

BEHAVIORAL RESPONSES TO MULTI-MODAL STIMULATION: THE  
ORGANIZATION OF THE FORAGING INSTINCT OF *MANDUCA SEXTA*

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BEHAVIORAL RESPONSES TO MULTI-MODAL STIMULATION: THE ORGANIZATION OF THE FORAGING INSTINCT OF *MANDUCA SEXTA*

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The main objective of this doctoral thesis is to understand the adult foraging behavior of the nocturnal hawkmoth *Manduca sexta*. Adult *M. sexta* feed from the floral nectar of certain plants, which benefit from the pollination services resulting from these feeding visits. This mutualism has resulted in co-adaptive processes which link flower phenotypes with animal foraging behavior. The distinctive attributes of flowers, such as colors and odors, can exert an attraction on certain animals, particularly the ones that mediate their pollination. Therefore, in order to understand the foraging behavior of *Manduca* I focus on how responses to typical floral features, such as visual display and fragrance, allow this insect to get a nectar meal. My approach is purely experimental; I use different laboratory set-ups while manipulating artificial flowers to study responses to olfactory, visual, and tactile stimulation with the hope of understanding the appetitive behaviors, innate releasing mechanisms and motor patterns that together constitute the feeding instinct of *Manduca*. Throughout this thesis I explore 1) the role of tactile stimulation during flower “handling”, and describe a type of non-associative learning which allows moths to improve their probing motor skills; 2) foraging at the stages preceding flower handling (approach and probing) by spatially and temporally decoupling olfactory and visual stimuli, which led to new insights on how successive motor patterns are released by different stimuli configurations as moths search for flowers; 3) the scale-dependent, contextual

use of respiratory CO<sub>2</sub> emanated from *Datura wrightii* flowers, and how dimorphic behavior suggests that nectar foraging and oviposition instincts are seamlessly integrated; 4) the visual associative learning capabilities of *Manduca*, and 5) how deficient larval nutrition, affecting the visual system, has an impact on foraging behavior. Results show that the effect of relevant stimuli cannot be disentangled from the context and scale in which they act. In fact, “context” appears to be *the* releasing factor of particular motor patterns, suggesting that the consideration of stimuli without context is misguided. Meanwhile, scale becomes significant only under the framework of a sequential, hierarchical organization of the behavioral mechanisms constituting the feeding instinct of *Manduca sexta*.

## BIOGRAPHICAL SKETCH

Joaquin Goyret was born son of Julio Goyret and Enriqueta Bonavita, and brother of Maria Eugenia in Montevideo, Uruguay. With his parents and Lucia, his second sister, he moved to Buenos Aires, Argentina, where he grew up and his two brothers, Ignacio and Gonzalo, were born. Even though he lived most of his life in Argentina and loves this country, he still finds himself Uruguayan. This fact is probably due to the influence of his grandmother Alba “Abu” Bonavita-Barbachán, and is manifested in his inclinations whenever Argentina and Uruguay are engaged in a soccer game.

Having been constantly encouraged to further develop his interest in the natural world by his school teacher “Gachi”, he thought of studying Chemistry after high school. Nevertheless, he had the luck of having XXXX as a neighbor, who, he himself a chemist, suggested his inquiring tendencies would be better fulfilled through the study of Biology, a career which in Argentina is oriented towards basic science, as opposed to the industry-oriented chemistry.

He obtained his *Licenciatura en Ciencias Biológicas* in the *Facultad de Ciencias Exactas y Naturales* of the *Universidad de Buenos Aires*, where he studied communication between worker honeybees during foraging. There he met Josué Núñez and Rodrigo De Marco, two friends who had an important influence on his scientific philosophy and encouraged him to go into Graduate School.

In 2004 he joined the lab of Robert Raguso in The University of South Carolina, where he studied aspects of the behavior of the hawkmoth *Manduca sexta* and met his partner, Samantha Smith. With her, he moved to Brooktondale, NY to continue his doctoral studies with Rob Raguso in the Department of Neurobiology and Behavior at Cornell University. During this period he decided to continue his postdoctoral studies with Almut Kelber in Lund University, Sweden.

To Iñaki, Juan, Imanol and the little ones to come

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Nothing would have been the way it was in South Carolina without having met people like Sue Carstensen, Linda Smith and Dick Vogt. There I was lucky enough to become a friend of Jim Byrum; his love for life has been and always will be an inspiration. I'll always remember the good times we shared in and out of Goatfeathers. I also had a very good time with some grad students; here I remember Kenny and Dan and the Friday parties at Mark & Laura's place. In and out of the lab, my first postdoc lab-mates Boris Schlumpberger and Glenn Svensson, were first class friends and colleagues. I am sure we caught the attention of some southern "smiling faces" with our heated discussions from science to politics. Kate was an excellent lab-mate and the most devoted TA I know, and I am very happy she is now a Professor to keep on doing her excellent job. Shanae, Shanice, Akila, Mike, Larissa, Annie and Poppy made everything livelier in the lab and I had very enriching experiences working with them. From Brooktondale, I want to thank Greg, Gina, and their beautiful girls Artemis and Esmeralda (good neighbors are everywhere, but seldom just across the street!). Here in Cornell, I'd like to thank Lynn Fletcher, Dan Fergus, Biz Turnell and Chris Wiley for being such good office partners, always ready for one of my "English questions". Rainee Kaczorowski, Derek Artz and Cristian Villagra were all good colleagues and friends. I am very thankful to Tom Seeley, Ton Eisner and Charlie

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**CHAPTER 1**  
**THE ROLE OF MECHANOSENSORY INPUT IN FLOWER HANDLING**  
**EFFICIENCY AND LEARNING BY *MANDUCA SEXTA*\***

***Abstract***

Nectar-foraging animals are known to utilize nectar guides – patterns of visual contrast in flowers – to find hidden nectar. However, few studies have explored the potential for mechanosensory cues to function as nectar guides, particularly for nocturnal pollinators such as the tobacco hornworm moth, *Manduca sexta*. We used arrays of artificial flowers to investigate the flower handling behavior (the ability to locate and drink from floral nectaries) of naïve moths, looking specifically at: (1) how the shape and size of flat (two-dimensional) artificial corollas affect nectar discovery and (2) whether three dimensional features of the corolla can be used to facilitate nectar discovery. In these experiments, we decoupled visual from tactile flower features to explore the role of mechanosensory input, putatively attained via the extended proboscides of hovering moths. In addition, we examined changes in nectar discovery times within single foraging bouts to test whether moths can learn to handle different kinds of artificial flowers. We found that corolla surface area negatively affects flower handling efficiency, and that reliable mechanosensory input is crucial for the moths' performance. We also found that three dimensional features of the corolla, such as grooves, can significantly affect the foraging behavior, both positively (when grooves converge to the nectary) and negatively (when grooves are unnaturally oriented). Lastly, we observed that moths can decrease nectar discovery time during a single foraging bout. This apparent learning ability seems to be possible only when reliable mechanosensory input is available.

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## ***Introduction***

One of the ‘mysteries of nature’ revealed by Sprengel’s landmark (Sprengel, 1793) publication was the concept of nectar guides – visually contrasting markings or aspects of flower morphology – that indicate the location of nectar to animal pollinators. The ubiquity of such markings, particularly those perceived in ultraviolet (UV) wavelengths, is one of the primary arguments made for the importance of contrasting flower colors to the visual perception and foraging behavior of insect pollinators (Menzel and Schmida, 1993; Chittka et al., 1994; Lunau et al., 1996). For example, honeybees show an innate proboscis extension reflex (PER) to UV marks at the center of *Helianthus rigidus* sunflowers, and probe at the periphery of the flower when the orientation of the ray florets is reversed (Daumer, 1958). However, it is unlikely that vision is the only sensory modality used by animals to find the nectar within flowers. Kevan and Lane (Kevan and Lane, 1985) showed that honeybees can detect differences in petal surface cell texture, and can learn such differences in conjunction with nectar rewards. Thus, tactile floral cues also could function as nectar guides (Leppik, 1956; Glover and Martin, 1998), especially for animals with poor vision, or those that forage under low light conditions, such as crepuscular or nocturnal hawkmoths (Lepidoptera: Sphingidae). Hawkmoths are abundant in tropical and warm-temperate habitats worldwide, where they constitute an important class of pollinators (Grant, 1983; Nilsson et al., 1987; Haber and Frankie, 1989). Olfactory and visual floral stimuli are known to attract several species within an appetitive context (Knoll, 1926; Kugler, 1971; Haber, 1984; Kelber, 1997). The European *Deilephila elpenor* and *Macroglossum stellatarum* utilize true color vision even under starlit conditions (Kelber and Hénique, 1999; Kelber et al., 2002), and modify their innate odor and color preferences through associative learning (Kelber, 1996; Balkenius and Kelber, 2004). *Manduca sexta*, a large nocturnal hawkmoth native to the Americas,

also can learn particular odors associated with nectar rewards (Daly and Smith, 2000; Daly et al., 2001a). The blue photoreceptors have been identified as the major visual mediators of feeding behavior in *M. sexta* (Cutler et al., 1995), whereas ultraviolet wavelengths were found to inhibit its feeding response (White et al., 1994). Floral odors attract *M. sexta* from a distance (3-m) in wind tunnel assays (Raguso and Willis, 2003; Raguso et al., 2005), and synergize with visual cues to activate feeding behavior (i.e. proboscis extension while hovering) in both naïve and wild moths (Raguso and Willis, 2002; Raguso and Willis, 2005). However, successful approach to floral nectar sources and release of feeding behavior must be followed by reliable nectar assessment of individual flowers. Locating the nectary within a flower (evaluating the energy resource) is as critical as searching efficiently in order to find that flower. The hovering flight of *M. sexta* is an energetically expensive activity (Heinrich, 1971; Ziegler and Schulz, 1986), thus, the efficiency with which these moths handle flowers should be subject to selective pressures. *Manduca sexta* has a broad geographical distribution with several generations per year and it visits a wide variety of flower types across its range (Fleming, 1970; Raguso et al., 2003; Nattero et al., 2003). These observations led us to ask whether *M. sexta* can handle some flower morphologies more easily than others, and whether they can *learn* to handle flowers more efficiently with time. Such abilities would be consistent with their generalist foraging behavior and would allow these moths to efficiently assess flower profitability, as do other generalist flower visiting insects, such as bumblebees (Lavery and Plowright, 1988; Chittka and Thomson, 1997) and *Pieris* butterflies (Lewis, 1986). The question remains as to which sensory modalities adult *M. sexta* might utilize for such a task. The diurnal hawkmoth *Macroglossum stellatarum* utilizes contrasting marks on the surface of flower corollas by preferentially placing its proboscis on such visual nectar guides (Knoll, 1922). Thus, *M. stellatarum* uses visual cues not only while searching

(in flight) for nectar sources (Kelber, 1997), but also while hovering a relatively short distance (proboscis length: 2.5-cm) in front of individual flowers. Owing to its long (8–10-cm) tongue, *M. sexta* also hovers at a distance from flowers while feeding, such that in most cases, its only physical contact with flowers is through the proboscis. Here we ask whether mechanosensory input to the proboscis is redundant or complementary to the visual stimuli used by *M. sexta* when freely foraging on artificial flowers. In the first experiment, we decoupled visual from tactile stimuli by placing flat square transparency film sheets over the corolla portion of plain-white artificial flowers to test whether these moths use mechanosensory stimuli to find nectar within individual flowers. If visual stimuli are sufficient, hawkmoths should show comparable handling efficiencies on the same flower models, whether or not they are covered with transparency film. We repeated this comparison among five different artificial flower morphologies, systematically varying corolla shape and surface area. In the second experiment, we tested whether groove-like folds, usually found in the corollas of flowers visited by hawkmoths, affect flower handling by *M. sexta*. We also evaluated flower handling performance in relation to different artificial flower morphologies by comparing total, successful and unsuccessful visits of individual moths foraging on arrays of 12 flowers. Finally, we examined whether moths can learn to handle different flower morphs more efficiently within a single foraging bout by examining the time they took to find nectaries as foraging bouts progressed.

## ***Materials and methods***

### *Animal care*

This study was carried out from September to December 2004 at the University of South Carolina, Columbia, SC, USA. We used 3- to 5-day-old *M. sexta* L. adults

reared from eggs provided by Dr Lynn Riddiford, University of Washington, Seattle, WA, USA. Larvae were fed *ad libitum* on an artificial diet (Bell and Joachim, 1976) and were kept, as pupae, under a 16-h:8-h light:dark cycle (24:21°C), in a humidified atmosphere. Male and female pupae were kept in separate incubators (Precision 818, Winchester, VA, USA) under the same ambient regime and emerged within 45x45x45-cm screen cages (BioQuip, Inc., Rancho Dominguez, CA, USA). Adults were starved for 3–4-days before being used in experiments.

#### *Experimental arena and flight assays*

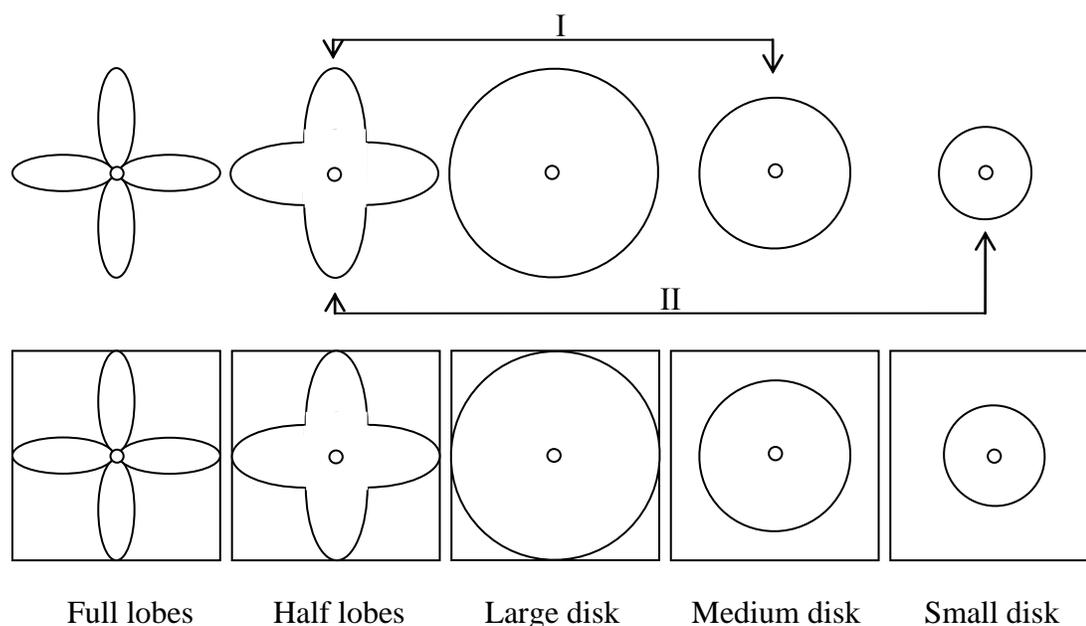
At the beginning of scotophase (15:00-h, temperature range: 22–25°C), naïve moths were placed individually within a closed Tedlar mesh flight enclosure (Bioquip; 2x2x2-m). The flight cage included an experimental floral array (20x30x45-cm) placed over a dark, odor-permeable box constructed by covering a matte-black-painted aluminum grid with black cheesecloth. To provide appropriate olfactory cues and humidity, we placed the cheesecloth-covered grid over two 200-ml glass beakers filled with water, each of which contained a cotton-tipped applicator swab impregnated with two drops of undiluted bergamot oil (Body Shop, Columbia, SC, USA). Thus, odor and water vapor passed through the cheese cloth and permeated the flight chamber. Bergamot oil is chemically similar to the odors of many hawkmoth-pollinated flowers (Kaiser, 1993; Knudsen and Tollsten, 1993; Mondello et al., 1998), and pilot experiments revealed it to be a potent releaser of feeding behavior in *M. sexta*. Visual floral stimuli were provided by a 3x4 array of artificial flowers (see below), in which each flower was separated from its neighbor by 10-cm. Artificial flowers were bathed in odor and water vapor that diffused freely through the cheesecloth. The flight enclosure was lit with a dim red light [wavelengths >600-nm (see Raguso and Willis, 2002)]. Each trial involved only one moth, which was allowed to fly freely. If the

moth did not find, approach or probe the flowers within 5-min, it was captured and discarded. If it found the flowers, it was allowed to forage for a maximum of 10-min after the first floral approach. Foraging bouts were recorded with a video camera (Sony Digital 8 –TRV120 Best Buy, Columbia, SC, USA) in ‘night-shot’ mode placed outside the flight enclosure.

### *Experiment 1*

In each trial, individual moths were offered different homogeneous arrays (12 flowers of the same morph) displayed as described above. We used light-grey paper with low UV reflectance (Kinkos ‘Grey fleck’; wavelength reflectance 80% of a barium sulfate ‘white’ standard above 420-nm, <50% below 400-nm) to construct five different flower morphs (Fig. 1.1A), as follows. Full lobes: four elliptical lobes or petals with a semi-major axis of 2.2-cm and a semi-minor axis of 0.8-cm each. Total area: 21.4-cm<sup>2</sup>. Flower span: 9-cm. Half lobes: four elliptical lobes with a semi-major axis of 3.2-cm and a semi-minor axis of 1.3-cm each. Every lobe overlaps with the adjacent ones leaving a squared center with sides of 2.7-cm. Half of each ellipse appears as a petal. Total area, 33.7-cm<sup>2</sup>; flower span, 9-cm. Large disk: a disk with a diameter of 9-cm and an area of 63.6-cm<sup>2</sup>. Medium disk: a disk with a diameter of 6.5-cm and an area of 33.2-cm<sup>2</sup>. Small disk: a disk with a diameter of 4-cm and an area of 12.57-cm<sup>2</sup>. Additionally, the corolla portion of each of the five flower morphs was covered with a square transparency film sheet (henceforth called transparency treatments; Fig. 1.1B), accounting for a total of 10 treatments. In this way, we could evaluate the foraging behavior under circumstances where no reliable tactile stimuli were available to the animals, but visual stimuli could be preserved. To control for any surface texture effect, flowers in the treatments lacking a transparency square above them (henceforth called no transparency treatments) were covered with transparency film that had been

cut to match the exact shape of the underlying paper flower. Each flower from every treatment offered 20- $\mu$ l of a 20% (w/w) sucrose solution in a nectary (5-cm long by 0.5-cm opening diameter pipette tip) placed at its center; nectaries were accessible to moths through a 0.5-cm opening cut into the transparency film in both treatments. Each nectary was attached to the flower such that it did not protrude above the flower surface.



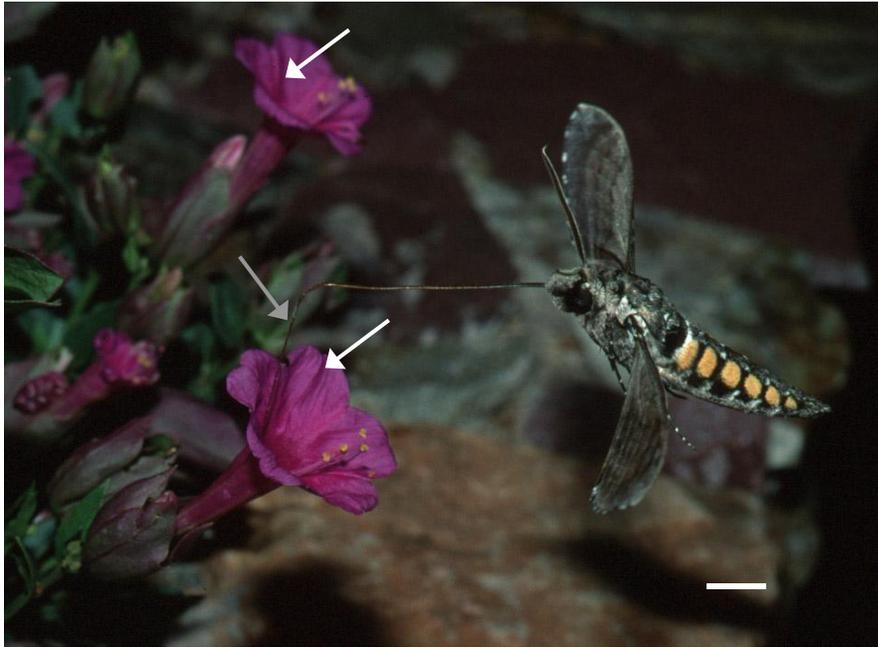
**Figure 1.1. Artificial flowers -Experiment #1.** Five different two-dimensional flower morphs tested in experiment 1. A) “No transparency” paper flowers, whose surfaces are covered with acetate film cut to their exact shape, to control for fine texture. B) “Transparency” flowers covered with a square (9x9 cm) sheet of acetate film. Arrows and brackets indicate *a priori* comparisons: I) Half Lobes vs. Medium Disks – compares flowers that have similar surface area but the different edge-to-center distances. II) Half Lobes vs. Small Disks –compares flowers with different surface area but same edge-to-center distances. Note: all flowers have accessible nectaries at their centers.

### *Variables recorded*

We recorded the foraging efficiency (number of successfully exploited flowers over 10-min) after each trial. The number and duration of total, successful and unsuccessful flower visits were recorded from video-tape playback and timed with a Mistral chronometer (Buenos Aires, Argentina) to a resolution of 1-s. Each flower visit began at the moment the proboscis made contact with the flower. Unsuccessful visits ended when the proboscis lost contact with the artificial flower without having reached the nectary. Successful visits were recorded until the proboscis was inserted into the nectary; when drinking time was recorded as the time elapsed until the proboscis was removed. The ratio of successful to total visits (successful visits/total visits; where total visits = unsuccessful visits + successful visits) was established as an indicator of the animals' efficiency when foraging on the different flower morphs. Given that we had recorded the time moths took visiting each flower, we tested whether moths could learn to handle the different flower morphs during a single foraging bout. Discovery time was defined by the time elapsed between the initiation of flower probing and the entry of the proboscis into the nectary. This does not include the time flying from one flower to another or drinking, but only the time spent probing at the flower's threshold. We measured discovery time for the first eight successful visits, as did Lewis (Lewis, 1986).

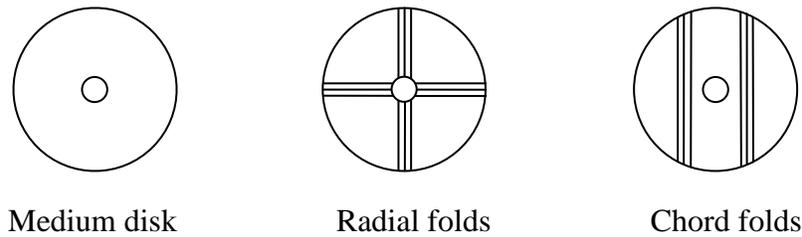
### *Experiment 2*

A second experiment was carried out to evaluate whether *M. sexta* can use morphological features of flowers involving a third dimension (i.e. depth) to improve its foraging efficiency. Many night-blooming flowers (e.g. *Datura*, *Mirabilis*) have conspicuously grooved petals, which could in theory be used as tactile guides for the moths' proboscides (Fig. 1.2).



**Figure 1.2.** *Manduca sexta* feeding from a flower of *Mirabilis multiflora* (Nyctaginaceae). Note the extended proboscis (grey arrow), the distance of the moth's body from the flower, and the radial grooves in the flower's perianth (white arrows). Scale bar (lower right corner) = 1 cm. Photo © Robert A. Raguso.

