No evidence for asymmetric sperm deposition in a species with asymmetric male genitalia

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Abstract

Background. Asymmetric genitalia have repeatedly evolved in animals, yet the underlying causes for their evolution are mostly unknown. The fruit fly *Drosophila pachea* has asymmetric external genitalia and an asymmetric phallus with a right-sided phallotrema (opening for sperm release). The complex of female and male genitalia is asymmetrically twisted during copulation and males adopt a right-sided copulation posture on top of the female. We wished to investigate if asymmetric male genital morphology and a twisted gentitalia complex may be associated with differential allocation of sperm into female sperm storage organs.

Methods. We examined the internal complex of female and male reproductive organs by micro-computed tomography and synchrotron X-ray tomography before, during and after copulation. In addition, we monitored sperm aggregation states and timing of sperm transfer during copulation by premature interruption of copulation at different time-points.

Results. The asymmetric phallus is located at the most caudal end of the female abdomen during copulation. The female reproductive tract, in particular the oviduct, re-arranges during copulation. It is narrow in virgin females and forms a broad vesicle at 20 min after the start of copulation. Sperm transfer into female sperm storage organs (spermathecae) was only in a minority of examined copulation trials (13 / 64). Also, we found that sperm was mainly transferred early, at 2 - 4 min after the start of copulation. We did not detect a particular pattern of sperm allocation in the left or right spermathecae. Sperm adopted a granular or filamentous aggregation state in the female uterus and spermathecae, respectively.

Discussion. No evidence for asymmetric sperm deposition was identified that could be associated with asymmetric genital morphology or twisted complexing of genitalia. Male genital asymmetry may potentially have evolved as a consequence of a complex internal alignment of reproductive organs during copulation in order to optimize low sperm transfer rates.

Introduction

Animal genitalia are often remarkably complex and reveal a high degree of variation between species (Eberhard, 1985)□. Sexual selection has been proposed as the chief driver for both the rapid diversification and the great morphological complexity of genital structures through sexual conflict between females and males at different levels of reproduction, including access to copulation, sperm competition inside the female, and control over post-mating sperm storage and usage for fertilization (Thornhill, 1983; Eberhard, 1985; Arnqvist, 1998; Birkhead & Pizzari, 2002; Chapman et al., 2003)□. In general, genitalia enable ejaculate transfer during copulation from the male into the female and mediate inter-sexual communication during copulation (Eberhard, 1985, 1994)□.

Asymmetric genitalia are observed in many species and must have recurrently evolved from symmetric ancestors (Huber, Sinclair & Schmitt, 2007; Huber, 2010; Schilthuizen, 2013)□. They have been associated with a specific interlocking of female and male genitalia (Kamimura et al., 2019; Holwell et al., 2015; Rhebergen et al., 2016; Richmond, Park & Henry, 2016)□ and with lateralized courtship and copulation behavior (Rhebergen et al., 2016; Orbach et al., 2020; Torres-Dowdall et al., 2020)□. However, the ultimate evolutionary cause for these transitions from symmetry to asymmetry are not fully understood (Schilthuizen, 2013)□. To help solve this puzzle, detailed analyses of the function and the internal configuration of asymmetric genitalia during copulation are helpful, as already evaluated in a few cases. In anseriform birds (waterfowl), the female vagina is coiled clockwise and has dead-end sacs that potentially function to avoid insemination via the male phallus, which is coiled in counter-clockwise direction (Brennan et al., 2007)□. Similarly, harbor porpoise *Phocoena phocoena (L.)* reveal strikingly asymmetric female and male genitalia (Orbach et al., 2020)□ and males attempt copulation by approaching a female exclusively on her left side, potentially to overcome female insemination or fertilization barriers. Lateralized mating behavior has also been shown to be associated with genital asymmetry in one-sided livebearer fish *Jenynsia lineata (Jenyns)* (Torres-Dowdall et al., 2020)□. The tip of the male gonopodium is either bent to the left or to the right side and the direction of this asymmetry was correlated with sidedness of mating attempts. Also, complementary antisymmetric genitalia have been suggested to increase reproductive success in snails of the subgenus *Amphidromus* s. str. The genitalia of these hermaphrodite species are coiled and favor copulation of interchiral pairs that better match for sperm transfer and that

70 circumvent sperm digestion (Schilthuizen et al., 2007; Schilthuizen & Looijestijn, 2009)☐. In

71 insects, the evolution of asymmetric genitalia was proposed to have evolved in response to

72 changes in mating position, potentially affecting the coupling efficiency of female and male

73 genitalia during copulation (Huber, 2010)☐. Changes in mating positions could evolve through

74 sexual conflict, for the male to gain control over copulation, or through the female to increase the

75 difficulty of insemination and to increase the potential to reject male gametes. This may impose

76 selection pressure on genitalia to become asymmetric to modify genital contacts. However, the

77 opposite is also possible, that asymmetric genitalia may provoke adaptive changes in mating

78 position. In addition, no experimental data exist to test this hypothesis and generally little is

79 known about the internal alignments of the intermittent phallus inside the female reproductive

80 tract. Such studies have, however, become feasible in recent years due to the advances in micro-

81 computed tomography (Mattei et al., 2015; Woller & Song, 2017; Dougherty & Simmons, 2017;

82 Gutiérrez et al., 2018)☐.

83   Males of *Drosophila pachea* Patterson & Wheeler have strikingly asymmetric genitalia.

84 They possess a pair of external genital lobes that are attached to the lateral plates of the male

85 genital arch (= epandrium: Rice et al., 2019). The left lobe being approximately 1.5 times longer

86 than the right lobe (Pitnick & Heed, 1994; Lang & Orgogozo, 2012)☐. In addition, the phallus is

87 asymmetrically bent, harbors ventrally asymmetric spurs and the male phallotrema (opening for

88 sperm release) is positioned at the right dorsal tip of the phallus (Acurio et al., 2019)☐. Apart

89 from this asymmetric genital morphology, *D. pachea* also mates in a right-sided copulation

