

## RESEARCH ARTICLE

# Complex multi-modal sensory integration and context specificity in colour preferences of a pierid butterfly

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## ABSTRACT

Innate colour preferences in insects were long considered to be a non-flexible representation of a floral ‘search image’ guiding them to flowers during initial foraging trips. However, these colour preferences have recently been shown to be modulated by multi-sensory integration of information. Using experiments on the butterfly *Catopsilia pomona* (common emigrant), we demonstrate that cross-modal integration of information not only affects colour preferences but also colour learning, and in a sex-specific manner. We show that spontaneous colour preference in this species is sexually dimorphic, with males preferring both blue and yellow while females prefer yellow. With minimal training (two training sessions), both males and females learned to associate blue with reward, but females did not learn green. This suggests that the aversion to green, in the context of foraging, is stronger in females than in males, probably because green is used as a cue to find oviposition sites in butterflies. However, females learned green after extensive training (five training sessions). Intriguingly, when a floral odour was present along with green during training, female colour preference during the subsequent choice tests resembled their innate preference (preference for yellow). Our results show that multi-sensory integration of information can influence preference, sensory bias, learning and memory in butterflies, thus modulating their behaviour in a context-specific manner.

**KEY WORDS:** Colour learning, Learning and memory, Sensory bias, Context dependency, Sexual dimorphism, Pieridae

## INTRODUCTION

Flower-visiting insects rely on a suite of signals advertised by flowers, such as colour, scent, shape, pattern and iridescence, as well as electric potential and heat emanating from the flower (Raguso, 2004; Schiestl and Johnson, 2013; Balamurali et al., 2015). Of these, colour and olfactory cues are considered the most important for facilitating insect–plant interactions. Insects learn to associate floral colour and scent with nectar and/or pollen, and depend on short- and/or long-term memory to find and persistently visit rewarding flowers. Flower-naïve insects, in contrast, are hypothesised to mainly rely on innate preferences for colour and scents during their initial foraging trips. These preferences are thought to be phylogenetic adaptations in the form of neural


representations of a floral ‘search image’ which guide them to flowers without any prior experience and further facilitate learning through experience (Giurfa et al., 1995; Lehrer et al., 1995; Lunau and Maier, 1995; Kelber, 1997; Cunningham, 2004). Innate preference is particularly important in solitary insects because exposure and learning of floral cues via social interactions are absent (Dötterl et al., 2011; Kinoshita et al., 2017).

The most studied sensory modality with respect to innate preference in flower-visiting insects is colour. Naïve flower-visiting insects have been shown to rely predominantly on colour cues while foraging (Lunau and Maier, 1995). Furthermore, some species of butterflies are reported to be exclusively dependent on colour cues while making foraging decisions (Anderson and Dobson, 2003; Ômura and Honda, 2005; Tang et al., 2013). Thus, colour preferences are thought to be inflexible and hardwired adaptations which provide the insects with a salient ‘search image’ to find flowers. Experiments have shown a preference for short-wavelength colours, primarily blue, in honeybees (Giurfa et al., 1995), bumblebees (Gumbert, 2000) and stingless bees (Dyer et al., 2016; Koethe et al., 2016; Balamurali et al., 2018). These preferences are reflected in the choices they make while they forage in natural habitats (Raine and Chittka, 2007; Dyer et al., 2019). Indeed, the preference for blue appears to be ubiquitous across insects, including crepuscular and nocturnal moths (Giurfa et al., 1995; Gumbert, 2000; Kelber, 1997; Goyret et al., 2008; Kandori and Yamaki, 2012; Yoshida et al., 2015; Satoh et al., 2016), with the exception of a few butterflies and most flies, which prefer red/yellow and yellow, respectively (Ilse, 1949; Swihart and Swihart, 1970; Lunau and Wacht, 1997; Weiss, 1997; Kinoshita et al., 1999; Kandori et al., 2009; Blackiston et al., 2011; Lunau, 2014). It is unclear why the preference for blue is so widespread, although one study in a Central European habitat reported blue flowers to be the most rewarding in terms of nectar quality and quantity (Raine and Chittka, 2007). The short-wavelength preference may also be a physiological constraint, as innate preferences are limited by the spectral sensitivity of photoreceptors and neuro-sensory filters in insects (Lunau and Maier, 1995).

Recent work has shown that colour preference in butterflies can be modulated by the presence of odour and the behavioural context (Yoshida et al., 2015; Balamurali et al., 2019). Flowers can be regarded as ‘sensory billboards’ which provide a multitude of cues to visitors, most importantly colour and scent (Raguso, 2004). It is beneficial for flower visitors to rely on multi-sensory cues rather than on a single sensory cue for efficient foraging. In fact, recent studies have shown that naïve insects rely on multi-modal ‘search images’ when searching for food (Riffel and Alarcón, 2013; Yoshida et al., 2015; Kinoshita et al., 2017; Balamurali et al., 2019). Even in the obligate fruit-feeding butterfly *Mycalopsis mineus*, colour preferences are exhibited only in the presence of odour cues (Balamurali et al., 2019). Foraging butterflies are shown to prioritise vision or olfaction or to use them synergistically (Tang et al., 2013).

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However, the mechanism by which this sensory cross-talk occurs is poorly understood.

Cross-modal integration of information has been shown to modulate colour preference in diurnal butterflies (Yoshida et al., 2015; Balamurali et al., 2019) and to negatively affect odour learning in diurnal hawkmoths (Balkenius and Kelber, 2006). In the diurnal hummingbird hawkmoth *Macroglossum stellatarum*, the preference for blue negatively affects the ability to learn floral scents (Balkenius and Kelber, 2006). However, in bees, cross-modal integration of visual and olfactory stimuli facilitates faster colour learning (Kunze and Gumbert, 2001; Kulahci et al., 2008; Leonard and Masek, 2014). Furthermore, honeybees scent mark spectrally similar non-rewarding flowers and avoid them in subsequent visits, demonstrating synergistic interaction of olfactory and visual stimuli and the salience of multi-modal stimuli in improving foraging (Giurfa et al., 1994). Interestingly, colour has also been shown to facilitate odour learning in bees (Gerber and Smith, 1998).

In this study, we investigated multi-sensory integration in the context of colour preference in a flower-visiting butterfly, *Catopsilia pomona* (Pieridae; lemon emigrant or the common emigrant). We infer that innate colour preference and associative colour learning are sexually dimorphic, and linked to differences in the ecology of the sexes, in particular to the need of females to find oviposition sites. We also show how odour strongly modulates colour preference, to the extent of overriding preferences learned in the absence of odour.

