Cryptic female choice in response to male pheromones in *Drosophila melanogaster*

Nicolas Doubovetzky[1], Philip Kohlmeier[1,2], Sanne Bal[1] and Jean-Christophe Billeter[1,*]

Affiliation

[1]Groningen Institute for Evolutionary Life Sciences, University of Groningen, 9474AG Groningen, The Netherlands.

[2]Current Address: University of Memphis, Department of Biological Sciences, Memphis, TN 38152-3530, USA

*To whom correspondence should be addressed: j.c.billeter@rug.nl

Abstract

Females control the paternity of their offspring by selectively mating with males they perceive to be of high quality. In species where females mate with multiple males in succession, females may bias offspring paternity by favoring the sperm of one male over another, a process known as cryptic female choice (CFC). While evidence of CFC exists in multiple taxa, the mechanisms underlying this process have remained difficult to unravel. Understanding CFC requires demonstration of a female-driven post mating bias in sperm use and paternity, and a causal link between this bias and male cues. Here, we show that in the vinegar fly, *Drosophila melanogaster,* mated females eject the ejaculate of the first male they mated with faster when exposed to the pheromones of an attractive male than in the presence of an unattractive one. Using transgenic males expressing fluorescent sperm, we show that exposure to attractive males between mating causes twice-mated females to bias sperm storage towards the second male, affecting paternity. Using pheromonal bioassays in combination with genetic manipulation of sensory systems, we show that females modulate ejaculate ejection latency in response to male pheromones heptanal and 11-cis-Vaccenyl acetate (cVA) sensed via olfactory receptor neurons OR35a, Or22a, Or65a and OR67d, demonstrating that polyandrous females use male pheromonal cues to modulate ejaculate ejection timing and impact paternity. We provide the first demonstration to our knowledge of a CFC mechanism allowing a female to increase or decrease the share of paternity of her first mate depending on the sensing of the quality of potential mates in her environment. These findings showcase that paternity can be influenced by events that go beyond copulation and highlights the importance of post-copulatory sexual selection.

Keywords

Cryptic female choice, post-mating sexual selection, sperm ejection, sperm storage, pheromones, olfaction, *Drosophila melanogaster*.

Introduction

Individuals of sexually reproducing species evolved multiple mechanisms to identify and select high quality mating partners. These traits include pre-copulatory mechanisms such as selecting highly fecund mates, or those with genetic quality that confer survivability and fecundity to their offspring. In species where females mate with multiple males in one reproductive episode, sperm from different males interact in the female reproductive tract. This creates an opportunity for competition between sperms, often leading the last male to mate to fathers most of the offspring - a phenomenon known as last male sperm precedence. Although often overlooked, polyandry also allows for females to apply post-copulatory mechanisms that bias sperm use towards one male or the other, affecting the share of offspring of each male – a poorly understood phenomenon called cryptic-female choice (CFC).

One CFC mechanism is ejaculate ejection, via which females expel sperm from a previous mating which can bias that male's paternity share (Eberhard et al., 1996; Eberhard et al., 2009; Firman et al., 2017). In the feral fowl, females have a higher chance of ejecting the ejaculate of a low-ranking male than a high-ranking one, which favors paternity by higher ranking males (Pizzari & Birkhead, 2000). In the Ulidiid flies *Euxesta bilimeki*, females partially or totally expel sperm transferred during mating depending on the male size (Rodriguez-Enriquez et al., 2013). Understanding the mechanisms that underlie CFC has however remained elusive because this process happens hidden in the female reproductive tract and requires demonstration of a female-driven post mating bias in sperm use and paternity, and a causal link between this bias and male cues (Firman et al., 2017).

Drosophila melanogaster is an ideal model to investigate mechanisms of CFC because it is a polyandrous species (Billeter & Wolfner, 2018; Firman et al., 2017) for which genetic tools have been developed to label spermatozoa of different males with different fluorescent markers and directly observing them in the female reproductive tract (Laturney et al., 2018; Lee et al., 2015; Manier et al., 2010). Moreover, the cues based on which females choose which male to mate with, a pre-copulatory choice, are also partially identified, including the male pheromones cis-Vaccenyl Acetate (cVA), Palmitoleic acid, and 7-Tricosene (Billeter et al., 2009; Grillet et al., 2006; Kohlmeier et al., 2021; Kurtovic et al., 2007). *D. melanogaster* females also naturally eject male ejaculates. Indeed, directly after mating females store a portion of the sperm from the male ejaculate in sperm storage organs located in their reproductive tract, a process that lasts a few hours, and then eject the unstored ejaculate from their reproductive tract (Laturney & Billeter, 2016; Lee et al., 2015; Manier et al., 2010). After this first ejaculate ejection, females can remate with a second male, which occurs in both lab and nature settings (Billeter & Wolfner, 2018;

Laturney & Billeter, 2016). With the arrival of the new ejaculate, second male sperm displaces a portion of the first male sperm from the female sperm storage organs, and these displaced sperms are then ejected by the female (Manier et al., 2010). The timing of this second ejaculate ejection correlates with paternity in a way where the longer females take to eject, the more displacement can occur, and the more eggs are sired by the second male (Lüpold et al., 2013). Although the mechanism remains unknown, it is presumed that in this case, females have more time to store sperm from the second male and for it to displace resident sperms from the first male.

Given that ejaculate ejection latency is associated with female sperm storage, we hypothesized that it could be a CFC mechanism diminishing storage of sperm from a recent mate in the presence of attractive males in order to favor the sperm of these potential future mates. In this study, we observed an effect of male quality on the timing of ejaculate ejection and offspring production in *D. melanogaster*. We show that females eject the ejaculate from their first mate earlier in the presence of an attractive male. When females remate, the advanced ejection correlates with a bias in paternity and sperm stored by the female, biasing paternity by the second male. We also identify that the male pheromones cVA and heptanal modulate these effects through detection by specific female odorant receptors. We thus propose a mechanism of cryptic female choice that bias paternity in response to male cues.

Results

Mated females modulate ejaculation latency and bias paternity depending on the quality of potential mates in their environment.

Cryptic female choice (CFC) is a post-copulatory mechanism that biases sperm use and impacts paternity ratios. We hypothesized that female modulation of ejaculate ejection latency is a CFC mechanism, allowing females to bias the paternity share of a given male depending on the female's mating opportunities. If true, we predicted that recently mated females should shorten the ejection latency of their first mate ejaculate when sensing a mate of high quality in their environment, resulting in a decrease in first mate share of paternity in favor of their next mate.

To identify high quality males, we assessed *Canton-S* (*CS*) female preference between males from two strains, *CS* and *Oregon-R* (*OR*). In a competitive mate choice experiment, we found that 82.7% of virgin females mated with *CS* over *OR* males (n=53; Fisher's exact test; $P<0.0001$, Supp fig 1A). Taking female preference as a proxy for male quality, this suggests that *CS* males are of higher quality than *OR* males to *CS* females. Next, to link preference with ejection latency, we tested if mate preference also impacted the ejaculate ejection latency given different postcopulatory conditions. We exposed single *CS* females recently mated to a *CS* male to either a *CS*, *OR*, or no

male and measured ejaculate ejection latency. We found that females that were exposed to *CS* males ejected about 60 minutes earlier compared to females exposed to *OR* males or females kept in isolation (Fig 1B). Recently mated females thus advance timing of ejaculate ejection from their first mate when exposed to high quality males.

To test whether modulation of ejaculate ejection latency for the first mate impacts paternity share, females were first mated with a *CS* male with Green Fluorescent Protein (GFP)-labelled sperm, then exposed to either *CS* or *OR* males until the first ejaculate ejection (before they could remate) and then immediately transferred in an arena with a *CS* male with Red Fluorescent Protein (RFP) labelled sperm for remating (Fig1.A). This produced double-mated females that either advanced or did not advance ejaculate ejection time after their first mating but otherwise mated with similar males, removing confounding factors such as differences in male sperm competitive abilities. Females exposed to *CS* or *OR* males between first and second mating ejected ejaculate from their second mates at similar times, indicating that all variation comes from the period during which once mated females were exposed to *CS* or *OR* males (Supp Fig 1F). These twice-mated females were then either isolated and the paternity of the resulting offspring determined to belong to the first or the second *CS* male based on GFP/RFP expression, or flash frozen to determine the number of sperms stored from GFP/RFP males (Fig 1A). We found that twice mated females exposed to *CS* males between first and second mating produced a lower proportion of offspring from their first mate than females exposed to *OR* males (Fig 1C). These data show that exposure to a preferred male after the first mating resulted in lower first mate paternity share. Females exposed to high quality *CS* males between first and second mating had an overall lower fecundity (Fig 1D) and, critically, less offspring from the first male compared to females exposed to *OR* males (Fig 1E), while the number of offspring from the second male remained similar in the two exposures (Fig 1F). These results show that *D. melanogaster* females bias paternity in favor of the second male when exposed to high-quality males in their post mating environment and, alternatively biasing paternity in favor of the first male when sensing low-quality mates. We thus show that *D. melanogaster* females can exert CFC.

