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

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Abstract

We investigated the ability of colour discrimination and learning in male adults of nocturnal cotton bollworm moth, *Helicoverpa armigera*, under a dim light condition. The naïve 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 yellow, an innately non-preferred colour, moths learned to select yellow over blue. The choice distribution between yellow and blue changed significantly from that of naïve 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 basic property of colour vision.

Keywords: 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 well respond to scents and even learn the scents of rewarding flowers (Burguiere et al., 2001; Cunningham et al., 2004; Fan et al., 1997).

Despite the prejudice that vision may not work well at night, vision is actually a viable sense also for nocturnal insects. Typical nocturnal insects have compound eyes of superposition type, whose optics makes the vision fully functional even in extremely dark environment (Stöckl et al., 2016; Warrant, 2008). The tobacco hawkmoth, *Manduca sexta*, is a classical model of nocturnal vision. *M. sexta* express three distinct visual pigment opsins (Chase et al., 1997), which respectively produce the ultraviolet, blue and green receptors in the retina (White et al., 1983a; White et al., 1983b; White et al., 2003). These three spectral receptors most likely serve the basis of 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 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 whose 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 *Helicoverpa armigera* (Noctuidae, Lepidoptera) is one of the world-wide pest species. The polyphagous larvae cause serious damage to many agricultural crops (Umeya and Okada, 2003). Studying the ability of discriminating colours in *H. armigera* will not only add information about nocturnal colour vision but also will contribute controlling the pest using light without having trouble caused by 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) has demonstrated that the eyes of *H. armigera* express three opsins each cluster with the ultraviolet (UV), blue and green-absorbing type of opsins, suggesting possible colour vision.

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

MATERIALS AND METHODS

Animals

Pupae of male *Helicoverpa armigera* Hübner (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 25ºC under a 14 h light :10 h dark regime. Day of the emergence was defined as day one. Emerged moths were kept in the plastic container and never fed until we started the experiments, for 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 done within 3 hours from 30 min after the light-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 (W × H × D=36 × 35 × 42 cm³). A plastic plate was put 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 shorter than 400 nm (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 disks of colour paper (Nihon-Shikisai Tonal Colour 93 colours; Fig. 1a) on black cardboard, covering the vertical plastic plate. Used colours were different among experiments (see below). Each colour disk was covered with low-reflection acrylic sheet. The stimuli were presented to the moth always with odour (except for the learning experiment using yellow and grey), because coexistence of visual and olfactory stimuli appears to promote feeding response in nocturnal moths (Raguso and Willis, 2002). For the olfactory stimuli, we put a small cotton bud with 10 µl of chamomile essential oil (Tree-of-Life, Tokyo, Japan) behind the plate. The cotton 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 (personal observation).

Innate colour preference

We conducted two sets of experiment using the four-disk pattern (Fig. 1b). First, we investigated moths' preference among red, yellow, green and blue, using one of the eight different arrangements at random for each individual. We observed the moth's behaviour for up to 10 min. Most naïve moths started flapping wings immediately after being released in the cage, and then began to fly. We recorded the colour of the disk they chose first as the innately preferred colour. A 'choice' was defined as when the moth flew to the disk and landed on it: we however could not record moths' proboscis extension under the dim light condition. Moths that landed on the net or that were tenaciously flying by the net were gently snapped for letting them fly in open space. Moths that did not fly well were rejected even in the middle of the observation time. Second, we presented a disk 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 disks randomly for each individual.

Colour learning (blue and yellow)

We first confirmed that the innate blue preference on the two-disk pattern (Fig. 1b) with blue and yellow by the same procedure with the above test. We then tested whether the preference can 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 disk, but not on the blue disk, and released a moth in the cage. We then guided flying moth towards the yellow disk without directly touching the moth by hands, and let it take sucrose solution. After the moth finished feeding and took off from the disk, we again put some sugar solution, and the moth was allowed to fly back voluntarily to the disk for feeding up to 10 times on day 1 and 5 times on day 2. If the moth visited the blue disk repeatedly, we again guided the moth towards the yellow disk. We repeated this training until the moth took sucrose solution for criterion number of times (10 or 5), or became unresponsive to either disk. The position of the yellow disk was opposite between two training days. We did the following tests only with moths that took sucrose solution on the yellow disk for 5 times on day 2.

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

Colour learning (yellow and grey)

We did another learning experiment using two disks, 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-disk 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 disk, and then guided the flying moth towards the disk. After the moth started to feed, we put a cotton puff soaked with 10% sucrose solution close to the moth by forceps, and let the moth take sucrose for 10 seconds. We repeated this training procedure 3 times for 2 days. Successfully trained moths visited the disk by themselves.