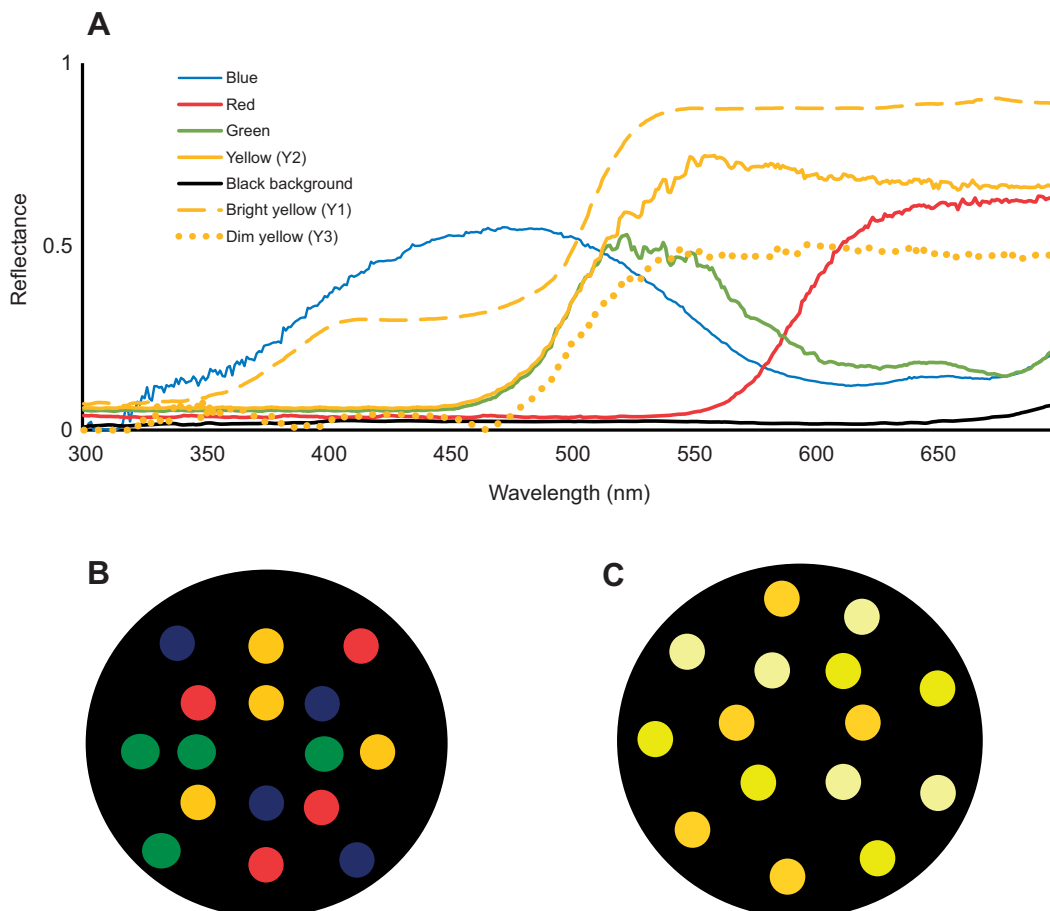
## MATERIALS AND METHODS

### Animals

*Catopsilia pomona* (Fabricius 1775) is widely distributed across South and South-East Asia to Australia. Eggs and larvae of this species were collected from its host plant *Cassia fistula* (Indian laburnum; Fabaceae) on the Indian Institute of Science Education and Research (IISER) Thiruvananthapuram campus in Vithura (8.67°N, 77.08°E), India. The eggs and larvae were kept separately in cages (0.6 m×0.3 m×0.51 m) containing young leaves of *C. fistula*. Upon pupation, the pupae were collected, moved to a separate cage and a laboratory stock population was established from the eclosed individuals. The butterflies were maintained in cages (0.6 m×0.3 m×0.51 m) and fed with 20% sucrose solution. Male and female butterflies were kept in separate cages and starved for 2 days after eclosion before being used in the experiments. All tested individuals were unmated.

### Stimuli

Coloured bond paper (80 GSM, FIS 10 colour photocopy paper, Farook International Stationery, UAE) was used to make blue, green and red stimuli as well as yellow stimuli of three different intensities. Spectral reflectance of the stimuli (Fig. 1A) was measured with a spectrophotometer (Maya 2000, Ocean Optics) using a reflectance probe connected to a pulsed xenon light source (PX-2, Ocean Optics). Coloured paper was cut into circular discs of 4 cm diameter to make the stimuli. Grey stimuli of similar dimensions were used to check the motivation of butterflies to feed before the trials began



**Fig. 1. Stimuli and experimental design.** (A) Spectral reflectance curves of the stimuli used. (B) Schematic representations of the four-colour test array used to test colour preference and learning. (C) The yellow array used to test the effect of stimulus brightness on colour preference.

(see ‘Experiments’, below). Geranium essential oil (Miaroma, Holland and Barrett, UK) was used as the floral odour in experiments where colour and odour were presented together.

### Experimental arena

A nylon mesh-covered cylindrical cage (20 cm in diameter and 25 cm in height) was used as the experimental arena. The top of the cage could be opened to access the arena to let the animals in and to change the stimuli. The floor of the cage was covered in black paper, which served as the background. Each stimulus was attached to the black background using 200  $\mu$ l pipette tips in the centre.

### Experiments

All experiments were performed outdoors under natural daylight illumination in the IISER Thiruvananthapuram campus. Pilot experiments were carried out to determine the ideal starvation period by subjecting freshly eclosed butterflies to starvation for 1, 2 and 3 days and checking for proboscis extension and subsequent feeding by exposing the animal to an achromatic grey stimulus with 30% sucrose solution presented in a 200  $\mu$ l pipette tip in the centre against a black background. Butterflies starved for 2 days were sufficiently motivated to feed and search the experimental arena. It was also clear from the pilot trials that the butterflies needed to be pre-trained to the achromatic grey stimulus after starvation to motivate them to search the arena. Thus, butterflies were allowed to feed for 5 s on the achromatic grey stimulus with 30% sucrose solution prior to experiments. Individuals that fed from the grey stimulus were released into test cages. Each individual was tested only once. Five experiments (see below) were carried out to test for:

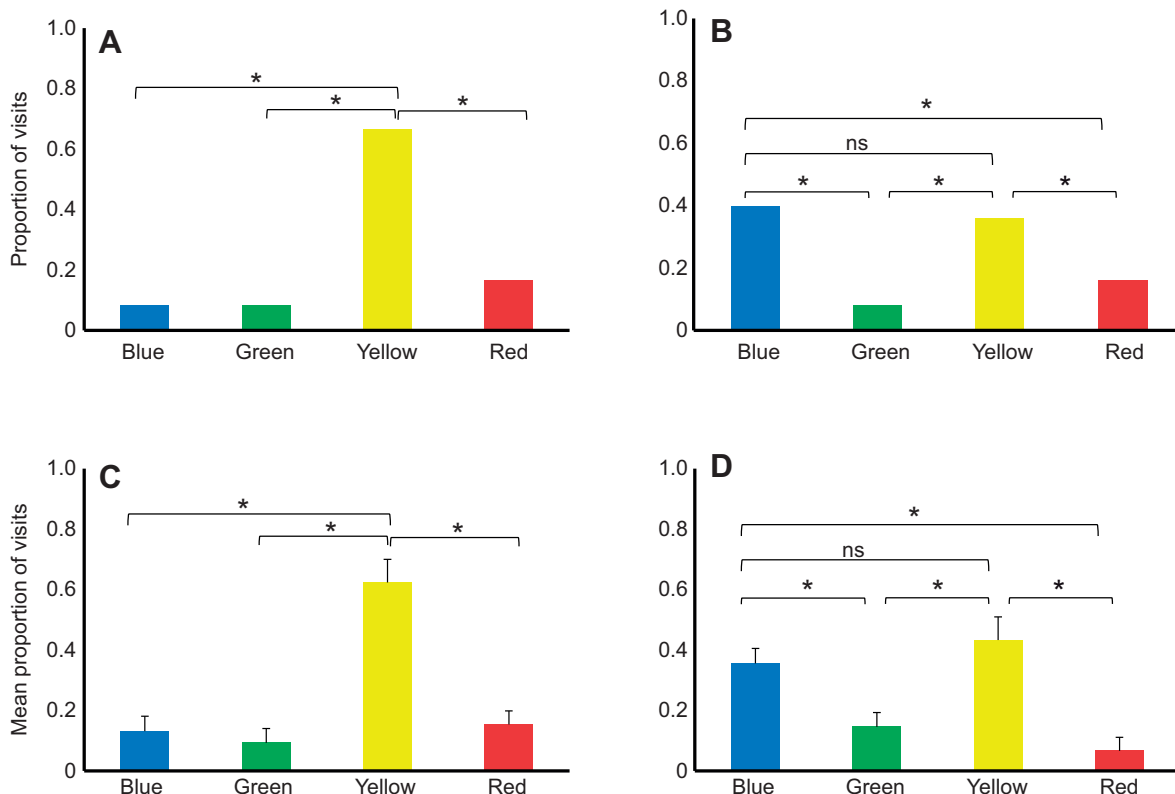
(1) innate colour preference, (2) effect of brightness on colour preference, associative colour learning with (3) minimal training or (4) extensive training and (5) associative colour learning in the presence of a floral odour in *C. pomona*. The relative position of the colour stimuli was randomised between trials to avoid possible positional bias, while the stimuli and background were changed between trials to avoid olfactory cues from previous test animals. For the experiments using floral odour, cotton soaked in 200  $\mu$ l of geranium essential oil was placed inside the arena 30 min prior to the tests to saturate the experimental arena with the odour. A butterfly was considered to have responded to a particular stimulus if it landed on the stimulus, and probed it by extending the proboscis. The first choice as well as total choices for 5 min were noted for each individual.

#### Experiment 1 – spontaneous colour preference

After 2 days of starvation, females ( $n=24$ ) and males ( $n=25$ ) were individually released into the experimental arena with four stimuli each of blue, green, yellow and red colour, randomly arranged on the black background (hereafter four-colour test array, Fig. 1B).

