Trends in
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Review

Developmental evolution in fast-forward: insect male genital diversification

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Insect male genitalia are among the fastest evolving structures of animals. Studying these changes among closely related species represents a powerful approach to dissect developmental processes and genetic mechanisms underlying phenotypic diversification and the underlying evolutionary drivers. Here, we review recent breakthroughs in understanding the developmental and genetic bases of the evolution of genital organs among *Drosophila* species and other insects. This work has helped reveal how tissue and organ size evolve and understand the appearance of morphological novelties, and how these phenotypic changes are generated through altering gene expression and redeployment of gene regulatory networks. Future studies of genital evolution in *Drosophila* and a wider range of insects hold great promise to help understand the specification, differentiation, and diversification of organs more generally.

Highlights

A growing number of genes, pathways, and developmental mechanisms underlying insect male genital diversification have been identified.

Co-option of gene regulatory networks appears to play an important role in the evolution of novel genital structures, including *Drosophila* posterior lobes and postgonadal sheath projections.

Changes in the specification of tissue boundary positions likely contribute to differences in the relative size of genital structures, such as the *Drosophila* claspers.

Differential growth of genital structures among species can be regulated through interactions with the extracellular matrix.

Individual genes and even mutations can have pleiotropic effects that influence the evolution of sexual traits.

The evolutionary significance of divergence in male genital morphology

Male genitalia evolve extremely rapidly and exhibit remarkable diversity [1]. This is particularly evident among insects, where even closely related species can often be distinguished by striking differences in genital structures [1,2] (Figure 1).

In *Drosophila*, the terminalia are composed of the analia and the genitalia (Figure 2). Rice et al. [3] recently proposed a standard nomenclature for terminal substructures to facilitate clearer comparisons among *Drosophila* species. The analia comprise the cerci (anal plates), while the genitalia are subdivided into the **phallic** (see Glossary) and **periphallic structures** (Figure 2) [3]. The phallus, or intromittent organ, includes the **aedeagus** (penis) and postgonites (dorsal paramere) [3]. The periphallic structures include the epandrial posterior lobes (**posterior lobes**), the epandrial ventral lobes (lateral plates), and surstyli (**claspers**) (Figure 2) [3]. The genitalia of most other insects also include a phallus and peripheral clasping structures. While the phalli of many insects may be homologous, the size and elaboration of phallic structures are highly variable. For example, some species of beetles and true bugs have large phalli relative to body size (e.g., [4,5]), and the aedeagus can be absent or highly sclerotized with spines and additional distal processes [6]. In Odonata, the true phallus is rudimentary, and the functional intromittent organ is located in the anterior part of the abdomen [7]. The number of phallic structures can also vary; some families of earwigs (Dermaptera) have paired phalli [8], as do mayflies (Ephemeroptera) and caddisflies (Trichoptera) [6].

Structures often called claspers are periphallic organs that aid to secure mating ([9] and references therein). However, similarity in function and lack of knowledge of their exact developmental origins means that terminology has been used inconsistently to refer to structure homology across distantly related groups of insects. For example, the claspers of *Drosophila* are unlikely to be homologous to the clasping structures of other insects [10]. Indeed, even among dipterans, it remains unclear whether dorsal claspers of Eremoneurans are homologous to the ventral

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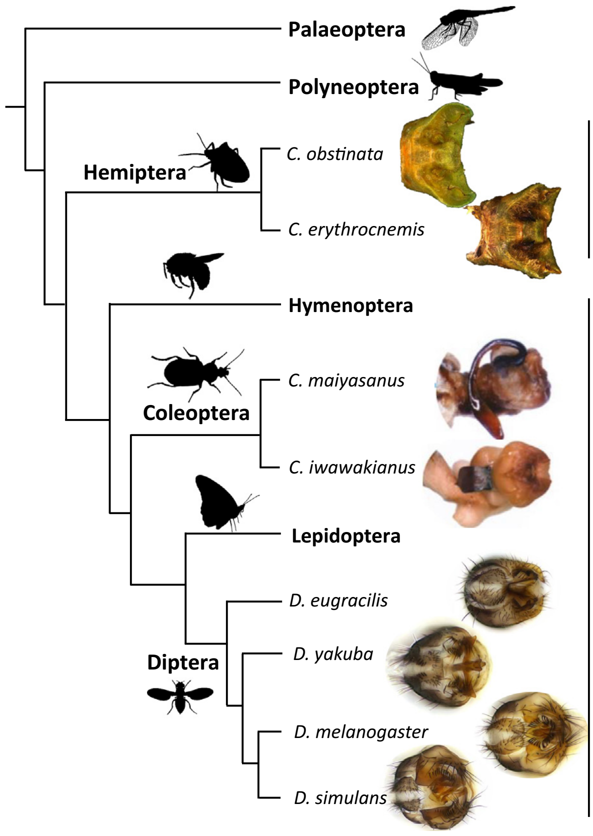

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Figure 1. Genital diversity among insect species. Males of *Chinavia obstinata* have smaller and less elaborate pygophores (additional clasping structures dorsolateral to the parameres) than *C. erythrocnemis* (ventral views; images credit: Bruno C, Genevcius, University of Sao Paulo). Despite these differences and substantial phylogenetic distance [98], these two species have been found to mate heterospecifically in the wild [99]. The male copulatory piece of *Carabus* ground beetles is an elaboration of the endophallus that helps stabilize genital coupling after intromission into the female and plays roles in postmating sexual selection and speciation [62]. The morphology of this structure varies greatly between closely related species, such as *C. iwawakianus* and *C. maiyasanus* (images credit: Teiji Sota, Kyoto University) and has been shown to have coevolved with the female vaginal appendix, an example of reproductive character displacement in the genital morphology of closely related species [31]. The size, shape, and sensory bristle number of the different structures of the *Drosophila* male epandrium have diverged rapidly between closely related species (images credit: Gavin Rice, University of Pittsburgh).

claspers of non-Eremoneurans [3]. The complexities in determining the homology of genital structures is exacerbated by their rapid evolution.

Sexual selection is thought to be the main driver of this fast evolution (Box 1). Species of the *D. melanogaster* clade evolved novel posterior lobes that have since diversified in shape and size (Figures 1 and 3) [11,12]. Functional mating behavior, surgical and genetic manipulation, and experimental evolution studies indicate that sexual selection acts on the posterior lobes during mounting and copulation and impacts reproductive fitness components, but natural selection also plays a role in their diversification [13–17]. Furthermore, females of these species have evolved oviscapt pouches morphologically complementary to conspecific male posterior lobes, suggesting coevolution [13,14,18]. This is consistent with sexual selection either due to sexually antagonistic coevolution or cryptic female choice (Box 1) but could also be explained by the lock-and-key hypothesis (Box 1), which would suggest a role in reproductive isolation between species [19–21].

The periphallic organs of insects often bear cuticular structures or spines. The number and location of these spines are remarkably diverse in *Drosophila*. Surgical shortening or ablation of specific sets of spines in *D. ananassae* and *D. montium* [9,22] affected mating success and in some cases reproductive fitness, suggesting that these structures may be used for sensory intersexual communication as well as grasping. In addition, the contact of male genital spines with the female genitalia can often result in wounding the female [23,24], suggesting a significant role of sexual

Glossary

Aedeagus: intromittent organ of male insects that is used to transfer sperm to the female.

