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► To cite this version:

Bénédicte M Lefèvre, Diane Catté, Virginie Courtier-Orgogozo, Michael Lang. Male genital lobe morphology affects the chance to copulate in *Drosophila pachea*. BMC Evolutionary Biology, 2021, BMC Ecol Evo, 21 (23). <hal-03133172>

HAL Id: hal-03133172

<https://hal.science/hal-03133172v1>

Submitted on 5 Feb 2021

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1 **Male genital lobe morphology affects the chance to copulate in *Drosophila pachea***

2 running title: *D. pachea* mate competition analysis

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7 running title: *D. pachea* mate competition analysis

8 Abstract

9 **Introduction:** Male genitalia are thought to ensure transfer of sperm through direct physical contact
10 with female during copulation. However, little attention has been given to their pre-copulatory role
11 with respect to sexual selection and sexual conflict. Males of the fruitfly *Drosophila pachea* have a
12 pair of asymmetric external genital lobes, which are primary sexual structures and stabilize the
13 copulatory complex of female and male genitalia. We wondered if genital lobes in *D. pachea* may
14 have a role before or at the onset of copulation, before genitalia contacts are made.

15 **Results:** We tested this hypothesis with a *D. pachea* stock where males have variable lobe lengths.
16 In 92 mate competition trials with a single female and two males, females preferentially engaged
17 into a first copulation with males that had a longer left lobe and that displayed increased courtship
18 vigor. In 53 additional trials with both males having partially amputated left lobes of different
19 lengths, we observed a weaker and non-significant effect of left lobe length on copulation success.
20 Courtship durations significantly increased with female age and when two males courted the female
21 simultaneously, compared to trials with only one courting male. In addition, lobe length did not
22 affect sperm transfer once copulation was established.

23 **Conclusion:** Left lobe length affects the chance of a male to engage into copulation. The
24 morphology of this primary sexual trait may affect reproductive success by mediating courtship
25 signals or by facilitating the establishment of genital contacts at the onset of copulation.

26 keywords: *Drosophila pachea*, primary sexual trait, mating behaviour, mate-competition
27 experiments, genitalia

28 **Introduction**

29 Males and females exhibit different reproduction strategies due to higher energy costs of
30 larger female gametes compared to smaller and more abundant male gametes [1]. This implies
31 male-male intrasexual mate-competition for siring the limited female gametes. In turn, females may
32 optimize reproduction by choosing males that confer survival and fecundity benefits to her and to
33 the offspring. This dynamics has been formalized into genetic models [2, 3] involving female
34 preferences for particular male characters and male mate competition, and can contribute to the
35 rapid evolution of female preferences and male sexual attributes. There is also evidence for male
36 mate-choice and female intra-sexual selection [4–6], showing that traditional sex-roles can change
37 between species and in individuals under diverse conditions. In general, sexual selection implies a
38 sexual conflict whenever male and female reproductive fitness strategies differ [7].

39 Across animals with internal fertilization, genitalia are usually the most rapidly evolving
40 organs [8]. Several hypotheses propose that the evolution of genitalia is based on sexual selection
41 and a consequence of sexual conflict between males and females at different levels of reproduction,
42 including competition of sperm from different males inside the female (sperm competition) [9],
43 female controlled storage and usage of sperm to fertilize eggs (cryptic female choice) [8, 10, 11], or
44 sexually antagonistic co-evolution of aggressive and defensive morphologies and/or behaviors
45 between male and female to control fertilization decisions [12, 13]. Male courtship is well known as
46 a behaviour to attract females before copulation begins, but has also been reported to occur during
47 or even after copulation [14, 15]. It is thought to be a widespread and key aspect of copulation in
48 the cryptic female choice scenario, where males stimulate the female to utilize his own sperm [8,
49 10]. A variety of insect species were reported where males had evolved elaborated male genital
50 structures that are apparently used for courtship throughout copulation to stimulate the female
51 through tapping or other physical stimuli [14, 15].

52 Traditionally, sexual traits are categorized as primary or secondary. Genital structures are
53 considered “primary” when they are directly used for the transfer of gametes during copulation or
54 when they contribute to the complexing of female and male copulatory organs [8, 16]. Other traits
55 that differ between sexes and that are linked to reproduction are considered “secondary” sexual
56 traits. For primary sexual traits, sexual selection is traditionally thought to act during or after
57 copulation [8, 15], while secondary sexual traits can be involved in pre-copulatory mate-choice via
58 visual, auditory or chemical long range signalling [17–25].

59 Primary genitalia could also be possibly involved in pre-copulatory mate choice. Supposing
60 that a particular male genital trait enhances or favours female fecundity, it might become preferred
61 by the female during pre-copulatory courtship or at the moment of genitalia coupling [26–28]. This
62 preference could rely on direct benefits to reduce energy investment or predation risk caused by
63 unsuccessful copulation attempts, or on indirect male genetic quality to be inherited into the
64 offspring generation. Several examples of primary genitalia used in pre-copulatory courtship
65 signalling are known in vertebrates. For example, male and female genital display, swelling, or
66 coloration changes are associated with sexual activity [29–31] and penile display was also observed
67 in lizards [32]. Human male penis size was found to influence male attractiveness to women [33]. In
68 diverse mammalian species, females have also evolved external and visible structures that resemble
69 male genitals, a phenomenon called andromimicry [31, 34, 35]. These structures are likely used in
70 visual signaling, so it is possible that the male analogous parts also do. Furthermore, females of
71 some live-bearing fish species were reported to prefer to mate with males with large gonopodia [36,
72 37]. These cases involve visual stimuli and large genital organs. Only a few studies examined the
73 roles of primary genitalia in invertebrates during courtship or at the onset of copulation before
74 phallus intromission is achieved [26–28, 38, 39]. Longer male external genitalia of the water strider
75 *Aquarius remigis* were associated with higher mating success [28]. In addition, male genital spine
76 morphology of *Drosophila bipectinata* and *Drosophila ananassae* were reported to evolve in

77 response to direct competition among males for securing mates in a so called ‘scramble
78 competition’ scenario [26, 27]. In general, the role of primary sexual traits in establishing
79 copulation or their role during courtship needs further investigation.

80 The fruitfly species *Drosophila pachea* is a promising model to study the evolution of
81 primary sexual traits, especially with respect to the evolution of left-right asymmetry. Male *D.*
82 *pachea* have an asymmetric aedeagus (intromission organ) and a pair of asymmetric external lobes
83 with the left lobe being approximately 1.5 times longer than the right lobe [40–42] (Figure 1). The
84 genital lobes have likely evolved during the past 3-6 Ma and are not found in closely related species
85 [43]. *D. pachea* couples also mate in a right-sided copulation position where the male rests on top
86 of the female abdomen with the antero-posterior midline shifted about 6-8 degrees to the right side
87 of the female midline [41, 42, 44]. Male and female genitalia form an asymmetric complex during
88 copulation and the asymmetric lobes stabilize this genital complex [44]. Furthermore, *D. pachea* is
89 among the *Drosophila* species that produce the longest (giant) sperm [45, 46], and their ejaculates
90 contain in average just about 40 sperm cells [47]. Thus, a particular right-sided mating position
91 could potentially be associated with optimal transfer of giant sperm during copulation [42].

