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Early-exposure to new sex pheromone blend alters mate preference in female butterflies and in their offspring

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Abstract

Insects use species-specific sex pheromone blends to attract members of the opposite sex who express the corresponding molecular receptors. Given this lock and key mechanism used for species identification and mate choice, it is currently not well understood how pheromone blends or receptor systems evolve. One possibility is that insects develop preferences for new sex pheromone blends via the process of learning, and that these learned preferences may be passed on to the next generation. We tested these hypotheses by exposing newly emerged *Bicyclus anynana* female butterflies to either wild type or to modified male sex pheromone blends. A few days later, we scored female mating outcome in a choice trial involving both male types. We also assessed the mating outcome of naïve offspring of females that underwent distinct odor learning trials to test for a potential inheritance of learned odor preferences. Naïve (parental) females mated preferentially with Wt-blend males, but females pre-exposed to new blends either shifted their preference to new-blend males, or mated equally with males of either blend type; the response depending on the new blend they were introduced to. Naïve daughters of females who were exposed to new-blend males behaved similarly to their experienced mothers. We demonstrate that females are able to learn preferences for novel pheromone blends in response to a short social experience, and pass that learned preference down to the next generation. This suggests that learning can be a key factor in the evolution of sex pheromone blend recognition and in chemosensory speciation.

31 **Significance statement**

32 While the diversity of sex pheromone communication systems across insects is well documented, the
33 mechanisms that lead to such diversity are not well understood. Sex pheromones constitute a species-
34 specific system of sexual communication that reinforces interspecific reproductive isolation. When odor
35 blends evolve, the efficacy of male-female communication becomes compromised, unless preference for
36 novel blends also evolves. We explore odor learning as a possible mechanism leading to changes in sex
37 pheromone preferences. We show that preferences for new blends can develop following a short learning
38 experience, and that these novel preferences can be transmitted to the next generation. To our
39 knowledge, this is the first investigation of sex pheromone blend preference learning impacting mate
40 choice and being inherited in an insect.

41

Introduction

The evolution of sexual communication via pheromones is a fascinating area of evolutionary biology because changes in pheromones or their perception may lead to assortative mating, reproductive isolation, and eventually speciation. In most insects, sex pheromones are critical to the process of finding and selecting a mate (1, 2). The composition and relative proportion of the sex pheromone blend components are typically species-specific, and, together with the corresponding specific reception molecules, play a fundamental role in interspecific reproductive isolation (3, 4). Recent studies in Lepidoptera, for instance, support a key role of this chemosensory system in speciation, where both male and female pheromone preferences have diversified along with the evolution of the respective blends (5-8). However, there is still very little understanding of the mechanisms originating divergence in mate preferences for new pheromone blends.

Learning to prefer a novel mate signal early in life could be a possible mechanism driving the evolution of new pheromone communication systems. Learned preferences for novel mate visual signals were previously shown in several arthropod species. In particular, early exposure to new ornamentations in spiders (9), fruit flies (10), or butterflies (11) all led to shifts in mate preferences in sexually mature older individuals. These premating experiences have thus been proposed to play a significant role in reproductive isolation (12, 13). Similar to visual signal learning, odor learning is known to happen routinely in an insect's life. For instance, honeybees learn pollen odors while foraging or after being exposed to pollen at an early age (14), and parasitoids learn the odors of their hosts when laying their eggs (15). Moths can also learn to associate a sex pheromone component with a food reward after a few proboscis extension conditioning trials (16). To date, however, there is no data on whether any insect can learn to prefer novel pheromone blends, via an early exposure, that results in a change in mating outcome. Learned pheromone preferences, over time, could eventually become genetically assimilated and fixed in a population, giving rise to populations of insects with novel pheromone blends and with specific sensitivity for those blends encoded at the genetic level.

A mechanism that could accelerate the process of genetic assimilation could be the transgenerational inheritance of acquired traits (17). Behavioral variations following an environmental experience have been hypothesized to be caused by epigenetic modifications that affect the expression of relevant genes, and which can be inherited through the germline (18). In particular, inheritance of learning and memory processes has already been shown in several species. Attraction of the nematode *Caenorhabditis elegans*

72 to olfactory signals after exposure to these cues was shown to be passed-down to their naïve offspring
73 for several generations (19, 20). A more detailed molecular mechanism of learned odor avoidance was
74 discovered in mice, where deterrence towards an odor was shown to be transmitted to the next
75 generation via the inheritance of a hypomethylated form of the odor receptor gene expressed in the
76 olfactory system (21). These examples illustrate how learning to avoid or prefer an odor might be
77 transmitted by epigenetic marks to the offspring via the germ line. If epigenetic modifications, such as
78 silencing marks, alter patterns of gene expression for a few generations, shielding these regions from
79 natural selection, then genetic mutations are free to also accumulate in these same regions, eventually
80 stabilizing the phenotype that was originally only environmentally induced.

