

## Look and touch: multimodal sensory control of flower inspection movements in the nocturnal hawkmoth *Manduca sexta*

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Accepted 8 August 2010

### SUMMARY

**A crucial stage in the interaction between pollinators and plants is the moment of physical contact between them, known as flower inspection, or handling. Floral guides – conspicuous colour markings, or structural features of flower corollas – have been shown to be important in the inspecting behaviour of many insects, particularly in diurnal species. For the nocturnal hawkmoth *Manduca sexta* tactile input has an important role in flower inspection, but there is no knowledge about the use of visual floral guides in this behaviour. I carried out a series of experiments to first, evaluate the putative role of floral guides during flower inspection and second, to explore how simultaneous tactile and visual guides could influence this behaviour. Results show that visual floral guides affect flower inspection by *M. sexta*. Moths confine proboscis placement to areas of higher light reflectance regardless of their chromaticity, but do not appear to show movements in any particular direction within these areas. I also recorded inspection times, finding that moths can learn to inspect flowers more efficiently when visual floral guides are available. Additionally, I found that some visual floral guides can affect the body orientation that moths adopt while hovering in front of horizontal models. Finally, when presented with flower models offering both visual and tactile guides, the former influenced proboscis placement, whereas the latter controlled proboscis movements. Results show that innate inspection behaviour is under multimodal sensory control, consistent with other components of the foraging task. Fine scale inspection movements (elicited by diverse floral traits) and the tight adjustment between the morphology of pollinators and flowers appear to be adaptively integrated, facilitating reward assessment and effective pollen transfer.**

Key words: flower handling, Lepidoptera, nectar guides, pollinator, mechanoreception, sensory ecology.

### INTRODUCTION

In recent years, there has been increasing interest in the use that animals make of multiple sensory modalities for particular tasks (Hebets and Papaj, 2005). However, Tinbergen (Tinbergen, 1950) and Baerends (Baerends, 1950) had already examined this idea, and proposed that the organization of instincts was based on different movements (i.e. motor patterns), sequentially arranged, and each influenced by particular stimuli configurations.

An interesting case is provided by the diverse adaptive responses that nectarivorous animals show to the variety of stimuli offered by flowers. For example, honeybees utilize different aspects of flower visual signals, using achromatic vision from afar, but recognizing flowers chromatically at close range (Giurfa et al., 1996). Floral odours elicit and maintain visual responses to flowers in many butterflies (Andersson and Dobson, 2003; Tinbergen, 1958), and bumblebees can better discriminate flowers by their olfactory and visual features when these are combined (Gegear, 2005; Odell et al., 1999). Nocturnal moths typically show positive anemotactic responses to floral odours, whereas subsequent visual detection elicits hovering flight and probing when close to the flower (Brantjes, 1978; Raguso and Willis, 2002).

These few examples illustrate how nectarivorous insects use flower features of different physical nature during the phases of flower detection, location and recognition, and describe how animals show specific responses to particular stimuli to *find* potential food sources. However, the instance of physical contact between animal and flower (i.e. flower inspection, or handling) is essential in determining the success, and continuation, of an interaction based

on reward assessment and pollen transfer (Darwin, 1876). The concept of nectar guides was seminal on this subject (Sprengel, 1793). This concept, later termed floral guides (Lunau and Dinkel, 2001), refers to contrasting markings or floral structures with an effect in the motion or positioning of a flower visitor, and its empirical examination has gradually shown the influence of visual floral guides on the behaviour of some pollinators.

Visual control by floral guides has been shown by Daumer (Daumer, 1958), who could misdirect honeybees' inspection behaviour on sunflowers (*H. rigidus*) by manipulating the position of their natural UV markings, and by Lunau and collaborators (Lunau et al., 2009), who showed that bumblebees and honeybees antennate colour marks on artificial flowers before landing. *Pieris rapae* butterflies (Kandori and Ohsaki, 1998) and drone flies *Eristalis tenax* (Lunau and Dinkel, 2001) also enhance their flower inspection efficiency by using visual floral guides on flowers. Additionally, visual marks also influence flower choice from a distance, and insects such as honeybees (Lehrer et al., 1995) and the hawkmoth *Macroglossum stellatarum* (Kelber, 2002) have a preference for radial patterns.

Beyond visual features, olfactory and tactile floral guides have also been proposed. Olfactory floral guides have been convincingly suggested in honeybees (Bolwig, 1954; Lex, 1954) and moths (Brantjes, 1976), but more direct evidence is needed. By contrast, the use of tactile floral guides (henceforth: tactile guides) is supported experimentally. Honeybees learn petal microtexture orientation, suggesting that they could use this flower feature as a tactile guide (Kevan and Lane, 1985). Moreover, the nocturnal hawkmoth

*Manduca sexta* has been recently shown to inspect flowers using tactile input from their 9 cm long proboscis (Goyret and Raguso, 2006).

