

## Better mate in the shade: enhancement of male mating behaviour in the cabbage butterfly, *Pieris rapae crucivora*, in a UV-rich environment

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### SUMMARY

Ultraviolet (UV) vision is widespread in a variety of animals, playing important roles in behaviours such as foraging and reproduction. Despite accumulated information about UV vision and UV-dependent behaviours of animals, little is known about the effect of temporal changes and local variations in UV light on UV-dependent behaviour. Here we report the mating behaviour of male cabbage butterfly, *Pieris rapae crucivora*, in environments with varying content of UV light. We first confirmed that the relative UV content is higher in shaded places than in sunny places. We furthermore arranged experimental areas with varying UV contents in the field, where we compared three aspects of male mating behaviour: visual localization of females, female-searching flight and copulation success rate. In all aspects males performed more actively in UV-rich environments: males searched females for longer, approached females preferentially in the shade and copulated there more frequently. Apparently, female-searching males detect females more easily in a UV-rich environment. The present findings should be taken into consideration when UV-dependent behaviours, visual mate choice in particular, are studied.

Key words: mate recognition, UV vision, photoreceptor, compound eye, colour vision, wing reflection spectra.

### INTRODUCTION

Humans cannot see ultraviolet (UV) light, but the eyes of many animals, notably insects, are furnished with photoreceptors sensitive to UV (Briscoe and Chittka, 2001; Kelber et al., 2003). UV light is often indispensable for certain behaviours, such as foraging (Barth, 1982; Church et al., 2001), mate recognition (Obara, 1970; Silberglied and Taylor, 1973) and mate choice (Rutowski, 1985; Bennett et al., 1996). However, little is known about whether and how these UV-related behaviours are affected by the temporal as well as local variations of UV conditions in the field. For example, the relative content of UV light is higher in shaded places than in direct sunlight because of scattering (Kok, 1972).

The Japanese subspecies of cabbage butterfly, *Pieris rapae crucivora*, is subjected to such changes in the UV environment. Its eyes contain at least six classes of spectral receptors, including UV receptors (Wakakuwa et al., 2006), and the males and females distinctly differ in wing reflectance in both the UV and visible wavelength range (Giraldo and Stavenga, 2007). The males visually recognize the females as potential mates based on the UV reflection of the wings (Obara and Hidaka, 1968; Obara, 1970). It is therefore probable that the visibility of female wings for males varies with the weather conditions or local light environment, i.e. depending upon whether the females are exposed to the sun or are in the shade. This raises the question of whether the light environment affects the UV-guided mating behaviour of males that compete for access to virgin females (Ohsaki, 1980; Suzuki, 1980; Thornhill and Alcock, 1983). To answer this we first confirmed that the relative UV content is much higher in shaded than in sunlit places, and investigated whether and how the UV content affects the mating behaviour of male *Pieris rapae crucivora*.

### MATERIALS AND METHODS

#### Animals

We used male and female adults of summer form *Pieris rapae crucivora* Boisduval reared in the laboratory under a light cycle of 14h:10h L:D, at 25°C.

#### Spectral measurements

A calibrated spectrophotometer (HSU-100S, Asahi Spectra) was used to measure spectra. The spectral composition of sunlight was measured with a MgO-coated reflection standard placed on a table in the field on a fine day. The reflection standard was shaded with cardboard when measuring the spectrum in the shade. The reflection spectra of wings of *Pieris rapae crucivora* were measured with the same method using an isolated hind wing of a freshly emerged female, ventral side up, instead of the reflection standard. The transmittance spectra of UV-opaque and UV-transparent plastic sheets (see below) were measured with the same spectrophotometer.

#### Behavioural experiments

##### Experiment 1: large cage experiments

We tested the female-locating behaviour of males in the sunlight and in the shade in a large outdoor cage measuring 9 m × 9 m × 2 m (experiment 1). Females that were presented to males were freshly emerged individuals killed by freezing, wings closed, and laid flat with the ventral side of the wings up. Such female dummies effectively elicit mating behaviour of males, which even respond to a piece of paper that reflects UV (Obara and Hidaka, 1968; Obara, 1970). To stimulate mating behaviour even further, we added cabbage plants as well as buckwheat plants as nectar sources.

