Double nexus—*Doublesex* is the connecting element in sex determination

Eveline C. Verhulst and Louis van de Zande

Corresponding author. E. C. Verhulst, Laboratory of Genetics, Wageningen University, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands. Tel.: +31317482272; Fax: +31317418094; E-mail: e.c.verhulst@gmail.com

**Abstract**

In recent years, our knowledge of the conserved master-switch gene *doublesex* (*dsx*) and its function in regulating the development of dimorphic traits in insects has deepened considerably. Here, a comprehensive overview is given on the properties of the male- and female-specific *dsx* transcripts yielding DSXF and DSXM proteins in *Drosophila melanogaster*, and the many downstream targets that they regulate. As insects have cell-autonomous sex determination, it was assumed that *dsx* would be expressed in every somatic cell, but recent research showed that *dsx* is expressed only when a cell is required to show its sexual identity through function or morphology. This spatiotemporal regulation of *dsx* expression has not only been established in *D. melanogaster* but in all insect species studied. Gradually, it has been appreciated that *dsx* could no longer be viewed as the master-switch gene orchestrating sexual development and behaviour in each cell, but instead should be viewed as the interpreter for the sexual identity of the cell, expressing this identity only on request, making *dsx* the central nexus of insect sex determination.

**Key words:** doublesex; sex determination; insects; dimorphism; transcription factor

**Introduction**

In many animals, males and females have distinct gender-related appearances such as size differences, ornamentation and colour. Some species have such extreme sexual dimorphisms, that it is sometimes hard to identify them as belonging to the same species based on phenotype alone. These extreme phenotypic differences make sexual dimorphism one of the most intriguing aspects of animal morphology, physiology and behaviour. This diversity is reflected in the underlying molecular mechanisms by an array of systems, from sex-specific gonadal hormones sealing sexual fate in mammals and other vertebrates, to cell-autonomous auto-regulatory splicing loops that maintain the sexual state in insects [reviewed in (1, 2)]. It had not been realized that the basis of sex determination harbours a common theme, until a large family of similar transcription factors was discovered. In *Drosophila melanogaster* and *Caenorhabditis elegans*, the homologous genes *doublesex* (*dsx*) and *male abnormal-3* (*mab-3*) were found, and more *doublesex/mab-3*-related genes (*Dmrt* genes) were subsequently identified in many other metazoa.

In insects, the sex determination cascade regulates the sex-specific expression and splicing of genes required for sex-specific development and behaviour. The primary signals are extremely variable in the insect order [3] but all relay their signal through a number of genes to regulate the sex-specific splicing of *dsx* resulting in male and female proteins [4–29]. These *dsx* splicing factors are conserved in many species [reviewed in (30, 31)] but in Lepidoptera and possibly Coleoptera different mechanisms operate [reviewed in (29, 32)]. As all insects have cell-autonomous sex determination mechanisms and sex dimorphism in particularly haplodiploids.

**Eveline C. Verhulst** is a postdoctoral fellow at the Laboratory of Genetics of Wageningen University. Her research interests are the evolution of sex determining mechanisms and sexual dimorphism in particularly haplodiploids.

**Louis van de Zande** is an associate professor at the Evolutionary Genetics Group of the Groningen Institute for Evolutionary Life Sciences of the University of Groningen, the Netherlands. His research focuses on the functional molecular aspects of biological complexity such as life history evolution (including ageing, photoperiodic diapause induction and sex determination).

© The Author 2015. Published by Oxford University Press. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
Determination, the sex determining cascade operates on a cell-to-cell basis and features a memory function [15].

For many years, research into insect sex determination has focused only on the presence and position of dsx in the sex determining cascade, but its function in sexual differentiation was studied primarily in D. melanogaster. However, recently, several papers have been published that focus on the function of dsx in the differentiation of many extreme sexual traits in non-model insects. In this review, we synthesize the research on D. melanogaster dsx and combine it with the description of the current status of dsx research in non-model organisms. We describe some major properties of the dsx gene and the male- and female-specific proteins, DSXM and DSX, which are translated from their sex specifically spliced transcripts. We outline the functional domains of these proteins and how these domains aid the mechanism by which dsx maintains its function as an integral part of insect sex determination pathways. In addition, the role of doublesex in development, which results in such widely divergent sex-specific and species-specific morphologies, will be discussed. Ultimately, we identify a common pattern in all insect dsx research that changes our view of the role of dsx in determining sex.