Claspers: appendage-like genital organs found in many insects that are used by males to grasp females during copulation. Like other periphallic structures, the homology of these structures is difficult to infer beyond very closely related species.

Co-option: when a gene regulatory network used in one aspect of development is redeployed in a new role.

Introgression mapping: using recombination to introduce genomic regions from one species to another with subsequent genotyping of breakpoints and phenotyping, allowing fine-scale mapping of genes underlying a morphological difference between the closely related focal species.

Periphallic structures: external male insect genital structures that are indirectly connected to the intromittent organ and develop from distinct primordia in the genital disc.

Phallic structures: external male insect genital structures encompassing the intromittent organ and directly connected structures.

Posterior lobes: novel extensions of the epandrium of the male genitalia in *D. melanogaster* and related species, which interact with the female during copulation.

Quantitative trait loci (QTL) mapping: statistical method to map phenotypic variation between pairs of species or strains to regions of the genome using markers such as single-nucleotide polymorphisms.

Reciprocal hemizygosity test: generation of pairs of hybrids where a copy of the candidate gene is only functional from one strain/species or the other in an otherwise identical background. Differences in a phenotype between the hybrids, consistent with the evolved parental strain/species difference, must be caused by the candidate gene.

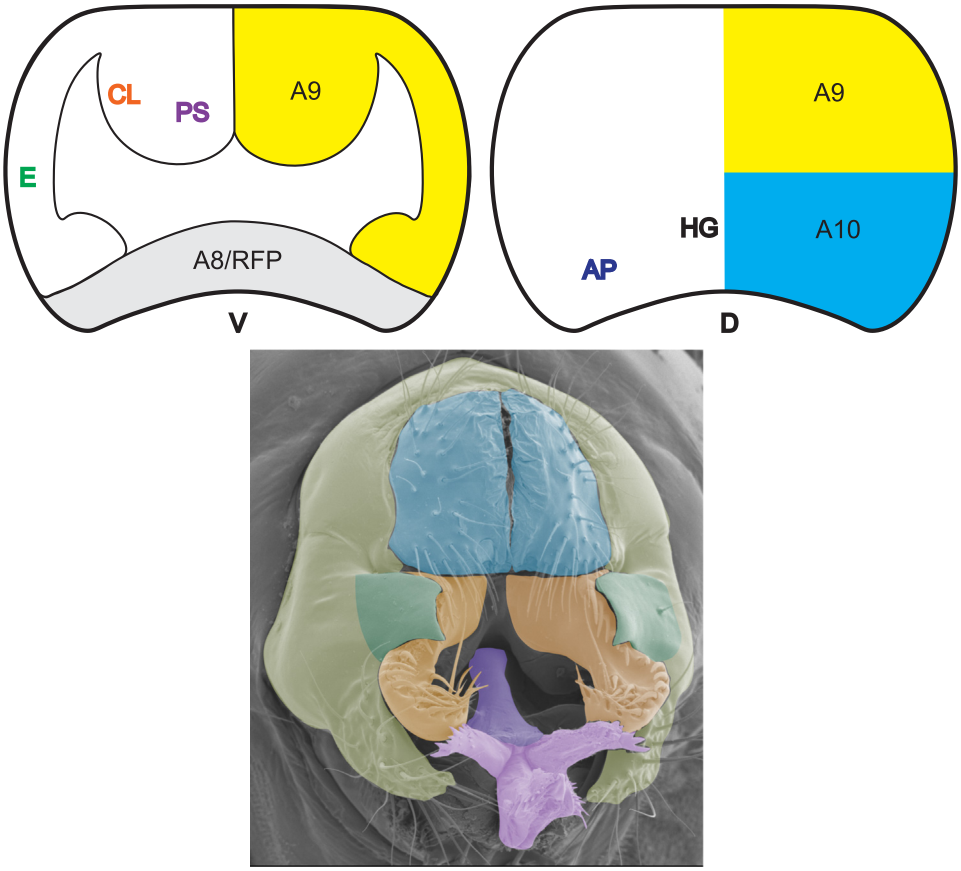

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Figure 2. Fate map of the *Drosophila* male genital disc to adult structures. The top schematics show the ventral (V; left) and dorsal (D; right) maps of the *Drosophila* third instar male genital disc. The segmental origins of the disc regions are indicated by A8, A9, and A10. Regions that will give rise to adult structures are indicated within the left-side half of the ventral and dorsal disc views and are colored to indicate the corresponding adult structures on the scanning electron microscopy image below. The repressed female primordia are not visible in the scanning electron microscopy image. The top schematics were adapted from [32,33]. The scanning electron microscopy image of *D. melanogaster* was kindly provided by Javier Figueras Jimenez. Abbreviations: AP, anal plates (blue); CL, claspers (orange); E, epandrium (green, with the posterior lobes shaded dark green); HG, hind gut; PS, phallic structures (dorsal postgonites and aedeagus, light purple; phallapodeme, dark purple); RFP, repressed female primordia.

conflict in the evolution of these structures. This is also the case for the other spikes present on insect phallic and periphallic structures [18,21,25], which may be used to secure coupling forcefully, remove rival's sperm, prevent future mating, or transfer seminal fluids into the female's circulatory system, manipulating female reproduction [24,26]. In *Carabus* beetles, the morphology of the copulatory piece (a sclerotized projection on the endophallus) is variable between and within species (Figure 1) and has been shown to match the shape of the female vaginal appendix [27–29]. Morphological incompatibility in heterospecific matings can result in female injury as well as insemination failure [28,29] even between allopatric populations of *C. maiyasanus* [30], supporting the lock-and-key hypothesis of genital evolution in these beetles [31] (Box 1).

In this review, we explore the development and evolution of male insect genitalia and theories for their diversification. We then discuss recent new insights into the developmental and genetic causes of this diversification in *Drosophila* and other insects. We also propose future research

Box 1. Hypotheses for genital evolution

Lock-and-key hypothesis

The lock-and-key hypothesis was originally proposed by Dufour (1844) as a mechanism of natural selection where genital divergence evolved to prevent hybridization, with conspecific males possessing the right 'key' to fit the female 'lock' [79]. This hypothesis was dismissed for several decades due to a reported lack of compelling evidence ([1,80] and citations therein). However, this was the result, at least partially, of a male-biased focus [81–83]. Although much harder to phenotype because of difficulties in accessing internal soft tissues, female genitalia have now been shown to display remarkable diversity [84–86]. In addition, more recent studies showed that mechanical and sensory incompatibilities between sexes of different species can affect reproductive fitness in some taxa (reviewed in [20,87,88]), leading to a renewed appreciation of the relevance of the lock-and-key hypothesis. These incompatibilities can arise because of sex-specific natural and/or sexual selection on the genitalia, which then drives coevolution in the other sex, and this may be facilitated by a shared genetic or developmental origin of the coevolved structures between sexes (E. McQueen, PhD thesis, University of Pittsburgh, 2021) [64,89].

