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SHORT COMMUNICATION

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Floral CO_2 emission may indicate food abundance to nectar-feeding moths

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Abstract As part of a study of the roles of the sensory subsystem devoted to CO_2 in the nectar-feeding moth *Manduca sexta*, we investigated CO_2 release and nectar secretion by flowers of *Datura wrightii,* a preferred hostplant of *Manduca. Datura* flowers open at dusk and wilt by the following noon. During the first hours after dusk, when *Manduca* feeds, the flowers produce considerable amounts of nectar and emit levels of CO_2 that should be detectable by moths nearby. By midnight, however, both nectar secretion and CO_2 release decrease significantly. Because nectar production requires high metabolic activity, high floral CO_2 emission may indicate food abundance to the moths. We suggest that hovering moths could use the florally emitted CO_2 to help them assess the nectar content before attempting to feed in order to improve their foraging efficiency.

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Introduction

The ability to sense subtle variations in ambient CO_2 concentration is well established among moths. CO_2 receptor cells are enclosed within sensilla located in a sensory organ, the labial-palp pit organ (LPO; e.g., Kent et al. 1986), in the distalmost segment of each labial palp. Morphological studies have shown that this organ contains up to 2,000 receptor cells projecting into the deutocerebrum (Kent et al. 1986), and physiological experiments have revealed that those sensory cells respond specifically to CO_2 (Bogner 1990; Guerenstein et al. 2002) with high sensitivity (Stange 1992). For most species of moths, however, the roles of sensory information about ambient CO_2 are unclear.

The existence of CO_2 gradients in their natural habitats has led to several hypotheses about the significance of CO_2 information for moths. The strongest evidence for the use of CO_2 information by moths came from a study of the use of local CO_2 gradients by adult *Cactoblastis cactorum* (Lepidoptera: Pyralidae; Stange et al. 1995; Stange 1997). The LPO of *C. cactorum* is larger in females than in males, and it was suggested that probing the surface of hostplants with the labial palps might inform female moths about metabolically more active parts of the plants in order to identify high-quality oviposition sites.

In the hawkmoth *Manduca sexta* (Lepidoptera: Sphingidae; hereinafter *Manduca*), the LPO is large and apparently not sexually dimorphic (Kent et al. 1986), suggesting that in this species, CO_2 information could be similarly important for both males and females. We speculated, therefore, that information about ambient CO_2 could be valuable for functions other than, or in addition to, oviposition. *Manduca* species, which feed as adults, possess a more elaborate LPO than that of moths that do not feed as adults (Kent et al. 1986). We hypothesize, therefore, that *Manduca* might use its CO_2-sensing system to detect the high metabolic activity of flowers and thus to locate profitable nectar sources.

Datura wrightii (Solanaceae) is a known, preferred hostplant of *Manduca*. Female adult moths use its leaves

as a substrate for oviposition, larvae feed on its fruits and leaves, and adult moths feed on the nectar of its flowers. In order to explore a role of the CO_2-sensing system in the feeding behavior of *Manduca*, we investigated the emission of CO_2 by the flowers of *Datura* and its possible association with their nectar secretion.

Materials and methods

Plants

These studies were conducted in Tucson, Ariz., USA, where both the moth *Manduca* and its hostplant *Datura wrightii* are native. The four plants used in most of this work belonged to a large (ca. 2 m^2), well-irrigated patch. In summer, when *Manduca* moths interact with their hostplants, *Datura* produces many large (ca. 17 cm long), white, trumpet-shaped flowers. These strongly scented flowers open at dusk and wilt by the following noon. Five narrow canals (spurs) running down the corolla tube to its base lead to the nectary of each flower (see S1, Electronic Supplementary Material, for a photograph of a dissected *Datura* flower). At night the nectar is secreted along the length of the spurs.

