

Behaviour towards an unpreferred colour: can green flowers attract foraging hawkmoths?

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SUMMARY

Naïve hawkmoths (*Manduca sexta*) learn from a single trial to approach and attempt to feed from an artificial flower of an innately unpreferred green colour even when a distractor flower with a preferred yellow colour is present. In some of the animals, the choice of the innately unpreferred colour during free-flight testing persists for several days despite not being rewarded and eventually leads to starvation. The results show that moths exhibit a very strong flower constancy that is not limited to the colours of nectar flowers.

Key words: colour, hawkmoths, flower constancy, foraging, long-term memory, one-trial learning.

INTRODUCTION

Nocturnal insects rely mainly on odour to forage and mate during low light levels (Balkenius and Kelber, 2006; Brantjes, 1978). Earlier experiments with colour and odour in nocturnal and diurnal hawkmoths have shown that vision is the primary modality for diurnal hawkmoths, while nocturnal species mainly depend on odour (Balkenius et al., 2006), although they can discriminate colour under very dim light conditions (Kelber et al., 2002). This ability depends on their superposition compound eyes that can accumulate light from numerous facet lenses to increase the chance of light capture.

The retina of *Manduca sexta* contains three visual pigments P520, P450 and P357 (Bennett and Brown, 1985) that make their photoreceptors sensitive to ultra-violet, blue and green wavelengths (Land and Nilsson, 2002; Stavenga and Arikawa, 2006; Warrant et al., 2003), and allows the feeding behaviour of *M. sexta* to depend on wavelength (Cutler et al., 1995; White et al., 1994).

Because the colour and odour of suitable nectar flowers depend on the particular living environment, and because hawkmoths migrate over long distances, the choice of target flower could favourably be modified by learning (Kelber, 1996). *Manduca sexta* have an innate preference for blue flowers (Cutler et al., 1995) but are most often observed foraging from white or yellow night-blooming flowers in the wild (Goyret et al., 2008). They can also be trained to select an initially unpreferred colour (Goyret et al., 2009). This implies that they can learn the colours of the flowers containing nectar during the night. Although *M. sexta* will spontaneously visit flowers of many different colours, it will never mistake the green background for a flower. This suggests that there are innate limitations on the colours that indicate a flower to the hawkmoth.

In bumblebees, it has been shown that in a short time span after a floral visit, the probability of visiting a flower of the same species is extremely high (Chittka et al., 1997; Chittka et al., 1999). This flower constancy can even make pollinators ignore flowers close to them that contain relatively more nectar than a flower of the right

kind further away (Chittka et al., 1999; Hill et al., 1997). One advantage of flower constancy is that it allows for motor learning specific to a particular flower. This decreases the time spent probing and finding the nectar as the insect already knows how the nectar should be retrieved. As a consequence, it minimises the cost of switching between flowers. Another aspect of flower constancy is that the insect can know that a particular type of flower is likely to contain nectar because it has previously been able to retrieve nectar from it.

Being constant to a particular flower type could also enable the use of visual search strategies based on a single visual feature such as a colour (Chittka et al., 1999). Flower constancy is also useful from the point of view of the flower, because it facilitates pollen transfer (Chittka et al., 1999; Raine and Chittka, 2006). If a single-colour search strategy is used, then it would be expected that processing of green is suppressed because it is a background colour in nature.

In the present study, we investigate if naïve *M. sexta* can learn to forage from green flowers after being rewarded while presented with a green colour. Is the flower constancy so strong that they will select an unpreferred green colour over a preferred yellow flower? What is the strength and persistence of the flower constancy? Moreover, we tested if there is any difference in the foraging behaviour with and without odour? Can a flower odour tell the hawkmoth that green is a flower? Finally, we tested how long the moths would persist in preferring green even when they were never rewarded.

MATERIALS AND METHODS

Animals

The animals used were both males and females of the hawkmoth *Manduca sexta* L. (Lepidoptera: Sphingidae). Larvae were reared on an artificial diet (Bell and Joachim, 1976) with 200 mg l⁻¹ of beta-carotene added (Raguso et al., 2007).