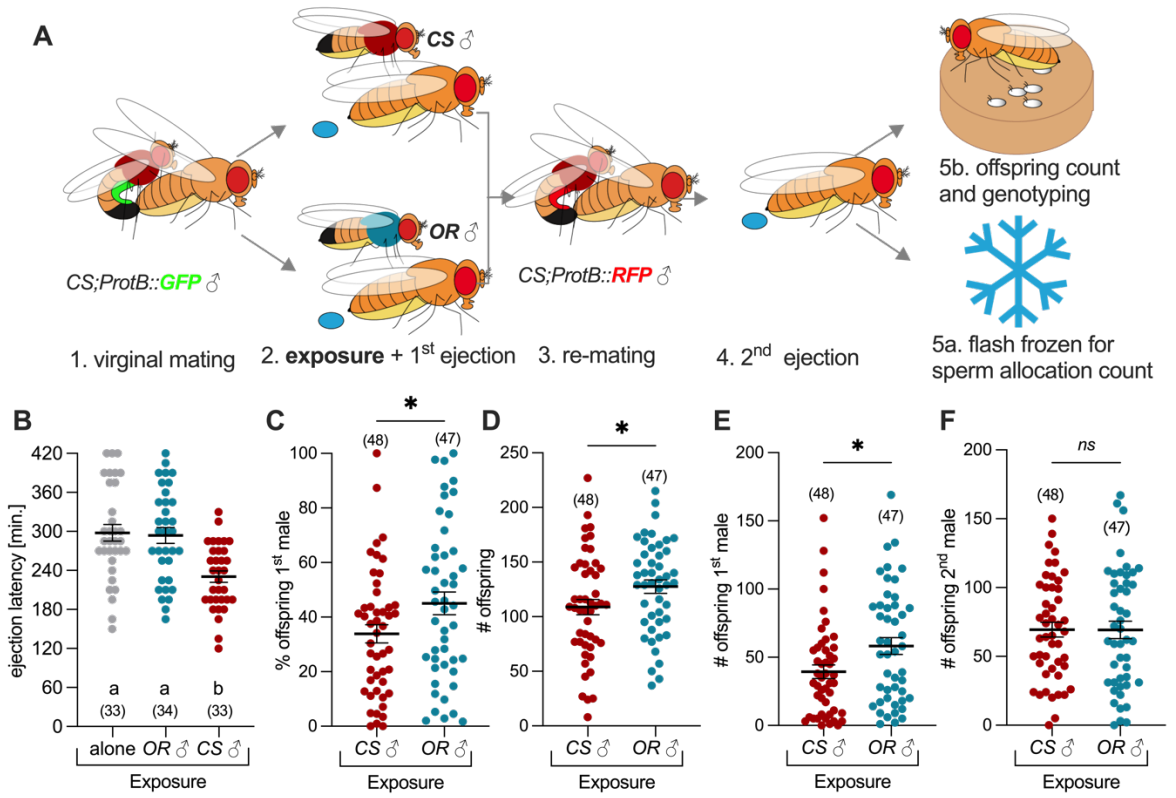


Figure 1 Twice-mated females modulate ejaculate ejection latency and bias paternity depending on the quality of males they are exposed immediately after their first mating. (A) Schematic of the experiment. (B) Ejaculate ejection latency of mated *Canton-S* (*CS*) females isolated, in the presence of an *Oregon-R* (*OR*) male or a *CS* male. (B-F) Offspring production of *CS* females twice mated with CS males as in (A) but exposed to either one *CS* or *OR* male in-between the two mating: (C) Percentage (%) of offspring from the first male, (D) Total number (#) of offspring, (E) Number of offspring from first male, and (F) Number of offspring from second male (mean ± S.E.M). Numbers between brackets indicate sample size. Mean ± S.E.M are indicated in the graphs. Scatter-plot labelled with the same letter are not statistically different. *n.s.*: non-significant; *:p<0.05; **:p<0.01; ***:p<0.001.For full statistical details see Supplementary Table S1.

Females bias sperm storage in the seminal receptacle depending on male quality

Females store sperm in two different specialized structures in their reproductive tract. The seminal receptacle is the main organ storing the majority of sperm, while the spermathecae store less sperm but for longer term (Pitnick et al., 1999; Schnakenberg et al., 2011, 2012)(Fig 2A). To understand how females bias paternity, we examined the proportion of the first male sperm (labeled with GFP) and second male sperm (labeled with RFP) in storage in twice mated females exposed to *CS* or *OR* males between first and second mating (as in Figure 1A). For this, we counted the sperm stored in the seminal receptacle and the spermathecae separately. Although the total number of sperm in storage was equal (Fig 2B). In the seminal receptacle, we observed a bias against the first male's sperm when the female was exposed to a *CS* male, and in favor of the first male in the presence of an *OR* male (Fig 2C). These biases result from both a lower number of first male's sperm (Fig

2D) and a higher number of second male sperm (Fig 2E) stored in the seminal receptacle when exposed to a *CS* male, and the opposite when exposed to an *OR* male. When analyzing sperm storage in the spermathecae, we observed an equal amount of sperm between groups (Fig 2F), but no bias in the proportion of first versus second male sperm (Fig 2G). There were also no differences in the number of sperm from the first (Fig 2H) or second male (Fig 2I). Difference in sperm allocation in the presence of *OR* or *CS* males between two mating is thus in keeping with differences in paternity observed depending on male exposure in the seminal receptacle but not in the spermathecae (Fig. 1). We conclude that females exert CFC by biasing sperm storage in the seminal receptacle.

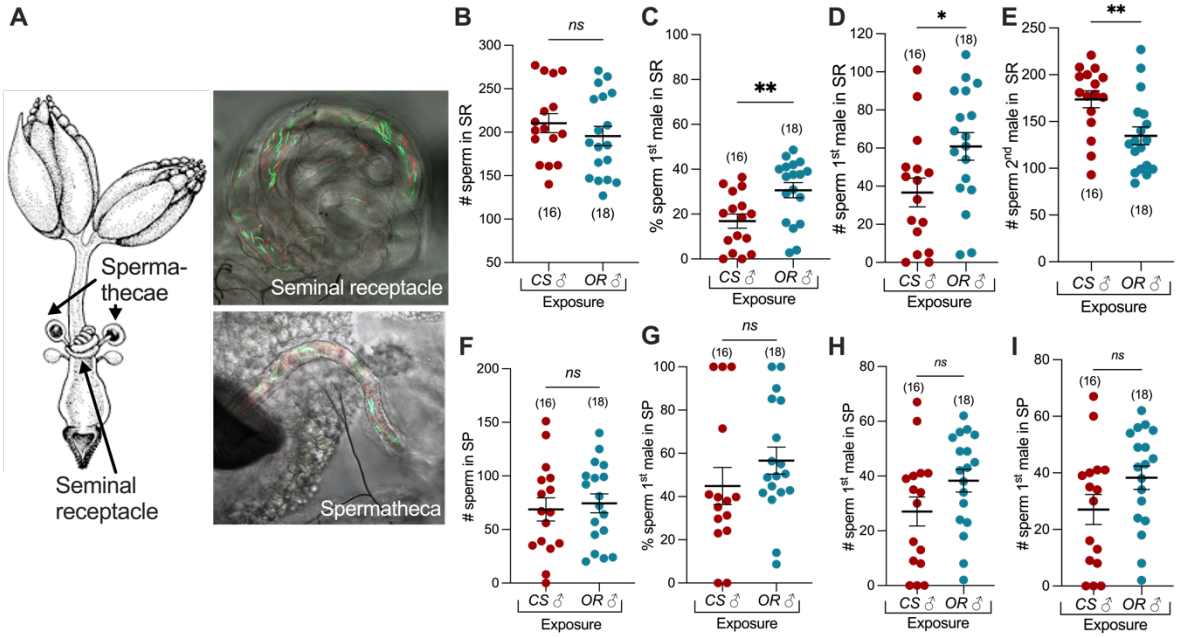


Figure 2 Sperm storage bias toward the first male when exposed to a low-quality potential mate between two mating. (A)Schematics of the female reproductive tract with confocal microscopy images detailed green and red sperms from different males in the seminal receptacle or the spermatheca. (B-I) number (#) or percentages (%) of sperms from first or second males in storage in the indicated sperm storage organ of a twice mated females exposed to either *CS* or *OR* males between the two mating (See Fig 1A). Numbers in between brackets indicate sample sizes. Mean ± S.E.M are indicated in the graphs. Scatter-plot labelled with the same letter are not statistically different. *n.s.*: non-significant; *:p<0.05; **:p<0.01; ***:p<0.001.For full statistical details see Supplementary Table S1.

Female modulate ejaculate ejection based on male olfactory cues

Having revealed a mechanism of CFC connecting male quality with modulation of ejaculate ejection latency affecting sperm storage and paternity, we explored the temporal structure of the effect of female exposure to males between first and second mating and the nature of the male cue detected by females. First, to examine the critical exposure window, we subdivided the 120

178 minute window after the end of mating (AEM) in which sperm storage takes place (Manier et al.,
179 2010). We introduced a single *CS* male in the arena 30, 60, 90, or 120 minutes AEM until the
180 female ejected, or kept the female isolated as a control, and recorded ejaculate ejection latency. We
181 found that females must be exposed to "high quality" *CS* males during the first 60 minutes AEM
182 to show a decrease in ejaculate latency (Fig. 3A). However, being in the presence of a male only
183 during the first hour AEM is not sufficient to observe a complete advancement of the timing of
184 ejaculate ejection (Fig 3B). Together, these results suggest that modulation of ejaculate ejection
185 latency requires continuous presence of males after the end of a female's first mating.

186 Secondly, to investigate the cue responsible for modulation of ejaculate ejection, we hypothesized
187 that advanced ejaculate ejection latency should be a phenomenon specific to the presence of
188 attractive males. Consistent with this, we found that ejection latency is not modulated in presence
189 of virgin females (Fig 3C), indicating that male-specific cues are likely involved. To test if females
190 where modulating ejection latency based on male quality, quantity or diversity, we measured the
191 ejection latency of females placed in the presence of one or six *CS* males or with males of different
192 strains or a mix of two males of each strain (Fig 3D). We found that the greater number or genetic
193 diversity of males did not affect female ejaculate ejection latency, narrowing the relevant cue down
194 to male attractiveness.