CO_2 emissions

Data were collected during August and September 2002. Emission of CO_2 by *Datura* flowers was measured from about half an hour before flower opening, which occurred at ca. 1900 hours, to ca. midnight (2400 hours) using an infrared gas analyzer (LI-7000, LI-COR, Lincoln, Neb., USA). Thirteen flowers were sampled repeatedly every 30 min. Two different sampling procedures were used, one procedure per flower. *Syringe method*: Plastic syringes were used to collect 5-ml air samples at the corolla opening ('flower') and from 7 cm lateral to the corolla opening ('plant patch control'). Samples were then injected into a septum port on the LI-7000 configured in a closed-loop. Airflow was maintained at 0.5 l min^{-1}, and calibration was achieved by injecting known concentrations of CO_2 (LI-COR 1998). *Closed-loop method*: Rates of release of CO_2 were measured by enclosing intact flowers in 1-l plastic chambers. Chambers were configured with an air input and an output port. A small electric fan mixed the air within the chambers. The temperature at the surface of the enclosed flowers was constantly recorded with a fine-wire thermocouple and stored in a data-logger (CR23X, Campbell Sci., Logan, Utah, USA). Chambers were fitted and sealed to the flower stems using modeling clay. Two flowers were monitored each night for 4 nights (eight flowers in total). Chambers remained on flowers until midnight. Between measurements ambient air was pumped continuously at 0.9 l min^{-1} through the chamber. For measurement of CO_2 release rates solenoid valves diverted the airflow to a closed loop that included the chamber and the LI-7000. The transient increase in the level of CO_2 in the loop (μl/s) was recorded using a portable computer. A single measurement lasted 2 min, and the CO_2 concentration in the loop did not exceed 600 μl/l. Plots of CO_2 concentration versus time illustrated a positive linear increase of CO_2 within the loop during the 2-min periods (r^2>0.96 in 98% of the measurements, n=95 on eight flowers). The slopes of those curves represented the floral CO_2 release rates. At dusk, when flowers opened, CO_2 release rates were very high because the CO_2-rich air in the closed flowers (see Results) was suddenly released. Thus, an abrupt increase in the slope of the plot of CO_2 concentration versus time was observed during the 2-min measuring periods that included flower opening. In these cases the curve was divided into two parts, and two CO_2-release rates were obtained over these single 2-min periods. At the end of the measurements, the flowers were cut, dried at 80°C, and weighed.

Nectar secretion

Nectar was collected between June and September 2002 using 1-ml plastic syringes with an 11-cm-long plastic-fused silica needle (MicroFil 28AWG, World Precision, Sarasota, Fla., USA). Nectar measurements were performed on flowers different from those from which CO_2 emissions were assessed although those flowers were from the same plants. Nectar secretion was assessed in two ways. *Nectar milking* involved repeated sampling of ten intact flowers. At flower opening all nectar was removed from the nectary through the five spurs, and flowers were immediately bagged with netting (1 mm mesh) until final nectar harvesting. The *nectar accumulated* method involved a one-time sampling of 51 bagged flowers from four plants, at different times after opening. Because of variability in the volume of nectar per flower between plants, data were normalized so that the maximum volume found in flowers of a certain plant was considered 100% for that plant. Care was taken for each method to avoid damage to the floral tissue of the spur and the nectary. The sugar concentration of the nectar of 10 flowers was assessed at different times after opening using a pocket refractometer (Bellingham and Stanley, Tunbridge Wells, UK).

Data on CO_2 emission and nectar secretion were analyzed using the Friedman ANOVA, a repeated-measures nonparametric ANOVA test (Zar 1999). Other comparisons were performed using the *t*-test or the nonparametric Mann-Whitney U test depending on the data dispersion.

