

Effect of elevated atmospheric CO₂ on oviposition behavior in *Manduca sexta* moths

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Abstract

Moths can detect changes in environmental carbon dioxide (CO₂) with extremely high sensitivity, but the role of CO₂ in the biology of these and other insects is not well understood. Although CO₂ has been demonstrated to influence egg-laying (oviposition) behavior of the pyralid moth *Cactoblastis cactorum* and nectar foraging of the sphingid moth *Manduca sexta*, information about the generalized role of CO₂ in the behavioral biology of these species is lacking. Comparative data are necessary to properly assess how the behaviors of different species may be modified by steadily rising levels of greenhouse gases in the environment. Experiments carried out in Biosphere 2 addressed whether changes in ambient CO₂ levels play a role in the oviposition behaviors of *M. sexta* moths. In the first series of experiments, oviposition was measured inside a flight cage with different levels of nearly ambient or elevated CO₂ (400, 800 or 1200 ppm). For each concentration, hostplants used as oviposition sites were grown from seed at a CO₂ level that matched the environment inside the flight cage. Under homogenous levels of CO₂, we observed no significant difference in oviposition behavior at the concentrations tested. In a second series of experiments, two groups of hostplants, each surrounded by a mini free-air CO₂ enrichment (FACE) ring, were assembled inside a flight cage. In this choice test, a dynamic plume of artificially high CO₂ was generated around one group of test plants, while ambient CO₂ was released around the second (control) group. After eggs were counted on both plant groups, *M. sexta* females showed a small preference for ovipositing on the control plants. Therefore, in contrast to *C. cactorum* females tested under similar dynamic flow conditions, *M. sexta* female oviposition was not strongly inhibited by elevated CO₂. To investigate this phenomenon further, we used electrophysiological recording and found that the CO₂ receptor cells in *M. sexta*, unlike those in *C. cactorum*, are not readily affected by elevated levels of ambient CO₂. These findings therefore suggest that elevated background levels of CO₂ affect the physiology of the CO₂ detection system of *M. sexta* to a lesser extent than that of *C. cactorum*, and this correlates well with the observed differences in oviposition behavior between the two species under elevated levels of environmental CO₂. Hostplants of *C. cactorum* are crassulacean acid metabolism plants that generate nocturnal CO₂ sinks on the cladode surfaces, whereas, *M. sexta* hostplants are nocturnal sources of respiratory CO₂. We hypothesize that the abrupt and continuing increase in global ambient CO₂ levels will differentially alter the behavior and physiology of moths that use CO₂ sinks and sources as sensory cues to find hostplants.

Keywords: *Datura*, FACE, labial palp, Lepidoptera, *Manduca*, oviposition and elevated CO₂

Received 21 May 2004; revised version received 9 January 2005 and accepted 27 January 2005

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Introduction

Research on effects of rising atmospheric carbon dioxide (CO₂), a principal driver of global climate change, has revealed a trend towards decreased hostplant quality with variable effects on different herbivorous insect feeding guilds: for example, leaf chewers tend to compensate by increased consumption (Williams *et al.*, 2000), leaf miners inadequately compensate by increased consumption, and sap feeders benefit with increased population and reduced development time (Coviella & Trumble, 1999; Whittaker, 1999; Karnosky *et al.*, 2003). However, little is known about direct effects of elevated CO₂ on the physiology and behavior of herbivorous insects.

Many insect species possess olfactory receptor cells that specifically detect ambient CO₂ levels, and it is known that the CO₂ receptor cells located in the labial palps (a paired mouthpart) of herbivorous Lepidoptera, are among the most sensitive (Stange, 1992). While CO₂ sensory systems displaying enormous structural diversity have been noted in different arthropods (Stange & Stowe, 1999), CO₂ sensory organs in invertebrates are most prevalent among the herbivorous Lepidoptera (Bogner, 1990; Stange, 1996, 1997). There is also ample evidence that detection of CO₂ concentration changes plays an important role in the behavioral biology of many insect species (Stange, 1996). The ecological importance of CO₂ detection to hematophagous or soil-dwelling insects is well documented (Stange, 1996), but for herbivores, it has been investigated thus far in only a few species (e.g. Stange, 1999). In moths, CO₂ appears to be involved in a number of different behaviors, including egg-laying (oviposition) by *Cactoblastis cactorum* (Lepidoptera: Pyralidae) (Stange *et al.*, 1995; Stange, 1997) and nectar foraging by the hawkmoth *Manduca sexta* (Lepidoptera: Sphingidae) (Guerenstein *et al.*, 2004a; Thom *et al.*, 2004).

Direct effects of rising atmospheric CO₂ concentration on the ability of herbivorous insects to detect CO₂ cues were reported by Stange & Wong (1993), who described an increase in the temperature dependence of CO₂ receptor cells in *Helicoverpa armigera* (Lepidoptera: Noctuidae) moths at CO₂ concentrations above pre-industrial levels (ca. 280 ppm). CO₂ receptor cells of *C. cactorum* also stop functioning at elevated CO₂ levels, resulting in adverse effects on oviposition. This special case, in which biological control of an introduced plant pest (*Opuntia stricta*) in SE Australia has been established for 70 years, is crucially dependent on detection by moths of microgradients of CO₂ concentration at the cactus cladode surface (Stange *et al.*, 1995; Stange, 1997). In the hawkmoth *M. sexta*, recent studies indicate that CO₂ plays an important role in signaling food-source

profitability to these nectar-feeding insects (Guerenstein *et al.*, 2004a; Thom *et al.*, 2004). These last two species are, therefore, particularly well suited for further studies of the behavioral consequences of rising environmental CO₂ levels.