Characteristics of doublesex

Discovery of doublesex and mab-3 led to identification of a family of DM-genes

In 1965, a recessive mutation was described in Drosophila that causes genetical males and females to develop as intersexes. Appropriately, this mutation, and consequently the whole gene, was termed doublesex (dsx) [33]. Molecular analysis revealed that the bifunctional nature of this gene and its role in somatic sexual differentiation is achieved by sex-specific alternative splicing of the dsx transcript resulting in sex-specific proteins [24, 34]. In the same period, and also based on the occurrence of a mutant phenotype, the male abnormal-3 (mab-3) gene was identified in C. elegans as required for male-specific functions [35]. Both genes share a DNA-binding motif that has a common evolutionary origin in sexual development, evidenced by the fact that the Drosophila male DSX protein is able to direct male-specific neuroblast differentiation in C. elegans [36, 37]. This DNA-binding motif was named dsx/mab-3-domain (DM-domain). Subsequently, a large array of proteins containing a DM-domain was identified in mammals and other vertebrates, resulting in the description of a large family of homologous DM-genes. These DM-genes were found to be expressed in gonad-precursor cells in both mice and chicken and may control testis development in particular [38]. All vertebrate and invertebrate genomes contain multiple DM-domain proteins some of which are not directly involved in sex determination (reviewed by [39]) but also function in other developmental processes (reviewed by [40, 41]). Still, this family of dsx/mab-3-related genes (Dmrt genes) appears to be involved in sex-specific differentiation in all cases studied, thus evidencing a common theme at the basis of the mechanism of sex determination.

Dsx has a DNA-binding domain and two oligomerization domains

The dsx gene and all its orthologs found so far contain two oligomerization domains: the DM-domain, a sex-independent domain shared with all Dmrt genes (see above), and the sex-specific OD2 domain that is restricted to dsx and its orthologs (Figure 1) [42, 43]. The DM domain consists of a DNA binding domain (DBD) and an oligomerization domain (OD1). It features two novel zinc modules containing intertwined CCHC and HCCC zinc-binding sites that bind to the DNA minor groove, and a nascent alpha-helix structure [36, 44]. The OD2 domain consists of a common non-sex-specific N-terminus and a sex-specific C-terminus resulting from sex-specific splicing of the dsx transcripts. In Drosophila, both DSXM and DSX proteins form asymmetric homodimers at low concentrations and tetramers and higher oligomers at higher concentrations, but on binding to DNA, only DSX dimers are formed [42, 43, 45]. OD1 and DBD are together required for specific binding to DNA as a dimer [43] and both full-length DSX proteins have similar DNA binding properties [46, 47], as the OD2 domain plays no role in DNA binding. This indicates that both DSX homodimers recognize and bind to the exact same DNA sequence and thus can compete with each other for DNA binding [42, 43]. Apart from OD1, the entire OD2 domain is independently involved in the dimerization of the full-length protein by coiled-coil interactions [43]. The observed 3-fold stronger dimerization of DSXM over DSX is likely caused by the male-specific C-terminus in OD2 [48]. Apparently, both common regions of the DSX and DSXM oligomerization domains form dimers or tetramers by holding both ends of the protein together. The ease with which the two common domains dimerize is reflected in the formation of heterodimers when both DSX and DSXM are present, which then inhibits their respective activity, at least in vitro [42, 43]. It is unclear if this could result in an effective dsx-null mutant in vivo. Still, in the normal DSX or DSXM homodimers, OD1 forms the dimeric DNA binding unit, and OD2 regulates the sex-specific functionality of the protein by sex-specific interaction with the transcriptional machinery, or maybe by forming sex-specific regulatory structures by increasing DNA binding cooperativity when DSX binds multiple regulatory sites of its target genes [43].

![Figure 1: Overview of the male and female D. melanogaster DSX protein showing the functional protein domains. At the N-terminus, the doublesex/mab3-domain (DM-domain) consisting of the DBD and the first oligomerization domain (OD1). Towards the C-terminus, the second oligomerization domain (OD2) with first, the OD2 common region that is present in both male and female isoforms; and second the male-specific OD2 domain; and the female-specific OD2 domain. Not drawn to scale.](http://bfg.oxfordjournals.org)
DSX^{F} requires intersex and sometimes hermaphrodite in *Drosophila*

DSX^{F} requires two co-factors for its female-specific function, which are encoded by the genes *intersex (ix)* and *hermaphrodite (her)* [49–51]. It was suggested by Siegal and Baker ([52] and references therein) that DSX^{F} requires IX because DSX^{F} lacks a transcription-activation domain. IX has a proline-, glycine-, glutamate- and serine-rich region, which resembles some known transcriptional activation domains [50]. DSX^{M}, on the other hand, contains a longer OD2 domain with a proline- and serine-rich tail, suggesting that DSX^{M} alone has the same functionality as the combination of DSX^{F} and IX [52]. Still, ix seems to be transcribed in males as well [51], but its function in males is unknown.

