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Mating increases *Drosophila melanogaster* females' choosiness by reducing olfactory sensitivity to a male pheromone

Authors: Philip Kohlmeier[1], Ye Zhang[2], Jenke A. Gorter[1], Chih-Ying Su[2] and Jean-Christophe Billeter[1,*]

Affiliations:

[1] Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, The Netherlands.

[2] Neurobiology Section, Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92093, USA.

*Correspondence to: j.c.billeter@rug.nl

Abstract:
Females that are highly selective when choosing a mate run the risk of remaining unmated or delaying commencing reproduction. Low female choosiness would therefore be beneficial when males are rare, but it would be maladaptive if males become more frequent; how can females resolve this issue? Polyandry would allow mating status-dependent choosiness, with virgin females selecting their first mate with little selectivity and becoming choosier thereafter. This plasticity in choosiness would ensure timely acquisition of sperm and enable females to increase offspring quality during later mating. Here, we show that *Drosophila melanogaster* females display such mating status-dependent choosiness by becoming more selective once mated, and identify the underlying neurohormonal mechanism. Mating releases juvenile hormone, which desensitizes OR47b olfactory neurons to a pheromone produced by males, resulting in increased preference for pheromone-rich males. Besides providing a mechanism to a long-standing evolutionary prediction, these data suggest that intersexual selection in *D. melanogaster*, and possibly in all polyandrous, sperm-storing species, is mainly the domain of mated females as virgin females are less selective. Juvenile hormone influences behaviour by changing cue responsiveness across insects, the neurohormonal modulation of olfactory neurons uncovered in *D. melanogaster* provides an explicit mechanism for how this hormone modulates behavioural plasticity.

Introduction

In most species, the costs associated with reproduction, such as energy investment into gamete production and non-reproductive postcopulatory periods, are higher in females than in males. A consequence of female-biased reproductive investment is that the selective pressure to choose high quality mating partners is higher in females than in males[1,2]. However, females might have difficulties finding a male that meets their criteria when male density or quality is low. Such male limitations can occur during seasonal changes, when migrating into a new habitat or as a result of habitat destruction. Under these conditions, high choosiness can become costly for females as it engenders the risk of delayed or reduced reproductive output[3], which can contribute to population extinction[4].

Theory suggests that these risks drive the evolution of mating status dependent choosiness: virgin females mate unselectively and become more choosy regarding male quality or attractiveness when re-mating[5,6]. Mating status dependent choosiness can be beneficial for females as it allows them to reduce the risk of dying as a virgin without lowering offspring

46 quality[6]. In contrast, a stable, non-plastic choosiness either increases the risk of virgin death
47 (if choosiness is constantly high) or reduces offspring quality (if choosiness is constantly
48 low). When potential mating partners are scarce, low virgin female choosiness relaxes sexual
49 selection on males and increases the likelihood of fertilization. If an already mated female
50 encounters males of higher quality or attractiveness later in life, increased post-mating
51 choosiness enables her to re-mate specifically with those males[6]. Elevated choosiness during
52 re-mating is particularly beneficial in species with last male sperm precedence, a phenomenon
53 in which most eggs are fertilized by the sperm of the last male the female mated with, because
54 it results in the production of offspring mostly from the higher quality male[6].

55 Despite strong theoretical support for its benefits, empirical evidence for the existence of
56 mating status dependent choosiness is scarce and has mainly been produced using indirect
57 measurements of female choosiness, such as receptivity[7–10]. Moreover, knowledge about the
58 underlying mechanisms that regulate transitions in choosiness is lacking. This gap in research
59 impairs our understanding of the dynamics that underlie sexual selection and how
60 environmental conditions impact the strength of sexual selection faced by males. The fruit fly
61 *Drosophila melanogaster* is an ideal model for closing this gap because it fulfils the
62 conditions for the evolution of plasticity in mate choice: females naturally mate with multiple
63 males[11], most eggs are fertilized by the last male they mated with[12], and population density
64 undergoes seasonal fluctuations leading to periods of reduced mate availability[13]. In this
65 study, we reveal that *D. melanogaster* female choosiness is higher in mated than in virgin
66 females. We deploy neurogenetic tools available in *D. melanogaster* to dissect the molecular
67 and neuronal mechanisms that underlie this plasticity. Our data demonstrate that this increase
68 in choosiness is mediated by a neurohormonal pathway that specifically leads to a post-
69 copulatory desensitization of Or47b Odorant receptor neurons, a class of olfactory sensory
70 neurons responding to a pheromone produced by males. These data thus demonstrate the
71 existence of mating status dependent choosiness and provide a mechanism for this plasticity.
72

73 ## Results

74

75 **Mating status modulates female mate choice through juvenile hormone.**
76 To test whether *D. melanogaster* females display mating status dependent choosiness, we
77 offered wildtype *Canton-S* females the choice to mate and re-mate with males of two
78 established wildtype strains, *Netherlands* (*NL*) and *Tai.* We found that female mate choice
79 was indeed mating status dependent as no preference for one of the two males was observed
80 in virgins whereas already mated females preferred *Tai* over *NL* males for re-mating (Fig.
81 1A). This finding additionally establishes *Tai* as the more attractive strain than *NL* due to their
82 higher mating success with mated females. In support of this we found that, when exposed to
83 only a single male, mating latency of *Canton-S* female virgin is independent of male genotype
84 but already mated females re-mate faster with a *Tai* than with a *Nl* male (Fig. S1). We then
85 transformed the mating preference data into a *Δvirgin→mated female choosiness score* based
86 on which we tested whether females display the predicted increase in female choosiness[6].
87 This score equals -1, if the female selected the *Tai* male as a virgin and the *NL* male as a
88 mated female (*i.e.* a decrease in choosiness because the male selected as virgin was more
89 attractive than the male selected as a mated female); equals 0, if the female selected the same
90 male genotype as virgin and mated female; and equals +1 if the female selected the *NL* male
91 as a virgin and the *Tai* male as a mated female (*i.e*. an increase in choosiness because the male
92 selected as virgin was less attractive than the male selected as a mated female). If mating
93 status dependent choosiness exists, this *Δvirgin→mated female choosiness* score would be
94 close to 0.5 with virgin females mating unselectively (*i.e.* equally choosing *NL* or *Tai*) and
95 100% of these females remating with *Tai* males. If mating status dependent choosiness does

