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Molecular mechanisms of secondary sexual trait development in insects

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Secondary sexual traits are those traits other than the primary gametes that distinguish the sexes of a species. The development of secondary sexual traits occurs when sexually dimorphic factors, that is, molecules differentially produced by primary sex determination systems in males and females, are integrated into the gene regulatory networks responsible for sexual trait development. In insects, these molecular asymmetric factors were always considered to originate inside the trait-building cells, but recent work points to external factors, such as hormones, as potential candidates mediating secondary sexual trait development. Here, we review examples of the different molecular mechanisms producing sexually dimorphic traits in insects, and suggest a need to revise our understanding of secondary sexual trait development within the insect lineage.

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Introduction

With the origin of male and female sexes within a species, associated with differential investment in sperm and eggs, came the origin of secondary sexual traits [1]. From an evolutionary point of view, these are sexually dimorphic traits other than the primary gametes that evolved secondarily in order to improve the chances of each sex to survive and reproduce [2•]. They encompass a suite of traits from the external genitalia, to courtship behaviours, to any other sex-specific morphological, physiological, or behavioural trait that maximizes the fitness of each sex and distinguishes one sex from the other.

Sexually dimorphic traits are quite prevalent in the animal kingdom, yet, knowledge about the molecular mechanisms that give rise to such diversity remains poorly understood. It was long believed that sex differentiation in vertebrates proceeded via steroid hormones secreted from the developing gonads [3–5], while sex determination and differentiation in insects proceeded in a cell-autonomous manner by read-outs of the sex chromosomes [6•,7]. However, accumulating evidence challenges this dichotomy and suggests that sex differentiation in vertebrates and insects could be occurring via both cell-autonomous and non-autonomous hormonal mechanisms [2•,8,9]. This largely changes our perspective of the development of sex specific traits across lineages and highlights possible additional mechanisms that explain the enormous diversity of sexually dimorphic traits seen in insects (Figure 1). In this review, we concentrate on the development of secondary sexual traits in insects. We begin with a brief overview of the different primary sex determination mechanisms in insects, which provide the first molecular asymmetries cuing the development of secondary sexual traits. We then highlight the different molecular mechanisms by which these dimorphic traits are determined. Finally, we propose the need to consider both cell-autonomous and non-autonomous mechanisms as equally probable processes that govern the formation of secondary sexual traits in insects.

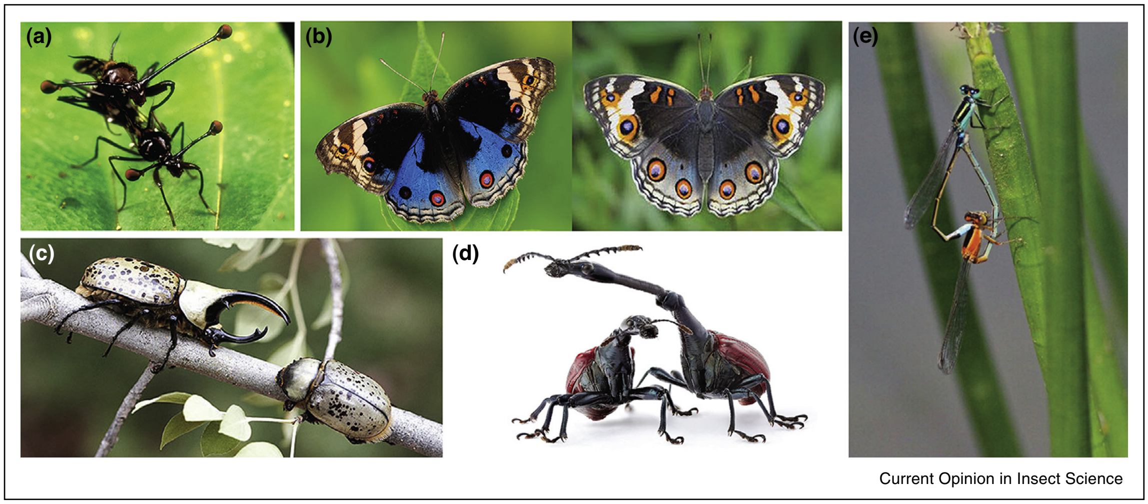
Sex determination systems in insects

A common theme in all the insect sex determination systems examined till date is that different initiation signals converge on similar downstream regulators of primary, as well as secondary, sexual trait development [10•,11,12] (Figure 2). These downstream regulators are highly conserved across animal lineages and belong to the Doublesex/mab-3 related transcription factor (*Dmrt*) family [reviewed in [11,13]].