The requirement for IX in female development may be conserved in insect sex determination as ix seems conserved through the metazoans. In transgenic *D. melanogaster*, expression of an ix ortholog of *Megaselia scalaris* or *Bombbyx mori* completely or partially rescues the sexual development of mutant ix females [52]. However, apart from a study in *B. mori* [53], no follow-up research has been done on the presence and function of ix homologs in insects, and the requirement of IX for DSX^{F} function outside *Drosophila* is unknown.

Her encodes a zinc-finger protein and is expressed independently of the sex determination cascade [54]. It is involved upstream in the D. melanogaster cascade, in parallel to or downstream of, *dsx* [49]. Homologs of her have not been reported outside *Drosophila*, indicating that at least some of the interactants of *D. melanogaster* DSX^{F} are derived. No association of HER and DSX^{M} has ever been reported.

*DSX* expression is regulated by HOX genes in *D. melanogaster*

The research on the sex combs, a sex- and species-specific morphological trait in *Drosophila*, has put the role of dsx as master-regulator into a new perspective. The sex comb is a recently evolved male trait found only in a small subset of *Drosophila* species [55]. Sex comb development requires the expression of the HOX gene *Sex combs reduced (Scr)* and *dsx* in a tightly restricted, sex-specific pattern at a critical time in development. In species without sex combs, Scr is expressed at equal levels in males and females throughout development [55, 56]. In D. melanogaster, in the absence of DSX^{M} or DSX^{F}, Scr is expressed at an intermediate level, which is sufficient to activate sex comb development [57]. Relative to this level, Scr is actively upregulated by DSX^{M}, both Scr and DSX^{M} being then required for sex comb development. In contrast, DSX^{F} is involved in repressing Scr in a female-specific pattern [57, 58]. On the other hand, a knockdown of Scr results in a strong reduction of dsx expression, indicating that SCR in turn is required for dsx expression. Hence, Scr and dsx form a positive feedback loop [57]. It was recently shown that early expression of Scr does not require DSX [58], making it not entirely clear how the feedback loop of Scr and dsx may precisely function, but the study by Tanaka et al. is the first that has identified a regulator of dsx expression after realization of sex determination [57].

The occurrence of interactions between DSX and a HOX gene is supported by studies on the dynamics of dsx expression in the posterior pupal abdomen [59, 60]. *Dsx* is expressed at low levels through the developing abdomen and is highly enriched in the posterior abdomen of both sexes compared with the anterior abdomen. In male pupae, dsx levels are even higher when compared with female pupae, and this coincides with the highest levels of Abd-B in this region. Disruption of Abd-B expression results in lower dsx expression, while ectopic expression of Abd-B leads to higher dsx expression, indicating that, as observed with Scr, the HOX-gene Abd-B regulates dsx expression. However, no indication was found here of dsx-mediated regulation of Abd-B expression [59, 60]. These studies gave the first hints that dsx expression may not be continuous and ubiquitous, but only observed when and where required. Yet, more research is required to identify additional (HOX-) genes that regulate dsx in a spatiotemporal manner and more importantly, to determine the possible evolutionary conservation in the regulation of dsx in different insect taxa.

**Doublesex function in sexual differentiation**

*Regulation of dimorphic trait development by DSX in Drosophila*

The mechanisms by which DSX^{F} and DSX^{M} can regulate sexual dimorphism have been studied primarily in *D. melanogaster* and appear diverse. In some cases, DSX^{F} and DSX^{M} are antagonistic, but in other cases, both have a context-dependent instructive function (reviewed by [77]). In this section, we will highlight some of the research on the target genes of DSX and their mode of regulation in *D. melanogaster*. A summary of the DSX target genes and the activating or repressing mode of regulation is given in Table 1.

**Yolk protein genes**

The first identification of transcriptional regulation by DSX proteins came from studies on the yolk protein genes (Yp), showing the binding of DSX^{F} and DSX^{M} [35, 46, 61] to the fat body enhancer (FBE) in between two Yp [78, 79] thereby regulating their expression. Together with IX and HER, DSX^{F} promotes expression of both genes while DSX^{M} alone represses gene expression [46, 50, 51, 61, 62, 63]. A 13-bp palindromic sequence ([G/A]NNAC[A/T][A/T][A/T][GNN][C/T]) was identified in the FBE consisting of two motifs around a central A/T [42]. Based on these data, Luo et al. defined a palindromic consensus motif (GCCAAATGGTGG) in the genomes of different *Drosophila* species and some other Dipterans [80]. A number of *D. melanogaster* genes that were known DSX targets were found to be associated with this sequence, and putative novel DSX targets were identified. By examining more distantly related species, Luo et al. also showed that a 13-bp sequence with variation around the core palindromic sequence ACA[A/T][G/T]G is present in *B. mori* and the mosquitoes *Aedes aegypti*, *Anopheles gambiae* and *Culex pipiens*. However, no such motifs were found in *Tribolium castaneum*, *Apis mellifera* and *Nasonia vitripennis* and other insect species, indicating that DSX-binding sites are evolving within the insect order [80] and that further studies into the binding sites of DSX proteins are required.

