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

Emilie Dion [1,*], Li Xian Pui [1] and Antónia Monteiro [1, 2,*]

[1] National University of Singapore, Department of Biological Sciences, 14 Science Drive 4, Singapore, 117543

[2] Yale-NUS-College, 6 College Avenue East, Singapore 138614

[*] Correspondence to ED dion.emilie@ymail.com and AM antonia.monteiro@nus.edu.sg

Keywords: sex pheromone evolution, learning, mate choice, butterflies, plasticity, trans-generational inheritance

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 premating 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 *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
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. anynana* 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. anynana*.

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

6

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

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: χ^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: χ^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

9

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   5µg of MSP2 (Cayman Chemical, n°9001996) diluted in 2µL 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

13

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

369 **Acknowledgment**

375

376 **Author contributions**

14

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.

382 **References**

383 1. Johansson BG & Jones TM (2007) The role of chemical communication in mate choice. *Biol. Rev.*
384 82(2):265-289.
385 2. Gomez-Diaz C & Benton R (2013) The joy of sex pheromones. *EMBO reports* 14(10):874-883.
386 3. Smadja C & Butlin RK (2009) On the scent of speciation: the chemosensory system and its role in
387 premating isolation. *Heredity (Edinb)* 102(1):77-97.
388 4. Cande J, Prud'homme B, & Gompel N (2013) Smells like evolution: the role of chemoreceptor
389 evolution in behavioral change. *Curr. Opin. Neurobiol.* 23(1):152-158.
390 5. Bacquet PMB, Brattström O, Wang H-L, Allen CE, Löfstedt C, Brakefield PM, & Nieberding CM
391 (2015) Selection on male sex pheromone composition contributes to butterfly reproductive
392 isolation. *Proceedings of the Royal Society of London B: Biological Sciences* 282(1804).
393 6. Bacquet PMB, de Jong MA, Brattström O, Wang H-L, Molleman F, Heuskin S, Lognay G, Löfstedt
394 C, Brakefield PM, Vanderpoorten A, & Nieberding CM (2016) Differentiation in putative male sex
395 pheromone components across and within populations of the African butterfly *Bicyclus anynana*
396 as a potential driver of reproductive isolation. *Ecology and Evolution* 6(17):6064-6084.
397 7. Groot AT, Dekker T, & Heckel DG (2016) The Genetic Basis of Pheromone Evolution in Moths.
398 *Annu. Rev. Entomol.* 61:99-117.
399 8. Liénard MA & Löfsted C (2016) Small Ermine moths. Role of pheromone in reproductive isolation
400 and speciation. . *Pheromone communication in moths. Evolution, behavior and application.* , eds
401 Allison JD & Cardé RT (University of Californa Press, Oakland), pp 211-224.
402 9. Hebets E (2003) Subadult experience influences adult mate choice in an arthropod: Exposed
403 female wolf spiders prefer males of a familiar phenotype. *Proceedings of the National Academy*
404 *of Sciences* 100(23):13390-13395.
405 10. Verzijden MN, Abbott JK, von Philipsborn AC, & Loeschcke V (2015) Male *Drosophila*
406 *melanogaster* learn to prefer an arbitrary trait associated with female mating status. *Current*
407 *Zoology* 61(6):1036-1042.
408 11. Westerman EL, Hodgins-Davis A, Dinwiddie A, & Monteiro A (2012) Biased learning affects mate
409 choice in a butterfly. *Proc Natl Acad Sci U S A* 109(27):10948-10953.
410 12. Verzijden MN, ten Cate C, Servedio MR, Kozak GM, Boughman JW, & Svensson EI (2012) The
411 impact of learning on sexual selection and speciation. *Trends Ecol. Evol.* 27(9):511-519.
412 13. Gilman RT & Kozak GM (2015) Learning to speciate: The biased learning of mate preferences
413 promotes adaptive radiation. *Evolution* 69(11):3004-3012.
414 14. Arenas A & Farina WM (2014) Bias to pollen odors is affected by early exposure and foraging
415 experience. *J. Insect Physiol.* 66:28-36.
416 15. De Jong R & Kaiser L (1991) Odor learning by *Leptopilina boulardi*, a specialist parasitoid
417 (Hymenoptera: Eucoilidae). *J. Insect Behav.* 4(6):743-750.
418 16. Hartlieb E, Hansson BS, & Anderson P (1999) Sex or Food? Appetetive Learning of Sex Odors in a
419 Male Moth. *Naturwissenschaften* 86(8):396-399.
420 17. Klosin A & Lehner B (2016) Mechanisms, timescales and principles of trans-generational
421 epigenetic inheritance in animals. *Curr. Opin. Genet. Dev.* 36:41-49.
422 18. Jensen P (2013) Transgenerational epigenetic effects on animal behaviour. *Progress in*
423 *Biophysics and Molecular Biology* 113(3):447-454.
424 19. Remy JJ (2010) Stable inheritance of an acquired behavior in Caenorhabditis elegans. *Curr. Biol.*
425 20(20):R877-R878.
426 20. Remy JJ & Hobert O (2005) An interneuronal chemoreceptor required for olfactory imprinting in
427 C. elegans. *Science* 309(5735):787-790.