In *Drosophila melanogaster*, sex determination begins with the different number of X chromosomes in males and females. A higher expression level of several X-linked activating transcription factors leads to the activation of *Sex-lethal* in females, the primary sex determination signal [reviewed in [14]], whereas in males, this gene remains off. This differential activation of *Sex-lethal* leads to sex-specific differences in the splicing of downstream products in the sex determination pathway like *transformer* (*tra*) and the transcription factor *doublesex* (*dsx*) [15,16] (Figure 2).

In the honeybee, *Apis mellifera*, sex is determined by a haplo-diploid mechanism dependent on the *complementary sex determination* (*csd*) gene. This multi-allelic gene

Figure 1



Sexually dimorphic traits among insects. **(a)** Male and female stalk-eyed fly. Males have longer eyestalks than females (Photo credit: Mark W Moffett). **(b)** Male (left) and female *Junonia orithya* showing sexually dimorphic colours and patterns (Photo credit: SK Khew and Horace Tan). **(c)** The two sexes of the Western Hercules Beetle (*Dynastes granti*) with males having large horns as compared to females (Photo credit: Alex Wild). **(d)** Sexually dimorphic giraffe weevil (*Trachelophorus giraffa*) where males display much longer necks (Photo credit: André De Kesel). **(e)** Damselflies (*Ischnura senegalensis*) showing sexually dimorphic colouration, with orange females and blue males (Photo credit: Vivek Sarkar).

occurs in the sex determination locus (SDL) of honeybees and is not sex-specifically spliced. Instead, females who are always heterozygous at this locus, produce functional *csd* proteins while males, either hemizygous or homozygous, produce non-functional *csd* proteins which lead to default male development [17]. The *feminizer* (*fem*) gene, a paralog of *csd*, also located in the SDL, is the downstream target of *csd* proteins in females. *fem* has sequence similarity to *D. melanogaster tra*, and, like *tra*, is also sex-specifically spliced into a functional female protein and a non-functional male protein [18]. The female protein isoform of *fem* maintains female specific development by splicing *dsx* in a sex-specific way [19] (Figure 2).

Likewise, in the silkworm *Bombyx mori*, sex determination occurs via genes contained on the sex chromosomes. ZW females have active *fem* piRNA synthesis, coded for in the W chromosome, which is absent in ZZ males. This piRNA leads to feminization by preventing the translation of a masculanization gene, *masc*, present on the Z chromosome. In the absence of this *fem* piRNA, male development occurs [20••]. Male and female development occur downstream of this process, again via sex-specific splicing of *dsx* [20••,21] (Figure 2).

