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Refutation of traumatic insemination in the *Drosophila bipectinata* species
complex: Hypothesis fails critical tests

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17 **Abstract**

18 Traumatic insemination (TI) is a rare reproductive behaviour characterized by the transfer of sperm
19 to the female via puncture wounds inflicted across her body wall. Here, we challenge the claim made
20 by Kamimura (2007) that males of species of the *Drosophila bipectinata* complex utilize a pair of
21 claw-like processes (“claws”) to traumatically inseminate females: the claws are purported to
22 puncture the female body wall and genital tract, and to inject sperm through the wounds into the
23 genital tract, bypassing the vaginal opening, the route of sperm transfer occurring in other
24 *Drosophila*. This supposed case of TI is widely cited and featured in prominent subject reviews. We
25 examined high-resolution scanning electron micrographs of the claws and failed to discover any
26 obvious “groove” for sperm transport. We demonstrated that sperm occurred in the female
27 reproductive tract as a single integrated unit when mating flies were experimentally separated,
28 inconsistent with the claim that sperm are injected via paired processes. The aedeagus in the
29 *bipectinata* complex was imaged, and shown to deliver sperm through the vaginal opening. Laser
30 ablation of the sharp terminal ends of the claws failed to inhibit insemination. The results refute the
31 claim of TI in the *Drosophila bipectinata* species complex.

32

33 **Key words:** *Drosophila bipectinata* species complex; traumatic insemination hypothesis; copulation;
34 aedeagal lateral processes; genital claws; anchoring; sperm delivery

35 1. Introduction

36 Traumatic insemination (TI) is a form of mating behaviour during which males employ
37 specialized “devices”, such as spines and stylets, to puncture the female body wall and transfer sperm
38 through the wound(s) (Lange et al., 2013). This extraordinary behaviour is distinguished from other
39 forms of “traumatic mating”, where only non-sperm components of the ejaculate, or no ejaculate at
40 all, transfer to the female through male-inflicted wounds (Blanckenhorn et al., 2002; Siva-Jothy,
41 2009; Lange et al., 2013; Reinhardt et al., 2015).

42 Though rare, TI *sensu stricto* has arisen independently in a number of animal groups
43 (Reinhardt et al., 2015), and the evolutionary drivers of this unusual form of insemination are of
44 considerable interest and the focus of ongoing debate (Eberhard 1985, 1996; Arnqvist and Rowe,
45 2005; Tataric and Cassis, 2010; Lange et al., 2013; Tataric et al., 2014; Dougherty et al., 2017;
46 Brand et al., 2021). The identification of *bona fide* cases of TI in animals will be important not only
47 for accurately documenting the taxonomic distribution of this remarkable form of mating (Reinhardt
48 et al., 2015), but also for facilitating the comparative method to test hypotheses about the selective
49 pressures favoring its emergence in evolution (Futuyma and Kirkpatrick, 2017, p. 69). Distinguishing
50 the various forms of traumatic mating also lies at the heart of our ability to predict specific selective
51 challenges females may face during mating, and hence to interpret immunological, anatomical, and
52 behavioural features as potential counter-adaptations to variable forms of male-induced harm in the
53 broader context of sexual conflict theory (Johnstone and Keller, 2000; Hosken et al., 2003; Morrow
54 et al., 2003; Arnqvist and Rowe, 2005; Rönn et al., 2007; Siva-Jothy, 2009; Dougherty et al., 2017).

55 Among terrestrial arthropods, TI is particularly prevalent in the hemipteran infraorder
56 Cimicomorpha, which includes the well-studied human bed bug, *Cimex lectularius*. Males of this
57 species pierce the ventral surface of a female’s abdomen with a curved, needle-like, hollow stylet (or
58 paramere) (Usinger, 1966) that possesses a sizeable pore near its tip (Fig. 1), through which sperm
59 and seminal fluid are injected into the female (Davis, 1956; Carayon, 1966). Cases of TI have also
60 been convincingly demonstrated in the plant bug genus *Coridromius* (Tataric & Cassis, 2010), the

61 spider *Harpactea sadistica* (Řezáč, 2009), and the marine flatworm *Pseudocerus bifurcus* (Michiels
62 and Newman, 1998; and see Brand et al., 2021).

63 It has been claimed by Kamimura (2007) (henceforth K2007) that TI occurs in species of the
64 *Drosophila bipectinata* complex, a small taxonomic grouping of four very similar species in the
65 *ananassae* subgroup of the *melanogaster* species group, that includes: *D. bipectinata* Duda, 1923; *D.*
66 *parabipectinata* Bock, 1971; *D. malerkotliana* Parshad and Paika, 1965; and *D. pseudoananassae*
67 Bock, 1971 (Bock, 1971). Specifically, males of these species are purported to use a pair of claw-like
68 phallic structures, called “basal processes” by K2007, but which we call aedeagal lateral processes
69 following Rice et al. (2021), as explained below, to pierce the female body wall and reproductive
70 tract, and inject sperm into her reproductive tract through the wound sites. If true, then TI in this
71 complex would be an astonishing evolutionary innovation within the genus, family and order.

72 A wide range of species of *Drosophila* and related genera have been used to investigate
73 dipteran reproductive biology in detail. This body of knowledge both underscores the astounding
74 nature of the TI claim and provides a thorough understanding of the “typical” route of sperm transfer,
75 storage and use, which is particularly well understood for the model organism, *D. melanogaster*. In
76 this species, mating lasts approximately 20 min during which the male and female attach through the
77 union and integration of various male and female genital structures (Eberhard and Ramirez, 2004;
78 Jagadeeshan and Singh, 2006; Mattei et al., 2015). The intromittent organ in males is the aedeagus,
79 and the ejaculate, comprised of sperm and seminal plasma, pass via the tip of the aedeagus into the
80 female uterus (bursa) via her gonopore (Bairati, 1968; Fowler, 1973; Manier et al., 2010; Mattei et
81 al., 2015). The transferred ejaculate fills and can considerably swell the bursa in this and other
82 species (Patterson, 1946; Lefevre and Jonsson, 1962; Markow and Ankney, 1988; Alonso-Pimentel
83 et al., 1994). Upon dissection of the female after the termination of copulation, the ejaculate can
84 readily be visualized intact by teasing the bursa open and releasing the sperm mass associated with
85 its “waxy” plug material (Alonso-Pimentel et al., 1994; Pitnick and Markow, 1994; Polak et al.,
86 1998). As sperm entry into storage within the female requires some time to begin (approximately 4

87 minutes in *D. melanogaster*) after the end of copulation (Manier et al., 2010), the dissected sperm
88 mass may be discerned intact with its full complement of sperm, so long as it is dissected from the
89 bursa quickly after copulation (Pitnick and Markow, 1994; Manier et al., 2010; Polak and Rashed,
90 2010; Tyler et al., 2021).

91 In K2007's study, adult males were allowed to ingest food containing rhodamine-B
92 fluorescent dye, and were mated to virgin females. Mating pairs were flash frozen in liquid nitrogen
93 5 minutes after the onset of copulation (uninterrupted copulations last, on average, approximately
94 10.6 min in *D. bipectinata*, Polak et al., 2021, and see below), and abdomens of coupled pairs
95 removed, mounted and observed. A laser scan micrograph showing two areas of coloration adjacent
96 to the tips of the basal processes (figure 1 (c) (ii) in K2007) was evidence put forward for the
97 occurrence of TI. On the basis of these images, Kamimura claimed that the two claw-like processes
98 pierce the female's body wall, and transfer sperm through the wounds into the female reproductive
99 tract, bypassing the opening of the vagina (gonopore). The following is the relevant excerpt from
100 K2007 (pp. 403–404): "TI clearly occurs in the *bipectinata* complex, as the basal processes pierce the
101 pockets during copulation and sperm is ejaculated through the wounds but not through the genital
102 orifice...". No direct evidence for the passage of sperm via such a route was provided. The following
103 statement likewise gave us pause (p. 404): "The basal processes of this group have a groove on the
104 dorsal surface which may transport semen." The problem also here is that no visual evidence for such
105 a groove was presented.

