Evolution of sexual development and sexual dimorphism in insects

Ben R. Hopkins[1*] & Artyom Kopp[1]

1. Department of Evolution and Ecology, University of California – Davis, Davis, USA

* Corresponding author

Corresponding author email: brhopkins@ucdavis.edu

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.

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. Why sex-determining signals diversify so rapidly and the extent to which the rate of their diversification varies across taxa 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 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 [36–39]. 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. 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 [25,39,40]. 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* [41–45]. 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 [42,46,47]. 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 [44]. 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 [48].

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 [49]. 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. Male-specific abdominal pigmentation in *D. melanogaster* 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 level of modularity in the development of a single trait that *dsx's* mode of action provides may provide a high level of evolutionary lability, allowing sub-elements to evolve independently and, crucially, without disrupting conserved sexual differentiation programs [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, recent analysis of a broader set of species has revealed the presence of multiple, trans-species *dsx* polymorphisms, suggesting that the divergence in *dsx* alleles instead reflects allelic turnover, where alleles from a polymorphic ancestor are subsequently replaced by their own allelic descendants [60]. Resolving which force is at play 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 *invective* [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 are increasingly recognized as critical to the development and maintenance of some dimorphic traits [65,66]. 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 [65,67,68]. 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* [69]. 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 [70–74]. 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 [73]. 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 coregulating 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*. As we broaden our taxonomic sampling, 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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Sharma *et al.* identify the dominant male-determining gene of the house fly *Musca domestica* (*Mdmd*). This recently-evolved gene acts upstream of the widely conserved *transformer/doublesex* splicing cascade, demonstrating that new sex-determining signals can co-opt deeply conserved sexual differentiation pathways. *Mdmd* evolved by duplication and neofunctionalization of a generic splicing factor, suggesting that the *tra*/*dsx* pathway may predispose insects to evolving sex-determining mechanisms based on alternative splicing. *Mdmd* can translocate from the Y chromosome to different autosomes, potentially facilitating the evolution of new sex chromosomes.

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Meccariello *et al.* identify the dominant male-determining gene of the Medfly *Ceratitis capitata* (*MoY*). Importantly, this gene is unrelated to the male-determining gene *Mdmd* previously identified in *Musca domestica* by Sharma *et al.* (2017), and appears to be limited to the Tephritidae family. However, like *Mdmd*, *MoY* acts by regulating the sex-specific splicing of *tra* – although the exact molecular function of MoY remains to be determined.

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Zou *et al.* identify the gene (*wom*) that mediates haplodiploid sex determination in the parasitoid wasp *Nasonia vitripennis*. *wom* is unrelated to the *csd* locus that was shown to mediate haplodiploid sex in honeybees (Beye *et al.* 2003); moreover, *csd* acts zygotically, whereas *wom* acts via sex-specific parental imprinting. This contrast suggests that the molecular mechanisms of haplodiploid sex determination in Hymenoptera may turn out to be as diverse as the mechanisms of male-heterogametic (XY) sex in Diptera.

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This paper attempts to reconstruct the evolutionary history of the canonical insect sexual differentiation pathway. In the ancestral condition, *dsx* is expressed only in males and promotes male-specific traits; in the derived condition found in the Holometabola, *dsx* actively promotes both male and female differentiation via alternatively spliced isoforms. Wexler *et al.* show that hemimetabolous insects orders represent different stages in the transition from the transcription-based to the splicing-based mode of sexual development. They suggest that the canonical *tra*/*dsx* pathway evolved via merger between expanding *dsx* function (from males to both sexes) and narrowing *tra* function (from a broad-range splicing factor to a dedicated regulator of *dsx*).