81 In order to contribute to this field of research we tested whether female butterflies can shift their mate
82 preferences after being exposed to males with novel sex pheromone blends, and whether learned
83 preferences can be transmitted to the next generation. We performed these experiments on *Bicyclus*
84 *anynana* butterflies, the only Lepidopteran in which mate preference learning has been shown to take
85 place (11). In particular, females can learn preferences for novel male wing patterns in males if they are
86 exposed to them for a short period after emergence (11). This learned preference, however, only happens
87 if these males express the correct pheromone blend (22). Three male sex pheromones (MSP) have been
88 identified in this species: (Z)-9-tetradecenol (MSP1), hexadecanal (MSP2) and R6, R10, R14-
89 trimethylpentadecan-2-ol (MSP3). They are produced after emergence in specialized wing glands (23, 24).
90 In *B. anynana* of the wet season form, females are the choosy sex (25, 26). Young virgin females frequently
91 reject courting mates before reaching sexual maturity, being exposed to male sex pheromones during this
92 process (23, 27). This particular life history, thus, lends the performance of pheromone learning
93 experiments in this butterfly ecologically relevant.

94 To decide on the type of blend manipulations to do for this experiment we investigated how pheromone
95 blends vary across closely related species. In insects, new sex pheromone blends may evolve in their
96 composition by loss or gain of single components, or by variation in the ratios of components (28). A
97 comparative MSP blend study across *Bicyclus* species showed that close relatives vary both quantitatively
98 and qualitatively in their MSP blend. Sympatric pairs of species display larger differences in component
99 amount and identity than allopatric pairs, suggesting that the MSP blend is involved in pre-mating
100 reproductive isolation in this genus of butterfly (5). We decided, thus, to vary the amounts of blend
101 components in our odor learning experiment in *B. anynana*.

Our experimental design and questions were as follows: We created two New Blends (NB) by either preventing the release of MSP2 and reducing the amounts of MSP1 and MSP3, thus creating a "reduced" blend (called NB1); and by increasing the amount of MSP2, producing an "enriched" blend (called NB2). We exposed immature females to males with these new blends and to males with the respective control manipulations (called Wt1 or Wt2), and observed the mating outcome of the same females in the presence of the two types of male a few days later (Fig. 1). We tested whether 1) new blend males are less attractive to naïve females; 2) females learn to prefer males with new blends and; 3) learned female preferences for one of the blends are transmitted to their offspring.

Results

Male manipulations altered the levels of MSPs

MSP2 was previously suggested to be the most relevant pheromone to female choice because males producing higher absolute or relative amounts of MSP2 had a higher mating success (29). By blocking the pheromone gland on the male hindwing or by perfuming the wing with MSP2, we created two novel blends, NB1 and NB2 respectively, that were different from Wt1 and Wt2 control blends (Fig. 1). In particular, MSP2 was absent in NB1 males, and was increased by 50 fold in NB2 males (30 minutes after perfuming) (Figs. 1b, 1c and S1). Total amounts of MSP1 and MSP3 were reduced by an average of 70% and 60% respectively in NB1 males, compared to Wt1 males (Fig. 1b).

New blend males are not attractive to naïve females

The innate sex pheromone preference of females without any social experience was monitored in a mate-choice assay, where the identity of the male (NB1 versus Wt1; or NB2 versus Wt2) that mated first with that female was scored. Naïve females showed an innate mating bias for the wild type blends in both experiments, with 77% and 70% of the tested females mating first with Wt1 and Wt2 males, respectively (Figs. 2a and 3).

Premating exposure to novel MSP blends modified female innate mating bias.

To test if female mating outcome changed after a short social experience, we exposed different females to either NB1, NB2, or to their corresponding control wild type males, and scored mating outcome in a mate choice assay a few days later. Because the males used for exposure and mate choice were from 4 to 6 days old, we used mixed models to measure the effects of these variable along with the effect of exposure treatment on female mating outcome (see method section for full details). Female premating

132 exposure treatment significantly affected subsequent mating outcomes in both experiment 1 (MERL:
133 χ^2=16.7, Df=4, p=2.2 e^{-4}) and experiment 2 (GLM, F= 6.7, Df=2, p=1.8 e^{-3}). In particular, 90% of the females
134 pre-exposed to Wt1-males mated with Wt1-males, showing a strong significant preference for the Wt1-
135 blend, whereas females pre-exposed to NB1-males showed no mating bias, mating randomly with either
136 male (only 51% mated with Wt1 males; Fig. 2a). These two mating outcomes were significantly different
137 (Post-hoc tests from MERL, adjusted p = 0.018). In the "enriched blend" treatment (Fig. 3), NB2-exposed
138 females mated predominantly with NB2-males (70%), while naïve females mated predominantly with
139 Wt2-males (70%). These two mating outcomes were also significantly different (Post-hoc tests from GLM,
140 adjusted p=0.001). Females pre-exposed to Wt2-males showed no mating bias, 51% of them accepting
141 the NB2-male first for mating.

