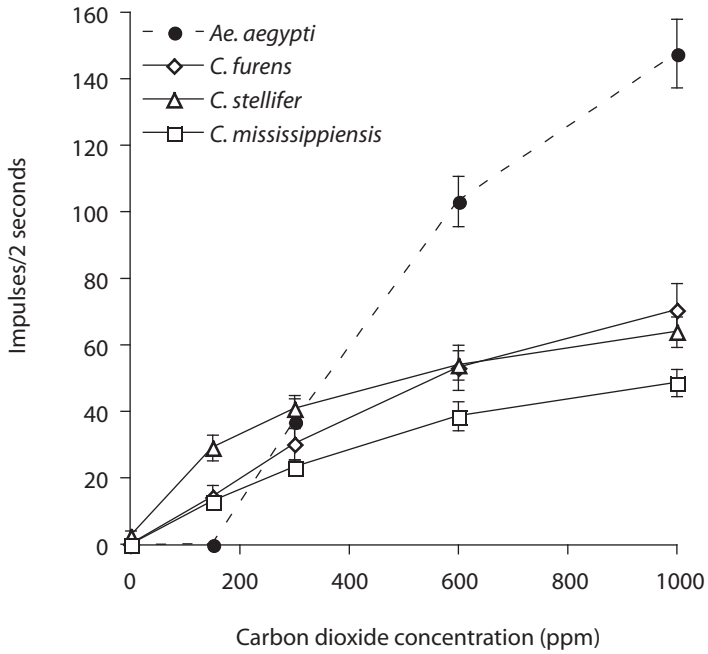


*Figure 8. Concentration-response functions from three species of* Culicoides *[*C. furens *(n=15),* C. mississippiensis *(n=3) and* C. stellifer *(Coquillett) (n=6)] in response to graded stimulations of $CO_2$. For comparison, also included is the concentration response function for* Ae. aegypti *(n=13) (Grant* et al. *1995).*

compared to *Culicoides* suggesting that the mosquito would be able to detect a smaller change in $CO_2$ concentration. Although the threshold and slope of the concentration functions were different, they do overlap with each other near ambient levels (350 ppm $CO_2$).

The significance of a lower threshold of sensitivity to $CO_2$ in *Culicoides* is unclear. Ambient atmospheric levels of $CO_2$ are currently about 350 ppm. So, it seems unlikely that having the ability to detect lower $CO_2$ concentrations than ambient would enhance the insect's host-seeking ability. Many nematoceran insects are autogenous and utilise sugar feeding for survival (Stewart and Kline 1999). The environment contains many sources and sinks for $CO_2$. Actively photosynthesising plants may produce local reductions in the concentration of $CO_2$. Thus, it is possible that these insects are using their enhanced $CO_2$ sensitivity to find $CO_2$ levels below ambient where plants that contain nectar for sugar feeding are located. *Culicoides mississippiensis* is known to sugar feed from the plant Yaupon Holly, *Ilex vornitoria* Aiton, during the day and possibly during the night (Stewart and Kline 1999). Therefore, it is possible that low concentrations of $CO_2$ in conjunction with other volatiles may serve as a plant attractant for sugar feeding. In a similar manner, higher concentrations of $CO_2$ may serve as a host-seeking attractant for blood feeding.

Sensitivity to low concentrations of $CO_2$ also may have no behavioural significance. It has been suggested (Stange 1997, Stange and Wong 1993) that certain species of moths, which also have $CO_2$-sensitive neurons, actually are adapted to lower, pre-industrialisation levels of $CO_2$. Although our methods differed from those of Stange in characterising $CO_2$ sensitivity, perhaps the sensitivity to lower concentrations of $CO_2$ in *Culicoides* simply reflects an earlier sensitivity, which has not yet been genetically adapted to fit the dramatic increase caused by human-based emissions of $CO_2$.

