

## SHORT COMMUNICATION

# Innate preference and learning of colour in the male cotton bollworm moth, *Helicoverpa armigera*

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## ABSTRACT

We investigated colour discrimination and learning in adult males of the nocturnal cotton bollworm moth, *Helicoverpa armigera*, under a dim light condition. The naive moths preferred blue and discriminated the innately preferred blue from several shades of grey, indicating that the moths have colour vision. After being trained for 2 days to take nectar at a yellow disc, an innately non-preferred colour, moths learned to select yellow over blue. The choice distribution between yellow and blue changed significantly from that of naive moths. However, the dual-choice distribution of the trained moths was not significantly biased to yellow: the preference for blue is robust. We also tried to train moths to grey, which was not successful. The limited ability to learn colours suggests that *H. armigera* may not strongly rely on colours when searching for flowers in the field, although they have the basic property of colour vision.

**KEY WORDS:** Behaviour, Colour vision, Nocturnal moth

## INTRODUCTION

Nocturnal moths appear to use olfaction for searching night-blooming flowers, which typically have strong scents but not vivid colours (Baker, 1961). In fact, flower-visiting nocturnal moths respond well to scents and even learn the scents of rewarding flowers (Burguiere et al., 2001; Cunningham et al., 2004; Fan et al., 1997).

Despite the preconception that vision may not work well at night, vision is actually a viable sense for nocturnal insects. Typical nocturnal insects have compound eyes of superposition type, the optics of which enable fully functional vision even in an extremely dark environment (Stöckl et al., 2016; Warrant, 2008). The tobacco hawkmoth, *Manduca sexta*, is a classic model of nocturnal vision. *Manduca sexta* express three distinct visual pigment opsins (Chase et al., 1997), which produce the ultraviolet (UV), blue and green receptors in the retina (White et al., 1983a,b, 2003). These three spectral receptors probably form the basis of the trichromatic system, as in honeybees (Menzel and Backhaus, 1989; Wakakuwa et al., 2005). Behaviourally, *M. sexta* can switch colour preference from the innately preferred colour to the rewarded colour after a feeding experience (Goyret et al., 2008). However, this result is not sufficient to demonstrate that *M. sexta* have true colour vision, the ability to discriminate visual stimuli based on the chromatic content irrespective of the brightness. The first nocturnal insect in which colour vision was demonstrated is the elephant hawkmoth, *Deilephila elpenor* (Balkenius and Kelber, 2004; Kelber et al.,

2002). Foraging *D. elpenor* can discriminate colours even under starlight, under which humans are completely colour-blind. Some nocturnal hymenopterans (bees, wasps and ants) have also been shown to have colour vision (Warrant, 2008).

The cotton bollworm moth, *Helicoverpa armigera*, is a world-wide pest species. The polyphagous larvae cause serious damage to many agricultural crops (Umeya and Okada, 2003). Studying the ability of *H. armigera* to discriminate colours will not only add information about nocturnal colour vision but also contribute to controlling the pest using light without the problems associated with chemical insecticides. In fact, green illumination at night effectively suppresses the activity of *H. armigera* (Yabu et al., 2014), but the underlying mechanism is not clear (Nomura et al., 1965). A previous study (Yan et al., 2014) demonstrated that the eyes of *H. armigera* express three opsins, which cluster with the UV-, blue- and green-absorbing type of opsins, suggesting possible colour vision.

Here, we report that *H. armigera* have an innate colour preference, and that they can learn and discriminate coloured discs depending on the chromatic content. We thus conclude that *H. armigera* have colour vision.

## MATERIALS AND METHODS

### Animals

Pupae of male *Helicoverpa armigera* (Hübner 1809) (Noctuidae, Lepidoptera) were obtained from Sumika Technoservice (Hyogo, Japan). The pupae were individually kept in plastic containers (80 mm in diameter, 45 mm in depth) with water-soaked filter paper and maintained at 25°C under a 14 h light:10 h dark regime. Day of the emergence was defined as day 1. Emerged moths were kept in the plastic container and not fed until we started the experiments, as starvation was necessary for moths to respond in our setup. We used 5–7 day old moths for the innate preference test and 4–5 day old moths for the learning ability test. All experiments were completed within 3 h, beginning 30 min after lights off, because *H. armigera* are most active at the beginning of scotophase (Cunningham et al., 1998; Saito, 2000). Each moth was used in only one test.