92 The aim of this study was to test if the asymmetric genital lobes of *D. pachea* would have an
93 effect on pre-copulatory mate-choice, in addition to their role in stabilizing the complex of male and
94 female genitalia during copulation. Previously, we found that males originating from one of our *D.*
95 *pachea* laboratory stocks possess short and rather symmetric lobes [41] (Figure 1), while others
96 have the typical size asymmetry. The variability in left genital lobe length that we observed within
97 this fly stock enabled us to test if lobe length might affect pre-copulatory courtship or mate
98 competition. We selected *D. pachea* males with short left lobes and produced a stock with an
99 increased variance of left lobe length. Next, we tested sibling males of this “selection stock” in
100 mate-competition assays for their success to engage first into copulation with a single female. We
101 also tested whether copulation success in our assay would be affected by male courtship vigour.

102 Then, we surgically shortened the length of the left lobe in males that had developed long left lobes,
103 to further test whether left lobe length affects copulation success. Finally, we assessed whether lobe
104 length affects ejaculate allocation into female storage organs.

105 **Results**

106 **Genital lobe lengths differ between *D. patchea* stocks**

107 In our laboratory stock 15090-1698.01 (Additional datafile 1: Table S1), *D. patchea* males
108 display a characteristic left-right size asymmetry of genital lobes with the left lobe being
109 consistently larger than the right lobe (Figure 1a,c, Figure 2a, Additional datafile 1: Table S2,
110 Additional datafile 2). Similarly, in stock 15090-1698.02, most males reveal larger left lobes, but a
111 few individuals are observed with particularly small lobes that are rather symmetric in length
112 (Figure 1b-d, Figure 2b). We selected for males with short lobe lengths and crossed them with
113 sibling females for 50 generations (Methods). The resulting stock was named “selection stock”. We
114 observed an increased variance of left lobe length compared to the source stock (Levene’s test:
115 selection stock / 15090-1698.02: $DF = 1/147$, $F = 11.506$, $P = 0.0008913$) (Figure 2c, Additional
116 datafile 2). In contrast, the right lobe length differed only marginally among the two stocks
117 (Additional datafile 1: Table S2) and the variances of right lobe lengths were not significantly
118 different among them (Levene’s test, $DF = 1/147$, $F = 0.5164$, $P = 0.4735$).

119 **Male genital left lobe length, male courtship and failed copulation attempts have an effect on** 120 **copulation success**

121 We performed a mate competition assay (Methods) with two males and a single female to
122 test if differences in genital lobe length and courtship behavior may influence the chance of a male
123 to copulate first with a single female. This first engagement into copulation will be referred to as
124 “copulation success”. We filmed courtship and copulation behavior (Additional datafile 1: Figure

125 S1) of two males of the selection stock and a single female of stock 15090-1698.01 or 15090-
126 1698.02. We annotated 111 trials where both males simultaneously courted the female (Methods)
127 (51 and 60 trials with females of stocks 15090-1698.01 or 15090-1698.02, respectively). Then, we
128 dissected the genitalia of both males to determine lobe lengths. We examined 54 additional trials
129 with two courting males from the selection stock that had partially amputated left lobes and females
130 of stock 15090-1698.01. This was done to test if other characters might co-vary with left lobe length
131 and could potentially also have an effect on copulation success. We shortened the left lobes of both
132 males in order to be able to neglect possible effects of the caused wound. The relative courtship
133 activity or vigor of the two males in each trial was compared by quantifying periods that each male
134 spent touching the female ovipositor or the ground next to it with the proboscis (mouth-parts),
135 hereafter referred to as “licking behavior” (Methods). Licking was chosen over other behaviors
136 because it occurs throughout courtship of *D. pachea* (Additional datafile 1: Figure S2) and was
137 relatively easy to spot. We counted the sum duration of these periods (sum licking duration) and the
138 number of licking periods (number of licking sequences). A copulation was considered to take
139 place, once a male had mounted the female abdomen and achieved to settle into an invariant
140 copulation position (Methods).

141 In the majority of trials (130/165), the first mounting attempt of a male resulted in a stable
142 copulation. We did not observe any male behavior that could indicate an attempt of the non-
143 copulating male to directly usurp the female as observed for *D. ananassae* or *D. bipectinata* [26,
144 27]. However, in 35 trials, a total of 51 failed mounting attempts were observed (Methods). These
145 were not proportionally higher among trials with lobe amputated males (9 failed mounting attempts
146 in 7 / 54 trials 13%) compared to unmodified males (42 failed mounting attempts in 28 / 111 trials
147 25%). Thus, the partial lobe amputation probably did not decrease the ability of a male to mount a
148 female. Only 15/51 failed mounting attempts lasted 8 seconds or longer. Among those, the non-
149 mounted male continued to court the female in 10/15 attempts. However, this was also observed in

150 131/165 successful mounting attempts and these proportions are not significantly different (χ^2 -test,
151 χ^2 : 1.4852, df = 1, P = 0.2230). Altogether, mounting failure was not found to be significantly
152 associated with the courtship activity of the other male.

153 Different variables were tested to have an effect on male copulation success by using a
154 Bradley Terry Model [48] (Methods). In trials with unmodified males, copulation success was
155 positively associated with left lobe length and the sum licking duration of the males during
156 courtship (Table 1). The effect of sum licking duration is illustrated when comparing the ratio of
157 sum licking duration and total courtship duration between the copulating and the non-copulating
158 male (Figure 3a). This ratio was higher in the copulating male compared to the non-copulating
159 male. In particular, we identified a positive interaction of failed mounting attempts and female age,
160 indicating that the negative effect of failed mounting attempts is pronounced in young females but
161 not in older females. However, we observed failed mounting attempts in only 35 trials (Additional
162 datafile 1: Figure S3) and the model estimates were sensitive to outlier observations (Additional
163 datafile 1: Figure S10). Therefore, we relied on a simpler but more robust model that only takes
164 sum licking duration and left lobe length into account (Table 1). More observations are required to
165 evaluate the interaction of failed mounting attempts and female age.

166 In trials with males that underwent lobe surgery, left lobe length was not significantly
167 associated with copulation success (Table 2) or with male courtship behaviors. Thus, we cannot
168 exclude the possibility that left lobe length co-varies with yet another unknown selected trait that
169 reveals an advantage for the male to copulate. Since the copulating male still showed an increased
170 sum licking duration compared to the non-copulating male (Figure 3), the non-significant model fit
171 may thus reflect insufficient statistical power due to a smaller number of observations compared to
172 trials with unmodified males.

173 **Courtship duration is increased when two males court a female simultaneously**

174 Regardless of the stock for the female used, courtship durations appeared to be longer when
175 both males courted the female simultaneously compared to trials where only one male courted
176 (Mann-Whitney-Wilcoxon test, $W = 1906.5$, $N = 48/165$, $P = 4.7 \times 10^{-8}$) (Figure 3b). We also
177 observed that courtship duration was strikingly increased in females that were older than 11 days
178 (after emerging from the pupa) compared to younger females (Additional datafile 1: Figure S4)
179 (Mann-Whitney test, $W = 660$, females younger than 12 days / females 12 days or older = 33 / 132,
180 $P = 6.333 \times 10^{-10}$). Thus, older females may be less likely to copulate and may also discriminate less
181 between males based on failed mounting attempts (see above).

