

Plant scents modify colour preference
in foraging swallowtail butterflies

Mina Yoshida

Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

Department of Evolutionary Studies of Biosystems,
School of Advanced Sciences,
SOKENDAI (The Graduate University for Advanced Studies)

2015

Acknowledgments

Firstly, I would like to express my sincere gratitude to my supervisor Dr. Michiyo Kinoshita for the continuous support of my whole Ph.D. research and study more than 5 years. I am also deeply grateful to my co-supervisor Dr. Kentaro Arikawa for his fruitful comments, advices and suggestions on my research. Without of these two grateful supervisors, I was not able to experience such wonderful time.

Besides my supervisors, I would like to thank Dr. Hisashi Ômura and Ms. Yuki Itoh for analyzing odorants of many plants and making their synthetic mimics and Dr. Hisashi Ohtsuki for supporting statistical analysis of the behavioural data.

In addition, I would like to extend my gratitude to Dr. Finlay Stewart for carefully reading the manuscripts every time and putting comments. I am also grateful Dr. Hisaharu Koshitaka for helping to rear butterflies and take care of the plants.

My sincere thanks also go to all of my fellow colleagues in SOKENDAI and the members of laboratory for stimulating discussions and fun I have had in my school life.

Lastly, I wish to express special thanks to my family and all friends for their supports.

June 2015

Abstract

Flower constancy, where pollinators restrictedly visit flowers of single species or morph within a species, involves mainly learning ability of flower traits: colour, shape, scent, taste and so on. Integration between different sensory modalities in flower foraging must be important for naïve pollinators that are still not fully understood. I therefore carefully examined effect of particular scents in innate colour preference of foraging swallowtail butterfly, *Papilio xuthus*, a potential pollinator for reddish flowers. When recording the colour to which a naïve *P. xuthus* first chooses for foraging among four coloured discs (blue, green yellow and red), I found more than half individuals visited the blue disc in the experimental room without any source of particular odorant. With ambient plant odorant, clear differences in innate colour preference emerged between different odorants as well as between sexes. Scents of essential oils of bitter orange and lily flower increased females visited the red disc, whereas odorant of host plants, *Citrus unshiu*, increased attractiveness to green in females. In contrast to females, effects of any odorant in males were quite limited. In females whose antennae were painted with mascara, the odorants' effect disappeared, indicating that odorants detected by the antennae altered innate colour preference. Because no clear sexual dimorphism exist in visual ability, presumably either olfactory system or integration of visual and olfactory information involves the sexual dimorphism in colour preference modified by odorants. At least in female *Papilio* butterflies, flower constancy for reddish flowers might relate to innate colour preference modified by flower scents and their learning ability of red colour.

Table of Contents

Acknowledgments	2
Abstract	3
Table of Contents	4
Chapter 1. General Introduction	5
Chapter 2. Innate Colour Preference of Foraging Swallowtail Butterfly	10
2.1. Introduction	11
2.2. Material and Methods.....	13
2.3. Results	27
2.4. Discussion.....	38
Chapter 3. Conclusion and General Discussion.....	43
References	47

Chapter 1

General Introduction

Foraging is one of the most important behaviours for virtually all animals. There are various different strategies, achieved by a wide range of mechanisms (Kevan and Baker 1983, Goulson 1999, Goulson 2000, Chapman 2009). By using multisensory systems, animals obtain information about food sources from the environment and can therefore forage more efficiently. For example, when we search an apple, we use vision for detecting the red and also use olfaction for detecting the sweet scents, to confirm the apple more precisely. In flower foraging insects, a nocturnal elephant hawkmoth, *Deilephila elpenor* and a diurnal hawkmoth, *Macroglossum stellatarum*, use olfaction when they are away from nectar (flying quickly in a zig-zag pattern), and use vision when they are close to nectar (flying in a straight line) (Balkenius, Rosén et al. 2006). Among of sensory systems, vision and olfaction are of particular importance for foraging and have been extensively investigated in many insect species.

Research on insect vision has particularly a long history (Briscoe and Chittka 2001). Insects can associate certain visual features with rewards. Researchers have been using this ability to investigate their visual perception. Colour vision is one of important visual ability for flower foragers and defined as ability to discriminate chromatic contents of objects with regardless of their brightness. Honeybees, *Apis mellifera*, have been intensively studied with respect to colour vision over the years. Karl von Frisch (1914) demonstrated colour vision in honeybees by proving their ability to discriminate blue from some shades of grey. Since Frisch demonstrated the ability of the colour vision, several studies have demonstrated colour vision in the other insect species including lepidopteran (Kelber and Pfaff 1999, Briscoe and Chittka 2001, Blackiston, Briscoe et al. 2011, Kinoshita and Arikawa 2014, Leonard and Masek 2014). In nocturnal hawkmoth, *D. elpenor*, can discriminate a training colour from eight different

shades of grey in dark, where human cannot use colour vision (Kelber, Balkenius et al. 2002). Japanese yellow swallowtail butterfly, *Papilio xuthus*, is the first demonstrated insect that has the ability to discriminate red as colour (Kinoshita, Shimada et al. 1999).

To have colour vision an animal must have two or more photoreceptor types with different spectral sensitivities in its retina. Colour vision in honeybees is based upon three types of spectral receptors, peaking at UV, blue, and green wavelength region, that is trichromatic (Menzel and Backhaus 1989) . As well as bees, hawkmoths, *M. stellatarum*, also have UV, blue and green receptors (Lunau and Maier 1995). Interestingly, butterfly species often have more spectral types of receptor adding UV, blue and green types. For example, Eastern pale clouded yellow butterflies, *Colias erate*, have eight photoreceptor types and interestingly there is a sexual difference in variations of red receptors (Ogawa, Kinoshita et al. 2013). Butterflies, therefore, could have more sophisticated colour vision comparing moths. These red receptors provide butterflies to see red as colour, which bees and moths cannot detect as independent colour.

Besides visual information, floral scents are also crucial cues for searching flowers (Andersson and Dobson 2003). Volatile organic compound from flowers is received by sensilla containing olfactory receptors on antennae. Olfactory information is first sent to the first olfactory ganglion (antennal lobe) and then projects to the mushroom body and the lateral horn for processing (Hallberg, Hansson et al. 2003, Kinoshita, Shimohigashi et al. 2014). Function of olfactory cue in flower foragers has been studied mainly in honeybees and hawkmoths (Gerber and Smith 1998, Srinivasan, Zhang et al. 1998, Andersson 2003, Andersson and Dobson 2003, Balkenius, Rosén et al. 2006, Chittka and Raine 2006). Honeybees can detect and learn a wide range of floral volatiles from various plant species (Leonard and Masek 2014). The moth olfaction has been

extensively studied because of their clear sexual dimorphism. In most moth species, female antennae are rather simple, while male antennae are large and have a high density of sensilla (Hansson 1995). Males have to detect sex pheromone released by females. The studies on olfaction are inclined to the mating behaviour that is elicited by the detection of pheromone. On the other hand, antennae of butterflies seemed to have no clear sexual differences, and studies are not inclined to even mating behaviour but on foraging behaviour. In white cabbage butterflies, *Pieris rapae*, only a single chemical compound is known to trigger the foraging behaviour (Honda, Ômura et al. 1998).

The integration of vision and olfaction is also important when considering the foraging, because insects use both sensory modalities. This point has received some attention even though studies of multi sensory integration are very limited in Lepidopteran insects. In naïve red admiral butterflies, *Vanessa indicia*, priority of vision versus olfaction are tested and revealed that they make feeding decisions based more on colour than scent when visiting flowers, that is, vision has priority over olfaction (Ômura and Honda 2005). Nocturnal hawkmoths, *Manduca sexta*, visit flowers having strong smell with light colour and floral scents play important roles in various aspects of their foraging behaviour (Dobson 2006). In addition, a naïve *M. sexta*, only visit white real flower even if there are scented white artificial flowers or non-scented real flowers (Raguso and Willis 2005), indicating that for naïve *M. sexta*, both visual and olfactory cues are crucial to elicit proper foraging behaviour. They detect and orient flower scents from noise of other odorants at a distance, and require flower fragrance together with visual cue at the same time to elicit and complete proper foraging behaviour (Raguso and Willis 2005, Andersson 2006, Riffell, Shlizerman et al. 2014). In contrast to nocturnal moths, floral scent may play a role in triggering searching behaviour or in

attraction at close range in diurnal Lepidoptera, *V. indica* (Ômura and Honda 2005) and *M. stellatarum* (Balkenius and Kelber 2006) can learn odorant only when it is presented with visual stimuli, indicating that their foraging behaviour is dominant in visual cue.

Japanese yellow swallowtail butterflies, *Papilio xuthus*, strongly depends on vision in its foraging as other butterflies. Important phenomena of colour vision; colour constancy and colour contrast and other visual properties; brightness and polarization vision have also been demonstrated by behavioural experiments (Kinoshita and Arikawa 2014). Visual systems of *P. xuthus* are most well understood among flower visiting insects (Arikawa 2003). Structure and physiology of the eye have been studied in detail as well as molecular basis of vision (Arikawa 2003). Contrast to bee and moth colour vision, colour vision of *P. xuthus* is tetrachromatic based on UV, blue, green and red receptors among six classes of spectral receptor over the retina (Arikawa 2003). The ommatidia of *P. xuthus* are divided into three types possessing the different combination of spectral receptor classes. In addition to the organization of the retina, recently, both visual and olfactory inputs into the mushroom body from visual center, the optic lobe, are discovered in *P. xuthus* (Kinoshita, Shimohigashi et al. 2014). This new finding indicates integration of vision and olfaction may occur in *P. xuthus* brain

Here I studied integration of vision and olfaction in foraging of native *P. xuthus*, because its first visitation might be important for flower constancy of solitary flower foragers like butterflies not bees. Which modality has priority and how these two otherwise interact in foraging behaviour are opened questions in butterflies. I focused on their foraging behaviour, and performed behavioural experiments.