The concept of floral guides can thus be extended beyond the conspicuous visual features that inspired it. Flowers are complex structures that offer different smells, shapes, colours and textures concomitantly. The question remains as to whether (and how) pollinators can integrate multiple sensory inputs during flower inspection, as they do in the instances of flower detection and recognition.

*Manduca sexta* typically forages on fragrant, whitish, large trumpet-shaped flowers with their long proboscis while hovering (Baker, 1961; Grant, 1983; Haber and Frankie, 1989). Their foraging activity occurs when light conditions are poor (dusk and night), but their large refracting superposition eyes allow them to capture significant amounts of light without severely compromising spatial resolution. However, although this large eye size results in a narrow interommatidial angle (approximately 1 deg), the acceptance angle of the photoreceptor is comparatively wide, approximately 3 deg (Eric Warrant, personal communication). These angles suggest an inability to distinguish two different objects separated by less than 3–4 mm (hovering distance to the flower: 6 cm). Proboscis placement is erratically directed towards flowers when they are uniformly coloured, and tactile cues aid in finding the corolla opening of natural and artificial flowers (personal observation) (Goyret and Raguso, 2006). The hovering flight displayed by *M. sexta* during flower inspection is energetically expensive (Heinrich, 1971), which would impose significant selective pressures to minimize inspection times. Visual guides such as radial or circular lines could facilitate correct initial proboscis placement, enhancing inspection efficiency. These marks occur in some *Petunia*, *Datura* and *Nicotiana* flowers, typically visited by this and other hawkmoths.

In this study, I present naïve moths with artificial flowers showing conspicuous visual markings alone or in combination with tactile guides to test the following hypotheses. H<sub>1</sub>: the inspection behaviour of *M. sexta* is affected by the presence of visual guides on the corolla surface; H<sub>2</sub>: visual guides control placement of the proboscis within the corolla, while tactile guides control its subsequent movements. While H<sub>1</sub> proposes the possibility of visual guides affecting flower inspection, H<sub>2</sub> proposes a functional interaction between tactile and visual responses in this behaviour. Finally, inspection times were recorded in successive visits on different model flowers to test a third hypothesis, H<sub>3</sub>: visual nectar guides have an effect on flower inspection learning, as does tactile input (Goyret and Raguso, 2006).

## MATERIALS AND METHODS

Experiments were performed from July to December of 2008 in Cornell University, Ithaca, New York, USA. I used 3–5-day-old adult *Manduca sexta*, Linnaeus 1763, reared from eggs obtained from a colony maintained in Cornell University by Marta del Campo. Larvae were fed an artificial diet (Bell and Joachim, 1976) modified after Goyret et al. (Goyret et al., 2009) under a 16h:8h light:dark cycle (25°C:21°C) in a humidified (ca. 60% RH) chamber. Male and female pupae were kept in separate incubators (Precision 818, Winchester, VA, USA) in 45 cm×45 cm×45 cm screen cages under the same ambient regime (BioQuip, Inc., Rancho Dominguez, CA, USA). Adults were starved for 3–4 days and were flower-naïve before being used in experiments.

### Experimental set-up

The experimental arena consisted of a cage (1.2 m×1.2 m×1.2 m) the sides of which were covered with a black and green cotton cloth

(fern motifs on black background). The ceiling of the cage was covered with an opaque Plexiglas plate with cheesecloth draped over it to serve as a light diffuser. The cage was illuminated from above with an arrangement of 50 cool white and 50 warm white LEDs (LEDtronics, Inc., Torrance, CA, USA) equally distributed in a checkerboard fashion. Illuminance was set to 0.03 lux (corresponding to dim moonlight conditions). A metal structure (height × depth × width: 40 cm×40 cm×50 cm) was placed at the centre of the cage and covered with the same black and green cloth used for the cage sides. This cloth was punctured to allow diffusion of humidity from two water filled beakers (200 ml) and odours emanating from two cotton swab applicators impregnated with bergamot oil (Body Shop, Ithaca, NY, USA). Bergamot oil volatiles are known to be attractive to *M. sexta* adults and increase their foraging behaviour (Goyret and Raguso, 2006). An array of 3×4 artificial flowers was fixed at the top of this structure. Each flower had a diameter of 6.5 cm and was held by a 10 cm drinking straw. The nectary of each flower consisted of a 6-cm-long pipette tip inserted at the centre of the corolla filled with 25 µl of a 20% w/w sucrose solution (0.5 cm opening diameter; the tip was sealed with Super Glue (Super Glue Corp., Rancho Cucamonga, CA, USA). At the beginning of the scotophase, naïve adult moths were placed individually inside the experimental arena. Each moth was allowed to fly freely for a maximum of 180 s, and if it did not probe on the flowers during that period, it was recorded as 'non-responsive'. If it probed, it was allowed to forage for an additional 210 s, and its foraging behaviour was recorded with a video camera (30 frames<sup>-1</sup>; Sony Digital 8 TRV 120).

### Experimental flower models

Each moth was presented once with a single, homogeneous flower array (i.e. treatment; 12 flower models of the same type). Treatments were run in parallel and pseudo-randomly.