90 posture, with the male antero-posterior midline being shifted about 6-8° to the right side of the

91 female midline (Lang & Orgogozo, 2012; Rhebergen et al., 2016; Acurio et al., 2019)☐. This

92 one-sided mating posture is associated with asymmetric genitalia coupling and an asymmetric

93 twist of the female ovipositor (Rhebergen et al., 2016)☐, potentially resulting in a specific

94 positioning of the male phallotrema inside the female reproductive tract. Right-sided mating and

95 male genital asymmetry have co-evolved in *D. pachea* and closely related species, but so far it

96 has not been determined whether right-sided mating behavior may have provoked the evolution

97 of morphological asymmetry or vice versa (Acurio et al., 2019)☐. Within the genus *Drosophila*,

98 *D. pachea* belongs to the *nannoptera* species group, which consists of four described species: *D.*

99 *nannoptera* Wheeler has fully symmetric genitalia but copulates in a right-sided mating posture,

100 *D. acanthoptera* Wheeler has an asymmetric phallus but mates in a symmetric posture, and *D.*

101   *wassermani* Pitnick & Heed has asymmetric anal plates and an unknown copulation posture.

102   Thus, right-sided mating and male genital asymmetry appears to have evolved repeatedly in this

103   species group.

104   Sperm storage and usage for fertilization has been associated with sexual conflict and the

105   evolution of sexual characters with respect to sperm selection from multiple copulations, sperm

106   viability and temporal unlocking of copulation and fertilization (Orr & Zuk, 2012; Edward,

107   Stockley & Hosken, 2015)☐. *Drosophila* species store sperm after copulation in two types of

108   sperm storage organs, the tubular seminal receptacle and the paired spherical spermathecae

109   (Pitnick, Markow & Spicer, 1999)☐. Both types of organs are connected to the anterior end of

110   the female uterus. In particular, *D. pachea, D. acanthoptera* and *D. wassermani* were reported to

111   store sperm exclusively in the spermathecae (Pitnick, Markow & Spicer, 1999)☐. Sperm length

112   also varies enormously among *Drosophila* species and range from 0.3 mm in *D. persimilis*

113   Dobzhansky & Epling to 58 mm in *D. bifurca* Patterson & Wheeler (Pitnick, Markow & Spicer,

114   1995)☐. In particular, *D. pachea* and its sister species *D. nannoptera* also produce giant sperm

115   with lengths of 16.5 and 15.7 mm, respectively (Pitnick & Markow, 1994)☐. Migration

116   capacities of such long sperm require further investigation and it is unknown how giant sperm is

117   organized post copulation inside female reproductive tract.

118   It is known that in certain Diptera females preferentially store sperm in specific

119   spermathecae for high-quality males (Otronen, 1998; Hellriegel & Bernasconi, 2000)☐. It is

120   therefore conceivable that the asymmetric male genitalia in *D. pachea* may have evolved to

121   enhance the chances that sperm would be deposited in or near such preferential sperm storage

122   organs. The aim of our study was to investigate if male genital asymmetry results in an

123   asymmetric internal configuration of the copulatory complex in *D. pachea* which may allow

124   sperm to be directed specifically towards one of the female spermathecae. We carried out micro-

125   computed tomography (microCT) and synchrotron-based X-ray computed tomography of snap-

126   frozen *D. pachea* mating couples before and during copulation to investigate the positioning of

127   reproductive organs as well as ejaculates during copulation. Furthermore, we investigated when

128   sperm transfer occurs during copulation and what aggregation states ejaculate mass assumes

129   during and after transport into the spermathecae.

Materials & Methods

Drosophila pachea maintenance and virgin collection

Drosophila pachea stock 15090-1698.02 from the San Diego *Drosophila* Species Stock Center (now The National Drosophila Species Stock Center, College of Agriculture and Life Science, Cornell University, USA) was maintained in 25 x 95 mm plastic vials containing 10 mL food medium (60 g/L brewer's yeast, 66.6 g/L cornmeal, 8.6 g/L agar, 5 g/L methyl-4-hydroxybenzoate and 2.5% v/v ethanol). In addition, 40 µL of 5 mg/mL 7-dehydrocholesterol (dissolved in ethanol) was mixed into the food medium with a spatula (standard *D. pachea* food). Flies were transferred to fresh vials every 2 to 4 days. In order to isolate virgin individuals, adult flies at 0-3 day after emerging from the puparia were 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 female or male individuals until they reached sexual maturity, about 14 days for males and 4 days for females (Pitnick, 1993)□.

micro-CT scanning

Sexually mature virgin couples of *D. pachea* were introduced each into a 2-mL reaction tube (Eppendorf) that was roughened on the inside. Flies were observed at room temperature for at most 1 hour or until copulation had ended. As soon as a couple started to copulate, time was recorded and the couple was snap-frozen by submerging the Eppendorf tube for 20 sec in liquid nitrogen after one of the following intervals: 2 min, 8 min, 15 min, 20 min, and 120 min after the start of copulation. Tubes were filled with cold (-20°C) absolute ethanol and were stored at -20°C for about a week. Finally, the absolute ethanol was replaced with 80% ethanol and samples (mating complexes) were stored at room temperature. A total of 40 samples were prepared for micro-computed tomography scanning (micro-CT) by critical point-drying, including 25 mating complexes, nine single females two hours after copulation start (ACS) and six virgin females (Table 1). First, the complexes were fixed by successive 20-min incubations in 90% ethanol, 96% ethanol, and twice in absolute ethanol. Samples were then dried in the Automated Critical Point Dryer EM CPD300 (Leica) (program 1, parameters: 'CO2 IN' speed slow, delay 120 sec; 'Exchange' speed 5, cycles 12; 'gas OUT' heat slow, speed slow). Samples were subsequently mounted on an aluminium stub with the male's dorsal side (mating complexes) or the female dorsum (for single female specimen) glued to the stub surface. The ventral side of the female