96 not exist, then both virgin and mated females would select the attractive *Tai* male for mating
97 and hence, *Δvirgin→mated female* choosiness score would equal 0. In our experiment, the
98 *Δvirgin→mated female choosiness* score was 0.52 which demonstrates an increased choosiness
99 in females after mating (Fig 1A).
100
101 Post-mating increases in female choosiness have previously been reported in a few
102 species[7,14,15]. However, it remained unclear whether such shifts in choosiness resulted from
103 the acquisition of experience by mated females, who might have had limited information on
104 male quality as virgins, or are mediated by a dedicated mechanism that increases selectivity
105 post-mating irrespective of experience[6,14]. To distinguish between these two potential
106 explanations, we investigated the molecular mechanisms that trigger this post-mating shift in
107 choosiness. *D. melanogaster* females undergo well documented physiological and behavioural
108 changes in response to the first mating[16]. The majority of these female post-mating responses
109 are independent of experience since they are triggered by the receipt of sex peptide (SP), a
110 peptide in the male ejaculate that binds to SP receptor (SPR)-expressing neurons in the female
111 uterus[17–20]. We found that this SP/SPR pathway also regulates mating status dependent
112 choosiness: females which were mated as virgins with mutant males lacking SP were less
113 selective during remating than females that were mated as virgins with control males
114 transferring SP (Fig. 1B). Similarly, mutant females lacking *SPR* increased choosiness after
115 the first mating less strongly than control females (Fig. 1C). Together, these results
116 demonstrate that mating status dependent choosiness is a SP-dependent post-mating response
117 in *D. melanogaster*.

118 One of the downstream effects triggered by mating, and potentially by SP[21] is the increased
119 biosynthesis of Juvenile Hormone (JH)[22,23]; a pleiotropic hormone that, among others,
120 stimulates oogenesis[24]. We tested the hypothesis that a post-mating increase in choosiness is
121 mediated by elevated JH titres by treating virgin females with the JH analogue Methoprene. In
122 support of this hypothesis, Methoprene-treated virgin females displayed a stronger preference
123 for *Tai* males than virgin females treated with the ethanol vehicle alone (Fig. 1D). Taken
124 together, these data demonstrate that both the SP/SPR pathway and an increase in JH titres are
125 necessary to increase female choosiness. This increase in choosiness is likely to be controlled
126 by a dedicated, experience-independent mechanism because, firstly, the experience of mating
127 did not increase choosiness in females in which the SP/SPR pathway was disrupted and
128 secondly, an application of Methoprene was sufficient to increase choosiness in virgins
129 without the experience of mating. We thus provide empirical evidence for the existence of
130 mating status dependent choosiness in *D. melanogaster.*

131 **Or47b olfactory neuron is the neuronal substrate for state-dependent mate choice.**

132 The observed preference for *Tai* over *NL* males in mated females raises the question of how
133 females distinguish between these males. We focused on pheromones since they influence
134 female mate choice and different *D. melanogaster* strains display stable quantitative
135 differences in pheromone profiles[25–27]. As pheromones are often sensed by olfactory receptor
136 neurons (ORNs)[28–33], we hypothesized that the olfactory system is required for females to
137 increase choosiness after mating. In support of this hypothesis, we found that the choosiness
138 of *Orco* mutant females, whose ORNs are non-responsive to odour stimulation[34], increased
139 less strongly after mating compared to females in which *Orco* expression was rescued (Fig.
140 2A), showing that responsive ORNs are necessary for a post-mating increase in female
141 choosiness.

142 To identify the specific ORNs that mediate mating status dependent choosiness, we tested the
143 mate choices of females mutant for either of the three ORs documented to respond to male
144 pheromones in *Drosophila melanogaster: Or47b, Or67d and Or88a*[28,29,31–33] . While *Or88a*
145 and *Or67d* mutant females displayed increased choosiness after mating, *Or47b* mutant
146 females did not increase choosiness between virgin and mated state (Fig 2B). These data show
147 that mating status dependent choosiness is modulated through olfaction and specifically
148 requires a functioning *Or47b* OR.

149 How can OR47b ORNs mediate changes in female choosiness? Previous studies demonstrated
150 that changes in the sensitivity of a chemosensory neuron to its ligand or changes in firing rate
151 can modulate responsiveness to specific cues and by that behaviour[32,35–38]. We thus
152 hypothesized that mating status dependent choosiness is based on a post-mating change in the
153 physiology of the ORNs expressing *Or47b*. To test this, we performed single-sensillum
154 recordings in virgin and mated females on the OR47b, OR67d, and OR88a ORNs. The spike
155 response of OR47b ORNs in response to their ligand Palmitoleic acid (PA)[32] was lower in
156 mated than virgin females, with a ~50% difference at the highest PA dose (Fig $2C_{1-2}$). This
157 post-mating neuronal desensitization is specific to OR47b ORNs, as neither OR67d nor
158 OR88a ORNs responses to their pheromone ligands changed after mating (Fig. 2D&E). These
159 data suggest that mating status dependent choosiness is specifically mediated by a mating
160 status-dependent sensitivity of OR47b ORNs.

161 **JH specifically acts on OR47b ORNs and desensitizes its response to Palmitoleic Acid.**
162 We then asked whether the reduced post-mating sensitivity of OR47b ORNs is caused by
163 elevated JH titres, which we found to be sufficient to increase choosiness in virgin females
164 (Fig 1D). To answer this, we treated virgin females with Methoprene and measured the
165 spiking response of OR47b ORNs to PA. Methoprene decreased OR47b ORN sensitivity in
166 virgin females suggesting that elevated JH titres, which are characteristic of mated
167 females[22,23], are sufficient to lower OR47b ORN sensitivity (Fig. $3A_{1-2}$). To assess whether JH
168 reduces OR47b ORN sensitivity to PA by directly acting on these neurons, we knocked down
169 the JH receptor *Methoprene-tolerant* specifically in OR47b ORNs. In these females, OR47b
170 ORN sensitivity to PA remained at virgin level after mating, which shows that the binding of
171 JH to these neurons is necessary for their post-mating desensitization (Fig. $3B_{1-2}$). We then
172 tested whether knocking down *Methoprene-tolerant* in OR47b ORNs does not only inhibit the
173 post-mating decrease in neuronal sensitivity but also the increase in choosiness. Indeed,
174 females in which *Methoprene-tolerant* was knocked down specifically in OR47b ORNs
175 displayed a weaker increase in choosiness after mating compared to females with an
176 unmanipulated *Methoprene-tolerant* expression (Fig. 3C).
177
178 Our data suggest a model in which a post-mating increase in JH titres desensitizes OR47b
179 ORNs, which results in a reduced sensitivity to the aphrodisiac pheromone PA in mated
180 compared to virgin females. Therefore, males that produce large quantities of PA will be more
181 likely to be accepted by mated females for re-mating than males who produce low quantities.
182 In support of this, we found that *Tai* males produce larger quantities of PA than *NL* males
183 (Fig. 3D), as well as most other known pheromones (Table S1). This model predicts that
184 elevated JH titres in females and differences in PA quantities between males are sufficient to
185 explain mating status dependent choosiness. To test this, we applied additional PA to *NL*
186 males and pitted these PA^{+}*NL* males against vehicle only treated *NL* males for mating. We
187 exposed these males to two types of virgin females: Methoprene-treated virgins, which
188 display the higher choosiness of mated females, and ethanol-treated virgins, which display the
189 lower choosiness of virgin females (see Fig. 1D). Methoprene-treated virgin females selected
190 PA^{+}*NL* males more frequently for mating than vehicle-treated virgin females (Fig. 3E). This

