Comparative analysis on visual and olfactory signals of *Papilio xuthus* (Lepidoptera: Papilionidae) during foraging and courtship

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Abstract

In this study, we examined the roles of visual and olfactory responses during foraging and courtship of the butterfly *Papilio xuthus*. Our results showed that *P. xuthus* was sensitive to color in the range of 350–500 nm. Visits of *P. xuthus* females and males to blue, purple, and red artificial cloth flowers were $\approx$ 54.90% and $\approx$ 39.22%, $\approx$ 19.61% and $\approx$ 35.29%, and $\approx$ 9.80% and $\approx$ 19.61%, respectively. Application of 10% honey on these artificial flowers resulted in an increase of 3.41 and 3.26 fold in flower visits by the butterfly in comparison with controls. When 10% honey water was sprayed on colorless artificial flowers, flower visits reduced seven fold for females and two fold for males, indicating that both visual and olfactory perceptions play important roles during foraging. During courtship, four types of chasing were observed in a natural population of *P. Xuthus*. The four types are males chasing females (49%), males chasing males (25%), females chasing males (13%), and females chasing females (10%). However, when odorless artificial mimics of butterflies were used, no significant differences were observed among these types of chasing, indicating that olfactory perception was crucial for the butterfly during courtship. Profiling volatile organic chemicals and individual bioassays revealed that α -farnesene might play an important role in distinguishing males from females during courtship of the butterfly.

Main Text

Visual and chemical cues are critical during butterfly foraging and courtship, but their roles vary in individual butterfly species. The eyes of most butterflies contain visual pigments that can sense a range of light spectrum including ultraviolet (300–400 nm), blue (400–500 nm), long-wave (500–600 nm), and red (760–622 nm)¹. During foraging, different taxa of butterflies use different visual cues to locate food source. Most butterflies prefer blue², and some butterflies of *Papilio* and *Pieris* species prefer purple. *Tirumala limniace* and some *Pieris* and *Vanessa* prefer yellow, whereas *Danaus chrysippus*, *Cethosia biblis* and *Cethosia cyane* are more sensitive to red²⁻⁸. During courtship, butterflies distinguish females from males of the same species primarily based on visual signals, including wing characteristics (color, spots, and size), wing surface reflection spectrum, and polarized light. Dichroic butterflies identify opposite sex by the color of wings under natural light⁹⁻¹⁰. *Pieris rapae* males identify females using visual cues of their wings¹¹. *Heliconius Cydno* males recognize females by their polarized aurora¹².

Chemical cues are also important in localizing food sources¹³⁻¹⁶. Butterflies can also distinguish the opposite sex from the same species as well as individuals from heterogenetic species based on chemical cues¹⁷⁻²⁰. Some butterflies, such as *Kallima inachus* and *Danaus genutia*, hardly have any vision. Therefore chemical signals are their only cues during foraging and courtship. VOCs from individual butterflies are critical for them to distinguish opposite sex from the same species and individuals from different species²¹⁻²³. For example, VOCs from the females and males of *Papilio machaon* are significantly different and serve as important cues in identifying opposite sex from the same species²⁴.

Four types of relationships between visual and chemical communication have been identified during butterfly foraging and courtship: (1) vision was given priority over olfaction; (2) olfaction was given priority over vision; (3) olfaction and vision were equally important; and (4) only olfaction was used. However, what determines the contribution of visual and chemical perception in foraging and courtship is not yet clear.

In this study, we conducted a series of assays to determine the importance of visual and chemical cues for the butterfly *P. xuthus* during foraging and courtship. We used butterfly mimics to separate the impact of visual and chemical cues on butterfly foraging and mating behavior. We also analyzed wing color patterns between the females and males, profiles of VOCs from males and females, and tested selected individual VOCs on butterfly behavior. Our results represent an initial analysis on the contribution of visual and chemical cues to foraging and mating behavior in this butterfly species.

