Biased learning affects mate choice in a butterfly

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Abstract

Early acquisition of mate preferences or mate-preference learning is associated with signal diversity and speciation in a wide variety of animal species. However, the diversity of mechanisms of mate-preference learning across taxa remains poorly understood. Using the butterfly Bicyclus anynana we uncover a mechanism that can lead to directional sexual selection via mate-preference learning: a bias in learning enhanced ornamentation, which is independent of preexisting mating biases. Naïve females mated preferentially with wild-type males over males with enhanced wing ornamentation, but females briefly exposed to enhanced males mated significantly more often with enhanced males. In contrast, females exposed to males with reduced wing ornamentation did not learn to prefer drab males. Thus, we observe both a learned change of preference, but females brieﬂy exposed to wild-type males over males with enhanced wing ornamentation. The form of imprinting that would apply to most insects, there is no genetic learning, including biases in learning, in the evolution of wing-pattern variation in this genus. The diversity of mechanisms of mate-preference learning is associated with signal diversity and speciation in a wide variety of animal species. However, the diversity of mechanisms of mate-preference learning across taxa remains poorly understood. Using the butterfly Bicyclus anynana we uncover a mechanism that can lead to directional sexual selection via mate-preference learning: a bias in learning enhanced ornamentation, which is independent of preexisting mating biases. Naïve females mated preferentially with wild-type males over males with enhanced wing ornamentation, but females briefly exposed to enhanced males mated significantly more often with enhanced males. In contrast, females exposed to males with reduced wing ornamentation did not learn to prefer drab males. Thus, we observe both a learned change of preference, but females brieﬂy exposed to wild-type males over males with enhanced wing ornamentation. The form of imprinting that would apply to most insects, there is no genetic learning, including biases in learning, in the evolution of wing-pattern variation in this genus.

Keywords: biased learning, sexual imprinting, mate preference, reproductive isolation, Bicyclus anynana, nymphalid
Therefore, the only difference between the males used in either the premating exposure period or the mating trial was the number of UV-reflective spots on the dorsal surface of the forewing (Fig. 1F). Females exposed to a single extraornamented (Sp-like) male mated preferentially with extraornamented males ($\chi^2 = 7.95$, $P = 0.005$), and females exposed to normal-ornamented (Wt-like) males mated preferentially with normal-ornamented males ($\chi^2 = 5.01$, $P = 0.025$) (Fig. 2B). Neither male courting nor male activity level during the premating exposure was correlated with mating outcome (Table S1), and all behaviors were rejected as significant parameters for a stepwise nominal logistic model of mating outcome (all effect test $P$ values $>0.1$).

This shift in preference in response to a premating exposure to an adult male with enhanced wing ornamentation demonstrates that preexisting mating biases can be modified by premating experience in a butterfly. The brevity of the exposure period required to produce a change in mating outcome, coupled with the lack of a correlation between male courting behavior and female response to exposure, suggests that *B. anynana* is capable of a sexual imprinting-like behavior, in that females responded to the presence of the male and not to a specific male-female interaction that occurred during the training period (6–8, 20). Our findings differ from previous mate-preference learning studies in invertebrates, in which a stereotypic courting experience was necessary to produce an observable exposure effect (12–14, 21–23). Although the use of the triad mating assay introduces male competition as a potential confounding factor for our results, the only parameter that differed between the treatments was female experience, suggesting that the observed shift in mating outcome is the result of a shift in female behavior, not male-male competitive ability. This shift in mating outcome in response to premating social experience is a unique demonstration of a learned mate preference in Lepidoptera, and a unique demonstration of a modification of a preexisting mating bias by premating experience in an invertebrate, whereas similar modifications were also recently shown in a vertebrate species (24).

Lepidopterans are currently used as models for investigating genetic linkages between genes for trait preference and genes for trait production (25–28). The presence of a learned-mate preference in this group highlights the importance of considering rearing environment when designing experiments to test the genetic basis of behavioral traits. Previous studies examining genes

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Fig. S1 for reflectance spectra of modified UV spots. Therefore, the only difference between the males used in either the premating exposure period or the mating trial was the number of UV-reflective spots on the dorsal surface of the forewing (Fig. 1F). Females exposed to a single extraornamented (Sp-like) male mated preferentially with extraornamented males ($\chi^2 = 7.95$, $P = 0.005$), and females exposed to normal-ornamented (Wt-like) males mated preferentially with normal-ornamented males ($\chi^2 = 5.01$, $P = 0.025$) (Fig. 2B). Neither male courting nor male activity level during the premating exposure was correlated with mating outcome (Table S1), and all behaviors were rejected as significant parameters for a stepwise nominal logistic model of mating outcome (all effect test $P$ values $>0.1$).

