

1 **Repeated evolution of asymmetric genitalia and right-**
2 **sided mating behavior in the *Drosophila nanoptera***
3 **species group**

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15

Research article

16 **Abstract**

17 **Background:** Male genitals have repeatedly evolved left-right asymmetries, and the
18 causes of such evolution remain unclear. The *Drosophila nannopectera* group contains four
19 species, among which three exhibit left-right asymmetries of distinct genital organs. In the
20 most studied species, *Drosophila pachea*, males display asymmetric genital lobes and
21 they mate right-sided on top of the female. Copulation position of the other species is
22 unknown.

23 **Results:** To assess whether the evolution of genital asymmetry could be linked to the
24 evolution of one-sided mating, we examined phallus morphology and copulation position in
25 *D. pachea* and closely related species. The phallus was found to be symmetric in all
26 investigated species except *D. pachea*, which display an asymmetric phallus with a right-
27 sided gonopore, and *D. acanthoptera*, which harbor an asymmetrically bent phallus. In all
28 examined species, males were found to position themselves symmetrically on top of the
29 female, except in *D. pachea* and *D. nannopectera*, where males mated right-sided, in
30 distinctive, species-specific positions. In addition, the copulation duration was found to be
31 increased in *nannopectera* group species compared to closely related outgroup species.

32 **Conclusion:** Our study shows that gains, and possibly losses, of asymmetry in genital
33 morphology and mating position have evolved repeatedly in the *nannopectera* group. Current
34 data does not allow us to conclude whether genital asymmetry has evolved in response to
35 changes in mating position, or vice versa.

36

37 **Keywords:** phallus asymmetry, *Drosophila nannopectera* species group, one-sided mating
38 position, copulation behavior

39 **Background**

40 Changes in behavior are thought to play important roles in animal evolution [1–3]□.
41 How new behaviors evolve and how they are encoded in the genome is little understood.
42 New behaviors can initiate secondary evolutionary shifts in morphology, physiology or
43 ecology (“behavioral drive”) [1–9]□, for example when they bring an organism into contact
44 with new environmental factors. Behavior can also prevent evolutionary changes because
45 plasticity in behavior might enable individuals to adjust for changed environmental
46 conditions [10–12]□. Other investigations suggest that behavior and morphology are both
47 subject to natural selection and that their responses to changes in the environment are
48 perhaps independent [13, 14]□, or that behavior could simultaneously impede and drive
49 evolutionary diversification of different characters [12, 15, 16]□. So far, it appears that the
50 effects of behavioral changes on the evolution of morphological traits cannot be
51 generalized and that they require case-specific assessments.

52 The evolution of left-right asymmetric genitalia in insects is a case where
53 morphology was proposed to have evolved in response to changes in mating behavior
54 [17]□. Asymmetric genitalia are observed in many species and phylogenetic studies
55 indicate that they have evolved multiple times independently from symmetric ancestors
56 [18, 19]□. While most extant insect species copulate with the male being on top of the
57 female abdomen, the ancestral mating position in insects is inferred to be a configuration
58 with the female on top of the male [18, 20, 21]□. The extant male-on-top configuration has
59 likely evolved multiple times in insects [20]□. Such changes in mating position probably
60 altered the efficiency of male and female genital coupling, and may have led to the
61 evolution of genital asymmetries to optimize the coupling of genitalia [17]□.

62 The *nannoptera* species group belongs to the genus *Drosophila* and consists of four
63 described species that feed and breed on rotten pouches of columnar cacti of the genus

64 *Stenocereus* and *Pachycereus* in Northern and Central America [22–24]□. These species
65 are particularly interesting to study the evolution of genital asymmetry because distinct
66 genital structures were identified to be asymmetric in three out of the four described
67 species of this group. *D. acanthoptera* males have asymmetric phallus, *D. pachea* males
68 have a pair of asymmetric external lobes with the left lobe being approximately 1.5 times
69 longer than the right lobe [25, 26]□, and in the sister species *D. wassermani* males have a
70 pair of asymmetric anal plates (cerci) [25]□. In contrast, no asymmetries are known in the
71 fourth described species *D. nannopectera* [27]□. The four species separated about 3-6 Ma
72 and lineage-specific changes likely led to the distinct and elaborated asymmetries in each
73 species [28]□. Interestingly, *D. pachea* mates in a right-sided copulation position where
74 the male rests on top of the female abdomen with its antero-posterior midline shifted about
75 6-8 degrees to the right side of the female midline [26, 29]□. This one-sided mating
76 posture is associated with asymmetric coupling of female and male genitalia during
77 copulation, with the male genital arch being rotated about 6 degrees towards the female's
78 right side. Apart from our previous investigations of the *D. pachea* copulation position [26,
79 29]□, little is known about mating positions in other *Drosophila* species. In Diptera, several
80 mating positions are known and all involve a symmetric alignment of male and female
81 genitalia. Male and female genitalia are usually inversely positioned relative to each other
82 with the dorsal surface of the aedeagus (phallus) contacting the ventral side of the female
83 reproductive tract [30]□. *D. melanogaster*, *D. simulans* and *D. sechellia* were reported to
84 adopt such a symmetric copulation posture, with the male aligned along the female midline
85 [31–33]□. A one-sided mating position was generated artificially in *D. melanogaster* by
86 unilateral ablation of a long bristle located on the genital claspers [31]□. In any case, no
87 data is currently available regarding mating positions of the closely related species of *D.*
88 *pachea*.

89 The observation of a right-sided mating posture and asymmetric male genitalia in *D.*
90 *pachea* led us to wonder whether morphological asymmetry in the *nannoptera* group
91 species might have evolved in response to the evolution of one-sided mating [17]. We
92 therefore decided to investigate copulation position and aedeagus asymmetry in species
93 closely-related to *D. pachea*, and to reconstruct their most likely evolutionary history.

94

95 **Results**

96 **The phallus of *D. pachea* is asymmetric**

97 The shape of the aedagus/phallus of *D. pachea* has not been described previously.
98 We examined the aedeagus of two dissected *D. pachea* males using scanning electron
99 microscopy (SEM) and found that both were strikingly asymmetric (Fig. 1). Aedeagi were
100 strongly bent, dorsally flattened and pointed at the dorsal tip. Their ventral region bore two
101 ventrally pointing asymmetric spurs, one positioned apically, the other sub-apically. The
102 gonopore was positioned dorso-apically on the right side of the aedeagus. The aedeagal
103 parameres broke off during dissection and were not visualized. In order to corroborate the
104 SEM observations, we dissected and examined 10 aedeagi of *D. pachea* males using light
105 microscopy. Apical and subapical spurs, as well as a right-sided gonopore, were
106 consistently observed in all preparations (n=10, Supplementary Fig.1). Our results indicate
107 that the *D. pachea* phallus is directionally asymmetric (Fig. 2b).

108

109 **Aedagus asymmetry is observed in *D. acanthoptera* but not in *D. nannoptera*, *D.* 110 *machalilla* and *D. bromeliae***

111 We compared aedeagus shapes in several species that are closely related to *D.*
112 *pachea* (Fig. 2). As previously described [27], the aedeagus of *D. acanthoptera* was

113 found to be asymmetrically bent (n=10). Two asymmetric spurs were found at the ventral
114 apical tip of the aedeagus, with the right spur being consistently longer than the left spur
115 (Fig. 2e, Supplementary Fig.2). However, in contrast to *D. pachea*, no dorso-apical
116 gonopore was observed on the right side of the apex. Aedeagi of *D. nannoptera* males
117 (Fig. 2k, Supplementary Fig. 3) were found to be symmetric (n=15). The ventral side of the
118 apex revealed two apical elongations with slightly variable lengths at the left and right side
119 (n=15, Supplementary Fig.3). The variation in length was not directional and thus
120 considered to reflect random fluctuating asymmetry. The ventral tip of the aedeagus of *D.*
121 *machalilla* (*atalaia* species group) (n=10) displayed two lateral hooks (Fig. 2n,
122 Supplementary Fig.4), of the same length on both sides. The aedeagus of *D. bromeliae*
123 showed two lateral symmetric ridges (n=10) (Fig. 2q, Supplementary Fig. 5). In summary,
124 aedeagus asymmetry was only observed in *D. pachea* and *D. acanthoptera*, and distinct
125 phallus structures were found to be asymmetric in these species.

126 ***D. pachea* and *D. nannoptera* males mate right-sided**

127 The position of the male during copulation has not been described for any of the
128 closely related species of *D. pachea*. In this study, we assessed copulation postures in *D.*
129 *pachea* and nine related species: *D. acanthoptera* and *D. nannoptera* (sister species of *D.*
130 *pachea*), *D. machalilla* and *D. bromeliae* (representatives of close outgroup lineages), *D.*
131 *buzzatii* and *D. mojavensis* (members of the *repleta* species group), as well as
132 representatives of other *Drosophila* species groups (*D. tripunctata*, *D. willistoni* and *D.*
133 *melanogaster*). Phylogenetic relationships between the ten studied species were
134 estimated with a Bayesian phylogeny (Supplementary Fig. 6) based on a previously
135 published sequence dataset [28], supplemented with publicly available sequence data
136 (this study) for *D. tripunctata* and *D. willistoni*. The obtained phylogeny is congruent with

137 previous findings [28] that *D. nannoptera*, *D. acanthoptera* and *D. pachea* form a
138 monophyletic group with a short internode branch length between the split of the *D.*
139 *nannoptera* lineage and the separation of *D. acanthoptera* and *D. pachea*. Also, *D.*
140 *machalilla* and *D. bromeliae* form two close outgroup lineages of the *nannoptera* clade [28,
141 34, 35], followed by the *repleta* group species *D. buzzatii* and *D. mojavenis* [28].