191 demonstrates that high JH titres increase female choosiness for males with larger amounts of
192 PA, while virgin JH levels lead to females which lack a preference for males with larger
193 amounts of PA. Taken together, these data show that a specific post-mating decrease in
194 OR47b ORNs sensitivity mediated by JH increases mate choosiness towards pheromone-rich
195 males.
196
197 **Genetic mechanisms regulating OR47b ORN sensitivity**
198 Via which mechanisms does JH reduce Or47b sensitivity to PA in mated females? The JH
199 receptor Methoprene-tolerant functions as a JH-dependent transcription factor[39] that
200 influences gene expression[40–42]. In *D. melanogaster* males, binding of JH to Methoprene-
201 tolerant increases OR47b ORN firing rate in response to PA stimulation by upregulating the
202 expression of the male-specific transcription factor Fruitless^{M} (Fru^{M})[43]. Increased expression
203 of Fru^{M} results in increased expression of *pickpocket25 (ppk25)*, an ion channel subunit that
204 amplifies electrical signals generated after ligand binding[42,43]. This pathway is responsible for
205 mediating increased sensitivity of O47b ORNs to PA as males age or when they are part of a
206 social group, resulting in increased courtship intensity[32,42,43].
207
208 The *Or47b*-JH neurohormonal pathway we have identified in females thus has a male
209 counterpart. However, JH has opposite effects in the two sexes as JH binding increased
210 OR47b ORN sensitivity in males but decreases Or47b ORN sensitivity in females. This
211 predicts that the mechanism via which JH influences Or47b ORN sensitivity differs between
212 males and females in line with their different functions; increasing courtship intensity in
213 males and increasing choosiness in females. To test this, we used RNA-sequencing and
214 compared antennal gene expression between virgin and mated females. We identified a total
215 of 340 differentially expressed genes with 188 of them being more strongly expressed in
216 virgin and 152 of them being more strongly expressed in mated females (Fig 4A&B). In line
217 with what has been described in males[43], we found that *fruitless* female-specific transcripts
218 (*fru^{F}*) -derived from the same promoter as *fru^{M}* [44] - are more abundant in mated females,
219 which are characterized by elevated JH titres, than in virgin females (FDR < 0.0001, Fig. 4A).
220 However, *ppk25* (or other members of that gene family) did not change in expression between
221 virgin and mated females suggesting that in females, a change in *fruitless* expression does not
222 translate into a change in *ppk25* expression and that a decrease in OR47b ORN sensitivity is
223 not mediated through modulation of *ppk* genes (Fig. 4C & Fig. S2). The Or47b-JH
224 neurohormonal pathways thus has different downstream elements in males and females.
225
226 Besides changes in *ppk* genes, ORN firing rate can also be decreased by upregulating genes
227 encoding odorant degrading enzymes that inactivate pheromones before they can bind to their
228 respective receptor[45,46]. The observed decrease in OR47b ORN sensitivity is unlikely to be
229 caused by an increased degradation of PA as none of the candidate odorant degrading
230 enzymes were upregulated after mating (Fig. S2). Moreover, we found no olfactory or
231 ionotropic receptor genes including *OR47b*, (Fig. 4C & S2) to be differentially expressed in
232 the antennae of virgin and mated females which indicates that changes in receptor expression
233 are not mediating OR47b ORN sensitivity. The mechanisms through which JH affects Or47b
234 ORN sensitivity are thus different than in males and await further investigation.

235 Discussion

236 Theory has predicted the existence of mating status dependent choosiness as a solution to the
237 risk of females delaying mating due to high choosiness[6]. In this study, we provide
238 experimental evidence for this long-standing prediction and reveal a neurohormonal
239 mechanism through which *Drosophila melanogaster* females adjust choosiness. In particular,
240 increased JH titres, which are characteristic for mated females[22,23], causes a decrease in

241 sensitivity of the *OR47b* ORN and by that renders mated females more selective for males
242 with higher PA quantities.