Tests were done on day 3. First, we stimulated the moths' feeding motivation by letting them to take a small amount of 10% sucrose solution on the disk. We then presented a two-disk 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 disks.

Statistical analysis

We omitted moths that did not make any choices within a criterion observation time from statistical analysis. We used 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 naïve and yellow-trained moths, and that between yellow and grey-trained moths in colour learning experiments. The response to the colour disks was also compared between yellow- and grey-trained moth by using Fisher's exact test.

RESULTS AND DISCUSSION

Innate colour preference and colour vision

When presented four colours, 85% of naïve moths visited blue for the first time (Fig. 2a). The second preferred colour was yellow. The subjective brightness is lowest in 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 greys, no matter whether their subjective brightness is similar (grey 2) with blue, or brighter (grey 1) and dimmer (grey 3) than blue (Fig. 1a). We thus concluded that the moths discriminated disks based on their chromatic contents irrespective of the brightness: they have colour vision.

Innate preference for blue has been reported also 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 colour 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 blue target on grey background, but select yellow target on blue background (Kelber, 1997). This can be explained at least in part by the phenomenon called colour induction, i.e. perception of target colour is affected by the colour of its surrounding area. *P. xuthus* trained to visit orange target on black/grey background select yellow-orange target on green background: the green background "induces" reddish colour, which makes yellow-orange target appears 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 two days, the choice distribution between yellow and blue changed significantly from that of naïve moths (Fisher's exact test, $p < 0.05$; Fig. 3a left). However, more than half of the yellow-trained moths still visited blue for their first choice. The choice distribution during 5 min observation was also not significantly biased to yellow (Fig. 3a right).

These results suggest that the learning ability in *H. armigera* appears to be 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 rewarding flowers. We could only test males due to some technical reasons, 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 to 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 un-preferred. On the other hand, 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 to begin with; none of the eight grey-trained moths visited any disk more than 4 times, whereas six out of thirteen yellow-trained moths did (Fisher's exact test, $p < 0.05$).

The "grey" papers we used have reduced reflectance in UV (Fig. 1a). These papers are achromatic for humans but may be not for animals with UV sensitivity. In fact "grey" papers without UV reflection can be discriminated by honeybees (Chittka, 1999; Frisch, 1914; Vorobyev et al., 1999), *Manduca sexta* (Goyret et al., 2008), and by *P. xuthus* to some extent (Kinoshita et al., 1999). Convincing demonstration of learning ability of achromatic targets appears to be difficult (Chittka, 1999; Swihart, 1971; Vorobyev et al., 1999). Failure to train *H. armigera* to non-UV reflecting grey papers may reflect 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., 2011). We also tested *H. armigera* for intensity discrimination using blue disks of several intensities. They appear to select the brightest one, implying they discriminate intensity differences. But because the feeding activity was low, and therefore we could not train the moths to visit disks of certain intensity. Maybe the intensity and spectrum of the illumination was not best 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 colour disks with olfactory stimuli using chamomile essential oil that may enhance the foraging activity, assuming that the synergy between visual and olfactory cues is important also 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. Analyzed the data: A.S. Wrote the paper: A.S. and K.A., with suggestions from M.K.

