

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

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10 inheritance

11

12 **Abstract**

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

30

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
36 for novel blends also evolves. We explore odor learning as a possible mechanism leading to changes in
37 sex pheromone preferences. We show that preferences for new blends can develop following a short
38 learning 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

42 **Introduction**

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

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

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

72 *Caenorhabditis elegans* to olfactory signals after exposure to these cues was shown to be passed-down
73 to their naïve offspring for several generations (19, 20). A more detailed molecular mechanism of
74 learned odor avoidance was discovered in mice, where deterrence towards an odor was shown to be
75 transmitted to the next generation via the inheritance of a hypomethylated form of the odor receptor
76 gene expressed in the olfactory system (21). These examples illustrate how learning to avoid or prefer
77 an odor might be transmitted by epigenetic marks to the offspring via the germ line. If epigenetic
78 modifications, such as silencing marks, alter patterns of gene expression for a few generations, shielding
79 these regions from natural selection, then genetic mutations are free to also accumulate in these same
80 regions, eventually stabilizing the phenotype that was originally only environmentally induced (22).

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 *anyana* 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
87 happens if these males express the correct pheromone blend (23). Three male sex pheromones (MSP)
88 have been 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 (24,
90 25). In *B. anyana* of the wet season form, females are the choosy sex (26, 27). Young virgin females
91 frequently reject courting mates before reaching sexual maturity, being exposed to male sex
92 pheromones during this process (24, 28). This particular life history, thus, lends the performance of
93 pheromone learning 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 (29). A
97 comparative MSP blend study across *Bicyclus* species showed that close relatives vary both
98 quantitatively and qualitatively in their MSP blend. Sympatric pairs of species display larger differences
99 in component amount and identity than allopatric pairs, suggesting that the MSP blend is involved in
100 pre-mating reproductive isolation in this genus of butterfly (5). We decided, thus, to vary the amounts of
101 blend components in our odor learning experiment in *B. anyana*.

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

110

111 **Results**

112 **Male manipulations altered the levels of MSPs**

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

120 **New blend males are not attractive to naïve females**

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

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

127 To test if female mating outcome changed after a short social experience, we exposed different females
128 to either NB1, NB2, or to their corresponding control wild type males, and scored mating outcome in a
129 mate choice assay a few days later. Because the males used for exposure and mate choice were from 4
130 to 6 days old, we used mixed models to measure the effects of these variable along with the effect of
131 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 $\chi^2=16.7$, Df=4, $p=2.2 \times 10^{-4}$) and experiment 2 (GLM, $F= 6.7$, Df=2, $p=1.8 \times 10^{-3}$). In particular, 90% of the
134 females pre-exposed to Wt1-males mated with Wt1-males, showing a strong significant preference for
135 the Wt1-blend, whereas females pre-exposed to NB1-males showed no mating bias, mating randomly
136 with either male (only 51% mated with Wt1 males; Fig. 2a). These two mating outcomes were
137 significantly different (Post-hoc tests from MERL, adjusted $p = 0.018$). In the “enriched blend” treatment
138 (Fig. 3), females pre-exposed to Wt2-males showed no mating bias, 51% of them accepting the NB2-
139 male first for mating. NB2-exposed females mated predominantly with NB2-males (70%), while naïve
140 females mated predominantly with Wt2-males (70%). These two mating outcomes were also
141 significantly different (Post-hoc tests from GLM, adjusted $p=0.001$).

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
145 mothers of these female offspring, despite differences in early odor exposure, all mated with Wt males
146 to control for this variable. Offspring of females exposed to Wt1 males mated preferentially with Wt1
147 males (72%; Pearson’s test: $\chi^2=9.7$, $p=0.003$), whereas offspring of females exposed to NB1 males did
148 not show any mating bias, mating randomly with both male types (57% mated with Wt1-males;
149 Pearson’s test: $\chi^2=0.8$, $p=0.4$), as did their mothers (Fig. 2b). The percentage of matings with NB1 males
150 was 15% higher in offspring of NB1-exposed females than in offspring of Wt1-exposed females (Fig. 2b).
151 For a difference of this magnitude (i.e., effect size) to be significant across offspring types, the sample
152 size would need to be increased to an average of 275 tested female offspring in each group
153 (Supplementary Table 2).