#### Experiment 2 – effect of brightness on colour preference

Females ( $n=18$ ) and males ( $n=18$ ), starved for 2 days, were checked for feeding motivation as described above and released into the experimental arena with 15 stimuli, five each of three yellow stimuli with varying brightness: Y1 (bright yellow stimuli; 89% peak reflectance), Y2 (yellow used in experiment 1; 74% peak reflectance) and Y3 (dim yellow; 55% peak reflectance) (hereafter yellow test array; Fig. 1C).



**Fig. 2. Experiment 1 – spontaneous colour preference.** Proportion of first choices made by females (A;  $n=24$ ) and males (B;  $n=25$ ), and the mean ( $\pm$ s.e.m.) proportion of total choices of these individuals (C, females; D, males) in experiment 1 using the four-colour test array. \* $P<0.008$ ; ns, not significant.

**Experiment 3 – associative colour learning with minimal training**

Females ( $n=18$ ) and males ( $n=20$ ) were starved for 2 days after eclosion and trained twice to a blue stimulus containing 30% sucrose solution. Likewise, another group of females ( $n=20$ ) and males ( $n=19$ ) were trained twice to a green stimulus with 30% sucrose solution. During training, each butterfly was held against the respective training stimulus and allowed to feed for 10 s, after which it was released back into its cage. This training procedure was repeated after 2 h. Then, 2 h after the second training session, each butterfly was individually released into the experimental arena with the four-colour test array and their choices were noted.

**Experiment 4 – associative colour learning with extensive training**

Females starved for 2 days ( $n=17$ ) were trained five times to a green stimulus with 30% sucrose solution and with inter-training intervals of 1 h. An hour after the last training session, they were individually released into the experimental arena with the four-colour test array and choices were noted.

**Experiment 5 – associative colour learning in the presence of floral odour**

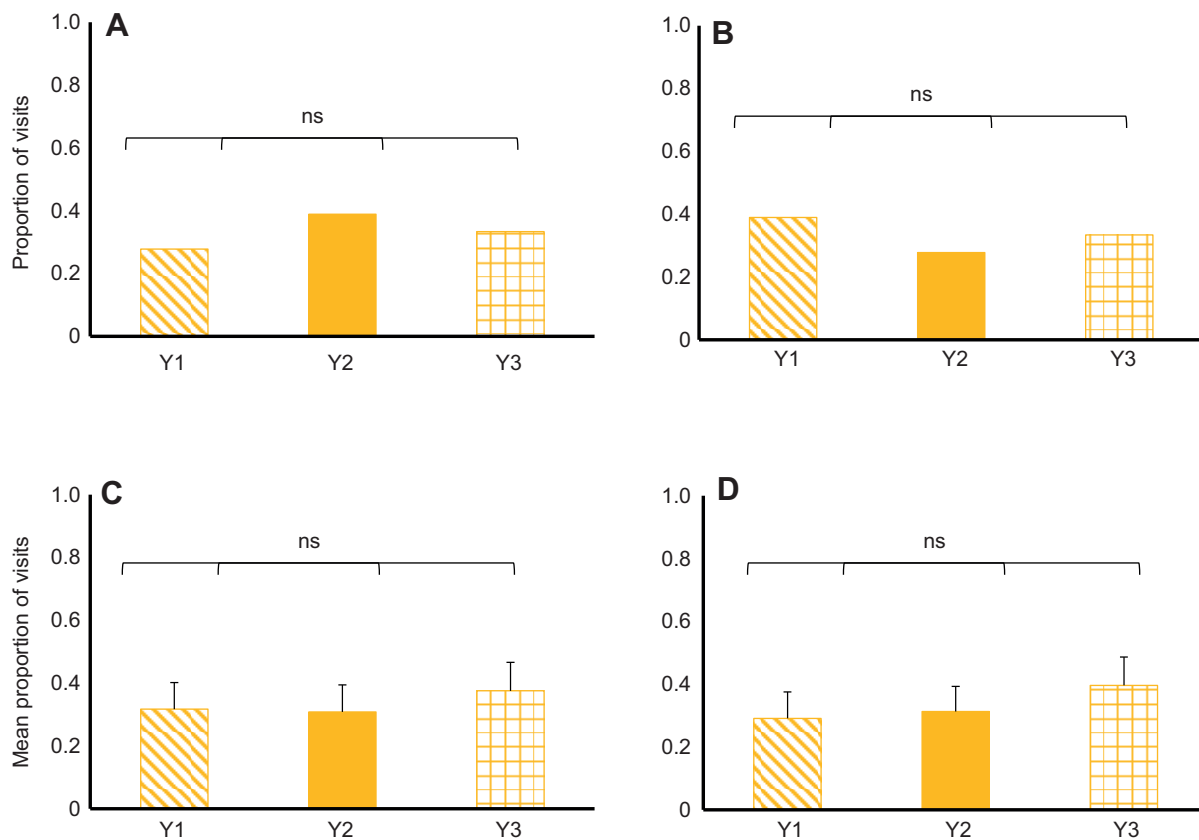
Starved females ( $n=17$ ) and males ( $n=15$ ) were trained twice to a green stimulus with 30% sucrose solution in the presence of geranium oil and with an interval of 2 h between each training session. Then, 1 h after the second training session, they were individually released into the experimental arena with the four-colour test array, which was saturated with geranium odour, and choices were noted as described above.

**Statistical analysis**

Butterflies that did not make any choice within 10 min of introduction into the experimental arena were not considered for analysis. First choices and the total choices of individual butterflies were analysed separately. All analyses were done separately for males and females. Chi-square tests were employed to determine whether the number of first choices differed significantly across colours. There was significant variation in the number of total choices made by individual butterflies within experiments (Fig. S1A,B). We converted the responses to proportions and used Kruskal–Wallis tests to check whether total choices differed significantly across colours. When one of the tests indicated a significant difference, it was followed by *post hoc* pair-wise comparisons using chi-square tests with Bonferroni correction to check for differences between pairs of colours. All analyses were carried out in R v 3.5.0 (<http://www.R-project.org/>).

**RESULTS****Experiment 1 – spontaneous colour preference**

The number of first choices was significantly different from random (i.e. 25%) across the four colours in both females ( $n=24$ ;  $\chi^2=96.24$ , d.f.=3,  $P<0.0001$ ; Fig. 2A) and males ( $n=25$ ;  $\chi^2=28.64$ , d.f.=3,  $P<0.0001$ ; Fig. 2B). First visits by naive females were to yellow primarily (67%), while first visits by naive males were mostly to blue (40%) or yellow (36%). Pair-wise comparisons indicated that yellow was preferred over the other three colours by females, but males preferred both blue and yellow over green and red (Fig. 2A,B; Table S1). The number of total visits differed across colours in females



**Fig. 3. Experiment 2 – effect of brightness on colour preference.** Proportion of first choices made by females (A;  $n=18$ ) and males (B;  $n=18$ ), and the mean ( $\pm$ s.e.m.) proportion of total choices of these individuals (C, females; D, males) in experiment 2 using the yellow array. Y1, bright yellow stimuli with 90% peak reflectance; Y2, yellow stimuli with 74% peak reflectance; and Y3, dim yellow stimuli with 55% peak reflectance. ns, not significant.

(79 visits;  $H=26.39$ , d.f.=3,  $P<0.0001$ ; Fig. 2C) and males (73 visits;  $H=10.67$ , d.f.=3,  $P=0.01$ ; Fig. 2D). Pair-wise comparisons of total visits indicated that females preferred yellow, while males preferred blue and yellow over the other colours (Fig. 2C,D; Table S1).

### Experiment 2 – effect of brightness on colour preference

The first visits made by females ( $n=18$ ) and males ( $n=18$ ) did not differ significantly across the three yellow stimuli of varying intensity (females:  $\chi^2=1.82$ , d.f.=3,  $P>0.05$ ; Fig. 3A; males:  $\chi^2=1.22$ , d.f.=3,  $P>0.05$ ; Fig. 3B). The number of total choices by both sexes also did not differ across the stimuli (females: 55 visits;  $H=0.28$ , d.f.=3,  $P=0.86$ ; Fig. 3C; males: 71 visits;  $H=1.01$ , d.f.=3,  $P=0.60$ ; Fig. 3D).

