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Evolution of sexual development and sexual dimorphism in insects

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Abstract

Most animal species consist of two distinct sexes. At the morphological, physiological, and behavioural levels the differences between males and females are numerous and dramatic, yet at the genomic level they are often slight or absent. This disconnect is overcome because simple genetic differences or environmental signals are able to direct the sex-specific expression of a shared genome. A canonical picture of how this process works in insects emerged from decades of work on *Drosophila*. But recent years have seen an explosion of molecular-genetic and developmental work on a broad range of insects. Drawing these studies together, we describe the evolution of sexual dimorphism from a comparative perspective and argue that insect sex determination and differentiation systems are composites of rapidly evolving and highly conserved elements.

Introduction

Anisogamy is the definitive sex difference. The bimodality in gamete size it describes represents the starting point of a cascade of evolutionary pressures that have generated remarkable divergence in the morphology, physiology, and behaviour of the sexes [1]. But sexual dimorphism presents a paradox: how can a genome largely shared between the sexes give rise to such different forms? A powerful resolution is via sex-specific expression of shared genes. In the latter part of the 20th century, experiments in the fruit fly *Drosophila melanogaster* helped construct a canonical picture of the mechanisms through which this is achieved in insects. In this review, we discuss how recent discoveries at each stage of sex determination and differentiation both challenge and expand upon that canon.

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Conflict of Interest

No conflict of interest exists.

Intellectual Property

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

The canonical view of insect sex determination and differentiation

In the canonical *Drosophila* sexual differentiation pathway [reviewed by 2,3], sex is largely defined at the level of the individual cell. Cell autonomy hinges on the ability of two autosomal transcription factors to produce sex-specific isoforms. Key among these factors is *doublesex* (*dsx*), which functions in a wide range of somatic tissues; the other, *fruitless* (*fru*), is mainly involved in sex-specific differentiation of the nervous system. The male and female isoforms of Dsx share a common DNA-binding domain but possess sex-specific C-termini. Thus, the two isoforms can have sex-biased [e.g. 4] or even opposite [e.g. 5] effects on the expression of their target genes.

In the canonical pathway, male isoforms of *dsx* and *fru* are produced by default, with female-specific isoforms requiring the splicing factor *transformer* (*tra*) and its partner *transformer-2* (*tra-2*). Although *tra-2* is active in the soma of both sexes, functional Tra protein is only produced in females. Female-specific splicing of *tra* is activated by *Sex lethal (Sxl*), a sex-determining master switch that also controls dosage compensation via its regulation of *male-specific lethal 2* (*msl-2*). *Sxl* expression is activated by the dosage of several X-linked regulatory proteins, which in turn depends on the number of X-chromosomes [6]. Consequently, while *D. melanogaster* has X and Y chromosomes, it is not the presence of Y that specifies maleness, but rather the number of X's – one in males, and two in females (Fig. 1).

Challenging the canon: rapid evolution of primary sex signals

Sex determination systems diversify rapidly among species [7]. Insects are no exception. Haplodiploid honeybees use zygosity at the sex-determining locus, booklice paternal genome elimination, and butterflies ZW chromosome systems with females as the heterogametic sex [8,9]. The speed and relative freedom with which sex determining signals evolve has been best studied in Diptera, where species are known to have gained and lost heteromorphic sex chromosomes, replaced original sex chromosomes with new ones, incorporated other chromosomal elements into the original sex chromosome, or transitioned from male to female heterogamety [10–13]. But it is not the sex chromosomes themselves that define sex, but rather the sex determining signals they encode. Indeed, evolution of new sex determining signals may initiate changes in sex chromosome structure as well as switches from old to new sex chromosomes.