142 For each species, we introduced a single virgin female and a single virgin male into
143 a circular mating chamber and recorded the couple until copulation ended or for 45 min
144 when no copulation was detectable. We obtained 315 movies, of which 111 were used for
145 assessing courtship duration, 146 for copulation duration and 124 for copulation posture
146 analysis (supplementary dataset 3). Most movies were discarded because no copulation
147 occurred or individuals had damaged wings or legs (all reasons listed in supplementary
148 dataset 3). As previously described [36–38], copulation duration varied significantly
149 among species (ANOVA, $df_1 = 9$, $df_2 = 136$, $F = 73.38$, $p < 2e-16$) (Table 1). We could
150 reproduce a previously reported trend that copulation duration in *nannoptera* group
151 species was remarkably long compared to *D. mojavenis* and *D. buzzatii* of the *repleta*
152 group, with copulation duration of $88.49 \text{ min} \pm 35.18 \text{ min}$ for *D. acanthoptera*, 29.58 ± 7.86
153 min for *D. pachea* and $11.9 \pm 4.2 \text{ min}$ for *D. nannoptera* (mean \pm SD). In comparison,
154 copulation duration of *D. buzzatii* $1.79 \pm 0.65 \text{ min}$ and *D. mojavenis* $2.3 \pm 0.35 \text{ min}$ (mean
155 \pm SD) of the *repleta* species group was shorter and similar to *D. machalilla* $2.28 \pm 0.53 \text{ min}$
156 and *D. bromeliae* $0.92 \pm 0.28 \text{ min}$ (mean \pm SD) (Table 1).

157 To assess mating posture, we calculated the angle between a line drawn through
158 the male head midline and the female scutellum tip and a second line drawn through the
159 female head midline and the female scutellum tip (Supplementary Fig. 7A). The angle was
160 set positive when male head lies on the right side of the female and negative when on the
161 left. The camera view relative to the fly couple position within the mating cell may affect the

162 measured angle in each experiment but the sign of the average mating angle taken from
163 different recordings for each species should accurately reflect the one-sidedness of the
164 male mating position. As a consequence, we expected a one-sided copulation position to
165 produce a consistent positive or negative distribution of angle values, while symmetric
166 mating positions should result in an angle distribution around zero.

167 To compare mating angles between species, it is necessary to examine copulation
168 postures at the same corresponding time point during copulation. At copulation start, the
169 male position on top of the female was found to be greatly variable between couples, even
170 within a single species, so this time point was not considered appropriate for our
171 comparative analysis. Since copulation duration varies greatly between species, finding
172 another comparable time point across species was not trivial. We subdivided copulation
173 into two phases, an initial phase where the male is on top of the female abdomen but
174 consistently moving legs and abdomen, and a second phase when the male maintains an
175 invariant position relative to the female, which can sometimes walk or move its legs
176 (Supplementary Fig. 6). The “settling time point” is defined as the time point between the
177 first and second phase, when the male adopts an invariant position relative to the female.
178 For our cross-species analysis we chose to assess copulation angle at two time points: (1)
179 right after the male had settled into an initial invariant copulation position (the settling time
180 point) and (2) at 10% of elapsed time between the settling time point and the end of
181 copulation (10% stable copulation time point). For species with a mean copulation duration
182 > 2.5 min, > 15 min or > 60 min, we also measured the angles every 2.5 min, 5 min or 10
183 min, respectively. This allowed us to follow mating postures of each species over the
184 course of copulation.

185 Significant one-sided mating positions were observed in *D. pachea* and *D.*
186 *nannopectera*, both at the settling time point and at the 10% stable copulation time point

187 (Fig.3a,b, Table 2). No significant one-sided copulation postures were detected in *D.*
188 *acanthoptera* and the other seven tested species including *D. melanogaster* (Fig. 3a,b).

189 Over the course of copulation, mating angles continued to range over zero for *D.*
190 *melanogaster* and *D. acanthoptera* (Fig. 3c,d), indicating a relatively steady symmetric
191 copulation position without any left- or right-sidedness. Similar to previous investigations
192 [26, 29], *D. pachea* revealed right-sided angles that were highest at the beginning of
193 copulation at 0-10 min after settling (Fig. 3e). At later time points, the angles tended to
194 range over zero. In *D. nannoptera*, mating angles tended to be right-sided throughout
195 copulation (Fig. 3f). In summary, *D. pachea* and *D. nannoptera* revealed a right-sided
196 copulation posture whereas all the other tested species displayed a symmetric mating
197 posture.

198 **Male *D. nannoptera* tilt to the right side of the female abdomen during copulation**

199 To further investigate the right-sided copulation posture in *D. nannoptera* and better
200 observe the male position relative to the female dorso-ventral midline, we filmed the
201 couples from a frontal perspective (Supplementary Fig. 8). In particular, we assessed the
202 inclination of the male body relative to the female dorso-ventral axis by measuring the
203 angle P4-P5-P6, with P4 as the medial most dorsal edge of the female head (often visible
204 by the ocelli), P5 being the most ventral medial position of the female head (the female
205 proboscis) and P6 as the medial most dorsal edge of the male head (often visible by the
206 ocelli) (Supplementary Fig. 8).

207 *D. nannoptera* mating positions were on average strikingly right-sided
208 (Supplementary Fig. 8), with a considerable variation of observed angles, ranging from
209 slightly left- to strongly inclined right-sided ($-8.42^\circ - 57.7^\circ$) over the course of copulation.
210 Left-sided angles were only observed during the first two minutes of copulation. On

211 average, the male tended to initially adopt a right-sided copulation posture with an angle of
212 $10.36^\circ \pm 6.88^\circ$ (mean \pm SD) (n=25) between 0-1 min after copulation start (Table 3). Over
213 the course of copulation, the angle then increased to $27.16^\circ \pm 10.81^\circ$ (n=29) between 3-4
214 min after copulation start (Table 3), which was visible by an inclination of the male head
215 towards the female's right side. This tilt-movement was not observed in *D. pachea*, where
216 all males remained on top of the female abdomen [29]. We therefore conclude that *D.*
217 *pachea* and *D. nannoptera* adopt distinct copulation postures, even though both of them
218 are right-sided.

219

220 Discussion

221 Phallus asymmetries differ between *D. pachea* and *D. acanthoptera*

222 The currently published data suggest that genital asymmetries are rare among
223 *Drosophila* species. The genus *Drosophila* encompasses over 1500 described species
224 [39] and only 8 species have been shown without doubt to display an asymmetric
225 phallus: *D. marieaehelenae* and *D. hollisae* of the flavopilosa group [40, 41], *D.*
226 *asymmetrica* and *D. quinarensis* of the guarani group [42, 43], *D. endobranhia* of the
227 canalinea group [44], *D. acuminanus* and *D. freilejoni* of the onychophora group [27,
228 45, 46] and the *nannoptera* group species *D. acanthoptera* [27]. Genital asymmetry
229 might be more widespread than what is reported in the literature across *Drosophila*, as
230 certain species are only described based on a few specimens, and subtle asymmetric
231 characters might have been overlooked and interpreted as fluctuating variation between
232 left and right sides. Here, we compared aedeagus morphology of at least 10 specimens of
233 five different species that belong to the *nannoptera* species group and closely related
234 species. We did not detect aedeagus asymmetry in the tested species outside of the

235 *nannopectera* species group and found that within the *nannopectera* group only *D.*
236 *acanthoptera* and *D. pachea* but not *D. nannopectera* reveal striking left-right asymmetries
237 (Fig. 4). We did not evaluate aedeagus asymmetry of *D. wassermani*, as this species is not
238 available for examination and our attempts to catch specimen in the wild were not
239 successful (see materials and methods). Asymmetries differed between *D. pachea* and *D.*
240 *acanthoptera*. Whereas ventral spurs on the *D. pachea* aedeagus were apart from each
241 other, with one being apical and the other subapical, *D. acanthoptera* aedeagus had a pair
242 of apical spurs that differed in length. In addition, the gonopore was visible and right-
243 sided in *D. pachea* while it was not visible in *D. acanthoptera*. Our results thus highlight
244 that the asymmetric phallus structures of *D. pachea* and *D. acanthoptera* are derived
245 morphologies that have little in common and diversified independently after the split of the
246 two species about 3-6 Ma ago [28]. It is impossible to infer whether the asymmetries
247 observed in both species derived from a pre-existing asymmetric phallus in their ancestor
248 or if asymmetry evolved *de novo* in both lineages.

249 The outer genitalia (epandrium) has been reported to be asymmetrical in *D. pachea*
250 (where the left lobe is longer than the right lobe [25, 26] and in *D. wassermani* (where the
251 right anal plate is larger than the left one [25]). Our inspection of a few dissected
252 epandria of *D. nannopectera*, *D. acanthoptera* and *D. machalilla* revealed no obvious
253 asymmetry (Fig. 2). However, a quantitative comparison remains to be done to confirm the
254 absence of asymmetry in the epandrium of these species.