Thus, three treatments were designed. The first was 'medium disks', the same flat flowers used in Experiment 1. The second and third were paper disks of the same diameter as medium disks, with two groove-like folds (see Fig. 1.3). In the second treatment, the folds were oriented parallel to each other ('chord grooves') and were placed 1.5-cm apart from the origin (nectary) of the disk (Fig. 1.3). In the third treatment, the folds were placed as two orthogonal diameters of the disk ('radial grooves'), intersecting at the nectary (Fig. 1.3).



**Figure 1.3. Artificial flowers with 3-D features -Experiment #2. Medium Disk:** Same disk as in Fig. 1. *Radial Folds:* Medium disk with two groove-like folds along two perpendicular diameters of the disk. *Chord Folds:* Medium disk with two groove-like folds along two parallel chords, each 1.5 cm apart from the origin of the disk.

#### *Statistical analysis*

Response levels of male and female *M. sexta* to different flower morphs in Experiment 1 were tested by means of log-likelihood tests (*G*-tests using the  $G_h$  test statistic). Foraging efficiency, measured with the variables, emptied flowers and ratio of successful/total visits, was tested with the Kruskal–Wallis non-parametric test using a corrected  $\alpha$ -level of significance of 0.005. Thus, we performed ten statistical tests using the same set of data: six for emptied flowers, three for ratio of successful/total visits and one linear regression). Discovery time as a function of the sequence of feeding attempts was tested to fit the classic exponential decline learning curve described by Hilgard and Bower (Hilgard and Bower, 1966). A corrected  $\alpha$ -level of significance of 0.008 was used in these tests (six regression analyses). Because the variables measuring moths' foraging success on model flowers (emptied flowers and ratio of successful to total visits) showed equivalent results in Experiment 1 (see Results), we only analyzed emptied flowers data in Experiment 2. Two *a priori*

comparisons were planned (control group, i.e. medium disks vs radial grooves, and control vs chord grooves). Our evaluation of emptied flowers and the appropriate contrasts were performed using one-way analysis of variance (ANOVA) and *t*-tests, respectively, because the assumptions of the model (normality and homogeneity of variances) were met.

## **Results**

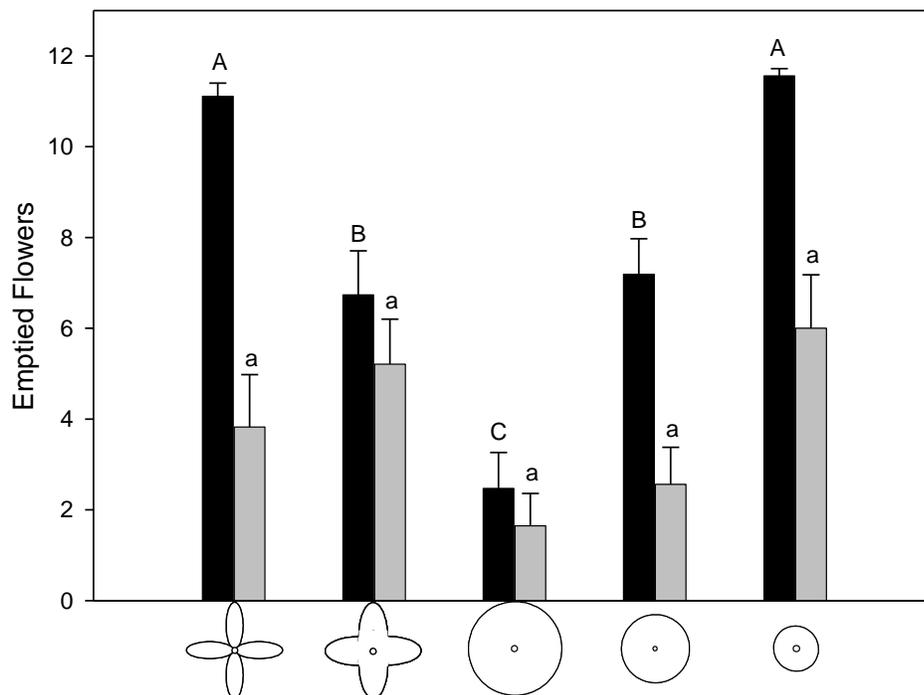
### *Experiment 1*

Inside the flight cage, 71.4% of the experimental animals (N=172) approached and probed the artificial flowers, with no significant gender differences observed (females: 66.4%; males: 76.2%; Gh=1.81; P=0.6). There were no differences in the overall proportions of responses to the different flower morphs, either with or without square transparency film (Gh=5.85; P=0.56; Table·1.1). Variation in flower shape and size did not account for any difference in initial feeding responses (i.e. approaches and probes). The presence of the square transparency film had a significant effect on the number of artificial flowers that moths successfully exploited (‘emptied flowers’) during each foraging bout (Kruskal–Wallis test; transparency vs no transparency:  $\chi^2_{(1,0.005)}=18.43$ ;  $P<0.0001$ ; Table·1.1, Fig·1.4). In addition, variation in emptied flowers was significantly affected by flower morphology among the no transparency treatments (Kruskal–Wallis test; within no transparency:  $\chi^2_{(4,0.005)}=44.64$ ;  $P<0.0001$ ). This effect was not observed among transparency treatments; in this case differences were not as pronounced, only accounting for a trend (Kruskal–Wallis median test; within transparency:  $\chi^2_{(4,0.005)}=10.18$ ;  $P=0.04$ ). Among the no transparency treatments, moths clearly were more successful when handling full lobe and small disk morphs (see Fig·1.4). As flower surface area increased from small disk (12.6·cm<sup>2</sup>) to large disk (63.6·cm<sup>2</sup>), moth performance declined (see regression analysis below).

**Table 1.1. Variables recorded in relation to flower handling by *Manduca sexta* on different flower morphs.** All values are means  $\pm$  SEM. Numbers in parenthesis reflect number of replicates for each treatment. Abbreviations: FL = Full lobe, HL = Half lobe, LD = Large disc, MD = Medium disc, SD = Small disk.

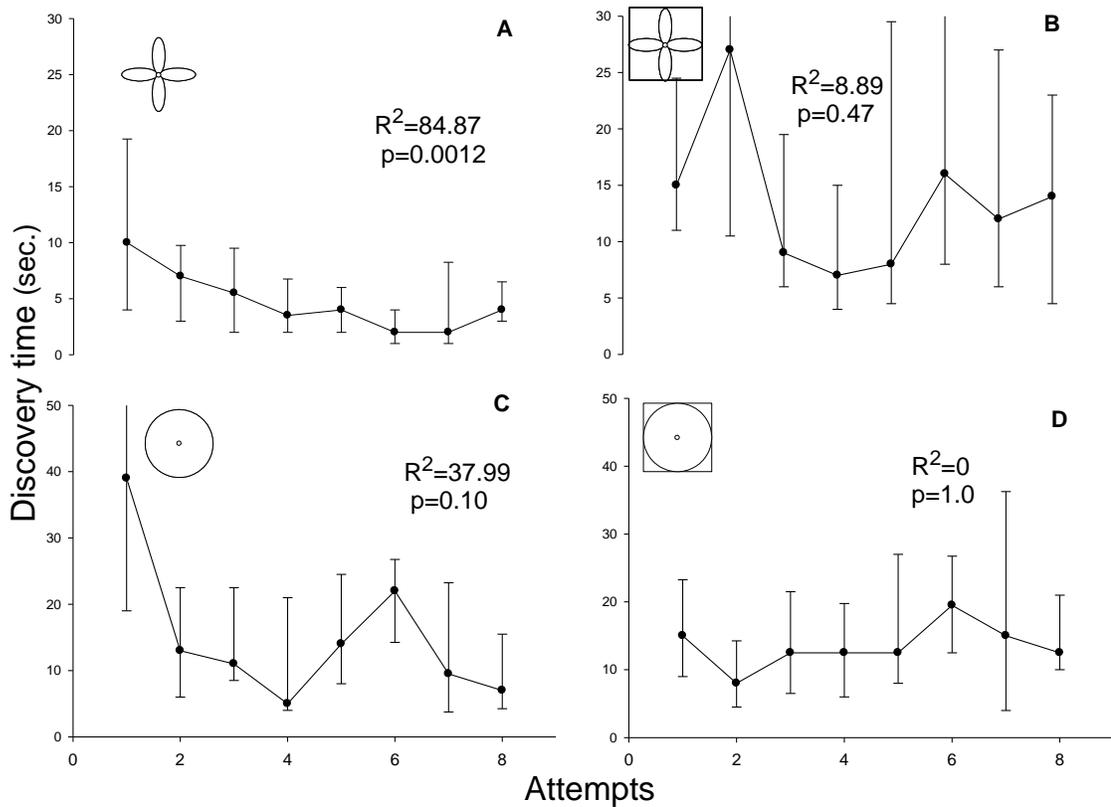
	No Transparency Film					Transparency Film				
	FL(16)	HL(18)	LD(17)	MD(16)	SD(16)	FL(17)	HL(19)	LD (17)	MD (16)	SD (16)
Mean # of visits	85.11 $\pm$ 8.7	73.20 $\pm$ 15.4	48.25 $\pm$ 10.3	81.69 $\pm$ 10.5	77.88 $\pm$ 11.1	65.53 $\pm$ 16.4	74.88 $\pm$ 9.2	54.44 $\pm$ 10	39.20 $\pm$ 6.9	85.11 $\pm$ 11.81
Mean # of Successful visits	50.39 $\pm$ 5.9	21.4 $\pm$ 4.4	5.81 $\pm$ 2.2	18.38 $\pm$ 3.7	51.18 $\pm$ 6	8.63 $\pm$ 2.7	13.47 $\pm$ 3.6	6.89 $\pm$ 2.8	4.13 $\pm$ 1.4	22.69 $\pm$ 5.6
Mean number of Failed visits	34.72 $\pm$ 5.6	51.8 $\pm$ 7.9	42.44 $\pm$ 9.5	63.31 $\pm$ 6.5	26.71 $\pm$ 4.2	60.94 $\pm$ 13.9	61.41 $\pm$ 8.8	47.56 $\pm$ 14.1	35.07 $\pm$ 8.8	41.63 $\pm$ 8.6
Mean ratio Successful visits/ Total visits	0.6 $\pm$ 0.04	0.24 $\pm$ 0.04	0.08 $\pm$ 0.02	0.21 $\pm$ 0.02	0.65 $\pm$ 0.05	0.08 $\pm$ 0.02	0.15 $\pm$ 0.03	0.09 $\pm$ 0.03	0.07 $\pm$ 0.02	0.26 $\pm$ 0.07

This same effect was observed when comparing full lobe (21.4-cm<sup>2</sup>), half lobe (33.7-cm<sup>2</sup>) and large disk (Kruskal–Wallis test; full lobe vs half lobe:  $\chi^2_{(1,0.005)}=6.07$ ;  $P=0.014$ ; large disk vs medium disk:  $\chi^2_{(1,0.005)}=16.63$ ;  $P<0.0001$ ). Because surface area is not the only flower feature that varied among treatments, we tested whether the minimum distance from the edge to the nectary (center) of the flower could affect moths' performance independently. Half lobe and medium disk flowers have similar surface areas (33.7-cm<sup>2</sup> and 33.2-cm<sup>2</sup>, respectively) but different edge-to-center distances (2-cm and 3.25-cm, respectively).



**Figure 1.4. Foraging efficiency within a single foraging bout versus different 2-D flower models.** Mean  $\pm$  SEM number of emptied flowers after a 10 minute foraging bout by individual *Manduca sexta* inside the flight chamber. In each treatment (abscissa) an array of 12 artificial flowers of the same morph was present. Black bars indicate responses to artificial flowers without square transparency film. Gray bars indicate responses to the same artificial flowers covered with a square transparency film. Different letters denote statistically significant differences with a corrected  $\alpha$ -level for significance of 0.008 (see text).

Similarly, half lobe and small disk flowers have the same minimum edge-to-center distance (2·cm) but different surface areas (33.7·cm<sup>2</sup> and 12.6·cm<sup>2</sup>, respectively). Surface area appeared to be a more important flower feature than edge-to-center distance, as feeding effectiveness did not differ significantly between half lobe and medium disk flowers (Kruskal–Wallis test;  $\chi^2_{(1,0.005)}=0.005$ ;  $P=0.945$ ), but differed significantly between half lobe and small disk flowers (Kruskal–Wallis test;  $\chi^2_{(1)}=18.34$ ;  $P<0.0001$ ;  $\alpha$ -level: 0.005). Moreover, the number of emptied flowers was significantly correlated with flower surface area ( $a+\beta x=y$ ;  $a=14.33$ ;  $\beta=-0.75$ ;  $R^2=0.56$ ;  $F_{(1,0.005,84)}=105.5$ ;  $P<0.0001$ ). Analysis of the ratio of successful/total visits yielded the same results as obtained from the analysis of emptied flowers (Kruskal–Wallis tests; transparency vs no transparency:  $\chi^2_{(1)}=30.48$ ;  $P<0.0001$ ; within transparency:  $\chi^2_{(4)}=59.29$ ;  $P<0.0001$ ; within no transparency:  $\chi^2_{(4,0.005)}=7.39$ ;  $P=0.12$ ). Contrasts between flower morphs on this variable show the same significance levels as those on the emptied flowers variable. Discovery times generally decreased when moths foraged on the artificial flowers (no transparency; exponential decline fit:  $R^2=84.35$ ;  $P=0.001$ ) as illustrated by Fig.·1.5A (full lobe with no transparency; exponential decline function fit:  $R^2=84.87$ ;  $P=0.001$ ). Nevertheless, this was not the case for the large disk treatment (Fig.·1.5C; exponential decline function fit:  $R^2=37.99$ ;  $P=0.10$ ). When flowers were covered by a transparency film, discovery times did not conform to a typical learning curve (transparency treatment; exponential decline function fit:  $R^2=56.58$ ;  $P=0.031$ ; corrected  $\alpha$ -level of significance: 0.005), as shown in Fig.·1.5B,C (full lobe with transparency:  $R^2=8.89$ ;  $P=0.47$ ; large disk with transparency:  $R^2=0.0$ ;  $P=1.0$ ).



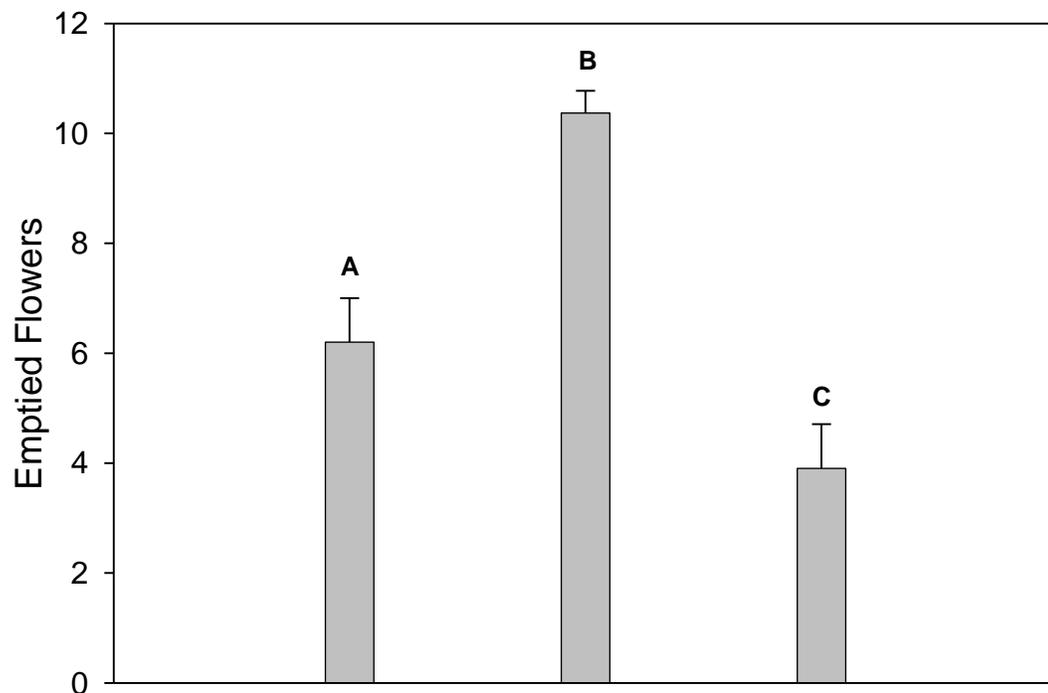
**Figure 1.5. Discovery time versus number of attempt.** Probing time between feeding attempts for 4 different treatments: (A) Full Lobe, (B) Full Lobe with Transparency film, (C) Large Disk and (D) Large Disk with Transparency film. Data points are medians, whiskers represent first and third quartiles. Statistical values refer to goodness-of-fit to an exponential decline function (one factor), a classical “learning curve”. Moths exploiting Full Lobe flowers with no transparency film show exponentially decreasing discovery times. When exploiting Large Disks, or either shape with transparency film, moths show larger variances in their responses, which do not fit an exponential decline function.

### Experiment 2

Flower morphology was significantly associated with the number of emptied flowers when moths foraged on different flower arrays (ANOVA:  $F_{(2,57)}=21.11$ ;  $P<0.0001$ ).

Both kinds of grooved artificial flowers affected the performance of foraging moths,

but in opposite ways (Fig. 1.6). Moths performed worse on flowers with chord grooves than on flat control flowers (medium disk vs chord grooves:  $F_{(1,39)}=78.9$ ;  $P<0.0001$ ), whereas moth performance on flowers with radial grooves was significantly better than on flat control flowers (medium disk vs radial grooves:  $F_{(1,37)}=328.11$ ;  $P<0.0001$ ).



**Figure 1.6. Foraging efficiency during a single foraging bout versus different flower models with 3-D features.** Three-dimensional corolla features affect foraging efficiency by *Manduca sexta*. The vertical bars indicate mean  $\pm$  SEM number of flowers emptied after a 10 minute foraging bout by individual moths inside the flight chamber. In each treatment (abscissa) an array of 12 artificial flowers of the same morph was present. Different letters denote statistically significant differences (see text).

## ***Discussion***

### *Behavioral sequence of flower foraging and its distinct sensory modalities*

The foraging behavior of *Manduca sexta* appears to follow a sequential pattern involving different sensory modalities at each stage (Raguso and Willis, 2003). Thus, a moth under appetitive motivation will first fly upwind when encountering an appropriate fragrance (Brantjes, 1978). At closer range, flower approach by *M. sexta* is guided by either olfactory or visual stimuli, whereas proboscis extension requires the combination of visual and olfactory cues (Raguso and Willis, 2002; Raguso and Willis, 2005). Here we show that mechanoreception is an additional sensory modality that contributes to the final stage of the feeding sequence, once the proboscis is extended and moths must locate and drink from floral nectaries, a process frequently referred to as ‘flower handling’.

In our experiments, moths were effectively and equally attracted to the different artificial paper flowers, regardless of the fact that they differed in shape and size (which in turn, greatly affected performance), when paper flower arrays were presented with Bergamot oil as an olfactory stimulus (see Results). This result indicates that probing responses (i.e. emptied flowers) to different treatments were not confounded by innate differences in attractiveness, and that no biases in moth preference or attraction were associated with the transparency films used to de-couple visual and mechanical stimuli.

### *Vision and mechanoreception during flower probing*

What is the innate probing strategy of *M. sexta*? Is the proboscis guided visually or are there other sensory systems involved? The use of different artificial flowers affected the efficiency with which *M. sexta* foraged on them. The ‘lobes’ series and the ‘disks’ series (both of which include the large disk treatment) showed improvements in moth

performance correlated with decreased surface area. As surface area increases, edge-to-center distance also increases, but the *a priori* comparisons (Figs. 1.1, 1.4) strongly suggest that for the set of artificial flowers used in this study, surface area was the main corolla feature affecting performance. Furthermore, this hypothesis is supported by the significant linear regression between surface area and performance (i.e. emptied flowers). On flat disk flowers with no surface features, probing by naïve *M. sexta* is ineffectual on larger disks, as the moths probe across the disk's surface and rarely find the centrally located nectary. Similarly, Knoll (Knoll, 1926) showed that when *Hyles lineata livornica* hawkmoths forage on artificial flowers, they probe the entire surface of the paper models. Our findings suggest that the innate strategy of *M. sexta* is to perform a 'random walk' of probing across the flower's surface.

The disruption of reliable tactile information clearly interferes with flower handling by *M. sexta*, showing that mechanoreception, in addition to vision and olfaction, is involved in nectar feeding by these moths (Fig. 1.4). Tactile cues constitute an important component of many flower-pollinator systems (Kevan and Lane, 1985; Borg-Karlsom, 1990), but are rarely investigated from a behavioral standpoint.

Interestingly, in the treatments with transparency film, we observed an overall reduced performance to the point where variation in flower shape had no significant effect on handling efficiency. Moths performed equally poorly on the different flower models without reliable tactile information, despite the fact that visual differences were preserved. Further investigation of the influence of mechanoreception on probing behavior led to Experiment 2, in which we found that corolla grooves positively affect the handling performance when they converge at the nectary and negatively affect it when they are incorrectly oriented (Fig. 1.6). This suggests that three-dimensional features have a hierarchical precedence on nectar-searching behavior at the flower handling scale as proposed by Brantjes and Bos (Brantjes and Bos, 1980). At this

level, the spatial resolution of *M. sexta*'s eyes does not allow for accurate feedback about proboscis position (A. Kelber, personal communication). The low signal-to-noise ratio of the visual modality at this scale could have imposed selective pressures for *M. sexta* to efficiently assess floral nectar content by other means. Such means include mechanoreception, as suggested by Leppik (Leppik, 1956) for some butterflies and as showed in this study, and probably gustation, given the responses of chemoreceptive sensilla positioned along the tip of the lepidopteran proboscis (Krenn, 1998; Kelber, 2003).

Spengel (Spengel, 1793) introduced the concept of nectar guides as floral features that could be used by pollinators to visually locate the nectar. Subsequent experiments revealed that the diurnal hawkmoth *Macroglossum stellatarum* (Knoll, 1922), bumblebees (Manning, 1956; Kugler, 1966), honeybees (Daumer, 1958; Free, 1979) and bee-flies (Johnson and Dafni, 1998), among other insects, successfully utilize visual nectar guides. Here we show that the utility of Spengel's idea extends beyond the visual system, as the tactile sensitivity of the proboscis of *M. sexta* allows these moths to exploit the physical features of flowers in order to find nectar (Figs. 1.2, 1.4, 1.6). Our experiments, unlike those of Knoll (Knoll, 1922; Knoll, 1926), varied the contours of artificial flowers, rather than testing moth responses to natural flowers. Further experiments will be required to test whether visual nectar guides of color contrast can be used by *M. sexta*.

#### *Context dependence of the floral visual display*

This study indicates that once moths approach a flower patch, they extend their proboscides towards a visual target and then appear to rely on mechanosensory input. At this point, when probing is relatively random, any irregularity on the corolla

surface could guide moths' searching behavior, such that the proboscis 'rides' along the length of petal grooves, nectary openings or the margins of highly divided corollas. The funnel-shaped flowers of *Datura wrightii*, a favored nectar source of *M. sexta* in the Sonoran Desert (Raguso et al., 2003) are comparable in diameter to the large disk models in Experiment 1, but previous experiments indicate that *Datura* flowers are learned very quickly by naïve *M. sexta* (Desai and Raguso, 2001), which is not the case when foraging on our large disks (Fig. 1.4). It appears that the decrement in flower handling by *M. sexta* on flowers with high surface area is offset by floral depth. However, attraction from a distance is enhanced by the increased visual display provided by flowers with larger diameters (Knoll, 1922). Tubular flowers appear to offer a compromise solution to this hypothetical trade-off, while simultaneously providing for high nectar volumes and appropriate physical contact between the body of the moth and the sexual organs of the flower (Nilsson, 1988). It is tempting to consider how differences in handling efficiency associated with corolla form might impact competition between night blooming flowers for hawkmoths as pollinators (see Haber and Frankie, 1989), however, most flowers in nature are likely to be visited by experienced moths. Additional experiments will be needed to determine whether the handling differences identified in this study have an impact on subsequent foraging decisions.

