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Behavioral responses of *Drosophila* to biogenic levels of carbon dioxide depend on life-stage, sex and olfactory context

Cécile Faucher[1,2], Manfred Forstreuter[3], Monika Hilker[2] and Marien de Bruyne[1,*]

[1]*Freie Universität Berlin, Neurobiologie, Königin-Luise-Strasse 28-30, D-14195 Berlin, Germany,* [2]*Freie Universität Berlin, Angewandte Zoologie, Haderslebener Strasse 9, D-12163 Berlin, Germany and* [3]*Technische Universität Berlin, Ökologie, Königin-Luise-Strasse 22, D-14195 Berlin, Germany*

*Author for correspondence at address 1 (e-mail: mdebruyn@zedat.fu-berlin.de); present address: Monash University, Biological Sciences, Wellington Road, Clayton VIC 3800, Australia

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Summary

Drosophila melanogaster **(Meigen) detects and uses many volatiles for its survival. Carbon dioxide (CO_2) is detected in adults by a special class of olfactory receptor neurons, expressing the gustatory receptor *Gr21a*. The behavioral responses to CO_2 were investigated in a four-field olfactometer bioassay that is new for *Drosophila*. We determined (1) whether the sensitivity of this response changes with odor context, and (2) if it depends on sex and life stage. When CO_2 was added to ambient air in one field and tested against ambient air in the three other fields, individually observed adults avoided CO_2 (0.1–1% above ambient), but did not respond to a low rise of 0.02%. We relate this behavior to measurements of CO_2 production in bananas and flies. When 0.02% CO_2 was combined with the odor of apple cider vinegar in one field of the olfactometer and tested against ambient air in the three other fields, the addition of CO_2 did not affect the attractiveness of apple cider vinegar alone. However, this combination of CO_2 and vinegar became repellent when it was tested against vinegar at ambient CO_2 concentrations in the three other fields. This 'odor background effect' was female-specific, revealing a sexually dimorphic behavior. The new assay allowed us to test larvae under similar conditions and compare their behavior to that of adults. Like adults, they avoided CO_2, but with lower sensitivity. Larvae lacking neurons expressing *Gr21a* lost their avoidance behavior to CO_2, but kept their positive response to vinegar odor. Hence, Gr21a-expressing neurons mediate similar behaviors in larvae and adults.**

Key words: olfaction, behavior, fruit, carbon dioxide, *Drosophila melanogaster*, larvae, receptor, odor context.

Introduction

Insects are well known to orientate by a wide range of chemical cues to locate suitable resources. They evolved highly sensitive olfactory systems to optimize behavioral processes, such as host location (Bruce et al., 2005), finding mates (Löfstedt, 1993) or locating suitable resting sites (Syed and Guerin, 2004), but also avoidance of inappropriate hosts (Vallat and Dorn, 2005). It has long been known that *Drosophila melanogaster* (Meigen) is attracted to fruit odors and certain volatiles (Reed, 1938; Hoffmann, 1985; Zhu et al., 2003). However, other behavioral studies suggest that several highly concentrated odorants are avoided by adults (Devaud et al., 2001; Heimbeck et al., 2001; Wang et al., 2003), whereas larvae are still attracted by many of those compounds at high doses (Cobb, 1999; Larsson et al., 2004). Relatively little is known about how flies determine the 'hedonic value' of olfactory stimuli, or how this depends on sex and life-stage in this holometabolous insect.

Recent research in this species has focused on the molecular, physiological, and neurological basis of olfaction (for reviews, see Keller and Vosshall, 2003; Hallem and Carlson, 2004; de Bruyne and Warr, 2006). Volatiles are detected by olfactory receptor neurons (ORNs), housed in sensilla on the third segment of the antennae and on the maxillary palps. More than 40 functional classes of ORNs have been characterized (de Bruyne et al., 1999; de Bruyne et al., 2001; Couto et al., 2005). Odorants activate specific receptors belonging to a large family of olfactory receptor (OR) genes (Hallem et al., 2004). In most ORN classes, expression of a single receptor gene determines the unique odorant response spectrum of that particular ORN. ORNs vary in their tuning breadth, responding to many or only a few odorants. Likewise, odorants vary in the number of ORNs that they excite (de Bruyne et al., 2001; Stensmyr et al., 2003; Hallem et al., 2004). However, specificity of an ORN's response increases with decreasing odorant concentration. Therefore, in natural odor plumes at a certain distance from an odor source, the number of ORN classes activated in a flying insect may be much lower than when it is walking near the

source. Among ORNs, the ab1C neuron is unique because it is stimulated exclusively by carbon dioxide, and it is the only ORN class that responds to it (de Bruyne et al., 2001; Suh et al., 2004).

Carbon dioxide (CO_2) is present in the atmosphere at ~0.03%, a concentration that can fluctuate considerably with time of day and between different habitats (Gillies, 1980; Zollner et al., 2004). In vertebrates, CO_2 regulates breathing: it is detected by chemoreceptors in the blood stream and in the brain stem (Lahiri and Forster, 2003). Mammals perceive high doses of CO_2 in the air *via* free nerve endings of the trigeminal system (Shusterman, 2002). For insects, it can be considered an olfactory stimulus, since it is detected by ORNs projecting to the antennal lobe, where olfactory information is integrated in spherical neuropile areas called glomeruli. In both moths and flies, CO_2-sensitive ORNs innervate a single glomerulus (Guerenstein et al., 2004; Suh et al., 2004). Many insects have developed special dendritic structures for detecting it with high sensitivity as, for example, has been observed in a tephritid fly (Hull and Cribb, 1997) and a moth, with a threshold as low as 0.005% (Stange, 1992).