142 **Female offspring had similar preferences as their exposed mothers**

143 To test for inheritance of learned preferences, we submitted each naïve offspring of NB1-exposed and of
144 Wt1-exposed females to mate choice trials with a single NB1 and a single Wt1 male. Note that the mothers
145 of these female offspring, despite differences in early odor exposure, all mated with Wt males to control
146 for this variable. Offspring of females exposed to Wt1 males mated preferentially with Wt1 males (72%;
147 Pearson's test: χ^2=9.7, p=0.003), whereas offspring of females exposed to NB1 males did not show any
148 mating bias, mating randomly with both male types (57% mated with Wt1-males; Pearson's test: χ^2=0.8,
149 p=0.4), as did their mothers (Fig. 3b). The percentage of matings with NB1 males was 15% higher in
150 offspring of NB1-exposed females than in offspring of Wt1-exposed females (Fig. 3b). For a difference of
151 this magnitude (i.e., effect size) to be significant across offspring types, the sample size would need to be
152 increased to an average of 275 tested female offspring in each group (Table S2).

153 In all experiments, the age of males used for the pre-mating exposure, mating trial, and the position of
154 the black dot placed on the wings to differentiate NB2 and Wt2 males (experiment 2) did not significantly
155 affect mating outcome (Table S3).

156 **Discussion**

157 **Females learned to prefer a mutant pheromone blend**

158 Naïve females mated preferentially with males with a Wt blend over males with either of the mutant
159 blends tested. These results demonstrate the ability of the olfactory circuitry to distinguish the different
160 blends and confirm that the specific male sex pheromone composition and ratios of components are
161 important for *B. anynana* mate selection (23, 27, 29). We demonstrate, however, that an early and brief

162 exposure of females to novel pheromone mutant blends alters their subsequent mating patterns. Initially
163 unattractive males, lacking MSP2 and producing less MSP1 and MSP3, became as attractive as Wt males
164 after a short early-exposure of females to their mutant blend. More strikingly, females mated
165 preferentially with originally unattractive males with high amounts of MSP2, after they were exposed to
166 this new blend. The changes in mating outcome are likely to have resulted from a change in female
167 behavior rather than from alterations in male-male competition or male behavior during the mate choice
168 trial due to the male's different odors. This is because the mate-choice experimental set-up with both
169 males was identical in every treatment. In addition, the shift in the butterflies' mate preference was not
170 influenced by mate-choice copying (30), as all females were isolated from each other and from the males
171 since the pupal stage, and visually isolated from each other at every point in the experiment, including
172 during mate choice trials. These results lead us to conclude that female preference for a male pheromone
173 odor blend in *B. anynana* is not fixed but plastic, and influenced by early pheromone odor experiences.
174
175 Female preference learning was stronger towards NB2 than NB1 blends. In particular, NB2-exposed
176 females preferred NB2 over Wt2 males, but NB1-exposed females only lost their preference bias towards
177 Wt1 males, mating randomly with either male type. Previous work showed that males with either higher
178 absolute or relative levels of MSP2 to other MSP components had higher mating success. MSP2 was, thus,
179 proposed as the most relevant MSP to female choice (29). Consequently, it might be harder for females
180 to overcome the unattractiveness of NB1 compared to NB2 because NB1 is a highly divergent blend
181 lacking MSP2, whereas NB2 has increased amounts and relative ratios of MSP2. The neurological
182 mechanisms involved in this learning asymmetry, however, are still unclear.
183
184 **Alterations of the chemosensory system may be responsible for the change in female blend preference**
185 Brief exposures to odors were previously shown to impact the expression of olfactory receptors, odorant
186 binding proteins, and the development of brain olfactory centers in honeybees and moths (31-34). In the
187 bee, qRT-PCR analysis revealed that 6 floral scent receptors were differentially expressed in the antenna
188 depending on the scent environment they experienced (31). A brief one-minute exposure of male moths
189 to female sex pheromones led to the up-regulation of a pheromone-binding protein in the male antennae,
190 an enlargement of the antennal lobe, and an increase in the volume of the mushroom bodies in the male
191 brain, which resulted in a higher sensitivity of the exposed males to the female blend (32-34). The brief
192 exposure of *B. anynana* females to the new male pheromone blend may have led to similar changes in
193 the female brain. The mechanisms in place, however, require future exploration.