182 **Lobe length is not associated with the amount of ejaculate in female sperm storage organs**

183 To test whether lobe length influences the amount of ejaculate deposited into the female
184 after copulation, we dissected a random subset of females (54 of stock 15090-1698.01 and 24 of
185 stock 15090-1698.02) in trials with non-modified lobes that revealed a copulation. Neither right
186 lobe length, left lobe length, sum licking duration or female age (days after emerging from the
187 pupa) was significantly associated with ejaculate content in the spermathecae (Table 3). However,
188 male adult age and copulation duration had a negative effect. Given that *D. pachea* males become
189 fertile about 13 days after emerging from the pupa [49], our results indicate an optimal period of
190 male ejaculate transfer at the beginning of their reproductive period. Copulation duration had a
191 negative effect on sperm transfer (Table 3), indicating that failed sperm transfer may result in
192 prolonged attempts and longer copulation duration.

193 **Discussion**

194 **Left lobe length affects the chance of a male to copulate**

195 We observed that most females copulated first with the male that had the longer left lobe
196 when two males courted simultaneously in our trials. These results suggest that a long left lobe
197 might not only stabilize an asymmetric genital complex during copulation [44], but also increases
198 the chance of a male to engage into copulation. With our data, we cannot exclude that the sensing of
199 the lobe length by the female occurs just before intromission, while male and female genitalia are
200 contacting each other or if it is used even during courtship before genital contacts establish. Other
201 factors, unrelated to lobe length, can also bias copulation. For example, females might prefer to
202 copulate with males of larger body size or that display particular courtship behaviors. In our study,
203 we found no significant effect of tibia length on copulation success, which was used as an
204 approximation for overall body size. However, the mutation(s) associated with lobe length variation
205 in this stock are unknown. It is possible that one or several alleles associated with lobe length still
206 segregate in this stock, even after 50 generations of inbreeding. If such segregating mutations have
207 pleiotropic effects on other male characters (morphological, physiological or behavioural), these
208 traits are expected to co-vary together with lobe length and the effect of lobe length that we detected
209 might actually be due to the other factors. In trials with artificially shortened left lobes , males were
210 taken from the selection stock but had the expected wild-type lobe asymmetry. Left lobe length
211 difference was then artificially introduced in those males, so that it would not be expected to co-
212 vary with the relative phenotypic expression of the supposed underlying mutation. In such trials, left
213 lobe length after artificial length reduction was not associated with copulation success so that we
214 cannot rule out the possibility that left lobe length in our initial experiment with unmodified males
215 affects copulation success indirectly via a pleiotropic mutation affecting both, lobe length and other
216 traits. It is possible that we failed to detect a significant effect because of a reduced variation in left
217 lobe length among the lobe-modified males, compared to the initial experiment with non-ablated

218 males. In addition, we could not measure left lobe length on living males before lobe amputation.
219 Therefore, we could not test whether the lobe length "before" length reduction influences male
220 mating success. Furthermore, lobe amputation did not only cause a length reduction of the left lobe
221 but also removed the bristles located at the distal tip. Laser ablation experiments have shown that
222 these bristles contribute to the stabilization of the mating complex during copulation [44]. The
223 bristles may also be important in pre-copulatory events, but we could not measure this since they
224 were removed from both males in our trials. Future mate-choice experiments could be carried out
225 with an unmodified male and a male where only those bristles are ablated [44].

226 **Male intra-sexual competition is potentially reflected by male courtship intensity**

227 Previous analyses in *D. ananassae* and *D. bipectinata* relate male genital spines to pre-
228 copulatory abilities to achieve a copulation due to increased male intrasexual competitiveness in so
229 called "scramble competition" or vigorous copulation attempts with females that are optionally
230 already copulating with another male [26, 27]. In *D. pachea*, this behavior was not observed and
231 most mounting events of a male resulted in a stable copulation. Once a copulation started, the non-
232 copulating male was never observed to separate the couple. Instead, it continued to display
233 courtship behaviors such as licking, wing vibration or touching the female side in the majority of
234 cases. In addition, male mounting was only observed upon female wing opening and oviscapt
235 protrusion. This indicates that mounting in *D. pachea* is a complex behavior involving both sex
236 partners. One possibility might be that upon mounting the chance of a *D. pachea* male to engage
237 into copulation thus may largely depend on male-female communication rather than on male male
238 competition.

239 Total courtship durations were increased in trials where both males courted the female
240 simultaneously compared to trials with only one male courting. This indicates that females
241 potentially require more time to accept one of the males for copulation in cases where two males
242 court simultaneously. Alternatively, longer courtship durations might indeed rely on male-male

243 competition, causing mutual disturbances in courtship display. We detected a strong association of
244 copulation success with the sum licking duration, which we used as an approximation for overall
245 courtship activity or vigor of each male. This is achieved by maintaining its position close to the
246 female ovipositor in competition with the other male, suggesting that licking behavior in part reports
247 male-intrasexual conflict.

248 **Potential female benefits gained from choosing males with long left lobes**

249 Female mate choice on male sexual traits was suggested to rely at least in part on the way a
250 sexual trait is displayed or moved [50]. This implies that a certain quantity but also accuracy of
251 locomotor activity would matter in courtship and might better reflect overall male quality and “truth
252 in advertising” than a morphological character or ornament alone. Our data suggest that male
253 settling upon mounting the female might be crucial for a successful copulation since we identified a
254 negative effect of failed mounting attempts on copulation success and a positive interaction of failed
255 mounting attempts with female age, possibly indicating that young females are more discriminative
256 compared to older females with respect to male mounting performance. However, this effect was
257 only based on 35 trials (Additional datafile 1, Figure S3) and model estimates were sensitive to
258 outliers. Future work must further evaluate this trend. Female mate choice is hypothesized to be
259 based on direct benefits, affecting fecundity and survival of the female, but also on indirect benefits
260 that relate to genetic quality, fecundity and survival of the progeny [50, 51]. Left lobe length and
261 male mounting performance might therefore influence female mate-choice through enhancing the
262 quality of male courtship signals and by enhancing the coupling efficiency of male and female
263 genitalia upon mounting. It remains to be resolved what these courtship signals could be and how
264 they might be presented to the female. We did not observe direct contact of the female with male
265 genitalia prior to the male mounting attempts at copulation start. A possible pre-copulatory signal
266 mediated by genital lobes could therefore be visual or vibratory. During *Drosophila* courtship,
267 males perform abdomen shakes (“quivers”) that generate substrate-borne vibratory signals [52].