154 In all experiments, the age of males used for the pre-mating exposure, mating trial, and the position of
155 the black dot placed on the wings to differentiate NB2 and Wt2 males (experiment 2) did not
156 significantly affect mating outcome (Supplementary Table 3).

157 **Discussion**

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

159 Naïve females mated preferentially with males with a Wt blend over males with either of the mutant
160 blends tested. These results demonstrate the ability of the olfactory circuitry to distinguish the different
161 blends and confirm that the specific male sex pheromone composition and ratios of components are

162 important for *B. anynana* mate selection (24, 28, 30). We demonstrate, however, that an early and brief
163 exposure of females to novel pheromone mutant blends alters their subsequent mating patterns.
164 Initially unattractive males, lacking MSP2 and producing less MSP1 and MSP3, became as attractive as
165 Wt males after a short early-exposure of females to their mutant blend. More strikingly, females mated
166 preferentially with originally unattractive males with high amounts of MSP2, after they were exposed to
167 this new blend. The changes in mating outcome are likely to have resulted from a change in female
168 behavior rather than from alterations in male-male competition or male behavior during the mate
169 choice trial due to the male's different odors. This is because the mate-choice experimental set-up with
170 both males was identical in every treatment. In addition, the shift in the butterflies' mate preference
171 was not influenced by mate-choice copying (31), as all females were isolated from each other and from
172 the males since the pupal stage, and visually isolated from each other at every point in the experiment,
173 including during mate choice trials. These results lead us to conclude that female preference for a male
174 pheromone odor blend in *B. anynana* is not fixed but plastic, and influenced by early pheromone odor
175 experiences.

176
177 Female preference learning was stronger towards NB2 than NB1 blends but it is still unclear why this
178 was the case. In particular, NB2-exposed females preferred NB2 over Wt2 males, but NB1-exposed
179 females only lost their preference bias towards Wt1 males, mating randomly with either male type.
180 Previous work showed that males with either higher absolute or relative levels of MSP2 to other MSP
181 components had higher mating success. MSP2 was, thus, proposed as the most relevant MSP to female
182 choice (30). Here our data for naïve female mating outcome showed that females actually discriminate
183 against males with very high levels of MSP2, but upon exposure to these high levels, females
184 subsequently mate more frequently with these males. We propose that it might be harder for exposed
185 females to overcome the unattractiveness of NB1 compared to NB2 because NB1 is a highly divergent
186 blend lacking MSP2, whereas NB2 has increased amounts and relative ratios of MSP2. Another
187 possibility for this asymmetry in learning, which will need additional testing in future, is that female
188 exposure to enhanced blends (with additional components) relative to Wt blends, leads to overall
189 stronger mate discrimination ability, whereas exposure to weaker blends relative to Wt, leads to loss of
190 mate discrimination abilities. When females are exposed to low amounts or absence of components (as
191 it is the case for NB1- and Wt2-exposed butterflies), they are less discriminatory and mate randomly
192 with either male. However, when females are exposed to higher levels of blend components (such as
193 Wt1-and NB2-exposed females), they discriminate between NB and Wt males, preferring the blend they

194 have been exposed to. We also note that an increase in MSP2 amount alone (as in NB2 males) is
195 sufficient to trigger a change in female discriminating abilities, confirming that this component is
196 important in *B. anynana* mate choice. The neurological mechanisms involved in this process, however,
197 are still unclear.

