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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Abstract

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

Key Words: Homology, Drosophila, Genitalia, rapid evolution

Introduction:

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

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

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

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

98 itself has posed several challenges in this regard. In particular, the homology of the various
99 phallic processes, pointed outgrowths, remains controversial (Figure 2, Supplementary videos)
100 (reviewed in Rice et al., 2019). These outgrowths have been implicated in sexual conflict
101 between males and females, and in some species have been shown to physically interact with
102 corresponding pockets in the female genitalia (Kamimura, 2016; Muto, Kamimura, Tanaka, &
103 Takahashi, 2018; Yassin & Orgogozo, 2013) or induce copulatory wounds (Kamimura, 2007).
104 Male seminal proteins can enter the female circulatory system through these copulatory
105 wounds, and are associated with increased ovulation and reduced remating rates (Avila, Sirot,
106 Laflamme, Rubinstein, & Wolfner, 2011; Kamimura, 2010). To better understand how genital
107 morphology may coevolve we must better establish which homologous tissues have been
108 modified in each sex.
109 Two major sources have contributed to confusion regarding homology of phallic
110 processes. The first is the relationship of the postgonal sheath (referred to as aedeagal sheath
111 in Rice et al., 2019) with respect to these processes (Figure 2 H-I). Several authors consider the
112 postgonal sheath and postgonal processes (referred to as postgonites in Rice et al., 2019) of *D.*
113 *melanogaster* as substructures of a unified tissue that was usually referred to as the “posterior
114 parameres” (Bock & Wheeler, 1972; Okada, 1954; Tsacas, Bocquet, Daguzan, & Mercier, 1971).
115 While others designated the postgonal sheath as a separate tissue from the postgonal
116 processes (Al Sayad & Yassin, 2019; Bächli, Vilela, Andersson Escher, & Saura, 2004; Lachaise et
117 al., 2004). The three-dimensional nature and the presence of transparent cuticle has made it
118 difficult to determine the precise connection points of the processes to the tissues of the
119 phallus. Determining whether these processes were formed by a single or separate primordium

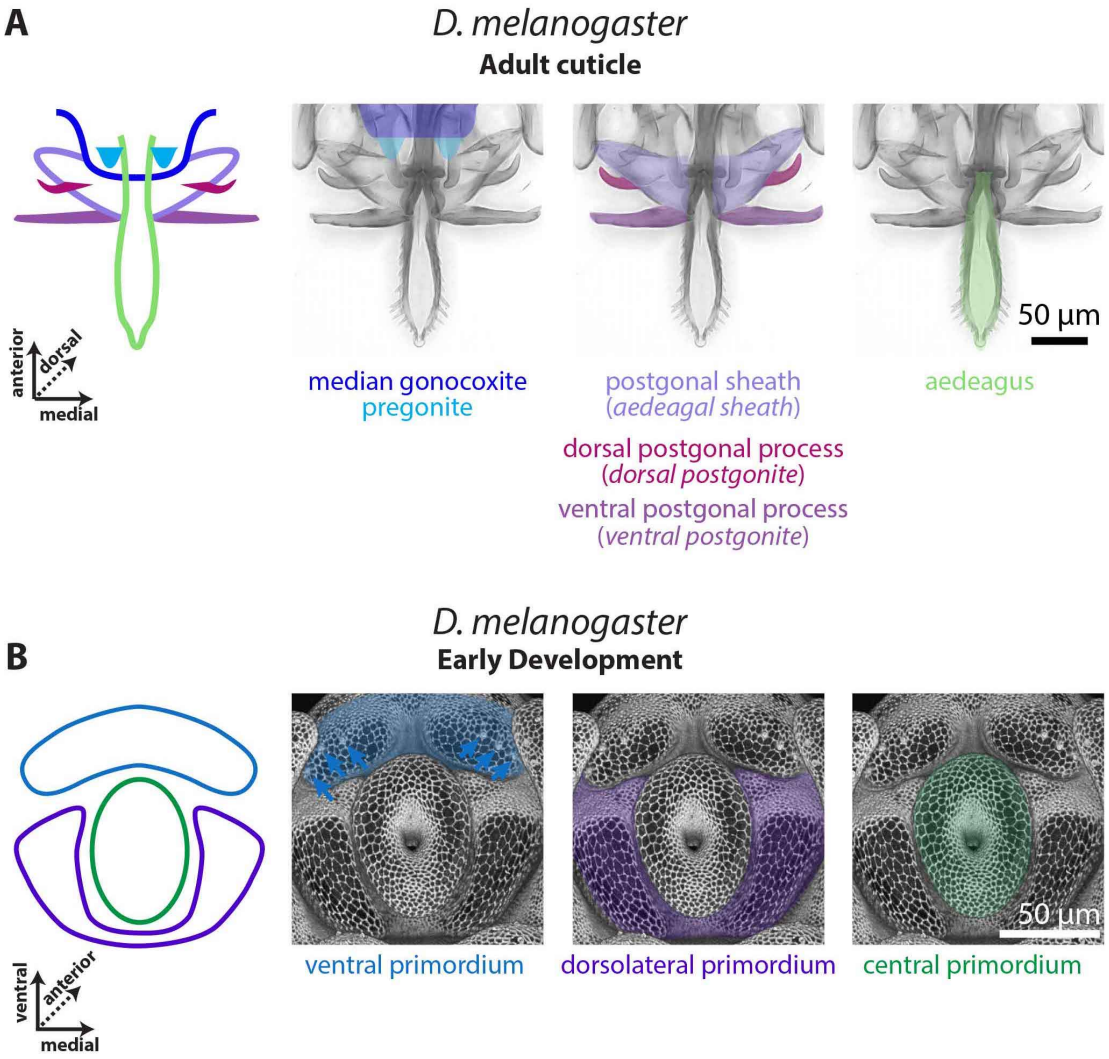
would help resolve this discordance. The second source of confusion is in regard to the nomenclature used to compare the phallic processes in different members of the *melanogaster* species group. The term “basal process” has been used to refer to a number of pointed outgrowths that are attached to different phallic tissues in different species (Kamimura, 2007, 2010, 2016; Kamimura & Mitsumoto, 2011, 2012a, 2012b; Kamimura & Polak, 2011). Such a designation implies a concept of homology independent of the exact anatomical position. Yassin & Orgogozo (2013) sought to provide distinct terms, such as “spurs” and “hooks” for outgrowths emanating from the same tissue, implying the potential for non-homology. Building upon our recent revision of the male terminalia nomenclature of *D. melanogaster* (Rice et al., 2019), developmental studies presented here allow us to provide a more detailed, homology-informed nomenclature for these structures.

In this study, we characterized both the adult morphology and the development of the pupal genitalia in five members of the *melanogaster* species subgroup and three outgroup members from the larger *melanogaster* species group. This analysis allows us to determine whether processes are homologous or of different origins. Tracing the development of the phallus by confocal microscopy showed that all processes arise from three distinct pupal primordia in all species. We found both that several similarly shaped processes arise from distinct primordia, whereas in other cases, distinct processes arise from different parts of the same primordium. In light of these analyses, we refined the identity and terminology of the phallic processes and identify distinct homology groups. We map these different morphologies on previously established phylogenies and identified multiple gain, loss, and homoplastic events

141 in the history of these diverse structures. Thus, our results demonstrate how developmental

142 approaches can resolve unclear relationships among rapidly evolving structures.