268 Perhaps the quiver frequency or amplitude could be affected by the length of the left lobe, thus
269 producing a modified vibration signal. Alternatively, asymmetric lobes might be associated with
270 lateralized courtship. For example, the beetle species *Sitophilus oryzae* and *Tribolium confusum*
271 were observed to perform left-biased copulation attempts, which led to higher mating success over
272 right-biased males [53]. However, in our study the male always mounted the female from the rear,
273 along the female midline. We also noted that the licking side upon mounting was most commonly
274 the rear of the female abdomen, but changed frequently in response to female abdomen movements
275 and due to the presence of the other male. Therefore, we did not identify any lateralized male pre-
276 copulatory behavior. This is in contrast with the lateralized posture of *D. pachea* males later during
277 copulation, with the male being shifted about 6-8° to the right side of the female abdomen [41, 42,
278 44].

279 Alternatively, left lobe length might potentially associate with direct copulation benefits for
280 male and female by ensuring the efficiency of ejaculate transfer during copulation. Males of *D.*
281 *pachea* produce giant sperm and previous estimates revealed that a *D. pachea* male transmits only
282 about 44 ± 6 sperm cells per copulation [47], while the maximum female sperm storage capacity in
283 the spermathecae was estimated to be much higher and to be about 304 sperm cells [47]. Wild-
284 caught *D. pachea* females were found to contain sperm from at least 3-4 males based on
285 spermathecae filling levels [47]. As asymmetric lobes stabilize the genital complex [44], they might
286 have a positive effect on the amount of sperm transferred per copulation. However, we have not
287 observed any effect of lobe length on ejaculate presence in the spermathecae after copulation. We
288 note that we did not test for the presence of seminal fluid in the female outside of the spermathecae.
289 Also, our quantification method was not precise and sample size was limited. Accurate direct sperm
290 counts requires radio-labeling methods [47], which was not applicable in our experimental setup. In
291 future approaches, improved sperm counts should be applied to test for a correlation between lobe
292 length, female-male copulation complex stability and sperm quantity in female spermathecae.

293 Based on our data, we note that left lobe length affects the chance of a male to establish
294 genital contacts that lead to a firm complex of female and male genitalia, congruent with previous
295 observations in other insects [26–28, 54]. In particular, the male genital spines in *D. ananassae* and
296 *D. bipectinata* were observed to play a role in pre-copulatory events but had no measurable
297 influence later on with respect to sperm transfer or fecundity [26, 27, 54].

298 Interestingly, copulation duration negatively affected ejaculate presence in the female. This
299 observation suggests that the couple separates once successful sperm transfer is achieved. However,
300 not all copulations reveal sperm transfer. In occasions of failure the copulation duration may
301 therefore be prolonged to further attempt to mate. Copulation duration varies largely among
302 Drosophila species from fractions of a minute to up to about 2 h in *D. acanthoptera* [42, 49].
303 Copulation duration in *D. pachea* is thus comparatively long with respect to other species of the
304 genus, pointing to presumably complex copulatory interactions affecting sperm transfer. Future
305 studies must analyze the internal processes of sperm transfer during *D. pachea* copulation to better
306 understand this process and its possible limitations.

307 Indirect paternal effects on the growth and survival of offspring could potentially play a role
308 in *D. pachea* female choice for males with long left lobes. It has been demonstrated that mate
309 choice based on particular male sexual characters correlates with offspring survival in diverse
310 species [18, 20–23, 55]. However, our experiment did not allow us to test for these effects because
311 females were sacrificed after the mating experiment and no progeny was obtained. We assessed
312 ejaculate filling levels instead of the amount of female progeny because *D. pachea* females rarely
313 lay eggs after a single insemination and about four copulations are necessary for a female to start
314 oviposition [56]. To test for indirect effects, future studies should ideally use progeny males from
315 wild caught *D. pachea* females and test if subtle lobe length variation in those males would
316 correlate with offspring survival in single couple crosses. A similar approach was used by Hoikkala

et al. (1998) [55], who found that *Drosophila montana* courtship song frequency of wild caught males correlated with the survival rate of the male's progeny.

Age of reproductive activities appear to be non-overlapping in sibling males and females

The amount of ejaculate present in the female spermathecae after a single copulation was negatively affected by the age of the copulating male. Similarly, it was previously found that the number of progeny in single couple crosses (presumably involving multiple copulations) was also dependent on male adult age in *D. pachea* [57]. Males in *D. pachea* need about 10-14 days at 25° C after emergence to become sexually mature [49, 56, 57]. This is related to adult testis growth and production of giant sperm [47]. It was shown that the relatively long time to reach male sexual maturity impacts the proportion of sexually active *D. pachea* adults (operational sex ratio), which is female-biased [49]. Our findings suggest that males potentially have a maximum fertility period at the beginning of their sexual maturity at approximately 13-16 days after emerging from the pupa. The detected reduction of transferred ejaculate in males older than 16 days implies an additional male reproductive limitation.

It was hypothesized that the delay in male sexual maturity might potentially lead to decreased sibling mating in *D. pachea* [56]. In *D. melanogaster*, sibling matings yield fewer progeny compared to crosses with unrelated individuals [57]. In our study, the female was not a sibling of the two males. Courtship durations were shortest in trials with “young” virgin females, between 6 and 11 days. Given that females reach sexual maturity at about 4 days after emerging from the pupa [49, 57], our observation suggests that young females engage more quickly into copulation compared to older females. Overall, female and male maximum reproductive activities appear to be non-overlapping among siblings of opposite sex and similar age. Such an effect can be relevant in the wild if a cactus rot is colonized by a single previously fertilized female.

340 **Conclusion**

341 Longer male left genital lobes potentially enhance the probability of *D. pachea* males to
342 engage into copulation, which is expected to confer a reproductive advantage over males with
343 shorter left lobes. The chance of a male to copulate first with a female was also affected by the
344 relative male courtship activity and potentially by mounting attempt failures, especially for females
345 at the beginning of their reproductive activity. The evolution of left lobe length might not only
346 relate to its previously known function as a stabilizing device during copulation, but also through
347 pre-copulatory mate choice by increasing the chances to copulate and to increase the coupling
348 efficiency of genitalia upon mounting. Lobes do not appear to impact sperm transfer in later
349 copulatory events. In addition, reproductive ages are non-overlapping between the two sexes
350 potentially decreasing the amount of sibling mating in this species.

351 **Methods**

352 **Fly stock establishment and maintenance**

353 Two *D. pachea* stocks 15090-1698.01 and 15090-1698.02 were retrieved from the San
354 Diego Drosophila Species Stock Center (now The National Drosophila Species Stock Center,
355 College of Agriculture and Life Science, Cornell University, USA) (Additional datafile 1: Table
356 S1). Flies were maintained in 25 x 95 mm plastic vials containing 10 mL of standard Drosophila
357 medium (60 g/L brewer's yeast, 66.6 g/L cornmeal, 8.6 g/L agar, 5 g/L methyl-4-hydroxybenzoate
358 and 2.5% v/v ethanol) and a ~ 10 x 50 mm piece of bench protection sheet (Bench guard). As *D.*
359 *pachea* requires 7-dehydrocholesterol for proper development [58–60], we mixed the medium of
360 each vial with 40 µL of 5 mg/mL 7-dehydrocholesterol, dissolved in ethanol (standard *D. pachea*
361 food). Flies were kept at 25°C inside incubators (Velp) with a self-made light installation for a 12 h
362 light: 12h dark photo-periodic cycle combined with a 30-min linear illumination change between

light (1080 lumen) and dark (0 lumen). We used males of stock 15090-1698.02 to generate a new stock with increased proportions of males with short genital lobes. For this, we chose 3 males with apparently symmetric (aberrant) genital lobes and crossed them with 3-4 sibling virgin females. We repeated the selection with the progeny for a total of 36 generations. Then, we discarded males with clearly visible asymmetric (wild-type) lobes from the progeny for another 14 generations to derive the final stock (selection stock).