## Diapause and $CO_2$ detection

*Culex pipiens* is a vector of several medically important viruses in the United States including West Nile. Under environmental conditions of short day length and low ambient temperatures during late larval and early pupal stages, the mosquitoes are induced to enter diapause (Spielman and Wong 1973). Diapausing adults demonstrate a slower rate of development of primary ovarian follicles. In addition, host-seeking and feeding behaviours are reduced. Because of these differences we sought to determine if the $CO_2$-sensitivity of palp receptor neurons was affected by diapausing status. To test this, we recorded responses from the $CO_2$-sensitive peripheral sensory neurons in the maxillary palps of adult female mosquitoes to graded stimulation with $CO_2$ in diapausing and non-diapausing populations. Non-diapausing *Cx. pipiens* were reared from pools of egg rafts to adults in environmental chambers at 18 °C, 15:9 h light-dark cycle, and 75% RH. To induce diapause, alternate groups selected from the same raft pools were reared with short days 7:17 h light-dark cycle. To confirm the effectiveness of our rearing protocols, primary ovarian follicle and germarium lengths were measured in a portion of each cohort to determine diapause status, and the stage of ovarian development was determined according to Christopher (1911).

Our electrophysiological data (Figure 9) indicate that the diapause state does not appear to affect the sensitivity of these sensory neurons. Diapausing and non-diapausing female *Cx. pipiens* have similar electrophysiological responses to stimulation with 0, 150, 300, 600, and 1000 ppm $CO_2$. These finding suggest that the reduction in host-seeking behaviour observed in diapausing *Cx. pipiens* is not determined by changes in the $CO_2$ sensitivity of peripheral sensory neurons. We note that these data suggest that the changes in behaviour that occur with diapause must arise because of factors that affect some other stage of central nervous system processing (see Chapter 4, this volume).

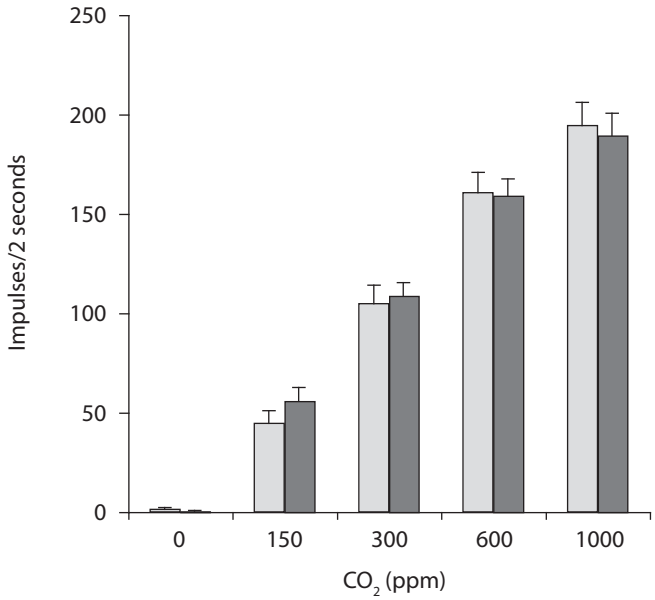


*Figure 9. Mean + SE concentration-response from $CO_2$-sensitive neurons on female* Cx. pipiens *that were reared under temperature and light conditions to produce non-diapausing adults (light grey; n=35) and diapausing adults (dark grey; n=26). Those adults reared under diapausing condition were not statistically different from those reared under non-diapausing conditions at any concentrations tested.*