198

199 **Alterations of the chemosensory system may be responsible for the change in female blend** 200 **preference**

201 Brief exposures to odors were previously shown to impact the expression of olfactory receptors,
202 odorant binding proteins, and the development of brain olfactory centers in honeybees and moths (32-
203 35). In the bee, qRT-PCR analysis revealed that 6 floral scent receptors were differentially expressed in
204 the antenna depending on the scent environment they experienced (32). A brief one-minute exposure
205 of male moths to female sex pheromones led to the up-regulation of a pheromone-binding protein in
206 the male antennae, an enlargement of the antennal lobe, and an increase in the volume of the
207 mushroom bodies in the male brain, which resulted in a higher sensitivity of the exposed males to the
208 female blend (33-35). The brief exposure of *B. anynana* females to the new male pheromone blend may
209 have led to similar changes in the female brain. The mechanisms in place, however, require future
210 exploration.

211

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

213 Both empirical and theoretical studies have highlighted how the learning of a trait or a mate preference
214 can impact assortative mating and population divergence (12, 36). Depending on the specific ecological
215 conditions, type of trait, or learning process, models predict that mate preference learning can lead to
216 reproductive isolation (e.g. (13, 37). Moth and butterfly sex pheromone blends are highly species-
217 specific, ensuring the precise recognition of a compatible mate. These blends are generally thought to
218 be under stabilizing selection because altered signals are less attractive and are thus selected against
219 (29). However, the learning process that we describe here, by allowing males with divergent blends to
220 reproduce, may mitigate the strength of stabilizing selection, and create opportunities for pheromone
221 blends and reception systems to evolve. A recent study suggested that quantitative and qualitative
222 variations observed in blends within and between natural *B. anynana* populations are potentially
223 catalyzing ongoing speciation (6). The odor learning ability of *B. anynana* females has probably
224 maintained the high variance in MSP amounts measured in different stock populations (24, 25, 30), as
225 well as the variance in MSPs detected across natural populations (6). Furthermore, the use of

226 multimodal signals in mate selection in *B. anynana*, where females use both olfactory and visual signals
227 to assess mate quality (11, 23, 28), may facilitate pheromone learning and the evolution of the MSP
228 blends. The presence of species-specific visual cues on the male wings likely increases a female's
229 acceptance of odor-unattractive males from the same species, and decreases the risks of females
230 learning new blends from hetero-specifics that could lead to hetero-specific mating. Thus, learning to
231 prefer novel odors or odor blends may be a key starting point in the process of reproductive isolation
232 and speciation, especially if this preference can be transmitted to the next generation via the germ line.

233

234 **Transgenerational inheritance of pheromone preferences may facilitate the evolution of assortative** 235 **mating and speciation**

236 Naïve female offspring of mothers exposed to NB1 blends stopped avoiding NB1 blends, as did their
237 naïve mothers, indicating that habituation towards this new blend was transgenerationally inherited.
238 Daughters of females exposed to Wt-blend males, however, did not increase their preference for Wt-
239 blend males. This lack of transmission of a more extreme preference for Wt blends in female offspring
240 could be explained by an exhaustion of genetic variation, since exposure of females to wild type
241 butterflies has been repeatedly done presumably since the origin of this species. Because all F1
242 individuals were kept completely isolated from their conspecifics until mate choice, a change in F1
243 female preference is also not a result of social transmission, but more likely mediated via epigenetic
244 mechanisms.

245

246 The transgenerational inheritance of acquired behaviors remains a controversial topic despite the
247 growing number of empirical work supporting it, including mechanistic studies. For instance, first- and
248 second-generation naïve *Drosophila melanogaster* offspring displayed a preference toward the alcoholic
249 odors their parent where trained to like. Disruption of the F0 olfactory receptors and specific neuron
250 inputs into the mushroom bodies abolished the change in offspring response, identifying potential
251 targets of epigenetic transmission (38). In addition, a number of studies have revealed that DNA
252 methylation regulates memory formation and learning processes in insects (*e.g.* in bees (39, 40)) but
253 have not investigated whether these marks can be inherited to the next generation. Inheritance of a
254 differentially methylated odor receptor gene, however, was shown to take place in mice that learned to
255 avoid a specific odor (21). We speculate that in our system, genes involved in odor perception and/or
256 processing may have mediated the transmission of odor preferences to female offspring via yet

257 unknown epigenetic mechanisms. A transmission of acquired pheromone odor preferences may favor
258 assortative mating and chemosensory speciation.