A particular feature of the *Drosophila* ab1C neuron is the expression of a gustatory receptor, Gr21a (Scott et al., 2001; Suh et al., 2004; Couto et al., 2005). However, there is no direct evidence that this receptor is indeed involved in CO_2 detection. The Gr21a receptor is also expressed in larvae, in a single bilateral neuron innervating the terminal organ and projecting to the larval antennal lobe (Scott et al., 2001). In *Drosophila* larvae, chemicals are detected mainly by the dorsal organ, which houses olfactory neurons, and the terminal organ with a largely gustatory function (Cobb, 1999; Oppliger et al., 2000; Python and Stocker, 2002). Whereas adult flies possess ~1200 ORNs on the antenna and ~120 on the palps (Stocker, 2001), there are only 21 olfactory neurons in the central dome of the dorsal organ, which have been shown to express OR genes (Kreher et al., 2005; Couto et al., 2005). Externally, the larva has another 42 putative gustatory neurons in the dorsal, terminal and ventral organs (Python and Stocker, 2002). Although taste and olfaction may not be easily separable in larvae, the small number of identifiable neurons and robust behaviors render it a suitable model system for chemoperception. To our knowledge, nothing is known about CO_2 perception in larvae.

CO_2 is ubiquitous and, as a product of respiration and degradation of organic matter, also rather unspecific as an ecological signal. Nevertheless, it has been shown to play various roles in insect chemical ecology (Stange, 1996). For example, honeybees ventilate their hive in response to high concentrations of CO_2 (Seeley, 1974). Lower doses are used by blood-feeding insects to locate their host (Gillies, 1980; Pinto et al., 2001; Barrozo and Lazzari, 2004; Dekker et al., 2005). Herbivorous insects use CO_2 to locate leaves, damaged fruits, flowers or roots (Hibbard and Bjostad, 1988; Stange et al., 1995; Stange, 1999; Thom et al., 2003).

What role could the detection of CO_2 play in the ecology of *Drosophila*? *Drosophila* flies aggregate on fallen fruits where they feed, mate and oviposit (Spieth, 1974; Wertheim et al., 2002). These substrates generally harbor microorganisms, which contain valuable nutrients. Both living fruit tissue and the process of its fermentation produce CO_2. Its concentration will vary among fruits and their stage of ripening. However, even though *Drosophila* flies use CO_2-producing substrates, they are repelled by CO_2 (Suh et al., 2004). Such a repellent effect of CO_2 may be explained by avoidance of the anesthetic and toxic effects of CO_2 (Badre et al., 2005) or by avoidance of stressed conspecifics emitting CO_2 (Suh et al., 2004).

In this study, we investigated the mechanisms of CO_2 avoidance by observing the orientation of individual flies walking in a four-field olfactometer, a new assay to test behavioral responses of *Drosophila* to odors. We first tested different doses of CO_2 and determined the threshold of behavioral sensitivity. Then we examined the response to CO_2 at threshold level when combined with a fruit odor, apple cider vinegar, and found an increase in sensitivity, which was sex-specific. We related this effect to differences in the walking activity of males and females. In addition, we conducted measurements of CO_2 emission from bananas to explore a possible role of the detection of CO_2 in orientation to fruits and compared it with the concentrations used in the bioassays. We also established that larvae show behavioral responses to CO_2 and vinegar, comparable to adults. Finally, the CO_2 avoidance vanishes in larvae lacking the chemosensory neuron expressing the Gr21a receptor, but not the attraction to vinegar.

Material and methods

Fly stocks and rearing conditions

Drosophila melanogaster (Meigen) flies were reared at 25°C, 50–60% relative humidity in 68 ml vials on standard yeasted cornmeal–syrup medium with a 12 h:12 h L:D photoperiod. Wild-type flies were Canton S (CS-5) (Helfand and Carlson, 1989). On the day of the experiment, flies were 4–7 days old and starved on agar overnight for 17–24 h. The genetic background for P-element insertions was w^{1118}. The *Gr21a* receptor driver line *w; P{Gr21a-GAL4}/CyO* was a gift from Kristin Scott. Gal4-driven apoptosis was induced by crosses with *w;; P{UAS-rpr}/TM3 Sb* and membrane bound GFP was from *y w P{UAS-mCD8::GFP.L}; Pin/CyO* flies obtained from the Bloomington Stock Center.

Behavioral paradigm

Fly orientation in odor fields was studied in a four-field olfactometer (Meiners and Hilker, 1997) as modified from Vet et al. (Vet et al., 1983). It consisted of a four-pointed star-shaped arena (Fig. 1), 1 cm high and 30 cm wide (from one tip to another). Air was pumped into the arena at the four corners and exited from a central hole in the base. A nylon mesh prevented flies from entering the nozzle in the corner. The converging airflows defined four separate odor fields as demonstrated by the use of smoke (not shown). We used room air, cleaned over an activated-charcoal filter, and used rotameters (Supelco, Bellefonte, PA, USA) to keep the flow in