Chapter 2

Innate Colour Preference of Foraging Swallowtail Butterfly

2.1. Introduction

Many flower visiting insects restrictedly visit flowers of single species or morph within a specie and is called flower constancy (Linsley and MacSwain 1958, Waser 1986, Goulson and Cory 1993, Hill, Wells et al. 1997, Chittka, Thomson et al. 1999, Hirota, Nitta et al. 2012). For example, honeybees frequently visit green flowers and swallowtail butterflies visit reddish flowers in the field (Tanaka 1991). Foraging to the limited flower species needs less time and can reduce the energy for searching food, which is beneficial for insects (Goulson 2000). Flower constancy is strongly associated with learning ability. Flower foragers such as bees, flies, and butterflies can learn to associate food rewards with particular colours or scents. Having flower constancy might not be only related to learning ability but also related to the innate colour preference. In the field, insects may rely their first visit on the innate preference especially in solitary species and learn sensory information of the first flower that provides efficient nectar.

The innate colour preference was reported in many flower visiting insects (Ilse and Vaidya 1956, Scherer and Kolb 1987, Goulson and Cory 1993, Giurfa, Nunez et al. 1995, Lunau and Maier 1995, Kelber 1997, Ômura and Honda 2005, Blackiston, Briscoe et al. 2011). Flower naïve honeybees, *A. mellifera*, were trained on achromatic stimuli to get reward, and then colour preference tests were performed. As result, honeybees were mostly attracted to UV-blue and green colour stimuli (Giurfa, Nunez et al. 1995). As honeybees, bumble bees, *Bombus terrestris*, (Gumbert 2000), flies, *Eristalis tenax*, (Lunau and Maier 1995) also prefer blue region well. In naïve hawkmoths, *M. stellatarum*, when eight colour patches were presented (violet, blue, blue-green, yellow-green, yellow, orange, red and white), blue was most selected colour

(Kelber 1997, Riffell, Alarcón et al. 2008). As in hawkmoths, butterflies: *Battus philenor*, *Aglais urticae* and *Gonepteryx rhamni* basically prefer blue colour (Weiss 1997, Andersson 2003). On the other hand, some Papilionidae butterflies were reported to prefer either yellow or red colours (Andersson and Dobson 2003).

Among studies of innate colour preference of flower visiting insects, Japanese yellow swallowtail butterflies, *Papilio xuthus*, have sexual dimorphism in the innate colour preference; females prefer yellow and red while males prefer blue (Kinoshita, Shimada et al. 1999). Because no clear sexual dimorphism has been found in the visual system, I hypothesized that particular olfaction stimuli would affect the colour preference, and set two questions in foraging behaviour of naïve *P. xuthus*.

1. What causes the sexual dimorphism of colour preference in *P. xuthus*?
2. How are vision and olfaction integrated in foraging behaviour?

To answer these questions, I first performed confirmation tests of sexual dimorphism in the colour preference of *P. xuthus*. I then tried to understand mechanisms of sensory integration and performed behavioural experiments for investigating effects of scents to foraging behaviour.

2.2. Material and Methods

Animals

Laboratory-raised summer form Japanese yellow swallowtail butterflies, *Papilio xuthus* were used for all tests. For collecting eggs, females were captured mostly in Kanagawa, Japan and if necessary mated artificially with males. Females laid eggs on *Citrus* leaves and eggs had been at the room temperature until hatched. Hatched larvae were fed on fresh *Citrus* leaves under a 14 hours light and 10 hours dark photoperiod at 28 ± 3 °C. Pupae had been in an experimental room, where temperature was 30 ± 2 °C. I kept each emerged adult separately in a styrofoam box covered with gauze at 30 ± 2 °C for 2 or 3 days without any foods until behaviour experiments. All tested butterflies were naïve, meaning that they had no experience of foraging behaviour and mating.

To reveal whether the odorant received by antennae was related to innate colour preference, we painted bilateral antennae using clear mascara on the day of emerged and performed preference tests using these butterflies.

Experimental setup

All experiments were performed in a small cage (Height 46 cm × Width 62 cm × Depth 42 cm, **Figure 1**). The cage was illuminated by 12 halogen lamps (30w, JCV100V-300WGS, Ushio Lighting), 6 fluorescent tubes (32w, FHF32EX-N-H, Panasonic) and 4 xenon lamps (100w, SET-140F, SERIC). The light intensity varied from about 6000 lux at the floor of the cage to about 8000 lux at the ceiling. I measured the irradiation spectrum as a reflection spectrum from a standard white material by a

spectrometer (HSU-100S/SC, Asahi Spectra Japan, **Figure 2-A**).

Visual stimuli

The stimulus for colour preference tests consisted of four coloured discs; blue green yellow and red, $\Phi 5$ cm (Tonal Colour 93, Japan Colour Enterprise Co., Ltd, Tokyo, Japan) on black background (**Figure 3**). For avoiding position effects, I prepared three patterns with different arrangements of the colours and selected arrangement of the pattern at random for each tested butterfly. During experiments, the pattern was covered with an artificial quartz glass. The reflectance spectra of the four colours were measured using the spectrometer (**Figure 2-B**).

Behavioural experimental protocol

One naïve and starved butterfly was released into the cage with the four-colour pattern on the floor in each test. I recorded the colour of the disc which the butterfly first landed on with extended its proboscis as its innate preference colour. Mostly, experiments were carried out in the morning. If the individual did not visit any coloured disc within 5 minutes of the release, I tested it in the afternoon or next day again.

Olfactory stimuli

I used 7 different odorant sources (3 essential oils, 2 potted plants and 2 synthetic mimics) in the colour preference tests. Three essential oils were Neroli (flower of bitter orange, *Citrus aurantium* ssp *amara*, Rutaceae, Essential Therapeutics, Australia),

Lavender (*Lavender angustifolia*, Lamiaceae, Essential Therapeutics, Australia), and Lirio (*Lilium* ssp, Katsuragi, Japan). Two potted plants were flowering *Hibiscus rosa-sinensis* (Malvaceae) and *Citrus unshiu* (Rutaceae). Two synthetic mimics were scent of Neroli and odorant of *C. unshiu* (for details, see next section). Essential oils and synthetic mimics were set under the cage and the potted plants were placed behind a screen to one side of the cage. Neither position was visible to the tested butterflies, meaning that there was no visual effect of odour introduction.

The quantity and methods of olfactory introducing differed according to odorant. For Neroli and Lavender, 200 µl of essential oil diffused through the entire period of these colour preference tests (about 2 weeks). For Lirio and 2 synthetic mimics, I put 5µl of odorant on a small piece of filter paper 30 minutes before beginning tests, and changed it every hour. For *H. rosa-sinensis* and *C. unshiu*, six potted trees were in the experimental room through the entire period of tests (about 2 weeks). A fan worked to diffuse odorants in the whole room.

After finishing experiments with one particular odorant, the experimental room had been opened and ventilated for at least 5 days to completely remove previous odorant before switching to a new one. Before starting next experiments using another odorant, I performed the control colour preference tests (non-odorant test) to ensure whether there was no effect of previous odorant.

Making synthetic mimics

To examine which volatile organic compounds (VOCs) were effective in colour preference, VOCs in each odorant were collected by dynamic headspace adsorption

method and determined by gas chromatography-mass spectrometry analysis. Then, I performed the preference tests to investigate which VOCs affect the innate colour preference of *P. xuthus* using synthetic mimics (for details, see former section).

Sample preparation

VOCs of *C. unshiu* and *H. rosa-sinensis* were collected by dynamic headspace sampling in the laboratory. Undamaged plant branches (with 30 leaves for *C. unshiu*, and with an 1 fresh flower and 5-7 leaves for *H. rosa-sinensis*) put into an 100 ml Erlenmeyer flask containing 30 ml of distilled water were placed in a separable glass container (200 mm×110 mm ID). Plant VOCs were trapped with a Supelco ORBO 32 adsorbent tube (100 mm×8 mm OD, 600 mg of activated charcoal) for 8 hours by using a membrane pump at a flow rate of 5 l/min. The container had an air inlet equipped with an activated charcoal filter, from which purified ambient air was allowed into the container. Sampling was repeated twice using different branches for each plant. To distinguish between plant VOCs and contaminants, the inner air of blank container was collected as a blank sample in the same manner. Collected volatiles were extracted from the adsorbents with 10 ml of purified isopentane/diethyl ether (4:1 v/v). The extracts were concentrated by N₂ flow to 100 µl and stored at -20 °C until chemical analyses.

VOCs in essential oils of Neroli, Lirio, and Lavender were collected by static headspace sampling with a solid phase microextraction (SPME) syringe and a 100 mm polydimethylsiloxane fiber (Supelco). An aliquot of each essential oil (100 µl) was deposited in a glass test tube (130 mm×19 mm OD) and the headspace volatiles were adsorbed with a SPME fiber for 2 min. Moreover, the inner air of blank test tube was also collected as a blank sample in the same manner. After collecting, these SPME samples were then employed for chemical analyses.

Chemical analyses

Gas chromatography-mass spectrometry (GC-MS) analyses were run on a Shimadzu QP5000 mass spectrometer coupled with a Shimadzu GC-17A gas chromatograph. I analyzed all samples by splitless injection (1 min, a helium flow of 2.2 ml/min) using a Varian CP-Wax 58CB capillary column (25 m×0.25 µm ID, 0.25 µm film thickness) at an injector temperature of 230 °C and an EI ionization voltage of 70 eV. GC-MS analyses for ORBO 32 samples were conducted using oven temperature program of 40 °C (2 min isothermal) and raising rate of 5 °C/min to 230 °C, while those for SPME samples were performed using oven temperature program of 40 °C (1 min isothermal) and raising rate of 5 °C/min to 200 °C. Plant VOCs were determined by subtracting the compounds (possible contaminants) detected in blank samples and identified by comparing retention times and mass spectra of authentic samples and those from literatures (**Table 1-3, Figure 4**).

Synthetic mimics

I prepared two types of synthetic mimics: scent of Neroli and odorant of *C. unshiu* as mixture of chemical compound. Authentic samples were commercially purchased from Tokyo Chemical Industry (Tokyo, Japan) and Sigma-Aldrich (St. Louis, USA) and were mixed in volume ratio (**Table 4**).

Statistical analysis

Multinomial tests were used to confirm whether the distribution of visits among the four colours was random. In the cases where it was non-random ($p < 0.05$), binomial tests adjusted by Bonferroni's correction (because binomial tests were performed 6

times) were used for all colour pairs to confirm whether the visitation between these two colours were different.

Fisher's exact tests (2×4) were used for comparing the distribution of individuals visiting among four colours between under a particular odorant and the control. In the case where the distribution was different ($p < 0.05$), for each colour X we aggregated data (colour X vs. the other colours) and performed Fisher's exact test to confirm that the visitation pattern to which colour was different.

