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## Resolving Between Novelty and Homology in the Rapidly Evolving Phallus of *Drosophila*

Running Title: **Defining homology in a rapidly evolving tissue**

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34 **Abstract**

35 The genitalia present some of the most rapidly evolving anatomical structures in the animal  
36 kingdom, possessing a variety of parts that can distinguish recently diverged species. In the  
37 *Drosophila melanogaster* group, the phallus is adorned with several processes, pointed  
38 outgrowths, that are similar in size and shape between species. However, the complex three-  
39 dimensional nature of the phallus can obscure the exact connection points of each process.  
40 Previous descriptions based upon adult morphology have primarily assigned phallic processes  
41 by their approximate positions in the phallus and have remained largely agnostic regarding  
42 their homology relationships. In the absence of clearly identified homology, it can be  
43 challenging to model when each structure first evolved. Here, we employ a comparative  
44 developmental analysis of these processes in eight members of the *melanogaster* species group  
45 to precisely identify the tissue from which each process forms. Our results indicate that adult  
46 phallic processes arise from three pupal primordia in all species. We found that in some cases  
47 the same primordia generate homologous structures whereas in other cases, different  
48 primordia produce phenotypically similar but remarkably non-homologous structures. This  
49 suggests that the same gene regulatory network may have been redeployed to different  
50 primordia to induce phenotypically similar traits. Our results highlight how traits diversify and  
51 can be redeployed, even at short evolutionary scales.

52

53 **Key Words:** Homology, *Drosophila*, Genitalia, rapid evolution

54 **Research Highlight:**

55 By incorporating developmental analysis, we find that genital structures previously identified as  
56 homologs are novel structures. This highlights how developmental analysis can help resolve  
57 contentious claims of homology.

## 58 **Introduction:**

59           Most studies of developmental evolution depend on our ability to precisely compare the  
60 same body parts in different species or populations. Rigorously establishing such homology  
61 relationships allows us to identify novel traits, which are often defined by their lack of  
62 homology (Reviewed in Moczek, 2008; G. Wagner, 2007). Many of the current model systems  
63 for the study of novelty focus on traits that arose in the distant past (Bruce & Patel, 2020; Clark-  
64 Hachtel & Tomoyasu, 2020; Emlen, Szafran, Corley, & Dworkin, 2006; Hinman, Nguyen,  
65 Cameron, & Davidson, 2003), making the investigation of their molecular origins difficult. These  
66 traits likely arose through a multitude of genetic changes and exist in organisms that are less  
67 amenable to genetic studies. Recently evolved traits found in the rapidly evolving tissues of  
68 model organisms can provide qualitative changes in morphology produced by genomes that are  
69 easily compared and modified. However, rapidly evolving anatomical structures pose a distinct  
70 challenge. When differences between the anatomy of two species are numerous, it can be  
71 difficult to disentangle which structures are ancestral and which represent newly derived  
72 novelties. Thus, while macroevolutionary novelties often appear as clear discontinuities in the  
73 evolutionary record, the more molecularly tractable micro- and mesoevolutionary novelties  
74 require us to consider their relationships in a rich and complicated comparative context  
75 (Abouheif, 2008; Church & Extavour, 2020). Overcoming this challenge is thus critical to develop  
76 a genetic portrait of morphological novelty.

77           Most assertions of homology are defined through establishing that the structure in  
78 question connects to an unambiguously homologous tissue in both species (Moczek, 2008).  
79 Contentious claims of homology often revolve around the question of whether a set of traits

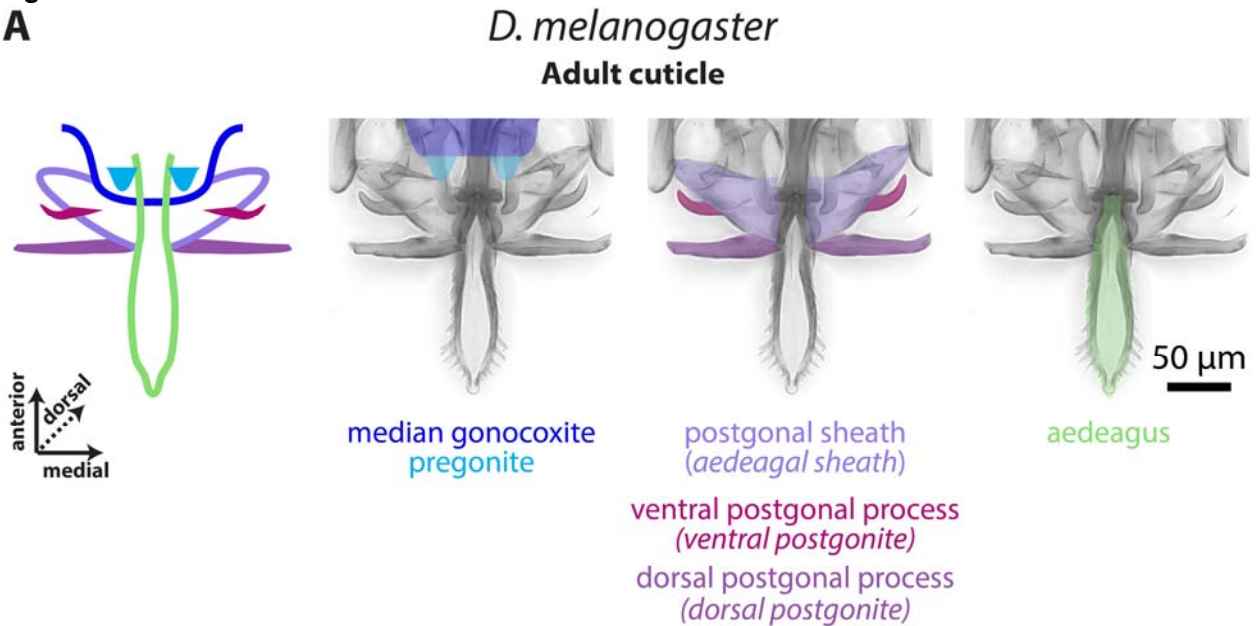
80 are formed by the same cells or tissues. These assertions can be strengthened through  
81 developmental analysis, where the primordium that initially forms the trait in question can be  
82 determined (Tanaka, Barmina, & Kopp, 2009). This type of analysis is especially important in  
83 complex three-dimensional traits, as resolution in the X, Y, and Z axes may be required. The  
84 high spatial resolution of confocal microscopy generates three-dimensional renderings of entire  
85 body parts, allowing us to define the position of structures relative to tissues that have  
86 straightforward homology assignments (Klaus, Kulasekera, & Schawaroch, 2003). Many  
87 developing tissues progressively become more complex over developmental time. The  
88 formation of specific traits is often established only after the tissue that encompasses that trait  
89 is identifiable, providing clear anchor points in a conserved tissue to establish homology. Thus,  
90 developmental trajectories provide a rich context in which to disentangle ambiguous  
91 relationships among rapidly evolving structures.

92         The terminalia (genitalia and analia) of drosophilids host an extensive assortment of  
93 rapidly evolving morphological structures. Variation of terminal structures is often one of the  
94 most reliable ways to differentiate species of *Drosophila* based on adult morphology (Bock &  
95 Wheeler, 1972; Hsu, 1949; Markow & O'Grady, 2006; Okada, 1954). The male genital structures  
96 are often divided into two major compartments: the periphallic parts surrounding the anus,  
97 which mostly play a role in grasping the external surface of the female genitalia (Acebes, Cobb,  
98 & Ferveur, 2003; Jagadeeshan & Singh, 2006; Kamimura & Mitsumoto, 2011; Masly &  
99 Kamimura, 2014; Mattei, Riccio, Avilaa, Wolfner, & Denlinger, 2015; Rhebergen, Courtier-  
100 Orgogozo, Dumont, Schilthuizen, & Lang, 2016; Robertson, 1988; Yassin & Orgogozo, 2013), and  
101 the phallic parts (Figure 1), which comprise the intromittent organ. While the homology of the

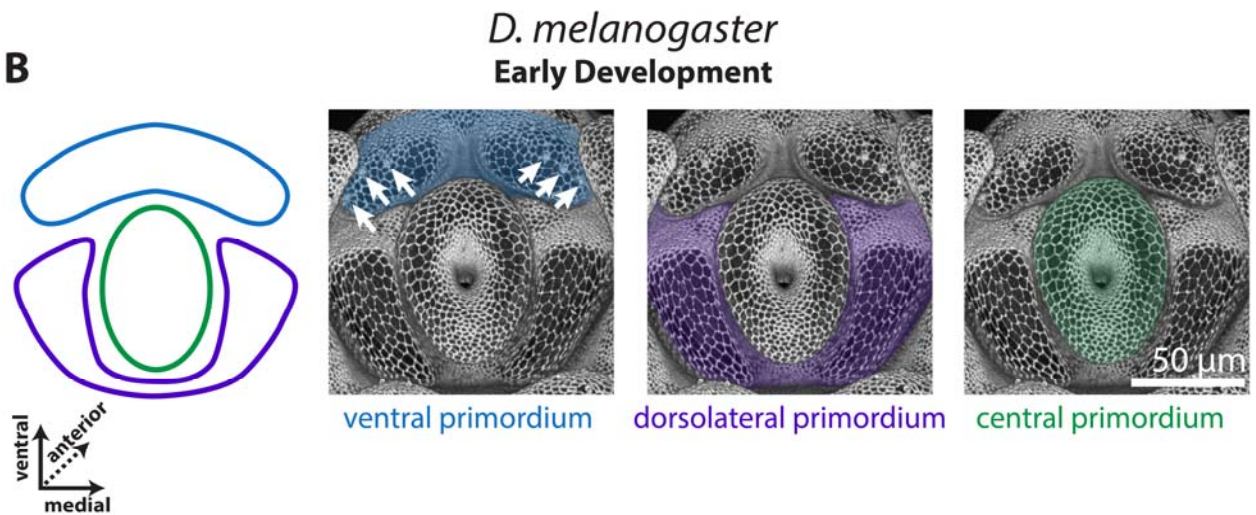
102 periphallid organs has always been relatively straightforward, the phallus itself has posed  
103 several challenges in this regard. In particular, the homology of the various phallic processes,  
104 pointed outgrowths, remains controversial (Figure 2, Supplementary videos) (reviewed in Rice  
105 et al., 2019). These outgrowths have been implicated in sexual conflict between males and  
106 females, and in some species have been shown to physically interact with corresponding  
107 pockets in the female genitalia (Kamimura, 2016; Muto, Kamimura, Tanaka, & Takahashi, 2018;  
108 Yassin & Orgogozo, 2013) or induce copulatory wounds (Kamimura, 2007). Male seminal  
109 proteins are associated with increased ovulation and reduced remating rates and can enter the  
110 female circulatory system through these copulatory wounds (Avila, Sirot, Laflamme, Rubinstein,  
111 & Wolfner, 2011; Kamimura, 2010). To better understand how genital morphology may  
112 coevolve we must better establish which homologous tissues have been modified in each sex.

113 **Figure 1:**

**A**



**B**



114

115 **Figure 1:** The *D. melanogaster* pupal phallus is produced by three primordia

116

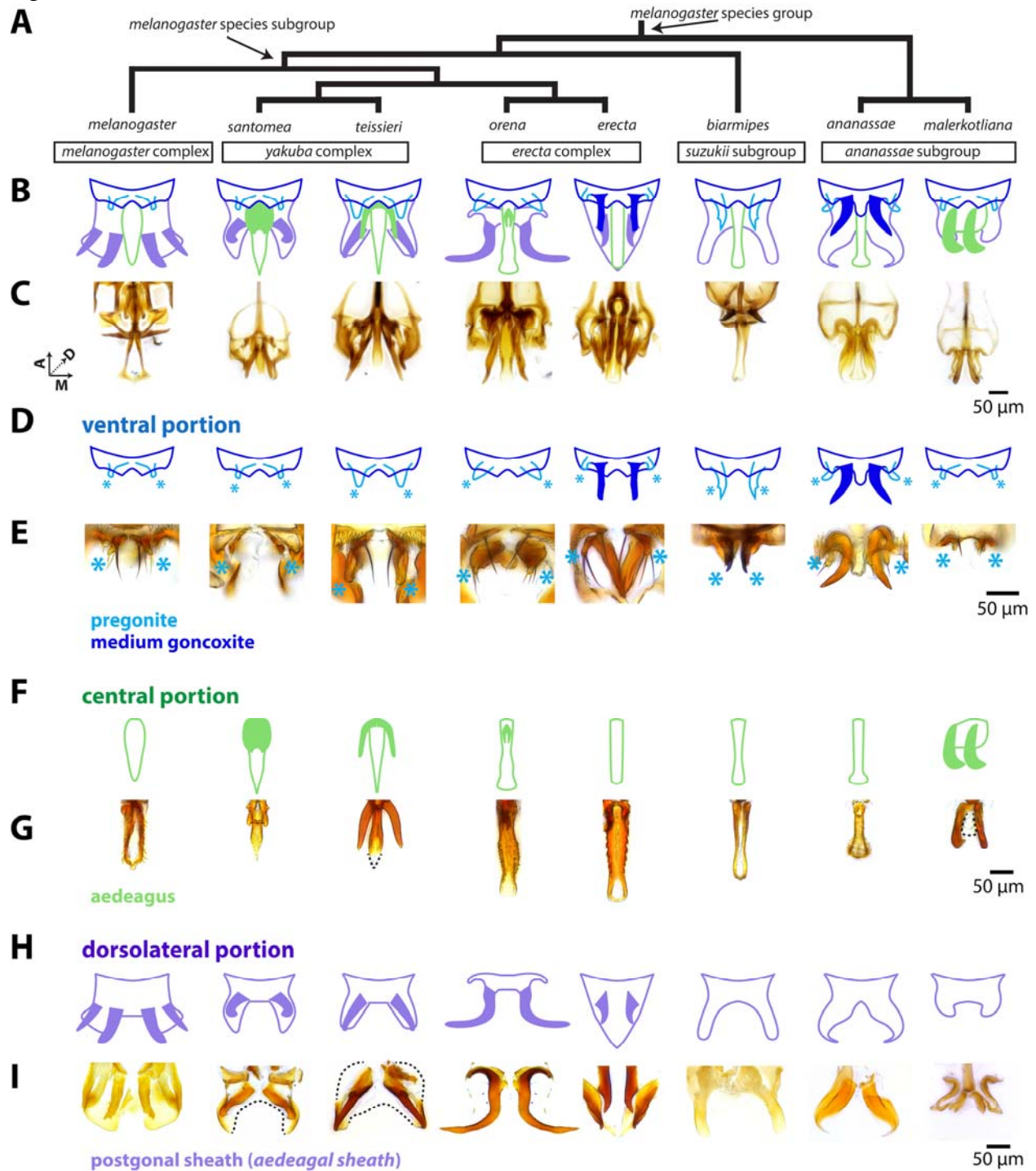
117 **A)** Left: A schematic representation of the adult phallus of *D. melanogaster*, the median  
 118 gonocoxite outlined in dark blue, the pregonites highlighted in light blue, the postgonal sheath  
 119 in light purple, the ventral postgonal process in magenta, the dorsal postgonal process in violet,  
 120 and the aedeagus in light green. Right: the adult phallus of a *yw;+:+* line of *D. melanogaster*. **B)**  
 121 Left: A schematic representation of the early developing pupal genitalia of *D. melanogaster*.  
 122 The primordia of developing phallus with the ventral primordium in blue, the dorsolateral  
 123 primordium in purple, and the central primordium in green. Right: Developing pupal phallus of  
 124 a *D. melanogaster* arm-GFP transgenic line. Apical cellular junctions are shown, highlighting the



125 overall morphology. White arrows indicate the position of the pregonal bristles. Aedeagal  
126 sheath is an alternative term for postgonal sheath, ventral postgonite is an alternative term for  
127 ventral postgonal process, and dorsal postgonite is an alternative term for dorsal postgonal  
128 process. The images of the developing phallus are shown with ventral view on top to match the  
129 orientation of the phallus during copulation.