## $CO_2$ responses from receptor neurons in autogenous mosquitoes

*Culex pipiens* forms a complex of mosquitoes whose taxonomic status is still unclear. *Culex pipiens* was first described by Linneaus in 1758 and *Cx. molestus* by Forskal in 1775 (Vinogradova 2000). Morphologically these two mosquitoes are very similar. Physical differences such as comparative measures of maxillary palp to proboscis length in males have been suggested as a potential diagnostic trait. However, such lengths appear quite variable from population to population, greatly reducing their predictive value. In our hands, this metric discriminated potential *Cx. molestus* Forskal from *Cx. pipiens* with approximately 85% accuracy. Although considered by some a distinct species or subspecies, many are inclined to consider *Cx. molestus* as an 'intraspecific category, i.e. a variety, eco-physiological race or ecotype' (Vinogradova 2000). Whatever the taxonomic classification, *Cx. molestus* are autogenous and are able to produce eggs without taking a blood meal, whereas *Cx. pipiens* are anautogenous and require a blood meal for egg production. Both *Cx. pipiens* and *Cx. molestus* produce an egg raft, however, the size of the rafts differ considerably. *Culex molestus* (sugar-fed) rafts contained 78.9±7.0 (SE, n=13) eggs, whereas an average blood-fed *Cx. pipiens* raft contained 190.7±7.7 (SE, n=3) eggs.

Since the life cycle of the autogenous *Cx. molestus* does not require blood feeding, we were interested in characterising the $CO_2$-sensitive receptor neurons in their palps and comparing their sensitivity with those found in *Cx. pipiens*. The females of both groups of mosquitoes possess maxillary palps with similar basiconic sensilla. Although no detailed study of fine structure of female maxillary palps has been done, *Cx. molestus* and *Cx. pipiens* palps and sensilla appear

very similar. However, recordings from the $CO_2$-sensitive neurons revealed some interesting differences in their overall sensitivity to $CO_2$ stimulation. *Culex molestus* are significantly less sensitive as compared to *Cx. pipiens* (Figure 10). Extrapolating from the dose-response curves; both *Cx. molestus* and *Cx. pipiens* have similar thresholds of slightly over 100 ppm $CO_2$; however the slopes of the concentration response functions are different. These results are interesting because as was the case with the difference between young and older *Ae. aegypti* and *Cx. pipiens*, the group that shows reduced or no host-seeking behaviours possessed sensory neurons less sensitive to $CO_2$. *Culex molestus* can be induced to blood feed and we are currently conducting experiments to determine if the nutrient source (sugar or blood) affects the receptor neurons of the resulting offspring. Preliminary data from *Cx. molestus* suggest that aspects of the $CO_2$ sensitivity is dependent on diet.

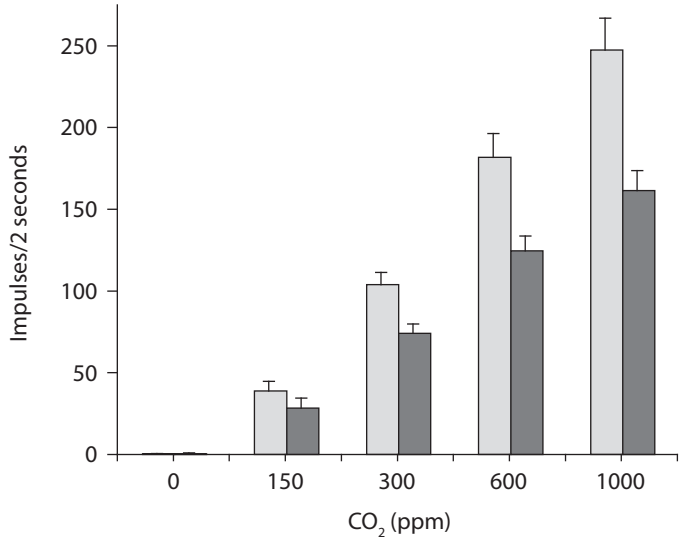


*Figure 10. Mean + SE concentration-response relationships for female* Cx. p. pipiens *(light grey; n=15) and* Cx. p. molestus *(dark grey; n=20). All recording were from insects 5-8 days post emergence. Values were statistically different at 150, 300, 600 and 1000 ppm $CO_2$ (t-test, two tailed;* $P<0.01$*).*