The animals were kept under a 16h:8h light:dark cycle at 23–25°C, 40–50% relative humidity. The training and tests were

made within the first 2 h of their dark period. Experiments were performed on 3–4 days post-emergent naïve moths to increase feeding motivation.

Stimuli

The environment was illuminated from above with a halogen spotlight filtered to 0.5 cdm^{-2} (neutral density filters, LEE filters, 210 0.6ND; Andover, Hants, UK).

All experiments were made in a flight cage ($50 \text{ cm} \times 50 \text{ cm} \times 80 \text{ cm}$) with two artificial flowers (Pfaff and Kelber, 2003) made from syringes (diameter 21 mm) fitted with collars (diameter 30.5 mm) in green or yellow (Fig. 1A) bearing a $5 \mu\text{l}$ aliquot of a phenylacetaldehyde odour solution (a general flower odour). In a control experiment, the brightness of the yellow artificial flower was reduced using a filter (neutral density filters, LEE filters, 210 0.6ND, Fig. 1B). The spectra of the light reflected from the collars were measured using a calibrated Ocean Optics S2000 spectrophotometer (Dunedin, FL, USA) (Fig. 1B). The stimuli were centrally placed 35 cm above the cage floor and 25 cm apart from each other.

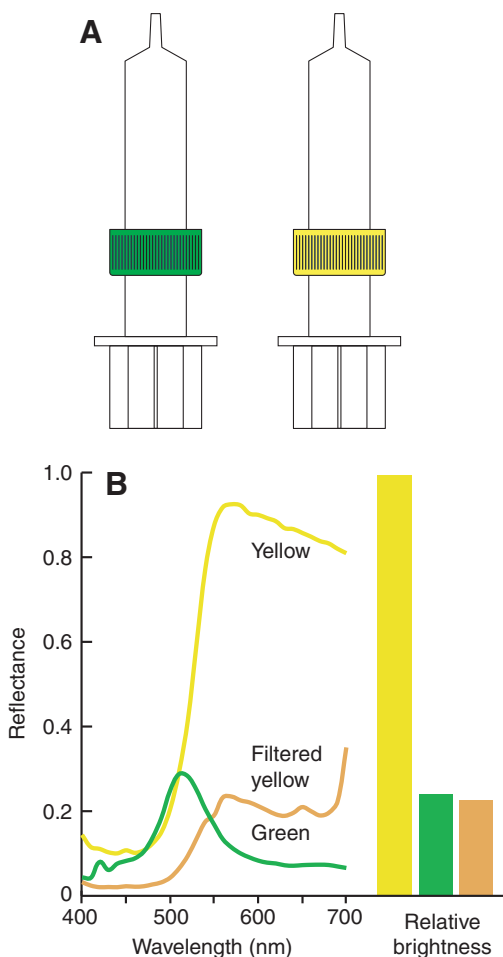


Fig. 1. (A) The artificial flowers with a green or yellow collar. (B) Reflection curves for the green, yellow and the filtered yellow artificial flowers together with the relative brightness of the three feeders within the wavelengths visible to the moths.

Experimental procedure

The naïve animals were divided into six groups. Each moth was tested individually. The first group was used for preference tests where animals were released into the cage without being pre-exposed to any colour or odour. Their first choice of one of the stimuli was recorded. It was considered a choice when the moth hovered in front of the artificial flower and extended its proboscis in an attempt to feed. Animals that did not make a choice within five minutes were removed from the experiment. No reward was present during the preference tests.

The second group was the experimental group. As *M. sexta* do not spontaneously approach green stimuli, it was necessary to manually move the moth towards the artificial green flower to train them. In order to not harm the animals, they were allowed to first enter a plastic tube (diameter 1.4 cm) with a small opening in the front that enabled the head to get through. This tube is then moved towards the artificial flower in a way that makes the moth receive the same visual stimulation as if it had been flying. The moth was subsequently held at a distance from the artificial flower appropriate for the length of its proboscis with the artificial flower stimulus presented at the centre of the visual field.