243 **Mating status dependent choosiness influences the dynamics of sexual selection**
244 Our demonstration of the existence of mating status dependent choosiness combined with the
245 well documented high last male sperm precedence in *Drosophila* suggests that the strength of
246 sexual selection is influenced by an interaction between female mating status and the
247 availability of attractive males in this species. These mechanisms predict that if population
248 density is low or if the environment limits the production of attractive males, mating status
249 dependent choosiness can relax the strength of sexual selection on male traits and makes it
250 more likely that a virgin female will mate with the first male they encounter[6]. This ability to
251 reproduce even when population density is low can for instance be beneficial when migrating
252 into new environments. Conversely, if population density is high or if the environment allows
253 the production of attractive males, sexual selection on male traits in largely determined by
254 mated females because genes will be passed on predominantly from males attractive enough
255 to be selected for mating by non-virgin, *i.e.* selective, females[6]. The conventional practice of
256 using virgin females for studies on sexual selection is thus likely to have masked the strength
257 and dynamics of selective pressure imposed by *D. melanogaster* females and potentially of
258 polyandrous species in general. Our study thus stresses the fundamental importance of
259 factoring female mating status and availability of attractive males when studying sexual
260 selection.
261
262 **JH as a widespread modulator of behaviour**
263 JH-dependent modulation of sensory sensitivity is found across insects where it governs
264 state-dependent changes in behaviour. For instance, an increase in JH titres with age increases
265 ORN sensitivity to a stimulatory pheromone in *D. melanogaster* males, which results in
266 elevated courtship behaviour in older males and resembles one factor that contributes to the
267 higher mating success of older compared to younger males[32]. JH dependent pheromone
268 responsiveness in the moth *Agrotis ipsilon* modulates male responsiveness to sex pheromones,
269 and by that the timing of reproduction[47]. In this case, JH does not influence the pheromone
270 responsiveness of ORNs but instead of olfactory antennal lobe interneurons showing that JH
271 can impact behaviour through modulation of both cue sensing as well as the processing of
272 sensory input. In the locust *Locusta migratoria,* JH influences expression of olfactory-related
273 genes, such as ORs, potentially influencing the attraction to or repulsion of volatiles from
274 conspecifics and thus, gregarious versus solitary behaviour[48]. Non-reproductive division of
275 labour in the eusocial honeybee *Apis mellifera* is influenced by JH-dependent responsiveness
276 to task related cues. Foragers have both, a higher JH titre and sucrose responsiveness than
277 nurses[49,50] and treating nurses with JH analogues increases sucrose responsiveness[51] and
278 induces foraging[52]. Our study adds an adaptative change in mate choice to the list of
279 behaviours modulated by JH. JH and its modulation of the sensing or processing of cues is
280 thus emerging as a wide-spread and versatile neurohormonal mechanism modulating state-
281 dependent beneficial behaviours that has been recycled in different species with different
282 levels of social organization. To what extent such neurohormonal mechanisms interact with
283 pathways that perceive the experience of mating remains to be studied.
284
285 **The evolution of sex specific ORN functioning**
286 We show here that JH is responsible for a mating status-dependent change in olfactory
287 sensitivity allowing for adaptation by females to the risk of delaying reproduction due to high
288 choosiness. The net result of increased JH post-mating is a decrease in Or47b sensitivity in
289 mated females. This decrease is beneficial since it increases choosiness in females who have
290 already secured sperm for fertilization from their first mate. This female response however

291 should create a conflict with Or47b ORN functioning in males, in which elevated JH-titres
292 increase -not decrease- the neurons sensitivity. If JH had similar effects on OR47b ORNs in
293 both sexes, it would be disadvantageous for one of the two sexes because either male
294 courtship intensity would decrease with age or females would not be able to increase
295 choosiness after the first mating. Such opposite olfactory responses of the sexes to the same
296 stimulus (PA) and modulator (JH) bears the hallmark of the resolution of intra-locus sexual
297 conflict. In the course of evolution, a gene may acquire a function that is beneficial to one sex
298 but costly to the other resulting in opposite reproductive values when in a male than when in a
299 female genome. In such situation, evolutionary modelling predicts strong selection for the
300 evolution of mechanisms restricting expression of the gene to the sex where it is beneficial[53].
301 Such sex-specific expression can be achieved by placing the gene's transcription under the
302 control of transcription factors of the sex-determination pathway. One such transcription
303 factor in *Drosophila* is Fruitless^{M}, which is produced in the nervous system of males only[54].
304 The promoter regulating *fru*^{M} transcription in Or47b neurons has been previously shown to
305 increase activity in response to elevated JH in males[43]. The resulting Fru^{M} *protein* then
306 initiates the transcription of *pickpocket25* ion channels[42], which results in an enhanced
307 amplification of the electrical signals in OR47b ORNs generated after PA binding[42]. Our
308 RNA-sequencing data suggest that, in females, JH has a similar effect on the expression of *fru*
309 but not on the expression of any *pickpocket* genes downstream of *fru*. This observation is
310 consistent with intra-locus sexual conflict resolution because of the sex-specific expression of
311 *fru*[53]. The *fru*^{F} transcripts made in females are not translatable into a transcription factor due
312 to insertion of an early stop codon[44,55]. This can explain why unlike in males, in females, the
313 upregulation of *fru* does not result in transcriptional changes of any *pickpocket* genes. How JH
314 exactly decreases Or47b ORN in females remains to be determined, but our study firmly
315 establishes JH and Or47b ORN as the mechanism regulating beneficial changes in female
316 choosiness. Such mechanisms can include posttranslational modifications of proteins involved
317 in modulating neuronal sensitivity, such as *orco* dephosphorylation[56], and yet undescribed
318 mechanisms as 40.2% of those genes differentially expressed between the antennae of virgin
319 and mated females were uncharacterized or non-annotated proteins.

320

321 ## Materials and Methods

322 Rearing conditions

323 Flies were reared at 25°C in a 12:12hr light/dark cycle (LD 12:12). Rearing was done on food
324 medium containing agar (10g/L), glucose (167mM), sucrose (44mM), yeast (35g/L),
325 cornmeal (15g/L), wheat germ (10g/L), soya flour (10 g/L), molasses (30 g/L), propionic acid
326 (5 ml of 1M) and Tegosept (2g in 10ml ethanol). Virgin adults were collected using CO_2
327 anaesthesia and aged in same-sex groups of 15 in food vials (2.3cm X 9.3cm) for 5-7 days
328 until testing. Flies were aged in the same light and temperature conditions as rearing. To
329 ensure consistency in male quality, *NL* and *Tai* males were collected only for two days after
330 hatching started from their culture bottles.

331

332 List of flies

Full genotype	Short name	Source	Identifier
w^{1118}	*w*^{1118}	Vienna *Drosophila* Resource Centre	VDRC: 60000
Canton-S	*CS*	Billeter lab	
Oregon-R	*OR*	Billeter lab	
Wildtype: Netherlands	*NL*	Global Diversity Lines, Andrew Clarke	

Wildtype: Tai	*Tai*	Jean-François Ferveur	
+;+; SP^0 */TM3, Sb;+*	*Sp*^{0}	Reference [17]	BDSC: 77892
+;+;Delta^{130}*/Tm3, Sb;+*	*Δ130*	Reference [17]	
w$^-$*,Df(1R)SPR/FM7;+;+;+*	*SPR*$^-$	Reference [57]	BDSC: 7708
w$^-$*;Orco-gal4;Orco*^{2}*;+*	*Orco-gal4*	Reference [58]	
w$^-$*;UAS-Orco;Orco*^{2}*;+*	*UAS-Orco*	Reference [34]	
+;+;Orco^2*;+*	*Orco*$^-$	Reference [34]	BDSC: 23130
+;Or47b^3*;+;+*	*Or47b*$^-$	Reference [31,32]	BDSC: 51307
w$^-$*;p{Or47b-gal4,w*$^+$*};+;+*	*OR47b-gal4*	Reference [59]	BDSC: 9983
y$^-$*,w*$^-$*;UAS-Methoprene-tolerant RNAi;+;+*	*UAS-Met-RNAi*		VDRC:100638
w$^-$*;+; OR88a*$^-$*;+*	*OR88a*$^-$	Reference [33]	
+;+;Or67d$^-$*gal4;+*	*OR67d*$^-$	Reference [29]	