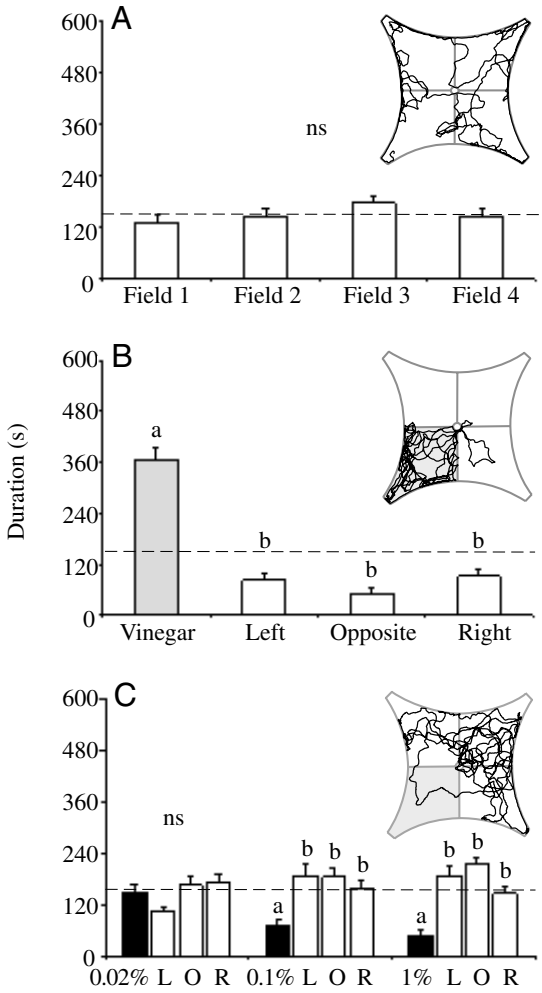

Fig. 1. *Drosophila* flies are attracted to apple cider vinegar but avoid CO_2. Time spent by flies in each of the fields in a four-field olfactometer during a 600 s experiment. (A) Control situation when only air is delivered from the four corners (N=57). (B) Apple cider vinegar odor (N=39) is added to the air in one field (grey bar). (C) CO_2 of different concentrations ($N_{0.02\%}$=38; $N_{0.1\%}$=42; $N_{1\%}$=35) is added to the air in one field (black bars). The orientation of the fields is indicated relative to the field laced with the test odor: L, left, O, opposite, R, right. Insets show examples of 10 min tracks of single flies for control, vinegar, and 1% CO_2 respectively. The broken line at 150 s indicates an equal amount of time in all fields. Deviations from equal distribution were tested with a Friedman-ANOVA (P<0.001; ns, no significant difference). Fields with different letters above the bars are significantly different from each other (Wilcoxon–Wilcox test; P<0.05 for 0.1% CO_2, P<0.001 for 1% CO_2 and vinegar). Values are means ± s.e.m.

each arm entering the four arena fields at a constant rate of 145 ml min^{-1}. Each air-stream was first humidified by passing through a glass flask with distilled water (200 ml). It then passed through a 50 ml glass flask that could contain an odor source. Carbon dioxide was delivered from a gas bottle by adding it to one of the four flows, upstream of the last flask. The CO_2 flow was controlled by a rotameter with a precision valve. Teflon tubing was used throughout. Experiments were conducted in complete darkness to exclude orientation to visual cues. The arena was illuminated by infrared LEDs, and each run was monitored by a video camera. A single fly was introduced into the central hole *via* a small tube. After the fly entered the arena its walking activity and its location was recorded for 10 min, using 'The Observer' software (Noldus, Wageningen, The Netherlands). Flies that did not enter the arena within 2 min were discarded. The olfactometer was cleaned with ethanol and distilled water after approx. 10 runs to avoid the accumulation of fly-derived chemicals in the arena. Males and females were tested in different arenas.

For testing larvae, the olfactometer was modified as follows: a smaller arena (15 cm width) with a lower flow rate (50 ml min^{-1}) was used, and the base was covered with 1.5% agar. Ten crawling larvae were gently introduced from a central hole in the top of the arena. Third instar larvae were used exclusively: they were collected as described previously (Monte et al., 1989) and kept on agar in a Petri dish for 5–60 min prior to the experiment. The number of larvae in each field was counted every 2.5 min for 10 min.

Odor stimuli

Different volumes of 5% CO_2 in synthetic air (Air-Liquide, Duesseldorf, Germany) were mixed into the air-flow of the arm entering the test field of the olfactometer. Thus, CO_2 concentrations were raised by adding 0.02%, 0.1% or 1% to the background. Control fields were supplied with charcoal-filtered air at ambient CO_2 concentrations. We regularly monitored the background concentration with a portable CO_2 sensor (Testo 445, Lenzkirch, Germany) and kept it between 0.07% and 0.1% by ventilating the room between experiments. As a complex fruit-derived attractive odor, we used apple cider vinegar made from bio-organically grown apples (Bio-Zentrale, Stubenberg, Germany). 20 μl of vinegar, diluted 50% in distilled water, was loaded onto a piece of filter paper and placed in the 50 ml glass flask before each run. The test stimulus was delivered two to three times to one field, and then moved to another, to avoid bias due to odor contamination or its absolute orientation.

CO_2 gas exchange measurements on fruits and flies

Carbon dioxide gas exchange by bananas and flies was measured using a mini cuvette system (CMS 400; Walz, Effeltrich, Germany). The system was equipped with an input humidity control (KF-18/2 and RSV-42; Walz), a measuring gas cooler and a CO_2/N_2 gas mixing system (GMA-2; Walz). The measurements were made in constant environmental conditions. Air temperature, relative humidity (RH), CO_2 concentration and wind speed were adjusted inside the gas exchange cuvette to 25°C, 55% RH, 0.0347% CO_2 and a speed of 1.9 m s^{-1}. The open gas exchange system was connected to a differential nondispersive infrared gas analyzer (IRGA) for water vapor and CO_2 (BINOS 100; Fisher-Rosemount, Hasselroth, Germany).

A Peltier-controlled climate unit (GK 022; Walz) with a flanged Plexiglas cuvette (MK-022/A; Walz), expanded by a

removable Plexiglas upper section (total air volume of 1000 cm^3) was provided with air taken from outside the laboratory. Incoming dry air was cleaned and humidified by first circulating it through water. Relative humidity was adjusted by passing the saturated air with water vapor through the input humidity control (dew point temperature 15.4°C). The CO_2 concentration was controlled by passing air over soda lime columns, retaining the naturally occurring CO_2, and adding the concentration needed from a CO_2 gas container. The system flow rate through the cuvette was regulated by thermal mass flow meters and set at 2200 ml min^{-1} for bananas and 500 ml min^{-1} for flies. Environmental conditions inside the cuvette were continuously monitored with a microprocessor-controlled data acquisition system. Gas exchange rates were calculated after Field et al. (Field et al., 1989) and Forstreuter (Forstreuter, 2002).