Primary sex-determining signals have evolved many times independently and act via different mechanisms. For example, *Drosophila*'s system of measuring X-chromosome dosage via *Sxl* appears to be restricted to the *Drosophilinae* [14,15]. A phylogenetically diverse array of Dipterans instead use dominant male-determining genes ('M-factors'), as in the case of the mosquitos *Anopheles gambiae* (*Yob*) and *Aedes aegypti* (*Nix*), the Medfly *Ceratitis capitata* (*MoY*), and the housefly *Musca domestica* (*Mdmd*) (Fig. 1). These M-factors are all unrelated to each other, reflecting their independent evolution [16–20]. Other non-homologous M-factors no doubt exist in other fly groups [13]. Where closely related species share a homologous M-factor, its sequence can diverge rapidly (e.g. *Aedes Nix*)[21]. In *M. domestica*, individuals can even vary in which chromosome encodes the M-factor –

Mdmd has been detected on four of the six chromosomes (Y, II, III, and V) in different populations [16,22]. In most cases the origin of M-factors is unknown. An exception is *Mdmd*, which arose through the duplication and subsequent neofunctionalization of *CWC22* (*nucampholin*), a spliceosomal factor gene [16]. *Aedes Nix* also encodes a potential splicing factor, suggesting this may be a common starting point for M-factors [18].

A pattern similar to the diversity of unrelated M-factors in Diptera may be found in Hymenoptera. Although all hymenopterans are haplodiploid, the ploidy signal is mediated by different genes and via different mechanisms. In honeybees, sex is determined zygotically by the *csd* locus, a paralog of *tra* [23]. But in the wasp *Nasonia vitripennis*, sex depends on the maternal imprinting of an unrelated gene, *wom* [24]. *wom* is a recently evolved chimeric gene, not found even in all species of the same family (Pteromalidae), suggesting that the proximate mechanisms of haplodiploid sex determination may be as varied as in the case of XY heterogametic systems. Rapid diversification of sex-determining signals may be due to intragenomic sexual conflict, sex ratio distorters, changing links between environmental conditions and sex-specific fitness, and other evolutionary factors [7]. The extent to which the rate of their diversification varies across taxa, and the reasons behind this variation, remain key questions for future work.

Challenging the canon: translating primary sex signals into the sex-specific splicing of *dsx*

Downstream, the story is different. Diverse sex determination inputs, from X chromosome dosage to M-factors to haplodiploidy, converge on the *tra-dsx* splicing cascade, which is present in early-branching insect clades like cockroaches and certainly ancestral to the Holometabola [25]. But even this deeply conserved mechanism is not universal. The entire order Lepidoptera have lost the *tra* gene, but maintain sex-specific *dsx* activity [26]. How, then, is the sex-specific splicing of *dsx* achieved? Studies of the silkworm *Bombyx mori* provide an answer. In this species, females are the heterogametic sex, bearing both Z and W chromosomes; males have two Zs. The Z-chromosome carries the *Masculinizer* (*Masc*) gene, which encodes a CCCH-tandem zinc finger protein that regulates maleness via its control of the sex-specific splicing of *dsx* [27,28]. The homologues of *Masc* in *Trilocha varians* and *Plutella xylostella* are similarly required for sex-specific splicing of *dsx*, suggesting deep conservation of this mechanism within Lepidoptera [29,30].

Masc functions by regulating the male-specific transcription of RNA-binding protein 3 (RBP3/Aret), which binds to one of the two *dsx* exons that are skipped in males and directly interacts with RBP1/Lark, which binds to the other [31]. The W chromosome encodes a dominant feminizing factor, a PIWI-interacting RNA (piRNA) produced from the *Feminizer* precursor [27]. *Fem* piRNA guides the assembly of a protein complex that suppresses *Masc* expression to promote the female-specific splicing of *dsx* [32]. piRNAs are thought to principally function in protecting the germline from transposons, which makes this derived role in Lepidopteran sex determination surprising. But while the participation of piRNAs appears novel, gene regulation by small RNAs during sex determination is not. Indeed, miR-1–3p appears to perform a role in the oriental fruit fly *Bactrocera dorsalis* that is

opposite to that of *Fem* in silkworms [33]. miR-1–3p, which is transcribed at high levels in males, transduces an uncharacterized Y-linked M-factor signal to promote the canonical male-specific splicing of *tra*, which in turn converges on the conserved sex-specific splicing of *dsx*. The mechanistic simplicity and efficiency with which small RNAs can regulate the expression of their target genes may make them readily evolvable, and therefore common, intermediaries between rapidly evolving primary sex determination signals and regulators of *dsx* splicing.

tra has also not been detected in the genomes of a small number of non-Lepidopteran insect species, including *Aedes, Anopheles*, and other mosquitos [26]. If these species have lost *tra*, it remains to be seen how *Nix, Yob*, and other such M-factors control *dsx* splicing in its absence (Fig. 1).