Fisher's exact tests (2×4) were used to compare the distributions of visit among four colours between the sexes. In the case where the distribution was different ($p < 0.05$), for each colour X we aggregated data (colour X vs. the other colours) and performed Fisher's exact test to confirm that the visitation pattern to which colour was different.

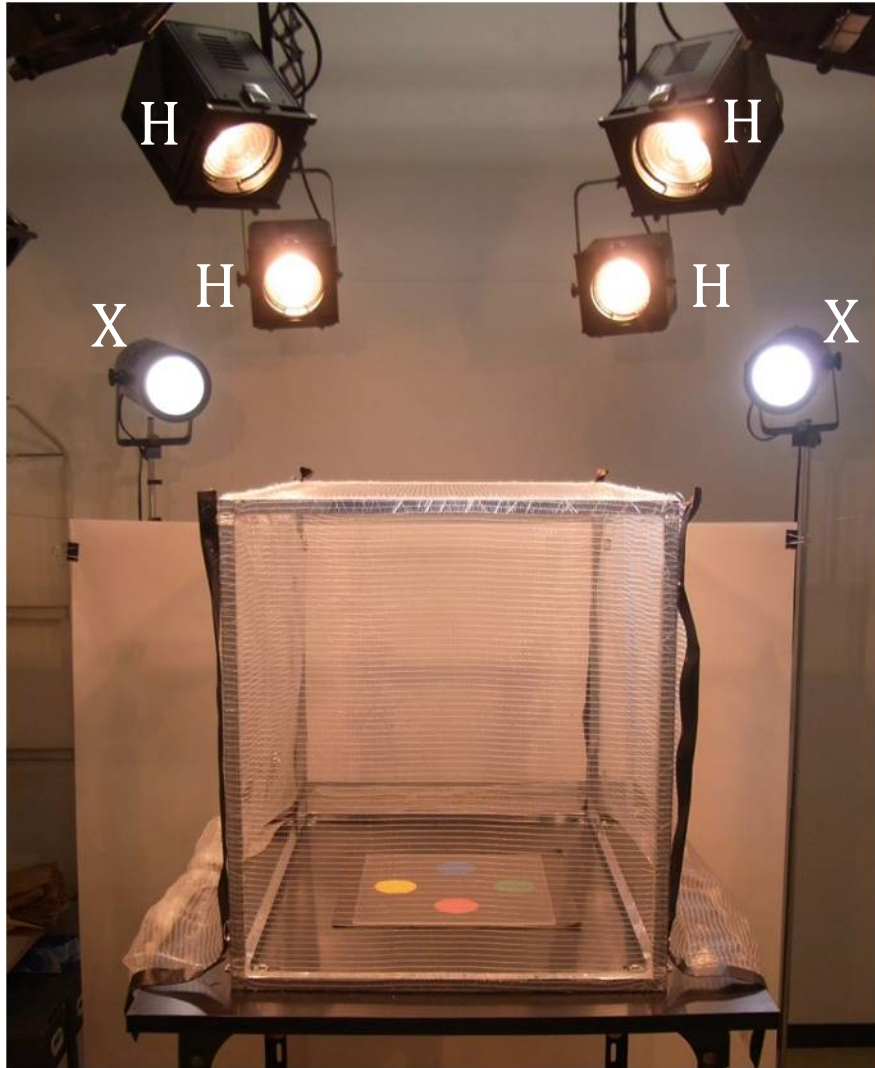


Figure 1. Experimental setup used for all behavioural tests in the room. Halogen lamps (H) and xenon lamps (X) and illuminates the cage.

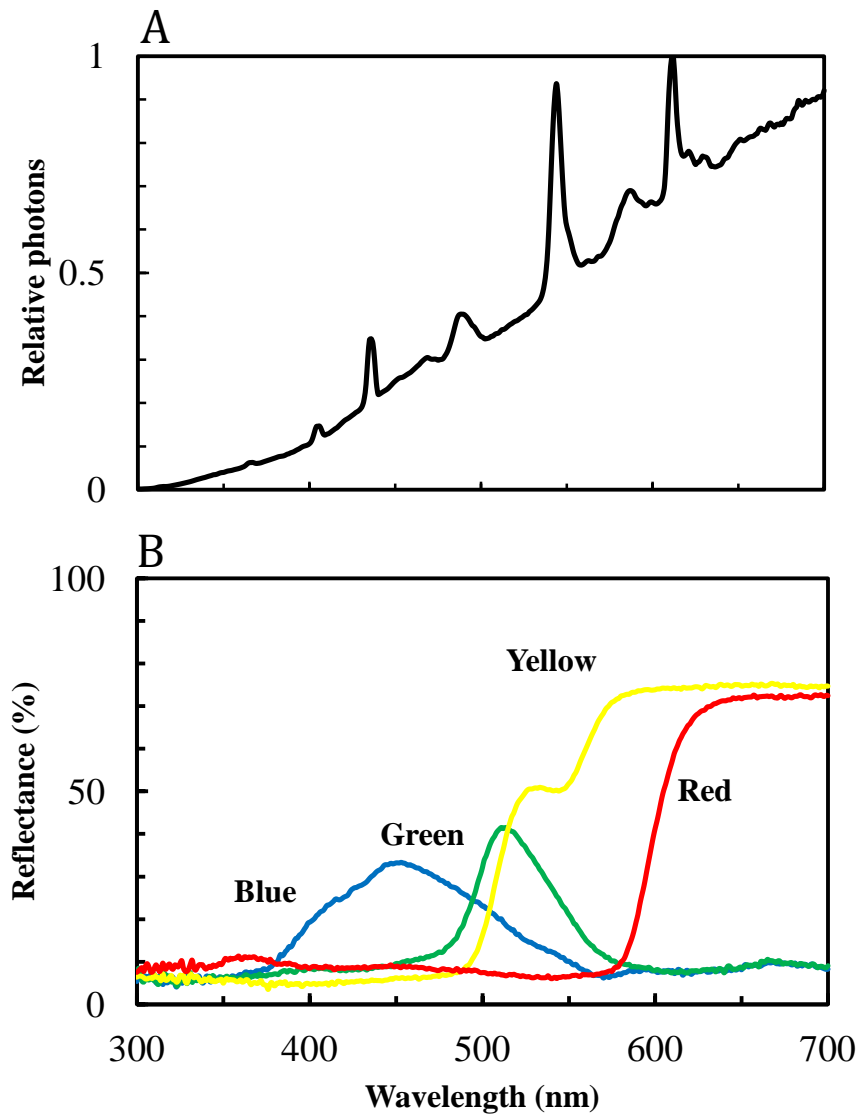


Figure 2. A: Irradiation spectrum of the illumination, B: Reflectance spectra of the four coloured discs

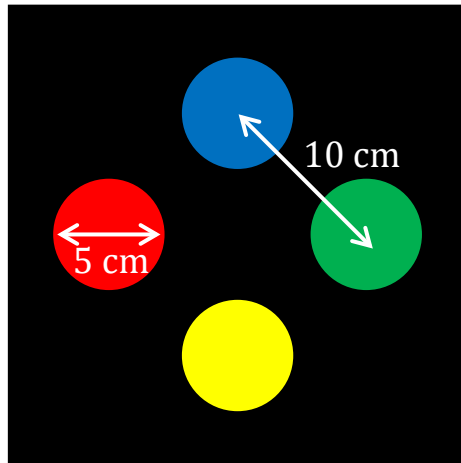


Figure 3. The four-colour pattern used for preference tests

Table 1. Relative abundance of volatile organic compounds from fresh leaves of *Citrus unshiu*

No.	RT (min)	Compound	Relative abundance ^a		
			Sample 1	Sample 2	Average
1	4.3	3-Heptanone	4.3	1.6	2.9
2	4.6	α-Terpinene	0.5	2.8	1.6
3	5.0	Limonene	7.5	7.7	7.6
4	5.5	Dodecane	6.4	N.D. ^b	3.2
5	6.0	γ-Terpinene	100	100	100
6	6.2	β-Ocimene	2.5	2.9	2.7
7	6.5	<i>p</i>-Cymene	26.4	28.0	27.2
8	6.8	α-Terpinolene	2.2	2.4	2.3
9	7.8	(Z)-3-Hexenyl acetate	N.D.	7.6	3.8
10	8.3	6-Methyl-5-hepten-2-one	3.6	2.4	3.0
11	9.7	Nonanal	1.2	N.D.	0.6
12	10.3	Tetradecane	1.9	N.D.	0.9
13	13.6	Linalool	8.2	1.2	4.7
14	14.3	Isocaryophyllene	N.D.	4.0	2.0
15	14.5	β-Caryophyllene	N.D.	3.5	1.8
16	16.2	α-Caryophyllene	N.D.	1.1	0.5
17	18.4	Germacrene D	N.D.	0.7	0.4

^a Calculated based on the total ion intensity of γ -terpinene as 100.

^b Not detected (below 0.2 in relative abundance, if any).

Table 2. Relative abundance of volatile organic compounds from fresh flower and leaves of *Hibiscus rosa-sinensis*

No.	RT (min)	Compound	Relative abundance ^a		
			Sample 1	Sample 2	Average
1	4.6	3-Heptanone	N.D. ^b	18.7	9.3
2	5.7	Dodecane	6.4	3.0	4.7
3	8.1	Tridecane	29.8	9.8	19.8
4	8.7	6-Methyl-5-hepten-2-one	17.9	5.9	11.9
5	10.1	Nonanal	4.6	5.0	4.8
6	10.7	Tetradecane	40.8	29.7	35.2
7	13.0	Pentadecane	12.8	13.5	13.1
8	15.4	Hexadecane	100	100	100
9	16.1	Acetophenone	37.5	51.8	44.7
10	17.7	Heptadecane	13.8	18.9	16.4

^a Calculated based on the total ion intensity of hexadecane as 100.

^b Not detected (below 2.0 in relative abundance, if any).