## Potential effects of changes in atmospheric levels of $CO_2$

We are becoming increasingly convinced that our environment is changing (Gore 2006). Pressures associated with post-industrial development and methods of environmental management have led to large changes in the levels of chemicals in our environment. One of these chemicals is $CO_2$, the ambient level of which has been increasing for several centuries. Pre-industrial revolution environmental levels of $CO_2$ were estimated at or below 200 ppm. Current ambient atmospheric levels range between 330 and 350 ppm. Predictions of future levels have been as high as 500 ppm. Increasing levels of environmental $CO_2$ as well as other greenhouse gasses are believed by some to contribute to an elevation in global temperatures. There are many consequences of global warming including sea level elevation and wide-spread regional climate change. Some have speculated that such environmental changes in temperature could lead to a shifting of

habitat range for many species including mosquitoes (Eisen *et al.* 2008). Such a change in range could potentially affect the transmission of vector-borne diseases. Diseases that are prevalent in tropical climates might become more prevalent in temperate areas with significantly more public health impact.

Given that $CO_2$ is one of the primary activators-attractants for biting insects and that many biting insects possess highly specialised sensory neurons to detect $CO_2$, we suggest that alterations in $CO_2$ levels alone could also have effects on host-seeking behaviour. We know that the threshold of response of these sensory neurons lies around 100-120 ppm $CO_2$. Based on the response characteristics of these neurons, we question how potentially increasing environmental levels of $CO_2$ could affect the ability of the sensory neurons to process $CO_2$ changes and consequently how they will modulate orientation behaviour. We know that when these neurons are challenged with graded stimulation with increasing doses of $CO_2$, the background $CO_2$ concentration is important. In other words, a stimulus pulse of 1000 ppm $CO_2$ in a background of 150 ppm $CO_2$ elicits a significantly different response compared to a similar stimulus pulse of 1000 ppm $CO_2$ from a background of 600 ppm $CO_2$ (Figure 5). The signal received by the CNS is different. At present we do not know how this change in signal will be interpreted by the mosquito CNS and what behavioural consequences may result. In addition, little is understood about the effects of changes in long-term stimulation of these sensory neurons. The $CO_2$-sensitive system seems to be rather plastic and it is likely that the overall consequences for behaviour will not be obvious. However, it is clear now that a more thorough understanding of the system would be useful.

## Molecular studies of $CO_2$ detection

With the advent of molecular methods of study there have been many exciting advances in our understanding of the cellular mechanism underlying olfactory detection in insects. Early and continuing studies have been conducted with the model systems *Drosophila* (Dobritsa *et al.* 2003, Hallem *et al.* 2006, Jones *et al.* 2007, Rutzler and Zwiebel 2005). With the sequencing of the *Anopheles* genome in 2002 (Holt *et al.* 2002) much work has focused on *An. gambiae* (Goldman *et al.* 2005, Hallem *et al.* 2004, Hill *et al.* 2002, Kwon *et al.* 2007, Lu *et al.* 2007). Recent published work has begun to unravel and identify the molecular nature of the receptor proteins involved in $CO_2$ detection. Vosshall and her colleagues (Jones *et al.* 2007) report that receptor neurons in *Drosophila* co-express two receptor proteins necessary for $CO_2$ detection. These proteins Gr21and Gr63a have analogues in mosquitoes, GPRGR22 and GPRGR24. Both of these are expressed in olfactory neurons found in maxillary palp sensilla of *An. gambiae* (Jones *et al.* 2007). This work also indicates that the expression of these two proteins in $CO_2$-insensitive cells confers sensitivity and the removal of Gr63a causes mutant flies to lose this sensitivity both at a behavioural and sensory level. Work from other labs has implicated a third receptor (AgGR23) whose addition clearly augments $CO_2$ responsiveness (Lu *et al.* 2007). The roles of each of these proteins remains to be determined in the presumed cascade of events that leads to the detection and processing of this important environmental cue. It is tempting to speculate that these proteins will have differential effects on the sensitivity and overall rate of response to $CO_2$.