Development of secondary sexually dimorphic traits

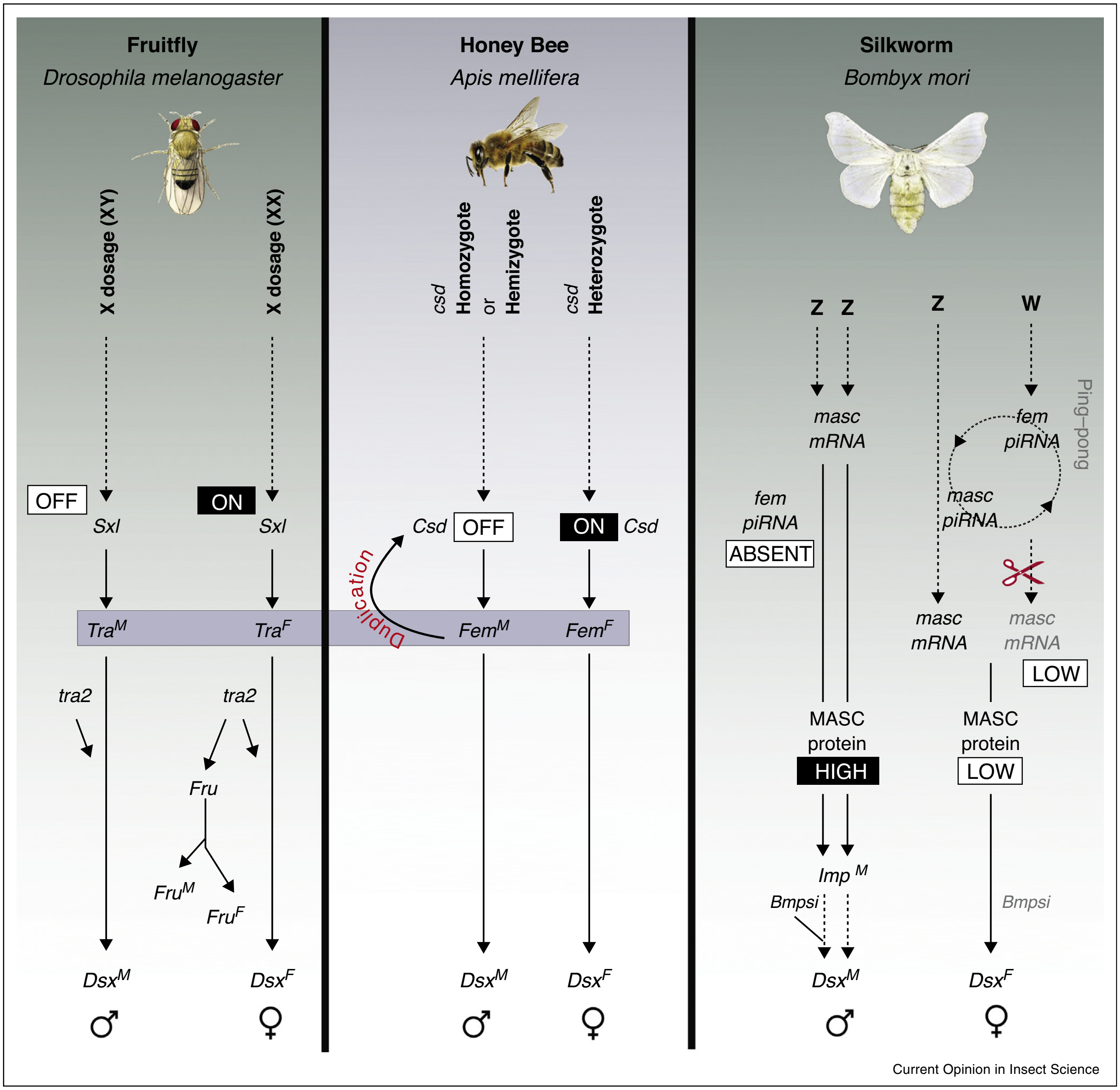
To understand the development of secondary sexual traits it is necessary to understand trait development integrated with knowledge of primary sex determination mechanisms. This includes investigating molecular asymmetries, that is, sexually dimorphic factors that result from these early sex-determining mechanisms, which can later affect the development of other traits in the same organism.

The development of sexually dimorphic traits appears to be controlled by non-sex-specific temporal and spatial factors interacting with sexually dimorphic factors [22]. Until recently, the sexually dimorphic factors involved were considered to be sex-specific hormones in vertebrates and cell-autonomous factors, such as the *Dmrt* transcription factors discussed above, in insects. However, it is becoming evident that both these mechanisms regulate secondary sexual trait development across animal lineages [2•,9]. More specifically, the role of hormonal control in insects has been overlooked, as detailed below.

Sex-specific behaviours and physiologies

In insects, sex-specific behaviours and physiologies such as courtship dances, songs, and pheromones, play important roles in reproduction. Most of the knowledge on the molecular mechanisms that govern these traits comes from studies of *D. melanogaster*. The discovery of *fruitless* (*fru*) in *D. melanogaster* in the 1960s led to the identification of a bifurcation in the sex-determination pathway and the notion that sex-specific *fru* expression in neural tissues was sufficient to direct sexually dimorphic behaviours [23,24]. Cell-autonomous *fru* male (FruM) expression can create sexually dimorphic neural circuits [25,26], that can lead to sex-specific behaviours in response to the same stimulus. For example, the male pheromone cVA, when bound to its odorant receptor, leads to courtship

Figure 2



An overview of the primary sex-determination pathways of the fruitfly *Drosophila melanogaster*, the honeybee *Apis mellifera* and the silkworm *Bombyx mori*. Despite diversity in the top regulators of the different pathways, they all lead to sex-specific splicing of the conserved downstream regulator *doublesex*. *Imp*^{M}: male isoform of the *Bombyx mori* homolog of *IGF-II mRNA binding protein*; *Bmpsi*: *Bombyx mori* homolog of *P-element somatic inhibitor*. Refer to text for details of the pathways.
Source: Figure modified from Ref. [10].