### Experiment 3 – associative colour learning with minimal training

#### Blue training

First visits were significantly different across the four test colours in females ( $n=18$ ) and males ( $n=20$ ) after blue training (females:  $\chi^2=138.7$ , d.f.=3,  $P<0.0001$ ; Fig. 4A; males:  $\chi^2=88.0$ , d.f.=3,  $P<0.0001$ ; Fig. 4B). Pair-wise comparisons of first visits revealed a preference for the trained blue stimulus in females (72%) and males (65%) (Fig. 4A,B; Table S2). The total number of visits made by females and males was also significantly different among colours (females: 47 visits;  $H=32.94$ , d.f.=3,  $P<0.0001$ ; Fig. 4C; males: 40 visits;  $H=28$ , d.f.=3,  $P<0.0001$ ; Fig. 4D). Pair-wise comparisons of total choices showed that yellow was preferred over the other three colours by both females and males (Fig. 4C,D; Table S2).

### Green training

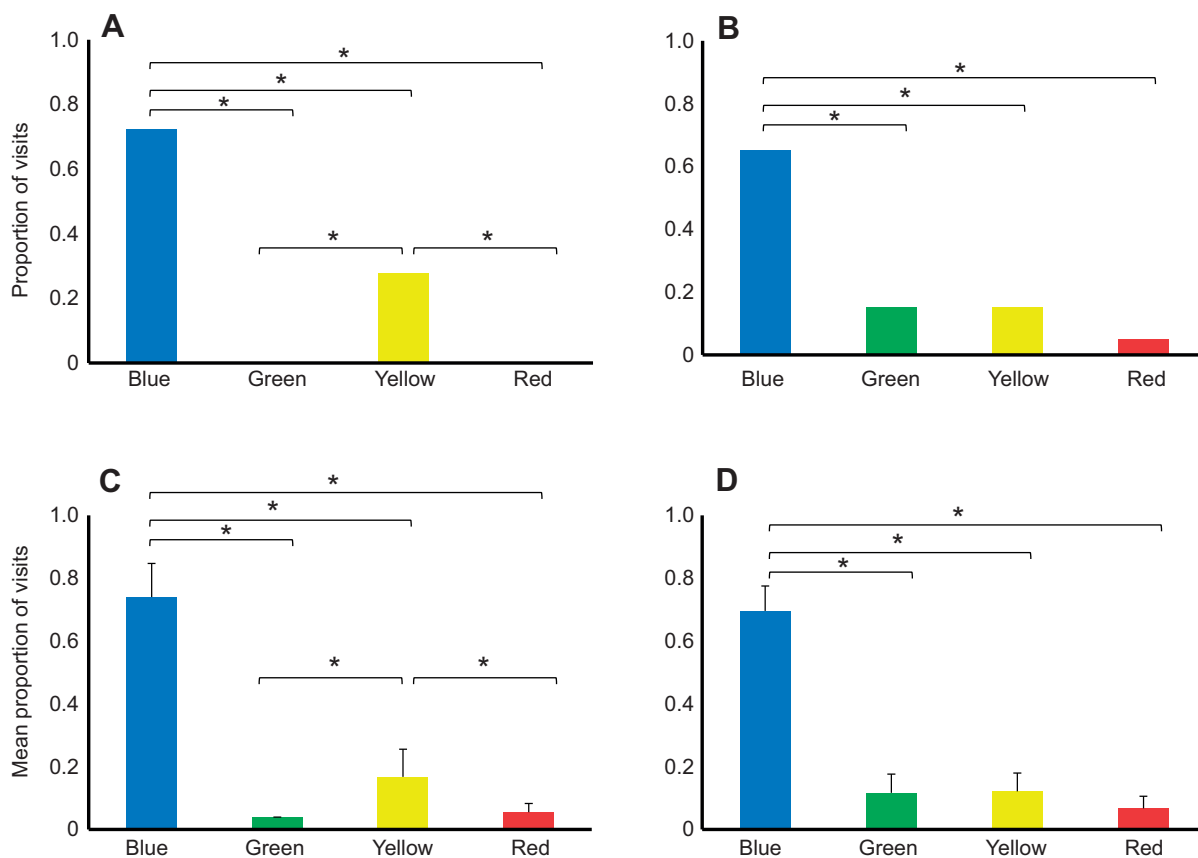
First visits made by females and males differed significantly among the test colours after green training (females:  $n=20$ ;  $\chi^2=82$ , d.f.=3,  $P<0.0001$ ; Fig. 5A; males:  $n=19$ ;  $\chi^2=161.81$ , d.f.=3,  $P<0.0001$ ; Fig. 5B), with the number of first visits to green being significantly higher than that to all other colours (Fig. 5A,B; Table S3). The total number of visits also differed significantly among colours in females (124 visits;  $H=27.58$ , d.f.=3,  $P<0.0001$ ; Fig. 5C) and males (87 visits;  $H=23.2$ , d.f.=3,  $P<0.0001$ ; Fig. 5D). However, pair-wise comparisons indicated that females made significantly more visits to green and yellow, while males visited green significantly more than the other stimuli (Fig. 5C,D; Table S3).

### Experiment 4 – associative colour learning with extensive training

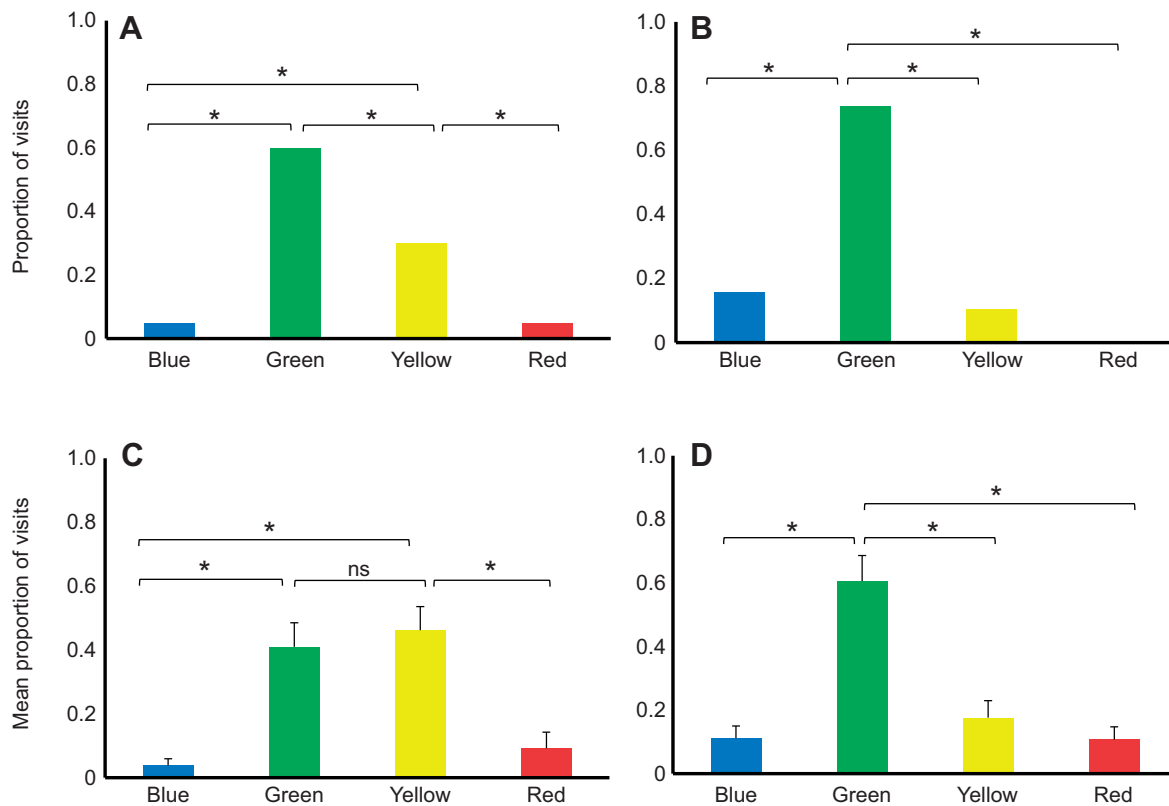
First and total visits to the colours differed significantly in females ( $n=17$ ) after five trainings to the green stimuli (first visits:  $\chi^2=142.56$ , d.f.=3,  $P<0.0001$ ; Fig. 6A; total visits: 58 visits;  $H=29.40$ , d.f.=3,  $P<0.0001$ ; Fig. 6B). Pair-wise comparisons indicated that green was visited significantly more than the other colours during the first and total visits (Fig. 6A,B; Table S4).