#### Experiment 1: visual guides

In all nine treatments, flower models were flat disks of 6.5 cm diameter, that differed only in their colouration. Treatments were as follows. Plain white: disks coloured homogeneously white; Black stripes: white disk with two parallel 0.5-cm-wide stripes running 1.5-cm away from the centre; Black cross: two black stripes crossing at right angles at the centre of the flower (Fig. 1A); Plain black: disks coloured homogeneously black; White stripes, and White cross: the colour negatives of the above-described Black stripes and Black cross, respectively (Fig. 1B); Plain blue: disks coloured homogeneously blue; Blue stripes, and Blue cross: same as before, but using blue lines on white disks (Fig. 1C).

In experiment 1, I tested whether heterogeneously coloured flowers could affect inspecting behaviour while tactile input was the same across all treatments (H<sub>1</sub>; see Introduction). *M. sexta* is known to probe on white objects, but there is no evidence that they probe on black ones. Therefore, I chose the colour blue, which although darker than white, is preferred in dual choice experiments (Goyret et al., 2008). It was predicted that if moths use the visual guides offered, they would show different foraging efficiencies on the patterned and the plain coloured models.

#### Experiment 2: tactile versus visual

In this experiment the inspecting behaviour was compared between the treatment White cross, as described before, and White cross with grooves, which was the same flower model but with two straight grooves, 0.5 cm wide and ca. 0.2 cm deep, running parallel 1 cm away from the centre (Fig. 1D) (see Goyret and Raguso, 2006).

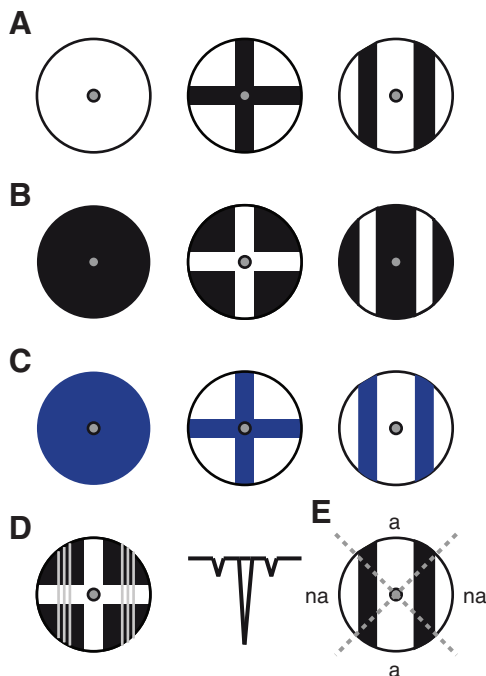


Fig. 1. Flower models used in experiments (not to scale). All models had a diameter of 6.5 cm with a pipette tip inserted centrally, serving as a nectar reservoir (opening, 0.5 cm; depth, 6 cm). From left to right: (A) Plain white, Black cross and Black stripes. (B) Plain black, White cross and White stripes. (C) Plain blue, Blue cross and Blue stripes. (D) In experiment 2 moths were also presented with a White cross with two parallel grooves (White cross with grooves), here in superior view (left, grooves in grey, dashed lines) and lateral view (right) to illustrate the three-dimensionality of this model, which is otherwise visually identical to White cross flowers. (E) Illustration of the criterion used to determine whether the body length of a probing moth was aligned (a) or not aligned (na) in-between the parallel visual guides in experiment 1 (tested both for Black stripes and Blue stripes; see Fig. 3).

Knowing that parallel grooves impair the moths' inspecting efficiency without visual guides, I predicted that the presence of tactile guides would diminish the effect of visual guides, unless visual control was stronger than the misleading tactile input.

#### Variables recorded

Responsiveness was recorded as the percentage of moths that probed at the flower array (for 20 s or more) out of the total number of moths subjected to the same treatment (i.e. flower type). Latency was measured as the time elapsed from the moment the moth took off until it probed on the first flower (maximum allowed: 180 s). Foraging time was measured as the time flying and/or probing since the first probe (max: 210 s). Foraging efficiency for each treatment was recorded as the number of emptied flowers during 210 s of foraging (emptied flowers). Probing time was measured as the time spent inspecting flowers before each of the first nine successful events (i.e. proboscis getting inside the nectary), not accounting for periods of flight in between flower visits (after Lewis, 1986). These measurements allowed me to evaluate whether moths can decrease the time spent probing as they forage on flower models as an indicator of inspection learning (Lewis, 1986). This was performed for Plain white, Black cross, Black stripes, and White cross treatments.