**Abdominal pigmentation genes**

In *D. melanogaster*, males show conspicuous abdominal pigmentation, which is absent in females. Just as with Yp expression regulation, DSX^{F} and DSX^{M} act antagonistically in controlling this sex-specific abdomen pigmentation [64, 65]. The bric-a-brac (*bab*) gene is a repressor of abdominal pigmentation, whereas the HOX gene *Abdominal-B (Abd-b)* is an activator of abdominal pigmentation and, in addition, represses *bab* expression. DSX^{F} promotes *bab* expression by binding to a dimorphic cis-regulatory-element (CRE), thereby overruling the repressing action of Abd-B on *bab*. This results in repression of posterior
pigmentation in females. DSX\textsuperscript{M} binds to the same CRE to directly repress \textit{bab} expression and consequently, promotes male-specific pigmentation [65]. This mechanism of sexually dimorphic pigmentation has only arisen in the \textit{D. melanogaster} species group and evolved through multiple fine-scale changes within the dimorphic CRE [65].

### Pheromone genes

In Drosophilids with dimorphic pheromone production, female-specific pheromones are produced via the activity of the desaturase DESAT-F under control of DSX\textsuperscript{F} in combination with other cis-regulatory factors [69]. DSX\textsuperscript{M} does not repress \textit{dsatF} expression, indicating that DSX does not always act antagonistically. DSX\textsuperscript{F} binds a CRE upstream of \textit{dsatF} to control dimorphic expression, but it is unclear whether DSX\textsuperscript{M} binds to the same CRE but without effect. Within the Drosophilids, frequent evolutionary changes in this CRE site partly explain the gain and loss of direct DSX\textsuperscript{F} regulation of \textit{dsatF}, which is correlated with transitions from dimorphic to monomorphic expression of \textit{dsatF} [69].

### Gustatory sense organs genes

The effect of \textit{dsx} on shaping dimorphic tissues can be dependent on spatial determinants as was discovered in examining the sex-specific development of gustatory sense organs (GSOs) in the foreleg of \textit{D. melanogaster} [66]. Males have more GSOs than females on Segments 1-4 of the tarsus (T1-T4), and this is controlled by \textit{dsx}. In T1 and T3, DSX\textsuperscript{M} promotes the number of GSOs in males, whereas DSX\textsuperscript{F} seems to have no effect in females. In T2, DSX\textsuperscript{M} stimulates the number of GSOs in males, whereas DSX\textsuperscript{F} has no effect in females [66]. Thus, DSX\textsuperscript{M} and DSX\textsuperscript{F} have apparently different modes of action within close tissue proximity. To fully understand this developmental process, more research is required to determine the specific target genes and the regulatory mechanism exerted by DSX\textsuperscript{F} and DSX\textsuperscript{M} on these GSO target genes.

### Genital and abdominal development genes

The sex-specific differentiation of the genital imaginal disc is actively regulated by both DSX\textsuperscript{F} and DSX\textsuperscript{M} in two separate steps.
3.3 Regulation of dimorphic trait development by DSX in other insects

The occurrence of orthologs of *dsx* and the conservation of its function in insects outside of Drosophila has been known for years. However, many of the studied cases show the presence of multiple protein isoforms of *dsx*, with some isoforms being non-sex-specific and (partially) missing the O2D domain [4, 10, 12, 13, 76, 83]. This contrasts with the case of *Drosophila*, which features only one male- and one female-specific isoform [24]. In the past couple of years, the search for the dsx target genes in other insect species has been boosted by the availability of many complete genome sequences [29], and in some cases, the function of (some of) the dsx isoforms has been identified. In this section, the spatiotemporal regulation of dsx and the downstream targets of DSX are discussed in non-model insect species, see also Table 1.