In the first experiment (experiment 1a), conducted in the large cage, we placed two female dummies laid flat, 15 cm apart, in the centre of a piece of black cardboard measuring 25 cm × 30 cm

(Fig. 1A). One dummy was shaded by placing a 60 cm × 60 cm wooden board 1.8 m above the ground. The black cardboard with the female dummies was rotated every 15 min by 180° to eliminate any possible differences due to local factors. In this experimental arrangement, we assumed that the males visually compared the two female dummies at the same time.

In the second experiment (experiment 1b), we placed two female dummies, each flat in the centre of a piece of black cardboard (25 cm × 30 cm), and placed the cardboards 2.0 m apart (Fig. 1B). One of the dummies was shaded, and the positions of the two dummies were interchanged every 15 min. In this situation, we assumed that the males do not see the two female dummies at the same time, so that the males could not visually compare them simultaneously.

In each experiment, we released about 280 virgin males in the cage, and observed their mating behaviour to the female dummies. The observed behaviours included: approach to the dummies as close as about 5 cm, contact with the dummies, and copulatory attempt

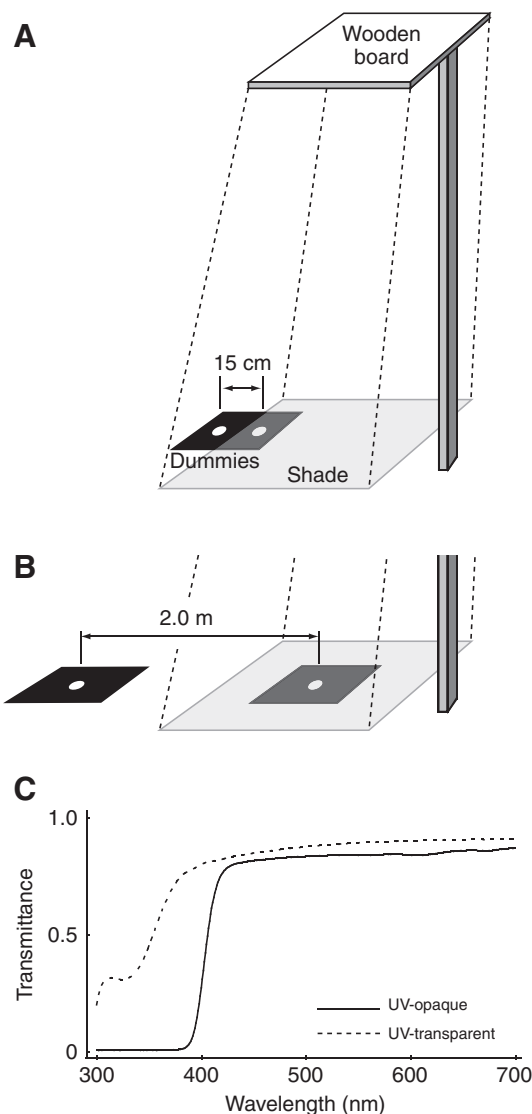


Fig. 1. (A,B) Schematic drawing of the experimental set-up for positioning of female dummies in the shade and in the sunlight. (C) Transmittance spectra of the UV-opaque (solid, Cut-Ace-Clean, MKV Platech) and the UV-transparent (dotted, Clean-Ace-Daichi, MKB platech) plastic sheets.

with the dummies. We counted the number of males that expressed any one of these behaviours. The counted males were immediately removed from the cage during or after the behaviour; consequently, the data obtained are all derived from males naïve to the experiment.