106 Another source of our skepticism regarding K2007's claim comes from our own observations
107 of the ejaculated sperm mass in *D. bipectinata* made previously (Polak and Rashed, 2010; Tyler et
108 al., 2021). Upon dissection from the female bursa immediately after copulation, the sperm in *D.*
109 *bipectinata*, which are 1.63 mm long (Tyler et al., 2021) and, for reference, slightly shorter (by c.
110 12%) than in *D. melanogaster* (1.85 mm, (Manier et al., 2013)), invariably occur as a *single and*
111 *strongly integrated mass* in association with characteristic "waxy" plug material, similar in
112 appearance to that seen in *D. melanogaster*. This observation challenges the TI hypothesis of

113 insemination via paired “basal processes”, because if TI occurs we would expect the injected sperm
114 mass within the bursa immediately after copulation to be discernable as two more-or-less distinct
115 units, but we have consistently observed only a single, highly integrated mass (Tyler et al., 2021).
116 The sperm tails in *D. bipectinata* are long and the sperm mass consequently is difficult to disentangle
117 (Tyler et al., 2021), so the idea that two separate masses injected via the paired “basal processes”
118 would then dynamically coalesce during mating, or shortly thereafter, and form a single
119 homogeneous mass with associated plug material, seems unlikely.

120 Here, we challenge the claim that TI occurs in the *bipectinata* complex. Our results provide
121 observational and direct experimental evidence contradicting such a claim: the evidence supports
122 sperm transfer occurring via the route of sperm delivery in other *Drosophila*, that is, through the
123 female gonopore into the reproductive tract. In the present section we first offer a reinterpretation of
124 the male phallic architecture of species within the *bipectinata* complex, following the standardized
125 nomenclature proposed for *D. melanogaster* (Rice et al., 2019) and refined for a wider group of
126 species following developmental studies (Rice et al., 2021). On the one hand, we agree with K2007
127 that the paired claw-like structures, the purported piercing organs of TI, are not a “bifid aedeagus”, a
128 terminology adopted by earlier authors. Okada ((Okada, 1954), pl. 3, fig. 14) illustrated the *D.*
129 *bipectinata* male terminalia and labelled the paired structures “aedeagus”, and Parshad and Paika
130 used the term “bifid” in describing the same paired structures (Parshad and Paika, 1964). Bock
131 (1971) used the descriptor “aedeagus bifid and bare” to distinguish the *bipectinata* complex, and
132 Bock and Wheeler (1972) and Gupta (1973) followed this usage. On the other hand, we also agree
133 with Rice et al. ’s (2021) suggestion that the claw-like structures should be termed “aedeagal lateral
134 processes”, rather than K2007’s “basal processes”. Rice et al. (2021) showed that the claws have a
135 distinct developmental origin, deriving from the *lateral* portions of the central primordium of the
136 phallus observable during metamorphosis in the pupa. Since most of the cells of the central
137 primordium normally give rise, in related *Drosophila* subgroups, to the aedeagus (not to postgonites,

138 pregonites, or postgonal sheath) the term *aedeagal lateral process* was proposed by Rice et al.
139 (2021), a term we adopt here.

140 As a first step in our test of the TI hypothesis, we examined scanning electron micrographs of
141 the aedeagal lateral processes (claws) at varying orientations and magnifications to search for the so-
142 called “groove”—the alleged conduit for sperm delivery. We then addressed two additional
143 predictions. The first is that the sperm mass should be observable as two more-or-less distinct units
144 upon transfer to the female, as mentioned above. To this end, we dissected and examined the
145 ejaculated sperm masses extracted from the female reproductive tract both immediately after the
146 terminus of full-length, uninterrupted copulations, and after pairs were interrupted 6–8 min after the
147 onset of coupling. Reproductive structures of both sexes were also examined after copulation
148 interruption to elucidate the path of sperm transfer to the female. Finally, we used ultraprecise laser
149 surgery (Polak and Rashed, 2010) to ablate the terminal ends of both claws in individual males, thus
150 eliminating their pointed tips. If the claws serve to transfer sperm by piercing across the female’s
151 body wall and reproductive tract, then males with surgically ablated piercing devices should fail to
152 transfer sperm.

153

154 **2. Material and methods**

155 **(a) Source and culture of flies**

156 *Drosophila bipectinata* Duda and *D. parabipectinata* Bock cultures were established with field-
157 caught flies captured from the surface of fallen fruits in Taiwan (25°2'30.24" N, 121°36'39.37" E). A
158 *D. malerkotliana* Parshad and Paika culture was established with flies from Thailand (8°54'22.24"N
159 98°31'43.51"E). Flies were cultured in half-pint glass bottles on standard cornmeal-agar medium
160 within an environmental chamber under controlled light and temperature conditions (12h light
161 (24°C):12h dark (22°C)). Adult virgin flies for mating trials were harvested under a light stream of
162 humidified CO₂ within 8 h of emergence, and housed in groups of 10–15 flies in 8-dram food vials
163 containing cornmeal-agar medium. All flies used in mating trials were 4–6 d old. Material for

164 morphological examination and imaging of genitalia were also derived from ethanol-preserved
165 specimens held in the Australian Museum, Sydney (AMS K.380306–07).

166

167 **(b) Laser surgery**

168 The laser surgical protocol is described in detail elsewhere (Polak and Rashed, 2010). Briefly, young
169 males (< 24 h of age) were anesthetized with CO₂ in an acrylic chamber with a thin glass bottom.

170 The male was positioned ventral side down in the chamber, so the external genitalia were visible
171 from below and accessible to the laser light. The chamber was mounted on a Prior (Rockland, MA,

172 USA) H117 motorized stage fitted to an Olympus (Center Valley, PA, USA) IX71 inverted light

173 microscope. Individual pulses of light ($\lambda=532$ nm) from a Vector 532-1000-20 Q-switched laser

174 (Coherent, Santa Clara, CA, USA) focused through an Olympus UPlanApo 20x objective were used

175 to ablate 1/4 to 1/3 of both lateral processes (claws) of individual males. After surgery, the fly was

176 gently aspirated out of the chamber, and allowed to recover in groups of 3–5 males in food vials for

177 at least 3 d until individually paired with virgin females. Uncut control males were treated identically

178 to that as above, except that 1–2 large bristles near the apex of the abdomen were laser-ablated on

179 both sides of the body; their claws were untouched by laser light. These constitute the co-called

180 “surgical control” group used in previous work (Grieshop and Polak, 2012, 2014; Rodriguez-

181 Exposito et al., 2020).

182

183 **(c) Mating trials and dissections**

184 All mating trials were conducted in the morning between 8:00 am (lights on) and 11:00 am. Virgin

185 males (4–6 d of age) were each individually paired with a virgin female (3–4 d old). The onset and

186 termination of copulation were recorded, and copulation duration was taken as the difference

187 between these time points. Immediately after the termination of copulation (when the male

188 dismounted), the female was killed with ether fumes and dissected in a drop of phosphate-buffered

189 physiological saline (PBS) under an Olympus SZX12 stereomicroscope (Olympus Corp. of the

190 Americas, Center Valley, PA, USA). The female bursa was gently teased open using fine biology-
191 grade forceps. As the sperm mass was released into the saline, it was ascertained whether it was in
192 the form of a single mass or > 1 mass. On two separate mornings, copulations were interrupted using
193 a fine paintbrush to separate the pair. Immediately after the pair was separated, the female was
194 dissected and the sperm mass, if present, was released from the bursa and examined, as above. A
195 total of 12 copulations with different individuals were interrupted between 6 and 8 minutes after the
196 start of mating. One copulation was interrupted at 4 min; no ejaculate could be detected within the
197 female.