259

260 **Conclusion**

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

268

269 **Methods**

270 **Husbandry**

271 *Bicyclus anynana* is an African butterfly that produces alternative seasonal phenotypes in response to
272 environmental cues (41). To avoid the seasonal variations in courtship behavior (27), eye size and UV
273 light perception (42), and sex pheromone production (25), we performed all experiments with wet
274 season butterflies, all reared at 27°C, 80% humidity and 12:12h light:dark photoperiod. Larvae were fed
275 young corn plants, and adults mashed banana. Sex was determined at the pupal stage, and females
276 were placed in individual containers stored in a separated incubator, devoid of males or male sex
277 pheromones until a male exposure or a mating trial. Upon emergence, males were put in age-specific
278 cages. They were all naïve, virgin, aged from 4 to 6 days old during the experiment and had dorsal
279 forewing eyespot UV-reflective pupils (as their absence in males is strongly selected against by females
280 (26)). The two males presented to each female for a mating trial had the same age and similar wing size.
281 The experimental procedure is described in Fig. 1.

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

283 Males were prepared following the method described in (28). The ventral hindwing androconia and
284 yellow hair pencil were both coated with transparent non-viscous nail solution (Revlon Liquid Quick
285 Dry). The hindwing dark hair patch, which overlaps the forewing androconia, was left uncoated. This
286 treatment prevents the emission of MSP2 produced by hindwing glands only (24), and causes the

287 reduction of MSP1 and MSP3 total amounts by an average of 70% and 60% respectively (Fig. 1b) (25).
288 The hindwing ventral side of Wild type (Wt1) males received the same treatment to control for the odor
289 of the nail solution. Males were prepared ~16 hours prior to exposure or mate choice trials (Figs. 1a and
290 1b).

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

292 5 μ g of MSP2 (Cayman Chemical, n°9001996) diluted in 2 μ L of hexane were applied to each hindwing
293 androconia of NB2 males. Wild type control males (Wt2) received the same volume of solvent only in
294 the same wing location (Fig. 1c). Hexane was used as a solvent as it didn't impact naïve female choice
295 (tested in a mate choice assay, described in the Supplementary file 1). The high load of synthetic
296 hexadecanal was chosen to maximize the difference between MSP2 amounts of NB2 and Wt2 butterflies
297 until several hours after application of the solution (Supplementary Fig. 1). The evaporation rate of
298 hexadecanal was determined by gas chromatography from 30 minutes to 8 hours after perfuming.
299 Between perfuming and MSP extraction, two males were placed together in one cylindrical hanging net
300 cage, under identical temperature, humidity and light conditions than the ones used for the mate choice
301 experiment (see "Mate choice assays" below and Supplementary procedure 1). Males were allowed to
302 rest 30 minutes after perfuming until used for exposure or mate choice trials. At the end of this period,
303 NB2 males had similar amounts of MSP2 and MSP3 on their wings (Fig. 1c).

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

305 The female butterfly was released in a cylindrical hanging net cage (30cm diameter, 40cm height) less
306 than an hour after emergence (on day 0). The exposure was done manually by retaining the male
307 between the head and the thorax with narrow-tipped featherweight forceps for 3 minutes. The males
308 were presented directly to the females in a similar way as the natural courtship behavior (same distance
309 and orientation). In this position, male fluttering, the first step of the courtship sequence, helped the
310 volatilization of the pheromones and could be encouraged by a gentle squeezing of the forceps (Fig. 1d).
311 This procedure allowed a direct and controlled exposure of the females, and was non-harmful to the
312 males. After exposure, the female remained isolated until day 2, when mate choice assays were
313 conducted (Fig. 1a). Each female (naïve included) was allocated an identification number which doesn't
314 indicate the treatment she was submitted to, so that the investigator was unaware of the sample group
315 allocation during the mate choice experiment and when assessing its outcome.