We carried out experiment 1a from 07:15 h to 08:15 h on a sunny day in mid summer (16th July, 2004) and experiment 1b from 08:30 h to 11:00 h on the same day. We designed this procedure based on the previous findings that the males start searching females around 06:30 h on a sunny day and that the number of female-searching males increases over time, peaking between 09:00 h and 11:00 h; at the peak, nearly three times more male flies are searching for females (Hirota and Obara, 2000).

#### Experiment 2: open field experiment

We designed experiment 2 to test whether and how the male mating behaviour was affected by a varying UV environment. Here we observed the male mating behaviour in the presence or absence of UV. For this purpose, we covered an 8 m × 8 m area of a cabbage field (20 m × 30 m) with a UV-opaque plastic sheet (Cut-Ace-Clean, MKV Platech, Tokyo Japan), which transmits visible light but not UV light (Fig. 1C). We covered another 8 m × 8 m area of the same field similarly with a UV-transparent plastic sheet (Clean-Ace-Daichi, MKV Platech, Tokyo Japan; Fig. 1C). The two areas were 2 m apart and we hereafter call these areas the UV-absent and the UV-present areas, respectively.

The sheets were placed 1.4 m above the ground, and because there were no walls, the butterflies could freely move into and out of the areas; the temperatures of these areas were virtually the same (30.5–31.5°C and 30.0–31.0°C in the UV-present and -absent areas, respectively). In an ‘inter-generations period’, when wild individuals were virtually absent, we released about 300 fresh males into the cabbage field in the evening 1 day before the experiment. The released males stayed around the cabbage field for several days, thus allowing observation of their mating behaviour for an extended period. The experiment was carried out from 08:00 h to 12:00 h on 22 July, 2005.

In the morning of the next day, the released males started to search for females by flying zigzag among the cabbage leaves, although there were no females. The time of female-searching behaviour was measured by visually following a flying male and recording the time the male entered the UV-present area, apparently then searching for females, and subsequently recording the time the male left the area. Some males took up a resting position on a substrate within the area and stayed there for a prolonged period. In such cases, we concluded that the males were no longer performing the female searching behaviour. After the male left the area or alighted on a substrate, we repeated the same observation in the UV-absent area. We performed these observations in the two areas alternately, and thus recorded in each area the behaviour of total 79 cases (not necessarily 79 individuals, because some individuals may have flown a number of times in and out of the areas).

#### Experiment 3: small arena experiment

To measure the frequency of actual copulation, we carried out experiment 3. We prepared two frames, each measuring 45 cm × 5 cm × 55 cm, one with the ceiling and four walls covered with the UV-opaque plastic sheet, and the other covered with the UV-transparent plastic sheet (Fig. 1C). We hereafter refer to the former and the latter as the UV-absent and UV-present arenas, respectively. We put 47 males and 64 females in each arena, and counted the number of mated pairs in a period of 75 min. The arenas were placed in the shade to avoid excessive warming. The

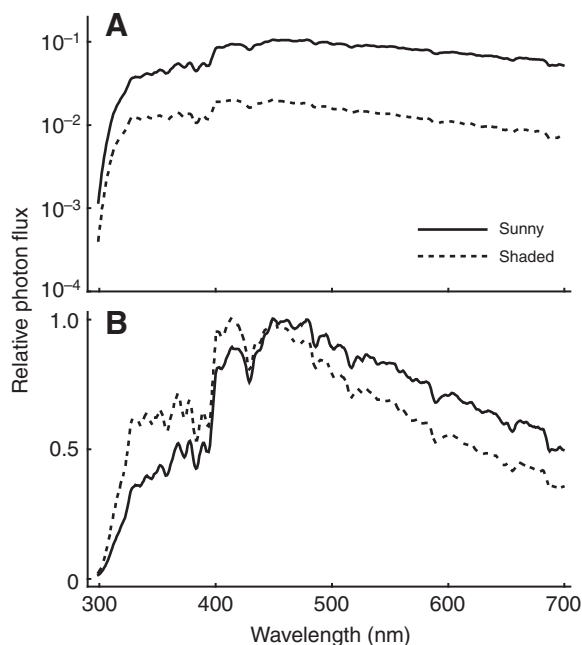


Fig. 2. (A) Logarithmic plots of irradiation spectra measured as the reflection of an MgO-coated surface in direct sunlight (solid line) and in the shade (dotted line). (B) Same spectra as in A, normalized and plotted on a linear scale.

experiment was carried out from 12:30 h to 13:45 h on the same day as experiment 2.

