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Is mate choice in *Drosophila* males guided by olfactory or gustatory pheromones?

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choice; olfaction ; sex pheromone; taste

1 *Drosophila melanogaster* flies use both olfactory and taste systems to detect sex pheromones
2 and select the most suitable mate for reproduction. In nature, flies often face multiple
3 potential partners and should have an acute sensory ability to discriminate between different
4 pheromonal bouquets. We investigated both the pheromones and the chemosensory neurons
5 influencing *Drosophila* mate choice. We measured various courtship traits in single tester
6 males simultaneously presented with two target male and/or female flies carrying different
7 pheromonal bouquets (pairs of control flies of the same or different sex, same-sex target pairs
8 of pheromonal variant strains). The courtship traits reflected the perception of either olfactory
9 cues perceived before or gustatory cues perceived after the first physical taste contact. Our
10 results suggest that male mate choice exists in *D. melanogaster* and that male discrimination
11 between potential mates could be a two-step process involving chemical cues perceived
12 before and after the first gustatory contact. In addition, when a male was simultaneously
13 presented with two potential sexual partners, the olfactory and gustatory cues he used
14 depended on the pheromonal patterns of both flies, but his response could also depend on
15 additional effects resulting from the simultaneous perception of the two flies, leading to a
16 nonlinear choice of a sexual partner. Moreover, some tester males with genetically altered
17 gustatory receptor neurons strongly changed their partner preference, indicating that the fly's
18 peripheral nervous system is essential for pheromonal detection and mate choice.

19

19 For animals, it is crucial to select the fittest mating partner to produce progeny and the
20 ‘grossest blunder’ in sexual behaviour would be to mate with a partner that does not ensure
21 the greatest chance of producing the most viable offspring (Fisher 1930, page 130). In higher
22 animals, both sexes are adapted to avoid this blunder and their nervous system is built in such
23 a way that mating with an unsuitable partner is usually inhibited by differences in the
24 ‘appearance or behaviour’ of this individual (Fisher, page 130). However, these signals could
25 instead indicate the high genetic quality of the bearer and constitute ‘viability indicators’
26 (Andersson 1994; Kokko 2001). In many animals, chemical signals are often used as viability
27 indicators detected by smell and taste which allow individuals to select potential mates (Wyatt
28 2003).

29 In *Drosophila*, as in many dipterans, most known sex pheromones are cuticular hydrocarbons
30 (CHs; Wicker-Thomas 2007) which are detected by the olfactory and/or gustatory sensory
31 systems. As in other insects, olfactory and gustatory receptors in *Drosophila* are found in
32 neurons housed in various sensory appendages (Dethier 1976). These neurons can perceive
33 chemical stimuli, transduce them and convey the corresponding information to the central
34 nervous system, which in turn will trigger the appropriate behavioural response (Wang et al.
35 2004).

36 In *Drosophila melanogaster*, gustatory receptors and gustatory receptor neurons (GRNs) are
37 relatively well characterized (Hiroi et al. 2004; Thorne et al. 2004; Marella et al. 2006; Moon
38 et al. 2006; Dahanukar et al. 2007; Jiao et al. 2007; Slone et al. 2007; Kent & Robertson
39 2009). More specifically, a small group of GRNs present on the labial palps and legs carrying
40 the Gr66a receptor are involved in the taste detection of both bitter substances and 7-tricosene
41 (7-T), a male CH which acts as a sex pheromone inhibiting male and stimulating female flies
42 (Ferveur & Sureau 1996; Grillet et al. 2006; Moon et al. 2006; Lacaille et al. 2007, 2009).
43 Both types of compound induce a similar aversive dose-dependent effect on male courtship

44 and related feeding behaviours (Lacaille et al. 2007, 2009). We hypothesized that the *Gr66a* -
45 expressing taste neurons were initially used to detect and respond to toxic food, and
46 subsequently co-opted into the new function of detecting pheromonal stimuli carried by other
47 males to avoid homosexual interactions (Lacaille et al. 2007). This hypothesis does not
48 exclude the possibility that other groups of taste neurons and/or sensory modalities are also
49 involved in the male avoidance response. We also found that while males with altered GR66a
50 neurons showed no, or very little, avoidance (Lacaille et al. 2007), the genetic feminization of
51 the same neurons (in otherwise male flies) enhanced their avoidance response to 7-T and
52 bitter molecules (Lacaille et al. 2009).

53 In the field, *Drosophila* adults of different species and strains aggregate on food patches
54 where courtship, mating and oviposition occur (Wertheim et al. 2005, 2006). Aggregation
55 behaviour is influenced by food sensory stimuli, social interaction and individual experience
56 (Tinette et al. 2004). As they live in heterotypic groups, *Drosophila* flies must have an
57 accurate sensory system to discriminate the appropriate partner from all potential ones. This
58 process of discrimination probably involves multiple sensory signals.

59 The genetic dissection of *D. melanogaster* courtship behaviour has allowed researchers to
60 unravel the mechanisms of interindividual communication involving species- and sex-specific
61 sensory signals (Greenspan & Ferveur 2000; Hall 2002). Visual, olfactory, gustatory, acoustic
62 and/or tactile stimuli are exchanged by pairs of mature *D. melanogaster* flies (reviewed in
63 Greenspan & Ferveur 2000) and the degree of specificity of these signals could increase as
64 courtship progresses (Arienti 1993; Cobb & Ferveur 1996). Since it was first described by
65 Sturtevant (1915), most studies of *D. melanogaster* courtship have exclusively focused on
66 male behaviour while female precopulatory behaviour has often been assigned the role of an
67 ‘accept - or - reject’ switch leading to mating. However, (Lasbleiz et al. 2006) showed that the
68 female constantly interacts with the male during courtship and is important for mating

69 success. This is consistent with the costs of reproduction to *Drosophila* females (Ikeda 1974;
70 Turner & Anderson 1983; Chapman et al. 1995; Sgro & Partridge 1999; Chapman 2001;
71 Lung et al. 2002). However, reproductive costs also reduce life span and future fertility in
72 male *D. melanogaster* (Cordts & Partridge 1996). Although male mate choice remains poorly
73 documented, available evidence suggests that it is widespread among insects and other
74 animals (Bonduriansky 2001; Kraaijeveld et al. 2007). The most commonly observed male
75 mate choice is precopulatory mate choice which tends to maximize a male's expected
76 offspring from each mating by favouring female phenotypes associated with high fecundity or
77 reduced sperm competition intensity (Bonduriansky 2001). Male insects typically choose
78 females based on easily detectable phenotypic indicators of fecundity (Cordts & Partridge
79 1996), possibly including volatile and contact chemical cues.

80 In *D. melanogaster*, sex pheromones are CHs with little or no volatility (Ferveur 2005;
81 Wicker-Thomas 2007) and are therefore mainly perceived by gustation (Stocker 1994;
82 Shanbhag et al. 2001; Boll & Noll 2002; Lacaille et al. 2007) but sometimes also by short-
83 distance olfaction (Ferveur 2005; Grillet et al. 2006; Benton et al. 2009). Therefore, the
84 choice of a mate by a *Drosophila* male could depend on both smell and taste. In the present
85 study, we addressed three questions: (1) do male flies use CHs to choose between two
86 partners simultaneously present, (2) do they use volatile or nonvolatile cues and (3) are Gr66a
87 neurons involved in this process?

88

89 <H1>METHODS

90

91 <H2>*Fly husbandry*

92 All *D. melanogaster* strains were raised and tested at 24 ± 0.5 °C and $65 \pm 5\%$ relative
93 humidity on a 12:12 h light:dark cycle. Stocks were maintained on alcohol-free medium

94 mixed with killed yeast in 150 ml glass vials. When 1-2 h old, flies were sexed under light
95 carbon dioxide anaesthesia at 2 - 4 h after lights on, and were kept in fresh-food vials either
96 isolated (males) or in groups of five (females) until the age of 4 days.

97

98 <H2>Genetics

99 <H3>Target flies

100 The targets consisted of mature male and female flies from two wild-type strains, Canton-S
101 (CS) and Tai and from one mutant strain, *desat1*. CS and Tai strains had been maintained in
102 the laboratory for several decades, and the *desat1* strain for more than one decade.

103 In these three strains, both sexes strongly diverge in their cuticular hydrocarbon production.

104 CS is a wild-type strain (caught in the U.S.A.) used in many laboratories and representative of
105 cosmopolitan *D. melanogaster* strains: males produce high levels of 7-tricosene (7-T) and low
106 levels of 7-pentacosene (7-P) whereas females predominantly produce 7,11-dienes (7,11-
107 hepta- and nonacosadiene: 7,11-HD, 7,11-ND; Antony & Jallon 1982; Jallon 1984). Tai is a
108 variant strain (caught in the Ivory Coast) where males show low 7-T and high 7-P levels
109 whereas females produce much higher levels of 5,9-hepta- and nonacosadiene (5,9-HD, 5,9-
110 ND) than of 7,11-dienes (Jallon 1984; Jallon & Pechine 1989; Savarit & Ferveur 2002). Flies
111 of the *desat1* mutant strains almost completely lack unsaturated cuticular hydrocarbons
112 (monoenes and dienes) but produce high levels of cuticular alkanes with 23 and 25 carbons
113 (23-Lin, 25-Lin; Marcillac et al. 2005).