Challenging the canon: not all insects rely on sex-specific *dsx* isoforms for sexual differentiation

dsx is an arthropod-specific paralog from the wider *doublesex/mab-3 related* (*Dmrt*) family of transcription factors [34]. Members of this ancient gene family appear to be the only conserved element of sexual differentiation pathways across Metazoa [35,36]. Despite this conservation, using sex-specific isoforms of a *Dmrt* gene to direct both male and female development is an insect innovation; vertebrates, nematodes, mites, and crustaceans instead use male-specific transcription of *Dmrt* genes to direct elements of male-specific development [36–40]. How did this transition from sex-specific transcription to the canonical sex-specific splicing of *dsx* occur?

Recent work suggests two key processes were at play [25]. Firstly, the expansion of *dsx* function from a “male gene” that overrides a default female pathway to a bifunctional switch actively required in both sexes [25,40]. Male and female *dsx* isoforms are present as far back in the insect phylogeny as cockroaches, but outside of the Holometabola the female isoforms appear dispensable for female differentiation [25,39,41]. Why female isoforms first evolved and how they later came to play critical functions in female sexual differentiation remains unknown. Secondly, while *dsx* function expanded, *tra* function narrowed. As in the canonical *Drosophila* pathway, basal insects such as cockroaches require *tra* for both female-specific differentiation and the sex-specific splicing of *dsx*. But they use *tra* differently. In these basal groups, *tra’s* role in female development is independent of *dsx* and does not involve the production of sex-specific *tra* isoforms [25]. Thus, *tra* appears to have transitioned from controlling female development via at least partly *dsx*-independent mechanisms to being a dedicated regulator of *dsx*. The selective forces behind these transitions, as well as any consequences that non-canonical variants of the *tra-dsx* cascade have for the manifestation of sexual dimorphism, remain significant outstanding questions.

Expanding the canon: changes in the expression and targets of *dsx* underlie the origin and diversification of sex-specific traits

Two processes are required for the evolution of sexually dimorphic traits in insects, and *dsx* is central to both (Fig. 2). One is the establishment of sex-specific identity in a previously monomorphic tissue. This process is facilitated by the cell-autonomous nature of *dsx* function: *dsx* transcription gives cells the capacity for sex-specific differentiation – but not all cells transcribe *dsx* [42–46]. From this sexual mosaicism emerges a prediction about the origin of new sexually dimorphic traits: by changing which cells express *dsx*, tissues can acquire (or lose) sex-specific functions. There is good evidence in support of this: the evolution of novel male-specific grasping structures in *Drosophila* legs, and the male-specific scent organs in *Bicyclus* butterflies, are both associated with the evolution of new spatial domains of *dsx* expression [43,47,48]. Localized upregulation of *dsx* also precedes the appearance of visible dimorphism in developing *Trypoxylus dichotomus* beetle horns, suggesting that the establishment of sexual identity by *dsx* early in the development of novel traits is critical to their dimorphic nature [45]. The evolutionary malleability in the spatiotemporal control of *dsx* expression that these studies demonstrate is afforded by modular enhancers. In *Drosophila*, several distinct enhancers have been identified that are collectively required for sex-specific development of leg sensory organs [49].

Controlling the pattern of *dsx* expression in time and space lays the foundations for sexual dimorphism, but not the endpoint. The second process therefore is the establishment of a repertoire of *dsx* target genes. Work on the development of dung beetle (*Onthophagus*) horns suggests that this repertoire can expand and shift rapidly [40]. Moreover, it needn't be the target genes that change, it can also be the direction of the regulatory effect conferred by *dsx*. A rare sex-reversal in the dimorphism of *O. sagittarius* horns appears to be driven by the two *dsx* isoforms swapping regulatory roles relative to the ancestral state: male *dsx* evolving from stimulating horn growth to repressing it, and female *dsx* evolving the reverse [50].