Pleiotropy hypothesis

The pleiotropy hypothesis postulates that genital divergence can evolve neutrally as a result of natural selection on other phenotypic traits if the underlying genes have pleiotropic effects on genitalia [90]. This hypothesis has been largely rejected as an important driver of changes in genitalia because it is unclear why the genes involved in genital development would be more susceptible to pleiotropy than other tissues [2]. However, as our understanding of the GRNs underlying genital evolution and the genetic correlations between different traits [91] and the genitalia in the different sexes (E. McQueen, PhD thesis, University of Pittsburgh, 2021) [89] improves, we should be able to re-evaluate the importance of this hypothesis.

Sexual selection hypothesis

The sexual selection hypothesis is by far the hypothesis most supported by evidence [1,2,76,80,92], but the proximal mechanisms through which sexual selection drives genital divergence are likely to be taxa specific and differ for different genital structures (e.g., phallic versus periphallic) and mating systems (e.g., depends on levels of polygamy and competition for mates) [2]. In species with polyandry, sperm of multiple males compete to fertilize the eggs of a female, and this can drive the evolution of male genitalia to enhance sperm transfer, storage, or displacement [93,94]. This can also result in indirect fitness benefits to females through producing more competitive sons, known as the 'sexy sons' or the Fisherian selection hypothesis. Cryptic female choice, through which sperm from males with preferred genital traits during or after mating is selected, has received much attention but has been difficult to demonstrate [92,95]. Of all the possible mechanisms, sexual conflict, where male genitalia evolve to manipulate female reproduction, has arguably received the most empirical support [1,2,76,80], probably because the detrimental fitness effects to the females are more directly observable (but see [96]).

that could help to better understand the genetic and evolutionary mechanisms for the rapid evolution of insect genitalia more broadly.

The development of male genital structures in *Drosophila*

The development of male terminalia is best understood in *D. melanogaster* [32]. These organs develop from the genital imaginal disc, which is composed of cells from abdominal segments A8, A9, and A10. In males, the genitalia and analia develop from A9 and A10, respectively (Figure 2). Differentiation into male or female structures is regulated by the sex determination pathway through the male and female Doublesex isoforms (reviewed in [32]).

In the genital disc, the primordia of the different adult structures are already marked by the expression of specific genes. *caudal* expression marks the anal primordia that will give rise to the anal plates, while the Hox gene *Abdominal-B* (*Abd-B*) marks the genital region (reviewed in [32]). During the third instar, the primordia of the genital structures expand in cell number and start to undergo remodeling, and subsequently during pupal stages, they evert and grow and are shaped to assume their adult form.

Insect genitalia are considered to be derived ventral appendages [33,34]. Loss of *Abd-B* expression in genital disc cell clones transforms genital structures into leg structures [35]. This is consistent with findings supporting the idea that the 'ground state' of the *Drosophila* ventral appendage

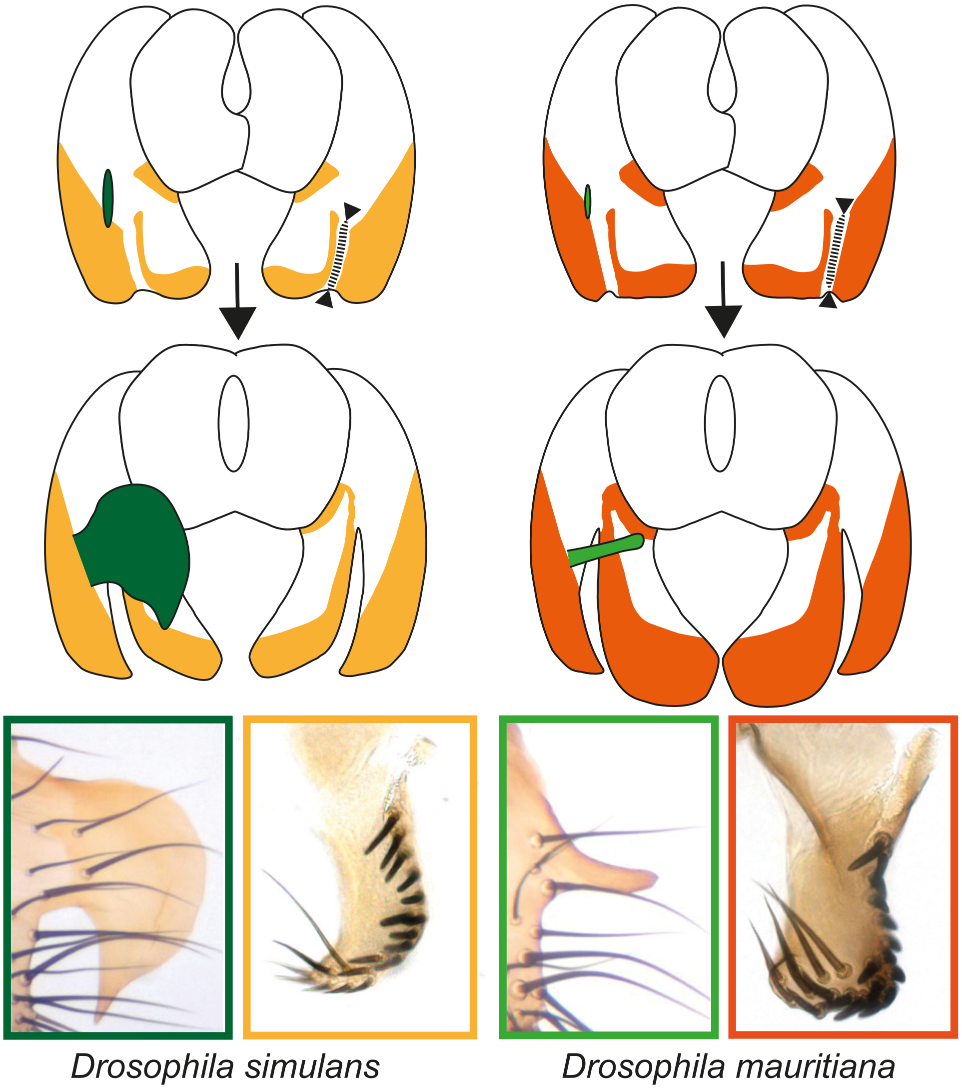

Figure 3. Development of differences in epandrial posterior lobe and clasper morphology between *Drosophila simulans* and *D. mauritiana*. The top schematics show developing genitalia at approximately 28 h after puparium formation (hAPF). Spatial expression of *tartan (trn)* is indicated by light orange and dark orange in *D. simulans* and *D. mauritiana*, respectively [41]. The position of the boundary between the lateral plate and clasper primordia is indicated by arrowheads and a dashed line on the right-hand side of the primordia of each species. The larger primordium of the posterior lobe of *D. simulans* is indicated by a dark green ellipse compared with the smaller primordium of *D. mauritiana* in light green on the left-hand side of the developing genitalia of each species. The middle images show the developing primordia of each species at approximately 46 hAPF when the larger claspers and smaller posterior lobes (light green) of *D. mauritiana* are visible compared with the smaller claspers and larger epandrial posterior lobes of *D. simulans* (dark green). *trn* expression is again indicated in light orange (*D. simulans*) and dark orange (*D. mauritiana*). The bottom images show the adult structures of each species. Adapted from [41,78].

was a proximal segment and distal leg-like segments, and that Hox input produced specific appendages, for example, *Abd-B*, in the genitalia [35,36]. Intriguingly, several genes of the distal leg gene regulatory network (GRN) are also deployed in developing genitalia, and it would be interesting to determine how *Abd-B* reprograms their roles and interactions for the development of derived genital morphology [33,34,37–39].