114

115 <H3>Tester males

116 Tester males (whose behaviour was measured) belonged to the CS strain or were genetically
117 manipulated for their Gr66a-Gal4-expressing neurons (Lacaille et al. 2009): *66a-Gal4/+* and
118 *66a-Gal4/UAS-tra* males.

119 The Gr66a transgenic males carry, on chromosome III, a single copy of the *Gr66a-Gal4*
120 transgene which contains a promoter of the gustatory receptor *Gr66a* gene fused with the
121 yeast *Gal4* sequence (Dunipace et al. 2001). We used the *Gr66a-Gal4* transgene to target and
122 activate the *UAS-transformer* (*UAS-tra*; Ferveur et al. 1995) reporter transgene which
123 combines the upstream activation sequence (UAS, specifically activated by Gal4; Brand &
124 Perrimon 1993) fused with the cDNA of the *transformer* sex determination gene, which
125 allowed us to feminize autonomously the Gal4-expressing neurons (see Lacaille et al. 2009).

126

127 <H2>Behaviour

128 <H3>Behavioural tests

129 Each single tester male fly was given a choice of two target flies. We measured several
130 behavioural parameters reflecting the male's ability to perceive both volatile and contact
131 pheromones. These parameters included the courtship latency, the first target chosen, the total
132 duration of courtship (or courtship index, CI) directed towards each target (i.e. the proportion
133 of time that the courting male spends vibrating one wing, curving his abdomen, licking the
134 female genitalia or attempting to copulate; no qualitative difference was noted between the
135 courtship sequences of the different courting males), and the discrimination index (DI) which
136 was adapted from the Malogolowkin - Cohen index (Malogolowkin-Cohen et al. 1965) and
137 computed as follow: $DI_{xy} = (CI_x - CI_y) / (CI_x + CI_y)$, where CI_x and CI_y are CIs towards the 'x' or
138 the 'y' target. Besides providing a comparison of the attractiveness of both targets in each
139 pair, the DI alone is insufficient to describe precisely the discrimination ability of tester
140 males. Therefore we also recorded the frequency of males courting each target, and the
141 number of transitions between targets, and we designed two parameters to allow us to
142 characterize both the direction and the intensity of the discrimination, independently of the
143 chosen target. The global choice was computed by classifying the tester males into three

144 groups depending on whether they did not prefer either target, they courted the x target more
145 or the y target more. The global choice intensity was quantified using the absolute values of
146 the DI. Together, the last three parameters provide a good indicator of the ‘robustness’ of the
147 discrimination. Courtship latency and first target chosen are relevant to chemoperception prior
148 to the first gustatory contact whereas the other parameters provide a measure of the tester
149 male behaviour after his first gustatory contact.

150

151 All behavioural tests took place 1 - 4 h after lights on. When tests simultaneously involved
152 male and female targets, they were carried out under a dim red light (25 W with a Kodak
153 Safe-light filter n°1) to remove all sexually dimorphic visual stimuli (Boll & Noll 2002).
154 Tests with same-sex targets were carried out under a white light, so the flies could also see the
155 targets, but, as the two targets were similar, visual cues should theoretically not have
156 influenced the discrimination process. In each pair, a notch made with a microscissor in one
157 wing of either target allowed us to distinguish genotypes. To eliminate behavioural feedback
158 and to reduce the effect of acoustic signals (Ferveur et al. 1995), all target flies were
159 decapitated 30 min before the test under carbon dioxide anaesthesia.

160

161 Four-day-old tester males were individually aspirated (without anaesthesia) under a watch
162 glass used as an observation chamber (1.6 cm³). After a 5 min acclimation period, a pair of 4-
163 day-old decapitated target flies was introduced and the CI that the tester male directed
164 towards each of them was measured for 10 min. After each observation, the watch glasses
165 were cleaned using detergent, then pentane and finally purified water. For each behavioural
166 test, $N = 28 - 32$.

167

168 We conducted three types of test. First and to assess the validity of our discrimination
169 parameters, we carried out a control experiment in which we put a single CS tester male with
170 a pair of same-sex CS flies. We also measured the ability of tester males of three genotypes
171 (CS, *66a-Gal4/+* and *66a-Gal4/UAS-tra*) to discriminate the sex pheromones of control flies.
172 The behaviour that they directed towards a decapitated target male and a decapitated target
173 female from a wild-type strain (CS) was measured under red light. Second, to assess the
174 ability of the three tester males to discriminate the quality of male cuticular pheromones, we
175 put them with target males of the three variant strains CS, Tai (T) and *desat1* (D) with
176 different predominant pheromones. Target females were paired in the three possible
177 combinations and we measured the ability of the three tester males (CS, *66a-Gal4/+* and *66a-*
178 *Gal4/UAS-tra*) to discriminate each genotype within these pairs. Third, we put tester males
179 with target females from CS, Tai (T) and *desat1* (D) strains, which also have different
180 predominant cuticular compounds.

181

182 <H3>*Statistical analysis of behavioural data:*

183 Except for percentages (first target chosen, frequency of males courting each target and global
184 choice intensity), all other parameters are expressed as their mean \pm SEM. Several statistical
185 treatments were used according to the parameter analysed. The courtship latency, CI, DI,
186 number of transitions between targets and the global choice intensity were tested with Kruskal
187 - Wallis analysis of variance of ranks completed by Dunn's multiple pairwise comparisons
188 (two tailed with Bonferroni correction). The significance of the first target chosen was tested
189 with a binomial test and that of the courting males' frequency with a *z* test. The differences in
190 global choice between the tester genotypes were tested with a chi-square test (with a
191 computation of significance by cell) and differences in global choice intensity with a Mann –

192 Whitney *U* test. All statistical analyses were conducted with XLSTAT 2007 (Addinsoft, New
193 York, U.S.A.).

194

195 <H2>*Chemical analysis*

196 <H3>*Extraction and gas chromatography analysis:*

197 Cuticular hydrocarbons (CHs) from 4-day-old individuals from the different tester and target
198 flies were analysed by gas chromatography following hexane extraction and adding synthetic
199 C26 and C31 hydrocarbon internal markers, according to standard procedures (Ferveur 1991).
200 Analyses were performed with a Varian CP3380 chromatograph, fitted with a flame-
201 ionization detector, with a CP-sil/5CB capillary column (Varian, 25 m x 0.32 mm internal
202 diameter) and with a split-splitless injection system (operating with a split flow of 60 ml/min
203 and a septum purge of 3 ml/min, opening of the split port 30 s after injection). Hydrogen was
204 used as carrier gas (50 cm/s velocity at room temperature). The injector and detector
205 temperatures were 260 and 280 °C, respectively. The column was held isothermally at 140 °C
206 for 2 min, then programmed to increase at a rate of 5 °C/min to 280 °C. The data were
207 automatically computed and recorded using PC software (Star 5.2, Varian Inc., Palo Alto,
208 CA, U.S.A.).

209

210 <H3>*Statistical analysis of target cuticular profiles:*

211 For each series of target pairs (pairs formed either by CS wild-type flies of the two sexes or
212 by heterotypic flies of the same sex), the absolute amounts of CHs were used to compute an
213 agglomerative hierarchical clustering (AHC; using the Pearson correlation coefficient as a
214 similarity index and the unweighted pair-group average linkage method, at a level of 0.95)
215 allowing us to exclude correlated CHs from further analysis. In each group of correlated
216 compounds we retained the most abundant CH as representative of this cluster.

217 For each type of target pair, we conducted a discriminant Analysis (DA) using the absolute
218 amounts of the uncorrelated CHs as quantitative variables and the sex or the strain (depending
219 on the target pair) as a qualitative variable. For each type of target pair, we also conducted a
220 forward stepwise DA of the quantitative variables (with an entry threshold value of $P = 0.05$)
221 which led to a 100% correct classification of the individuals. The results of these different
222 DAs were summarized by their confusion matrices after cross-validation, and by the selected
223 CHs for the forward stepwise DA. The AHCs and DAs were computed using XLSTAT 2007.

224

225 <H1>RESULTS

226

227 <H2>Discrimination of sex pheromone

228 In the control test, no significant difference was found with either male or female pairs,
229 supporting the validity of our parameters (Fig. 1). Moreover, tester CS males directed a much
230 stronger courtship towards females than towards males ($H_3 = 36.09$, $P < 0.0001$; Fig. 1c).
231 Also, both the frequency of courting males ($z = 4.85$, $P < 0.0001$; Fig. 1e), and the global
232 choice intensity were higher with target females than target males ($U = 225.50$, $P < 0.0001$;
233 Fig. 1h). Examination of the global choice parameter shows that 100% of CS tester males
234 courted target females, while only 37% of testers courted target males ($\chi^2_2 = 29.23$,
235 $P < 0.0001$; Fig. 1g).