## RESULTS

### Light environments and wing reflectance

We measured the spectral composition of daylight in sunny and shaded conditions. Fig. 2A shows a logarithmic plot of a set of raw data obtained from both conditions. Apparently, the sunny place

was about ten times brighter than the shaded place. The normalized spectra intersect at around 450 nm, with a higher relative content of shorter wavelength light in the shade (Fig. 2B).

### Experiment 1: higher detectability of females in the shade

In Experiment 1, where males were allowed to choose between sunlit and shaded dummies, males significantly preferred the female dummy placed in the shade. When the dummies were placed 15 cm apart in experiment 1a (Fig. 3A), a total of 21 males responded, 17 of which responded to the female in the shade, whereas four responded to the female in the sun ( $\chi^2=8.05$ ,  $P<0.05$ ). When the dummies were placed 2 m apart in experiment 1b (Fig. 3B), 128 males responded: 80 responded to the dummy in the shade, and 48 to the dummy in direct sunlight ( $\chi^2=8.00$ ,  $P<0.05$ ). The number of responding males in experiment 1b was larger, because the experiment was conducted for a longer period than experiment 1a and also later in the morning, when more males were actively searching females (Hirota and Obara, 2000).

Fig. 3C,D show un-normalized and normalized reflection spectra of female wings, respectively, in sunny and shaded places. The absolute reflection intensity was of course higher under the sun (Fig. 3C), but because of the higher content of short wavelength light in the shade (Fig. 2A,B), the relative reflection of short wavelength light is higher in the shade (Fig. 3D).

### Experiment 2: active female search in the UV-rich environment

In experiment 2, 76 of 79 males in the UV-present area performed active zigzag flight and left the area while continuing the search for females; only three males alighted on a substrate within the area and stopped the search for females (Fig. 4A). In the UV-absent area, 58 of 79 males performed active zigzag flight, whereas 21 males sat on a substrate within the area and stopped the search behaviour ( $\chi^2=14.20$ ,  $P<0.001$ ). However, the average duration of the female-searching flight was virtually the same in the UV-present (mean  $\pm$  s.d.;  $16.7\pm 9.1$  s,  $N=76$ ) and the UV-absent areas ( $16.7\pm 12.1$  s,  $N=58$ , Mann-Whitney  $U$ -test,  $P=0.587$ ).