143 **Figure 1:**



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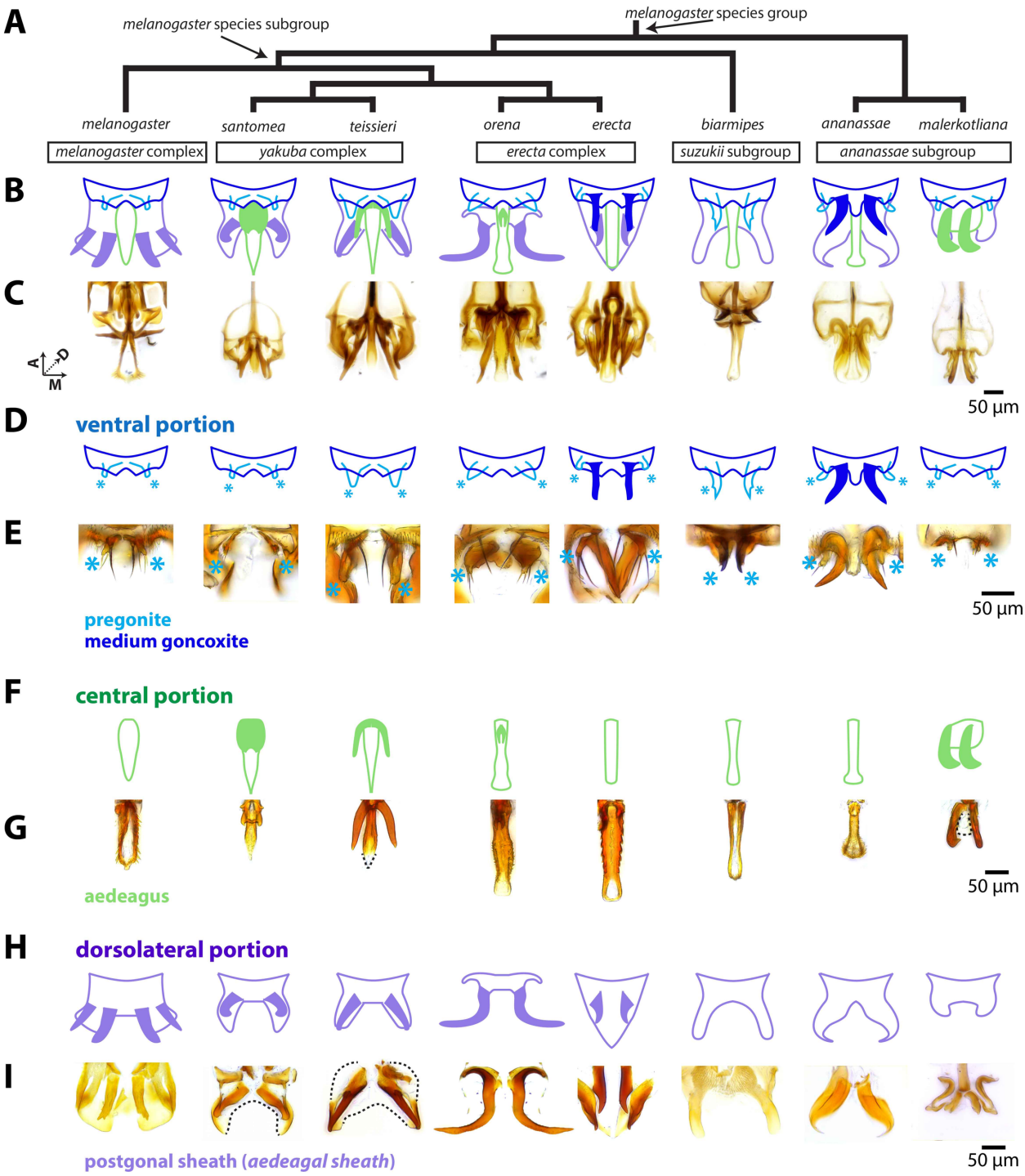
145

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

147

148 **A)** Left: A schematic representation of the adult phallus of *D. melanogaster,* the median
149 gonocoxite outlined in dark blue, the pregonites highlighted in light blue, the postgonal sheath
150 in light purple, the dorsal postgonal process in magenta, the ventral postgonal process in violet,
151 and the aedeagus in light green. Right: the adult phallus of a *yw;+:+* line of *D. melanogaster*. **B)**
152 Left: A schematic representation of the early developing pupal genitalia of *D. melanogaster*.
153 The primordia of developing phallus with the ventral primordium in blue, the dorsolateral
154 primordium in purple, and the central primordium in green. Right: Developing pupal phallus of
155 a *D. melanogaster* arm-GFP transgenic line. Apical cellular junctions are shown, highlighting the
156 overall morphology. Blue arrows indicate the position of the pregonal bristles. Aedeagal sheath
157 is an alternative term for postgonal sheath, ventral postgonite is an alternative term for ventral
158 postgonal process, and dorsal postgonite is an alternative term for dorsal postgonal process.

159 **Figure 2**



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

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163 **A)** Phylogeny for eight species of the *melanogaster* species group based on Obbard et al., 2012
164 with nodes that contain the *melanogaster* species subgroup and *melanogaster* species group

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

Materials and Methods:

Drosophila strains:

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

Sample collection, dissection, and fixation:

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

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

Immunohistochemistry and microscopy:

After fixation developing pupal genitalia of all species except *D. melanogaster* were stained with rat anti-E-cadherin, 1:100 in PBT (DSHB Cat# DCAD2,RRID:AB_528120) overnight at 4°C, followed by an overnight at 4°C incubation with anti-rat 488, 1:200 (Invitrogen, Carlsbad, CA) to visualize apical cell junctions. For *D. melanogaster*, an *armadillo*-GFP tagged line (Bloomington stock number #8556) was used to visualize apical cell junctions (Huang et al., 2012). Fluorescently labeled samples were mounted in glycerol mounting solution (80% glycerol, .1M Tris, pH 8.0) on microscope slides coated with poly-L-lysine (Thermo Fisher Scientific #86010). Samples for all species except *D. melanogaster* were imaged at 20X on a Leica TCS SP8 confocal microscope. *D. melanogaster* samples were imaged at either 20X or 40X on an Olympus Fluoview 1000. As the imaged structures are three-dimensional in nature, we used the program MorphoGraphX (de Reuille et al., 2015) to render and manipulate images in three-dimensions. This allowed us to rotate the samples to better present the most informative perspectives of the various phallic structures.

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

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

Establishing designated early, middle, and late timepoints:

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

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

Results:

Unpigmented cuticle reveals undescribed connection points in the phallus

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

While imaging, we observed that parts of the postgonal sheath in the *melanogaster* species subgroup and *D. malerkotliana* were partially translucent, and only easily visible after microdissection. It is this translucent tissue of the postgonal sheath that physically connects to the postgonal processes in *D. melanogaster*. These observations highlight that, due to their transparency, determining the exact connection points between the processes and the rest of

279 the phallus can be difficult to visualize by traditional light microscopy approaches. To test
280 whether the different connection points of the phallic processes observed in the adult reflect
281 separate homology groups we investigated whether these phallic processes were initially
282 produced by the same or different primordia during development.
283
284 *Phallic structures develop from three primordia in D. melanogaster*
285 To date, the morphogenesis of the three-dimensional adult phallic structures from the
286 epithelium of the larval genital disc has been investigated only in *D. melanogaster* (Ahmad &
287 Baker, 2002; Epper, 1983). Additionally, using surgical fragmentation of the larval genital disc,
288 Bryant & Hsei, 1977 provided a fate map for the different adult structures. They showed that
289 the phallus is situated at the subcenter of the symmetrical imaginal disc and is surrounded on
290 each side by a primordium that will produce the medium gonocoxite and pregonites. However,
291 the sequence and timing of the appearance of the various substructures of the phallus during
292 development, remains unknown. By finding the key points in development where substructures
293 first emerge, we can determine the primordium from which each process initially form.
294 Early in *D. melanogaster* pupal development (see timepoint designation in the Materials
295 and Methods) the phallus is separated into three primordia: ventral, dorsolateral and central
296 (Figure 1B). As the pupal phallus continues to develop from this point, the ventral primordia
297 form a pair of processes (Figure 1A). This pair develops into the small processes known as the
298 pregonites that can be recognized from the presence of the minute bristles (Figure 1B arrows).
299 The dorsolateral primordia produce two processes (one dorsal and one ventral) (Figure 3B).
300 These processes ultimately develop into the ventral and dorsal postgonal processes (referred to

as ventral postgonite and dorsal postgonite in Rice et al., 2019) (Figure 1A). The remaining parts of the dorsolateral tissue develop into the large flaps of the postgonal sheath (Figure 1A). The central primordium of *D. melanogaster* develops into the aedeagus and lacks a process (Figure 1A).

