Odour influences whether females learn to prefer or to avoid wing patterns of male butterflies

Erica L. Westerman*, Antónia Monteiro 1

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Mating displays are often composed of multiple signals in multiple sensory modalities, with each individual signal contributing to the attractiveness of the displaying individual. Adult mate preferences for some of these signals are learned during premating, or juvenile, social experience with a sexually mature individual. While learned mate preferences have been described in multiple taxa, it is still unclear how the different display signals perceived during the learning period influence the development of adult mate preferences. Of particular interest is whether a learned mate preference for a sexual signal in one modality is context dependent (i.e. dependent on a second signal in either the same or a different sensory modality). Here we test whether a signal in one modality (odour) influences the interpretation (aversion or preference learning) of signals in a different modality (wing colour patterns) using the butterfly Bicyclus anynana. Previously we showed that female B. anynana learn to prefer additional ornaments, UV-reflective white spots, on the wings of males if they are exposed to these ornaments on the first day after eclosion from pupae. We now show that females exposed to males with manipulated odour do not learn to prefer these additional ornaments, and learn to avoid the wild-type male ornamentation. This aversion learning, where animals learn to avoid visual signals previously coupled with unattractive odours and to prefer visual signals previously coupled with attractive odours, demonstrates that visual mate preference learning in a butterfly is context dependent, and that specific sexual signals may have epistatic effects on mate preference development.

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Juvenile social experience of particular conspecific signals (such as song or visual phenotype) is known to influence adult mate preference in a variety of taxonomic groups with varying levels of parental care (ten Cate & Vos 1999; Verzijden et al. 2012). However, how different signals influence adult preference formation is not well understood. Controlled laboratory studies that isolate and examine mate preference learning of specific signals in specific modalities in species without parental care have demonstrated that subadult female spiders can learn a preference for a particular male visual signal (leg coloration) when exposed to directed courtship by those males (Hebets 2003; Hebets & Vink 2007), and that the mere presence of a sexually mature male with an enhanced visual signal (increased number of wing spots in butterflies) induces preference learning of that visual signal in immature females (Westerman et al. 2012). However, because these, and similar studies, control for variation in signals other than the focal signal, it remains unclear whether, and how, preference learning in single sensory modalities may be augmented or modified by sexual signals in other sensory modalities.

Although the role of multiple signals in the learning of specific signals has not yet been explored in mate preference development, signals in a particular modality have been found to influence a receiver’s response to signals in a second modality in other ecological contexts, such as toxin avoidance. The presence of a novel odour can induce innate avoidance of aposeмatically coloured prey items in birds (Rowe & Guilford 1996; Jetz et al. 2001). In addition, studies of taste aversion learning and aposematic signalling have demonstrated that bitter tastes (often associated with odours) enhance avoidance learning of visual aposematic signals in both birds and mammals (Galef & Osborne 1978; Palmerino et al. 1980; Rowe & Skelhorn 2005). These studies show that it is possible for a signal in one modality (taste or odour) to influence the learned response of a signal in a second modality (coloration), particularly in the context of toxin avoidance. It is currently unclear, however, whether multimodal signals play a similar role in mate preference learning.

Here we examine whether odours (sex pheromones) influence the learning of visual signals in butterflies, a lineage whose members have extremely diverse colour patterns. Mate preference
learning of visual signals was recently described in a member of the butterfly genus *Bicyclus* (Westerman et al. 2012; results summarized in Table 1). This is a clade where multiple species live in sympatry and display a diversity of visual signals (Condamin 1973; Brakefield & Reistma 1991), an ecological scenario that occurs for multiple butterfly species (Estrada & Jiggins 2008; Estrada et al. 2011). The diverse visual signals of sympatric species are inappropriate models for visual mate preference learning by immature individuals. So, we examined whether a signal in a separate modality, such as odour, could restrict the pool of appropriate visual models to fewer individuals, such as those sharing a familiar odour (i.e. conspecifics).