195 To determine how females sense male attractiveness cues, we measured the ejection latency of
196 females' mutant for different sensory modalities in the presence or absence of a *CS* male (Fig 3E).
197 Females with a mutation in *inactive*, affecting hearing, *ninaB*, affecting vision, *GR32a*, a gustatory
198 receptor for a male pheromone, and *ppk23*, an ion channel associated with male pheromone
199 sensing (Thistle et al., 2012), all showed a wild type response where they reduced ejaculate ejection
200 latency in the presence of a *CS* male compare to isolated controls. However, *Orco* mutant females,
201 who have partially impaired olfaction, did not (Fig 3E). We confirmed this result by silencing *Orco*
202 neurons using *Kir2.1* (Fig 3F) and found that females of both control groups showed early ejection
203 in the presence of *CS* males compared to isolated controls while the females with *Orco* neurons
204 silenced did not. Taken together, these results suggest that females modulate timing of ejection in
205 response to male pheromones perceived through the olfactory system.

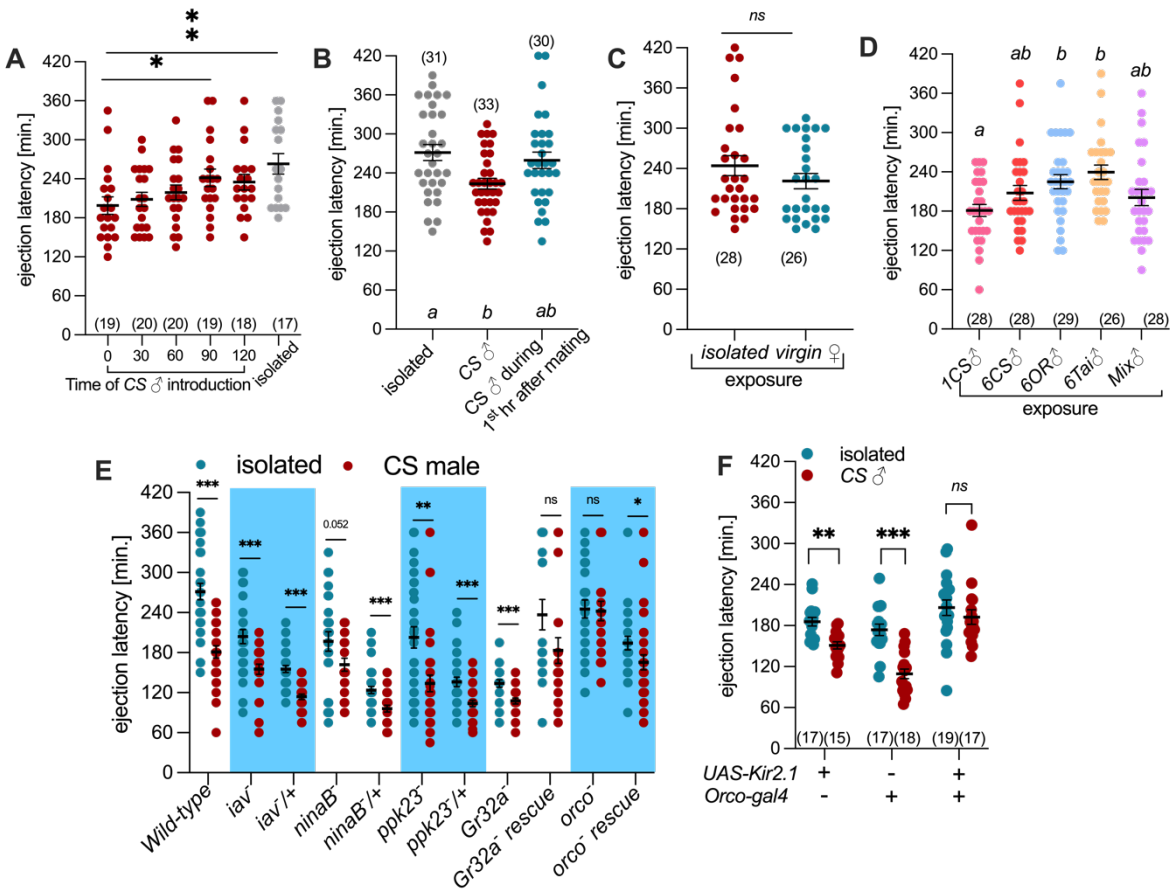


Figure 3: Females sense high quality males via olfaction. (A) Mean time of ejaculate ejection of a recently mated female exposed to a *CS* male, indicated by "o" or at the indicated times after she finished mating. Males were kept in the arena until ejection was observed. The "isolated" condition indicates a female that was not exposed to males. (B) Ejection latency of a females who was exposed for 1 hour with a *CS* male, after which the male was removed and the female transferred to a clean arena. (C) Mean ejection latency of a recently mated female exposed to a virgin female or left isolated. (D) Ejection latency of a recently mated females exposed to males from different strains. Mix indicates 2 *OR*, 2 *CS* and 2 *Tai* males. Experiments are performed as in Fig 1B. (E) Ejection latency of mutant females of the indicted genotypes, exposed to either a *CS* male directly after the end of their first mating or kept in isolation. (F) Females in which *Orco*-expressing neurons are silenced by means of Kir2.1 expression, and their controls, exposed to either a CS male directly after the end of their first mating or kept in isolation. Numbers in between brackets indicate sample sizes. mean ± S.E.M are indicated in the graphs. Scatter-plot labelled with the same letter are not statistically different. *n.s.:* non-significant; *:p<0.05; **:p<0.01; ***:p<0.001.For full statistical details see Supplementary Table S1.

Females modulate timing of ejaculate ejection in response to a mix of heptanal and cVA

To test the influence of male pheromones on the modulation of ejaculate ejection latency, we generated males that do not produce cuticular hydrocarbons (CHCs), the main *Drosophila* pheromone, by genetically ablating the oenocytes (*oe*$^-$), the cells that synthesize CHCs (Billeter et al., 2009). We exposed recently mated females to *oe*$^-$ males and observed that females did not advance ejection as they do when exposed to a *CS* or a control male of the same genotype as *oe*$^-$

227 but without the ablation. This indicates that CHCs produced by the oenocytes produce one or
228 more of the male cues used by females to modulate ejaculate ejection timing (Fig. 4A). Given that
229 the most prominent male CHC, 7-Tricosene (7-T), is also expressed in higher quantity in *CS* than
230 *OR* (Jallon, 1984), we tested the hypothesis that 7-T is the cue that females use for CFC by applying
231 7-T on *oe*$^-$ males and testing whether this would result in a faster ejaculate ejection in females
232 exposed to these males than when exposed to untreated *oe*$^-$ males. We measured ejection latency
233 of females in the presence of *oe*$^-$ males perfumed with 1000ng of 7-T, which is an average amount
234 in *CS* males, and observed a reduction in female ejaculate ejection latency similar to that observed
235 when females are exposed to *CS* males (Fig. 4A). Recently, we reported that 7-T is oxidized into a
236 smaller molecule, heptanal, which is sensed by olfactory system and is used by females to choose
237 egg-laying sites occupied by conspecifics (Verschut et al., 2023). To determine whether 7-T or its
238 oxidization product heptanal is sensed by females to modulate ejaculate ejection latency, we
239 perfumed *oe*$^-$ male with 1000ng of heptanal (Fig. 4A) and found that females show a decreased
240 ejection latency in the presence of the heptanal perfumed males compared to controls,
241 recapitulating the "high quality male"-exposed phenotype.

242 Because flies deposit pheromonal residues in their environment (Duménil et al., 2016; Keesey et
243 al., 2017; Verschut et al., 2023), we next tested whether male pheromones alone can modulate
244 female ejaculate ejection without the male interacting with the females. For this, we exposed
245 recently mated females to pheromonal extracts of *CS*, *OR* males or a solvent control, placed on a
246 filter paper in a cup covered with a mesh, and measured the timing of ejection of females exposed
247 to these extracts between their first and second mating. We observed reduced ejection latencies of
248 females in the presence of *CS* pheromone extracts compared to *OR* pheromone extracts or a
249 hexane control, demonstrating that male pheromones are sufficient to modulate female ejaculate
250 ejection timing (Fig. 4B). To isolate the specific pheromone responsive for this effect, females
251 were exposed to individual pheromones in a dose response manner. Females exposed to cVA, 7-
252 T or heptanal alone did not advance timing of ejection at any doses (Supp Fig. 4A,B,C). As cVA
253 acts in combination with other pheromones and is still expressed in oe$^-$ males (Billeter et al., 2009;
254 Laturney & Billeter, 2016; Verschut et al., 2023), we surmised that females respond to heptanal
255 perfumed *oe*$^-$ because of the resulting combination of cVA and heptanal. To test this hypothesis,
256 we exposed females to a mix of heptanal and cVA. Females indeed advanced ejaculate at specific
257 doses of heptanal and cVA demonstrating that the cue indicative of male quality is a combination
258 of cVA and heptanal (Fig 4C).

259

260 Next, we investigated which odorant receptor neurons (ORN) females use to sense male
261 quality/cVA-heptanal and adjust ejaculate ejection in response. We silenced different ORNs by
262 driving expression of *UAS-Kir2.1* with ORN specific genetic driver lines. *ORN22a*, *ORN13a*, and
263 *ORN35a*, are necessary for females to respond to heptanal in the context of egg-laying site choice
264 (Verschut et al., 2023). Silencing ORN22a+ (Fig4.D) and ORN35a+ (Fig 4E) neurons resulted in
265 females no longer advancing timing of ejaculate ejection in the presence of a *CS* male. However,
266 we observed advanced ejection latency in the presence of a *CS* male when ORN13a neurons were
267 silenced (Fig 4F). Together, these data demonstrate that ORN22a and ORN 35a are the main input
268 pathways for the modulation of ejaculate ejection in response to heptanal. As an out-group control,
269 we also tested ORN47b, which we recently showed to be involved in mated female mate choice
270 in response to palmitoleic acid (Kohlmeier et al., 2021), but this ORN does not appear necessary
271 for the modulation of ejaculate ejection (Fig 4G). We next tested the two documented receptors
272 for cVA, *ORN65a* and *ORN67d* (van der Goes van Naters & Carlson, 2007). Females with silenced
273 ORN65a (Fig4.H) or ORN67d (Fig 4I) neurons did not significantly decrease ejection latency in
274 the presence of a *CS* male, indicating that they are responsible for the sensing of cVA in the context
275 of ejaculate ejection modulation. We confirm the involvement of OR22a and OR35a (Fig 4J,K) by
276 exposing the flies to a mix of heptanal and cVA inducing a decrease of the timing of ejaculate
277 ejection in control flies. In both cases females with silenced ORN22a 35a neurons did not advance
278 timing of ejection. We conclude that female adjust ejaculate ejection latency in response to male
279 quality by a combination of heptanal receptors OR22a and OR35a and cVA receptors OR65a and
280 OR67d.