255

256 **Long copulation duration is specific to the *nannopectera* group species**

257 We observed that *nannopectera* species copulated considerably longer than any
258 representative species of the close outgroup lineages (Fig. 4). This trend was previously
259 reported by Pitnick and Markow (1991) [36, 37], where the authors compared

260 copulation duration of *nannoptera* group species with *repleta* group and other species.
261 Here we included two additional closely related species, *D. machalilla* and *D. bromeliae*,
262 and observed that their copulation durations were relatively short. Our observations
263 therefore indicate that a long copulation duration is specific to the *nannoptera* group.

264

265 **Right-sided mating positions differ between *D. pachea* and *D. nannoptera***

266 We assessed copulation postures of *D. pachea* and a range of related species to
267 track the conservation of right-sided mating position in the *nannoptera* group. Two aspects
268 of copulation behavior made cross-species comparisons difficult. First, copulation duration
269 was extremely diverse and ranged from less than a minute in *D. bromeliae* to more than
270 two hours in *D. acanthoptera*. Second, the movements of the male and female during
271 copulation varied between species. In *D. melanogaster* and *D. willistoni*, we observed
272 vigorous movements of the male during copulation accompanied by female hindleg
273 kicking. These phases were interrupted by periods without movements. In contrast, males
274 of most other species initially moved upon mounting the female and then settled into an
275 invariant copulation posture relative to the female. We chose to compare mating postures
276 across species once the couple adopted the invariant position, at the settling time point,
277 and at 10% of elapsed time between the settling time point and the end of copulation (10%
278 stable copulation time point). These two time points were assumed to represent
279 comparable moments during copulation.

280 At the two measured time points during copulation, the angle between the male
281 midline and the female midline during copulation was distributed symmetrically around
282 zero, indicating a symmetric mating position in all tested species except *D. pachea* and *D.*
283 *nannoptera*. Our previous data from *D. pachea* [26, 29] was re-analysed in this study

284 with a different measurement approach and led to the same conclusion as our earlier
285 reports. In addition, we found that *D. nannoptera* adopts a right-sided mating position with
286 angle values that were slightly higher than in *D. pachea* (Fig. 3). Assessment of homology
287 of behaviors is difficult compared to morphological characters, because Owen's position
288 criterium for homology [47] does not exist for behavioral traits. Observation of similar
289 behaviors does not necessarily mean common descent [6]. Our precise examination of
290 the mating position of *D. nannoptera* from a frontal perspective revealed that *D.*
291 *nannoptera* males strongly tilt to the female's right side during copulation, a behavior that
292 is not observed in *D. pachea* [26, 29]. Therefore, mating postures can be regarded as
293 distinct between the two species. Interestingly, a comparable tilting behavior during
294 copulation was observed in experiments with *D. pachea* males that had surgically modified
295 external genital lobes [29]. Male lobes are considered to be important in grasping the
296 female abdomen beneath the oviscapt valves and to keep *D. pachea* upright on the female
297 abdomen. A hypothetical scenario is thus that the ancestral mating position in shared
298 ancestor of the two species may have been right-tilted but the evolution of asymmetric
299 external lobes in *D. pachea* led to a derived right-sided copulation posture, which is
300 upright. Alternatively, right-sided mating position may have evolved independently in the
301 two lineages leading to *D. pachea* and *D. nannoptera*. In all scenarios, at least two
302 evolutionary changes in mating position must be considered to account for the distinct,
303 species-specific right-sided mating positions in the *nannoptera* group.

304

305 **Asymmetry in mating position and in phallus have evolved in different branches of** 306 **the *nannoptera* group phylogeny**

307 Across the *nannoptera* group, we find no striking correspondence between right-
308 sided mating posture and asymmetric male genitalia. For example, *D. acanthoptera* has an

309 asymmetric aedeagus but mates in a symmetric overall posture. On the opposite, no
310 directional asymmetry is detected in the male (external and internal) genitalia of *D.*
311 *nannoptera*, but males adopt a right-sided copulation posture (Fig. 4). Based on our
312 phylogeny, *D. nannoptera* presents the earliest branching lineage within the *nannoptera*
313 group. In this sense, right-sided mating postures could have originated earlier during
314 evolution than asymmetric morphologies and may have been lost in *D. acanthoptera*.
315 However, the internode branch length between the split of the *D. nannoptera* lineage and
316 the separation of *D. acanthoptera* and *D. pachea* is short and statistical support is weak
317 [28]. Thus, phylogenetic relationships within the *nannoptera* group remain to be
318 resolved and it is more appropriate to regard all *nannoptera* species as sister species.

319 So far, we conclude that both right-sided copulation behavior and asymmetric male
320 genitalia evolved within the *nannoptera* species group and that diversification of both traits
321 have involved lineage-specific evolutionary changes. They may have evolved by
322 modifications of pre-existing right-sided mating behavior and/or asymmetric genital
323 morphologies already present in the ancestor. Alternatively, they can have appeared *de*
324 *novo* in each extant lineage.

325

326 **One-sided mating and asymmetric phallus are correlated with giant sperm and**
327 **female sperm storage, respectively**

328 Asymmetric genital morphology and right-sided mating behavior may also be
329 associated with other characters that are special to the *nannoptera* species group. *D.*
330 *pachea* and *D. nannoptera* are among the *Drosophila* species that produce the longest
331 (giant) sperm [48, 49] (Fig. 4). The association of right-sided mating with giant sperm
332 production actually holds better than with asymmetric male genital morphology because *D.*

333 *acanthoptera* has an asymmetric aedeagus but has relatively small sperm [48]□□ and
334 mates in a symmetric overall posture (Fig. 4). A specific one-sided mating posture might
335 be necessary for optimal transfer of giant sperm. Examining mating postures in *Drosophila*
336 species which harbor even longer sperm (*D. bifurca* 58 mm, *D. kanekoi* 24 mm, *D. hydei*
337 23 mm, *D. eohydei* 18 mm) [50, 51]□ would be interesting to test further the possible
338 association between sperm length and one-sided mating.

339 The species *D. pachea*, *D. acanthoptera* and *D. wassermani* are also special in the
340 way the female stores sperm after copulation (Fig. 4). They are the only *Drosophila*
341 species that store sperm exclusively inside the spermathecae but not in the seminal
342 receptacle as most other species [49]□. In contrast, *D. nannoptera* stores sperm
343 exclusively inside the seminal receptacle [49]□□. Morphological phallus asymmetry is thus
344 observed in those species that reveal exclusive sperm storage in the spermathecae. Male
345 specimen of *D. wassermani* are thus required to analyse their phallus shapes to confirm
346 this trend. On the other hand, it is hard to generalize from our observations as only three
347 species are concerned.

348

349 **Conclusion**

350 Phallus asymmetries were identified in *D. pachea* and *D. acanthoptera* of the *nannoptera*
351 species group and distinct structures were observed to be asymmetric in both species. An
352 increased copulation duration was found to be specific to *nannoptera* group species and
353 was not observed in the closely related outgroup species *D. machalilla* and *D. bromeliae*.
354 Right-sided mating positions were detected in *D. pachea* and *D. nannoptera* and were
355 found to be distinct between them. Our data does not allow us to conclude whether the
356 evolution of the right-sided copulation position may have promoted the evolution of genital

357 asymmetry, or vice versa. Our results nevertheless indicate that asymmetry in genital
358 morphology and in copulation behavior have evolved through multiple evolutionary steps in
359 the *nannoptera* group, revealing a complex history of sexual trait changes, maybe in
360 relationship with the evolution of giant sperm and unique sperm storage in the *nannoptera*
361 group.

362

363 **Methods**

364 **Fly sampling and maintenance**

365 An isofemale stock of *Drosophila machalilla* was established from a collection of A.
366 A. in December 2015 at San Jose Beach (01°13'46.4"S, 80°49'14.6"W) Ecuador, using a
367 modified version of the fly traps described in [52]. Our baits contained rotten pieces of
368 the columnar cactus *Armatocereus carwrightianus* and yeast solution. The *D. machalilla*
369 stock was raised on standard *Drosophila* medium (60 g/L brewer's yeast, 66.6 g/L
370 cornmeal, 8.6 g/L agar, 5 g/L methyl-4-hydroxybenzoate and 2.5% v/v ethanol) and a piece
371 of fresh *Opuntia ficus-indica* (prickly pear opuntia) or *Hylocereus undatus* (dragon fruit) in
372 the medium. The isofemale stock was raised for two generations before experiments
373 started and it was maintained for a total of 36 generations.

374 We also intended to collect *D. wassermani* in August 2016 in Oaxaca, Mexico. Six
375 localities were sampled based on previous records: Reserva de la Biosfera Tehuacan-
376 Cuicatlan (18°11'21.30" N, 97°14' 51.7" W), Huajuapán de León (17°48'25.6" N, 97°14'
377 56.7" W), San Luis del Río (16°46'30" N, 96°10' 49.9" W), and four sites along the
378 Carretera Internacional 190: Kms 73 (16°42'57.2" N, 96°19'41.9" W), 89 (16°40'41.3" N,
379 96°14'41.7" W), 102 (16°42'11.3" N, 96°11'32.4" W) and 111 (16°39'48.4" N, 96°07'31.8"
380 W). We used banana traps, cactus baits that contained rotten organ pipe cactus

381 *Stenocereus prionosus* and mixed food traps that additionally contained banana and
382 yeast. Besides the invasive species *Zaprionus indianus* and cosmopolitan species *D.*
383 *melanogaster* and *D. simulans*, we identified several species of the *repleta* group, about
384 100 individuals of *D. nanoptera*, three males of *D. wassermani* and one female of *D.*
385 *acanthoptera*. Unfortunately, we were not successful in establishing iso-female strains
386 from *D. nanoptera* and *D. acanthoptera*.