160   pointed upward and was therefore visible. Twelve scans of different mating complexes were

161   performed with the Zeiss Xradia 520 Versa micro-CT scanner at Naturalis Biodiversity Center in

162   order to adjust experimental conditions. Scan 14 was used to build the model of the female

163   reproductive tract (Table S1). A total of 28 single fly specimen or mating complexes (Table S1)

164   were scanned using Synchrotron Radiation X-ray Tomographic Microscopy (SRXTM) at the

165   TOMCAT (X02DA) beamline of the Swiss Light Source, Paul Scherrer Institut, Switzerland. Of

166   those, 26 scans could be used for data analysis (Table 1, Table S1). Absorption and phase

167   contrast scanning modes were performed at 10, 20, and 40-fold magnification and 10 KeV, with

168   200 ms to 600 ms exposure time (Table S1). Projections were post-processed and rearranged into

169   flat- and darkfield-corrected sinograms, and reconstruction was performed on a Linux PC farm,

170   resulting in isotropic voxel dimensions of 0.65 μm, 0.33 μm and 0.16 μm.

171   **Ejaculate quantification in female spermathecae**

172   In the laboratory, *D. pachea* copulation lasts for about 30 - 40 min (Pitnick, 1993; Lang

173   & Orgogozo, 2012; Rhebergen et al., 2016; Acurio et al., 2019; Lefèvre et al., 2021). To further

174   examine when sperm transfer would take place during copulation, we interrupted copulation of a

175   single couple by vigorous shaking at various time points after copulation start and examined the

176   presence of ejaculate about 24 h later in the female sperm storage organs (Table S2). We

177   prepared mating complexes as described above and monitored the mating progress by annotation

178   of the courtship and copulation duration. The former was defined as the period from the first

179   male "licking" courtship behavior (Spieth, 1952)□ to the start of copulation. Copulation start

180   was defined as the moment when the male mounted the female and copulation end was

181   considered to be the moment when the male had dismounted, with genitalia and forelegs being

182   fully detached from the female. Copulation was interrupted at 4 min, 8 min, 12 min, 18 min, and

183   24 min after copulation start by shaking the tube until the couple separated. The male was stored

184   in 96% ethanol and the female was transferred into a food vial for 12-16 hours at 25°C. The

185   number of eggs laid were counted (Table S2) and the female paired spermathecae were prepared

186   by opening the dorsal side of the abdomen and then separating them from the reproductive tract.

187   The remainder of the female's body was stored together with the corresponding male in 96%

188   ethanol.

189   For imaging, spermathecae were transferred into a transparent dissection dish, filled with

190   phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM

191   KH₂PO₄, pH 7.4) and examined in lateral view with a VHX2000 (Keyence) microscope

192   equipped with a 100-1000x VH-Z100W (Keyence) zoom objective at 500 fold magnification.

193   For data analysis, file-names were replaced by a three-digit random number so that sperm

194   presence was quantified blindly with respect to the annotated copulation duration. Ejaculate

195   presence or absence in the spermathecae was annotated based on visual inspection according to

196   previously published protocols (Jefferson, 1977; Lefèvre et al., 2021)□.

197   The uterus and / or the spermathecae of females of relevant synchrotron X-ray

198   tomographic microscopy samples contained an apparent ejaculate mass that mainly formed

199   spheres of about 5 μm. We counted these spheres to approximate sperm abundance and to

200   compare the relative deposition of sperm in the left and right spermatheca (Table S1). For

201   quantification, we used Imaris 9.9 software (https://imaris.oxinst.com/) to manually superimpose

202   artificial spherical objects with identified sperm mass spheres, using the Imaris "spot" option.

203

Results

The male phallus is asymmetric

206   The phallus of *D. pachea* has a complex and asymmetric shape (Acurio et al., 2019)□

207   (Figure 1). In dorsal/rostral view, the distal part (apex) is rather flat but pointed at the apical tip

208   (Figure 1B). It is strongly curved or folded at the base. The phallotrema is positioned dorso-

209   apically on the right side of the phallus. In lateral view (Figure 1A, C), a broadened ridge on the

210   apical side and a slender ridge on the more distal side are observed. The ejaculatory duct is also

211   located at the right side of the phallus and ends in the right-sided apical phallotrema (Figure 1A,

212   B). In contrast, the uterus of a virgin female appears to be bilaterally symmetrical (Figure 2A). It

213   appears to be S-shaped and forms a major sack with a dorsal and a ventral blind protrusion

214   (Figure 2B). The anterior part of the uterus forms a narrow tube that leads to the ovaries (ovaries

215   are not included in the 3D-model).

The reproductive tract rearranges during copulation

217   The uterus re-arranges and re-orients during copulation, similar to previous observations

218   in females *D. melanogaster* Meigen (Adams & Wolfner, 2007; Mattei et al., 2015)□ (Figure 2).

219   Uterus and oviduct appear to be elongated along the antero-posterior axis of the female. The

220   ventral blind protrusion, found in virgin females, is not detected and two constrictions are

221 observed that allowed us to subdivide the female reproductive tract into three compartments

222 (Figure 2E, configuration at 20 min after copulation start): 1) the vagina, which is the most

223 caudal part, 2) a balloon-shaped medial uterus, and 3) a broadened oviduct (Figure 2D). The

224 vagina appears to be elastic. It harbors and adopts the shape of the dorsal male phallus, which

225 does not reach into the uterus but stretches the vagina in opposite dorsal-caudal direction. The

226 male genital lobes (Figure 2E) are positioned externally against the ventral female abdomen wall

227 (not shown in the model). Together with the phallus they appear to be used to lever or clamp the

228 male abdomen against the female abdomen (Figure 2D, E). The caudal positon of the phallus tip

229 with the male phallotrema is most distantly to the anterior parts of the female reproductive tract.