#### *Flower handling improves with experience*

We analyzed whether *M. sexta* could learn to improve its handling abilities (i.e. reduce the time to find nectar) during a single foraging bout. Indeed, *M. sexta* adults improve their handling of artificial flowers within an extended feeding bout (Fig. 1.5). We analyzed improvement in flower handling overall (with and without transparency film) and within two specific treatments – full lobe and large disk – as examples of flowers

that elicited high and low performances, respectively. Flower handling did not improve on model flowers in which the nectary was difficult to find (large circles), nor when square transparency films prevented the acquisition of reliable mechanosensory information (see Fig. 1.5B,D). This suggests that reliable tactile information is needed not only to forage efficiently (Fig. 1.3), but also to learn to forage more efficiently (slope of learning curves, see Results and Fig. 1.5).

Learned improvement in flower handling has been shown in a variety of nectivorous insects, including other lepidopterans (Lewis, 1986; Hartlieb, 1996; Cunningham et al., 1998) and hymenopterans (Harder, 1983; Laverty and Plowright, 1988; Chittka and Thomson, 1997). This ability gives animals the opportunity to decrease the time they spend on individual flowers and thus, directly increase their foraging efficiency and caloric intake (Pyke et al., 1977; Hughes and Seed, 1981).

Learned flower handling (and its attendant constraints, i.e. the inability to learn more than one or a few floral species) has been hypothesized to account for facultative flower specialization through the advantage that generalist pollinators gain by learning to handle a particular floral species (Darwin, 1895; Lewis, 1986). This is supported by Lewis' (Lewis, 1986) observation that *Pieris rapae* butterflies trained to one flower type find it more difficult (than do naïve butterflies) to learn a second flower type. Moreover, bumblebees can associate the morphology of artificial flowers with their color (Chittka and Thomson, 1997). On natural flowers, preference for flowers that are more easily handled is shown by the specialist bumble bee *Bombus consobrinus* (Laverty and Plowright, 1988), and by two species of hummingbirds and bumblebees for bluecolored (over albino) flowers of *Delphinium nelsonii* (Waser and Price, 1983). We have shown that naïve *Manduca sexta* hawkmoths are equally likely to feed from several different homogeneous arrays of artificial flowers with different morphologies. However, we did not explicitly test whether the moths have innate preferences for

flower morphology in a dual choice setting, nor whether they develop preferences for different flower models *after* learning to handle them. Future studies should test whether naïve *M. sexta* prefer specific flower morphologies when faced with a mixed array, and if so, whether such preferences can be modified through experience.

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**CHAPTER 2**  
**THE EFFECT OF DECOUPLING OLFACTORY AND VISUAL STIMULI ON**  
**THE FORAGING BEHAVIOR OF *MANDUCA SEXTA*\***

***Abstract***

*Manduca sexta*, a nectivorous nocturnal hawkmoth, can be attracted by a range of stimuli including floral volatiles and visual display, carbon dioxide and water vapor. Several studies on this and other flower-visiting insects have shown how olfactory and visual stimulation play (or do not play) a role in attraction and feeding. Nevertheless, these studies have consistently manipulated stimuli in a ‘presence–absence’ manner. Here, we experimentally decoupled the presentation of both stimuli spatially and temporally in a wind tunnel, rather than entirely eliminating either one, and found that responses to these stimuli are more flexible and complex than previously asserted. *M. sexta* was most responsive when both cues were present and emanated from the same source. When stimuli were spatially separated, responsiveness levels were comparable to those elicited by a single stimulus. However, transient olfactory stimulation either before or after visually guided approach (temporal decoupling) enhanced responsiveness to an odorless visual target. Additionally, searching times were increased by either a transient olfactory stimulation before take-off or by having the flower model spatially separated from the odor source tracked by the moths. Finally, in a dual-choice experiment, moths showed a strong bias for the visual display over the odor plume, suggesting the former to be the ultimate indicator of a nectar source. Our manipulation of floral cues shows that the feeding behavior of *M. sexta*, and probably of other nectivorous insects, is based not only on the sensory stimulation *per se* but also on the temporal and spatial pattern in which these stimuli are perceived.

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## ***Introduction***

The use of multiple sensory modalities empowers animals to respond efficiently to variable and complex environments (reviewed by Hebets and Papaj, 2005). In goal-seeking tasks such as close-range searching, where effective stimuli are often emitted by the target (e.g. food, shelter, hosts), multiple sensory inputs provide animals with several advantages, including behaviorally flexible ‘contingency plans’ conferred by redundant inputs (Brantjes, 1978; Raguso, 2004). Another advantage of multi-modal communication is the reinforcement of highly specialized information content, such as hostspecificity or flower constancy, due to the integration of sensory modalities (Gegeer, 2005; Hebets and Papaj, 2005). For example, cabbage moths (*Mamestra brassicae*) orient more frequently to the combination of visual and olfactory host-plant cues than to either cue presented alone (Rojas and Wyatt, 1999). *Diachasmimorpha longicaudata*, the hymenopteran parasitoids of tephritid fruit flies, show different responses when stimulated by different fruit signals in a wind tunnel, landing 5-fold more often on appropriately scented visual targets than on odorless guava fruit models (Jang et al., 2000). Björklund et al. found similar, but in this case additive, effects when using visual and olfactory cues from conifer seedlings to attract the pine weevil *Hylobius abietis* (Björklund et al., 2005). Thus, stimulation of more than one sensory system can elicit additive as well as synergistic responses (see Raguso and Willis, 2002).

The interplay between olfactory and visual cues is known to mediate the sequence of feeding behaviors (i.e. from flower approach to proboscis extension) of several species of moths (Brantjes, 1978; Naumann et al., 1990; Raguso and Willis, 2002) and butterflies (Tinbergen, 1958; Andersson and Dobson, 2003; Omura and Honda, 2005), but little is known about how these substantially different sensory systems interact during the decision-making process(es) of foraging lepidopterans. The butterfly

*Vanessa indica* is more attracted to scented than to unscented paper flowers when their color is relatively unattractive (e.g. purple), but prefers unscented yellow flower models over scented purple flower models in choice tests (Omura and Honda, 2005). The innate attraction of these butterflies to yellow is stronger than their attraction to a scented but unattractive colored flower model. Balkenius and Kelber documented a similar sensory bias in a study of odor learning by the diurnal hawkmoth *Macroglossum stellatarum* (Balkenius and Kelber, 2006), which shows appetitive conditioning to sugar-rewarded odors associated with unattractive flower colors (e.g. yellow) but cannot learn to distinguish between differently scented blue flowers, which they innately prefer. These authors (Balkenius et al., 2006) have shown that the ecology of the animal is an important factor regarding the weight given to the different sensory cues. Thus, the nocturnal hawkmoth *Deilephila elpenor* responds preferentially to floral scent over visual targets in choice assays within a wind tunnel, but the diurnal *M. stellatarum* shows the converse preference for visual stimuli. However, it is also possible that the feeding response of *D. elpenor* is odor guided because these moths also feed from fermented fruit and sap without strong visual contrast (Newman, 1965). These studies indicate that Lepidoptera generally use multi-modal sensory inputs during nectar foraging but that the integration of such cues may be complex and hierarchical.

*Manduca sexta*, a crepuscular, nectar-feeding hawkmoth native to the Americas, has been well studied as a model system for flight energetics and biomechanics (Tu and Daniel, 2004), visual and olfactory neurophysiology and development (White et al., 2003; Reisenman et al., 2005). These moths are known to be attracted by a range of sensory stimuli, including floral odors and visual display (Brantjes, 1978; Raguso and Willis, 2002; Raguso and Willis, 2005), water vapor (Raguso et al., 2005), carbon dioxide (Thom et al., 2004) and hostplant volatiles

(Mechaber et al., 2002). Behavioral events associated with foraging are released by an apparently synergistic interplay between olfactory and visual cues, such that the combined signal elicits proboscis extension (while hovering) in both naïve and wild *M. sexta* (Raguso and Willis, 2002; Raguso and Willis, 2005). In these studies, *M. sexta* moths approached either visual targets or odor sources, but only extended their proboscides towards a visual target when olfactory cues were present. These authors concluded that odor and visual cues were both needed for feeding by *M. sexta*, but could not distinguish between an odor-gated visual approach and simultaneous olfactory–visual stimulation of feeding. Are these sensory inputs perceived as a single composite signal with an enhanced predictive value for a nectar source, or does odor ‘activate’ a visually guided search behavior?

In previous studies of feeding behavior by *M. sexta* and other Lepidoptera, experimental manipulation was limited to the presence or absence of visual and/or olfactory floral stimuli, and thus was insufficient to acquire fine-scale information on how the integration of olfactory and visual signals affects foraging decisions. For example, visual contact with flower targets can be temporarily obstructed, and olfactory stimulation can be intermittently affected by wind turbulence in the natural environments in which hawkmoths forage for nectar (see Eisikowitz and Galil, 1971). Thus, in the present work, we address an important gap in studies of lepidopteran foraging behavior by spatially and temporally manipulating the presentation of visual and olfactory stimuli to naïve *M. sexta* moths.

In the first experiment, we spatially decoupled the presentation of olfactory and visual stimuli in a laminar flow wind tunnel, by creating an odor plume and a visual target (artificial flower) separated by different incremental distances. We used this design to test the following hypotheses:

Hypothesis1A – olfactory stimulation in the form of an odor plume spatially restricts moths’ responsiveness to probing at the odor source;

Hypothesis1B – once olfactory stimulation occurs within an odor plume, probing may occur at visual targets within or outside of the plume.

In the second experiment, we temporally decoupled olfactory and visual stimuli by presenting moths with a discrete odor puff at different times in the presence of an odorless visual target. In each manipulation, we quantitatively evaluated the moths’ decisions to probe at a visual target or not, contrasting the following hypotheses:

Hypothesis2A – moths require simultaneous olfactory and visual stimulation to probe at artificial flowers in a wind tunnel;

Hypothesis2B – feeding behavior by *M. sexta* shows a sequential pattern, with olfactory stimulation releasing or ‘gating’ a visually guided searching and probing behavior [after Knoll (Knoll, 1922; Knoll, 1926) and Brantjes (Brantjes, 1978)].

In the third experiment, we challenged moths to choose between the visual target and the odor source separated by 40-cm, to determine whether they show an innate bias for either modality at the final stage of the searching behavior (i.e. probing):

Hypothesis3A – *M. sexta* favors probing on olfactory over visual cues when presented with a binary choice, as has been shown for another nocturnal hawkmoth, *D. elpenor* (Balkenius et al., 2006);

Hypothesis3B – *M. sexta* favors probing on visual over olfactory cues, suggesting visual information to be the ultimate nectar source indicator.

Our results are discussed in the framework of multi-modal sensory usage by foraging *M. sexta* and other Lepidoptera.

### ***Materials and methods***

This study was carried out during August and September 2005 (experiments 1 and 2) and January 2006 (experiment 3) at the University of South Carolina, Columbia, SC, USA.

### ***Animals***

We used 3–5-day-old *Manduca sexta* L. adults reared from eggs provided by Dr Lynn Riddiford, University of Washington, Seattle, WA, USA. Larvae were fed *ad libitum* on an artificial diet (Bell and Joachim, 1976) and were kept as pupae under a 16-h:8-h light:dark, 24:21°C cycle. Moths were separated by sex as pupae and were housed in different incubators (Precision 818; Winchester, VA, USA) under the same ambient regime and emerged within 45x45x45-cm screen cages (BioQuip, Inc., Rancho Dominguez, CA, USA). Adults were starved for 3 to 4-days before being used in experiments to increase their appetitive motivation.

### ***General procedure in the wind tunnel and recorded variables***

At the beginning of scotophase (15:00-h), the naïve, starved adult moths were placed individually at the downwind end of a 3x1.5x1.5-m laminar flow wind tunnel, with a flow rate of  $1\cdot\text{m}\cdot\text{s}^{-1}$ . Each moth was allowed to fly freely inside the wind tunnel for 5-min, during which its behavior was recorded. In experiments 1 and 2, we recorded whether or not moths approached (i.e. hovered in front of) and probed an artificial flower at least once with their extended proboscides. Both variables were expressed as proportions of the number of animals flown in each treatment. We also recorded the amount of time (approach time, in s) during which moths flew inside the tunnel before probing the artificial flower. In experiment 3 (choice experiment; see below), we recorded the proportions of moths that probed on the artificial flower (visual stimulus)

vs the odor source as their initial response when these stimuli were spatially decoupled. We also recorded the total number of choices and total time probing (s) at each stimulus, as well as the latency (time elapsed before the first choice, in s).

### *Sensory stimuli*

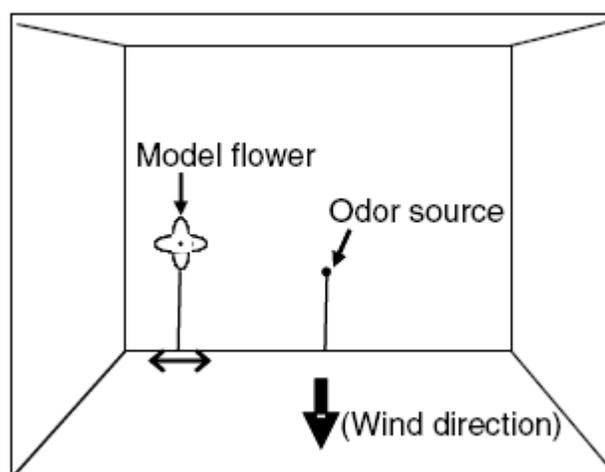
A cotton swab was soaked with 25- $\mu$ l of bergamot essential oil (Body Shop, Columbia, SC, USA) for each experimental trial that included an olfactory stimulus and was refreshed every 15-min in order to maintain odor intensity. This odor source is a reliable feeding stimulant for *M. sexta* (Goyret and Raguso, 2006) and, like many night-blooming flowers visited by this species, is dominated by linalool and related monoterpenoid odors (see Raguso and Pichersky, 1999). The wooden stem of the swab (2.5-cm) was affixed to a 3-cm<sup>3</sup> piece of dark gray modeling clay at a 45° angle to the black ring stand and 1-cm below the flower. In the treatments testing visual cues without odor, a scentless cotton swab was affixed to the ring stand to present the same amount of visual contrast.

The visual stimulus consisted of a white artificial flower with a paper perianth (9-cm in diameter; no reward was present) positioned on the vertical ring stand at a height of 50-cm against a black background. Spectrophotometer readings of flowers (not shown) revealed that the paper absorbed UV wavelengths but reflected light nearly uniformly from 400 to 700-nm.

Volatile analysis (not shown) using solid phase microextraction combined with gas chromatography–mass spectrometry revealed that the artificial flower did not emit volatile compounds. In the treatments without a white flower, we constructed a black flower to control for turbulence effects on the odor plume. White and red tungsten lamps were positioned above the wind tunnel, providing diffuse illumination through a white cotton sheet (see below).

*Experiment 1: spatial decoupling of visual and olfactory cues*

We manipulated the presence and relative position of olfactory and visual stimuli at the upwind end of the tunnel (see Table 2.1 for treatment summary).



**Figure 2.1. Transverse section of wind tunnel.** Upwind view of the inside of the wind tunnel (3x1.5x1.5 m) showing the odor source (i.e. cotton swab) and the artificial flower (diameter, 9 cm), which could be displaced by moving it left or right in the same plane (as shown by double-ended arrows).

In the first four treatments, by moving the artificial flower to the right or left of the centrally positioned odor source (Fig. 2.1), we wished to observe whether probing behavior varies with increasing distance between stimuli. The fifth and sixth treatments allowed us to compare responses when only one stimulus was present. The

seventh treatment was designed to measure baseline responses by the moths to the ancillary structures utilized in the other treatments (ring stand, cotton swab and tape). Light intensity measured within the wind tunnel ranged from 0.011·lx to 0.023·lx (approximate conditions of a bright starlit night).

**Table 2.1. Summary of treatments used in Experiment 1.** O = olfactory, V = visual. Stimuli were placed at the end of a 3x1.5x1.5 meters wind tunnel. Where there is no artificial white flower (treatments Odor and Negative control) we placed instead a black flower matching the background to have same wind turbulence effect as in other treatments. Where there is no odor present, we placed the same cotton swab as in other treatments but without soaking it with bergamot essential oil.

<b>Treatment [N]</b>	<b>Description</b>
Positive Control (O+V)[22]	Artificial flower next to a scented cotton swab
O+V@10 [23]	Artificial flower with a scented cotton swab 10 cm apart
O+V@20 [22]	Artificial flower with a scented cotton swab 20 cm apart
O+V@40 [23]	Artificial flower with a scented cotton swab 40 cm apart
Visual [25]	Artificial flower with unscented cotton swab
Odor [21]	Scented cotton swab without artificial flower
Negative Control [25]	Neither artificial flower nor cotton swab present

*Experiment 2: temporal decoupling of visual and olfactory cues*

Given that the spatial separation of visual and olfactory cues also implies a non-simultaneous presentation for which we had no control, we designed a second

experiment in which these cues were decoupled temporally. Here, we always presented the white artificial flower at the upstream end of the wind tunnel but manipulated the timing of the olfactory stimulation, either before releasing the moth (downwind puff), during the whole trial (odor plume) or at the flower (flower puff; treatments are summarized in Table-2.2). We used a different set of syringes, tubing and artificial flowers to avoid odor contamination.

Compared with pilot experiments, feeding responses in the positive control of experiment 1 were less probable, thus, in this experiment, light intensity was increased to 0.054-lx [approximate conditions of a (half)moonlit night] by the addition of a second white bulb. Increased illumination could affect the conspicuousness of the visual target, but given the positive and negative controls in this experiment, we could still evaluate the effect of the temporal sequence of stimulation (see Discussion).

### *Experiment 3: stimulus preference in a dual-choice set-up*

We performed a choice experiment using the set-up from the ‘Visual at 40-cm’ (O+V@40) treatment of experiment 1. Instead of having the odor source at the center of the wind tunnel and the flower at 40-cm to its left or right side, here we randomly placed each stimulus 20-cm apart from the center but in opposite directions. We analyzed the relative feeding responses towards the visual display (artificial flower) and odor source (scented cotton swab) with a larger sample of moths ( $N=56$ ), to see whether they showed an innate preference for either the visual or the olfactory stimulus. Light conditions were set as described above for experiment 1.

**Table 2.2. Summary of treatments used in experiment 2.** Puffs and cotton swab could be either scented or unscented as stated. Down-stream puff was applied directly with a 30 ml syringe that had a cotton swab inside that could be either scented or unscented. Flower puff was applied in the exact way, but through a piece of Tygon™ tubing that ended in the center of the flower model to avoid disturbing the moths. The cotton swab at the flower was always present and could be either scented (positive control) or unscented (other treatments). Abbreviation: @=at.

<b>Treatment [N]</b>	<b>Down-stream puff</b>	<b>Cotton swab at flower</b>	<b>Flower puff</b>	<b>Stimuli delivery sequence</b>
Odorless (Negative control) [25]	Air alone	Dry (no odor)	Air alone	Baseline response to visual display alone
Odor@Start [24]	Air saturated with bergamot oil volatiles	Dry (no odor)	Air alone	Transient olfactory stimulation before visual display
Odor@Flower [19]	Air alone	Dry (no odor)	Air saturated with bergamot oil volatiles	Transient olfactory stimulation during visual display
Fragrant flower (Positive control) [23]	Air alone	Impregnated with bergamot oil	Air alone	Continuous olfactory stimulation

### *Statistical analysis*

In experiments 1 and 2, the categorical variables ‘approach’ and ‘probing’ were analyzed by means of a log-likelihood test (G-test) when testing overall treatment effects and by using binomial tests when comparing pairs of proportions (binomial distributions). An  $\alpha$ -level of significance of 0.0045 was adopted for experiment 1 to preserve a global  $\alpha$ -value of 0.05, because we performed 11 statistical tests on these data. Approach time was analyzed as a continuous dependent variable using one-way analysis of variance (ANOVA) (with treatments as factors – see Table-2.1) because

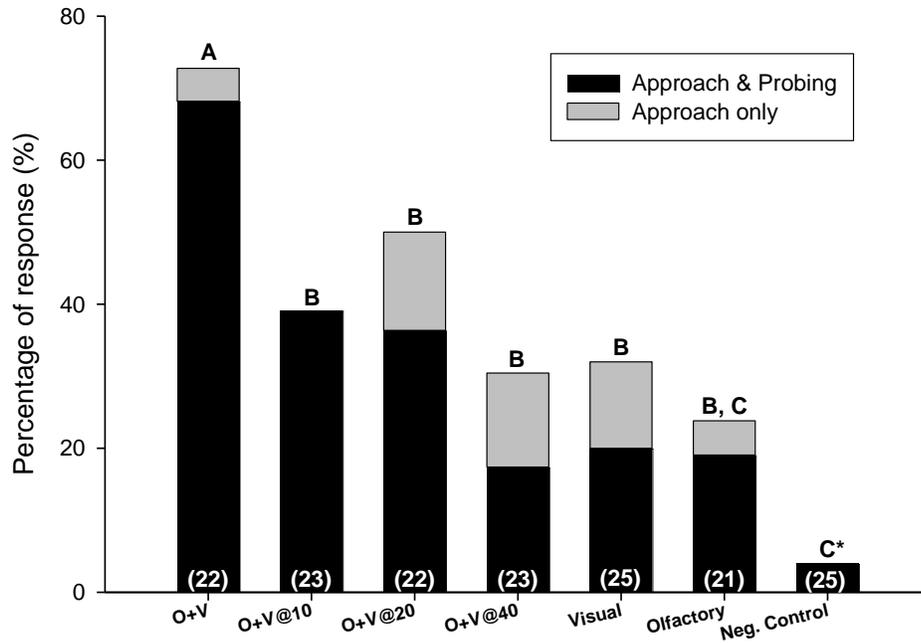
data met the assumptions of this test, and an orthogonal *a priori* comparison was performed (positive control vs treatments with spatially separated stimuli – 10, 20 and 40-cm apart).

In experiment 2, mean ranks of ‘approach time’ data were analyzed using the Kruskal-Wallis non-parametric test, because the data were refractory to transformation. In experiment 3, the dependent variables ‘total visits’ and ‘total visit time’ were square root and log transformed, respectively, for ANOVA. Finally, initial moth choice was analyzed using the binomial test with the null hypothesis of equal attraction to olfactory and visual stimuli [ $P(\text{odor source})=P(\text{visual target})=0.5$ ].