Virgin fly selection

Virgin flies at 0-1 d after emerging from the pupa were CO₂ anaesthetised on a CO₂-pad (INJECT+MATIC Sleeper) under a stereo-microscope Stemi 2000 (Zeiss), separated according to sex and maintained in groups of 20-30 individuals. Males and females were raised until reaching sexual maturity, about 14 days for males and 4 days for females at 25° C [49]. This allowed us to use virgin individuals in each mating experiment. Males were anaesthetised on the CO₂ pad (see above), sorted according to lobe morphology (asymmetric and symmetric lobes) and isolated into single vials at least two days before each mating experiment took place.

Mate-competition assay

We used a single virgin female of wild-type stock 15090-1698.01 or 15090-1698.02 and two sibling males of the selection stock that visually differed in lobe length when inspected with a binocular microscope (Additional datafile 1: Figure S5). This selection was done to increase the average pairwise difference of lobe lengths between the two males. Specimens were introduced into a white, cylindrical mating cell (Additional datafile 1: Figure S1) with a diameter of 20 mm, a depth of 4 mm and a transparent 1 mm Plexiglas top-cover. Optionally, mating cells were concave with a diameter of 20 mm and a depth of 4 mm at the center (Additional datafile 3: trials 1-16). Flies were transferred without CO₂ anaesthesia using a fly aspirator: a 7 mm diameter silicone tube closed at

386 the tip with cotton and a 1000 μ L wide bore (3 mm) micro-tip. Movie recordings were started as
387 soon as the chamber was immediately put under the camera (see below).

388 All mating trials were carried out inside a temperature and humidity controlled climate
389 chamber at $25^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ and $80\% \pm 5\%$ or $60\% \pm 5\%$ (trials 1-15) humidity. We used digital
390 cameras MIRAZOOM MZ902 (OWL) or DigiMicro Profi (DNT) to record copulation and
391 courtship behaviour. The MIRAZOOM MZ902 (OWL) camera was mounted on a modified
392 microscope stand (191348, Conrad) equipped with a 8-cm LED white light illumination ring (EB-
393 AE-COB-Cover, YM E-Bright) and a platform to hold four individual cylindrical mating cells
394 (Additional datafile 1: Figure S1). Trial 16 (Additional datafile 3) was filmed with the OWL camera
395 and a concave shaped mating cell that was put on a flat plastic cover on top of the microscope stand.
396 For trials 1-15, we used concave shaped mating cells that were put onto the stand of the DigiMicro
397 Profi (DNT) camera. Data was acquired with the programs Cheese (version 3.18.1)
398 (<https://wiki.gnome.org/Apps/Cheese>) or GUVVIEW (version 0.9.9) GTK UVC (trials 1-15) on
399 an ubuntu linux operating system in webm or mkv format. Up to four trials were recorded in a
400 single movie, which was split after recording and converted into mp4 format with Avidemux 2.6
401 (<http://www.avidemux.org/>) to obtain a single movie per experiment.

402 We waited for a copulation to take place between one of the males and the female (see
403 below). The mating cell was then shortly recovered from the climate chamber in order to remove
404 the non-copulating male with an aspirator and to transfer it into a 2-mL reaction tube filled with
405 70% ethanol. This was performed after the copulating male appeared to have settled into a stable
406 mating posture on the female abdomen, which was controlled by live-imaging. The copulating male
407 and the female were isolated from the mating cell after copulation had ended and were also isolated
408 into single 2-mL reaction tubes filled with 70% ethanol. Optionally, the female was kept alive for
409 12-24 h in a vial containing 5-10 mL grape juice agar (24 gr/L agar, 26 gr/L sucrose, 120 mg/L
410 Tegosept, 20% v/v grape juice, and 1.5% v/v ethanol). The presence of eggs on the plate was

411 systematically checked but never observed. Females were finally sacrificed to prepare
412 spermathecae.

413 **Left lobe surgery**

414 Epandrial lobe surgery was done on 5-6 day-old *D. patchea* adult males of the selection stock
415 following Rhebergen et al. (2016). Males were anaesthetised on a CO₂ pad (see above) and then
416 further immobilized with a small copper wire, which was slightly pressed onto the male abdomen.
417 The left epandrial lobe was shortened to various lengths with micro dissection scissors (SuperFine
418 Vannas Scissors, World Precision Instruments). Flies were let to recover for at least 7 days on
419 standard *D. patchea* food at 25° C in groups. No mortality was observed in males that underwent
420 lobe surgery, similar to what was observed by Rhebergen et al. (2016). Partially amputated lobes
421 ranged in length from lacking at least their most distal tip to more reduced stumps whose length
422 approximated the corresponding right lobe.

423 **Adult dissection and imaging**

424 Adults were dissected in water with forceps (Forceps Dumont #5, Fine Science Tool) inside
425 a transparent 25 mm round dissection dish under a stereo-microscope (Zeiss Stemi 2000). Male
426 genitalia were isolated by piercing the abdomen with the forceps between the genital arch and the
427 A6 abdominal segment and thereby separating the genitalia from the abdomen. We also dissected
428 the left anterior leg of each dissected male. Female spermathecae were recovered after opening the
429 ventral abdomen with forceps and removal of the gut and ovaries. The spermathecae were isolated
430 and separated according to left and right sides and immediately examined using a microscope (see
431 below). All dissected tissues were stored in 200 µL storage solution (glycerol:acetate:ethanol, 1:1:3)
432 at 4° C.

433 For imaging, male genitalia were transferred into a dissection dish filled with pure glycerol
434 and examined with a VHX2000 (Keyence) microscope equipped with a 100-1000x VH-Z100W

435 (Keyence) zoom objective at 300-400 fold magnification. Genitalia were oriented to be visible in
436 posterior view. The left and right lateral spines, as well as the dorsal edge of the genital arch were
437 aligned to be visible in the same focal plane. In some preparations, the genital arch broke and lobes
438 were aligned without adjusting the position of the dorsal genital arch. The experiment was
439 discarded from analysis in cases where lobes could not be aligned. Male lobe lengths were
440 measured on acquired images as the distance between the base of each lateral spine and the tip of
441 each lobe (Figure 1c,d) using ImageJ version 1.50d (<https://imagej.nih.gov/ij>). Male legs were put
442 on a flat glycerol surface with the inner side of the tibia facing to the camera. Legs were imaged at
443 200 fold magnification with the VHX 2000 microscope (Keyence) as described above.