The moth *M. sexta* is an established model organism for studies on insect olfaction (Hildebrand, 1996), and major advances in our understanding of the CO₂ sensory system in this species have recently been reported (Kent *et al.*, 1986, 1999; Guerenstein *et al.*, 2004b; Thom *et al.*, 2004). The CO₂-sensing system of *M. sexta* bears many similarities to that of other moths, yet, there are also interesting differences. For example, the CO₂ receptor cells of all moths studied thus far, are clustered within a special organ in each labial palp, the labial-palp-pit organ (LPO). *M. sexta* LPOs contain ca. 2000 CO₂ receptor cells and show no apparent sexual dimorphism (Kent *et al.*, 1986), whereas LPOs of female *C. cactorum* house ca. 200 CO₂ receptor cells, but are vestigial in males (Stange *et al.*, 1995).

As part of a larger program to study insect olfaction and the mechanisms underlying detection of plant volatiles by flying insects, we sought a suitably large environment with controlled atmosphere and airflow in which we could accurately manipulate the CO₂ conditions in the vicinity of hostplants while simultaneously observing odor-modulated insect behaviors. Given the suitability of *M. sexta* for studies on CO₂ detection (Guerenstein *et al.*, 2004b; Thom *et al.*, 2004) and the important consequences that rising atmospheric CO₂ could have on the biology of insects and other organisms (Stange, 1997), we obtained access to the controlled environments available at Biosphere 2 Laboratory (B2L) in Oracle, Arizona and combined behavioral and electrophysiological methods to explore the following: (1) oviposition behavior of *M. sexta* females to determine whether elevated CO₂ affects egg deposition, as demonstrated in *C. cactorum* and (2) how the physiology of *M. sexta* CO₂ receptor cells correlates with any observed behavioral effects. Our results provide new evidence that elevated CO₂ may differentially affect species that rely on CO₂ sinks and sources as behavioral cues.

Materials and methods

Experimental approach

The lower savanna (LS) mesocosm inside B2L, where climate controlled nocturnal experiments could be conducted most of the year with minimal interference, presented itself as an optimal location for CO₂ experiments with the *M. sexta*–*Datura wrightii* (jimson weed) hostplant system. *M. sexta* CO₂ detection experiments

inside B2L were modeled largely after free-air CO₂ enrichment (FACE) ring experiments with *C. cactorum* moths (Stange, 1997). Simplified versions of FACE ring technology, developed over the past decade as a method for modifying a local CO₂ terrestrial environment in different ecosystems (Lewin *et al.*, 1994), were constructed inside B2L for *M. sexta* oviposition dual-choice experiments. In addition, nonchoice experiments were also performed in intensively managed forest mesocosm (IFM) of B2L to measure oviposition differences under future ambient CO₂ conditions using hostplants grown under conditions that may have induced long-term physiological effects because of increased CO₂.

Moths

M. sexta L. (Lepidoptera: Sphingidae) (tobacco hornworm) hawkmoths were obtained from the rearing facility of the Arizona Research Laboratories Division of Neurobiology of the University of Arizona. Larvae were reared on artificial diet (modified from that of Bell and Joachim, 1976) under long-day photoperiod regimen (17 L:7 D) at 25–26 °C and 40–50% relative humidity (RH) (Prescott *et al.*, 1977; Tolbert *et al.*, 1983). Pupae were segregated by sex and held in a rearing room under reverse-photoperiod conditions (14 L:10 D) and with a superimposed temperature cycle: 26 °C L:24 °C D. Two to five days before adult eclosion, pupae were transferred to fiberglass-screen cages (31 × 31 × 32 cm³) under ambient Arizona light conditions. One or 2 days after emergence, female and male adults were placed together in a cage for mating (confirmed visually and assessed by observed retention of coupling after gentle handling) at 23 °C. Behavioral tests were performed with female moths that had eclosed 2–3 days prior to testing and had mated 1 day prior to testing. At no time prior to experimentation were moths exposed to plant odor. Each moth was used for only one nocturnal behavior experiment.

B2L LS

Experiments were conducted in the B2L LS, a hydrological transition zone between B2L desert and rainforest mesocosms consisting primarily of edible species including acacias for galagos, large-seeded grasses for finches, and fruit-bearing trees for galagos and birds. Irrigated *D. wrightii* and *Brachiaria mutica* grass were dominant in a clearing in the LS when moth experiments were conducted.

B2L air-handling machines conditioned the air at a maximum rate of 9500 m³ min⁻¹ (ca. 28 °C L:20 °C D) and consistently forced it across the LS. Soap bubbles

(ca. diameter 5 cm) were used to characterize the air movement over the LS. The trajectories and speeds of the bubbles were measured and used as an airflow visualization tool (smoke generation was not permissible inside B2L). With all air-handlers turned on, the average wind speed in the middle of the LS was ca. 0.5 m s⁻¹. Similar values were obtained using a hand-held anemometer.

B2L LS served as a superb, 'semi-natural,' large wind tunnel by providing a steady, gentle, unidirectional airflow without uncontrollable climate interferences of the outside world. The area of LS (ca. 100 m²; trapezoidal shape) mimicked natural conditions, yet the containment allowed control of wind, temperature, and humidity. The synthetic, functioning ecosystems inside B2L produced a desirable environment for observing insect behavior.

LS soils consisted of acid psamment and grassland haplustalf and closely approximated the natural soils supporting *D. wrightii* in the desert Southwest, USA. Temperature, RH, and CO₂ concentration were constantly monitored. Environmental conditions in B2L LS typically consisted of: a diurnal CO₂ concentration fluctuation between ca. 200 and 450 ppm because of plant respiration (CO₂ reached daily maximum levels at ca. 7:00 hours and minimum levels at ca. 18:00 hours, roughly corresponding to light/dark conditions), a diurnal temperature fluctuation between ca. 20 and 28 °C (highest in daytime), and a diurnal RH fluctuation between ca. 60% and 90% (highest in daytime).