## **Results**

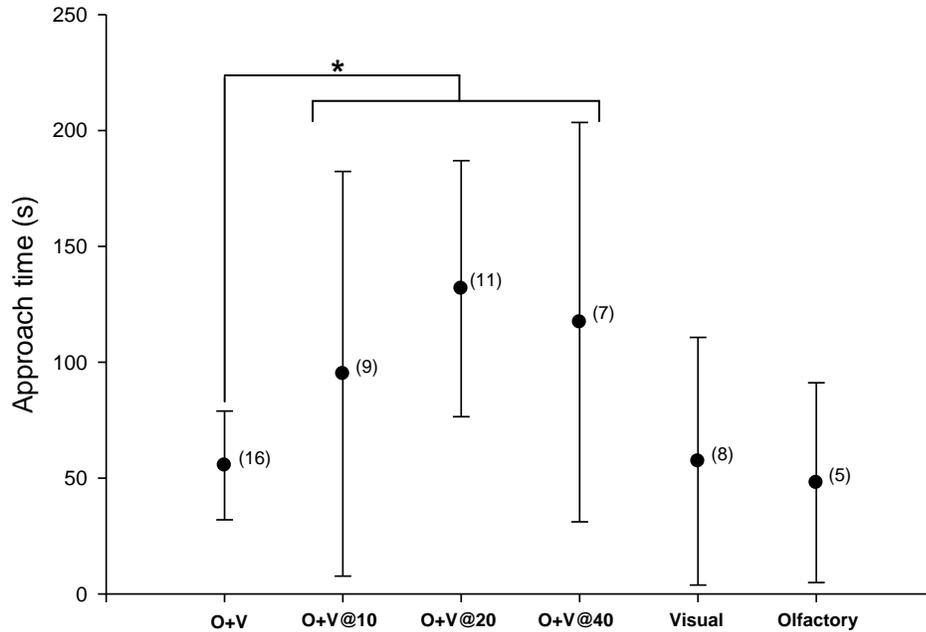
### *Experiment 1: spatial decoupling of visual and olfactory cues*

All experimental moths took off and flew in the wind tunnel, and 72% responded by approaching and probing at the positive control. An analysis of Approaches and Probing responses showed significant effects of the treatments on both variables (Approaches,  $Gh=31.14$ ,  $P<0.0001$ ; Probing,  $Gh=30.37$ ,  $P<0.0001$ ;  $N=161$ ) (Fig. 2.2). When the artificial flower was spatially separated from the odor source, we observed a significantly decreased response (probing) to each treatment (O+V@10,  $P=0.0005$ ,  $N=45$ ; O+V@20,  $P<0.0001$ ,  $N=44$  and O+V@40,  $P<0.0001$ ,  $N=45$ ; binomial tests) (Fig. 2.2). A similar decrease in probing was observed when visual or olfactory stimuli were presented alone (Visual,  $P<0.0001$ ,  $N=47$ ; Olfactory,  $P<0.0001$ ,  $N=43$ ; binomial tests). The only treatment that did not differ statistically from the negative control was the Olfactory treatment (odor alone;  $P=0.008$ ,  $N=46$ ,  $\alpha=0.0045$ ; see Materials and methods; Fig. 2.2).



**Figure 2.2. Spatial decoupling of stimuli.** Percentages of individual moths (sample replicates in parentheses) that approached only (grey bars) or approached *and* probed (black bars) at 7 different spatial manipulations of sensory stimuli in a wind tunnel. Different letters denote significant differences between treatments for the “approach and probing” variable. \*Based on the response of one moth

Analysis of variance of Approach time showed a marginally non-significant effect of treatment ( $F_{5,56}=2.35$ ,  $P=0.055$ ) (Fig. 2.3), but the *a priori* comparison showed that the mean approach time to the flower in the positive control (55-s) was significantly shorter (by half) than the approach time when the artificial flower was spatially separated from the odor source (10, 20 and 40-cm treatments; mean approach time=114.67-s;  $F_{1,42}=6.99$ ,  $P=0.01$ ; Fig. 2.3).

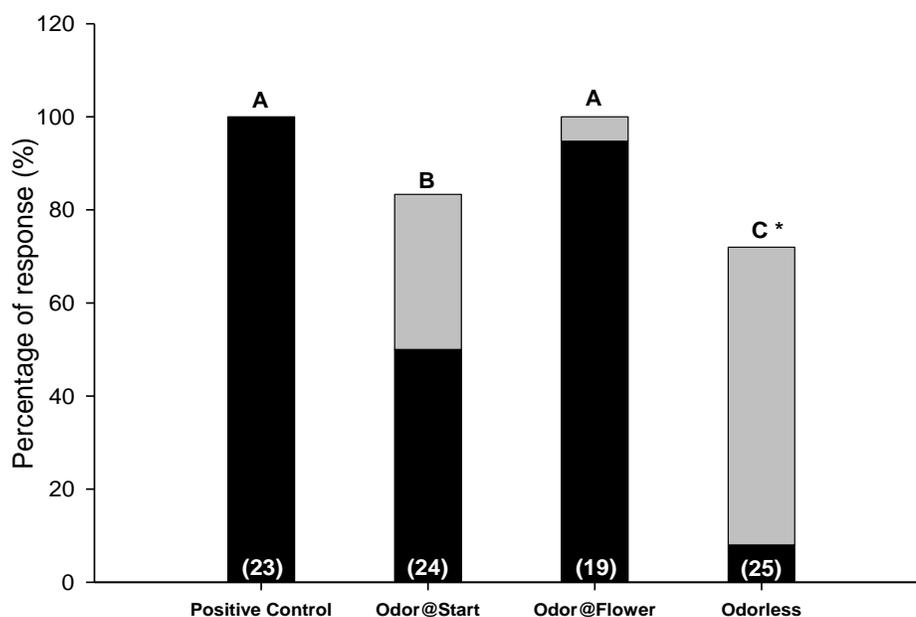


**Figure 2.3. Approach times for spatially decoupled stimuli.** Mean  $\pm$  Confidence Interval ( $\alpha=0.05$ ) of time elapsed between take off and approach to the visual target under different conditions of visual and olfactory stimuli presentation (see Methods and Table 2.2 for experimental and statistical details). Asterisk denotes significant differences for the “approach time” variable between the positive control and the treatments in which odor and visual stimuli were spatially separated. Numbers in parentheses are moths that approached the visual target, and thus represent a subset of sample sizes given in Fig. 2.2.

*Experiment 2: temporal decoupling of visual and olfactory cues*

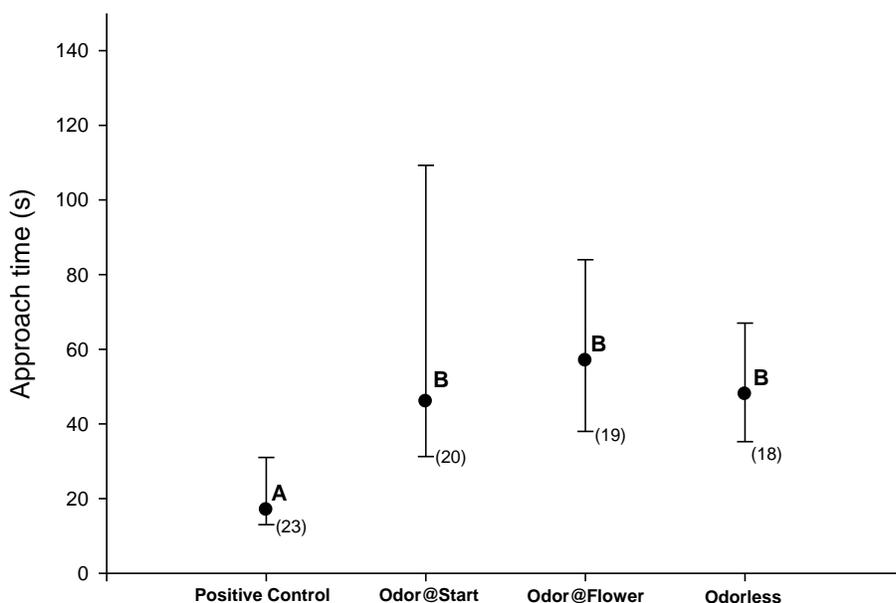
In experiment 2, all 23 moths exposed to the positive control (no temporal separation between stimuli) responded by probing, which shows a 28% increase from the exact control in experiment 1. This was probably due to our deliberate increase in light

intensity, since responsiveness to this treatment in the first experiment was lower than usual. Nevertheless, variation in the Approach & Probing variable was significantly associated with treatment ( $Gh=60.64$ ,  $P<0.0001$ ,  $N=91$ ; Fig. 2.4). The approaches and probing responses to the positive control differed significantly from treatments in which odor was absent (Negative control – or ‘Odorless’ –  $P<0.0001$ ,  $N=48$ ) or present only at the start (Odor@Start,  $P<0.0001$ ,  $N=47$ ) (Fig. 2.4) but not when odor was presented as the moth approached the flower (Odor@Flower,  $P=0.16$ ,  $N=42$ ; binomial tests). Probing responses in the Odor@Start treatment were significantly fewer than in the Odor@Flower treatment ( $P<0.0001$ ,  $N=43$ ) (Fig. 2.4) but significantly more frequent than was observed at Odorless flowers ( $P<0.0001$ ; binomial tests) (Fig. 2.4).



**Figure 2.4. Temporal decoupling of stimuli.** Percentages of individual moths (sample replicates in parentheses) that approached only (grey bars) or approached *and* probed (black bars) at 4 different temporal manipulations of stimuli in a wind tunnel (see Methods and Table 2.2). Different letters denote significant differences between treatments for the probing variable. \*Based on the response of two moths.

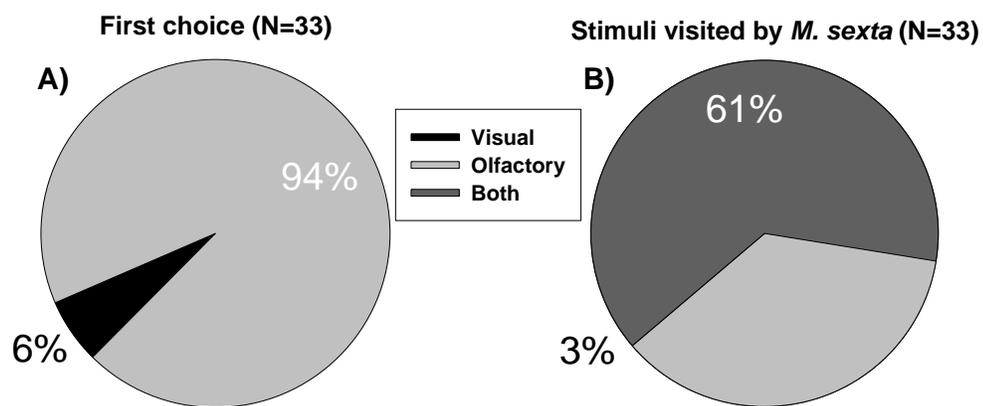
Approach time was also strongly affected by treatment (Kruskall-Wallis test;  $H_3=26.12$ ,  $P<0.0001$ ,  $N=80$ ; Fig. 2.5). Mean ranks of approach times were significantly lower in the positive control than in all other treatments (vs Odor@Start,  $H_1=15.6$ ,  $P=0.0001$ ,  $N=43$ ; vs Odor@Flower,  $H_1=17.94$ ,  $P=0.0001$ ,  $N=42$ ; vs Odorless,  $H_1=14.45$ ,  $P=0.0001$ ,  $N=41$ ; Fig. 2.5). No significant differences were found when comparing this variable between Odor@Start and Odor@Flower treatments ( $H_1=0.03$ ,  $P=0.87$ ,  $N=39$ ) (Fig. 2.5).



**Figure 2.5. Approach times for temporally decoupled stimuli.** Median  $\pm$  first and third quartiles of the time elapsed between take off and approach to the visual target under different conditions of odor presentation (see Methods and Table 2.2 for experimental and statistical details). Different letters denote significant differences between treatments for the “approach time” variable. Numbers in parentheses are moths that approached the visual target, and thus are a subset of the sample sizes given in Fig. 2.4.

### Experiment 3: stimulus preference in a dual-choice set-up

Slightly more than half (55%) of all experimental moths responded by approaching and probing the test stimuli, with 94% of the first choices to the visual target and only 6% of first choices to the odor source. These proportions differed significantly ( $P < 0.0001$ ,  $N = 33$ ; binomial test; Fig. 2.6A). When all probing events for each moth were evaluated, we found that 61% probed only the visual target, 3% probed only the odor source and 36% probed both stimuli ( $N = 33$ ) (Fig. 2.6B). When we analyzed total number of probes to the visual target ( $3.41 \pm 0.49$ ) and to the odor source ( $1.62 \pm 0.16$ ), we found significant differences between means ( $F_{1,43} = 5.60$ ,  $P = 0.023$ ; ANOVA with square root transformation). We also analyzed total visit time and found that moths spent more time probing the visual target ( $43.56 \pm 10.54 \cdot s$ ) than the odor source ( $10.85 \pm 3.52 \cdot s$ ;  $F_{1,43} = 4.65$ ,  $P = 0.037$ ; ANOVA with log transformation).



**Figure 2.6. Choice between stimuli.** A) First choice made by single *M. sexta* flying in a wind tunnel. Different proportions are statistically significant (Binomial test;  $p < 0.0001$ ). B) Stimuli visited by single *M. sexta* within a 5 min foraging bout in the wind tunnel (unrewarded flower model). Mean Number of visits  $\pm$  S.E.M.: Visual target =  $3.41 \pm 0.49$ ; Odor source =  $1.62 \pm 0.16$  ( $F_{(1, 43)} = 5.60$ ;  $p = 0.023$ ; ANOVA with square root transformation).

## ***Discussion***

### *Spatial decoupling of olfactory and visual stimuli*

Results from the spatial decoupling experiment (experiment 1) show that when *M. sexta* tracks an odor plume in a turbulence-free environment, a separation as small as 10-cm between the odor source and the visual target can significantly diminish the probing probabilities from those observed when both signals occur together (Fig. 2.2). Remarkably, when floral signals were spatially separated, mean approach and probe responses were comparable to treatments in which only single stimuli were available (Fig. 2.2). The lack of spatial concomitance between olfactory and visual stimuli appears to impact the likelihood that a moth evaluates a visual target as a feeding site when both signals are present (Hypothesis<sub>1A</sub>; see Introduction). However, approaches and probes did occur when odor and visual cues were separated and when each was presented alone (Fig. 2.2), thus Hypothesis<sub>1B</sub>, while less well supported by our data, cannot be rejected. Additionally, mean approach times and their variances were also greater when stimuli were spatially uncoupled than when both stimuli were present at the same position or when only one stimulus was available (Fig. 2.3). This could imply a prolonged (or different) integration process triggered when both visual and olfactory stimuli are present but are contradictory in spatial terms. Of course, the threshold distances defined in our study are relevant only to the laminar flow wind tunnel, which is designed to reduce or eliminate turbulence (Willis and Arbas, 1991; Vickers, 2000). Floral scent plumes are unlikely to indicate point sources in natural blooming populations and are likely to show turbulence and complex three-dimensional structure (Murlis et al., 1992). Manipulative experiments in which the visual and olfactory floral cues of *Datura wrightii* were decoupled (Raguso and Willis, 2005) showed that the spatial threshold of visual–olfactory synergism for wild *M. sexta* foraging in open desert environments is on the scale of meters, rather than

centimeters. Taken together, these studies demonstrate the contextual importance of odor in the perception of bright visual targets as nectar sources by *M. sexta*.

### *Temporal decoupling of olfactory and visual cues*

In experiment 2, we temporally decoupled olfactory and visual stimulation to determine whether *simultaneous* perception of both modalities is necessary to trigger the probing response with high probabilities (Hypothesis<sub>2A</sub>; see Introduction). The alternative view, described by Brantjes (Brantjes, 1973) and implied by Knoll (Knoll, 1922; Knoll, 1926), is that odor ‘gates’ or releases a sequence of visually guided feeding behaviors, for which odor is superfluous (Hypothesis<sub>2B</sub>; see Introduction). Instead of rejecting either of these hypotheses, our results lead us to provisionally accept both, which indicates that they are not mutually exclusive. Indeed, we have observed an unexpected flexibility in feeding behavior by *M. sexta*, such that olfactory stimulation before, during or after visual stimulation is sufficient to elicit probing. Thus, an odor plume can guide a moth to its source (the flower) when sustained, but it also can increase a moth’s responsiveness to a visual target when transient (see below). Moths that approached the visual target in the absence of odor showed very low probabilities of proboscis extension, but this behavior could be reversed by a transient odor puff administered as moths hovered in front of the flower model (Fig. 2.4). Such responses increased probing probability to the levels of the positive control, where moths could be guided by both olfactory and visual inputs. These results support the hypothesis that simultaneous visual and olfactory stimulation has the highest probability of triggering proboscis extension (Hypothesis<sub>2A</sub>). Nevertheless, odor-gated visual foraging can occur in *M. sexta*, increasing responsiveness of naïve moths above that occurring in the absence of odor (Hypothesis<sub>2B</sub>; Fig. 2.4). Transient presentation of the olfactory stimulus before flight led to longer latencies (Fig. 2.5),

which behaviorally could be explained by a repetitive up- and downwind, looping flight pattern observed solely under this treatment. Unfortunately, we did not record flight tracks to quantify this flight behavior, which differs fundamentally from the upwind casting flight tracks limited to the vertical plane of a continuous odor plume, as shown by Raguso and Willis (Raguso and Willis, 2003). As described by Brantjes (Brantjes, 1973), olfactory stimulation appears to ‘arrest’ moths into an odor-plume search and at the same time increases the chances of probing upon encounter with a visual target (Fig. 2.4).

#### *Choosing between olfactory and visual signals of a flower*

In experiment 3, we forced moths to choose between decoupled visual and olfactory floral cues, providing a distinct test of the necessity for concomitance of stimuli and addressing the potential for sensory hierarchies observed by Omura and Honda (Ômura and Honda, 2005) and Balkenius and Kelber (Balkenius and Kelber, 2006) for other nectar-feeding Lepidoptera. We found that first visits by *M. sexta* overwhelmingly favored the visual target over the odor source (Fig. 2.6A), suggesting that Hypothesis<sub>3A</sub> should be rejected in favor of Hypothesis<sub>3B</sub> (see Introduction). Balkenius et al. used a wind tunnel to perform similar experiments with two nectar feeding European hawkmoth species (Balkenius et al., 2006). In their study, *M. stellatarum*, a diurnal hawkmoth species, approached a visual target instead of an odor source, while the nocturnal *D. elpenor* more frequently approached the odor source. *M. stellatarum* can feed on scentless flower models (Kelber, 1997), while *D. elpenor* has been shown to require olfactory stimulation to feed from flowers (Brantjes, 1978). Although few moths in our experiment (6%) probed the odor source first, nearly 40% of all moths did so at least once during their trial flights (Fig. 2.6B), providing additional evidence that the sensory requirements and preferences of naïve *M. sexta*,

however strong, are not rigid. The sensory flexibility of foraging hawkmoths is also apparent in the experimental demonstration that appetitive conditioning can override or reverse innate sensory preferences (Kelber, 1996; Balkenius and Kelber, 2006). The results of preference experiments are likely to shift as individual moths gain foraging experience, particularly for hawkmoths that feed from rotting fruit and sap as well as floral nectar (e.g. *Amphion floridensis*), for whom visual cues may be conditionally useful but not essential. Even *M. sexta* will feed from a scentless feeder after it has been in the flight cage for one or two nights (J.G., personal observation). We are now addressing how quantitative aspects of the visual stimulus, such as color, size, contrast and light intensity, as well as larval diet quality, could affect the tendency of *M. sexta* to probe at the visual target rather than at the odor source.

#### *Multi-sensory cues in the study of sensory ecology*

Our experiments allowed us to identify a non-linear relationship between visual and olfactory stimuli and nectar feeding behavior in *M. sexta*. We also showed that the dynamics of sensory stimulation, besides the stimulation *per se*, represent a fundamental component in the decision-making process of *M. sexta*. Olfactory–visual integration appears to be flexible, in the sense that it allows *M. sexta* to search and probe under different temporal patterns of stimulation rather than requiring a prefixed order or concomitance of stimulus presentation. The diversity of growth forms, floral density and sources of odor (flowers vs leaves) in plants pollinated by nocturnal hawkmoths (Haber and Frankie, 1989; Raguso and Willis, 2005) suggests that wild foraging *M. sexta* should encounter variance in the spatial and temporal concomitance of floral visual displays and odor plumes. Our results show that *M. sexta* would be able to identify and feed from flowers under such conditions.

Sensory flexibility, rather than the exception, may prove to be the rule for opportunistic, generalist flower-feeding animals, especially when foraging under different photic conditions or when preferred colors or odors are not available. Recent studies indicate considerable flexibility in the sensory information used by glossophagine bats (von Helversen et al., 2000; von Helversen et al., 2003; Winter et al., 2003) and bumblebees (Saleh et al., 2006) in nectar foraging behavior. Thus, in behavioral studies it may be more fruitful to address sensory systems as interacting sub-systems whose properties contribute to an animal's functional relationship with its environment, rather than as isolated components of their perceptual apparatus.

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**CHAPTER 3**  
**CONTEXT- AND SCALE-DEPENDENT EFFECTS OF FLORAL CO<sub>2</sub> ON**  
**NECTAR FORAGING BY *MANDUCA SEXTA*\***

***Abstract***

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<sub>2</sub>), may function as pollinator attractants. Night-blooming flowers of *Datura wrightii* show transient emissions of up to 200 ppm above-ambient CO<sub>2</sub> at anthesis, when nectar rewards are richest. Their main hawkmoth pollinator, *Manduca sexta*, can perceive minute variation (0.5 ppm) in CO<sub>2</sub> 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<sub>2</sub>, visual, and olfactory stimuli using a laminar flow wind tunnel. Responses in no-choice assays were scale-dependent; CO<sub>2</sub> functioned as an olfactory distance-attractant redundant to floral scent, as each stimulus elicited upwind tracking flights, but it 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<sub>2</sub> over those with ambient CO<sub>2</sub>, whereas females showed similar bias only in the presence of host plant (tomato) leaf volatiles. Nevertheless, all moths probed both flowers regardless of their first choice. While floral CO<sub>2</sub> unequivocally affects male appetitive responses, the context-dependence of female responses suggests that they may use floral CO<sub>2</sub> as a distance indicator of host plant quality during mixed feeding-oviposition bouts on *Datura* and *Nicotiana* plants.

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## ***Introduction***

Anthophilous insects use information from a variety of sensory channels to locate flowers and feed from them (Barth, 1991). 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 (Raguso, 2004a). While flower colors and patterns (Kelber, 2002; Weiss, 1997), whole-flower and nectar odors (Raguso, 2004b), and even corolla shape and texture are known features used by foraging insects and other nectivorous animals (Goyret and Raguso, 2006; Waser, 1983), few studies have explicitly addressed the role of flower respiratory carbon dioxide (CO<sub>2</sub>) as a stimulus involved in plant-pollinator interactions.

Our knowledge of how insects utilize CO<sub>2</sub> 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<sub>2</sub> to locate their animal hosts from a distance (Stange, 1996). Discontinuous CO<sub>2</sub> plumes modulate host-seeking behavior by mosquitoes (Gillies, 1980; Takken and Knols, 1999), suggesting that CO<sub>2</sub> acts as a long-range orientation stimulus (Zwiebel and Takken, 2004). Tsetse flies and biting midges also use CO<sub>2</sub> as a long-range attractant which can synergize the attractiveness of other host odors (Bhasin et al., 2000; Gibson and Torr, 1999). However, CO<sub>2</sub> also functions as a close-range feeding stimulus (on the host's skin) for mosquitoes (Stange, 1996).

Carbon dioxide synergizes the attractiveness of some human skin odors to female yellow fever mosquitoes (Dekker et al., 2005), and has been suggested to synergize triatomine bug responses to L(+)-lactic acid (Barroso and Lazzari, 2004).

