Context- and scale-dependent effects of floral CO₂ on nectar foraging by *Manduca sexta*

Joaquín Goyret*[†][‡], Poppy M. Markwell[§], and Robert A. Raguso*[†]

*Department of Biological Sciences, University of South Carolina, Columbia, SC 29208; and [§]Oberlin College, Oberlin, OH 44074

Typically, animal pollinators are attracted to flowers by sensory stimuli in the form of pigments, volatiles, and cuticular substances (hairs, waxes) derived from plant secondary metabolism. Few studies have addressed the extent to which primary plant metabolites, such as respiratory carbon dioxide (CO_2), may function as pollinator attractants. Night-blooming flowers of *Datura wrightii* show transient emissions of up to 200 ppm above-ambient CO_2 at anthesis, when nectar rewards are richest. Their main hawkmoth pollinator, *Manduca sexta*, can perceive minute variation (0.5 ppm) in CO_2 concentration through labial pit organs whose receptor neurons project afferents to the antennal lobe. We explored the behavioral responses of *M. sexta* to artificial flowers with different combinations of CO_2, visual, and olfactory stimuli using a laminar flow wind tunnel. Responses in no-choice assays were scale-dependent; CO_2 functioned as an olfactory distance-attractant redundant to floral scent, as each stimulus elicited upwind tracking flights. However, CO_2 played no role in probing behavior at the flower. Male moths showed significant bias in first-approach and probing choice of scented flowers with above-ambient CO_2 over those with ambient CO_2, whereas females showed similar bias only in the presence of host plant (tomato) leaf volatiles. Nevertheless, all males and females probed both flowers regardless of their first choice. While floral CO_2 unequivocally affects male appetitive responses, the context-dependence of female responses suggests that they may use floral CO_2 as a distance indicator of host plant quality during mixed feeding-oviposition bouts on *Datura* and *Nicotiana* plants.

fragrance | odor | olfaction | pollination | Sphingidae

Anthophilous insects use information from a variety of sensory channels to locate flowers and feed from them (1). Thus, a crucial task for studying insect–plant interactions is to identify which components of the environment provide the sensory inputs used by insects, and to what extent context and scale affect their information content (2). Whereas flower colors and patterns (3, 4), whole-flower and nectar odors (5), and even corolla shape and texture are known features used by foraging insects and other nectivorous animals (6, 7), few studies have explicitly addressed the role of flower respiratory carbon dioxide (CO_2) as a stimulus involved in plant–pollinator interactions.

Our knowledge of how insects use CO_2 as a sensory cue is derived primarily from studies of insects that vector diseases or attack crop plants. For example, many haematophagous insects use CO_2 to locate their animal hosts from a distance (8). Discontinuous CO_2 plumes modulate host-seeking behavior by mosquitoes (9, 10), suggesting that CO_2 acts as a long-range orientation stimulus (11). Tsetse flies and biting midges also use CO_2 as a long-range attractant that can synergize the attractiveness of other host odors (12, 13). However, CO_2 also functions as a close-range feeding stimulus (on the host's skin) for mosquitoes (8). CO_2 synergizes the attractiveness of some human skin odors to female yellow fever mosquitoes (14) and has been suggested to synergize triatomine bug responses to L(+)-lactic acid (15).

Some herbivorous insects show similar responses to CO_2, although typically at closer range to its source than haemato-

phagous insects. CO_2 alone is sufficient to guide *Diabrotica virgifera* beetle larvae toward corn roots (16), and larvae of noctuid (*Helicoverpa armigera*) (17) and pyralid (*Elasmopalpus lignosellus*) (18) moths orient to above-ambient CO_2 sources. Females of the pyralid moth *Cactoblastis cactorum* search for CO_2 sinks on photosynthetic stem surfaces of *Opuntia stricta*, as indicators of high carbon fixation activity (19). In this case, CO_2 gradients are used as close-range stimuli, as is true for tephritid flies that oviposit on fruit wounds, which provide a localized source of CO_2 and other olfactory oviposition stimulants (20). Finally, responses to CO_2 may be context-dependent. Tephritid flies respond to CO_2 when presented with a fruit-like visual stimulus (20). Within a certain concentration range, *Drosophila melanogaster* adults and larvae are repelled by CO_2, and it has been suggested that these responses depend on the olfactory context (21).