130

Figure 2:



131

132 **Figure 2:** The rapidly evolving phallus is composed of three main components

133

134 **A)** Phylogeny for eight species of the *melanogaster* species group based on Obbard et al., 2012

135 with nodes that contain the *melanogaster* species subgroup and *melanogaster* species group

136 indicated by arrows. **B)** A schematic breakdown of the adult phalluses of each species. **C)** Light  
137 microscopy images of the whole adult phallus for each species. Image stacks that show the  
138 relative position of each part can be found in Supplementary videos. **D)** Schematic  
139 representation of the ventral portion of the phallus (dark blue) which contains the pregonites  
140 (light blue) and processes (filled in dark blue) in *D. erecta* and *D. ananassae*. Light blue asterisks  
141 designate the position of the pregonites. **E, G, I):** Light microscopy images of microdissections of  
142 the adult phallus (here in lateral view, distal end pointing downward) separated in the ventral,  
143 central, and dorsolateral portions. **E)** Microdissections of the ventral portion processes shows  
144 the processes of *D. erecta* and *D. ananassae* are connected to the pregonites. Light blue  
145 asterisks designate the position of the pregonites. **F)** Schematic representation of the central  
146 portion of the phallus (light green), which contains processes (filled in light green) in *D.*  
147 *santomea*, *D. teissieri*, *D. orena*, and *D. malerkotliana*. **G)** Microdissections of the aedeagus  
148 confirm that the processes are physically attached to aedeagus. The aedeagus of the *D.*  
149 *malerkotliana* is translucent (dashed line) with only the process sclerotized. **H)** Schematic  
150 representation of the dorsolateral portion of the phallus (light purple), which contains two pairs  
151 of processes (filled in light purple) in *D. melanogaster*, and one pair in *D. santomea*, *D. teissieri*,  
152 *D. erecta*, and *D. orena*. **I)** Microdissection confirms that the processes are physically attached  
153 to postgonal sheath. In *D. santomea*, *D. teissieri*, *D. erecta*, and *D. malerkotliana* portions of the  
154 anterior postgonal sheath are translucent (outlined with dashed lines). The image of the *D.*  
155 *orena* dorsolateral portion was created by copying and mirroring one side of the structure, as it  
156 was difficult to flatten intact for imaging. Aedeagal sheath is an alternative term for postgonal  
157 sheath.

158           Two major sources have contributed to confusion regarding homology of phallic  
159 processes. The first is the relationship of the postgonal sheath (referred to as aedeagal sheath  
160 in Rice et al., 2019) with respect to these processes (Figure 2 H-I). Several authors consider the  
161 postgonal sheath and postgonal processes (referred to as postgonites in Rice et al., 2019) of *D.*  
162 *melanogaster* as substructures of a unified tissue that was usually referred to as the “posterior  
163 parameres” (Bock & Wheeler, 1972; Okada, 1954; Tsacas, Bocquet, Daguzan, & Mercier, 1971).  
164 While others designated the postgonal sheath as a separate tissue from the postgonal  
165 processes (Al Sayad & Yassin, 2019; Bächli, Vilela, Andersson Escher, & Saura, 2004; Lachaise et  
166 al., 2004). The three-dimensional nature and the presence of transparent cuticle has made it  
167 difficult to determine the precise connection points of the processes to the tissues of the  
168 phallus. Determining whether these processes were formed by a single or separate primordium  
169 would help resolve this discordance. The second source of confusion is in regard to the  
170 nomenclature used to compare the phallic processes in different members of the *melanogaster*  
171 species group. The term “basal process” has been used to refer to a number of pointed  
172 outgrowths that are attached to different phallic tissues in different species (Kamimura, 2007,  
173 2010, 2016; Kamimura & Mitsumoto, 2011, 2012a, 2012b; Kamimura & Polak, 2011). Such a  
174 designation implies a concept of homology independent of the exact anatomical position.  
175 Yassin & Orgogozo (2013) sought to provide distinct terms, such as “spurs” and “hooks” for  
176 outgrowths emanating from the same tissue, implying the potential for non-homology. Building  
177 upon our recent revision of the male terminalia nomenclature of *D. melanogaster* (Rice et al.,  
178 2019), developmental studies presented here allow us to provide a more detailed, homology-  
179 informed nomenclature for these structures.

180           In this study, we characterized both the adult morphology and the development of the  
181 pupal genitalia in five members of the *melanogaster* species subgroup and three outgroup  
182 members from the larger *melanogaster* species group. This analysis allows us to determine  
183 whether processes are homologous or of different origins. Tracing the development of the  
184 phallus by confocal microscopy showed that all processes arise from three distinct pupal  
185 regions that likely represent primordia for three subdivisions of the phallus in all species. We  
186 found both that several similarly shaped processes arise from distinct primordia, whereas in  
187 other cases, distinct processes arise from different parts of the same primordium. In light of  
188 these analyses, we refined the identity and terminology of the phallic processes and identify  
189 distinct homology groups. We map these different morphologies on previously established  
190 phylogenies and identified multiple gain, loss, and homoplastic events in the history of these  
191 diverse structures. Thus, our results demonstrate how developmental approaches can resolve  
192 unclear relationships among rapidly evolving structures.

## 193 **Materials and Methods:**

### 194 *Drosophila strains:*

195 To study the evolution of the phallic processes in the *melanogaster* species subgroup we used  
196 the following species, representing all major complexes: *D. santomea* (Lachaise et al., 2000), *D.*  
197 *teissieri* (Tsacas, 1971), *D. orena* (Tsacas & David, 1978), *D. erecta* (Tsacas & Lachaise, 1974), *D.*  
198 *melanogaster* (Meigen, 1830) from the *melanogaster* species subgroup and the following  
199 outgroup species *D. biarmipes* (Malloch, 1924) from the *suzukii* subgroup, *D. ananassae*  
200 (Doleschall, 1858), and *D. malerkotliana* (Parshad & Paika, 1964) from the *ananassae* subgroup.  
201 Previous work has investigated the function of the copulatory anatomy of all species we  
202 analyzed (Kamimura, 2007, 2016; Muto et al., 2018; Yassin & Orgogozo, 2013). Stocks were  
203 obtained from both the National Drosophila Species Stock Center at Cornell (*D. santomea*  
204 (14021-0271.01), *D. teissieri* (14021-0257.01), *D. orena* (14021-0245.01), *D. erecta* (14021-  
205 0224.01), *D. biarmipes* (14023-0361.09), *D. ananassae* (14024-0371.13), the Bloomington  
206 Drosophila Stock Center *D. melanogaster* armadillo-GFP, arm-GFP, (Bloomington stock number  
207 #8556), and from the lab of Dr. Thomas Williams, *D. malerkotliana*.

208

### 209 *Sample collection, dissection, and fixation:*

210 Male white pre-pupae were collected at room temperature and incubated in a petri dish  
211 containing a moistened Kimwipe at 25°C prior to dissection. After incubation, pupae were  
212 impaled in their anterior region and immobilized within a glass dissecting well containing  
213 Phosphate Buffered Saline (PBS). The posterior tip of the pupa (20–40% of pupal length) was  
214 separated and washed with a P200 pipette to flush the pupal terminalia into solution. Samples

215 were then collected in PBS with 0.1% Triton-X-100 (PBT) and 4% paraformaldehyde (PFA, E.M.S.  
216 Scientific) on ice, and multiple samples were collected in the same tube. Samples were then  
217 fixed in PBT + 4% PFA at room temperature for 30 min, washed three times in PBT at room  
218 temperature, and stored at 4°C.

219

#### 220 *Immunohistochemistry and microscopy:*

221 After fixation developing pupal genitalia of all species except *D. melanogaster* were stained  
222 with rat anti-E-cadherin, 1:100 in PBT (DSHB Cat# DCAD2,RRID:AB\_528120) overnight at 4°C,  
223 followed by an overnight at 4°C incubation with anti-rat 488, 1:200 (Invitrogen, Carlsbad, CA) to  
224 visualize apical cell junctions. For *D. melanogaster*, an *armadillo*-GFP tagged line (Bloomington  
225 stock number #8556) was used to visualize apical cell junctions (Huang et al., 2012).  
226 Fluorescently labeled samples were mounted in glycerol mounting solution (80% glycerol, .1M  
227 Tris, pH 8.0) on microscope slides coated with poly-L-lysine (Thermo Fisher Scientific #86010).  
228 Samples for all species except *D. melanogaster* were imaged at 20X on a Leica TCS SP8 confocal  
229 microscope. *D. melanogaster* samples were imaged at either 20X or 40X on an Olympus  
230 Fluoview 1000. As the imaged structures are three-dimensional in nature, we used the  
231 MorphoGraphX program (de Reuille et al., 2015) to render and manipulate images in three-  
232 dimensions. This allowed us to rotate the samples to better present the most informative  
233 perspectives of the various phallic structures.

234 For light microscopy of adult phallic microdissections, samples were mounted in PVA  
235 Mounting Medium (BioQuip) until fully cleared and imaged at 20X magnification on a Leica DM  
236 2000 with a Leica DFC450C camera and the resulting images were enhanced using Adobe

237 Photoshop. For light microscopy images and videos of the whole phallus, genitalia were  
238 dissected in water, cleared overnight in 10% KOH at RT. These were then mounted in a drop of  
239 Dimethyl Hydantoin Formaldehyde (Steedman, 1958) on a coverslip and oriented using 2  
240 mounting needles before the resin hardened. Coverslip were positioned on a microscope slide,  
241 the hard drop facing away from the microscope lens. Images were acquired on Ti2-Eclipse  
242 Nikon microscope equipped with a 20x plan apochromatic lens and a 5.5 M sCMOS camera  
243 (PCO, Kelheim, Germany). Each preparation was imaged as a z-stack (z-step = 2  $\mu$ m). The stacks  
244 are presented as raw images. Stacks of images were also projected into single extended depth-  
245 of-field images using Helicon Focus software (HeliconSoft) and the resulting projections were  
246 enhanced using Adobe Photoshop.

247

248 *Establishing landmarks for early, middle, and late timepoints:*

249 We used confocal microscopy to chart a time course of the developing phallus (Figures S1-S3).  
250 To compare the development of the phallus of these species, we needed to examine whether  
251 all analyzed species develop at the same rate after pupal formation. Due to the large-scale  
252 changes in the phallus of these species, we used two stable features found outside of the  
253 phallus to calibrate developmental timing. In all analyzed species, the epandrial ventral lobe  
254 (lateral plate) and surstylus (clasper) first appear as a single continuous structure early in  
255 development, but then separate from each other as development progresses (Figure S2). We  
256 use the beginning of this separation as a landmark for the “early” developmental timepoint. We  
257 also used the midpoint of this progression to approximate the “mid” timepoint. This  
258 intermediate timepoint is useful in showing which tissue the phallic processes protrude from



259 during development. In all species, the adult cerci (anal plates) directly abut against one  
260 another but during “early” and “mid” development, these structures are separated from one  
261 another by a large gap (Figure S3). We designate “late” timepoint as directly preceding the  
262 closing of this gap between the cerci.

263 **Results:**

264 *Unpigmented cuticle reveals undescribed connection points in the phallus*

265           In order to better understand how the processes surrounding the aedeagus are  
266 physically connected to the neighboring tissues of the phallus, we imaged whole (Figure 2 B,C)  
267 and micro-dissected adult phalluses in eight members of the *melanogaster* species group  
268 (Figure 2 D-I). The phallus of each species can be partitioned into three discrete parts. The  
269 ventral portion (Figure 2 D,E) contains the pregonites, an outgrowth that contains three  
270 bristles, and the median gonocoxite (the central section of the shield shaped hypandrium). The  
271 central portion (Figure 2 F,G) contains the aedeagus, through which sperm is transferred. The  
272 dorsolateral portion contains the postgonal sheath (referred to as aedeagal sheath in (Rice et  
273 al., 2019)), a flat sheet that wraps around the aedeagus, and the pair of processes known as the  
274 postgonal processes (referred to as postgonites in Rice et al., 2019) (Figure 2 H,I). Analysis of  
275 these dissections support the designation of the postgonal sheath and postgonal processes as a  
276 single tissue (Bock & Wheeler, 1972; Okada, 1954; Tsacas et al., 1971). Furthermore, we found  
277 that certain species had processes connected to different portions of the phallus— a ventral  
278 portion (*D. erecta*, *D. ananassae*), a central portion (*D. santomea*, *D. teissieri*, *D. orena*, and *D.*  
279 *malerkotliana*), and a dorsolateral portion (all members of the *melanogaster* subgroup).

280           While imaging, we observed that parts of the postgonal sheath in the *melanogaster*  
281 species subgroup and *D. malerkotliana* were partially translucent, and only detectible after  
282 microdissection. It is this translucent tissue of the postgonal sheath that physically connects to  
283 the postgonal processes in *D. melanogaster* (Figure 2I). These observations highlight that, due to  
284 their transparency, determining the exact connection points between the processes and the

285 rest of the phallus can be difficult to visualize by traditional light microscopy approaches. To  
286 test whether the different connection points of the phallic processes observed in the adult  
287 reflect separate homology groups we investigated whether these phallic processes were  
288 initially produced by the same or different primordia during development.

289

### 290 *Phallic structures develop from three primordia in D. melanogaster*

291 To date, the morphogenesis of the three-dimensional adult phallic structures from the  
292 epithelium of the larval genital disc has been investigated only in *D. melanogaster* (Ahmad &  
293 Baker, 2002; Epper, 1983). Additionally, using surgical fragmentation of the larval genital disc,  
294 Bryant & Hsei, 1977 provided a fate map for the different adult structures. They showed that  
295 the phallus is situated at the subcenter of the symmetrical imaginal disc and is surrounded on  
296 each side by a primordium that will produce the medium gonocoxite and pregonites. However,  
297 the sequence and timing of the appearance of the various substructures of the phallus during  
298 development, remains unknown. By finding the key points in development where substructures  
299 first emerge, we can determine the primordium from which each process initially forms.