198

199 **(d) Imaging phallic structures**

200 Genitalia were dissected from alcohol-preserved specimens, or from fresh material from culture,
201 using a common procedure. Under a stereomicroscope, reproductive structures from fresh material
202 for examination and imaging were dissected or extruded in a few drops of $1\times$ phosphate buffered
203 saline (PBS) on a depression slide and imaged immediately. For male genitalic structures, genitalia
204 were dissected into 70% ethanol and teased free of attached pieces of exoskeleton and soft tissue
205 using fine tweezers and dissecting probes. The specimen then was gently boiled in 1N KOH for ≈ 8
206 min to dissolve soft tissue and to improve observation of the hard parts. Digital images of fresh
207 material and boiled genital structures were captured with a Leica M205 Stereomicroscope (Leica
208 Microsystems, Buffalo Grove, IL, USA). The light microscope images were used for 1) imaging
209 freshly dissected reproductive structures (sperm masses, female uterus, male aedeagus and female
210 oviscape), 2) visualizing, describing and annotating phallic and periphallid structures, and 3) for
211 confirming the integrity of laser-surgical manipulations. For scanning electron micrograph (SEM)
212 acquisition, specimens of fly genitalia were dissected and treated with KOH as above. They were
213 then rinsed in distilled water, air-dried, mounted on conductive carbon adhesive tabs atop an
214 aluminum post, adjusted for proper orientation, sputter coated with gold-palladium film, and imaged
215 with a SCIOS Dual-Beam Scanning Electron Microscope (ThermoFisher, Waltham, MA, USA).

216 SEMs were used primarily for examining the claws for possible sperm conduit architecture, and for
217 producing exemplars of the laser cuts. To check for sperm conduits, multiple images of the same
218 structure were taken at different magnifications (typically between 350–3500×) and different
219 orientations effected by motorized tilting of the specimen within the microscope chamber. Genitalic
220 preparations of a total of 28, 29, and 3 different individuals of *D. parabipectinata*, *D. bipectinata* and
221 *D. malerkotliana*, respectively, were imaged, and a total of 166 SEMs were examined and archived.

222

223 **3. Results**

224 **(a) Phallic architecture of the *bipectinata* species complex**

225 The phallic structures of the *bipectinata* complex are represented here by *D. bipectinata* and
226 *D. parabipectinata*; the same, or very similar, morphology is present in the other two species of the
227 *bipectinata* complex (Bock, 1971). Naming the parts of the copulatory apparatus is difficult because
228 they are exceptionally evolutionary labile, their homologies obscure (but see Rice et al., 2021), and
229 their function often speculative.

230 The male hypandrium of the *bipectinata* complex species was imaged within the body (Fig.
231 2A). The aedeagal lateral processes (claws) (Fig. 2) are articulated with the apex of the phallapodeme
232 (articulation arrowed in Fig. 2D). The aedeagal lateral processes are large, curved, apically pointed
233 and bare (Fig. 2C, D; Fig. 3A–E), approximately 90 μm in length from base to tip. They are
234 bilaterally symmetrical, and arise from the lateral portions (“shoulders”) (Fig. 2A, D, E), not the
235 center, of the apex of the phallapodeme (this comports with the ontogeny described by Rice et al.,
236 2021).

237 We see no connection between the base of the pregonites and the claws (Fig. 2D, E), which
238 confirms that the claws are also not “basal extensions” of the pregonites that exist in closely allied
239 species in the *D. ananassae* complex (Bock and Wheeler, 1972; McEvey and Schiffer, 2015); in such
240 species a very clear nexus exists between the pregonite and a large structure that curves and extends
241 caudally from its base, the basal extension (McEvey and Schiffer, 2015).

242 The pregonites are small, rounded and J-shaped (Fig. 2E), with very small apical setae or
243 “pregonal bristles” (Rice et al., 2019; Fig. 3A, B). They are derived from ventral primordial cells and
244 are, therefore, developmentally separate from the aedeagal lateral processes and the postgonal
245 sheath. The postgonal sheath (*sensu* Rice et al., 2021) is membranous, folds and bends freely, and it
246 is loosely symmetrical, lobe-like and dorsal to the aedeagal lateral processes (Fig. 3A–D). When
247 viewed via light microscopy it is largely membranous and transparent (Fig. 2C), with hardened outer
248 ridges and leaf-like structure connected to the base of each claw (Fig. 2B–E). The postgonal sheath
249 arises from the dorsolateral primordial cells, which, in other species, develop into postgonites
250 (posterior parameres) (Rice et al., 2021). Postgonites are absent in the four species of the *bipectinata*
251 complex.

252

253 **(b) The traumatic insemination hypothesis**

254 We examined SEMs of the claws, including their dorsal, ventral and lateral surfaces (Fig. 3).
255 The claws are bare and smooth on all surfaces, and when the phallopodeme is not extended, they are
256 “cloaked” by the sheath dorsally (Fig. 3A, B). Critically, we could identify no channel, groove or
257 fold, medially, laterally, dorsally or ventrally, on the claws that would function as a conduit for
258 sperm. In some preparations (not shown) we observed the tip of one or both claws to have an
259 irregular depression or lesion (typically $\leq 1 \mu\text{m}$ in diameter), which could be a result of abrasion
260 given the often irregular (torn) edges of these spots.

261 When females were dissected immediately upon the termination of copulation, the sperm
262 within the bursa invariably occurred as a single mass (Fig. 4A). Out of a total of 12 matings
263 interrupted at 6–8 min after the start of mating, 11 produced a sperm mass within the female; in all
264 these cases the sperm likewise occurred as a single mass within the bursa (Fig. 4B). In 3 cases, the
265 sperm mass was small and appeared irregular in shape, amorphous, not smoothly oval or rounded,
266 but nevertheless unquestionably as a single unit.

267 The aedeagus (intromittent organ, phallus, penis) was discovered when anaesthetized
268 copulating pairs were gently pulled apart while submerged in saline solution. This organ in *D.*
269 *biplectinata* is translucent, membranous and pliable, and it appears to have a textured (scaly) surface
270 (Fig. 5A). Sperm were readily identified emanating from the tip of the aedeagus (Fig 5A, B), and
271 could be gently drawn out of the aedeagus using fine stainless steel minuten pin probes. The
272 aedeagus itself arises from between the bases of the aedeagal lateral processes (Fig. 5B), and was not
273 obviously apparent in any of our KOH-boiled preparations. In our SEMs, a reticulated mat of tissue
274 between the bases of the claws could be discerned (Fig. 3F), which we interpret to be the collapsed
275 aedeagus. In the female of the separated pair, sperm was observed emanating from the female
276 gonopore (the orifice of her reproductive tract through which eggs also exit) (Fig. 5C), and likewise
277 could be pulled further out of the gonopore with minuten pin probes (arrowed Fig. 5D). These
278 observations of sperm emanating simultaneously from the male aedeagus and female gonopore in
279 real time as pairs were gently pulled apart during mating establishes the route of sperm transfer in *D.*
280 *biplectinata*.

281 Of the 30 total copulations with “cut” males (those whose claw tips were surgically ablated
282 (Fig. 6)), 22 (73%) resulted in sperm transfer to the bursa (Table 1). In all of these 22 cases, the
283 sperm dissected from the bursa immediately at the end of copulation occurred as a single mass (Fig.
284 4C, D). Among the males that transferred ejaculate, mean (SE) copulation duration did not differ
285 significantly between cut (10.59 (0.730) min, n = 22) and uncut control (10.12 (1.713) ± 1.77 min, n
286 = 4) ($t = 0.25$, $df = 24$, $P = 0.80$) males. Variance in copulation duration for cut males (12.941) was
287 greater than that for uncut males (3.314), but not significantly so ($P > 0.10$). Overall, mean
288 copulation duration between *D. biplectinata* and *D. parabiplectinata* did not differ significantly ($t =$
289 0.47, $df = 33$, $P = 0.64$).

290 Among the 8 cut males (out of 30) that copulated but failed to transfer sperm to the female
291 bursa, 2 males were observed to dismount but could not disengage their genitalia from that of the
292 female, and remained fastened to the female in an end-to-end position. What appeared to be ejaculate

293 seeped out from between one pair, and remained attached to the male's terminalia after the pair
294 finally separated (Fig. 7). This viscous, whitish mass contained sperm, verifying that it was leaked
295 ejaculate.