### Apparatus

All experiments were done in a cage covered with nylon net (36×35×42 cm W×H×D). A plastic plate was placed vertically at one wall of the cage, which was illuminated with a 20 W incandescent bulb through a diffuser from approximately 30 cm above the cage top. The illumination contained little light of <400 nm wavelength (Fig. 1A, inset). The intensity was approximately 2.0 lx at the plate surface. Temperature and humidity were set approximately at 25°C and >50%, respectively.

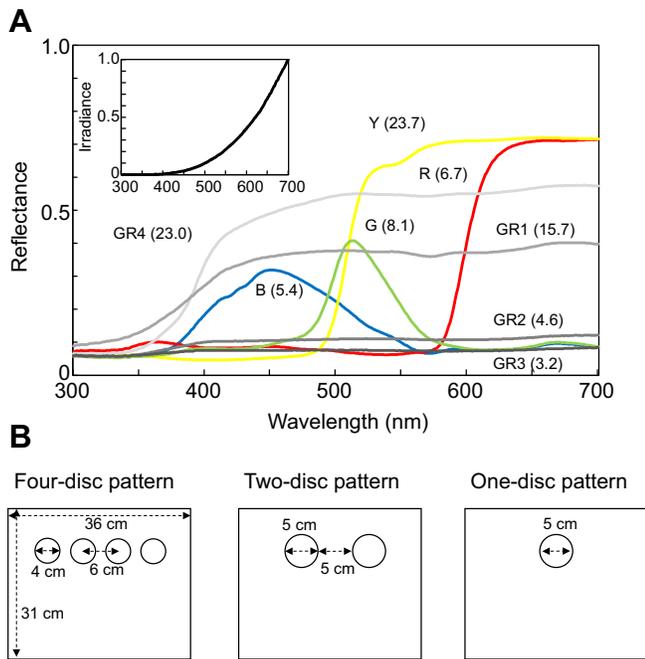
### Stimuli

Visual stimuli were presented by placing discs of coloured paper (Nihon-Shikisai Tonal Colour 93 colours; Fig. 1A) on black cardboard, covering the vertical plastic plate. The reflectance

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**Fig. 1. Experimental setup.** (A) Reflectance spectra of coloured paper relative to the white standard. Inset, the irradiance spectrum of the arena illumination. B, blue; G, green; Y, yellow; R, red; GR, grey. The numbers in parentheses indicate the subjective brightness for *Helicoverpa armigera* of coloured paper  $i$  ( $B_i$ ) under the present conditions, which was predicted by:

$$B_i = \int_{300}^{700} I(\lambda) S(\lambda) R_i(\lambda) d\lambda,$$

where  $I(\lambda)$  is the illumination spectrum,  $R_i(\lambda)$  is the reflectance spectrum of the coloured paper  $i$ ,  $S(\lambda)$  is the spectral sensitivity of *H. armigera* compound eye determined by electroretinographic recording (Yabu et al., 2014) and  $\lambda$  is the wavelength. (B) Patterns presented in the experiments. A four-disc pattern was used in the innate preference experiments. A two-disc pattern and one-disc pattern were used in the learning experiments.

spectra were measured with a spectrometer (HSU-100F, Asahi Spectra Co., Ltd., Tokyo, Japan) relative to a white standard (SRS-99-020, Labsphere, Inc., USA). The colours used differed among experiments (see below). Each coloured disc was covered with a low-reflection acrylic sheet. The stimuli were always presented to the moth with odour (except for the learning experiment using yellow and grey), because the combination of visual and olfactory stimuli appears to promote the feeding response in nocturnal moths (Raguso and Willis, 2002). For the olfactory stimuli, we put a small cotton bud with 10  $\mu$ l of chamomile essential oil (Tree-of-Life, Tokyo, Japan) behind the plate. The cotton bud was changed every few hours. We chose chamomile (Asteraceae) for the odour source, because *H. armigera* is a pest of Asteraceae plants (Gu et al., 2001) and the moths take nectar from their flowers (A.S., personal observation).