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This is a foundational work that showed that in the silkworm *Bombyx mori* (which, like other Lepidoptera, has female-heterogametic, ZW mode of sex determination), female sex is specified by the dominant *Fem* locus located on the W chromosome. However, *Fem* is not a conventional protein-coding gene; rather, it encodes a Piwi-interacting RNA (piRNA). The *Fem* piRNA silences the *Masc* gene, which is located on the Z chromosome and promotes male development via male-specific *dsx* splicing; thus, *Fem* activity leads to female-specific *dsx* splicing and female development. This highly unusual mechanism shows that a wide variety of sex determination systems can all act by regulating sex-specific *dsx* splicing.

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Although sexually dimorphic development in Lepidoptera, as in other holometabolous insects, depends on sex-specific *dsx* splicing, all examined Lepidoptera lack *tra*, the key regulator of *dsx* splicing in other Holometabola. Zheng *et al.* identify a group of RNA-binding proteins that promote male-specific *dsx* splicing in the silkworm *Bombyx mori*. One of these proteins itself has sex-specific isoforms produced under the control of the *Masc* gene, which promotes male development in *Bombyx*. This work suggests that Lepidopteran sexual differentiation is still based on a cascade of alternative splicing, but a new set of regulators has taken over this function from the ancestral *transformer*.

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Morita *et al.* show that localized expression of *dsx* in the developing head horns of rhinoceros beetles precedes the onset of sexually dimorphic horn formation. Moreover, they find that the regulatory role played by *dsx* in modelling the developing horn, such as driving tissue growth, death, or movement, depends on both the spatial and temporal context. Alongside work on *Drosophila,* this study further suggests that region-specific expression of *dsx* may be an essential and general precursor to the development of sexually dimorphic traits in insects.

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the origin and diversification of male sexual ornaments in the Drosophila immigrans species group. *Evol Dev* 2018, **20**:78–88.

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Prakash and Monteiro show that the spatial regulation of *dsx* underlies the development of male-specific scent organs in *Bicyclus* butterflies. Moreover, they show that *dsx* controls the sexually dimorphic development of different sub-elements of these organs via different modes: some structures require the male-specific *dsx* isoform, while others develop by default but are repressed by the female *dsx* isoform. The lability in *dsx* expression and function provides a mechanism for the striking divergence that a single sexually dimorphic trait can show between species.

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Rice *et al.* identify *cis*-regulatory sequences that control the precise spatio-temporal expression of *dsx* that underlies the development of the sex comb, a male-specific leg ornament displayed by some *Drosophila* species. They also show that a different enhancer controls the development of sex-specific chemosensory organs, suggesting that modular control of *dsx* transcription allows insects to develop as mosaics of sexually dimorphic and monomorphic structures. Evolutionary changes in *dsx* enhancers can expand its expression into new tissues, thereby conferring the sexual identity upon which sexually dimorphic development is based.

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butterflies is regulated by a doublesex-orchestrated gene network. *Commun Biol* 2019, **2**:257.

Female-limited Batesian mimicry in the swallowtail butterfly *Papilio polytes* is controlled by a tightly linked cluster of genes (the H locus) that includes *dsx*. Iijima *et al.* show that the mimetic (H) and non-mimetic (h) alleles at this locus regulate several transcription factors and signaling molecules in opposite directions in the developing wing, resulting in a binary switch between mimetic and non-mimetic color patterns. The mechanism that limits mimicry to females is not entirely clear, but could depend on lower expression of *dsx* in males (and in *hh* females) compared to *HH* and *Hh* females.

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Female-limited Batesian mimicry is present in many species of the genus *Papilio* (swallowtail butterflies), and alternative *dsx* alleles are associated with mimetic and non-mimetic wing color patterns in several of those species. Does this reflect an ancestral polymorphism, or independent origin of mimetic *dsx* alleles in different

species? The work of Palmer and Kronforst suggests that the answer may be "a bit of both": *dsx*-dependent mimicry was likely present in the last common ancestor of four distantly related *Papilio* species, but the shared ancestral alleles have been largely replaced by their own allelic descendants.

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In an important counterpoint to other studies of *Papilio* swallowtail butterflies, this report shows that female-limited mimicry is not always controlled by *dsx*. In the African mocker swallowtail *P. dardanus*, this trait is controlled instead by a chromosomal region that includes the paralogous transcription factors *engrailed* and *invected*. What limits the action of this "supergene" to females remains to be determined.