### 3.3.1 Silk moths—female-specific genes

In *B. mori*, one female and one male isoform were published in 2001 [6, 7]. Recently, however, more male and female isoforms have been discovered but the functional difference between all these proteins is still unclear [84, 85]. Ecotopic expression of DSXF1 in males has no effect on morphology, which may suggest the requirement for a co-factor such as IX [53, 71]. Transgenic females expressing dsxM1 do show intersex phenotypes [71], as DSXMX1 has no requirement for IX. The presence of a DSXF1 isoform in males activates the expression of the female-specific genes vitellogenin (vg) and hexameric storage protein 1 (sp1), and represses the expression of the male-specific gene pheromone-binding protein (pbp) [72]. As expected, ectopic expression of DSXM1 in females showed the reverse pattern for vg and pbp expression [71]. Apparently, DSXF1 activates vg expression while DSXM1 represses vg expression by binding to the palindromic core sequence (ACATTGT) in the promoter region of vg [71, 72]. The activation of vg and sp1 expression by DSXf was also shown in the wild silk moths Antheraea assama and Antheraea mylitta [83].

### 3.3.2 Bombyx mori—abdominal morphology genes

In *B. mori*, only females have a chitin plate, which is formed by degeneration of the eighth abdominal segment (A8) and is essential for copulation. Expression of dsxM1 in transgenic females results in the formation of an abnormal chitin plate, indicating that the normal formation of a male-specific A8 is under developmental control of DSX1 [73]. Moreover, these transgenic females show an increase of Abd-B expression in their posterior abdomen, resembling that of wild-type males. This suggests that DSX1 induces Abd-B expression in their posterior abdomen, which then develop male-specific structures. In female cells, DSXX actively represses bnl expression, most likely by directly binding to the upstream region of bnl that contains multiple putative DSX-binding sites [67].

Further shaping of the male-specific posterior abdomen is also controlled by DSXM in conjunction with Abd-B. The reduction of adult male segment A7 is achieved by DSXM through repression of *wg* and promotion of extramachetae (*emc*) [60, 70]. Female development of the posterior abdomen is controlled by DSX together with Abd-B by repressing *emc* [60].

### Concluding remark

All these studies indicate that DSX1 and DSXM1 indeed bind to the same DSX-binding site or dimorphic CRE sites of their target genes, but their mode of actions likely depends on the sex-specific OD2 that probably interacts with HOX-genes, other transcription factors or regulatory proteins. Small changes in binding sites can then have a huge effect on the sex-specific regulation of that particular gene, making evolutionary changes in dimorphic traits relatively straightforward.

### 3.3.3 Tribolium castaneum—vitellogenin gene

A more direct approach was used to identify dsx target genes in *T. castaneum* [12]. Knocking down different dsx isoforms revealed a number of target genes, including vg, which is also a dsx target in *D. melanogaster* and *B. mori* with a similar regulation. DSX1 increases vg expression, whereas DSXM1 represses vg expression [12]. In addition, the presence of a 13-bp consensus sequence, with the palindromic core ACA[AT]TGT, was identified in eight more target genes, suggesting that these genes might be direct dsx targets as well [12]. The presence of the 13-bp consensus binding site is noteworthy, as this motif was not identified in *T. castaneum* by Luo et al. [80].

### 3.3.4 Horned beetles—exaggerated horn development

The regulation of *dsx* in exaggerated beetle horn development has been studied in two beetle genera, dung beetles (*Onthophagus*) and rhinoceros beetles (*Trypoxylus*) [10, 11]. The location and size of horn development differs between species and sexes in both genera, and also depends on the nutritional status of the male. In *Onthophagus* taurus, males in good nutritional condition have large horns, whereas males in bad condition have small horns. Knockdown of *dsx* resulted in a significant reduction in horn size in both types of males, but was more dramatic in large males. This suggests that *dsx* function depends on nutritional condition. Female dung beetles...
have a posterior ridge on the head that is proportional to body size. After larval dsx knockdown, this ridge develops into small horns, particularly in large females [10]. Thus, in O. taurus, DSXM promotes horn development while DSXF inhibits horn development, and both are influenced by nutritional status. However, in the closely related Onthophagus sagittarius, females have a single thoracic horn and a posterior head horn that both lack in males. Dsx knockdown in females results in a reduction of thoracic horn size, development of male-specific anterior head horns and transformation of the large single female posterior head horn to a smaller branched horn. In males, dsx knockdown results in development of a small thoracic horn, and development of a large, branched posterior horn, but it has no effect on the anterior head horn [10]. The function of DSXM and DSXF are, therefore, reversed in O. sagittarius compared with O. taurus in development of the thoracic horn growth. In addition, the DSXM function is reversed for posterior head horn, but DSXF promotes transformation of the posterior head horn. This suggests that DSX is not simply promoting horn growth in Onthophagus but features complex regulation. Besides, DSX can quickly reverse its function or gain novel functions as seen from these two closely related species [10]. A similar complex regulation by DSX on horn development was seen in Trypoxylus dichotomus [11]. Here, females have no horns at all, and males have a thoracic horn and a head horn. Dsx knockdown reduces the size of the head horn in males, while the thoracic horn disappears completely. In females, dsx knockdown results in the development of a small head horn, suggesting that DSX regulates horn formation in different ways for the two horn morphologies [11].