300 Early in *D. melanogaster* pupal development (see timepoint determination in the  
301 Materials and Methods) the phallus is separated into three stereotypic regions that likely  
302 represent primordia: ventral, dorsolateral and central (Figure 1B). As the pupal phallus  
303 continues to develop from this point, the ventral primordia form a pair of processes (Figure 1A).  
304 This pair develops into the small processes known as the pregonites that can be recognized  
305 from the presence of minute bristles (Figure 1B arrows), while the remainder of the primordia  
306 forms the median gonocoxite (Figure S4). The dorsolateral primordia produce two processes

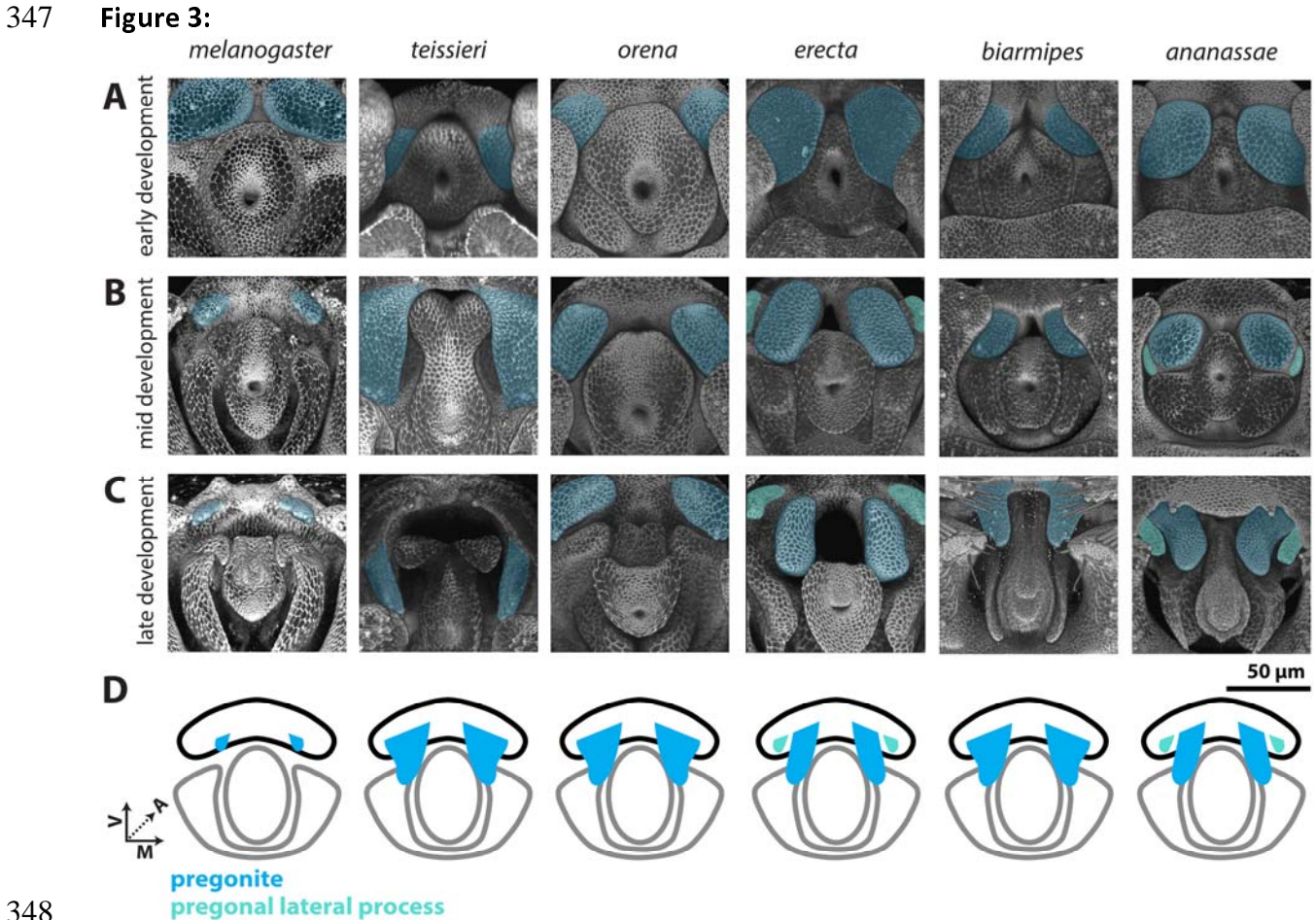
307 (one dorsal and one ventral) (Figure 3B). These processes ultimately develop into the ventral  
308 and dorsal postgonal processes (referred to as ventral postgonite and dorsal postgonite in Rice  
309 et al., 2019) (Figure 1A) The remaining parts of the dorsolateral tissue develop into the large  
310 flaps of the postgonal sheath (Figure 1A, Figure S5). The central primordium of *D. melanogaster*  
311 develops into the aedeagus and lacks a process (Figure 1A, Figure S6). *The three primordia are*  
312 *conserved across species*

313         Several studies have analyzed pupal development of the terminalia in species outside of  
314 *D. melanogaster*, but did not investigate phallic structures (Glassford et al., 2015; Hagen et al.,  
315 2019; Smith, Davidson, & Rebeiz, 2020). To determine whether the features of phallic  
316 development observed in *D. melanogaster* are conserved in members of the *melanogaster*  
317 species group, we produced a developmental time course for the remaining seven species  
318 studied here (Figure S1-S3). Our time course indicates that all adult phallic organs develop from  
319 three regions that are similar in size and shape to the ones described in *D. melanogaster* and  
320 thus likely represent homologous primordia. The ventral, dorsolateral, and central primordia  
321 produce the median gonocoxite (Figure S4), pregonal sheath (Figure S5), and aedeagus (Figure  
322 S6), respectively in all species. Nonetheless, significant interspecific differences were observed  
323 regarding the timing of development (Figure S1). As we only used one strain per species, we  
324 cannot comment if these are particular properties of the strains/laboratory conditions we used  
325 or are general differences between the species. We found that most species had early, mid, and  
326 late developmental timepoints within a six-hour window of each other (Figure S1-S3).

327

328 *Different processes emerge from different primordia*

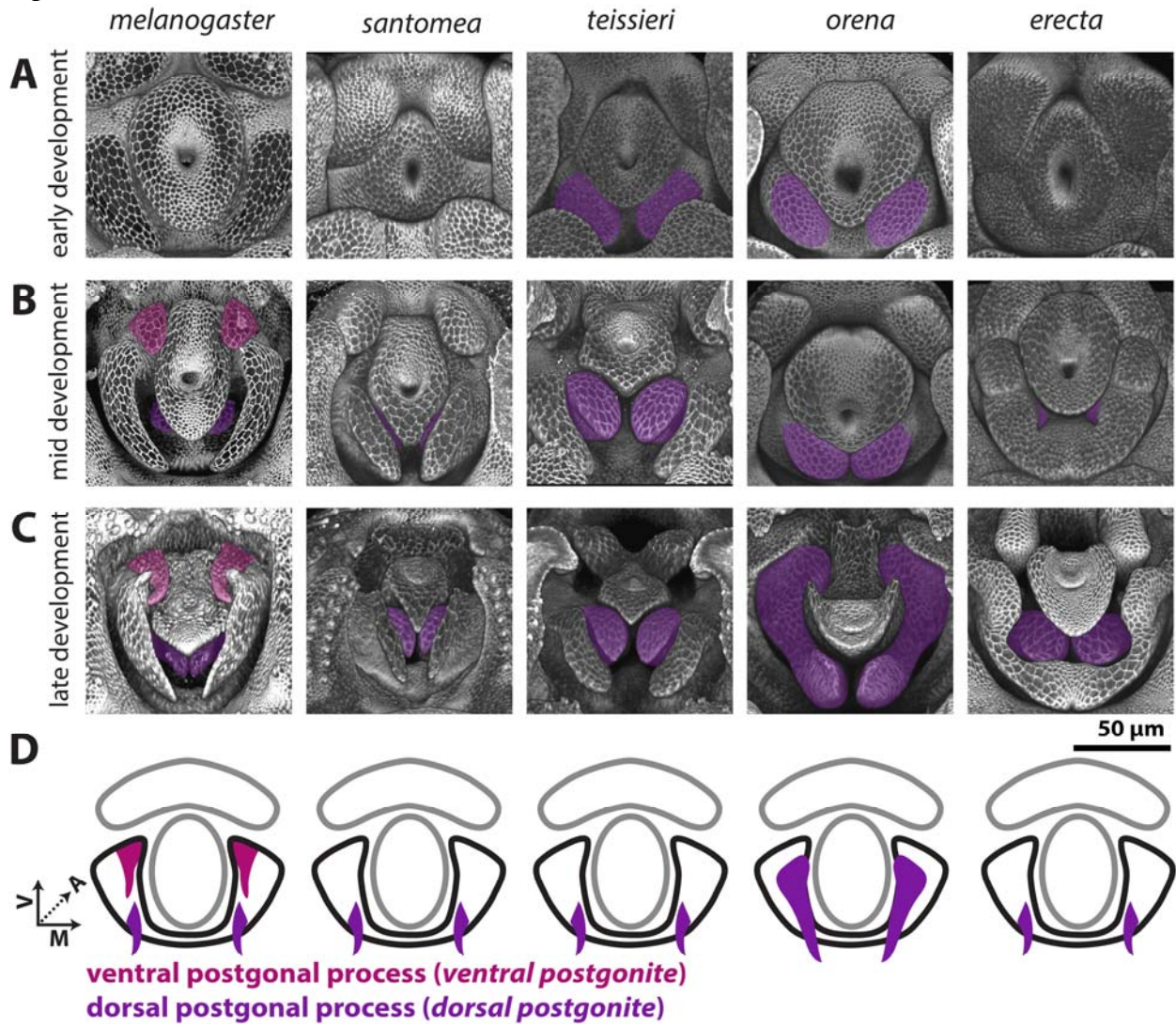
329           The developmental analysis of the eight species used in this study allowed us to test  
330 whether the phallic processes seen in the adults of each species were produced by the same  
331 primordia. We began by investigating the ventral primordium (Figure 3, Figure S4), which  
332 develops into the pregonites in all analyzed species. While the size of the pregonites varies  
333 between species, during mid-development (Figure 4B) we can identify recognizable outgrowths  
334 from the ventral primordium, consistent with a highly conserved developmental trajectory.  
335 Interestingly, an additional pregonal process is found in two distantly-related species, *D. erecta*  
336 and *D. ananassae*. Both *D. erecta* and *D. ananassae*, produce two processes from their ventral  
337 primordia, a large pregonal medial process and a second smaller pregonal lateral process which  
338 contains the three pregonal bristle cells (Figure S7) and overall resembles the pregonites of  
339 other species. To determine whether this additional process was produced by duplication or  
340 fission of the pregonite we inspected early pupal timepoints. We found that initially a single  
341 process is formed (Figure 3A), which during mid-development asymmetrically splits along the  
342 medial-lateral axis to form the distinct lobe-like pregonal medial process (Figure 3B). These  
343 asymmetric projections then extend in late development to form the larger pregonal medial  
344 process and smaller pregonite (Figure 3C). Thus, although the ventral primordium produces the  
345 pregonite in all species we examined, in *D. erecta* and *D. ananassae* the ventral primordium is  
346 split into the pregonite and a pregonal medial process.



348  
349 **Figure 3:** Processes developing from the ventral primordium are found in all members of the  
350 *melanogaster* species group.  
351  
352 **A-C)** Signal from the apical cellular junctions was used to highlight the overall morphology of  
353 developing pupal genitalia. **D.** *teissieri*, *D. orena*, *D. erecta*, *D. biarmipes*, and *D. ananassae* were  
354 stained for ECAD while apical cell junctions were visualized in *D. melanogaster* by detecting  
355 *arm-GFP* (see methods). **A)** Early in development, a pair of processes, that will form the  
356 pregonites, can be visualized in the ventral primordia in all species shown (light blue). **B)** By  
357 mid-development, large processes can be found in all species shown except *D. melanogaster*. In  
358 *D. erecta* and *D. ananassae*, the pregonite is split into a large pregonal medial process and a  
359 small lateral bristle-bearing process (teal). **C)** By late development, the pregonites have  
360 extended to their full adult size and shape. The pregonites are connected to the medial-ventral  
361 portion of the median gonocoxite, see Figure S4. **D)** Schematic representations of the  
362 pregonites (blue) and pregonal lateral process (teal) showing their approximate size, number,  
363 and connections to the medial gonocoxite (outlined in black). All images have the same axes, V  
364 (Ventral), A (Anterior), M (Medial) and are the same scale.

365           The dorsolateral primordium (Figure 4, Figure S5) showed a number of large  
366   evolutionary changes within the *melanogaster* species group. We found that no species, other  
367   than *D. melanogaster*, develop the ventral process that forms the ventral postgonal process  
368   (Figure 4). By contrast, all members of the *melanogaster* species subgroup form dorsal  
369   postgonal processes. Outside of the *melanogaster* subgroup, we did not find any modifications  
370   of the dorsolateral primordium, which develops into a single thin, strongly sclerotized structure  
371   in those species that resembles the postgonal sheath of *D. melanogaster*. However, the size,  
372   and shape of these homologous structures significantly differ among species (Figure 2 H,I),  
373   ranging from the flat rod-like processes in *D. biarmipes*, to the strongly pointed sinuate  
374   processes in *D. ananassae*, and the minute, transparent sclerites in *D. malerkotliana*.

375 **Figure 4:**



376

377 **Figure 4:** Processes produced by the dorsolateral primordium are found only in the  
 378 *melanogaster* subgroup.