387 All other stocks were retrieved from the San Diego Drosophila Species Stock
388 Center or were provided by Jean David (Supplementary Table 1). Flies were maintained at
389 25 °C, except for *D. melanogaster*, *D. tripunctata* and *D. willistoni*, which were either
390 maintained at 22 °C or 25 °C (see supplementary dataset 3 for details). Flies were kept in
391 vials with 10 mL of standard *Drosophila* food medium (see above) inside incubators with a
392 12 h light: 12h dark photo-periodic cycle combined with a 30-min linear illumination change
393 between light (1080 lumen) and dark (0 lumen). For maintenance of *D. pachea*, we mixed
394 standard *Drosophila* food medium in the food vial with 40 µL of 5mg/mL 7-
395 dehydrocholesterol (dissolved in ethanol) [53].

396

397 **SEM analysis of the *D. pachea* aedeagus**

398 Virgin males of at least 14 days after hatching from the pupa were transferred into a
399 2 mL reaction tube, snap frozen in liquid nitrogen and stored in ethanol at -20°C. For
400 dissection, frozen and fixed males were placed in 80% ethanol at room temperature and
401 the aedeagus was dissected out with fine needles. Tissues were dried using an EM
402 CPD300 automated critical point dryer (Leica) and mounted on aluminium stubs with the
403 distal end facing upwards and coated with platinum/palladium (20 nm). Each aedeagus
404 was SEM-imaged with a JSM-7500F field emission scanning electron microscope (Jeol) at
405 270x magnification.

406

407 **Analysis of aedeagus asymmetry by light microscopy**

408 The terminal segments of the male abdomen were picked out with fine forceps and
409 boiled for 10 min in two drops of 30% KOH. Genital parts were further dissected on a
410 microscope slide (Thermo Scientific Menzel) in a drop of water using 0.1 mm Minutien
411 Pins (Fine Science Tools) under the stereo-microscope K-500 (VWR). Dissected
412 structures were mounted in pure glycerol on 1.5 mm concave microscope slides
413 (Marienfeld). Images were acquired with a light microscope VHX2000 (Keyence) equipped
414 with a zoom lens VH-Z100UR/W at 350-550 fold magnification. For storage, male genitalia
415 were mounted in DMHF medium on microscope slides (Entomopraxis).

416

417 **Phylogenetic analysis**

418 We used data of eight species from a multi-locus dataset of Lang et al. (2014)
419 [28], and added corresponding sequences for *D. willistoni* and *D. tripunctata*
420 (Supplementary Table 2, Supplementary Datafile 2, BEAST input file in DRYAD). For *D.*
421 *tripunctata*, only mitochondrial data was available at GenBank
422 (<https://www.ncbi.nlm.nih.gov/nucleotide/>) and missing data was annotated by '?'.
423 Phylogenetic analysis was performed in BEAST [54] according to the settings described
424 in Lang et al (2014) [28]. Markov-Chain Monte-Carlo (MCMC) runs were performed with
425 a chain length of 10^7 generations and recorded every 1000 generations. MCMC output
426 analysis was carried out using TreeAnnotator [54] and the tree was visualized and edited
427 with FigTree [55]. We chose a strict molecular clock and set priors for most recent
428 common ancestors according to the divergence estimates of Lang et al. (2014) [28] for
429 the splits of *D. nanoptera* - *D. pachea* 3.7 ± 1.5 Ma, *D. bromeliae* - *D. pachea* 8 ± 3 Ma.
430 The divergence estimate for all analyzed species was set to 40 ± 5 Ma [56].

431

432 **Copulation recording**

433 Emerged flies (0-14 h) were anesthetized with CO₂, separated according to sex and
434 transferred into food vials in groups of either 5 females or 5 males using a Stemi 2000
435 (Zeiss) stereo microscope and a CO₂-pad (Inject+Matic sleeper). Flies were maintained at
436 22°C or 25°C until they reached sexual maturity (Supplementary Table 1). Males of *D.*
437 *bromeliae*, *D. melanogaster*, *D. pachea* and *D. nannoptera* were isolated into single vials
438 for at least two days before the experiment was performed. For video recording, one male
439 and one female were introduced with a self-made fly aspirator by mouth suction into a
440 circular plastic mating cell with a diameter of 10-12 mm, a depth of 4mm and a transparent
441 1-mm Plexiglas cover [26]. For copulation recording of *D. acanthoptera*, flies were let to
442 initiate copulation in a food vial and were then rapidly transferred to the mating cell.

443 Movies were recorded in a climate controlled chamber [26] at 22 or 25 ± 0.1°C
444 and 60% or 85% ± 5% humidity (supplementary datafile 3). Flies were filmed from above
445 using digital microscope cameras 191251-62 (Conrad), DigiMicro Profi (DNT) or
446 MIRAZOOM MZ902 (OWL). Movies were recorded with the program GUVVIEW (version
447 0.9.9) GTK UVC or Cheese (version 3.18.1) (<https://wiki.gnome.org/Apps/Cheese>) at a
448 resolution of 800 X 600 pixels on a Linux Ubuntu operating system. Movies were recorded
449 until copulation ended or for at least 45 min when no copulation was detectable. After
450 movie recording, flies were dissected or stored in ethanol at -20°C.

451

452 **Multiple species mating position analysis**

453 Each movie name consisted of a three-letter abbreviation for the species filmed, an
454 additional two-digit number that also indicated the species and a two-digit number for each
455 respective experiment. Movies were analyzed with the video editor OpenShot 1.4.3 (Open

456 Shot Studios, Texas, USA). Courtship start, copulation start, the settling time point and the
457 end of copulation were annotated manually by two different persons, except for movies of
458 *D. pachea* and *D. melanogaster*, which were annotated only by one person
459 (supplementary datafile 3). Courtship was defined to start when the male displayed at least
460 three consecutive typical courtship behaviors, such as tapping the female, following the
461 female's abdomen, licking the female oviscapt or the ground beneath the female
462 abdomen, wing rowing (*D. melanogaster*) or other wing vibrations [57]□□. Courtship was
463 defined to end with the start of copulation, when a male started to mount the female
464 abdomen. Only cases where the male remained mounted on the female for at least 15 sec
465 were counted as copulation starts. Copulation was defined to end when the male had
466 completely descended from the female abdomen with the forelegs detached from the
467 female dorsum and female and male genitalia being separated. As mentioned above, the
468 male moved its legs and abdomen for a certain time period (considered as the settling
469 phase) until adopting an invariant abdomen posture at the settling time point
470 (supplementary datafile 3, Supplementary Fig. 6). The remaining copulation period was
471 defined as the stable copulation period (Supplementary Fig. 6). In fact, this period was
472 often interrupted by periods of vigorous movements in *D. melanogaster*, *D. tripunctata*
473 and *D. willistoni*. In the other species, males remained rather invariant on the female
474 abdomen after the settling time point.

475 We video-recorded 315 movies, of which 111 were used for assessing courtship
476 duration (supplementary dataset 3). Reasons for discarding 204 movies for courtship
477 duration measurements were: wrong handling of the camera or the software, damaged
478 files: 4; incomplete recording of courtship: 43; fly leg or wing damaged: 27; no copulation
479 after 45 min of experiment start: 129; wing damaged and incomplete recording of
480 courtship: 1. A total of 146 movies were used for the analysis of copulation duration.

481 Reasons for discarding 169 movies for copulation duration measurements were: wrong
482 handling of the camera or the software, damaged files: 4, incomplete recording of
483 copulation: 7, fly leg or wing damaged: 27, no copulation after 45 min of experiment start:
484 129; multiple reasons: 2 (supplementary dataset 3). From these 146 movies, we had to
485 exclude 22 movies for the assessment of the copulation posture because landmark
486 positions could not be observed. This was mainly due to couples being recorded from the
487 ventral view. As a result, 124 experiments were used for assessment of the copulation
488 posture (supplementary dataset 3). One additional movie was discarded for posture
489 assessment at the 10% stable copulation time point because the female head was not in
490 the camera field of view.

491 Movie names were replaced by a seven-digit random number (supplementary
492 datafile 5) so that mating postures were quantified in a blind fashion with respect to the
493 species name. Time points for position analysis (supplementary dataset 3, supplementary
494 Fig. 6) were calculated with a custom R script and exported values were used as an input
495 for a bash script to extract images from each movie at particular time points with avconv
496 (libav tools, <https://www.libav.org>).