### Experiment 5 – associative colour learning in the presence of floral odour

First visits of females and males were significantly different from random choices (females:  $n=17$ ;  $\chi^2=73.24$ , d.f.=3,  $P<0.0001$ ; Fig. 7A; males:  $n=15$ ;  $\chi^2=69.12$ , d.f.=3,  $P<0.0001$ ; Fig. 7B). Pairwise comparisons indicated that females visited yellow and



**Fig. 4. Experiment 3 – associative colour learning with minimal training: blue stimuli.** Proportion of first choices made by females (A;  $n=18$ ) and males (B;  $n=20$ ), and the mean ( $\pm$ s.e.m.) proportion of total choices of these individuals (C, females; D, males) after two training sessions to blue stimuli in experiment 3 using the four-colour test array. \* $P<0.008$ .



**Fig. 5. Experiment 3 – associative colour learning with minimal training: green stimuli.** Proportion of first choices made by females (A;  $n=20$ ) and males (B;  $n=19$ ), and the mean ( $\pm$ s.e.m.) proportion of total choices of these individuals (C, females; D, males) after two training sessions to green stimuli in experiment 3 using the four-colour test array. \* $P<0.008$ ; ns, not significant.

males visited green significantly more compared with the other colours (Fig. 7A,B; Table S5). Total choices were also significantly different across all colours in females (40 visits;  $H=13.95$ , d.f.=3,  $P=0.002$ ; Fig. 7C) and males (38 visits;  $H=16.81$ , d.f.=3,  $P=0.0007$ ; Fig. 7D). Pair-wise comparisons revealed that females visited yellow while males visited green significantly more when compared with the other three colours (Fig. 7C,D; Table S5).

## DISCUSSION

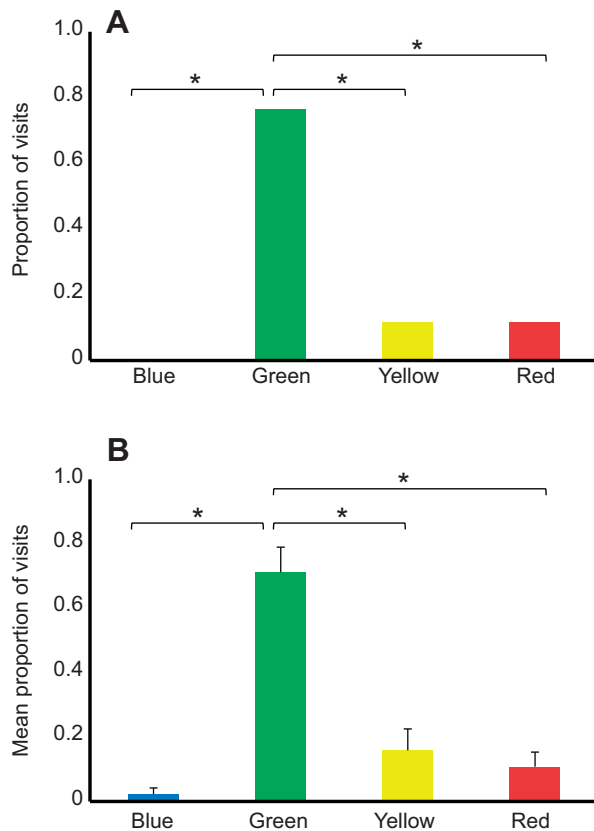
### Spontaneous colour preference in *C. pomona*

Spontaneous colour preferences are sensory biases which are thought to aid flower-visiting insects in recognising rewarding flowers and oviposition substrates, and further facilitate associative learning of colour cues in respective behavioural contexts (Gould, 1984; Giurfa et al., 1995; Lunau and Maier, 1995; Kinoshita et al., 1999; Weiss and Papaj, 2003; Satoh et al., 2016; Balamurali et al., 2019). Naive *C. pomona* females preferred yellow in the four-colour test array in our experiments, while males showed a bimodal preference for blue and yellow over the other colours. Moreover, the pattern of choices for the four colours was similar across first and total visits, suggesting strong bias for the preferred colours. The preference for blue and yellow in the context of foraging is common across Lepidoptera including nocturnal moths (Ilse, 1928; Weiss, 1991; Kelber, 1997; Ômura and Honda, 2005; Goyret et al., 2008; Kandori et al., 2009; Satoh et al., 2016; Kinoshita et al., 2017). A few species have also been shown to prefer orange, red and purple, the latter of which is a combination of blue and red (Ilse, 1928; Ilse and Vaidya, 1956; Swihart and Swihart, 1970; Scherer and Kolb, 1987a; Blackiston et al., 2011; Kandori and Yamaki, 2012). Interestingly, studies have also revealed that innate colour

preference exhibited by flower-visiting insects, especially lepidopterans, is affected by the background colour, the number of stimuli used, as well as by the behavioural context, cross-modal integration and sex (Kinoshita et al., 1999; Blackiston et al., 2011; Kandori and Yamaki, 2012; Yoshida et al., 2015; Balamurali et al., 2019). Thus, the preferences exhibited in laboratory experiments using a single sensory modality under tight control might not be reflected in natural environments where preferences might be more flexible and modulated by sex and multi-sensory integration (Kinoshita et al., 2017). In contrast, the yellow preference exhibited by *C. pomona* in our experiments is likely to be a sensory bias to recognise conspecifics, as the wings of females reflect broadly in the yellow region of the visible spectrum, while male wings are lime-green (Fig. S1C). The orange preference in the monarch butterflies is speculated to have developed as a sensory bias for conspecific coloration, which is also expressed in the foraging context (Blackiston et al., 2011). However, empirical evidence is lacking so far for a sensory bias in one context being co-opted into another.

### Sex-specific differences in spontaneous colour preference

Sex-specific colour preferences have also been reported in other flower-visiting butterflies (e.g. Kinoshita et al., 1999; Kandori et al., 2009; Kandori and Yamaki, 2012) and in a fruit-feeding butterfly (Balamurali et al., 2019). However, the mechanisms underpinning these differences remain unclear. These differences may be caused by differences in the visual system. For example, in *Pieris rapae* (small cabbage white) males, one of the three short-wavelength receptors has a double-peaked spectral sensitivity and coexists with a screening pigment which fluoresces under blue-violet and UV



**Fig. 6. Experiment 4 – associative colour learning with extensive training.** Proportion of first choices made by females (A;  $n=17$ ) and the mean ( $\pm$ s.e.m.) proportion of total choices (B) after five training sessions to green stimuli in experiment 4 using the four-colour test array. \* $P<0.008$ .

light, while these are absent in females (Arikawa et al., 2005). This imparts *P. rapae* males with better discrimination ability in the UV-violet region of the spectrum and is hypothesised to function for better discrimination of females. Moreover, the distribution of photoreceptors across eye regions in a species can differ between sexes, which in turn affect their behavioural ecology (Bernard and Remington, 1991). Furthermore, eye morphology (size and structure) and the pattern of opsin expression are dimorphic in the nymphalid butterfly *Bicyclus anynana* (squinting bushbrown; Everett et al., 2012). The role of such sexual differences in the visual system on spontaneous colour preference remains unclear and would be interesting to investigate in *C. pomona*.

#### Effect of brightness on spontaneous colour preference

Although colour is the most important visual cue that many flower-visiting insects rely on, their visual systems can also detect and discriminate achromatic or brightness contrast, which is important for visually guided behaviours such as pattern discrimination and landing (Lehrer et al., 1995; Kelber et al., 2003; Koshitaka et al., 2011). However, lepidopteran and hymenopteran insects are thought to ignore achromatic contrast when colour information is present. Even nocturnal moths have been shown to rely on chromatic rather than brightness cues (Kelber et al., 2002; Satoh et al., 2016). Diurnal hawkmoths and swallowtail butterflies (Papilionidae) can learn to discriminate brightness of stimuli only with extensive training, but they primarily rely on chromatic information (Kelber, 2005; Kinoshita et al., 2012). Honeybees ignore brightness cues altogether, indicating the salience of