444 We prepared the female paired sperm storage organs (spermathecae) at 12 h - 24 h after
445 copulation (54 females from stock 15090-1698.01 and 24 females from stock 15090-1698.02,
446 additional file 2) and examined the presence of ejaculate. *D. pachea* has giant sperm [47], which
447 makes direct sperm counts difficult. Therefore, we determined an apparent average spermathecae
448 filling level per female based on visual inspection, similar to Jefferson (1977) [57]. Female
449 spermathecae were arranged on the bottom of the transparent plastic dissection dish, filled with
450 water. Images were acquired using transmission light in lateral view with the Keyence VHX2000
451 microscope at 400-500 fold magnification. Ejaculate was directly visible inside the transparent
452 spermathecae. Ejaculate filling levels were annotated for each spermatheca separately to match
453 three different categories: 0, 1/6 or 1/3 of its total volume. Then, the average filling level for both
454 spermathecae was calculated for each female.

455 **Annotation of courtship and copulation behaviour**

456 Videos were analysed with the OpenShot Video Editor software, version 1.4.3
457 (<https://www.openshot.org>) to annotate the relative timing of courtship and copulation in our
458 experiments (Additional datafile 1: Figure S2, Additional datafile 3). Data was manually entered
459 into spreadsheets. We annotated the beginning of male courtship as the start of at least three

consecutive male courtship behaviours according to Spieth (1952) [61], such as male touching the female abdomen with the forelegs, wing vibration, male following the female, and male licking behavior (see above). We estimated licking behavior throughout courtship of each male in trials where both males courted the female simultaneously. Licking occurs abundantly throughout courtship of *D. pachea* (Additional datafile 1, Figure S3) and was therefore chosen to assess the relative courtship contribution of the two males. We calculated the sum duration of all licking events per male and trial and the number of licking sequences. However, this behavior could not be quantified in 7 trials because the males changed positions very fast so that they could not be unambiguously distinguished (Additional datafiles 3,4). In all analysed trials, licking behaviour was observed throughout *D. pachea* courtship from shortly after courtship start to the start of copulation (Additional datafile 1: Figure S2). Both males reached at least once the licking position in all trials (Additional datafile 1: Table S3). This indicates that the female had physical contact with the proboscis of both males before copulation started (Additional datafile 1: Figure S2).

The beginning of copulation was defined as the moment when the male mounted the female abdomen. However, we only scored a copulation start if the male also settled into an invariant copulation posture. Upon mounting, the male moves its abdomen tip forth and back along the female oviscape. This behavior lasts for up to about 3 minutes and during this time the male grasps the female wings and abdomen with its legs. This behavior was previously described as settling period and ends with a characteristic right-sided, invariant copulation posture [41, 42, 44]. Any copulation that ended within the first minutes after mounting without settling was considered to be a “failed mounting attempt” (Additional datafile 4). The end of copulation was considered as the moment when male and female genitalia were separated and the male had completely descended with the forelegs from the female abdomen.

483 Copulation duration (Additional datafile 1: Table S3) was comparable to previous analyses
484 of *D. pachea* copulation durations [41, 44, 47, 57]. We observed that copulation ended upon
485 isolation of the non-copulating male in 3 trials with females of stock 15090-1698.01 and in 4 trials
486 with females of stock 15090-1698.02, probably indicating premature copulation end due to
487 specimen handling. However, the estimates were included into the dataset and had little impact on
488 average copulation duration when comparing mean and median values.

489 **Mate-competition analysis**

490 Our aim was to compare at least 50 mate-competition trials, where both males courted the
491 female simultaneously and where copulation was observed. In total, we carried out 98 and 89 trials
492 with females of stocks 15090-1698.01 and 15090-1698.02, respectively (Additional datafile 1:
493 Table S2). We performed another 62 trials with males that underwent surgical manipulation of the
494 left lobe and females of stock 15090-1698.01 (Additional datafile 1: Table S4). In total, we removed
495 36 trials from the analysis because either copulation was not observed until 1 h after recording
496 started (21 trials), flies escaped, got injured or died inside the mating cell (4 trials) or the dissections
497 of male genitalia failed (11 trials, Additional datafile 1: Table S4). We thus examined 152 trials with
498 unmodified males and 61 trials with males that had undergone genital surgery, (see below,
499 Additional file 1: Table S4). Among those, we observed that both males courted the female
500 simultaneously in 111 and 54 trials, respectively. Statistical assessment with the Bradley Terry
501 Model (see below) further required a complete dataset so that we excluded 20 additional trials with
502 missing values, 19 trials with unmodified males (left lobe length: 2 trials, right lobe length: 2 trials,
503 licking behavior and/or failed mounting attempts: 7 trials, tibia length: 8 trials) and one trial with
504 lobe-modified males (no information on tibia length). In total, we considered 92 trials with
505 unmodified males and 53 trials with lobe-modified males.

506 Data analysis was performed in R version 3.6 [62]. Male copulation success (see above) was
507 evaluated with a Bradley Terry Model [48] using the R package BradleyTerry2 [63]. This model is

508 suitable for logit fits to pairwise comparison data since it takes into account the special non-random
509 (pairwise) structure of the dataset. We incorporated data (Additional datafile 3) into a list with three
510 objects (Pacdata, available on DRYAD): 1) “contest” with the pairings of males, a trial ID and a
511 factor indicating trials with males that had modified left genital lobes, 2) trial-specific variables
512 “conditions” containing female age, female stock, courtship duration and a factor indicating which
513 male initiated courtship, 3) male-specific predictor variables “male_predictors” with left and right
514 genital lobe lengths, male tibia length, sum licking duration, number of licking sequences and failed
515 mounting attempts. We developed the statistical model (Additional datafile 1: Tables S5-11) by
516 evaluating the effect of different variables on copulation success and by comparing pairs of nested
517 models, using Chi-square tests with the generic anova() function [63]. Outlier observations were
518 considered when the standardized model residuals were greater than 2. The effect of corresponding
519 trials on model fits was evaluated by comparison to additional model estimates without these outlier
520 trials. We estimated correlations of male specific predictor variables and among numeric trial
521 specific variables with the corrplot R package (Additional datafile 1: Figure S6). We found a
522 significant correlation of the male predictor variables ‘sum licking duration’ and ‘number of licking
523 sequences’. In addition, the trial specific variables ‘female age’ and ‘courtship duration’ were
524 significantly correlated (Additional datafile 1: Figure S4). Correlated parameters were not
525 incorporated into the same model for assessing the best fitting model (Additional datafile 1: Tables
526 S5-S12). We tested trial specific variables in interaction with all male-specific variables, but only
527 found an interaction of female age with failed mounting attempts in the dataset with unmodified
528 males (Additional datafile 1: Figure S4, Table S8, Table 1). We also tested for interactions between
529 male specific predictor variables but did not find any. The best fitting model for the datasets was:
530 $\text{copulation success} \sim \text{sum licking duration} + \text{left_lobe length}$.

531 Spermathecae filling levels were evaluated with a linear model using the lm() function:
532 $\text{spermatheca filling level} \sim \text{left lobe length} + \text{right lobe length} + \text{sum licking duration} + \text{Number of}$

licking sequences + copulation duration + female age + male age, and significance of each variable
was evaluated with an F-test using anova().