It is clear from the studies reviewed above that CO_2 may function alone or in concert with host odors, at a distance or at close range, via several behavioral mechanisms. Recent studies suggest that CO_2 may also contribute to the interactions between flowers and insect pollinators. Floral CO_2 is primarily associated with elevated respiratory activity in thermogenic flowers, including deceptive flowers that mimic carrion and other decaying brood sites (22, 23). Although CO_2 emission is a by-product of elevated floral temperature, the compound stimulus of CO_2, heat, and foul odors most accurately mimics microbial activity in rotting meat (24, 25). Less well studied are the patterns of CO_2 production in nondeceptive flowers that proffer nectar as an energetic reward. In principle, if newly opened, unvisited flowers emit elevated CO_2, nectivorous insects could use such information to find the most profitable flowers or patches thereof (26). If floral CO_2 levels were ephemeral, insects might use them as more "honest" indicators of nectar availability in real time than floral color or scent (2), in much the same way that tarsal secretions are used as flower-marking cues by foraging bees (27, 28). Given that CO_2 is a component of ambient air in plant communities, flowers would need to emit amounts that could be detected by the insect with a sufficiently high signal-to-noise ratio.

These conditions are met in a night-blooming plant, *Datura wrightii* [Solanaceae (29, 30)], and its primary pollinator, the crepuscular hawkmoth, *Manduca sexta* (Sphingidae), in the Sonoran Desert. Hawkmoth-pollinated flowers often undergo dramatic bud elongation, nectar secretion, scent biosynthesis, and emission during the 6–12 h before opening (31, 32). Flowers of *D. wrightii* open explosively at dusk, releasing concentrations of CO_2 up to 200 ppm above ambient levels (29). Adult *M. sexta*

Table 1. Alternative hypotheses and critical predictions for the behavioral role(s) of floral CO_2 in nectar foraging by *M. sexta* from experiment 1 (no-choice assays): Scale-dependence of CO_2-mediated moth foraging behavior

Predictions	H_{A0}	H_{A1}	H_{A2}	H_{A3}
Tracking	V = V+CO_2	V < V+CO_2	V = V+CO_2	V < V+CO_2
Probing	V = V+CO_2	V = V+CO_2	V < V+CO_2	V < V+CO_2

Hypotheses: H_{A0}, CO_2 has no behavioral effect; H_{A1}, CO_2 is an orientation cue only; H_{A2}, CO_2 is a local feeding stimulus only; H_{A3}, CO_2 is both an orientation cue and a local feeding stimulus.

moths can detect such differences with their CO_2-sensing organ [labial-palp pit organ (LPO)], whose threshold may be as low as 0.5 ppm (33, 34). In the aforementioned *C. cactorum*, males have vestigial LPOs, whereas females use their well developed LPOs to detect gradients of CO_2 concentration for oviposition on *Opuntia* cactus. In contrast, the LPOs of *M. sexta* are well developed in both sexes (35), suggesting that they contribute to adult nectar foraging rather than, or in addition to, oviposition (36). Thom and collaborators (26) performed dual-choice assays with naïve male *M. sexta* moths in a flight cage, in which the moths preferred to feed from an artificial flower emitting higher than ambient CO_2 concentrations (765 ppm) over one emitting ambient levels of CO_2 (438 ppm). These findings provide direct evidence that floral CO_2 affects the foraging behavior of adult Lepidoptera.

In this study, we examined the behavioral mechanisms of CO_2-mediated nectar foraging in *M. sexta* by addressing three scale- or context-related questions. (*i*) Do *M. sexta* moths use floral CO_2 as an orientation cue or a local feeding stimulus? (*ii*) How does CO_2 interact with visual and olfactory cues during foraging? (*iii*) Do the behavioral responses of moths to floral CO_2 change in the presence of host-plant volatiles? We explored these questions by presenting naïve adult moths with subsets of sensory stimuli in a laminar flow wind tunnel, scoring tracking, approaching, and probing behaviors to distinguish between alternative hypotheses on the behavioral role(s) of floral CO_2 (Table 1, Table 2, and Fig. 1). Additionally, we performed a choice experiment similar to that published by Thom *et al.* (26), but in a laminar flow wind tunnel, in which we tested the foraging behavior of unmated females in addition to males. Because male moths already had been shown to choose scented paper flowers with above-ambient CO_2 (19), we simply expected female moths to behave similarly. When they did not, we repeated this experiment with the addition of tomato plant volatiles to test whether female responses to floral CO_2 depend on contextual information provided by host-plant odors.