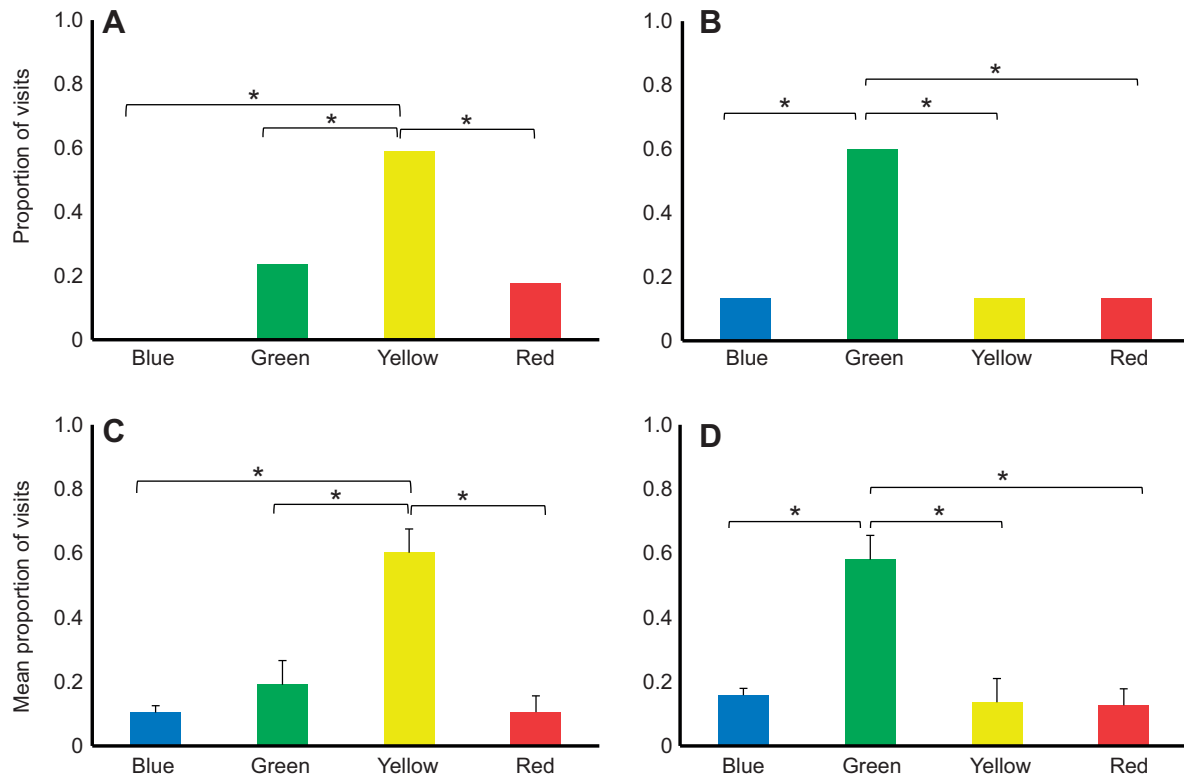
chromatic information over achromatic cues (Reser et al., 2012; Ng et al., 2018). Moreover, spontaneous colour preference in bees, butterflies and moths is independent of brightness of stimuli (Giurfa et al., 1995; Gumbert, 2000; Kelber, 1997; Kinoshita et al., 1999; Satoh et al., 2016). In our study, *C. pomona* visited all three yellow stimuli that varied in brightness equally (experiment 2), indicating that this species is guided solely by chromaticity rather than the brightness of the stimulus, thus corroborating the findings in several other insects. It has been shown that processing chromatic and achromatic information together requires highly specialised neural processing such as hierarchical and parallel processing, as found in primates (Nassi and Callaway, 2009), and the lack of such neural processing capabilities may preclude butterflies from using the two cues together.

#### Colour learning in *C. pomona*

Our results demonstrate that both females and males rapidly associated blue stimuli with reward after minimal training, suggesting robust colour associative learning. Visitation patterns were similar to all colour stimuli in terms of first choices and total visits after just two reward–stimuli associations, indicating fast learning. Colour associative learning is ubiquitous in flower-visiting insects as it helps them to learn and exploit ephemeral floral resources. Though butterflies and moths associate colour with reward (Swihart, 1971; Lewis and Lipani, 1990; Weiss, 1995; Kelber, 1996; Blackiston et al., 2011), the rate at which colours are learnt is influenced by innate colour preferences, prior experience with other colours and even sex (Swihart, 1971; Kinoshita et al., 1999; Kandori et al., 2009; Blackiston et al., 2011; Satoh et al., 2016). Furthermore, the number of training trials and the inter-training interval required to learn vary between species. For example, *P. rapae* learnt to associate colour with reward with just a single training (Lewis and Lipani, 1990), while *Battus philenor* (pipevine swallowtail) needed up to 10 visits to learn floral colours (Weiss, 1997). *Catopsilia pomona*, in contrast, needed two reward–stimuli associations to learn colours, which is comparable to the learning rate in *P. rapae* and honeybees (Lewis and Lipani, 1990; Menzel, 1993). The training paradigm used can influence colour learning in honeybees. For example, bees learn perceptually different colours well with either absolute or differential conditioning (Giurfa, 2004), but can learn similar colours only with differential conditioning (Giurfa, 2004; Dyer and Chittka, 2004). In our experiment, it is unlikely that the training paradigm influenced the results for two reasons: (1) we did not use spectrally close wavelengths as stimuli in our experiments and (2) the extensive training resulted in better training performance with green, though green is often associated with oviposition rather than feeding (Weiss and Papaj, 2003). By training just twice to the rewarded blue stimulus, 74% females learnt the colour and only 17% visited the innately preferred yellow, suggesting that innate colour preference has little influence on colour learning. Though 70% of males learnt the blue stimulus with just two trials, they also exhibited a spontaneous preference for blue and yellow (experiment 1). Thus the influence of spontaneous colour preference on colour learning could not be entirely ruled out in males in our study.

#### Sex-specific differences in colour learning and context dependency

Sex-specific differences in colour learning were apparent in our results. After training twice to the rewarded green stimuli, 74% of first visits and 61% of total visits by males were to green. However, such strong associative learning with green was absent in females,



**Fig. 7. Experiment 5 – associative colour learning in the presence of floral odour.** Proportion of first choices made by females (A;  $n=17$ ) and males (B;  $n=15$ ), and the mean ( $\pm$ s.e.m.) proportion of total choices of these individuals (C, females; D, males) after two training sessions to green stimuli in the presence of floral odour in experiment 5 using the four-colour test array. \* $P<0.008$ ; ns, not significant.

which visited green significantly more than the other colours only during first visits (60%), but showed a bimodal preference for yellow (46%) and green (41%) during subsequent visits. However, females strongly preferred blue after training sessions to blue stimuli, indicating that the robustness of learning differs across colours. Female butterflies are known to prefer green in an egg-laying context (Ilse, 1937; Scherer and Kolb, 1987b; Weiss and Papaj, 2003; Yoshida et al., 2015). The association of green with host plants in female butterflies is so strong that females of *Colias erate* (eastern pale clouded yellow) have three long-wavelength receptors which increases their sensitivity in the long-wavelength region of the spectrum and helps them locate larval host plants (Ogawa et al., 2013). In contrast, males have just one long-wavelength receptor. Mated *B. philenor* females trained to colour stimuli coated with host plant extract exhibited a bias towards green stimuli, suggesting the salience of green as an oviposition substrate cue rather than a floral cue (Weiss and Papaj, 2003). A study on *Danaus plexippus* (monarch butterfly) showed that green was learnt more slowly than other colours although that study analysed data from both males and females together and ignored sex-specific differences (Blackiston et al., 2011). Sex-specific differences and context specificity in associating floral odour and sex pheromones with sugar reward have been reported in the cotton leafworm moth *Spodoptera littoralis*, with males associating female pheromones with sugar reward at a significantly slower rate than females (Hartlieb et al., 1999). Our study is the first to show sex-specific differences in green learning. We surmise that the aversion to green in a foraging context is stronger in females, because females use green as a cue to find host plants, while males do not. However, our study was performed with unmated females and we hypothesise that

mated *C. pomona* females are likely to learn green more readily and form stronger associations with green than unmated females. This requires investigation in future studies and may show how preferences are rewired over the ontogeny of individuals.

#### Effect of extensive training on colour learning

Interestingly, when *C. pomona* females were trained extensively (five stimuli–reward associations) to associate reward with green, their learning became more robust: 76% of the first choices and 71% of total choices were to green stimuli. This indicates that the initial difficulty in associating green as a floral cue is overcome by repeated stimuli–reward associations. Extensive training also has been shown to improve associative learning of green in *D. plexippus* (Blackiston et al., 2011). Our results suggest that in *C. pomona*, even non-preferred colours and colours that are unattractive in the context of foraging can be learnt with extensive training. Thus, our results suggest that sensory bias might help inexperienced flower-visiting insects recognise flowers and host plants, but it is the flexibility in learning that helps them respond rapidly to spatio-temporally varying resources.