The proboscis was manually unrolled to allow the administration of 20% sugar solution. This was necessary because *M. sexta* never extend their proboscis unless they are hovering, whether they have been trained or not. Only a single learning trial was used where the moth was presented with the artificial green flower starting 5 s before being given sugar solution for 15 s (Fig. 2A). After training, the moth was released in the flight cage and allowed to make a single choice between the two artificial flowers. The test phase was subsequently repeated once every day to see if their choices would change. The locations of the two colours were randomly interchanged between days. The moths were never fed except during the initial training phase. The moths were kept in separate small cans with the same light cycle as during their pupal stage.

The third group was treated in exactly the same way as the experimental group, except that the timing of the stimulus presentation was different (Fig. 2B). This backward-paired group

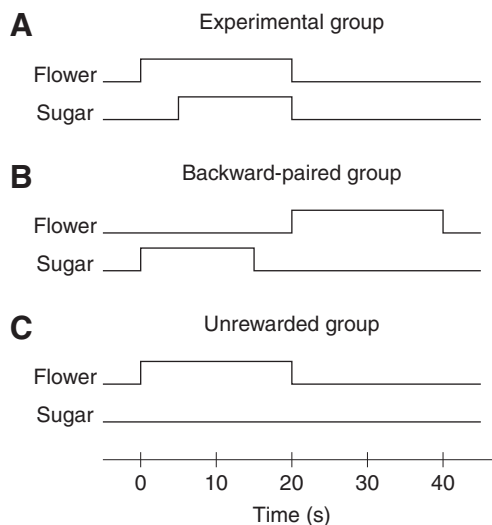


Fig. 2. Timing of the presentation of the artificial flower and the sugar solution. (A) Experimental group. (B) Backward-paired group. (C) Unrewarded group.

was used to test whether learning resulted from the pairing of the green artificial flower and the sugar solution rather than the presentation of the two stimuli independently of their temporal relationship. Also the fourth unrewarded group was treated in the same way as the experimental group, except that it was not rewarded with any sugar solution when presented with the artificial green flower (Fig. 2C). The fifth group also received identical training to the experimental group but no odour was present.

Finally, we performed a control experiment to determine whether the animals used brightness rather than colour to identify the rewarded stimulus. The experimental procedure was exactly the same as for the experimental group above except that we manipulated the brightness of the stimuli. Because the green stimulus is less bright to the moths, they could conceivably have learned to approach the darkest stimulus rather than the green colour. To test this, we made the brightness of the two stimuli almost the same (Fig. 1B). If the moths had learned to select stimulus based on brightness, they would not be able to distinguish between the two test stimuli. However, if they had learned the colour regardless of brightness, they would select the green stimulus.

Data evaluation

The choices of the moths were analysed using Fisher's exact test using the statistics package R (R Development Core Team, 2009).

RESULTS

Preference group

The naïve animals that were allowed to choose between a yellow and a green stimulus in the preference test always selected the yellow. These choices are significantly different from random selection (Fisher's exact test, $P < 0.01$, $N = 20$; Fig. 3A).

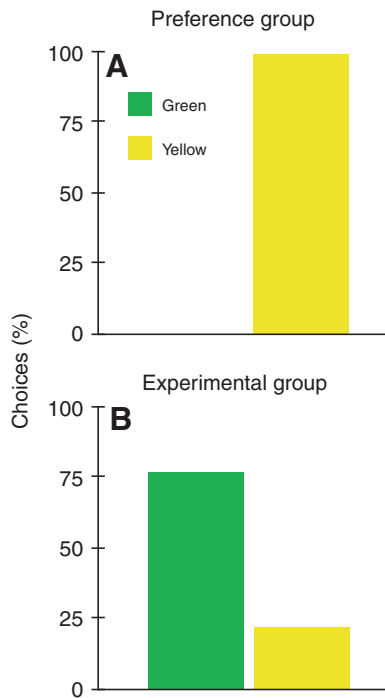


Fig. 3. Stimulus choices of the moths. (A) Choices of the yellow and green stimuli during the preference test. (B) Most moths in the experimental group selected the green stimulus after one training trial.

Experimental group

The animals in the experimental group were exposed to a single presentation of the green colour combined with sugar solution. As a result, the moths made almost completely reversed choices in the following test with a green and a yellow artificial flower compared with the preference group (Fisher's exact test, $P < 0.0001$, $N = 52$; Fig. 3B). This shows that a single learning trial changed the behaviour of most of the moths from selecting the innately preferred yellow foraging colour to the unpreferred green colour, thus proving the ability of moths to learn the green colour.