379

380 **A-C)** Signal from the apical cellular junctions was used to highlight the overall morphology of

381 developing pupal genitalia. *D. santomea*, *D. teissieri*, *D. orena*, and *D. erecta*, were stained for

382 ECAD while *D. melanogaster* samples used arm-GFP. **A)** Early in development, the dorsolateral

383 primordium is a smooth lobe like structure in all analyzed species. **B)** By mid-development, all

384 shown species form processes in the dorsal portion of the dorsolateral primordium (violet). *D.*

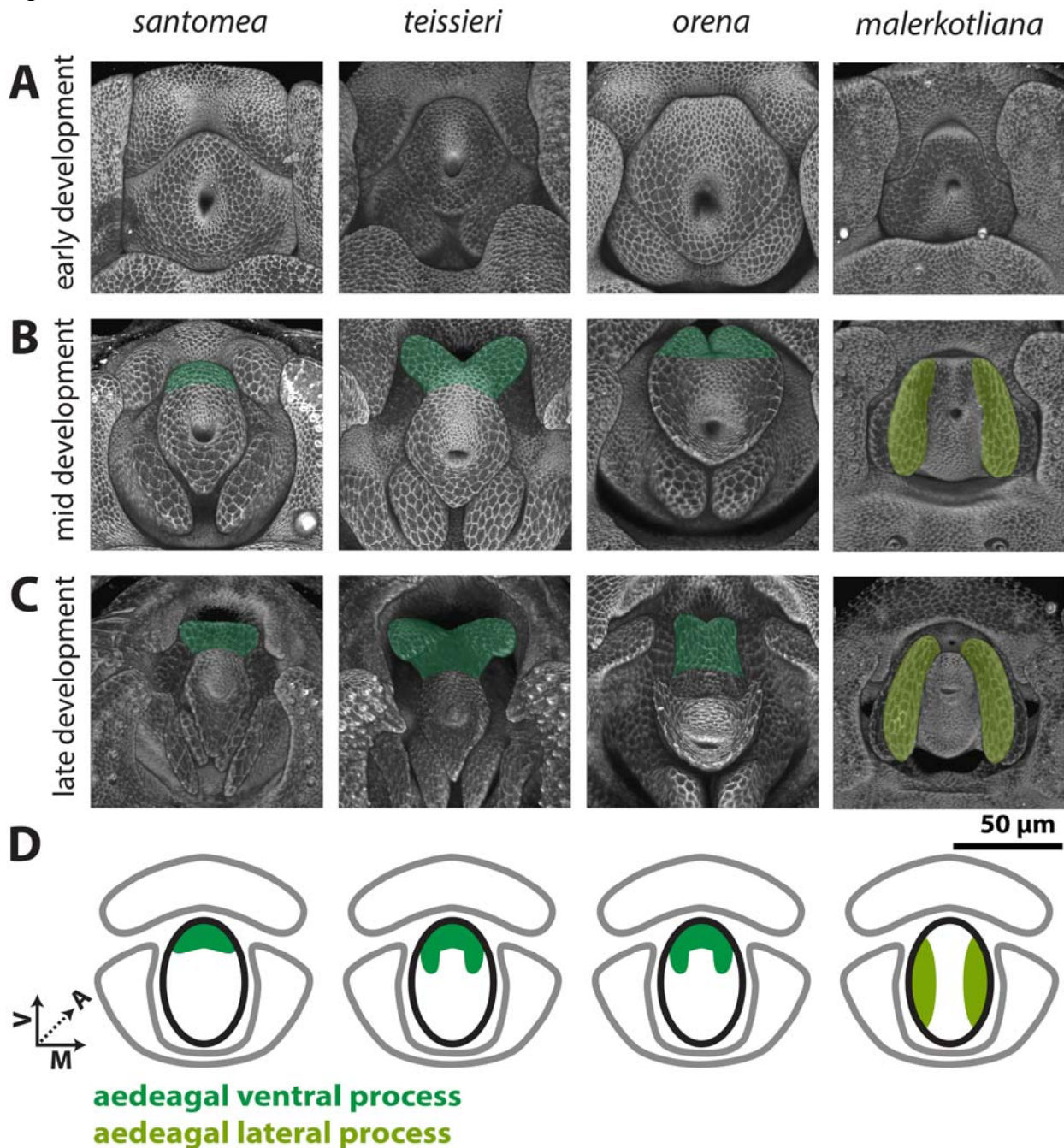
385 *melanogaster* also forms an additional pair of processes in the ventral portion of dorsolateral



386 primordium (magenta). **C**) By late development, the dorsal and ventral processes have  
387 extended to a long thin shape. Both the ventral and dorsal processes are connected to the  
388 medial-anterior part of the postgonal sheath which is formed by the remaining tissue of the  
389 dorsolateral primordium. **D**) Schematic representations of the dorsal (violet) and ventral  
390 (magenta) postgonal process showing where they connect to the postgonal sheath (outlined in  
391 black). All images have the same axes, V (Ventral), A (Anterior), M (Medial) and are the same  
392 scale. Ventral postgonite is an alternative term for ventral postgonal process, and dorsal  
393 postgonite is an alternative term for dorsal postgonite (Table 1).

394           While the central primordium (Figure 5, Figure S6) forms a simple aedeagus that lacks  
395 processes in *D. melanogaster*, we note processes which develop in *D. santomea*, *D. teissieri*, *D.*  
396 *orena* and *D. malerkotliana*. Early in development, the central primordia of all species analyzed  
397 are similar in size and shape (Figure 5A). However, during mid-development, in *D. santomea*, *D.*  
398 *teissieri*, and *D. orena*, the ventral side of the central primordium elongates to form a process  
399 (Figure 5B). The process of *D. teissieri* and *D. orena* splits along the ventral midline to form a  
400 pair of processes, while in *D. santomea*, it forms one rounded structure. These processes  
401 further elongate in late development to more closely resemble the size and shape of their adult  
402 counterparts (Figure 5C).

403 **Figure 5:**



404  
405 **Figure 5:** Processes developing from the central primordium are found in the *yakuba/erecta*  
406 and *bipectinata* complexes.

407  
408 **A-C)** Signal from the apical cellular junctions (ECAD) was used to highlight the overall  
409 morphology of developing pupal genitalia. **A)** Early in development, the central primordium  
410 forms a flat donut-shaped structure in all species shown. **B)** By mid-development, the ventral  
411 portion of the aedeagus is extended in *D. santomea*, *D. teissieri*, and *D. orena* in what will form  
412 the aedeagal ventral process (dark green). In *D. malerkotliana* the lateral edges of the central

413 primordium extend anteriorly in what will form the aedeagal lateral process (yellow-green). **C)**  
414 By late development, the aedeagal ventral process and the aedeagal lateral process further  
415 extend from the aedeagus. **D)** Schematic representations of the aedeagal ventral process (dark  
416 green) and aedeagal lateral process (yellow-green) showing where they connect to the  
417 aedeagus (outlined in black). All images have the same axes, V (Ventral), A (Anterior), M  
418 (Medial) and are the same scale.

419 Okada, 1954 and Bock & Wheeler, 1972 suggested that the aedeagus in the  
420 *melanogaster* species group were of two types: fused like in *D. ananassae* and split like in *D.*  
421 *malerkotliana*. Indeed, we did not observe any process in the central primordium of *D.*  
422 *ananassae*, whereas a pair of processes develops in *D. malerkotliana*. Kamimura, 2007  
423 suggested that the aedeagus of *D. malerkotliana* has degenerated and was replaced by a pair of  
424 lateral processes. During early development, the central primordium of *D. malerkotliana* is  
425 similar to all other analyzed species (Figure 5A). However, by mid-development, the lateral  
426 sides of the central primordium extend, forming a pair of processes, while the medial-dorsal  
427 and medial-ventral sides of the central primordium fail to extend (Figure 5B). Late in  
428 development, the proximal-dorsal side of the lateral process constricts, conferring a hook like  
429 shape (Figure 5C). As this substructure is produced from the lateral portions of the central  
430 primordium and not from the ventral portion, it is likely non-homologous to the aedeagal  
431 ventral processes of the *yakuba* and *erecta* complexes. We therefore propose the term  
432 aedeagal lateral process for this substructure of *D. malerkotliana*.

433 **Discussion:**

434           The rapid evolution of morphological structures is an attractive subject for study, as it  
435 allows us to glimpse at the molecular and genetic causes of remodeled and restructured  
436 anatomical forms. Here, we examined some of the most rapidly evolving morphologies of  
437 *Drosophila melanogaster* and its close relatives. Despite decades of research, many of the  
438 intricate phallic processes have eluded our ability to clearly classify their homology  
439 relationships. By studying the developmental trajectories of these processes in multiple species,  
440 we have better defined their physical connections, and clarified which structures most likely  
441 share ancestry. This research highlights the distinct challenges in studying novelties at  
442 mesoevolutionary scales, specifically that traits may be rapidly gained and lost between closely  
443 related species making it difficult to discern between true homology and convergence  
444 (Abouheif, 2008).

445

446 *Classification and nomenclature of rapidly evolving phallic structures*

447           Our results suggest that the great diversity of the phallic structures of the eight species  
448 studied here cluster into three homology groups corresponding to the three pupal primordia,  
449 leading us to propose revised naming conventions. First, our developmental analysis supports  
450 the notion, initially suggested by Okada, 1954, that the weakly sclerotized postgonal sheath and  
451 strongly sclerotized postgonal processes in *D. melanogaster*, are both parts of the same tissue,  
452 which *Drosophila* systematists called the “posterior paramere” e.g. (Bock & Wheeler, 1972;  
453 Tsacas et al., 1971). Because the term “posterior paramere” is itself synonymous to the term  
454 “postgonite” in Dipteran systematics (Tsacas et al., 1971; van Emden & Hennig, 1970), we

455 suggest using the term “postgonite” to encompass the combined tissue produced by the  
456 dorsolateral primordia in species of the *melanogaster* group (including both the postgonal  
457 sheath and the processes), and the term “postgonal processes” to designate the strongly  
458 sclerotized branches emerging from this tissue in the *melanogaster* subgroup. Second, our  
459 results also show that the structures previously called the “basal processes” (Kamimura, 2007,  
460 2010, 2016; Kamimura & Mitsumoto, 2011, 2012a, 2012b; Kamimura & Polak, 2011), develop  
461 from different primordia and are therefore most likely non-homologous. We suggest therefore  
462 to give them distinct names that directly relate to the tissues that produce them: aedeagal  
463 ventral process in species of the *yakuba* complex (synonymous to Yassin & Orgogozo, 2013  
464 phallic spur) and *D. orena* (synonymous to Yassin & Orgogozo, 2013 phallic hook), the pregonal  
465 medial process in *D. erecta* and *D. ananassae*, and aedeagal lateral process in *D. malerkotliana*  
466 and species of the *biplectinata* complex (Table 1). Future work that establishes the extent of cell  
467 migration between or with the three regions that we designate as primordium will further  
468 improve our resolution of the homology of these phallic processes.

469

#### 470 *Evolution of the phallic structures*

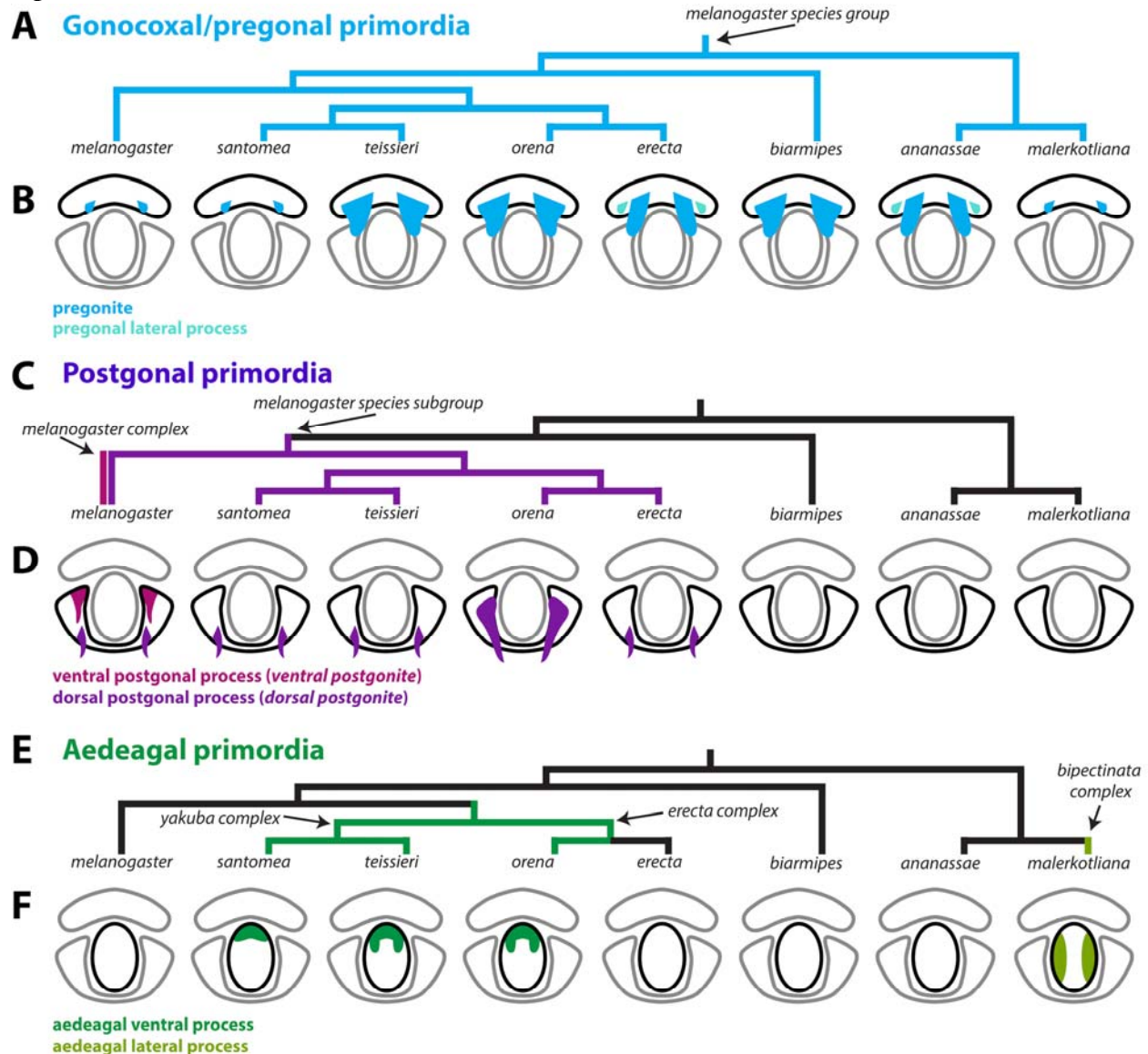
471 Mapping character states over robust phylogenies provide the opportunity to  
472 distinguish novel from recurrent (homoplastic) states as well as derived states (synapomorphic)  
473 from ancestral (symplesiomorphic) ones. Our findings have led us to propose a model for the  
474 evolution of the phallic processes found in the *melanogaster* species subgroup (Figure 6). For  
475 example, our demonstration of the development of an additional pregonal process in *D. erecta*  
476 and *D. ananassae* (Figure 3, S4) is likely recurrent, as illustrations from Bock & Wheeler, 1972

477 suggest that this configuration of the pregonites might have recurrently evolved in this clade.  
478 Reversals to ancestral states through secondary losses represent another mechanism of  
479 recurrent evolution. The lack of the aedeagal ventral processes in *D. erecta*, is more likely due  
480 to loss rather than an independent gain of the aedeagal ventral process in *D. orena*.



481

**Figure 6:**



482

483 **Figure 6:** A model of the evolution of phallic processes in the *melanogaster* species group

484

485 **A,C,E)** Phylogeny of the 8 analyzed species based on Obbard et al., 2012. **A)** Parsimony suggests

486 that the pregonites originated outside of the *D. melanogaster* species group. **B)** Light blue

487 represents the pregonites and teal represents the pregonal lateral process. **C)** Parsimony

488 suggests that the ventral postgonites originated in the *melanogaster* complex (*D. melanogaster*,

489 *D. simulans*, *D. mauritiana*, *D. sechellia*) and that the dorsal postgonal process originated in the

490 *melanogaster* subgroup. **D)** Magenta represents the ventral postgonal process, violet

491 represents the dorsal postgonal process. **E)** Parsimony suggests that the aedeagal ventral

492 process originated at the base of the *erecta* and *yakuba* complexes. Additionally, parsimony

493 suggests that the aedeagal lateral process originated in the bipectinata complex. **F)** Dark green

494 represents the aedeagal ventral process and yellow-green represents the aedeagal lateral

495 process. Ventral postgonite is an alternative term for ventral postgonal process, and dorsal  
496 postgonite is an alternative term for dorsal postgonite. Future developmental analysis across  
497 more members within and outside of the *D. melanogaster* species group will be required to  
498 better establish when these distinct phallic processes first originated.