Mate choice assays

Disposable mating arenas were produced by piercing a hole (diameter = app. 3mm) into the side wall of a 35x10 mm Petri dish (Greiner Bio-One) and by covering the bottom of the dish with 3ml of fly food. Fifteen age-matched *NL* and *Tai* males were transferred to a colouring vial and the vial was rotated for 3 seconds. Colouring vials were produced by giving 0.5g of green or pink fluorescent powder (Slice of the moon™) into a fresh vial and rotating it until the inner surface of the vial is entirely covered. Residual powder was removed by tapping vials upside down. Colours were assigned randomly on every experimental day. One *NL* and one *Tai* male were transferred into a mating arena using a mouth pipettor and allowed to acclimatize for 30 minutes. A single female was added to the arena and was inspected for mating every 3 to 5 minutes for mating. In the experiment testing whether elevated JH levels delay mating latency, flies were checked once per minute. The male selected by the female was inspected for coloration using a UV flashlight (EFI 4, Perel). Virgin females that did not mate within 120 minutes were discarded. After mating ended, males were removed and single females were kept individually in the mating arena for 24hr. The next day, new mating arenas including one *NL* and one *Tai* male were prepared as described above. Mated females were transferred individually to the new mating arena and remating choice was recorded. Females that did not remate within 10 hr were discarded. As *Or47b* mutant females displayed low receptivity, remating was observed for 30hr. All experiments were performed at 25°C in a 12:12LD. To suppress potential leaky expression of *UAS-Met-RNAi*, all females in this experiment were raised at 18°C and kept at 18°C during the isolation phase between first mating and remating. To compensate for daily fluctuations, data collection for each experiment was done over several days. Lab bench was cleaned with acetone before and after every experiment to remove potential pheromonal residues. The tip of the mouth pipettor was renewed after each genotype to prevent pheromone transfer between groups.

Methoprene treatment

Canton-S virgin females were starved for 7hrs by keeping them in groups of 15 in a vial without food but with a moist cotton ball to prevent desiccation. 15µl 0.25% (v/v) Methoprene (Sigma-Aldrich) dissolved in 100% ethanol were spread over the surface of food in a vial, a validated method for delivering methoprene[60]. To ensure equal spreading, we used

366 vials with an even food surface and no bubbles. To control for vehicle, 15 µl of 100% ethanol
367 were used. Residual ethanol was evaporated by keeping the vials unplugged for 2hrs. Starved
368 *Canton-S* female virgins were transferred to the Methoprene or ethanol feeding vials and flies
369 were tested after 18 hours.
370
371 <u>Single-sensillum recordings</u>
372 A fly was wedged into the narrow end of a truncated plastic 200µl pipette tip to expose the
373 antenna, which was then stabilized between a tapered glass microcapillary tube and a
374 coverslip covered with double-sided tape. Single-unit recordings were performed essentially
375 as described [61]. Briefly, electrical activity of the ORNs was recorded extracellularly by
376 placing a sharp electrode filled with artificial haemolymph solution [62] in the at4 sensillum
377 (Or47b ORN and Or88a recordings). For at1 (Or67d ORN) sensillum recordings, 0.6X
378 sensillum lymph Ringer solution [63] was used. The reference electrode filled with the same
379 respective solution was placed in the eye. No more than three sensilla from the same antenna
380 were recorded per fly.
381
382 <u>Odour stimuli</u>
383 Both palmitoleic acid (4.5µl per filer disc) and *c*VA (10 µl per filer disc) were diluted in
384 ethanol and delivered via a 500-ms air pulse at 250 ml min-1 directly to the antenna from a
385 close range, as previously described [61]. Ethanol was allowed to evaporate for 1 hour in a
386 vacuum desiccator prior to experiments. Methyl palmitate was diluted in paraffin oil, applied
387 as 100µl aliquots on filter discs and delivered to the antenna via a 500-ms air pulse at 200 ml
388 min-1 through the main airstream (2000 ml min-1).
389
390 <u>Cuticular hydrocarbon and fatty acid quantification and treatment of flies with synthetic</u>
391 <u>pheromones</u>
392 Cuticular hydrocarbon (CHC) and Fatty acid quantification were performed as in [26] and [32],
393 respectively. Ten *Tai* or *NL* male flies were anaesthetized and placed in a 2-ml glass vial
394 (Sigma-Aldrich) containing 200 µl of an internal standard made of hexane spiked with 10
395 µg/ml of octadecane (C18) and 10 ng/ml of hexacosane (C26). Vials were vortexed at
396 minimum speed for 2min and the flies were removed using a clean metal pin. The resulting
397 extract was split in half (2 x 100µl) and evaporated under a nitrogen flow. One half was used
398 for fatty acid quantification; it was resuspended in 80 µl methanolic acid (Sigma-Aldrich) and
399 incubated at 60°C for 1 hour for trans-esterification, after which Methanolic acid was
400 evaporated under a Nitrogen flow and the sample resuspended in 100 µl Hexane. The other
401 half for CHC analysis was directly resuspended in 100 µl hexane.

402 Two µl of the resulting CHC or fatty acid extracts were analysed using an Agilent 7890 gas
403 chromatograph with a flame ionization detector (GC-FID), an Agilent DB-1 column
404 (Diameter: 0.180 mm; film 0.18 µm) and a split-splitless injector set at 250°C with 40ml/min
405 splitless flow. Injector valve opened 1.5 min after injection in splitless mode with helium as
406 carrier gas (flow: 37.2 cm/sec). The oven temperature program begins at 50 °C for 1.5 min,
407 ramping at 10°C /min to 150 °C, then ramping at 4 °C /min to 280°C and holding for 5
408 minutes. ChemStation software (Agilent technologies) was used to quantify compounds based
409 on peak areas relative to internal standard C18 and C26. Palmitoleic acid was only detectable
410 in the trans-esterified fatty acid samples and was identified based on both retention time [32] and
411 by spiking extracts with synthetic Palmitoleic acid.