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associated with reproductive isolation in butterflies used either wild-caught adults or laboratory-reared animals from emergence cages containing both males and females (25–28). Therefore, although the observed mating preferences may be because of genetic linkage between genes for trait preference and genes for trait production, the alternative hypothesis of preference learning immediately following emergence cannot be ruled out.

Test of Biased Learning. Mate-preference learning is predicted to homogenize populations and prevent trait evolution because of a high rate of exposure to (and consequent learned preferences for) the common phenotype (29). However, biased learning might counteract homogenization of preference and lead to trait evolution if individuals are biased toward learning specific categories of phenotypes, such as ornament enhancements (30, 31). We therefore tested for the presence of a bias in female mate-preference learning by exposing Wt females to Wt males manipulated to display reduced-ornamentation, mimicking the inferred ancestral B. anynana male phenotype [one dorsal UV-reflective forewing spot per wing (18)] (Fig. 1F), and assessing whether females learned preferences for this reduced-ornamentation phenotype as readily as they learned a preference for an enhanced-ornamentation phenotype.

When given a choice between males with the ancestral phenotype (one spot per forewing) and males with the extant phenotype (two spots per forewing), naïve Wt females mated randomly to display reduced-ornamentation, mimicking the inferred ancestral B. anynana male phenotype [one dorsal UV-reflective forewing spot per wing (18)] (Fig. 2C), and, in addition, there was no effect of exposure to either the B. anynana Wt phenotype ($\chi^2 = 0.618, P = 0.433$), or the ancestral phenotype ($\chi^2 = 0.00, P = 1.00$) on mating outcome, and male behavior during the exposure period did not influence mating outcome in either treatment (Table S1) [stepwise nominal logistic model with full factorial design rejected all behaviors as significant parameters (all effect test $P$ values $>0.1$)]. A power analysis comparing effect sizes for learning increased vs. decreased visual signals is presented in Table S2.

We conclude that not all visual signals are learned equally by B. anynana (Table S2), and that females do not have a preexisting mating bias against deviations from the population average, because we did not observe a preexisting bias for two- or one-spotted males. The observed biased learning for enhanced male ornaments provides a possible mechanism for the preferential spread and fixation of enhanced phenotypes in a population without parental care, even when these phenotypes represent a minority of visual models in the population. Further study of a female’s response to premating exposure to multiple phenotypes simultaneously, or sequentially, will be necessary to determine the importance of biased learning in promoting assortative mating in a variety of social scenarios.

Possible causes for biased learning in B. anynana, where females learn to prefer four spots (over two) better than one spot (over two), could include: (i) a better perceptual ability to distinguish between four and two spots compared with one and two spots, or (ii) an easier learning of enhanced signals over reduced signals. To test these two hypotheses we conducted choice trials between normal males and modified males with zero spots (Fig. 1I and J). By creating the same total difference in ornament number formerly permitting preference learning, we directly address the hypothesis that a difference of two spots is easier to learn than a difference of one. A failure of females to change their preference when exposed to nonornamented males would lend support to the hypothesis that signal enhancement is easier to learn than signal reduction in this species.

Naïve females mated randomly with zero- and two-spot males ($\chi^2 = 1.007, P = 0.316$). Females exposed to nonornamented (zero spot) males also mated randomly with either male type.
(χ² = 0.361 P = 0.548) (Fig. 2D) (see Table S2 for power analysis). However, unlike females exposed to four- or one-spot males, the mating outcome of females exposed to zero-spot males was influenced by the training male’s behavior (females exposed to males who frequently displayed their dorsal wing surfaces during the training period learned to prefer the zero-spot males better) (Table S3). The asymmetric response of females to early exposure to males with zero, one, and four spots suggests that, although these sexual ornaments are both gained and lost in the Bicyclus lineage (18) (Fig. 3A), signal enhancement may be easier to learn than signal reduction for B. anynana.