499           In the *melanogaster* subgroup, all species contain a strongly-sclerotized dorsal  
500 postgonal process which develops as a localized extension within the dorsolateral primordium.  
501 The development of a strongly-sclerotized ventral postgonal process is a definitive novelty in *D.*  
502 *melanogaster* and allied species of the *melanogaster* complex. Although we did not find  
503 structures resembling the dorsal postgonal processes in members outside of the *melanogaster*  
504 species subgroup that we studied here, polarization remains difficult. Indeed, Okada, 1954 and  
505 Bock & Wheeler, 1972 reported the presence of “basal processes of the posterior parameres”  
506 in multiple members of the *melanogaster* species group. Similarly, Bächli et al., 2004 illustrated  
507 the presence of “ribbon-shaped process” in several members of the *obscura* group which is  
508 sister to the *melanogaster* species group. Further taxonomic sampling and better phylogenetic  
509 resolution of those clades are required to draw a more complete picture of the evolution of the  
510 postgonal differentiation outside the *melanogaster* subgroup. The novel structures described  
511 here may present an excellent model to study the molecular mechanisms producing novelty.

512           Although we do not address the function of the phallic processes, other research groups  
513 have demonstrated that the rapid evolution of male genital sclerites in arthropods were most  
514 likely driven by selection (Eberhard, 1985; Hosken, Archer, House, & Wedell, 2019; Simmons,  
515 2014). These included groups as diverse as spiders (Huber, 2005), damselflies (Cordero-Rivera,  
516 2017), waterstriders (Rowe & Arnqvist, 2012), moths (McNamara, Dougherty, Wedell, &  
517 Simmons, 2019) and beetles (Simmons & Fitzpatrick, 2019). This unique mechanism is  
518 remarkable given the diverse developmental origin of the rapidly evolving male structures in  
519 these groups, being pedipalps in spiders (Quade et al., 2019), cerci in damselflies (McPeck,  
520 Shen, & Farid, 2009), pregenital segments in waterstriders (Perry & Rowe, 2018), chitinous

521 spermatophores in butterflies (Sánchez & Cordero, 2014), or aedeagii of appendicular and non-  
522 appendicular origins in bugs and beetles (Aspiras, Smith, & Angelini, 2011). However, the exact  
523 form of selection, e.g., cryptic female choice, sexual conflict, etc., remains debated in the  
524 literature (Ah-King, Barron, & Herberstein, 2014; Brennan & Prum, 2015; Eberhard, 1985;  
525 Masly, 2012).

526           In *Drosophila*, the various processes of the phallus have been implicated in copulatory  
527 wounding of the female (Kamimura, 2007, 2016; Muto et al., 2018; Yassin & Orgogozo, 2013).  
528 Furthermore, studies have found that some of the phallic processes pivot from pointing  
529 posteriorly to pointing laterally, when the phallus is everted during copulation, thus directing  
530 how they interact with the female reproductive tract (Kamimura, 2010). The ability to pivot  
531 during copulation correlates with the homology groups we have found in this study. The ventral  
532 and dorsal postgonal processes, and pregonites pivot during copulation while the aedeagal  
533 ventral process does not change orientation. This may be due to the direct connection of the  
534 aedeagal ventral process to the aedeagus. Surprisingly the aedeagal lateral process, which is  
535 also directly connected to the aedeagus, pivots laterally during copulation, which may only be  
536 possible due to the loss of aedeagal sclerotization, making the tissue between the aedeagal  
537 lateral processes flexible. Co-evolution between the phallic processes and the female genitalia  
538 has been suggested and several novel modifications of the female genitalia have been  
539 identified (Kamimura, 2007; Yassin & Orgogozo, 2013). A developmental analysis of the female  
540 genitalia of these species along with three-dimensional analysis of copulating flies similar to  
541 studies in *D. melanogaster* (Mattei et al., 2015; Shao et al., 2019) would provide vital context  
542 for the potential co-evolution of novel male and female structures.

543 *Developmental mechanisms underlying phallic evolution*

544           A major challenge in the evo-devo field has been to identify the molecular mechanisms  
545 driving morphological novelty (Linz, Hu, & Moczek, 2020; Moczek, 2008; Rebeiz, Patel, &  
546 Hinman, 2015; G. P. Wagner & Lynch, 2010). While macroevolutionary novelties have been the  
547 focus of coarse-grained molecular study (Bruce & Patel, 2020; Clark-Hachtel & Tomoyasu, 2020;  
548 Emlen et al., 2006; Hinman et al., 2003; Prud'Homme et al., 2011), much hope has been placed  
549 on rapidly diverging structures in molecularly amenable systems (Rebeiz & Tsiantis, 2017).  
550 Recent work in *Drosophila* genital evolution has highlighted how quickly changes in cellular  
551 morphology (Green et al., 2019; Smith et al., 2020), and genetic networks (Glassford et al.,  
552 2015; Hagen et al., 2021; Nagy et al., 2018) can lead to shifts in morphology in closely related  
553 species. Our work highlights distinct underexplored challenges to interpreting and advancing  
554 these model systems. The ambiguous ancestry of similar parts which appear in different  
555 locations causes us to consider multiple models to explain their emergence. These parts may  
556 arise by parallelism – a predisposition to drive similar new structures by co-opting the same  
557 networks (Abouheif, 2008). Alternately, it is entirely possible that these structures are indeed  
558 ancestral but have undergone massive tissue reorganizations to reposition their attachment  
559 points. Such repositioning could be caused by moving the location of a critical signal or  
560 transcription factor within the tissues. Alternately, these structures could be specified before  
561 the discernable tissues of the phallus are separated, and their migration could be caused by  
562 differences in tissue folding. Under this scenario, we would anticipate that critical tissue  
563 patterning regulators of these processes are activated before these tissues become discernable.  
564 Finally, it is entirely possible that completely different networks account for the appearance of

565 these unique structures. Developmental genetic analysis of the genes that produce the phallic  
566 processes described above will aid us in distinguishing these models. Recent work has identified  
567 several genes that are spatially restricted to the pregonites and postgonal processes of *D.*  
568 *melanogaster* providing an ideal set of candidates to examine (Vincent et al., 2019). Thus, we  
569 envision that detailed mechanisms of parallelism, repositioning, and novelty will emerge from  
570 studying systems where both network architecture is accessible, and genetic manipulations can  
571 be introduced to test the sufficiency of these mechanisms to produce these novel  
572 morphological structures.

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583 a truly inspirational scientist and friend; we were privileged to know him.

584

585 **Data Availability Statement:**

586 The data that support the findings of this study are available from the corresponding author  
587 upon reasonable request.

## 588 **References:**

- 589
- 590 Abouheif, E. (2008). Parallelism as the pattern and process of mesoevolution. *Evolution and*  
591 *Development*, 10(1), 3–5. <https://doi.org/10.1111/j.1525-142X.2007.00208.x>
- 592 Acebes, A., Cobb, M., & Ferveur, J. F. (2003). Species-specific effects of single sensillum ablation  
593 on mating position in *Drosophila*. *Journal of Experimental Biology*, 206(17), 3095–3100.  
594 <https://doi.org/10.1242/jeb.00522>
- 595 Ah-King, M., Barron, A. B., & Herberstein, M. E. (2014). Genital Evolution: Why Are Females Still  
596 Understudied? *PLoS Biology*, 12(5), 1–7. <https://doi.org/10.1371/journal.pbio.1001851>
- 597 Ahmad, S. M., & Baker, B. S. (2002). Sex-Specific Deployment of FGF Signaling in *Drosophila*  
598 Recruits Mesodermal Cells into the Male Genital Imaginal Disc, 109, 651–661.
- 599 Al Sayad, S., & Yassin, A. (2019). Quantifying the extent of morphological homoplasy: A  
600 phylogenetic analysis of 490 characters in *Drosophila*. *Evolution Letters*, 3(3), 286–298.  
601 <https://doi.org/10.1002/evl3.115>
- 602 Aspiras, A. C., Smith, F. W., & Angelini, D. R. (2011). Sex-specific gene interactions in the  
603 patterning of insect genitalia. *Developmental Biology*, 360(2), 369–380.  
604 <https://doi.org/10.1016/j.ydbio.2011.09.026>
- 605 Avila, F. W., Sirot, L. K., Laflamme, B. A., Rubinstein, C. D., & Wolfner, M. F. (2011). Insect  
606 seminal fluid proteins: Identification and function. *Annual Review of Entomology*, 56, 21–  
607 40. <https://doi.org/10.1146/annurev-ento-120709-144823>
- 608 Bächli, G., Vilela, C., Andersson Escher, S., & Saura, A. (2004). *The Drosophilidae (Diptera) of 252*  
609 *Fennoscandia and Denmark. Fauna Entomologica Scandinavica*. Brill.
- 610 Bock, I. R., & Wheeler, M. R. (1972). The *Drosophila melanogaster* species group. *The University*  
611 *of Texas Publication*, VII(7213), 1–102.
- 612 Brennan, P. L. R., & Prum, R. O. (2015). Mechanisms and evidence of genital coevolution: The  
613 roles of natural selection, mate choice, and sexual conflict. *Cold Spring Harbor Perspectives*  
614 *in Biology*, 7(7), 1–21. <https://doi.org/10.1101/cshperspect.a017749>
- 615 Bruce, H. S., & Patel, N. H. (2020). Knockout of crustacean leg patterning genes suggests that  
616 insect wings and body walls evolved from ancient leg segments. *Nature Ecology and*  
617 *Evolution*, 4(12), 1703–1712. <https://doi.org/10.1038/s41559-020-01349-0>
- 618 Bryant, P. J., & Hsej, B. W. (1977). Pattern formation in asymmetrical and symmetrical imaginal  
619 discs of *Drosophila melanogaster*. *American Zoologist*, 17(3), 595–611.  
620 <https://doi.org/10.1093/icb/17.3.595>
- 621 Church, S. H., & Extavour, C. G. (2020). Null hypotheses for developmental evolution.  
622 *Development (Cambridge)*, 147(8), 1–6. <https://doi.org/10.1242/DEV.178004>
- 623 Clark-Hachtel, C. M., & Tomoyasu, Y. (2020). Two sets of candidate crustacean wing  
624 homologues and their implication for the origin of insect wings. *Nature Ecology and*  
625 *Evolution*, 4(12), 1694–1702. <https://doi.org/10.1038/s41559-020-1257-8>
- 626 Cordero-Rivera, A. (2017). Sexual conflict and the evolution of genitalia: male damselflies  
627 remove more sperm when mating with a heterospecific female. *Scientific Reports*, 7(1), 1–  
628 8. <https://doi.org/10.1038/s41598-017-08390-3>
- 629 de Reuille, P. B., Routier-Kierzkowska, A. L., Kierzkowski, D., Bassel, G. W., Schüpbach, T.,  
630 Tauriello, G., ... Smith, R. S. (2015). MorphoGraphX: A platform for quantifying  
631 morphogenesis in 4D. *ELife*, 4(MAY). <https://doi.org/10.7554/eLife.05864>



- 632 Doleschall, C. L. (1858). Derde bijdrage tot de kennis der Dipteren fauna van Nederlandsch  
633 Indie. *Natuurkundig Tijdschrift Voor Nederlandsch Indie*, 17, 73–128.
- 634 Eberhard, W. G. (1985). *Sexual Selection and Animal Genitalia*. Cambridge, Mass: Harvard  
635 University Press.
- 636 Emlen, D. J., Szafran, Q., Corley, L. S., & Dworkin, I. (2006). Insulin signaling and limb-patterning:  
637 Candidate pathways for the origin and evolutionary diversification of beetle “horns.”  
638 *Heredity*, 97(3), 179–191. <https://doi.org/10.1038/sj.hdy.6800868>
- 639 Epper, F. (1983). The Evagination of the Genital Imaginal Discs of *Drosophila melanogaster* II.  
640 Morphogenesis of the Intersexual Genital Disc of the Mutant doublesex-dominant (dsx D).  
641 *Roux's Archives of Developmental Biology*, 192, 280–284.
- 642 Glassford, W. J., Johnson, W. C., Dall, N. R., Smith, S. J., Liu, Y., Boll, W., ... Rebeiz, M. (2015). Co-  
643 option of an Ancestral Hox-Regulated Network Underlies a Recently Evolved  
644 Morphological Novelty Article. *Developmental Cell*, 34(5), 520–531.  
645 <https://doi.org/10.1016/j.devcel.2015.08.005>
- 646 Green, J. E., Cavey, M., Médina Caturegli, E., Aigouy, B., Gompel, N., & Prud'homme, B. (2019).  
647 Evolution of Ovipositor Length in *Drosophila suzukii* Is Driven by Enhanced Cell Size  
648 Expansion and Anisotropic Tissue Reorganization. *Current Biology*, 29(12), 2075–2082.e6.  
649 <https://doi.org/10.1016/j.cub.2019.05.020>
- 650 Hagen, J. F. D., Mendes, C. C., Blogg, A., Payne, A., Tanaka, K. M., Gaspar, P., ... Nunes, M. D. S.  
651 (2019). Tartan underlies the evolution of *Drosophila* male genital morphology. *Proceedings*  
652 *of the National Academy of Sciences of the United States of America*, 116(38), 19025–  
653 19030. <https://doi.org/10.1073/pnas.1909829116>
- 654 Hagen, J. F. D., Mendes, C. C., Booth, S. R., Figueras Jimenez, J., Tanaka, K. M., Franke, F. A., ...  
655 McGregor, A. P. (2021). Unraveling the Genetic Basis for the Rapid Diversification of Male  
656 Genitalia between *Drosophila* Species. *Molecular Biology and Evolution*, 38(2), 437–448.  
657 <https://doi.org/10.1093/molbev/msaa232>
- 658 Hinman, V. F., Nguyen, A. T., Cameron, R. A., & Davidson, E. H. (2003). Developmental gene  
659 regulatory network architecture across 500 million years of echinoderm evolution.  
660 *Proceedings of the National Academy of Sciences of the United States of America*, 100(23),  
661 13356–13361. <https://doi.org/10.1073/pnas.2235868100>
- 662 Hosken, D. J., Archer, C. R., House, C. M., & Wedell, N. (2019). Penis evolution across species:  
663 divergence and diversity. *Nature Reviews Urology*, 16(2), 98–106.  
664 <https://doi.org/10.1038/s41585-018-0112-z>
- 665 Hsu, T. C. (1949). The external genital apparatus of male *Drosophilidae* in relation to  
666 systematics. *The University of Texas Publication*, 4920, 80–142.
- 667 Huang, J., Huang, L., Chen, Y. J., Austin, E., Devor, C. E., Roegiers, F., & Hong, Y. (2012).  
668 Differential regulation of adherens junction dynamics during apical-basal polarization.  
669 *Development*, 139(3), 4001–4013. <https://doi.org/10.1242/jcs.086694>
- 670 Huber, B. A. (2005). Sexual selection research on spiders: Progress and biases. *Biological*  
671 *Reviews of the Cambridge Philosophical Society*, 80(3), 363–385.  
672 <https://doi.org/10.1017/S1464793104006700>
- 673 Jagadeeshan, S., & Singh, R. S. (2006). A time-sequence functional analysis of mating behaviour  
674 and genital coupling in *Drosophila*: Role of cryptic female choice and male sex-drive in the  
675 evolution of male genitalia. *Journal of Evolutionary Biology*, 19(4), 1058–1070.