Results

Experiment 1. Tracking, approaching, and probing behaviors all were significantly affected by treatment [Gh = 78.36, $P < 0.0001$; Gh = 15.05, $P = 0.0018$; and Gh = 27.35, $P < 0.0001$, respectively; $n = 130$ (Fig. 2*A*)]. Tracking behavior (Fig. 2*A*,
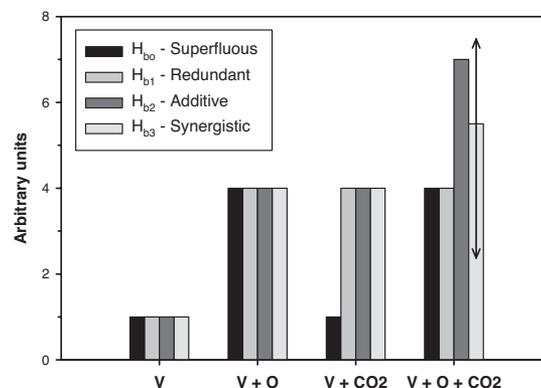

Fig. 1. Visualization of predicted outcomes for alternative hypotheses on the behavioral function of floral CO_2 in nectar foraging behavior by *M. sexta*, as tested in experiment 1. The vertical axis represents a unit-free relative measure of moth response (e.g., tracking and probing). The horizontal axis contrasts the predicted outcomes when moths are exposed to artificial flowers with different subsets of floral stimuli, including visual target (V) with floral odor (V+O), carbon dioxide (V+CO_2), or the combination thereof (V+O+CO_2). Hypothesis names reflect how CO_2 would interact with floral odor. Here, synergism is broadly defined as a nonadditive (+ or −) interaction with odor, as indicated by the two-headed arrow within the bar. The potential for strict synergism, in which only the combination of odor and CO_2 elicits the appropriate behavior, has already been falsified for tracking, floral approach, and probing in previous experiments (7, 43, 44).

black bars) essentially was not observed in the V (visual display as an attractant) treatment but was elicited either by adding odor (bergamot oil) (V+O: Gh = 30.51, $P < 0.0001$, $n = 66$) or above-ambient CO_2 emissions to the corolla opening (V+CO_2: Gh = 54.25, $P < 0.0001$, $n = 67$). Surprisingly, model flowers emitting CO_2 elicited the most tracking displays (V+O vs. V+CO_2: Gh = 4.35, $P = 0.037$, $n = 63$), and additive effects were not observed with the addition of odor (V+CO_2 vs. V+O+CO_2: Gh = 0.58, $P = 0.45$, $n = 64$). Conversely, approaches (Fig. 2*A*, light gray bars) increased with the presence of odor (Gh = 5.16, $P = 0.02$) but were not significantly affected by the addition of CO_2 to the visual display (Gh = 3.42, $P = 0.06$, $n = 67$). The highest levels of approaches were observed when odor was present (V+O vs. V+O+CO_2: Gh = 2.44, $P = 0.12$). Finally, probing responses were not affected by CO_2, either alone (V vs. V+CO_2: Gh = 0.036, $P = 0.85$) or in addition to odor (V+O vs. V+O+CO_2: Gh = 0.39, $P = 0.53$), but there was a gender difference in the V+O treatment, where males were more responsive than females (85% and 45% of responsive animals, respectively; Gh = 5.49, $P = 0.02$). We did not find any other significant asymmetries, but in the V treatment there was a trend for females to be less responsive than males (11% and 31%, respectively). These tendencies are not apparent in the treatments with CO_2 (V+CO_2: 24% of males and 20% of females; V+O+CO_2: 71% of males and 67% of females).

There was no significant treatment effect on the latency time [two-way ANOVA; $F_{(3, 74)} = 0.51$, $P = 0.67$] but a strong gender

Table 2. Alternative hypotheses and critical predictions for the behavioral role(s) of floral CO_2 in nectar foraging by *M. sexta* from experiment 1 (no-choice assays): Multimodal interactions between CO_2, visual, and olfactory stimuli in moth feeding

Predictions	H_{B0}	H_{B1}	H_{B2}	H_{B3}
Probing	V+O = V+O+CO_2	V < V+O, V+CO_2	V+O+CO_2 =	V+O+CO_2 ≠
	V = V+CO_2	V+O = V+CO_2	(V+O) + (V+CO_2)	(V+O) + (V+CO_2)
		V+O = V+O+CO_2		