Genes can be added to or lost from the repertoire of *dsx* targets by the gain (or loss) of Dsx binding sites in their enhancers, or by structural changes in Dsx protein domains [51]. For example, transitions from sexual monomorphism to dimorphism (and vice versa) in the pheromone profile of *Drosophilid* flies have been partly driven by gain (and loss) of a Dsx binding site in the enhancer of the hydrocarbon-processing enzyme *desat-F* [4]. Because Dsx targets may be co-regulated by other transcription factors, multiple cues alongside sex, such as position and developmental stage, may be integrated. In the case of male-specific abdominal pigmentation in *D. melanogaster*, sexual dimorphism evolved via the gain of a Dsx binding site in the enhancer of *bric á brac* (*bab*), a gene that is also regulated by the position-specifying HOX gene *Abd-b* [5,52]. Combinatorial changes in the spacing, polarity, and number of transcription factor binding sites within *bab* enhancers are associated with inter- and intra-specific changes in the position and extent of sex-specific pigmentation across *Drosophila* species [5,53].

Changes in the targets and regulatory effects of Dsx are likely to represent a major channel through which sexually dimorphic traits diversify. The cell-autonomous nature of Dsx action, combined with the co-regulation of its downstream targets by cell-type

and developmental stage specific transcription factors, affords a high level of modularity to the development of a given sex-specific trait. Such modularity may provide a high level of evolutionary lability, allowing different aspects of sexual dimorphism to evolve independently and, crucially, without disrupting conserved sexual differentiation programs in other tissues [53,54].

Expanding the canon: *dsx*, a master regulator of sex-limited intraspecific polymorphisms

Due to the modular control of its expression, a broad and evolving set of target genes, and the ability to switch roles between activator and suppressor, *dsx* can control wide-ranging morphological change within as well as between species. Some swallowtail butterflies (*Papilio*) have multiple discrete female morphs, some of which mimic the warning coloration of toxic model species, while the males are monomorphic. The differences between female morphs of *P. polytes* are controlled by different *dsx* alleles, which act as a switch between a default, male-like colour pattern and different mimetic morphs [55,56]. In *P. polytes*, the *dsx-H* allele controls wing coloration by activating "mimetic" genes that include *Wnt1* and *Wnt6*, and repressing "non-mimetic" genes such as *abd-a* [57]. *dsx* mimicry alleles segregate within multiple *Papilio* species and show species-specific patterns of genetic differentiation [58–61]. This differentiation has been interpreted as pointing to independent evolutionary origins of *dsx* alleles in the genus *Papilio* [58,59]. However, the recent identification of *dsx* polymorphisms that are shared across *Papilio* species instead points to shared inheritance from a common polymorphic ancestor, rather than recurrent, convergent evolution at the same locus [60]. In contrast to incomplete lineage sorting, where ancestral polymorphisms are maintained across lineages, the pattern of genetic divergence observed among *Papilio dsx* alleles is best described by allelic turnover, where alleles from a polymorphic ancestor are subsequently replaced by their own allelic descendants [60]. Resolving the evolutionary history of these alleles is key to understanding the repeatability of *dsx*-dependent female-limited polymorphism. Indeed, evolutionary change in *dsx* is not the only route to female-limited mimicry polymorphism, as evidenced by the African mocker swallowtail *(Papilio dardanus*), where mimetic phenotypes are controlled by a polyalleic locus that contains the transcription factor genes *engrailed* and *invected* [62,63], and *Hypolimnas misippus* (Nymphalidae), where a novel, though unidentified, color patterning locus has been detected [64].

Challenging the canon: sexual differentiation affected by hormone signaling

Insects define sexual identity at the level of the individual cell, through cell-autonomous control of transcription and splicing. However, non-cell-autonomous, systemic hormonal inputs [reviewed by 65] are increasingly recognized as critical to the development and maintenance of some dimorphic traits [66,67]. For example, ecdysteroids and their receptors have been implicated in a variety of sex-specific processes in *Drosophila*, including ejaculate production, female post-mating gut growth, and courtship [66,68,69]. Available data currently support two mechanisms through which hormones can affect sexually dimorphic

trait development (Fig. 3). Firstly, through sex differences in hormone titer (Fig. 3a). At present, the only conclusive demonstration of this mechanism comes from sex-specific seasonal wing patterns in the butterfly *Bicyclus anynana* [70]. Early in development, dry season morphs of both sexes express the Ecydsone Receptor (EcR) in a similar number of dorsal eyespot cells. Later, the titer of the hormone 20-hydroxyecdysone diverges between the sexes, inducing a corresponding divergence in the rate of division of eyespot cells that ultimately generates sex differences in eyespot size.