Five yellow bananas from the same bunch [ripening stage 5 (Commonwealth Scientific and Industrial Research Organization, 1972)] were kept for 21 days at 25°C in an unused room, shielded from direct sunlight. Fruits were completely black at the end of the experiment. Each day they were weighed and carefully placed in the gas exchange cuvette where the difference between the input and output of CO_2 and water were measured continuously with the IRGA after a few minutes of system calibration.

To measure CO_2 gas exchange by normally respiring or stressed flies, 10 males and 10 females were kept in a small metal cage (4×3×3 cm) inside the gas exchange cuvette. The cage could be shaken by alternately activating two solenoid magnets. CO_2 gas exchange was measured continuously at a frequency of 10 s. After 5 min of adaptation, flies were stressed for 1 min by moving the cage 1 cm every 0.6 s.

Data analysis and statistics

To test whether the flies in a single treatment allocated equal amounts of time to each of the four fields, we used a non-parametric test for dependent data (Friedman-ANOVA, $P<0.05$) and differences were attributed to fields using Wilcoxon–Wilcox as a *post-hoc* test. We used a Mann–Whitney *U*-test to compare times allocated to the test fields of different treatments. All experiments were done with similar numbers of male and female flies. Data were grouped together when differences between the sexes were not significant.

Results

Adult flies stay in vinegar odor but avoid CO_2

To quantify behavioral responses of *Drosophila* to odors, we used an arena in which individual flies can move freely from odor-free to odor-laden air (Fig. 1). Four converging air streams created separate fields, and the fly's behavior was observed in complete darkness on a video screen. When charcoal-cleaned room air was delivered to all four fields, most flies explored the whole arena, dividing their time more or less equally between the four fields (Fig. 1A). Most of the time flies walked in 1–20 s bouts interrupted by equally long stops, but we observed occasional jumps. When the apple cider vinegar odor was added to one field, flies walked mainly in this field (Fig. 1B). They repeatedly made sharp turns upon leaving the odor-laden air, returning to the test field. By contrast, when the air contained 1% additional CO_2 they clearly avoided the test field and spent significantly more time in the three control fields with only room air (Fig. 1C). We did not see any change in the preference for vinegar or avoidance of CO_2 during the 10-min period. We then tested two lower concentrations of CO_2 to determine the threshold for this behavioral response (Fig. 1C). The flies still significantly avoided the test field when 0.1% CO_2 was added, but failed to respond to a 0.02% CO_2 increase above background. Electrophysiological recordings from the ab1C neurons demonstrated that they are sensitive enough to detect a 0.02% CO_2 pulse in a carbon dioxide-free air-stream (M. de Bruyne, unpublished observations). Therefore, either this low stimulation is detected but does not induce a behavioral response or the presence of CO_2 in the room air background prevents its detection.

Apple cider vinegar enhances the behavioral sensitivity to CO_2

In a natural situation, CO_2 is unlikely to occur as an isolated stimulus. Thus flies may respond to a low dose of CO_2 when combined with other odors. We tested 0.02% CO_2 in combination with vinegar in one of the four fields and tested it against air at ambient CO_2 concentrations. Vinegar plus 0.02% CO_2 was found to be as attractive as vinegar alone (Fig. 2A). Thus, under these circumstances the combination did not induce a substantially different behavior. However, the slight (non-significant) reduction in attractiveness suggested to us that CO_2 might affect the behavior when flies are constantly stimulated by vinegar. When 0.02% CO_2 plus vinegar was offered in a single field and tested against vinegar odor in the three other fields, significant avoidance of CO_2 was observed. Since preliminary analyses revealed sexual differences, we separated the results for males and females (Fig. 2B). Only females avoided the field with 0.02% CO_2, whereas males clearly did not. When analyzing the data in Fig. 1 and Fig. 2A separately for the sexes, no significant differences were detectable between sexes (data not shown). Clearly, the results shown in Fig. 2 demonstrate that a 0.02% difference in CO_2 is detectable when vinegar is offered as a background odor in the entire arena and that it affects males and females differently. Thus we can conclude that this concentration of CO_2 is detected by female flies, but does not elicit any behavior on its own.

Carbon dioxide activates both males and females, vinegar only females

To investigate the effects of vinegar and CO_2 on the activity of flies, we analyzed how long the flies were walking when apple cider vinegar or 0.1% CO_2 was applied in all four fields of the olfactometer and compared it with the control (room air) (Fig. 3). Female flies spent significantly more time walking in the arena filled with apple cider vinegar odor than in the arena

