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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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 (Sxy) 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 complementarity sex determination (csd) gene. This multi-allelic gene

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 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 orthya 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) Damsel flies (Ischnura senegalensis) showing sexually dimorphic colouration, with orange females and blue males (Photo credit: Vivek Sarkar).

Sexual development 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 dmx 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 masculization 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 dmx [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 [23,25,26]. 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
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 \textit{fru} being the sole sexual dimorphic factor in control of insect sexual behaviour \cite{31}. In particular, \textit{dsx} can play a role in the central nervous system (CNS) in determining sex-specific behaviours, independently of, or in association with \textit{fru} \cite{32}. For example, the co-expression of DsxM and FruM in male neurons of the CNS is needed for complete courtship song production in \textit{D. melanogaster} \cite{33}. In addition, two clusters of DsxM expressing neurons in the CNS of females regulate pre-mating receptivity to courtship signals, in a \textit{fru} independent manner \cite{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 \cite{35,36}. Females with lower ecdysteroids courted wild-type males \cite{36}, and males with lower ecdysteroid signalling showed an increase in male-male courtship \cite{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 \cite{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 \textit{D. melanogaster} behaviours \cite{39}. \textit{takeout}, a gene similar in sequence to \textit{Juvenile hormone binding protein}, is expressed specifically in male fat bodies and is activated by both FruM and DsxM either directly or indirectly \cite{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 \cite{39}. In addition, sexually dimorphic locomotory activity in \textit{D. melanogaster}, that is, in the different number of active and inactive phases, is controlled by the insulin-signalling pathway in coordination

![Figure 3](current-opinion-in-insect-science-a-schematic-of-the-different-cell-autonomous-and-non-autonomous-controls-of-a-behavioural-and-b-morphological-traits-in-insects-discussed-in-this-review.png)
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 DsxM 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. domestica*, 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.
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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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest


This paper overviews secondary sexual trait determination mechanisms in vertebrates and insects, and proposes the need to consider both autonomous and hormonally-induced development in determining sexually dimorphic traits in both vertebrates and insects.


The study by Robineff et al. was the first to show that Drosophila melanogaster bodies are composed of a mosaic of cells that are gender neutral and cells that know their sex via the expression of the downstream regulators of the sex-determination pathway, doublesex and fruitless. This work shows that sex-specific factors are not expressed in all cells simultaneously but are controlled by elaborate spatial and temporal mechanisms during the course of development.


Herpin et al. provides a nice review of the known sex-determination systems and their evolution in vertebrate and invertebrate lineages, also highlighting the extensive plasticity in the evolutionary history of one of the key biological pathways in animal development.


The Kiuchi et al. paper has advanced our understanding of insect sex determination by identifying a PiWI-interacting RNA (piRNA), produced from a precursor sequence Fem located on the female-specific W chromosome of the silkworm Bombyx mori. This piRNA production is necessary for the female-specific splicing of the downstream gene doublesex and hence female determination. In addition, the Fem piRNAs silence the transcripts of the Msc gene. Msc is required for masculinization and is located on the Z chromosome.


29. Meier N, Käppeli SC, Hediger Niessen M, Billicter JC, Goodwin SF, Bopp D: Genetic control of courtship behavior in the housefly;
The authors identify circuits in the female Drosophila melanogaster brain which are involved in regulating pre-mating receptivity to males by processing courtship-related signals. The neurons in these clusters express doublesex but lack fruitless expression thereby highlighting the fact that sex-specific behaviours in D. melanogaster can be controlled in a fruitless independent manner.


45. Tanaka K, Barmina O, Sanders LE, Arbetman MN, Kopp A: Evolution of sex-specific traits through changes in HOX-dependent doublesex expression. *PLoS Biol* 2011. Tanaka et al. show that the gain of doublesex expression in novel tissues is one way of evolving sexual dimorphisms in those tissues. Their work also identifies one of the genes that spatially and temporally regulates dsx.


63. Gotoh H, Miyakawa H, Ishikawa A, Ishikawa Y, Sugiyama Y, Emlen DJ, Lavine LC, Miura T: Developmental link between sex and nutrition; doublesex regulates sex-specific mandible growth via juvenile hormone signaling in stag beetles. *PLoS Genet* 2014, 10:1-9. The work of Gotoh et al. identifies that doublesex is involved in the determination of sex-specific mandible morphologies in the stag beetle *Cyclommatus metallifer*. In addition, the authors show that doublesex can specifically modulate mandible tissue sensitivity to juvenile hormone thereby affecting their growth. This provides a foundation for
understanding the development of secondary sexual traits that are nutrition dependent.


Androgenic glands in crustaceans are male-specific glands that are sources of hormones. In this work, Ventura et al. show that one of these hormones, a insulin-like peptide, plays a role in spermatogenesis and secondary sexual character development in the prawn Macrobrachium rosenbergii. By silencing the gene that codes for this peptide, the authors show effects on spermatogenesis and regeneration of secondary sexual traits in males.


This study identifies, for the first time, the existence of a crustacean female sex hormone (CFSH) in the blue crab, Callinectes sapidus, and in a related species. This hormone is female-specific and knockdown experiments highlight the importance of this hormone in the development of female secondary characters such as gonopores and ovigerous setae.