236 Under red light, the time taken to initiate courtship (courtship latency; Fig. 2a) varied
237 significantly according to the sex of the target flies and the genotype of the tester male
238 ($H_5 = 10.98$, $P = 0.05$). Both CS and *66a-Gal4/UAS-tra* tester males courted the target
239 female faster than the target male ($H_2 = 16.25$, $P < 0.0001$; Fig. 2a) whereas *66a-Gal4/+*
240 tester males showed no difference in courtship latency. This observation is consistent with the
241 finding that most CS and *66a-Gal4/UAS-tra* males first courted the target female (binomial

242 test: CS: $P = 0.001$; *66a-Gal4/UAS-tra*: $P < 0.0001$; Fig. 2b), in contrast to *66a-Gal4/+* tester
243 males which showed no significant first choice ($P = 0.570$).

244 After the first contact, *66a-Gal4/+* tester males showed a lower heterosexual CI, and a higher
245 homosexual CI than CS and *66a-Gal4/UAS-tra* tester males ($H_5 = 101.55$, $P < 0.0001$; Fig.
246 2c). Consequently, the DI of the *66a-Gal4/+* males was much lower than those of the two
247 latter males ($H_2 = 23.29$, $P < 0.0001$; Fig. 2d). However, the different ability to discriminate
248 and to court flies of both sexes did not change the total time that males spent courting (72 -
249 81% of the total duration; $H_2 = 5.34$, $P = 0.069$).

250 The comparison of the courting males' frequency towards each target fly (Fig. 2e) also
251 allowed us to evaluate the intensity of discrimination. While more than 91% of males of the
252 three genotypes courted the target female, the difference in frequency between heterosexual
253 and homosexual courtship varied among tester male genotypes: it was not significant for *66a-*
254 *Gal4/+* males ($z = 1.08$, $P = 0.281$) while it was highly significant for both CS ($z = 2.98$, $P =$
255 0.0001) and *66a-Gal4/UAS-tra* males ($z = 5.60$, $P < 0.0001$).

256 The variation in the number of transitions shown by the three tester males confirmed these
257 differences: *66a-Gal4/+* tester males showed more transitions than both CS and *66a-*
258 *Gal4/UAS-tra* males ($H_2 = 14.29$, $P = 0.001$; Fig. 2f). The higher stability showed an inverse
259 relationship with the CI differences. Finally, the global choice parameters (Fig. 2g, h) reflect
260 the general variation in the behavioural parameters. Whereas both CS and *66a-Gal4/UAS-tra*
261 testers showed a clear and somewhat equivalent heterosexual preference, the choice shown by
262 *66a-Gal4/+* testers was not so marked. This differed significantly from the choice made by
263 the other two tester types ($\chi^2_2 = 10.90$, $P = 0.028$): the *66a-Gal4/+* tester males chose the
264 female target less often (and consequently the male target more often) than the other two
265 tester males. Similarly, the *66a-Gal4/UAS-tra* tester males showed the highest intensity of

266 global choice whereas the *66a-Gal4/+* males showed the lowest ($H_2 = 22.61$, $P < 0.0001$;
 267 Fig. 1h).

268

269 <H2>Discrimination of male pheromones

270 In the test with male targets, the parameters reflecting the male behaviour before the first
 271 physical contact (courtship latency - $H_{17} = 55.17$, $P < 0.001$ - and first choice; Fig. 3a, b)
 272 differed according to both the tester and the target males. With [T | CS] pairs, Tai males
 273 always induced a faster courtship than CS males (Fig. 3a) and were the first chosen target
 274 (binomial test: $P < 0.004$; Fig. 3b). With [T | D] pairs, the preference for the Tai target male
 275 was only significant for CS and *66a-Gal4/+* tester males (binomial test: CS: $P = 0.006$; *66a-*
 276 *Gal4/+*: $P = 0.025$; Fig. 3a, b). With [CS | D] pairs, the *66a-Gal4/+* tester males slightly
 277 preferred CS target males, whereas CS and *66a-Gal4/UAS-tra* testers slightly preferred *desat1*
 278 target males. However the first choice was only significant for *66a-Gal4/UAS-tra* testers
 279 (binomial test: $P = 0.039$; Fig. 3b).

280 After the first contact, Tai target males induced higher CIs (Fig. 3c), resulting in higher DIs
 281 (Fig. 3d), except for *66a-Gal4/UAS-tra* testers with [T | D] targets. In this case, Tai and
 282 *desat1* males induced similar CIs resulting in a low discrimination of the [T | D] pair. A
 283 similar pattern was found for the frequency of courting males (Fig. 3e). The summed CI
 284 towards both targets was high when a Tai target was present ($H_8 = 112.47$, $P < 0.0001$), and
 285 low in the case of [CS | D] targets.

286 With these [CS | D] targets, *66a-Gal4/UAS-tra* testers showed fewer transitions ($H_8 = 16.81$,
 287 $P = 0.032$; Fig. 3f) than in any other situation, indicating that their courtship was very stable
 288 although not very high. Conversely, the most transitions were found in *66a-Gal4/UAS-tra*
 289 testers with [T | D] targets. Finally, the global choice parameters were consistent with the
 290 previous parameters: (1) Tai target males were always preferred except by *66a-Gal4/UAS-tra*

291 testers associated with the [T | D] targets and (2) no strong preference was shown with
292 [CS | D] targets.

293

294 <H2>***Discrimination of female pheromones***

295 With female targets, male behaviour before physical contact (courtship latency - $H_{17} = 29.79$,
296 $P < 0.028$ – and first choice; Fig. 4a, b) differed with the combination of target females used.

297 In [CS | D] pairs, CS females always induced a faster courtship than *desat1* female (Fig. 4a)

298 and were more often chosen first (binomial test: $P < 0.03$; Fig. 4b). With [T | CS] pairs, only

299 *66a-Gal4/+* testers preferred CS females ($P < 0.001$; binomial test: $P = 0.0002$; Fig. 4a, b),

300 whereas *66a-Gal4/UAS-tra* testers preferred Tai females and CS testers showed no preference

301 (binomial test: $P = 0.05$; Fig. 4b). These differences are reflected by the difference in

302 courtship latencies (Fig. 4a). With [T | D] pairs, CS and *66a-Gal4/+* testers slightly preferred

303 the Tai target female whereas *66a-Gal4/UAS-tra* testers slightly preferred the *desat1* target.

304 The CIs of the three tester males were similar with [CS | D] pairs: CS females always induced

305 a higher CI than *desat1* females (Fig. 4c). With [T | D] pairs, CS and *66a-Gal4/+* testers

306 slightly preferred Tai females whereas *66a-Gal4/UAS-tra* testers preferred *desat1* females.

307 With [T | CS] pairs, CS and *66a-Gal4/+* testers courted CS females more intensively than Tai

308 females whereas *66a-Gal4/UAS-tra* testers courted the Tai target more intensively. The total

309 amount of courtship did not vary between the testers with the [CS | D] pairs, and was similar

310 with both [T | D] and [T | CS] pairs: CS < *66a-Gal4/+* < *Gr66a-Gal4/UAS-tra* ($H_8 = 20.88$,

311 $P = 0.007$).

312 Independently of tester genotypes, the CS female target was courted more often than the

313 *desat1* female in the [CS | D] pair (z test: $z = 2.419, 4.536$ & 4.526 , $P < 0.02$; Fig. 4e). In the

314 [T | CS] pairs, the CS target was also courted more than the Tai female by the *66a-Gal4/+*

315 male (z test: $z: 5.477; P < 0.0001$), while it was courted less by the *66a-Gal4/UAS-tra* males
 316 (z test: $z: 2.739; P = 0.006$).

317 The number of transitions differed significantly ($H_8 = 66.26, P < 0.0001$; Fig. 4f). While CS
 318 and *66a-Gal4/UAS-tra* testers showed a high number of transitions with the [CS | D] pairs,
 319 *66a-Gal4/+* testers showed few transitions with [CS | D] pairs. Again the global choice
 320 parameter reflected well the situation described above: it showed no variation with the
 321 [CS | D] female pairs (the CS target was always preferred), and varied significantly with the
 322 [T | D] and the [T | CS] pairs ([T | D]: $\chi^2_2 = 18.86, P = 0.001$; [T | CS]: $\chi^2_2 = 35.26,$
 323 $P < 0.0001$; Fig. 4g). The most obvious differences in global choice were (1) the preference
 324 of the *66a-Gal4/UAS-tra* tester males for the *desat1* female in the [T | D] pairs and for the Tai
 325 target in the [T | CS] pairs, and (2) the preference of the *66a-Gal4/+* tester males for the CS
 326 female in the [T | CS] pairs. All the global choice intensities showed a similar level, except for
 327 both the CS testers with the [CS | D] pairs and *66a-Gal4/+* testers with the [T | D] pairs which
 328 showed a lower robustness of global choice ($H_8 = 52.78, P < 0.0001$; Fig. 4h).