The second mechanism is through changes in the sensitivity of a developing tissue to a fixed hormone titer (Fig. 3b). Sex- and trait-specific sensitivity to insulin/IGF, juvenile hormone, and ecdysone signalling pathways is variously thought to underlie dimorphic horn and mandible growth in a number of beetle species [71–75]. Work in the stag beetle (*Cyclommatus metallifer*) has shown that sex-specific isoforms of *dsx* differentially regulate the sensitivity of mandible cells to juvenile hormone, promoting exaggerated growth in males and repressing it in females [74]. This illustrates the interplay between cell-autonomous and hormonal inputs into the development of sexually dimorphic traits. Rather than serving as alternative ways of generating sexual dimorphism, systemic hormones may act by co-regulating the target genes of *dsx* and *tra*. In other cases, the hormone titers themselves may be controlled via *dsx*- and *tra*-dependent mechanisms in hormone-secreting cells.

Conclusion

A canonical view of sex determination and differentiation in insects emerged from work on *D. melanogaster*. But as we broaden our taxonomic sampling, this canon is repeatedly challenged and expanded. The evolutionary history of insect sexual development increasingly appears to conform to the developmental hourglass model: while sex-determining signals and downstream target genes diverge rapidly, *doublesex* acts as a conserved linchpin, defining and expanding sex-specific identity into new tissues to dramatic and beautiful effect.

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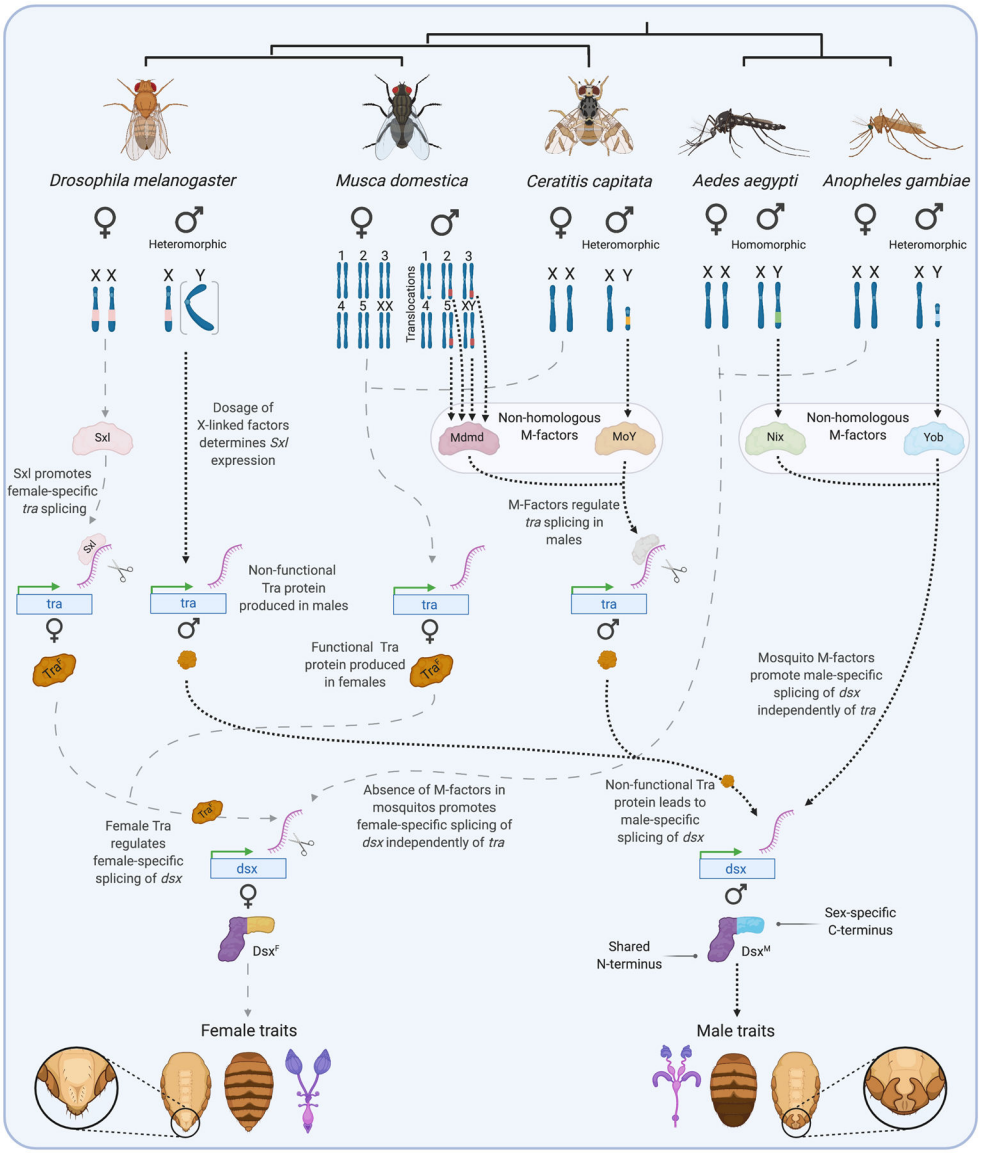