296

297 **4. Discussion**

298 In their authoritative review of copulatory wounding, Lange et al. (2013) listed a set of criteria for
299 establishing the existence of traumatic mating in a given species, and here, building upon this work
300 and that of Tataric et al. (2014), we assemble a set of criteria for establishing the occurrence of TI.
301 We suggest that evidence for TI should minimally include: *i*) a specific wounding structure(s) that
302 demonstrably breaches the female body wall; *ii*) physical features of said structure(s), such as a
303 canal, lumen, groove and/or pore, for the transfer and delivery of spermatozoa; and *iii*) the transfer of
304 spermatozoa across the female body wall.

305 Several studies have demonstrated TI by fulfilling these criteria (e.g., Davis, 1956; Carayon,
306 1966; Řezáč, 2009; Tataric and Cassis, 2010), a paradigmatic example of which occurs in bed bugs
307 (Cimicidae) (Carayon, 1966; Usinger, 1966; Benoit, 2011). Copulation in several cimicid species has
308 been observed directly, and it has been unambiguously documented to involve males breaching the
309 female's body wall with their needle-like "parameres", often stabbing the female multiple times
310 during a single mating event, and demonstrably transferring sperm into her body cavity (Carayon,
311 1966). The needle-like paramere, with its readily discernible channel and subterminal pore (Fig. 1),
312 are phenotypic features that reflect the paramere's function in TI (Siva-Jothy, 2006).

313 In contrast, we contend that none of the above criteria for demonstrating TI were
314 convincingly met by K2007. In the first place, whereas K2007 claims that integumental penetration
315 is achieved by the claws, stating that they "...pierce the pockets during copulation..." (p. 403), there
316 was no direct evidence presented for physical penetration of the female body wall (nor the genital
317 tract for that matter). The presence of melanized patches in mated females was presented, but this is
318 not decisive evidence for penetration of the integument. Such scarring cannot exclude other possible

319 causes such as surface injury without perforation, and in any event such lesions are known to occur
320 during mating without insemination in other species (Merrit, 1989; Blankenhorn et al., 2002;
321 Kamimura, 2010; Lange et al., 2013). The second criterion (the functional morphology of the organ)
322 was not fulfilled either, as convincing visual evidence for a structure that could guide and transfer
323 sperm across the female body wall was also not provided, and according to the present investigation,
324 does not exist (and see below). Finally, although K2007 claimed that "...sperm is ejaculated through
325 the wounds but not through the genital orifice" (p. 404), pink areas of coloration in a laser scan
326 micrograph were presented as evidence for this claim, which, to us, is insufficient since the presence
327 of sperm within these pink "clouds" was not confirmed, let alone evidence of sperm transfer via the
328 claws to the reproductive tract.

329 Here, we examined SEMs of the dorsal, lateral and ventral surfaces of the claws in *D.*
330 *parabipectinata* and *D. bipectinata*, and discerned no obvious "groove" that could transport sperm.
331 We also evaluated the assertion that the paired claws serve to inject sperm into the reproductive tract,
332 by testing the prediction that immediately after and/or during mating, the ejaculatory material within
333 the female should be discernable as two distinct masses. This prediction failed, as sperm invariably
334 occurred as a single mass. This outcome aligns with previous work on the reproductive biology of *D.*
335 *bipectinata*: in all cases, the sperm dissected from the bursa immediately after copulation occurred as
336 a single mass (Tyler et al., 2021).

337 The aedeagus (intromittent organ, phallus, penis) in the *bipectinata* complex has been
338 notoriously difficult to detect and characterize. Throughout the genus *Drosophila*, the aedeagus is
339 usually a tubular organ with an external opening, a phallosome or gonopore. The organ itself is
340 usually membranous, often expanded apically and hirsute or irregularly papillate (Bock and Wheeler,
341 1972). K2007 suggested that in the *bipectinata* species complex, the aedeagus had diminished to "a
342 degenerate, transparent, tube-like true aedeagus" (p. 403), while Rice et al. (2021) referred to the
343 aedeagus as "translucent" and outlined the area where it ought to be located in *D. malerkotliana*,
344 between the paired lateral processes (figure 2G in Rice et al., 2021). Here, for the first time we have

345 imaged the everted aedeagus (in *D. bipectinata*) through adopting a method of gently separating
346 copulating pairs while under ether anesthesia and submerged in saline solution. Pulling pairs apart
347 during coupling extruded the male aedeagus and clearly showed sperm emanating from its tip and
348 simultaneously from the female vaginal opening (her gonopore). Sperm emanating from these male
349 and female structures in real time identifies the path of sperm transfer to the female, and explains
350 why the sperm mass invariably consists of a single integrated unit, and why males with cut claw tips
351 were able to inseminate females. The aedeagus is a translucent, membranous, and highly pliable
352 tube-like structure that appears to readily collapse upon itself. These characteristics suggest why the
353 aedeagus has been difficult to detect in previous works.

354 We also tested the prediction of the TI hypothesis that after experimentally eliminating the
355 sharp terminal ends of the claws, insemination should be inhibited. This prediction also failed, as a
356 plurality of males without these sharp ends successfully inseminated females. In matings with ablated
357 males (claw tips removed), 73% resulted in insemination, and in *all* these cases, the sperm occurred
358 as the typical single mass within the female reproductive tract. In all of our experimental males with
359 cut claws, the efficacy of the surgery was verified afterwards; all surgeries had successfully removed
360 the apical third to apical half of both aedeagal lateral processes (claws).

361 Taken together, the results refute the hypothesis of TI in the *bipectinata* species complex, and
362 therefore in *Drosophila*, and for that matter in Diptera as far as we know. Sperm transfer in the
363 *bipectinata* complex occurs from the male aedeagus into the female reproductive tract via her
364 gonopore, and comports with knowledge about other *Drosophila* species including *D. melanogaster*.
365 An alternative possibility is that the claws may serve to transfer (or secrete) fluid, and therefore
366 function in “traumatic secretion transfer” (Lange et al., 2013), evidence for which occurs in the seed
367 beetle *Callosobruchus maculatus* (Hotzy et al., 2012) and blowfly *Lucilia cuprina* (Merritt, 1989),
368 possibly accounting for the pink areas highlighted in K2007’s images. This idea, however, is
369 speculative, and we have no evidence for or against it.

370 From our observations of matings in *D. bipectinata* and *D. parabipectinata*, the most likely
371 functions of the claws that we can discern are at least three-fold, all of which are mechanical in
372 nature. We emphasize, however, that additional experiments are needed to fully characterize the
373 function of these remarkable structures, but which are beyond the scope of the present study. Here,
374 our primary focus was to address the TI hypothesis in and of itself.

375 A first potential function we may deduce from our data is that the claws serve to assist the
376 male in achieving copulation by facilitating the grasping of the female. In 10 out of 30 cases of
377 copulations with cut males, males were observed to mount the female, probe the female terminalia
378 with their own genitalia, but failed to achieve genital coupling in these attempts (some later did). A
379 grasping function has been demonstrated for the sharp (periphallic) spines emanating from the male
380 ventral cercal lobes in *D. bipectinata* (Polak and Rashed, 2010) and *D. ananassae* (Grieshop and
381 Polak, 2012). More generally, male genital clasping devices, such as spines, hooks, inflatable
382 organs/structures, and other interlocking features, occur in a wide range of invertebrate species to
383 function in achieving union and genital integration, holding the female securely, and protecting her
384 from rival males (Thornhill and Alcock, 1983; Eberhard, 1985; Gwynne, 1998; Simmons, 2001).

385 A second apparent function may be to assist in the opening of the female gonopore. In 2 of
386 the above 10 instances of cut males failing to couple, males were observed to use their periphallic
387 structures (surstyli or claspers) to probe and apparently attempt to part the female ovipositor, but
388 failed to do so, and failed to achieve union. These observations suggest that the female ovipositor
389 could not be opened as a result of the males lacking intact claws.