#### Multi-modal interaction in learning

*Catopsilia pomona* males learned to associate green with reward when they were minimally trained and tested, in both the presence and the absence of floral odour, suggesting that odour does not affect colour learning in males. However, females visited yellow stimuli (60% of total visits) significantly more than all the other stimuli, including the trained green stimuli (19% of the total visits) after minimal training in the presence of floral odour. This indicates the strong influence of multi-modal integration of information in



*C. pomona* females. Mated female *Battus philenor* butterflies trained to oviposit on green stimuli in the presence of host plant extract quickly learned to associate green with oviposition substrate (Weiss and Papaj, 2003). Interestingly, females trained to oviposit on other colours also learned to associate those colours, but made a higher number of wrong choices, and mostly to green, suggesting a bias towards green in the context of oviposition. However, when a subset of these butterflies was trained to associate nectar with another colour, they learned to associate the paired colour with nectar and the wrong choices were influenced by the trained colour from the oviposition context, suggesting confusion in learning. In our experiments, the females were unmated and starved; therefore, it is likely that the context in which they were tested is nectar foraging, and green colour is not easily learned in a foraging context.

It is intriguing that despite having the ability to learn colours fast (as indicated by blue learning), females revert to their spontaneously preferred colour in the presence of floral odour. Synergistic integration of visual and olfactory stimuli has been shown to improve learning of the rewarding stimuli in honeybees and moths (Kunze and Gumbert, 2001; Raguso and Willis, 2002; Kulahci et al. 2008; Leonard et al., 2011; Kantsa et al., 2017). The presence of visual stimuli has also been shown to enhance learning and memory of olfactory stimuli in restrained bees (Gerber and Smith, 1998). In contrast, the presence of the innately preferred blue has been shown to hinder floral odour learning in a diurnal hawkmoth (Balkenius and Kelber, 2006). Bumblebees learn faster and show consistently higher accuracy when trained to multi-modal stimuli than to unimodal stimuli (Kulahci et al., 2008). Hence, it is intriguing that despite having the ability to learn colours fast (as indicated by blue learning), *C. pomona* females revert to their spontaneously preferred colour after being trained and tested in the presence of floral odour. We surmise that females find it difficult to associate green with foraging, and rather than mediating a synergetic interaction, the presence of floral odour inhibits visual learning, leading to the butterflies reverting to their innate preference. We conclude that the modulatory effect of olfactory cues is not limited to spontaneous colour preference but extends to learning, in addition to being sex and context dependent.

## Perspective

Our results show that *C. pomona* depends on multiple sensory modalities while making foraging decisions, and this is reflected in both spontaneous preferences and learning. This multi-modal sensory integration is coupled with flexibility in learning, and could together help insects adapt to spatio-temporal variation in resources. Investigating the effect of cross-modal integration across behavioural contexts such as mating and ovipositing can offer insight into the neural mechanisms underpinning decision making in insects. For example, the synergistic effect of odour on colour learning in the context of mating has been demonstrated in *B. anynana*, in which females learn male wing patterns robustly in the presence of male odours, but in the presence of manipulated male odours they in turn learn to avoid males (Westerman and Monteiro, 2013). Our results show for the first time that context dependency and cross-modal integration of information affect colour learning in a sex-specific manner in a butterfly.

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## Competing interests

The authors declare no competing or financial interests.

## Author contributions

Conceptualization: G.S.B., H.S., U.K.; Methodology: G.S.B., H.S., U.K.; Validation: G.S.B., U.K.; Formal analysis: G.S.B.; Investigation: G.S.B., S.R.; Writing - original draft: G.S.B.; Writing - review & editing: G.S.B., S.R., H.S., U.K.; Visualization: G.S.B., S.R., U.K.; Funding acquisition: U.K.

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## Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.223271.supplemental>

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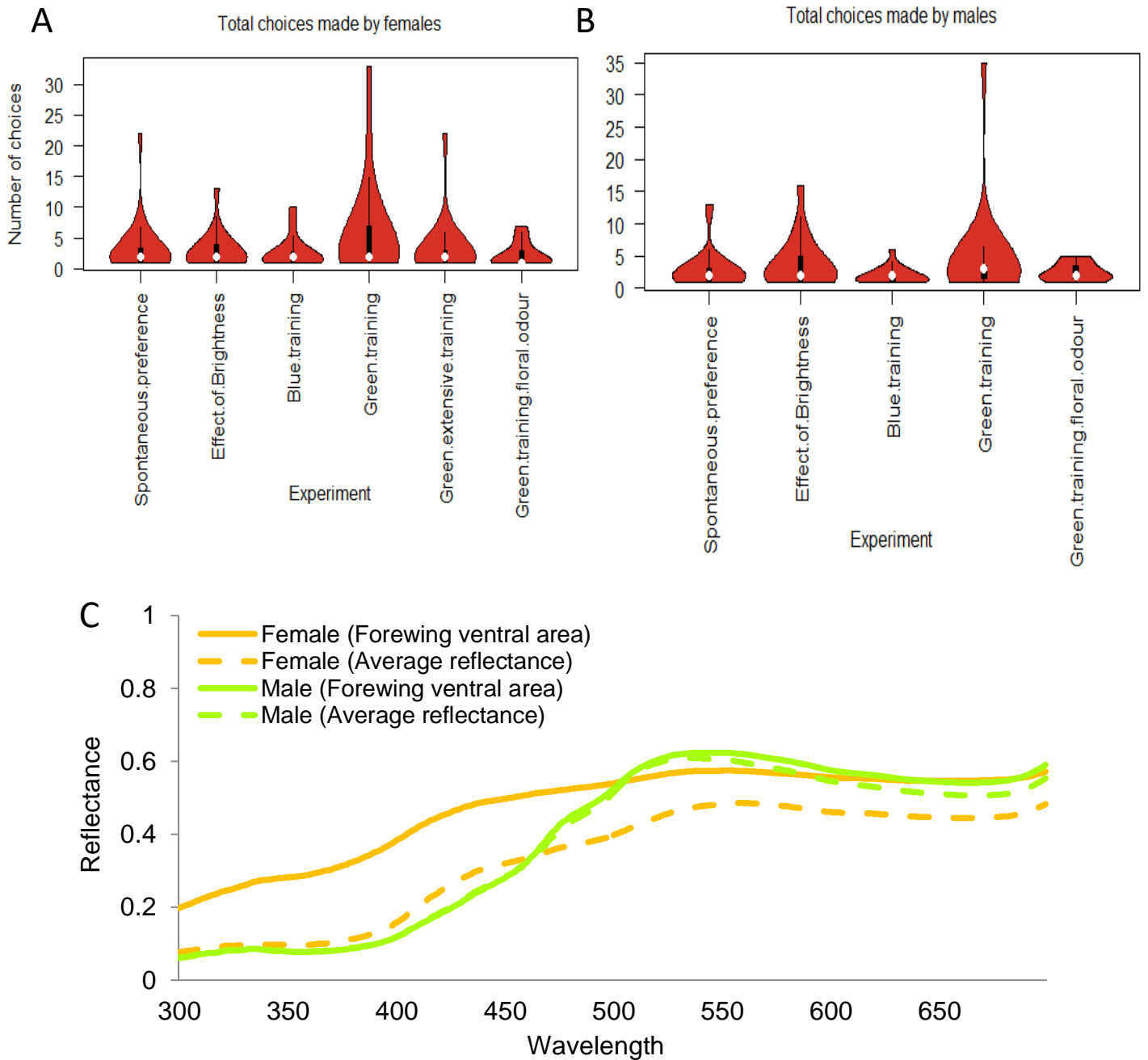


Figure S1. Violin plots for the distribution of total choices made by A) females and B) males in the experiments. The white circle represents the median and the black rectangle depicts the interquartile range. C) Spectral reflectance curves of *C. pomona* male (n=2) and female (n=2) wings. Reflectance was measured from eight different spots from each butterfly and averaged. Females showed two distinct patterns of reflectance – UV-reflecting white in the ventral region of the forewing and yellow reflectance in other regions. On the other hand males did not show any difference in reflectance across the wing regions sampled.