Our findings demonstrate that: (i) B. anynana females with no adult social experience have a preexisting mating bias against ornament enhancement, (ii) their preexisting mating bias can be changed by premating social experience, and (iii) they possess a bias in learned enhanced signals over reduced signals.

This biased learning in B. anynana differs from previous proposed mechanisms of mate-choice learning. B. anynana learning is not the result of associations of positive or negative experiences with particular signals, other than spot number itself, because trainer male behavior did not vary significantly between treatments (Table S4) (13, 21). This biased learning also does not reflect a simple sensory bias (32), as the preexisting mating bias and the learned preference operate in opposite directions. The observed bias in female response to premating experience also differs from that observed in Schizocosa spiders, in that B. anynana females shifted their mate choice only when exposed to enhanced males, whereas Schizocosa shifted mate choice to enhanced males regardless of the male phenotype they experienced (12). The shift in B. anynana is also independent of mate-choice copying (33), because all females were isolated from each other and unable to observe and copy the male selection of other females at any point during the experiment. Our results, therefore, suggest that the B. anynana female response to premating experience is dependent on the male phenotype they experience, not the simple presence of a conspecific male or mate copying. Our findings therefore illustrate how mate preferences may arise from the interaction of an intrinsic capacity to learn with a range of extrinsic social stimuli.

Differences in signal conspicuousness or in signal weighting by experienced females could produce the observed bias in learning-enhanced ornamentation. Wings with white, UV spots may be more conspicuous and therefore easier to learn than wings without these spots. If this is the case, then mate-choice learning should be primarily associated with ornament enhancement in this group, and not observed in lineages that have recently undergone ornament losses. Alternatively, premating social experience could alter signal weighting. B. anynana, like many animals, uses both olfactory and visual signals to assess mate quality (16, 17, 34, 35). Sex pheromone production is variable in male B. anynana and changes with age (35, 36). Although naïve females appear to weigh presence or absence of visual and olfactory signals equally when males are matched in age (34), it is unknown whether this weighting can be changed by premating social experience. Both early exposure and shifts in relative signal conspicuousness are known to alter the relative weighting of olfactory and visual signals in mate preference in wolf spiders, damselflies, and fruitflies (13, 14, 37, 38). Exposure to a male with an enhanced visual signal could cause females to later overemphasize the visual component of male display relative to all other signals, but exposure to a male with fewer visual signals could cause females to later emphasize pheromones or other as yet unexplored signals. In this latter scenario the mating outcomes of female B. anynana would be reflecting preferences for other, nonvisual, traits instead of an inability of females to learn preferences for reduced signals.

The wing-pattern learning bias in B. anynana resembles song-learning biases in birds, where birds learn some songs (often conspecific) more readily, or instead of, other songs (often heterospecific) in their acoustic environment (39–43). Song-learning biases are suggested to be genetically determined perceptual biases that may facilitate species-specific oblique imprinting (41). Oblique imprinting has historically been undervalued in mate preference and sexual ornament evolution because of the lack of genetic connections between the phenotype of the learning individual and that of the model (5, 29), which could include individuals from other species. However, genetically determined perceptual biases that restrict the pool of suitable learning models to conspecics or closely related individuals reduce the likelihood of an individual learning a preference for an inappropriate phenotype. Oblique imprinting of songs in birds, for example, produces very low error rates in mating with heterospecifics (10, 12–14, 21, 22, 44). Although the learning bias in B. anynana was observed using artificially modified conspecific individuals as models, the observed learning bias for additional ornaments would prevent B. anynana from forming mating preferences for close relatives that are living in sympathy, such as male Bicyclus campas and Bicyclus angulosus, with fewer dorsal forewing eyespots (18, 45) (Fig. 3A). Furthermore, oblique imprinting and subadult mate-preference learning may be particularly important in species where cross-generational changes in environment result in alternating phenotypes or seasonal polyphenisms. If individuals look different in each season, then

![Fig. 3. Hypothesized role of mate choice learning on the evolution of butterfly ornament diversity.](image-url)
learning a mate preference, rather than having a genetically
determined mate preference, may allow successful mate recog-
nition or mate selection in these species (46). Further research
on mate-choice learning in species with and without seasonal
polyphenisms is needed to determine the importance of mate-
choice plasticity in maintaining this form of environmentally
induced morphological plasticity.