Table 3. Chemical compositions of volatile organic compounds in essential oils of Neroli, Lirio and Lavender

No.	RT (min)	Compound	Content (%)			Identification ^a
			Neroli	Lirio	Lavender	
1	1.9	α-Pinene	3.5	2.8	2.1	Std
2	2.1	α-Thujene	1.3	N.D. ^b	N.D.	Ref
3	2.3	Camphene	0.3	0.5	0.1	Std
4	2.9	β -Pinene	21.1	16.4	9.3	Std
5	3.1	Sabinene	2.6	N.D.	2.0	Ref
6	3.5	δ-3-Carene	0.2	0.2	0.4	Ref
7	3.8	β -Myrcene	2.0	1.8	1.8	Std
8	4.5	Limonene	26.1	40.6	21.9	Std
9	4.5	1,8-Cineol	2.4	1.0	5.2	Std
10	5.2	β -Ocimene	0.1	0.1	0.9	Std
11	5.6	3-Octanone	0.3	1.0	1.0	Std
12	5.8	p-Cymene	10.3	15.7	13.3	Std
13	6.1	Hexyl acetate	0.6	1.3	2.3	Std
14	7.0	(Z)-3-Hexenyl acetate	0.1	0.1	0.1	Std
15	7.5	6-Methyl-5-hepten-2-one	0.1	0.2	0.6	Std
16	7.9	1-Hexanol	0.05	N.D.	0.2	Std
17	8.5	1-Octen-3-yl acetate	0.4	1.3	5.4	Std
18	8.9	(unknown)	N.D.	N.D.	0.2	
19	9.5	(unknown)	0.03	0.2	0.6	
20	9.8	cis-Linalool oxide furanoside	2.2	1.4	1.7	Std
21	10.2	1-Octen-3-ol	N.D.	N.D.	0.9	Std
22	10.5	trans-Linalool oxide furanoside	1.6	1.2	1.4	Std
23	11.3	Camphor	N.D.	0.1	0.6	Std
24	12.7	Linalool	20.6	7.1	16.3	Std
25	12.8	Linalyl acetate	3.5	4.5	3.0	Std
26	13.0	(unknown)	N.D.	N.D.	0.6	
27	13.1	Bornyl acetate	N.D.	N.D.	0.6	Std
28	13.3	β -Caryophyllene	N.D.	N.D.	1.9	Std
29	13.7	Terpinen-4-ol	0.1	0.1	2.6	Std
30	13.9	(unknown)	N.D.	N.D.	1.6	
31	14.5	(unknown)	N.D.	1.7	N.D.	
32	15.6	(unknown)	N.D.	N.D.	0.3	
33	15.8	Borneol	N.D.	N.D.	1.1	Std
34	16.1	α-Terpineol	0.5	0.1	0.3	Std
35	17.0	Carvone	N.D.	0.1	N.D.	Std

^a Std and Ref denote identification based on authentic samples and published data (Adams 2009), respectively.

^b Not detected (below 0.03%, if any).

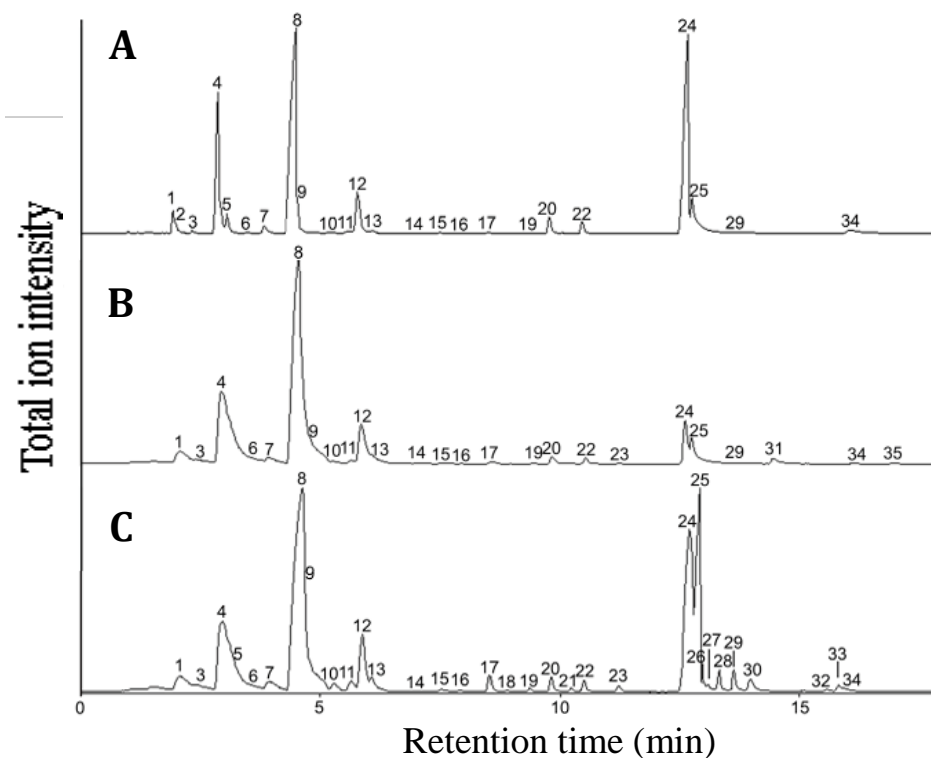


Figure 4. Total ion chromatograms of headspace samples from essential oil. **A:** Neroli, **B:** Lirio, **C:** Lavender. Peak numbers correspond to number in Table 3.

Table 4. Chemical compositions of synthetic mimics of Neroli oil and plant volatiles of *Citrus unshiu*

Compound	Relative abundance ^a	
	<i>C. unshiu</i> mimic	Neroli mimic
α -Pinene	—	14
Camphene	—	1
β -Pinene	—	81
β -Myrcene	—	8
3-Heptanone	3	—
α -Terpinene	2	—
Limonene	8	100
α -Terpinene	100	—
<i>p</i> -Cymene	30	40
γ -Terpinolene	1	—
(<i>Z</i>)-3-Hexenyl acetate	4	—
6-Methyl-5-hepten-2-one	3	—
Nonanal	1	—
<i>cis/trans</i> -Linalool oxide furanoside	—	15
Linalool	5	79
Linalyl acetate	—	13
α -Terpineol	—	2
β -Caryophyllene	4	—

^a Calculated based on the abundance of γ -terpinene for *C. unshiu* mimic and that of limonene for Neroli mimic (see Tables 4-1 and 4-3).

2.3. Results

Behavioural experiments in Japanese yellow swallowtail butterflies

When a naive butterfly was released in a small cage with 4 coloured discs, it started to approach the disc in a few minutes and landed on with its unrolled proboscis (called “visit”, **Figure 5**). More than 60% tested individuals visited on a disc within 5 minutes.

Innate colour preference without odorant (Control test, Figure 6-A)

In colour preference tests without any source of particular plant scent, more than half individuals in both sexes visited the blue disc (**Table 5**), which was significantly higher than those on other 3 colours (**Table 6**, Female; blue-green: $p < 0.001$, blue-yellow: $p = 0.029$, blue-red: $p < 0.001$, Male; blue-green: $p < 0.001$, blue-yellow: $p = 0.004$, blue-red: $p < 0.001$). There were no significant differences between sexes (**Table 8**).

Colour preference with floral scent (Figure 6-B)

In tests with floral scent, I performed colour preference tests using 4 types of floral scents; Neroli, Lirio, Lavender and *Hibiscus rosa-sinensis*. As results, the scent of Neroli was significantly effective in colour preference comparing to the control only in females (**Table 7**, $p < 0.001$). In the Neroli tests, individuals visited blue, yellow and red, that is, there were no significant differences between these 3 colours in females (**Table 6**). Ratio of individuals, which visited the blue disc was significantly decreased and that of the red disc was significantly increased comparing to the control (**Table 7**, blue: $p =$

0.001, red: $p < 0.001$). On the other hand, about half of males visited the blue disc as in control tests (**Table 5**), indicating that Neroli scent seemed to be much less effective for males and there was no significant difference comparing with the control (**Table 7**). About the sexual dimorphism, there was significant difference between sexes (**Table 8**, $p = 0.021$) and these were caused because less females visited blue (**Table 8**, $p = 0.025$) whereas more females visited red (**Table 8**, $p = 0.018$) than males.

Similarly with Neroli, scent of Lirio was significantly effective in colour preference in females (**Table 7**, $p = 0.009$) comparing to the control. Female visited more to the red disc comparing to the control (**Table 7**, $p = 0.001$). On the other hand, in males, more than half of tested males visited the blue discs as in the control (**Table 5**), meaning that Lirio scent seemed to be much less effective for males and there were no significant difference comparing with the control (**Table 7**, $p = 0.912$). About sexual dimorphism, there were significant difference between sexes (**Table 8**, $p = 0.014$) and these were caused because less females visited blue (**Table 8**, $p = 0.047$) whereas more females visited red (**Table 8**, $p = 0.016$) than males.

In contrast to Neroli and Lirio, we could not find any odorant effect of Lavender and flowering *H. rosa-sinensis*, meaning that more than half individuals visited the blue disc (**Table 5**) and no significant difference comparing with the control in both sexes (**Table 7**). There was also no significantly sexual dimorphism comparing with the control, both Lavender and *H. rosa-sinensis* (**Table 8**, $p = 0.551$).

Colour preference with host plant odorant (Figure 6-C)

In tests with host plants, *Citrus unshiu*, odorant in colour preference tests, selection for green became not significantly different from that for blue in female (**Table 6**, $p =$

0.633). Because odorant of *C. unshiu* significantly increased ratio of females visiting the green disc, and decreased that visiting the blue disc comparing to the control (**Table 7**, blue: $p = 0.038$, green: $p = 0.001$). Interestingly, this result was almost same as colour preference in the odorant of *C. unshiu* combined with scents of Neroli and female visited more to the green disc comparing to the control (**Table 7**, $p = 0.006$). It appears that odorant of *C. unshiu* somehow masked effects of Neroli.

Odorant of *C. unshiu* combined with scents of Neroli shows slightly different effects in colour preference in males. Ratio of males visiting to the blue disc further increased with odorant of *C. unshiu* and Neroli (**Table 7**, $p = 0.002$) and those visiting to the yellow disc decreased (**Table 7**, $p = 0.008$) comparing to the control. About sexual dimorphism, more females significantly visit green than males (**Table 8**, $p < 0.001$), indicating that there were also clear sexual dimorphisms in the colour preference with *C. unshiu* either with or without Neroli.