194

195 **Learning to prefer a mutant blend male may have important evolutionary consequences**

196 Both empirical and theoretical studies have highlighted how the learning of a trait or a mate preference
197 can impact assortative mating and population divergence (12, 35). Depending on the specific ecological
198 conditions, type of trait, or learning process, models predict that mate preference learning can lead to
199 reproductive isolation (*e.g.* (13, 36). Moth and butterfly sex pheromone blends are highly species-specific,
200 ensuring the precise recognition of a compatible mate. These blends are generally thought to be under
201 stabilizing selection because altered signals are less attractive and are thus selected against (28). However,
202 the learning process that we describe here, by allowing males with divergent blends to reproduce, may
203 mitigate the strength of stabilizing selection, and create opportunities for pheromone blends and
204 reception systems to evolve. A recent study suggested that quantitative and qualitative variations
205 observed in blends within and between natural *B. anynana* populations are potentially catalyzing ongoing
206 speciation (6). The odor learning ability of *B. anynana* females has probably maintained the high variance
207 in MSP amounts measured in different stock populations (23, 24, 29), as well as the variance in MSPs
208 detected across natural populations (6). Furthermore, the use of multimodal signals in mate selection in
209 *B. anynana,* where females use both olfactory and visual signals to assess mate quality (11, 22, 27), may
210 facilitate pheromone learning and the evolution of the MSP blends. The presence of species-specific visual
211 cues on the male wings likely increases a female's acceptance of odor-unattractive males from the same
212 species, and decreases the risks of females learning new blends from hetero-specifics that could lead to
213 hetero-specific mating. Thus, learning to prefer novel odors or odor blends may be a key starting point in
214 the process of reproductive isolation and speciation, especially if this preference can be transmitted to
215 the next generation via the germ line.

216

217 **Transgenerational inheritance of pheromone preferences may facilitate the evolution of assortative**
218 **mating and speciation**

219 Naïve female offspring of mothers exposed to NB1 blends stopped avoiding NB1 blends, as did their naïve
220 mothers, indicating that habituation towards this new blend was transgenerationally inherited. Daughters
221 of females exposed to Wt-blend males, however, did not increase their preference for Wt-blend males.
222 This lack of transmission of a more extreme preference for Wt blends in female offspring could be
223 explained by an exhaustion of genetic variation, since exposure of females to wild type butterflies has
224 been repeatedly done presumably since the origin of this species. Because all F1 individuals were kept

225 completely isolated from their conspecifics until mate choice, a change in F1 female preference is also not
226 a result of social transmission, but more likely mediated via epigenetic mechanisms.
227
228 The transgenerational inheritance of acquired behaviors remains a controversial topic despite the growing
229 number of empirical studies supporting it, including mechanistic studies. For instance, first- and second-
230 generation naïve *Drosophila melanogaster* offspring displayed a preference toward the alcoholic odors
231 their parent where trained to like. Disruption of the F0 olfactory receptors and specific neuron inputs into
232 the mushroom bodies abolished the change in offspring response, identifying potential targets of
233 epigenetic transmission (37). In addition, a number of studies have revealed that DNA methylation
234 regulates memory formation and learning processes in insects (*e.g.* in bees (38, 39)) but have not
235 investigated whether these marks can be inherited to the next generation. Inheritance of a differentially
236 methylated odor receptor gene, however, was shown to take place in mice that learned to avoid a specific
237 odor (21). We speculate that in our system, genes involved in odor perception and/or processing may
238 have mediated the transmission of odor preferences to female offspring via yet unknown epigenetic
239 mechanisms. A transmission of acquired pheromone odor preferences may favor assortative mating and
240 chemosensory speciation.
241

242 **Conclusion**

243 We have demonstrated the learning, and the inheritance of new behavioral responses to new sex
244 pheromone blends by female *B. anynana* butterflies, calling into question the belief that sexual chemical
245 communication is under stabilizing selection. Over time, as new pheromone blends appear, and new
246 learned sex pheromone preferences for those blends develop, new populations of insects may evolve with
247 specific sensitivity for those blends encoded at the genetic level. Learning to prefer a new sex pheromone
248 blend could be the starting point of the evolution of chemosensory communication, especially if the
249 learned preferences can be inherited.
250

251 **Methods**

252 **Husbandry**

253 *Bicyclus anynana* is an African butterfly that produces alternative seasonal phenotypes in response to
254 environmental cues (40). To avoid the seasonal variations in courtship behavior (26), eye size and UV light
255 perception (41), and sex pheromone production (24), we performed all experiments with wet season

256 butterflies, all reared at 27°C, 80% humidity and 12:12h light:dark photoperiod. Larvae were fed young
257 corn plants, and adults mashed banana. Sex was determined at the pupal stage, and females were placed
258 in individual containers stored in a separated incubator, devoid of males or male sex pheromones until a
259 male exposure or a mating trial. Upon emergence, males were put in age-specific cages. They were all
260 naïve, virgin, aged from 4 to 6 days old during the experiment and had dorsal forewing eyespot UV-
261 reflective pupils (as their absence in males is strongly selected against by females (25)). The two males
262 presented to each female for a mating trial had the same age and similar wing size. The experimental
263 procedure is described in Fig. 1.