Results

Response to visual and chemical cues during foraging

Generally, males of most butterflies are more active than females during flower visits. However, both males and females of *P. xuthus* were very active during foraging. In the absence of any chemical cue, both females and males mainly visited blue (440–475 nm), purple (380–420 nm), and red flowers (615–630 nm) (Fig. 2A-B). Together, 84.31% of flower visits by females and 94.12% of flower visits by males were to blue, purple, and red flowers. The preference of females and males toward flowers with these three colors was significantly different. Specifically, visits to blue, red, and purple flowers by females versus males were 54.90% versus 39.22% ($F = 12.34$; $df = 5.00$; $P < 0.05$); 9.80% versus 19.61% ($F = 9.38$, $df = 5.00$; $P < 0.05$); and 19.61% versus 35.29% ($F = 14.77$, $df = 5.00$; $P < 0.05$), respectively.

Chemical cues had a significant impact on butterfly behavior. After spraying 10% honey water on otherwise odorless, artificial flowers, total number of flower visits increased 3.33 times. More specifically, visits to flowers with honey water by females and males increased 3.41 and 3.26 times, respectively, in comparison to the same flowers without honey water (Fig. 2C). On the other hand, flower colors also impacted responses of butterflies to honey water. Total number of butterfly visits by both males and females increased 33.78 times to flowers applied 10% honey water in comparison with honey water alone controls. Males were more sensitive to flower and honey combinations than females, with 83.00 times increase in male visits and 24.85 times increase in female visits to flowers with 10% honey water than controls with 10% honey water alone (Fig 2C). These results showed that *P. xuthus* relied on both visual and chemical cues during foraging.

Response to color and odor during courtship

Four types of chasing behavior of natural *P. xuthus* populations were observed during courtship, namely, males chasing females, males chasing males, females chasing males, and females chasing females (Fig. 3A). Nearly half (49%) of chasings were males chasing females, but only 13% of chasings were

females chasing males ($F = 23.34$, $df = 5.00$; $P < 0.01$). Interestingly, there were also 25% of chasings between males and 10% of chasings between females ($F = 17.53$, $df = 11.00$; $P < 0.01$). On the hand, *P. xuthus* individuals hardly chased *D. chrysippus* individuals, indicating that *P. xuthus* could identify conspecific butterflies and reject heterogeneous butterflies in nature.

When butterfly mimics were used, results were significant different. The frequency of males chasing female mimics reduced to 19.67%, whereas the frequency of females chasing male mimics increased to 27.9%. On the other hand, the frequency of males chasing male mimics increased to 24.6%, and females chasing female mimics increased to 27.9% (Fig. 3A). Unlike in the natural population, no significant difference was found among the frequencies of the four types of chasing behavior ($F = 0.89$, $df = 11.00$; $P > 0.05$). Similarly, *P. xuthus* hardly chased *D. chrysippus* mimics, indicating that *P. xuthus* butterflies identify heterogeneous conspecific butterflies but could not distinguish females and males of their own if only patterns of colors are available.

Analysis of wing color spectrum

It is very difficult to distinguish females from males of *P. xuthus* because their color and stripes are very similar (Fig. 3B). The color spectrum of wings from both males and females is in the range of visual and ultraviolet light, with only a few differences observed on the ventral surface of the wings (Fig. 3C-D). The results may explain that the butterflies could not recognize their partners by vision alone during courtship.

Body VOCs and their roles in courtship

During courtship, chemical cues play important roles in identifying sex partners in insects, especially in butterflies because females and males of butterflies are very similar in patterns of both color and shape. To examine potential chemical cues, we measured 29 VOCs obtained from *P. Xuthus* individuals. The 29 VOCs belonged to 11 different categories. No gender-specific VOCs were observed in either females or males. The top three categories of the identified VOCs included alcohols (38.39%; 37.52%) and esters (34.19%; 32.15%) (Table 1). We selected three abundant chemicals, 2-ethylhexanol (alcohol), 2-ethylhexyl acetate (ester) and the aromatic α -farnesene, to test their ability to attract butterflies during courtship. The total number of males chasing mimics increased significantly after the mimics were sprayed the three chemicals, while no significant change was observed in the number of females chasing mimics after similar spraying (Fig. 4A). Again *P. Xuthus* butterflies hardly chased *D. chrysippus* mimics when the three VOCs were applied on the mimics (Fig. 4A-B), suggesting that patterns of colors were the main cues in identifying heterologous conspecific butterflies.