- 676 <https://doi.org/10.1111/j.1420-9101.2006.01099.x>
- 677 Kamimura, Y. (2007). Twin intromittent organs of *Drosophila* for traumatic insemination.
- 678 *Biology Letters*, 3(4), 401–404. <https://doi.org/10.1098/rsbl.2007.0192>
- 679 Kamimura, Y. (2010). Copulation anatomy of *Drosophila melanogaster* (Diptera: Drosophilidae):
- 680 Wound-making organs and their possible roles. *Zoomorphology*, 129(3), 163–174.
- 681 <https://doi.org/10.1007/s00435-010-0109-5>
- 682 Kamimura, Y. (2012). Correlated evolutionary changes in *Drosophila* female genitalia reduce the
- 683 possible infection risk caused by male copulatory wounding. *Behavioral Ecology and*
- 684 *Sociobiology*, 66(8), 1107–1114. <https://doi.org/10.1007/s00265-012-1361-0>
- 685 Kamimura, Y. (2016). Significance of constraints on genital coevolution: Why do female
- 686 *Drosophila* appear to cooperate with males by accepting harmful matings? *Evolution;*
- 687 *International Journal of Organic Evolution*, 70(7), 1674–1683.
- 688 <https://doi.org/10.1111/evo.12955>
- 689 Kamimura, Y., & Mitsumoto, H. (2011). Comparative copulation anatomy of the *Drosophila*
- 690 *melanogaster* species complex (Diptera: Drosophilidae). *Entomological Science*, 14(4),
- 691 399–410. <https://doi.org/10.1111/j.1479-8298.2011.00467.x>
- 692 Kamimura, Y., & Mitsumoto, H. (2012a). Genital coupling and copulatory wounding in
- 693 *drosophila teissieri* (diptera: Drosophilidae). *Canadian Journal of Zoology*, 90(12), 1437–
- 694 1440. <https://doi.org/10.1139/cjz-2012-0186>
- 695 Kamimura, Y., & Mitsumoto, H. (2012b). Lock-and-key structural isolation between sibling
- 696 *Drosophila* species. *Entomological Science*, 15(2), 197–201.
- 697 <https://doi.org/10.1111/j.1479-8298.2011.00490.x>
- 698 Kamimura, Y., & Polak, M. (2011). Does surgical manipulation of *drosophila* intromittent organs
- 699 affect insemination success? *Proceedings of the Royal Society B: Biological Sciences*,
- 700 278(1707), 815–816. <https://doi.org/10.1098/rspb.2010.2431>
- 701 Klaus, A. V., Kulasekera, V. L., & Schawaroch, V. (2003). Three-dimensional visualization of
- 702 insect morphology using confocal laser scanning microscopy. *Journal of Microscopy*,
- 703 212(2), 107–121. <https://doi.org/10.1046/j.1365-2818.2003.01235.x>
- 704 Lachaise, D., Capy, P., Cariou, M.-L., Joly, D., Lemeunier, F., & David, J. R. (2004). Nine relatives
- 705 from one African ancestor: population biology and evolution of the *Drosophila*
- 706 *melanogaster* subgroup species. In R. S. Singh, M. K. Uyenoyama, & S. K. Jain (Eds.), *The*
- 707 *Evolution of Population Biology* (pp. 315–344). Cambridge University Press.
- 708 <https://doi.org/10.1017/cbo9780511542619.019>
- 709 Lachaise, D., Harry, M., Solignac, M., Lemeunier, F., Benassi, V., & Cariou, M. L. (2000).
- 710 Evolutionary novelties in islands: *Drosophila santomea*, a new *melanogaster* sister species
- 711 from Sao Tome. *Proceedings of the Royal Society B: Biological Sciences*, 267(1452), 1487–
- 712 1495. <https://doi.org/10.1098/rspb.2000.1169>
- 713 Linz, D. M., Hu, Y., & Moczek, A. P. (2020). From descent with modification to the origins of
- 714 novelty. *Zoology*, 143(August), 125836. <https://doi.org/10.1016/j.zool.2020.125836>
- 715 Malloch, J. R. (1924). Two *Drosophilidae* from Coimbatore. *Memoirs of the Department of*
- 716 *Agriculture in India. Entomological Series*, 8(6), 63–65.
- 717 Markow, T. A., & O’Grady, P. M. (2006). *Drosophila: A Guide to Species Identification and Use.*
- 718 *Academic Press.* London.
- 719 Masly, J. P. (2012). 170 Years of “Lock-and-Key”: Genital Morphology and Reproductive

- 720 Isolation. *International Journal of Evolutionary Biology*, 2012, 1–10.  
721 <https://doi.org/10.1155/2012/247352>
- 722 Masly, J. P., & Kamimura, Y. (2014). Asymmetric mismatch in strain-specific genital morphology  
723 causes increased harm to drosophila females. *Evolution*, 68(8), 2401–2411.  
724 <https://doi.org/10.1111/evo.12436>
- 725 Mattei, A. L., Riccio, M. L., Avilaa, F. W., Wolfner, M. F., & Denlinger, D. L. (2015). Integrated 3D  
726 view of postmating responses by the *Drosophila melanogaster* female reproductive tract,  
727 obtained by micro-computed tomography scanning. *Proceedings of the National Academy  
728 of Sciences of the United States of America*, 112(27), 8475–8480.  
729 <https://doi.org/10.1073/pnas.1505797112>
- 730 McNamara, K. B., Dougherty, L. R., Wedell, N., & Simmons, L. W. (2019). Experimental evolution  
731 reveals divergence in female genital teeth morphology in response to sexual conflict  
732 intensity in a moth. *Journal of Evolutionary Biology*, 32(5), 519–524.  
733 <https://doi.org/10.1111/jeb.13428>
- 734 McPeck, M. A., Shen, L., & Farid, H. (2009). The correlated evolution of three-dimensional  
735 reproductive structures between male and female damselflies. *Evolution*, 63(1), 73–83.  
736 <https://doi.org/10.1111/j.1558-5646.2008.00527.x>
- 737 Meigen, J. W. (1830). *Systematische Beschreibung der bekannten europäischen zweiflügeligen  
738 Insekten*. Schulze.
- 739 Moczek, A. P. (2008). On the origins of novelty in development and evolution. *BioEssays*, 30(5),  
740 432–447. <https://doi.org/10.1002/bies.20754>
- 741 Muto, L., Kamimura, Y., Tanaka, K. M., & Takahashi, A. (2018). An innovative ovipositor for  
742 niche exploitation impacts genital coevolution between sexes in a fruit-damaging  
743 *Drosophila*. *Proceedings of the Royal Society B: Biological Sciences*.  
744 <https://doi.org/10.1098/rspb.2018.1635>
- 745 Nagy, O., Nuez, I., Savisaar, R., Peluffo, A. E., Yassin, A., Lang, M., ... Courtier-Orgogozo, V.  
746 (2018). Correlated Evolution of Two Copulatory Organs via a Single cis-Regulatory  
747 Nucleotide Change. *Current Biology*, 28(21), 1–8.  
748 <https://doi.org/10.1016/j.cub.2018.08.047>
- 749 Obbard, D. J., MacLennan, J., Kim, K. W., Rambaut, A., O’Grady, P. M., & Jiggins, F. M. (2012).  
750 Estimating divergence dates and substitution rates in the drosophila phylogeny. *Molecular  
751 Biology and Evolution*, 29(11), 3459–3473. <https://doi.org/10.1093/molbev/mss150>
- 752 Okada, T. (1954). Comparative morphology of the drosophilid flies. I. Phallic organs of the  
753 melanogaster group. *Kontyu*, 22, 36–46.
- 754 Parshad, R., & Paika, I. J. (1964). Drosophilid survey of India. II. Taxonomy and cytology of the  
755 subgenus *Sophophora* (*Drosophila*). *Research Bulletin of the Panjab University. Science.*,  
756 15, 225–252.
- 757 Peluffo, A. E., Nuez, I., Debat, V., Savisaar, R., Stern, D. L., & Orgogozo, V. (2015). A major locus  
758 controls a genital shape difference involved in reproductive isolation between *Drosophila*  
759 *yakuba* and *Drosophila santomea*. *G3: Genes, Genomes, Genetics*, 5(12), 2893–2901.  
760 <https://doi.org/10.1534/g3.115.023481>
- 761 Peluffo, A., Hamdani, M., Vargas-Valderrama, A., David, J., Mallard, F., Graner, F., & Courtier-  
762 Orgogozo, V. (2021). A morphological trait involved in reproductive isolation between  
763 *Drosophila* sister species is sensitive to temperature. *BioRx*.

- 764 <https://doi.org/10.1101/2020.01.20.911826>
- 765 Perry, J. C., & Rowe, L. (2018). Sexual conflict in its ecological setting. *Philosophical Transactions*
- 766 *of the Royal Society B: Biological Sciences*, 373(1757).
- 767 <https://doi.org/10.1098/rstb.2017.0418>
- 768 Prud'Homme, B., Minervino, C., Hocine, M., Cande, J. D., Aouane, A., Dufour, H. D., ... Gompel,
- 769 N. (2011). Body plan innovation in treehoppers through the evolution of an extra wing-like
- 770 appendage. *Nature*, 473(7345), 83–86. <https://doi.org/10.1038/nature09977>
- 771 Quade, F. S. C., Holtzheimer, J., Frohn, J., Töpperwien, M., Salditt, T., & Prpic, N. M. (2019).
- 772 Formation and development of the male copulatory organ in the spider Parasteatoda
- 773 tepidariorum involves a metamorphosis-like process. *Scientific Reports*, 9(1), 1–12.
- 774 <https://doi.org/10.1038/s41598-019-43192-9>
- 775 Rebeiz, M., Patel, N. H., & Hinman, V. F. (2015). Unraveling the Tangled Skein: The Evolution of
- 776 Transcriptional Regulatory Networks in Development. *Annual Review of Genomics and*
- 777 *Human Genetics*, 16(1), 103–131. [https://doi.org/10.1146/annurev-genom-091212-](https://doi.org/10.1146/annurev-genom-091212-153423)
- 778 [153423](https://doi.org/10.1146/annurev-genom-091212-153423)
- 779 Rebeiz, M., & Tsiantis, M. (2017). Enhancer evolution and the origins of morphological novelty.
- 780 *Current Opinion in Genetics and Development*, 45, 115–123.
- 781 <https://doi.org/10.1016/j.jgde.2017.04.006>
- 782 Rhebergen, F. T., Courtier-Orgogozo, V., Dumont, J., Schilthuizen, M., & Lang, M. (2016).
- 783 *Drosophila pachea* asymmetric lobes are part of a grasping device and stabilize one-sided
- 784 mating. *BMC Evolutionary Biology*, 16(1), 1–24. [https://doi.org/10.1186/s12862-016-0747-](https://doi.org/10.1186/s12862-016-0747-4)
- 785 [4](https://doi.org/10.1186/s12862-016-0747-4)
- 786 Rice, G., David, J. R., Kamimura, Y., Masly, J. P., Alistair, P., Nagy, O., ... Yassin, A. (2019). A
- 787 standardized nomenclature and atlas of the male terminalia of *Drosophila melanogaster*.
- 788 *Fly*, 13(1–4), 51–64. <https://doi.org/10.1080/19336934.2019.1653733>
- 789 Robertson, H. (1988). Mating Asymmetries and Phylogeny in the *Drosophila melanogaster*
- 790 Species Complex, 42.
- 791 Rowe, L., & Arnqvist, G. (2012). Sexual selection and the evolution of genital shape and
- 792 complexity in water striders. *Evolution*, 66(1), 40–54. [https://doi.org/10.1111/j.1558-](https://doi.org/10.1111/j.1558-5646.2011.01411.x)
- 793 [5646.2011.01411.x](https://doi.org/10.1111/j.1558-5646.2011.01411.x)
- 794 Sánchez, V., & Cordero, C. (2014). Sexual coevolution of spermatophore envelopes and female
- 795 genital traits in butterflies: Evidence of male coercion? *PeerJ*, 2014(1), 1–12.
- 796 <https://doi.org/10.7717/peerj.247>
- 797 Shao, L., Chung, P., Wong, A., Siwanowicz, I., Kent, C. F., Long, X., & Heberlein, U. (2019). A
- 798 Neural Circuit Encoding the Experience of Copulation in Female *Drosophila*. *Neuron*,
- 799 102(5), 1025–1036.E6. <https://doi.org/10.1016/j.neuron.2019.04.009>
- 800 Simmons, L. W. (2014). Sexual selection and genital evolution. *Austral Entomology*, 53(1), 1–17.
- 801 <https://doi.org/10.1111/aen.12053>
- 802 Simmons, L. W., & Fitzpatrick, J. L. (2019). Female genitalia can evolve more rapidly and
- 803 divergently than male genitalia. *Nature Communications*, 10(1), 1–8.
- 804 <https://doi.org/10.1038/s41467-019-09353-0>
- 805 Smith, S. J., Davidson, L. A., & Rebeiz, M. (2020). Evolutionary expansion of apical extracellular
- 806 matrix is required for the elongation of cells in a novel structure. *ELife*, 9.
- 807 <https://doi.org/10.7554/eLife.55965>