Some herbivorous insects show similar responses to CO<sub>2</sub>, although typically at closer range to its source than haematophagous insects. Carbon dioxide alone is sufficient to guide *Diabrotica virgifera* beetle larvae towards corn roots (Bernklau and Bjostad,

1998), and larvae of noctuid (*Helicoverpa armigera*) (Rasch and Rembold, 1994) and pyralid (*Elasmopalpus lignosellus*) (Huang and Mack, 2001) moths orient to above-ambient CO<sub>2</sub> sources. Females of the pyralid moth *Cactoblastis cactorum* search for CO<sub>2</sub> sinks on photosynthetic stem surfaces of *Opuntia stricta*, as indicators of high carbon fixation activity (Stange et al., 1995). In this case, CO<sub>2</sub> 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<sub>2</sub> and other olfactory oviposition stimulants (Stange, 1999). Finally, responses to CO<sub>2</sub> may be context-dependent. Tephritid flies respond to CO<sub>2</sub> when presented with a fruit-like visual stimulus (Stange, 1999). Within a certain concentration range, *Drosophila melanogaster* adults and larvae are repelled by CO<sub>2</sub>, and it has been suggested that these responses depend on the olfactory context (Faucher et al., 2006).

It is clear from the studies reviewed above that CO<sub>2</sub> 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<sub>2</sub> may also contribute to the interactions between flowers and insect pollinators. Floral CO<sub>2</sub> is primarily associated with elevated respiratory activity in thermogenic flowers, including deceptive flowers that mimic carrion and other decaying brood sites (Seymour et al., 2003) (Patiño et al., 2002). Although CO<sub>2</sub> emission is a by-product of elevated floral temperature, the compound stimulus of CO<sub>2</sub>, heat and foul odors most accurately mimics microbial activity in rotting meat (Angioy et al., 2004; Stensmyr et al., 2002). Less well studied are the patterns of CO<sub>2</sub> production in non-deceptive flowers that proffer nectar as an energetic reward. In principle, if newly opening, unvisited flowers emit elevated CO<sub>2</sub>, nectivorous insects could use such information to find the most profitable flowers or patches thereof (Thom et al., 2004). If floral CO<sub>2</sub> levels were ephemeral, insects might utilize them as more “honest” indicators of nectar availability in real time than floral

color or scent (Raguso, 2004a), in much the same way that tarsal secretions are utilized as flower marking cues by foraging bees (Giurfa and Núñez, 1992; Saleh et al., 2006). Given that CO<sub>2</sub> 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; (Guerenstein et al., 2004b), (Raguso et al., 2003a), 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 hours prior to opening (Cruden et al., 1983), (Haber and Frankie, 1989). Flowers of *D. wrightii* open explosively at dusk, releasing concentrations of CO<sub>2</sub> up to 200 ppm above ambient levels (Guerenstein et al., 2004b). Adult *M. sexta* moths can detect such differences with their CO<sub>2</sub>-sensing organ (Labial-palp Pit Organ –LPO), whose threshold may be as low as 0.5 ppm (Guerenstein et al., 2004a; Stange, 1992). In the aforementioned *C. cactorum*, males have vestigial LPOs, whereas females use their well developed ones to detect gradients of CO<sub>2</sub> concentration for oviposition on *Opuntia* cactus. In contrast, the LPOs of *M. sexta* are well developed in both sexes (Kent et al., 1986), suggesting that they contribute to adult nectar foraging rather than, or in addition to, oviposition (Abrell et al., 2005). Thom and collaborators (Thom et al., 2004) 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<sub>2</sub> concentrations (765 ppm) over one emitting ambient levels of CO<sub>2</sub> (438 ppm). These findings provided the first direct evidence that floral CO<sub>2</sub> affects the foraging behavior of adult Lepidoptera.

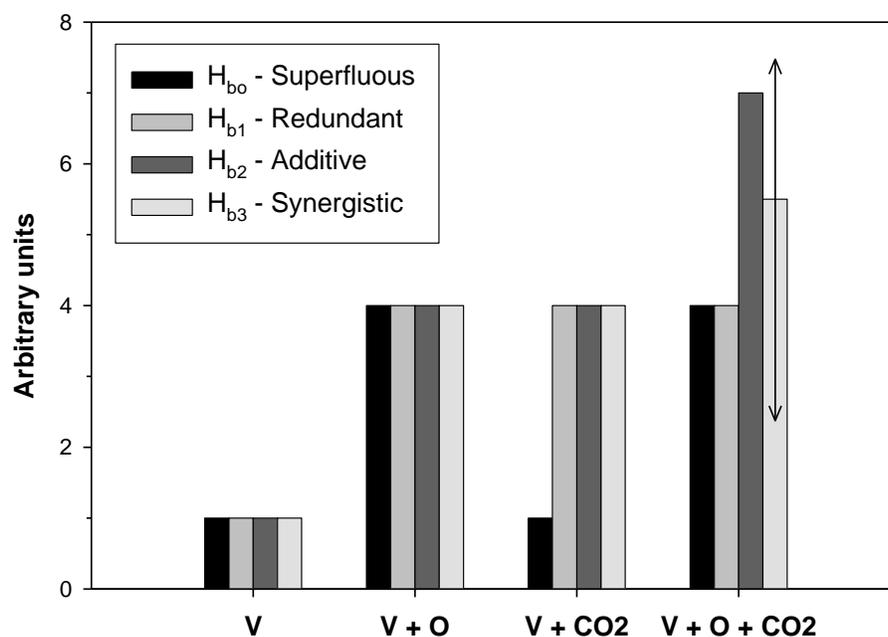
In this study we examined the behavioral mechanisms of CO<sub>2</sub>-mediated nectar foraging in *M. sexta* by addressing three scale- or context-related questions: 1) do *M. sexta* moths use floral CO<sub>2</sub> as an orientation cue or a local feeding stimulus? 2) how does CO<sub>2</sub> interact with visual and olfactory cues during foraging? and 3) do the behavioral responses of moths to floral CO<sub>2</sub> 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 in order to distinguish between alternative hypotheses on the behavioral role(s) of floral CO<sub>2</sub> (Table 3.1, Fig. 3.1).

**Table 3.1. Alternative hypotheses and critical predictions for the behavioral role(s) of floral CO<sub>2</sub> in nectar foraging by *M. sexta*. No choice assays (experiment 1).** Scale-dependence of CO<sub>2</sub>-mediated behavior. Hypotheses:  $H_{A0}$ , CO<sub>2</sub> has no behavioral effect;  $H_{A1}$ , CO<sub>2</sub> is an orientation cue only;  $H_{A2}$ , CO<sub>2</sub> is a local feeding stimulus only;  $H_{A3}$ , CO<sub>2</sub> is both an orientation cue and a local feeding stimulus.

<b>Predictions:</b>	$H_{A0}$	$H_{A1}$	$H_{A2}$	$H_{A3}$
Tracking	$V=V+CO_2$	$V<V+CO_2$	$V=V+CO_2$	$V<V+CO_2$
Probing	$V=V+CO_2$	$V=V+CO_2$	$V<V+CO_2$	$V<V+CO_2$

Additionally, we performed a choice experiment similar to that published by Thom et al. (Thom et al., 2004), but in a laminar flow wind tunnel, in which we tested the foraging behavior of unmated females in addition to males. As male moths already had been shown to choose scented paper flowers with above-ambient CO<sub>2</sub> (Thom et al., 2004), 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<sub>2</sub> are dependent on contextual information provided by host plant odors.



**Figure 3.1. Visualization of predicted outcomes for alternative hypotheses on the behavioral function of floral CO<sub>2</sub> in nectar foraging behavior by *M. sexta* (experiment 1).** The vertical axis represents a unit-free relative measure of moth response (e.g. tracking, 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<sub>2</sub>) or the combination thereof (V+O+CO<sub>2</sub>). Hypothesis names reflect how CO<sub>2</sub> would interact with floral odor. Here, synergism is broadly defined as a non-additive (+ 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<sub>2</sub> elicits the appropriate behavior, has already been falsified for tracking, floral approach and probing by previous experiments (Raguso & Willis 2002, Goyret & Raguso 2006, Goyret et al. 2007).

**Table 3.2. Alternative hypotheses and critical predictions for the behavioral role(s) of floral CO<sub>2</sub> in nectar foraging by *M. sexta*. No-choice assays (experiment 1).** Multimodal interactions between CO<sub>2</sub>, visual, and olfactory stimuli in moth feeding. Hypotheses:  $H_{B0}$ , addition of CO<sub>2</sub> has no effect (superfluous);  $H_{B1}$ , addition of CO<sub>2</sub> is redundant to floral odor;  $H_{B2}$ , addition of CO<sub>2</sub> is additive to floral odor;  $H_{B3}$ , addition of CO<sub>2</sub> is synergistic (+ or -) to floral odor.

Predictions:	$H_{B0}$	$H_{B1}$	$H_{B2}$	$H_{B3}$
Probing	$V+O=V+O+CO_2$ $V=V+CO_2$	$V<V+O, V+CO_2$ $V+O=V+CO_2$ $V+O=V+O+CO_2$	$V+O+CO_2 =$ $(V+O)+(V+CO_2)$	$V+O+CO_2 \neq$ $(V+O)+(V+CO_2)$

### *Materials and Methods*

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

#### *Animals*

We used *Manduca sexta* adults reared from eggs provided by Dr. Lynn Riddiford, University of Washington, Seattle, WA. Larvae were fed ad libitum on an artificial diet (Bell and Joachim, 1976) and were kept as pupae under a 16:8 light/dark, 24/21°C cycle. Moths were separated by sex as pupae, and were housed in different incubators (Precision 818, Winchester, VA) under the same ambient regime and emerged within 45 x 45 x 45cm screen cages (BioQuip, Inc., Rancho Dominguez, CA). 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 (not shown) revealed that the paper absorbed UV wavelengths but reflected light nearly uniformly from 400 to 700 nm. Volatile analysis (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  $\mu$ l of bergamot essential oil (Body Shop, Columbia, SC) and was refreshed every 30 min in order to standardize odor intensity. This odor source is a reliable feeding stimulant for *M. sexta* (Goyret and Raguso, 2006) and, like many night-blooming flowers visited by this species, is dominated by linalool and related monoterpenoid odors (Raguso and Pichersky, 1999). The wooden stem of the swab (2.5 cm) was affixed to a 1 cm<sup>3</sup> piece of dark grey 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<sub>2</sub>): Carbon dioxide was delivered from a CO<sub>2</sub> tank (National Welders Inc., Charlotte, NC, USA) through Teflon tubing directly into a blue aquarium “air stone” positioned at the bottom of the artificial flower. Treatments with no CO<sub>2</sub> emission were set up in the same way, but with the CO<sub>2</sub> tank in the closed position. Ambient and ‘flower-emitted’ CO<sub>2</sub> levels were measured with a Licor 840 CO<sub>2</sub>/H<sub>2</sub>O detector (Licor, Inc., Lincoln, NE, USA) previously calibrated with 3 different CO<sub>2</sub> concentrations (392, 798, 1032 ppm  $\pm$  2%). We obtained the following calibration curve:  $R = (A - 8.1199) / 0.0432$ ; where A is the actual CO<sub>2</sub> concentration and R is the one read by the Licor detector. This linear regression was statistically significant ( $p < 0.05$ ) with an  $R^2 = 0.99$ . Carbon dioxide emission from the artificial flower was set at a flow of 10 mL/min, achieving a CO<sub>2</sub> concentration at the flower model opening of  $787 \pm 33$  ppm (mean  $\pm$  s.e.m.), based on concentrations measured by Thom and collaborators (Thom et al., 2004).

### *General procedure in the wind tunnel and recorded variables*

At the beginning of scotophase naïve, starved adult moths were placed individually at the down-wind end of a 3x1.5x1.5m wind tunnel with a laminar flow rate of 1m/s. Each moth was allowed to fly freely inside the wind tunnel for 5 minutes, during which its behavior was recorded.

### *Experiment 1 (No Choice Assay)*

In order 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 up-wind end of the wind tunnel. The first treatment encompassed the artificial flower alone (only visual display as an attractant, henceforth: ‘V’). The following two treatments consisted of the addition of either odor (henceforth: ‘V+O’) or CO<sub>2</sub> (henceforth: ‘V+CO<sub>2</sub>’). Finally, the fourth treatment consisted of the addition of both odor and CO<sub>2</sub> (henceforth: ‘V+O+CO<sub>2</sub>’). In all cases, the surrogate flower was identically attached to a ring-stand, with an air-stone diffuser. In treatments lacking CO<sub>2</sub>, 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: 1) showed the stereotypic anemotactic “zigzag” odor plume tracking behavior (Vickers, 2000), 2) approached (i.e. hovered in front of) and 3) probed at the artificial flower at least once with their extended proboscides. We also recorded the amount of time (Latency, sec) during which moths flew inside the tunnel before probing the artificial flower, and the time spent probing (Probing time, sec).

### *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 from each other and equidistant to the release site of the moths, at the up-wind end of the wind tunnel. This distance effectively decouples visual and olfactory stimuli under our test conditions (Goyret et al. 2007), reducing the potential for ambiguous binary choices by moths. The physical characterization of CO<sub>2</sub> plumes is beyond the scope of this paper, 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<sub>2</sub> emission) vs. the one emanating odor plus above ambient CO<sub>2</sub>. As in experiment 1, we also recorded Latency and Probing time at each flower model.

### *Experiment 3 (Binary Choice Assay with Hostplant)*

Given the gender asymmetries found in experiment 2 (see Results section below), the possible role that CO<sub>2</sub> may play in oviposition behavior (Abrell et al., 2005), and the observation that female *M. sexta* may show mixed bouts of nectar feeding and oviposition (Adler & Bronstein 2004), we decided to investigate responses to floral CO<sub>2</sub> in the presence of stimuli related to oviposition (host plant volatiles). Thus, in this experiment we replicated the design of experiment 2, but now we positioned four tomato plants (*Solanum lycopersicum*; cultivar: “Better boy”, South Carolina Farmers Market, Columbia, SC) one meter up-stream from the flowers. This provided the odor of undamaged host plant vegetation in addition to bergamot oil and CO<sub>2</sub>. 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.

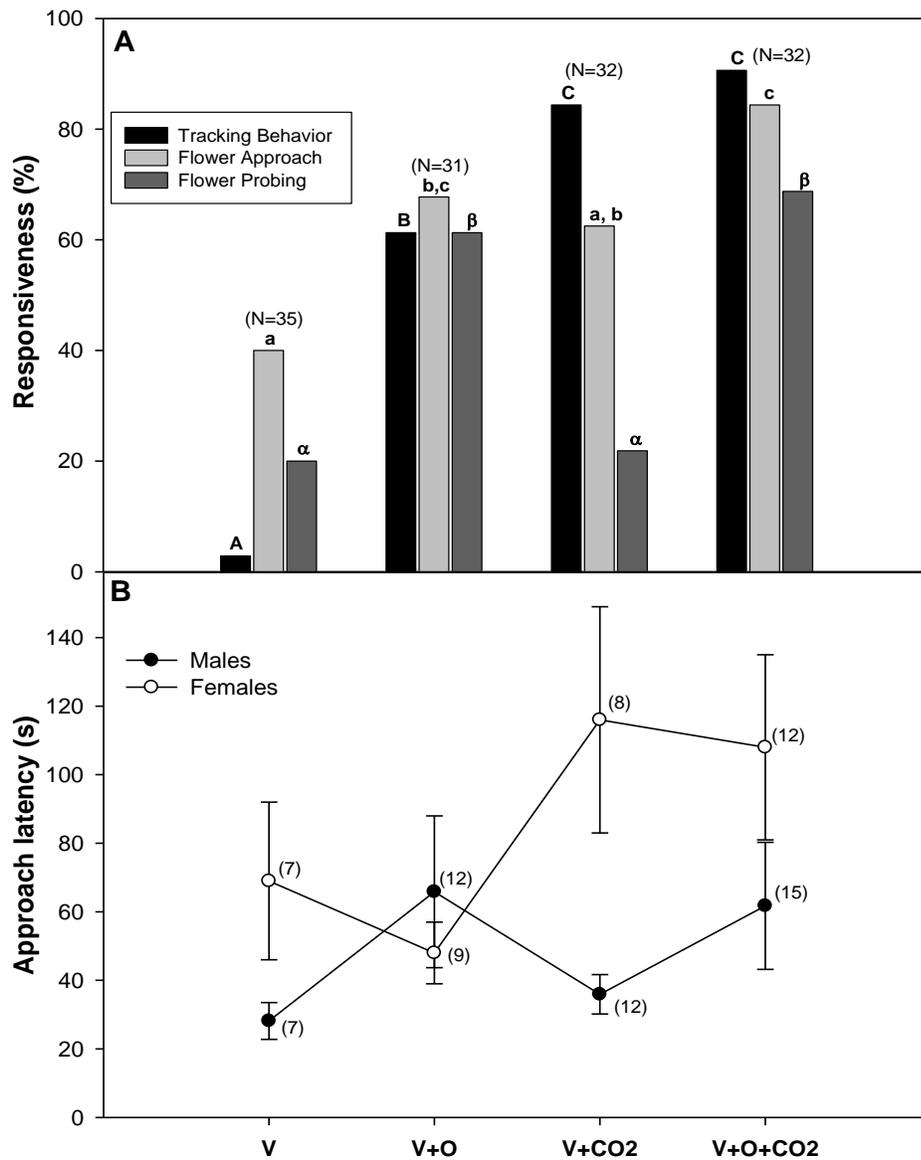
### *Statistical Analysis*

The effects of treatment on the display of tracking, approach and probing behaviors were analyzed by means of G-tests. Latency and Probing times were analyzed by means of a 2-way ANOVA, accounting for treatment, gender and their interaction. For this analysis, data from experiment 1 were log-transformed. Chi-squared tests of independence were performed to determine any deviation from a random choice pattern in experiments 2 and 3.

### **Results**

#### *Experiment 1*

Tracking, approaching and probing behaviors all were significantly affected by treatment (Gh=78.36;  $p < 0.0001$ ; Gh=15.05;  $p = 0.0018$ ; Gh=27.35;  $p < 0.0001$ , respectively. N=130; Fig. 3.2A). Tracking behavior (Figure 3.2A, black bars) essentially was not observed in the 'V' treatment, but was elicited either by adding odor ('V+O'; Gh=30.51;  $p < 0.0001$ ; N=66) or above-ambient CO<sub>2</sub> emissions to the corolla opening ('V+CO<sub>2</sub>'; Gh=54.25;  $p < 0.0001$ ; N=67). Surprisingly, model flowers emitting CO<sub>2</sub> elicited the most tracking displays ('V+O' vs. 'V+CO<sub>2</sub>'; Gh=4.35;  $p = 0.037$ , N=63) and additive effects were not observed with the addition of odor ('V+CO<sub>2</sub>' vs. 'V+O+CO<sub>2</sub>', Gh=0.58;  $p = 0.45$ ; N=64). Conversely, approaches (Figure 3.2A, light-grey bars) increased with the presence of odor (Gh=5.16;  $p = 0.02$ ) but were not significantly affected by the addition of CO<sub>2</sub> 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<sub>2</sub>'; Gh=2.44;  $p = 0.12$ ). Finally, probing responses were not affected by CO<sub>2</sub>, either alone ('V' vs. 'V+CO<sub>2</sub>'; Gh=0.036;  $p = 0.85$ ) or in addition to odor ('V+O' vs. 'V+O+CO<sub>2</sub>' Gh=0.39;  $p = 0.53$ ), but there was a gender difference



**Figure 3.2. Responses of adult *M. sexta* in a wind tunnel with a single surrogate flower.** (A) Responsiveness (%) of the recorded behaviors. Categories in the abscissa represent the different sets of stimuli offered by the surrogate flower: ‘V’: Unscented visual stimulus, ‘V+O’: Scented visual stimulus; ‘V+CO<sub>2</sub>’: Unscented visual stimulus with above-ambient levels of CO<sub>2</sub>; ‘V+O+CO<sub>2</sub>’: Scented visual stimulus with above-ambient levels of CO<sub>2</sub>. Different type of letters (Capitol, lower-case and Greek) are used for comparisons between treatments for each behavior. Different letters indicate significant differences (see Results). (B) Latencies to approach (i.e. time from take-off to flower approach – in seconds) of male and female moths. Data points and error bars represent mean ± s.e.m. Number of replicates for each treatment in parentheses.

in the 'V+O' treatment, where males were more responsive than females (85% and 45% of responsive animals respectively;  $G_h=5.49$ ;  $p=0.02$ ). 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<sub>2</sub> (for 'V+CO<sub>2</sub>': 24%-males- and 20%-females; for 'V+O+CO<sub>2</sub>': 71%-males- and 67%-females).

There was no significant treatment effect on the latency time (2-way ANOVA;  $F_{(3, 74)}=0.51$ ;  $p=0.67$ ) but a strong gender effect ( $F_{(1, 74)}=7.25$ ;  $p=0.009$ ), with females and males averaging  $87\pm 13$  seconds and  $51\pm 9$  seconds respectively (mean  $\pm$  s.e.m.). This was probably due to the females' tendency for increased latencies in the presence of CO<sub>2</sub> ('V+O vs. V+CO<sub>2</sub>':  $F_{(1, 15)}=4.40$ ;  $p=0.05$ ; 'V+O vs. V+O+CO<sub>2</sub>':  $F_{(1, 19)}=3.52$ ;  $p=0.08$ ; see Figure 3.2B). No significant interaction between gender and treatment was detected ( $F_{(3, 74)}=0.84$ ;  $p=0.48$ ). Probing times were affected neither by treatment nor by 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*

Roughly 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<sub>2</sub>' flower instead of the 'V+O' flower (Chi-squared test;  $p=0.04$ ;  $N=60$ ; Figure 3.3). However, when analyzing gender differences we found this result to be due exclusively to male behavior (Chi-squared test for males;  $p=0.002$ ;  $N=28$ . For females;  $p=1.00$ ;  $N=32$ ). Females took  $64\pm 15$  seconds from take off to probing on the 'V+O+ CO<sub>2</sub>' flowers, and  $61\pm 18$  seconds on 'V+O' flowers. For males, latency times were  $48\pm 7$  s and  $28\pm 7$  s respectively on these treatments. However, latencies did not differ significantly between gender when analyzed via ANOVA ( $F_{(1, 58)}=1.91$ ;  $p=0.17$ ).