When the test was repeated each day, many of the moths in the experimental group persisted in selecting the green artificial flower for six days, indicating that the experience of the single learning trial had resulted in long-term memory processes (Fig. 4A). The choices of the moths each day after the training were significantly different from their initial preferences during the first three days (Fisher's exact test, day 1: $P < 0.0001$, day 2: $P < 0.0001$, day 3: $P < 0.0001$, Fig. 4A) but not for the subsequent three days (Fisher's exact test, day 4: $P = 0.078$, day 5: $P = 0.52$, day 6: $P = 1$, Fig. 4A).

As the moths that selected the green artificial flower over the rewarding yellow flower were never fed, their persistence in selecting the trained stimulus eventually led to their starvation. Fig. 4B shows the number of surviving animals in the group that continued to select green. This result shows that many moths adhere to their flower constancy even when it is clearly maladaptive and a rewarding flower of a colour that would normally be preferred is present.

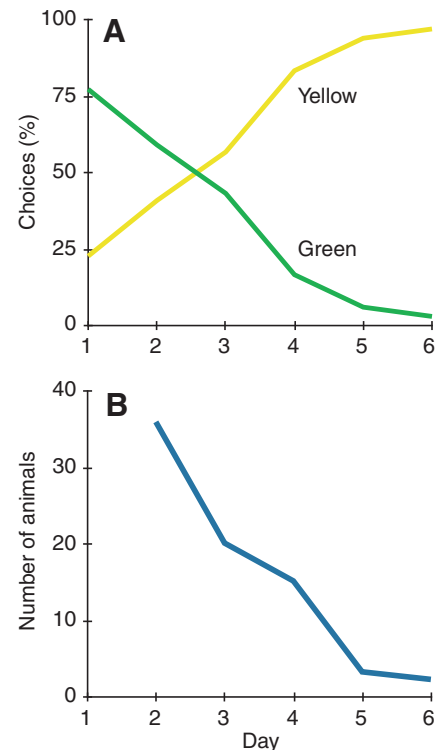


Fig. 4. (A) Preference changes over time. The choices of the green and yellow stimuli each day after a single learning trial with a green stimulus. (B) Survival over time for the animals that persistently selected green. This shows that a single learning trial can lead to permanent maladaptive behaviour.

Backward-paired group

In the backward-paired control group where the sugar solution was administered before the presentation of the green artificial flower, all of the moths selected the yellow colour during the tests, indicating that this training method did not result in any learning (Fisher's exact test, $P < 0.0001$, $N = 10$; Fig. 5A). This shows that the presentation of the flower and the sugar solution must occur in an ecologically valid temporal relationship with the visual stimulus present before the sugar solution is received.

Unrewarded group

To determine whether the handling of the moths was not responsible for the learning effects, the second control group received identical treatment to the experimental group but did not receive any sugar reward. The choices of the unrewarded control group are significantly different from the experimental group as the animals always choose the yellow stimulus just like the naïve animals (Fisher's exact test, $P < 0.0001$, $N = 10$; Fig. 5B).

No odour group

To test the role of the odour, we also trained and tested moths without any odour. The results were not significantly different from the experimental group (Fisher's exact test, $P = 0.26$, $N = 15$; Fig. 5C), indicating that the odour did not affect the learning.

Brightness group

Fig. 5D shows the result of the control experiment where the brightness of the yellow stimulus was reduced to coincide with the green training stimulus. The moths always selected the stimulus with the trained green colour, thus ruling out that they had learned brightness. Fisher's exact test showed that their choices were significantly different from random selection ($P < 0.05$, $N = 10$; Fig. 5D).