The three primordia are conserved across species

Several studies have analyzed pupal development of the terminalia in species outside of *D. melanogaster*, but did not investigate phallic structures (Glassford et al., 2015; Hagen et al., 2019; Smith, Davidson, & Rebeiz, 2019). To determine whether the features of phallic development observed in *D. melanogaster* are conserved in members of the *melanogaster* species group, we produced a developmental time course for the remaining seven species studied here (Figure S1-S3). We found that all adult phallic organs develop from three primordia similar to the ones described in *D. melanogaster*. Nonetheless, significant interspecific differences were observed regarding the timing of development (Figure S1). As we only used one strain per species, we cannot comment if these are particular properties of the strains/laboratory conditions we used or are general differences between the species. We found that most species had early, mid, and late developmental timepoints within a six-hour window of each other, while *D. orena* and *D. teissieri* showed particularly divergent developmental timing (Figure S1-S3).

Different processes emerge from different primordia

322 The developmental analysis of the eight species used in this study allowed us to test
323 whether the phallic processes seen in the adults of each species were produced by the same
324 primordia. We began by investigating the ventral primordium (Figure 3, Figure S4), which
325 develops into the pregonites in all analyzed species. While the size of the pregonites varies
326 between species, during mid-development (Figure 4B) we can identify recognizable outgrowths
327 from the ventral primordium, consistent with a highly conserved developmental trajectory.
328 Interestingly, an additional pregonal process is found in two distantly-related species, *D. erecta*
329 and *D. ananassae*. Both *D. erecta* and *D. ananassae,* produce two processes from their ventral
330 primordia, a large pregonal medial process and a second smaller lateral process which contains
331 the three pregonal bristle cells and overall resembles the pregonites of other species. To
332 determine whether this additional process was produced by duplication or fission of the
333 pregonite we inspected early pupal timepoints. We found that initially a single process is
334 formed (Figure 3A), which during mid-development asymmetrically splits along the medial-
335 lateral axis to form the distinct lobe-like pregonal medial process (Figure 3B). These asymmetric
336 projections then extend in late development to form the larger pregonal medial process and
337 smaller pregonite (Figure 3C). Thus, although the ventral primordium produces the pregonite in
338 all species we examined, in *D. erecta* and *D. ananassae* the ventral primordium is split into the
339 pregonite and a pregonal medial process.
340 The dorsolateral primordium (Figure 4, Figure S5) showed a number of large
341 evolutionary changes within the *melanogaster* species group. We found that no species, other
342 than *D. melanogaster*, develop the ventral process that forms the ventral postgonal process
343 (Figure 4). By contrast, all members of the *melanogaster* species subgroup form dorsal

postgonal processes. Outside of the *melanogaster* subgroup, we did not find any modifications of the dorsolateral primordium, which develops into a single thin, strongly sclerotized structure in those species that resembles the postgonal sheath of *D. melanogaster*. However, the size, and shape of these homologous structures significantly differ among species (Figure 2 H,I), ranging from the flat rod-like processes in *D. biarmipes*, to the strongly pointed sinuate processes in *D. ananassae*, and the minute, transparent sclerites in *D. malerkotliana*.

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

Okada, 1954 and Bock & Wheeler, 1972 suggested that the aedeagus in the *melanogaster* species group were of two types: fused like in *D. ananassae* and split like in *D. malerkotliana*. Indeed, we did not observe any process in the central primordium of *D. ananassae*, whereas a pair of processes develops in *D. malerkotliana*. Kamimura, 2007 suggested that the aedeagus of *D. malerkotliana* has degenerated and was replaced by a pair of lateral processes. During early development, the central primordium of *D. malerkotliana* is similar to all other analyzed species (Figure 5A). However, by mid-development, the lateral

366 sides of the central primordium extend, forming a pair of processes, while the medial-dorsal
367 and medial-ventral sides of the central primordium fail to extend (Figure 5B). Late in
368 development, the proximal-dorsal side of the lateral process constricts, conferring a hook like
369 shape (Figure 5C). As this substructure is produced from the lateral portions of the central
370 primordium and not from the ventral portion, it is likely non-homologous to the aedeagal
371 ventral processes of the *yakuba* and *erecta* complexes. We therefore propose the term
372 aedeagal lateral process for this substructure of *D. malerkotliana*.

Figure 3:

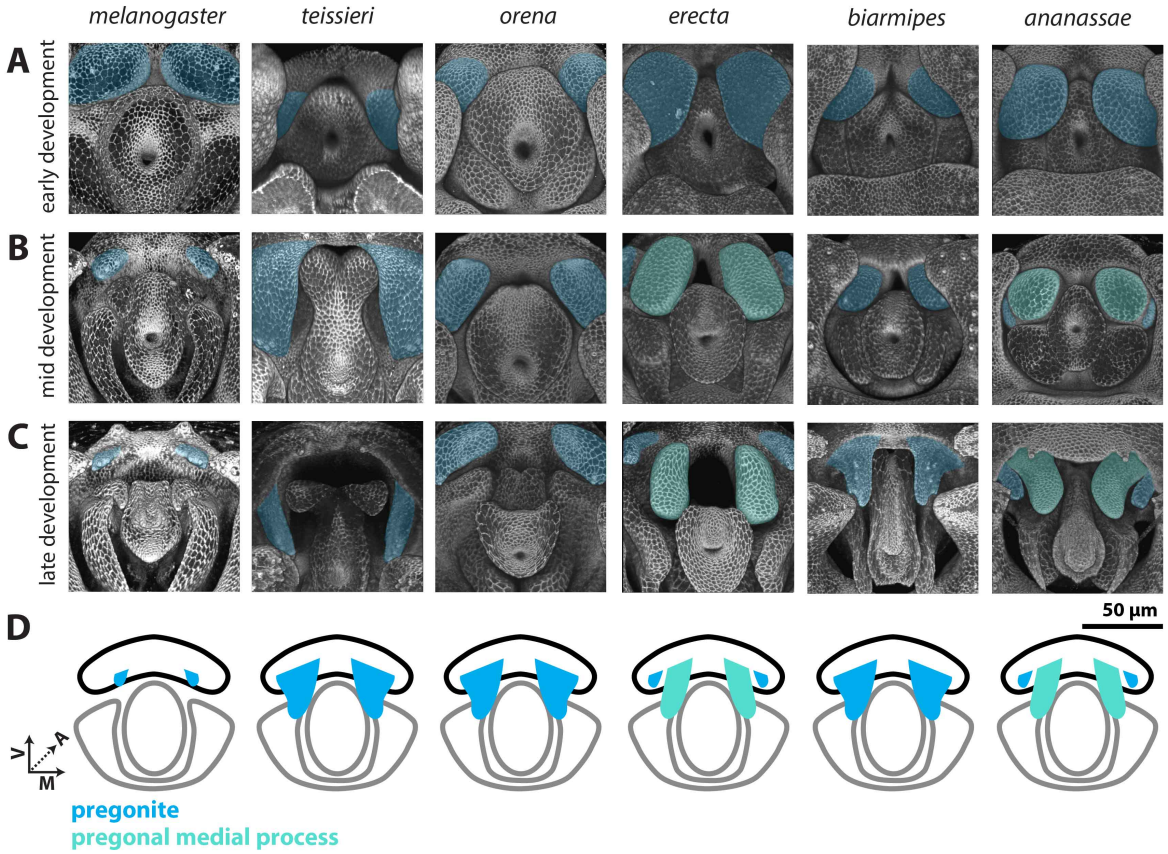

Figure 3: Processes developing from the ventral primordium are found in all members of the *melanogaster* species group.