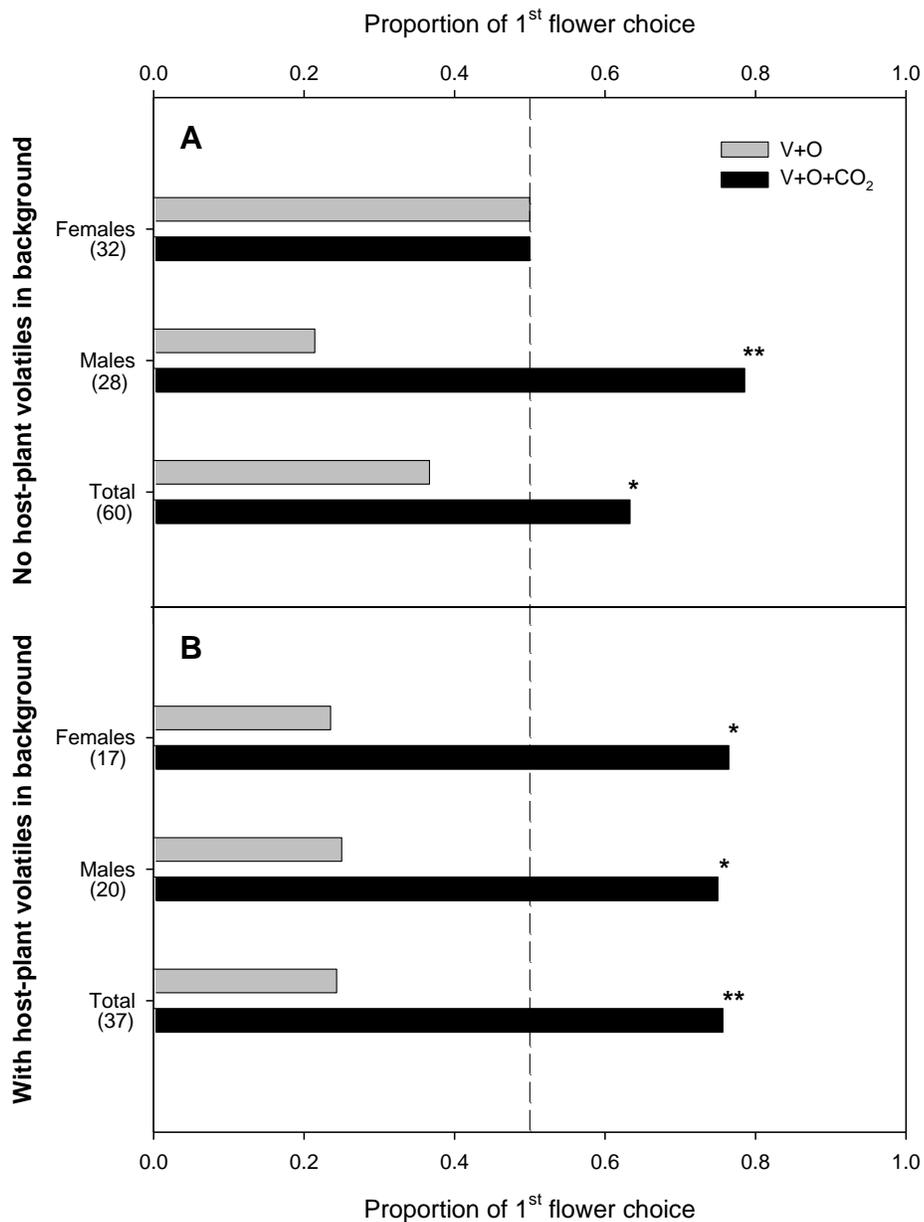
Analysis also showed that neither females nor males differed significantly in latency times when responding to the different flower models (Females:  $F_{(1; 30)}=0.01$ ;  $p=0.91$ ; Males:  $F_{(1;26)}=2.00$ ;  $p=0.17$ ), but females tended to probe the ‘V+O+ CO<sub>2</sub>’ flower for a longer time ( $7\pm 1$  s vs.  $4\pm 1$  s;  $F_{(1; 30)}=3.87$ ;  $p=0.06$ ), while males did not show any probing time difference between the two flower models ( $7\pm 2$  s probing on the ‘V+O+ CO<sub>2</sub>’ flower and  $9\pm 4$  s 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<sub>2</sub>’ flower (28 out of 37; Chi-squared test,  $p=0.002$ ). The addition of background tomato plant volatiles did not change first choice preferences in males (Fig. 3.3), which first chose the ‘V+O+ CO<sub>2</sub>’ flower (Chi-squared 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 towards the ‘V+O+ CO<sub>2</sub>’ flower over the ‘V+O’ flower (Chi-squared test,  $p=0.018$ ,  $N=17$ ; Fig. 3.3).

### *Discussion*

Over 200 years ago, Sprengel (Sprengel, 1793) first recognized that foraging animals utilize diverse components of floral display (e.g. color, shape, texture, 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 (Chittka and Thompson, 2001). Few studies, beyond those of thermogenic “trap flowers” (Seymour et al., 2003) and figs (Galil et al., 1973), have considered the potential importance of more



**Figure 3.3. Proportion of first flower choices by adult *M. sexta*.** Dual choice assays between two scented surrogate flowers, of which only one emitted above-ambient CO<sub>2</sub> levels (V+O+CO<sub>2</sub>). Assays were performed in a wind tunnel either without (upper panel) or with (lower panel) four tomato plants at the up-stream end. \* Significant departure from random probabilities with an  $\alpha$ -level of 0.05. \*\*Same, with an  $\alpha$ -level of 0.005.

basic floral cues, such as respiratory CO<sub>2</sub>, to pollinator behavior. The results of our experiments, consistent with those of Thom et al. (Thom et al., 2004), confirm that floral CO<sub>2</sub> can be used as a foraging stimulus by *Manduca sexta*, a nectivorous insect with high metabolic demands (Heinrich, 1971), 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<sub>2</sub> accumulation in hives (Seeley, 1974) and thermogenic flowers, respectively (Seymour et al., 2003).

Nectar foraging by naive *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 (Brantjes, 1973; Raguso and Willis, 2002) floral approach is elicited by olfactory and/or visual stimuli (Raguso and Willis, 2002) that are most effective when combined (Goyret et al., 2007), after which probing is guided by visual and tactile inputs (Goyret and Raguso, 2006). Upon closer inspection, these behavioral “rules” are somewhat flexible, depending upon the spatial and temporal scale (Goyret et al., 2007) and the context in which specific stimuli are perceived (e.g. after appetitive conditioning; (Daly et al., 2001; Daly and Smith, 2000)). With this framework in mind, we explored how CO<sub>2</sub> might interact with other floral stimuli in behavioral assays with *M. sexta*.

#### *Scale-dependence of responses to CO<sub>2</sub> in no-choice assays*

Our first approach (experiment 1) was to decouple floral traits to examine how CO<sub>2</sub> affects nectar foraging by *M. sexta* at artificial flowers with different subsets of sensory stimuli. As outlined in Table 3.1, our goal was to determine the scale (if any) at which CO<sub>2</sub> affects appetitive responses, using no-choice behavioral assays. The

tracking and probing behaviors observed (Figure 3.1a) allow us to reject the hypotheses stating that CO<sub>2</sub> has no behavioral effect (H<sub>A0</sub>) or acts as a local feeding stimulus (H<sub>A2</sub>). Instead, our results support the hypothesis that CO<sub>2</sub> acts as an orientation stimulus (Table 3.1, H<sub>A1</sub>). The CO<sub>2</sub> plume evoked the typical zig-zag casting flight pattern indicative of odor-guided behavior in *M. sexta* (Mechauber et al., 2002; Raguso et al., 2003a; Willis and Arbas, 1991) and was slightly more likely to elicit upwind casting flight than was floral odor. These results suggest that floral CO<sub>2</sub> is perceived by *M. sexta* as an odor, a hypothesis that is consistent with the fact that CO<sub>2</sub>-receptor cells in the LPO project their axons through the suboesophageal ganglion into both antennal lobes (Guerenstein et al., 2004a), the primary centers for the processing of olfactory inputs from the antennae.

Even though CO<sub>2</sub> alone ('V+CO<sub>2</sub>') had an effect on tracking behavior, approaches to the flower did not differ significantly from the negative control (i.e. 'V', Fig.3.1a). Probing behavior was unaffected by the presence of CO<sub>2</sub>, with and without floral odor, which allows us to reject the hypotheses of redundant (H<sub>B1</sub>), additive (H<sub>B2</sub>) and synergistic (H<sub>B3</sub>) effects in favor of the null hypothesis (H<sub>B0</sub>; Table 3.1). Thus, in no-choice assays, CO<sub>2</sub> affects the foraging behavior of naive *M. sexta* as an orientation stimulus. Additionally, CO<sub>2</sub> 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<sub>2</sub> (Fig. 3.1b). Also, females were less responsive than males when no CO<sub>2</sub> 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<sub>2</sub> effectively elicits orientation towards flowers with no apparent effect on the probing response at close range, we designed experiment 2 in order to evaluate whether or not the tracking response elicited by CO<sub>2</sub> 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<sub>2</sub> (V+O+CO<sub>2</sub>), but that males significantly preferred the latter (Fig. 3.3a). When host-plant (tomato vegetation) odors were added upstream of the flowers (experiment 3), males maintained the CO<sub>2</sub>-bias observed in experiment 2, but females now chose to probe the flower with above-ambient CO<sub>2</sub> in the same proportion as did males (Fig. 3.3b). Our assays, like those of Thom et al. (Thom et al., 2004), presented moths with a binary choice between single flowers with markedly different local CO<sub>2</sub> 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 which did not overlap near their sources, whereas Thom et al. (Thom et al., 2004) mounted artificial flowers on *Datura wrightii* plants in a small flight cage, with no air flow. These observations suggest that *M. sexta* can perceive differences in CO<sub>2</sub> on the scale of a single plant, amidst background contributions from its vegetation (e.g. Abrell et al. 2005). It remains to be tested whether moths can distinguish between closely spaced individual flowers with different CO<sub>2</sub> 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<sub>2</sub>, whether host plant odors are present or not (Fig. 3.3), suggesting that their responses to floral CO<sub>2</sub> 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. 3.3). Mechauber et al. (Mechauber et al., 2002) 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, three days after eclosion. Thus, our unmated females – all of them three or more days post-eclosion- 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*; (Mechauber et al., 2002)), and derive a ten-fold fitness benefit in number of eggs matured when they consume nectar as adults (Ziegler, 1991). The observation that female *M. sexta* lay more eggs on plants with experimentally enhanced nectar volumes (Adler and Bronstein, 2004) prompted Kessler and Baldwin (Kessler and Baldwin, 2007) to suggest that females use nectar as a proxy measure of host plant quality. In experiment 2, CO<sub>2</sub> had no bearing on the first flower probed by females when host-plant odors were absent, while the addition of tomato odors in experiment 3 resulted in a first choice preference for the above-ambient CO<sub>2</sub> 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<sub>2</sub> were probed. Following Kessler and Baldwin (Kessler and Baldwin, 2007) and considering the positive correlation between nectar production and CO<sub>2</sub> emission found by Guerenstein and collaborators (Guerenstein et al., 2004b) we suggest that

floral respiratory CO<sub>2</sub>, when presented in the context of host plant odors, is used by female moths as a long-distance indicator of host plant quality, releasing up-wind orientation flight as shown here. Both floral nectar and CO<sub>2</sub> 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<sub>2</sub>; then, floral nectar could act as a local indicator of host plant quality as proposed by Kessler and Baldwin (Kessler and Baldwin, 2007).

#### *Conditionality and opportunism in the use of floral stimuli*

We have documented two dimensions of sensory conditionality in the use of floral CO<sub>2</sub> by *M. sexta* during nectar foraging. The first dimension was scale-dependence, such that CO<sub>2</sub> functioned as a redundant stimulus (with floral odor) for distance orientation and floral approach, but was superfluous as a local feeding stimulus (Fig. 3.1). This pattern calls to mind the phenomenon of post-pollination color change, in which older flowers turn color but remain turgid and are retained on the plant (rev. by Weiss (Weiss, 1995)). Such flowers, while unrewarding, enhance pollinator attraction from a distance, but are ignored once the insects arrive at the inflorescence, due to learned color preferences (Weiss, 1991). The second dimension of conditionality was context-dependence, such that male first-visit preference for flowers with above-ambient CO<sub>2</sub> 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. 3.2). For these hawkmoths, responses to floral CO<sub>2</sub> clearly depend upon the sex of the moth and the conditions under which it receives this stimulus, as non-host plant odor plumes attract both sexes of *M. sexta* in the absence of CO<sub>2</sub> (Goyret et al., 2007). The context-dependent responses of female moths to the

combined stimulus of host plant odor and CO<sub>2</sub> are conflated with non-appetitive behaviors likely to be associated with oviposition as discussed above.

Given the multitude of scales, contexts and mechanisms by which CO<sub>2</sub> functions in plant-insect interactions, the conditionality of its significance in nectar foraging by *M. sexta* is not surprising. The perception of CO<sub>2</sub> as a floral odor by *M. sexta* adds to the small but growing list of “secondary functions” (e.g. pollinator attraction, herbivore deterrence) attributable to primary plant metabolites among plant-insect interactions (Berenbaum, 1995; Brenes-Arguedas et al., 2006). Floral CO<sub>2</sub> is a stimulus whose relevance to moth foraging behavior depends upon the specific task at hand (e.g. oviposition) and the extent to which other floral information is available. In night blooming plants such as *Datura wrightii*, floral CO<sub>2</sub> might provide the most honest indication of nectar profitability to foraging moths, as it decreases markedly within the first hour after anthesis (Guerenstein et al., 2004b), when nectar feeding by *M. sexta* is most intense (Raguso et al., 2003b; Raguso and Willis, 2005), while floral odor and visual display remain unchanged after nectar has been drained from individual flowers (Guerenstein et al., 2004b; Thom et al., 2004). Future experiments should test the extent to which moths’ foraging behavior in a natural setting reflects local differences in floral CO<sub>2</sub>, and whether females are capable of utilizing such information at non-host nectar sources such as *Oenothera caespitosa* (Onagraceae) and *Mirabilis longiflora* (Nyctaginaceae). We have found increased CO<sub>2</sub> levels at the corolla openings of these night blooming plants ( $1912 \pm 195$  p.p.m.; N=8, and  $1281 \pm 173$  p.p.m.; N=9, respectively), which also are visited by *Manduca sexta* and related hawkmoths (Grant and Grant, 1983; Gregory, 1964).

At the same time, our results call for more detailed investigations of the role of CO<sub>2</sub> in mixed nectar foraging-oviposition bouts shown by female *M. sexta* in nature.

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## CHAPTER 4

### WHY DO *MANDUCA SEXTA* FEED FROM WHITE FLOWERS? INNATE AND LEARNT COLOUR PREFERENCES IN A HAWKMOTH\*

#### *Abstract*

Flower colour is an important signal used by flowering plants to attract pollinators. Many anthophilous insects have an innate colour preference that is displayed during their first foraging bouts and which could help them locate their first nectar reward. Nevertheless, learning capabilities allow insects to switch their colour preferences with experience and thus, to track variation in floral nectar availability. *Manduca sexta*, a crepuscular hawkmoth widely studied as a model system for sensory physiology and behaviour, visits mostly white, night-blooming flowers lacking UV reflectance throughout its range in the Americas. Nevertheless, the spectral sensitivity of the feeding behaviour of naïve moths shows a narrow peak around 450 nm wavelengths, suggesting an innate preference for the colour blue. Under more natural conditions (i.e. broader wavelength reflectance) than in previous studies, we used dual choice experiments with blue- and white-colored feeders to investigate the innate preference of naïve moths and trained different groups to each colour to evaluate their learning capabilities. We confirmed the innate preference of *M. sexta* for blue and found that these moths were able to switch colour preferences after training experience. These results unequivocally demonstrate that *M. sexta* moths innately prefer blue when presented against white flower models and offer novel experimental evidence supporting the hypothesis that learning capabilities could be involved in their foraging preferences, including their widely observed attraction to white flowers in nature.

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## ***Introduction***

The initial foraging success of anthophilous insects depends upon their sensory capabilities, mainly vision and olfaction, in locating rewarding flowers (Brantjes 1976; Frisch von 1967). Many insects use their visual system to locate flowers (Chittka and Menzel 1992) and show an innate preference for certain flower colours (Giurfa et al. 1995; Kelber 1997). This preference is seen as a feature of an innate search image—which may include flower colour, pattern, shape and odour—necessary for inexperienced foragers to locate their first nectar source (Giurfa et al. 1995; Kelber 1996, 1997). Nevertheless, many flower-visiting insects can switch colour preferences through experience (e.g. honeybees (Menzel et al. 1974); bumblebees (Gumbert 2000); butterflies; *Pieris rapae* (Lewis and Lipani 1990); *Battus philenor* (Weiss 1997); and the diurnal hawkmoth, *Macroglossum stellatarum* (Kelber 1996). Because nectar availability is highly variable (Bell 1986), the ability to switch from innate preferences would allow nectivorous insects to track the current floral market (review: Chittka et al. 1999).

*Manduca sexta* is a crepuscular, nectar-feeding hawkmoth common to guilds of long-tongued Sphingidae that pollinate white, night-blooming flowers throughout the Americas (Baker 1961; Galliot et al. 2006; Grant 1983; Haber and Frankie 1989; Hoballah et al. 2007; Moré et al. 2007; Raguso et al. 2003). Richard White and colleagues (Cutler et al. 1995; White et al. 1994) used artificial flowers to explore the spectral sensitivity of feeding behaviour by *M. sexta*, according to the absorption maxima of its three visual pigments (P357 [ultraviolet = UV], P450 [blue], P520 [green]). Flower models that reflected or transmitted UV wavelengths were shown to inhibit feeding, consistent with the observation that “white” flowers visited by *M. sexta* and similar hawkmoths in nature generally lack UV reflectance (White et al. 1994; Raguso et al. 2003). In contrast, feeding behaviour by naive *M. sexta* showed a

narrow peak in the (blue) 450 nm wavelength (and a low secondary peak in the—green —560 nm wavelength; Cutler et al. 1995), suggesting an innate preference for blue. When presented with two flowers with the same spectral properties, these moths consistently preferred the most highly reflective of the two flowers, suggesting that *M. sexta* evaluate multiple components of visual stimuli.

It seems counterintuitive that an animal which mostly encounters and feeds from white (non-UV) flowers should show an innate preference for blue. Here, we use dual choice experiments to examine the responses of naïve *M. sexta* adults to white (non-UV-reflective) vs. blue artificial flowers and test the hypothesis that *M. sexta* possess the ability to alter their innate colour preference through visual learning. Our results support the idea that this capability could be involved in their natural foraging decisions. Although olfactory conditioning has been studied in *M. sexta* (e.g. Daly et al. 2001), our study provides the first example of visual learning in this important model system for sensory physiology. Finally, we present evidence showing that larval diet, previously shown to affect the anatomy and physiology of the adult visual system (Bennett and White 1989), does not affect foraging preferences (innate and learnt) under our experimental paradigm.

### ***Materials and Methods***

Experiments were run during October–November of 2005 at Lund University, Lund, Sweden (henceforth: LU) and during July–August of 2006 at the University of South Carolina, Columbia, SC, U.S.A. (henceforth: USC).

### ***Animals***

We reared *M. sexta* from eggs provided by Dr. Lynn Riddiford, from her laboratory colony at the University of Washington, Seattle, WA, USA, under a photoperiod of

16:8 L:D cycle. The amount of photopigment precursor in larval diets previously was shown by Bennett and White (1989) to affect the visual system anatomically and physiologically. Thus, we tested whether moths reared on different larval diets showed differences in innate colour preference and/or visual learning abilities. Larvae were fed artificial diet (Bell and Joachim 1976) or *Nicotiana tabacum* plants (seed lot GL350, GoldLeaf Seed Co., Hartsville, SC). Additional animals were reared on artificial diet supplemented with 800 mg of  $\beta$ -carotene/L. Moths were starved for 3–4 days after eclosion to increase feeding motivation. Females were used because they survive for longer periods than males, making them more suitable for training (see below). Moths were tested within the first 2 h of their scotophase (dark period).

#### *Flight cage, feeders and general procedure*

The flight cages (45×45×45 cm) were covered with white cloth (constituting the feeders' background), with a transparent plastic sheet on one side, through which the moths' behaviour was recorded. Fifteen white LED lamps (Ledtronics, Inc., CA, USA) provided a uniform illumination of 0.08 cd m<sup>-2</sup> (similar to twilight). We placed two artificial feeders (Fig. 4.1; see Pfaff and Kelber 2003) in the flight cage. Feeders were placed 20 cm from each other, above black covered water bottles, 25 cm above the cage floor (vertically). One feeder was white to the human eye with low UV reflectance (henceforth: white), and the other feeder was dark blue to the human eye (henceforth: blue; Fig. 4.2c, as measured with a S2000 spectrophotometer, Ocean Optics Inc., Dunedin, FL, USA). We also provide reflectance spectra of a blue flower, *Pulmonaria obscura* (Boraginaceae) and two white flowers, *Datura wrightii* and *Oenothera neomexicana* (Fig. 4.2a, as measured with a SI440 CCD array UV–VIS Spectrophotometer—Spectral Instruments, Tucson, AZ). *O. neomexicana* and *D. wrightii* flowers were previously used to demonstrate the synergistic effect of visual

and odour cues mediated nectar feeding by *M. sexta* (Raguso and Willis 2002, 2005) and are representative of the white (no UV), night-blooming flowers visited by *M. sexta* and similar hawkmoths throughout the Americas (White et al. 1994). *P. obscura* is a typical blue flower, visited by bees and diurnal hawkmoths (Chittka et al. 1994; Müller 1881), and very similar in colour to our blue feeder (Fig. 4.2b and d). Additionally, we give a quantitative description of colour differences as perceived by *M. sexta* by providing the colour loci of flowers and feeders (Fig. 4.2b and d, respectively) within the perceptual colour space of *Deilephila elpenor*, a related sphingid moth (Johnsen et al. 2006).

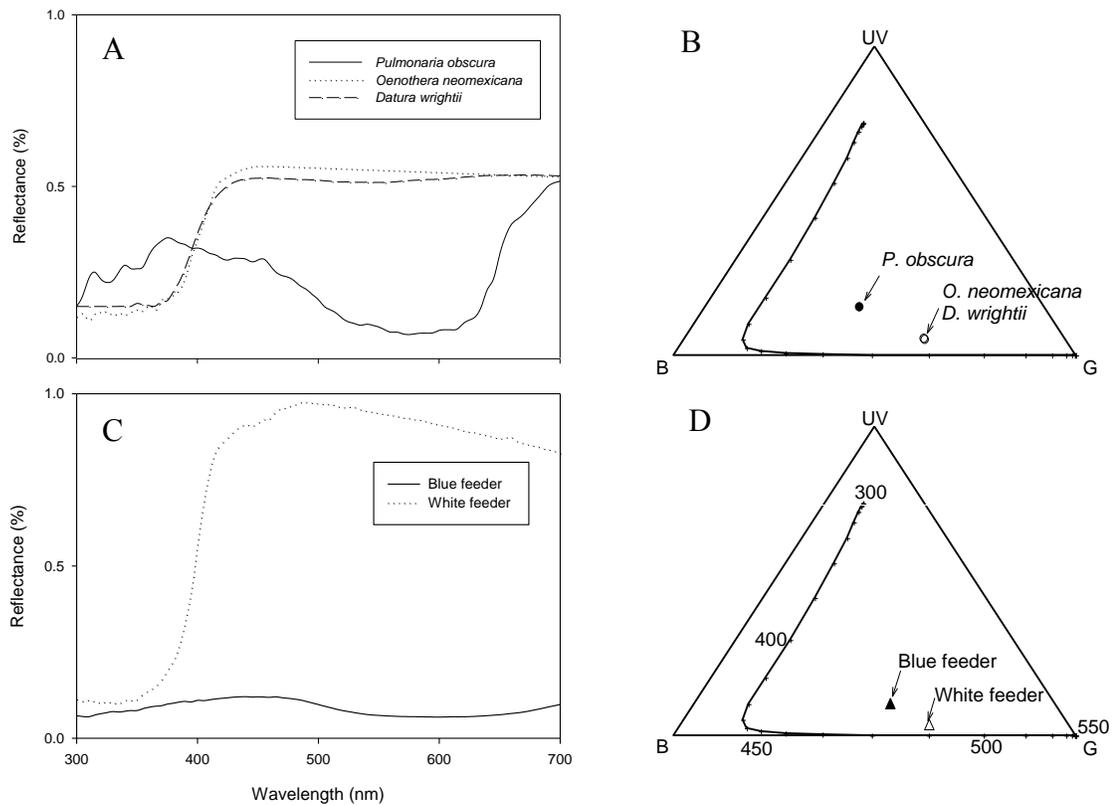


**Figure 4.1. *Manduca sexta* feeding from a blue feeder.** The receptacle is positioned 25 cm. above the cage floor.

### *Naïve preference, training and test*

Naïve moths were released individually and observed for up to 5 min. The first feeding choice between the two rewarding feeders (“naïve preference”) and its latency (i.e. time from takeoff to probing) were recorded. Afterwards, groups of 10 to 15 individuals were placed within additional flight cages containing two feeders exactly as described, but with only one feeder containing sucrose solution (20% w/w). Each group of moths was trained to blue or white feeders (with only the blue or only the white feeder being rewarding, respectively), but not both. After 5 days, during which rewarding feeders were daily replenished, trained moths were kept without feeders in their training cage for 1 day, and were tested the following day as in the naïve preference experiment. However, in this case both feeders were empty and we recorded up to five choices, allowing moths to fly for up to 15 min. Throughout the experiment, the relative position of the feeders was changed randomly, always symmetrically in relation to the cage. To increase moths’ feeding responsiveness, the sucrose solution used in the naïve preference test was scented with 25 µl/L of pure Bergamot oil extract, as discussed by Goyret et al. (2007). Training phases were run with unscented sucrose solutions.