390 A third non-mutually exclusive function we believe is one for anchoring—to brace the male
391 genitalia to allow the intromittent organ (aedeagus) to evert through the female gonopore into the
392 vagina. A firm integration of the genitalia should assure transfer of ejaculate from the aedeagus to the
393 female. Passage of the aedeagus through the female gonopore could also be facilitated or provided by
394 the physical opening of the gonopore by the abduction of the claws. A relevant observation here is
395 that there were 2 cases (out of 8) in which cut males remained “adhered” to the female after

396 dismounting, suggesting that ejaculate failed to transfer to the female because of poor union of the
397 genitalia, seeped from between the pair, and acted as an adhesive that then unnaturally prolonged
398 genital coupling. Sperm seepage is consistent with clawless males being unable to adequately insert
399 the aedeagus into the gonopore and/or effectively maintain their genitalia integrated with that of the
400 female during copulation. Ejaculate seepage during coupling was directly observed in one case (Fig.
401 7).

402 When TI occurs in a given species, it is predicted to impose a number of potential costs to
403 females, some of which will be unique relative to other forms of copulatory wounding. In cases of
404 TI, we may expect females to incur specific fitness costs associated not only with wound healing,
405 immune system activation and risk of infection of the hemocoel and internal organs, but also with the
406 physiological challenges stemming from the introduction of seminal fluid and spermatozoa into the
407 hemolymph (Davis, 1956; Morrow and Arnqvist, 2003; Siva-Jothy, 2009). This latter cost could also
408 encompass loss of control over fertilization that would normally be available to females where
409 insemination occurs via the female reproductive tract (Eberhard, 1996; Beani et al., 2005). Our
410 ability to predict such costs to females, and to interpret aspects of female reproductive anatomy,
411 physiology, and behaviour, that evolve in response to sexual conflict linked to mating (Lessels, 2006;
412 Parker, 2006; Yassin and Orgogozo, 2013), rests upon accurately describing and classifying the
413 highly varied forms of copulatory trauma that exist in animals. The results of the present study lead
414 us to reject the assertion that TI occurs in species of the *Drosophila bipectinata* complex.

415

416 **Competing interests.** The authors declare that they have no competing interests.

417

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424

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Table 1. Number of ejaculates transferred to the female bursa as a single mass in male *D.*

parabipectinata and *D. bipectinata*. The aedeagal lateral processes (claws) were cut with an ultraprecise surgical laser; the uncut treatment group consisted of surgical controls. Cut males had approximately 1/3–1/2 the distal ends of both aedeagal lateral processes ablated (Fig. 6). All females were dissected immediately after pairs separated. In all cases of ejaculate transfer, the sperm occurred as a single mass within the female bursa irrespective of whether the male was cut or uncut.

Species	Surgical treatment	Number pairs set up	Number copulations	Number copulations resulting in ejaculate transfer	Number of ejaculates as a single mass
<i>D. parabipectinata</i>	Cut	8	6	5	5
	Uncut	2	2	2	2
<i>D. bipectinata</i>	Cut	34	24	17	17
	Uncut	3	3	3	3
Total	Cut	42	30	22	22
	Uncut	5	5	5	5

Figures

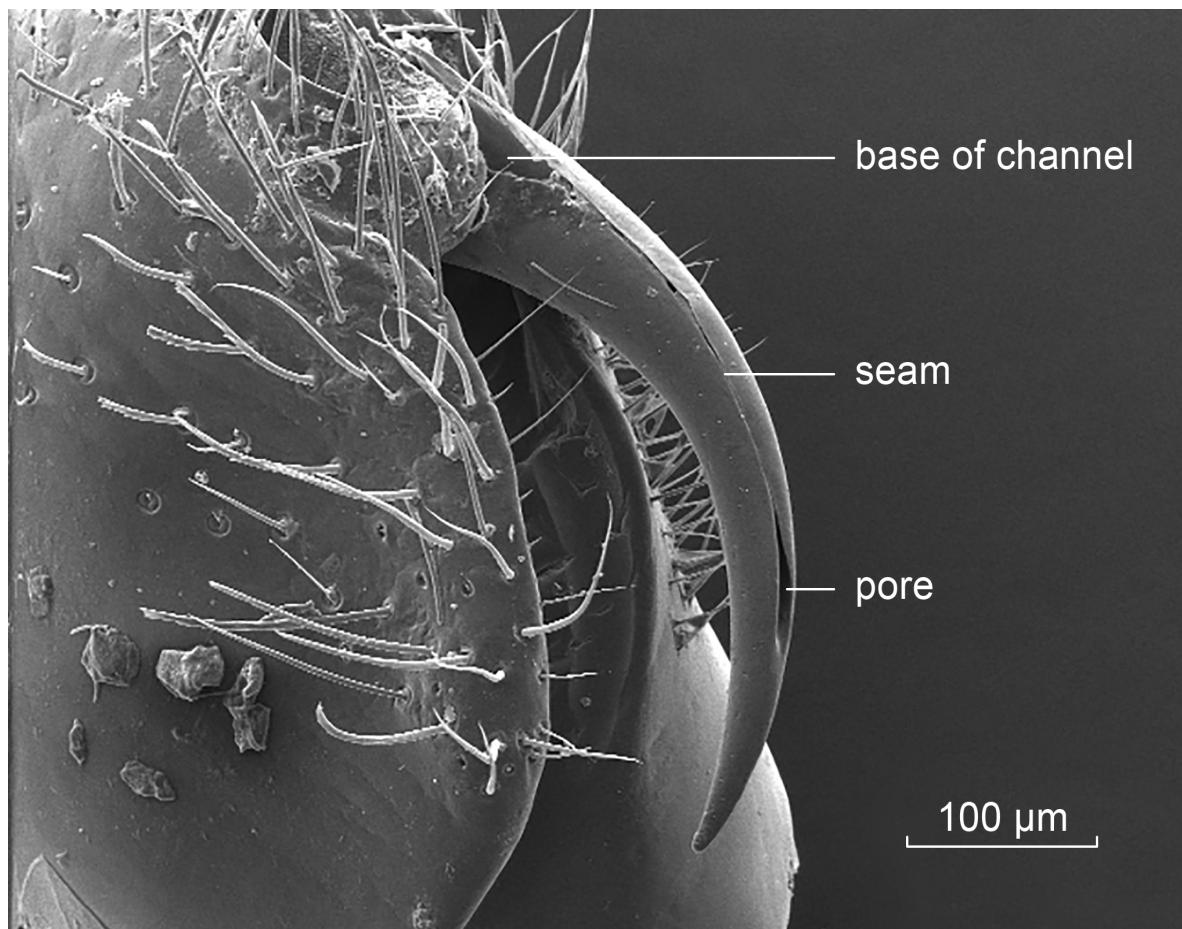


Figure 1. Scanning electron micrograph (200 \times) of the curved and tapered copulatory organ (the paramere, Usinger, 1966) of the bed bug, *Cimex lectularius* L. During mating, the paramere can be seen in real time to puncture the female abdominal integument (Usinger, 1966). The channel (groove) of the paramere extends from its base to the subterminal pore (c. $65 \times 10 \mu\text{m}$); the aedeagus is everted through the channel and ejaculate injected into the female (Davis, 1956; Usinger, 1966).

