Flexible responses to visual and olfactory stimuli by foraging *Manduca sexta*: larval nutrition affects adult behaviour

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Here, we show that the consequences of deficient micronutrient (β -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 *M. sexta* were reared on natural (*Nicotiana tabacum*) and artificial laboratory diets containing different concentrations of β -carotene (standard diet, low β -carotene, high β -carotene and cornmeal). This vitamin-A precursor has been shown to be crucial for photoreception sensitivity in the retina of *M. 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 on β -carotene-deficient artificial diets. Strikingly, moths reared on artificial diets supplemented with increasing amounts of β -carotene (low β and high β) showed increasing probabilities of response to scentless feeders. We discuss these results in relationship to the use of complex, multi-modal sensory information by foraging animals.

Keywords: Lepidoptera; olfaction; vision; hawkmoth; sensory ecology

1. 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 & 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, naive animals can show ‘appetitive behaviour’, displayed as a seemingly random survey of the environment, which increases their chances of encountering relevant stimuli that release innate, adaptive-feeding responses. Such responses may include motor patterns such as upwind flight in an odour plume, or proboscis extension towards a visual target (Lorenz 1981), and can be released through one or various sensory cues. For example, nectivorous bats from the genus *Glossophaga* use echolocation (von Helversen *et al.* 2003) and olfactory cues (von Helversen *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 & Honda 2005) and the hawkmoths *Macroglossum stellatarum* and *Deilephila elpenor* (Balkenius *et al.* 2006).

The use of apparently redundant stimuli (e.g. odour and visual display) offers searching pollinators the potential to use different sensory channels to mediate

foraging responses under different contexts and at different scales (upwind flight from a distance, proboscis extension close to the target; see Jang *et al.* 2000; Balkenius *et al.* 2006; Goyret *et al.* 2007). 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 & Gumbert 2001) and *B. impatiens* (Gegear & 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 (Hebets & Papaj 2005).

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 (von Helversen *et al.* 2000), and honeybees and *V. indica* butterflies can forage on scentless artificial flowers (Giurfa *et al.* 1995; Ômura & Honda 2005). The diurnal hawkmoth *M. 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, *D. elpenor*, are mediated primarily by olfactory stimulation (Balkenius *et al.* 2006), despite this species’ acute capacity for night colour vision (Kelber *et al.* 2002). Thus, there is substantial evidence that single modality input can be sufficient to elicit appetitive responses.

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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, hygro-reception, mechanoreception and CO₂ detection (Raguso & Willis 2002; Thom *et al.* 2004; Goyret & Raguso 2006). Nevertheless, visual and olfactory stimulations have been shown to be necessary and sufficient to elicit the complete sequence of foraging responses in laboratory-reared and wild adult *M. sexta* (Raguso & Willis 2002, 2005). Bennett & 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 stimulations to feed (Raguso & Willis 2002, 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 *et al.* (2007) investigated the effect of larval diet composition on adult moth foraging behaviour, 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 β -carotenes. These results support the general hypothesis that deficient intake of β -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 β -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 naive adult *M. sexta* moths reared on five different diets, including live tobacco plants, a natural host plant and the commonly used (wheat germ based) artificial diet with direct and indirect β -carotene supplements. We designed this study to test the following hypotheses:

(H₀) Null: larval diets have no effect on naive adult-feeding behaviour.

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

(H₁) The previously observed feeding requirement of laboratory-reared adult *M. sexta* for combined olfactory and visual inputs is an artefact of β -carotene deficiency.

Prediction: diet effects should be more evident when moths depend primarily on visual stimulation (unscented feeders).

(H₂) Differences in foraging behaviour 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 β -carotene content (see §2) should not affect foraging behaviour.

2. MATERIAL 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, SC, USA. (henceforth: USC).

(a) *Animals and diets*

We reared *M. sexta* in the University of South Carolina from eggs provided by Dr Lynn Riddiford, from her laboratory colony at the University of Washington, Seattle, WA, USA, under a photoperiod of 16 L : 8 D cycle. This source of moths was used for all experiments.