Colour preference in antennae covered butterflies (Figure 6-D)

To confirm whether plant scents were detected by antennae affected the colour preference, I carried out colour preference tests using individuals whose antennae were covered by mascara. We found that more than half of individuals in both sexes visited the blue disc even there were *C. unshiu* in the room (not antennae covered butterflies visited more green, **Table 5**). These results were not significantly different from results of the control (**Table 7**, Female: $p = 0.475$, Male: $p = 0.163$). This was also true in females under condition with the existence of plants and scent of Neroli (**Table 5, 7**).

Measurement of volatile organic compounds (VOCs)

Either 24, or 24 or 32 VOCs were identified in the headspace volatiles from the essential oils: Neroli, Lirio and Lavender (**Table 3, Figure 4**). In these flower scents, several monoterpenoids including β -pinene, limonene, *p*-cymene and linalool were the major VOCs. The chemical compositions of VOCs from Neroli and Lavender correspond to previous reports (Da Porto, Decorti et al. 2009, Gholivand, Piryaei et al. 2013). That of Lirio was similar to that of *Lilium sulphureum* except for the absence of several aromatic compounds such as isoeugenol and methyl benzoate (Kong, Sun et al. 2013). The scent of Lavender essential oil contained more abundant oxygenated aliphatic compounds than those from Neroli and Lirio.

In contrast to essential oils, the scent of potted flowering *Hibiscus rosa-sinensis* is less perceivable by human nose, which is supported by chemical analysis. The scent was mainly composed of linear alkanes instead of general flower volatiles. Among these VOCs identified, hexadecane was the major compound of *H. rosa-sinensis* plant (**Table 2**). By using the ion intensity of linalool (100 ng) as reference, the emission amounts of hexadecane from *H. rosa-sinensis* was estimated only 37 ng/h. This small emission of VOCs might relate to no clear effect in colour preference with *H. rosa-sinensis*.

Although trees of *Citrus unshiu* also smell little, 17 compounds were identified (**Table 1**). These compounds correspond to those reported previously (Choi 2003, Miyazawa, Fujita et al. 2010). The emission amount from *C. unshiu* was estimated 112 ng/h and γ -terpinene was the predominant component. Female could determine this compound or some of VOCs of green volatile from *C. unshiu* as larval host plants.

Colour preference with the synthetic mimics scent (Figure 6-E)

I made synthetic mimics of scent from Neroli and plant volatile of *C. unshiu* as mixtures of 10 and 11 authentic samples respectively (**Table 4**) and used them for colour preference tests. Synthetic mimic of Neroli was effective in females but not in males. Ratio of females visited between the blue disc and the yellow disc was not significantly different (there were significantly difference between blue and yellow in the control, **Table 6**), even though the colour preference does not significantly change (meaning that they continuously prefer blue, **Table 7**). These result indicated that these synthetic mimics did not reproduce the clear colour preference effects of natural scents, suggesting that minor VOCs including major ones might be essential to this behaviour as in other butterfly species.

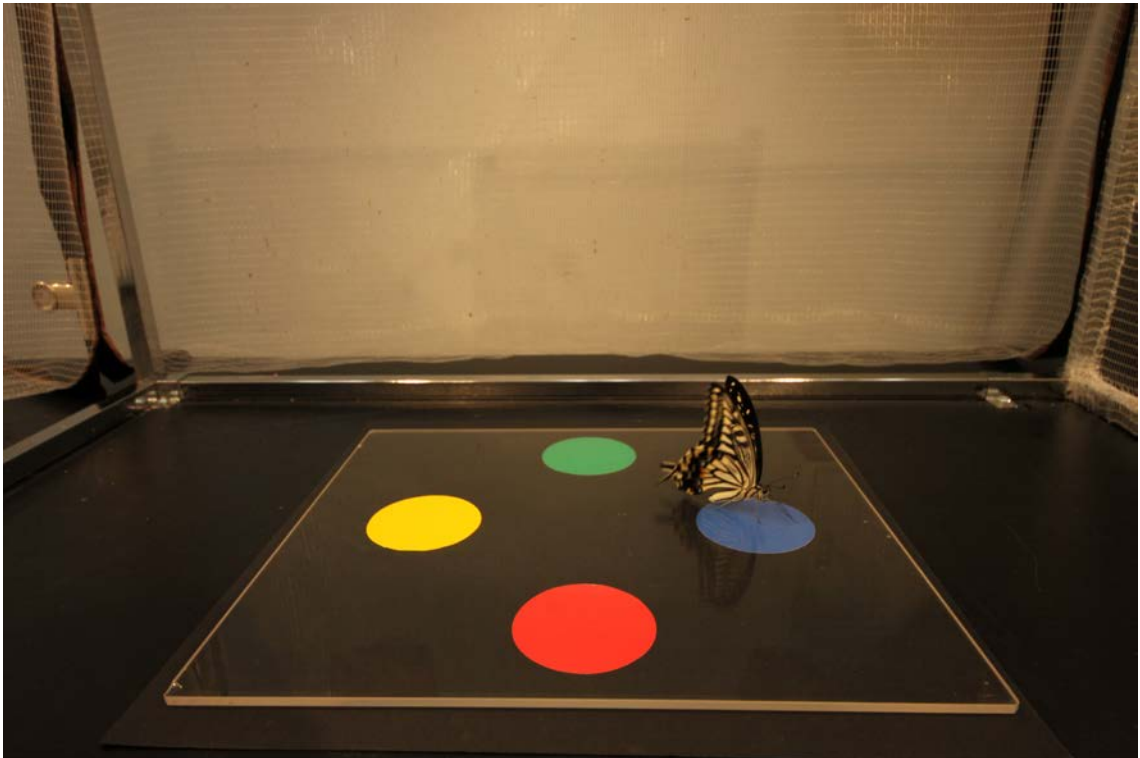


Figure 5. A naïve butterfly was landing on and extending its proboscis on the blue disc.

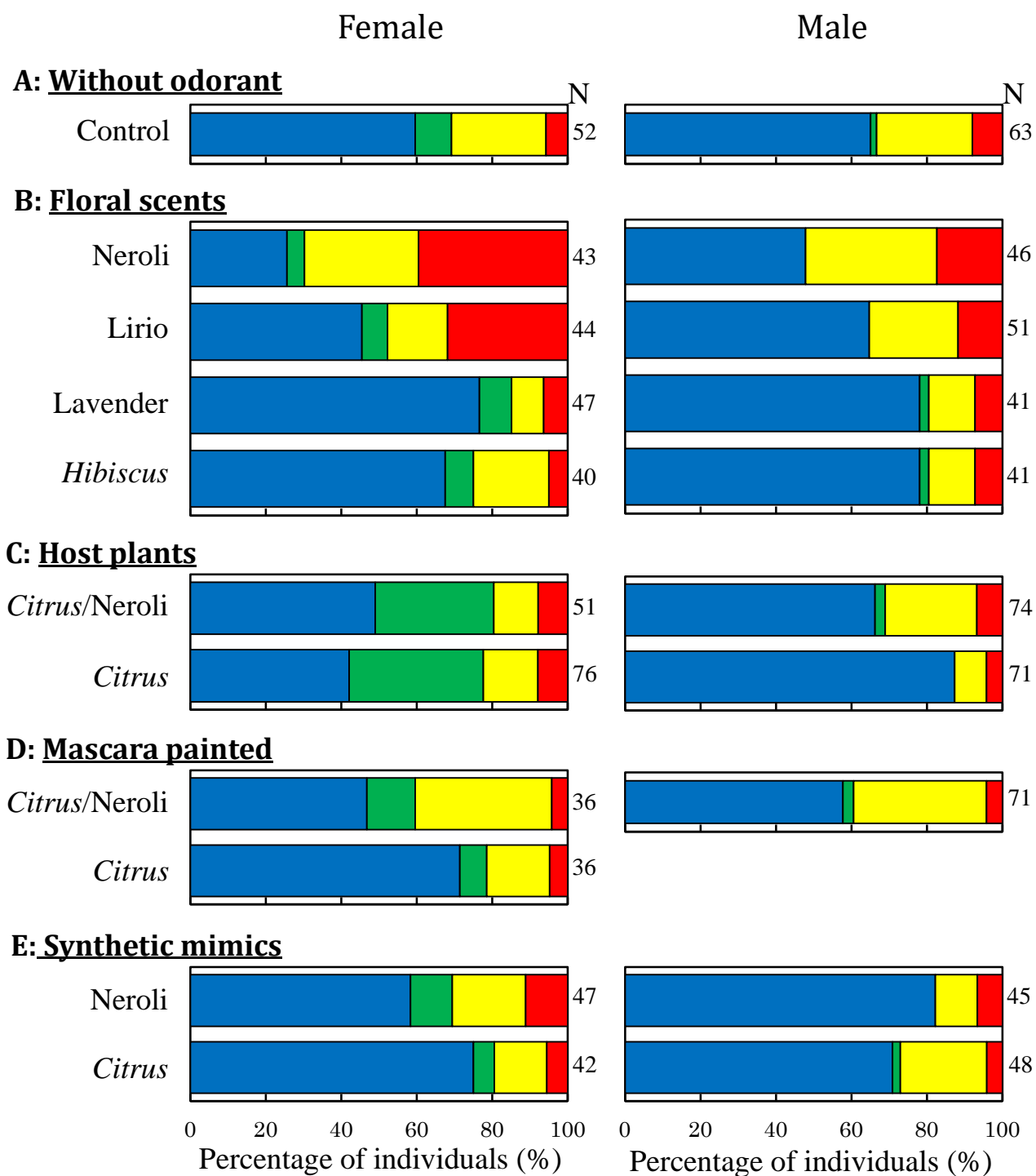


Figure 6. Innate colour preference of foraging *Papilio xuthus*. **A:** test without any odorant (control), **B:** test with floral scents, **C:** test with hot plant odorant, **D:** test in antennae painted butterflies, **E:** test with synthetic mimic odorant

Table 5. Number of individuals visiting each colour in different ambient odour conditions