230 Ejaculate needs to pass through the vagina and along the phallus towards the uterus in order to

231 reach the female spermathecae (Figure 2E). The vagina is dorsally pointed at its anterior end

232 where it resembles the curved phallus base (Figure 2E). The expanded medial uterus is separated

233 from the anterior oviduct by a constriction, which also links the ducts to the spermathecae and

234 the seminal receptacle at the dorsal and ventral side, respectively (Figure 2D – F). The oviduct

235 forms a broad vesicle of the size of the uterus, whereas it is narrow in virgin females and in *D.*

236 *melanogaster* (Adams & Wolfner, 2007)□ (Figure 2B, E) (ovaries were not included in the

237 model).

238 **Different sperm morphologies in the female reproductive tract**

239   Similar to previous findings (Pitnick, Markow & Spicer, 1999)□, no sperm cells were

240 found in the seminal receptacle. By means of high-quality scans using SRXTM it was possible to

241 visualize sperm mass within the female reproductive tract (Figure 3, Table S1). The sperm cells

242 appear to adopt different aggregation states in different parts of the uterus. They are spherically

243 coiled or granular in the medial uterus (Figure 3A), while they are filamentous when entering the

244 spermathecae (Figure 3B) and eventually re-adopt a spherical organization in the spermathecae

245 (Figure 3C). No distinction could be made between the sperm tails and heads and it is not clear if

246 the identified spheres are filled or rather hollow "bubbles" that aggregate sperm tissue at the

247 surface. Overall, it is not possible to deduce if the identified shapes correspond to single cells,

248 but the number of spheres in the uterus and in the spermathecae appear to correlate with the total

249 amount of sperm mass.

250 **Sperm transfer can occur at 2 min after copulation start and is found in both spermathecae**

251   We observed sperm presence in female spermathecae by dissections of females about 12-

252 24 h after copulation. The spermathecae of virgin females (n=9) were empty. Apparent sperm

253 masses were identified after 4 min of copulation (2/12 trials) (Figure 4). Overall, sperm transfer

254 into the spermathecae occurred only in a minority of trials (13/64). Highest frequencies of

255 ejaculate presence were observed after 8 min and 12 min (4/14 and 4/11 observations,

256 respectively) of copulation. Unexpectedly, longer copulation duration of 18 min and 24 min

257 yielded fewer cases of sperm presence in the spermathecae (2/12 observations and 1/15

258 observations, respectively). In all 13 trials with observable sperm transfer, both spermathecae

259 revealed apparent sperm mass. In order to quantify different sperm levels in our synchrotron X-

260 ray tomography scans, we examined the spherical organization of sperm mass in the uterus and

261 in the spermathecae and counted the relative abundance of spheres in the left and right

262 spermathecae (Table S1). Upon copulation, sperm was present in the female reproductive tract in

263 the majority of scans (14/19 scans), but in different parts of the reproductive tract: in the uterus,

264 spermathecae, spermathecal tubes, and in the broadened anterior part of the uterus (Tables S1,

265 S3). Sperm presence in the uterus was even detected in one couple at 2 min after copulation start

266 (1/4 interpretable scans) and spermathecae contained sperm at least 8 min after copulation start

267 (1/3 interpretable scans) (Table S1). Sperm sphere abundance varied between the left and the

268 right spermatheca (Table S1) but no particular bias of sperm storage towards the left or the right

269 spermatheca was observed among couples or at different time points after copulation start.

270 ## Discussion

271 **Genital asymmetry is not reflected in sperm allocation**

272   Although we were unable to precisely quantify sperm, we observed no left or right-sided

273 bias in the way sperm were deposited into the female reproductive tract. The distance between

274 the male phallotrema and the female receptacles remains large at all stages of copulation, and it

275 appears impossible for the asymmetric phallus to preferentially deposit sperm unilaterally in the

276 female. Sperm allocation, may therefore not be controlled by the intermittent phallus

277 morphology, but by female structures or by components of the male ejaculate. Copulatory and

278 post-copulatory uterus rearrangements in *D. melanogaster* females are induced by male

279 accessory gland proteins and are associated with sperm storage and fertilization (Adams &

280   Wolfner, 2007; Mattei et al., 2015)□. Selective sperm storage has been associated with female

281   muscular activity in female yellow dung flies *Scathophaga stercoraria* (L.), which was found to

282   be sensitive to CO_2 anesthetization and was proposed to rely on active female muscular

283   movements (Hellriegel & Bernasconi, 2000)□. In a specific case, external and non-intermittent

284   male genital asymmetry was found to indirectly stimulate postcopulatory sperm distribution in

285   females of the fly *Dryomyza anilis* Fallén. During copulation, the male taps the female external

286   genitalia after insemination with a pair of genital claspers (= surstyli: Rice et al., 2019), which

287   can be symmetric or asymmetric. Male tapping affects sperm distribution in female sperm

288   storage organs (Otronen & Siva-Jothy, 1991; Otronen, 1997)□ and males with asymmetric small

289   claspers reveal higher fertilization success compared to males with large symmetric lobes since

290   asymmetric male tapping probably affects sperm distribution in the spermathecae (Otronen,

291   1998)□. However, we know of no cases where the male phallus morphology influences sperm

292   storage patterns in fly species. Phallus asymmetry in *D. pachea* may therefore rather ensure a

293   relatively low basal or minimal efficiency of ejaculate transfer and stabilize the complex of male

294   and female genitalia during copulation, but appears to be unrelated to postcopulatory sperm

295   storage.