Butterflies chased butterfly mimics in a way similar to that observed in the natural population during courtship after the mimics were sprayed with α -farnesene (Fig. 4B). The abundance of α -farnesene in females was higher than that in males 3-5 days after emergence (Fig. 4C). The high abundance of α -farnesene might be responsible for distinguishing males from females in natural butterfly populations. Application of the other two volatiles resulted in increased total number of chasing but did not result in increased chasing between different sex partners specifically, suggesting that α -farnesene could be a

chemical cue for distinguishing males from females, whereas 2-ethylhexanol and 2-ethylhexyl acetate could be cues for distinguishing *P. Xuthus* from other butterfly species.

Discussion

Lepidopterans generally use multi-modal sensory cues during nectar foraging, but the integration of such cues may be complex and hierarchical²⁵.² pointed out that there are four types of signal perception during butterfly foraging and courtship, including perception based predominantly on vision, perception based predominantly on olfaction, perception based equally on vision and olfaction, and perception based on olfaction alone. To examine the signal perception mechanism of the butterfly *P. Xuthus*, we tested the responses of *P. xuthus* to visual cues, chemical cues, and the combination of visual and chemical cues. We found that *P. xuthus* used primarily visual cues during flower visits when visual cues and chemical cues were separated. However, synergism was observed when visual and chemical cues were present together during flower visits. Using visual cues as the predominant signals during foraging by butterflies may be due to colorful flowers are easy to identify in nature. On the other hand, chemical cues from VOCs in nature are abundant, complex, and unstable, which may need to invest more genetic resource to identify and distinguish the information from various VOCs. Butterflies may have gradually evolved and developed visual perception as the main signal recognition due to its less genetic cost.

Similarly, butterflies use a variety of visual and chemical cues to identify opposite sex during courtship²⁶⁻²⁸. Most butterflies first use visual signals to distinguish females and males, and then use chemical signals to differentiate them further^{27,29}. Our results indicated that visual perception of *P. xuthus* outclassed its olfactory perception during courtship. *P. xuthus* could accurately distinguish individual butterflies from other species solely based on visual cues. However, visual cues were not enough to distinguish males from females of its own species, likely due to very similar colors and patterns of wings on both *P. xuthus* males and females. The frequency of males chasing females was only 19% in the absence of chemicals. The frequency of males chasing females increased to 40% when honey water was applied, suggesting that chemical cues played an important role in identification of opposite sex during courtship.

Possible chemical cues include sex hormones and other body VOCs^{17-20, 30-31}. Analyses of VOCs on the butterfly *P. xuthus* identified several abundant VOCs, which could play roles in identification of mates during courtship. Even though sex-specific VOCs were not identified, several VOCs with quantitative differences between males and females were found. The quantitative differences in VOCs might serve as cues for chemical perception during mating. Indeed, application of two abundant VOCs, 2-ethylhexanol and 2-ethylhexyl acetate, resulted in increased total courtship chases among *P. Xuthus* individuals. Application of the aromatic volatile α -farnesene resulted in increased frequency of males chasing females specifically. These three chemicals exhibited no effects on chasing by *P. xuthus* to *D. chrysippus* butterflies. These observations indicated that chemical cues play important roles in distinguishing butterflies from different species, and between males and females from the same species.

Materials And Methods

No specific permits were required for the field studies described.

Test site: Observation was carried out in a net house (8 × 8 × 5 m) in Kunming (102 ° 45 ' E, 25 ° 03 ' N), Yunnan Province, southwestern China. The altitude was 1930 m. Annual average temperature was 15–16 °C. Annual average rainfall was 900–1000 mm.