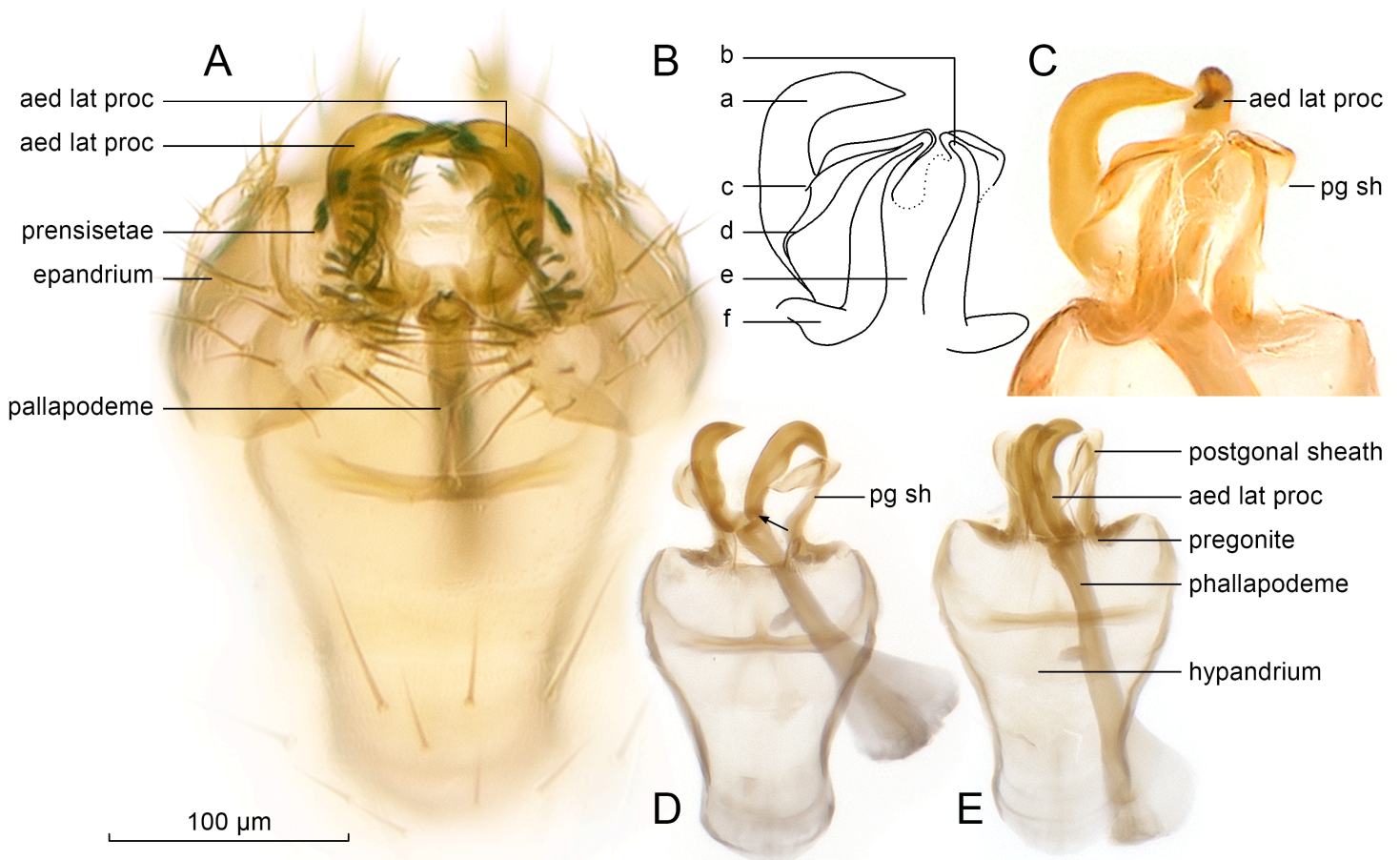


Figure 2. Light microscope images of the phallic structures of the *bipectinata* complex. **(A)** Posterior end of the undissected male body viewed from below; the hyandrium is inside the body, surrounded by the epandrium. **(B, C)** Drawing and magnified view, after dissection, of the posterior end of the hyandrium, showing the aedeagal lateral processes (aed lat proc) and the postgonal sheath (pg sh). The sheath surrounds the phallic structures dorsally; it is largely membranous (e) but ridges and thickened processes (b, c, d, f) are evident within. **(D, E)** Views of the hyandrium in its entirety. Images show the aedeagal lateral processes and postgonal sheath in two phallapodeme orientations (projected and withdrawn, respectively), as well as the articulation of the aedeagal lateral process (arrowed in D) at the posterior end of the phallapodeme. Specimens: (A) *D. bipectinata* (Cape Tribulation, Australia | 16.104°S 145.455°E | 2011 | M. Polak & S.F. McEvey); (B, C) *D. bipectinata* (Taipei, Taiwan | 25°2'30.24"N, 121°36'39.37"E | 2017 | M. Polak); (D, E) *D. parabiptinata* (Christmas Island [nr Java] | 10°30'S 105°35'E | 2003 | S.F. McEvey et al. [Australian Museum]).

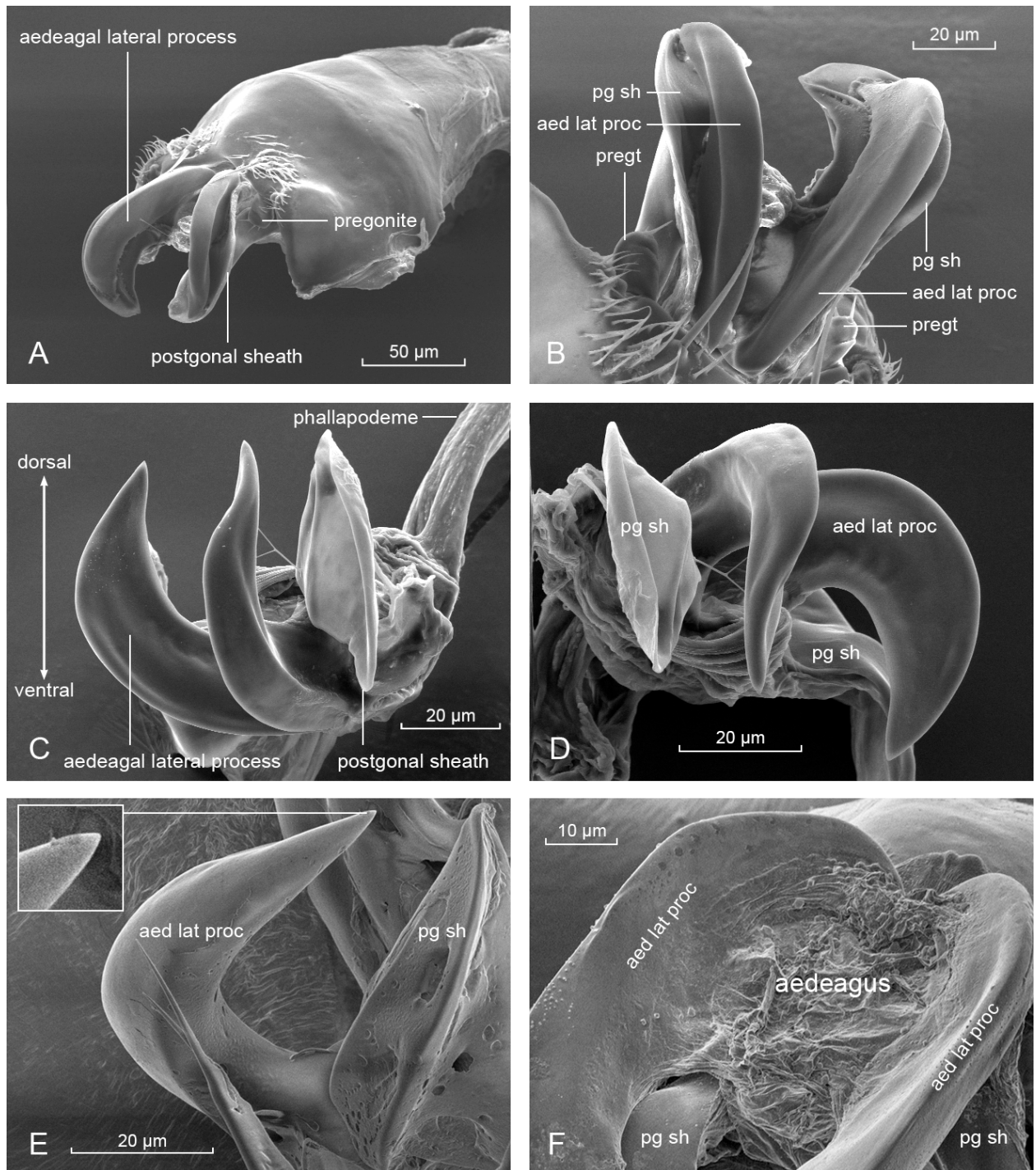


Figure 3. Scanning electron micrographs of the aedeagal lateral processes (aed lat proc) in the *bipectinata* complex. Aedeagal lateral processes were imaged at different orientations and magnifications to allow detailed examination of all surfaces, which failed to reveal purported conduits (grooves or channels) for sperm transport. **(A)** Ventral surface of hypandrium with the pair of aedeagal lateral processes, sheathed in the postgonal sheath (pg sh); the small pregonite (pregt) with apical setae arises from the gonocoxite. **(B)** Strong, rounded, bare, ventral surfaces of the aedeagal lateral processes with the postgonal sheath extending beyond (and possibly protecting) the sharp tips. Pregonite (pregt) with apical sensilla. **(C)** Smooth, bare, seamless, ventral and lateral surfaces of the aedeagal lateral

processes; no gonopore (phallotrema) present; thickened edges and internal “ribs” of the postgonal sheath visible (see also D, and Fig. 2B, C). **(D)** Ventral and lateral faces of the aedeagal lateral processes, in relation to the lobes of the postgonal sheath. **(E)** Surfaces of aedeagal lateral process. Inset shows intact tip, which occasionally carries a lesion, possibly an abrasion (not shown). **(F)** Reticulated mat of tissue between the bases of the aedeagal lateral processes, interpreted to be the collapsed aedeagus (see Fig. 5A, B). Specimens: (A–D) *D. parabiplectinata*, (E, F) *D. biplectinata* (Taipei, Taiwan | 25°2'30.24"N, 121°36'39.37"E | 2017 | M. Polak).