Results

The syringe method of CO_2 sampling showed that at the beginning of the night, shortly after flower opening, the air at the entrance of the *Datura* flowers had a higher CO_2 concentration than that beside the flowers within the plant patch (Fig. 1a). The floral CO_2 levels decreased during the night (Friedman ANOVA test, χ_r^2=18.99, df=10, P=0.040, n=5), and thus, after ca. 4 h from flower opening, the air at the flower entrance had a CO_2 concentration similar to that of the surrounding air in the patch. Results from the closed-loop method confirmed that CO_2 emission from *Datura* flowers decreased significantly a few hours after opening (Friedman ANOVA test comparing CO_2 emission at approximate times 15, 150 and 300 min after flower opening, χ_r^2=16.00, df=2, P<0.001, n=8; Fig. 1b). CO_2 release peaked at the moment of floral opening (which is achieved within a few seconds). This is because the CO_2 concentration in the closed flowers is high (mean±SEM=2,037±101 μl/l, n=30, unpublished data), and opening of flowers results in the rapid emission of CO_2. The CO_2 release rate during the night was positively correlated with the temperature of the flowers (linear regression flower 1: F=201.68, df_{error}=9, P<0.001; linear regression flower 2: F=138.11, df_{error}=11, P<0.001; Fig. 1b, inset). Flowers did not produce detectable amounts of heat, as the flower temperature values did not exceed the ambient temperature (data not shown).

Nectar secretion by repeatedly sampled *Datura* flowers also decreased significantly a few hours after opening (Friedman ANOVA test, χ_r^2=6.00, df=2, P=0.0498, n=10; Fig. 1c). The secretion of nectar ca. 200 min after flower opening was significantly lower than that during the first hour after opening (multiple comparisons test for Fried-