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Bhardwaj *et al.* provide the first conclusive evidence that sexual divergence in the titer of a hormone can drive sexual dimorphism in an insect trait. They show that females of a seasonal morph of the butterfly *Bicyclus anynana* have higher levels of circulating 20-hydroxyecdysone during larval development compared to males. This higher level leads to increased proliferation in the cells that give rise to the wing eyespot, resulting in larger eyespots in females.

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*72. Lavine MD, Gotoh H, Hayes A, Corley Lavine L: **The Insulin Signaling Substrate Chico and the Ecdysone Response Element Broad Both Regulate Growth of the Head Horns in the Asian Rhinoceros Beetle, Trypoxylus dichotomus**. *Integr Comp Biol* 2019, **59**:1338–1345.

Lavine *et al.* provide evidence that the insulin signalling pathway – a pathway with known sensitivity to nutritional inputs – is a key mediator of the sexually dimorphic and condition-dependent growth of rhinoceros beetle horns. They also show for the first time that horn growth in this species is mediated by the ecdysone signalling pathway. These discoveries pave the way for elucidating the crosstalk between hormonal pathways and cell-autonomous regulatory mechanisms that collectively mediate the development of sexually dimorphic traits.

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Gotoh *et al.* show that the expression of *dsx* has sex-specific and opposing effects on mandible growth in the stag beetle *Cyclommatus metallifer*: male *dsx* isoforms stimulate exaggerated mandible growth, while female *dsx* isoforms suppress it. Loss of *dsx* expression leads to intermediate mandible growth. The authors further show that *dsx* appears to achieve these effects by modulating the sensitivity of developing mandible cells to juvenile hormone. This work illustrates the interplay between cell-autonomous and hormonal inputs into the development of sexually dimorphic traits.

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Figure legends

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 ($\mathrm{Tra}^{\mathrm{F}}$) 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 ($\mathrm{Tra}^{\mathrm{M}}$). The presence of a premature stop codon renders $\mathrm{Tra}^{\mathrm{M}}$ 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 a new downstream target gene due to sequence changes in that gene's regulatory region. Incorporation of this new target into the gene regulatory network that controls the development of the male-specific structure leads to the 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 eye spot 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.