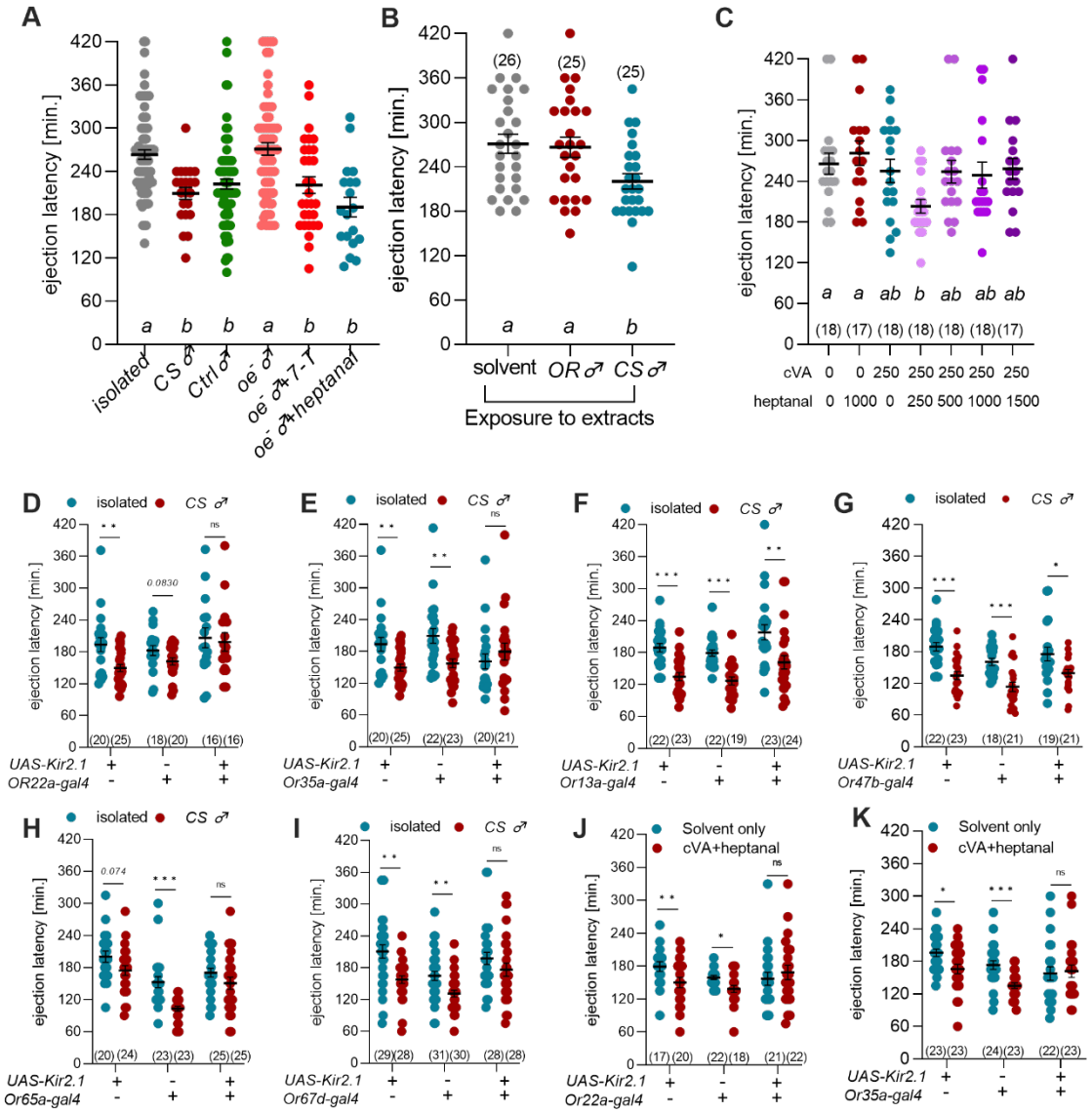


Figure 4: Females modulated ejection latency based on heptanal and cVA. (B) mean ejection latency of a recently mated female exposed to extracts from the equivalent of one OR or one CS male. (A) Mean ejection latency of a recently mated female exposed to males of the indicated genotypes. Genotype with "+ 7-T" where perfumed with 1000ng of this compound. Genotype with "+heptanal" were perfumed with 10000 ng of this compound. (C) mean ejection latency of a recently mated female exposed to cVA and heptanal at a variety of concentrations. (D-I) Females in which Specific Odorant receptors neurons are silenced by means of Kir2.1 expression, and their controls, exposed to either a CS male directly after the end of their first mating or kept in isolation. (J-K) same as D-I but with the males replaced by a blotting paper containing 250ng of heptanal and 250ng of cVA. Numbers in between brackets indicate sample sizes. mean ± S.E.M are indicated in the graphs. Scatter-plot labelled with the same letter are not statistically different. *n.s.*: non-significant; *:p<0.05; **:p<0.01; ***:p<0.001.For full statistical details see Supplementary Table S1.

Discussion

We provide the first demonstration, to our knowledge, of a mechanism for cryptic female choice in response to male cues in *D. melanogaster*. We show that *D. melanogaster* females advance timing of ejaculate ejection when exposed to a high-quality male after their first mating, leading to decreased sperm stored from the first male and an increase in paternity share of their second mate. Females assess male quality based on olfaction, sensing two male pheromones, cVA and heptanal, an oxidization product of 7-T. Our results highlight that social cues happening outside of mating itself can influence paternity.

Females exert male selection at multiple stages of reproduction in *D. melanogaster*

We recently showed that virgin *D. melanogaster* females mate less selectively as virgins than as mated females (Kohlmeier et al., 2021). This behavior may allow females to relax sexual selection in environments where there are few males available or where conditions forces males to be of poor quality, allowing low selectivity virgin females to mate as soon as possible to begin offspring production. Once female have secured sperm from their first mating, they become more selective and will mate with higher quality males if they encounter them (Kokko & Mappes, 2005). Because of last male sperm precedence, the second male will sire most of the offspring, reducing paternity form the first male. The cryptic female choice mechanism we report here further extends the importance of post-copulatory events for female fitness by showing that females can influence paternity by modulating ejaculate ejection timing and biasing the ratio of sperms they keep from their first mate depending on their perception of higher quality males in their environment. Critically, we show that males do not need to be physically present for this effect as the sensing of specific male pheromones in the physical absence of a male is sufficient to modulate ejaculate ejection timing. Given that *D. melanogaster* naturally deposit pheromones where they dwell (Duménil et al., 2016; Keesey et al., 2017; Verschut et al., 2023), we speculate that the sensing of environmental pheromones allows recently mated females to determine the presence of high-quality males without having to physically interact with them. Indeed, those recently mated females are still in the process of storing sperm from their first mate and are not yet attractive to males for further mating because they have also acquired anti-aphrodisiac pheromones from their first mate (Laturney & Billeter, 2016; Manier et al., 2010). These anti-aphrodisiac pheromones are jettisoned by the female once the sperm storage process is over and they ejected the ejaculate from their first mate; a point at which female may begin remating (Laturney & Billeter, 2016). Advancement of

327 ejaculate ejection in presence of high-quality males may thus allow females to prepare themselves
328 to allocate a higher ratio of paternity when they eventually remate with those high-quality males.

329 A picture is emerging where *D. melanogaster* female adapt reproductive behaviors to match their
330 social environment. Indeed, by mating with low discrimination as a virgin, they ensure they acquire
331 sperm as soon as possible. By having the ability to adjust how much sperm from the first male
332 they keep, they can adjust investment in that first male depending on their perception of mating
333 opportunities with higher quality males. *D. melanogaster* female have thus multiple levels of controls
334 over who sires their offspring allowing them to maximize fitness in different demographic
335 conditions; relaxing sexual selection when male quality is low and making it more stringent when
336 male quality is high.

337 **Female advance ejaculate ejection when sensing specific male pheromones**

338 Our findings reveal that female advance ejaculate ejection in response to the sensing of male
339 pheromones cVA and heptanal, a derivative of 7-Tricosene (Verschut et al., 2023). There are two
340 remarkable aspects in the involvement of these two pheromones in CFC. The first is that cVA and
341 7-T are male pheromones that normally function together to repel other males; they are indeed
342 naturally expressed by males and are also part of an anti-aphrodisiac cocktail transferred by males
343 to recently mated females, which renders them unattractive (Billeter et al., 2009; Laturney &
344 Billeter, 2016). In this context, males sense 7-T via Gustatory receptor Gr32 expressed in their
345 legs that get activated when touching males or mated females, while cVA is detected via their
346 olfactory system (Billeter & Levine, 2013; Laturney & Billeter, 2016; Wang et al., 2011). We show
347 here that females also use these pheromones to advance ejaculate ejection latency. However, they
348 do not sense 7-T per se, but its breakdown product heptanal, sensed via olfaction. This indirect
349 detection of 7-T via olfaction in females as opposed to direct detection by gustatory receptor in
350 males, allows 7-T to take a different function to males and females. Indeed, detection by olfaction
351 is typically at a distance, allowing females to sense males without physically interacting with them.
352 That females use olfactory cues rather than gustatory ones requiring physical contact, gives support
353 to our proposal that females advance ejaculate ejection in response to their evaluation of males
354 they will potentially mate with in the future.