D. wrightii hostplants were grown from seeds in 15 cm pots using a 3:2:1 (by volume) mixture of Sunshine All-Purpose potting mix (Bellevue, WA, USA), vermiculite, and silica sand and fertilized with Osmocote[®] 14-14-14 (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA). Plants were grown in a nearby greenhouse and later in a downwind portion of the LS. Healthy, nondamaged, flower- and bud-less plants of similar structure (ca. 0.5 m diameter × 0.5 m height), and leaf count were randomly chosen for rotation through the experiments. Experiments were conducted within two 40-day time periods: March–April and November–December 2003. Oviposition experiments were started 1–2 h preceding sunset.

Flight cage and FACE rings

M. sexta moths were released inside a cage constructed of amber-colored Lumite[®] screen (18 × 14 mesh in⁻¹; 35% shading, SI Performance Technology, Gainesville, GA, USA) and a 1 in diameter PVC[®] pipe frame (2.8 × 2.8 × 6.3 m³, height × width × length, respectively, 49.4 m³). A closable entrance on the downwind side allowed access for scientists. Two FACE rings were

constructed within the cage in excavated circles so that the tops of the rings were flush with the soil floor of the cage. FACE ring dimensions were 1.3 m diameter and 5.0 m apart (center to center; see Fig. 1a). Flexible ventilator duct tubing (15 cm diameter) and rigid 'Y' pieces were painted black and sealed with plumber's caulking to create FACE rings for delivering CO₂-air mixtures. One hundred holes (ca. 3 mm) were pierced on top of each ring with a nail to allow the CO₂-air mixture to escape from the ring and produce a rising 3-D active space above each ring. An AC Tube axial fan (W2S130 series, 5.9 in diameter × 2.2 in depth, 115 V, 225 ft³ min⁻¹, 62 dB A, 50/60 Hz, Newark Electronics[®], Chicago, IL, USA) was mounted in the 'Y' piece sidearm of each ring to force the CO₂-air mixture from outside the cage into the ring and out through the 3 mm holes. A silicon tube, 1/4 in O.D. was used to deliver CO₂ and was randomly inserted into one of the two FACE ring sidearms upstream of the fan from outside the cage at the beginning of each experiment. This design allowed CO₂ (99.998%, Praxair Distributions Inc, Tucson, AZ, USA or BOC Gases, Murray Hill, NJ, USA) to be delivered at 2.0 and 3.5 L min⁻¹ to mix with ambient air forced into the rings by the fans. Test and control fans were turned on for the duration of each experiment. For a description of FACE ring technology, see Hendrey *et al.* (1999).

Before each experiment, three potted *D. wrightii* hostplants were placed on the soil floor inside each FACE ring and positioned so that their foliage was retained in the vertical space inside the FACE rings. This design presented six plants (two groups of three

plants) to the moth for oviposition during each experiment (the six plants were chosen from a pool of ca. 25 plants for each experiment). One group randomly received forced ambient air and added CO₂, and the control group received only forced ambient air. This experimental FACE ring design was based largely on that described by Stange (1997). We controlled for possible bias because of the position of the plants in the cage.

Measurement of CO₂ level around FACE rings

CO₂ concentrations above each FACE ring (with hostplants present) were measured between 19:00 and 21:00 hours with, and without, added CO₂ using a LI-7500 open-path CO₂/H₂O analyzer (Li-Cor Biosciences, Lincoln, NE, USA). Measurements of 30 s duration were systematically taken at five points within a horizontal plane at three heights (30, 60, and 90 cm) above the ring. Figure 1b shows raw data collected above hostplants in the center of the ring with and without added CO₂. Data averages from Fig. 1b are included in Table 1. Large fluctuations of CO₂ concentration were introduced by the FACE rings with added CO₂, but control FACE rings produced relatively stable CO₂ levels. The introduced fluctuations were very similar to conditions imposed by FACE rings for *C. cactorum* (Stange, 1997). Within each plane, a measurement was taken directly above the center of the ring, and at four points over the circumference of the FACE ring (at 0°, 90°, 180°, and 270° around the ring). Values for fluctuating CO₂ concentrations were averaged over 30 s at each height. Average CO₂ levels (ppm ± SD) from the center of the ring in