- 808 Steedman, H. F. (1958). Dimethyl Hydantoin Formaldehyde: A new Water-soluble Resin for Use  
809 as a Mounting Medium. *Journal of Cell Science*, 99(4), 451–452.  
810 <https://doi.org/10.1163/187529266X00220>
- 811 Tanaka, K., Barmina, O., & Kopp, A. (2009). Distinct developmental mechanisms underlie the  
812 evolutionary diversification of *Drosophila* sex combs. *Proceedings of the National Academy*  
813 *of Sciences*, 106(12), 4764–4769. <https://doi.org/10.1073/pnas.0807875106>
- 814 Tsacas, L. (1971). *Drosophila teissieri*, nouvelle espece africaine du groupe melanogaster et  
815 note sur deux autres especes nouvelles pour l’Afrique (Dipt. Drosophilidae). *Bulletin de La*  
816 *Société Entomologique de France*, 76, 35–45.
- 817 Tsacas, L., Bocquet, C., Daguzan, M., & Mercier, A. (1971). Comparaison des genitalia males de  
818 *Drosophila melanogaster*, de *Drosophila simulans* et de leurs hybrids. *Annales de La*  
819 *Société Entomologique de France*, 7, 75–93. Retrieved from  
820 <https://ci.nii.ac.jp/naid/10030580910/>
- 821 Tsacas, L., & David, J. (1978). Une septieme espece appartenant au sous-groupe *Drosophila*  
822 *melanogaster* Meigen: *Drosophila orena* spec. nov. du Cameroun. (Diptera: Drosophilidae).  
823 *Beiträge Zur Entomologie*, 28, 179–182.
- 824 Tsacas, L., & Lachaise, D. (1974). Quatre nouvelles especes de la Cote-d’Ivoire du genre  
825 *Drosophila*, groupe melanogaster, et discussion de l’origine du sous-groupe melanogaster  
826 (Diptera: Drosophilidae). *Annales de l’Université d’Abidjan Série E: Ecologie*, 7, 193–211.
- 827 van Emden, F., & Hennig, W. (1970). *Taxonomists’ glossary of genitalia of insects*. (S. L. Tuxen,  
828 Ed.) (2nd ed.). Munksgaard, Copenhagen.
- 829 Vincent, B. J., Rice, G. R., Wong, G. M., Glassford, W. J., Downs, K. I., Shastay, J. L., ... Rebeiz, M.  
830 (2019). An atlas of transcription factors expressed in the *Drosophila melanogaster* pupal  
831 terminalia. *G3: Genes/Genomes/Genetics*, 9(December), 3961–3972.  
832 <https://doi.org/10.1101/677260>
- 833 Wagner, G. (2007). The developmental genetics of homology. *Nature Reviews Genetics*, 8(6),  
834 473–479. <https://doi.org/10.1038/nrg2099>
- 835 Wagner, G. P., & Lynch, V. J. (2010). Evolutionary novelties. *Current Biology*, 20(2), 48–52.  
836 <https://doi.org/10.1016/j.cub.2009.11.010>
- 837 Yassin, A., Gibert, P., & Capy, P. (2021). In memoriam: Jean R. David (1931-2021). Retrieved  
838 from <https://genestogenomes.org/in-memoriam-jean-r-david-1931-2021/>
- 839 Yassin, A., & Orgogozo, V. (2013). Coevolution between Male and Female Genitalia in the  
840 *Drosophila melanogaster* Species Subgroup. *PLoS ONE*, 8(2).  
841 <https://doi.org/10.1371/journal.pone.0057158>  
842

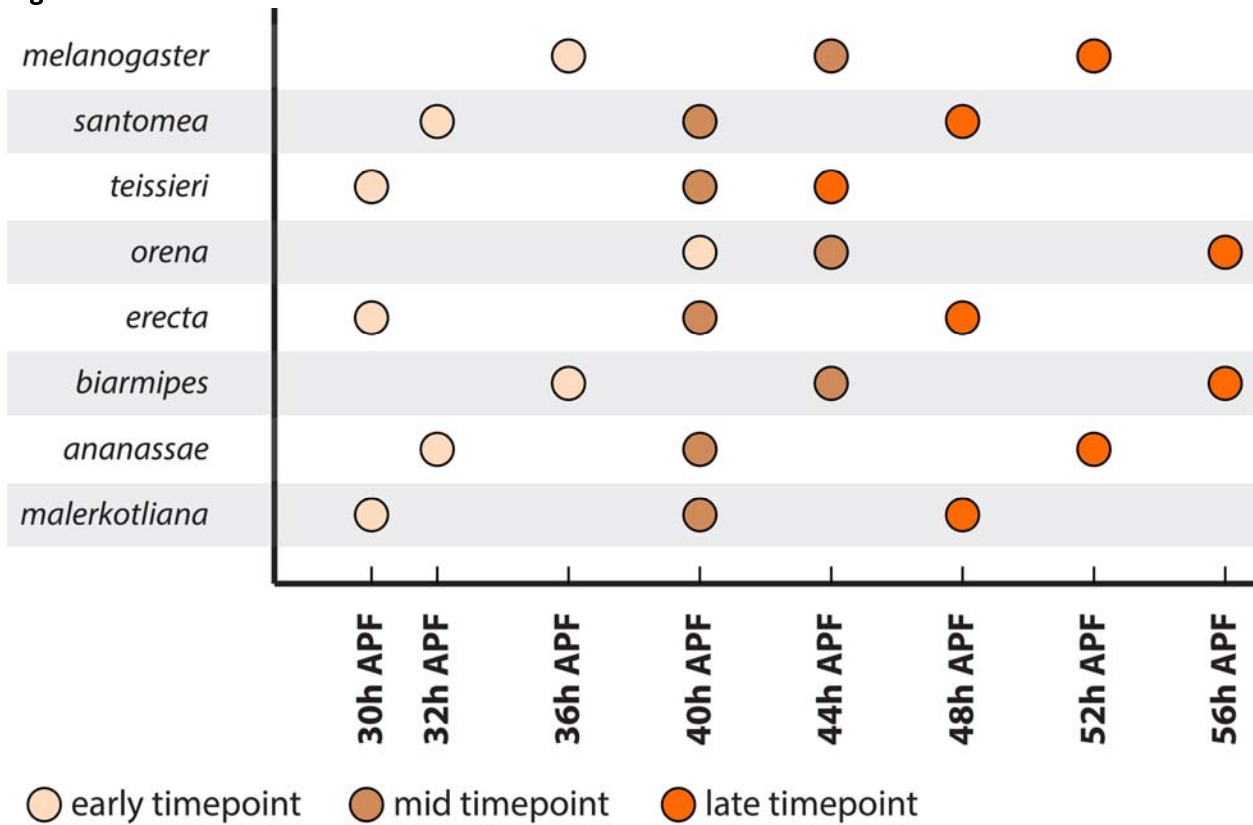
843 **Tables:**

<b>New nomenclature</b>	<b>Previous terminology</b>
postgonite	posterior paramere (Bock & Wheeler, 1972; Tsacas et al., 1971)
dorsal postgonal process	dorsal postgonite (Rice et al., 2019; Vincent et al., 2019) dorsal branch (Kamimura, 2010, 2016; Kamimura & Mitsumoto, 2011) dorsal paramere (Bryant & Hsei, 1977)
ventral postgonal process	ventral postgonite (Rice et al., 2019; Vincent et al., 2019) ventral branch (Kamimura, 2010; Kamimura & Mitsumoto, 2011) ventral paramere (Bryant & Hsei, 1977)
aedeagal ventral process	phallic spur (Yassin & Orgogozo, 2013) phallic hook (Yassin & Orgogozo, 2013) ventral branch (Kamimura, 2012, 2016; Kamimura & Mitsumoto, 2012b, 2012a; A. E. Peluffo et al., 2015; A. Peluffo et al., 2021)
aedeagal lateral process	basal process (Kamimura, 2007; Kamimura & Polak, 2011)
pregonal medial process	basal process (Kamimura, 2007), ventral branch (Kamimura, 2016)
Postgonal sheath	Aedeagal sheath (Rice et al., 2019; Vincent et al., 2019)

844 Table 1: Table of correspondence between terms previously used in publications and proposed  
845 nomenclature.

846 **Supplemental figures:**

847 **Figure S1:**



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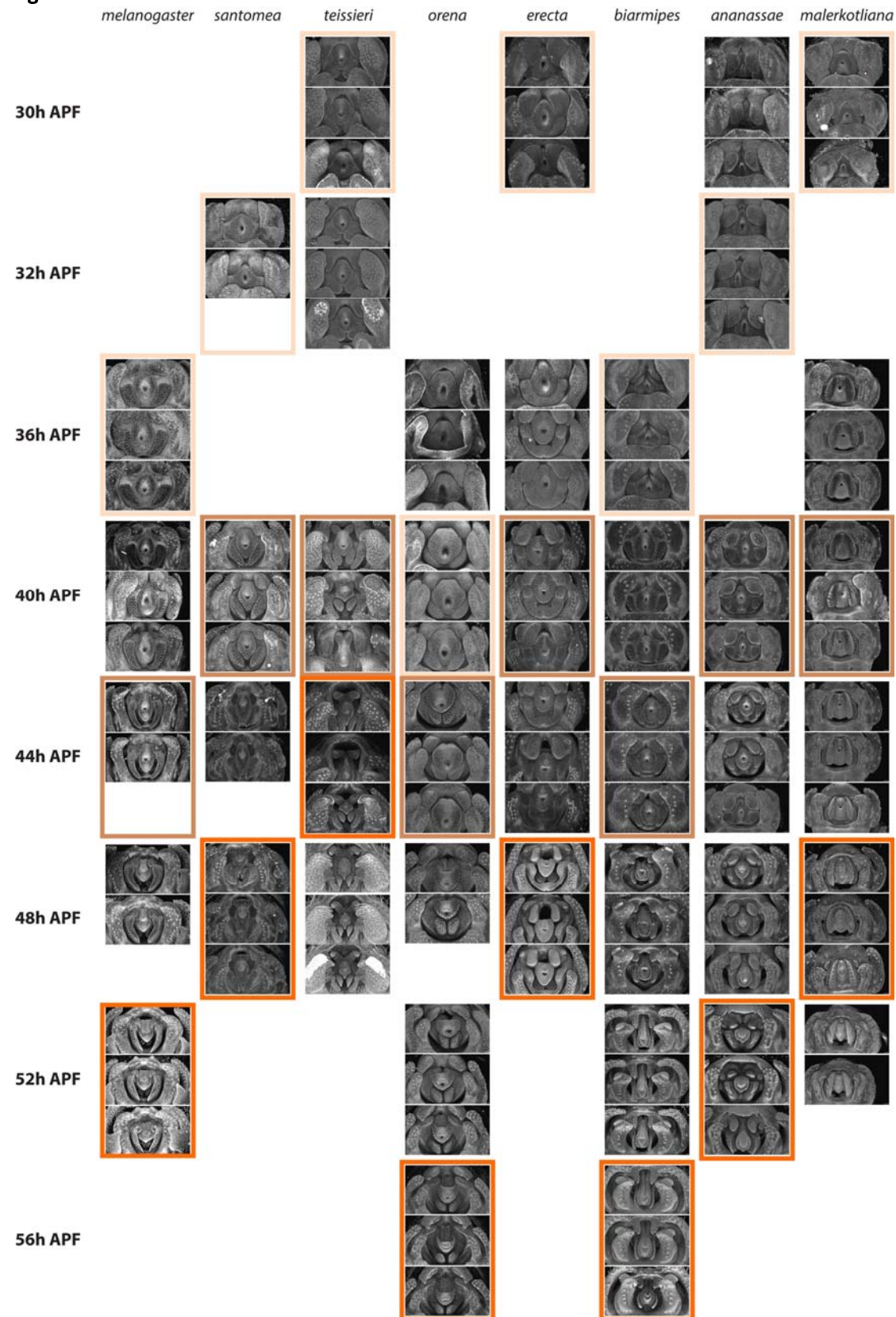
850 **Figure S1:**

851

852 **A summary of our designations of early (beige) mid (brown) and late (orange) developmental**  
853 **timepoints for each species.** The early timepoint was designated when a cleavage between the  
854 epandrial ventral lobe (lateral plate) and surstylus (clasper) first forms. The mid timepoint was  
855 designated by when the cleavage of the epandrial ventral lobe and surstylus reached half of its  
856 total length. The late time point was designated as directly preceding when the cerci (anal  
857 plates) close over the gap between them.

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Figure S2:



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860 **Figure S2:**

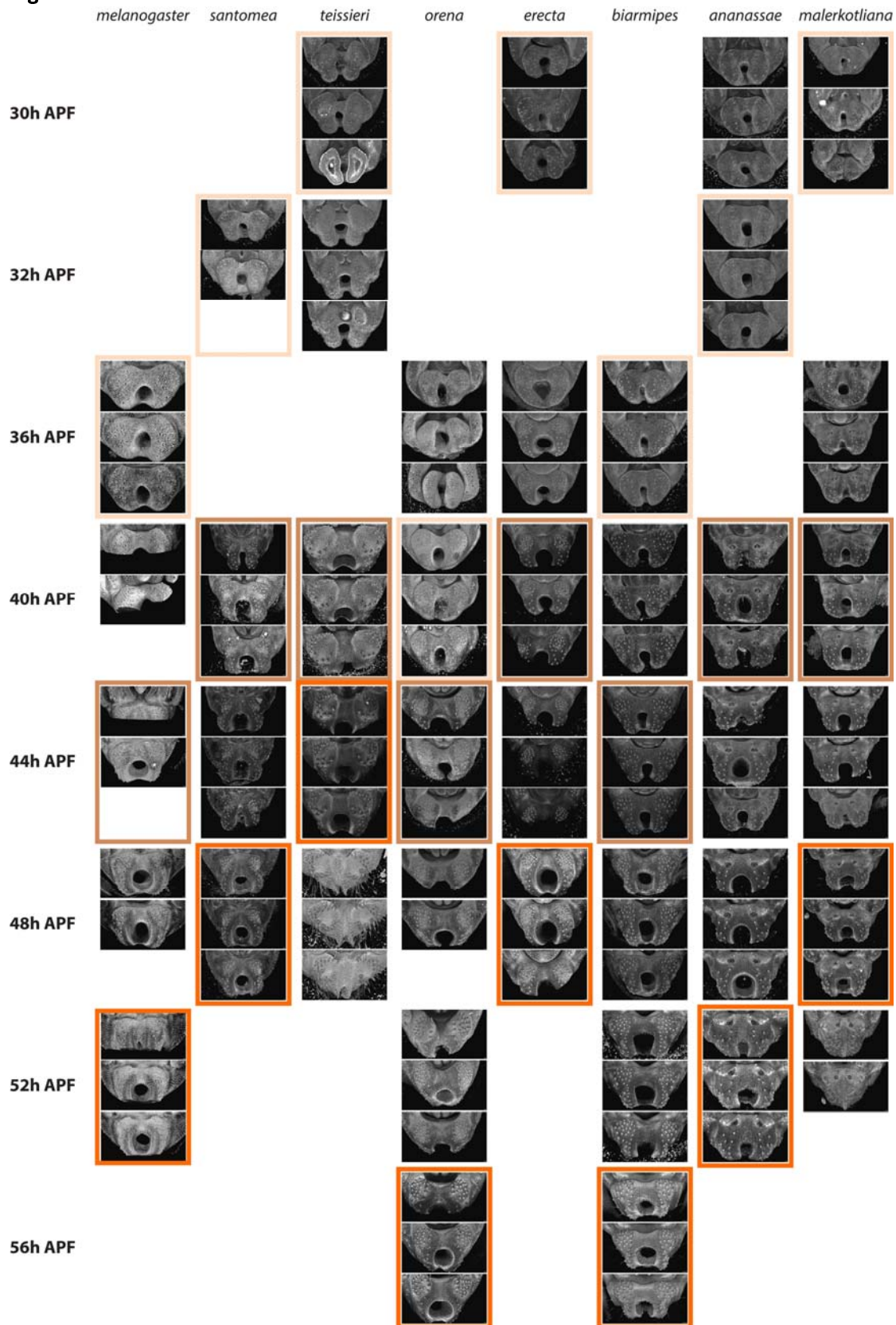
861

862 **Full ECAD time course of ventral genitalia across the *melanogaster* species group.**

863 Developing pupal genitalia of *D. santomea*, *D. teissieri*, *D. orena*, *D. erecta*, *D. biarmipes*, *D.*  
864 *ananassae*, and *D. malerkotliana* stained for ECAD, apical cellular junctions, highlighting the  
865 overall morphology. Note that *D. melanogaster* samples use a transgenic line *arm-GFP* that also  
866 labels the apical cell junctions. Colored boxes highlight our three designated developmental  
867 timepoints for each species: early (beige) mid (brown) and late (orange) as shown in Figure S1.