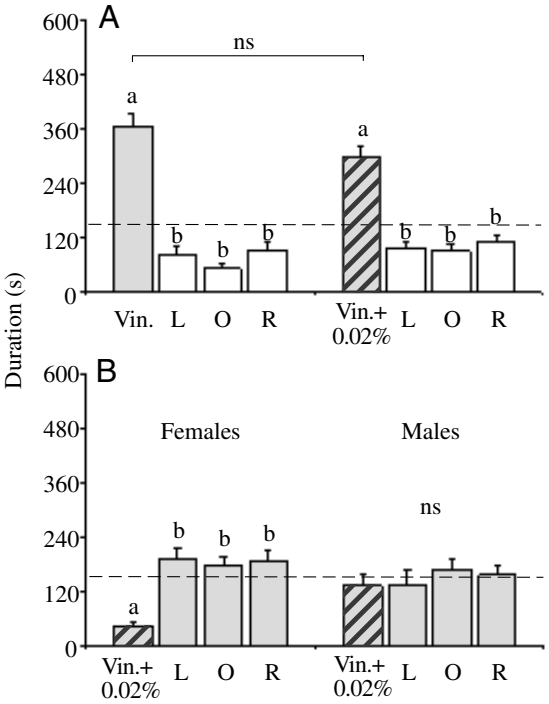

Fig. 2. Apple cider vinegar makes females more sensitive to CO_2. Distributions of time spent in four fields of the olfactometer as in Fig. 1. (A) 0.02% CO_2 (black hatched bars) added to apple cider vinegar (vin.; grey bars; N=53, Friedman-ANOVA: P<0.001; Wilcoxon–Wilcox: P<0.001) is as attractive as apple cider vinegar alone (same data as in Fig. 1B; ns, no significant difference, Mann–Whitney U-test). (B) When all fields contain apple cider vinegar odor (grey bars) as background, a field laced with 0.02% CO_2 (hatched bars) is avoided, but only by females ($N_{females}$=29, N_{males}=17, Friedman-ANOVA, P<0.001; Wilcoxon–Wilcox: P<0.001). Values are means ± s.e.m.

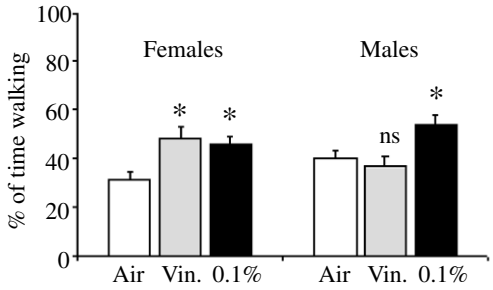

Fig. 3. Effects of vinegar and CO_2 backgrounds on walking activity of male and female flies. Percentage of time walking is indicated for males and females when all four fields contain air only (Air), apple cider vinegar (Vin.) or 0.1% CO_2. For females, N_{air}=30; $N_{vinegar}$=22; N_{CO_2}=21; for males, N_{air}=27; $N_{vinegar}$=20; N_{CO_2}=20. *Significant difference compared to air (Mann–Whitney U-test, P<0.01 for vinegar, P<0.05 for CO_2); ns, no significant difference. Values are means ± s.e.m.

containing only room air. For males, the walking time in vinegar background was not different from room air. However, 0.1% CO_2 added to the ambient in the four fields elicited an increase in walking time for both sexes. Apple cider vinegar seems to be an activator for females only, whereas CO_2 activates both males and females, making it likely that sexual differences in CO_2 perception are due to differential modulation by the vinegar background.

Fruits emit CO_2, producing concentrations above the behavioral threshold

Because female flies are more sensitive to CO_2 when combined with an attractive odor derived from fruit, we measured CO_2 production by ripening fruits (Fig. 4). The average CO_2 emission from single bananas over a period of 21 days is shown in Fig. 4A. Bananas changed from just ripe to over-ripe during this period. Initially, CO_2 production was quite high at ~200 μl min^{-1} from a single fruit. Over the next 3 weeks, there was a considerable reduction (6.5-fold) in CO_2 production with the final rate at 30 μl min^{-1}. The reduction in CO_2 emission can be only partly explained by the loss in weight that we also observed in these bananas (Fig. 4B). Weight loss was only 1.7-fold, and the CO_2 emission curve does not show the same linear decline. If banana headspace were carried by the flow of 145 ml min^{-1} that we normally use for a single olfactometer field, the resulting CO_2 concentration would be about 0.1% in the case of a yellow banana and 0.02% in the case of a black one. This is within the range of detection. In the field we would expect the concentrations to be lower, for ripe bananas probably dropping below the detection threshold.

A small group of flies (N=20) produced considerably less CO_2 (~0.89 μl min^{-1}) than bananas did (Fig. 4C). Suh et al. reported that flies, when stressed by shaking, emit an odor containing CO_2 as one component (Suh et al., 2004). Indeed, when we agitated our flies, their CO_2-emission roughly doubled, confirming their analysis.

Larvae also avoid CO_2, but with less sensitivity

Larvae of *Drosophila* develop on fruits in different stages of ripeness and/or fermentation. They are therefore continuously exposed to CO_2. We wondered whether larvae can detect CO_2 and whether they avoid it as adults do. To address that question, we used a slightly adapted version of the same paradigm we used for adult flies: the arena was smaller and covered with agar, and the position of 10 larvae was noted at four time points of a 10 min period, instead of continuously recording the behavior of a single insect. In a control situation (room air only in all fields), after 10 min, larvae distributed themselves equally over the four fields (Fig. 5A). Adding 0.1% CO_2 to one field did not affect the larval distribution after a 10 min period, whereas an addition of 1% CO_2 reduced their number significantly (Fig. 5B). Therefore, larvae are able to detect CO_2 and, like adults, avoid it. However, compared to adults, their sensitivity seems to be lower (compare Fig. 1C). In both control and CO_2 experiments, the distribution at the three preceding measuring points was not significantly different from the one at 10 min (not shown). Like adults,