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

Figure 4:

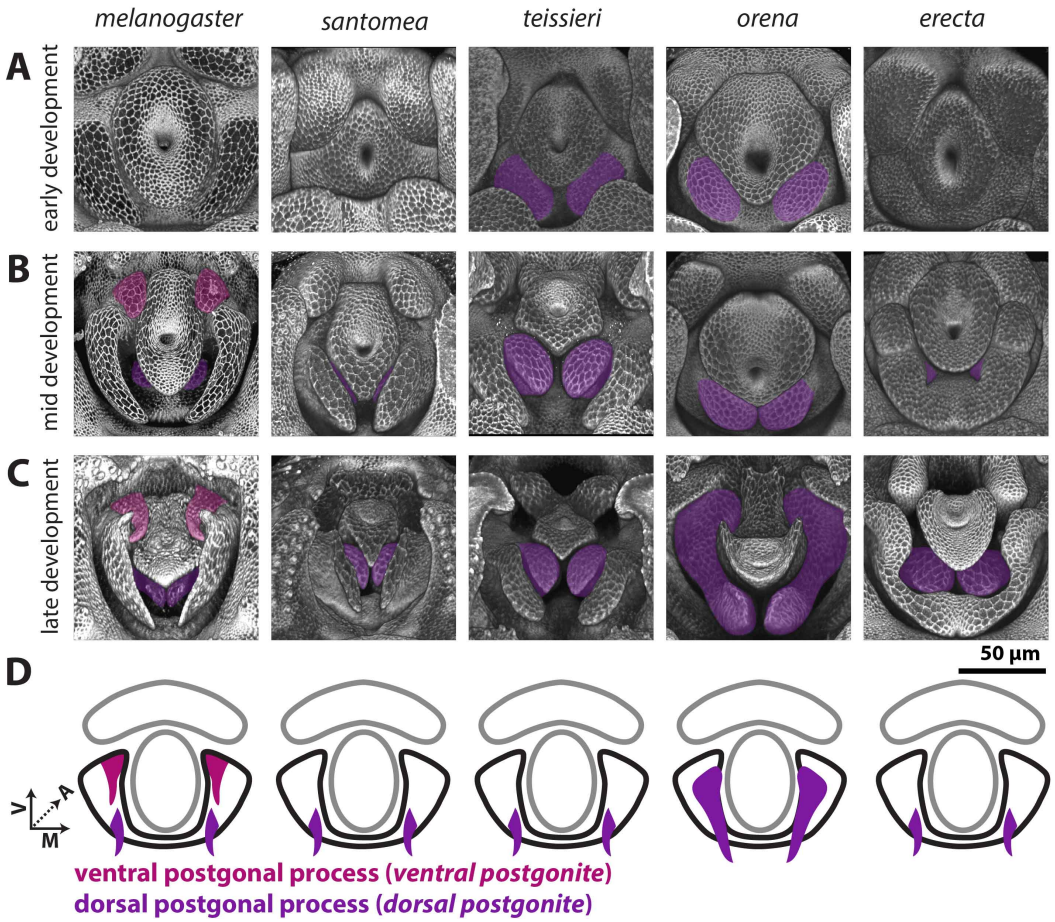

Figure 4: Processes produced by the dorsolateral primordium are found only in the *melanogaster* subgroup.

A-C) Signal from the apical cellular junctions was used to highlight the overall morphology of developing pupal genitalia. *D. santomea*, *D. teissieri*, *D. orena*, and *D. erecta*, were stained for ECAD while *D. melanogaster* samples used arm-GFP. **A)** Early in development, the dorsolateral primordium is a smooth lobe like structure in all analyzed species. **B)** By mid-development, all shown species form processes in the dorsal portion of the dorsolateral primordium (violet). *D. melanogaster* also forms an additional pair of processes in the ventral portion of dorsolateral primordium (magenta). **C)** By late development, the dorsal and ventral processes have extended to a long thin shape. Both the ventral and dorsal processes are connected to the medial-anterior part of the postgonal sheath which is formed by the remaining tissue of the dorsolateral primordium. **D)** Schematic representations of the dorsal (violet) and ventral (magenta) postgonal process showing where they connect to the postgonal sheath (outlined in black). All images have the same axes, V (Ventral), A (Anterior), M (Medial) and are the same scale. Ventral postgonite is an alternative term for ventral postgonal process, and dorsal postgonite is an alternative term for dorsal postgonite.

Figure 5:

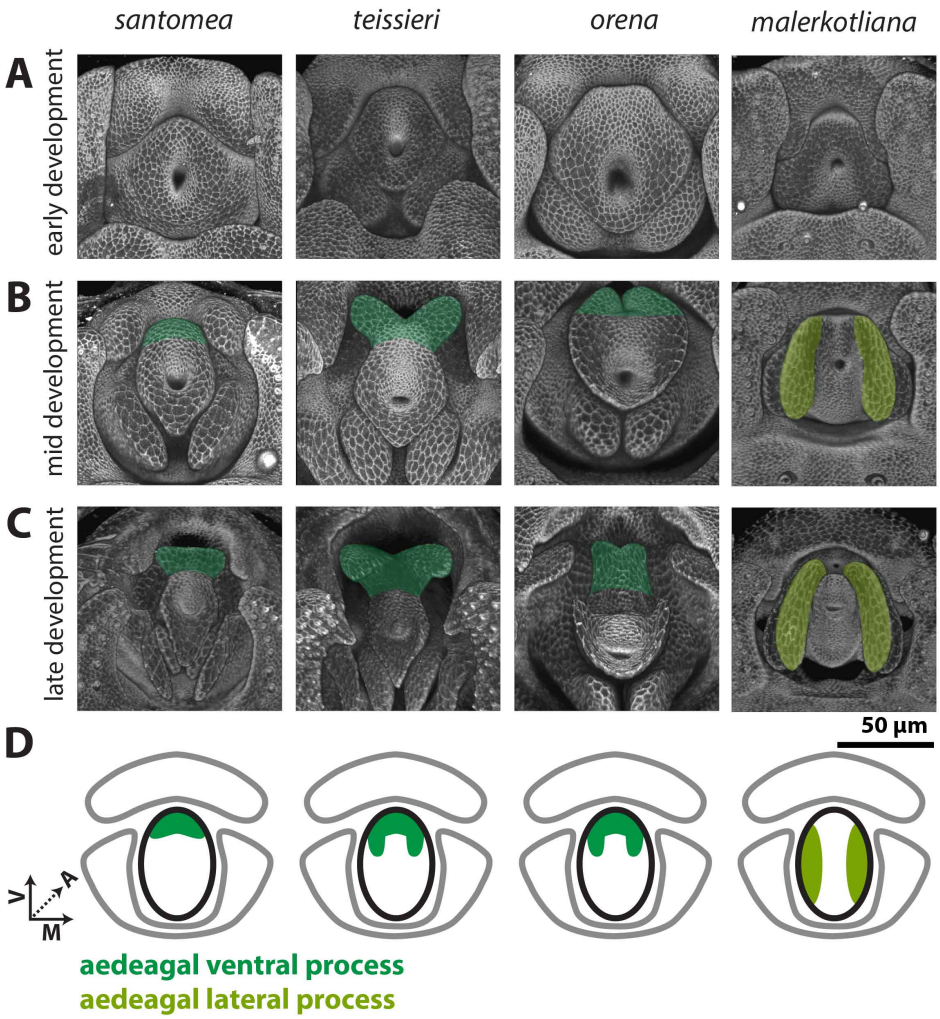

Figure 5: Processes developing from the central primordium are found in the *yakuba*/*erecta* and *bipectinata* complexes.

A-C) Signal from the apical cellular junctions was used to highlight the overall morphology of developing pupal genitalia. *D. santomea*, *D. teissieri*, *D. orena*, and *D. erecta*, were stained for ECAD. **A)** Early in development, the central primordium forms a flat donut-shaped structure in all species shown. **B)** By mid-development, the ventral portion of the aedeagus is extended in *D. santomea*, *D. teissieri*, and *D. orena* in what will form the aedeagal ventral process (dark green). In *D. malerkotliana* the lateral edges of the central primordium extend anteriorly in what will form the aedeagal lateral process (yellow-green). **C)** By late development, the aedeagal ventral process and the aedeagal lateral process further extend from the aedeagus. **D)** Schematic representations of the aedeagal ventral process (dark green) and aedeagal lateral process (yellow-green) showing where they connect to the aedeagus (outlined in black). All images have the same axes, V (Ventral), A (Anterior), M (Medial) and are the same scale.