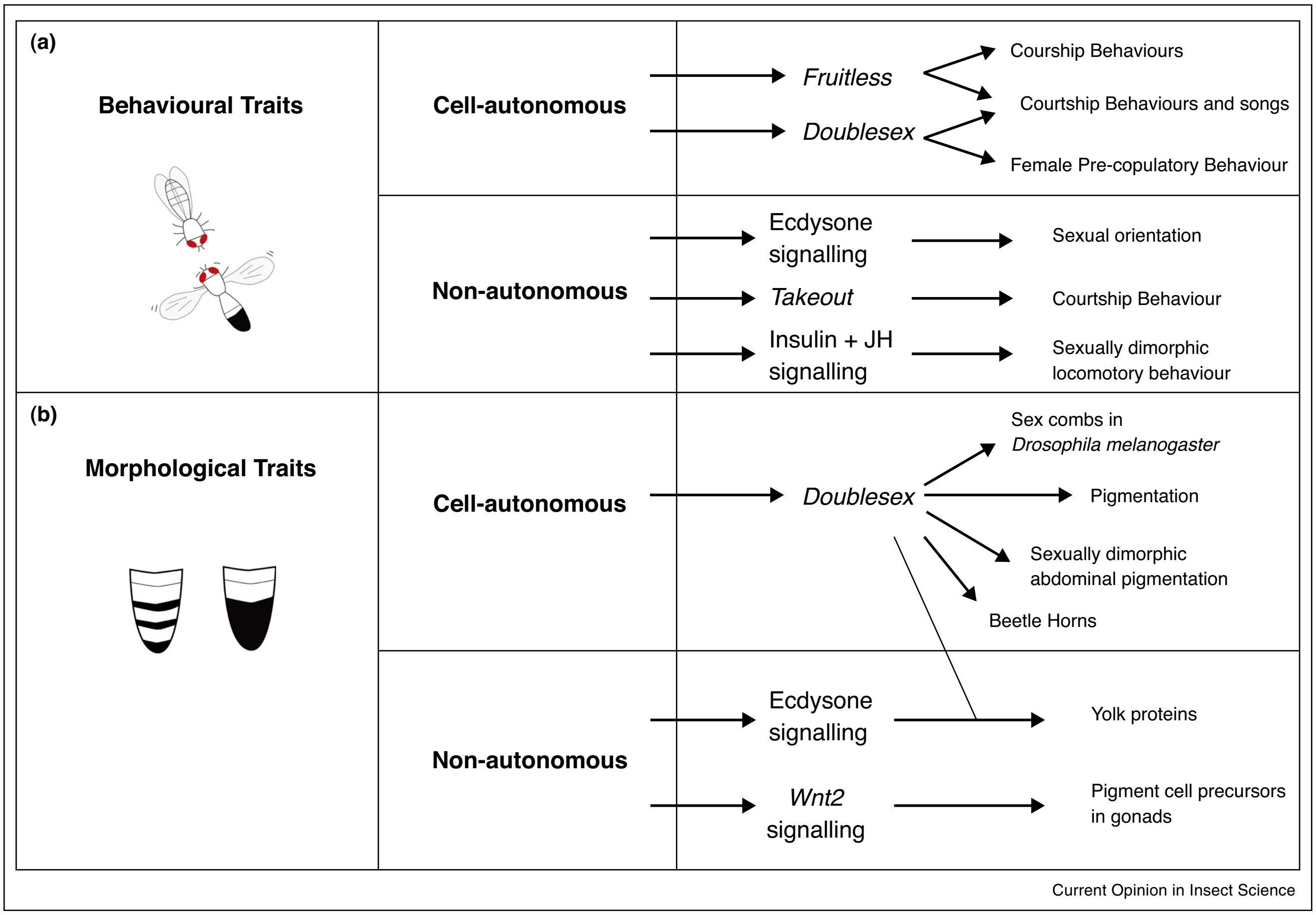
inhibition in males and sexual receptivity in females [27]. In another example, FruM decreases the threshold for neural activity in a sexually monomorphic wing vibration 'song' circuit, allowing song initiation in males only [28]. The function of *fru* also seems to be conserved in other species. For instance in the housefly *Musca domestica*, sex-specific splicing of *fru* occurs as in *D. melanogaster*, and FruM is involved in determining male courtship behaviours [29]. In the German cockroach, *Blattella germanica*, a more basal hemimetabolous insect, *fru* again appears to have a role in specifying male mating behaviour [30].