412 For application of Palmitoleic Acid (PA) on *NL* flies, 0.5µl of a 333ng/µl solution of PA in
413 Acetone (Sigma-Aldrich) was pipetted directly onto the dorsal abdomen of male flies
414 anesthetized on ice, using a positive displacement micropipette with capillary pistons

415 (Microman, Gilson). Control males received the same treatment but only with Acetone. After
416 PA/Acetone application, both PA-treated and control-treated males were placed back in food
417 vials for 30 min to recover before being subjected to mate choice assays with Ethanol- or
418 Methoprene-treated virgin females as described above.
419
420 RNA-sequencing of virgin and mated female antennae
421 Mated females were produced by mating 10 *Canton-S* females (5-6 days old) with 15 *NL*
422 males in a vial for 2h. After 2h, males were removed and females were kept in the vial for
423 24hrs. Virgin females were kept in vials in groups of 10 for 24hrs. Antennae of 200 mated or
424 virgin females were pooled in 150µl Trizol (Ambion), homogenized with RNase-free micro
425 pestles, and stored at -80°C. Whole mRNA extraction and low input library construction were
426 performed by Novogene (Cambridge, UK). A total of 6 libraries (3 virgin and 3 mated female
427 samples) were indexed, pooled and sequenced on a single lane of an Illumina Novogene 6000
428 resulting in 68.3 ± 4.8 million (mean ± S.E.) 150bp long paired end reads per sample. Quality-
429 filtered and adapter-trimmed raw reads were mapped to the *D. melanogaster* genome (Release
430 6 plus ISO1 MT, August 2014) using *STAR* 2.7[64]. Differentially expressed genes were
431 identified by running a pairwise comparison between virgin and mated female samples in
432 *edgeR* 3.30.3[65]. Read counts were normalized for sequencing depth by dividing the read
433 counts by the total number of million reads in the library.
434
435 Statistical analysis
436 For experiments, in which we compared choosiness among females of the same mating status
437 (*e.g.* Fig. 1B), we ran a binomial Generalized Linear Mixed Model including selected male
438 (*Tai vs. NL*) as a response variable, genotype/treatment as an explanatory factor and date of
439 experiment as a random factor. For experiments in which we tested whether females display
440 an increase in choosiness, we used ordinal logistic regression to test whether the ordinal
441 *Δvirgin→mated female choosiness* score (see Results section for how this score was
442 calculated) differs between females of different genotypes. To additionally test whether the
443 *Δvirgin→ mated female choosiness* score is different from 0, we used a one sample t-test. All
444 analyses were run in *R* v.4.0.0 loaded with the packages *car*, *MASS*, *lme4* and *lmerTest*.

445 For dosage curve for odour concentration, two-tailed t tests comparing the experimental and
446 control groups were performed for each concentration and the p- value is indicated on the
447 figure by asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). These data are presented as mean ±
448 SEM and plotted in Igor Pro 6.32A (https://www.wavemetrics.com/products/
449 igorpro/igorpro). Dosage response curves were fitted with Hill equation for illustration
450 purposes.
451
452 **Data availability**: Raw sequencing reads are available on NCBI Short Read Archive
453 (https://www.ncbi.nlm.nih.gov/sra/SRX9940561[accn]); Ref PRJNA694952. All other raw
454 data are available on Dataverse-NL (reference: https://doi.org/10.34894/454UBX).
455
456 **Code availability**: R scripts and the gene expression analysis code are available on
457 Dataverse-NL (reference: https://doi.org/10.34894/454UBX).
458
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613
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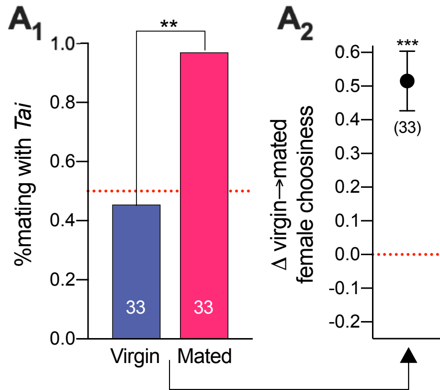
Figure Legends

Figure 1. Increased choosiness between virgin and mated state in *Drosophila melanogaster* females. Choosiness of virgin and/or mated females of the indicated genotypes that mated with *Tai* males when exposed to one *NL* and one *Tai* male. Blue bars indicate virgin females and magenta bars mated females. (A) *Canton-S* females mate choice indicated in percentages (A_1) and translated to change in choosiness (*Δvirgin→mated female choosiness*) (A_2) measured as the proportion of females that selected *Tai* males for mating after transitioning from virgin to mated state (mean ± standard error). Asterisks indicate a statistically significant increase in choosiness, compared to 0 (dashed red line). (B) Percentage of *Canton-S* mated females that mated with *Tai* males after having mated as virgins with males of the indicated genotypes. What is referred to as SP^- in the text is $SP^o/\Delta 130$. (C) Difference in choosiness between virgin and mated females of the indicated genotypes (mean ± standard error). (D) Virginal mate choice of females either fed the Juvenile Hormone analogue Methoprene or the Ethanol vehicle. Numbers in histograms and between brackets indicate sample sizes. Histograms labelled with the same letter are not statistically different. *n.s.*: non-significant; *:$p<0.05$; **:$p<0.01$; ***:$p<0.001$. Data were analysed using binomial generalized linear mixed model (A_1, B, D), one-sided t-test (A_2), and ordinal logistic regression (C). For detailed statistical analysis see Table S2.

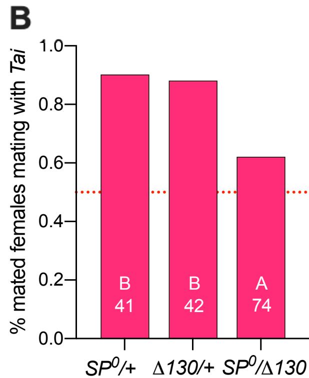
Figure 2. Or47b ORN is necessary for plastic female choosiness and decreases in sensitivity to palmitoleic acid after mating. (A) Difference in choosiness between virgin and mated females of the indicated genotypes (mean ± standard error). (B) Difference in choosiness between virgin and mated females of the indicated genotypes (mean ± standard error). (C_1) Representative traces (top), raster plots (middle), and peri-stimulus time histograms (bottom), of single-unit recordings of at4 sensillum housing Or47b ORN, in virgin and mated *Canton-S* females in response to a palmitoleic acid puff (shaded grey). Line width: s.e.m. Gray bar: stimulus duration. (C_2) Dose-response curves comparing Or47b ORN spike responses to PA in mated and virgin females. (D) Dose-response curves comparing Or67d ORN spike responses to cis-Vaccenyl Acetate in mated and virgin females. (E) Dose-response curves comparing Or67d ORN spike responses to Methyl Palmitate in mated and virgin females. Error bars indicate s.e.m. *n.s.*: non-significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. Data were analysed using ordinal logistic regression (A, B) and two-tailed t-tests (C – E). Number of replicates per group are indicated in the histograms or between brackets. For detailed statistical analysis see Table S2.