While Abd-B acts upstream in the development of genital structures, until recently, little was understood about how the development of the different external structures was regulated, even in *Drosophila*, apart from a few genes. Vincent et al. helped to address this by performing RNA sequencing and generating an atlas of the spatial expression of the top 100 highest expressed transcription factors in the developing pupal genitalia of *D. melanogaster* [37]. This valuable resource combined with studies of the development and evolution of specific structures, such as the claspers and posterior lobes [12,40–42], now provide an excellent platform to understand the regulation of these processes better at the genetic and cellular levels.

Development of genitalia in other insects

In contrast to *Drosophila*, the development of genitalia has only been studied sporadically among other insects and consequently is relatively poorly understood. Nevertheless, several key studies of beetles, parasitic wasps, and true bugs have helped to reveal ancestral features of the development of these structures. Although some adult structures develop from the larval ectoderm rather than internalized imaginal discs in beetles, unlike in *Drosophila*, it has been observed that genitalia of the horned beetle *Onthophagus taurus* and the carabid beetle *Carabus maiyasanus* develop from imaginal disc-like tissues [43,44]. In these beetles, these disc-like structures grow during the last (third) larval instar before undergoing differentiation to form the adult structures, again like *Drosophila* [43,44]. Analysis of gene expression profiles across larval and pupal stages in *Carabus* suggests that differentiation of genital structures follows a similar timescale and uses the same key regulatory factors as *Drosophila* [44]. Furthermore, a recent study identified two additional male-specific isoforms of Dsx in *Nasonia vitripennis* and showed that this gene is required to suppress female genital differentiation and promotes the growth of male genital structures, including the aedeagus in the larval stages of this parasitic wasp [45].

Again, consistent with findings in *Drosophila*, studies of gene expression and the genetic regulation of male genital development in other insects suggest that some insect male genital structures are homologous and likely to be derived serial homologs of walking appendages. Several key genes involved in leg development in *Drosophila*, including *dachshund* and *Distal-less*, are expressed during genital differentiation in *Carabus*, and knockdown of ‘leg’ development genes in *Onthophagus* beetles and the milkweed bug *Oncopeltus fasciatus* perturbed male genital development [43,44,46,47]. It appears that *Abd-B* specifies genital versus leg identity generally in insects ([48] and references therein), and knockdown of *Abd-B* in the firefly *Photuris pennsylvanica* transformed the genitalia to legs like in *Drosophila* [35,49].

Development of insect male genital diversity

Identifying the genetic basis for the diversification of complex structures requires a developmental framework. Understanding how and when homologous structures arise and diverge during morphogenesis and the development of novelties as well as the cellular processes involved can help focus gene expression analysis and filtering of candidate genes to reveal the underlying genetic basis. Two recent studies have compared the development of anal and genital structures across *Drosophila* species [12,50].

Rice et al. studied the development of phallic structures in eight *Drosophila* species [50]. They showed that the phallic structures develop from three prepupal primordia in all species surveyed, and they were able to trace the development of novel and conserved structures and infer their homology [50]. For example, the dorsolateral portion develops into one tissue made up of the aedeagal sheath and the dorsal postgonites in all species examined, while the *D. melanogaster* complex has evolved novel ventral postgonites [23]. Their finding that similar additional structures can develop from the same or different primordia raises interesting questions about the genetic basis of these structures, including whether they arise through the redeployment of the same GRNs in different primordia and the underlying genetic and/or cellular mechanisms [50].

Urum et al. generated a staging system for the development of periphallic structures in *D. melanogaster* pupae and used this to describe the morphogenesis of these structures in 11 other *Drosophila* species [12]. Focusing on the anal plates, claspers, and lateral plates, including the posterior lobes, this important systematic study provided new insights into the developmental and evolutionary timelines as well as underlying mechanisms for differences in these structures [12]. For example, they showed that the lateral plate and clasper primordia start to separate at around 28 h after puparium formation (hAPF), and the position of the cleavage furrow reflects the relative size of the adult structures across these *Drosophila* species [12] (Figure 3). This suggests that the genes that specify the position of the cleavage furrow influence the development and evolution of clasper and lateral plate size within and among species (Figure 3 and see following section) [41,51].

Urum et al. also showed that differences in the posterior lobe primordia of *D. simulans* compared with other species, prefiguring the difference in the adult size of this structure, could also be detected by 28 hAPF (Figure 3) [12]. Furthermore, they pinpointed that this morphological novelty likely arose in the ancestor of the *D. melanogaster* and *D. yakuba* complexes before diversifying so dramatically in the former complex (Figures 1 and 3).

Much less is understood about the development of the genitalia of other insects, never mind how these processes have evolved to generate genital diversity. However, there have been a few pioneering studies of beetles [52,53]. Terada et al. showed that the longer copulatory piece of male *C. maiyasanus* than of *C. iwawakianus* (Figure 1) is due to the earlier initiation of development and longer growth time of this structure in the former species [53]. Indeed, higher growth rate also underlies the exaggerated male genitalia of *C. uenoi* [54]. This suggests that heterochrony and differential growth time and rate are likely common features of developmental changes underlying genital evolution.

The growth of insect bodies can be plastic in response to different conditions during development. Like other tissues, the growth and adult size of insect genitalia is regulated by the insulin signaling pathway, and it has been shown that this has evolved to modulate genital nutritional plasticity and promote reproductive success [55–57] (Box 2).

The genetic basis for differences in male genital morphology

The genetic basis of differences in male genital morphology among *Drosophila* species has been studied for decades using **quantitative trait loci (QTL) mapping**, genome-wide association studies, **introgression mapping**, and experimental evolution [14,58–61]. This work has shown that differences in genital structures are highly polygenic and generally additive.