Test butterfly: *Papilio xuthus* was obtained from artificial breeding populations and used for the observation.

Artificial flowers: Artificial cotton flowers of seven colors were used for the test. The seven colors were red, orange, yellow, blue, purple, green, and white corolla. The size (8.56 ± 0.18 cm in diameter and 2.73 ± 0.04 cm in depth; Fig. 1A) and shape of the artificial flowers were the same. A spectrometer (USB2000+, Ocean Optics, Inc., USA) was used to measure the reflection spectrum of flower colors. The spectrometer was calibrated against a MgO-coated surface as a reference and the spectral reflection of the fake flowers was in the wavelength range of 379–780 nm (Fig. 1B)

Butterfly mimics: Artificial butterfly wings were adhered to an insect body made of black wooden strips. Butterfly mimics were wrapped in transparent plastic film. The butterfly mimics were the same in size as natural butterflies.

Reflection measurements of natural butterflies and butterfly mimics: The reflection characteristics of natural butterflies and butterfly mimics were measured using a USB2000+ spectrometer with a PX-2 light source (Ocean Optics, Inc., USA). Proofreading was performed for the magnesium oxide surface.

Measurement of color attraction: Seven artificial flowers in a nosegay were placed 0.5 m above the ground. The distance between adjacent bouquets was 80 cm. Female and male butterflies of *P. xuthus* (20 pairs each) were released into the net house, and their behavior of visiting the fake flowers of seven colors was observed. The position of these flowers was moved clockwise once every 30 min.

Measurement of odor attraction: Corollas from flowers were removed, leaving only their branches and leaves on. Honey water (10%) was then sprayed on these branches and leaves, and butterfly response to the chemical was examined and recorded.

Measurement of combined impact of both visual and chemical cues: On the artificial flower bouquets, 10% honey water was sprayed, and flower visits of male and female butterflies were recorded. Honey water was sprayed every hour to avoid its evaporation. All tests were observed continuously for two days.

Observation of visual response behavior during courtship: In the net house, 20 pairs of *P. xuthus* (♂:♀ = 1:1) were released into the net house for observing their courtship behavior for two days. Additionally, 10 pairs of another butterfly species, *Danaus chrysippus*, were also released to the same net house to determine any inter-species courtship between *P. xuthus* and *D. chrysippus*.

To separate impact of visual and chemical cues on butterfly behavior during courtship, mimics of *P. xuthus* and *D. chrysippus* were used to test the discrimination of *P. xuthus* based only on butterfly mimic colors. Four pairs of butterfly mimics (♂:♀= 1: 1) for each species were hung randomly at the height of 1.5~2.0 m above the ground with 80 cm apart from each other. Then 20 pairs of virgin *P. xuthus* butterflies (♂:♀= 1: 1) were released into the net house and their chasing behavior toward the mimics was recorded. The mimics were rotated clockwise every 30 min. All tests were observed continuously for 2 days.

Measurement of volatile organic chemicals (VOCs): To measure the impact of volatiles, the method solid-phase microextraction (SPME) was used to extract VOCs from female and male butterflies separately. Samples were then analyzed using GC-MS (Thermo Scientific TRACE GC ULTRA and Thermo Scientific ITQ 900 MS; Borg-Karlson & Mozuraitis, 1996). Three days after eclosion when mating started, *P. xuthus* individuals were put into an 80 mL wide mouth bottle. A PDMS/DVB extraction needle with 65 µm diameter (Supelco, Bellefonte, PA, USA) was then inserted into the bottle for 1 h. A SPME extraction needle was inserted at the GC inlet (250 °C, 10 min) before GC-MS analysis. A TR-5MS column, with inner diameter 0.25 mm, thickness 0.25 µm, and length 30 m, was used to analyze VOCs. The program for GC-MS analysis was 2 min at 40 °C, 2 min at 120 °C (achieved by heating at 4 °C /min), and 5 min at 230 °C (by heating 5 °C /min). The inlet temperature was 250 °C and the pressure of the carrier gas He was 69 kPa.