DISCUSSION

When *M. sexta* are allowed to choose between a yellow and a green stimulus in a preference test they always selected the yellow one (Fig. 3A). This is natural because the nectar flowers of hawkmoths are never green in nature. However, when we trained the animals with a single short exposure to the green artificial flower combined with sugar solution, their choices were almost completely reversed in a following test (Fig. 3B). This contrasts with earlier experiments with hawkmoths where typically many learning trials were necessary. Goyret et al. trained *M. sexta* with a white rewarded feeder and a blue unrewarded feeder for five days before testing the animals (Goyret et al., 2008). Another species of hawkmoth, *Macroglossum stellatarum*, has been shown to learn to discriminate between a red and a blue stimulus in a single day but 15 visits to the flowers were necessary before obtaining significant learning results (Kelber, 1996).

There are two crucial differences between our study and previous colour learning experiments with hawkmoths that may explain the faster learning. The first is that our naïve animals were only presented with the single training stimulus, while in previous studies, the moths had to choose between the rewarded stimulus and an unrewarded, innately preferred, colour. In the study by Kelber (Kelber, 1996), the moths were even initially rewarded at the preferred colour to increase motivation and this would probably reduce the learning speed. Some earlier demonstrations of one-trial colour learning in honeybees also used a single training stimulus and tests with several stimuli (Menzel and Erber, 1974; Giurfa et al., 1995), although single-trial learning has been shown in bumblebees also with two

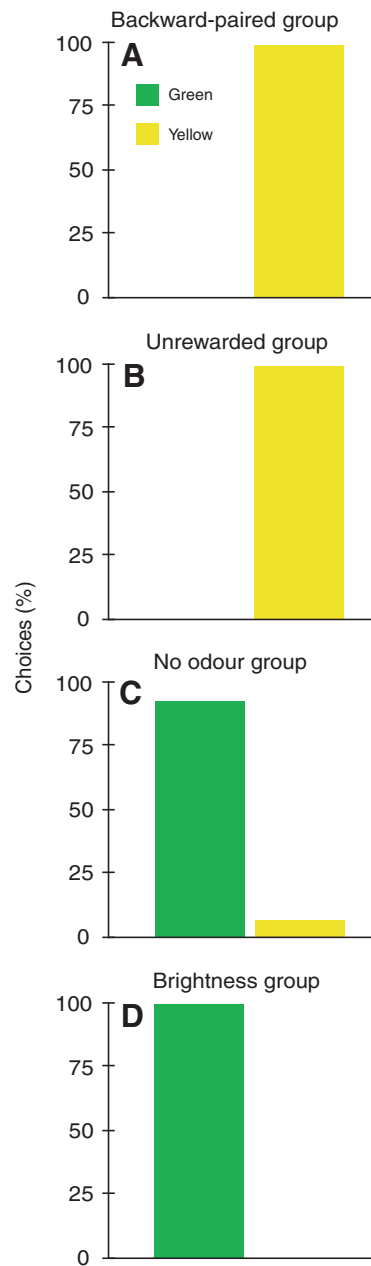


Fig. 5. The four control groups. (A) The moths received sugar solution before the presentation of the green colour and this did not result in any learning as they selected only the yellow stimulus. (B) The moths did not receive any reward but were otherwise treated in the same way as the experimental group. The moths behaved exactly as the naïve untrained animals and selected the yellow stimulus every time. (C) The animals received identical training to the experimental group except that no odour was presented. The absence of odour did not prevent learning as the animals learned to select the green stimulus. (D) The moths selected the trained green colour although the brightness of the yellow test stimulus was more similar.

different stimuli present during training (Ings et al., 2009). The second difference to earlier experiments was that our moths were constrained during training while previous experiments have used free-flying animals. It is reasonable to believe that free-flying animals are faced with a larger number of competing stimuli that could slow down learning.

As the choice of the green stimulus was not rewarded during testing, it would be expected that the choice of this stimulus would be quickly extinguished. We tested this by allowing the trained animals to choose once between a green and a yellow stimulus on each subsequent day. Surprisingly, the choice of the green stimulus was not completely extinguished until day 6, indicating that the experience of the single learning trial had resulted in long-term memory processes (Fig. 4A).

This contrasts sharply with earlier colour learning experiments in insects where multiple trials were necessary for long-term effects. Typically 3–5 trials were used (Menzel, 1999; Hori et al., 2006; Niggebrügge et al., 2009). This can lead to long-term effects within 2 min if the trials follow each other quickly (Menzel and Müller, 1996). A similar result is also found for olfactory learning where it has recently been shown that five conditioning trials lead to synaptic changes in the mushroom body of the honeybee (Hourcade et al., 2010).