329

330 <H2>Chemical differences

331 The *D. melanogaster* cuticular profile consists of 58 compounds including the classical
 332 monoenes (9-, 7-, 5-tricosenes; 9-, 7-, 5-pentacosenes; 7-heptacosene; 7-nonacosene), dienes
 333 (7,11-hepta- and 7, 11-nonacosadiene), linear (L) and methyl branched alkanes (Br)
 334 previously described (Everaerts et al., in press). Although the cuticular hydrocarbon profiles
 335 were very similar in the three genotypes of tester males (data not shown), the target flies' CHs
 336 were very different. For the sake of clarity, we only show the absolute quantities for the six
 337 principal cuticular compounds with a putative pheromonal role in *D. melanogaster*, namely 7-
 338 and 5-tricosene (7-T, 5-T), 9-, 7-pentacosene (9-P, 7-P), and 7,11-hepta- and 7,11-
 339 nonacosadiene (7,11-HD and 7,11-ND; Table 1). For each combination of target flies, the

340 results of the different DAs are shown with their confusion matrices after cross-validation,
341 and with the CHs selected by the forward stepwise DAs (Table 2).

342 The cuticular profile of the male/female CS targets allowed us to classify correctly 100% of
343 male and female individuals. However, among all CHs the forward stepwise DA indicated
344 that only five were informative enough to obtain this result, namely the 7-T > 5-P > 27-Br >
345 7,11-ND > 7-N (CHs are classified according to their contribution to the discriminatory
346 power of our model).

347 With the chemical data obtained with heterotypic target pairs, the DAs reached a 13.3 - 86.7%
348 correct classification of individuals. Depending upon the combination pair, the forward
349 stepwise DAs indicates that three or four CHs are sufficient to obtain a 100% correct
350 classification.

351 The compound 7-T contributed strongly to the discrimination between target males of the
352 [CS | D] and [T | CS] pairs. Moreover 6-docosene (6-D) and a branched heptacosene (Br-H)
353 could be involved in the [CS | D] pair whereas 9- and 5-P could be used in the [T | CS] pair.
354 On the other hand, 7-T seemed not to be used for discrimination in the [T | D] male pair: Tai
355 and *desat1* males could be discriminated by 25-Br, 5-P and Br-H.

356

357 For female targets, the sets of selected CHs varied more according to the pairs, but they never
358 included dienes. The DA discriminatory power was very low with the [T | D] pair of target
359 females.

360

361 <H1>DISCUSSION

362

363 <H2>Male ability to use pheromones to discriminate potential mates

364

365 In the present study, we did not observe any significant difference between the discrimination
366 or the courtship of the CS testers towards the two targets of a same-sex wild-type pair
367 (Fig. 1). However, and as expected, the female targets elicited a stronger and steadier
368 courtship than male targets. Also, CS males could discriminate at a short distance the male
369 and female targets within heterosexual CS pairs (Fig. 2). After the first gustatory contact, the
370 CS tester males courted the female more intensively and more steadily than the male target.
371 Heterotypic pairs of males triggered varied discrimination patterns in CS tester males. Before
372 the first gustatory contact, they first courted the Tai target (within both [T | D] and [T | CS]
373 pairs), while they showed no preference within the [CS | D] pair. The response shown by CS
374 testers after the first gustatory contact is globally consistent with their pregustatory choice:
375 they courted the Tai male targets more within the [T | D] and the [T | CS] pairs, whereas
376 within [CS | D] pairs, they courted the *desat-1* male target more intensively. Heterotypic pairs
377 of female targets also triggered a variable pattern of response in wild-type tester males. While
378 the CS tester oriented first to the CS female within the [CS | D] female pairs, no preference
379 was shown with the [T | CS] and [T | D] female pairs. However, after the first gustatory
380 contact, the CS testers courted the CS female targets more intensively within the [CS | D] and
381 [T | CS] female pairs, and the Tai female targets more intensively within the [T | D] female
382 pairs.

383 In insects, sex chemical communication often relies on both volatile and contact pheromones
384 (Wyatt 2003). In *D. melanogaster*, most known sex pheromones are CHs with little or no
385 volatility (Ferveur 2005; Wicker-Thomas 2007). These compounds are mainly perceived with
386 the gustatory organs located on the fore tarsi and mouthparts (Stocker 1994; Shanbhag et al.
387 2001; Boll & Noll 2002; Lacaille et al. 2007). However, some of the lightest cuticular
388 compounds may be volatile enough to be detected at a short distance by the olfactory organs
389 on the head (antennae and maxillary palps; Ferveur 2005; Grillet et al. 2006; Benton et al.

390 2009). Therefore, the choice of a sexual partner by a *Drosophila* male could depend
391 successively on smell and taste. First, and at a short distance, olfactory perception could allow
392 a fly to discriminate some chemical features of a potential sex partner. Then after the first
393 gustatory contact, the pheromones perceived with taste appendages could modulate (by either
394 enhancing or inhibiting) male courtship ardour.

395 To deal with the hypothesis of such a two-step discrimination process, we used two series of
396 ‘pregustatory’ and ‘postgustatory’ parameters. The courtship latency and the first chosen
397 target depend on the chemoperception prior to the first gustatory contact, while the number of
398 transitions, the CI, the DI, the frequency of males courting and the ‘global choice parameters’
399 allowed us to quantify the behaviour of the tester male after his first gustatory contact.

400 Except for the total CI, all parameters were related to the differential response of the tester
401 male towards each of the two target flies. The total CI for both targets allowed us to check
402 whether some combinations of targets induced a global change in the tester male’s ardour.

403 To date, the preference of a male tested in a choice procedure has always been scored using
404 either DI or CI. The ‘global choice parameters’ allowed us to describe more completely the
405 discriminatory ability of tester males. The comparison of these ‘global choice parameters’
406 with a same-sex pair of control targets revealed strong differences (Fig. 1g, h), whereas the
407 DIs were not different (Fig. 1d). With control female pairs, although all testers courted, only
408 25% directed a similar courtship to both targets, whereas the other 75% continuously courted
409 only one female. Conversely, with control male pairs, only 3% of males courted both male
410 targets while 34% courted only one male. The weak DIs induced by a same-sex pair of
411 control flies indicates that either: (1) tester males are randomly attracted by one target whose
412 attractiveness is sufficient to prevent courtship of the other target, or that (2) both targets
413 induce a weak response in tester males. Therefore, the ‘global choice parameters’ not only

414 reflect the robustness of the tester male choice but also provide information that completes the
415 ‘number of transitions’ parameter to assess the courtship stability of the tester male.

416 Whereas in choice experiments, *G66a-Gal4/UAS-tra* males were unable to discriminate
417 between Tai and *desat1* male targets (this study), in no-choice experiments they court Tai
418 more intensively than *desat1* male targets (Lacaille et al. 2007, 2009). Several studies have
419 already shown that choice and no-choice mating experiments may yield diverging results (e.g.
420 Ryan & Rand 1993; Gupta & Sundaran 1994; Wade et al. 1995; Coyne et al. 2005). The
421 respective advantages and disadvantages of choice versus no-choice mating experiments are
422 still a matter of debate (see Casares et al. 2005; Noor & Ortiz-Barrientos 2006). While it has
423 been argued that the design of choice experiments is more realistic than that of no-choice
424 experiments (Spieth & Ringo 1983; Alipaz et al. 2005a, b), Coyne et al. (2005) suggested that
425 when *Drosophila* of different strains or species congregate, the flies could evaluate potential
426 mates individually and sequentially reject unfavourable partners. In *D. bipectinata* sexual
427 activity is different between choice and no-choice situations, with no relationship with the sex
428 ratio (Singh & Sisodia 1999). Theoretically, the simultaneous presentation of two concurrent
429 stimuli allows us to obtain a more sensitive measure of the preference for one of them, while
430 their separate presentation provides a more rigorous test (Martin & Bateson 1993). However,
431 simultaneous presentation may be distracting to the subject, which may be ‘trapped’ by its
432 first choice whether or not it was random, and withdrawing from one target may be
433 incorrectly interpreted as approaching the other (Martin & Bateson 1993). However, mate
434 choice is a discrimination process between several objects. Consequently, only the
435 simultaneous presentation of two targets could yield a simplified but valuable representation
436 of what happens in the field where a subject could be trapped by, or diverted from, a stimulus
437 by another one. Both these effects could occur during male courtship with two target flies in
438 comparison with courtship towards a single female target and have to be taken into account in

439 evaluating male mate choice. Furthermore, to be more realistic future work should involve
440 more than two target females belonging to various strains.

441 The present work clearly shows that wild-type CS male flies are able to discriminate at a short
442 distance between wild-type sex pheromones. We have also shown that they are able to
443 discriminate between males and females of various strains, and that their preference could
444 change after the first gustatory contact, especially towards female targets.