Hypotheses: H_{B0}, addition of CO_2 has no effect (superfluous); H_{B1}, addition of CO_2 is redundant to floral odor; H_{B2}, addition of CO_2 is additive to floral odor; H_{B3}, addition of CO_2 is synergistic (+ or −) to floral odor.
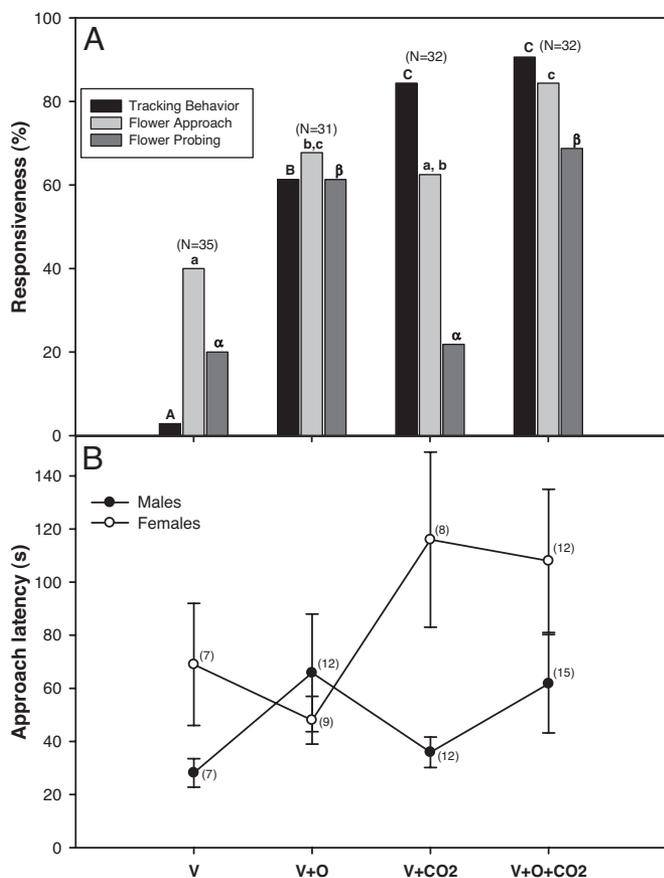
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Fig. 2. Behavioral responses of adult *M. sexta* in a wind tunnel with a single surrogate flower. (*A*) Responsiveness, expressed in percentage, of the recorded behaviors. Categories in the abscissa represent the different sets of stimuli offered by the surrogate flower: Different type of letters (uppercase, lowercase, and Greek) are used for comparisons between treatments for each behavior. Different letters indicate significant differences with an α-level of 0.05 (see *Results*). (*B*) Latencies to approach (i.e., time from takeoff to flower approach, in seconds) of male and female moths. Data points and error bars represent means $\pm$ SEM. Number of replicates for each treatment in parenthesis.

effect $[F_{(1, 74)} = 7.25, P = 0.009]$, with females and males averaging 87 ± 13 sec and 51 ± 9 sec, respectively (mean $\pm$ SEM). This was probably due to the females' tendency for increased latencies in the presence of CO_2 [V+O vs. V+CO_2: $F_{(1, 15)} = 4.40, P = 0.05$; V+O vs. V+O+$CO_2$: $F_{(1, 19)} = 3.52, P = 0.08$ (see Fig. 2*B*)]. No significant interaction between gender and treatment was detected $[F_{(3, 74)} = 0.84, P = 0.48]$. Probing times were affected by neither treatment nor gender [ANOVA; treatment: $F_{(1, 41)} = 0.02, P = 0.90$; gender: $F_{(1, 41)} = 3.08, P = 0.09$; interaction: $F_{(1, 41)} = 0.51, P = 0.48$].

Experiment 2. Approximately 74% of the males and 82% of the females tested flew and probed at the experimental flowers, showing a significant preference of first approaches to the V+O+CO_2 flower instead of the V+O flower [χ^2 test; $P = 0.04$, $n = 60$ (Fig. 3*A*)]. However, when analyzing gender differences, we found this result to be due exclusively to male behavior (χ^2 test for males: $P = 0.002, n = 28$; χ^2 test for females: $P = 1.00$, $n = 32$). Females took 64 ± 15 sec from take-off to probing on the V+O+CO_2 flowers and 61 ± 18 sec on V+O flowers. For males, latency times were 48 ± 7 sec and 28 ± 7 sec, respectively, on these treatments. However, latencies did not differ significantly between gender under ANOVA [$F_{(1, 58)} = 1.91, P = 0.17$]. Analysis also showed that neither females nor males differed
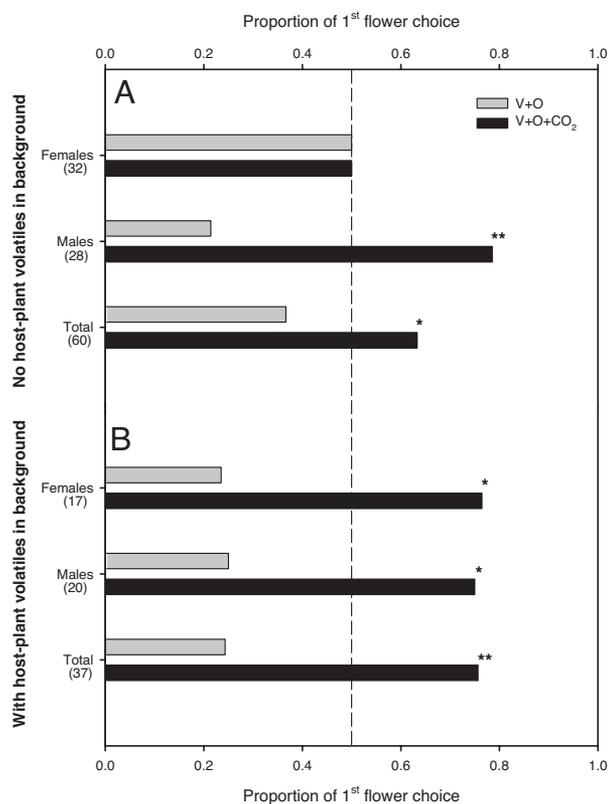