21. Dias BG & Ressler KJ (2014) Parental olfactory experience influences behavior and neural structure in subsequent generations. *Nat. Neurosci.* 17(1):89-96.

22. Schlichting CD & Wund MA (2014) Phenotypic plasticity and epigenetic marking: an assessment of evidence for genetic accommodation. *Evolution* 68(3):656-672.

23. Westerman EL & Monteiro A (2013) Odour influences whether females learn to prefer or to avoid wing patterns of male butterflies. *Anim. Behav.* 86(6):1139-1145.

24. Nieberding CM, Vos Hd, Schneider MV, Lassance J-M, Estramil N, Andersson J, Bang J, Hednström E, Löfsted C, & Brackefield PM (2008) The male sex pheromone of the butterfly *Bicyclus anynana*: towards an evolutionary analysis *Plos ONE* 3(7):e2751.

25. Dion E, Monteiro A, & Yew JY (2016) Phenotypic plasticity in sex pheromone production in *Bicyclus anynana* butterflies. *Scientific Reports* 6:39002.

26. Robertson KA & Monteiro A (2005) Female *Bicyclus anynana* butterflies choose males on the basis of their dorsal UV-reflective eyespot pupils. *Proc. R. Soc. Lond. [Biol].* 272(1572):1541-1546.

27. Prudic KL, Jeon C, Cao H, & Monteiro A (2011) Developmental plasticity in sexual roles of butterfly species drives mutual sexual ornamentation. *Science* 331(6013):73-75.

28. Costanzo K & Monteiro A (2007) The use of chemical and visual cues in female choice in the butterfly *Bicyclus anynana*. *Proc. R. Soc. Lond. [Biol].* 274(1611):845-851.

29. Symonds MRE & Elgar MA (2008) The evolution of pheromone diversity. *Trends Ecol. Evol.* 23(4):220-228.

30. Nieberding CM, Fischer K, Saastamoinen M, Allen CE, Wallin EA, Hedenstrom E, & Brakefield PM (2012) Cracking the olfactory code of a butterfly: the scent of ageing. *Ecol. Lett.* 15(5):415-424.

31. Dugatkin LA (1992) Sexual selection and imitation: females copy the mate choice of others *Am. Nat.* 139(6):1384-1389.

32. Claudianos C, Lim J, Young M, Yan S, Cristino AS, Newcomb RD, Gunasekaran N, & Reinhard J (2014) Odor memories regulate olfactory receptor expression in the sensory periphery. *Eur. J. Neurosci.* 39(10):1642-1654.

33. Anderson P, Hansson BS, Nilsson U, Han Q, Sjoholm M, Skals N, & Anton S (2007) Increased behavioral and neuronal sensitivity to sex pheromone after brief odor experience in a moth. *Chem. Senses* 32(5):483-491.

34. Guerrieri F, Gemeno C, Monsempes C, Anton S, Jacquin-Joly E, Lucas P, & Devaud J-M (2012) Experience-dependent modulation of antennal sensitivity and input to antennal lobes in male moths (*Spodoptera littoralis*) pre-exposed to sex pheromone. *The Journal of Experimental Biology* 215(13):2334-2341.

35. Anton S, Chabaud M-A, Schmidt-Büsser D, Gadenne B, Iqbal J, Juchaux M, List O, Gaertner C, & Devaud J-M (2016) Brief sensory experience differentially affects the volume of olfactory brain centres in a moth. *Cell Tissue Res.* 364(1):59-65.

36. Servedio MR (2015) Advances on the interplay of learning and sexual selection. *Current Zoology* 61(6):1004-1007.

37. Servedio MR & Dukas R (2013) Effects on population divergence of within-generational learning about prospective mates. *Evolution* 67(8):2363-2375.

38. Williams ZM (2016) Transgenerational influence of sensorimotor training on offspring behavior and its neural basis in *Drosophila*. *Neurobiol. Learn. Mem.* 131:166-175.

39. Biergans SD, Claudianos C, Reinhard J, & Galizia CG (2017) DNA methylation mediates neural processing after odor learning in the honeybee. *Scientific Reports* 7.

40. Biergans SD, Claudianos C, Reinhard J, & Galizia CG (2016) DNA methylation adjusts the specificity of memories depending on the learning context and promotes relearning in Honeybees. *Frontiers in Molecular Neuroscience* 9.