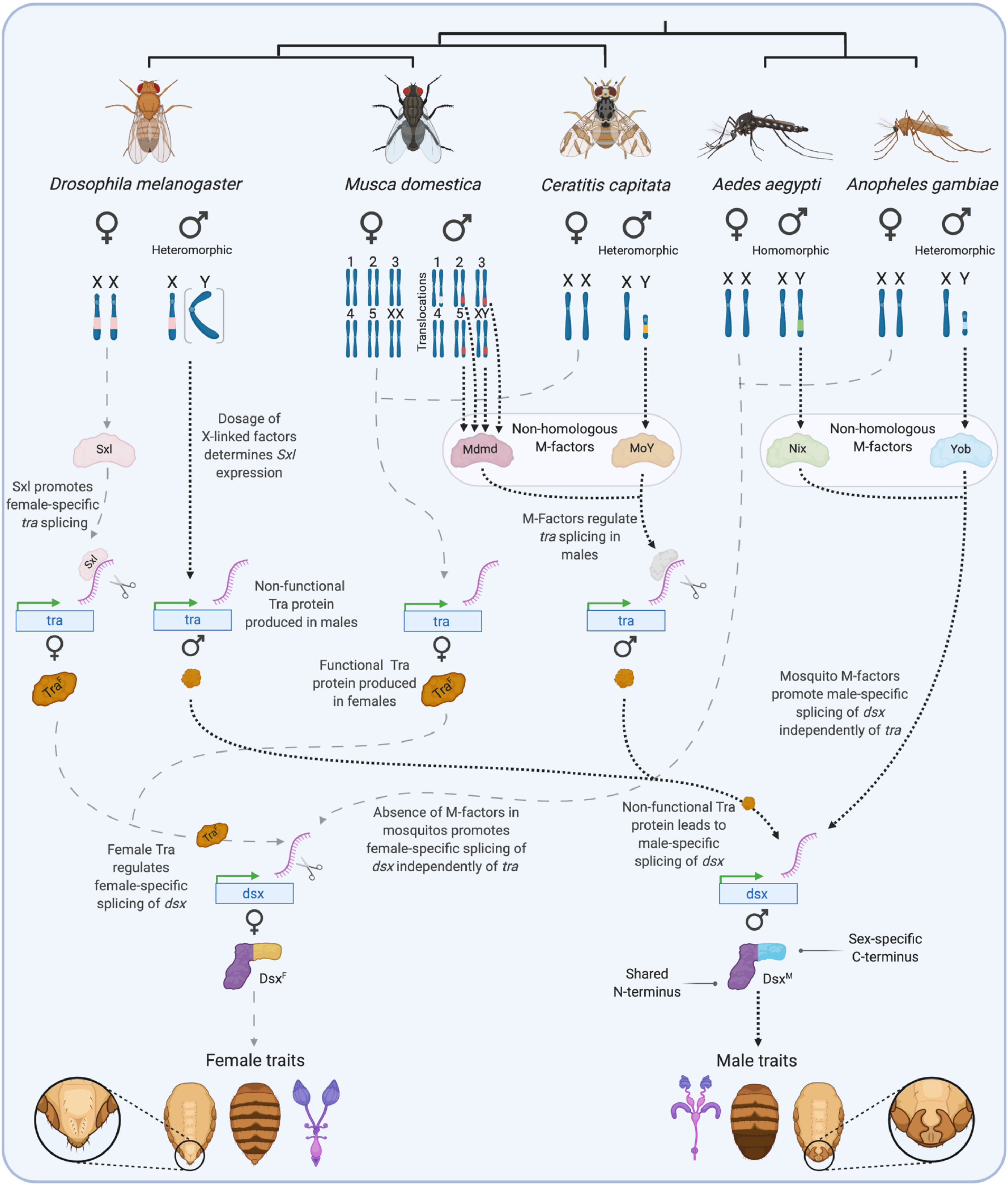
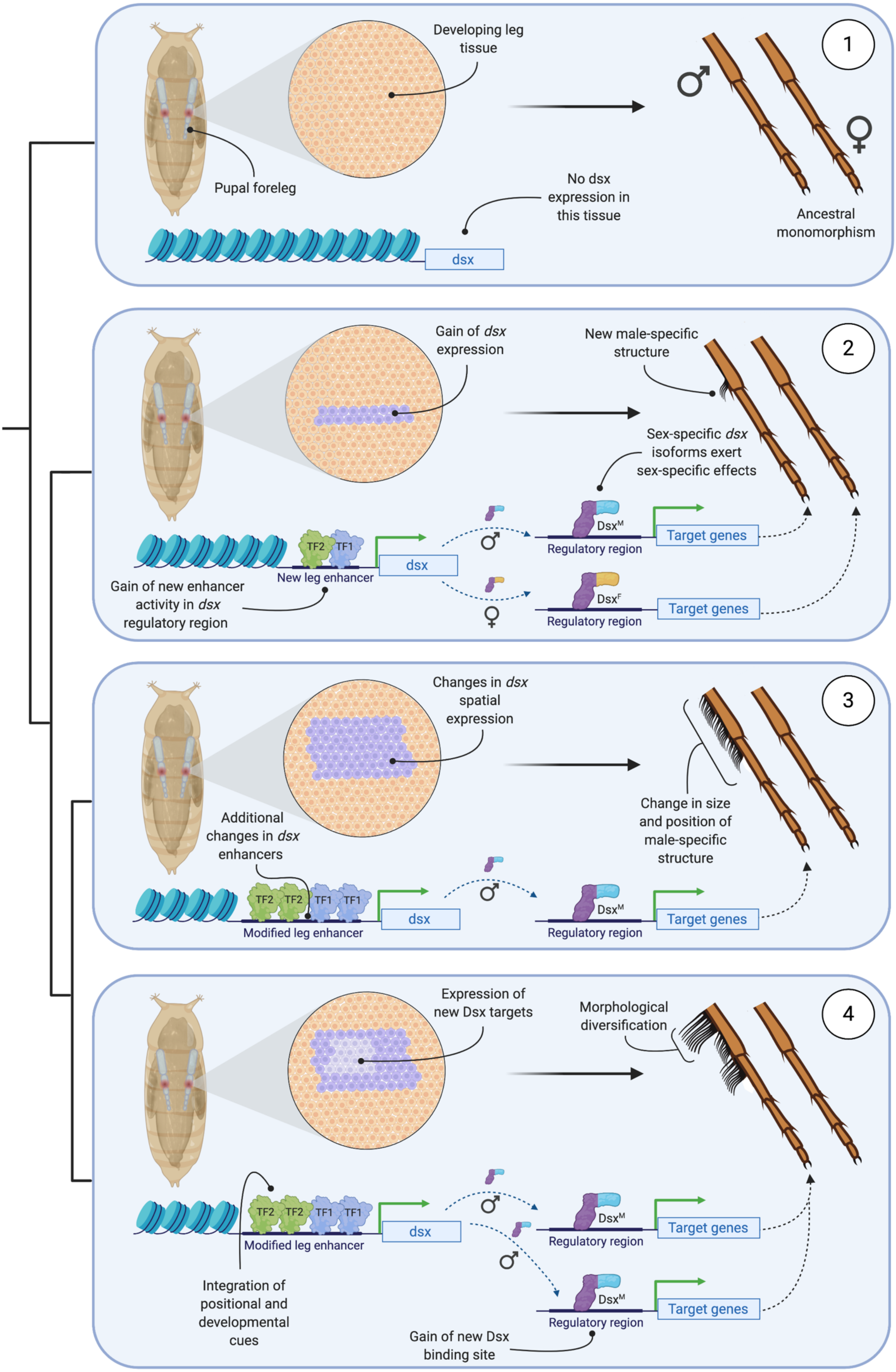