445

446 <H2>***Discrimination varies with tester male and target flies***

447 The three tester males showed very different abilities to discriminate male and female CS
448 targets. While CS and *66a-Gal4/UAS-tra* tester males preferred the CS target female, *66a-*
449 *Gal4/+* males did not discriminate between the sexes. These differences were similar before
450 and after the first gustatory contact.

451 With [T | CS] males, the three testers preferred the Tai male target, but with the [T | D] males
452 *66a-Gal4/UAS-tra* testers did not discriminate between the targets. In comparison with the
453 other two male pairs, the [CS | D] pair induced the lowest CIs and discrimination. Moreover,
454 the behavioural response induced by the [CS | D] pair varied with the male tester genotype:
455 CS and *66a-Gal4/UAS-tra* testers preferred the *desat1* male target before and after gustatory
456 contact while the *66a-Gal4/+* males preferred the CS target male before the first gustatory
457 contact but showed no preference after this contact.

458 With female pairs, the response of tester males also varied before and after the first gustatory
459 contact. With [CS | D] pairs, the three testers showed similar responses before and after
460 gustatory contact: the CS female was always preferred. With [T | CS] pairs, males maintained
461 a similar response before and after gustatory contact. However, the CS female target was
462 preferred by CS and *66a-Gal4/+* testers whereas the Tai female target was preferred by *66a-*
463 *Gal4/UAS-tra* testers. Prior to the contact with [T | D] female pairs, *66a-Gal4/+* and CS

464 testers slightly preferred the Tai target whereas the *66a-Gal4/UAS-tra* males slightly preferred
465 the *desat1* target. After gustatory contact, the respective preferences of the CS and the *66a-*
466 *Gal4/UAS-tra* males were enhanced whereas *66a-Gal4/+* testers did not show any preference.

467

468 <H2>*Role of cuticular hydrocarbons*

469 Except in the control experiment, the target flies diverged strongly in their main CHs (Table
470 1), some of which are known or suspected to play a pheromonal role in the behaviour of CS
471 males (Table 1 and references therein).

472 The ability of the tester males to discriminate between CS male and female targets mostly
473 relies on the sexual dimorphism of their principal CHs: 7-tricosene (7-T) in males and 7,11-
474 dienes in females (Antony & Jallon 1982; Jallon 1984; Marcillac et al. 2005). Although it is
475 not known whether *Drosophila* males can perceive female dienes, some information related to
476 the perception of 7-T by *Drosophila* flies is available. In particular a subset of taste neurons
477 expressing the Gr66a receptor is involved in the 7-T detection (Lacaille et al. 2007, 2009).
478 This CH is suspected to play a reciprocal pheromonal role in both sexes: its gustatory
479 perception tends to inhibit male courtship (Ferveur & Sureau 1996; Svetec & Ferveur 2005;
480 Lacaille et al. 2007), whereas its olfactory perception by the female enhances her receptivity
481 (Grillet et al. 2006). Although its low volatility may be increased by male motion, the amount
482 of dispersed 7-T probably remains very low. This suggests that the olfactory perception of 7-
483 T by females would entail a lower detection threshold than its gustatory perception by control
484 males. This fits with two studies (Grillet et al. 2006; Lacaille et al. 2009). Our present results
485 also indicate that *66a-Gal4/+* males weakly discriminate sex pheromones. This was reflected
486 by the high number of transitions they showed between the two targets. Moreover the *66a-*
487 *Gal4/+* males showed the same total CI as CS and *66a-Gal4/UAS-tra* males, but their CI with
488 the target male was twice that of the latter two males. Consequently, their CI with the female

489 target was lower than that shown by CS and *66a-Gal4/UAS-tra* testers. Therefore, it is
490 possible that the weak discrimination ability of *66a-Gal4/+* males is due to the decrease in
491 their homosexual inhibition rather than in their heterosexual ardour.

492 The three tester males' responses towards males varied after the first contact. The Tai target
493 male was always preferred to the CS target male. While the preference of the CS and *66a-*
494 *Gal4/UAS-tra* tester males could be driven by the lower amount of 7-T produced by the Tai
495 males than by the CS males (Table 1), the *66a-Gal4/+* testers could be stimulated by the
496 higher amount of 7-pentacosene (7-P) produced by Tai males since they are unable to detect
497 7-T (Lacaille et al. 2007; Table 1). There have been suspicions that 7-P is an excitatory
498 compound for CS males (Antony et al. 1985; Ferveur 1997). Furthermore, 7-P is thought to
499 act synergistically with 9-pentacosene (9-P) to stimulate males attempting to copulate
500 (Ferveur & Sureau 1996; Siwicki et al. 2005). This preference for Tai males over CS males
501 also confirms a previous result obtained with a no-choice design: CS, *Gr66a-Gal4/+* and
502 *Gr66a-Gal4/UAS-tra* courted Tai target males more intensively than CS target males
503 (Lacaille et al. 2007, 2009).

504 With [T | D] male pairs, the *66a-Gal4/UAS-tra* tester males did not show any preferential
505 response to either target, while CS and *66a-Gal4/+* tester males preferentially chose the Tai
506 target. Since Tai and *desat1* males produce very similar amounts of 7-T (Table 1), the
507 preferential choice of CS and *66a-Gal4/+* testers could be induced by the highest amount of
508 7-P. On the other hand, *66a-Gal4/UAS-tra* males, which seem to be very sensitive to 7-T,
509 could be equally inhibited by the similar amounts of this substance carried by the two target
510 males (Table 1).

511 Compared to other target combinations, [CS | D] male pairs induced the lowest CIs and DIs.
512 This could be caused by (1) the presence of a large amount of 7-T on the CS target, and/or (2)
513 the absence or low amounts of stimulating chemicals (7-P or 7,11-dienes) on both CS and

514 *desat1* targets (Antony & Jallon 1982; Jallon 1984; Marcillac et al. 2005; Table 1). Before
515 gustatory contact, CS and *66a-Gal4/UAS-tra* testers preferred the *desat1* male target, while
516 the *66a-Gal4/+* testers preferred the CS male target. After the first gustatory contact, the *66a-*
517 *Gal4/+* testers did not prefer either target, while the other two testers persisted in preferring
518 the *desat1* male target. Furthermore, the preference of the *66a-Gal4/UAS-tra* testers for the
519 *desat1* male target was even enhanced after gustatory contact. These variations could also be
520 related to the 7-T gustatory detection deficiency of the *66a-Gal4/+* tester males and to the
521 gustatory oversensitivity of the *66a-Gal4/UAS-tra* males to the same compound.

522 For male target pairs, the statistical analysis (stepwise DAs) of the cuticular profiles is
523 consistent with our behavioural data: 7-T seems to be the most important CH used by tester
524 males to discriminate either between CS males and females, or between CS and Tai or CS and
525 *desat1* males. However, 7-T seems not to be required by males to discriminate target females.

526 Among pairs of target females, only the [CS | D] pairs induced clear behavioural responses in
527 the three tester males: the CS target female was always preferred. CS females produce high
528 levels of 7,11-dienes which are thought to stimulate male courtship (Antony & Jallon 1982;
529 Jallon 1984; Ferveur & Sureau 1996), while the *desat1* females mostly lack these CHs
530 (Marcillac et al. 2005). The 7,11-dienes were clearly preferred by CS males in a choice
531 experiment (Marcillac & Ferveur 2004). The preference of CS males for CS females also
532 supports the general expectation of Dobzhansky & Mayr (Dobzhansky & Mayr 1944; Mayr &
533 Dobzhansky 1945) that species and strains of *Drosophila* will generally show positive
534 assortative mating.

535 With the [T | D] female pairs, except for a possible involvement of the 7,11 nonacosadiene
536 (7,11-ND) in the preference of the CS tester for the Tai female target, our results could not be
537 easily related to any main female CHs. The *desat1* females possess larger amounts of 9-P and
538 7-P than the Tai females and these compounds are thought to stimulate male courtship

539 (Ferveur & Jallon 1996; Ferveur & Sureau 1996; Siwicki et al. 2005). Therefore, the
540 preference of the CS males for the Tai females and of *66a-Gal4/UAS-tra* males for the *desat1*
541 females could reflect a differential sensitivity to pentacosenes.

542 The [T | CS] female pairs triggered responses that were maintained before and after the
543 gustatory contact, but they differed between the three tester males: the CS female was
544 strongly preferred by the *66a-Gal4/+* males and slightly preferred by the CS tester males,
545 whereas the *66a-Gal4/UAS-tra* tester males preferred the Tai female. Since CS females
546 possess more dienes, 7-P and 7-T than Tai females, the excitatory effects of the two former
547 substances could explain the preference of CS and *66a-Gal4/+* tester males. Conversely, the
548 preference of the *66a-Gal4/UAS-tra* males for Tai females could be related to their strong
549 avoidance of 7-T in CS females.