List of abbreviations

Ma million years ago
DF degree of freedom
N number of observations
P probability

Declarations

Ethics approval and consent to participate

“Not applicable” --- This research focuses on invertebrate insects. There are no ethical
considerations mentioned for these species according to EU Directive 86/609-STE123.

Consent for publication

“Not applicable”

Availability of data and material

The data sets supporting the results of this article are available in the DRYAD repository,
https://datadryad.org/stash/share/jzbHRT070QV55ejT_BxIcopEoG-wVDjRI_HpQK0ergY

Competing interests

The authors declare no competing financial interests

Funding

This study was supported by the CNRS and by a grant of the European Research Council
under the European Community’s Seventh Framework Program (FP7/2007-2013 Grant Agreement

no. 337579) given to VCO. BL was supported by a pre-doctoral fellowship Sorbonne Paris Cité of the Université Paris 7 Denis Diderot and by a fellowship "Initiatives d'excellence" (Idex ANR-18-IDEX-0001) of the Labex *Who Am I?* project (ANR-11-LABX-0071). The funding body had no influence on the design of the study, on collection, analysis, and interpretation of data, and in writing the manuscript.

Authors' contributions

ML and BL designed the experiments. BL and DC recorded fly copulation and performed light microscopy analysis of *Drosophila* male genitalia. BL and ML analysed the data. ML, VCO and BL wrote the manuscript. All authors have read and approved the manuscript.

Acknowledgements

We thank Therese A. Markow for discussions. We are very grateful to Alexandre Peluffo for help with the statistical analysis, Claus Lang for technical support and Juliette Royer for dissections of *D. pachea* male genitalia.

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700 **Supporting Information**

701 **Additional datafile 1:** Supplementary Figures and Tables, **Figure S1:** Camera device for movie
702 recording, **Figure S2:** Courtship and copulation duration in competition mating experiments,
703 **Figure S3:** Failed copulation attempts compared to female age, **Figure S4:** Courtship duration
704 increases with female age, **Figure S5:** Pairwise lobe length differences of males used in the
705 competition mating experiment, **Figure S6:** Pearson correlations of male specific predictor
706 variables, **Table S1:** *Drosophila pachea* resources, **Table S2:** Genital lobe lengths in *D. pachea*
707 stocks, **Table S3:** Courtship duration, male licking behavior and copulation duration in trials where
708 both males courted the female, **Table S4:** Competition mating trials used for courtship analysis,
709 **Tables S5-S12:** Bradley Terry Model Fit.

710 **Additional datafile 2:** Raw measurements of lobe lengths in different *D. pachea* stocks

711 **Additional datafile 3:** Measurements of mating behaviour, genital lobe lengths and spermathecae

712 filling levels of individuals used in the competition mating experiments.

713 **Additional datafile 4:** Dataset for male licking behavior and male mounting.

714 **Tables**

715 **Table 1: Bradley–Terry (BT) model examining the effects of left lobe length, sum licking**

716 **duration and failed mounting attempts on copulation success**, in 92 trials with unmodified males.

717 Model: copulation success ~ sum licking duration + left lobe length, null deviance: 127.539 on 92 degrees

718 of freedom, residual deviance: 98.416 on 90 degrees of freedom. Influential trials 87 and 98 were detected to

719 have only marginal effects on model estimates.

variable	estimate	std. error	z value	P (> z)
sum licking duration	0.714251	0.206293	3.462	0.000536
left lobe	0.007511	0.003254	2.308	0.020981

720 **Table 2: Bradley–Terry (BT) model examining the effects of left lobe length, sum licking**

721 **duration, and number of licking sequences on copulation success**, in 53 trials with males that had

722 surgically modified left genital lobes. Model: copulation success ~ sum licking duration + left lobe length,

723 null deviance: 73.474 on 53 degrees of freedom, residual deviance: 68.163 on 51 degrees of freedom

variable	estimate	std. error	z value	P (> z)
sum licking duration	0.241981	0.166135	1.457	0.145
left lobe	0.007070	0.004615	1.532	0.126

724 **Table 3: Male age and copulation duration affect spermathecae filling levels.** Linear model

725 (spermathecae filling level ~ sum licking duration + left lobe length + right lobe length + copulation

726 duration + female age + male age), 32 observations were deleted due to missingness: sum licking

727 duration 30 values, left lobe length 1 value), right lobe length 1 value, multiple R²: 0.4975, adjusted

728 R²: 0.4201, F-statistic: 6.434 on 6 and 39 DF, P-value: 8.956e-05

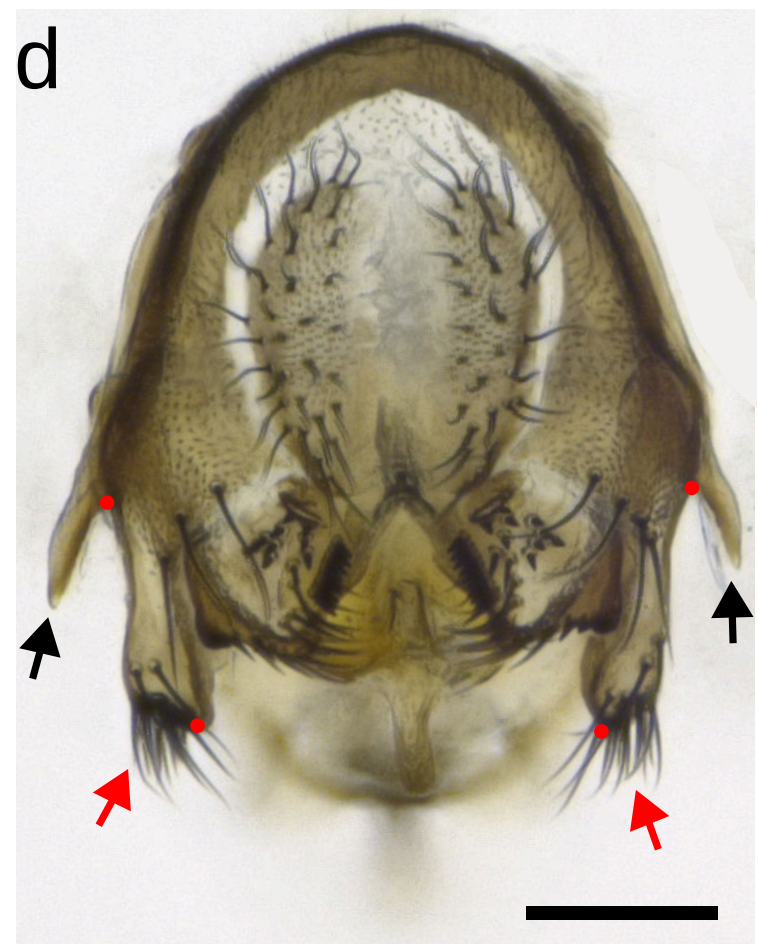
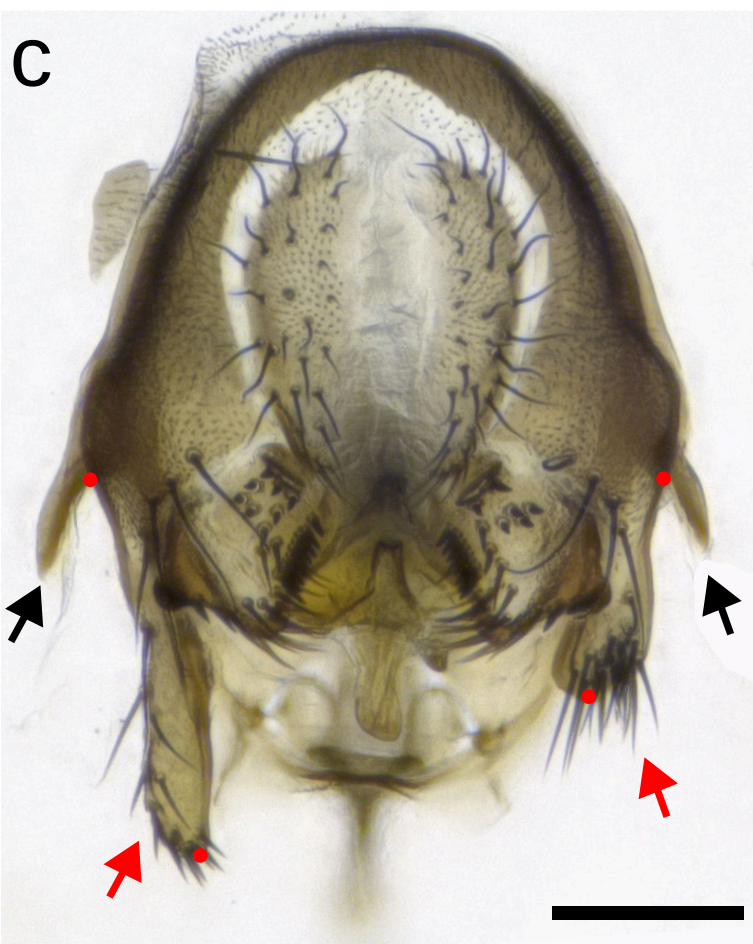
variable	F value	P
sum licking duration	0.8658	0.357838
left lobe length	0.0447	0.833711
right lobe length	2.4460	0.125901
copulation duration	11.3116	0.001737
female age	0.3300	0.568960
male age	23.6079	1.954e-05