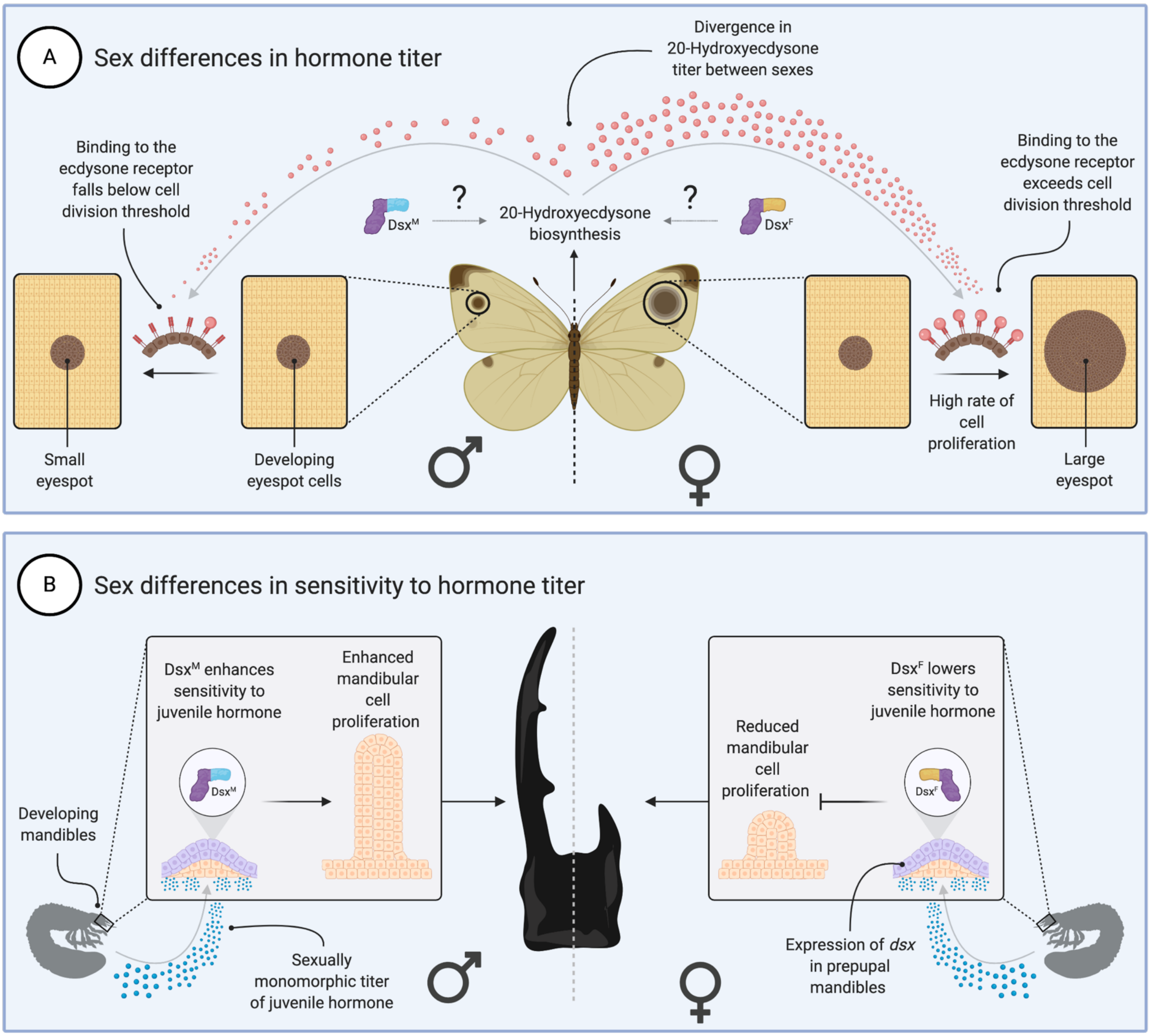