550 The preference pattern shown by the *66a-Gal4/UAS-tra* males towards the three combinations
551 of female pairs is more difficult to interpret: they preferred the CS females in [CS | D] pairs,
552 the *desat1* females in [T | D] pairs and the Tai females in [T | CS] pairs. This nonlinear pattern
553 of preference suggests that discrimination involves more than one compound and that the
554 feminization of the Gr66a neurons could lead to an alteration in the ‘sensorial representation’
555 of the pheromonal blend, especially resulting in the higher sensitivity to some components
556 even at very low doses on the fly cuticle.

557

558 In conclusion, our results show that male mate choice exists in *D. melanogaster* and that male
559 discrimination between potential mates could involve chemical cues perceived before and
560 after the first gustatory contact. They also suggest that when a male is simultaneously
561 presented with a choice of pheromonal cues produced by two potential sexual partners, he
562 will use different cues depending on the CH patterns of both flies, and not the same cues for a
563 given fly. Furthermore, his behavioural response could depend on additional effects caused by

564 the simultaneous perception of the two target flies (like the ‘trapping’ and ‘diverting’ effects
565 explained above). This can lead to a nonlinear, thus unpredictable choice of a sexual partner.
566 Moreover, the initial partner choice preference based on olfactory cues can, in some cases, be
567 changed according to the choice of gustatory cues perceived later during the courtship ritual.
568 Finally, the choice of a sexual partner based on pheromonal cues can clearly be modified in
569 males with altered gustatory receptor neurons. This clearly indicates that the male fly’s
570 peripheral gustatory system is essential for pheromonal detection and mate choice. Further
571 experiments should help to elucidate the involvement of the olfactory system in male mate
572 choice, for example using *Or83b* mutants (Larsson et al. 2001).

573

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575

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579

580

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- 754
- 755

755 **FIGURE CAPTIONS**

756 **FIGURE 1:** Behaviour that single CS tester males directed towards a pair of same-sex
757 CS flies. A single CS tester male fly was given a choice between two decapitated target
758 males or females from a wild-type strain (CS), under white light. Eight behavioural
759 parameters reflecting the male's ability to perceive both volatile and contact
760 pheromones were measured, namely: (a) the courtship latency; (b) the first target
761 chosen; (c) the courtship index towards each target; (d) the discrimination index; (e) the
762 frequency of males courting each target; (f) the number of transitions between targets;
763 (g) the global choice and (h) the global choice intensity. Except for percentages (b, e, g),
764 parameters are expressed as their mean + SEM. Different lowercase letters indicate
765 significant differences between target flies (Kruskal - Wallis analysis, completed by a
766 Dunn's multiple pairwise comparison). , *** $P < 0.001$; z test in (e) and Mann - Whitney U
767 test in (h). Different < and > symbols indicate significant differences between cells; chi-
768 square test in (h). In (g), '∅' indicates males that had no preference. See Methods for
769 further details.

770

771 **FIGURE 2:** Ability of tester males of three genotypes (CS, *66a-Gal4/+* and *66a-*
772 *Gal4/UAS-tra*) to discriminate the sex pheromones of control flies. A single tester male
773 fly was given a choice between a decapitated target male and a decapitated target
774 female from a wild-type strain (CS), under red light. For parameters and statistics see
775 Fig. 1, except for the global choice intensities (h) which were compared using the
776 Kruskal - Wallis analysis (completed by a Dunn's multiple pairwise comparison). In (g),
777 '∅' indicates males that had no preference. See Methods for further details.

778

779 **FIGURE 3:** Ability of tester males of three genotypes (CS, *66a-Gal4/+* and *66a-*
780 *Gal4/UAS-tra*) to discriminate the quality of male cuticular pheromones. The three
781 tester males were put with target males of three variant strains with different
782 predominant cuticular hydrocarbons: CS, Tai (T), and *desat1(D)*, under white light.
783 Targets were paired in the three possible combinations: [CS | D], [T | D] and [T | CS].
784 For parameters and statistics see Figs 1 and 2. In (g), '∅' indicates males that had no
785 preference. See Methods for further details.

786

787 **FIGURE 4:** Ability of tester males of three genotypes (CS, *66a-Gal4/+* and *66a-*
788 *Gal4/UAS-tra*) to discriminate the quality of female cuticular pheromones. The three
789 tester males were put, under white light, with target females of three variant strains that
790 diverged in their predominant cuticular hydrocarbons: CS, Tai (T) and *desat1(D)*.
791 Targets were paired in the three possible combinations: [CS | D], [T | D] and [T | CS].
792 For parameters and statistics see Figs 1 and 2. In (g), '∅' indicates males that had no
793 preference. See Methods for further details.

794 **TABLE 1:** Cuticular hydrocarbons (CHs) in the male and female flies from the Canton-S (CS), Tai and *desat1* strains.

CH	Males				Females			Sex pheromonal role in CS strain
	CS	Tai	<i>desat1</i>	CS	Tai	<i>desat1</i>		
7-T	707 ± 36	143 ± 9	166 ± 11	125 ± 15	<i>tr</i>	29 ± 2	Inhibits male courtship ^{1,2,3} Enhances female receptivity ⁴	
5-T	46 ± 2	52 ± 3	25 ± 1	<i>tr</i>	<i>tr</i>	<i>tr</i>	Inhibits male courtship ^{2,5}	
9-P	101 ± 4	105 ± 3	238 ± 15	170 ± 7	52 ± 4	154 ± 8	Acts in synergy with 7-P to stimulate attempting to copulate ^{2,6} Acts in synergy with 7,11-dienes to stimulate male courtship ⁷	
7-P	125 ± 12	1107 ± 62	56 ± 3	158 ± 15	11 ± 1	66 ± 5	Acts in synergy with 9-P to stimulate attempting to copulate ^{2,6}	
7,11-HD	<i>tr</i>	<i>tr</i>	<i>tr</i>	469 ± 35	127 ± 8	109 ± 13	Stimulates male courtship ^{2,8}	
7,11-ND	<i>tr</i>	<i>tr</i>	<i>tr</i>	190 ± 22	129 ± 12	72 ± 122	Stimulates male courtship ²	

795 The methods used for the cuticular hydrocarbon (CH) extraction and their gas chromatographic analysis are described in the Methods. CHs are
796 listed according to their retention time, and their amount expressed as mean ± SEM, in ng/insect. *tr* = traces. Sources: **1:** Jallon 1984; **2:** Ferveur &
797 Sureau 1996; **3:** Lacaille et al. 2007; **4:** Grillet et al. 2006; **5:** Greenspan & Ferveur 2000; **6:** Siwicki et al. 2005; **7:** Ferveur 1997; **8:** Antony & Jallon 1982.