#### Hover alignment

While performing the experiments I noticed moths had a strong tendency to align their body-length axis in between the parallel visual guides of the Black stripes and Blue stripes treatments. Therefore, in order to quantify this phenomenon the probing time spent in an 'aligned' position was recorded for each moth on these treatments. This was done by randomly choosing five non-consecutive seconds of the probing time of each moth with a random number generator (Microsoft Office Excel 2007) and recording the time spent 'aligned' to a resolution of 1/30 s. A probing moth was determined to be 'aligned' if its body-length axis, (1) overlapped the diameter-line central to the two visual guides or (2) subtended with it an angle smaller than 22.5 deg to either left or right. This divided the flower in four sectors of equal size (separated by dotted lines in Fig. 1E), two opposing sectors where the moth was considered aligned and the other two opposing sectors where the moth was considered not aligned ('a' and 'na', respectively in Fig. 1E).

#### Statistic analysis

Responsiveness to the different flower types in experiments 1 and 2 was tested by means of log-likelihood *G*-tests. In experiment 1, latency (log-transformed) and emptied flowers complied with the ANOVA assumptions of normal distribution of errors and homogeneous variances; therefore, this test was applied (one-factor model). *Post-hoc* comparisons were run through a Duncan test, with Plain white treatment as control, and three LSD *post-hoc* tests (Fisher's least significant difference). Foraging time had to be tested with the non-parametric Kruskal–Wallis ANOVA by ranks test. In experiment 2, latency was evaluated by means of a one-way ANOVA, but emptied flowers and foraging time did not meet normality and homoscedastic assumptions, and were analysed by means of a Mann–Whitney test for two independent samples. Because of the number of tests performed,  $\alpha$ -level of significance for each test was adjusted to an  $\alpha=0.005$  to maintain a global  $\alpha$ -level proximate to 0.05.

Hovering alignment (time 'aligned') to Black stripes and Blue stripes was analyzed by a one-sample *t*-test against the expected value of 2.5 s (random hovering orientation). Comparison between alignment times for Black and Blue stripes was performed by an independent samples *t*-test.

Ability to improve inspection efficiency was evaluated by means of a goodness-of-fit test of the time spent inspecting before each of the first nine nectary discoveries to an exponential decline function, which describes a classic learning curve (Lewis, 1986). Statistical analysis followed procedures described by (Sokal and Rohlf, 1994) and was performed using SPSS 16 (SPSS Inc., Chicago, IL, USA) and SigmaPlot 9 (Systat Software Inc., San Jose, CA, USA).

## RESULTS

### Experiment 1: visual guides

A total of 234 moths were flown, of which 178 (76%) probed the flower arrays. There were no differences between treatments for this variable ( $G_6=3.52$ ;  $P=0.74$ ).

The average latency time for all analyzed treatments was  $32 \pm 3$  s (mean  $\pm$  s.e.m.), and the ANOVA (log-transformed) showed no significant treatment effect for this variable ( $F_{7,170}=1.60$ ;  $P=0.138$ ). Foraging time was shorter in the Black stripes treatment (i.e. they stopped foraging sooner), in which the moths probed for  $99 \pm 18$  s, whereas for all other treatments average foraging time was  $183 \pm 5$  s (mean  $\pm$  s.e.m.; Kruskal–Wallis test; with Black stripes:  $H_7=36.97$ ;  $P<0.0001$ ; without Black stripes:  $H_6=7.95$ ;  $P=0.159$ ). Plain black was not included in the statistical analyses because moths did not

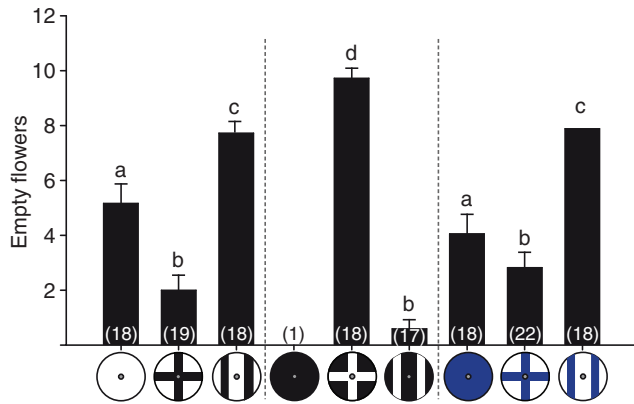


Fig. 2. Mean ( $\pm$  s.e.m.) number of emptied flowers by single moths during a foraging bout of 210 s. Regardless of colour pattern, moths tended to probe on white, brighter areas. Flower models had a diameter of 6.5 cm (area, 33.2 cm<sup>2</sup>; not to scale). Different letters denote significant differences (see text for statistic results). Numbers in parentheses are the number of moths probing and/or feeding in each treatment.

show any responses to these flowers (only 1 moth out of 18 probed, but for less than 5 s).