Work by Ditzen *et al.* (2008) has utilised the molecular understanding of receptor protein systems to understand the mode of action of the repellent DEET (N,N-diethyl-meta-toluamide). They report that DEET strongly inhibits the 1-octen-3-ol response of *An. gambiae* and *Drosophilia melanogaster* Meigen by mediating a response in the OR83b receptor protein and the mosquito analogue GPROR7 receptor complex. However, using electrophysiological techniques the detection and sensitivity of the $CO_2$ receptor neuron appears to be unaffected. These responses confirm that the

addition of DEET strongly inhibited the octenol response but did not affect the $CO_2$ sensitivity. In a similar fashion we also observed that DEET did not modulate the $CO_2$ response in *An. gambiae* Giles (Figure 11). More recent work in *Cx. quinquefasciatus* has suggested an alternative mechanism for DEET action on the octenol response. Syed and Leal (2008) have proposed that DEET acts through another channel by directly stimulating receptor neurons found in a specific antennal trichoid sensillum (Pickett *et al.* 2008, Syed and Leal 2008). Molecular understanding of receptor mechanisms will lead to further elucidation of the mode of action of attractants and repellents. Such understanding should enhance the development of semiochemicals and ultimately lead to the development of novel control strategies.

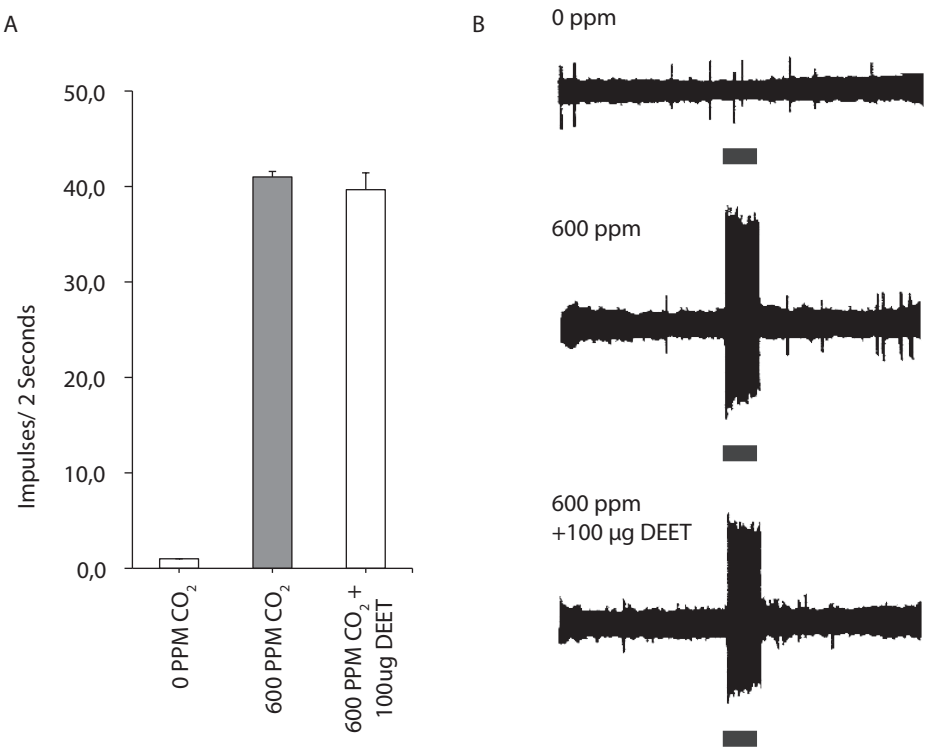


*Figure 11. Electrophysiological responses from basiconic sensilla on the maxillary palps of female* An. gambiae. *(A) Mean + SE responses (n=3) to 2 s stimulations of 0 ppm $CO_2$, 600 ppm $CO_2$ and 600 ppm $CO_2$+100 µg of DEET. Preparations were held in a background environment of 0 ppm $CO_2$ (these data were presented at the 1998 Association for Chemoreception Sciences meetings).*