296   **Phallus asymmetry is associated with a specific orientation inside the female uterus**

297   We identified a curious orientation of the *D. pachea* male phallus inside the female

298   uterus, which is strikingly different from previous findings in *D. melanogaster*. While in *D.*

299   *melanogaster* and closely related species, the phallus points anteriorly towards the uterus and

300   spermathecae (Yassin & Orgogozo, 2013; Mattei et al., 2015)□, it points to the caudal female

301   body wall in *D. pachea*. The *D. pachea* phallus is curved at its base. It appears that the phallus

302   functions on a mechanical basis to anchor the male abdomen on top of the female. Sperm is

303   released from this most caudal position of the phallus and needs to pass the phallus base to reach

304   the anterior uterus and female sperm storage organs. This is an intriguing observation since

305   direct ejection of ejaculate into more anterior parts of the uterus would seem more appropriate

306   for efficient gamete transfer. The copulation complex in *D. pachea* is shifted 6-8° to the right

307   side with respect to the female antero-posterior midline (Rhebergen et al., 2016)□. Extrapolating

308   this shift to the dorsal apex of the male phallus, the right-sided phallotrema is located in a medial

309   position (Figure 5). The dorsally pointed phallus structures stretch the dorsal vagina (like a

310   covering sheet). The ventrally pointing asymmetric phallus spurs (Acurio et al., 2019)□ must be

311 directed towards the caudal body wall and can potentially function as a grasping device to ensure

312 an internal grip of the phallus. Alternatively, they may provide ventral flaps that avoid a close

313 covering of the ventral vagina and therefore may stabilize a ridge in which ejaculate may be

314 transported. Overall, the asymmetric phallus morphology may function to stretch the vagina in a

315 specific configuration to enable efficient sperm release.

316 **The uterus reshapes during sperm transfer**

317 The uterus assumes a different shape during copulation compared to a virgin uterus,

318 similar to previous observations in *D. melanogaster* (Adams & Wolfner, 2007; Mattei et al.,

319 2015)☐☐. However, broadening of the anterior part of the uterus was only observed in *D.*

320 *pachea*. While it enlarges, the constriction at the junction to the spermathecal ducts remains

321 narrow. This could potentially induce a vacuum and could either induce transport of the egg or

322 perhaps result in a suction force to transport ejaculate towards the anterior part of the uterus.

323 However, we have no data on the specific temporal dynamics of this re-arrangement. Future

324 studies must analyze uterus shapes with progression of sperm and the eggs. Female *D. pachea*

325 are rarely observed to lay eggs after a single copulation (Pitnick, 1993; Lefèvre et al., 2021)☐,

326 indicating that uterus reshaping might be related to other processes.

327 **Low efficiency transfer of a complex ejaculate mass**

328 Sperm transport into female spermathecae was observed only in a minority of mating

329 trials. One possible explanation for this low rate is that our laboratory conditions may not

330 provide optimal ecological parameters for this species and this may influence copulatory

331 processes. Also, we use a laboratory stock that has been maintained for > 20 years in captivity

332 and which might reveal different sperm transfer frequencies with respect to wild *D. pachea*.

333 Previous reports on *D. pachea* ejaculate transfer did not report that sperm is not always released

334 from the male phallus during copulation (Pitnick, 1993; Pitnick & Markow, 1994)☐.

335 Nevertheless, our observations might indicate that ejaculation is, in fact, rare in *D. pachea*.

336 Males of *D. pachea* were reported to transfer only 44 ± 6 sperm cells per copulation (Pitnick &

337 Markow, 1994)☐, which was found to be far below the female sperm storage capacity. Males

338 produce giant sperm of >16 mm length (Pitnick & Markow, 1994) and the male investment into

339 spermatogenesis was argued to be high compared with other *Drosophila* species. Thus, males

340 might be selective with regard to the females to whom they would allocate their limited amount

341   of gametes (Pitnick & Markow, 1994)☐. Male gametes might even be so rare, that the females

342   would require to mate multiple times to reproduce at all. Wild-caught *D. pachea* females were

343   reported to contain sperm from at least 3-4 males and females are known to remate with various

344   males (Pitnick & Markow, 1994)☐. Alternatively, sperm might be discarded or absorbed by the

345   female during or shortly after copulation or the female may prevent the male from ejaculating.

346   Both might relate to female controlled cryptic mate-choice (Eberhard, 1996)☐. However, in the

347   majority of synchrotron X-ray tomography scans, sperm was present at least in some parts of the

348   female reproductive tract. Our data indicates that sperm release from the phallus and sperm

349   transport into the spermathecae might be two independent physiological processes. Again, the

350   overall number of gametes in each ejaculate is low and females often remate. Thus, the relative

351   loss of gametes per copulation is also low. Overall, the reproductive strategies for both sexes

352   may imply a high level of promiscuity to compensate for a rather inefficient sperm transfer

353   process during copulation.

354   However, when copulation does result in sperm transfer, it is released early during

355   copulation, from at least 2 min after the start of copulation (the entire copulation lasts up to 40

356   minutes). Similar results were reported from remating experiments in *D. melanogaster* (Gilchrist

357   & Partridge, 2000)☐, where males required about 4-8 min to replace sperm from a previous

358   mating. Intriguingly, longer copulation duration of *D. pachea*, of 18 min and 20 min yielded

359   fewer cases of sperm transfer compared to shorter copulation durations of 8 min or 12 min.

360   Previous analyses also revealed that copulation duration was negatively associated with sperm

361   transfer rates in *D. pachea* (Lefèvre et al., 2021). Male dependent sperm allocation is not

362   expected to show this pattern and was not observed in our X-ray computed tomography scans

363   where the majority of scans revealed sperm presence in other parts of the female reproductive

364   tract. Our observations indicate that sperm can be dumped by the female and this might occur if

365   copulation takes too long, which can be a parameter of male performance during copulation

366   (Eberhard, 1994)☐. Alternatively, it could be argued that our data for the longer copulation

367   durations are biased towards couples where the males continued copulating *because* they had not

368   yet been able to transfer ejaculate. However, the investigated time-points were below the

369   maximum copulation duration of 30-40 min seen in this species (Pitnick, 1993; Lang &

370   Orgogozo, 2012; Rhebergen et al., 2016; Acurio et al., 2019; Lefèvre et al., 2021). We did not

371   observe any couple that ended copulation before they were artificially separated.