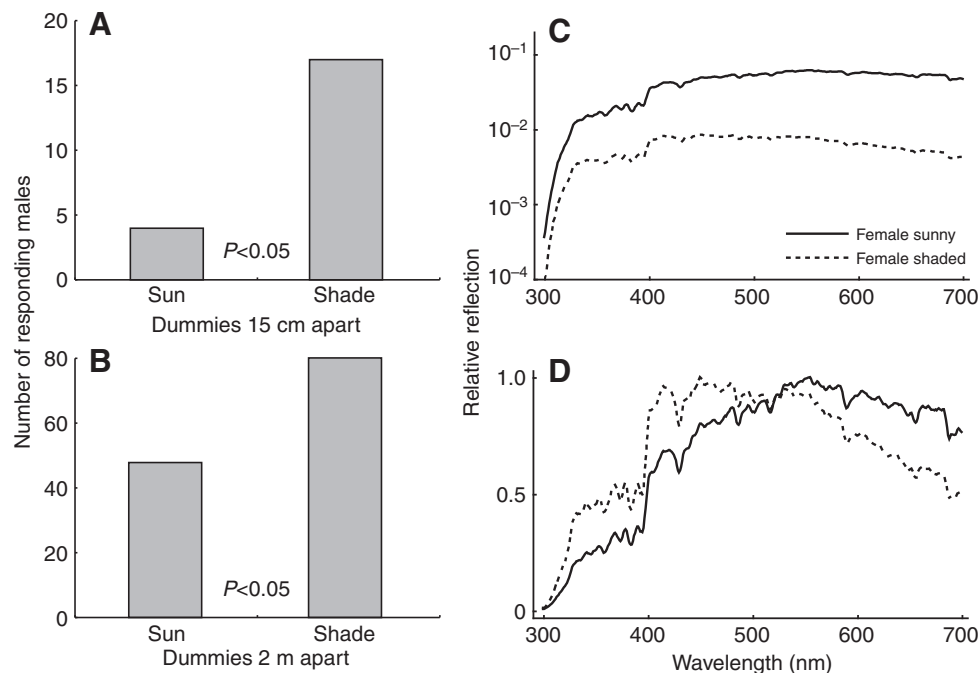


Fig. 3. Male mating responses to female dummies in direct sunlight and in the shade when (A) the two dummies were placed 15 cm apart (see Fig. 1A) and (B) when the two dummies were placed 2 m apart (see Fig. 1B). (C) Logarithmic plots of reflection spectra of the ventral side of a female wing in the sunlight (solid line) and in the shade (dotted line). (D) Same spectra as in C, normalized and plotted on a linear scale.

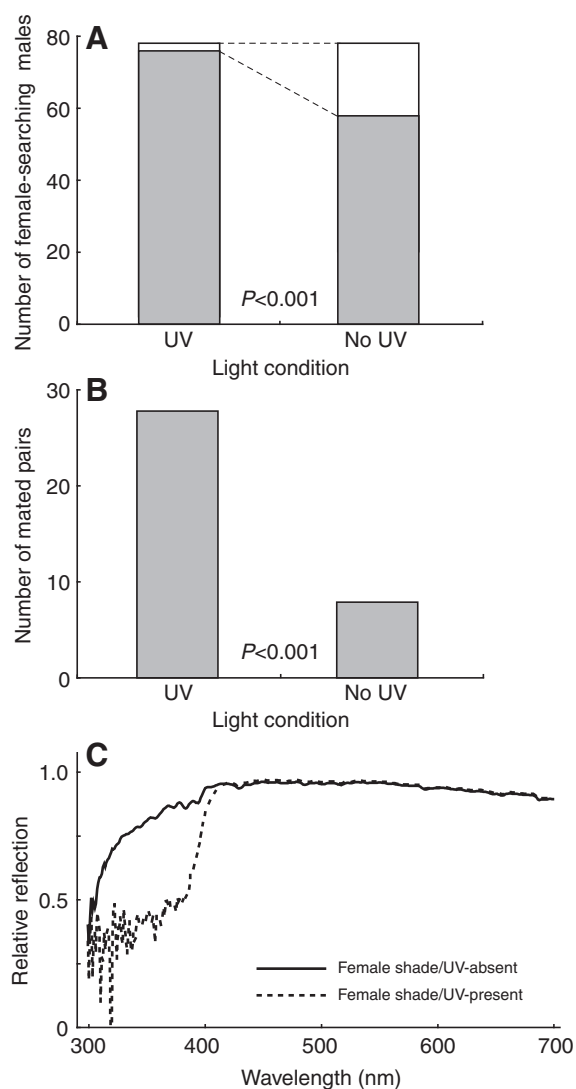


Fig. 4. Male mating behaviour in the UV-present and the UV-absent areas. (A) Female-searching flights in the open cabbage field. Grey regions of the bars show the number of males continuing to search; white regions show the number of males that stopped searching. (B) Number of mated pairs in closed arenas. (C) Logarithmic plots of the reflection spectra of the ventral side of a female hind wing in the UV-present (solid) and in the UV-absent (dotted) arenas set in the shade.