## The use of $CO_2$ in insect trapping

Carbon dioxide has long been used as an attractant for female mosquitoes and other biting insects in trapping technology. For most, but not all species of biting insects, $CO_2$ serves to selectively attract female biting insects in a concentration dependent manner (Figure 12A-B). For several species, a significant but smaller number of males are also attracted. Reports in the 1960s

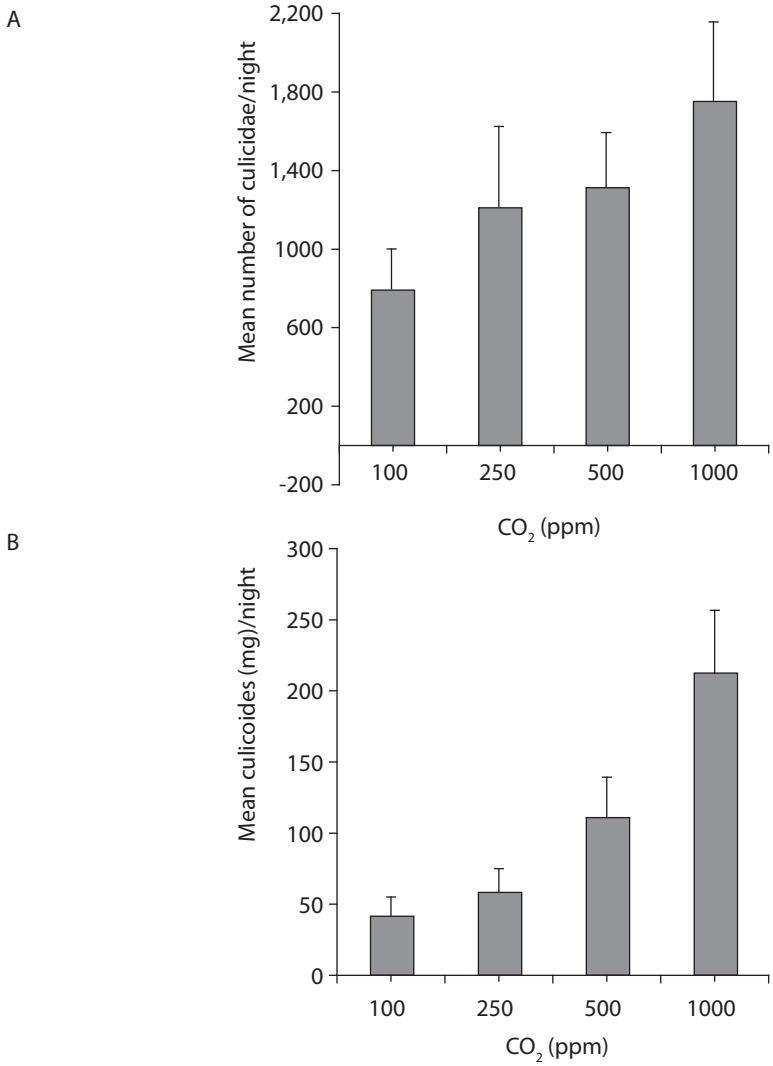


*Figure 12. Mean + SE concentration-response relationship of trap catch of (A) mosquitoes and (B) biting midges as a function of $CO_2$ release in field experiments conducted in Cedar Key, FL, USA. The trap was a passive counter-flow trap whose $CO_2$ source was a compressed tank. Release concentration amounts were determined by restrictive line orifices and regulated pressure. Each point represents eight nights of trapping.*