355 The second remarkable aspect of the discovery that cVA and heptanal modulate ejaculate ejection
356 is that these pheromones have not so far been implicated as major contributors to female mate
357 choice, at least not for mated females. Indeed, the change in female mate selectivity between virgin
358 and mated females is mediated by a different pathway involving odorant receptor neurons Or47b
359 and male pheromone palmitoleic acid (Kohlmeier et al., 2021). Our results indicate that this

360 pathway is not involved in CFC as silencing Or47b ORN in females still results in advanced
361 ejaculate ejection in the presence of high-quality males. We see two possible hypotheses that may
362 explain why females have evolved different pathways for mate selection and ejaculate ejection. The
363 first is the temporal aspect of these two decisions. Mate choice requires close temporal and spatial
364 association between the cue and a mate, while our data show that ejaculate ejection is a slow build
365 up requiring long exposure to male cues. These spatio-temporal differences in decision making
366 might have resulted in the evolution of two separate pathways, one for evaluating present mating
367 opportunities and one for evaluating future mating opportunities. A second related hypothesis is
368 that the cues themselves, palmitoleic acid for mate choice and cVA/heptanal for ejaculation
369 ejection, may have different volatility and/or stability, as well as spatial distributions. A potential
370 lower volatility/stability, or a lack of deposition of palmitoleic acid in the environment would
371 prevent it from exerting an environmental effect and would thus remain a cue physically associated
372 with a male. cVA and 7-T are however documented to be deposited in the environment, and are
373 both detectable at a distance (Duménil et al., 2016; Keesey et al., 2017; Nojima et al., 2021; Tolassy
374 et al., 2023).

375 **Females bias paternity through sperm allocation in the seminal receptacle.**

376 Here we have identified the specific male pheromones, and the female olfactory receptor neuron
377 detecting them, that influence the timing of females ejaculate ejection. We have also identified a
378 connection between ejaculation latency and a bias in both sperm storage and paternity. We have
379 therefore identified both the input and output mechanisms of a CFC. While we do not provide
380 the full pathway linking sensing of male cues and changing sperm storage, our data and previously
381 published findings allow speculating on the nature of this pathway. Female olfactory system is the
382 input that starts the chain of event leading to differential sperm storage in response to male
383 exposure. Lee *et al.* (2015) previously demonstrated that female neurons expressing the Dh44
384 peptide function to temporally block ejaculate ejection, allowing females sufficient time to store
385 sperm before eventually ejecting the surplus ejaculate a few hours after mating. It is likely that
386 there exists a connection between olfactory processing and Dh44-expressing neurons, whereby
387 detection of pheromones indicative of the presence of a high-quality male by the olfactory system
388 may directly or indirectly reduce inhibition of ejaculate ejection by Dh44 neurons resulting in
389 advancement of ejaculate ejection by an hour. In absence of high-quality males, Dh44 neurons
390 would regulate a default ejection a few hours after mating, ensuring maximum storage of sperm
391 from their first mate. While there is no direct connection between Dh44 neurons and the female
392 reproductive tract, the two sperm storage sites are innervated by descending neurons from the
393 brain, including octopaminergic/thyraminergic neurons (Avila et al., 2012). Interestingly, blocking

394 functioning of these neurons impairs sperm storage (Avila et al., 2012), and can even bias sperm
395 use in twice mated females (Chen et al., 2022). This strongly suggest that these neurons are effector
396 of CFC, who might receive input from neurons integrating male pheromones sensing and timing
397 by Dh44 neurons. Future work will doubtless elucidate the neuronal circuit integrating male cues
398 with sperm storage and ejaculate ejection latency.

399 Material and Methods

400 *Drosophila* strains and Rearing conditions

401 *Drosophila melanogaster* strains were reared in incubators set at 25°C with a 12:12h light–dark cycle
402 (LD 12:12) on food medium containing agar (10 g l−1), glucose (167 mM), sucrose (44 mM), yeast
403 (35 g l−1), cornmeal (15 g l−1), wheatgerm(10 g l−1), soya flour (10g l−1), molasses (30 g l−1),
404 propionic acid (5 ml of 1 M) and Tegosept (2 g in 10 ml of ethanol). Virgin adults were collected
405 using CO_2 anesthesia and aged in same-sex groups of 15 in food vials (25×95 mm) containing
406 food medium (23×15 mm) for 5 to 7 days before testing.

407 GFP and RFP males were obtained by backcrossing the original line with *Canton-S* or *Oregon-R*
408 strains for 18 generations.

Name	Genotype	Source/identifier
Canton-S	*CS*	Billeter lab
Oregon-R	*OR*	Billeter lab
w^{1118}	w^{1118}	Vienna Drosophila Resource Center VDRC: 60000
Tai	*Tai*	Jean-François Ferveur
Orco-	w^{-}; +; $orco^{2}$	BDSC: 23130
Orco rescue	w^{-}; +; *Orco-, pBac{Orco}*	(Silbering et al., 2011)
LAV^{-}	LAV^{1}; +; +	(Gong et al., 2004)
$NinaB^{-}$		(Von Lintig et al., 2001)
$Ppk23^{-}$		(Thistle et al., 2012)

Gr32a⁻		(Miyamoto & Amrein, 2008)
GR32a- rescue		(Miyamoto & Amrein, 2008)
UAS-Kir2.1	*w⁻; +; p{UAS-kir2.1:eGFP}*	BDSC: 6595
Or13a-gal4	*w⁻: P{w[+mC]=Or13a GAL4.F}40,1; +*	BDSC: 9945
Or47b-gal4	*w⁻; P{w[+mC]=Or47b-GAL4.7.467}Bacc[15.5A]*	BDSC: 9983
OR65a-gal4	*w⁻: P{w[+mC]=Or65a GAL4.F}72.5; +*	BDSC: 9993
OR67d-gal4	*w⁻; P{w[+mC]=Or67d-GAL4.F}57.2; +*	BDSC: 9998
OR35a-gal4	*w⁺⁻; P{w[+mC]=Or35a-GAL4.F}109.3; +*	Made from BDSC: 9968
OR22a-gal4	*w⁺; P{w[+mC]=Or22a-GAL4.7.717}14.2; +*	Made from BDSC: 9951
UAS-Stinger	*+ ; p{UAS-Stinger}; +*	(Barolo et al., 2004)
UAS-hid	*+ ; p{UAS-hid,w⁺},p{UAS-stinger,w+}/CyO; +*	Billeter *et al.*, 2009
PromE-gal4	*+; p{PromE(800)-gal4,w⁺},p{Tub-gal80[ts]}; +*	Billeter *et al.*, 2009
Canton-S GFP-labeled sperm	*w⁺; p{ProtB-ProtamineB::GFP}; +*	Made from Laturney *et al.*, 2018
Canton-S mCherry-labeled sperm	*w+; p{ProtB-ProtamineB::mCherry}; +*	Made from Laturney *et al.*, 2018

409

410 **Mate-choice assay**

411 Mate-choice assays were performed as detailed in (Kohlmeier et al., 2021; Verschut et al., 2023).
412 Briefly, coloring vials are made by putting 0.5g of green or pink fluorescent powder (Slice of the

413 moon) in an empty vial and rotating it until the surface gets covered. The residual powder was
414 removed by tapping down the vial. 15 males of both strains were transferred in a different coloring
415 vial, the vial was rotated for 3s. A male of each of the two strains are then individually transferred
416 in a mating arena for 30min to recover. Colors were randomly assigned on every experimental day.
417 Females are then individually added to the arena and inspected for mating every 10min for 60min.
418 The flies that did not mate within 60min were discarded.

419 **Ejaculate ejection timing assay**

420 *Canton-S* virgin female (or different genotype based on experiment) were individually mated at
421 25°C with a single *Canton-S* (CS) male in a mating arena (35 x 10mm Petri dish, Greiner Bio-One,
422 containing 2mL of fly food) at ZT1 under light condition. After mating, females were checked
423 with a UV flashlight (Perel EFL41UV) to ensure the presence of the mating plug indicative of
424 matin (Lung & Wolfner, 2001). Females that did not mate within 2 hours were discarded; that
425 corresponded to less than 10% of trials. Mated females were transferred individually using a mouth
426 pipette in a fresh mating arena alone, with a new male or pheromones. Ejection of the mating plug
427 was checked every 15min with a UV flashlight for 7h after the end of mating by checking its
428 presence in the arena and on the female genitals. Experiments were performed in a temperature-
429 controlled chamber at 25°C on a bench cleaned daily with ethanol/water and 100% Acetone to
430 avoid accumulation of pheromonal residues.

431 **Twice-mated female assay**

432 The beginning is exactly similar to the ejaculate ejection timing assay. After ejection, females were
433 transferred in a new mating arena with a GFP-labeled sperm male in *Canton-S* background.
434 Remating with the new male was visually inspected every 10min for 4 hours. Females that did not
435 remate after 4 hours were discarded; that corresponded to less than 20% of trials. After remating,
436 the presence of the new mating plug was checked and the female was transferred alone to a new
437 mating arena until ejection of the 2nd mating plug. Ejection of the mating plug was checked every
438 15min. After the 2nd ejection, females were individually transferred in a fresh food vial every 2 days
439 for 7 days. Offspring coming from these vials were counted and their paternity assessed as in
440 Laturney *et al.* (2018) by the red fluorescence in the ocelli due to *M{3xP3-RFP.attP}ZH-102D*
441 insertion when they are fathered by the male from GFP-labeled sperm lines using a fluorescent
442 stereo microscope (Leica MZ10 F). For sperm counting experiments, females followed the same
443 protocol but were first mated with a mCherry-labeled sperm male backcrossed in a *Canton-S*
444 background. After the 2nd ejection they were placed individually in a 0.5mL Eppendorf tube and
445 were immediately flash-frozen in liquid nitrogen directly after ejection and stored at -20°C until

446 dissection. The fluorescent-labeled sperm male in *Canton-S* background were obtain by
447 backcrossing the original lines to *Canton -S* for 18 generations.