Mate signalling is multimodal in *Bicyclus anynana*: females use volatile sex pheromones as well as visual signals in mate choice decisions (Costanzo & Monteiro 2007; Nieberding et al. 2008, 2012), as do other insects (Datta et al. 2008; Fujii et al. 2011). Here we tested whether pheromones interact with learning of visual signals in *B. anynana* and whether they influence a female’s response to visual learning models. We manipulated the odour of males as models for the learning of visual signals and assessed female preference responses in subsequent mate choice trials. In addition, we assessed the effect of odour manipulations on specific categories of wing patterns (containing additional or fewer dorsal forewing eyespots), which *B. anynana* females were previously shown to learn differentially (Westerman et al. 2012).

**METHODS**

**Study Species**

*Bicyclus anynana* is a subtropical African butterfly that has been maintained in the laboratory since 1988. A colony was established in New Haven, CT, U.S.A., from hundreds of eggs collected from a laboratory colony in Leiden, The Netherlands (originally established from 80 gravid females collected in Malawi). The species has two eyespots on the ventral and dorsal forewing surfaces, seven eyespots on the ventral surface of the hindwing, and zero to three (rarely up to five) eyespots on the dorsal surface of the hindwing. In males, the white, UV-reflective scales at the centre of the two dorsal forewing eyespots, but no other eyespot traits, have previously been demonstrated to be important in female mate selection (Robertson & Monteiro 2005; Prudic et al. 2012; Westerman et al. 2012).

While *B. anynana* is seasonally plastic in morphology and behaviour (Brakefield & Reistma 1991; Prudic et al. 2012), all butterflies used in this study were reared in a walk-in, climate-controlled chamber under wet season conditions (27°C, 80% humidity and a 12:12 h light:dark cycle) to remove any effect of seasonal phenotype on mating outcome or learning ability. Larvae were fed on young corn plants, and adults were fed on banana slices, and food was provided ad libitum. Female pupae were sexed and removed from the walk-in chamber and isolated in a separate climate-controlled chamber (Darwin Chambers Co., St Louis, MO, U.S.A.) to prevent exposure to male pheromones after emergence. Butterflies were reared continuously in overlapping generations and emerged daily in our laboratory populations.

All behavioural assays (training events and choice trials) were conducted under sunlamps and in front of east-facing windows at 25–28°C (for further details of the set-up, see Westerman et al. 2012). All treatments were conducted from September 2011 to April 2012 and used butterflies from multiple generations. Behavioural assays were conducted using cylindrical hanging net cages (30 cm diameter × 40 cm height). Butterflies were removed and isolated before noon on the day of eclosion (day 1). After emergence, males were put in sex- and age-specific cages, while females were isolated from all other butterflies (males and females) until use in a training event or mating trial.

**Altering Male Odour**

To eliminate or reduce male pheromone emission, we first chilled males in a cooler for 2 min to reduce male activity and the potential for butterfly stress, then painted over the pheromone-producing organs (androconia) on both fore- and hindwings with Revlon Liquid Quick Dry nail solution (Revlon, New York, NY, U.S.A.). Control males were painted on anterior portions of the dorsal forewing that did not contain androconia. Males with Revlon Liquid Quick Dry on their wings showed similar rates of behaviour as unmanipulated males (for further details of these methods, see Costanzo & Monteiro 2007).

**Altering Male Wing Patterns**

We again chilled males in a cooler for 2 min to reduce male activity and the potential for butterfly stress. We then either (1) painted two spots of white UV-reflective paint (White, Fish Vision™, Fargo, ND, U.S.A.) directly on top of the natural white UV-reflective spots (‘two-spot males’), or in between the natural spots (‘four-spot males’), or (2) painted two spots of black paint (Enamel Glossy Black 1147, Testors, Rockford, IL, U.S.A.) directly on top of the natural UV-reflective spots (‘zero-spot males’) or next to the two UV-reflective spots (‘two-spot males’). Paint manipulation did not influence male behaviour. This was the same paint as that used in the Westerman et al. (2012) study. For further details on UV spectra of spots, wing manipulation and absence of effect of wing manipulation on male behaviour, see Westerman et al. (2012).