demonstrated the utility of adding $CO_2$ to surveillance traps (Carestia and Savage 1967, Newhouse *et al.* 1966, Vickery, Jr. *et al.* 1966, Wright 1967). Often $CO_2$ was added in the form of dry ice that sublimates to gas. In CDC style traps, blocks of dry ice are added above the traps in an insulated chamber with a port releasing $CO_2$ into the trap stream. In some instances, a bag of dry ice could be simply hung in the vicinity of the trap attracting insects to the general area. Several pounds of dry ice are often sufficient to generate $CO_2$ for 12-14 hours. Alternately, regulated cylinders

containing compressed $CO_2$ could be used to release the gas at a standard rate (often 500 mls/minute). This rate could be adjusted to allow a standard 20 pound tank to last for days to weeks. This works well for surveillance system where trapping periods are relatively short. To further enhance catch, other attractants such as 1-octen-3-ol, lactic acid or ammonia are added. These supplemental attractants are often used to 'tailor' catches to specific problem species, such as anthropophilic day-biters.

In the 1990s, newer technology for commercial traps appeared on the market. They were designed to operate continuously for longer periods of time. These traps generated $CO_2$ by catalytically combusting propane on a bed of coated beads. Heat generated from this burn process is converted to electricity, which is used to power fans that dispersed the $CO_2$ attractant plume and vacuumed approaching insects into nets. An added advantage to this technology was the co-generation of heat and moisture, two addition attractants for many biting insects (Kline 2002). The advantages of such traps are several-fold. First, the trap is able to generate attractants in the form of $CO_2$, heat and moisture and collect insects continuously for over one month on a standard 20-lb 'barbeque grill' sized propane tank. Second, by generating its own power, the trap was not reliant on battery or line voltage. Consequently this allowed traps to be placed at some distance from living areas. The rational is that proper placement of the trap at some distance from commonly used areas can result in biting insects being drawn away from areas where they would most likely encounter a host for a blood meal. Third, these traps tended to be very specific usually attracting few non-target insects.

The release of $CO_2$ from traps can be modulated in several ways to tune attractiveness. First, the overall concentration released can be modified. Generally larger amounts of $CO_2$ will result in larger catches. Second, the strategy of release can affect catch. For example, counter flow release systems, where the plume dispensing the $CO_2$ stream is countercurrent to the aspiration stream, enhances species diversity and numbers in the catch. Third, the temporal nature of the $CO_2$ release can affect catch rate. Pulsing the attractant can be used to release discrete amounts of $CO_2$ at specific intervals or times of day. This aspect of release is complicated by the fact that even a continuous release of $CO_2$ is easily and quickly broken into pockets by a turbulent stream of wind. The importance of such discontinuous signals has been studied and discussed for field (Murlis and Jones 1981); bioassay (Dekker *et al.* 2001) and sensory (Grant *et al.* 1997) systems. In pulsing systems, several variables can be modulated including: concentration of $CO_2$ release, duration of release and interval between pulses. All these must be evaluated to determine optimal release conditions. Clearly traps can serve as an important tool to monitor population fluctuations and to assay for potential health risks by collecting and testing a variety of hematophagous insects for potentially dangerous viruses and other pathogens.

## Conclusion

In this brief review we summarised our current understanding of the properties and role of $CO_2$ sensitive chemosensory systems in modulating host-seeking behaviours in mosquitoes. We see that these orientation behaviours are complicated and potentially plastic. They represent a dynamic system whose sensitivity may be modulated by environmental factors. Under what conditions does the system's sensitivity change? We have shown here that the age of the insect as well as the blood feeding tendencies affect $CO_2$ sensitivity, but there are many other developmental and environmental conditions that may also modulate the response. For example, does the infected state of an individual mosquito change peripheral sensitivity?

There are many steps in the chain of events that lead from the detection of an environmental cue to a change in animal behaviour. Each provides a potential point of attack where treatment may alter the behavioural outcome. A complete understanding of this chain of events in disease vector organisms can provide new approaches to mechanisms that may be effective in decreasing disease transmission. We suggest that a failure to appreciate this fact supports another cautionary tale with widespread impact.

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