Figure 6:

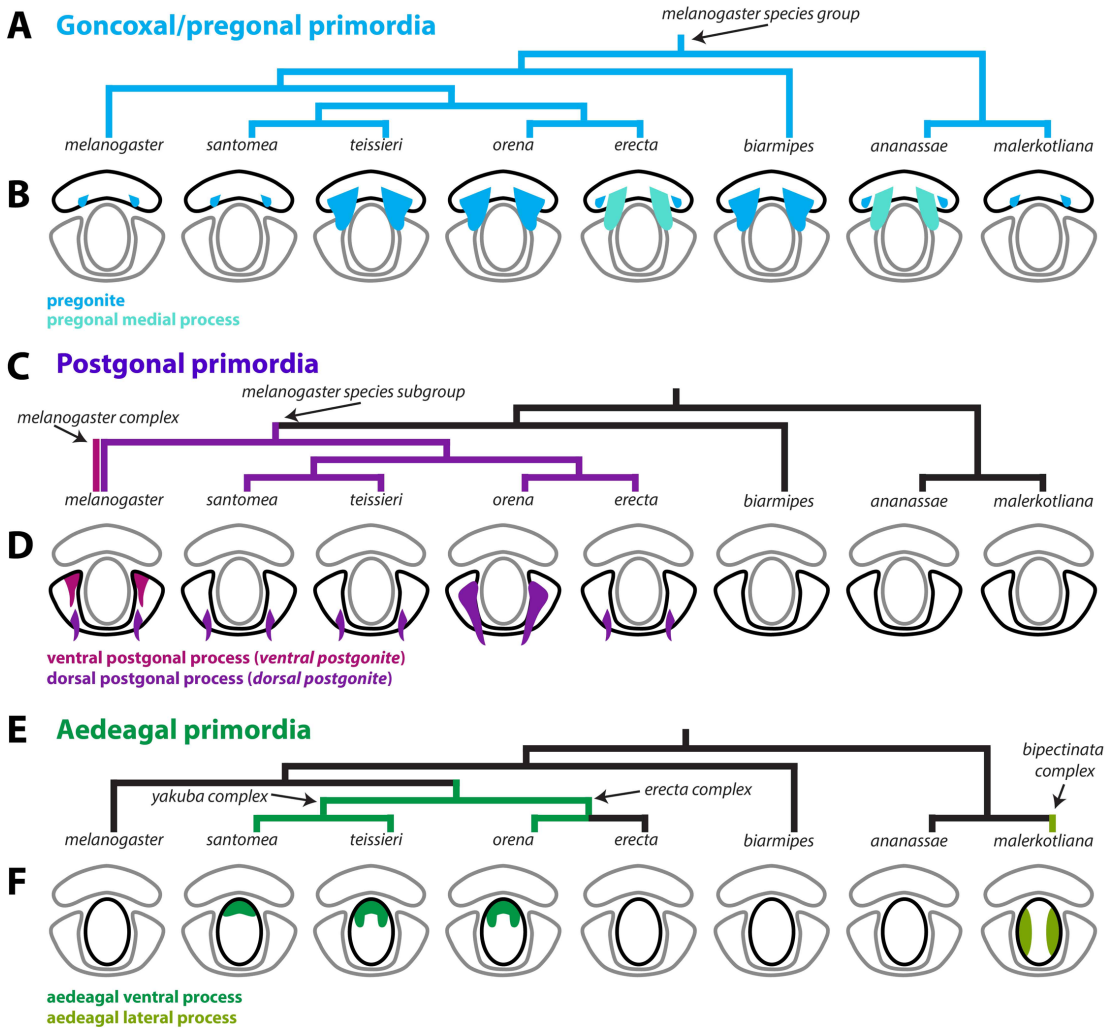

Figure 6: Model of the evolutionary of phallic processes in the *melanogaster* species group

A,C,E) Phylogeny of the 8 analyzed species based on Obbard et al., 2012. **A)** Parsimony suggests that the pregonites originated outside of the *D. melanogaster* species group. **B)** Light blue represents the pregonites and teal represents the pregonal medial process. **C)** Parsimony suggests that the ventral postgonites originated in the *melanogaster* complex (*D. melanogaster*, *D. simulans*, *D. mauritiana*, *D. sechellia*) and that the dorsal postgonal process originated in the melanogaster subgroup. **D)** Violet represents the ventral postgonal process, magenta represents the dorsal postgonal process. E) Parsimony suggests that the aedeagal ventral process originated at the base of the *erecta* and *yakuba* complexes. Additionally, parsimony suggests that the aedeagal lateral process originated in the bipectinate complex. **F)** Dark green represents the aedeagal ventral process and yellow-green represents the aedeagal lateral process. Ventral postgonite is an alternative term for ventral postgonal process, and dorsal postgonite is an alternative term for dorsal postgonite.

Discussion:

The rapid evolution of morphological structures is an attractive subject for study, as it allows us to glimpse at the molecular and genetic causes of dramatically remodeled and restructured anatomical forms. Here, we examined some of the most rapidly evolving morphologies of *Drosophila melanogaster* and its close relatives. Despite decades of research, many of the intricate phallic processes have eluded our ability to clearly classify their homology relationships. By studying the developmental trajectories of these processes in multiple species, we have better defined their physical connections, and clarified which structures most likely share ancestry. This research highlights the distinct challenges in studying novelties at mesoevolutionary scales.

Classification and nomenclature of rapidly evolving phallic structures

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

464 sheath and the processes), and the term “postgonal processes” to designate the strongly
465 sclerotized branches emerging from this tissue in the *melanogaster* subgroup. Second, our
466 results also show that the structures previously called the “basal processes” (Kamimura, 2007,
467 2010, 2016; Kamimura & Mitsumoto, 2011, 2012a, 2012b; Kamimura & Polak, 2011), develop
468 from different primordia and are therefore most likely non-homologous. We suggest therefore
469 to give them distinct names that directly relate to the tissues that produce them: aedeagal
470 ventral process in species of the *yakuba* complex (synonymous to Yassin & Orgogozo, 2013
471 phallic spur) and *D. orena* (synonymous to Yassin & Orgogozo, 2013 phallic hook), the pregonal
472 medial process in *D. erecta* and *D. ananassae,* and aedeagal lateral process in *D. malerkotliana*
473 and species of the *bipectinata* complex (Table 1).
474
475 *Evolution of the phallic structures*
476 Mapping character states over robust phylogenies provide the opportunity to
477 distinguish novel from recurrent (homoplastic) states as well as derived states (synapomorphic)
478 from ancestral (symplesiomorphic) ones (Figure 6). For example, our demonstration of the
479 development of an additional pregonal process in *D. erecta* and *D. ananassae* is likely recurrent,
480 as illustrations from Bock & Wheeler, 1972 suggest that this configuration of the pregonites
481 might have recurrently evolved in this clade. Reversals to ancestral states through secondary
482 losses represent another mechanism of recurrent evolution. The lack of the aedeagal ventral
483 processes in *D. erecta*, is more likely due to loss rather than an independent gain of the
484 aedeagal ventral process in *D. orena*.