264 **Experiment 1: Prevention of MSP2 release from males**

265 Males were prepared following the method described in (27). The ventral hindwing androconia and yellow
266 hair pencil were both coated with transparent non-viscous nail solution (Revlon Liquid Quick Dry). The
267 hindwing dark hair patch, which overlaps the forewing androconia, was left uncoated. This treatment
268 prevents the emission of MSP2 produced by hindwing glands only (23), and causes the reduction of MSP1
269 and MSP3 total amounts by an average of 70% and 60% respectively (Fig. 1b) (24). The hindwing ventral
270 side of Wild type (Wt1) males received the same treatment to control for the odor of the nail solution.
271 Males were prepared ~16 hours prior to exposure or mate choice trials (Figs. 1a and 1b).

272 **Experiment 2: Increase of MSP2 amount in males**

273 5µg of MSP2 (Cayman Chemical, n°9001996) diluted in 2µL of hexane were applied to each hindwing
274 androconia of NB2 males. Wild type control males (Wt2) received the same volume of solvent only in the
275 same wing location (Fig. 1c). This high load of synthetic hexadecanal was chosen to maximize the
276 difference between MSP2 amounts of NB2 and Wt2 butterflies until several hours after application of the
277 solution (Fig. S1). The evaporation rate of hexadecanal was determined by gas chromatography from 30
278 minutes to 8 hours after perfuming. Between perfuming and MSP extraction, two males were placed
279 together in one cylindrical hanging net cage, under identical temperature, humidity and light conditions
280 than the ones used for the mate choice experiment (see "Mate choice assays" below and Supplementary
281 procedure 1). Males were allowed to rest 30 minutes after perfuming until used for exposure or mate
282 choice trials. At the end of this period, NB2 males had similar amounts of MSP2 and MSP3 on their wings
283 (Fig. 1c).

284 **Female exposure to New Blend or Wild type males**

285 The female butterfly was released in a cylindrical hanging net cage (30cm diameter, 40cm height) less
286 than an hour after emergence (on day 0). The exposure was done manually by retaining the male between

287 the head and the thorax with narrow-tipped featherweight forceps for 3 minutes. The males were
288 presented directly to the females in a similar way as the natural courtship behavior (same distance and
289 orientation). In this position, male fluttering, the first step of the courtship sequence, helped the
290 volatilization of the pheromones and could be encouraged by a gentle squeezing of the forceps (Fig. 1d).
291 This procedure allowed a direct and controlled exposure of the females, and was non-harmful to the
292 males. After exposure, the female remained isolated until day 2, when mate choice assays were conducted
293 (Fig. 1a).

294 **Mate choice assays**

295 All experiments were done at 24°C, 60% humidity, under UV and white light, in cylindrical hanging net
296 cages. Mate choice of naïve and exposed females was started on day 2, around 9:30am (Fig. 1a). One Wt
297 and one NB male were placed in the same cage along with the female. Female's abdomens were pre-
298 dusted with fluorescent orange powder which is transmitted to the male upon copulation, allowing the
299 identification of the mating partner. Males were checked for presence of powder every 2 hours to prevent
300 multiple mating. Assays were ended after 8 hours after the beginning of the experiment. The latter time
301 point corresponds to MSP2 amounts becoming similar between Wt2 and perfumed males (Fig. S1). To
302 differentiate NB2 and Wt2 males, a black dot was applied with a sharpie pen randomly at the top or the
303 bottom of their ventral hindwing. NB1 and Wt1 males were recognizable thanks to the light grey color of
304 the nail solution covering the androconia or the corresponding area of the wing on the opposite side.

305 **Testing the transgenerational inheritance of mate choice preferences**

306 An additional group of females were exposed to either NB1 or WT1 males, following the same exposure
307 protocol as described above. We didn't test the preference of offspring of females that choose NB1 males,
308 but instead, each female was mated with a single naïve Wt males in a separate cage. This procedure was
309 followed to prevent possible confounding effects of the mate choice experiment and any predisposed
310 genetic preferences that females may have. The male was removed after mating and the female given a
311 corn plant for egg collection. Each female and its offspring (F1 individuals) constituted a family. F1 pupae
312 were sexed, and the females were submitted to the exact same isolation procedure as naïve females until
313 mate choice assays between a NB1 and a Wt1 male, tested on day 2 using identical procedures as
314 described above (Fig. 1). Around 5 females were tested from the 13 Wt1 and the 11 NB1 families.

315 **Statistical analyses**

316 Results from experiment 1, including offspring mate choice, and from experiment 2 were analyzed
317 separately using R v. 3.2.4 (42) implemented in RStudio v.1.0.136 (43). P-values were obtained by

likelihood ratio tests of full regression models tested against simplified models with specific factors removed.