316 **Mate choice assays**

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

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

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

339 **Statistical analyses**

340 Results from experiment 1, including offspring mate choice, and from experiment 2 were analyzed
341 separately using R v. 3.2.4 (43) implemented in RStudio v.1.0.136 (44). P-values were obtained by
342 likelihood ratio tests of full regression models tested against simplified models with specific factors
343 removed.

344 A Mixed Effect Logistic Regression (MELR) was used to analyze females and their offspring mate choice
345 (experiment 1), as this model includes both fixed and random effects. *Female mate choice* was the
346 binomial response (NB1 male chosen or not). The *family* identity was implemented as a random factor in
347 the model. Each female from the parental generation, taken from our stock population cage, was

348 considered as belonging to different families. The fixed factors included the female *treatment* (NB1-
349 exposed females, offspring of NB1-exposed females, Wt1-exposed females, offspring of Wt1-exposed
350 females, and naïve females) and the *age of males used for mate choice* (4, 5 or 6 days old). The analysis
351 was followed by a pairwise comparison of the significant fixed effects using Tukey Contrasts. Because
352 naïve females (including offspring) were not exposed, the effect of *male age during exposure* (4, 5 or 6
353 days old) on female choice was analyzed separately with a binomial logistic regression. Packages lme4
354 (45) and multcomp (46) were used.

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

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

364

365 **Data availability**

366 The datasets generated during the current study have been submitted to the Institutional repository of
367 the National University of Singapore ScholarBank@NUS (<http://scholarbank.nus.edu.sg/>).

368

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375

376 **Author contributions**

377 ED and AM designed the study; ED and LXP performed the experiments and analyzed the data; ED, LXP
378 and AM wrote the manuscript.

379

380 **Competing financial interests**

381 The authors declare no competing financial interests.

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489 **Figure Legends**

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

499 **Figure 2. Mating outcome of females after exposure to the “reduced” (NB1) and wild type pheromone**
500 **blends, and mating outcome of their naïve offspring.** (a) Mating outcomes shifted after females were
501 exposure to a male with a reduced blend. Most naïve females, and females exposed to Wt1 blend mated
502 with Wt1 males, but females exposed to NB1 males mated with these males at significantly higher rates
503 than Wt1-exposed females. (b) Offspring of females exposed to Wt1 mated preferentially with Wt1
504 males, similarly to naïve and Wt1-exposed females from the parental generation. However, offspring of
505 NB1-exposed females mated equally with either male type. Asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)
506 indicate statistically significant preferences for the Wt1 blend using Pearson’s χ^2 test. The dotted line at
507 50% illustrates random mating. The horizontal bar above the plot shows a significant difference in
508 mating outcome between the two treatments (from the Tukey post-hoc test, adjusted p value is
509 indicated). The “n” on each bar indicates the total number of female tested. Post-hoc test results
510 providing adjusted p values comparing the different treatments are shown in Supplementary Table 1a.