Fig. 3. Proportion of first flower choices by adult *M. sexta* in dual-choice assays between two scented surrogate flowers, of which only one emitted above-ambient CO_2 levels (V+O+CO_2). Assays were performed in a wind tunnel either without (experiment 2; *A*) or with (experiment 3; *B*) four tomato plants at the upstream end. * and **, significant departure from random probabilities with α-levels of 0.05 and 0.005, respectively.

significantly in latency times when responding to the different flower models [females: $F_{(1, 30)} = 0.01, P = 0.91$; males: $F_{(1, 26)} = 2.00, P = 0.17$], but females tended to probe the V+O+CO_2 flower for a longer time [7 ± 1 sec vs. 4 ± 1 sec; $F_{(1, 30)} = 3.87$, $P = 0.06$], whereas males did not show any probing time difference between the two flower models [7 ± 2 sec probing on the V+O+CO_2 flower and 9 ± 4 sec on the V+O flower; $F_{(1, 26)} = 0.58, P = 0.45$].

Experiment 3. In this experiment, 95% of the males and 85% of the females that were tested flew and probed at the experimental flowers. In 76% of those that probed, the first choice was the V+O+CO_2 flower (28 of 37; χ^2 test, $P = 0.002$). The addition of background tomato plant volatiles did not change first choice preferences in males (Fig. 3), which first chose the V+O+CO_2 flower (χ^2 test, $P = 0.025$, $n = 20$) as in experiment 2. Nevertheless, tomato volatiles appeared to have a strong effect on female moths, which departed from their random choice (experiment 2) to show a significant bias toward the V+O+CO_2 flower over the V+O flower (χ^2 test, $P = 0.018, n = 17$) (Fig. 3).

Discussion

More than 200 years ago, Sprengel (37) first recognized that foraging animals use diverse components of floral display (e.g., color, shape, texture, and fragrance) at different physical scales to find and evaluate flowers as potential food sources. To date, most studies of floral traits and their impact on the cognitive aspects of pollinator behavior have focused on these quintessentially floral stimuli (38). Few studies, beyond those of thermogenic "trap flowers" (22) and figs (39), have considered the

potential importance of more basic floral cues, such as respiratory CO_2, to pollinator behavior. The results of our experiments, consistent with those of Thom *et al.* (26), confirm that floral CO_2 can be used as a foraging stimulus by *M. sexta*, a nectivorous insect with high metabolic demands (40), and may well be perceived as a relevant floral cue by other flower-visiting insects, including mutualists and enemies. Field assays with honey bees and *Cyclocephala* beetles would be an appropriate test of this hypotheses, given their abundance and often destructive presence at *Datura* flowers (30), and their known responsiveness to CO_2 accumulation in hives (41) and thermogenic flowers (22), respectively.

Nectar foraging by naïve *M. sexta* follows a sequence of behavioral events in response to sensory stimuli at different distances from the flower. For example, upwind flight is released by a floral odor plume (42, 43) and floral approach is elicited by olfactory and/or visual stimuli (43) that are most effective when combined (44), after which probing is guided by visual and tactile inputs (7). Upon closer inspection, these behavioral "rules" are somewhat flexible, depending on the spatial and temporal scale (44) and the context in which specific stimuli are perceived [e.g., after appetitive conditioning (45, 46)]. With this framework in mind, we explored how CO_2 might interact with other floral stimuli in behavioral assays with *M. sexta*.