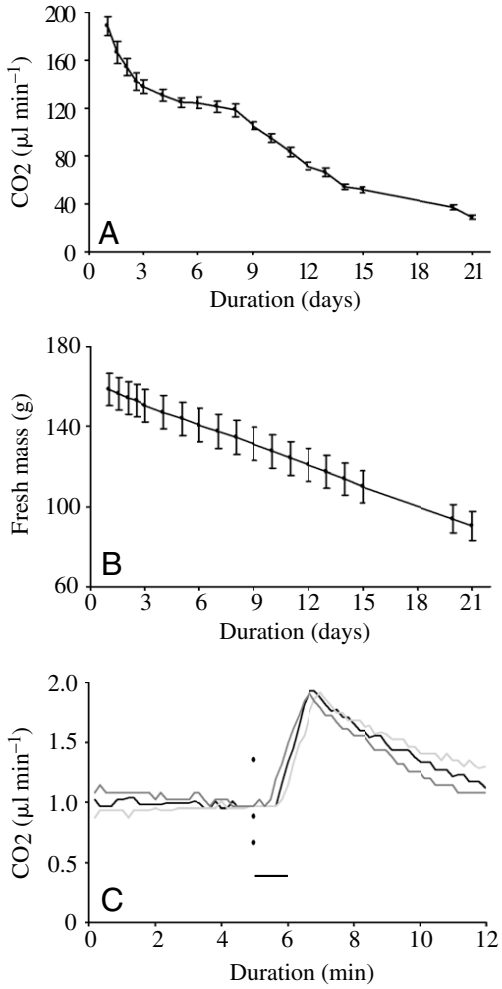

Fig. 4. Bananas and flies emit carbon dioxide. (A) Mean CO_2 emission for a single banana (N=5) over a period of 21 days. (B) Decrease in fresh mass of the bananas is linear over the same period. Values are means ± s.d. (C) CO_2 emission from three groups of 20 flies. The horizontal bar indicates 1 min of shaking, which induces a sharp rise in CO_2 emission. The three curves are normalized to their mean at a point before shaking. The dots indicate the absolute values for the three curves at that time. Note the differences in scale of both axes compared to A. The delay observed between the start of the stimulation and the increase in emission is caused by the design of the system.

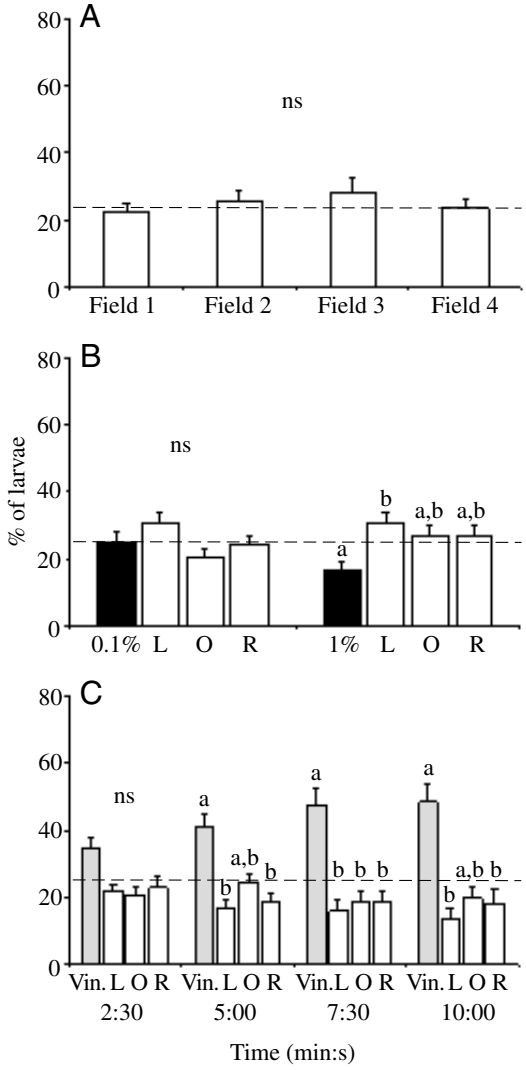

Fig. 5. *Drosophila* larvae avoid CO_2 and prefer vinegar. (A) Mean distribution of groups of 10 larvae after 10 min when air is delivered in the four fields (N=18). (B) Mean distributions after 10 min when CO_2 is added to one field (black) at two concentrations ($N_{0.1\%}$=24; $N_{1\%}$=25). (C) Mean distribution at four time points when one field (gray) is laced with vinegar odor (N=17). Abbreviations and statistics are as in Fig. 1. The broken line at 25% indicates an equal distribution in all fields. In contrast to the results in Figs 1 and 2 the test field is not significantly different from all control fields (Friedman-ANOVA, P<0.01 for CO_2, P<0.001 for vinegar; Wilcoxon–Wilcox: P<0.05); ns, no significant difference. Values are means ± s.e.m.

larvae showed significant preference for the field supplied with vinegar odor (Fig. 5C). However, this effect is significant only 5 min after exposure. It is still present after 10 min.

A single bilateral neuron, expressing the Gr21a receptor, is responsible for CO_2 detection in larvae

The Gr21a receptor that is expressed in adult ab1C neurons also labels a bilateral neuron in the terminal organ of larvae (Scott et al., 2001), which is generally considered a gustatory organ. We used the Gr21a-Gal4 driver to manipulate the sensory neurons (Fig. 6A). To demonstrate a role for these cells in the detection of CO_2, we genetically ablated them by expression of the apoptotic gene *reaper* (*rpr*) driven by *Gr21a-Gal4*. These *Gr21a-rpr* larvae, lacking the *Gr21a*-expressing neurons, did not show CO_2 avoidance, whereas their genetic controls, carrying only the *Gr21a* driver or the *UAS-rpr* construct were repelled by CO_2 (Fig. 6B), in a similar way to wild-type larvae (see Fig. 5B). By contrast, *Gr21a-rpr* larvae showed a behavior to apple cider vinegar like that of wild-type larvae (Fig. 6C). This clearly demonstrates that these two *Gr21a*-expressing neurons mediate CO_2 detection and avoidance behavior in larvae.