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

Although we do not address the function of the phallic processes, other research groups have associated these with sexual conflict. The various processes of the phallus have been implicated in copulatory wounding of the female (Kamimura, 2007, 2016; Muto et al., 2018; Yassin & Orgogozo, 2013). Furthermore, studies have found that some of the phallic processes pivot from pointing posteriorly to pointing laterally, when the phallus is everted during copulation, thus directing how they interact with the female reproductive tract (Kamimura, 2010). The ability to pivot during copulation correlates with the homology groups we have found in this study. The ventral and dorsal postgonal processes, and pregonites pivot during copulation while the aedeagal ventral process does not change orientation. This may be due to

the direct connection of the aedeagal ventral process to the aedeagus. Surprisingly the aedeagal lateral process, which is also directly connected to the aedeagus, pivots laterally during copulation, which may only be possible due to the loss of aedeagal sclerotization, making the tissue between the aedeagal lateral processes flexible. Co-evolution between the phallic processes and the female genitalia has been suggested and several novel modifications of the female genitalia have been identified (Kamimura, 2007; Yassin & Orgogozo, 2013). A developmental analysis of the female genitalia of these species along with three-dimensional analysis of copulating flies similar to studies in *D. melanogaster* (Mattei et al., 2015; Shao et al., 2019) would provide vital context for the potential co-evolution of novel male and female structures.

Developmental mechanisms underlying phallic evolution

A major challenge in the evo-devo field has been to identify the molecular mechanisms driving morphological novelty (Linz, Hu, & Moczek, 2020; Moczek, 2008; Rebeiz, Patel, & Hinman, 2015; G. P. Wagner & Lynch, 2010). While macroevolutionary novelties have been the focus of coarse-grained molecular study (Bruce & Patel, 2020; Clark-Hachtel & Tomoyasu, 2020; Emlen et al., 2006; Hinman et al., 2003; Prud'Homme et al., 2011), much hope has been placed on rapidly diverging structures in molecularly amenable systems (Rebeiz & Tsiantis, 2017). Our work highlights distinct underexplored challenges to interpreting and advancing these model systems. The ambiguous ancestry of similar parts which appear in different locations causes us to consider multiple models to explain their emergence. These parts may arise by parallelism – a predisposition to drive similar new structures by co-opting the same networks (Abouheif,

2008). Alternately, it is entirely possible that these structures are indeed ancestral but have undergone massive tissue reorganizations to reposition their attachment points. Such repositioning could be caused by moving the location of a critical signal or transcription factor within the tissues. Alternately, these structures could be specified before the discernable tissues of the phallus are separated, and their migration could be caused by differences in tissue folding. Under this scenario, we would anticipate that critical tissue patterning regulators of these processes are activated before these tissues become discernable. Finally, it is entirely possible that completely different networks account for the appearance of these unique structures. Developmental genetic analysis of the genes that produce the phallic processes described above will aid us in distinguishing these models. Recent work has identified several genes that are spatially restricted to the pregonites and postgonal processes of *D. melanogaster* providing an ideal set of candidates to examine (Vincent et al., 2019). Thus, we envision that detailed mechanisms of parallelism, repositioning, and novelty will emerge from studying systems where both network architecture is accessible, and genetic manipulations can be introduced to test the sufficiency of these mechanisms to produce these novel morphological structures.

Acknowledgments:

We would like to thank Deepak Dharmadhikari for his help with imaging, the Cornell Species and Bloomington Stock Centers for providing fly strains used in this study, Ben Vincent and the Rebeiz lab for their comments on the project and manuscript, Virginie Courtier-Orgogozo, Masanori Toda, Yoshitaka Kamimura and the Terminalia consortium for their insights on this project. We would also like to thank TaxoDros and the Japan Drosophila Database for their work cataloging resources for the original species descriptions for those analyzed.

552 **Tables:**

New nomenclature	Previous terminology
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)

553 Table 1: Table of correspondence between terms previously used in publications and proposed
554 nomenclature.

Supplemental figures:

Figure S1:

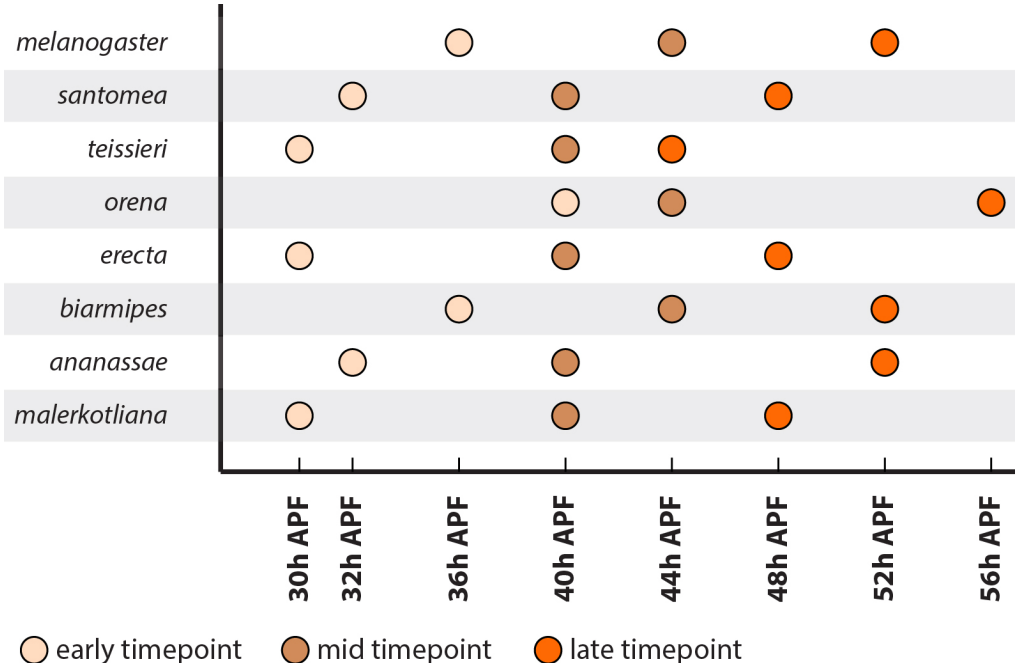

Figure S1:

A summary of our designations of early (beige) mid (brown) and late (orange) developmental timepoints for each species.