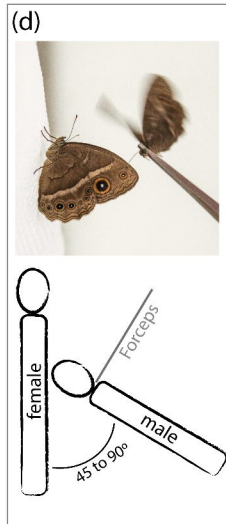
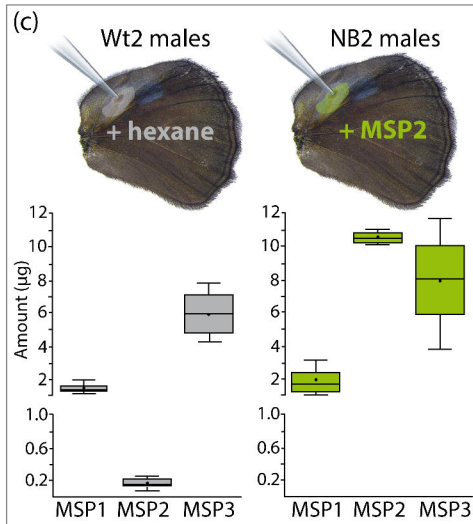
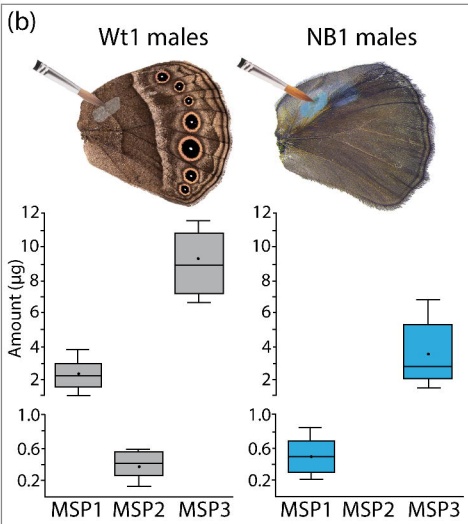
511 **Figure 3. Mating outcome of females after exposure to the “enriched” (NB2) and wild type**
512 **pheromone blends.** In experiment 2, females shifted their mating outcome after exposure to a Wt2
513 male or a male perfumed with a novel pheromone blend containing more MSP2. Most naïve females
514 mated with Wt2 males, females exposed to the Wt2 blend mated equally with both male types, and
515 females exposed to the new blend mated with new blend males at significantly higher rates than naïve
516 females. The dotted line at 50% illustrates random mating. Asterisks (* $p < 0.05$; ** $p < 0.01$) represent
517 non-random mating outcomes using Pearson’s χ^2 test. The horizontal bar above the plot shows a
518 significant difference in mating outcome between the two designated treatments (from the Tukey post-
519 hoc test, adjusted p value is indicated). The “n” on top of each bar indicates the total number of female

520 tested. Post-hoc test results providing adjusted p values comparing the different treatments are shown
521 in Supplementary Table 1b.

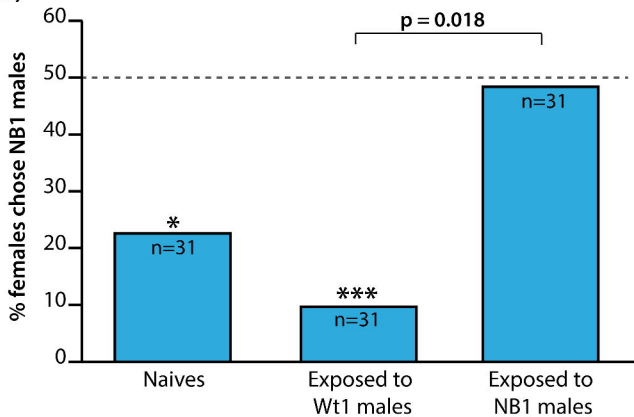
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(a)

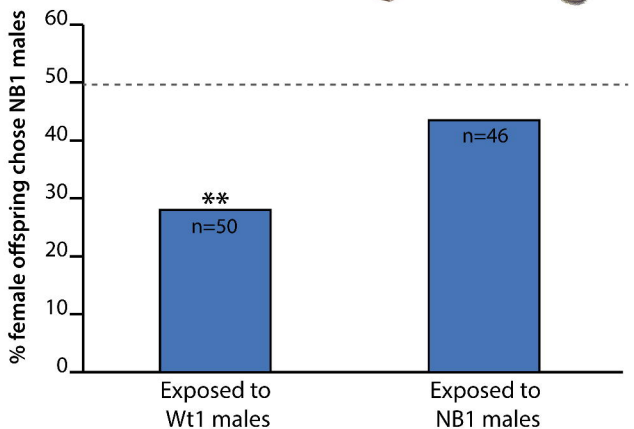
TIMELINE	Day -1	Day 0	Day 1	Day 2	Every day
EXPERIMENTAL STEPS	Preparation of Wt1 and NB1 males for exposure the next day	Female emergence and exposure Perfuming of Wt2 and NB2 males for exposure the same day	Preparation of Wt1 and NB1 males for mate choice the next day	Mate choice Perfuming of Wt2 and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



(a)



(b)



Treatment of females of the parental generation