Odorant	Blue	Green	Yellow	Red	Total
Female					
No odorant	31	5	13	3	52
Neroli	11	2	13	17	43
Lirio	20	3	7	14	44
Lavender	36	4	4	3	47
<i>Hibiscus rosa-sinensis</i>	27	3	8	2	40
<i>Citrus unshiu</i>	25	16	6	4	51
<i>C. unshiu</i> , Neroli	32	27	11	6	76
Artificial Neroli	22	6	17	2	47
Artificial <i>C. unshiu</i>	30	3	7	2	42
Mascara <i>C. unshiu</i> Neroli	21	4	7	4	36
Mascara <i>C. unshiu</i>	27	2	5	2	36
Male					
No odorant	41	1	16	5	63
Neroli	22	0	16	8	46
Lirio	33	0	12	6	51
Lavender	32	1	5	3	41
<i>H. rosa-sinensis</i>	32	1	5	3	41
<i>C. unshiu</i>	49	2	18	5	74
<i>C. unshiu</i> , Neroli	62	0	6	3	71
Artificial Neroli	37	0	5	3	45
Artificial <i>C. unshiu</i>	34	1	11	2	48
Mascara <i>C. unshiu</i> Neroli	41	2	25	3	71

Table 6. Result of statistical analysis for distribution of individuals visiting four colours in each ambient odorant condition

Odorant	Four colours ^a	Blue ^b Green	Blue ^b Yellow	Blue ^b Red	Green ^b Yellow	Green ^b Red	Yellow ^b Red
Female							
No odorant	0.001	< 0.001	0.029	< 0.001	0.289	1	0.064
Neroli	0.046	0.067	1	1	0.022	0.002	1
Lirio	0.039	0.001	0.057	1	1	0.038	0.568
Lavender	< 0.001	< 0.001	< 0.001	< 0.001	1	1	1
<i>Hibiscus rosa-sinensis</i>	0.001	< 0.001	0.006	< 0.001	0.68	1	0.328
<i>Citrus unshiu</i>	0.010	0.633	0.003	< 0.001	0.157	0.035	1
<i>C. unshiu</i> , Neroli	0.003	1	0.006	< 0.001	0.042	0.001	0.997
Artificial Neroli	0.004	0.011	1	< 0.001	0.104	0.867	0.002
Artificial <i>C. unshiu</i> ,	< 0.001	< 0.001	0.001	< 0.001	1	1	0.539
Mascara <i>C. unshiu</i> , Neroli	0.032	0.003	0.038	0.003	1	1	1
Mascara <i>C. unshiu</i> ,	< 0.001	< 0.001	< 0.001	< 0.001	1	1	1
Male							
No odorant	< 0.001	< 0.001	0.004	< 0.001	0.001	0.656	0.08
Neroli	< 0.001	< 0.001	1	0.048	< 0.001	0.023	0.455
Lirio	< 0.001	< 0.001	0.007	< 0.001	0.001	0.094	0.714
Lavender	< 0.001	< 0.001	< 0.001	< 0.001	0.656	1	1
<i>H. rosa-sinensis</i>	< 0.001	< 0.001	< 0.001	< 0.001	0.656	1	1
<i>C. unshiu</i> ,	< 0.001	< 0.001	0.001	< 0.001	0.001	1	0.032
<i>C. unshiu</i> , Neroli	< 0.001	< 0.001	< 0.001	< 0.001	0.094	0.750	1
Artificial Neroli	< 0.001	< 0.001	< 0.001	< 0.001	0.188	0.750	1
Mascara <i>C. unshiu</i> , Neroli	< 0.001	< 0.001	0.192	< 0.001	< 0.001	1	< 0.001

^a Multinomial test; null hypothesis = visitations are random

^b Binomial test (Bonferroni's-correction); null hypothesis = visitation between two colours is different

Table 7. Result in the test of independence in individuals visiting each colour between two conditions; without particular odorant and with ambient odour

Odorant	Four colours ^a	Blue ^b	Green ^b	Yellow ^b	Red ^b
Female					
Neroli	< 0.001	0.001	0.304	0.367	< 0.001
Lirio	0.009	0.119	0.455	0.201	0.001
Lavender	0.163	-	-	-	-
<i>Hibiscus rosa-sinensis</i>	0.91	-	-	-	-
<i>Citrus unshiu</i>	0.004	0.038	0.001	0.103	0.464
<i>C. unshiu</i> , Neroli	0.025	0.189	0.006	0.069	0.489
Artificial Neroli	0.578	-	-	-	-
Artificial <i>C. unshiu</i> ,	0.475	-	-	-	-
Mascara <i>C. unshiu</i> ,	0.475	-	-	-	-
Mascara <i>C. unshiu</i> ,, Neroli	0.797	-	-	-	-
Male					
Neroli	0.153	-	-	-	-
Lirio	0.912	-	-	-	-
Lavender	0.379	-	-	-	-
<i>H. rosa-sinensis</i>	0.379	-	-	-	-
<i>C. unshiu</i> ,	1	-	-	-	-
<i>C. unshiu</i> ,, Neroli	0.011	0.002	0.470	0.008	0.295
Artificial Neroli	0.163	-	-	-	-
Artificial <i>C. unshiu</i> ,	0.845	-	-	-	-
Mascara <i>C. unshiu</i> ,, Neroli	0.536	-	-	-	-

^a Fisher's exact test; null hypothesis = the whole visitation pattern is different

^b Fisher's exact test; null hypothesis = visitation pattern for each colour is different

Table 8. Result in the test of independence in individuals visiting each colour under different odour conditions between sexes

Odorant	Four colours ^a	Blue ^b	Green ^b	Yellow ^b	Red ^b	Female	Male
None	0.313	-	-	-	-	52	63
Neroli	0.021	0.025	0.231	0.409	0.018	43	46
Lirio	0.014	0.047	0.096	0.253	0.016	44	51
Lavender	0.668	-	-	-	-	47	41
<i>Hibiscus</i>	0.551	-	-	-	-	40	41
<i>Citrus</i> trees	< 0.001	0.041	< 0.001	0.062	0.54	51	74
<i>Citrus</i> trees, Neroli	< 0.001	< 0.001	< 0.001	0.189	0.282	76	71
Artificial Neroli	< 0.001	< 0.001	0.015	0.004	0.479	47	45
Artificial <i>Citrus</i> trees	0.645	-	-	-	-	42	48
Mascara <i>Citrus</i> trees, Neroli	0.078	-	-	-	-	36	71

^a Fisher's exact test; null hypothesis = the whole visitation pattern is different

^b Fisher's exact test; null hypothesis = visitation pattern for the colour is different

2.4. Discussion

Innate colour preference

Naïve *P. xuthus* innately preferred blue in this experiments. The previous study demonstrated that naïve females of *P. xuthus* preferred yellow and red, while males were more attracted to blue (Kinoshita, Shimada et al. 1999), which contradict the new result. Comparing experimental conditions between these studies, I assumed that this contradiction would be due to scent of lemon trees with flowers in the room for rearing larvae, and other experiments on egg-laying behaviour. Another possibility is different light condition of UV light by xenon lamps and intensity, because the room was brighter than old experiment particularly in UV wavelength region.

Why do they prefer blue? The action spectrum of foraging behaviour of trained *P. xuthus* shows high sensitivity at blue wavelength region (Koshitaka, Kinoshita et al. 2004). In addition, spectral sensitivity determined by electroretinogram from the middle part of the compound eye also peaks at 480 nm (Arikawa, Inokuma et al. 1987). These data indicate that blue wavelength region is relatively brighter for butterflies, although blue colour disc is not the brightest one. Calculation data using colour disc brightness, irradiance spectrum of illumination and spectral sensitivity of *P. xuthus* determined by electroretinogram recording indicated that the yellow disc was brightest colour through *P. xuthus* eyes (Kinoshita, Shimada et al. 1999). These indicated that *P. xuthus* innately prefer blue not because that was brightest, but because they preferred blue as the colour. I argue that innate preference was determined in not receptor levels but in higher levels. Innate colour preference to blue was common in bee, fly, hawkmoth, and also butterfly

species (Lunau and Maier 1995, Kelber 1997), although the mechanism for this innate colour preference is still unclear.

Innate colour preference is affected by particular scent

Females prefer red when scents of Neroli or Lirio exists, indicating that olfactory information could modify the decision based on colour preference and influenced to their foraging behaviour. *P. xuthus* were hard to be trained to a specific odorant (data not shown), but olfactory information is important for them.

Innate colour preference modified by flower scent presumably contributes flower constancy in *P. xuthus*. Butterfly species showed flower constancy as in bee species, *Pieris napi* (Goulson and Cory 1993) and *Thymelicus flavus* (Goulson, Ollerton et al. 1997) had good ability to learn visual cues (Weiss 1991, Hill, Wells et al. 1997). Innate preference must influence more to flower constancy in solitary insects than social bees, because they learn and use the flower trait, which they first visit based on their innate preference, for searching flowers in future foraging. Therefore the nature of foraging flower in *P. xuthus*, that visit reddish flower such as daylily *Hemerocallis fulva* in Japan (Hirota, Nitta et al. 2012), can be explained in the following way. In the field, the naïve female *P. xuthus* could get nectar on reddish flowers according to the innate colour preference modified by floral scents, and learn its colour and repeatedly visit flowers of similar colours after the first experience. This hypothesis partially explains the fact that *P. xuthus* are potential pollinators for reddish flowers (Hirota, Nitta et al. 2012).

In nature, not only female but also male *P. xuthus* appear to visit reddish flowers. What factors do contribute to the fact that male *P. xuthus* visit reddish flowers? The one of possibilities might be related to the fact that green of leaves would modify colour

appearance of flowers by colour contrast phenomenon. In *P. xuthus* colour vision, complimentary colours of green were violet which have high reflectance spectrum at blue and red wavelength region, instead of that between green and red in human vision (Kinoshita, Takahashi et al. 2008). It is meaning that violet trained butterflies might visit reddish violet when coloured discs are on green background. Considering that, colour preference to blue in male *P. xuthus* probably shift to reddish colour by greenish background.

P. xuthus and other butterfly species can discriminate red as colour based on red receptor in the retina (Kelber and Pfaff 1999, Kinoshita, Shimada et al. 1999, Zaccardi, Kelber et al. 2006). The ability to discriminate red and preference for red may provide them possibility to find reddish flowers which cannot be preferred much by other insect species such as bees and flies that lack the red receptors in the retina. But Speckled Wood butterflies, *Pararge aegeria*, also innately prefer red even though they are lack of red receptors (Scherer and Kolb 1987), and the red receptor is useful not for detecting red colour but also discriminating yellow and green. These facts mean that probably there may be no relation between having red receptor and preferring red.