We performed binomial tests (paired comparisons) and G-tests (overall treatment effects) to determine whether naïve preferences, the effect of training and the effect of diet in naïve and trained moths’ preferences differed from null expectations. We used ANOVA to test for diet effects on latency times of naïve moths.



**Figure 4.2. Reflectance spectra and colour loci.** (A) Flower reflectance spectra of typical white flowers with low UV reflectance visited by *Manduca sexta* (*D. wrightii* and *O. neomexicana*) and one blue flower (*P. obscura*). (B) Colour loci of flowers shown in a) in the perceptual colour space of *Deilephila elpenor*, a related hawkmoth. (C) Reflectance spectra of the two feeders used in this study. (D) Colour loci of the feeders shown in (C). Colour loci for flowers and feeders were calculated using the methods described in Johnsen et al. (2006).

## Results

Nearly 75% of the naïve moths fed in the flight cage. In their first foraging bout, naïve *M. sexta* showed a latency time of  $65 \pm 9$  s (Latency, mean  $\pm$  s.e.m., Table 1) and a strong preference for blue (38 moths out of 47—80%— $p < 0.0001$  in a binomial test against random probabilities, i.e.  $P(\text{Blue}) = 0.5$ ; Table 1 and Fig. 4.3a).

A total of 65 moths were trained (LU and USC), of which 58 survived and 56 responded in the tests. Thirty-eight moths were trained to the white feeder and tested (23 in LU and 15 in USC). After training to the white feeder, most moths changed from their innate blue preference to a learnt, effective white preference; 20 out of 23 in LU and 12 out of 15 in USC chose the white feeder ( $p=0.0002$  and  $p=0.014$ , respectively; Binomial test; see Table 1 and Fig. 4.3b). This learnt preference was maintained at least until the fifth unrewarded choice (Binom. test;  $p=0.033$ ; Fig. 4.3c). To control for any unspecific change in colour preference due to aging, 18 moths were trained at USC to the blue feeder, and were tested as previously described (see “Materials and methods”). Fourteen moths chose the blue feeder after training, showing that preference reflects training experience, not age ( $p=0.012$ ; Binomial test; Table 1 and Fig. 4.3b). Finally, no effects of larval diet were seen on innate preferences, nor on learning performance (see Table 1 for statistical results).

### ***Discussion***

Our results confirm that foraging *M. sexta* moths display an innate bias for blue (Fig. 4.3a), as was shown previously by White and collaborators (Cutler et al. 1995) using different experimental conditions and stimuli. Here, we show, for the first time, that blue is not only preferred to other spectral colours but also to white (without UV), the colour of typical night-blooming, hawkmoth-pollinated flowers. Moths were reared on three larval diets known to differ in photopigment precursor content, which affects the responsiveness of naïve adult moths to artificial flowers and feeders (Raguso et al. 2007; Goyret et al. unpublished data) and induces deficiencies in visual sensitivity and on the anatomy of the photoreceptor membrane (Bennett and White 1989). Nevertheless, colour preference and latency times were not affected by larval diet in our experiments (see Table 1).

**Table 4.1. Statistical results.** Number of replicates (N), colour choice (in %) for naïve and trained moths and feeding latency time (s) for naïve moths (columns). Animals reared on tobacco were not trained to blue. The “Total” row represents the sum of all individuals trained and tested (N), the average colour choice (%) and the average  $\pm$  standard error of mean for the feeding latency time (s). The fact that there are no data for “Tobacco moths” trained to the blue feeder in Lund, Sweden, is denoted by the “x” marks. <sup>a</sup>G-test for effect of larval diet on naïve choice: Gh=2.73; p=0.098. <sup>b</sup>(Mean  $\pm$  s.e.m.) ANOVA for effect of larval diet on naïve latencies:  $F_{(2;46)}=0.92$ ; p=0.406. \*Pool of 5 animals reared on Std diet plus 10 reared on  $\beta$ -carotene enhanced diet (marks could not be distinguished at testing). \*\*Pool of 16 animals reared on Tobacco plus 7 reared on Std diet (marks could not be distinguished at testing). <sup>c</sup> G-test for effect of larval diet on test choice: Gh=0.324; p=0.569.

	Naïve moths			Test of moths trained to White		Test of moths trained to Blue	
	N	Blue choice (%)	Naïve latency (s)	N	White choice (%)	N	Blue Choice (%)
Standard diet (USC)	15	73.33 <sup>a</sup>	48 $\pm$ 15 <sup>b</sup>	15*	80.0 <sup>c</sup>	18	77.78
$\beta$ -carotene (USC)	15	73.33 <sup>a</sup>	69 $\pm$ 18 <sup>b</sup>				
Tobacco (LU)	17	94.12 <sup>a</sup>	77 $\pm$ 14 <sup>b</sup>	23**	86.96 <sup>c</sup>	x	xxx
Total	47	80.85	65 $\pm$ 9	38	84.47	18	77.78

### *Learnt preferences*

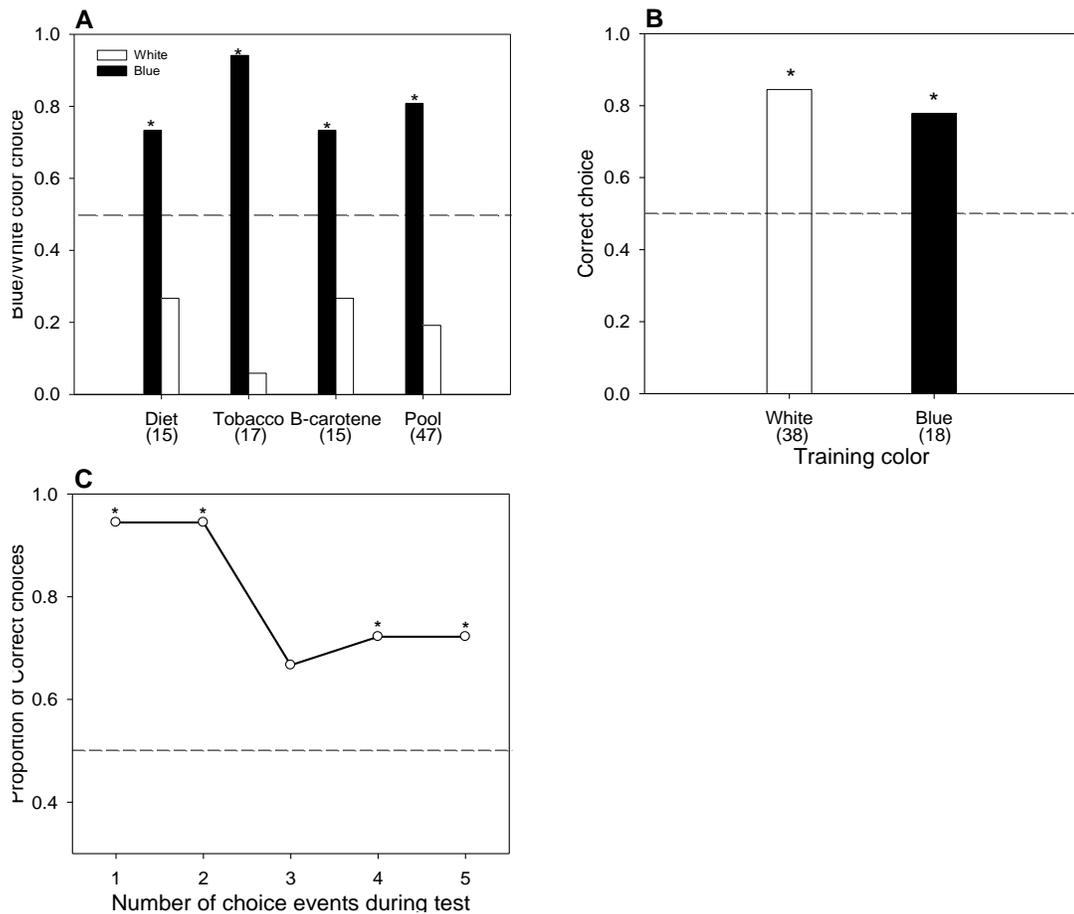
Despite their innate blue preference, *M. sexta* also investigate flower-like objects (such as feeders) of different colours (Fig. 4.3a) and can “override” instinctive blue bias through experience (Fig. 4.3b). Recent work by Raine and Chittka (2007) suggests that innate colour biases can be adaptive for local bumble bee (*Bombus terrestris*) populations that prefer violet flowers in regions of Europe where flowers of this colour are most rewarding. However, at present, there is no evidence that an innate blue preference would guide newly emerged *M. sexta* to abundant, profitable blue-coloured flowers in any environment in which this species has been observed (see below). A naïve searching behaviour would be more adaptive (considering a correlation between fitness and nectar intake) if guided by an innate search image which allows for some degree of generalization, thus, broadening the range of potential food sources. This is particularly relevant given the high variability of nectar availability (Bell 1986). Learning abilities, as demonstrated in this study, could allow animals to forage more efficiently by focusing their foraging efforts on certain floral species with reliable nectar rewards. Also, this “flower constancy” would increase the chances of successful pollen export to conspecifics (Chittka et al. 1999).

Even though the colour of our feeders occupy distinctive loci in the perceptual colour space of nocturnal hawkmoths (Fig. 4.2d), and thus could be distinguished by their spectral properties, we cannot conclusively state that the observed change in colour preference was based solely on the moths’ ability to discriminate the different spectral composition of the stimuli, because differences in intensity and background-contrast also could be associated with our feeders (Kelber 2005). Nevertheless, the innate colour preference must be based on more receptor types than the blue photoreceptors, as concluded by Cutler et al. (1995). Both feeders reflect blue wavelengths; the white feeder reflects approximately nine times more light around the 450-nm wavelengths

(blue) than the blue feeder (Fig. 4.2b). If innate feeding behaviour was mediated mainly by blue receptors, moths should have shown at least random probabilities to feed from either flower model (if not a bias to the brighter white feeder). Instead, naïve moths chose the blue feeder with lower but predominantly blue reflectance. This result, taken together with previous data showing true colour vision in a related nocturnal sphingid moth, *D. elpenor* (Kelber et al. 2002) under similar light intensity, supports the hypothesis that the crepuscular *M. sexta* used colour vision to differentiate between the two feeders. Additionally, the fact that we tested the moths in rather dim light suggests that the preference for blue is independent of background light intensity.

#### *Why blue?*

Blue flowers have one property that makes them a good choice for anthophilous insects in general: with the exception of the blue sky, most blue objects in a natural setting are flowers, providing colour contrast with the background in trichromatic insect visual perception (Kevan et al. 1996). Naïve diurnal hawkmoths (*M. stellatarum*) initially ignore colours other than blue (or, occasionally, yellow) but after being rewarded from blue flowers, they probe objects of other colours as well (Kelber 1996). The innate preference of *M. sexta* for blue is less rigid, as naïve moths respond to flower models other than blue if that colour is not available (Goyret et al. 2007; Goyret and Raguso 2006). Additionally, olfactory stimulation increases the responsiveness of *M. sexta* to contrasting (even scentless) objects (Goyret et al. 2007). This kind of behavioural flexibility, combined with the learning abilities shown here, suggests that *M. sexta* can switch from their innate blue preference to an effective preference for the visually conspicuous and fragrant white flowers that they encounter in nature.



**Figure 4.3. Feeding responses by naïve (A) and trained (B-C) *Manduca sexta* females.** (A) Innate colour choice in a dual choice experiment. First three pairs of bars show results from moths reared on different diets. Fourth bar represents the pooled results. (B) First choices made by trained moths. Proportion of moths that “chose correctly” in the first probing event of the testing phase (y-axis) as a function of the training colour (x-axis). (C) Proportion of correct choices by trained moths for each of the first 5 probing events. Only the 18 females trained to white that probed 5 consecutive times during testing are considered. Numbers in parentheses indicate the number of independent replicates. Asterisks denote significant departure from random choice (dashed line;  $\alpha=0.05$ ) in a binomial test (-see results and Table 4.1).

*M. sexta* inhabits diverse habitats from forest to desert throughout much of the Western Hemisphere (Hodges 1971; Janzen 1988; Moré et al. 2003); it is difficult to deduce the ancestral nectar sources on which this species evolved its feeding preferences. However, female *M. sexta* mix oviposition with nectar feeding from *Datura* and *Nicotiana* host plants (e.g. Adler and Bronstein 2004; Kessler and Baldwin 2007). Thus, host flowers may have provided the initial floral stimuli to which ancestral populations of *M. sexta* responded; when rewarding, these flowers are white without UV. Two exceptions are the small yellow or blue, buzz-pollinated flowers of tomato (*Solanum lycopersicum*) or horse nettle (*Solanum carolinense*), which are essentially nectarless (Elle 1998). Floral surveys in Costa Rican tropical forests (White et al. 1994), the deserts of south-western USA (Raguso et al. 2003), and northern Argentina (Moré et al. 2003) confirm that flowers visited by *M. sexta* and related moths are white without UV, or yellow with UV absorbing targets, but never blue. This pattern is exemplified by species of Convolvulaceae in subtropical Argentina. The blue, morning-opening, bee-pollinated flowers of *Ipomoea indica* offer relatively meagre nectar rewards (3–7mg sugar/flower) and have closed by dusk, whereas the white (no UV), night-blooming flowers of *Ipomoea alba* are more profitable (17–23 mg sugar/flower) and open at dusk, when they are visited heavily by *M. sexta* and related moths (Galletto and Bernardello 2004).

If innate preference for blue flowers is not adaptive in *M. sexta*, what might explain its maintenance? One possibility is that innate blue preference is a phylogenetic artefact in the Sphingidae or some larger subset of Lepidoptera. Although innate colour preferences have been investigated in a number of butterflies and moths, the density of sampling at present is unsuitable for phylogenetic mapping, given that visual pigments and colour preference can vary within genera (Bernard and Remington 1991; Briscoe and Chittka 2001; Kelber et al. 2003; Stavenga and Arikawa 2006). While most

species show some preference for blue, others also show yellow or red preferences, as reviewed by Weiss (2001). Innate blue preference is adaptive in *M. stellatarum*, a diurnal hawkmoth which visits many genera of rewarding blue flowers in Southern Europe (Herrera 1990a, b) where blue is an honest indicator of nectar quantity (Raine and Chittka 2007). However, its diurnal activity represents a derived condition in the Sphingidae (Hodges 1971) and its lineage (Macroglossinae) is distinct from that of *Manduca* (Sphinginae). Similarly, the butterflies (Papilionoidea) also represent a derived lineage of the Lepidoptera (Wiegmann et al. 2000) and are unlikely to provide inference concerning the ancestral state of the lineage giving rise to the Sphingidae. In sum, the innate preference of *M. sexta* for blue flowers remains a mystery in light of continent-wide patterns of visitation to guilds of white (without UV), night-blooming flowers. We have shown that blue preference is labile in *M. sexta*, which allows them to switch rapidly to white, rewarding flowers through learning. Innate preference for blue need not represent a constraint in nature, if it does not impede naïve adult *M. sexta* from exploring the market of differently coloured flowers, or if the innate response to floral odour (Brantjes 1978; Raguso and Willis 2002) helps to overcome such bias. Future experiments should utilize no-choice assays and odour–colour combinations to test these hypotheses.

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**CHAPTER 5**

**FLEXIBLE RESPONSES TO VISUAL AND OLFACTORY STIMULI BY  
FORAGING *MANDUCA SEXTA*: LARVAL NUTRITION AFFECTS ADULT  
BEHAVIOUR\***

*Abstract*

Here we show that the consequences of deficient micro-nutrient ( $\beta$ -carotene) intake during larval stages of *Manduca sexta* are carried across metamorphosis, affecting adult behaviour. Our manipulation of larval diet allowed us to examine how developmental plasticity impacts the interplay between visual and olfactory inputs on adult foraging behaviour. Larvae of *Manduca sexta* were reared on natural (*Nicotiana tabacum*) and artificial laboratory diets containing different concentrations of  $\beta$ -carotene (Standard diet, Low $\beta$ -carotene, High $\beta$ -carotene, and Cornmeal). This vitamin-A precursor has been shown to be crucial for photoreception sensitivity in the retina of *Manduca sexta*. After completing development, post-metamorphosis, starved adults were presented with artificial feeders that could be either scented or unscented. Regardless of their larval diet, adult moths fed with relatively high probabilities on scented feeders. When feeders were unscented, moths reared on tobacco were more responsive than moths reared in  $\beta$ -carotene deficient artificial diets. Strikingly, moths reared on artificial diets supplemented with increasing amounts of  $\beta$ -carotene (Low $\beta$ , High $\beta$ ) showed increasing probabilities of response to scentless feeders. We discuss these results in relation to the use of complex, multi-modal sensory information by foraging animals.

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## ***Introduction***

Nectivorous animals are responsible for the cross-pollination of most flowering plants, which have evolved multiple mechanisms ensuring pollen movement between conspecifics (Barth, 1991; Harder and Barrett, 2006). Plants advertise their nectar with flowers capable of attracting foraging animals, which rely heavily on environmental information gathered through their sensory systems. Initially, animals find and recognize flowers through innate responses to one or various sensory cues. For example, nectivorous bats from the genus *Glossophaga* use echolocation (Helvesen et al., 2003) and olfactory cues (Helvesen et al., 2000) to locate flowers; bumblebees (*Bombus impatiens*) use visual and olfactory input during foraging (Kulahci et al., 2008), as do *Vanessa indica* butterflies (Ômura and Honda, 2005) and the hawkmoths *Macroglossum stellatarum* and *Deilephila elpenor* (Balkenius et al., 2006).

The use of apparently redundant stimuli (e.g. odour, visual display) offers searching pollinators the potential to use different sensory channels to mediate foraging responses under different contexts and at different scales (up-wind flight from a distance, proboscis extension close to the target; see Balkenius et al., 2006; Goyret et al., 2007; Jang et al., 2000). Also, foragers show more accuracy in learning experiments when visual and olfactory cues are available than when they can use only one cue, as was shown independently for *Bombus terrestris* (Kunze and Gumbert, 2001) and *Bombus impatiens* (Gegear and Lavery, 2005; Kulahci et al., 2008). Searching and evaluating potential nectar sources through more than one of their physical properties can confer upon foraging animals an adaptive behavioural flexibility.

Nevertheless, most foragers have the capacity to show innate appetitive responses in reduced experimental settings, in which only one physical aspect of the flower is presented (i.e. only visual display, or only floral odour). Thus flower visiting

*Glossophaga* bats can innately respond to some flower odours (Helvesen et al., 2000), and bumblebees and *Vanessa indica* butterflies can forage on scentless artificial flowers (Ômura and Honda, 2005; Spaethe et al., 2001). The diurnal hawkmoth *Macroglossum stellatarum* shows very specific innate responses to the visual display of flowers, including corolla colour and pattern (Kelber, 1996; 1997). Conversely, innate feeding responses by the nocturnal European hawkmoth, *Deilephila elpenor*, are mediated primarily by olfactory stimulation (Balkenius et al., 2006), despite this species' acute capacity for night colour vision (Kelber et al., 2002). In most cases studied thus far, single modality input can be sufficient to elicit appetitive responses. Interestingly, single modality feeding has not been observed for *Manduca sexta*, a crepuscular-nocturnal hawkmoth (Lepidoptera: Sphingidae) native to the Americas. These moths use a variety of sensory modalities while nectar-foraging, including vision, olfaction, hygrometry, mechanoreception and CO<sub>2</sub> detection (Goyret and Raguso, 2006; Raguso and Willis, 2002; Thom et al., 2004). Nevertheless, visual and olfactory stimulation have been shown to be necessary and sufficient to elicit the complete sequence of foraging responses in laboratory-reared and wild adult *Manduca sexta* (Raguso and Willis, 2002; Raguso and Willis, 2005).

Bennett and White (1989), showed that carotenoid deficiency in laboratory larval diet leads to reduced visual sensitivity and anatomical abnormalities in the photoreceptor membranes of adult *M. sexta*. Could visual deficiencies have been directly responsible for former results showing dependence on both olfactory and visual stimulation to feed (Raguso and Willis, 2002; Raguso and Willis, 2005)? Do olfactory cues more strongly influence behavioural responses when visual input is inadequate or poor? Although they did not directly address these questions, Raguso and collaborators investigated the effect of larval diet composition on adult moth foraging behaviour (Raguso et al., 2007), marginally finding that female (but not male) moths were more

likely to respond to natural flowers (i.e. both visual and olfactory stimuli present) when their larval diet had been supplemented with  $\beta$ -carotenes. These results support the general hypothesis that deficient intake of  $\beta$ -carotenes during larval development can affect adult moth behaviour, but do not shed light on our specific question: Does the use of olfactory cues increase in priority when visual input is inadequate? In other words; is the documented lack of response by *M. sexta* to scentless visual targets an artefact of visual deficiencies caused by inadequate larval  $\beta$ -carotene intake? This question provides an opportunity to further investigate the interaction between the two most conspicuous floral cues used by pollinators.

Here, we quantitatively evaluate foraging responses to scented and unscented artificial flowers by naïve adult *M. sexta* moths reared on 5 different diets, including live tobacco plants, a natural host plant, and the commonly used (wheat germ based) artificial diet with direct and indirect  $\beta$ -carotene supplements. We designed this study to test the following hypotheses:

(H<sub>0</sub>) Null: Larval diets have no effect on naïve adult feeding behaviour.

Prediction: Within each treatment (scented and unscented feeders) there should be no behavioural differences between moths reared on different diets.

(H<sub>1</sub>) The previously observed feeding requirement of lab-reared adult *M. sexta* for combined olfactory and visual inputs is an artefact of  $\beta$ -carotene deficiency.

Prediction: diet effects should be more evident when moths depend primarily on visual stimulation (unscented feeders); when feeders offer olfactory stimulation, diet effects should be less pronounced. Additionally, treatment effects (scented or unscented feeders) should be more distinct for moths reared on  $\beta$ -carotene deficient diets.

(H<sub>2</sub>) Alternatively, differences in foraging behaviour could reflect less specific factors, such as feeding motivation or behavioural state due to metabolic disparities associated with different macronutrient content of the different diets.

Prediction: Diets differing only in  $\beta$ -carotene content (see Methods) should not affect foraging behaviour.