497 The angle was measured using three landmarks on the female and male body: the
498 anterior tip of the female head along its mid-line (P1), the distal tip of the female scutellum
499 (P2) and the most posterior medial point of the male head (P3) (supplementary Fig. 7a). In
500 cases where images were too dark, positions of P1 and P3 were approximated as the
501 anterior and posterior mid distances between the eyes and the position of the scutellum tip
502 (P2) was approximated by the medial dorsal point at the body constriction observed
503 between the third thoracic and first abdominal segment. Position landmarks were placed
504 manually on each image using imageJ and data analysis was done using R. Briefly,
505 coordinates (supplementary dataset 4) were rotated and scaled, so that all P1 points were

506 superimposed and all P2 points as well (supplementary Fig. 7B-K). The angle P1-P2-P3
507 (Supplementary Fig. 7A) was used to measure one-sidedness of mating positions (Fig. 3).
508 Repeatability of landmark positioning was assessed by two independent rounds of
509 coordinate acquisition for all species at one specific time point during copulation, the 10%
510 stable copulation time point (see text) (2x 124 images). Variation in angle estimates was
511 found to be attributable mostly to individual images and not to replicate measurement
512 (ANOVA, linear model: angle ~ image + replicate, image: df1 = 122, df2 = 123, F = 87.174,
513 $p < 2e-16$, replicate: df1 = 1, df2 = 244, F = 0.077, $p = 0.782$).

514 Hypothesis testing was performed in R to compare mating postures across species
515 (Fig. 3) with the null hypothesis: angle = 0, using the functions glm for generalized linear
516 model fits, and glht to derive estimated contrasts.

517

518 **Analysis of the *D. nanoptera* copulation posture**

519 Flies were reared and isolated before copulation as described above. One female
520 and one male were CO₂ anesthetized and transferred onto a white plastic support (mating
521 cap) and were caged with a transparent plastic cylindrical 25 mm x 7 mm cap. Once
522 courtship was observed, mating caps were put on a motorized horizontally turning stage
523 (0-30 rpm) (grinding stone 8215, Dremel) in front of a camera MIRAZOOM MZ902 (OWL)
524 and copulation was recorded with the camera being put into an optimized frontal view
525 towards the female head by rotating or turning the mating cap. The transparent cap was
526 optionally removed once copulation had started. The yield of informative experiments with
527 these settings was poor as we performed 167 mating experiments but only 29 experiments
528 were informative for our data analysis (supplementary datafile 6, reasons for discarding
529 the experiments are listed). Images were extracted with avconv (see above) every 15-30
530 sec or when the flies were visible in a frontal view. We measured the inclination of the

531 male body relative to the female dorso-ventral axis by using three landmarks: P4 as the
532 medial most dorsal edge of the female head (often visible by the ocelli), P5 being the most
533 ventral medial position of the female head (the female proboscis) and P6 as the medial
534 most dorsal edge of the male head (often visible by the ocelli) and measuring the angle
535 between the lines drawn through P4-P5 and P5-P6 (Supplementary Fig. 8, supplementary
536 datafile 7).

537 **List of abbreviations**

538 SEM scanning electron microscopy

539 **Declarations**

540 **Ethics approval and consent to participate**

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

543 **Consent for publication**

544 “Not applicable”

545 **Availability of data and material**

546 The data sets supporting the results of this article will be made available in the DRYAD
547 repository.

548

549 **Competing interests**

550 The authors declare no competing financial interests

551 **Funding**

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

554 Agreement no. 337579) given to Virginie Courtier-Orgogozo. FTR was financed by a Kees
555 Bakker Award, awarded by the Stichting Professor Dr. K. Bakker Fonds.

556

557 **Authors' contributions**

558 ML and VCO desiged the experiments, AA collected fly specimen, AA, SP and ML
559 recorded fly copulation, AA performed light microscopy analysis of *Drosophila* male
560 genitalia, FTR performed SEM analysis of *D. pachea* male genitalia. ML, AA and SP
561 analyzed the movie datasets, ML and VCO wrote the manuscript with AA.

562 **Acknowledgements**

563 We are grateful to Sadjo Sidikou and Pierre Quevreur for movie recordings, to
564 Bertie Joan van Heuven and Menno Schilthuisen for assistance and interpretation of SEM
565 analysis and to Martina Manns for discussions of *D. nanoptera* mating positions. We
566 greatly thank Álvaro Barragán, Violeta Rafael and Santiago Palacios for their help in the
567 field work made in Ecuador (Research Permit No.003-15 IC-FAU-DNB/MA), Therese
568 Markow and Alejandro Oceguera-Figueroa for their help with fly collections in Mexico,
569 Jean David for *Drosophila* laboratory strains and Alexandre Peluffo for help with the
570 statistical analysis.

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718 **Supporting Information**

719 **Supplementary Datafile 1:** Length measurements at the left and right sides of the ventral
720 aedeagus tip of *D. acanthoptera*, *D. nannoptera*, *D. machalilla* and *D. bromeliae*

721 **Supplementary Datafile 2:** Multilocus DNA sequence dataset for the molecular phylogeny
722 shown in supplementary Fig. 6.

723 **Supplementary Datafile 3:** Multi-species analysis of courtship and copulation periods,
724 shown in supplementary Fig. 6.

725 **Supplementary Datafile 4:** Landmark position measurements, used to calculate angle
726 values for the multi-species mating position analysis, shown in Fig. 3.

727 **Supplementary Datafile 5:** Randomization of experiment names for the multi-species
728 mating position analysis. Original names and random number substitutes are listed for
729 each movie.

730 **Supplementary Datafile 6:** Copulation times of couples filmed for the position analysis of
731 *D. nannoptera* from a frontal perspective, shown in supplementary Fig. 8.

732 **Supplementary Datafile 7:** Angle measurements for the position analysis of *D.*
733 *nannoptera* from a frontal perspective, shown in Fig. 3.

734 **Figures and Tables**

735 **Figure 1: The aedeagus of male *Drosophila pachea* is asymmetric.** SEM images of a
736 single phallus in lateral-dorsal and dorsal-apical view. Note the asymmetric position of two
737 subapical spurs, located on the ventral side of the aedeagus, and the asymmetric position
738 of the gonopore. The white arrows point to the gonopore. The scale bar is equivalent to
739 100 μm .

740 **Figure 2: Genital and aedeagus shapes in *D. pachea* and closely related species.**

741 External genitalia and aedeagus shapes are compared across closely related species of
742 *D. pachea*. Aedagus asymmetries are only found in *D. acanthoptera* and *D. pachea* (**a**)
743 ventral view of a *D. acanthoptera* male. The black frame indicates the position of male
744 genitalia and the box with a dashed frame shows a magnification with an erected penis.
745 (**b-d, e-g, h-j, k-m, n-p**) Lateral views of male specimen and male genitalia of *D.*
746 *acanthoptera*, *D. pachea*, *D. nanoptera*, *D. machalilla*, and *D. bromeliae*, respectively. (**c,**
747 **f, i, l, o**) Male terminalia in lateral and posterior view. (**d, g, j, m, p**) Aedeagus in lateral
748 and ventral view. The scale bar is 100 μm .

749 **Figure 3: The copulation position of *D. nanoptera* and *D. pachea* is asymmetric.**
750 Copulation angles of *D. pachea* couples and of nine related *Drosophila* species; aca: *D.*
751 *acanthoptera*, bro: *D. bromeliae*, buz: *D. buzzatii*, mac: *D. machalilla*, mel: *D.*
752 *melanogaster*, moj: *D. mojavensis*, nan: *D. nanoptera*, pac: *D. pachea*, tri: *D. tripunctata*,
753 wil: *D. willistoni*. (**a,b**) Copulation angle at the settling time point (settling, see material and
754 methods) and at the 10% stable copulation time point (10pct), respectively. Stars indicate
755 significant rejection of the null hypothesis: angle = 0 (Table 2, GLM fit angle~species **:
756 $p < 0.001$, ***: $p < 0.0001$). Numbers below each boxplot indicate the number of
757 observations. The dashed lines indicate an angle of zero degrees. (**c-e**) Copulation angles
758 over the course of copulation of *D. melanogaster* (mel), *D. acanthoptera* (aca), *D. pachea*
759 (pac) and *D. nanoptera* (nan). n indicates the number of observations. Grey lines connect
760 points obtained from the same copulation couple over time. The dashed lines indicate an
761 angle of zero degrees.

762 **Figure 4: Evolution of sexual characters in the *nanoptera* species group.** The
763 cladogram was established based on the phylogeny of this study (supplementary Fig. 6),
764 combined with data from Lang et al (2014) [28]. AS, asymmetric states; S, symmetric

765 states; nd, not determined, sp, spermathecae; rec, female seminal receptacle.

766 **Supplementary Figure 1: The aedeagus of *D. pachea* is asymmetric.** Preparations in
767 ventral view **(a)** The red circle indicates the right-sided position of the gonopore. **(a-j)** Ten
768 preparations. The scale bar is 100 μm .

769 **Supplementary Figure 2: The aedeagus of *D. acanthoptera* is asymmetric.**
770 Preparations in ventral view. **(a)** The red lines indicate the length measurements of ventral
771 apex spurs (see materials and methods). **(a-j)** preparations. **(k)** Length measurements of
772 apical spurs. The dashed line corresponds to the 1:1 length ratio of left and right spurs.
773 The scale bar is 100 μm .

774 **Supplementary Figure 3: No asymmetry is detected in the aedeagus of *D.***
775 ***nannoptera*.** Preparations in ventral view **(a)** The red lines indicate the length
776 measurements of ventral apex spurs (see materials and methods). **(a-o)** Fifteen
777 preparations. **(p)** Length measurements of apical spurs. The dashed line corresponds to
778 the 1:1 length ratio of left and right spurs. The scale bar is 100 μm .