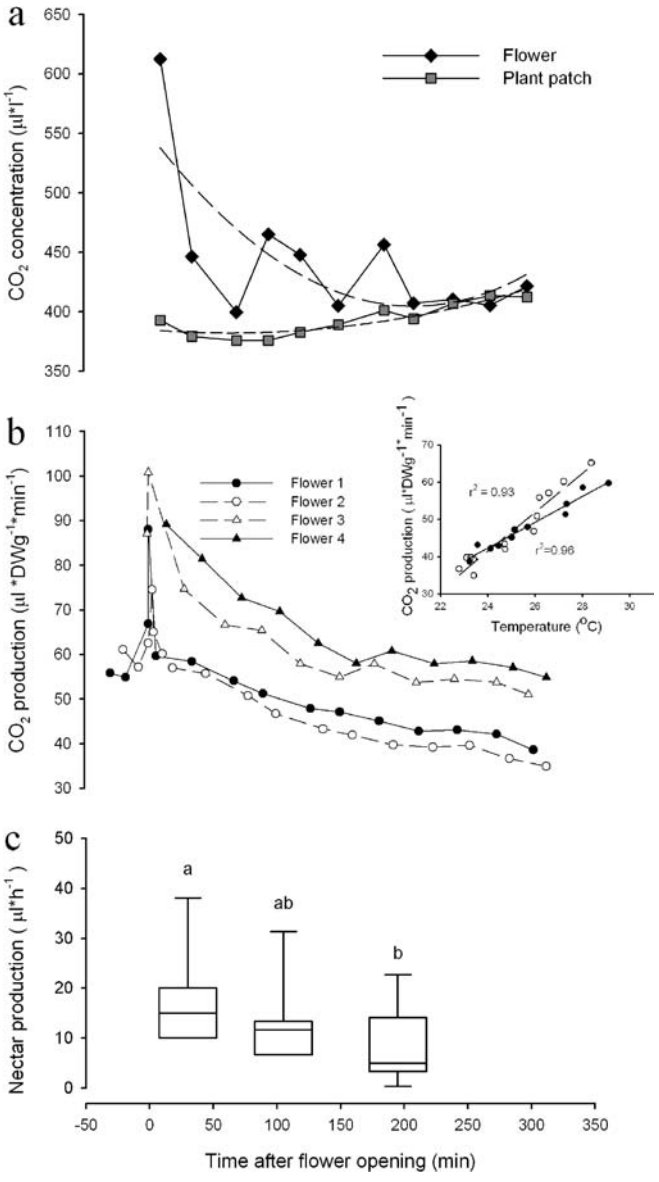

Fig. 1 Nocturnal dynamics of CO_2 release (**a**, **b**) and nectar secretion (**c**) by *Datura wrightii* flowers. Both CO_2 release and nectar secretion decreased during the night. **a** Syringe method: The floral CO_2 levels decreased significantly during the night (P=0.040) while control values increased slightly (P=0.056). Values shown are medians of five samples. Data dispersion is not shown for the sake of clarity. Trend lines (*dashed lines* representing polynomial functions of order 2) are shown only for descriptive purposes. **b** Closed-loop method. *Ordinate* Microliters of CO_2 produced per gram of flower dry weight (DW) per minute. Data from only four of the eight flowers investigated are presented for the sake of clarity (all flowers decreased their CO_2 production during the night). Values shown are single data points and represent flower CO_2 release rates throughout 2-min sampling periods. *Inset* CO_2 release rates from flower 1 and 2 positively correlated to the temperature values recorded on the respective flowers. **c** The *lines* within the boxes mark the medians for ten flowers sampled repeatedly (milked). The *lower and upper boundaries of the boxes* indicate the 25th and 75th percentiles and the *bars* below and above the boxes indicate the 10th and 90th percentiles, respectively

man ANOVA, q=3.32, df=∞, P<0.05, n=10). A similar result was obtained when the relative volume of nectar accumulated was measured in singly sampled flowers at different times ranging from –60 to +270 min from flower opening [means±SEM: –60 min: 25.5±3.9% (n=17), –30 min: 29.4±9.1% (n=7), 0 min: 32.2±6.1% (n=11), 180 min: 73.6±8.2% (n=9), 270 min: 76.3±9.2% (n=7)]. A considerable amount of nectar (median =80 µl, 25th and 75th percentiles =60 and 120 µl, respectively, n=21 flowers) was already present in all flowers inspected at the time of opening, as previously found in many hawkmoth-pollinated flowers (Cruden et al. 1983; Martínez del Río and Búrquez 1986). Milked and singly sampled flowers contained volumes of nectar that were similar in the two experimental groups when compared at opening time (median for flowers milked =90 µl, 25th and 75th percentiles =72.5 and 120 µl, respectively, n=10; median for nectar accumulated procedure =60 µl, 25th and 75th percentiles =40 and 170 µl, respectively, n=11; Mann-Whitney U test, U=42.50, P=0.379) and also at 4–4.5 h after opening (median of accumulated values at time 4 h for flowers milked =132.5 µl, 25th and 75th percentiles =116.25 and 170 µl, respectively, n=10; median for nectar accumulated procedure at time 4.5 h =130.0 µl, 25th and 75th percentiles =110 and 215 µl, respectively, n=7; Mann-Whitney U test, U=35.00, P=1). The sugar concentration of the nectar from 10 *Datura* flowers at opening time (mean±SEM=23.1±1.2%) was not different from that 120 min later (mean±SEM =23.1±0.5%; t-test, t=0.0, df=9, P=1). In addition, preliminary data suggest that this concentration may be stable at least until midnight, as already reported for another hawkmoth hostplant (Martínez del Río and Búrquez 1986).

Discussion

Floral odors contribute to the food-seeking and feeding behaviors of moths, and some of the volatile compounds involved in those behaviors have been identified (Dobson 1994; Raguso et al. 1996). CO_2 is suspected of helping to evoke behaviors that lead to nectar feeding in bees and moths (Lacher 1964; Raguso and Willis 2002). To our knowledge, however, no investigation of a possible role of CO_2 in the interaction between insects and flowers has been reported.

The elevated emission of CO_2 from *Datura* flowers could be detected by the CO_2-sensing organ, the LPO, of adult moths such as *Manduca*. During ca. 3 h after sunset, the CO_2 concentration at the opening of the flowers remained more than 15 µl/l above the background level in the plant patch (Fig. 1a). The response threshold of the CO_2-sensing system of moths may be as low as 0.5 µl/l at normal ambient CO_2 levels (Stange 1992). Thus, a moth passing near the opening of a flower would detect a change in CO_2 concentration. By midnight, however, floral CO_2 release decreases to levels comparable to those resulting from the respiration of the surrounding leaves and soil.