A Mixed Effect Logistic Regression (MELR) was used to analyze females and their offspring mate choice (experiment 1), as this model includes both fixed and random effects. *Female mate choice* was the binomial response (NB1 male chosen or not). The *family* identity was implemented as a random factor in the model. Each female from the parental generation, taken from our stock population cage, was considered as belonging to different families. The fixed factors included the female *treatment* (NB1-exposed females, offspring of NB1-exposed females, Wt1-exposed females, offspring of Wt1-exposed females, and naïve females) and the *age of males used for mate choice* (4, 5 or 6 days old). The analysis was followed by a pairwise comparison of the significant fixed effects using Tukey Contrasts. Because naïve females (including offspring) were not exposed, the effect of *male age during exposure* (4, 5 or 6 days old) on female choice was analyzed separately with a binomial logistic regression. Packages lme4 (44) and multcomp (45) were used.

The factors that contributed to *female mate choice* in experiment 2 were analyzed with a logistic regression, fitting a Generalized Linear Model (GLM) with quasi-binomial errors to control for over-dispersion, and a logit-link function. Fixed factors used in the model included *treatment* (NB2-exposed females, Wt2-exposed females and naïve females), *male age during exposure* and *male age for mate choice* (in both steps, they were 4, 5 or 6 days old), and the *position of the black mark* used to identify NB2 and Wt2 males (the bottom or the top of the wing).

Finally, in both experiments, actual preference for the NB or the WT blend was tested using a Pearson's χ^2 test in R. Blends were considered as preferred by females if mate choice differed significantly from random mating (50:50).

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454

455 **Figure Legends**

456 **Figure 1. Experimental procedure**. (a) The timeline of the experiment indicating when each step was
457 performed. (b) Coating of the male androconia (NB1 males) prevented the release of MSP2 and reduced
458 the total amount of MSP1 and MSP3 per male. (c) The average total amount of MSP2 per NB2 male, 30
459 minutes after perfuming with synthetic hexadecanal, is increased compared to Wt2 males. In each graph,
460 the horizontal line and the point in each box are the median and the mean amount, respectively. The 25th
461 and 75th percentiles are contained within the outline of the boxes, and the horizontal lines above and
462 below each box show the 1.5 times inter-quartile range of the data. 5 to 10 males were used to measure
463 MSP amount in each treatment. (d) Schematics of the female exposure where the bottom panel illustrates
464 the position of both male and female individuals from a top view.

465 **Figure 2. Mating outcome of females after exposure to the "reduced" (NB1) and wild type pheromone**
466 **blends, and mating outcome of their naïve offspring**. (a) Mating outcomes shifted after females were
467 exposure to a male with a reduced blend. Most naïve females, and females exposed to Wt1 blend mated
468 with Wt1 males, but females exposed to NB1 males mated with these males at significantly higher rates
469 than Wt1-exposed females. (b) Offspring of females exposed to Wt1 mated preferentially with Wt1 males,
470 similarly to naïve and Wt1-exposed females from the parental generation. However, offspring of NB1-
471 exposed females mated equally with either male type. Asterisks (* $p<0.05$; ** $p<0.01$; ** $p<0.001$) indicate
472 statistically significant preferences for the Wt1 blend using Pearson's χ^2 test. The dotted line at 50%
473 illustrates random mating. The horizontal bar above the plot shows a significant difference in mating
474 outcome between the two treatments (from the Tukey post-hoc test, adjusted p value is indicated). The
475 "n" on each bar indicates the total number of female tested. Post-hoc test results providing adjusted p
476 values comparing the different treatments are shown in Table S1a.