### Innate colour preference

We conducted two sets of experiment using the four-disc pattern (Fig. 1B). First, we investigated the moths' preference among red, yellow, green and blue, using one of the eight different arrangements at random for each individual. We observed each moth's behaviour for up to 10 min. Most naive moths started flapping their wings immediately after being released in the cage, and then began to fly. We recorded the colour of the disc they chose first as the innately preferred colour. A 'choice' was defined as when

the moth flew to the disc and landed on it: however, we could not record proboscis extension under the dim light conditions. When moths landed on the net or tenaciously flew by the net, we gently tapped on the net to get them to fly in open space. Moths that did not fly well were rejected even in the middle of the observation time. Second, we presented a disc of the innately preferred colour, i.e. blue (see Results), with three shades of grey (grey 1–3). The test procedure was identical to the first experiment. We changed the relative position of the discs randomly for each individual.

### Colour learning (blue and yellow)

We first confirmed the innate blue preference using the two-disc pattern (Fig. 1B) with blue and yellow by the same procedure as the above test. We then tested whether the preference could be modified through yellow learning.

Training was done on day 1 and 2 of the experiment. We put small droplets of 5% sucrose solution on the acrylic sheet covering the yellow disc, but not on the blue disc, and released a moth into the cage. We then guided the flying moth towards the yellow disc without directly touching the moth by hand, and let it take the sucrose solution. After the moth finished feeding and took off, we again put some sugar solution on the yellow disc, and the moth was allowed to fly back voluntarily to the disc for feeding up to 10 times on day 1 and 5 times on day 2. If the moth visited the blue disc repeatedly, we again guided the moth towards the yellow disc. We repeated this training until the moth took sucrose solution for the criterion number of times (10 or 5), or became unresponsive to either disc. The position of the yellow disc was swapped on the two training days. We did the following tests only with moths that took sucrose solution from the yellow disc 5 times on day 2.

Tests were done on day 3 using the two-disc pattern with blue and yellow targets. We first stimulated feeding by letting the moth to take a small amount of 10% sucrose solution on the yellow disc. Immediately after the moth took off from the disc, we removed the sucrose by changing the pattern to have the yellow disc on either side randomly. We then recorded the number of visits to each disc for 5 min.

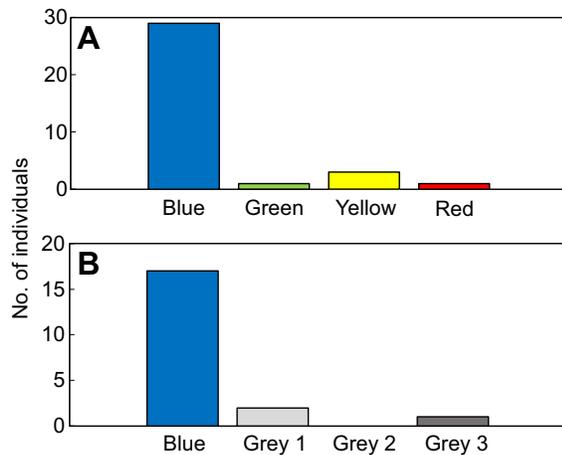
### Colour learning (yellow and grey)

We did another learning experiment using two discs, yellow and grey 4: the subjective brightness of grey 4 was similar to that of yellow (Fig. 1A). Moths were randomly allocated to either yellow or grey training. Individual training was done on day 1 and 2 using one-disc pattern (Fig. 1B) with either yellow or grey 4. We first put some small droplets of 10% sucrose solution on the acrylic sheet covering the disc, and then guided the flying moth towards the disc. After the moth started to feed, we put a cotton bud soaked with 10% sucrose solution close to the moth using forceps, and let the moth take the sucrose for 10 s. We repeated this training procedure 3 times for 2 days. Successfully trained moths visited the disc by themselves.

Tests were done on day 3. First, we stimulated feeding by letting the moths take a small amount of 10% sucrose solution on the disc. We then presented the two-disc pattern (Fig. 1B) with yellow and grey 4. We observed the moth's behaviour for up to 5 min, and recorded the first five choices between the two discs.