Other than *Drosophila*, most work to identify genes contributing to divergence in male genital morphology has also been conducted in *Carabus* beetles (Figure 1). Fujisawa et al. found that interspecific changes in phallus morphology are also polygenic, and QTLs associated with these differences showed increased levels of sequence divergence [62]. Analysis of differentially expressed genes in these beetles during genital development identified promising candidate genes that may underlie these differences [44,63,64]. Genevcius et al. also recently used a candidate gene approach to analyze genital evolution in Hemipterans (Figure 1). They found correlation between genital size and mutation rates of genes involved in a range of developmental

Box 2. Plasticity in genital morphology

Unlike other body parts, the genitalia of many insects show relatively little size plasticity in response to differences in development conditions, such as nutrition or temperature [97]. This means that genital structures are hypoallometric. For example the posterior lobes of small male *D. melanogaster* with small wings are larger than expected compared with the lobes of large males with large wings [56]. It is thought that this general 'one size fits all' rule is driven by the negative fitness consequences for males with the 'wrong size' of genital structures, perhaps due to mechanical fit and/or stimulatory effects on females [56,97]. It was previously shown that the reduced nutritional response of posterior lobe development is caused by insensitivity to growth control by insulin signaling levels as the result of low expression levels of the transcription factor FOXO [57]. Dreyer and Shingleton explored this further by increasing the levels of FOXO expression during posterior lobe development and testing the effect on lobe morphology and male reproductive success [56]. This made posterior lobe development sensitive to insulin signaling, and consequently males had smaller posterior lobes than usual. Importantly, they also demonstrated the likely selective explanation for reduced sensitivity to insulin signaling during genital development by showing that males with larger posterior lobes had greater mating success than males with small lobes [56]. This is consistent with previous observations that males with small lobes transferred less sperm than males with larger lobes [16], but cryptic female choice (Box 1) could also help explain these results.

The insulin signaling pathway is likely a general mechanism for the regulation of male insect genital growth and plastic developmental responses to differences in nutrition. Casasa and Moczek observed that while the aedeagus of male *O. taurus* is also hypoallometric, they found that knockdown of FOXO further reduced sensitivity [55]. This suggests that in this beetle, FOXO levels in the developing genitalia regulate a larger nutritional response than in *Drosophila*, and, therefore, lineage-specific differences in the response of genital development to nutrition can be tuned via the insulin signaling pathway [55].

processes, demonstrating that the genetic basis of rapid genital evolution is complex and in this case may be driven by relaxed constraints on pleiotropic genes [65].

However, no causative genes have yet been conclusively identified for genital differences in insects apart from *Drosophila*. Indeed, despite ever-growing knowledge of the development of male genitalia in *D. melanogaster* and other *Drosophila* species combined with extensive mapping and gene expression analysis, only a handful of the causative genes for differences in the morphology of genital structures have been characterized.

Evolution and diversification of the posterior lobes

As mentioned in the preceding text, the posterior lobes are novel structures that evolved before the divergence of the *D. melanogaster* and *D. yakuba* complexes and subsequently diversified in shape and size [11,12] (Figures 1–3). **Co-option** of an Abd-B and Pox neuro GRN from the larval posterior spiracles likely underlies the appearance of posterior evolution, as many of the genes and interactions are shared during the development of these distinct structures [11]. Posterior lobe growth is caused by cell elongation, which is mediated by interactions with the extracellular matrix and involves the protein Dumpy [40]. Furthermore, diversification of posterior lobe morphology likely involves differences in patterns of cell growth mediated by the extracellular matrix as well as specification of the initial number of cells assigned to the posterior lobe [12,42].

Ridgway et al. recently identified one of the genes underlying the difference in posterior lobe morphology between *D. simulans* and *D. mauritiana* [42] (Figure 3). Using introgression mapping and differential gene expression analysis, they identified *Sox21b* as a candidate gene. They then used CRISPR/Cas9 to generate null alleles of either *D. mauritiana* or *D. simulans Sox21b* and carried out a **reciprocal hemizygosity test** [66] to verify that *Sox21b* contributed to posterior lobe divergence. Males with *D. simulans Sox21b* also copulated with *D. simulans* females for longer, demonstrating an impact on mating behavior. Knockdown of *Sox21b* in *D. melanogaster* resulted in enlargement of the posterior lobes, and, consistent with this, *Sox21b* expression is lower in the *D. simulans* genital disc than in the *D. mauritiana* genital disc, implying that this transcription factor represses posterior lobe growth [42]. Therefore, it is likely that changes in a *Sox21b* genital enhancer underlie its role in the posterior lobe size difference between *D. simulans* and *D. mauritiana* [42]. It will be interesting to determine how *Sox21b* interacts with other genes involved in posterior lobe development, for example, by identifying the direct targets of this transcription factor in the posterior lobe GRN, and to pinpoint the other evolved nodes that contribute to posterior lobe divergence.

tartan and clasper size evolution

Using similar approaches to those of Ridgway et al. [42], Hagen et al. showed that the gene *tartan* (*trn*) contributes to the clasper size difference between *D. mauritiana* and *D. simulans* [41]. While this contribution of *trn* could be explained by higher expression of *trn* in the developing claspers, this gene encodes a leucine-rich repeat transmembrane protein that specifies compartment boundaries in developing tissues through interactions with another transmembrane protein, Tenascin-major [51,67]. Intriguingly, *trn* is expressed at the lateral plate and clasper primordia boundary that later cleaves as these structures separate (Figure 3). Therefore, an attractive hypothesis is that Trn helps to define this boundary, and changes in the location of Trn expression and/or interactions contribute to the clasper size difference between *D. mauritiana* and *D. simulans* and potentially among other *Drosophila* species (Figure 3) [12,41,68].

A pleiotropic mutation underlies loss of hypandrial bristles and gain of sex comb bristles in *D. santomea*

Using mapping between *D. santomea* and *D. yakuba* and reporter assays in *D. melanogaster*, Nagy et al. found that at least three *D. santomea*-specific substitutions, clustered downstream of the *scute* (*sc*) promoter, were associated with the loss of hypandrial bristles in *D. santomea* [69]. One of these mutations pleiotropically affected another sexually dimorphic structure, the sex combs on male forelegs, which are crucial during mating [70] and exhibit a higher bristle number in *D. santomea.* This mutation is located within a binding site for Abd-B. The authors proposed that the *D. santomea*-specific mutation likely decreases Abd-B binding affinity, reducing *sc* expression and resulting in fewer hypandrial bristles [69]. However, in the forelegs, where *Abd-B* is not expressed, the same substitution may affect the binding of another transcription factor, resulting in increased *sc* levels and more sex comb bristles [69].

Shavenbaby and phallus evolution in *D. eugracilis*

Male *D. eugracilis* genitalia are distinguished by trichome-like projections on the postgonadal sheath of the phallus that are not present on this structure in related species [71]. The GRN that regulates trichome formation on the larval cuticle and a range of adult structures, including the legs, is understood in great detail [72–75]. *shavenbaby* (*svb*) encodes a transcription factor that regulates scores of downstream genes to control trichome patterning and morphology, and this gene is a ‘hotspot’ for the evolution of larval trichomes [72,73]. Rice et al. showed that *svb* loss in *D. eugracilis* reduced the length of these projections [71]. Moreover, ectopic *svb* expression in developing *D. melanogaster* genitalia induces trichome formation [71]. These results provide a perspective on genital development and evolution beyond a single gene by showing that the Svb-regulated GRN has likely been co-opted to generate the large apical projections on the male *D. eugracilis* postgonadal sheath. This elegant work again suggests that co-option of GRNs from other developing tissues plays a key role in the diversification of male genital structures.

Challenges

While great progress has been made in understanding the genetics underlying the regulation of development and the diversification of male genital structures, mostly in *Drosophila*, several challenges need to be addressed in future work.