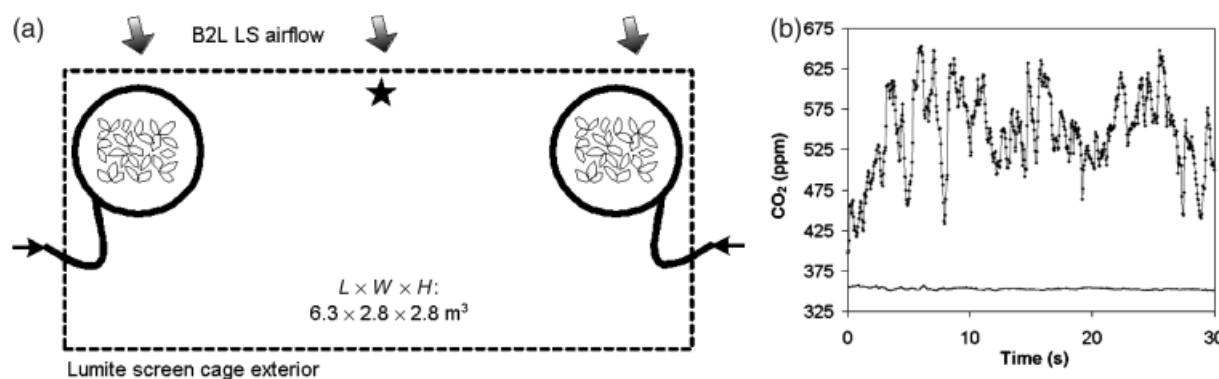


Fig. 1 (a) Top view (scale ca. 1 : 77) of Lumite[®] screen cage (17.6 m²) constructed in Biosphere 2 Laboratory (B2L) Lower Savanna (LS) for *Manduca sexta* nocturnal oviposition experiments. Free-air carbon dioxide (CO₂) enrichment (FACE) rings were symmetrically constructed on the upwind side of the cage with gas delivery points located outside of the cage (small arrows indicate ambient air intake on both cage sides and CO₂ delivery on treated side). The star indicates the point where the moth was released each evening on the east cage wall. (b) Example of 30 s CO₂ concentration (ppm) measurements collected using a Li-Cor 7500 open path CO₂/H₂O analyzer 30 cm above FACE rings with, and without (control plants), added CO₂ (2.0 L min⁻¹) on March 10, 2003 between 19:42 and 19:50 hours in the presence of *Datura wrightii* plants. Large CO₂ concentration fluctuations were introduced above treated plants by FACE rings receiving added CO₂ (◆, diamonds), but FACE rings around control plants produced stable (solid line) CO₂ levels. Average values are listed in Table 1.

Table 1 Average CO₂ levels (ppm ± SD) in excess of ambient concentration above plants in FACE ring experiments

	Control plants	Treated plants	Treated plants
CO ₂ delivered	None	2.0 L min ⁻¹	3.5 L min ⁻¹
<i>Height above</i>			
<i>FACE ring (cm)</i>			
90	4 ± 1	12 ± 20	35 ± 69
60	7 ± 1	134 ± 51	110 ± 104
30	9 ± 1	208 ± 51	740 ± 77

CO₂, carbon dioxide; FACE, free air CO₂ enrichment.

excess of ambient concentration above the plants are given in Table 1. CO₂ concentrations measured above control plants in FACE rings with no added CO₂ reflect CO₂ emissions by plant dark respiration. To obtain 'excess CO₂' values, ambient CO₂ level was subtracted from the measurements. The ambient background CO₂ concentration, which increases daily inside B2L LS between ca. 18:00 and 7:00 hours because of respiratory buildup from LS and thornscrub trees and shrubs, and mangroves and decreases between ca. 7:00 and 15:00 hours because of photosynthetic draw-down, has been subtracted from sequential Li-Cor measurements to obtain values shown in Table 1. The ambient CO₂ level in B2L LS was monitored ca. 8 m above the LS soil by GC analysis (every 15 min; not shown). Minute-by-minute ambient values were extrapolated from the CO₂ concentration curves by approximation using polynomial regression with a multiple regression correlation coefficient $R^2 = 1$. Subtraction of ambient [CO₂] values from averaged Li-Cor measurements, taken sequentially above plants inside FACE rings, yielded excess concentration levels shown in Table 1 and confirmed that the test plants were surrounded by elevated CO₂.

Li-Cor CO₂ concentration measurements taken between 19:00 and 21:00 hours above FACE rings in the presence of hostplants with added CO₂ demonstrated the effectiveness of turbulent elevated CO₂ delivery. CO₂ concentrations measured at increasing heights above the FACE rings for CO₂-treated plants tended to decrease in turbulence and concentration as the rising CO₂-air mixture quickly dispersed. Excess concentration levels for control-plant FACE rings also decreased when measured higher up, but fluctuation signals were still visible; for example, at 90 cm the CO₂ concentration fluctuated above control plants between 355 and 360 ppm, above the ambient level of 353. Hostplant-respiration CO₂ (approximated by excess values in 'control plants' column of Table 1) at 90 cm was still masked by added, fluctuating CO₂. A 30 s measurement collected 30 cm above a FACE ring and hostplants receiving 2.0 L min⁻¹ CO₂ showed a fluctu-

ating CO₂ concentration with a range of ca. 250 ppm between ca. 400 and 650 ppm in Fig. 1b. The control comparison for this measurement in Fig. 1b revealed CO₂ fluctuation presumably produced by plant and soil respiration, with a range of ca. 8 ppm around a 353 ppm average, 9 ppm above the ambient level (see also Table 1). Noise from added CO₂, under these conditions, largely masked CO₂ changes from hostplant respiration. Introduced fluctuations from added CO₂ were very similar to conditions imposed by FACE rings for *C. cactorum* (Stange, 1997).

Oviposition experiments with elevated, homogenous CO₂ levels

Nonchoice experiments were designed to measure oviposition differences under future ambient CO₂ conditions wherein any differences in hostplant physiology were also expressed and may have influenced oviposition behavior synergistically. *M. sexta* oviposition experiments were conducted with *D. wrightii* hostplants inside amber-colored Lumite[®] screen cages (20 × 20 mesh in⁻¹; 32% shading; 2 × 2 × 2 m³, BioQuip Products Inc., Gardena, CA, USA) with 2.5 cm diameter PVC[®] pipe frames under three elevated, homogenous CO₂ environments (400, 800, and 1200 ppm CO₂) inside the managed-forest mesocosm (IFM) of B2L (see Barron-Gafford *et al.*, 2005 for IFM description). Logistical restraints on changing IFM CO₂ concentrations precluded design of choice experiments. Experimental cages contained two similar *D. wrightii* hostplants. Plants were cultivated as described above, but these plants were raised in the elevated, homogenous CO₂ IFM environments of 400, 800, and 1200 ppm CO₂, respectively. Moths were released singly (into a single CO₂ concentration IFM environment) in the same manner described below for LS FACE experiments. The two hostplants offered as oviposition sites were grown in the same CO₂ concentration IFM environment as the cage experiment. The following day the moth was retrieved, and total numbers of eggs per moth were determined and summed (Fig. 2). Data from experiments in which the total egg count was below 50, or the moth escaped during the test, were discarded. Oviposition-preference percentages were calculated by using egg medians from 400 ppm CO₂ as control and egg medians from 800 ppm CO₂ as test.