**SUPPLEMENTARY TABLE 1**

Pair-wise comparisons	Females		Males	
	First choice Chi-square tests	Total choices Chi-square tests	First choice Chi-square tests	Total choices Chi-square tests
Blue vs Green	$\chi^2 = 0$ , df = 1, $p=1$	$\chi^2 = 0.72$ , df = 1, $p=0.39$	$\chi^2 = 21.33$ , df = 1, $p<0.001$	$\chi^2 = 8.65$ , df = 1, $p=0.003$
Blue vs Yellow	$\chi^2 = 46.41$ , df = 1, $p<0.001$	$\chi^2 = 32.01$ , df = 1, $p<0.001$	$\chi^2 = 0.21$ , df = 1, $p=0.65$	$\chi^2 = 0.68$ , df = 1, $p=0.512$
Blue vs Red	$\chi^2 = 3.24$ , df = 1, $p=0.07$	$\chi^2 = 0.14$ , df = 1, $p=0.71$	$\chi^2 = 10.28$ , df = 1, $p=0.001$	$\chi^2 = 19.56$ , df = 1, $p<0.001$
Green vs Yellow	$\chi^2 = 46.41$ , df = 1, $p<0.001$	$\chi^2 = 39.5$ , df = 1, $p<0.001$	$\chi^2 = 17.81$ , df = 1, $p<0.001$	$\chi^2 = 13.52$ , df = 1, $p<0.001$
Green vs Red	$\chi^2 = 3.24$ , df = 1, $p=0.07$	$\chi^2 = 1.5$ , df = 1, $p=0.22$	$\chi^2 = 2.66$ , df = 1, $p=0.11$	$\chi^2 = 2.9$ , df = 1, $p=0.08$
Yellow vs Red	$\chi^2 = 29.76$ , df = 1, $p<0.001$	$\chi^2 = 28.6$ , df = 1, $p<0.001$	$\chi^2 = 7.69$ , df = 1, $p=0.005$	$\chi^2 = 25.92$ , df = 1, $p<0.001$

Table S1: Pair-wise comparisons between colours in females (n=24) and males (n=25) (Experiment 1). Chi-square tests were done with Bonferroni correction to compare first choices and total visits. Corrected  $p=0$ .

## SUPPLEMENTARY TABLE 2

Pair-wise comparisons	Females		Males	
	First choice Chi-square tests	Total choices Chi-square tests	First choice Chi-square tests	Total choices Chi-square tests
Blue vs Green	$\chi^2 = 72$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 62.8$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 31.25$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 41.02$ , df = 1, <b><math>p &lt; 0.001</math></b>
Blue vs Yellow	$\chi^2 = 19.36$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 35.8$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 31.25$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 41.02$ , df = 1, <b><math>p &lt; 0.001</math></b>
Blue vs Red	$\chi^2 = 72$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 60.2$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 51.42$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 51.54$ , df = 1, <b><math>p &lt; 0.001</math></b>
Green vs Yellow	$\chi^2 = 28$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 8.04$ , df = 1, <b><math>p = 0.004</math></b>	$\chi^2 = 0$ , df = 1, $p = 1$	$\chi^2 = 0$ , df = 1, $p = 1$
Green vs Red	$\chi^2 = 0$ , df = 1, $p = 1$	$\chi^2 = 0.111$ , df = 1, $p = 0.74$	$\chi^2 = 5$ , df = 1, $p = 0.02$	$\chi^2 = 1.31$ , df = 1, $p = 0.25$
Yellow vs Red	$\chi^2 = 28$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 6.54$ , df = 1, $p < 0.01$	$\chi^2 = 5$ , df = 1, $p = 0.02$	$\chi^2 = 1.31$ , df = 1, $p = 0.25$

Table S2: Pair-wise comparisons between colours in females (n=18) and males (n=20) (Experiment 3-Blue training). Chi-square tests were done with Bonferroni correction to compare done to compare first choices and total visits. Corrected  $p = 0.008$ . Significant results are in bold font.

## SUPPLEMENTARY TABLE 3

Pair-wise comparisons	Females		Males	
	First choice Chi-square tests	Total choices Chi-square tests	First choice Chi-square tests	Total choices Chi-square tests
Blue vs Green	$\chi^2 = 46.53$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 30.42$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 37.37$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 34.72$ , df = 1, <b><math>p &lt; 0.001</math></b>
Blue vs Yellow	$\chi^2 = 17.85$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 35.28$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 1.38$ , df = 1, $p = 0.239$	$\chi^2 = 1.69$ , df = 1, $p = 0.193$
Blue vs Red	$\chi^2 = 0$ , df = 1, $p = 1$	$\chi^2 = 1.92$ , df = 1, $p = 0.166$	$\chi^2 = 16$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 0$ , df = 1, $p = 1$
Green vs Yellow	$\chi^2 = 10$ , df = 1, <b><math>p = 0.001</math></b>	$\chi^2 = 0.28$ , df = 1, $p = 0.596$	$\chi^2 = 48.76$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 23.4$ , df = 1, <b><math>p &lt; 0.001</math></b>
Green vs Red	$\chi^2 = 46.53$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 20.48$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 74$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 34.72$ , df = 1, <b><math>p &lt; 0.001</math></b>
Yellow vs Red	$\chi^2 = 17.85$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 24.89$ , df = 1, <b><math>p &lt; 0.01</math></b>	$\chi^2 = 10$ , df = 1, <b><math>p = 0.001</math></b>	$\chi^2 = 1.69$ , df = 1, $p = 0.193$

Table S3: Pair-wise comparisons between colours in females (n=20) and males (n=19) (Experiment 3 – Green training). Chi-square tests were done with Bonferroni correction to compare done to compare first choices and total visits. Corrected  $p = 0.008$ . Significant results are in bold font.

**SUPPLEMENTARY TABLE 4**

Pair-wise comparisons	Females	
	First choice Chi-square tests	Total choices Chi-square tests
Blue vs Green	$\chi^2 = 76$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 65.22$ , df = 1, <b><math>p &lt; 0.001</math></b>
Blue vs Yellow	$\chi^2 = 12$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 10.89$ , df = 1, <b><math>p &lt; 0.001</math></b>
Blue vs Red	$\chi^2 = 12$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 6.23$ , df = 1, $p = 0.112$
Green vs Yellow	$\chi^2 = 46.54$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 34.77$ , df = 1, <b><math>p &lt; 0.001</math></b>
Green vs Red	$\chi^2 = 46.54$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 43.9$ , df = 1, <b><math>p &lt; 0.001</math></b>
Yellow vs Red	$\chi^2 = 0$ , df = 1, $p = 1$	$\chi^2 = 0.926$ , df = 1, $p = 0.336$

Table S4: Pair-wise comparisons between colours in females (n=17) (Experiment 4). Chi-square tests were done with Bonferroni correction to compare done to compare first choices and total visits. Corrected  $p = 0.008$ . Significant results are in bold font.

## SUPPLEMENTARY TABLE 5

Pair-wise comparisons	Females		Males	
	First choice Chi-square tests	Total choices Chi-square tests	First choice Chi-square tests	Total choices Chi-square tests
Blue vs Green	$\chi^2 = 24$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 2.79$ , df = 1, $p = 0.094$	$\chi^2 = 30.26$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 23.83$ , df = 1, $p < 0.001$
Blue vs Yellow	$\chi^2 = 59$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 35.71$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 0$ , df = 1, $p = 1$	$\chi^2 = 0.133$ , df = 1, $p = 0.715$
Blue vs Red	$\chi^2 = 18$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 0.04$ , df = 1, $p = 0.827$	$\chi^2 = 0$ , df = 1, $p = 1$	$\chi^2 = 0.31$ , df = 1, $p = 0.577$
Green vs Yellow	$\chi^2 = 14.79$ , df = 1, <b><math>p = 0.001</math></b>	$\chi^2 = 21.27$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 30.26$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 26.89$ , df = 1, $p < 0.001$
Green vs Red	$\chi^2 = 0.85$ , df = 1, $p = 0.855$	$\chi^2 = 2.13$ , df = 1, $p = 0.144$	$\chi^2 = 30.26$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 28.52$ , df = 1, $p < 0.001$
Yellow vs Red	$\chi^2 = 21.83$ , df = 1, <b><math>p &lt; 0.001</math></b>	$\chi^2 = 33.81$ , df = 1, <b><math>p &lt; 0.01</math></b>	$\chi^2 = 0$ , df = 1, $p = 1$	$\chi^2 = 0.037$ , df = 1, $p = 0.847$

Table S5: Pair-wise comparisons between colours in females (n=17) and males (n=15) (Experiment 5). Chi-square tests were done with Bonferroni correction to compare done to compare first choices and total visits. Corrected  $p = 0.008$ . Significant results are in bold font.