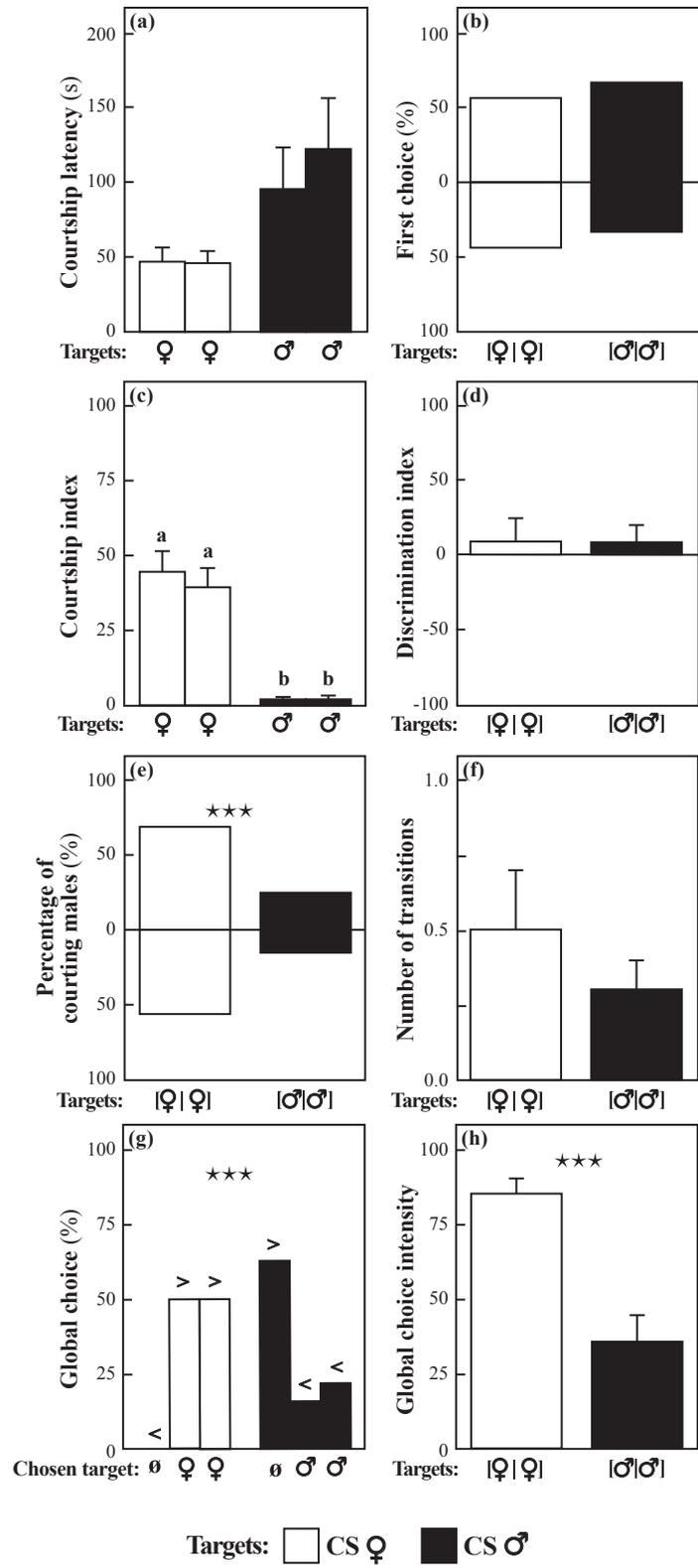
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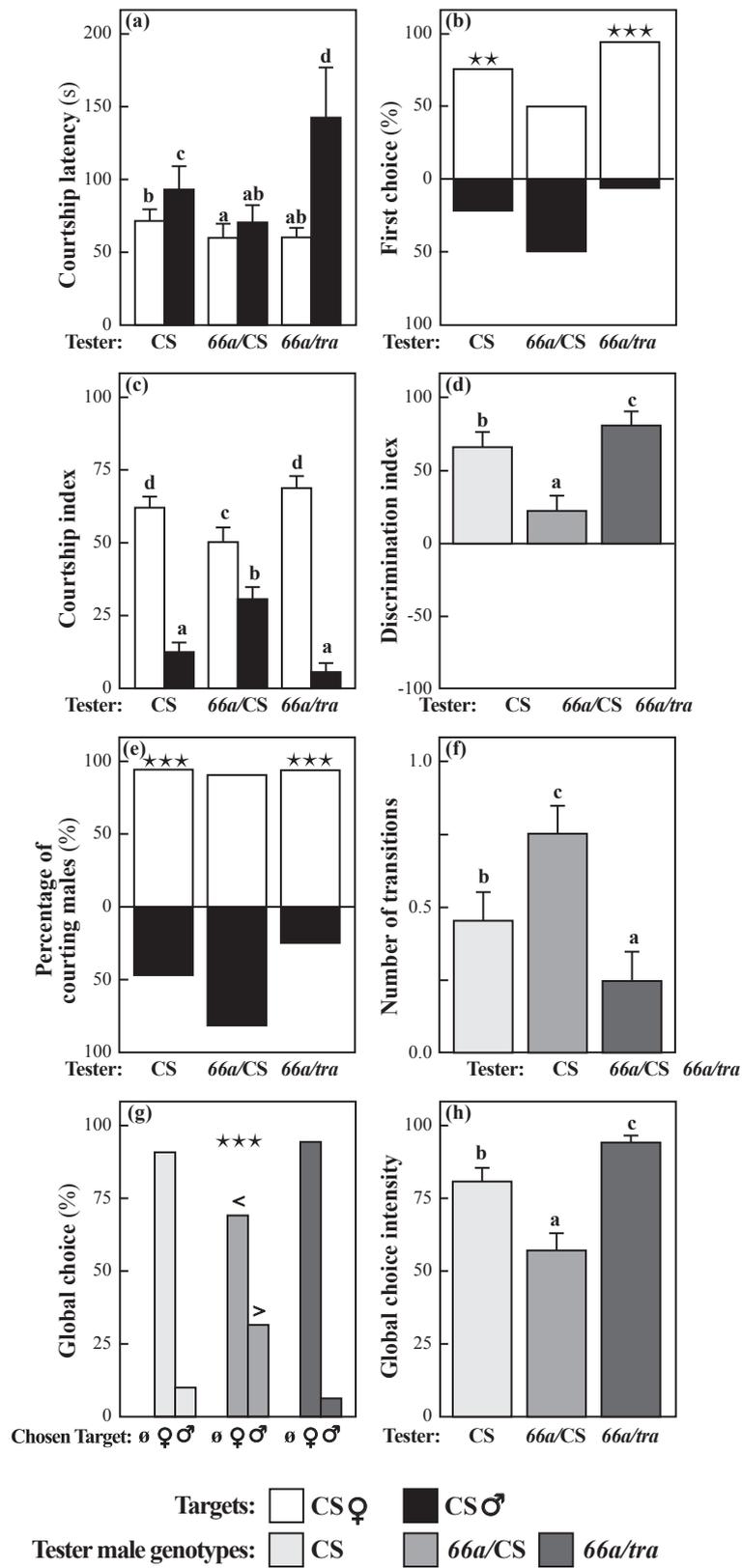
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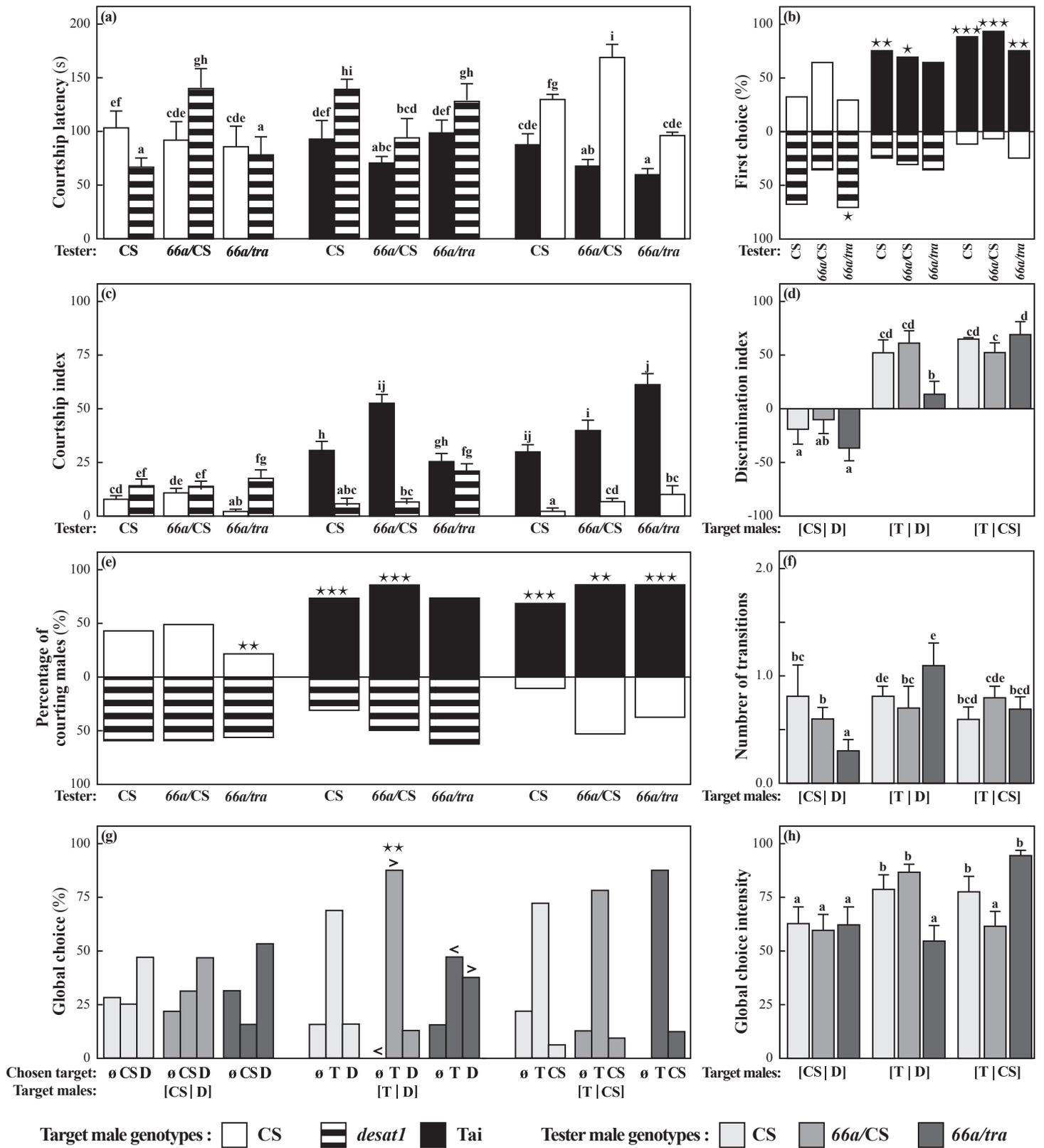
800 **TABLE 2:** Discriminant analyses (DAs) using either the sex or the strain as a
 801 qualitative variable and the absolute amounts of uncorrelated CHs as
 802 quantitative variables (without selection and with a forward stepwise
 803 selection of the quantitative variables)
 804