Oviposition-choice experiments in elevated, fluctuating CO₂

M. sexta oviposition-choice experiments were conducted with *D. wrightii* hostplants at two different CO₂ flow rates in FACE rings within the flight cage.

M. sexta moths are known to oviposit for 1–2 h beginning 1–2 h after sunset (Gilmore, 1938). Moths were released by placing them, singly, on the inner cage wall 1–2 h before sunset. Moths consistently remained perched after placement in the cage, and are presumed to have remained stationary before flight after sunset, typical of this nocturnal species. On several occasions, moths were observed after release for several hours after sunset. Moths were observed to wing-fan and fly ca. half of the time within 2 h after sunset. The following day the moth was retrieved and eggs were counted, removed, and summed for CO₂-treated and control plants. A total of 50 experiments were carried out. Experiments yielding a total egg count <50 or in which the moth escaped from the cage before the end of the test or CO₂ delivery from the FACE rings failed were discarded. Based on early field work and more recent experience with *M. sexta* behavior in the Hildebrand laboratory, we determined that females ovipositing fewer than 50 eggs were probably unhealthy. Also, larval herbivory on plants is known to induce volatiles that may affect oviposition (De Moraes *et al.*, 2001; Kessler & Baldwin, 2001). Thus, if larvae were discovered, results were discarded. Oviposition preferences are described as percentages of eggs laid on plants with added CO₂ with respect to the total number of eggs laid. Paired data from these two-choice experiments were analyzed with a Wilcoxon's signed ranks test (Fig. 3; Mozuraitis *et al.*, 2002).

Electrophysiological responses of CO₂ receptor cells

Stimulation. CO₂ receptor cells in the LPO of female *M. sexta* were subjected to prolonged exposure to different baseline levels of CO₂. CO₂ stimuli were delivered to the LPO by methods similar to those reported by Grant *et al.* (1995). Three-way valves were used to control the flow of two opposing gas streams, a high-flow (340 mL min⁻¹) and a low-flow (200 mL min⁻¹) stream. Fixed CO₂ concentrations were delivered from one of three gas cylinders (Praxair, Los Angeles, CA, USA), each containing metered amounts of CO₂, purified oxygen (20%), and nitrogen (to fill the remaining volume). Stimulation time was determined by the opening of the high-flow stream. During at least 1 min before CO₂ delivery, this flow was vented to the room to flush the line with the desired CO₂ concentration. Using this method, the low-flow stream was allowed to reach the palp only when the high-flow stream was closed. Stimuli lasted 6 min, and the number of receptor cell spikes per 2 s period was registered every 10 s. The responses of CO₂ receptor cells to three CO₂ levels (0, 280, and 2990 ppm) were recorded. The CO₂ concentration sequence was randomized.

Method for recording from CO₂ receptor cells. A moth was restrained in a close-fitting plastic tube so that only the head protruded from one end, and one labial palp was secured to a wax platform using U-shaped pins. Extracellular recordings were obtained from CO₂ receptor cells by means of sharp tungsten wire microelectrodes (Grant *et al.*, 1995, and references therein). A reference tungsten electrode was placed in the second segment of the labial palp, and a recording electrode was carefully inserted into the LPO cavity (in the third, distalmost, segment of the labial palp) until action potentials were recorded. The electrical signals from the receptor cells were amplified (50 000 ×; Grass P511K, Grass Instruments, Quincy, MA, USA), filtered, monitored on an oscilloscope, and simultaneously digitized and recorded with a computer using Axoscope 8.0 software (Axon Instruments, Foster City, CA, USA).

Results

Responses of moths to hostplants surrounded by high, homogenous CO₂

The number of eggs deposited on plants grown and maintained under continuously elevated CO₂ levels showed little variation at concentrations from 400 to 1200 ppm. The number of eggs oviposited by *M. sexta* released in an environment of 400 ppm ambient, nonfluctuating CO₂ (median = 104, *n* = 10) did not significantly differ from egg numbers deposited on plants in either 800 ppm ambient CO₂ (median = 100, *n* = 8) or 1200 ppm ambient CO₂ (median = 94, *n* = 4, *P* = 0.86, Kruskal–Wallis ANOVA, Fig. 2).

Responses of moths to hostplants surrounded by high, fluctuating CO₂

We conducted another series of tests (this time offering a choice) using elevated, but fluctuating levels of CO₂ around a set of test plants located ca. 5 m from a group of control (no CO₂ added) plants (Fig. 1a). We found that increasing the flow of CO₂ through the FACE ring from 2.0 to 3.5 L min⁻¹ led to a ca. 3.5-fold increase in the average level of CO₂ in excess of ambient concentration, measured 30 cm above the test ring (Table 1). At both flow rates, FACE rings provided a fluctuating, elevated CO₂ active space, and a relatively stable (CO₂), but equally turbulent, control active space (see 2.0 L min⁻¹ measurement in Fig. 1b).