First, the genetic basis of genital evolution is highly polygenic, and understanding the genetic architecture of such complex traits remains challenging due to the interplay of multiple genes and environmental factors. Mapping approaches can reveal large-effect genes, but it remains difficult to identify other genes with minor effects [68]; therefore, more studies combining genetic, developmental, and experimental evolution approaches are needed. Indeed, better characterizing the GRNs underlying genital development using single-cell RNA sequencing as well as approaches like Cleavage Under Targets and Release Using Nuclease (CUT&RUN) and RNA interference followed by RNA sequencing, for example, to identify direct target genes of transcription factors like Abd-B and Sox21b, would provide a better understanding of genital development and evolution.

Second, much of our understanding of the genetic and developmental bases of genital evolution comes from comparing *Drosophila* species pairs. Further comparisons among *Drosophila* species are needed to identify commonalities and differences in the evolution of genital structures [12,50]. Furthermore, given advances in genomics and functional tools (e.g., CRISPR/Cas9), the evolution of differences in genital morphology among a wider range of insects can now be studied, with *Carabus* ground beetles and butterflies representing particularly promising systems [76].

Third, as with the evolution of other phenotypes, it remains challenging to infer and test the evolutionary forces that underlie genital diversification. Therefore, it is important to examine the functional and behavioral consequences of these changes to help understand why they have been selected and test assumptions [42,56,77].

Concluding remarks

Recent studies have advanced our knowledge of the genetic and developmental bases of male genital evolution, although many questions need to be addressed further (see Outstanding questions). These studies have not only described the genetic architecture and identified genes, pathways, and developmental mechanisms underlying genital differences among species driven by sexual selection and other forces but also provided new insights into GRN structure, evolution and co-option, and phenotypic and behavioral evolution and speciation as well as organ growth and size specification more generally [78]. Therefore, future studies of the rapid diversification of male genital morphology have important implications for understanding genetics and developmental evolution more broadly.

Outstanding questions

Which other genes underlie differences in male genital morphology in *Drosophila* and other insects?

While a few genes have been identified that contribute to changes in genital morphology between *Drosophila* species, these differences are polygenic, and we need to identify the other genes involved to determine if they are part of the same GRNs or act in parallel and if they interact additively or epistatically. Furthermore, we need to identify genes underlying genital diversification in other species to compare with *Drosophila* and understand the genetic basis for rapid male insect genital evolution more broadly.

What is the role of pleiotropy in male genital divergence?

Cis-regulatory evolution is expected to drive morphological evolution by targeting gene expression changes to specific tissues, thereby reducing pleiotropic effects. Despite this, instances of pleiotropy of mutations responsible for male genital divergence highlight its significance (see the main text). Although challenging, characterizing the spatial and temporal expression profiles influenced by causative mutations is needed.

What is the contribution of standing genetic variation to species differences?

There is now an increasing realization that most genes expressed in relevant tissues can affect intraspecific variation even in relatively simple traits (omnigenic model) [100]. While the genetic architecture of interspecific male genital divergence is also highly polygenic, it remains unclear if the omnigenic model extends to macroevolutionary phenotypic changes.

What mutations in which genes underlie the co-option of GRNs in male genital novelties?

Co-option likely plays an important role in the evolution of male genital novelties, such as the epandrial posterior lobes of *D. melanogaster* and related species, but we still need to identify the mutations and genes responsible.

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Declaration of interests

The authors declare no competing interests.

References

1. Eberhard, W.G. (1985) *Sexual Selection and Animal Genitalia*, Harvard University Press
2. Hosken, D.J. and Stockley, P. (2004) Sexual selection and genital evolution. *Trends Ecol. Evol.* 19, 87–93
3. Rice, G. *et al.* (2019) A standardized nomenclature and atlas of the male terminalia of *Drosophila melanogaster*. *Fly (Austin)* 13, 51–64
4. Matsumura, Y. *et al.* (2017) Penetration mechanics of a beetle intromittent organ with bending stiffness gradient and a soft tip. *Sci. Adv.* 3, eaao5469
5. Wieczorek, K. *et al.* (2011) Comparative morphology of the male genitalia of Aphididae (Insecta, Hemiptera): part 1. *Zoomorphology* 130, 289–303
6. Snodgrass, R.E. (1994) *Principles of Insect Morphology*, Cornell University Press
7. Garrison, R.W. *et al.* (2006) *Dragonfly Genera of the New World: An Illustrated and Annotated Key to the Anisoptera*, Johns Hopkins University Press
8. Kamimura, Y. (2014) Pre- and postcopulatory sexual selection and the evolution of sexually dimorphic traits in earwigs (Dermaptera). *Entomol. Sci.* 17, 139–166
9. Rodriguez-Exposito, E. *et al.* (2020) Individual and synergistic effects of male external genital traits in sexual selection. *J. Evol. Biol.* 33, 67–79
10. Moreno-García, M. and Cordero, C. (2008) On the function of male genital claspers in *Stenomacra marginella* (Heteroptera: Largidae). *J. Ethol.* 26, 255–260
11. Glassford, W.J. *et al.* (2015) Co-option of an ancestral Hox-regulated network underlies a recently evolved morphological novelty. *Dev. Cell* 34, 520–531

Do the same or different genes underlie the coevolution of male and female genitalia?

While we have a growing understanding of the genes underlying the evolution of differences in male insect genitalia, much remains to be learned about the genetic basis of variation among females and the similarities and differences to males.