When analyzing emptied flowers I found a strong effect of visual guides in the foraging efficiency (i.e. emptied flowers) of naïve moths (ANOVA:  $F_{7,140}=34.15$ ;  $P<0.0001$ ; Fig. 2). *Post-hoc* comparisons between black and white treatments showed that moths probed on white lines on a black background, but avoided black lines on a white background (in all Duncan comparisons against Plain white,  $P<0.0001$ ; Fig. 2). Experiment 1 also included blue and white models. In this case, number of emptied flowers on Plain white differed from that of Blue cross and Blue stripes (Duncan:  $P<0.0001$ ), but not from Plain blue models ( $P=0.15$ ). The mean number of emptied flowers in Plain blue models was not significantly higher than in Blue cross (LSD:  $P=0.0982$ ) but was significantly lower than in Blue stripes (LSD:  $P<0.0001$ ; Fig. 2). Taken together, these results support the hypothesis that visual guides affect flower inspection efficiency in *M. sexta* ( $H_1$ ). When the nectary was included in white areas framed by coloured marks, moths frequently found the nectary, and could empty more flower models in the time they were allowed to forage.

Hovering alignment analyses showed that moths spent significantly more time in an aligned orientation when hovering in front of Black stripes flower models, spending 80% of the time in this situation [aligned time:  $4\pm 0.24$  s out of five randomly selected seconds, mean  $\pm$  s.e.m.; one-sample *t*-test (expected value: 2.5 s):  $t_{15}=6.175$ ;  $P<0.0001$ ; Fig. 3]. Even though moths showed a pronounced tendency, the time spent aligned when they probed on Blue stripes did not significantly depart from expected times (expected value: 2.5 s; aligned time:  $3.05\pm 0.24$  s, mean  $\pm$  s.e.m.; one-sample *t*-test:  $t_{15}=2.27$ ;  $P=0.039$ ; Fig. 3). When comparing alignment time between the two groups, an independent samples *t*-test showed that there was no difference between average times spent aligned to Black and Blue ( $t_{30}=-2.75$ ;  $P=0.01$ ; Fig. 3). A *G*-test was performed to evaluate whether moths approaching and first contacting a flower in an aligned position had higher probabilities of encountering the nectary. Out of 153 approaches to Black stripes models, 113 (74%) were aligned; of these, 51 ended in a successful visit and 62 in an unsuccessful one. Of the 40 approaches that were not aligned, 14 were successful and 26 were not. Thus, approach orientation

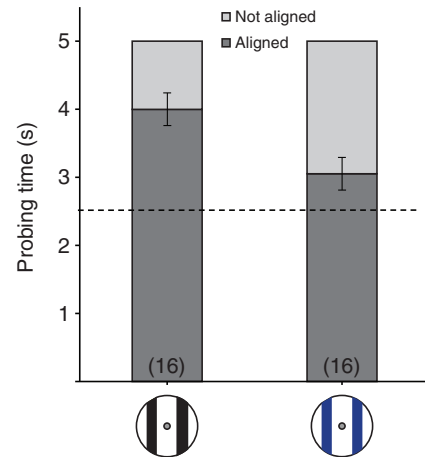


Fig. 3. Probing time spent aligned (dark grey bars) with parallel stripes of Black and Blue model flowers (out of 5 s recorded for each moth; resolution: 1/30 s; see text for details). Moths showed a tendency to probe flowers in alignment with stripes. This tendency was stronger for black stripes ( $P<0.0001$ ) than for blue stripes ( $P=0.039$ ). One-sample *t*-tests were performed under the null hypothesis of random orientation for each treatment (mean alignment, 2.5 s; horizontal dashed line). Number of replicates are in parentheses.

relative to the black stripes did not significantly increase the probability of a successful visit ( $G_h=1.26$ ;  $P=0.262$ ).

Tests of goodness of fit to an exponential decline function showed that moths did not learn to more efficiently inspect flowers without visual guides, or with 'misleading' visual guides (exponential decline regression; Plain white:  $R^2=0.39$ ,  $P=0.074$ ; Black cross:  $R^2=0.09$ ,  $P=0.562$ ; Fig. 4). Nevertheless, the presence of nectar guides that 'led to the nectary' facilitated inspection improvement with experience (Black stripes:  $R^2=0.59$ ,  $P=0.016$ ; White cross:  $R^2=0.64$ ,  $P=0.0095$ ).

## Experiment 2

There were no differences in responsiveness, with the flat White cross models being probed by 82% of moths and the White cross with grooves by 81% of total moths in that treatment (*G*-test;  $G_h=0.01$ ;  $P=0.914$ ).

Even though there was a trend for a longer latency on grooved models ( $44\pm 5$  s; mean  $\pm$  s.e.m.), there was no significant difference, with the latency on flat White cross flowers ( $22\pm 6$ ; mean  $\pm$  s.e.m.; ANOVA:  $F_{1,42}=3.96$ ;  $P=0.053$ ). Foraging times did not show differences between treatments either (Mann–Whitney test:  $U=159.5$ ;  $P=0.26$ ).

The parallel grooves significantly disrupted inspection efficiency, showing that even when moths almost exclusively probed on the white areas 'leading to the nectary' (cross), tactile guides interfered with this response (Mann–Whitney test:  $U=50.5$ ;  $P<0.0001$ ; Fig. 5). These results support hypothesis  $H_2$  (see Introduction and Discussion).