Observed behaviors consisted mostly of a mixture of directed and nondirected flight (Mechaber *et al.*, 2002) along with hovering flight and intermittent perching. Analysis of moths' preference for position of plants

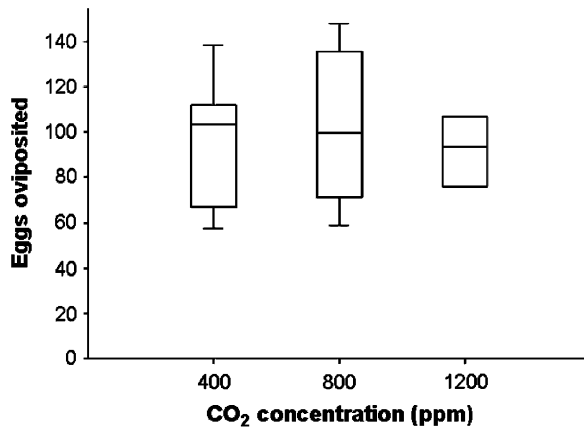


Fig. 2 Box plot of eggs oviposited by *Manduca sexta* under elevated, homogenous carbon dioxide (CO_2) levels (400 ppm, $n = 10$; 800 ppm, $n = 8$; 1200 ppm, $n = 4$). Lines within the boxes mark the median eggs oviposited by *M. sexta*. Lower and upper boundaries of the boxes indicate the 25th and 75th percentiles and bars below and above the boxes indicate the 10th and 90th percentiles, respectively. There is no statistical difference in the number of eggs laid by *M. sexta* under the different CO_2 levels.

inside the cage (Fig. 1a) by Fisher's exact test revealed no spatial bias ($P = 0.59$) and confirmed objective experimental conditions based on symmetry and random swapping of control and treated plant positions.

Experimental results at both CO_2 flow rates (and thus, different average, elevated CO_2 concentrations; listed in Table 1) demonstrated a tendency for mated *M. sexta* females to oviposit on the control plants (with no added CO_2). Oviposition-choice experiments conducted at the two flow rates showed similar tendencies by the moths (Fig. 3), indicating absence of a dose-response relationship. Oviposition-preference percentages of 40% and 44% were measured at CO_2 flow rates of 2.0 and 3.5 L min^{-1} , respectively, but these percentages were not consistently significant ($P = 0.04$ and $P = 0.17$, respectively; Wilcoxon's signed ranks test; Mozuraitis *et al.*, 2002). When the data were combined ($n_{\text{combined}} = 22$), however, the slight tendency to oviposit on control plants became significant ($P = 0.01$). Visual inspection of a combined data histogram implied unevenly dispersed data, but skewness and kurtosis analysis indicated an acceptable, normal data distribution, including one near-outlier point that was discarded.

Responses of CO_2 receptor cells to different homogenous levels of CO_2

We used electrophysiological recording techniques to examine the effects of elevated CO_2 on the response

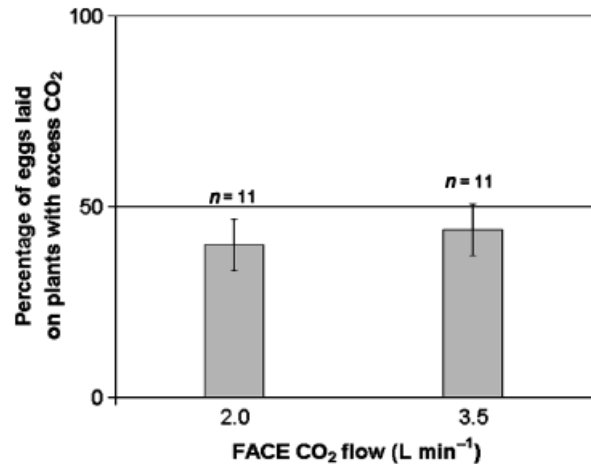


Fig. 3 Oviposition behavior of female *Manduca sexta* moths exposed to elevated, fluctuating carbon dioxide (CO_2) levels. A mean oviposition-preference percentage is shown for each CO_2 flow rate. SDs are indicated by vertical lines. Moths showed a slight tendency to lay more eggs on control hostplants, without added CO_2 .

properties of CO_2 receptor cells in the labial palps of female *M. sexta*. Receptor cells continuously exposed to different levels of CO_2 typically continued to produce spikes throughout the stimulation time, therefore, demonstrating a lack of sensory fatigue or complete adaptation during prolonged stimulation. The firing rate reached a plateau level that was concentration-dependent (Fig. 4). Therefore, the CO_2 receptor cells of *M. sexta* signaled a wide range of ambient CO_2 levels while still sensitive to fluctuations in CO_2 concentration after exposure to different levels of CO_2 (not shown).

Discussion

Both female and male *M. sexta* possess a large and sensitive CO_2 -detecting organ suggested to play a role in nectar foraging in both sexes (Thom *et al.*, 2004). It is not known, however, if CO_2 plays other roles in the biology of this species. *M. sexta* female moths oviposit nocturnally on plants from the Solanaceae and Martyniaceae families (Mechaber & Hildebrand, 2000), which assimilate CO_2 during the day and become sources of CO_2 at night. Many phytochemicals (attractants and repellents) mediate Lepidopteran oviposition behavior (Honda, 1995), and although upwind flight and directed orientation are clearly behaviors modulated by olfactory information about hostplant volatiles, the active compounds and their precise roles in regulating *M. sexta* oviposition behaviors have not yet been identified (Shields & Hildebrand, 2001; Mechaber *et al.*, 2002; Fraser *et al.*, 2003). Investigations of particular insect behaviors affected by elevated CO_2 could yield