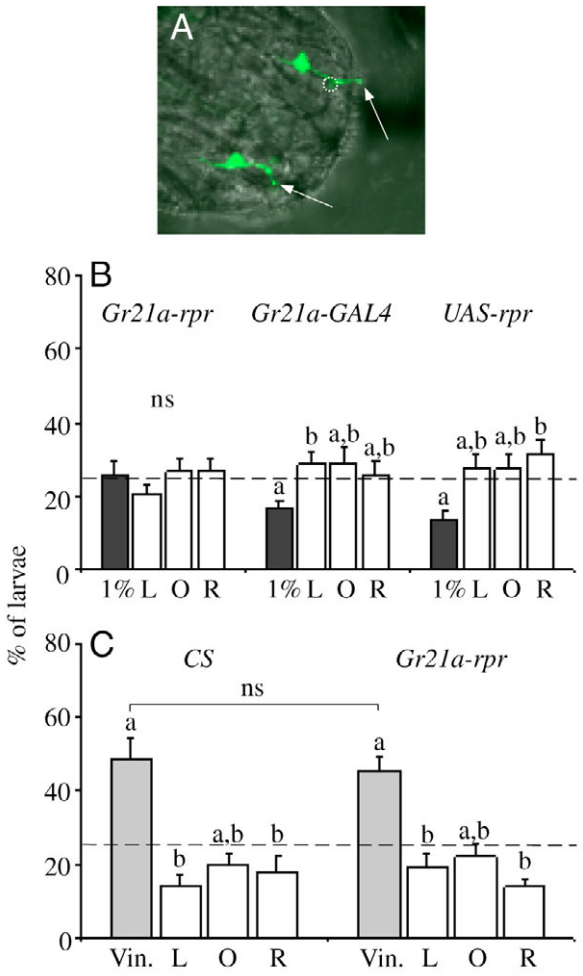

Fig. 6. The *Gr21a*-expressing neuron mediates CO_2 detection in larvae. (A) Confocal image of the anterior of a third instar larva expressing membrane-bound UAS-mCD8::GFP (green) driven by a *Gr21a*-Gal4 construct. Dotted line indicates the position of the dome of the dorsal organ in the transmission image; arrows point to the terminal organs. (B,C) Distributions of larvae in the four-field olfactometer as in Fig. 5. (B) Responses to 1% CO_2 by larvae lacking the *Gr21a*-expressing neurons due to *Gr21a*-driven expression of the apoptotic gene *reaper* (*rpr*) (*Gr21a-rpr*, *N*=22), compared to their genetic controls carrying only the driver construct (*Gr21a*, *N*=20) or the *reaper* construct (*rpr*, *N*=17). Significant avoidance is only seen in the controls. (C) Response to apple cider vinegar of larvae lacking the *Gr21a*-expressing neuron (*Gr21a*-rpr, *N*=18) is normal when compared to wild-type larvae (*CS*, same data as in Fig. 5C at 10:00 min; ns, no significant difference, Mann–Whitney *U*-test). Abbreviations and statistics are as in Fig. 1. The broken line at 25% indicates an equal distribution in all fields. In contrast to Figs 1 and 2 the test field is not significantly different from all control fields (Friedman-ANOVA, $P<0.05$ for CO_2, $P<0.001$ for vinegar; Wilcoxon–Wilcox: $P<0.05$); ns, no significant difference. Values are means ± s.e.m.

Discussion

We used a behavioral paradigm that is new to *Drosophila* and confirmed the CO_2 avoidance behavior that we (M. de Bruyne, unpublished observations) and others (Suh et al., 2004) observed in a T-maze paradigm. Male and female flies avoid CO_2 concentrations above 0.1%. By contrast, apple cider vinegar odor, is attractive. An unexpected, new finding of our study is that the response of flies to CO_2 depends on the background odor. When odor of vinegar is used as a background stimulus, the threshold for CO_2 avoidance was lowered to 0.02%. Interestingly, this shift of behavioral response in dependence of a background stimulus was found to be sex-specific, since only females changed their behavior. In contrast to this sex-specificity of *Drosophila* response to CO_2, we did not detect any specificity for the developmental stage. The larval response to CO_2 was found to be similar to the adult response.

The four-field olfactometer has been used in chemical ecology studies on several insect species (Vet et al., 1983; Quiroz and Niemeyer, 1998; Hilker et al., 2002; Saïd et al., 2005), but not to investigate *Drosophila* olfactory behavior (Devaud, 2003). This assay has distinct advantages. Individual flies undisturbed by manipulation and without interference by conspecifics can freely sample one or several odors. The measurements integrate many decisions rather than a single choice and include oriented (chemotactic) responses as well as changes in the parameters of locomotion (kinetic responses) (Kennedy, 1978).

Atmospheric CO_2 concentrations fluctuate from 0.03–0.04%. In natural microhabitats they can reach levels above 0.1% due to respiration of plants, animals and microorganisms (Gillies, 1980; Anderson and Ultsch, 1987; Zollner et al., 2004). We demonstrate here that CO_2 emission from bananas can raise concentrations in the air and that the ripening process leads to a drop in emission with time. The respiration rates we measured are in agreement with others (Golding et al., 1998), and produce concentrations that are within the range of detection by *Drosophila* flies. In our assay we measured behavioral responses of walking flies to CO_2 and vinegar. CO_2 signals from fruit are likely to be quickly diluted by air currents. Hence CO_2 may affect fly behavior close to or on the fruit. Flies may avoid CO_2 because its concentration is negatively correlated with ripening and because they prefer ripe fruits (Lachaise and Tsacas, 1983). The hawkmoth and the Queensland fruit fly also select suitable resources using CO_2 signals at close range (Thom et al., 2003; Stange, 1999).

Plant tissues are not the only source of changes in the CO_2 content of the air. *Drosophila* flies produce more CO_2 when taking flight or as a consequence of stress (Lehmann, 2001; Suh et al., 2004). However, we show that 20 stressed flies release 30 times less CO_2 than a ripe banana and 200 times less than an unripe one. Our demonstration of the sex-specific nature of CO_2 avoidance at low concentrations also makes a role of CO_2 in signaling stress in conspecifics less likely. Crucially, CO_2 on its own is a rather unspecific signal. Therefore, its effect on fly behavior is likely to vary with context.