448 **Sperm count**

449 Flash frozen female reproductive tract was dissected in PBS 1X and mounted on Vectashield
450 (Vector Laboratories) on a glass slide covered with a coverslip elevated by bridging it in between
451 two coverslips. The reproductive tracts were imaged with a Zeiss LSM 800 confocal microscope
452 with a 40x water immersion lens using a 587nm excitation wavelength for mCherry and 488nm
453 for GFP. Sperm are manually counted using Fiji software as in Laturney et al., (2018). Counts were
454 done blind, each female being given a random ID, and was a recounted blind again by another
455 experimenter.

456 **Pheromonal exposure:**

457 *CS* and *OR* males were anesthetized on ice in a plastic vial then transferred using forceps cleaned
458 by dipping them in n-hexane, methanol and n-hexane again in a 2ml glass vial. 10µL of hexane/flies
459 are pipetted in the glass vial for the extraction. The vial is vortexed for 3 minutes, every minute
460 the vial is checked to ensure that no flies are stuck above the hexane. The supernatant is pipetted
461 in a clean 2mL glass vial.

462 Filter paper discs made using a 3 mm diameter one-hole paper puncher out of cellulose
463 chromatography paper rolls. Using clean forceps, the discs were placed in a well, made of the cap
464 of 0.5mL reaction tube (Sarstedt). 10µL of pheromone solution were pipetted on the filter paper
465 and the well was put under a nitrogen flow for 5min to let the hexane evaporate. The wells were
466 then covered with foil paper, to avoid direct contact of flies with the pheromone patch, and, placed
467 at the center of mating arena. 2mL food was poured around the edges of the well make them stick
468 to the arena. Seven holes were made in the foil paper, small enough to not let a fly go through and
469 let the odour spread in the arena just before introducing a mated female inside.

470 **Perfuming:**

471 Oenocyte ablated flies were generated by crossing *+; PromE(800)-Gal4 [2M],Tub:Gal80^{ts} ;* + to +:
472 *UAS-StingerII, UAS-hid/CyO;* + flies (Billeter et al., 2009). Flies are reared at 18°C and transfer at
473 25°C after the adults have been collected for at least 5 days to allow the expression of the Gal4
474 and the ablation of the oenocytes. The pheromone of interest (or hexane for control) at 28 times
475 the desired concentration (7 flies are perfumed at the same time with a 25% transfer efficiency)
476 per fly is pipetted in a 2ml glass vial and briefly vortexed. The vial is placed under a nitrogen flow
477 until complete evaporation of the hexane. As in the CHCs extraction, flies are anesthetized on ice,

478 7 of them are transferred by their wings into the coated glass vial with clean forceps. The vial is
479 vortexed at low speed 3 times for 20s with a 20s interval. The flies are transferred into a fresh food
480 vial and an hour of recovery time is given to them before the start of the experiment.

481 **Gas chromatography:**

482 For hydrocarbon analysis, single flies were placed in glass microvials containing 50 µl of an internal
483 standard solution made by dissolving 10 ng/ml of octadecane (C18) and 10 ng/ml of hexacosane
484 (C26) in hexane. The vials were vortexed at minimum speed for 2min and the fly,ewas removed
485 using a clean metal pin. The resulting hydrocarbon extracts were analysed using an Agilent 7890
486 gas chromatograph with a flame ionization detector, an Agilent DB-1 column (Diameter: 0.180
487 mm; film 0.18 µm) and a split-splitless injector set at 250°C with 40ml/min splitless flow. Injector
488 valve opened 1.5 min after injection in splitless mode with helium as carrier gas (flow: 37.2
489 cm/sec). The oven temperature program begins at 50 °C for 1.5 min, ramping at 10°C /min to
490 150 °C, then ramping at 4 °C /min to 280°C and holding for 5 minutes. ChemStation software
491 (Agilent technologies) was used to quantify compounds based on peak areas relative to internal
492 standard C26 as described in ; Verschut et al., 2022).

493 **Statistics**

494 Statistical analyses were performed using R 4.3.0 software (The R Foundation for Statistical
495 Computing). Residuals were visually inspected for normality, and homogeneity of variances were
496 evaluated with the F-test. Some data were log transformed when normality was not respected (see
497 supp table). Wilcoxon-Mann-Whitney test and Student's t-test were used to compare ejection
498 latency, proportion of offspring, number of offspring and number of sperm between two groups
499 (isolated vs *CS* male or *CS* male vs *OR* male). Kruskal-Wallis test followed by Dunn's multiple
500 comparisons test and the Anova test followed by Tukey post-hoc comparisons or Dunnett's test
501 were used to compare ejection latency between more than two groups.
502
503 All graphs and some statistical analyses were performed using GraphPad Prism 9.5.1 software.
504

505 **Acknowledgment**

506 We thank Meghan Laturney for helpful feedback and comments on the manuscript. We thank
507 Thomas Verschut for his help with the pheromones and how to handle them. Thanks to Gerard
508 Overkamp for helping to solve technical issues with the experimental work. We thank Simon
509 Robledo-Cardona for helping setting up pilots for some these experiments. This work was funded

by the Netherlands Organization for Scientific Research (NWO) ALWOP.611 to J.C. Billeter and by a fellowship of the German Research Foundation awarded to P.K. (KO 5835/2-1).

References

Avila, F. W., Bloch Qazi, M. C., Rubinstein, C. D., & Wolfner, M. F. (2012). A requirement for the neuromodulators octopamine and tyramine in Drosophila melanogaster female sperm storage. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(12), 4562–4567. https://doi.org/10.1073/pnas.1117689109

Barolo, S., Castro, B., & Posakony, J. W. (2004). New Drosophila transgenic reporters: Insulated P-element vectors expressing fast-maturing RFP. *BioTechniques*, *36*(3), 436–442. https://doi.org/10.2144/04363st03

Billeter, J. C., Atallah, J., Krupp, J. J., Millar, J. G., & Levine, J. D. (2009). Specialized cells tag sexual and species identity in Drosophila melanogaster. *Nature*, *461*(7266), 987–991. https://doi.org/10.1038/nature08495

Billeter, J. C., & Levine, J. D. (2013). Who is he and what is he to you? Recognition in Drosophila melanogaster. *Current Opinion in Neurobiology*, *23*(1), 17–23. https://doi.org/10.1016/j.conb.2012.08.009

Billeter, J. C., & Wolfner, M. F. (2018). Chemical Cues that Guide Female Reproduction in Drosophila melanogaster. *Journal of Chemical Ecology*, *44*(9), 750–769. https://doi.org/10.1007/s10886-018-0947-z

Chen, D. S., Clark, A. G., Wolfner, M. F., & Clark, A. G. (2022). *Octopaminergic/tyraminergic*. *2216*, 0–3.

Duménil, C., Woud, D., Pinto, F., Alkema, J. T., Jansen, I., Van Der Geest, A. M., Roessingh, S., & Billeter, J. C. (2016). Pheromonal Cues Deposited by Mated Females Convey Social Information about Egg-Laying Sites in Drosophila Melanogaster. *Journal of Chemical Ecology*, *42*(3), 259–269. https://doi.org/10.1007/s10886-016-0681-3

Eberhard, William G. *Female Control: Sexual Selection by Cryptic Female Choice*. Vol. 69. Princeton University Press, 1996. https://doi.org/10.2307/j.ctvs32rx1

Eberhard, W. G. (2009). Postcopulatory sexual selection: Darwin's omission and its consequences. *Proceedings of the National Academy of Sciences of the United States of America*, *106 Suppl 1*, 10025–10032. https://doi.org/10.1073/pnas.0901217106

Eberhard, W. G., Biologia, E. De, Rica, U. D. C., Universitaria, C., & Rica, C. (2009). Criteria for Demonstrating Postcopulatory Female Choice Author (s): William G . Eberhard Published by : Society for the Study of Evolution Stable URL : http://www.jstor.org/stable/2640597. *Society*, *54*(3), 1047–1050.

Firman, R. C., Gasparini, C., Manier, M. K., & Pizzari, T. (2017). Postmating Female Control: 20 Years of Cryptic Female Choice. *Trends in Ecology and Evolution*, *32*(5), 368–382. https://doi.org/10.1016/j.tree.2017.02.010

Gong, Z., Son, W., Chung, Y. D., Kim, J., Shin, D. W., McClung, C. A., Lee, Y., Lee, H. W., Chang, D. J., Kaang, B. K., Cho, H., Oh, U., Hirsh, J., Kernan, M. J., & Kim, C. (2004).