12. Urum, A. *et al.* (2024) A developmental atlas of male terminalia across twelve species of *Drosophila*. *Front. Cell Dev. Biol.* 12, 1349275
13. House, C.M. *et al.* (2013) Sexual and natural selection both influence male genital evolution. *PLoS One* 8, e63807
14. House, C.M. *et al.* (2021) Sexual selection on the genital lobes of male *Drosophila simulans*. *Evolution* 75, 501–514
15. LeVasseur-Viens, H. *et al.* (2015) No evidence for external genital morphology affecting cryptic female choice and reproductive isolation in *Drosophila*. *Evolution* 69, 1797–1807
16. Frazee, S.R. and Masly, J.P. (2015) Multiple sexual selection pressures drive the rapid evolution of complex morphology in a male secondary genital structure. *Ecol. Evol.* 5, 4437–4450
17. Jagadeeshan, S. and Singh, R.S. (2006) A time-sequence functional analysis of mating behaviour and genital coupling in *Drosophila*: role of cryptic female choice and male sex-drive in the evolution of male genitalia. *J. Evol. Biol.* 19, 1058–1070
18. Yassin, A. and Orgogozo, V. (2013) Coevolution between male and female genitalia in the *Drosophila melanogaster* species subgroup. *PLoS One* 8, e57158
19. Frazee, S.R. *et al.* (2021) Interspecific introgression reveals a role of male genital morphology during the evolution of reproductive isolation in *Drosophila*. *Evolution* 75, 989–1002
20. Masly, J.P. (2012) 170 years of 'lock-and-key': genital morphology and reproductive isolation. *Int. J. Evol. Biol.* 2012, 247352
21. Masly, J.P. and Kamimura, Y. (2014) Asymmetric mismatch in strain-specific genital morphology causes increased harm to *Drosophila* females. *Evolution* 68, 2401–2411
22. Grieshop, K. and Polak, M. (2012) The precopulatory function of male genital spines in *Drosophila ananassae*[Doleschall] (Diptera: Drosophilidae) revealed by laser surgery. *Evolution* 66, 2637–2645
23. Grieshop, K. and Polak, M. (2014) Evaluating the post-copulatory sexual selection hypothesis for genital evolution reveals evidence for pleiotropic harm exerted by the male genital spines of *Drosophila ananassae*. *J. Evol. Biol.* 27, 2676–2686
24. Hotzy, C. *et al.* (2012) Phenotypic engineering unveils the function of genital morphology. *Curr. Biol.* 22, 2258–2261
25. Reinhardt, K. *et al.* (2015) Copulatory wounding and traumatic insemination. *Cold Spring Harb. Perspect. Biol.* 7, a017582
26. Tatarnic, N.J. *et al.* (2014) Traumatic insemination in terrestrial arthropods. *Annu. Rev. Entomol.* 59, 245–261
27. Sasabe, M. *et al.* (2010) QTL for the species-specific male and female genital morphologies in *Ohomopterus* ground beetles. *Mol. Ecol.* 19, 5231–5239
28. Kubota, K. *et al.* (2013) Mechanical reproductive isolation via divergent genital morphology between *Carabus insulicola* and *C. esakii* with implications in species coexistence. *Popul. Ecol.* 55, 35–42
29. Sota, T. and Kubota, K. (1998) Genital lock-and-key as a selective agent against hybridization. *Evolution* 52, 1507–1513
30. Xia, T. *et al.* (2023) Reproductive isolation via divergent genital morphology due to cascade reinforcement in *Ohomopterus* ground beetles. *J. Evol. Biol.* 36, 169–182
31. Nishimura, T. *et al.* (2022) Reproductive character displacement in genital morphology in *Ohomopterus* ground beetles. *Am. Nat.* 199, E76–E90
32. Estrada, B. *et al.* (2003) Development of the genitalia in *Drosophila melanogaster*. *Differentiation* 71, 299–310
33. Gorfinkiel, N. *et al.* (1999) *Drosophila* terminalia as an appendage-like structure. *Mech. Dev.* 86, 113–123
34. Ridgway, A.M. *et al.* (2024) Rewired gene interactions during development of serially homologous appendages in male and female *Drosophila*. *bioRxiv*, Published online October 21, 2024. https://doi.org/10.1101/2024.10.19.618997
35. Estrada, B. and Sanchez-Herrero, E. (2001) The Hox gene *Abdominal-B* antagonizes appendage development in the genital disc of *Drosophila*. *Development* 128, 331–339
36. Casares, F. and Mann, R.S. (2001) The ground state of the ventral appendage in *Drosophila*. *Science* 293, 1477–1480
37. Vincent, B.J. *et al.* (2019) An atlas of transcription factors expressed in male pupal terminalia of *Drosophila melanogaster*. *G3 (Bethesda)* 9, 3961–3972
38. Campbell, G. (2005) Regulation of gene expression in the distal region of the *Drosophila* leg by the Hox11 homolog, C15. *Dev. Biol.* 278, 607–618
39. Kojima, T. *et al.* (2005) A concerted action of a paired-type homeobox gene, *aristaless*, and a homolog of *Hox11/tlx* homeobox gene, *clawless*, is essential for the distal tip development of the *Drosophila* leg. *Dev. Biol.* 279, 434–445
40. Smith, S.J. *et al.* (2020) Evolutionary expansion of apical extracellular matrix is required for the elongation of cells in a novel structure. *Elife* 9, e55965
41. Hagen, J.F.D. *et al.* (2019) *tartan* underlies the evolution of *Drosophila* male genital morphology. *Proc. Natl. Acad. Sci. USA* 116, 19025–19030
42. Ridgway, A.M. *et al.* (2024) *Sox21b* underlies the rapid diversification of a novel male genital structure between *Drosophila* species. *Curr. Biol.* 34, 1114–1121
43. Moczek, A.P. and Nijhout, H.F. (2004) Trade-offs during the development of primary and secondary sexual traits in a horned beetle. *Am. Nat.* 163, 184–191
44. Nomura, S. *et al.* (2020) Gene expression during genital morphogenesis in the ground beetle *Carabus maiyasanus*. *Insect Sci.* 27, 975–986
45. Wang, Y. *et al.* (2022) Doublesex regulates male-specific differentiation during distinct developmental time windows in a parasitoid wasp. *Insect Biochem. Mol. Biol.* 142, 103724
46. Aspiras, A.C. *et al.* (2011) Sex-specific gene interactions in the patterning of insect genitalia. *Dev. Biol.* 360, 369–380
47. Sota, T. *et al.* (2018) Knockdown of *rotund* gene through larval RNA interference affects genital and elytral morphology in the ground beetle *Carabus maiyasanus* (Coleoptera: Carabidae). *Entomol. Sci.* 21, 469–474
48. Hughes, C.L. and Kaufman, T.C. (2002) Hox genes and the evolution of the arthropod body plan. *Evol. Dev.* 4, 459–499
49. Stansbury, M.S. and Moczek, A.P. (2014) The function of *Hox* and appendage-patterning genes in the development of an evolutionary novelty, the *Photuris* firefly lantern. *Proc. Biol. Sci.* 281, 20133333
50. Rice, G.R. *et al.* (2023) Resolving between novelty and homology in the rapidly evolving phallus of *Drosophila*. *J. Exp. Zool. B Mol. Dev. Evol.* 340, 182–196
51. Pare, A.C. *et al.* (2019) An LRR receptor–teneurin system directs planar polarity at compartment boundaries. *Dev. Cell* 51, 208–221
52. Matsumura, Y. *et al.* (2013) Parallel evolution of novelties: extremely long intromittent organs in the leaf beetle subfamily Criocerinae. *Evol. Dev.* 15, 305–315
53. Terada, K. *et al.* (2021) Heterochrony and growth rate variation mediate the development of divergent genital morphologies in closely related *Ohomopterus* ground beetles. *Evol. Dev.* 23, 19–27
54. Terada, K. *et al.* (2024) The development of extremely large male genitalia under spatial limitation. *Evol. Dev.* 26, e12488
55. Casasa, S. and Moczek, A.P. (2018) Insulin signalling's role in mediating tissue-specific nutritional plasticity and robustness in the horn-polyphenic beetle *Onthophagus taurus*. *Proc. Biol. Sci.* 285, 20181631
56. Dreyer, A.P. and Shingleton, A.W. (2019) Insulin-insensitivity of male genitalia maintains reproductive success in *Drosophila*. *Biol. Lett.* 15, 20190057
57. Tang, H.Y. *et al.* (2011) FOXO regulates organ-specific phenotypic plasticity in *Drosophila*. *PLoS Genet.* 7, e1002373
58. Zeng, Z.B. *et al.* (2000) Genetic architecture of a morphological shape difference between two *Drosophila* species. *Genetics* 154, 299–310
59. Masly, J.P. *et al.* (2011) The genetic basis of rapidly evolving male genital morphology in *Drosophila*. *Genetics* 189, 357–374
60. Hackett, J.L. *et al.* (2016) Mapping QTL contributing to variation in posterior lobe morphology between strains of *Drosophila melanogaster*. *PLoS ONE* 11, e0162573
61. Takahara, B. and Takahashi, K.H. (2015) Genome-wide association study on male genital shape and size in *Drosophila melanogaster*. *PLoS ONE* 10, e0132846
62. Fujisawa, T. *et al.* (2019) Genetic basis of species-specific genitalia reveals role in species diversification. *Sci. Adv.* 5, eaav9939
63. Nomura, S. and Sota, T. (2022) Evolutionary changes in gene expression profiles associated with the coevolution of male and female genital parts among closely related ground beetle species. *BMC Genomics* 23, 637