## DISCUSSION

### Visual guides and inspection behaviour

After finding the scented flower patch and extending their proboscis, *Manduca sexta* moths utilized non-graded visual marks on the flat corollas while inspecting flower models (Fig. 2). On the black and white models, moths directed their proboscis and maintained it on the white, brighter areas of the corolla, regardless of whether white

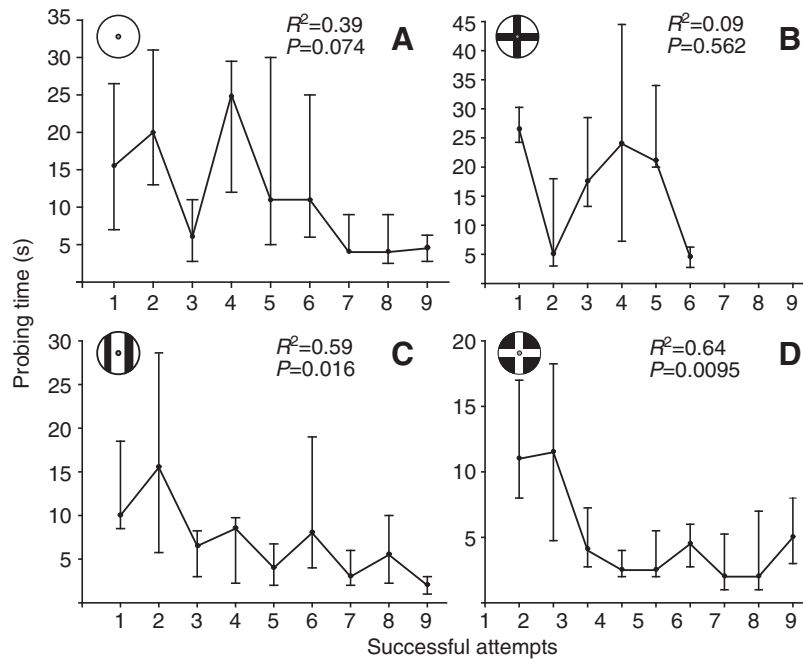


Fig. 4. Median  $\pm$  1st and 3rd quartiles of the time moths spent inspecting flowers to find the first nine nectaries.  $R^2$  and  $P$ -values are shown for a test of goodness of fit to an exponential decline function (1 factor; 2 parameters), a typical learning curve. (A) Plain white flower; (B) Black cross; (C) Black stripes; (D) White cross. Moths appear to improve inspecting efficiency with experience when foraging on flower models with the nectary over white areas (A,C,D). Nevertheless, among these, the fit is considerably better when black visual guides are present (C,D). Note the differences of scale on the y-axes.

was the colour of the background (main area) or of the contrasting markings (smaller areas; Fig. 2). In the blue and white models, moths also biased their probing towards the white, brighter areas (Fig. 2), strongly supporting the hypothesis of visual floral guides use by *M. sexta* ( $H_1$ ). It is noteworthy that, *a priori*, I regarded treatments with a cross (Black cross, Blue cross and White cross) as models where the visual guides 'lead to the nectary' and treatments with parallel stripes (Black stripes, Blue stripes and White stripes) as models where visual guides 'mislead' probing. The underlying hypothesis for these predictions was that probing movements would be directed by contrasting marks on the corolla surface. Overall, moths aimed their proboscis and probed persistently on the white, brighter areas of flower models regardless of their pattern, which by itself cannot predict moth behaviour. Thus, for *M. sexta*, it appears that pattern and contrast and/or colour features combined affect inspection efficiency.

When foraging on plain coloured models (white *or* blue) moths initially placed their proboscis approximately 1 cm from the edge, and upon contact, performed fast, short movements with the tip. Subsequently, body displacements while hovering dragged the 'vibrating' proboscis on, virtually, the whole surface of these models without any defined pattern. This 'searching' movements are functionally consistent with the goal-seeking characteristic of the flower inspection behaviour in a visually challenged naïve animal, and they resemble what Craig called an innate 'appetitive behaviour' (Craig, 1918). On bicolour models, the same innate inspecting pattern was displayed but narrowed to the white, brighter areas of the corolla. This behaviour suggests that visual guides indirectly affected probabilities of finding the nectary rather than guiding probing movements in a particular direction.