### Experiment 3: enhanced mating in the UV-rich environment

Within 75 min after introducing the individuals, 28 pairs successfully copulated in the UV-present arena, whereas only eight pairs copulated in the UV-absent arena (Fig. 4B;  $\chi^2=16.25$ ,  $P<0.001$ ). Fig. 4C presents the reflection spectra of female wings in two arenas set in the shade. The wings in the UV-absent area reflect very little UV.

## DISCUSSION

### UV-rich environment and male mating behaviour

The present study reconfirmed that the relative UV intensity is higher in the shade than in direct sunlight (Kok, 1972) (Fig. 2A,B), and revealed for the first time that the relative UV reflection of female wings is much higher in the shade, although the absolute UV

reflection is 10 times stronger in the sunlight (Fig. 3A,B). The illumination-dependent difference in the relative UV reflection of female wings affected the mating behaviour of male *Pieris rapae crucivora*: female-searching males responded more frequently to female wings placed in the shade (Fig. 3A,B).

The possibility that males prefer the shade itself is considered unlikely, as in experiment 1 carried out in a large cage, the males were allowed to visually compare two dummies while flying in the direct sunshine (cf. Fig. 1), particularly when the dummies were placed only 15 cm apart. Moreover, in experiment 2, carried out in an open field, more males searched for females actively in the UV-present area (Fig. 4A), which must be brighter than the UV-absent area for *Pieris*, because their eyes are furnished with a number of photoreceptors that are sensitive in the UV wavelength region (~400 nm) (Qiu and Arikawa, 2003; Arikawa et al., 2005).

The males prefer to locate mates in the shade and respond more readily to shaded females. The possible reasons for this include that the shaded females are more visible to the males. Also this is reasonable from the adaptive point of view. Newly emerged virgin females stay for a few hours on the underside of cabbage leaves, where they are shaded from direct sunlight (Hirota et al., 2001). This may promote female location by males and account for the facts that most females copulate before their initial flight after emergence, and that females flying in sunshine are most likely already mated and refuse approaching males by the characteristic mate refusal posture (Obara, 1964).

In addition, the UV environment also seemed to affect the male mating behaviour. UV-rich environments promoted the female-searching behaviour (Fig. 4A) and copulation (Fig. 4B). Such behavioural plasticity dependent on the UV environment will contribute to the enhanced mating success of males and therefore be favoured by natural selection.

### Visual mechanisms underlying male mating behaviour

Most probably, the females are more visible when staying in the shade. The richness of UV in the shade significantly enhances the UV reflection of female wings (Fig. 3D), which effectively stimulates the UV, DB and B receptors, i.e. the receptors with pronounced sensitivity in the wavelength region shorter than 400 nm (Qiu and Arikawa, 2003). As in other lepidopteran species (Kelber and Henique, 1999; Kelber and Pfaff, 1999; Kinoshita et al., 1999; Kelber et al., 2002), *Pieris rapae* also has colour vision (K.A. and Y. Nakatani, manuscript in preparation), which is the ability to discriminate visual stimuli based on the spectral contents regardless of the brightness. Therefore, the males of *Pieris* must be able to detect different reflection spectra as different colours. In fact, males preferred females with stronger UV reflection (summer form) to those with weaker UV reflection (spring/autumn form), both placed in direct sunlight (Obara et al., 2008). In the present case, the males selected the shaded female wings in preference to those under the sun even though the shaded ones were 10 times darker (Fig. 3A). Such discriminations must be achieved by their colour vision. Note that the DB receptor, which has a secondary sensitivity band peaking at 380 nm, is male-specific (Arikawa et al., 2005). Whether this receptor has any crucial function or not in mate detection is an interesting topic for further study.

We thank to D. G. Stavenga for critical reading of the manuscript. The study was financially supported by Grants-in-Aid for Scientific Research (No. 15405010 to YO and No. 18405008 to KA) from the JSPS and COE Research Grant (E-1) from the MEXT of Japan.

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