Drosophila melanogaster
Musca domestica
Ceratitis capitata
Aedes aegypti
Anopheles gambiae
♀
♂
Heteromorphic
Homomorphic
X X
X Y
1 2 3
4 5 XX
Translocations
1 2 3
4 5 XY
Sxl
Dosage of X-linked factors determines Sxl expression
Sxl promotes female-specific tra splicing
Non-homologous M-factors
Mdmd
MoY
Nix
Yob
M-Factors regulate tra splicing in males
tra
Non-functional Tra protein produced in males
Functional Tra protein produced in females
TraF
Mosquito M-factors promote male-specific splicing of dsx independently of tra
Female Tra regulates female-specific splicing of dsx
Absence of M-factors in mosquitos promotes female-specific splicing of dsx independently of tra
Non-functional Tra protein leads to male-specific splicing of dsx
dsx
DsxF
DsxM
Shared N-terminus
Sex-specific C-terminus
Female traits
Male traits

Figure 1

Developing leg tissue
Pupal foreleg
♂
♀
Ancestral monomorphism
No dsx expression in this tissue
dsx
1
Gain of *dsx* expression
New male-specific structure
Sex-specific *dsx* isoforms exert sex-specific effects
2
TF2
TF1
dsx
New leg enhancer
Gain of new enhancer activity in *dsx* regulatory region
♂
♀
Dsx^M
Dsx^F
Regulatory region
Target genes
Changes in *dsx* spatial expression
Change in size and position of male-specific structure
3
Additional changes in *dsx* enhancers
Modified leg enhancer
Expression of new Dsx targets
Morphological diversification
4
Integration of positional and developmental cues
Gain of new Dsx binding site

700 **Figure 2**



701

702 **Figure 3**