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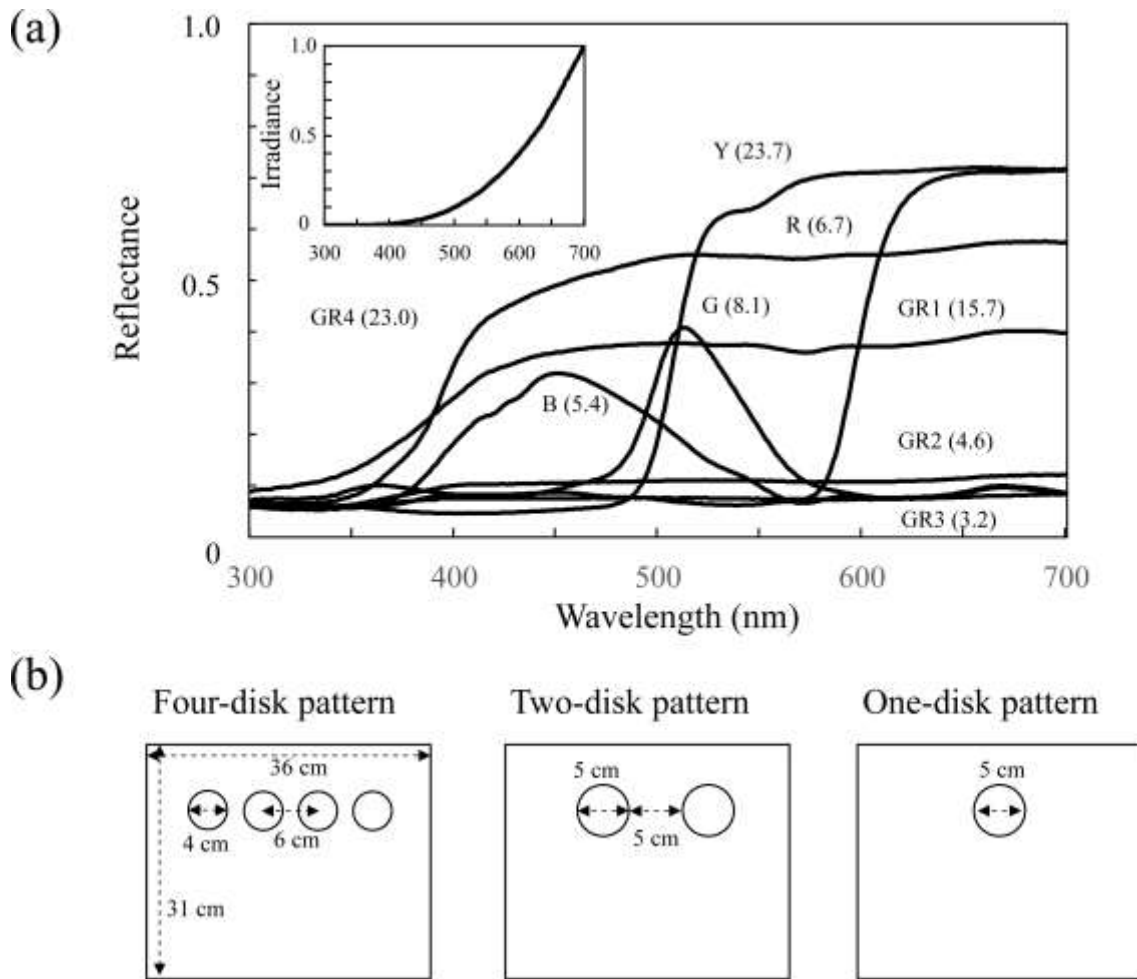
Figures