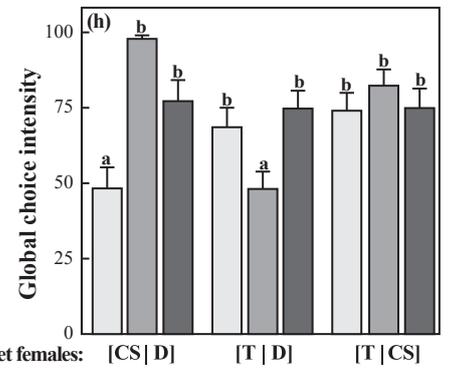
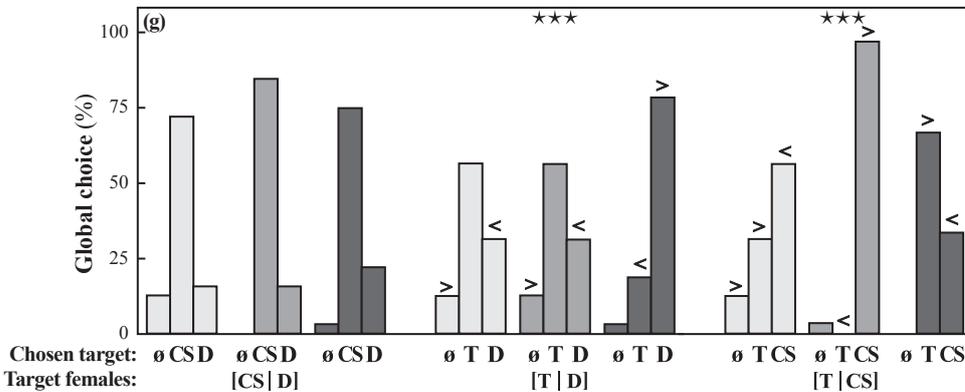
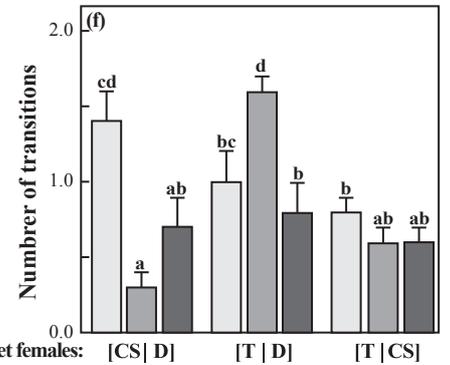
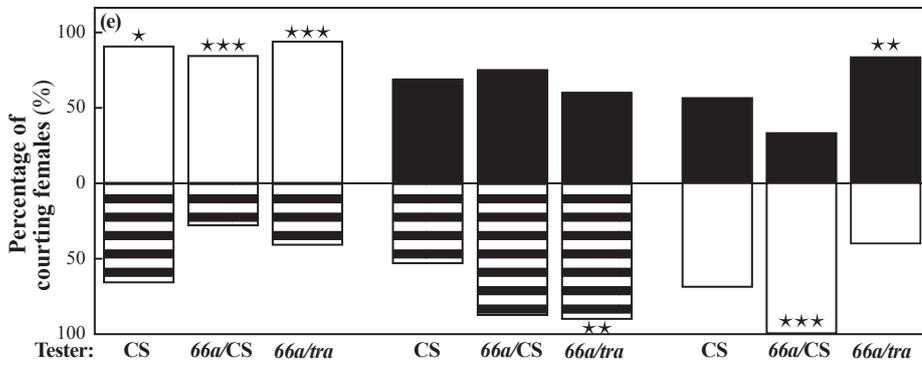
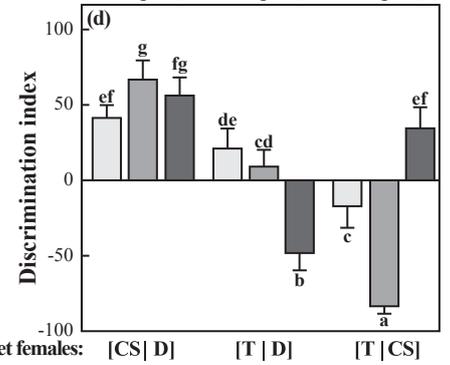
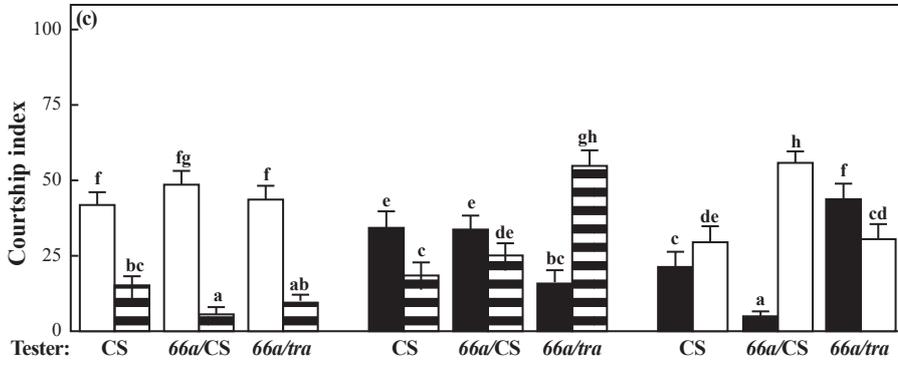
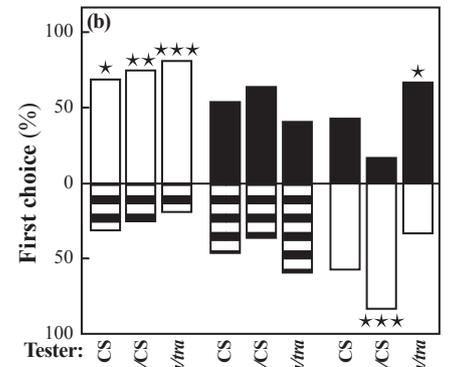
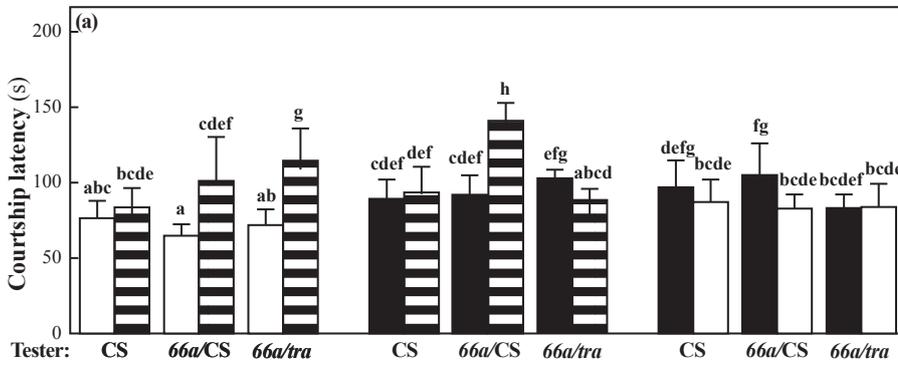
Pairs	DA	Stepwise forward DA
	% Correctly classified	Selected CHs (100% well-classified individuals)
Heterosexual pair	[100.0 100.0]	7-T > 5-P > 27-Br > 7,11-ND > 7-N
Male pairs		
[CS D]	[73.3 80.0]	7-T > 6-D > Br-H
[T D]	[46.7 80.0]	25-Br > 5-P > Br-H
[T CS]	[66.7 80.0]	7-T > 9-P > 5-P
Female pairs		
[CS D]	[73.3 73.3]	25-Br > 8-Te > 7-H > 27-L
[T D]	[40.0 13.3]	9-T > 5-P > 26-L > Br-H
[T CS]	[66.7 86.7]	23-L > 9-P > 5-P

805
 806 The results of the different DAs are summarized by their confusion matrices after
 807 cross-validation, and by the selected CHs for forward stepwise DA. The statistical
 808 method is detailed in the Methods. Selected CHs are listed according to their
 809 elution order: 6-D = 6-docosene; 9-T = 9-tricosene; 7-T = 7-tricosene; 23-L = n-
 810 tricosane; 8-Te = 8-tetracosene; 25-Br = methyl-branched pentacosane; 9-P = 9-
 811 pentacosene; 5-P = 5-pentacosene; 26-L = n-hexacosane; Br-H = branched-
 812 heptacosene; 27-Br = methyl-branched heptacosane; 7-H = 7-heptacosene; 27-L =
 813 n-heptacosane; 7,11-ND = 7,11-nonacosadiene; 7-N = 7-nonacosene.
 814
 815









Target female genotypes : CS *desat1* Tai Tester male genotypes : CS 66a/CS 66a/tra