More recently sex-specific behaviours have been connected with *dsx* as well as *fru*, and this led to the revision of *fru* being the sole sexual dimorphic factor in control of insect sexual behaviour [31]. In particular, *dsx* can play a role in the central nervous system (CNS) in determining sex-specific behaviours, independently of, or in association with *fru* [32]. For example, the co-expression of DsxM and FruM in male neurons of the CNS is needed for complete courtship song production in *D. melanogaster* [33]. In addition, two clusters of DsxF expressing neurons in the CNS of females regulate pre-mating receptivity to courtship signals, in a *fru* independent manner [34••].

Lately, hormonal cues have also been associated with sex-specific behavioural modifications in addition to the cell-autonomous mechanisms described above (Figure 3a). For instance, ecdysone signalling was implicated in both female and male courtship behaviours [35,36]. Females with lower ecdysteroids courted wild-type males [36], and males with lower ecdysteroid signalling showed an increase in male-male courtship [35,37]. Moreover, ecdysone receptors are co-expressed in FruM neurons and their targeted knockdown also leads to increased male–male courtship along with a decrease in the size of centres in the male brain that consolidate olfactory information [38••]. These results point to the influence of ecdysone signalling in determining sexual orientation in flies but conclusive details of whether titres of ecdysone vary between males and females, and the critical time in development or adulthood when they vary, to determine sexually dimorphic behaviours are still missing in this system.

Further hints of non-autonomous control of sexually dimorphic traits come from the role of the adult fat bodies, the sex of which affects *D. melanogaster* behaviours [39]. *takeout*, a gene similar in sequence to *Juvenile hormone binding protein*, is expressed specifically in male fat bodies and is activated by both FruM and DsxM either directly or indirectly [40]. Takeout protein is secreted into the hemolymph by male fat bodies and acts in the brain, along with other unidentified molecules, in promoting male specific behaviour [39]. In addition, sexually dimorphic locomotory activity in *D. melanogaster*, that is, in the different number of active and inactive phases, is controlled by the insulin-signalling pathway in coordination

Figure 3



A schematic of the different cell-autonomous and non-autonomous controls of **(a)** behavioural and **(b)** morphological traits in insects discussed in this review.

with Juvenile hormone (JH). Insulin signalling activates a key enzyme in the JH producing gland, the *corpus allatum*, to promote JH biosynthesis [41]. However, sexually dimorphic titres of JH in the hemolymph of males and females have not been directly measured.

Sexually dimorphic morphological traits

There is tremendous morphological diversity in pigmentation, size and shape between male and female insects (Figure 1). These differences aid in reproduction, camouflage, predator avoidance, mate signalling or competition for mates, and are likely driven by natural and/or sexual selection [42–44]. So far, most of the molecular mechanisms implicated in these sexually dimorphic traits appear to be cell-autonomous, and coordinated by the sex determination pathway (Figure 3b). In *D. melanogaster*, the local expression of male and female *dsx* isoforms interacts with gene regulatory networks that specify traits to create sex-specific morphologies. These include the presence of sex combs in the legs of males, and fewer abdominal segments and darker abdomens in males. For example, the hox gene *Scr* induces a leg-specific expression of *dsx* in both males and females but only the DsxM isoform maintains a positive feedback loop to *Scr* to regulate the formation of the male-specific sex combs [45••]. Similarly, the Hox gene *Abdominal-B* (*Abd-B*), expressed in the posterior abdominal segments of both sexes, regulates *dsx* expression positively with no reciprocal feedback. This *dsx* expression, however, is sex-biased, with higher expression in males during a limited period of development, the mechanistic basis of which is unknown [46]. Both *dsx* and *Abd-B* then control the repression of *wingless* expression in the A7 abdominal segment, leading to the loss of this segment in males only [47]. A final example involves abdominal pigmentation in flies that depends on direct *dsx* and *Abd-B* regulation of the *bab* locus, which is a repressor of pigmentation. In females, *bab* is directly activated by Abd-B and DsxF and therefore no pigmentation occurs [48]. In males, *bab* expression is repressed by DsxM, negating Abd-B's activating effect, and this leads to pigmentation in male abdomens [48].