We randomly assigned individuals to one of five different diets:

- (i) wheat germ-based artificial diet (after Bell & Joachim 1976; henceforth: standard diet), which is the most commonly used diet in *M. sexta* research (no β -carotene content),
- (ii) standard diet supplemented with 200 mg of β -carotene/L—211 $\mu\text{g g}^{-1}$ —(Sigma Aldrich, St. Louis, MO, USA—henceforth: low β),
- (iii) standard diet supplemented with 800 mg of β -carotene/L—844 $\mu\text{g g}^{-1}$ —(henceforth: high β),
- (iv) standard diet with yellow cornmeal instead of wheat germ as the main source of carbohydrates—793 $\mu\text{g g}^{-1}$ —(henceforth: cornmeal) and
- (v) intact, uncut *Nicotiana tabacum* plants (seed lot GL350, GoldLeaf Seed Co., Hartsville, SC—henceforth: tobacco)—880 $\mu\text{g g}^{-1}$ (in Ashton strain; see Mummery *et al.* 1976).

The concentrations of β -carotene listed above are provided for informational purposes; note that we did not determine assimilated quantities, which can be very different for each case (Mummery *et al.* 1976; Bennett & White 1989).

(b) *Treatments and procedures*

After eclosion, adult moths were starved for 3–4 days to increase feeding motivation. We used cubic cages (45 × 45 × 45 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., CA, USA) placed directly above each cage, providing a luminance of 0.08 cd m⁻² (similar to twilight). We placed two artificial feeders (see Pfaff & Kelber 2003) 20 cm from each other (with the midpoint between them at the centre of the cage), above black-covered water bottles and 25 cm above the cage floor (vertically). 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 per cent (w/w) sucrose sugar solution. In one of the two treatments (scented), both feeders were scented (25 $\mu\text{l l}^{-1}$ of pure bergamot oil extract in the sucrose solution), while in the other treatment (unscented), both feeders were unscented.

Moths were pseudo-randomly selected (random selection among the eclosed), individually placed in the flight cage and allowed to fly for up to 5 min. We recorded the responsiveness (percentage of responsive moths over the total number of moths subjected to the experiment—i.e. percentage of moths that extended their proboscis and contacted any of the two feeders with it) and latency (seconds elapsed from take-off to

Figure 1. 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 α -level of 0.0017 (after Bonferroni correction). n.s., non-significant diet effect of responsiveness to the scented feeders. Numbers in parentheses denote number of independent replicates.

first-probing attempt). We also recorded relative reflectance of adult moth eyes. The eyes of *M. 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 mm away from the eye (S2000, Ocean Optics Europe, Eerbeek, The Netherlands). These measurements were performed on moths reared on standard, cornmeal and tobacco diets.

(c) Statistical analysis

The responsiveness variable was analysed by means of *G*-tests and the latency variable by means of three-way analysis of variance (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 (five diets and two 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 α -level (0.0017) in order to maintain a global α -level of 0.05.

3. 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 versus females for the scented treatment and $G=0.006$; $p=0.94$; $n=243$; *G*-test males versus females for the unscented treatment). Treatments (i.e. scented versus 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; figure 1). Responsiveness to unscented feeders for the standard diet group was significantly lower than for all other treatments except the low β group (versus low β : $G=7.47$; $p=0.0063$; $n=118$; versus high β : $G=41.46$; $p=1.2 \times 10^{-10}$; $n=117$; versus cornmeal: $G=20.88$; $p=4.9 \times 10^{-6}$; $n=122$; versus tobacco: $G=49.49$; $p=2 \times 10^{-12}$; $n=120$; figure 1). The low β group differed from the high β group ($G=11.39$; $p=0.0007$; $n=79$; figure 1) and from the tobacco group ($G=14.95$; $p=0.0001$; $n=82$; figure 1), but not from the cornmeal group ($G=2.38$; $p=0.12$; $n=84$; figure 1). The high β group did not differ from the cornmeal group ($G=3.81$; $p=0.05$; $n=83$; figure 1), or either from the tobacco group ($G=0.20$; $p=0.66$; $n=81$; figure 1), which did show higher responsiveness levels than the cornmeal group ($G=5.95$; $p=0.015$; $n=86$; figure 1). For moths reared on artificial diets, the incremental direct addition of β -carotene reduced the disparities between treatments (standard diet: $G=30.48$; $p<0.0001$; $n=165$; low β : $G=4.09$; $p=0.04$; $n=80$; high β : $G=0.48$; $p=0.49$; $n=75$).