550 Two interdependent TRPV channel subunits, inactive and nanchung, mediate hearing in
551 Drosophila. *Journal of Neuroscience*, *24*(41), 9059–9066.
552 https://doi.org/10.1523/JNEUROSCI.1645-04.2004

553 Grillet, M., Dartevelle, L., & Ferveur, J. F. (2006). A Drosophila male pheromone affects female
554 sexual receptivity. *Proceedings of the Royal Society B: Biological Sciences*, *273*(1584), 315–323.
555 https://doi.org/10.1098/rspb.2005.3332

556 Jallon, J. M. (1984). A few chemical words exchanged by Drosophila during courtship and
557 mating. *Behavior Genetics*, *14*(5), 441–478. https://doi.org/10.1007/BF01065444

558 Keesey, I. W., Koerte, S., Khallaf, M. A., Retzke, T., Guillou, A., Grosse-Wilde, E., Buchon, N.,
559 Knaden, M., & Hansson, B. S. (2017). Pathogenic bacteria enhance dispersal through
560 alteration of Drosophila social communication. *Nature Communications*, *8*(1).
561 https://doi.org/10.1038/s41467-017-00334-9

562 Kohlmeier, P., Zhang, Y., Gorter, J. A., Su, C. Y., & Billeter, J. C. (2021). Mating increases
563 Drosophila melanogaster females' choosiness by reducing olfactory sensitivity to a male
564 pheromone. *Nature Ecology and Evolution*, *5*(8), 1165–1173. https://doi.org/10.1038/s41559-
565 021-01482-4

566 Kokko, H., & Mappes, J. (2005). Sexual selection when fertilization is not guaranteed. *Evolution*,
567 *59*(9), 1876–1885. https://doi.org/10.1111/j.0014-3820.2005.tb01058.x

568 Kurtovic, A., Widmer, A., & Dickson, B. J. (2007). A single class of olfactory neurons mediates
569 behavioural responses to a Drosophila sex pheromone. *Nature*, *446*(7135), 542–546.
570 https://doi.org/10.1038/nature05672

571 Laturney, M., & Billeter, J. C. (2016). Drosophila melanogaster females restore their
572 attractiveness after mating by removing male anti-aphrodisiac pheromones. *Nature*
573 *Communications*, *7*, 1–11. https://doi.org/10.1038/ncomms12322

574 Laturney, M., van Eijk, R., & Billeter, J.-C. (2018). Last male sperm precedence is modulated by
575 female remating rate in Drosophila melanogaster . *Evolution Letters*, *2*(3), 180–189.
576 https://doi.org/10.1002/evl3.50

577 Lee, K. M., Daubnerová, I., Isaac, R. E., Zhang, C., Choi, S., Chung, J., & Kim, Y. J. (2015). A
578 neuronal pathway that controls sperm ejection and storage in female drosophila. *Current*
579 *Biology*, *25*(6), 790–797. https://doi.org/10.1016/j.cub.2015.01.050

580 Lung, O., & Wolfner, M. F. (2001). Identification and characterization of the major Drosophila
581 melanogaster mating plug protein. *Insect Biochemistry and Molecular Biology*, *31*(6–7), 543–551.
582 https://doi.org/10.1016/S0965-1748(00)00154-5

583 Lüpold, S., Pitnick, S., Berben, K. S., Blengini, C. S., Belote, J. M., & Manier, M. K. (2013).
584 Female mediation of competitive fertilization success in Drosophila melanogaster.
585 *Proceedings of the National Academy of Sciences of the United States of America*, *110*(26), 10693–
586 10698. https://doi.org/10.1073/pnas.1300954110

587 Manier, M. K., Belote, J. M., Berben, K. S., Novikov, D., Stuart, W. T., & Pitnick, S. (2010).
588 Resolving mechanisms of competitive fertilization success in drosophila Melanogaster.
589 *Science*, *328*(5976), 354–357. https://doi.org/10.1126/science.1187096

590 Miyamoto, T., & Amrein, H. (2008). Suppression of male courtship by a Drosophila pheromone
591 receptor. *Nature Neuroscience*, *11*(8), 874–876. https://doi.org/10.1038/nn.2161

592 Nojima, T., Rings, A., Allen, A. M., Otto, N., Verschut, T. A., Billeter, J. C., Neville, M. C., &

Goodwin, S. F. (2021). A sex-specific switch between visual and olfactory inputs underlies adaptive sex differences in behavior. *Current Biology*, *31*(6), 1175-1191.e6. https://doi.org/10.1016/j.cub.2020.12.047

Pitnick, S., Markow, T., & Spicer, G. S. (1999). Evolution of multiple kinds of female sperm-storage organs in Drosophila. *Evolution*, *53*(6), 1804–1822. https://doi.org/10.1111/j.1558-5646.1999.tb04564.x

Pizzari, T., & Birkhead, T. R. (2000). *Female feral fowl eject sperm of subdominant males - Pizzari and Birkhead*. 787–789.

Rodriguez-Enriquez, C. L., Tadeo, E., & Rull, J. (2013). Elucidating the function of ejaculate expulsion and consumption after copulation by female Euxesta bilimeki. *Behavioral Ecology and Sociobiology*, *67*(6), 937–946. https://doi.org/10.1007/s00265-013-1518-5

Schnakenberg, S. L., Matias, W. R., & Siegal, M. L. (2011). Sperm-storage defects and live birth in drosophila females lacking spermathecal secretory cells. *PLoS Biology*, *9*(11). https://doi.org/10.1371/journal.pbio.1001192

Schnakenberg, S. L., Siegal, M. L., & Bloch Qazi, M. C. (2012). Oh, the places they'll go. *Spermatogenesis*, *2*(3), 224–235. https://doi.org/10.4161/spmg.21655

Silbering, A. F., Rytz, R., Grosjean, Y., Abuin, L., Ramdya, P., Jefferis, G. S. X. E., & Benton, R. (2011). Complementary function and integrated wiring of the evolutionarily distinct Drosophila olfactory subsystems. *Journal of Neuroscience*, *31*(38), 13357–13375. https://doi.org/10.1523/JNEUROSCI.2360-11.2011

Thistle, R., Cameron, P., Ghorayshi, A., Dennison, L., & Scott, K. (2012). Contact chemoreceptors mediate male-male repulsion and male-female attraction during drosophila courtship. *Cell*, *149*(5), 1140–1151. https://doi.org/10.1016/j.cell.2012.03.045

Tolassy, V., Cazalé-Debat, L., Houot, B., Reynaud, R., Heydel, J. M., Ferveur, J. F., & Everaerts, C. (2023). Drosophila Free-Flight Odor Tracking is Altered in a Sex-Specific Manner By Preimaginal Sensory Exposure. *Journal of Chemical Ecology*, *49*(3–4), 179–194. https://doi.org/10.1007/s10886-023-01416-3

van der Goes van Naters, W., & Carlson, J. R. R. (2007). Receptors and Neurons for Fly Odors in Drosophila. *Current Biology*, *17*(7), 606–612. https://doi.org/10.1016/j.cub.2007.02.043

Verschut, T. A., Ng, R., Doubovetzky, N. P., Le Calvez, G., Sneep, J. L., Minnaard, A. J., Su, C. Y., Carlsson, M. A., Wertheim, B., & Billeter, J. C. (2023). Aggregation pheromones have a non-linear effect on oviposition behavior in Drosophila melanogaster. *Nature Communications*, *14*(1), 1–15. https://doi.org/10.1038/s41467-023-37046-2

Von Lintig, J., Dreher, A., Kiefer, C., Wernet, M. F., & Vogt, K. (2001). Analysis of the blind Drosophila mutant ninaB identifies the gene encoding the key enzyme for vitamin A formation in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(3), 1130–1135. https://doi.org/10.1073/pnas.031576398

Wang, L., Han, X., Mehren, J., Hiroi, M., Billeter, J. C., Miyamoto, T., Amrein, H., Levine, J. D., & Anderson, D. J. (2011). Hierarchical chemosensory regulation of male-male social interactions in Drosophila. *Nature Neuroscience*, *14*(6), 757–762. https://doi.org/10.1038/nn.2800

635 **Supplementary table 1**
636

Fig	Test	Response variable	Explanatory factor	Results	Post-hoc test (only p-value <0.05)
1B	Anova	Ejection latency	Post-mating condition [male CS, male OR, isolated]	F=10.801, df=2, p=5.82e-05	Male CS-isolated: p=0.0002243, Male OR-isolated: p=0.9683661, Male OR- Male CS: p= 0.0004802
1C	Unpaired t-test	Proportion of offspring	Post-mating condition [male CS, male OR]	t = -2.0914, df = 93, p-value = 0.03922	NA
1D	Unpaired t-test	Number of offspring	Post-mating condition [male CS, male OR]	t = -2.0084, df = 93, p-value = 0.0475	NA
1E	Mann-Whitney exact test	Number of offspring from the 1st male	Post-mating condition [male CS, male OR]	U = 817.5, p-value = 0.0204, two-tailed	NA
1F	Unpaired t-test	Number of offspring from the 2nd male	Post-mating condition [male CS, male OR]	t = 0.0092772, df = 93, p-value = 0.9926	NA
2B	Mann-Whitney exact test	Proportion of offspring in SR	Post-mating condition [male CS, male OR]	U = 62, p= 0,0039, two-tailed	NA
2C	Mann-Whitney exact test	Number of sperm in SR	Post-mating condition [male CS, male OR]	U = 114,5, p= 0,3170, two-tailed	NA

2D	Unpaired t-test	Number of sperm in SR first male	Post-mating condition [male CS, male OR]	t = -2.3157, df = 32, p-value = 0.02714	NA
2E	Unpaired t-test	Number of sperm in SR 2nd male	Post-mating condition [male CS, male OR]	t = 2.9467, df = 32, p-value = 0.005949	NA
2F	Mann-Whitney exact test	Proportion of offspring in SP	Post-mating condition [male CS, male OR]	U= 85, p=0,0715, Two-tailed	NA
2G	Unpaired t-test	Number of sperm in SP	Post-mating condition [male CS, male OR]	t=0,4014, df=32, p-value=0,6908	NA
2H	Unpaired t-test	Number of sperm in SP first male	Post-mating condition [male CS, male OR]	t = -1.6899, df = 32, p-value = 0.1008	NA
2G	Unpaired t-test	Number of sperm in SP 2nd male	Post-mating condition [male CS, male OR]	t = 0.54826, df = 32, p-value = 0.5873	NA
3A	ANOVA	LOG transformed ejection latency	Time of male entrance [0min, 30min, 60min, 90min, 120min, isolated]	Df=5, F= 3.1999 , p-value = 0.00994	Dunnett's multiple comparisons test: 0 vs isolated: p=0,0025 0 vs. 30: p=0.7141 0 vs. 60: p=0.5032 0 vs. 90: p=0.0423 0 vs. 120: p=0.0914

