The effect of decoupling olfactory and visual stimuli on the foraging behavior of *Manduca sexta*

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Summary

Within an appetitive context, *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 the decision-making process based on these stimuli is more flexible and complex than previously asserted. *Manduca 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.

Key words: olfactory stimulus, sensory stimulus, temporal pattern, spatial pattern, temporal decoupling, hawkmoth.

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 host-specificity or flower constancy, due to the integration of sensory modalities (Gegear, 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; Ômura 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 (Ômura 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:

Hypothesis$_{1A}$ – olfactory stimulation in the form of an odor plume spatially restricts moths' responsiveness to probing at the odor source;

Hypothesis$_{1B}$ – 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:

Hypothesis$_{2A}$ – moths require simultaneous olfactory and visual stimulation to probe at artificial flowers in a wind tunnel;

Hypothesis$_{2B}$ – 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):

Hypothesis$_{3A}$ – *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);

Hypothesis$_{3B}$ – *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

Table 1. *Summary of treatments used in experiment 1*

Treatment (*N*)	Description
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

Stimuli were placed at the end of a $3\times1.5\times1.5$ m wind tunnel. Where there is no artificial white flower (treatments Odor and Negative control) we placed instead a black flower matching the background to ensure the 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. O=olfactory, V=visual.

same ambient regime and emerged within $45\times45\times45$ cm screen cages (BioQuip, Inc., Rancho Dominguez, CA, USA). Adults were starved for 3–4 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 $3\times1.5\times1.5$ m laminar flow wind tunnel, with a flow rate of 1 m 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 µl 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^{3} 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 1 for treatment summary). In the first four treatments, by moving the artificial flower to the right or left of the centrally positioned odor source (Fig. 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
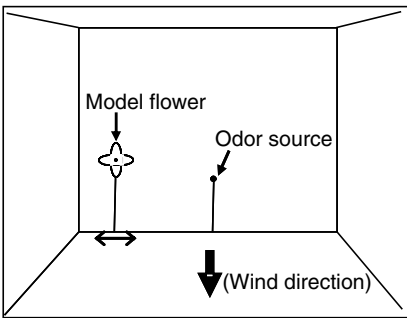

Fig. 1. Upwind view of the inside of the wind tunnel ($3\times1.5\times1.5$ m) showing the odor source (i.e. cotton swab) and the artificial flower (diameter, 9 cm), which could be displaced by moving it left or right in the same plane (as shown by double-ended arrows).

Table 2. *Summary of treatments used in experiment 2*

Treatment (*N*)	Downstream puff	Cotton swab at flower	Flower puff	Stimuli delivery sequence
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

Puffs and cotton swab could be either scented or unscented as stated. Downstream 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 same way, but through a piece of Tygon^{TM} 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.

(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).

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). 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.

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 α-level of significance of 0.0045 was adopted for experiment 1 to preserve a global α-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 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*(odor source)=*P*(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. 2A). 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. 2A). 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
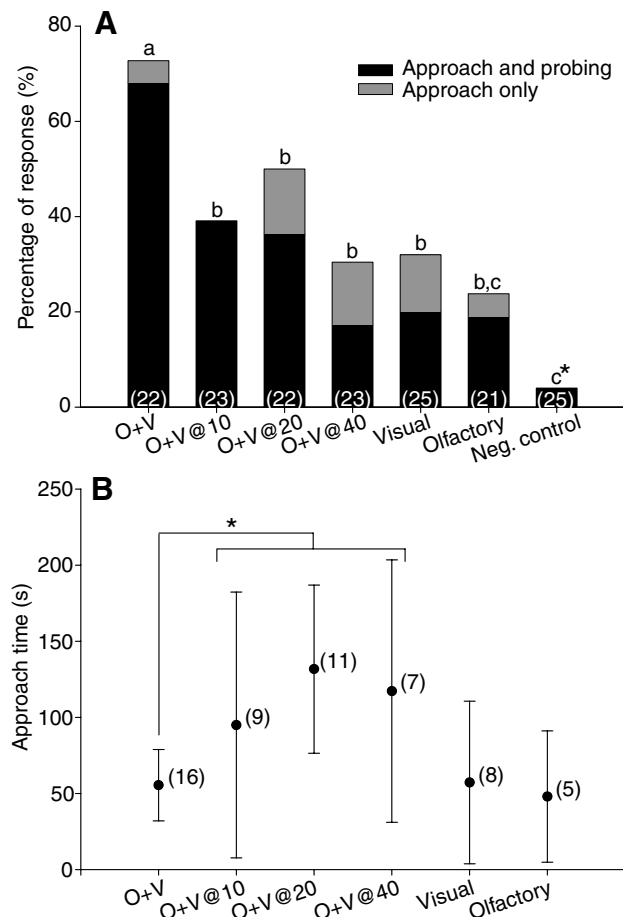
Fig. 2. (A) Percentages of individual moths (sample replicates in parentheses) that approached only (grey bars) or approached *and* probed (black bars) at seven different spatial manipulations of sensory stimuli in a wind tunnel. Different letters denote significant differences between treatments for the 'approach and probing' variable. *Based on the response of one moth. (B) Mean ± confidence interval (α=0.05) of time elapsed between take-off and approach to the visual target under different conditions of visual and olfactory cue presentation (see Materials and methods and Table 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. 2A.
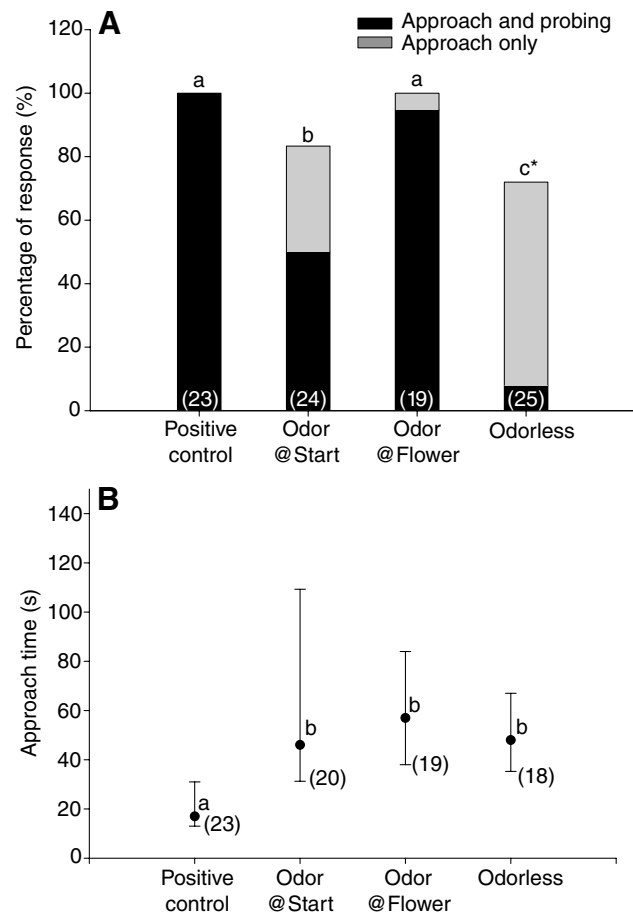