Fig. 3 Juvenile hormone reduces Or47b ORN sensitivity. (A_1) Sample traces of single-unit recordings of at4 sensillum housing Or47b ORN in vehicle and Methoprene (Met)-treated virgin females in response to a palmitoleic acid (PA) puff (shaded grey). (A_2) Dosage curves of Or47b ORN spike responses to dilutions of PA from control and Methoprene-treated virgin females (Mean ± s.e.m; n=9, from 4~5 flies). ($B_{1\text{-}2}$) Same as $A_{1\text{-}2}$, but in virgin and mated females of the indicated genotypes. (B_3) Comparison of Or47b ORN responses to PA (10^{-1}) in

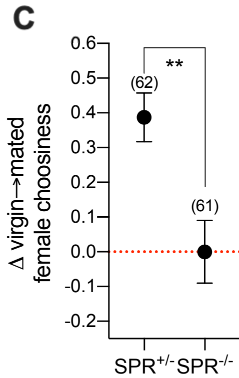
669 virgin and mated females of the indicated genotypes. Empty circles represent responses from
670 individual ORNs. (C) Difference in choosiness between virgin and mated females of indicated
671 genotypes (mean ± standard error). (D) Quantification of Palmitoleic acid in individual *NL*
672 and *Tai* males (Mean ± s.e.m). (E) Mate choice of virgin females treated with either
673 Methoprene (Met) or vehicle, towards *NL* males treated with PA. Numbers between brackets
674 indicate sample sizes. *: $p<0.05$; **:$p<0.01$; ***:$p<0.001$. Data were analysed using two-
675 tailed t-tests (A, B), ordinal logistic regression (C), unpaired two-tailed t-test (D), and
676 binomial generalized linear mixed model (E). For detailed statistical analysis see Table S2.
677 **Fig. 4**: **Transcriptomic differences between the antennae of virgin and mated females**.
678 (A) Heatmap of genes differentially expressed between virgin and mated females and
679 dendrogram of replicate mRNA antennal libraries based on differentially expressed genes
680 showing that they cluster by female mating status (virgin or mated). (B) Venn diagram
681 displaying the number of differentially expressed transcripts and of those transcripts with no
682 change in expression. (C) Normalized read counts of *fruitless* female-specific transcript (fru^F),
683 *pickpocket25* (*ppk25*) and *Or47b* in the antennae of virgin (blue) and mated (pink) females
684 (Mean ± s.e.m). *: *FDR*<0.05; **:*FDR*<0.01; ***:*FDR*<0.001. FDR values obtained from
685 gene expression analysis. For detailed statistical analysis see Table S2.



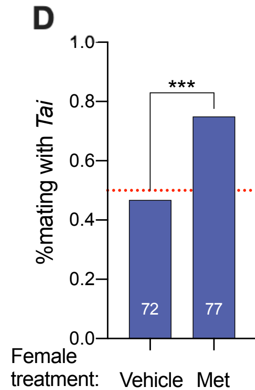
A1
%mating with Tai
**
33
33
Virgin
Mated



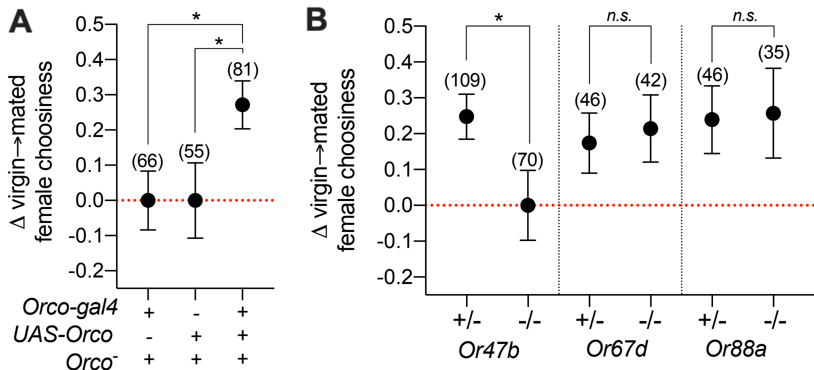
A2
Δ virgin→mated female choosiness
***
(33)
B
% mated females mating with Tai
B 41
B 42
A 74
SP0/+
Δ130/+
SP0/Δ130

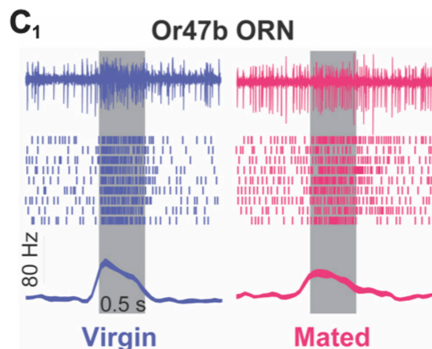


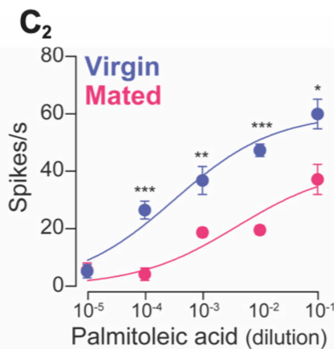
C
Δ virgin→mated female choosiness
**
(62)
(61)
SPR+/-
SPR-/-

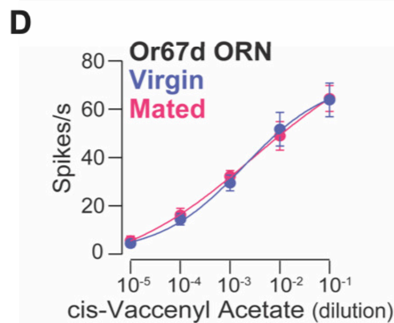


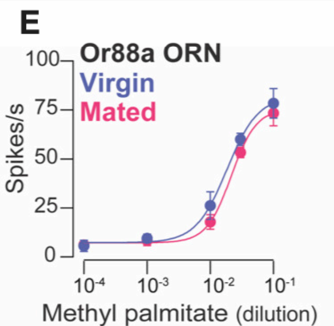
D
%mating with Tai
***
72
77
Female treatment:
Vehicle
Met