**Mate Choice Experiments**

All experiments followed the same basic design. The morning of emergence (day 1), virgin females were removed from the environmental chamber, isolated from all other butterflies, and either completely isolated for 2 days prior to preference trials (all ‘naïve’ treatments), or exposed to a single virgin male with manipulated androconia of a given spot phenotype for 3 h, then completely isolated until the preference trial on day 3 (all ‘exposure’ treatments). Trials were repeated 26–30 times, depending on treatment. We conducted two to four trials each day for two to four treatments (i.e. on no day were trials performed for only a single treatment) to control for an effect of day on either mating outcome or premating experience. Males used for the exposure treatments (i.e. ‘trainer males’) were painted and isolated in cages 1 day before their emergence.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Summary of results from Westerman et al. (2012) for mating outcomes of female <em>B. anynana</em> with different premating experience</th>
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<tbody>
<tr>
<td>Choice: two-spot vs four-spot male</td>
<td>Choice: two-spot vs zero-spot male</td>
</tr>
<tr>
<td>Premating experience</td>
<td>No experience</td>
</tr>
<tr>
<td>Male preference</td>
<td>No experience</td>
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Females either had no experience with males prior to the mate preference trial or were exposed during a brief premating trial to males with two or four dorsal forewing eyespots. Female were then given a choice between males with two versus four dorsal forewing eyespots or two versus zero dorsal forewing eyespots.

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female emergence, so that they had 24 h to acclimate to the cage before interacting with a newly emerged female. These males were 3 days old when exposed to females. All cages were labelled using a semi-random numbering system, and the treatment that corresponded to a given number on a given day was recorded in a separate notebook. This was done to ensure that the female’s exposure treatment during the premating observation period and during the mate choice trial was unknown to the observer. To determine whether any particular behaviour during the 3 h exposure period influenced mating outcome, we recorded all behaviour for both individuals, for 26 of the 30 trials for each treatment, using Spectator Go\textsuperscript{b} BIOBSERVE (Fort Lee, NJ, U.S.A.) data collection software. Using the Spectator Go\textsuperscript{b} BIOBSERVE data collection software, we calculated the number of incidents of each type of behaviour (flight, flutters, walks (short and long), parallel sitting, basking and courting), the proportion of total recorded events composed of each type of behaviour, and the amount of time spent performing each behaviour. We also performed a principal components analysis of the number of incidents of each type of behaviour performed for each sex.

In all preference trials, females were given a choice between two virgin males (range 2–4 days old) with manipulated wing patterns, but natural odour, and no prior experience with females. Males of each phenotype being tested were matched for age and wing size and introduced to the female’s cage on the morning of day 3. Immediately before the two males were introduced to the female cage, we dusted the female’s abdomen with fluorescent dust (PF-33 Clownfish Orange, Risk Reactor, Santa Ana, CA, U.S.A.), which is transferred to the male’s abdomen during copulation (Joron & Brakefield 2003), allowing us to determine mating outcome. Males were checked every morning, and the trial was ended when one male had orange dust on his abdomen, at which point his phenotype was recorded. We assessed male phenotype at this point to control for observer bias. The fact that male’s dorsal forewing phenotype was not visible when the males were sitting on the cage and that male dorsal forewing phenotype was very difficult to assess when the males were flying or fluttering their wings also helped control for observer bias.