Scale Dependence of Responses to CO_2 in No-Choice Assays. Our first approach (experiment 1) was to decouple floral traits to examine how CO_2 affects nectar foraging by *M. sexta* at artificial flowers with different subsets of sensory stimuli. As outlined in Tables 1 and 2, our goal was to determine the scale (if any) at which CO_2 affects appetitive responses, using no-choice behavioral assays. The tracking and probing behaviors observed (Fig. 2*A*) allow us to reject the hypotheses stating that CO_2 has no behavioral effect (H_{A0}) or acts as a local feeding stimulus (H_{A2}). Instead, our results support the hypothesis that CO_2 acts as an orientation stimulus (Table 1, H_{A1}). The CO_2 plume evoked the typical zigzag casting flight pattern indicative of odor-guided behavior in *M. sexta* (30, 47, 48) and was slightly more likely to elicit upwind casting flight than was floral odor. These results suggest that floral CO_2 is perceived by *M. sexta* as an odor, a hypothesis that is consistent with the fact that CO_2-receptor cells in the LPO project their axons through the suboesophageal ganglion into both antennal lobes (34), the primary centers for the processing of olfactory inputs from the antennae.

Even though CO_2 alone (V+CO_2) had an effect on tracking behavior, approaches to the flower did not differ significantly from the negative control (i.e., Fig. 2*A*, V). Probing behavior was unaffected by the presence of CO_2, with and without floral odor, which allows us to reject the hypotheses of redundant (H_{B1}), additive (H_{B2}), and synergistic (H_{B3}) effects in favor of the null hypothesis (Table 2 and Fig. 1, H_{B0}). Thus, in no-choice assays, CO_2 affects the foraging behavior of naïve *M. sexta* as an orientation stimulus. Additionally, CO_2 appears to have no effect or interaction with floral odor as a local feeding stimulus, either in terms of probing frequency or duration. Latency times did not show any treatment effect, although females tended to spend longer times than males in the wind tunnel before approaching the artificial flower when it emitted CO_2 (Fig. 2*B*). Also, females were less responsive than males when no CO_2 was present (see *Results*). We suspect a conflict or overlap in task specificity between female foraging and reproduction that will be discussed below in the light of experiments 2 and 3.

Context Dependence of Female Responses in Binary-Choice Assays. Having found that CO_2 effectively elicits orientation toward flowers with no apparent effect on the probing response at close range, we designed experiment 2 to evaluate whether the tracking response elicited by CO_2 might impact individual flower choice. Strikingly, we found that females showed no bias in their first probing choice between scented flowers (V+O) and scented flowers emitting above-ambient CO_2 (V+O+CO_2) but that males significantly preferred the latter (Fig. 3*A*). When host-plant (tomato vegetation) odors were added upstream of the flowers (experiment 3), males maintained the CO_2 bias observed in experiment 2, but females now chose to probe the flower with above-ambient CO_2 in the same proportion as did males (Fig. 3*B*). Our assays, like those of Thom *et al.* (26), presented moths with a binary choice between single flowers with markedly different local CO_2 concentrations. Our protocols differed (besides the fact that we flew females in addition to males) in that we presented two flowers in the wind tunnel with distinct odor plumes that did not overlap near their sources, whereas Thom *et al.* (26) mounted artificial flowers on *D. wrightii* plants in a small flight cage with no air flow. These observations suggest that *M. sexta* can perceive differences in CO_2 on the scale of a single plant, amidst background contributions from its vegetation (e.g., ref. 36). It remains to be tested whether moths can distinguish between closely spaced individual flowers with different CO_2 concentrations on the same plant.

Stimuli, Context, and Task Specificity in Female *M. sexta*. Male *M. sexta* show identical first-flower preferences for scented artificial flowers with CO_2, regardless of whether host plant odors are present (Fig. 3), suggesting that their responses to floral CO_2 are not influenced by the presence of host-plant volatiles. In contrast, female *M. sexta* showed clear first-choice biases only in the presence of tomato plant volatiles (Fig. 3). Mechaber *et al.* (48) demonstrated that virgin female *M. sexta* show host-plant tracking behavior in response to tomato leaf volatiles, and abdominal curling typical of oviposition when in contact with the plant, 3 days after eclosion. Thus, our unmated females—all of them 3 or more days posteclosion—were expected to show some response to host-plant volatiles. However, in experiment 3, females could not physically contact tomato plants, nor did they show reduced interest in the flowers due to the presence of host-plant odors; their approaches ended in floral probing while hovering rather than landing and abdominal curling. Host-plant volatiles had a dramatic influence on female responses, which strongly suggests that their feeding behavior is influenced by stimuli related to oviposition.