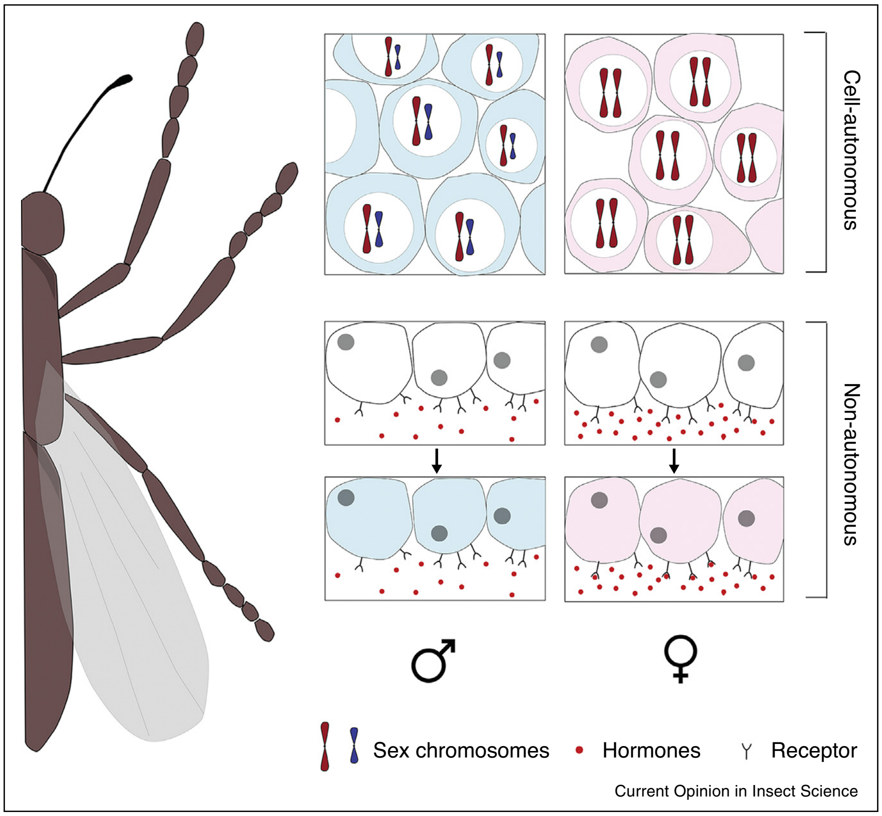
In beetles, sexually dimorphic horn morphologies are again determined by cell-autonomous expression of *dsx* isoforms. These sex-specific isoforms can either repress or activate horn development depending on developmental context, and have varying roles in different species [49,50]. *Scr*, the hox gene that activates *dsx* in the sex combs of flies, was also found to modulate horn development in a species- and sex-specific manner in *Onthophagus nigriventris* and *O. sagittarius* dung beetles [51], and it is conceivable that a regulatory relationship between *Scr* and *dsx* exists in beetle horns, similar to the one regulating leg sex combs in *D. melanogaster* [45••].

In contrast to these purely cell-autonomous mechanisms of dimorphic trait development, non-autonomous mechanisms have been identified regulating sexual traits in insects (Figure 3b). In *D. melanogaster*, for example, male-specific pigment cells that surround the gonad are recruited from the surrounding fat bodies non-autonomously via Wnt2 signalling. *Wnt2* expression is sexually dimorphic in the somatic gonadal cells during the critical recruitment period, and male-specific *Wnt2* expression leads to recruitment of pigment cell precursors only in males. This dimorphism appears to be related to *dsx* expression though it is unknown if *Wnt2* is a direct target of *dsx* [52••]. On the other hand, yolk protein synthesis in the fat bodies is a female-specific trait, which has both cell-autonomous and non-autonomous inputs controlling its expression [53–57]. In *D. melanogaster, dsx* isoforms in the fat body activate yolk protein synthesis in females and represses the same synthesis in males [54]. Furthermore, 20-hydroxyecdysone (20E) injections can stimulate yolk protein synthesis in males [58], indicating that males do not produce yolk proteins because they may have naturally lower titres of this hormone. However, a difference in 20E titres between the two sexes in *D. melanogaster* has not been conclusively shown [59,60]. In other species like the housefly *M. domesitca*, and the mosquito *Aedes aegypti*, ecdysteroid signalling also plays a role in yolk protein synthesis. The vitellogenin gene of *A. aegypti*, which codes for a precursor protein of egg yolk, has an enhancer that is directly bound by the active Ecdysone Receptor in cell culture [55]. In *M. domestica*, sexually dimorphic titres of ecdysteroids exist during the oogenic phase and a higher level of ecdysteroids in females correlate with higher levels of yolk proteins in the hemolymph [61,62]. In addition, a role for *dsx* has also been implicated in regulating yolk production in this species [56]. It has been proposed that the relative involvement of hormones and cell-autonomous control of yolk–protein synthesis in different insect species might be related to the different types of environmental cues and types of egg production taking place in these species [56].