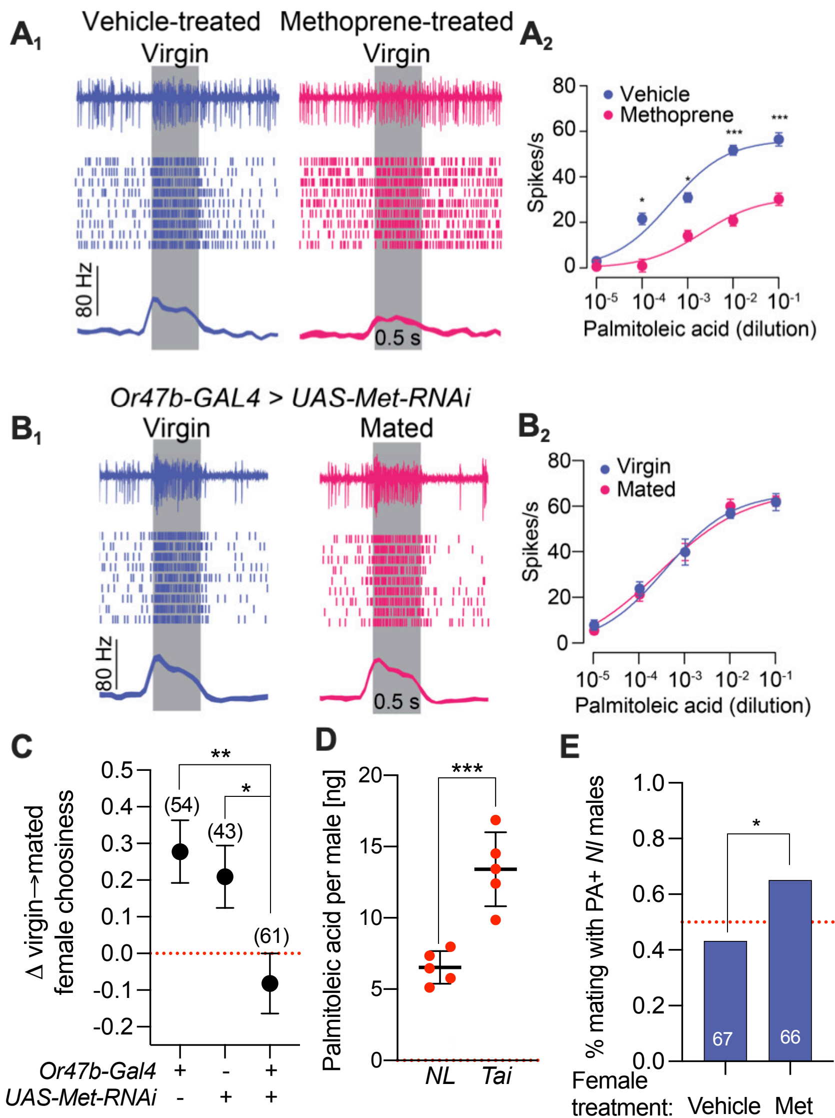

A
Δ virgin→mated female choosiness
(66)
(55)
(81)
*
*
Orco-gal4
UAS-Orco
Orco⁻
B
*
n.s.
n.s.
(109)
(70)
(46)
(42)
(46)
(35)
+/-
-/-
Or47b
Or67d
Or88a


C1
Or47b ORN
80 Hz
0.5 s
Virgin
Mated


C2
Virgin
Mated
Spikes/s
Palmitoleic acid (dilution)

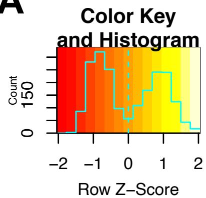

D
Or67d ORN
Virgin
Mated
Spikes/s
cis-Vaccenyl Acetate (dilution)

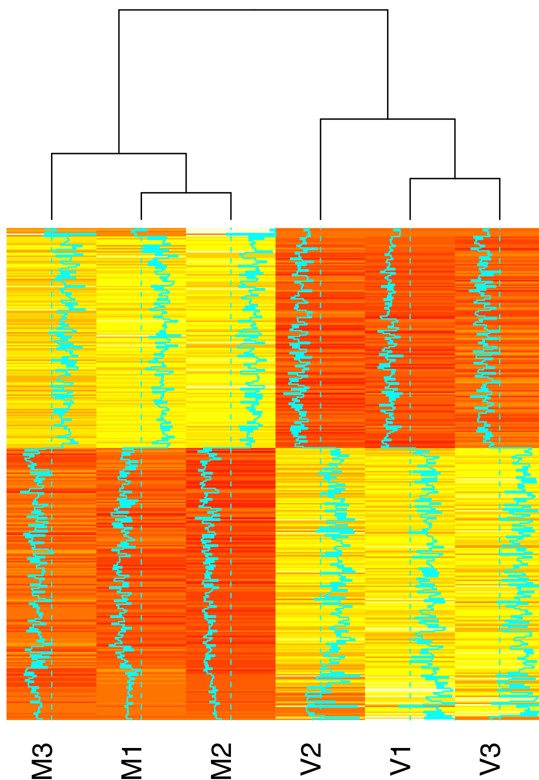

E
Or88a ORN
Virgin
Mated
Spikes/s
Methyl palmitate (dilution)



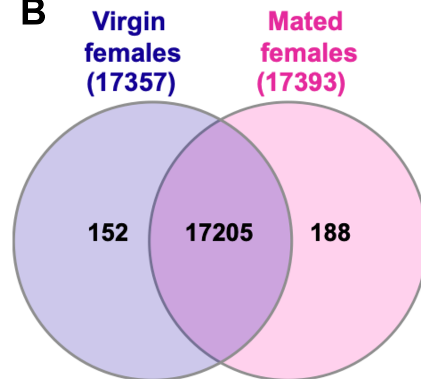
A1
Vehicle-treated Virgin
Methoprene-treated Virgin
80 Hz
0.5 s
A2
Vehicle
Methoprene
Spikes/s
Palmitoleic acid (dilution)
Or47b-GAL4 > UAS-Met-RNAi
B1
Virgin
Mated
80 Hz
0.5 s
B2
Virgin
Mated
Spikes/s
Palmitoleic acid (dilution)
C
Δ virgin→mated female choosiness
(54)
(43)
(61)
Or47b-Gal4
UAS-Met-RNAi
D
Palmitoleic acid per male [ng]
NL
Tai
E
% mating with PA+ Nl males
67
66
Female treatment:
Vehicle
Met

A





B



C