In tests with host plants, females visited green more frequently, and interestingly, similar effects were observed even when Neroli was presented together. In general, colour preference to green was observed in ovipositing behaviour by butterfly species, *Pieris brassicae* (Scherer and Kolb 1987) and *Papilio aegaeus* (Kelber 1999). But colour preference for green by host plants in this experiment was not the case, because no individual showed oviposition behaviour on the green disc. Increased preference for the green disc in females is assumed that females are sensitive to larval host plants and possibly approach green even as targets for foraging. So that female *P. xuthus*, even

though it is virgin, may keep close to the host plants by being attracted to green colour, when they detect odorant of the host plants, which is probably important in any situation for them to stay close to host plants. If they cannot get nectar on green leaves in the first trial of foraging, it is not a big matter for them because they can quickly switch to visit other colours of flowers and can learn it as colour associating with nectar.

More than half of individuals with their antennae covered in both sexes preferred blue even when there were host plants, indicating that ambient odorants are indeed received by antennae and affect the innate colour preference. And more, particular specific odorant from the plants were effective in the innate colour preference and difference of convergent pattern in volatile organic compounds (VOCs) among odorants was important for the preference, because the olfactory effects were different depending on the identity of the odorant. To clarify the odorant, we made two synthetic mimics (Neroli and *Citrus unshiu*) mixing major volatile organic compounds (VOCs). But both synthetic mimics did not work the same as the original scents, even though the proportion of each main compound referred to that in original scent. Electro-antennogram (EAG) measured in Papilionidae often shows response to minor VOCs of host plants rather than major ones (Baur, Feeny et al. 1993, Fadamiro, Chen et al. 2010, Li, Wakui et al. 2010). Therefore minor VOCs may be essential to induce the effect of odorant in colour preference in addition to major components in foraging behaviour of *P. xuthus*. This is not the case in *Manduca sexta*, showing foraging to the mimic mixture of nine compounds among 60 components can serve as the real flower scent (Riffell, Lei et al. 2009). Furthermore, mated female *Cydia molesta* requires only three general green leaf volatiles and the two specific aromatic compounds to recognize host plants (Piñero and Dorn 2007). But we have not measured it in *P. xuthus* yet.

Sexual dimorphism in innate colour preference

With particular plant scents, a clear sexual dimorphism in innate colour preference emerged. This behavioural sexual dimorphism might be attributed to the olfactory system or the integration of multi-sensory modalities. This is because effects of all odours were rather limited in males, scents affecting colour preference were detected by antennae and no sexual dimorphism in the visual abilities or retinal organization of *P. xuthus* has been found (Kinoshita and Arikawa 2014). Actually three enlarged sexually dimorphic glomeruli among about 60 glomeruli were found in antennal lobes of female *P. xuthus* (Kinoshita, Yoshida et al. 2012), although sexual dimorphism of the antennal lobe seems to be rather rare in other butterfly species (Rosparis 1983, Heinze and Reppert 2012). Those glomeruli are much smaller in male *P. xuthus*. Similarly, two large female glomeruli were found in the antennal lobes of the *Manduca sexta* (Rosparis and Hildebrand 2000). Considering those glomeruli in *M. sexta* were excited by linalool, which is one major component of volatiles from their host plants (King, Christensen et al. 2000), the sexually dimorphic glomeruli in *P. xuthus* possibly contribute to detect the odorant from the *C. unshiu*.

Effects of odorant in innate colour preference were more prominent in female. This might relate to difference in behaviours between sexes. Important behaviours in females may require detecting olfactory information from plants rather than those in males. Females have to be more sensitive to find nectar to let eggs mature enough in their ovary and search places for laying eggs. So that for females to be close host plants must be essential between times while they are foraging. On the other hand, sensory system of males must adapt for finding and mating with females.

Chapter 3

Conclusion and General Discussion

Conclusion

My findings can be summarized as follows.

1. Japanese yellow swallowtail butterflies, *Papilio xuthus* innately prefer blue.
2. Plant scents can modify the innate colour preference.
3. The olfactory effects are different depending on the identity of the odorant.
4. Effect of plant scents in innate colour preference is different between sexes.

Future perspectives

Multi-sensory integration is a hot topic at the moment, but good model animals are still limited. *P. xuthus* can be a potential model species for the study of multi-sensory integration. The fact that particular odorants modify the visually guided foraging behaviour in *P. xuthus* suggests integration of visual and olfactory cue through processing them in the brain. Olfactory information is sent to the mushroom body known as the center for olfactory learning and memory via antennal lobes in most insects (Farris 2013, Kinoshita, Shimohigashi et al. 2014). The mushroom body in *P. xuthus* receives both visual and olfactory inputs. Mushroom bodies of bees and ants possessing similar sensory inputs are assumed to be involved in the integration of multi sensory modalities in addition to olfactory learning and memory for their foraging (Gronenberg 1999, Ehmer and Gronenberg 2002, Farris 2013). Considering that *P. xuthus* can be efficiently trained with visual cue, its mushroom body may contribute to learning and memory of visual cue and integration of both visual and olfactory cue.

Here I found that innate preference was affected by odour, which has not been previously reported in flower visitors. This discovery offers a new viewpoint to understand the foraging strategies of solitary and short-living animals such as butterflies and moths. For solitary insects such as butterflies innate preference might strongly influence flower constancy, because their initial learning experiences would be determined by their innate preference. Similar experiments focusing on innate preference in other solitary insects would reveal how genetically programmed preference in different sensory modalities effect their strategy of foraging behaviour.

There is an issue how integration of visual and olfactory information in innate colour preference can work in natural habitat because I carried out all experiments under the fine controlled condition of the indoor room. For example, I use only one odorant source for each test, but there are many different odorants in many places in the field, which should interact in complicated ways. In addition, I also care about effect of background colour because background colour effects colour appearance in colour vision of *P. xuthus* (Kinoshita, Takahashi et al. 2008). I here used black background for the test, but in the field, the background is noisier and often green leaves. Presumably innate colour preference in natural habitat is affected by colour of background.

Neural mechanism underlying sexual dimorphic in innate colour preference induced by plant scents is completely open question. Why does innate colour preference change in only females? Large glomeruli in antennal lobes of female might correspond to large number of neurons in these glomeruli comparing others. This means that female antennae of *P. xuthus* could have more sensilla (e.g. linalool sensilla) than males. Observing ultra-structures of antennae could answer how antennae differ between sexes, while recording the electro antennogram would reveal which chemicals modulate innate

preference or difference in absolute sensitivity for particular odorants between sexes.

In conclusion, I found *P. xuthus* innately integrate vision and olfaction in foraging by behavioural experiments. *P. xuthus* strongly depends on vision, but they would visit flower and obtain nectar more precisely and efficiently by using also olfaction.

References

Andersson, S. (2003). "Foraging responses in the butterflies *Inachis io*, *Aglais urticae* (Nymphalidae), and *Gonepteryx rhamni* (Pieridae) to floral scents." Chemoecology **13**(1): 1-11.

Andersson, S. (2006). "Floral scent and butterfly pollinators." Biology of floral scent. Taylor & Francis Group, Boca Raton: 199-217.

Andersson, S. and H. E. Dobson (2003). "Behavioral foraging responses by the butterfly *Heliconius melpomene* to *Lantana camara* floral scent." Journal of Chemical Ecology **29**(10): 2303-2318.

Arikawa, K. (2003). "Spectral organization of the eye of a butterfly, *Papilio*." Journal of Comparative Physiology A **189**(11): 791-800.

Arikawa, K., K. Inokuma and E. Eguchi (1987). "Pentachromatic visual system in a butterfly." Naturwissenschaften **74**(6): 297-298.

Balkenius, A. and A. Kelber (2006). "Colour preferences influences odour learning in the hawkmoth, *Macroglossum stellatarum*." Naturwissenschaften **93**(5): 255-258.

Balkenius, A., W. Rosén and A. Kelber (2006). "The relative importance of olfaction and vision in a diurnal and a nocturnal hawkmoth." Journal of Comparative Physiology A **192**(4): 431-437.

Baur, R., P. Feeny and E. Städler (1993). "Oviposition stimulants for the black swallowtail butterfly: identification of electrophysiologically active compounds in carrot volatiles." Journal of Chemical Ecology **19**(5): 919-937.

Blackiston, D., A. D. Briscoe and M. R. Weiss (2011). "Color vision and learning in the monarch butterfly, *Danaus plexippus* (Nymphalidae)." Journal of Experimental Biology **214**(3): 509-520.

Briscoe, A. D. and L. Chittka (2001). "The evolution of color vision in insects." Annual Review of Entomology **46**(1): 471-510.

Chapman, R. (2009). "Foraging and Food Choice in Phytophagous Insects." Chemical Ecology. Eolss Publishers, Oxford.

Chittka, L. and N. E. Raine (2006). "Recognition of flowers by pollinators." Current Opinion in Plant Biology **9**(4): 428-435.

Chittka, L., J. D. Thomson and N. M. Waser (1999). "Flower constancy, insect psychology, and plant evolution." Naturwissenschaften **86**(8): 361-377.

Choi, H.-S. (2003). "Characterization of *Citrus unshiu* (C. unshiu Marcov. forma Miyagawa-wase) blossom aroma by solid-phase microextraction in conjunction with an electronic nose." Journal of Agricultural and Food Chemistry **51**(2): 418-423.

Da Porto, C., D. Decorti and I. Kikic (2009). "Flavour compounds of *Lavandula angustifolia* L. to use in food manufacturing: Comparison of three different extraction methods." Food Chemistry **112**(4): 1072-1078.

Dobson, H. E. (2006). "Relationship between floral fragrance composition and type of pollinator." Biology of Floral Scent, Taylor and Francis Group, Boca Raton: 147-198.

Ehmer, B. and W. Gronenberg (2002). "Segregation of visual input to the mushroom bodies in the honeybee (*Apis mellifera*)." Journal of Comparative Neurology **451**(4): 362-373.

Fadamiro, H., L. Chen, C. Akotsen-Mensah and W. N. Setzer (2010). "Antennal electrophysiological responses of the giant swallowtail butterfly, *Papilio cresphontes*, to the essential oils of *Zanthoxylum clava-herculis* and related plants." Chemoecology **20**(1): 25-33.