Ethical Note

All B. anynana butterflies were maintained in laboratory conditions as specified by U.S. Department of Agriculture permit PS26P-12-04897. All wing manipulations were performed on males that were chilled for 2 min to reduce movement and the potential for stress. In addition, during wing manipulation, males were held still using wax paper to prevent harm to the wings and the body. Males behaved normally as soon as the paint dried. For further details on wing manipulation method, please see Westerman et al. (2012) and Costanzo & Monteiro (2007). After their time as trainer males, all trainer male butterflies were maintained in cages in a climate-controlled, walk-in chamber maintained at wet season conditions with ample food and water until natural death. All focal females, and all males used in mate choice trials, were euthanized by freezing, so their bodies could be used for future morphological studies.

Statistical Analyses

We compared mating outcomes of females with and without premating experience using Pearson chi-square tests. Preferences for particular male treatments were deemed significant if mating outcomes differed significantly from random mating (50:50) using a sign test. We assessed the relationship between mating outcome and the quantity of trainer male behaviour (arcsine-transformed proportion of trainer-male-specific behaviour and composite behavioural variables determined by a principal components analysis) using logistic regression. To determine whether factors other than wing pattern and androconia manipulation during premating exposure influenced females’ responses to male visual ornamentation during mate choice trials, we tested for equal variances in behavioural data between treatments using an analysis of means for variances test and analysed the data using a Tukey–Kramer test (for behaviours with equal variance across treatments) or a Steel–Dwass all pairs test (for behaviours with unequal variance across treatments). All statistical analyses were performed in JMP (SAS Institute, Cary, NC, U.S.A.).

RESULTS

Males with Manipulated Odours are Unattractive to Females

Females isolated (visually and chemically) from males during pupation and kept in isolation until their mate choice trials (naïve females) mated more often with control males than with males whose androconia had been manipulated (80.77% of females mated with control males; sign test: N = 26, two-tailed P = 0.0017), confirming previous results that females have a pre-existing mating bias for males with intact androconia (Costanzo & Monteiro 2007; Nieberding et al. 2008). Therefore, the androconia manipulation (the addition of a nail coating solution to androconia, originally used to determine the importance of pheromones to female mate choice: Costanzo & Monteiro 2007; Nieberding et al. 2008, 2012) appeared to produce males that were ‘unattractive’ to our population of females. We describe males with manipulated androconia as having ‘less attractive odours’ (relative to control males) instead of having ‘no odour’ because Rutledge et al. (2010) found that female spiders differentiated between the odours of different finger nail coating solutions in mating trials, and that these chemicals, although volatile, have distinct gas chromatography spectra 18–24 h after application.

Exposure to Males with Manipulated Odours Affects Female Preference for Familiar Visual Phenotypes

Females that were housed in complete isolation from males until the mating trial (naïve females) tended to mate more often with two-spot males than with four-spot males (66.67%; sign test: N = 30, two-tailed P = 0.0679; Fig. 1a), suggesting a pre-existing bias for the wild-type wing pattern. Females exposed to a four-spot male with an unattractive odour during premating did not learn to prefer four-spot males, as they did not shift their pre-existing mating bias from two-spot to four-spot males (46.67% of females mated with four-spot males; Pearson chi-square test: $\chi^2_{1,59} = 1.111$, two-tailed $P = 0.218$; Fig. 1a). However, females given premating social experience with a wild-type-looking (two-spot) male with unattractive odour modified their pre-existing mating bias and mated preferentially with four-spot males when given a choice between two-spot and four-spot males with natural odours (pairwise mate preference of naïve versus exposed females: Pearson chi-square test: $\chi^2_{1,59} = 0.076$, two-tailed $P = 0.705$; 70% of exposed females mated with four-spot males: sign test: N = 30, two-tailed $P = 0.0428$; Fig. 1a). Thus, B. anynana females previously exposed to males with unnatural odours do not prefer the familiar visual phenotype when given a choice between two males with natural odours and either a familiar or an unfamiliar visual phenotype.