372   Sperm was observed to be differentially organized in different organs. In the medial part
373   of the uterus it had a granular shape while it was filamentous when entering the spermathecae. In
374   *D. melanogaster*, sperm was reported to assume a torus shape inside the female uterus and such
375   tori slowly rotate (Adams & Wolfner, 2007)□. It remains to be investigated at better resolution,
376   if granular shaped sperm in *D. pachea* also approximates these tori conformations. What also
377   needs further elaboration is whether these giant sperm are motile or are transported passively by
378   other male ejaculate components or by female peristalsis. The aggregation changes might be
379   related to the organization of the sperm tail, which may be non- or less motile. The filament
380   perhaps undergoes dynamic aggregations or crosslinks with other sperm tails or with different
381   parts of the tail. This could result in compaction and elongation of sperm mass and may explain
382   the different aggregation states observed in our microCT and synchrotron X-ray tomographic
383   scans.

Conclusions

385   The positioning of the asymmetric *D. pachea* male phallus was evaluated during
386   copulation inside the female reproductive tract. The twisted copulation complex likely positions
387   the right sided phallotrema into a medial location at the caudal end of the vagina. This particular
388   positioning of the male phallotrema, the inflation of the oviduct during copulation and the low
389   frequency of sperm transfer in different trials indicate a complex and inefficient sperm transport
390   into female sperm storage organs during and after copulation. No evidence was obtained that the
391   asymmetric male genitalia allow asymmetric sperm deposition inside the female reproductive
392   tract. Future investigations must unravel if transfer of giant sperm might be non-autonomous in
393   *D. pachea,* potentially mediated by re-arrangements of the female uterus and by different
394   aggregation states of sperm during transport through the female reproductive tract.

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519 Figure legends

520 **Figure 1: Asymmetric *Drosophila pachea* phallus morphology**. 3D-Model of the male phallus
521 (blue) and ejaculatory duct (light yellow). The terms left lateral, caudal, right lateral describe the
522 orientation with respect to the male abdomen. The scale bar is 100 µm.

523 **Figure 2: The female reproductive tract rearrangements during copulation**. 3D-Models of
524 virgin female *D. pachea* (A,B) and copulating couples (C-F). The female accessory glands and
525 ovaries are not included into the model, A) Virgin reproductive tract (pink) in caudal view,
526 ovipositor (magenta) and spermathecae with spermathecae ducts (orange-gold). (B) Virgin uterus
527 in lateral view (color codes as in A) and the seminal receptacle (yellow). (C) Copulating couple
528 in lateral view, exterior cuticles of female and male (grey) semi-transparent and relative location
529 of colored female reproductive tract and male external genitalia (green). (D-F) Copulation
530 complex of Figure 2 C at higher magnification: female reproductive tract (pink), spermathecae
531 and ducts (orange-gold), seminal receptacle (yellow) and male-phallus (blue), ejaculatory tract
532 (light yellow) and genital arch (green), red lines indicate assigned compartment borders of the
533 female reproductive tract into vagina, uterus and oviduct. (D) Dorsal view, (E) lateral view, (F)
534 ventral view. The scale is 100 µm.

535 **Figure 3: Sperm aggregates in the female reproductive tract.** (A) Female uterus harboring
536 granular sperm aggregates, indicated by blue arrows. (B) Spermatheca with filamentous sperm
537 (red arrows). Filamentous sperm that enters the spermatheca is indicated by yellow arrows. (C)
538 Transverse section of a spermatheca, spherical organization of sperm mass is highlighted by red
539 circles. The scale is 100 µm.

540 **Figure 4: Ejaculate transfer occurs early during copulation**. Fraction of female spermathecae
541 that revealed apparent sperm mass from copulations that were interrupted at different time points
542 after copulation start.

543   **Figure 5: Asymmetric positioning of the male phallus.** Contour drawings of the dorsal apex of

544   the *D. pachea* male phallus. (A) The male gonopore (filled black) is located at the right side of

545   the phallus. (B) The twisted complex of female and male genitalia (Rhebergen et al., 2016)☐ is

546   expected to shift the male apex by 6-8° which should result in a medial positioning of the male

547   gonopore with respect to the female antero-posterior midline (dashed line).

548

Number of scanned *D. pachea* copulation complexes and single females

Virgin female	Virgin male	Copulation complexes, after copulation start (ACS)				Mated female	Mated male
		2 min	8 min	15 min	20 min	2 h ACS	2 h ACS
2	3	5	4	4	3	4	1

Figure 1

Asymmetric *Drosophila pachea* phallus morphology.

3D-Model of the male phallus (blue) and ejaculatory duct (light yellow). The terms left lateral, caudal, right lateral describe the orientation with respect to the male abdomen. The scale bar is 100 μm.
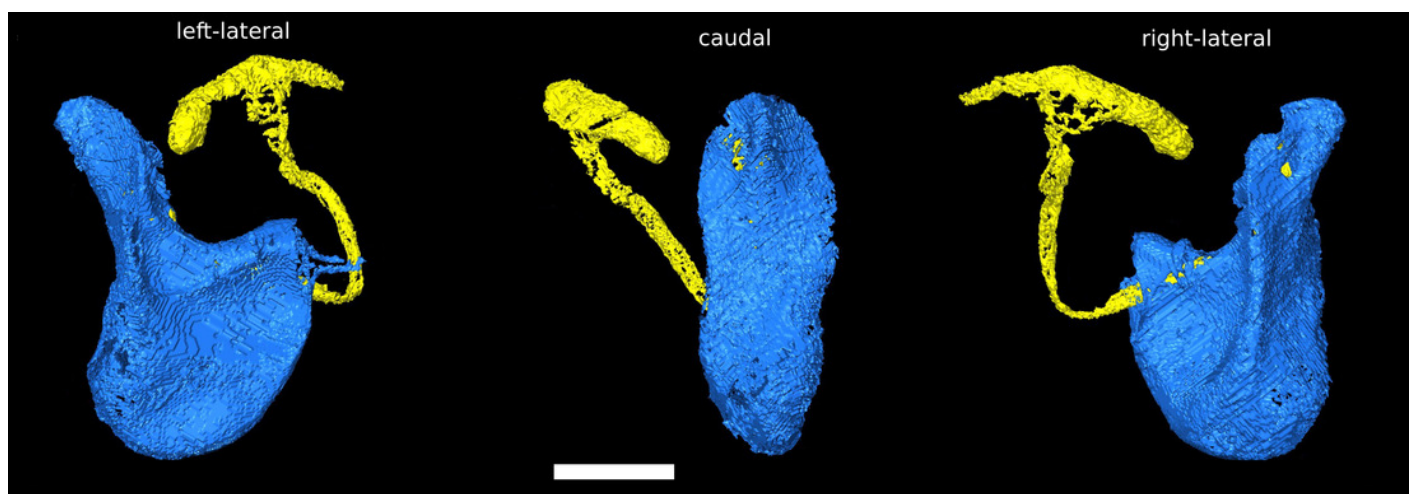
Figure 2