Although biases in mate-preference learning have not yet been
reported in species outside of *B. anynana*, they may have been
overlooked because of the long training periods used in other
mate-preference learning studies (13, 14, 47–49). Song-learning
biases are especially apparent in laboratory studies when training
periods or event number are reduced (reviewed in ref. 40). If
wing-pattern learning biases operate similarly to song-learning
biases we would expect learning biases to become apparent in
more taxa as experimental training event number and duration
are reduced. The possible role of mate-preference learning bia-
ses in reinforcing species boundaries is an exciting and largely
unexplored area.

One additionally intriguing question is whether biases in mate-
preference learning might also play a role in establishing species
boundaries in the first place. If this bias toward learning-en-
hanced signals arose in an early lineage of *Bicyclus*, predating
the current wing pattern, it is possible that the bias toward learning
enhancement permitted the spread and fixation of the enhanced
two-spot forewing *B. anynana* male wing pattern (Fig. 3B, Lower)
from a one-spot ancestor. Further enhancement of male orna-
mentation may have not yet occurred in male *B. anynana*
because of predation pressures against conspicuous wing patterns
in male butterflies (50). The apparent case with which sexual
ornaments are gained and lost in this genus (18)—and the high
diversity of sexual ornamentation in lepidopterans in general (51,
52)—raise the question of whether genetic variation in learning
biases are associated with both gains and losses of sexual orna-
ments in this group, either as secondary reinforcing mechanisms
or as causal drivers of divergence.

Variation in genetic perceptual biases—coupled with drift—are
hypothesized to have contributed to the high diversity in osine
bird song (53). In *Bicyclus*, natural selection is thought to operate
primarily on ventral wing surfaces, leaving dorsal wing surfaces
available for elaboration by sexual selection (50, 54). This signal
partitioning paired with biased signal learning could provide the
substrate for the incredible signal diversification observed in some
butterflies. However, further research across species is necessary
to clarify the extent to which genetic variation in learning bias
drives the evolution and diversification of sexual signals.

Conclusions

These findings demonstrate that a brief social interaction can
alter preexisting mating preferences and highlight the potential
for biased learning to drive assortative mating and potentially
reproductive isolation in species without parental care. Future
research on mate-choice learning should assess the role of
multiple other signals (olfactory, gustatory, tactile, and so forth)
play in visual signal learning, the prevalence of preference
learning in taxa with seasonal polyphenisms compared with other
taxa, and the role of biased learning in driving assortative mating
and speciation.

Materials and Methods

Animal Culture. *B. anynana* is a subtropical African butterfly that has been
maintained in the laboratory since 1988. A colony was established in New
Haven, CT from hundreds of eggs collected from a laboratory colony in
Leiden, The Netherlands (originally established from 80 gravid females col-
lected in Malawi in 1988). The species currently exhibits a sexually mono-
morphic wing pattern, in both natural and laboratory populations, with two
forewing eyespots on the ventral and dorsal surfaces (Fig. 1A and C), seven
hindwing eyespots on the ventral surface, and zero to three hindwing
eyespots on the dorsal surface. The white, UV-reflective scales at the center
of the dorsal wing eyespots in males, but no other eye spot trait, have pre-
viously been demonstrated to be important in female mate selection (16, 17).

Although *B. anynana* is seasonally polyphenic in morphology and be-
havior (17, 55), all butterflies used in this study were reared in wet season
conditions in a walk-in climate chamber at 27 °C, 80% humidity, and 12:12-h
light:dark photoperiod to remove any effect of seasonal phenotype on mat-
ing outcome or learning ability. Larvae were fed on young corn plants
and adults were fed on mashed banana. All behavioral assays (choice trials
and training events) were conducted under sunlamps and in front of east-
-facing windows at 25–28 °C. Behavioral assays were conducted using cylin-
drinal hanging net cages (30 cm diameter × 40 cm height). Butterflies eclosed
in strain-specific (Wt or Sp) emergence cages and were removed and isolated
before noon on the day of eclosion from pupa (day 1). The Sp line has been
maintained in isolation in the laboratory since 1993, and the two lines have
been maintained in the laboratory using similar mating regimes; that is, we
perform egg collections every generation for each line from line-specific
cages containing multiple adult individuals (> 50 individuals in both Sp and
Wt lines). After emergence males were put in sex- and age-specific cases
where they could not see females, and females were isolated from all other
butterflies (males and females) until use in a training event or mating trial.