Interestingly, naïve *M. sexta* moths choose to feed from plain blue feeders rather than plain white ones in dual choice assays, apparently using colour vision rather than monochromatic intensity for flower detection and approach (Goyret et al., 2008), as does the related hawkmoth *Deilephila elpenor* (Kelber et al., 2002). Yet, results here suggest that moths used intensity cues to place their proboscis while inspecting more realistically modelled flowers (i.e.

probed on the brighter areas). It remains to test directly the three alternative hypotheses that could explain these results: (1) moths switch from using colour cues while approaching, to using intensity cues during inspection, (2) moths change colour preference (blue to white) from one instance to the other, or, probably the most parsimonious hypothesis, (3) moths use monochromatic contrast cues in both foraging instances (i.e. approach and inspection). The preliminary behavioural observations and the results obtained in this study warrant a detailed quantification of proboscis movements as a function of intensity contrast (chromatic and achromatic; graded and non-graded) and different patterns to better understand what

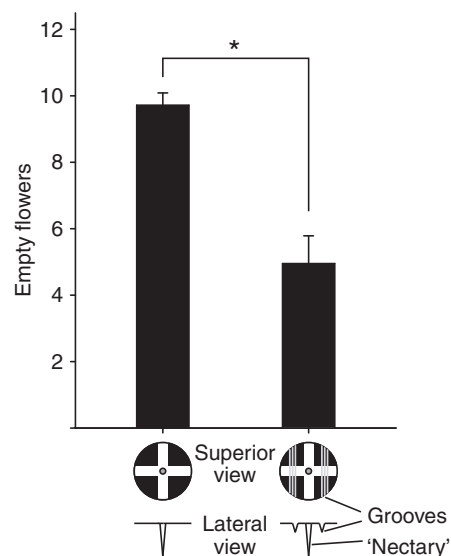


Fig. 5. Inspection efficiency (emptied flowers) when foraging on flat White cross models (left column) and the same model but with two parallel grooves (right column). X-axis shows the two treatments in lateral view (upper) and superior view (lower). \* $P < 0.0001$ ; see statistical details in Materials and methods and Results sections.

particular visual stimuli are used by moths and how (and if) they can affect inspection movements.

Finally, the presence of floral guides (which appear in some varieties of flowers pollinated by *M. sexta*) could make flowers more attractive, as it has been shown in bees (Free, 1970), bumblebees (Plowright et al., 2006), butterflies (Kandori and Ohsaki, 1998), drone flies (Lunau and Dinkel, 2001) and the diurnal hawkmoth *Macroglossum stellatarum* (Kelber, 2002). This was not tested, because I used homogeneous flower arrays consisting of one model at a time, but the similar responsiveness levels among the different treatments (excluding Plain black) suggest that differences in foraging efficiency were not linked to differences in attractiveness, but to inspection success rates (Fig. 2).

### Visual guides and hovering position

Some visual marks used in this study also elicited responses that were not directly associated with placement of the proboscis. This was the case for Black stripes and, to a lesser degree, for Blue stripes. When foraging on these flower models, moths tended to align their body-length axis with the parallel lines of the corolla (Fig. 3). A feasible hypothesis is that this body alignment in relation to the bilateral symmetry of these flowers could facilitate inspection, by indirectly affecting proboscis placement (on the mid line). Nevertheless, when investigating this phenomenon I found that approach orientation was not associated with the probability of success of the flower visit. Neither the distance of the moth to the flower, nor body angle relative to the horizontal plane appeared to be affected while probing (personal observation). Alternatively, this could be related to flight stability and motion detection. *M. sexta* is challenged to hover and keep its long proboscis in contact with flowers that can move by action of the wind. Nevertheless, they effectively track plain white flowers moving sinusoidally with frequencies of up to 2–3 Hz in the vertical and horizontal axes (Sprayberry and Daniel, 2007). We are now set to investigate whether contrasting visual marks on the corolla could have an effect on flower movement detection and tracking performance. During experiments and video analysis, it was noticeable that aligned hovering moths would frequently show fast, left–right oscillations of small amplitude, suggesting a regulatory process. The fact that alignment was more robust towards black lines than towards blue lines could suggest moths use achromatic contrast for this behaviour. Alternatively, the innate attractiveness of the blue colour (Cutler et al., 1995) could interfere with the orientation behaviour. These hypotheses remain to be tested.

### Visual guides and flower inspection improvement

Besides using visual guides to place their proboscis and orient their body while hovering, visual guides appeared to facilitate inspection improvement, resulting in decreasing probing times as the foraging bout proceeded (Fig. 4). Nevertheless, this was not always the case. When foraging on flowers such as Black cross models, initial discovery times were relatively long, and remained highly variable (Fig. 4B). This is deducible also from their low overall performance (Fig. 2). On models offering no visually contrasting marks, such as Plain white, there seemed to be a tendency to slowly decrease probing time averages, but with large variances (Fig. 4A). The case of models where the nectary was framed on a white background is very different in both studied cases (Black lines and White cross), showing a significant fit to a learning curve, with probing time decreasing rapidly along with its variance (Fig. 4C,D). These results support the hypothesis that visual guides can have a role in inspection learning performance ( $H_3$ ). Interestingly, only when moths have a relatively good initial performance (i.e. low probing

time in the first attempt) can they improve it. Lower initial performances appear to predict a difficulty to learn how to better exploit those models, even when several nectaries can be emptied. This aspect is very similar to what was previously found studying the role of mechanoreception in flower inspection learning (Goyret and Raguso, 2006). The prompt finding of the nectary provides the immediately preceding appetitive behaviour with the necessary feedback for motor calibration [i.e. inspection learning (Lorenz, 1973)]. *M. sexta* responded to visual and tactile floral features with an innate standard (i.e. fast, short proboscis-tip movements on white or brighter areas), which, under these particular experimental conditions, could be calibrated but could not be changed or re-set. Interestingly, the diurnal *M. stellatarum* is able to calibrate (when successful) and also to re-set (when initially unsuccessful) innate inspection patterns under very similar conditions (J.G. and A. Kelber, manuscript in preparation).