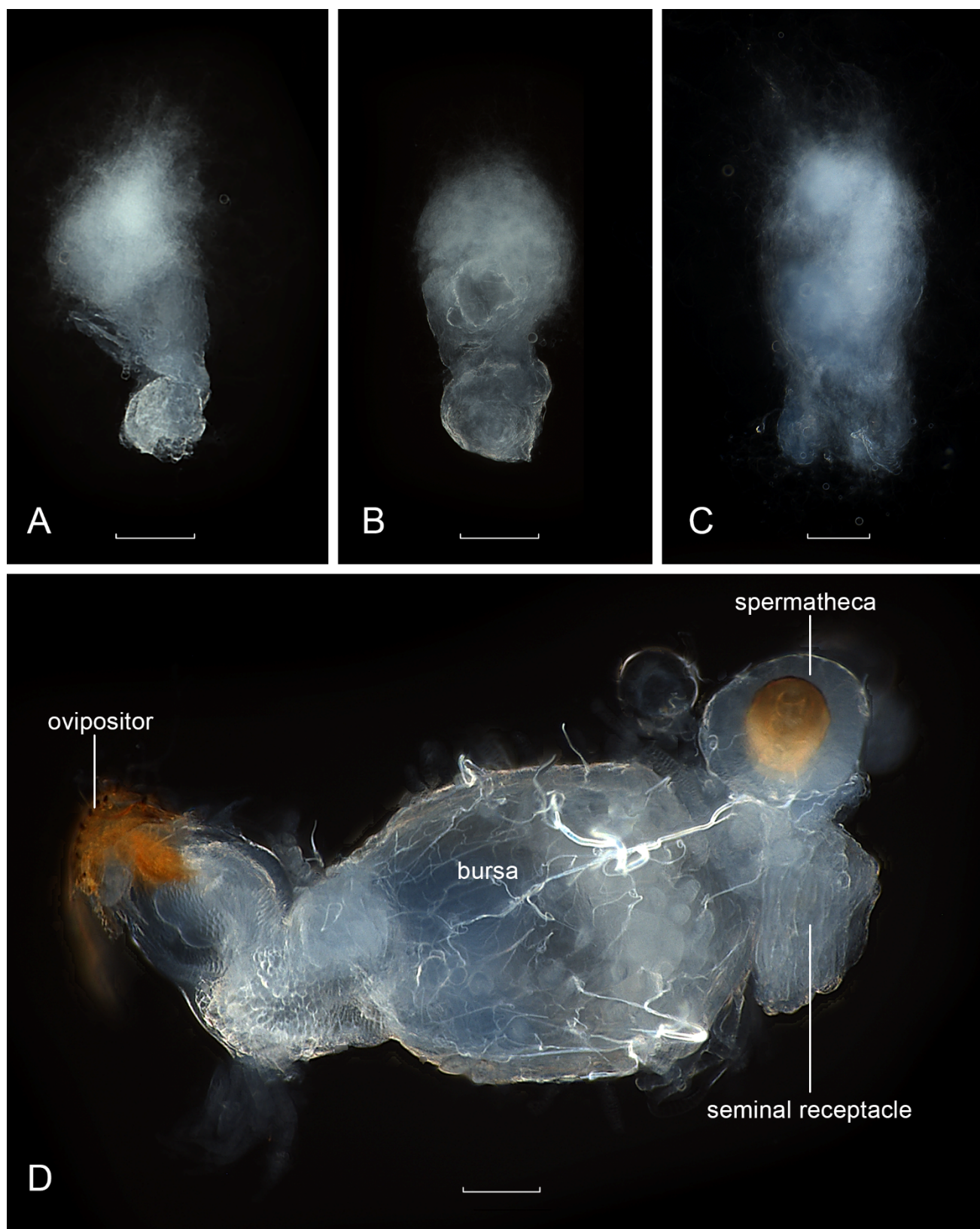


Figure 4. Light microscope images exemplifying the single sperm mass in *D. bipunctinata*. Masses were dissected intact from the female reproductive tract (A) immediately after the end of a full-

length, uninterrupted copulation, and **(B)** immediately after the pair was experimentally separated at 8 min or less after the onset of genital coupling; uninterrupted copulation duration in this species is on average about 10.6 min (Polak et al., 2021). At the base of each mass, the gelatinous ("waxy") component of the ejaculate is clearly visible, which is also present in *D. melanogaster* and in other species to varying degrees of expression (Bairati and Perotti, 1970; Alonso-Pimentel et al., 1994; Polak et al., 1998; Manier et al., 2010). **(C)** Sperm mass dissected from the bursa after copulation with a male with both aedeagal lateral process tips ablated. **(D)** Intact bursa of a female, full of sperm, after mating with a male with both aedeagal lateral process tips ablated. Scale bars = 200 μm .