Identification of VOCs was carried out by searching National Library of Standards and Technology (NIST, <http://webbook.nist.gov/chemistry/>) library combined with manual analysis of mass spectra. Retention indices were calculated and compared with corresponding values in the literature. The retention index (linear retention index, LRI or RI) was calculated as follows (Li 2017):

$$LRI = 100 \times n + \frac{100 \times (t_x - t_n)}{t_{n+1} - t_n}$$

LRI-retention index;

t_x —retention time of the component to be tested (min);

t_n —retention time of n-alkanes with n carbon atoms(min);

t_{n+1} —Retention time of n-alkanes with n+1 carbon atoms(min).

The area normalization method was used to determine the relative content of each volatile compound.

Analysis of selected VOCs on butterfly behavior

Three most abundant chemicals, 2-ethylhexanol (alcohol), 2-ethylhexyl acetate (ester), and α -farnesene (aromatic), were chosen for analysis of their impact on butterfly males and females. A 1% solution of each chemical was prepared and 0.5 mL of each solution was injected into a 0.6 mL finger tube that was attached to a butterfly mimic. Butterfly mimics were hung 1.8 m above the ground in the net house. Each mimic was placed one meter apart from each other. Twenty pairs of virgin butterflies three days after eclosion were released into the net house. Their chase behavior toward the butterfly mimics was observed and recorded. Butterfly mimics without chemicals were used as controls. Mimics of *D. chrysippus* were also used for observation of possible inter-species attraction.

Data analysis

The SPSS 18.0 software was used for data analysis. One-way ANOVA was used to identify significant differences during foraging and courtship events between males and females.

Declarations

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Author Contributions

X.M.C. designed the project with intellectual contributions from all authors; J.L. collected, analyzed the data and wrote the paper; all authors reviewed the final manuscript.

Competing Interests

The authors declare no competing interests.

Data availability

All data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Additional information

Correspondence and requests for materials should be addressed to XM. C.

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Tables

Table 1 Classification of VOCs in the adults of *P. xuthus*

VOCs	Contents(%)	
	♂	♀
Alkanes	6.91	6.05
Alkynes	0.48	0.31
Terpenes	4.65	10.54
Alcohols	38.39	37.52
Ethers	7.39	7.1
Aldehydes	1.60	0.25
Ketones	1.31	1.24
heterocycles	0.37	0.32
Acids	3.65	3.45
Esters	34.19	32.15
Arenes	1.06	1.07

Figures

Figure 1

Artificial flowers and their spectral reflectance. A. Artificial flowers with seven colors; B. Spectral reflectance of the seven colors

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Artificial flowers and their spectral reflectance. A. Artificial flowers with seven colors; B. Spectral reflectance of the seven colors

Figure 2

Response of the butterfly *P. xuthus* to artificial flowers with different colors and combination of different colors and odors. A. Response of *P. xuthus* to flowers with different colors. B. Response of *P. xuthus* to flowers sprayed with 10% honey water. C. Synergistic effects of colors and odor on butterfly behavior.

Figure 2

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Figure 3

Chase behavior of *P. xuthus* during courtship and wing reflection spectrum. A. Chase behavior of *P. xuthus* during courtship using both natural butterflies and butterfly mimics. B. Representative butterfly specimen under natural light. C. Reflective spectrum of the butterfly specimen. D. Images of butterfly specimen under UV light.

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Figure 4

Chasing behavior of the butterfly *P. xuthus* to VOCs during courtship. A. Chasing frequency between males and females to butterfly mimics applied with 2-ethylhexyl acetate and 2-ethyl-1-hexanol. B. Impact of α -Farnesene on chasing behavior of the butterflies *P. xuthus* and *P. bianor*. C. Abundance of α -farnesene in females and males of *P. xuthus*.

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