779 **Supplementary Figure 4: No asymmetry is detected in the aedeagus of *D. machalilla*.**
780 Preparations in ventral view **(a)** The red lines indicate the length measurements of ventral
781 apical hooks (see materials and methods). **(a-j)** Ten preparations. **(k)** Length
782 measurements of apical hooks. The dashed line corresponds to the 1:1 length ratio of left
783 and right hooks. The scale bar is 100 μm .

784 **Supplementary Figure 5: No asymmetry is detected in the aedeagus of *D. bromeliae*.**
785 Preparations in ventral view **(a)** The red lines indicate the length measurements of ventral
786 apex ridges (see materials and methods). **(a-j)** Replicate preparations. **(k)** Length
787 measurements of apex ridges. The dashed line corresponds to the 1:1 length ratio of left

788 and right ridges. The scale bar is 100 μm .

789 **Supplementary Figure 6: Courtship and copulation duration in *D. pachea* and**
790 **related species.** The phylogenetic relationships of analyzed species are indicated on the
791 left with a bayesian phylogeny based on a multilocus dataset of Lang et al. 2014 [28]□,
792 and additional data for *D. willistoni* and *D. tripunctata* (see material and methods,
793 supplementary Table 1). Numbers indicate posterior probabilities for node supports < 1.
794 Each line represents an experiment. Courtship is indicated in red, initial copulation with
795 variable positions in blue and copulation after the settling time point in grey. Experiments
796 are aligned by the settling time point. Time points at which the mating angle was
797 calculated are indicated as tick marks: the settling time point in yellow; 10% stable
798 copulation time point in green and measurements at later regular time intervals in black.

799 **Supplementary Figure 7: Multi-species mating position measurements.** A) Mating
800 couple of *D. buzzatii* the scale bar 500 μm . Landmarks P1 (1), P2 (2), and P3 (3) are
801 indicated. The dashed white lines (P1,P2) and (P2,P3) form an acute angle (semi-
802 transparent circle sectors) which was measured to assess copulation posture. B-K)
803 Position coordinates for angle measurements of *D. pachea* and nine related *Drosophila*
804 species; aca: *D. acanthoptera*, bro: *D. bromeliae*, buz: *D. buzzatii*, mac: *D. machalilla*, mel:
805 *D. melanogaster*, moj: *D. mojavensis*, nan: *D. nanoptera*, pac: *D. pachea*, tri: *D.*
806 *tripunctata*, wil: *D. willistoni*. Points P1 (orange circle) are placed at coordinates (0,1) and
807 P2 (red circle) at coordinates (0,0). P3 points are shown for the settling time-point in yellow
808 dots, for the 10% stable copulation time point in green dots and for later time points in
809 black circles. L) Correlation of angle values calculated from two replicate measurements
810 (n=124) at the 10% copulation time point (Pearson correlation coefficient = 0.988, df =
811 121, t = 69.231, p < 10e -16). The red dashed line indicates the linear regression line.

812 **Supplementary Figure 8: *D. nannoptera* tilts to the right side of the female abdomen.**

813 Frontal copulation angles (black circles) are plotted over the course of copulation. Positive
 814 and negative values indicate left-sided and right-sided angles, respectively. Grey lines
 815 connect points obtained from the same copulation couple over time. The dashed line
 816 indicates an angle of zero degrees. The position analysis from a frontal perspective is
 817 indicated on the image of the copulating couple on the right. Points and numbers indicate
 818 position landmarks P4 (4), P5 (5), and P6 (6). The dashed white lines (P1,P2) and (P2,P3)
 819 form an acute angle (semi-transparent circle sector) which corresponds to the frontal
 820 copulation angle. The scale bar is 500 μ m.

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822 **Table 1: Courtship and copulation duration**

<i>species</i>	courtship duration [min] (mean \pm SD)	range [min]	n	copulation duration [min] (mean \pm SD)	range [min]	n
<i>D. acanthoptera</i>	---	---	---	88.49 \pm 35.18	38.85 - 144.32	12
<i>D. pachea</i>	4.67 \pm 3.91	0.17 - 12.37	18	29.58 \pm 7.86	7.33 - 42.63	21
<i>D. nannoptera</i>	1.89 \pm 3.25	0.05 - 12.67	15	11.9 \pm 4.2	4.03 - 20.1	21
<i>D. machalilla</i>	1.97 \pm 3.44	0.08 - 11.85	13	2.28 \pm 0.53	1.07 - 3.55	18
<i>D. bromeliae</i>	2.07 \pm 2.4	0.23 - 8.37	10	0.92 \pm 0.28	0.65 - 1.73	12
<i>D. mojavensis</i>	1.56 \pm 2.71	0.13 - 5.63	4	2.3 \pm 0.35	1.83 - 2.57	4
<i>D. buzzatii</i>	2.87 \pm 5.26	0.08 - 18.87	15	1.79 \pm 0.65	1.13 - 3.42	17
<i>D. tripunctata</i>	5.17 \pm 5.98	0.68 - 13.82	4	33.34 \pm 9.54	20.47 - 42.15	4
<i>D. willistoni</i>	5.72 \pm 5.39	0.53 - 14.65	5	16.88 \pm 2.58	13.9 - 21.55	6
<i>D. melanogaster</i>	13.48 \pm 8.9	2.55 - 40.23	27	13.83 \pm 4.33	7.57 - 24.55	31

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828 **Table 2: Test for one-sided mating positions.** Fit: GLM (angle ~ species), family =

829 "gaussian", hypothesis: angle = 0, Bonferroni corrected p-values

species	settling			10% stable copulation time point replicate measurement 1			10% stable copulation time point replicate measurement 2		
	est. contrast	z value	p	est. contrast	z value	p	est. contrast	z value	p
<i>D. acanthoptera</i>	-1.9105	-0.282	1	0.5919	0.091	1	-0.4980	-0.075	1
<i>D. pachea</i>	21.4011	4.048	0.000517	18.8120	3.704	0.00212	17.8498	3.449	0.00564
<i>D. nanoptera</i>	32.5346	6.646	3.00e-10	34.4231	7.321	2.46e-12	35.0479	7.314	2.60e-12
<i>D. machalilla</i>	5.7585	0.851	1	6.5023	1.001	1	8.2219	1.242	1
<i>D. bromeliae</i>	-3.9911	-0.590	1	-2.9702	-0.457	1	-0.7399	-0.112	1
<i>D. mojavensis</i>	3.0034	0.268	1	4.2370	0.393	1	6.3650	0.580	1
<i>D. buzzatii</i>	2.4878	0.430	1	3.0853	0.555	1	1.2320	0.217	1
<i>D. tripunctata</i>	3.6189	0.323	1	-2.9770	-0.239	1	-5.4349	-0.429	1
<i>D. willistoni</i>	0.7288	0.080	1	0.4118	0.047	1	0.6928	0.077	1
<i>D. melanogaster</i>	6.6156	1.414	1	1.6010	0.356	1	0.7997	0.175	1

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831 **Table 3: *D. nanoptera* frontal mating angles.** The mean estimates for each time

832 interval were calculated with average values when multiple measurement points were

833 available for a given experiment

time interval after cop. start [min]	angle (mean ± SD)	n
0 - 1	10.36 ± 6.88	25
1 - 2	15.46 ± 8.82	29
2 - 3	23.44 ± 12.15	29
3 - 4	27.16 ± 10.81	27

4 - 5	29.1 ± 11.62	25
5 - 6	28.97 ± 11.92	21
6 - 7	32.48 ± 8.29	13
7 - 8	26.44 ± 10.24	5
8 - 9	23.21 ± 11.88	4
9 - 10	24.7 ± 13.66	3
10 - 11	18.65 ± 14.03	2
11 - 12	8.07 ± NA	1

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835 **Supplementary Table 1: Species Resources**

Species	Source	Stock number	collection locality	collection year
<i>D. acanthoptera</i>	Drosophila Species Stock Center	15090-1693.00	Oaxaca, Mexico	1976
<i>D. pachea</i>	Drosophila Species Stock Center	15090-1698.02	Sonora, Mexico	1996
<i>D. nanoptera</i>	Drosophila Species Stock Center	15090-1692.10 15090-1698.12	Oaxaca/Puebla, Mexico	1992
<i>D. machalilla</i>	Andrea Acurio		San Jose, Ecuador	2015
<i>D. bromeliae</i>	Drosophila Species Stock Center	15085-1682.00	Grand Cayman Island, UK	1985
<i>D. buzzatii</i>	Jean David		Bahia, Brazil	2010
<i>D. mojavensis</i>	Drosophila Species Stock Center	15081-1352.22	Catalina Island, USA	2002
<i>D. tripunctata</i>	Drosophila Species Stock Center	15020-2401,02	New Orleans, USA	1950
<i>D. willistoni</i>	Jean David		Rio de Janeiro, Brazil	2010
<i>D. melanogaster</i>	Drosophila Species Stock Center	14021-0231.07	Taiwan	1968

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837 **Supplementary Table 2: GenBank Accession Numbers of the phylogeny dataset**