The female reproductive tract rearrangments during copulation.

3D-Models of virgin female *D. pachea* (A,B) and copulating couples (C-F). The female accessory glands and ovaries are not included into the model, (A) Virgin reproductive tract (pink) in caudal view, ovipositor (magenta) and spermathecae with spermathecae ducts (orange-gold). (B) Virgin uterus in lateral view (color codes as in A) and the seminal receptacle (yellow). (C) Copulating couple in lateral view, exterior cuticles of female and male (grey) semi-transparent and relative location of colored female reproductive tract and male external genitalia (green). (D-F) Copulation complex of Figure 2 C at higher magnification: female reproductive tract (pink), spermathecae and ducts (orange-gold), seminal receptacle (yellow) and male-phallus (blue), ejaculatory tract (light yellow) and genital arch (green), red lines indicate assigned compartment borders of the female reproductive tract into vagina, uterus and oviduct. (D) Dorsal view, (E) lateral view, (F) ventral view. The scale is 100 μm.
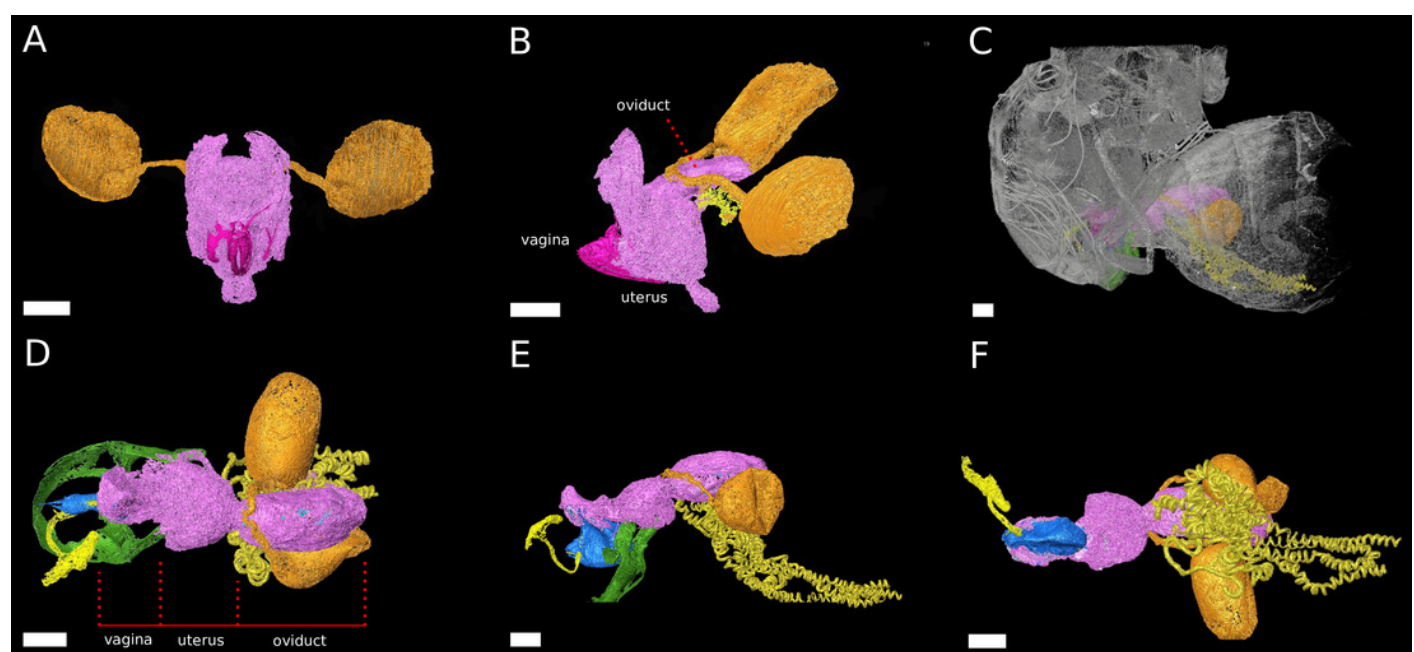
Figure 3

Sperm aggregates in the female reproductive tract.