Behavioral Assays. All experiments followed the same basic design. Virgin
females were isolated from all other butterflies the morning of emergence
(day 0) and either completely isolated for 3 d before preference trials (all
naïve treatments) or exposed to a single virgin male of a given phenotype
for 3 h the morning of emergence and then completely isolated until the
preference trial on day 3 (all exposure treatments).

In all preference trials virgin females were given a choice on day 3 between
two virgin males, matched in age and wing size, with no prior female ex-
perience. A randomly selected male of each phenotype being tested was
introduced to the female’s cage. Males ranged between 2 and 6 d old. Female
mating outcome was determined by dusting the females’ abdomen
with orange “rodent-tracking” florescent dust that is only transferred to
a male abdomen during copulation (56). 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.

Female Preexisting Mating Bias for Spot Number. We conducted mating trials
using both Wt and Sp naïve females. We tested Wt female preexisting
mating bias using Wt and Sp males (*n* = 40 females), and Sp female preex-
isting mating bias using Wt and Sp males (*n* = 42 females).

Preexisting Mating Bias Modified by Experience. We exposed Wt females to Sp
males and conducted preference trials using Wt and Sp males (*n* = 75 females).

Male Modification for Mating Trials. Males were painted with either: (i) two
spots of white, UV-reflective paint (Fish Vision white) (Fig. 1E and F); (ii) one
spot of black paint that did not reflect in the UV (Testors enamel glossy black
1147) (Fig. 1G and H); or (iii) two spots of black paint (Fig. 1I and J).

Quantification of Natural Eyespot Center Reflectance and White, UV-Painted
Spots. Using a bifurcated optical fiber connected to a spectrophotometer
(Ocean Optics USB2000+XR15 Sepsurmete Software), we shined white light
together through one branch and collected the reflectance spectra through the other
branch, from a constant area on the wing (less than 0.5 mm in diameter). *n* = 5
butterflies for natural UV spot (eyespot center), painted UV spot, and
painted extra spot. *n* = 3 butterflies for brown scales. Each butterfly was
measured three times.

Effect of Exposure to Increased Dorsal Forewing Spot Number. Naive, exposed
to two UV, and exposed to four UV, Wt virgin females were used in choice
trials between two and four painted UV spotted males. *n* = 25 females
per treatment.

Test of Learning Bias. Naive, exposed to two UV, and exposed to one UV, Wt
virgin females were used in choice trials between two- and one-spot modi-
fied Wt males *n* = 25 for naive females and females exposed to one spot
males, *n* = 26 for females exposed to control Wt males.

Test of Learning Signal Absence. Naive, exposed to two UV, and exposed to
zero UV, Wt virgin females were used in choice trials between two- and
zero-spot modified Wt males. *n* = 25 for naive females, *n* = 26 for females
exposed to ornament absent and control Wt males. Note that our results of
a nonsignificant preference for zero-sex and two-spot males using naive females are not significantly different from a previously reported naïve preference for two-spot males in the Robertson and Monteiro (16) study (between study $\chi^2 = 0.466, P = 0.4947$). The lack of significance in our preference trials (despite observing the same trend) may be because of the different lighting environments used in the two studies and the fact that here we also controlled for variation in number of dorsal hindwing spots, maintaining them constant in each choice trial. Irrespective of this difference between studies, the present experiments, all done under the same laboratory conditions, support our present conclusions.

**Effect of Male Behavior on Female Learning.** We recorded all bouts of male behavior during the training period for every trial. Males exhibited the following behaviors: (i) a stereotypic courting behavior (35), (ii) flights, (iii) wing flutters, (iv) walking on the cage, and (v) circling of females in flight. A principle components analysis of male behavior by male phenotype was performed using the first principle component (PC2: primarily composed of roughly equivalent loadings of flights, flutters, and walks) and second principle component (PC2: primarily composed of roughly equivalent loadings of circling and courting) were used as representatives of male behavior (Table S5). We also conducted a stepwise nominal logistic model with full factorial design to detect any combination of male behaviors that may have influenced female mating outcome.

**Statistical Analyses.** Mating outcomes between treatments were compared using a Pearson’s $\chi^2$ test, and treatment preferences were deemed significant if mating outcomes differed significantly from random mating (50:50). The relationship between PC1 and PC2 (Table S5) and mating outcome was assessed using logistic regression (Table S1). All statistical analyses were conducted in JMP 9.

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