Furthermore, mating outcomes of females exposed to four-spot males with unmanipulated androconia but with a nail coating solution added to a different part of the wing were similar to those of females exposed to four-spot males with blocked androconia.
(33.33% and 46.67% of exposed females mated with four-spot males, respectively; Pearson chi-square test: \( \chi^2 \approx 3.235 \), two-tailed \( P = 0.0663 \)), and did not learn to prefer four-spot males, but tended to mate more often with two-spot males (sign test: \( N = 30 \), two-tailed \( P = 0.0987 \); Fig. 1a). These results suggest that deviations from natural male odour, rather than an absence of sex pheromones per se, prevented females from learning a preference for males with enhanced ornamentation.

**Odour Does Not Affect Female’s Inability to Differentiate between Two-spot and Zero-spot Males**

Females that were isolated from males until the mating trials (naïve females) mated randomly with two-spot and zero-spot males (53.33% of naïve females mated with two-spot males; sign test: \( N = 30 \), two-tailed \( P = 0.8555 \); Fig. 1b). There was no effect of early exposure on this mating outcome, either when females were exposed to two-spot males with unattractive odours or when females were exposed to zero-spot males with unattractive odours (53.33% of naïve females versus 53.33% of females exposed to two-spot males mated with two-spot males; Pearson chi-square test: \( \chi^2 \approx 0.601 \), two-tailed \( P = 0.438 \); 53.33% of naïve females versus 50.00% of females exposed to zero-spot males mated with two-spot males; Pearson chi-square test: \( \chi^2 \approx 0.067 \), two-tailed \( P = 0.796 \); Fig. 1b).

**Trainer Male Behaviour Influences Female Learning**

During the training period, males and females showed the following behaviours. Males: (1) stereotypic courting behaviour (Nieberding et al. 2008); (2) flights; (3) wing flutters; (4) walking on the cage; (5) circling females; (6) sitting on the cage near to, and parallel with, the female (see Supplementary material, Table S1). Females: (1) flights; (2) wing flutters; (3) walking on the cage; (4) circling males; (5) sitting with their wings open (basking) and (6) sitting on the cage (with wings closed) near to, and parallel with, the male (see Supplementary Table S2). Principal components PC1 and PC2 were used as representative compound variables of male and female behaviours (see Supplementary Table S3).

To test whether particular male behaviours during the training period influenced later female mating outcome, we analysed behavioural data for treatments where females chose between two-spot and four-spot males and for treatments where females chose between two-spot and zero-spot males separately. When behavioural data were pooled for the first set of treatments, there was a significant positive effect of the proportion of the trainer male’s activity that was spent walking on the likelihood that a female would subsequently mate with that trainer type (logistic regression, choice between two-spot and four-spot males, whole model: \( \chi^2 \approx 4.75 \), \( P = 0.0293 \); proportion walking: \( \chi^2 \approx 4.36 \), \( P = 0.0370 \)). When behavioural data were pooled for the second set...
of treatments, no male activity was correlated with mating outcome (logistic regression, choice between two-spot and zero-spot males, whole model: \( \chi^2_{29} = 0.016, P = 0.900 \)). There was no statistically significant effect of any other measured aspect of male or female behaviour during the training period on mating outcome, either when data were pooled or when data from each treatment were analysed separately (see Supplementary material for details). An increase in female PC1 (mostly describing general activity; see Supplemental material) during the training period had no effect on later mate choice during the training period. Therefore, our finding that females’ avoidance learning was reduced when trainer males spent more time walking relative to flying, fluttering and courting may be due to a reduced display of the visual signal as well as reduced emission of pheromones. Butterfly pheromones are volatile chemicals (reviewed in Ando et al. 2004), and courting, flying or fluttering of male B. anynana near females exposes the females to both the male’s scent-producing organs as well as the male’s dorsal visual ornaments, which are normally hidden when walking. Males that spent more of the training period walking may therefore have exposed the females to less of the manipulated, unattractive odour and its associated visual signal, and therefore, the females did not effectively learn to avoid the visual signals of those individuals.