The slow extinction found in the present experiment differs from place learning in hawkmoths, which only persists for 24 h even without any extinction trials (Balkenius et al., 2004). This could reflect, that in the ecology of this animal, colours are more important predictors of nectar than a place. This flower constancy makes the animals persist in selecting the green stimulus even though the consequence is that they will never be fed. Although all animals will survive for at least two days (Fig. 4B), over the next four days, all animals that do not extinguish their behaviour and change their choice will eventually die from starvation. This shows that the consequences of the single learning trial are very strong and persist even when they are maladaptive.

The use of a choice behaviour to identify learning contrasts with the common use of the proboscis extension reflex (PER) as an index of learning processes in insects. The latter is not possible in constrained hawkmoths because they do not express a PER unless they are hovering, and training of constrained animals therefore does not appear to produce any learning. However, the results of our experiment show that constrained hawkmoths do learn colours even though they do not express this learning until later when they are released in the flight cage. This indicates that the choices made during foraging can be changed even at a time when no foraging behaviours are expressed. This is also a powerful demonstration that choice behaviour can be a much stronger indicator of learning than motor reflexes (Rescorla and Solomon, 1967).

Although stimulus learning could influence choice behaviour through transfer between a Pavlovian learning mechanism and instrumental learning (Sandoz et al., 2000), our results suggest that learned flower choice in free-flying moths depends directly on Pavlovian learning (Pavlov, 1927). The fact that the learned choices are expressed at the first testing trial also point in this direction. However, to further establish that a Pavlovian process is responsible for the learning, it would be necessary to record some form of response in the constrained animals. Even though constrained hawkmoths do not show any visible PER, it may be possible to measure the precursor to this response by inserting an electrode in the cibarial muscle. Unfortunately, this could harm the animals and would interfere with the tests in the flight cage. This procedure has also proved very unreliable in hawkmoths.

To exclude that the handling in the learning phase was responsible for the behavioural change, one control group received identical treatment to the experimental group but did not receive sugar solution. Another control group received the sugar solution before the green artificial flower was presented to it. None of the

control groups learned to select the green artificial flower. The choices of both control groups are significantly different from chance as the animals always chose the yellow stimulus just like the naïve animals in the preference test (Fig. 3B and Fig. 5). Nevertheless, we observed that the moths in the unrewarded control group sometimes approached the green stimulus before eventually selecting the yellow, something that the naïve moths never did. This may be a sign of a weak exposure effect (Bornstein, 1989).

Earlier experiments have suggested that odour is necessary both to promote proboscis extension (Raguso and Willis, 2002) and to motivate the animals to forage (Brantjes, 1978). Odour can also enhance responsiveness to a visual target (Goyret et al., 2007). This was the motivation for using scented artificial flowers in our experiments. However, the behaviour of the control group without odour shows that both colour learning and choice behaviour are possible without any odour even when a green colour is used. Apparently, the general flower odour did not make the green colour any more flower-like as the behaviour of the groups with and without odour was the same, suggesting that flower constancy mainly depends on colour in this species.

Another explanation for the identical behaviour with and without odour is that the flight cage is small, and we know that odour is most important far away from the flower while the visual stimulus is more important at a closer range (Goyret et al., 2007; Brantjes, 1978; Knoll, 1925). That colour is used for night-active hawkmoths is supported by studies showing their excellent colour vision under very dim light conditions (Kelber et al., 2002) and their ability to learn colours that are not initially preferred (Goyret et al., 2008). Earlier studies suggested that this species uses the blue receptor exclusively to detect flowers (Cutler et al., 1995). However, the result that the moths spontaneously select the yellow artificial flower over the green indicates that more than the blue receptor is used in foraging, as the green colour stimulates the blue receptor more than the yellow colour.

In conclusion, we have shown that restrained naïve hawkmoths that have been rewarded only a single time in front of a green flower will select that flower when they are later released in a flight cage. Even if they are never able to feed from the green flower, they are very reluctant to switch back to their initially preferred yellow flower. This shows that hawkmoths have flower constancy.

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