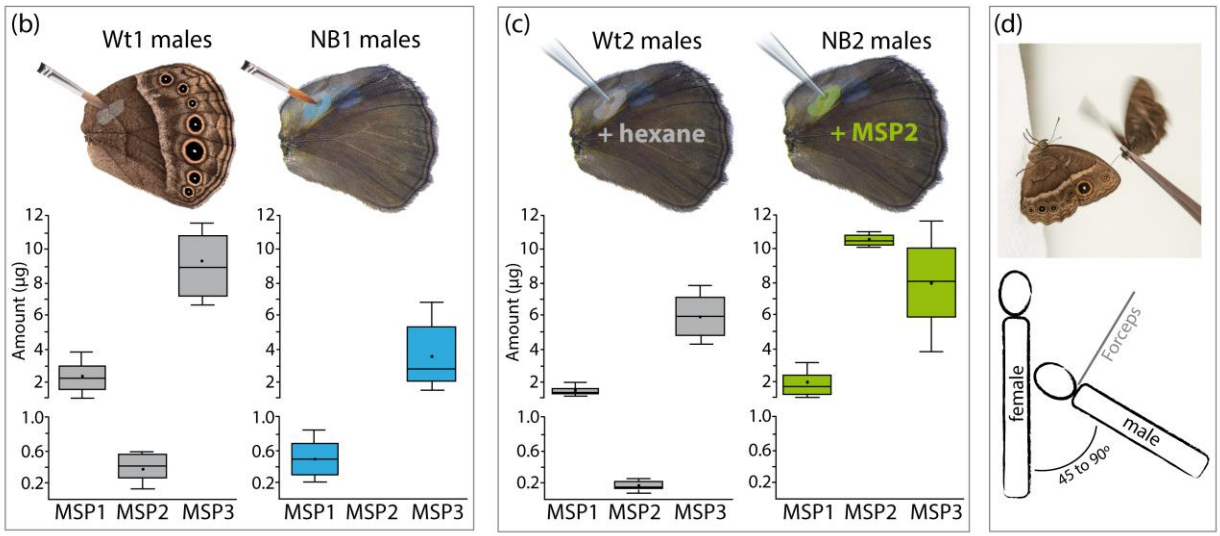
477 **Figure 3. Mating outcome of females after exposure to the "enriched" (NB2) and wild type pheromone**
478 **blends.** In experiment 2, females shifted their mating outcome after exposure to a Wt2 male or a male
479 perfumed with a novel pheromone blend containing more MSP2. Most naïve females mated with Wt2
480 males, females exposed to the Wt2 blend mated equally with both male types, and females exposed to
481 the new blend mated with new blend males at significantly higher rates than naïve females. The dotted
482 line at 50% illustrates random mating. Asterisks (* $p<0.05$; ** $p<0.01$) represent non-random mating
483 outcomes using Pearson's χ^2 test. The horizontal bar above the plot shows a significant difference in
484 mating outcome between the two designated treatments (from the Tukey post-hoc test, adjusted p value

is indicated). The “n” on top of each bar indicates the total number of female tested. Post-hoc test results providing adjusted p values comparing the different treatments are shown in Table S1b.

488 **Figure 1.**

(a)

TIMELINE	Day -1	Day 0	Day 1	Day 2	Every day
EXPERIMENTAL STEPS	Preparation of Wt1 and NB1 males for exposure the next day	Female emergence and exposure Perfuming of Wt2 and NB2 males for exposure the same day	Preparation of Wt1 and NB1 males for mate choice the next day	Mate choice Perfuming of Wt2 and NB2 males for mate choice the same day	Isolation of emerged males



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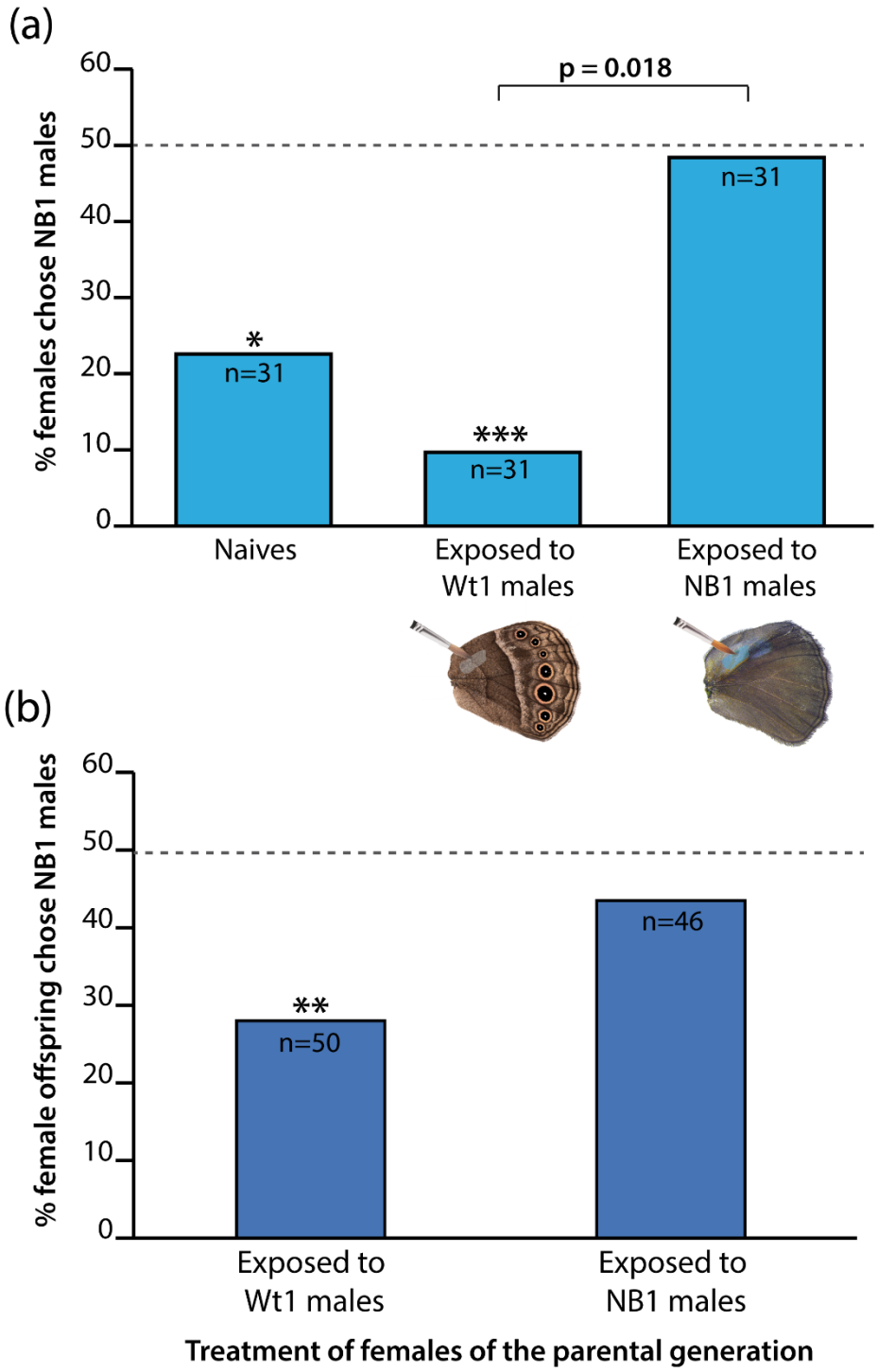
491 **Figure 2.**