We used apple cider vinegar to study the effect of an olfactory context. Apple cider vinegar is a natural blend of odors; a fruit fermentation product that is attractive to *Drosophila* adults and larvae (Fig. 1C and Fig. 5C). We first

investigated whether adding CO_2 to vinegar would change its attractiveness. In other insect species, combining CO_2 with attractive odorants has generally increased attraction (Barrozo and Lazzari, 2004; Dekker et al., 2005). In our olfactometer, a small increase of 0.02% CO_2 does not change the fly's behavior towards vinegar when tested against room air. However, that same concentration was avoided when vinegar odor was present in all four fields. The constant vinegar background apparently sensitizes the response of female *Drosophila* to CO_2. Mumm and Hilker also observed an increased sensitivity to changes in odor quality or quantity of its components depending on background odor in a parasitic wasp (Mumm and Hilker, 2005). Future studies need to elucidate whether this increase in sensitivity is specific to apple vinegar or whether it also occurs with other attractive odors or even to single odorants.

Whether this interaction of CO_2 and vinegar odor occurs at the level of the sensory neurons or in the brain is not known. Ziesmann recorded from termite olfactory sensilla containing neurons whose excitation by alcohols is blocked by the inhibitory effect of CO_2 (Ziesmann, 1996). In *Drosophila*, there is no evidence for an effect of CO_2 on receptor neurons sensitive to other odorants, nor for other odorants affecting the CO_2-sensitive ab1C neurons (de Bruyne et al., 2001), although mixtures of the two were not tested. Hence, we suggest that vinegar stimulation changes the processing of CO_2 in the brain.

We also observed that females are more active, i.e. they walk more, in a vinegar background than in clean air, whereas males do not show this increased activity (Fig. 3). Gender differences have been observed in basic elements of walking behavior without olfactory stimulation (Martin et al., 1999). Our data show a general tendency for males to be more active than females (Fig. 3, not significant). Whereas CO_2 increases activity levels equally for males and females, vinegar increases activity only in females. Such increases in activity are therefore not simply due to olfactory stimulation or gender *per se*, but rather reflect sex-specific differences in the processing of odor information. Differences between sexes in sensitivity to CO_2 have also been shown in sandflies where they are related to differential behaviors near the host: females feed on blood and males find mates (Pinto et al., 2001). We propose that the raised activity levels we observe in *Drosophila* due to vinegar stimulation play a role in the female-specific increase in sensitivity. Since vinegar odor activates females more than males, it might also have a role in a female-specific behavior such as oviposition.

Our study clearly shows that the response of *Drosophila* adults to CO_2 is dependent on other odors in the background. This finding opens new questions. For example, so far we do not know if CO_2 can also sensitize the response to vinegar, comparable to the way it sensitizes the response of some blood-sucking insects to host odors (Barrozo and Lazzari, 2004; Dekker et al., 2005). Future studies need to examine this question.

We report here for the first time that *Drosophila* larvae also detect CO_2 and that they avoid it. Most odorants appear attractive to larvae (Cobb, 1999) whereas many of them have been reported as repulsive to adults at similar doses (Devaud, 2003). However, most studies use different paradigms for adults and larvae. A distinct advantage of our four-field olfactometer is that larvae can be tested under the same circumstances as adults, allowing a direct comparison. Our data actually show that behavior is similar for both life-stages; both adults and larvae spend more time in vinegar odor but avoid CO_2. This raises the possibility that the properties of the neural network regulating such behaviors are conserved through metamorphosis, although sensory and motor pathways differ. The external chemosensory neurons of the larva are all replaced with new olfactory and taste neurons that grow from imaginal discs (Tissot and Stocker, 2000), and the antennal lobe is considerably reorganized (Jefferis et al., 2004). Kreher et al. showed that most larval ORNs located in the dorsal organ express OR genes that are not expressed in adult olfactory organs (Kreher et al., 2005). Thus, although CO_2-detecting sensory neurons degrade, the nature of the behavioral response endures. However, larvae are considerably less sensitive. It may well be that this is due to differences in the sensory neurons.

We demonstrate here that in larvae CO_2 is detected by a single (paired) neuron expressing the *Gr21a* gene, the same receptor gene that is expressed in CO_2-sensitive ORNs in adults (Suh et al., 2004; Couto et al., 2005). As in adults, this neuron functions like an olfactory neuron. The neuron is located in the terminal organ, thought to be a gustatory organ (Oppliger et al., 2000; Scott et al., 2001). However, its axon extends to the antennal lobe, the olfactory center and not to the suboesophageal ganglion, the gustatory center (Scott et al., 2001; Python and Stocker, 2002).

Whereas adult flies may use small increases in CO_2 to find appropriate feeding and/or oviposition sites, larvae, with their low mobility, are continuously exposed to high CO_2 levels in the medium they live in. Their higher behavioral threshold may be adaptive, and avoidance only induced to avoid toxic levels. Carbon dioxide can nevertheless be attractive for larvae of other insects such as moths and beetles (Hibbard and Bjostad, 1988; Rasch and Rembold, 1994). Although we observed CO_2 avoidance only at relatively high doses, larvae that lack *Gr21a* neurons do not show it. Hence it can be excluded that the avoidance we observed here is due to anesthetic or other physiological effects of CO_2 that occur at higher concentrations (Badre et al., 2005).

The ability to compare behavioral responses of larval and adult *Drosophila* to odorants will enable the exploration of the functional development of the relevant neuronal circuits. Combined with the extensive knowledge on defined neuronal classes in the olfactory system of this species it should lead to a better understanding of the neuronal basis of behavior. In spite of the relatively simple nature of the sensory input from a single class of neurons, our analysis reveals differences between the sexes and across metamorphosis. The avoidance of CO_2 by *Drosophila* flies apparently depends on odor context: an attractive odor can increase sensitivity. The

ubiquitous occurrence of CO_2 makes it difficult to assess a single biological role to this behavior. Nevertheless, we think the decrease in CO_2 production with fruit ripening, combined with the higher sensitivity of females, suggests a role in selection of profitable food sources.

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