(A) Female uterus harboring granular sperm aggregates, indicated by blue arrows. (B) Spermatheca with filamentous sperm (red arrows). Filamentous sperm that enters the spermatheca is indicated by yellow arrows. (C) Transverse section of a spermatheca, spherical organization of sperm mass is highlighted by red circles. The scale is 100 μm.
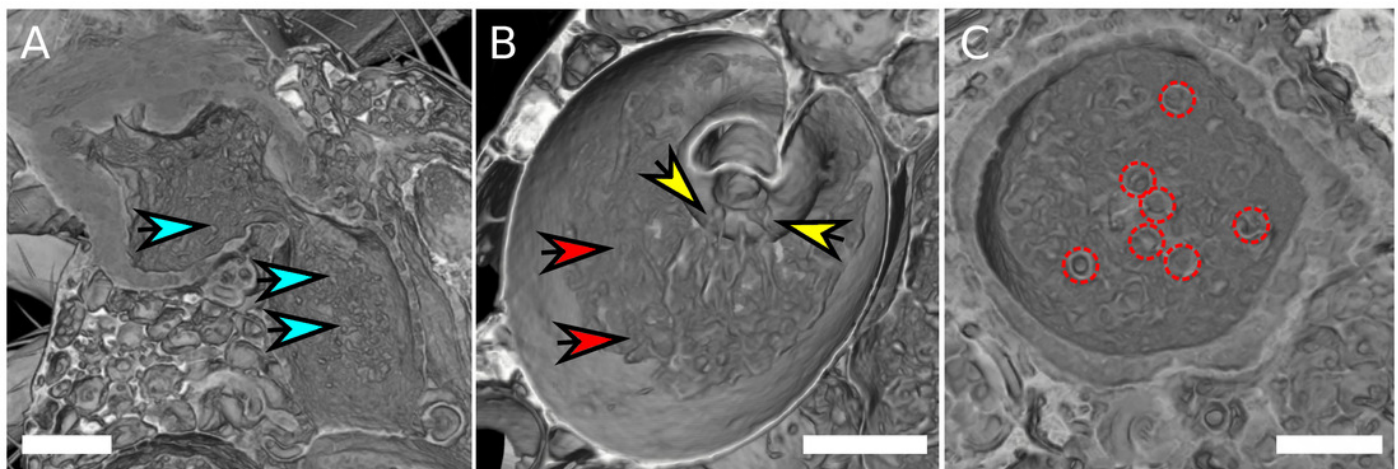
Figure 4

Ejaculate transfer occurs early during copulation.

Fraction of female spermathecae that revealed apparent sperm mass from copulations that were interrupted at different time points after copulation start
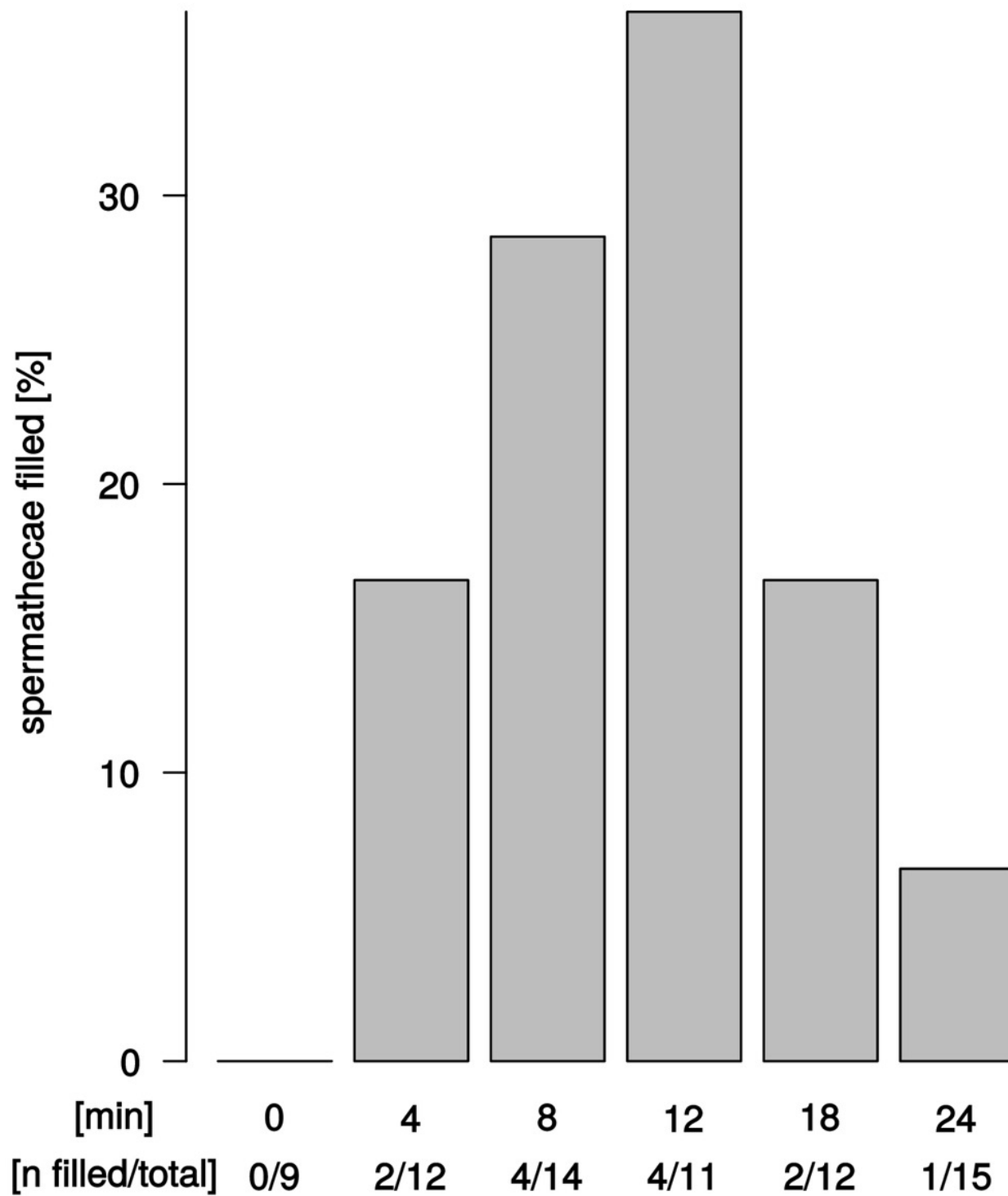
Figure 5

Asymmetric positioning of the male phallus.

Contour drawings of the dorsal apex of the *D. pachea* male phallus. (A) The male gonopore (filled black) is located at the right side of the phallus. B) The twisted complex of female and male genitalia (Rhebergen et al., 2016) is expected to shift the male apex by 6-8° which should result in a medial positioning of the male gonopore with respect to the female antero-posterior midline (dashed line).