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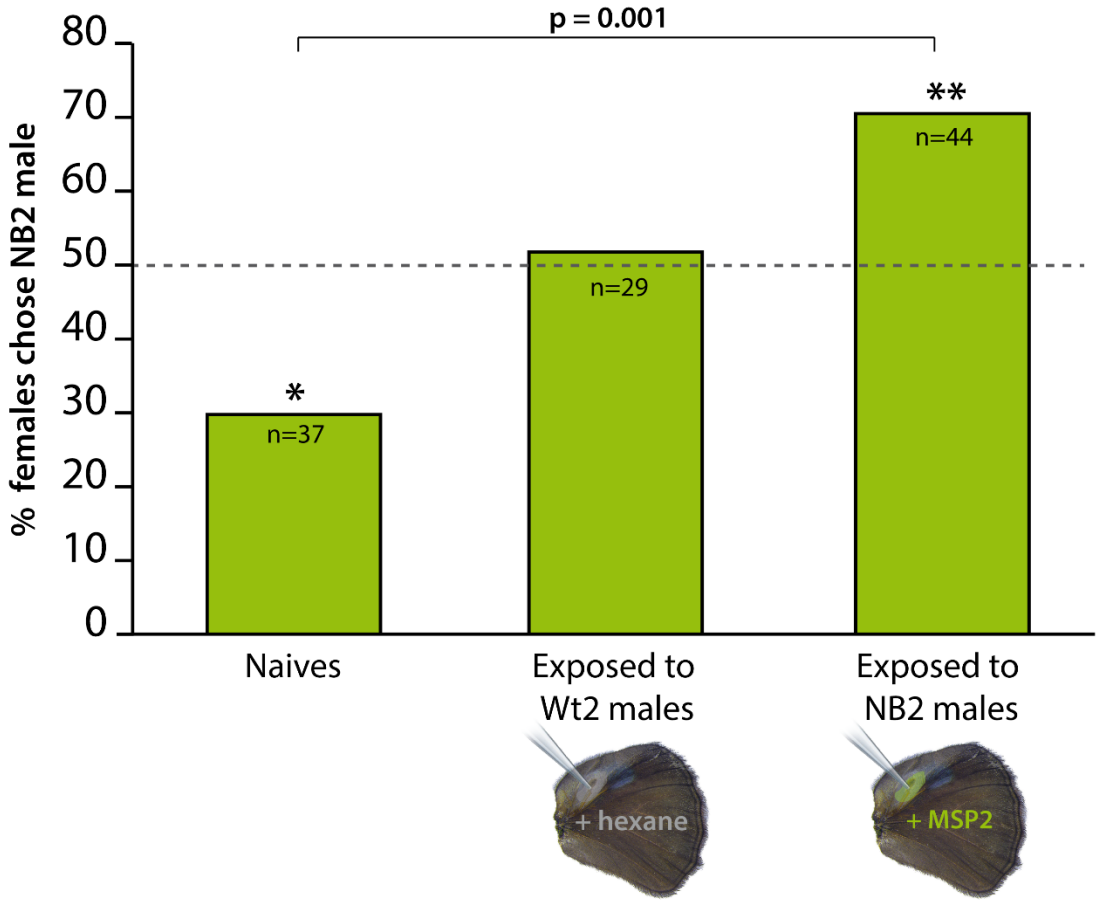
494 **Figure 3.**



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