### ***Materials and Methods***

Experiments were run during October-November of 2005 at Lund University, Lund, Sweden (henceforth: LU) and during July-December of 2006 at the University of South Carolina, Columbia, South Carolina, U.S.A. (henceforth: USC).

#### *Animals and diets*

We reared *Manduca sexta* from eggs provided by Dr. Lynn Riddiford, from her laboratory colony at the University of Washington, Seattle, WA, USA, under a photoperiod of 16:8 L:D cycle.

We randomly assigned individuals to one of five different diets:

- 1) Wheat germ based artificial diet (after Bell and Joachim, 1976; henceforth: Standard diet), which is the most commonly used diet in *M. sexta* research,
- 2) Standard diet supplemented with 200 mg of  $\beta$ -carotene/L (Sigma Aldrich, St. Louis, MO, USA -henceforth: Low $\beta$ ),
- 3) Standard diet supplemented with 800 mg of  $\beta$ -carotene/L (henceforth: High $\beta$ ),
- 4) Standard diet with yellow cornmeal instead of wheat germ as the main source of carbohydrates (henceforth: Cornmeal) and
- 5) Intact, uncut *Nicotiana tabacum* plants (seed lot GL350, GoldLeaf Seed Co., Hartsville, South Carolina –henceforth: Tobacco).

#### *Treatments and procedures*

After eclosion, adult moths were starved for 3-4 days to increase feeding motivation. We used cubic cages (45x45x45 cm) covered with white cloth on all sides but the

front one, which had a transparent plastic sheet in order to allow observation and data recording. Cages were illuminated by 15 white LED lamps (Ledtronics, Inc., California, USA) placed directly above each cage, providing a luminance of  $0.08 \text{ cd m}^{-2}$  (similar to twilight). We placed two artificial feeders (see Pfaff and Kelber, 2003) 20 cm from each other in the flight cage, above black covered water bottles, 25 cm above the cage floor (vertically). Both of these feeders could be either scented (25 $\mu\text{l/L}$  of pure Bergamot oil extract) or unscented (i.e. two treatments). For purposes other than the present experiment, one feeder was white to the human eye with low UV reflectance, and the other feeder was dark blue to the human eye. Both feeders contained a 20% (w/w) sucrose sugar solution.

Moths were individually placed in the flight cage and were allowed to fly for up to 5 minutes. We recorded the responsiveness (percentage) of animals probing at the feeders and latency (seconds) from take off to first probing attempt. We also recorded relative reflectance of adult moth eyes. The eyes of *Manduca sexta* possess a tapetum lucidum, a structure consisting of a network of trachea surrounding the receptors of each ommatidium, which reflects back incident light that has not been absorbed. The reflected light that is not absorbed by the photoreceptors after this second passage can be detected as light emanating from the animal's eye, known as "eye glow". We immobilized the moths and measured the relative (to a white ceramic standard) reflectance of the eye glow with an S2000 spectroradiometer probe orthogonally placed 2-4 millimetres away from the eye (S2000, OceanOptics Europe, Eerbeek, The Netherlands). These measurements were performed on moths reared on standard, cornmeal and tobacco diets.

#### *Statistical analysis*

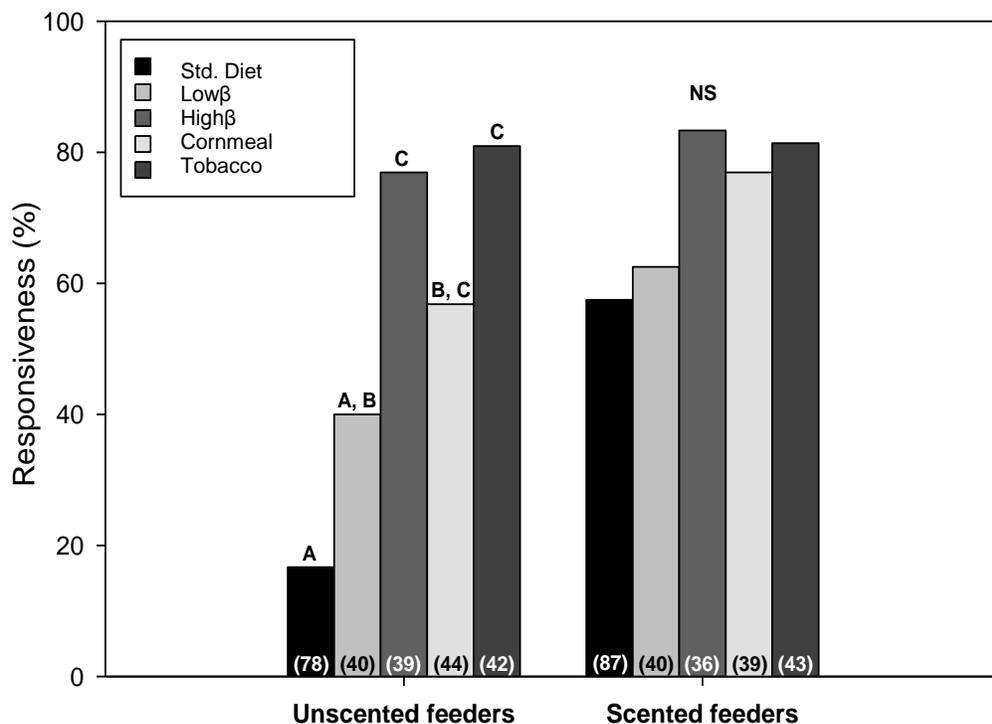
The responsiveness variable was analyzed by means of G-tests and the latency variable by means of 3-way ANOVA (gender, diet and treatment -scented and

unscented feeders), with data having been log-transformed to meet assumptions of the model. The number of replicates was substantially large, but due to the large number of factors (5 diets and 2 treatments), the number of tests (including G-tests and ANOVA) also was large. Accordingly, we evaluated statistical significance using Bonferroni's correction of each test's  $\alpha$ -level (0.0017) in order to maintain a global  $\alpha$ -level of 0.05.

### **Results**

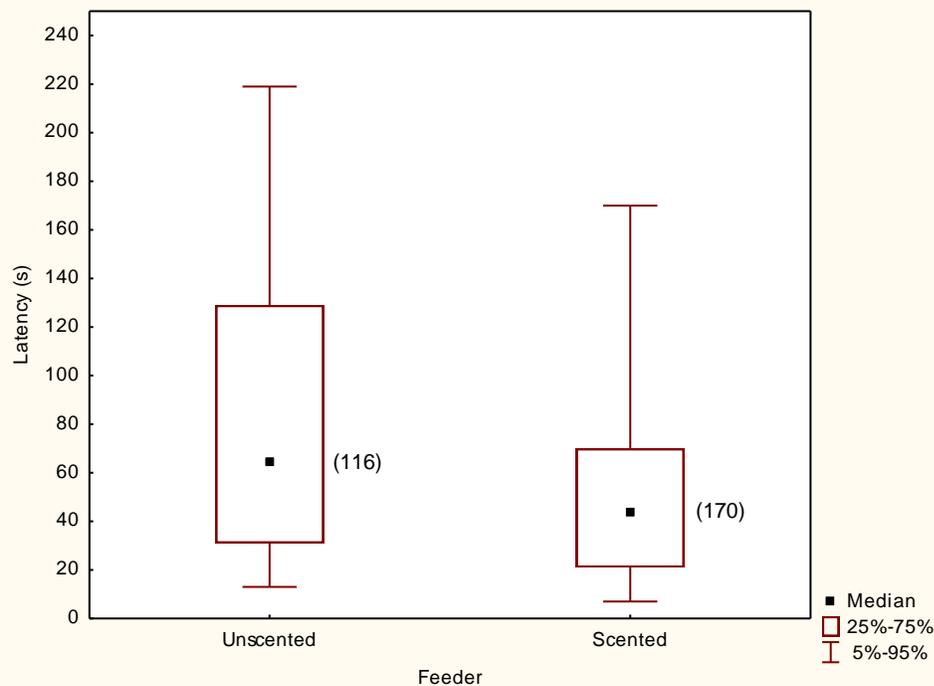
We flew a total of 488 moths, 245 to the treatment with scented feeders and 243 to the unscented feeders. Statistical analyses were performed on pooled data for males and females, because no significant differences were found between them ( $G=0.67$ ;  $p=0.41$ ;  $N=245$ ; G-test males vs. females for the scented treatment.  $G=0.006$ ;  $p=0.94$ ;  $N=243$ ; G-test males vs. females for the unscented treatment). Treatments (i.e. scented vs. unscented feeders) showed a significant effect on responsiveness ( $G=26.07$ ;  $p=2.6 \times 10^{-6}$ ;  $N=488$ ; G-test between treatments). Within each treatment, we found a diet effect for the group flown to unscented feeders ( $G=69.33$ ;  $p=5.9 \times 10^{-15}$ ;  $N=243$ ; G-test between diets), but not for the group flown to scented feeders ( $G=14.34$ ;  $p=0.0025$ ;  $N=245$ ; G-test between diets; Fig. 5.1). Responsiveness to unscented feeders for the standard diet group was significantly lower than for all other treatments except the low- $\beta$  group (vs. low $\beta$ :  $G=7.47$ ;  $p=0.0063$ ;  $N=118$ ; vs. high $\beta$ :  $G=41.46$ ;  $p=1.2 \times 10^{-10}$ ;  $N=117$ ; vs. cornmeal:  $G=20.88$ ;  $p=4.9 \times 10^{-6}$ ;  $N=122$ ; vs. tobacco:  $G=49.49$ ;  $p=2 \times 10^{-12}$ ;  $N=120$ ; Fig. 5.1). The low $\beta$  group differed from the high $\beta$  group ( $G=11.39$ ;  $p=0.0007$ ;  $N=79$ ; Fig. 5.1) and from the tobacco group ( $G=14.95$ ;  $p=0.0001$ ;  $N=82$ ; Fig. 5.1) but not from the cornmeal group ( $G=2.38$ ;  $p=0.12$ ;

N=84; Fig. 5.1). The high $\beta$  group did not differ from the cornmeal group ( $G=3.81$ ;  $p=0.05$ ; N=83; Fig. 5.1), nor from the tobacco group ( $G=0.20$ ;  $p=0.66$ ; N=81; Fig. 5.1), which did show higher responsiveness levels than the cornmeal group ( $G=5.95$ ;  $p=0.015$ ; N=86; Fig. 5.1). For moths reared on artificial diets, the incremental direct addition of  $\beta$ -carotene reduced the disparities between treatments (Std. diet:  $G=30.48$ ;  $p<0.0001$ ; N=165; low $\beta$ :  $G=4.09$ ;  $p=0.04$ ; N=80; high $\beta$ :  $G=0.48$ ;  $p=0.49$ ; N=75).



**Figure 5.1. Responsiveness as a function of diets and treatments.** Percentage of adult animals reared on different diets during their larval stages that probed on the experimental feeders when these were unscented and when they were scented. Different letters denote significant differences at an  $\alpha$ -level of 0.0017 (after Bonferroni correction). NS: Non significant diet effect of responsiveness to the scented feeders. Numbers in parentheses denote number of independent replicates.

Analysis of variance (ANOVA) for the latency variable showed no differences between genders ( $F_{(1; 266)}=0.55$ ;  $p=0.46$ ; Fig. 5.2), but we found strong treatment ( $F_{(1;266)}=14.81$ ;  $p=0.0001$ ) and diet effects ( $F_{(4; 266)}=5.34$ ;  $p=0.0004$ ). No significant interactions were detected (gender vs. treatment:  $F_{(1; 266)}=0.05$ ;  $p=0.83$ ; gender vs. diet:  $F_{(4; 266)}=0.28$ ;  $p=0.89$ ; diet vs. treatment:  $F_{(4; 266)}=1.21$ ;  $p=0.31$ ; gender vs. treatment vs. diet:  $F_{(4; 266)}=1.81$ ;  $p=0.13$ ). There were no diet effects when analysing the unscented treatment ( $F_{(4; 111)}=1.70$ ;  $p=0.16$ ; Fig. 5.2) but there was a diet effect within the scented treatment ( $F_{(4; 165)}=5.72$ ;  $p=0.0002$ ), which was due only to the difference between the high $\beta$  beta and the tobacco diets (Tukey H.S.D. for unequal replicates:  $p=0.0009$ ). Because all other diets within the unscented treatment did not differ significantly, we pooled diets for each treatment and present their mean  $\pm$  s.e.m. in Fig. 5.2.



**Figure 5.2. Latency times as a function of treatments.** Latency times (in seconds; medians, quartiles and 5-95% whiskers) animals spent from take-off to first probe. Data from the different diets are pooled for each treatment (unscented and scented feeders). \*\*\* $p<0.0001$ . Numbers in parentheses denote number of independent replicates.

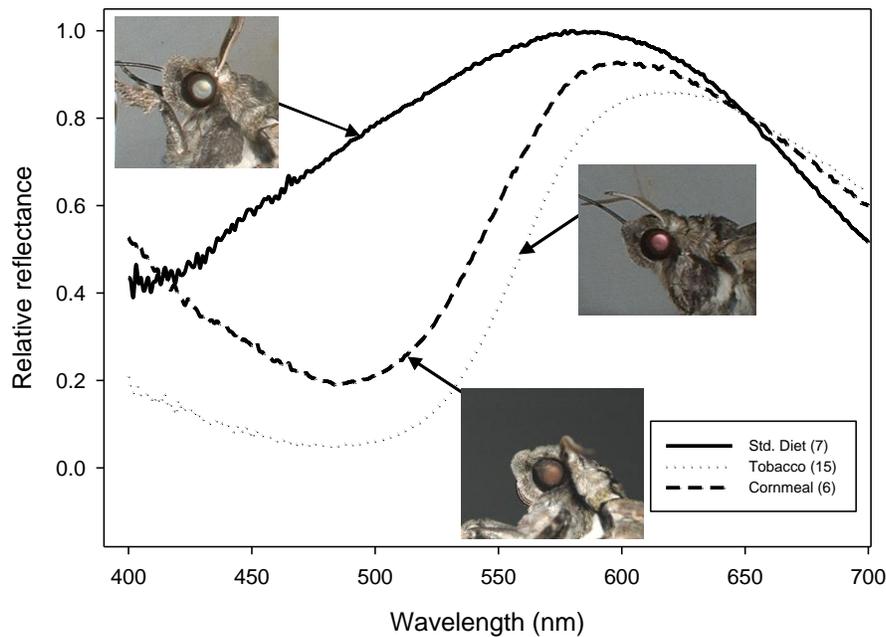
Spectroradiometry of the eye glow from animals reared on tobacco, standard diet and cornmeal diet showed quantitative differences (Fig. 5.3). Eye glow colour depends on the reflector (i.e. the tapetum, which in this case is assumed to be colourless, because tracheas act as mirrors) and the ommatidial pigments. In *Manduca sexta*, these pigments have sensitivity maxima at 357 nm, 450 nm, and 520 nm (White et al., 2003), absorbing much of the UV, blue and green light. Thus, wild moths typically present a reddish eye glow as a result of not having captured light at longer wavelengths and having absorbed most of the light at shorter wavelengths (White et al., 2003; personal observation). The eye glow of the standard diet group was whitish, absorbing significantly less shorter wavelengths than the tobacco group, which presented the typical reddish eye glow (Fig. 5.3). This difference had a peak at 510 nm, suggesting that large amounts of the P520 pigments are missing. When using cornmeal instead of wheat germ as the main carbohydrate source in the standard diet, the natural reflectance spectrum of the moths' eyes was "reconstituted", showing the same reflectance curve as in animals reared on tobacco, albeit at less efficient levels of absorbance (Fig. 5.3).

## ***Discussion***

### *$\beta$ -carotene and foraging behaviour*

Our experiments revealed that adult visual deficiencies are a direct result of inadequate larval nutrition, with clear and significant effects on adult foraging behaviour. This phenomenon has provided us with an unusual opportunity to study the intricacies of animal utilization of multimodal sensory information. How are multiple signals assessed in this system? How does apparently redundant sensory information impact an animal's foraging responses?

Regardless of larval diet, moths responded more frequently, and with lower latency times, to scented feeders than to scentless ones (Figs. 5.1 and 5.2). No significant diet effect on responsiveness was apparent when moths foraged on scented feeders, whereas a highly significant diet effect was observed when moths foraged on scentless feeders (Fig. 5.1).



**Figure 5.3. Eye glow spectrograms for 3 of the experimental diets.** Relative reflectance as a function of wavelength (nm) of the eyes of adult moths reared on either Standard diet, tobacco or cornmeal diet. The peak difference between standard diet animals and tobacco animals is at 510 nm, close to the maximum absorbance of the ‘green’ photoreceptor (P520).

Additionally, the apparent gradual augmentation in responsiveness that accompanied incremental supplements of  $\beta$ -carotenes to artificial diets (std. diet, low $\beta$  and high $\beta$ ) suggests that diet effects are not linked to differences in feeding motivation or

physiological state, which could arise from different macronutrient availability in the different larval diets. Instead, while adding little energetic value, the supplementation of  $\beta$ -carotene drastically changed moths' foraging behaviour in the scentless foraging scenario. Moreover, there were no consistent responsiveness differences between moths reared on cornmeal diet or tobacco leaves, which can be expected to differ from the standard diet in many ingredients besides  $\beta$ -carotene. These results lend support to accept hypothesis  $H_1$  and to reject the null ( $H_0$ ) and second alternative ( $H_2$ ) hypotheses presented in the Introduction. Altogether, our results strongly suggest that larval dietary  $\beta$ -carotene content specifically affects adult nectar foraging behaviour ( $H_1$ ).

When foraging on scented feeders, latency times averaged less than 60 seconds, whereas when odour was absent, moths spent an average of 85 seconds before probing (Figure 5.2). Our previous studies using naïve *M. sexta* (reared on standard diet) showed that floral odours can elicit both up-wind odour plume tracking and visually guided flower searching (Goyret et al., 2007). When olfactory stimulation was spatially or temporally decoupled from the visual target (i.e. odour was released from a different position, was interrupted or was absent), latency times increased and responsiveness diminished (Goyret et al., 2007). Formerly, we suspected that longer latencies could be related to longer, or different, integration processes provoked by contradictory stimulation (visual and olfactory cues separated in space or time). An alternative hypothesis suggested by the present results is that longer latency times might also be related to abnormal, peripheral sensory processes in the visual system. Below, we discuss this matter in the light of the morphological and physiological effects that  $\beta$ -carotene deficient larval diet has on the visual system of adult *M. sexta*.

### *β-carotene and the visual system*

Spectroradiometry of the eye glow (reflection of incident light by the tapetum) showed at first instance, that the eyes of moths reared on standard diet were less efficient at absorbing incident light than those of moths reared on tobacco plants or cornmeal (i.e. higher relative reflectance; Figure 5.3). This is consistent with measurements of rhodopsin content in the retina, which show that β-carotene deprived animals have 100-fold less retinal rhodopsin than moths reared on β-carotene fortified diets (Bennett and White, 1989). Tobacco-bred moths had a human red eye glow, due to absorption in the entire human visible spectrum except the ‘red wavelengths’ (> 600 nm). Standard diet-bred moths had a whitish eye glow, because their eyes did not efficiently absorb in the blue-green part of the spectrum (Figure 5.3). In fact, the peak difference between tobacco-bred and standard diet-bred moths is seen at 510 nm, in between of the 520 nm (rhodopsin) and the 490 nm (metarhodopsin) sensitivity peaks of their green sensitive receptors (P520). Replacement of the wheat germ of the Standard diet with the β-carotene rich cornmeal (Cornmeal diet) “reconstituted” the red reflectance spectrum of the eye glow of adult *M. sexta* (peak-difference at 503 nm, see Figure 5.3). Assuming that eye glow reflectance differences are due to missing pigments, we suggest that visual sensitivity has been affected. This result also strongly suggests that β-carotene, rather than some other component of tobacco leaves, is causally related to the differences in light absorption efficiency seen between standard diet-bred and tobacco-bred moths. In other words, β-carotene content in larval diets appears to be the main factor accountable for adult behavioural differences observed in our experiments.

*B-carotene, visual sensitivity and foraging behaviour*

Deficiencies in  $\beta$ -carotene are known to negatively affect the morphology of the photoreceptor membrane, retinal rhodopsin content and visual sensitivity in *Manduca sexta* (Bennett and White, 1989). On the other hand, *M. sexta* has consistently shown more significant responses to visual targets when olfactory stimuli also were provided (Goyret et al., 2007; Raguso and Willis, 2002; Riffell et al., 2008). Here, the combined results from our behavioural experiments and spectroradiometric measurements support the hypothesis that naive *M. sexta* have difficulty recognizing an odourless visual target as a nectar source when their vision is impaired by sub-optimal  $\beta$ -carotene intake during their larval stages.

This does not mean that floral odour is superfluous for foraging moths. Differences in latency reveal that even though moths bred on their (healthy) host-plant do not rigidly require olfactory cues to feed (Figure 5.1), odour still affects their feeding responses (Figure 5.2). Similarly, both vegetative and floral odours were observed to play important roles in nectar foraging by wild *M. sexta* adults that presumably fed on *Datura wrightii* leaves in the Sonoran Desert of Arizona (Raguso and Willis, 2005). On the other hand, the visual deficiencies resulting from low  $\beta$ -carotene intake neither impair flight performance (personal observation) nor do they affect innate colour preferences or colour learning (Goyret et al., 2008). Moreover, after being in the cage for one day, these same moths begin to respond to scentless feeders and are able to switch from innate preferences (blue) to learned ones (white). Thus, it appears that deficient diets can account for a loss in visual sensitivity, but that vision is far from being completely impaired in such animals.

In a previous study, we showed how decoupling visual from olfactory cues affects both responsiveness and latency to feed; of particular interest for the present study is the finding that moths unresponsive to scentless feeders started to feed when

transiently stimulated with odour (see Goyret et al., 2007). This showed that even transient olfactory stimulation can enhance responsiveness to scentless targets, probably through the elicitation of a visually guided search (see Brantjes, 1973). One interpretation of the present experiments is that visual targets were conspicuous enough for “visually healthy” moths to recognize them as potential nectar sources, but  $\beta$ -carotene deficient moths needed olfactory input to assess an ambiguous visual display as a nectar source. Here, we propose that olfactory input could also modulate thresholds for visual appetitive responses (e.g. intensity, contrast). We might test this hypothesis by evaluating responsiveness to visual targets under conditions of different light intensities. *Manduca sexta* show an increment in responsiveness from 72% to 100% when light intensity increases from 0.023 lx (starlit) to 0.054 lx (half-moonlit; Goyret et al., 2007). This is a non-trivial matter for moths with crepuscular and nocturnal habits, foraging under illumination conditions with irradiances that can vary by orders of magnitude (Johnsen et al., 2006).

In summary, we have shown first that low responsiveness of adult *Manduca sexta* to scentless feeders is directly linked to lower levels of light absorption (decreased visual sensitivity) associated with low intake of  $\beta$ -carotene during larval stages. Secondly, we have shown that visual sensitivity is affected throughout the visual spectrum, but predominantly in between the sensitivity peaks of the rhodopsin and metarhodopsin of the green receptor (520 nm and 490 nm). Besides the implications of our results for research on the model laboratory organism *Manduca sexta*, our discovery highlights the importance of subtle and flexible interplays between sensory modalities in a nectar-feeding insect. The fact that visually impaired moths remain capable of finding and feeding from flowers through olfactory input, reveals a functional overlap of sensory systems that we suspect is more widespread among flower-visiting animals, from bumblebees to glossophagine bats.

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