Our results suggest that the olfactory signal has an epistatic effect on the learning of the visual signal. If olfactory and visual signals were simply additive, we would have expected females to prefer familiar over unfamiliar male phenotypes in either modality (visual or olfactory). We based this prediction on two previous findings: (1) females prefer males with familiar visual and olfactory signals over males with unfamiliar visual and familiar olfactory signals when they have been previously exposed to males with modified visual signals but natural olfactory signals (Westerman et al. 2012) and (2) naïve females weigh the presence of preferred visual and olfactory signals equally (Costanzo & Monteiro 2007). However, when females experienced males with unnatural olfactory signals during the training period, they showed an unpredicted response to a familiar visual signal in subsequent choice trials. Females preferred males with both unfamiliar visual and olfactory signals (e.g. four-spot males with natural odours) over males with familiar visual signals and unfamiliar olfactory signals (e.g. two-spot males with natural odours) in all visual signal treatments. These results suggest that male odour sets the context for female visual mate preference learning. When an innately preferred visual signal (two-spot males) was associated with an unattractive, non-natural odour, the females learned to avoid mating with males displaying that visual signal and preferred to mate with males displaying unfamiliar visual signals. Odour, thus, appears to be guiding the learning of wing patterns.

The epistatic effect of olfaction on visual mate preference learning suggests that the mechanism underlying the learned preference or avoidance of male visual signals in B. anynana may involve pheromone receptors and may be similar to the mechanism...
regulating male courtship and female acceptance in Drosophila, or the mechanism of taste aversion learning in mammals, birds and insects. Stimulation of particular sex-specific neuronal pathways via input from pheromone- (CVA) specific receptors in the antennae of male and female Drosophila are known to inhibit courtship behaviour in males and induce acceptance behaviour in females (Datta et al. 2008; Ruta et al. 2010). Similarly, in taste aversion learning, neuronal signals from different chemical receptors (in the mouth or nose/antennae) can either induce learning attraction or avoidance of different foods (Galef & Osborne 1978; Rusiniak et al. 1979; Welzl et al. 2001; Marella et al. 2006). Sex pheromones in lepidopterans have been shown to be species-specific chemical cocktails that stimulate species-specific chemical receptors; a change in either the receptors or the chemical cocktail results in a change in the receiver’s behaviour (Estrada et al. 2010, 2011; Fuji et al. 2011). Future research examining the response of B. ananassae butterflies to heterospecific pheromones should determine whether species-specific pheromones can alter visual mate preference learning, as suggested in the present study. In addition, research on chemical receptors and the neuronal pathways associated with pheromones and learning in butterflies should expand our understanding of the connection between olfactory signals and visual mate choice learning.

Conclusions

The results of the present study combined with those of a previous study (Westerman et al. 2012) indicate that female B. ananassae butterflies learn to prefer novel (enhanced) visual signals via an imprinting-like mechanism only when these signals are coupled to an attractive odour during a brief premating exposure period. Females can also learn to avoid innately preferred visual signals when these visual signals are coupled with an unattractive odour during the premating period. Olfactory cues thus play an important role in visual mate preference development.

While this epistatic effect of olfactory signals on visual mate preference learning has not been described in any other species where mate preference learning occurs, these species tend to have multiple, multimodal sexual signals (e.g. Brooks 2002; Griffith & Ejima 2009; Utz et al. 2009), which could set the context for mate preference learning of specific sexual signals. Furthermore, the number of recent studies demonstrating mate choice learning in an increasingly diverse set of taxa, with and without parental care (ten Cate & Vos 1999; Verzijden et al. 2012), along with multiple reviews on mate choice learning and multimodal signalling (ten Cate & Vos 1999; Canolin 2003; Hebets & Papaj 2005; Verzijden et al. 2012), highlight the potential for multimodal signalling to have complex, nonadditive effects on preference development in multiple taxa.

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Supplementary Material

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References