Female *M. sexta* often mix nectar foraging and oviposition bouts on host plants with nectar-rich flowers [e.g., *Datura* (48)] and derive a 10-fold fitness benefit in number of eggs matured when they consume nectar as adults (49). The observation that female *M. sexta* lay more eggs on plants with experimentally enhanced nectar volumes (50) prompted Kessler and Baldwin (51) to suggest that females use nectar as a proxy measure of host-plant quality. In experiment 2, CO_2 had no bearing on the first flower probed by females when host-plant odors were absent, whereas the addition of tomato odors in experiment 3 resulted in a first-choice preference for the above-ambient CO_2 flower. However, only first choices appeared to be affected because moths continued to forage; and in all cases, scented flower models with and without CO_2 were probed. Following Kessler and Baldwin (51) and considering the positive correlation between nectar production and CO_2 emission found by Guerenstein and collaborators (29), we suggest that floral respiratory CO_2, when presented in the context of host-plant odors, is used by female moths as a long-distance indicator of host-plant quality, releasing upwind orientation flight as shown here. Both floral nectar and CO_2 could function as redundant plant-quality indicators, each affecting behavioral responses at different scales. A moth could be attracted from a distance to a plant by floral CO_2; then, floral nectar could act as a local indicator of host-plant quality as proposed by Kessler and Baldwin (51).

Conditionality and Opportunism in the Use of Floral Stimuli. We have documented two dimensions of sensory conditionality in the use of floral CO_2 by *M. sexta* during nectar foraging. The first dimension was scale dependence, such that CO_2 functioned as a redundant stimulus (with floral odor) for distance orientation and floral approach but was superfluous as a local feeding stimulus (Fig. 2*A*). This pattern calls to mind the phenomenon of postpollination color change, in which older flowers turn color but remain turgid and are retained on the plant [reviewed by Weiss (52)]. Such flowers, though unrewarding, enhance pollinator attraction from a distance but are ignored once the insects arrive at the inflorescence because of learned color preferences (53). The second dimension of conditionality was context dependence, such that male first-visit preference for flowers with above-ambient CO_2 was constant irrespective of host-plant odor, whereas female preference for such flowers was observed only when they were presented in the context of oviposition cues (Fig. 3). For these hawkmoths, responses to floral CO_2 clearly depend on the sex of the moth and the conditions under which it perceives this stimulus; non-host-plant floral odors attract both sexes of *M. sexta* in the absence of CO_2 (44). The context-dependent responses of female moths to the combined stimulus of host-plant odor and CO_2 are conflated with nonappetitive behaviors likely to be associated with oviposition, as discussed above.

Given the multitude of scales, contexts and mechanisms by which CO_2 functions in plant–insect interactions, the conditionality of its significance in nectar foraging by *M. sexta* is not surprising. The perception of CO_2 as a floral odor by *M. sexta* adds to the small but growing list of "secondary functions" (e.g., pollinator attraction and herbivore deterrence) attributable to primary plant metabolites among plant–insect interactions (54, 55). Floral CO_2 is a stimulus whose relevance to moth foraging behavior depends on the specific task at hand (e.g., oviposition) and the extent to which other floral information is available. In night-blooming plants such as *D. wrightii*, floral CO_2 might provide the most honest indication of nectar profitability to foraging moths because it decreases markedly within the first hour after anthesis (29), when nectar feeding by *M. sexta* is most intense (56, 57), whereas floral odor and visual display remain unchanged after nectar has been drained from individual flowers (26, 29). Future experiments should test the extent to which moths' foraging behavior in a natural setting reflects local differences in floral CO_2, and whether females are capable of using such information at non-host nectar sources such as *Oenothera caespitosa* (Onagraceae) and *Mirabilis longiflora* (Nyctaginaceae). We have found increased CO_2 levels at the corolla openings of these night-blooming plants [$1,912 \pm 195$ ppm ($n = 8$) and $1,281 \pm 173$ ppm ($n = 9$), respectively], which also are visited by *M. sexta* and related hawkmoths (58, 59). At the same time, our results call for more detailed investigations of the role of CO_2 in mixed nectar foraging–oviposition bouts shown by female *M. sexta* in nature.

Materials and Methods

This study was carried out during July and August 2006 and May 2007 at the University of South Carolina.

Animals. We used *M. sexta* adults reared from eggs provided by Lynn Riddiford (University of Washington, Seattle). Larvae were fed ad libitum on an artificial diet (60) and were kept as pupae under a 16:8 light/dark, 24/21°C cycle. Moths were separated by sex as pupae and housed in different incubators (Precision 818) under the same ambient regime and emerged within $45 \times 45 \times 45$-cm screen cages (BioQuip). Adults were starved for 3–4 days after eclosion to increase their appetitive motivation.