Locus, Accession Number

Species	<i>amy</i>	<i>amyrel</i>	<i>boss</i>	<i>fkh</i>	<i>marf</i>	<i>sinA</i>	<i>snf</i>	<i>wee</i>	ND2	COI	COII
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<i>D. acanthoptera</i>	KF632687	KF632675	JF736442	KF632652	KF632638	EU341611	JF736382	KF632612	KF632701	KF632601	AF183968
<i>D. pachea</i>	KF632697	KF632683	KF632672	KF632662	KF632648	KF632595	KF632634	KF632622	KF632709	KF632609	KF632600
<i>D. nannoptera</i>	KF632696	KF632682	JF736456	KF632661	KF632647	JF736334	KF632633	KF632621	KF632708	DQ47153	AF183971
<i>D. machalilla</i>	KF632694	KF632680	KF632671	KF632659	KF632645	KF632594	KF632631	KF632619	KF632706	KF632607	KF632599
<i>D. bromeliae</i>	KF632689	AY733049	KF632666	KF632654	KF632640	KF632591	KF632627	KF632614	KF632702	KF632602	AF478418
<i>D. buzzatii</i>	KF632690	KF632677	KF632667	KF632655	KF632641	EU341621	JF736384	KF632615	KF632703	KF632603	DQ20201
<i>D. mojavensis</i>	XM00200442	XM00200656	XM00199969	XM00199979	XM00200948	XM00200728	XM00201147	XM00200309	BK006339	BK006339	BK006339
	5	1	2	1	9	9	5	3			
<i>D. tripunctata</i>	---	---	---	---	---	---	---	---	EU493508	EF570023	EU493748
<i>D. willistoni</i>	CH963849	CH963719	CH964272	CH964232	CH963925	CH963876	CH964239	CH963920	consensus of: NW00203114 4 NW00203340 7 NW00203385 0 NW00203603 8 NW00203840 1	JQ679116	EU532097
<i>D. melanogaster</i>	NM079044	NM057914	NM080709	NM079818	AF355475	NM057377	NM078490	NM057687	NC024511	NC024511	NC024511

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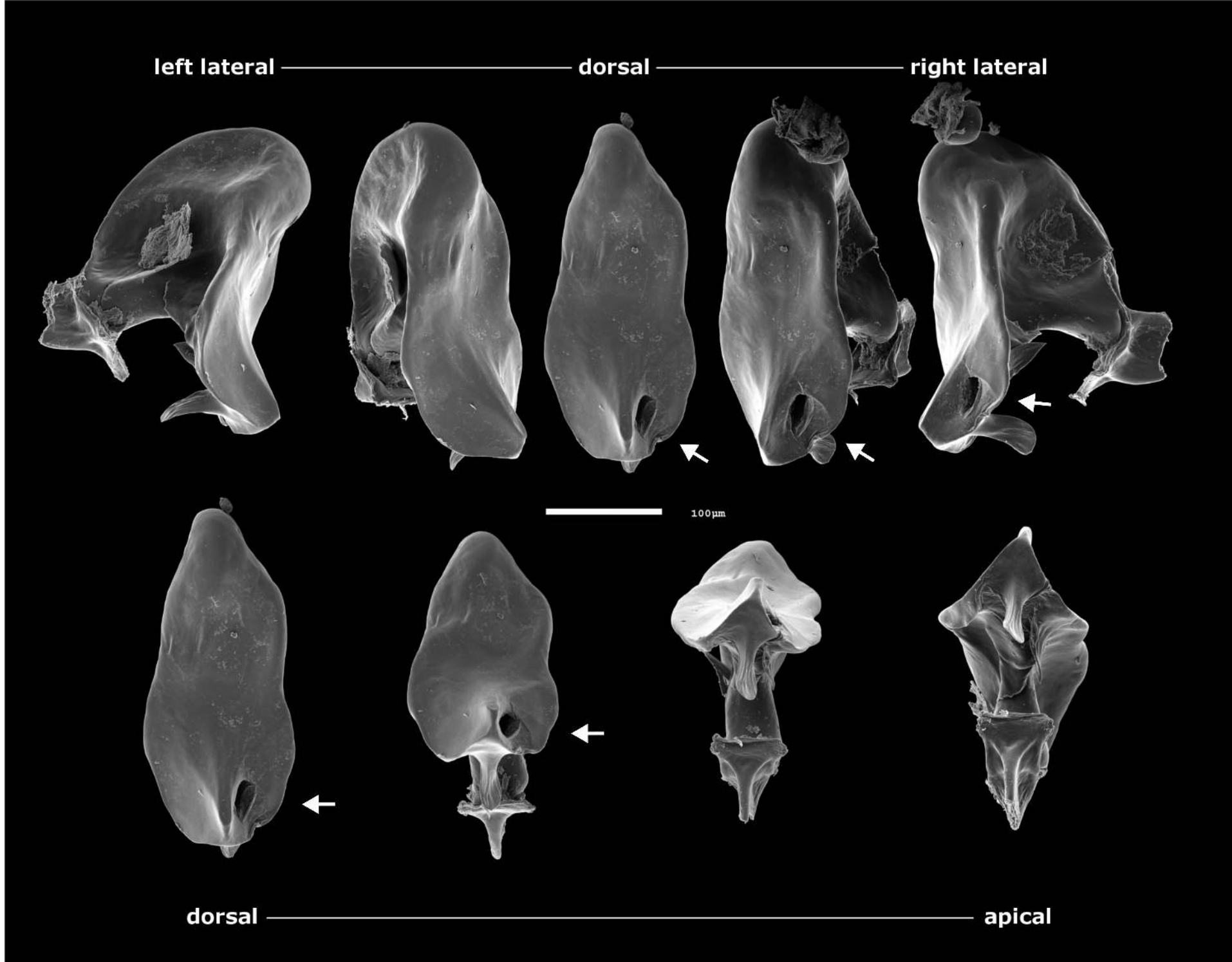
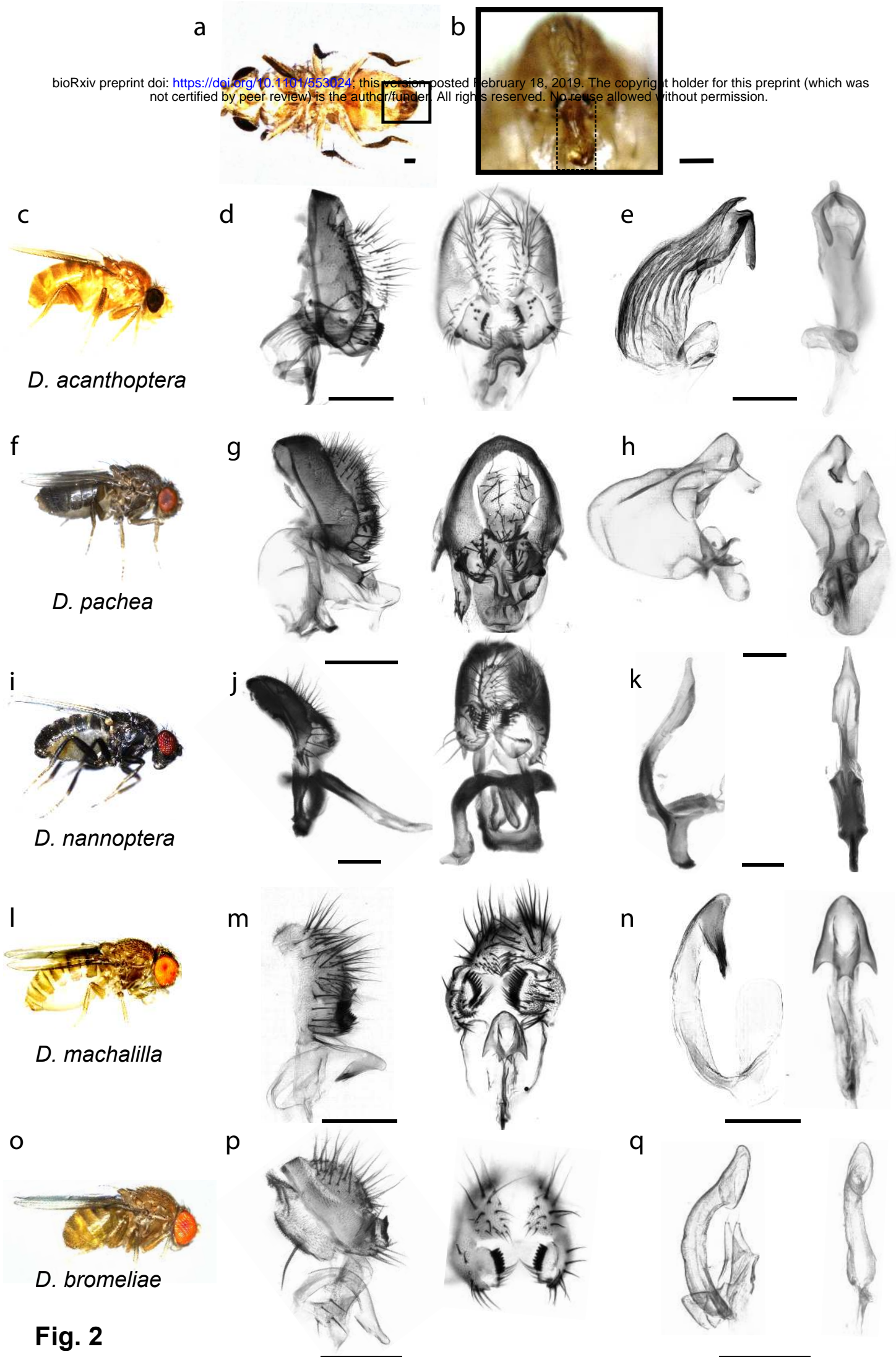