Fig. 1. (a) Reflectance spectra of colour paper relative to the white standard (SRS-99-020, Labsphere, Inc., USA). Inset shows the irradiation spectrum of the arena illumination. B, blue; G, green; Y, yellow; R, red; GR, grey. These spectra were measured using a spectrometer (HSU-100F, Asahi Spectra Co., Ltd., Tokyo, Japan). The numeral in the parenthesis indicated the subjective brightness for *H. armigera* of colour paper i, B_i, under the present condition, which was predicted by $B_i = \int_{300}^{700} I(\lambda)S(\lambda)R_i(\lambda)d\lambda$. $I(\lambda)$ is the illumination spectrum, $R_i(\lambda)$ is the reflectance spectrum of colour paper i, $S(\lambda)$ is the spectral sensitivity of *H. armigera* compound eye determined by electroretinographic (ERG) recording (Yabu et al., 2014) and λ is wavelength. (b) Patterns presented in the experiments. Four-disk pattern was used in the experiments of innate preference. Two-disk pattern and one-disk pattern were used in the learning experiments.
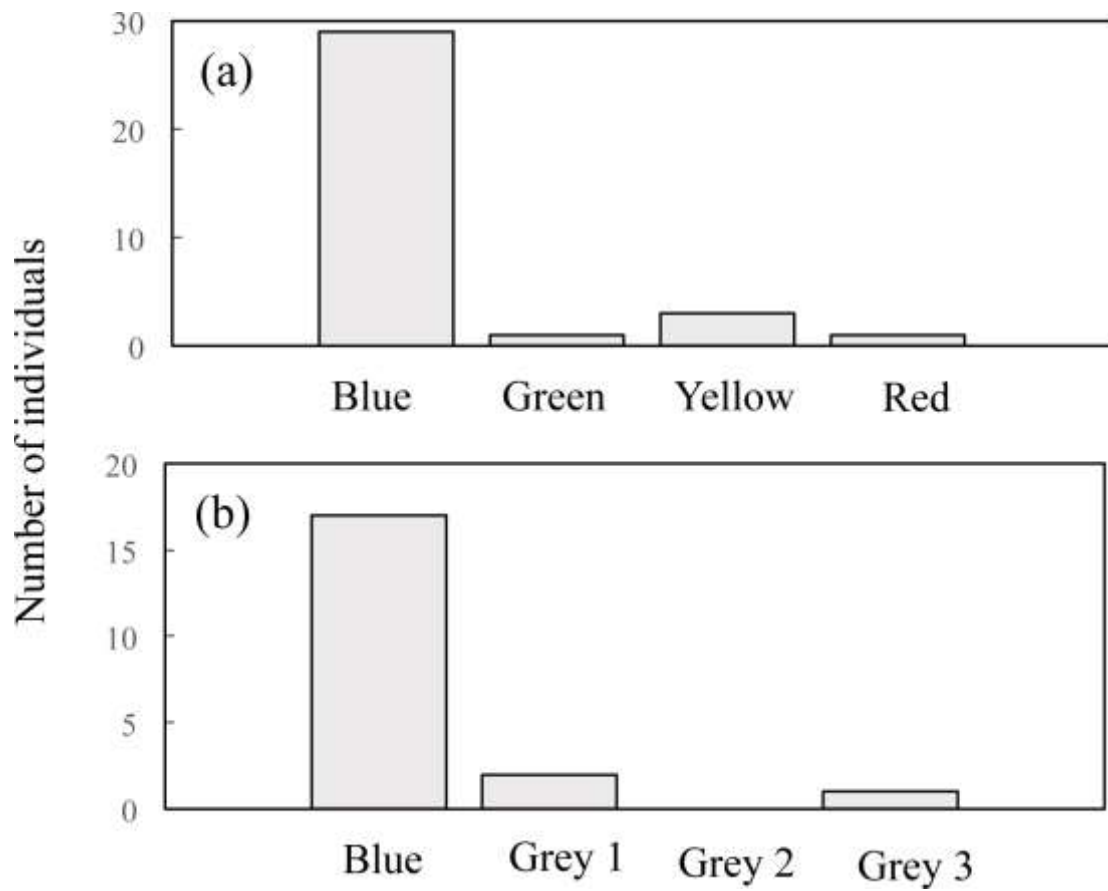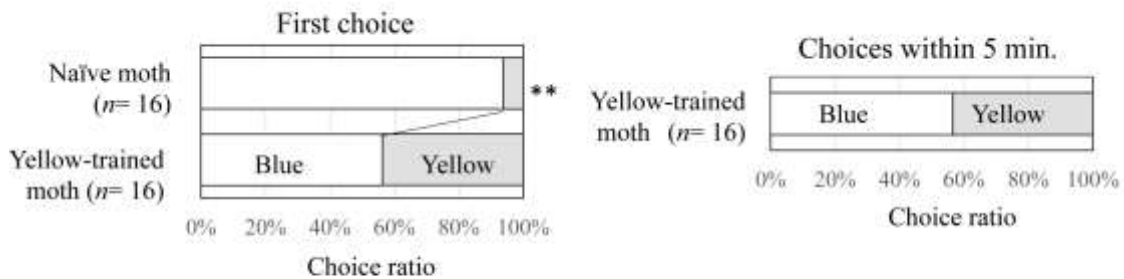
Fig. 2. First colour choice of naïve *Helicoverpa armigera* adults using four-disk pattern. (a) Choices among four colours. (b) Choices among blue and three shades of grey. Both choice distributions differed significantly from chance (chi-square test, $p < 0.001$).
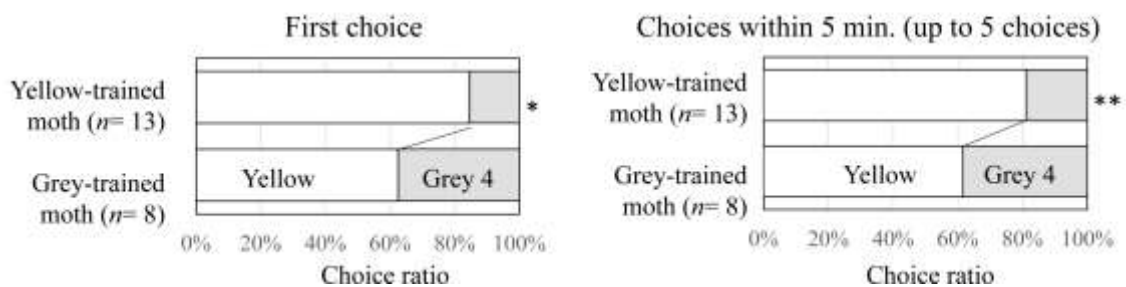
Fig. 3. Colour learning. (a) Choices of blue and yellow by naïve and yellow-trained moths. The right figure shows sum of all choices during 5 min observation time by yellow-trained moth (62 choices by 16 moths). (b) Choices of yellow and grey by yellow and grey-trained moths. The right figure shows sum of all choices during 5 min observation time (up to 5 choices) by yellow-trained moth (37 choices by 13 moths) and grey-trained moth (13 choices by 8 moths). *$p < 0.05$, **$p < 0.001$ (chi-square test).