476   41.   Brakefield PM & Reitsma N (1991) Phenotypic plasticity, seasonal climate and the population
477   biology of *Bicyclus* butterflies (*Satyridae*) in Malawi. *Ecol. Entomol.* 16(3):291-303.
478   42.   Everett A, Tong X, Briscoe A, & Monteiro A (2012) Phenotypic plasticity in opsin expression in a
479   butterfly compound eye complements sex role reversal. *BMC Evol. Biol.* 12(1):1-12.
480   43.   R Development Core Team (2008) R: A language and environment for statistical computing. ed
481   Computing RFfS (ISBN 3-900051-07-0, Vienna, Austria).
482   44.   RStudio Team (2016) RStudio: Integrated Development for R. http://www.rstudio.com/. ed Inc.
483   R (Boston, MA).
484   45.   Bates D, Mächler M, Bolker B, & Walker S (2015) Fitting Linear Mixed-Effects Models Using
485   lme4. *Journal of Statistical Software* 67(1):48.
486   46.   Hothorn T, Bretz F, & Westfall P (2008) Simultaneous inference in general parametric models.
487   *Biometrical Journal* 50(3):346-363.

488

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

19

520   tested. Post-hoc test results providing adjusted p values comparing the different treatments are shown

521   in Supplementary Table 1b.

522

(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

(b) Wt1 males   NB1 males

(c) Wt2 males + hexane   NB2 males + MSP2

(d)
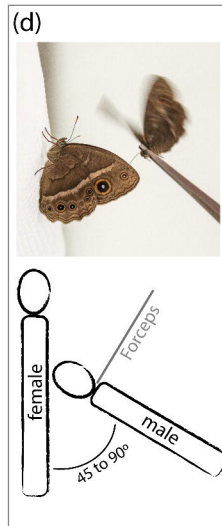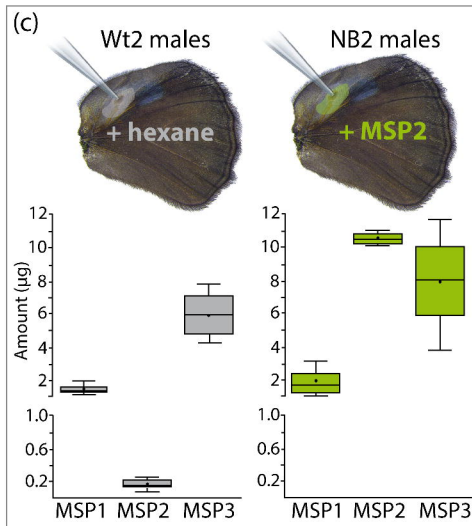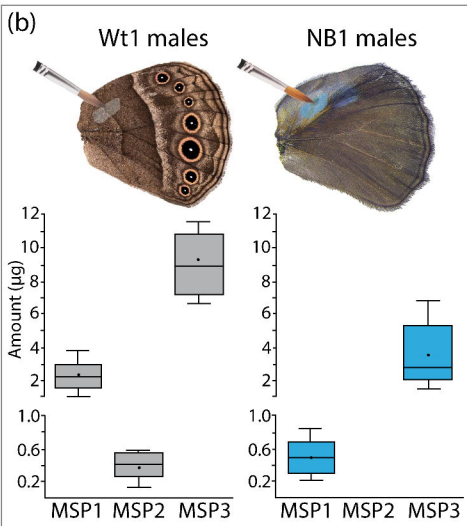
(a)
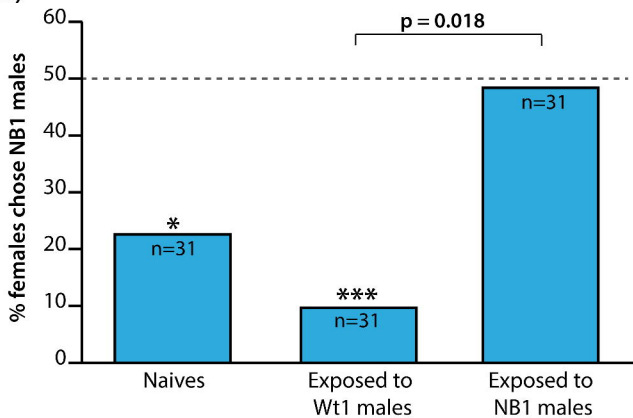
(b)
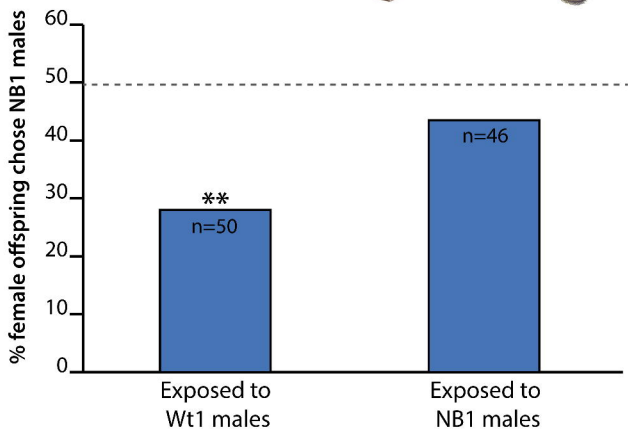
Treatment of females of the parental generation