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**Figure S3:**



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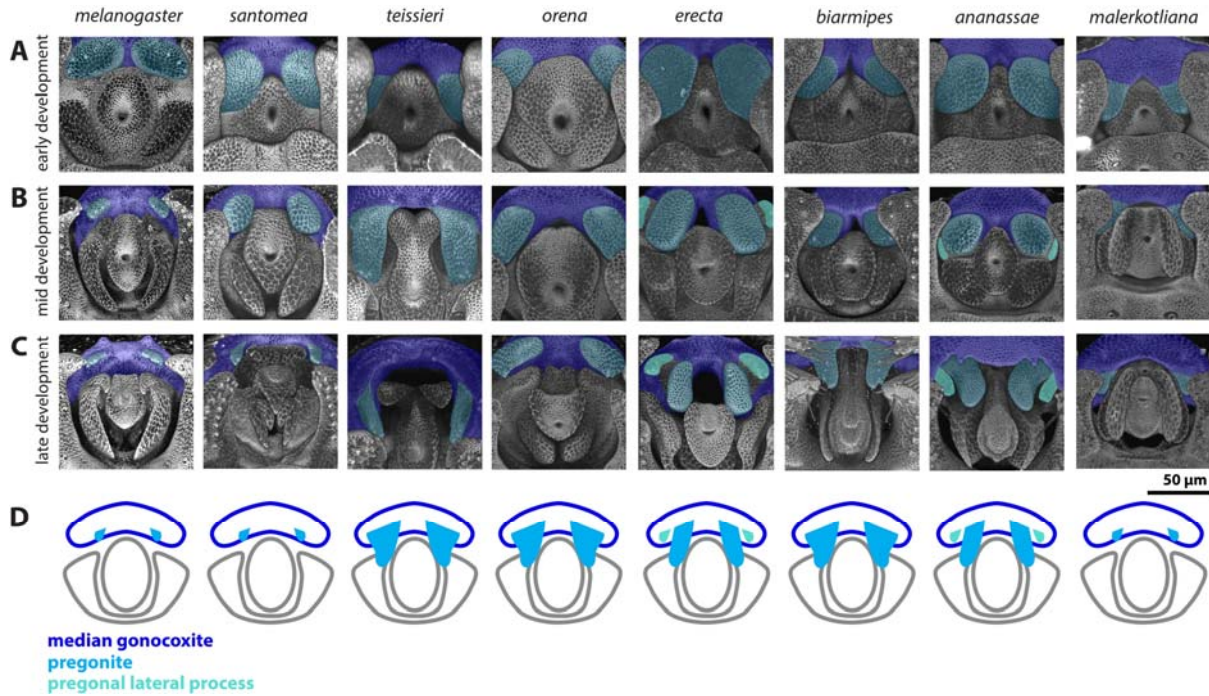
870 **Figure S3:**

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872 **Full ECAD time course of dorsal genitalia and analia across *melanogaster* species group.**

873 Developing pupal genitalia of *D. santomea*, *D. teissieri*, *D. orena*, *D. erecta*, *D. biarmipes*, *D.*  
874 *ananassae*, and *D. malerkotliana* stained for ECAD, apical cellular junctions, highlighting the  
875 overall morphology. Note that *D. melanogaster* samples use a transgenic line arm-GFP that also  
876 labels the apical cell junctions. Colored boxes highlight our three designated developmental  
877 timepoints for each species: early (beige) mid (brown) and late (orange) as shown in Figure S1.

878 **Figure S4:**  
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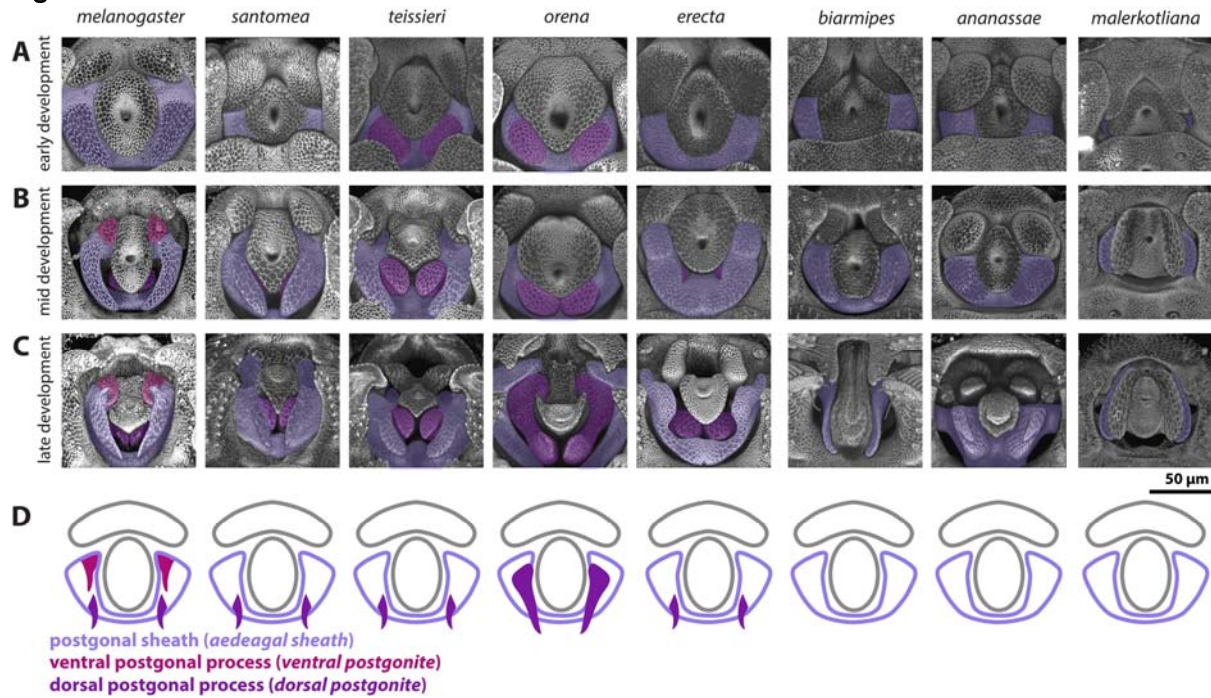


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881 **Figure S4:**  
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883 **Developing ventral primordia of all analyzed species. A-C)** The median gonocoxite is  
884 highlighted in dark blue, the pregonite is highlighted in with light blue, and the pregonal lateral  
885 process is highlighted in teal **D)** Schematic representation of the median gonocoxite (dark blue),  
886 the pregonite (light blue), pregonal lateral process (teal). Note that *D. melanogaster* samples  
887 use a transgenic line *arm-GFP*, while all other samples are stained for E-cadherin, both of which  
888 label apical cell junctions.

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**Figure S5:**



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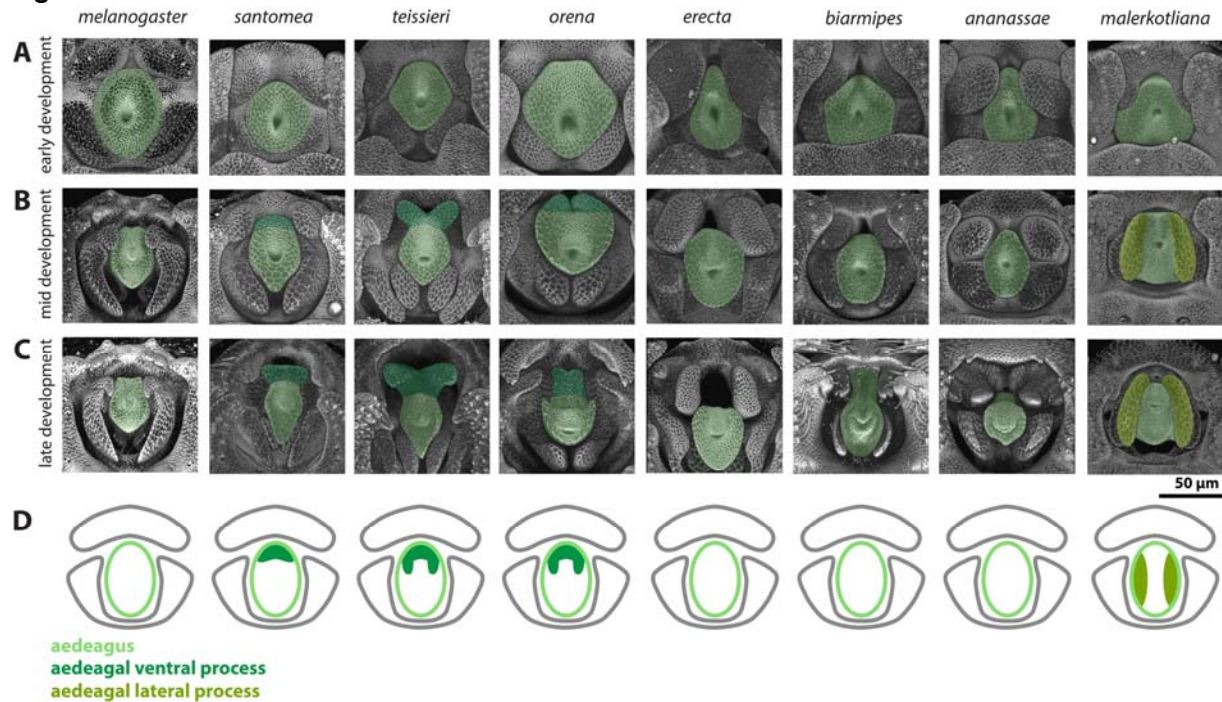
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**Figure S5:**

**Developing dorsolateral primordia of all analyzed species. A-C)** The postgonal sheath is highlighted in light purple, the ventral postgonal process is highlighted in magenta, and the dorsal postgonal process is highlighted in violet **D)** Schematic representations of the postgonal sheath (light purple), the ventral postgonal process (magenta), and dorsal postgonal process (violet). Aedeagal sheath is an alternative term for postgonal sheath, ventral postgonite is an alternative term for ventral postgonal process, and dorsal postgonite is an alternative term for dorsal postgonal process. Note that *D. melanogaster* samples use a transgenic line arm-GFP, while all other samples are stained for E-cadherin, both of which label apical cell junctions.

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**Figure S6:**



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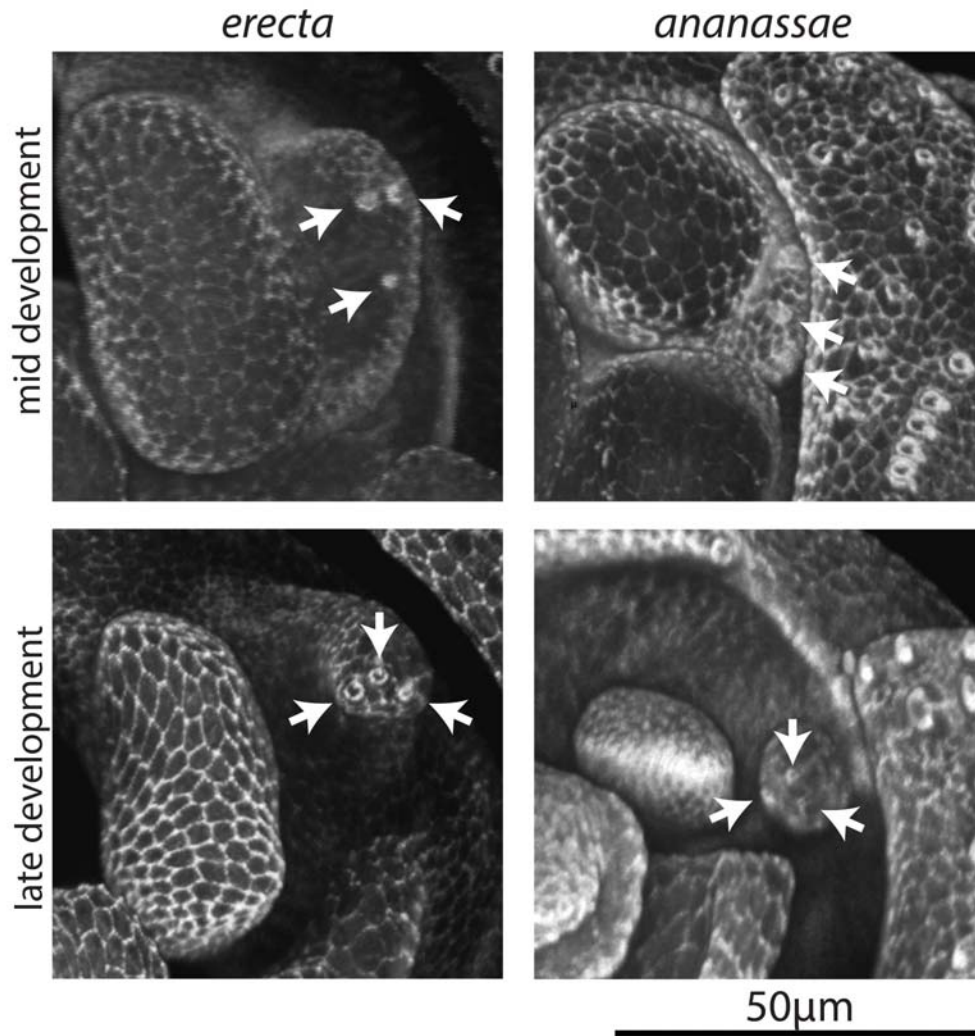
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**Figure S6:**

**Developing central primordia of all analyzed species. A-C)** The aedeagus is highlighted in light green, the aedeagal ventral process is highlighted in with dark green and the aedeagal lateral process is highlighted in yellow-green. **D)** Cartoon representations of the aedeagus (light green) aedeagal ventral process (dark green) and aedeagal lateral process (yellow-green). Note that *D. melanogaster* samples use a transgenic line *arm-GFP*, while all other samples are stained for E-cadherin, both of which label apical cell junctions.

913 **Figure S7:**



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916 **Figure S7:**

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918 **A zoom in on the developing pregonites of *D. erecta* and *D. ananassae*.** Apical cell junctions  
919 are labeled through ECAD staining. White arrows indicate the location of the three developing  
920 pregonal bristles found on the pregonal lateral process.