It has been suggested that CO_2 production in orchid flowers is associated with the production of fragrance (Hew et al. 1978). Preliminary data suggest that the nocturnal decrease in flower CO_2 emission reported here may not be accompanied by a decrease in scent production by *Datura* during the first half of the night. Benzyl alcohol, β-ocimene, and geraniol are among volatile compounds in *Datura*-flower headspace collections that evoke the strongest responses in *Manduca* antennae (A. Fraser, personal communication; see Fraser et al. 2003 for methods). The floral emission of these compounds did not appear to change consistently from opening time until midnight (n=4, unpublished results).

We found that, like CO_2 release, nectar secretion by *Datura* flowers decreases during the night as previously reported for other hawkmoth-pollinated plants (e.g., Martínez del Río and Búrquez 1986). As the nectar volumes obtained with the nectar-milking and nectar-accumulated methods around midnight are similar, nectar removal during the milking procedure apparently neither stimulated replenishment (Castellanos et al. 2002) nor inhibited secretion (Galetto and Bernardello 1993). Reabsorbtion of nectar (Búrquez and Corbet 1991) after the time of moths' feeding activity was not observed; singly sampled flowers contained high volumes of nectar even at midnight. Feeding activity of *Manduca* has been observed soon after sunset, particularly during the first 2 h after dusk (Madden and Chamberlin 1945; Casas et al. 1999). Therefore, *Manduca* appears to seek food at a time when the nectar secretion and CO_2 emission from *Datura* flowers are relatively high, and CO_2 could have informational value. There is already evidence from other hawkmoth-plant interactions that the feeding activity of moths peaks during the period of nectar secretion, that is, from about dusk to midnight (Cruden et al. 1983; Martínez del Río and Búrquez 1986; Willmott and Búrquez 1996).

The simultaneous decrease in nectar secretion and CO_2 emission suggests that at least some of the CO_2 released by *Datura* flowers derives from the metabolic activity required to produce nectar. The nocturnal dynamics of ambient temperature may affect nectar secretion and CO_2 release (see Fig. 1b, inset) and the feeding behavior of the moths, which do not feed on cold nights or during the colder periods of the night (e.g., Martínez del Río and Búrquez 1986). Fermentation by yeasts in the nectar (e.g., Herzberg et al. 2002) could also add to the basal floral CO_2 release (Drawert et al. 1987), and this supplementary production of CO_2 may also diminish with the drop in temperature during the night.

Datura flowers start to produce nectar before opening. Healthy, newly opened flowers emit relatively high levels of CO_2, which may signal a high probability that those flowers contain abundant nectar. On the other hand, unhealthy flowers may emit relatively low levels of CO_2 and contain little nectar. This could also be the case for recently visited flowers. *Manduca* usually hovers in front of the flowers while feeding (see S2, Electronic Supplementary Material, for a photograph of a *Manduca* moth hovering), and this activity probably causes air turbulence that could reduce the gradients of CO_2 and organic volatiles around a flower. This turbulence also may allow hovering moths to sense the CO_2 concentration of the air inside the flower. Thus, depletion of nectar and CO_2 from flowers may be coupled, and in case nectar secretion continues after a moth's visit, refilling of the nectary and spurs may be accompanied by reestablishment of a CO_2 gradient in front of the flower. We suggest that the moths could use the CO_2 signals from flowers to help them assess their nectar content before inspection of the spurs and nectary. Therefore, the moths would invest effort (e.g., in hovering activity) in probing only flowers that are promising.

Manduca moths can pollinate hostplant flowers without landing on them, and it was found that one hawkmoth visit could be sufficient for fertilization and fruit production (Willmott and Búrquez 1996). The high CO_2-emission rates of flowers secreting nectar may benefit the plant by attracting pollinators to healthy, non-pollinated flowers, and ultimately it may enhance plant reproduction. Therefore, floral CO_2 production may have had a role in the selection favoring plants producing copious nectar.

Behavioral studies in progress are testing the possible role of CO_2 as a foraging cue for adult *Manduca* (Thom et al. 2003). In addition, we are studying the processing of sensory information about CO_2 in the moth's brain (e.g., Guerenstein et al. 2002).

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