ANOVA for the latency variable showed no differences between genders ($F_{1,266}=0.55$; $p=0.46$; figure 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 of any class were detected (gender versus

Figure 2. Latency times (in seconds; medians (filled squares), quartiles (unfilled squares, 25–75%) 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). *** $p < 0.001$. Numbers in parentheses denote the number of independent replicates (note that the number of replicates differ from the ones in figure 1, because here we analyse only the fraction of responsive moths).

treatment: $F_{1,266} = 0.05$; $p = 0.83$; gender versus diet: $F_{4,266} = 0.28$; $p = 0.89$; diet versus treatment: $F_{4,266} = 1.21$; $p = 0.31$; gender versus treatment versus diet: $F_{4,266} = 1.81$; $p = 0.13$). There were no diet effects when analysing the unscented treatment ($F_{4,111} = 1.70$; $p = 0.16$; figure 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 β and the tobacco diets (Tukey's honest significant difference for unequal number of replicates: $p = 0.0009$). Because all other diets within the scented treatment did not differ significantly, we pooled diets for each treatment and present their medians, quartiles and 5–95% whiskers in figure 2.

Spectroradiometry of the eye glow from animals reared on tobacco, standard diet and cornmeal diet showed quantitative differences (figure 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 *M. sexta*, these pigments have sensitivity maxima at 357, 450 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; A. Kelber & J. Goyret 2005, 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 (figure 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 (figure 3).

4. DISCUSSION

(a) β -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 usage 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 (figures 1 and 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 (figure 1). Additionally, the apparent gradual augmentation in responsiveness that accompanied incremental supplements of β -carotenes to artificial diets (standard diet, low β and high β) 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 β -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 β -carotene. While we cannot speculate here on the assimilation capacity of pigments in the different diets, 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 β -carotene content specifically affects adult nectar-foraging behaviour (H_1).

When foraging on scented feeders, latency times averaged less than 60 s, whereas when odour was absent, moths spent an average of 85 s before probing (figure 2). Our previous studies using naive *M. sexta* (reared on standard diet) showed that floral odours can elicit both upwind 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 β -carotene-deficient larval diet has on the visual system of adult *M. sexta*.

(b) 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 in absorbing incident light than those of moths reared on

Figure 3. Relative reflectance as a function of wavelength (nm) of the eyes of adult moths reared on either standard diet (solid line), tobacco (dotted line) or cornmeal diet (dashed line). The peak difference between standard diet animals and tobacco animals is at 510 nm, close to the maximum absorbance of the 'green' photoreceptor (P520).

tobacco plants or cornmeal (i.e. higher relative reflectance; figure 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 & White 1989). Tobacco-bred moths had a human red eye glow, due to absorption in the entire human visible spectrum except the 'red wavelengths' (more than 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 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 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.

(c) β -carotene, visual sensitivity and foraging behaviour

Deficiencies in β -carotene are known to negatively affect the morphology of the photoreceptor membrane, retinal rhodopsin content and visual sensitivity in *M. sexta* (Bennett & White 1989). On the other hand, *M. sexta* has consistently shown more significant responses to visual targets when olfactory stimuli were also provided

(Raguso & Willis 2002; Goyret *et al.* 2007; Riffell *et al.* 2008). Here, the combined results from our behavioural experiments and spectroradiometric measurements support the hypothesis that naive *M. sexta* has difficulty recognizing an odourless visual target as a nectar source when their vision is impaired by suboptimal β -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 1), odour still affects their feeding responses (figure 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 & Willis 2005). On the other hand, the visual deficiencies resulting from low β -carotene intake neither impair flight performance (J. Goyret 2007, 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

β -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 and/or contrast; see Goyret *et al.* 2008). We might test this hypothesis by evaluating responsiveness to visual targets under conditions of different light intensities. *Manduca sexta* shows an increment in responsiveness from 72 per cent to 100 per cent when light intensity increases from 0.023 (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 *M. sexta* to scentless feeders is directly linked to lower levels of light absorption (decreased visual sensitivity) associated with low intake of β -carotene during larval stages. Second, 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 and 490 nm). Besides the implications of our results for research on the model laboratory organism *M. 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 bumble-bees to glossophagine bats.

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