Potential for hormones as sex-specific factors in insect secondary sexual trait development

The data highlighted above implicate hormones in the development of sexually dimorphic traits but it is still unclear in most insect species whether hormone titres themselves are asymmetric factors directing sex-specific secondary trait development. In beetles, sex-specific horn and mandible morphologies are dictated by *dsx*, however, the allometric relationship of these weapon sizes to body size is modulated sex-specifically via an interaction between the sex determination pathway and JH cued by nutrition, leading to different male morphs [49,63••]. While this interaction led to different horn or mandible sizes between male morphs, it underlies a potential to also influence trait size between sexes. However, sexually dimorphic hormone titres at critical horn developmental stages have not been measured in most species.

Figure 4



Alternative mechanisms for the control of secondary sex-specific trait development in insects. Both cell-autonomous read-outs of the sex chromosomes, and non-autonomous mechanisms of gene regulation via hormones and their receptors, affect secondary sexually dimorphic trait development in insects. In the cell-autonomous mechanism, each cell reads information contained in its sex chromosomes which then leads to sex-specific morphologies. In non-autonomous mechanisms, a dimorphic hormonal titre, determined outside the cell, leads to sex-specific trait development via asymmetries in hormonal signalling.

Another hormone signalling system that can play a major role in morphological trait development is the insulin signalling pathway. As mentioned before, this pathway has been implicated in sexually dimorphic locomotory behaviour of *D. melanogaster*. In addition, male-specific insulin-like peptides affect male sexual traits in crustaceans, such as the growth of the appendix masculina and spermatogenesis in the freshwater prawn, *Macrobrachium rosenbergii* [64••]. In *B. mori*, an insulin-like growth factor-like peptide displays sexually dimorphic titres during pupal development [65–67]. This peptide is secreted by the fat body, brain, and gonads and is involved in development of adult tissues, but has yet to be linked to secondary sexual trait development in this species [66].

The recent identification of sex-biased ecdysone titres in adult *D. melanogaster* and the male biased expression of the ecdysteroid induced *let-7* group of micro-RNAs, has also implicated hormones in the maintenance of adult behaviours and sexual fates [68,69]. Such dimorphic titres in hormones, however, have not been shown during pupal development, when most of the adult neural circuits and morphologies are determined. Taken together, these observations along with the recent identification of a female-specific sex hormone in crustaceans regulating secondary sexual trait development in this species [70•], provide sufficient foundation to hypothesize that similar insulin-like peptides or other hormones can act in the development of sex-specific traits in insects.

Conclusions and future work

The examples reviewed here show that the development of secondary sexual traits in insects can occur either by groups of cells reading out their sex chromosomes or by groups of cells responding to systemic signals such as hormones (Figure 4). The development of sexually dimorphic traits via dimorphism in hormone synthesis, however, is not yet established for any insect system. In order to conclusively implicate hormones in sexually dimorphic trait development in insects, including behavioural, physiological and morphological dimorphism, future work should focus on establishing clear sexual dimorphism in hormone titres between the sexes. This dimorphism may exist only during a critical stage of trait development, which may often be of short duration, thereby necessitating hormone profiling at short and regular intervals. Following this, hormonal manipulations, including the use of hormone antagonist in the sex with higher titres, or hormone gland extirpations, need to be performed to show a functional role for hormones in causing trait dimorphism.

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