



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



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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 aposematically 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

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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 used 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

Table 1
Summary of results from Westerman et al. (2012) for mating outcomes of female *B. anynana* with different premating experience

	Choice: two-spot vs four-spot male			Choice: two-spot vs zero-spot male		
	No experience	Two-spot male	Four-spot male	No experience	Two-spot male	Zero-spot male
Premating experience	No experience	Two-spot male	Four-spot male	No experience	Two-spot male	Zero-spot male
Mate preference	Two-spot male	Two-spot male	Four-spot male	Random mating	Random mating	Random mating

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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