64. Nomura, S. *et al.* (2021) Role of sex-concordant gene expression in the coevolution of exaggerated male and female genitalia in a beetle group. *Mol. Biol. Evol.* 38, 3593–3605
65. Genevcius, B.C. *et al.* (2022) Molecular and developmental signatures of genital size macro-evolution in bugs. *Mol. Biol. Evol.* 39, msac211
66. Stern, D.L. (2014) Identification of loci that cause phenotypic variation in diverse species with the reciprocal hemizygosity test. *Trends Genet.* 30, 547–554
67. Milan, M. *et al.* (2001) The LRR proteins capricious and tartan mediate cell interactions during DV boundary formation in the *Drosophila* wing. *Cell* 106, 785–794
68. Hagen, J.F.D. *et al.* (2021) Unraveling the genetic basis for the rapid diversification of male genitalia between *Drosophila* species. *Mol. Biol. Evol.* 38, 437–448
69. Nagy, O. *et al.* (2018) Correlated evolution of two copulatory organs via a single *cis*-regulatory nucleotide change. *Curr. Biol.* 28, 3450–3457
70. Ng, C.S. and Kopp, A. (2008) Sex combs are important for male mating success in *Drosophila melanogaster*. *Behav. Genet.* 38, 195–201
71. Rice, G. *et al.* (2024) Co-option of the trichome-forming network initiated the evolution of a morphological novelty in *Drosophila eugracilis*. *Curr. Biol.*, Published online October 22, 2024. https://doi.org/10.1016/j.cub.2024.09.073
72. Stern, D.L. and Frankel, N. (2013) The structure and evolution of *cis*-regulatory regions: the shavenbaby story. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 368, 20130028
73. Kittelmann, S. *et al.* (2021) A complex gene regulatory architecture underlies the development and evolution of cuticle morphology in *Drosophila*. *Curr. Opin. Genet. Dev.* 69, 21–27
74. Menoret, D. *et al.* (2013) Genome-wide analyses of shavenbaby target genes reveals distinct features of enhancer organization. *Genome Biol.* 14, R86
75. Kittelmann, S. *et al.* (2018) Gene regulatory network architecture in different developmental contexts influences the genetic basis of morphological evolution. *PLoS Genet.* 14, e1007375
76. Simmons, L.W. (2014) Sexual selection and genital evolution. *Austral Entomol.* 53, 1–17
77. Tanaka, K.M. *et al.* (2018) Mechanical incompatibility caused by modifications of multiple male genital structures using genomic introgression in *Drosophila*. *Evolution* 72, 2406–2418
78. Masly, J.P. and Azom, M.G. (2022) Molecular divergence with major morphological consequences: development and evolution of organ size and shape. *Essays Biochem.* 66, 707–716
79. Dufour, L. (1844) Anatomie générale des diptères. *Ann. Sci. Nat.* 1, 244–264
80. Hosken, D.J. *et al.* (2019) Penis evolution across species: divergence and diversity. *Nat. Rev. Urol.* 16, 98–106
81. Méndez, V. and Córdoba-Aguilar, A. (2004) Sexual selection and animal genitalia. *Trends Ecol. Evol.* 19, 224–225
82. Ah-King, M. *et al.* (2014) Genital evolution: why are females still understudied? *PLoS Biol.* 12, e1001851
83. Orbach, D.N. (2022) Gender bias in the study of genital evolution: females continue to receive less attention than males. *Integr. Comp. Biol.* 62, 533–541
84. Puniamoorthy, N. *et al.* (2010) Unlocking the 'black box': internal female genitalia in Sepsidae (Diptera) evolve fast and are species-specific. *BMC Evol. Biol.* 10, 275
85. Simmons, L.W. and Fitzpatrick, J.L. (2019) Female genitalia can evolve more rapidly and divergently than male genitalia. *Nat. Commun.* 10, 1312
86. Sloan, N.S. *et al.* (2024) Rapid divergent evolution of internal female genitalia and the coevolution of male genital morphology revealed by micro-computed tomography. *Proc. Biol. Sci.* 291, 20232883
87. Ballen-Guapacha, A.V. *et al.* (2023) Reproductive character displacement explains strengthening of mechanical barriers in damselflies. *bioRxiv*, Published online November 22, 2023. https://doi.org/10.1101/2023.10.26.564279
88. Nishimura, T. *et al.* (2022) Relationships between reproductive character displacement in genital morphology and the population-level cost of interspecific mating: implications for the Templeton effect. *Biol. J. Linn. Soc.* 138, 14–26
89. Genevcius, B.C. *et al.* (2020) Female-driven intersexual coevolution in beetle genitalia. *J. Evol. Biol.* 33, 957–965
90. Mayr, E. (1963) *Animal Species and Evolution*, Harvard University Press
91. Garlovsky, M.D. *et al.* (2022) Experimental sexual selection affects the evolution of physiological and life-history traits. *J. Evol. Biol.* 35, 742–751
92. Eberhard, W.G. and Lehmann, G.U.C. (2019) Demonstrating sexual selection by cryptic female choice on male genitalia: what is enough? *Evolution* 73, 2415–2435
93. Waage, J.K. (1979) Dual function of the damselfly penis: sperm removal and transfer. *Science* 203, 916–918
94. Leonard, J. and Cordoba-Aguilar, A. (2010) *The Evolution of Primary Sexual Characters in Animals*, Oxford University Press
95. Firman, R.C. *et al.* (2017) Postmating female control: 20 years of cryptic female choice. *Trends Ecol. Evol.* 32, 368–382
96. Eberhard, W.G. (2011) Experiments with genitalia: a commentary. *Trends Ecol. Evol.* 26, 17–21
97. Eberhard, W.G. (2009) Static allometry and animal genitalia. *Evolution* 63, 48–66
98. Genevcius, B.C. and Schwertner, C.F. (2014) Review and phylogeny of the *geniculata* group, genus *Chinavia* (Heteroptera: Pentatomidae), with notes on biogeography and morphological evolution. *Zootaxa* 3847, 33–56
99. Bianchi, F.M. *et al.* (2017) Heterospecific mating between distantly related species of stink bugs and its evolutionary implications. *J. Nat. Hist.* 51, 2701–2709
100. Boyle, E.A. *et al.* (2017) An expanded view of complex traits: from polygenic to omnigenic. *Cell* 169, 1177–1186