Figure 1. Divergent primary sex determination signals in Diptera converge on sex-specific *doublesex* splicing.

In the 5 Dipterans shown, sex is specified at the level of the individual cell by factors associated with sex (or proto-sex) chromosomes. These male- and female-defining chromosomes vary between species from being highly similar to each other (homomorphic) to highly divergent (heteromorphic) in morphology and gene content. In *D. melanogaster*, the number of X chromosomes determines the dosage of a set of X-linked factors that regulate the expression state of *Sex lethal* (*Sxl*). High dosage (XX) activates *Sxl* expression, the protein product of which promotes female-specific splicing of *transformer* (*tra*). The resulting female-specific isoform of Transformer protein (Tra^F) is required for the female-specific splicing of the transcription factor *doublesex* (*dsx*). Maleness is defined by the lower dosage of X-linked factors, rather than the presence of a Y-chromosome (e.g., X0 individuals are males). Having a single X chromosome leaves *Sxl* inactive in males, and the male-specific isoform of Transformer is produced (Tra^M). The presence of a

premature stop codon renders Tra^M non-functional, which in turn leads to the production of the male-specific isoform of *dsx*. *Musca domestica, Ceratitis capitata, Aedes aegypti*, and *Anopheles gambiae* each use independently evolved (non-homologous) dominant M-factors to determine maleness. These are encoded on the Y-chromosome in most cases, but translocations to autosomes (turning them into proto-sex chromosomes) have been detected in different *M. domestica* populations. Whether the M-factor found on chromosome 1 in one population of *M. domestica* (shown in white) is a derived *Mdmd* sequence or an independently evolved M-factor remains unclear. In *M. domestica* and *C. capitata*, the presence of M-factors leads to the production of non-functional Tra^M and therefore, as in *D. melanogaster*, the production of the male-specific isoform of Dsx. No *tra* homolog has been found in *Ae. aegypti* or *An. gambiae*. Their M-factors, *Nix* and *Yob* respectively, are therefore presumed to determine the male-specific splicing of *dsx* by an as of yet unknown, *tra*-independent mechanism. The male and female isoforms of Dsx share a DNA-binding N-terminus but bear different C-termini, allowing them to regulate downstream target genes in a sex-specific manner, leading to the development of sex-specific traits. Figure created using BioRender.