3B	ANOVA	Ejection latency	Post-mating condition [male CS, isolated, maleCS for 1st hour AEM]	F= 5.1655, df=2, p= 0.007501	TukeyHSD: Alone-1hrCSmale: p=0.7335201 Csmale-1hrCSmale: p=0.0623864 Csmale-Alone: p=0.0076787
3C	Mann-Whitney exact test	Ejection latency	Post-mating condition [virgin female CS, isolated]	U =303, p-value = 0.2932, two-tailed	NA
3D	ANOVA	Log transformed ejection latency	Post-mating condition [1male CS, 6malesCS, 6males OR, 6males tai, mix]	F=4,451, df=4, p=0,0021	TukeyHSD: 1CS♂ vs. 6OR♂: p=0,0261 1CS♂ vs. 6Tai♂: p=0,0018
3E wild type	Unpaired t test	Ejection latency	Post-mating condition [male CS, isolated]	t=5,789, df=57, p-value<0,0001	NA
3E iav-	Mann-Whitney exact test	Ejection latency	Post-mating condition [male CS, isolated]	U=179,5, p-value=0,0012, two-tailed	NA
3E iav/+	Mann-Whitney exact test	Ejection latency	Post-mating condition [male CS, isolated]	U=180, p-value <0,0001 , two-tailed	NA
3E ninaB-	Unpaired t test with Welch's correction	Ejection latency	Post-mating condition [male CS, isolated]	t=1,993, df=41,19 p-value=0,0529	NA

3E ninaB-/+	Mann-Whitney exact test	Ejection latency	Post-mating condition [male CS, isolated]	U=269, p-value =0,0005, two-tailed	NA
3E ppk23-	Mann-Whitney exact test	Ejection latency	Post-mating condition [male CS, isolated]	U=289,5, p-value =0,0014, two-tailed	NA
3E ppk23-/+	Mann-Whitney exact test	Ejection latency	Post-mating condition [male CS, isolated]	U=202, p-value =0,0003, two-tailed	NA
3E Gr32a-	Mann-Whitney exact test	Ejection latency	Post-mating condition [male CS, isolated]	U=111, p-value =0,0004, two-tailed	NA
3E Gr32a-rescue	Mann-Whitney exact test	Ejection latency	Post-mating condition [male CS, isolated]	U=119.5, p-value =0,0733 , two-tailed	NA
3E orco-	Mann-Whitney exact test	Ejection latency	Post-mating condition [male CS, isolated]	U=305, p-value =0,7109 , two-tailed	NA
3E orco-rescue	Mann-Whitney exact test	Ejection latency	Post-mating condition [male CS, isolated]	U=336, p-value =0,0108 , two-tailed	NA

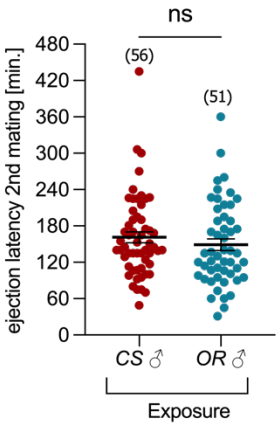
3F	Unpaired t test	Ejection latency	Post-mating condition [male CS, isolated]	Kir/+: t=4,166, df=30,p=0,0002 Orco/+: t=5,970, df=33, p<0,0001 orco/kir :t=0,8782, df=34, p=0,3860	NA
4A	ANOVA	Ejection latency	Post-mating condition [male CS CHCs, male OR CHCs, hexane]	F= 3.4526, df=2, p= 0.03749	TukeyHSD: hexane-CS CHC: p=0.0289712 OR CHC-CS CHC: p=0.4679140 OR CHC-hexane: p=0.3060676
4B	Kruskal-Wallis test	Ejection latency	Post-mating condition [isolated, male CS, male stinger, male oe-, male oe- +7T, male oe- + heptanal]	P <0.0001	Dunn's multiple comparisons test: isolated vs. CS♂: p=0,0018 isolated vs. Ctrl♂: p=0,0002 isolated vs. oe⁻♂: p>0,9999 isolated vs. oe⁻♂+7-T: p=0,0093 isolated vs. oe⁻♂+heptanal: p<0,0001
4C	ANOVA	LOG transformed ejection latency	Post-mating condition [hexane, 250cVA, 1000heptanal, 250cVA+250heptanal, 250cVA+500heptanal, 250cVA+1000heptanal, 250cVA+1500heptanal	F-value= 2.575, df=6, p-value: 0.02226	TukeyHSD: Hexane vs 250+250: p= 0.0452316 0+1000 vs 250+250: 0.0090598
4D	Unpaired t test	Ejection latency	Post-mating condition [male CS, isolated]	Kir/+: p=0,0044 OR22a/+: p=0,0830	NA

				OR22a/kir: p=0,7615	
4E	Mann-Whitney exact test	Ejection latency	Post-mating condition [male CS, isolated]	Kir/+: p=0,0044 OR35a/+: p=0,0024 OR35a/kir: p=0,2679	NA
4F	Mann-Whitney exact test	Ejection latency	Post-mating condition [male CS, isolated]	Kir/+: p<0,0001 OR13a/+: p<0,0001 OR13a/kir: p=0,0016	NA
4G	Mann-Whitney exact test	Ejection latency	Post-mating condition [male CS, isolated]	Kir/+: p<0,0001 OR47b/+: p=0,0001 OR47b/kir: p=0,0176	NA
4H	Mann-Whitney exact test	Ejection latency	Post-mating condition [male CS, isolated]	Kir/+: p=0,0741 OR65a/+: p<0,0001 OR65a/kir: p=0,1592	NA
4I	Mann-Whitney exact test	Ejection latency	Post-mating condition [male CS, isolated]	Kir/+: p=0,0007 OR67d/+: p=0,0029 OR67d/kir: p=0,1518	NA
4J	Mann-Whitney exact test	Ejection latency	Post-mating condition [cVA+heptanal, hexane]	Kir/+: p=0,0044 OR22a/+: p=0,0063 OR22a/kir: p=0,5085	NA

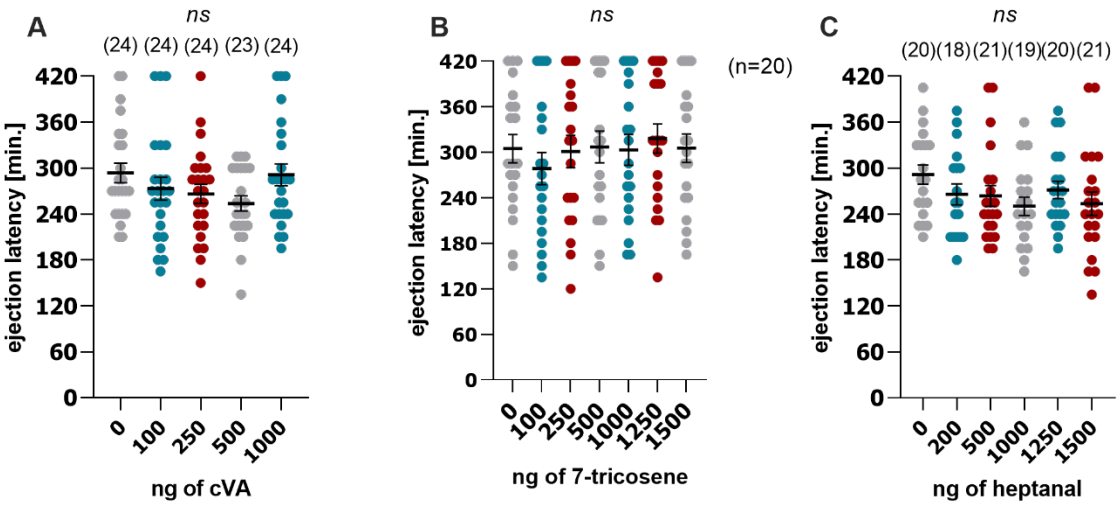
4K	Mann-Whitney exact test	Ejection latency	Post-mating condition [cVA+heptanal, hexane]	Kir/+: p=0,0171 OR35a/+: p=0,0001 OR35a/kir: p=0,6217	NA
Supp Fig1	Mann-Whitney exact test	Ejection latency	Post-mating condition [male CS, male OR]	P=0,2553	NA
Supp Fig4A	Kruskal-Wallis test	Ejection latency	Post-mating condition [hexane, cVA 100, cVA 250, cVA 500, cVA 1000]	P=0,3431	NA
Supp Fig4B	Kruskal-Wallis test	Ejection latency	Post-mating condition [hexane, 7-t 100, 7-t 250, 7-t 500, 7-t 1000, 7-t 1250, 7-t 1500]	P=0,9331	NA
Supp Fig4C	Kruskal-Wallis test	Ejection latency	Post-mating condition [hexane, heptanal 200, heptanal 500, heptanal 1000, heptanal 1250, heptanal 1500]	P=0,2531	NA

637

Supplementary figures



Supplementary figure 1: Female 2nd male ejaculate ejection latency is not affected by exposure to different types of males. Ejaculate ejection latency of females after a second mating exposed to the presence of *CS* or *OR* males between matings. Numbers in between brackets indicate sample sizes. mean ± S.E.M are indicated in the graphs. Scatter-plot labelled with the same letter are not statistically different. *n.s.*: non-significant; For full statistical details see Supplementary Table S1.



Supplementary figure 4: Ejection latency in response to male's pheromones. (A) Ejaculate ejection latency of female *CS* in response to different amount of cVA or to the solvent only (hexane). (B) Ejaculate ejection latency of female *CS* in response to different amount of 7-tricosene or to the solvent only (hexane). (C) Ejaculate ejection latency female *CS* in response to different amount of heptanal or to the solvent only (hexane). Numbers in between brackets indicate sample sizes. mean ± S.E.M are indicated in the graphs. Scatter-plot labelled with the same letter are not statistically different. *n.s.*: non-significant; *:p<0.05; **:p<0.01; ***:p<0.001.For full statistical details see Supplementary Table S1.