### Visual and tactile guides

In a previous study, where flat, plain white experimental flowers largely differed in shape and size, the only flower feature that appeared to influence inspection success was flower surface area, which negatively affected foraging efficiency (Goyret and Raguso, 2006). This strongly suggests that moths probed ‘randomly’ or ‘aimlessly’, and is consistent with the probing movements observed in this study. When adding a ‘grooved’ cross centred on the nectary, success rates dramatically increased, but when grooves were parallel to each other (and not intersecting the centre), moths could not find the nectary efficiently (Goyret and Raguso, 2006). In the present study, moths restricted their probing to the white, brighter areas of the corolla of flat flower models offering visual marks (Fig. 2). When both visual marks and grooves were present, success rates were strongly influenced by tactile guides (Fig. 5). Moreover, upon detection of a groove, probing behaviour changed from erratic short movements to a fast forward slide, following grooves through the previously avoided black areas. This supports the hypothesis that while visual floral guides can affect initial proboscis placement, tactile guides can control subsequent inspection movements ( $H_2$ ). It is interesting that these tactile stimuli, besides dominating inspection movements, appear to take priority over the visual stimuli used for flight stabilization while hovering (Sprayberry and Daniel, 2007; Wicklein and Strausfeld, 2000).

### Innate flower inspection behaviour

Instincts, or innate behaviours, appear to be organized in a sequence of discrete movements, each elicited by a specific stimuli configuration. Each of these responses usually increases probabilities of encountering the set of stimuli that will elicit the next movement, until the ‘goal’ is reached (Baerends, 1950; Mayr, 1988; Tinbergen, 1950). Naïve *M. sexta* respond to floral odours and air currents with an upwind zigzag flight (Brantjes, 1973; Willis and Arbas, 1991). This flight pattern increases the chances of visual detection of the odour source, which in turn, provokes a change in speed and a hovering flight pattern in front of the flower (Brantjes, 1978; Goyret et al., 2007; Raguso and Willis, 2003).

The innate flower inspection strategy of the nocturnal *M. sexta* involves an extension of the proboscis towards the visual target and an erratic, or ‘aimless’ probing. In a visually challenging context, this ‘appetitive behaviour’ (Craig, 1918) can effectively increase chances of finding the stimuli eliciting the next response. Upon tactile stimulation in the form of grooves or the corolla or nectary opening, moths ‘slide’ forward (see Results and Fig. 5) (Goyret and Raguso, 2006). The results of these experiments show that proboscis

placement is initially affected by corolla coloration (therefore restricting probing to the white or brighter areas), but that tactile cues control subsequent proboscis movements. This suggests that both inputs are processed sequentially and that the sequence of responses they elicit could be functionally integrated ( $H_2$ ). In fact, some *Nicotiana* and *Datura* flowers (among the preferred nectar sources for *M. sexta* in various environments), which have white, tubular corollas (Nattero et al., 2003; Raguso et al., 2003) show an either reddish or purplish ring along the perimeter of the nectary opening or around the corolla grooves [see pictures in Raguso and Willis (Raguso and Willis, 2003)]. Visually controlled avoidance of these markings could facilitate the encounter of the corolla opening and/or its grooves, leading to the deep nectaries.

Nevertheless, the question remains as to whether more natural floral marks such as colour gradation, intensity gradients or gradual changes in the density of small marks could influence not only proboscis placement, but also subsequent movement direction.

Pollination systems have been under strong selective pressures, which have led to physical adjustments between pollinators' body morphology and flower structures during their co-evolution [halictid bees (Benitez-Vieyra et al., 2006); hummingbirds (Campbell et al., 2002); hawkmoths (Moré et al., 2007; Nilsson, 1988)]. Here, I show experimentally that behavioural mechanisms during flower inspection could add to the functionality of these interactions. Different fine-scale motor responses elicited by multiple floral traits appear to be in concert with the adjusted morphology of pollinators and flowers required for both efficient foraging and effective pollen transfer.

#### ACKNOWLEDGEMENTS

I am very grateful to Tom Seeley and Tom Eisner for inspiring discussions about hawkmoth behaviour, and to Almut Kelber, Rob Raguso and two reviewers for their valuable comments and suggestions, which greatly improved the manuscript. I thank Raine Kaczorowski and Sarah Wigsten for help with video recordings, set up and animal care, and Miriam Henze for statistical advice. I am also grateful to the Department of Neurobiology and Behavior in Cornell University and The Wenner-Grenn Foundations of Sweden for their financial support.

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