Sensory Stimuli. *Artificial flower.* The visual stimulus consisted of a white conical paper cup (7 cm in diameter and 10 cm in depth; no reward present) attached perpendicularly to a black vertical ring stand at a height of 50 cm against a black background. Spectrophotometer readings of flowers (data not shown) revealed that the paper absorbed UV wavelengths but reflected light nearly uniformly from 400 to 700 nm. Volatile analysis (data not shown) using solid-phase microextraction combined with gas chromatography–mass spectrometry revealed that the artificial flower did not emit volatile compounds. *Olfactory stimulus.* A cotton swab was soaked with 25 μl of bergamot essential oil (Body Shop) and was refreshed every 30 min to standardize odor intensity. This odor source is a reliable feeding stimulant for *M. sexta* (7) and, like many night-blooming flowers visited by this species, is dominated by linalool and related monoterpenoid odors (61). The wooden stem of the swab (2.5 cm) was affixed to a 1-cm^3 piece of dark gray modeling clay, 2 cm inside the flower. When odor was not present, we arranged flowers in the same way but with a dry cotton swab.

Carbon dioxide (CO_2). Carbon dioxide was delivered from a CO_2 tank (National Welders) through Teflon tubing directly into a blue aquarium "air stone" positioned at the bottom of the artificial flower. Treatments with no CO_2 emission were set up in the same way but with the CO_2 tank in the closed position. Ambient and "flower-emitted" CO_2 levels were measured with a LI-COR 840 CO_2/H_2O detector previously calibrated with three different CO_2 concentrations (392, 798, and 1,032 ppm $\pm$ 2%). We obtained the following calibration curve: $R = (A - 8.1199)/0.0432$, where A is the actual CO_2 concentration and R is the one read by the LI-COR detector. This linear regression was statistically significant ($P < 0.05$) with an R^2 of 0.99. CO_2 emission from the artificial flower was set at a flow of 10 ml/min, achieving a CO_2 concentration at the flower model opening of 787 ± 33 ppm (mean $\pm$ SEM), based on concentrations measured by Thom and collaborators (26).

General Procedure in the Wind Tunnel and Recorded Variables. At the beginning of scotophase, naïve, starved adult moths were placed individually at the downwind end of a $3 \times 1.5 \times 1.5$-m wind tunnel with a laminar flow rate of 1m/sec. Each moth was allowed to fly freely inside the wind tunnel for 5 min, during which its behavior was recorded.

Experiment 1 (no-choice assay). To investigate moth responsiveness to different combinations of stimuli, we designed an experiment with four treatments in which a single artificial flower was presented at the upwind end of the wind tunnel. The first treatment encompassed the artificial flower alone (only V). The following two treatments consisted of the addition of either odor (V+O) or CO_2 (V+CO_2). Finally, the fourth treatment consisted of the addition of both odor and CO_2 (V+O+CO_2). In all cases, the surrogate flower was identically attached to a ring-stand, with an air-stone diffuser. In treatments lacking CO_2, the diffuser emitted ambient air at the same flow rate (10 ml/min).

We recorded "responsiveness," the percentage (%) of moths flown in each treatment that (*i*) showed the stereotypic anemotactic "zigzag" odor plume tracking behavior (62), (*ii*) approached the artificial flower (i.e., hovered in front of it), and (*iii*) probed at the artificial flower at least once with their extended proboscides. We also recorded the amount of time (latency, in seconds) during which moths flew inside the tunnel before probing the artificial flower, and the time spent probing (probing time, in seconds).

Experiment 2 (binary-choice assay). The second experiment was a dual-choice assay in which we presented individual moths with two artificial flowers that were 40 cm apart and equidistant to the release site of the moths at the upwind end of the wind tunnel. This distance effectively decouples visual and olfactory stimuli under our test conditions (44), reducing the potential for ambiguous binary choices by moths. The physical characterization of CO_2 plumes is beyond the scope of this article, but we confirmed that TiCl smoke plumes do not overlap for the first meter under our experimental conditions. We recorded the proportion of moths that probed the artificial flower emanating odor (no CO_2 emission) vs. the one emanating odor plus above ambient CO_2. As in experiment 1, we also recorded latency and probing time at each flower model.

Experiment 3 (binary-choice assay with host plant). Given the gender asymmetries found in experiment 2 (see *Results*), the possible role that CO_2 may play in oviposition behavior (36), and the observation that female *M. sexta* may show mixed bouts of nectar feeding and oviposition (50), we decided to investigate responses to floral CO_2 in the presence of stimuli related to oviposition (host-plant volatiles). Thus, in this experiment, we replicated the design of experiment 2 but now positioned four tomato plants (*Solanum lycopersicum* cv. "Better boy"; South Carolina Farmers Market, Columbia) 1 m upstream from the flowers. This provided the odor of undamaged host-plant vegetation in addition to bergamot oil and CO_2. Tomato plants were physically and visually separated from the flight arena of the wind tunnel by means of a fine, dark aluminum screen that spanned its entire transverse section.

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