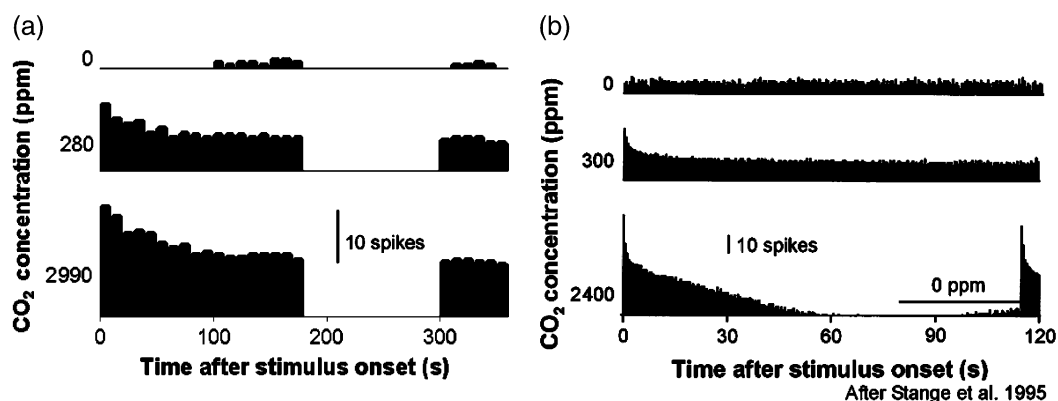


Fig. 4 Comparison of electrophysiological properties of carbon dioxide (CO₂) receptor cells in the moths *Manduca sexta* and *Cactoblastis cactorum*. (a) Rate-time histograms of action potentials (spikes) recorded from a single CO₂ receptor cell in *M. sexta*, during continuous 360 s stimulation (data gaps between times 180 and 300 s indicate no recorded data during that period) with CO₂ concentrations ranging from 0 to 2990 ppm. Prior to recording the unit was randomly exposed to one of the three CO₂ concentrations. Note at 2990 ppm CO₂, spike firing continued throughout the recording duration. (b) Rate-time histograms from a single CO₂ receptor cell in *C. cactorum*, during continuous 120 s stimulation with CO₂ concentrations ranging from 0 to 2400 ppm. Prior to recording, the unit was exposed to CO₂-depleted air. During part of the recording of the response to 2400 ppm, the unit was exposed to CO₂-depleted air (bar), to demonstrate reversibility of action potential suppression (adapted from Stange *et al.*, 1995). Note that data for *M. sexta* are presented in Hz, and the scales of both axes in (a) are different from those in (b).

better mechanistic knowledge of altered insect performance observed under these conditions (Percy *et al.*, 2002), and possibly lead to better predictions about the consequences of global climate change at higher trophic levels (Coviella & Trumble, 1999).

In this study, we took advantage of the suitability of B2L for measuring insect behavior in a realistic but carefully controlled environment. Given the important consequences that a rising CO₂ atmosphere could have on the biology of plants (Davey *et al.*, 2004) and herbivorous insects (e.g. herbivorous moths, Stange, 1997; Williams *et al.*, 2000), we asked if an altered CO₂ environment would interfere with oviposition behavior in *M. sexta* females. Thus, we explored a possible direct effect of increased environmental CO₂ on oviposition behavior of this moth.

Results from a first series of experiments using hostplants grown in nearly ambient or elevated CO₂ environments (400, 800, or 1200 ppm) indicate that oviposition in *M. sexta* was not affected under elevated, stable CO₂ conditions. These experiments suggest that oviposition in this species may not be affected directly by a global increase in ambient CO₂ levels, but the effects of such climate change on foraging behavior remain to be studied. We note that our oviposition experiments in homogenous, elevated CO₂ were designed and performed differently from those for *C. cactorum* (Stange, 1997; *C. cactorum* was given a temporal choice to oviposit in a higher or lower CO₂ concentration, whereas *M. sexta* was not offered a choice).

In a second series of experiments, we used two groups of hostplants surrounded by FACE rings with a plume of artificially high CO₂ (up to ca. 740 ppm above ambient; see Table 1) generated only around the test plants. The results suggest that ovipositing *M. sexta* moths slightly prefer plants surrounded by normal levels of CO₂: on average, 58% of eggs were laid on the control plants (oviposition preference = 42%; Fig. 5). These data suggest that as a population, *M. sexta* females tend to avoid plants surrounded by elevated CO₂. Although there is high similarity between the oviposition percentages at each flow rate (suggesting that the moths' behavioral response is likely saturated at 2.0 L min⁻¹) low *n*-values resulted in inconsistent significance levels between the results at each flow rate. Thus, while the pooled data suggest a role for CO₂ in the oviposition behavior of *M. sexta*, results from the two CO₂ flow-rate experiments analyzed separately indicate that this moth may not be affected by background CO₂ to the same extent as other species. The number of replicates in our data sets is limited because the B2L research site was closed permanently, precluding further experimentation.

Although *M. sexta* normally visits blooming *D. wrightii* plants, the plants used in these experiments did not have flowers. Because *D. wrightii* flowers emit high levels of CO₂ (Guerenstein *et al.*, 2004a), control-plant CO₂ values reported in Fig. 1b and Table 1 underestimate the relevant, natural situation for *M. sexta*. We anticipate that a blooming *D. wrightii* patch emits higher, and more fluctuating, CO₂ cues than those

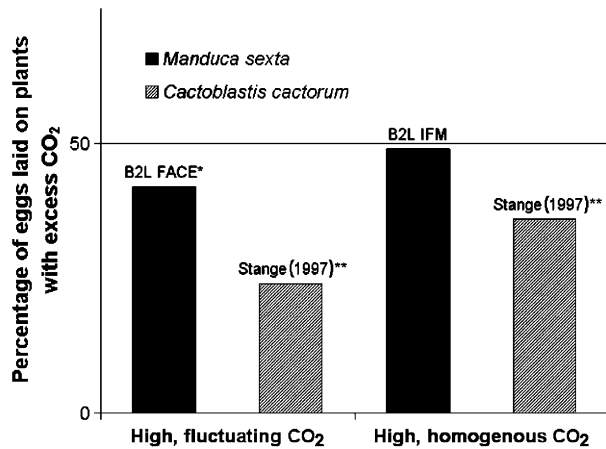


Fig. 5 Oviposition-preference percentages for female *Manduca sexta* moths on *Datura wrightii* hostplants under a high, fluctuating carbon dioxide (CO₂) background (median data from both free-air CO₂ enrichment (FACE) CO₂ flow rates combined; this work; $P = 0.01$) and under a high, homogenous CO₂ background (data from intensively managed forest mesocosm (IFM) experiments under 400 and 800 ppm CO₂; this work) and for female *Cactoblastis cactorum* moths on *Opuntia stricta* host cactus plants under a high, fluctuating CO₂ background and a high, homogenous CO₂ background (Stange, 1997). Oviposition-preference percentage = (number of eggs on plants with excess CO₂/total number of eggs) × 100. *M. sexta* avoidance of test plants (with excess CO₂) was small in comparison with that reported for *C. cactorum* tested under similar conditions. Asterisks denote statistical significance of results.