Farris, S. M. (2013). "Evolution of complex higher brain centers and behaviors: behavioral correlates of mushroom body elaboration in insects." Brain, Behavior and Evolution **82**(1): 9-18.

Gerber, B. and B. H. Smith (1998). "Visual modulation of olfactory learning in honeybees." Journal of Experimental Biology **201**(14): 2213-2217.

Gholivand, M. B., M. Piryaei and M. M. Abolghasemi (2013). "Analysis of volatile oil composition of *Citrus aurantium* L. by microwave-assisted extraction coupled to headspace solid-phase microextraction with nanoporous based fibers." Journal of Separation Science **36**(5): 872-877.

Giurfa, M., J. Nunez, L. Chittka and R. Menzel (1995). "Colour preferences of flower-naive honeybees." Journal of Comparative Physiology A **177**(3): 247-259.

Goulson, D. (1999). "Foraging strategies of insects for gathering nectar and pollen, and implications for plant ecology and evolution." Perspectives in Plant Ecology, Evolution and Systematics **2**(2): 185-209.

Goulson, D. (2000). "Are insects flower constant because they use search images to find flowers?" Oikos **88**(3): 547-552.

Goulson, D. and J. S. Cory (1993). "Flower constancy and learning in foraging preferences of the green-veined white butterfly *Pieris napi*." Ecological Entomology **18**(4): 315-320.

Goulson, D., J. Ollerton and C. Sluman (1997). "Foraging strategies in the small skipper butterfly, *Thymelicus flavus*: when to switch?" Animal Behaviour **53**(5): 1009-1016.

Gronenberg, W. (1999). "Modality-specific segregation of input to ant mushroom bodies." Brain, Behavior and Evolution **54**(2): 85-95.

Gumbert, A. (2000). "Color choices by bumble bees (*Bombus terrestris*): innate preferences and generalization after learning." Behavioral Ecology and Sociobiology **48**(1): 36-43.

Hallberg, E., B. S. Hansson and C. Lofstedt (2003). "10. Sensilla and Proprioceptors." Vol 2: Morphology, Physiology, and Development **4**: 267.

Hansson, B. (1995). "Olfaction in lepidoptera." Experientia **51**(11): 1003-1027.

Heinze, S. and S. M. Reppert (2012). "Anatomical basis of sun compass navigation I: the general layout of the monarch butterfly brain." Journal of Comparative Neurology **520**(8): 1599-1628.

Hill, P. S., P. H. Wells and H. Wells (1997). "Spontaneous flower constancy and learning in honey bees as a function of colour." Animal Behaviour **54**(3): 615-627.

Hirota, S. K., K. Nitta, Y. Kim, A. Kato, N. Kawakubo, A. A. Yasumoto and T. Yahara (2012). "Relative role of flower color and scent on pollinator attraction: experimental tests using F1 and F2 hybrids of daylily and nightlily." PLoS one **7**(6): e39010.

Honda, K., H. Ômura and N. Hayashi (1998). "Identification of floral volatiles from *Ligustrum japonicum* that stimulate flower-visiting by cabbage butterfly, *Pieris rapae*." Journal of Chemical Ecology **24**(12): 2167-2180.

Ilse, D. and V. G. Vaidya (1956). "Spontaneous feeding response to colours in *Papilio demoleus* L." Proceedings of the Indian Academy of Sciences-Section B, Springer.

Kelber, A. (1997). "Innate preferences for flower features in the hawkmoth *Macroglossum stellatarum*." Journal of Experimental Biology **200**(4): 827-836.

Kelber, A. (1999). "Ovipositing butterflies use a red receptor to see green." Journal of Experimental Biology **202**(19): 2619-2630.

Kelber, A., A. Balkenius and E. J. Warrant (2002). "Scotopic colour vision in nocturnal hawkmoths." Nature **419**(6910): 922-925.

Kelber, A. and M. Pfaff (1999). "True colour vision in the orchard butterfly, *Papilio aegeus*." Naturwissenschaften **86**(5): 221-224.

Kevan, P. and H. Baker (1983). "Insects as flower visitors and pollinators." Annual Review of Entomology **28**(1): 407-453.

King, J. R., T. A. Christensen and J. G. Hildebrand (2000). "Response characteristics of an identified, sexually dimorphic olfactory glomerulus." Journal of Neuroscience **20**(6): 2391-2399.

Kinoshita, M. and K. Arikawa (2014). "Color and polarization vision in foraging *Papilio*." Journal of Comparative Physiology A **200**(6): 513-526.

Kinoshita, M., N. Shimada and K. Arikawa (1999). "Colour vision of the foraging swallowtail butterfly *Papilio xuthus*." Journal of Experimental Biology **202**(2): 95-102.

Kinoshita, M., M. Shimohigashi, Y. Tominaga, K. Arikawa and U. Homberg (2014). "Topographically distinct visual and olfactory inputs to the mushroom body in the Swallowtail butterfly, *Papilio xuthus*." Journal of Comparative Neurology **523**(1): 162-182.

Kinoshita, M., Y. Takahashi and K. Arikawa (2008). "Simultaneous color contrast in the foraging swallowtail butterfly, *Papilio xuthus*." Journal of Experimental Biology **211**(Pt 21): 3504-3511.

Kinoshita, M., M. Yoshida and K. Arikawa (2012). "Odor of *Citrus* trees causes sexual dimorphism in innate color preference of swallowtail butterflies." Frontier Behavioral Neuroscience. Conference Abstract: Tenth International Congress of Neuroethology.

Kong, Y., M. Sun, H. Pan, Q. Zhang, C. Ai and Y. Wang (2013). "Floral scent composition of *Lilium sulphureum*." Chemistry of Natural Compounds **49**(2): 362-364.

Koshitaka, H., M. Kinoshita and K. K. Arikawa (2004). "Action spectrum of foraging behavior of the Japanese yellow swallowtail butterfly, *Papilio xuthus*." Acta Biologica Hungarica **55**(1): 71-79.

Leonard, A. S. and P. Masek (2014). "Multisensory integration of colors and scents: insights from bees and flowers." Journal of Comparative Physiology A **200**(6): 463-474.

Li, J., R. Wakui, S.-i. Tebayashi and C.-S. Kim (2010). "Volatile attractants for the common bluebottle, *Graphium sarpedon nipponum*, from the host, *Cinnamomum camphora*." Bioscience, Biotechnology, and Biochemistry **74**(10): 1987-1990.

Linsley, E. and J. MacSwain (1958). "The significance of floral constancy among bees of the genus *Diadasia* (Hymenoptera, Anthophoridae)." Evolution **12**(2): 219-223.

Lunau, K. and E. Maier (1995). "Innate colour preferences of flower visitors." Journal of Comparative Physiology A **177**(1): 1-19.

Menzel, R. and W. Backhaus (1989). Color vision honey bees: phenomena and physiological mechanisms. Facets of vision, Springer: 281-297.

Miyazawa, N., A. Fujita and K. Kubota (2010). "Aroma character impact compounds in Kinokuni mandarin orange (*Citrus kinokuni*) compared with Satsuma mandarin orange (*Citrus unshiu*)." Bioscience, Biotechnology, and Biochemistry **74**(4): 835-842.

Ogawa, Y., M. Kinoshita, D. G. Stavenga and K. Arikawa (2013). "Sex-specific retinal pigmentation results in sexually dimorphic long-wavelength-sensitive photoreceptors in the eastern pale clouded yellow butterfly, *Colias erate*." Journal of Experimental Biology **216**(10): 1916-1923.

Ômura, H. and K. Honda (2005). "Priority of color over scent during flower visitation by adult *Vanessa indica* butterflies." Oecologia **142**(4): 588-596.

Piñero, J. C. and S. Dorn (2007). "Synergism between aromatic compounds and green leaf volatiles derived from the host plant underlies female attraction in the oriental fruit moth." Entomologia Experimentalis et Applicata **125**(2): 185-194.

Raguso, R. A. and M. A. Willis (2005). "Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, *Manduca sexta*." Animal Behaviour **69**(2): 407-418.

Riffell, J. A., R. Alarcón, L. Abrell, G. Davidowitz, J. L. Bronstein and J. G. Hildebrand (2008). "Behavioral consequences of innate preferences and olfactory learning in hawkmoth–flower interactions." Proceedings of the National Academy of Sciences **105**(9): 3404-3409.

Riffell, J. A., H. Lei, T. A. Christensen and J. G. Hildebrand (2009). "Characterization and coding of behaviorally significant odor mixtures." Current Biology **19**(4): 335-340.

Riffell, J. A., E. Shlizerman, E. Sanders, L. Abrell, B. Medina, A. J. Hinterwirth and J. N. Kutz (2014). "Flower discrimination by pollinators in a dynamic chemical environment." Science **344**(6191): 1515-1518.

Rospars, J. P. (1983). "Invariance and sex-specific variations of the glomerular organization in the antennal lobes of a moth, *Mamestra brassicae*, and a butterfly, *Pieris brassicae*." Journal of Comparative Neurology **220**(1): 80-96.

Rospars, J. P. and J. G. Hildebrand (2000). "Sexually dimorphic and isomorphic glomeruli in the antennal lobes of the sphinx moth *Manduca sexta*." Chemical Senses **25**(2): 119-129.

Scherer, C. and G. Kolb (1987). "The influence of color stimuli on visually controlled behavior in *Aglais urticae* L. and *Pararge aegeria* L.(Lepidoptera)." Journal of Comparative Physiology A **161**(6): 891-898.

Srinivasan, M., S. Zhang and H. Zhu (1998). "Honeybees link sights to smells." Nature **396**(6712): 637-638.

Tanaka, H. (1991). "昆虫は何色の花を訪れるか." インセクトリウム **28**(11): 12-16.

Waser, N. M. (1986). "Flower constancy: definition, cause, and measurement." American Naturalist **127**(5): 593-603.

Weiss, M. R. (1991). "Floral colour changes as cues for pollinators." Nature **354**(6350): 227-229.

Weiss, M. R. (1997). "Innate colour preferences and flexible colour learning in the pipevine swallowtail." Animal Behaviour **53**(5): 1043-1052.

Zaccardi, G., A. Kelber, M. P. Sison-Mangus and A. D. Briscoe (2006). "Color discrimination in the red range with only one long-wavelength sensitive opsin." Journal of Experimental Biology **209**(10): 1944-1955.