**Stag beetle—exaggerated mandible growth**

The developmental interaction of dsx regulation and nutrition was studied in greater detail in the stag beetle (*Cyclommatus met-alifer*) [75]. The mandibles are an exaggerated male trait and, as in Onthophagus, mandible size is correlated with the body size of the male. Knockdown of dsx resulted in an intersex phenotype in males and females, the mandible size being dramatically reduced in males but slightly increased in females. The sensitivity of the mandibular tissue to Juvenile Hormone (JH) was previously shown in males, but ectopic expression of JH in female mandibular tissue did not lead to an increase in mandible size, suggesting a sex-specific tissue response to JH during development [86]. Supplementing a JH analog (JHA) to dsx knockout female induced mandible growth, which implicates that DSX sensitizes mandibular tissue to JH at a level comparable with male tissue sensitivity [75]. Dsx knockdown in males leads to a slight decreased sensitivity of supplemented JHA. The timing of increased dsx and dsxM expressions during the prepupal stages when mandible growth takes place in males, but is inhibited in females, is precise. This suggests that DSX inhibits mandible growth by repressing the sensitivity to JH, whereas DSXM promotes mandible growth by enhancing JH sensitivity [75].

**Nasonia vitripennis—wing size**

In the parasitic wasp, *Nasonia*, wing size is sex and species specific. *Nasonia* vitripennis males have small wings and cannot fly, whereas *Nasonia* giraulti males have large wings and do fly. The region responsible for this difference, us1, was mapped using positional cloning to the 5’ UTR of dsx [74]. The us1 region of *N. giraulti*, containing only the dsx 5’ UTR and no coding regions, was then backcrossed into a *N. vitripennis* background, resulting in a wing size increase that accounted for 44% of the interspecies difference [74]. An increase of DSXM expression was found in the developing wings of individuals with the vitripennis us1 (us1v) locus relative to individuals with the giraulti us1 (us1g) locus in the same background. This difference was not found in male legs or whole pre-pupae [74], suggesting that cis-regulation of dsx expression possibly by HOX genes could also have an effect on dimorphic and species-specific traits in other insect species.

**Papilio polytes—mimicry**

Recently, new research suggested another role for dsx in development, more precisely in sex-limited mimicry in the butterfly *Papilio polytes* [76]. The males of this species all have the same non-mimetic wing pattern, whereas the females have a wing pattern that either resembles the male-like non-mimetic pattern or mimics one of the different patterns in the toxic genus *Pachliopta*. Multiple genetic approaches pointed to dsx as the central gene in the female wing polymorphism and three female-specific isoforms were found, two expressed in the wings and one in the body. No splicing differences were found between the mimetic forms, and it seems that it is the variation in dsx expression levels of the two isoforms in the mimetic versus non-mimetic wings that controls the differences in wing pattern variation in females [76]. Strikingly, dsx expression in the mimetic wings has a strong spatial correlation to the adult wing patterns, showing that spatiotemporal dsx expression probably regulates the different mimetic forms, most likely by involving other regulatory elements [76, 87]. In addition to dsx expression differences, a number of coding changes were found between the mimetic and non-mimetic alleles located predominately in the first exon, but not in the DM-domain. These coding changes possibly lead to different protein structures, as the protein structure predictions shows that the non-mimetic DSX isoforms fold like other insects DSX proteins, whereas the mimetic DSX protein isoform structures are atypical. The allelic differences between the two forms are maintained by the reduced recombination caused by an inversion polymorphism of dsx [76]. Apparently, dsx has evolved a mechanism to regulate different phenotypes within one sex in addition to its normal function.

**Concluding remarks**

The comparison of Drosophila DSX targets and dsx regulation with that of other insects shows a partial overlap in target genes. For example, yolk proteins and vg are conserved female-specific genes that show the same mode of antagonistic regulation by DSX in different insect species. These conserved genes often even contain identical DSX binding sites. For other, more species-specific dimorphic traits, the DSX binding sites are unknown but, again, the regulatory role of DSX is often antagonistic. This indicates that in insects other than *Drosophila* also, DSXM and DSXF bind to the same binding site but probably assemble different additional factors for their sex-specific function. Evolutionary changes, therefore, are not restricted to the DNA binding sites of the target genes, but can also take place on the sex-specific OD2 part and the additional factors, which might thus explain the diversity found in dimorphic traits.