recorded for control plants in B2L LS. Moreover, our conclusions are limited by our experimental design, which tested plants only in a single plant life-stage. In FACE experiments, plants grown at ambient CO₂ levels were surrounded by a plume of increased CO₂. We, therefore, cannot exclude the possibility that an abrupt increase in CO₂ levels affected their physiology in a way that could have influenced moth behavior (e.g., by releasing odors not usually emitted). The correlation between moth oviposition behavior affected by CO₂ and physiology of CO₂ receptor cells, however, suggests that CO₂ is involved in oviposition behavior of moths.

FACE rings used in this work were admittedly much simpler and smaller (1.3 m diameter, comparable with mini-FACE rings used by Shaw *et al.*, 2002) than larger, multi-user FACE rings (see Osmond *et al.*, 2004 for a detailed discussion of experimental climate change systems available). FACE rings were simply designed because elaborate FACE ring control of environmental parameters (temperature, RH, CO₂ compensation for airflow) inside B2L would have been redundant, and space inside the moth cage was constrained.

The oviposition preference by *M. sexta* for test plants under homogenous, elevated CO₂ conditions (inside

B2L IFM) was 49% (Fig. 5), while the oviposition preference of this moth under fluctuating, elevated CO₂ conditions (using FACE rings) was somewhat less at 42% (Fig. 5; based on combined egg count data from the two different CO₂ flow rates). These values for *M. sexta* females stand in contrast to those observed for *C. cactorum* (Stange, 1997) under similar conditions of elevated CO₂. In the latter species, only 36% of the eggs deposited under homogenous conditions were found on the test plants, while even fewer, 24%, were deposited under a fluctuating CO₂ background (Fig. 5). Our data, therefore, indicate that *M. sexta* females show little avoidance of test plants surrounded by excess CO₂, and our electrophysiological data may help explain the contrasting findings with *C. cactorum* females.

As shown in Fig. 4, CO₂ receptor cells in *M. sexta* do not adapt completely or show signs of sensory fatigue during prolonged stimulation. These cells can function under a wide range of ambient CO₂ levels while still responding to rapid changes in CO₂ levels. In contrast CO₂ receptor cells in *C. cactorum* decrease or stop firing under increased, ambient CO₂, and are less sensitive to rapid changes in CO₂ levels (Fig. 4). Reduced input from CO₂ receptor cells may strongly interfere with brain processing of signals involved in oviposition behavior of moths, and this could explain low oviposition performance of *C. cactorum* under elevated CO₂ conditions.

It is important to point out that interspecific behavioral and physiological differences may also have an ecological correlate. *C. cactorum* oviposits on crassulacean acid metabolism (CAM) plants that generate relatively small CO₂ sinks (Stange *et al.*, 1995) whereas *M. sexta* oviposits on plants that are important sources of CO₂. *Opuntia* spp., CAM hostplants of *C. cactorum*, generate nocturnal CO₂ sinks on the cladode surfaces, whereas, *M. sexta* hostplants such as *D. wrightii* are nocturnal sources of respiratory CO₂. *M. sexta* may have evolved in an environment where CO₂ levels within a hostplant patch were higher, and CO₂ fluctuations larger, in comparison with the hostplant environment where *C. cactorum* typically oviposits. Hence, we hypothesize that the abrupt and continuing increase in global ambient CO₂ levels will have a greater effect on moths that use CO₂ sinks as cues than on those that use CO₂ sources.

In both species, the preference for oviposition on control plants was greater under conditions of high, fluctuating CO₂ than under homogenous, elevated CO₂. This suggests that CO₂ fluctuations are relevant to moth behavior and that unexpectedly large CO₂ fluctuations confuse the animal's system for sensing plant-derived CO₂. Such fluctuations are also likely to occur

unintentionally in FACE ring experiments conducted elsewhere, possibly causing effects on herbivores that will not occur in the future, homogeneously elevated CO₂ environment that such experiments attempt to emulate. The result could be analogous to the effect of sex-pheromone baits, which are widely used in the biological control of orchard pest insects, causing mating disruption by interfering with the orientation of males. It is possible that, like the system for pheromone detection, the extremely sensitive and selective CO₂-sensing system of pest insects could be exploited in future control regimens.

Acknowledgements

We thank Natalie Abrams, Tamara Montecute, Samuel Pegg, Selby Cull, Rod Carender, Joost van Haren, Judy Freeman, and Barbara Walss for technical assistance inside B2L; David Stumpf and Vincent Pawlowski for assistance in building a CO₂ delivery system for electrophysiological experiments; Heather Stein for laboratory assistance; A. A. Osman, Cassandra Mtine, and Suzanne Mackzum for rearing *M. sexta*; and Volker Witte, Barry Osmond, and members of the Hildebrand research group for valuable discussions. L. A. was partially supported by NSF (CHE-0216226). P. G. G. and J. G. H. were partially supported by NSF (IBN-0213032). W. L. M. and J. G. H. were partially supported by NSF (IBN-9983302). We thank Office of the Executive Vice Provost of Columbia University (Michael Crow, currently at Arizona State University) and Barry Osmond for a program enhancement grant to G. S. and a Chemistry Biosphere 2 Program grant to K. N. We also thank Edward P. Bass for supporting this work.

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