Fig. 1



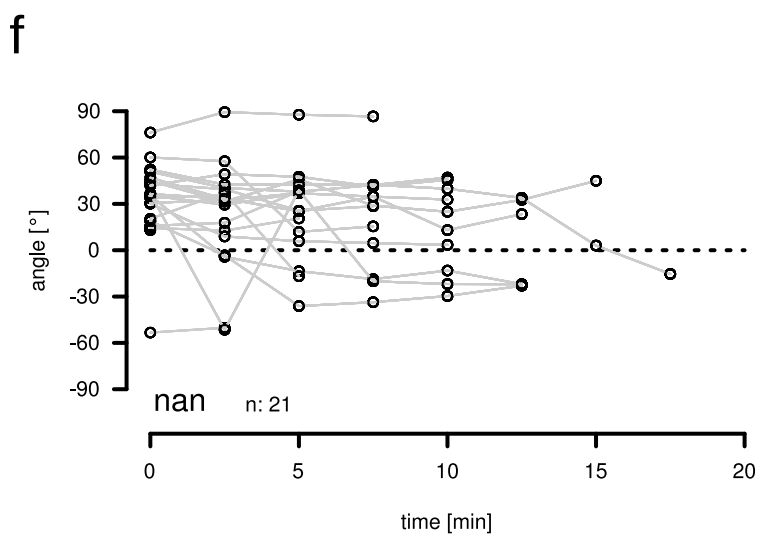
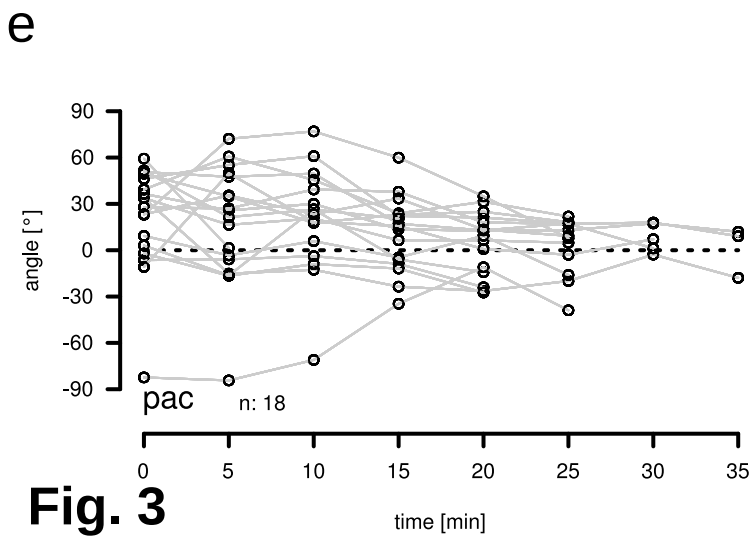
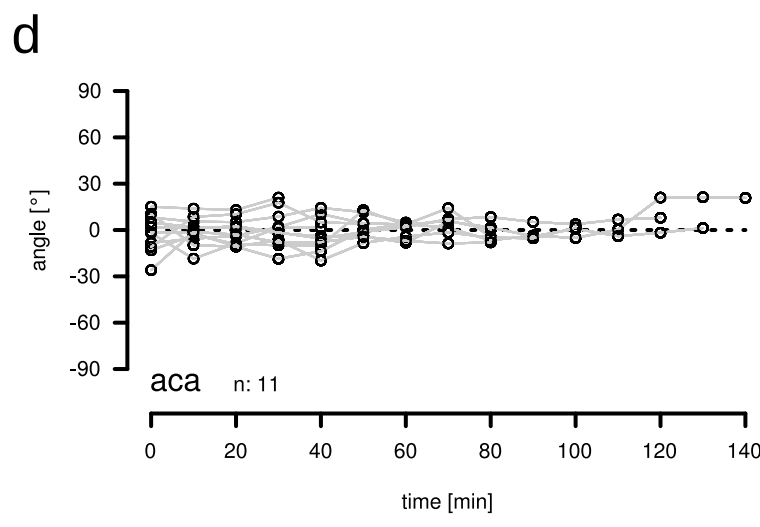
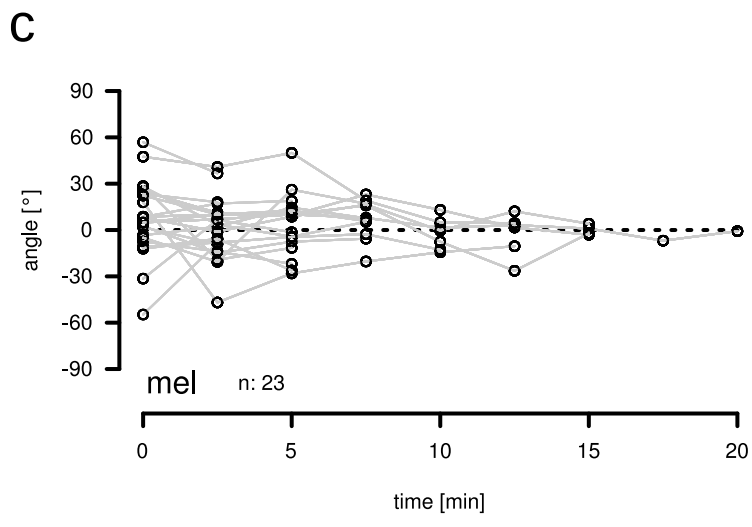
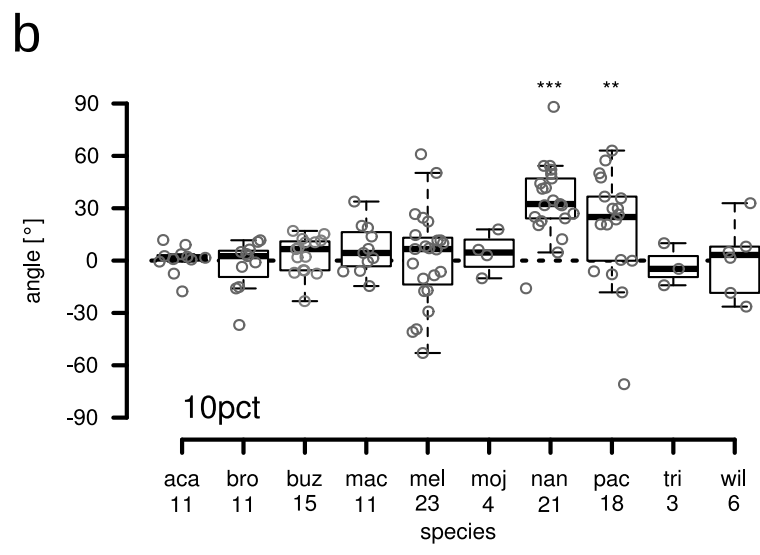
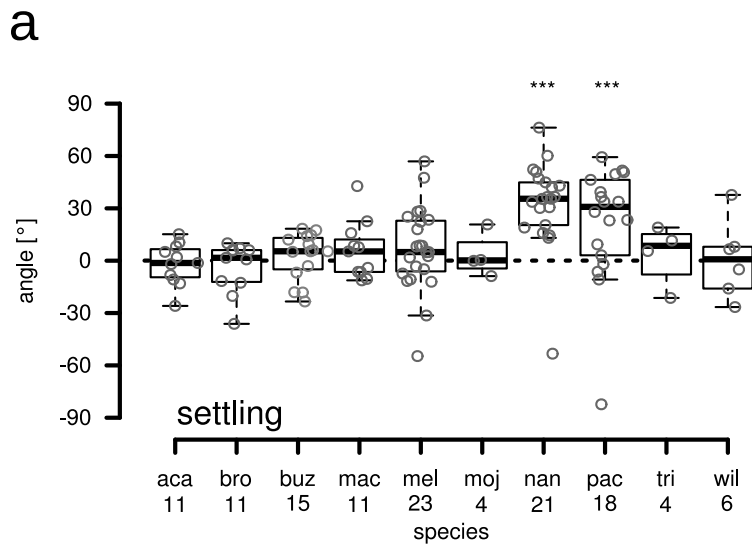


Fig. 3



	Aedagus	External Genitalia	Mating Position	Mating Duration	Sperm Length	Sperm Storage
<i>D. pachea</i>	AS	AS (lobes)	right-sided	30 min	16.6	sp
<i>D. wassermani</i>	nd	AS (anal plates)	nd	nd	4.5	sp
<i>D. acanthoptera</i>	AS	S	S	88 min	5.8	sp
<i>D. nanoptera</i>	S	S	right-tilted	12 min	15.7	rec
<i>D. machalilla</i>	S	S	S	< 3 min	nd	nd
<i>D. bromeliae</i>	S	S	S	< 3 min	nd	sp + rec
<i>D. mojavensis</i>	S	S	S	< 3 min	1.9	rec
<i>D. melanogaster</i>	S	S	S	14 min	1.9	sp + rec

Fig. 4