**Spatio-temporal regulation of DSX**

The general idea that emerged from earlier studies, primarily on *D. melanogaster*, was that dsx regulates sex-specific morphologies by either repressing or activating the expression of its target genes, while, as a sex determination master-switch, it was cell autonomous and expected to be expressed ubiquitously. However, more recent studies have shown that dsx isoforms are
not expressed constitutively, but are under complex spatiotem-
poral regulation, for example, by the somatic gonad identifica-
tion [88], in the central nervous system [89, 90], during neuron
development [91], coordinating dimorphic axon guidance [92],
regulating female receptivity [93], controlling female post-mat-
ing behaviour [94] and specifying male courtship behaviour (re-
viewed by [95]).

Specifically, Robinett et al. established a D. melanogaster
strain with a GAL4 insertion into the dsx gene, allowing them
to visualize all cells that express dsx during development [96].
This demonstrated that dsx is not expressed in all cells but ra-
ther forms a mosaic of expression patterns in developing and
adult individuals, regardless of their chromosomal sex com-
position (XX or XY). The sex determination cascade appears
to specify the sex of each cell, which is maintained as a molecu-
lar memory system (reviewed in [30]), but the regulation of dsx
expression sets the developmental route [96]. Shortly
hereafter, two studies in Drosophila showed that HOX genes
are responsible for strict regulation of dsx expression (see the
section ‘Dsx expression is regulated by HOX genes in
D. melanogaster’).

Evolution of doublesex

One of the questions now arising is how to reconcile the main-
tenance of the function of dsx as the nexus of sex determination
with its ever-evolving ability to regulate a variety of sexual mor-
phologies. When compiling the current data, three levels become
apparent on which selection for sexual dimorphic traits can act.
First, selection can result in changes in the coding region of
dsx itself. On the one hand, dsx is expected to be under strong purify-
ing selection, as deleterious mutations would have a devastating
effect on reproduction. On the other hand, DSX is also involved
in male courtship behaviour and genital development, both of

---

**Figure 2:** Doublesex (dsx) is at the interface of sex determination and sexual differentiation in insects. The different mechanisms of sex determination in insects are like the root of a tree and their effects are not noticeable until dsx is required for sex-specific instructions in the cell. The regulation by dsx on dimorphic traits development is diverse and extends to all aspects of sexual differentiation like the branches on a tree. ZZ/ZW, XX/XY, XX/XO and 2n/1n (haplodiploidy) represent the different chromosomal sex determining systems. Many sex determining systems converge on tra (sometimes termed feminizer) resulting in splicing of tra into a male- or female-specific transcript (reviewed in [29]). Only in females this transcript results in a functional TRA protein, which then splices dsx into a female-specific transcript. In males, no functional TRA protein is produced and dsx is spliced by default into a male-specific transcript. Cellular memory of the sex is maintained in most species by auto regulation of tra, but in Drosophila this is taken over by Sxl (reviewed in [30]). The dsx transcripts yield DSXM and DSXF proteins that regulate the downstream targets in a sex-specific manner. In B. mori, tra appears not present and dsx is spliced by default in the female-specific form. In B. mori males, a P-element somatic inhibitor (PSI) and IGF-II mRNA-binding protein (IMF) act together to splice dsx into the male-specific transcript (reviewed in [32]). In XX/XO individuals, little is known about the sex determination mechanisms. (A colour version of this figure is available online at: http://bfg.oxfordjournals.org)
which are often under sexual (positive) selection. Previous studies found only evidence for purifying selection, mainly in the common regions [19, 97], but, recently, multiple other studies also evidenced the role of positive selection in the evolution of dsx [76, 98, 99]. These modifications can have an effect on the secondary and tertiary structure of the DSX protein [76] and may lead to changes in the dimerization process and the interaction of DSX with other transcription factors [99]. Particularly, the male-specific exon accumulates the majority of the non-synonymous mutations over longer evolutionary time frames [98], which is in accordance with the fact that female genital morphology is more conserved than male genital morphology. As the common region is primarily under purifying selection, the main functionality of dsx can be maintained [19, 98, 99].

Second, in addition to selective forces acting on dsx directly, the cis-regulatory elements (CRE) of DSX target genes are also under selection [65, 69, 100]. Moreover, the idea that some DSX regions involved in dimerization and DNA binding are under positive selection [99], suggests that the evolution of the DSX binding domains and the DSX binding sites may even go hand-in-hand. In some studied cases, for instance the Drosophila pheromone genes, proto-sequences for the sexually dimorphic CRE sites are already present in monomorphic ancestor species and a relatively small number of mutations are required to make the transition to a DSX-sensitive CRE [65, 99–102].