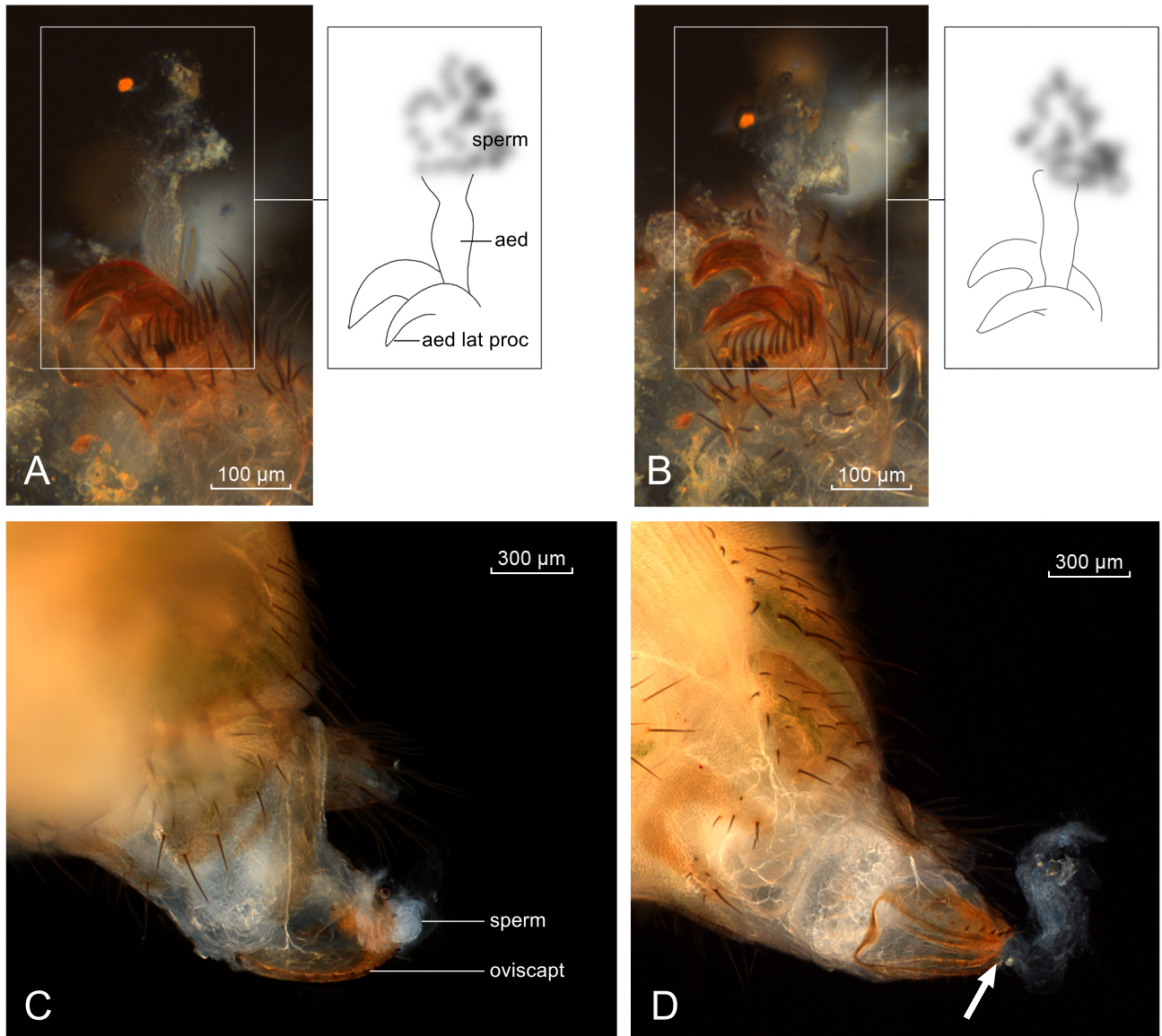


Figure 5. Light microscope images of the genitalia of a male-female pair *D. bipectinata* gently pulled apart during copulation and imaged, demonstrating the extruded aedeagus and female oviscape. **(A), (B)** The aedeagus of the male is clearly shown with sperm emanating from its tip; **(B)** the aedeagus arises from between the bases of the aedeagal lateral processes. **(C)** Sperm simultaneously seeping from the female gonopore, and **(D)** sperm teased and pulled further out from the gonopore (arrowed).

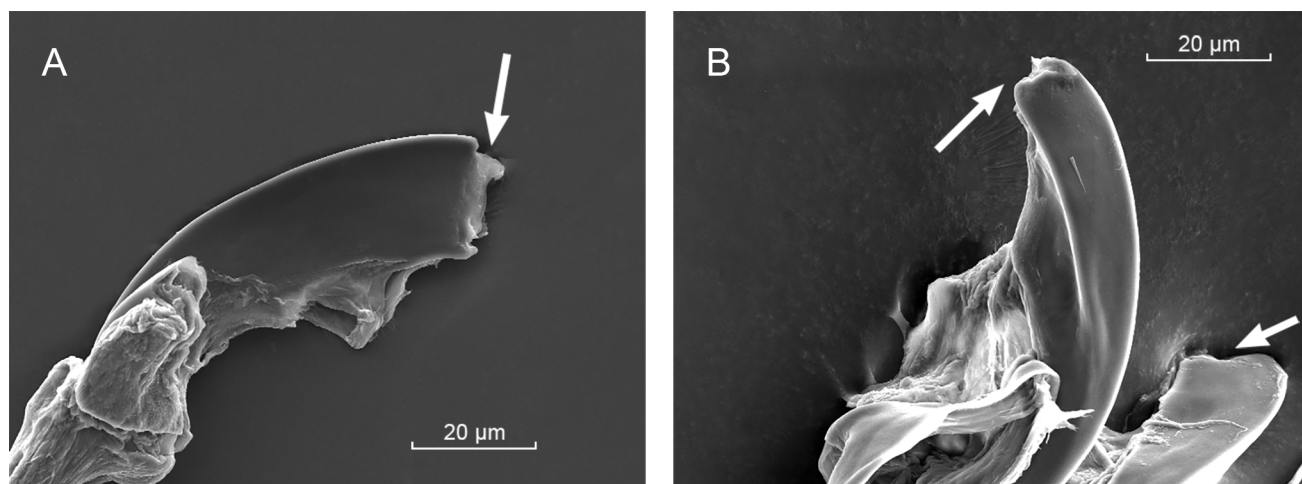


Figure 6. Scanning electron micrographs (1200 \times) of cut claws, or aedeagal lateral processes in *D. bipectinata*. Examples (A) and (B) of the processes experimentally blunted (arrowed) using ultra-precise laser surgery are shown. Both tip-ablated processes are visible in (B). From one third to one half of the distal ends of both aedeagal lateral processes of each male were ablated, completely eliminating their sharp terminal ends.

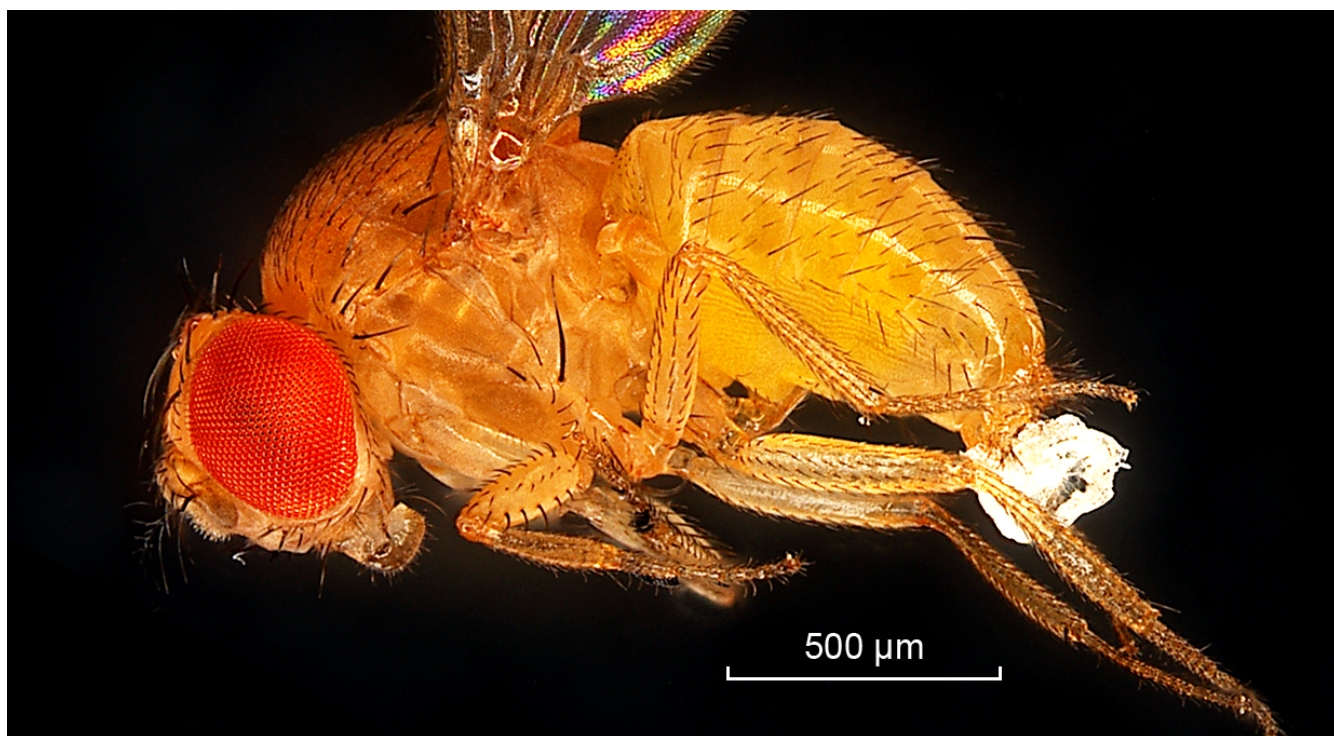


Figure 7. The ejaculate mass, adhered to the tip of a cut male's abdomen after copulation, had seeped out from between the pair during copulation. The tips of the aedeagal lateral processes in this *D. bipectinata* male had been ablated (Fig. 6) with a surgical laser prior to mating.