### Statistical analysis

We omitted moths that did not make any choices within the criterion observation time from statistical analysis. We used the chi-square test to determine whether the choice distribution differed significantly from the chance level. We also used Fisher's exact test to compare the choice distribution between naive and yellow-trained moths, and that between yellow- and grey-trained moths in colour learning



**Fig. 2. First colour choice of naive *H. armigera* adults using a four-disc pattern.** (A) Choices among four colours. (B) Choices among blue and three shades of grey. Both choice distributions differed significantly from chance (chi-square test,  $P < 0.001$ ).

experiments. The response to the coloured discs was also compared between yellow- and grey-trained moths using Fisher's exact test.

## RESULTS AND DISCUSSION

### Innate colour preference and colour vision

When presented with four colours, 85% of naive moths visited blue the first time (Fig. 2A). The second most preferred colour was yellow. The subjective brightness is lowest for blue (Fig. 1A), so the moths may have selected the darkest target. We tested this possibility by presenting blue with different shades of grey. As a result, 85% of moths selected blue (Fig. 2B), but not the greys, no matter whether their subjective brightness was similar to blue (grey 2), or brighter (grey 1) or dimmer (grey 3) than blue (Fig. 1A). We thus concluded that the moths discriminated discs based on their chromatic content irrespective of the brightness: they have colour vision.

Innate preference for blue has also been reported in a diurnal hawkmoth, *Macroglossum stellatarum* (Kelber, 1997; Telles et al., 2014), and in a swallowtail, *Papilio xuthus* (Yoshida et al., 2015), based on behavioural experiments using coloured paper. The preference for blue appears enigmatic because pure-blue flowers seem rare in the field (Chittka and Menzel, 1992; Gottsberger and Gottlieb, 1981), so this may be because the insects have detected the artificial blue as a 'super-normal' key stimulus (Tinbergen, 1951).

Interestingly, *M. stellatarum* select a blue target on a grey background, but select a yellow target on a blue background (Kelber, 1997). This can be explained at least in part by the phenomenon of colour induction, i.e. perception of the target colour is affected by the colour of its surrounding area. *Papilio xuthus* trained to visit an orange target on a black/grey background select a yellow–orange target on a green background: the green background 'induces' a reddish colour, which makes the yellow–orange target appear more reddish (=orange) for the butterfly (Kinoshita et al., 2008). If the same holds for *H. armigera*, the innate blue preference may be beneficial for visiting more common violet flowers blooming among green foliage.

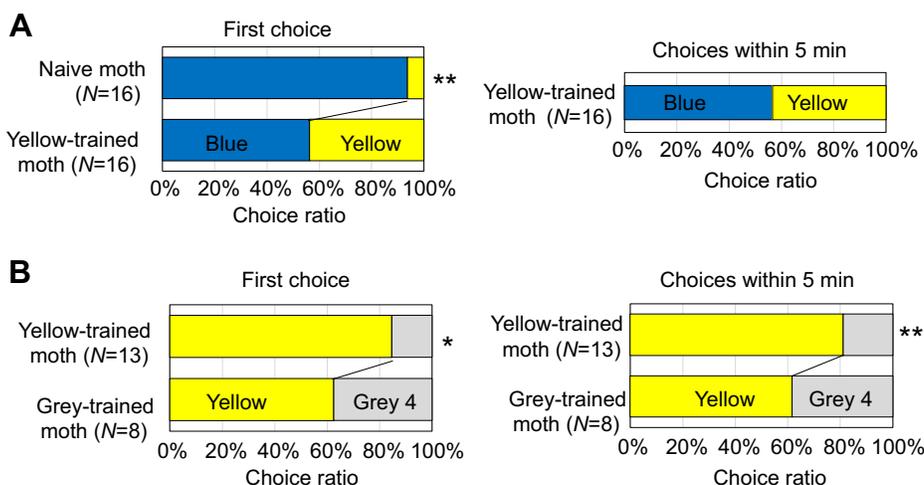
### Learning ability and the robustness of colour preference

The innate blue preference was confirmed in the dual-choice test using blue and yellow (Fig. 3A, left). After training with yellow for 2 days, the choice distribution between yellow and blue changed significantly from that of naive moths (Fisher's exact test,  $P < 0.05$ ; Fig. 3A, left). However, more than half of the yellow-trained moths still visited the blue disc for their first choice. The choice distribution during the 5 min observation period was also not significantly biased to yellow (Fig. 3A, right).