563 **Figure S2:**

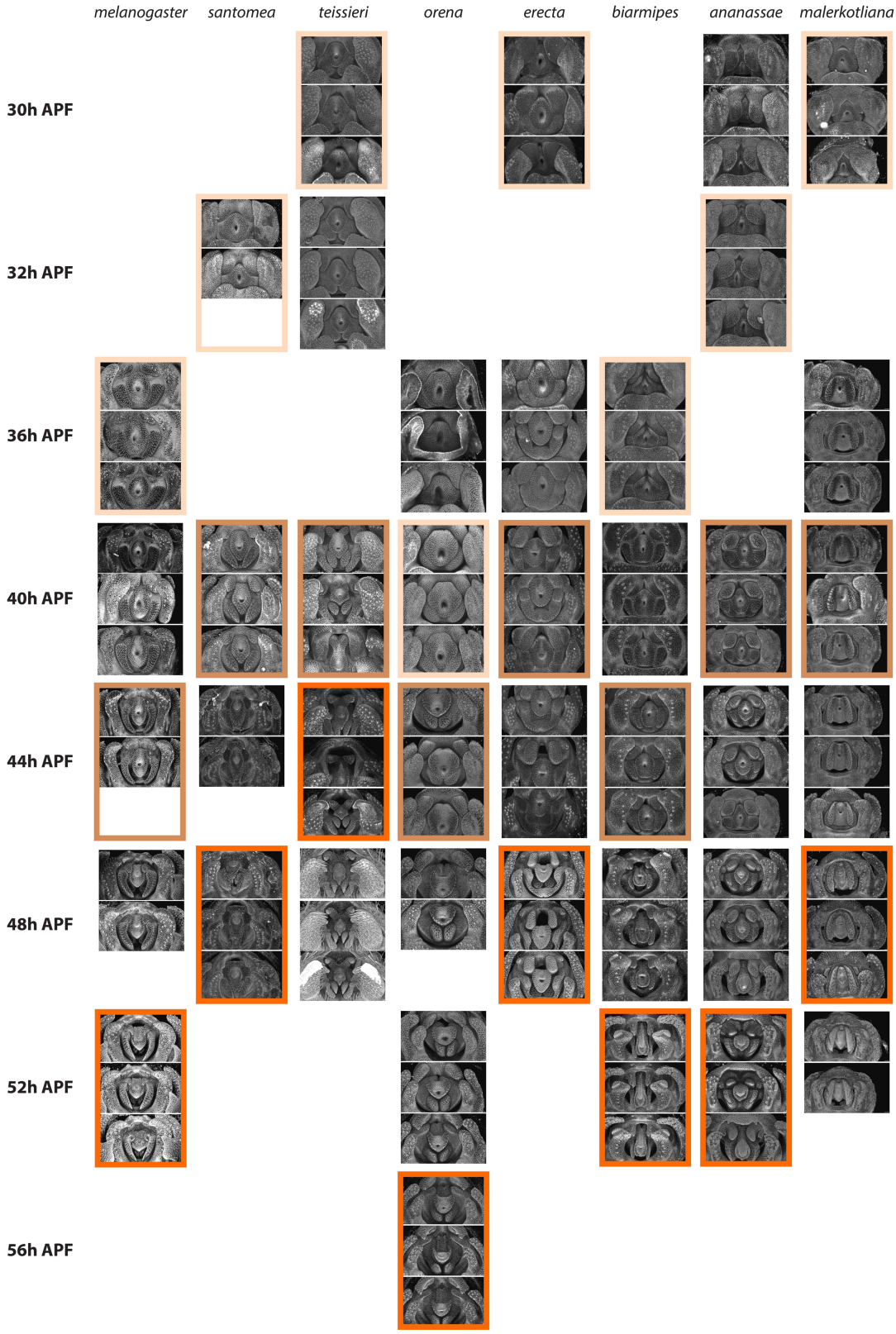

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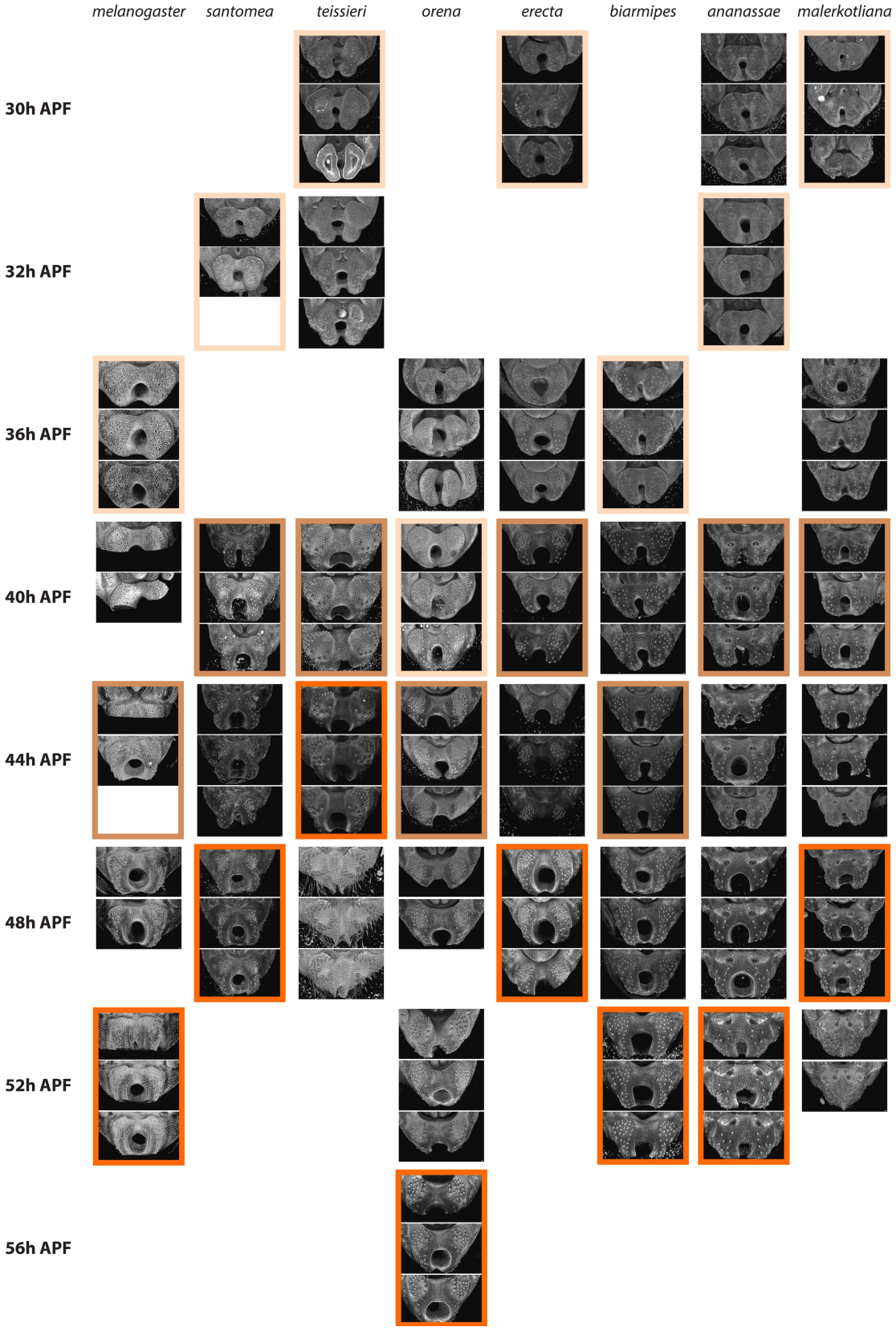
Figure S2:

Full ECAD time course of ventral genitalia across the *melanogaster* species group. Developing pupal genitalia of *D. santomea*, *D. teissieri*, *D. orena*, *D. erecta*, *D. biarmipes*, *D. ananassae*, and *D. malerkotliana* stained for ECAD, apical cellular junctions, highlighting the overall morphology. Note that *D. melanogaster* samples use a transgenic line arm-GFP that also labels the apical cell junctions.

Figure S3:



574 Figure S3:
575
576 Full ECAD time course of dorsal genitalia across *melanogaster* species group.
577 Developing pupal genitalia of *D. santomea*, *D. teissieri*, *D. orena*, *D. erecta*, *D. biarmipes*, *D.*
578 *ananassae*, and *D. malerkotliana* stained for ECAD, apical cellular junctions, highlighting the
579 overall morphology. Note that *D. melanogaster* samples use a transgenic line arm-GFP that also
580 labels the apical cell junctions.

Figure S4:

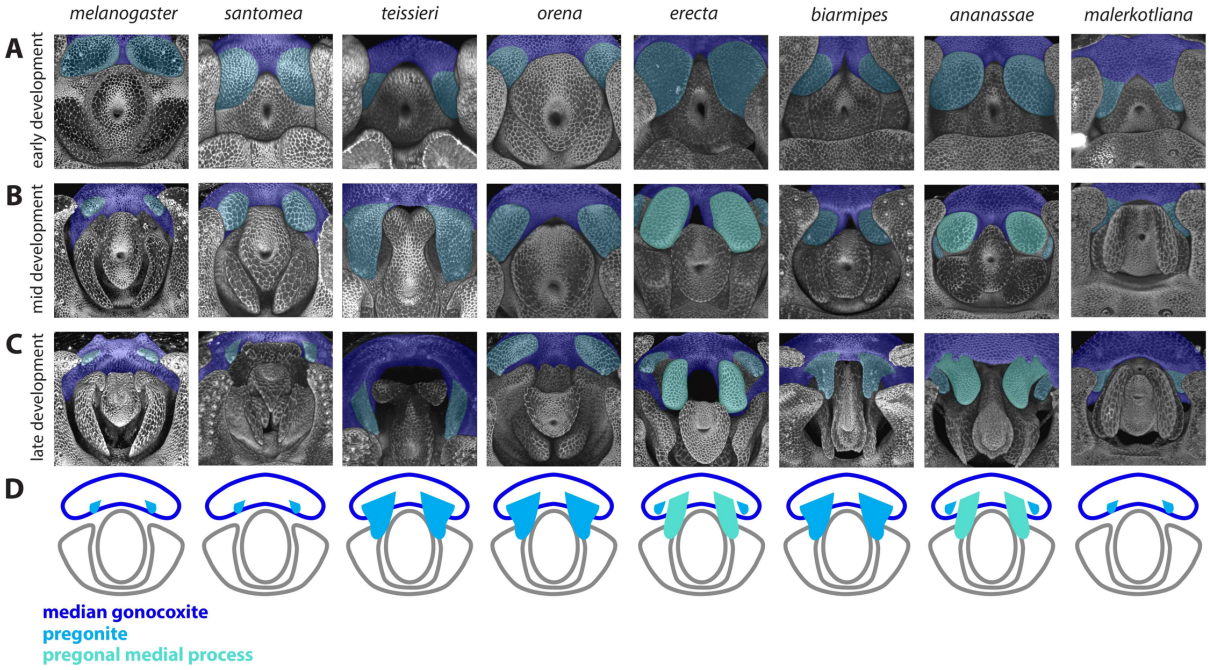


Figure S4:

Developing ventral primordia of all analyzed species. For *D. melanogaster* an arm-GFP line of was used to highlight the apical cellular junctions. **A-C)** The median gonocoxite is highlighted in dark blue, the pregonite is highlighted in with light blue, and the pregonal medial process is highlighted in teal **D)** Schematic representation of the median gonocoxite (dark blue), the pregonite (light blue), pregonal medial process (teal). Note that *D. melanogaster* samples use a transgenic line arm-GFP that also labels the apical cell junctions, while all other samples are stained for E-cadherin.

Figure S5:

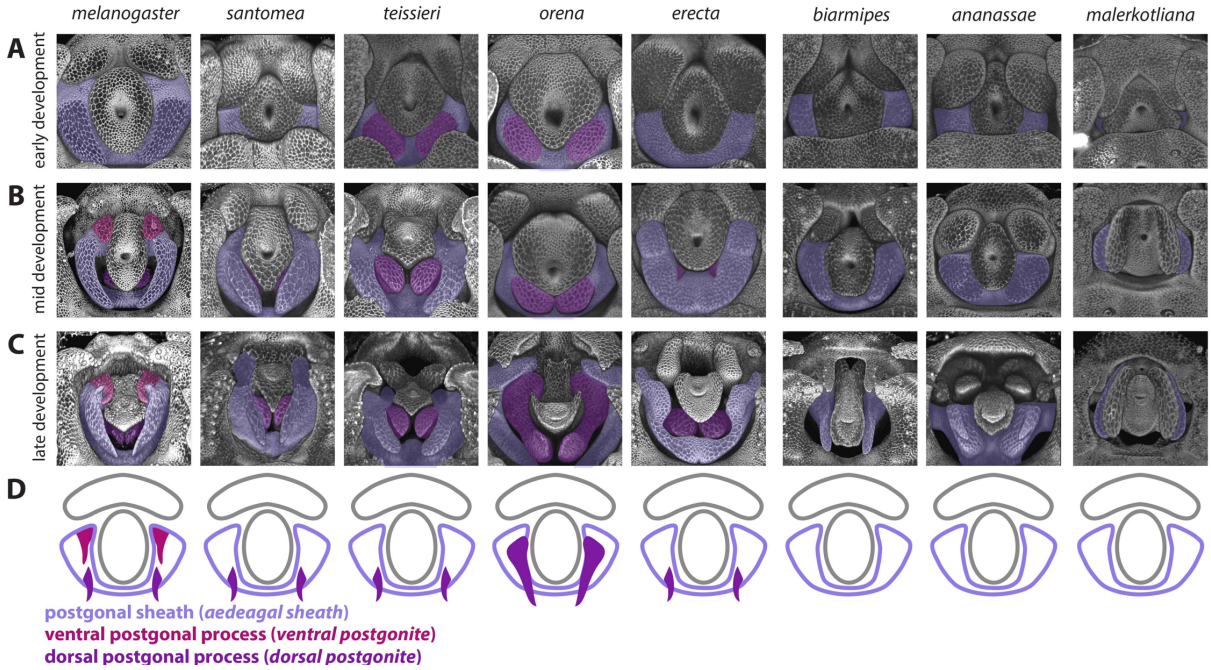
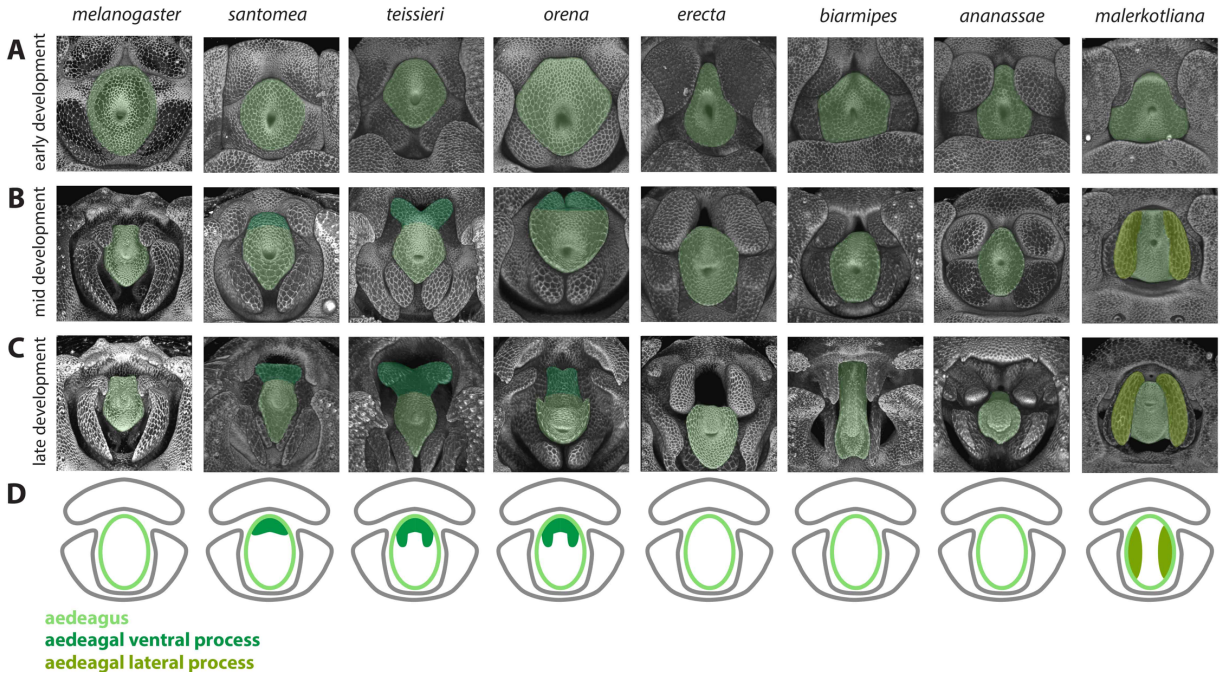


Figure S5:

Developing dorsolateral primordia of all analyzed species. For *D. melanogaster* an arm-GFP line of was used to highlight the apical cellular junctions. **A-C)** The postgonal sheath is highlighted in light purple, the ventral postgonal process is highlighted in with violet, and the dorsal postgonal process is highlighted in magenta **D)** Schematic representations of the postgonal sheath (light purple), the ventral postgonal process (violet), and dorsal postgonal process (magenta). 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 that also labels the apical cell junctions, while all other samples are stained for E-cadherin.

606 **Figure S6:**
607



608
609 Figure S6:
610
611 Developing central primordia of all analyzed species. **A-C)** The aedeagus is highlighted in light
612 green, the aedeagal ventral process is highlighted in with dark green and the aedeagal lateral
613 process is highlighted in yellow-green. **D)** Cartoon representations of the aedeagus (light green)
614 aedeagal ventral process (dark green) and aedeagal lateral process (yellow-green). Note that *D.*
615 *melanogaster* samples use a transgenic line arm-GFP that also labels the apical cell junctions,
616 while all other samples are stained for E-cadherin.

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