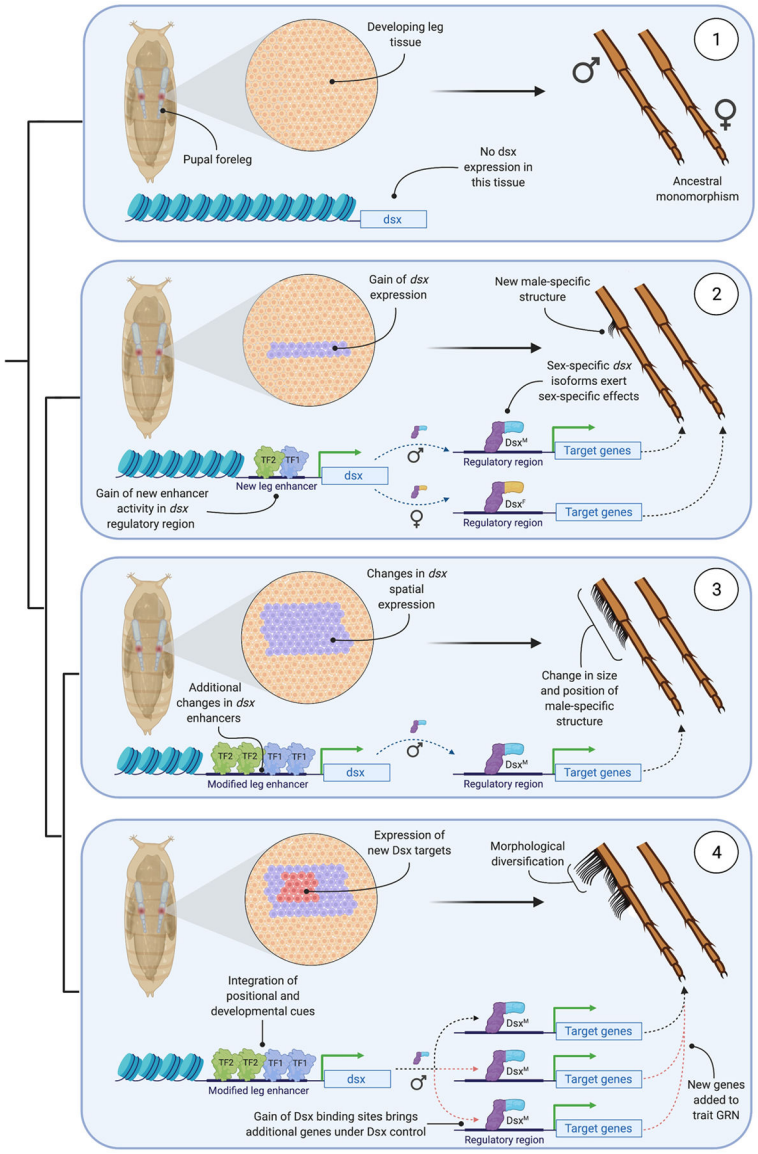

Figure 2. The origin and diversification of a new sex-specific trait.

This schematic describes a four-part model for the origin and subsequent morphological diversification of a sex-specific structure, in this case a modified row of bristles (a 'sex comb') on the male *Drosophila* foreleg. Species 1 displays the ancestral state of monomorphism. Here, developing leg cells do not express the transcription factor *doublesex* (*dsx*) and therefore lack the capacity for sex-specific differentiation. In species 2, changes in the sequence of the regulatory region controlling *dsx* expression enable the binding of position- and stage-determining transcription factors (TF). These TFs activate *dsx* expression in a subset of leg cells during a particular developmental window. *dsx* is alternatively spliced to give rise to male- and female-specific isoforms (Dsx^M and Dsx^F), which bind to the regulatory regions of target genes via a shared DNA-binding domain and impart sex-specific effects on target gene expression through sex-specific C-termini. The localized, sex-specific regulation of gene expression that results enables the development

of a novel structure only in males. In species 3, additional changes in the *dsx* enhancers generate changes in the binding of its upstream regulators. This leads to changes in the spatiotemporal pattern of *dsx* expression among developing leg cells, which in turn produces changes in the size and position of the male-specific structure. In species 4, Dsx has acquired new downstream target genes due to the gain of Dsx-binding sites in the regulatory regions of the new targets. Incorporation of the new targets into the gene regulatory network ('GRN') that controls the development of the male-specific structure leads to further morphological diversification. Figure created using BioRender.