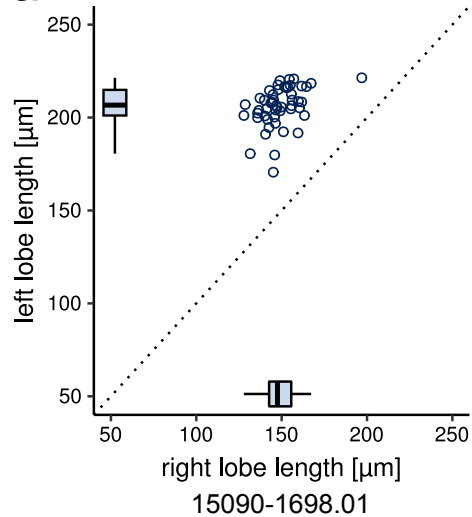
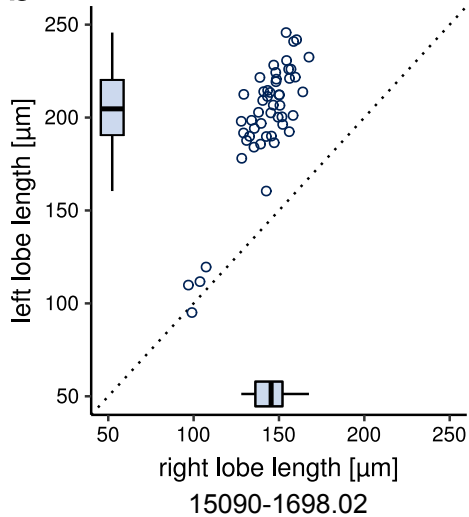
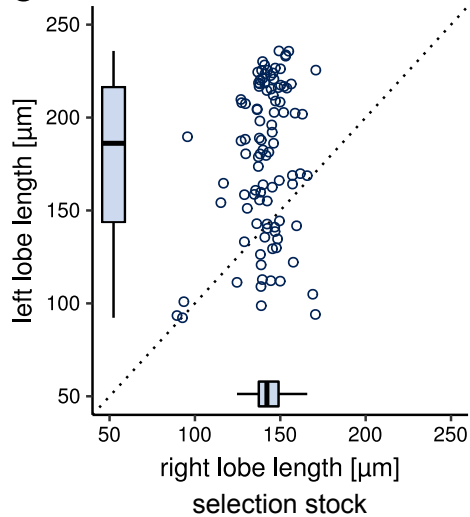
730 **Figure Legends**

731 **Figure 1: *Drosophila patchea* male genital lobes.** (a) Male of stock 15090-1698.02 in ventral view
732 and with asymmetric lobes. (b) Male of the selection stock with apparently symmetric lobes. The
733 scale bars correspond to 200 μ m. (c,d) Posterior view of a dissected male terminalia, (c) stock
734 15090-1698.02 with asymmetric lobes (d) selection stock. The scale bars correspond to 100 μ m,
735 dots indicate lobe length measurement points, red arrows point to the lobe tips and black arrows
736 indicate the lateral spines.

737 **Figure 2: Genital lobe lengths differ between *D. patchea* stocks.** Lengths of the left and right
738 epandrial lobe lobes are presented for (a) stock 15090-1698.01, (b) stock 15090-1698.02, and (c)
739 the selection stock. Sibling males of those used in the mating experiment are shown in panel (c).
740 Each point represents one male. The variance of left lobe length is increased in the selection stock
741 compared to the source stock 15090-1698.02 (Levene's test: selection stock / 15090-1698.02: DF =
742 1/147, F = 11.506, P = 0.0009), while the variance of the right lobe length were not significantly
743 different (Levene's test, DF = 1/147, F = 0.5164, P = 0.4735).

744 **Figure 3: Courtship durations and relative courtship activity.** (a) The ratio of sum licking
745 duration and total courtship duration in each trial shows that the copulating male (cop, grey box-
746 plots) had a higher average sum licking duration compared to the non-copulating male (non-cop,
747 white box-plots). Each point represents one male. (b) The total courtship duration was shorter in
748 trials with only one male displaying courtship signs (one, white box-plots) compared to trials with
749 both males courting simultaneously (both, grey box-plots) (Mann-Whitney-Wilcoxon test, female
750 stock 15090-1698.01, non modified males, W = 385, N = 25/51, P = 0.0053 ; females of stock
751 15090-1698.02, W = 219, N = 16/60, P = 0.0009 ; females stock 15090-1698.01, modified males, W
752 = 62.5, N = 7/54, P = 0.0044). Horizontal bars indicate pairwise comparisons with P-values ** <
753 0.01 or *** < 0.001. Each point represents one mating experiment.



a**b****c**

sum licking duration / total courtship duration