These results suggest that the learning ability in *H. armigera* is limited, unlike in *M. stellatarum* (Kelber, 1996) and *P. xuthus* (Kinoshita et al., 1999), where learning easily changes the innate colour preference. Because *H. armigera* change preference to certain odours more easily (Cunningham et al., 2004), olfaction is probably more important for this species when searching for rewarding flowers. For technical reasons, we could only test males, and females may respond differently, which remains for further investigation.

We could detect no significant difference in the choice distribution between yellow- and grey-trained moths (Fisher's exact test,  $P > 0.05$ ; Fig. 3B). The yellow-trained moths nevertheless became strongly selective for yellow when the yellow target was presented together with a grey target (Fig. 3B). This indicates that the moths have the ability to learn colours that are innately not preferred. In contrast, the grey-trained moths did not become selective to grey (Fig. 3B). The response of grey-trained moths was in fact lower than that of the yellow-trained moths to begin with; none of the eight grey-trained moths visited any disc more than 4 times, whereas six out of 13 yellow-trained moths did (Fisher's exact test,  $P < 0.05$ ).

The four shades of 'grey' paper we used have reduced reflectance in UV (Fig. 1A). These papers are achromatic for humans but may



**Fig. 3. Colour learning.** (A) Choices of blue and yellow by naive and yellow-trained moths. The right panel shows the sum of all choices during the 5 min observation time by yellow-trained moths (62 choices by 16 moths). (B) Choices of yellow and grey by yellow- and grey-trained moths. The right panel shows the sum of all choices during the 5 min observation time (up to 5 choices) by yellow-trained moths (37 choices by 13 moths) and grey-trained moths (13 choices by 8 moths). \* $P < 0.05$ , \*\* $P < 0.001$  (chi-square test).

not be for animals with UV sensitivity. In fact ‘grey’ paper without UV reflection can be discriminated by honeybees (Chittka, 1999; Frisch, 1914; Vorobyev et al., 1999), *Manduca sexta* (Goyret et al., 2008) and *P. xuthus* to some extent (Kinoshita et al., 1999). Convincing demonstration of a learning ability for achromatic targets appears to be difficult (Chittka, 1999; Swihart, 1971; Vorobyev et al., 1999). The failure to train *H. armigera* to non-UV-reflecting grey paper may indicate some species-specific ecological demands.

### Perspectives

In addition to colour vision, intensity vision is also useful for searching flowers in diurnal moths and butterflies (Kelber, 2005; Kinoshita et al., 2012). We also tested *H. armigera* for intensity discrimination using blue discs of several intensities. They appeared to select the brightest disc, implying they discriminate between different intensities. However, the feeding activity was low, and therefore we could not train the moths to visit discs of a particular intensity. Perhaps the intensity and spectrum of the illumination were not ideal for achromatic vision to function.

Cooperation of multiple sensory modalities has recently attracted attention (Balkenius et al., 2006; Goyret et al., 2007; Raguso and Willis, 2002; Yoshida et al., 2015). We presented coloured discs with olfactory stimuli using chamomile essential oil, which may enhance foraging activity, assuming that the synergy between visual and olfactory cues is also important in *H. armigera*, as in *M. sexta* (Raguso and Willis, 2002). Comparison of the effects of other scents on colour vision would be of particular interest.

### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Designed the experiments: A.S., K.A. and M.K. Performed the experiments: A.S. Analysed the data: A.S. Wrote the paper: A.S. and K.A., with suggestions from M.K.

### Funding

This work was supported by a National Agriculture and Food Research Organization (NARO) grant for SIP ‘Technologies for creating next generation agriculture, forestry, and fisheries’, and by a Japan Society for the Promotion of Science (JSPS) Kakenhi for Scientific Research A (26251036 to K.A.).

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