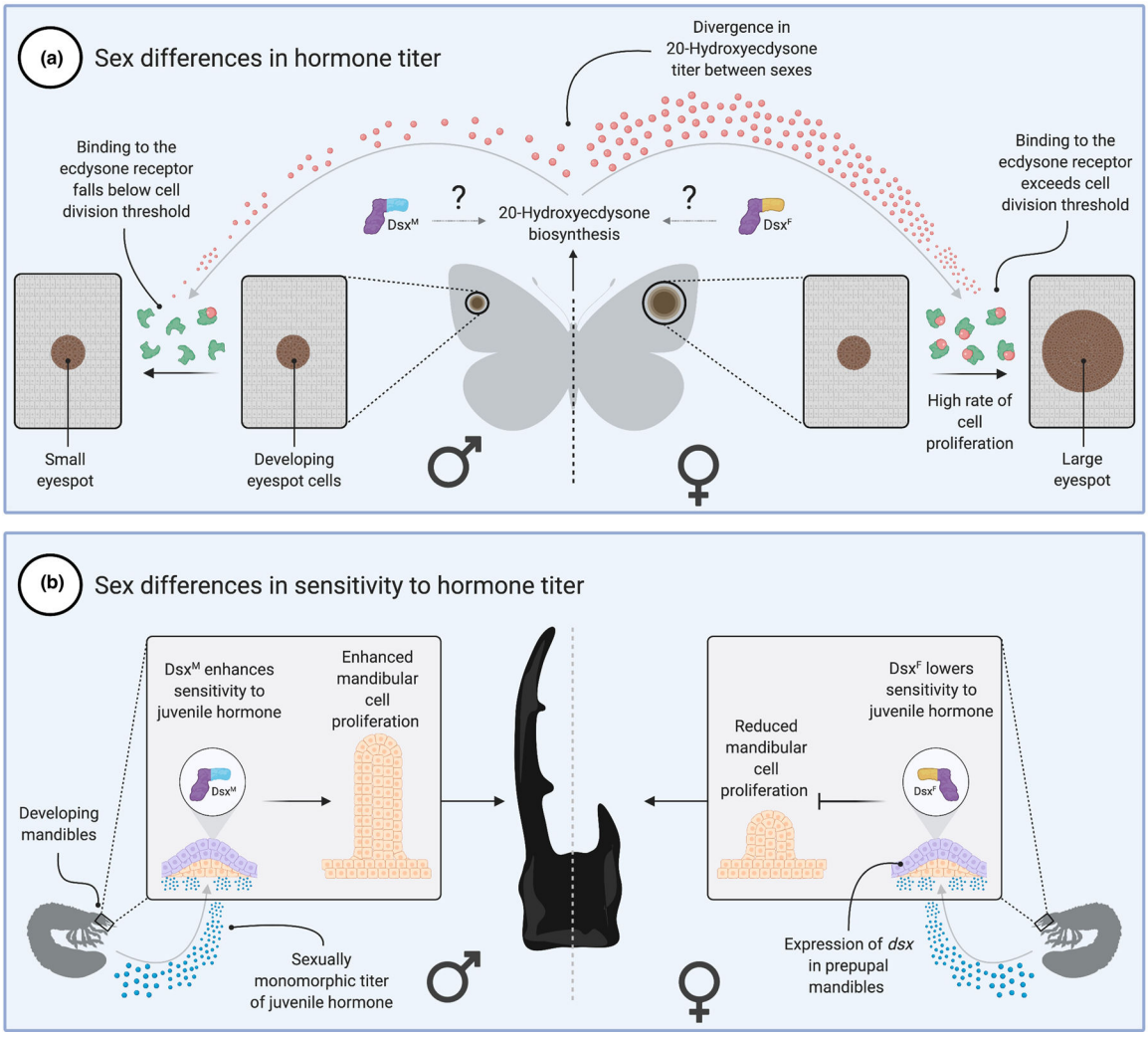

Figure 3. Hormonal inputs into insect sexual dimorphism.

Two principal mechanisms exist through which hormones can deliver sex-specific effects in insects. (A) Sex differences in hormone titer. Developing eyespot cells in the butterfly *Bicyclus anynana* express ecdysone receptor. The titer of circulating 20-hydroxyecdysone in females leads to a binding threshold being exceeded, which causes the cells to proliferate and the eyespot to grow. The lower titer in males fails to exceed the binding threshold and the cells fail to proliferate. What generates the divergence in hormone titer is unclear, but one potential mechanism is the direct or indirect regulation of enzymes in the ecdysone biosynthesis pathway by Dsx^M and/or Dsx^F. (B) Sex differences in sensitivity to hormones. Expression of *dsx* in the developing prepupal mandibles of the stag beetle *Cyclommatus metallifer* changes the sensitivity of mandibular cell proliferation to juvenile hormone. Dsx^M increases sensitivity, leading to enlarged mandibles in males. Dsx^F reduces sensitivity, leading to small mandibles in females. Figure created using BioRender.