Fig. 3. (A) Percentages of individual moths (sample replicates in parentheses) that approached only (grey bars) or approached *and* probed (black bars) at four different temporal manipulations of sensory stimuli in a wind tunnel (see Materials and methods and Table 2 for experimental and statistical details). Different letters denote significant differences between treatments for the probing variable. *Based on the response of two moths. (B) Median ± first and third quartiles of the time elapsed between take-off and approach to the visual target under different conditions of odor presentation (see Materials and methods and Table 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. 3A.

treatment that did not differ statistically from the negative control was the Olfactory treatment (odor alone; P=0.008, N=46, α=0.0045; see Materials and methods) (Fig. 2A).

Analysis of variance of Approach time showed a marginally non-significant effect of treatment ($F_{5,56}$=2.35, P=0.055) (Fig. 2B), 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. 2B).

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. 3A). The approaches and probing responses to the positive control differed significantly from
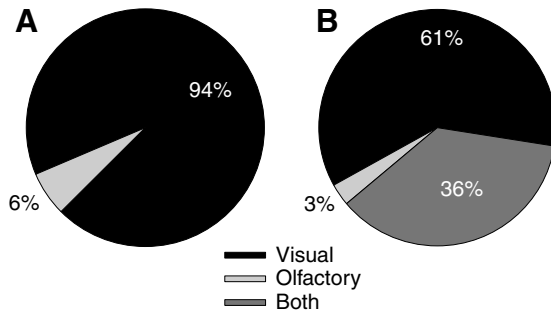
Fig. 4. (A) First choice made by single *M. sexta* flying in a wind tunnel (*N*=33). Different proportions are statistically significant (binomial test; *P*<0.0001). (B) Stimuli visited by single *M. sexta* within a 5 min foraging bout in the wind tunnel (unrewarded flower model) (*N*=33). Mean number of visits ± s.e.m.: visual target=3.41±0.49; odor source=1.62±0.16 ($F_{1,43}$=5.60, *P*=0.023; ANOVA with square root transformation).

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. 3A) 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. 3A) but significantly more frequent than was observed at Odorless flowers (*P*<0.0001; binomial tests) (Fig. 3A).

Approach time was also strongly affected by treatment (Kruskall-Wallis test; H_3=26.12, *P*<0.0001, *N*=80) (Fig. 3B). 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. 3B). 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. 3B).

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. 4A). 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. 4B). When we analyzed total number of probes to the visual target (3.41±0.49) and to the odor source (1.62±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±10.54 s) than the odor source (10.85±3.52 s; $F_{1,43}$=4.65, *P*=0.037; ANOVA with log 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. 2A). Remarkably, when floral signals were spatially separated, mean approach and probe responses were comparable to treatments in which only single stimuli were available (Fig. 2A). 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$_{1A}$; see Introduction). However, approaches and probes did occur when odor and visual cues were separated and when each was presented alone (Fig. 2A), thus Hypothesis$_{1B}$, 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. 2B). 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$_{2A}$; 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$_{2B}$; 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. 3A). 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$_{2A}$). Nevertheless, odor-gated visual foraging can occur in *M. sexta*, increasing responsiveness of naïve moths above that occurring in the absence of odor (Hypothesis$_{2B}$; Fig. 3A). Transient presentation of the olfactory stimulus before flight led to longer latencies (Fig. 3B), 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. 3A).

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 Ômura 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. 4A), suggesting that Hypothesis$_{3A}$ should be rejected in favor of Hypothesis$_{3B}$ (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. 4B), 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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