Third, the observation that spatiotemporal expression of dsx is one of the main regulators for dimorphic characters indicates that selection on CRE in the dsx promoter region may be of huge importance for the evolution of new sex-specific traits [74]. However, this area of research is less advanced, as only the HOX-genases Abd-B and Scr are now known to be implicated in dsx expression regulation [57, 58, 60, 70], and their mechanism of interaction is largely unknown.

**DSX is not a master-switch but a central nexus**

Doublesex has thus far been regarded as the final master-switch gene in the sex determination cascade of all studied insects. However, in the past couple of years, it has become clear that dsx is not the final master-switch in the sex determination cascades but rather the central switch at the interface of sex determination and sexual differentiation. The sex determination cascades start with a primary sex-determining signal, which is highly variable in insect species (e.g. csd, maternal imprinting or M-factor). This signal results in sex-specific splicing of downstream genes (Sex lethal, tra) (Figure 2) [30]. When the female state is induced, this state is memorized in the cell by a positive feedback-splicing loop [15]. Only in the female state, transformer (tra) splicing results in a functional TRA protein, which then splices dsx into a female-specific transcript. In males, no functional TRA protein is produced and dsx is spliced by default into a male-specific transcript [2]. In other systems, dsx splicing is instructed by P-element somatic inhibitor – mRNA-binding protein (PSI-IMP) [32]. Sexual differentiation starts when the sex-specific information provided by DSX° or DSX^4 to its target genes is used to differentiate tissues during development, leading to sex-specific traits.

Extending the metaphor of a tree as proposed by Clough and Oliver [103] to include all studied insect species, the difference between sex determination and sexual differentiation can be envisioned by comparing the variety of primary signals with the root of a tree (Figure 2). These signals and the resulting cascades operate underground and their actions are not (yet) visible. When dsx is expressed, it receives sex-specific input from multiple signal transferring genes, including tra and PSI-IMP [30, 32], ultimately resulting in either DSX^M or DSX^F. The transition from the roots (sex determining cascades) to the branches (sexual differentiation) is in the trunk of the tree, which is represented by the two DSX proteins (Figure 2). All the sex-determining actions in the roots take place during early development, and the effects of disrupting the sex-specific input to dsx in adults can be minor [104].

As has been summarized in this review, the regulation by DSX^M or DSX^F on dimorphic trait development is diverse and extends to all aspects of, often visible, sexual differentiation like the branches on the tree (Figure 2). As insects have cell-autonomous sex determination, it was expected that the entire tree was present in all cells, so that depending on the outcome of the sex determination cascade, either DSX^M or DSX^F protein would be found in all somatic cells to regulate their target genes. However, growing evidence suggests that regulation of dsx itself may be at the basis of sex- and species-specific morphological differences. Only when a cell needs sex-specific information, dsx is ordered to present this information. This also suggests that the role of the positive feedback-splicing loop may be of even more importance than previously thought [2]. After all, it is this cell-autonomous auto regulation that maintains the memory (male or female) of the cell should it require this information during development [15]. Therefore, as also noted by Clough and Oliver for Drosophila [103], in all studied insects dsx is not part of the sex determining cascade in the roots but rather represents the trunk of the tree connecting roots and branches. It gets the input from the omnipresent feeding tree root (the sex determination cascades), but, only at a specific time and place, dsx relays this information through the tree trunk to the required target genes, resulting in visible dimorphic traits in the tree branches. Taken together, dsx is not the sex determining master-switch gene but only a nexus for sexual differentiation.

**Key points**

- DSX^M or DSX^F regulates sex-specific morphologies by either repressing or activating the expression of its target genes.
- This mode of activation or repression by DSX differs between target genes, sexes and species.
- Changes of cis-regulatory-elements in the promoter regions of target genes can lead quickly to novel dimorphic binding sites for DSX resulting in fast evolution of dimorphic phenotypes.
- Dsx was thought to be ubiquitously expressed and cell autonomous, however dsx expression is regulated spatiotemporally, often by HOX-genes, and provides sexual information to the cell only when required.

**Funding**

This work is part of the research program Innovational Research Incentives Scheme, financed by the Netherlands Organization for Scientific Research (NWO) Grant No. ALW 863.13.014 to ECV. We thank two anonymous reviewers for their constructive comments and suggestions.

**References**

1. Wilhelm D, Palmer S, Koopman P. Sex determination and gonadal development in mammals. *Physiol Rev* 2007;87:1–28.
2. Verhulst EC, Van de Zande L, Beukeboom LW. Insect sex determination: it all evolves around transformer. Curr Opin Genet Dev 2010;20:376–83.

3. Sánchez L. Sex-determining mechanisms in insects. Int J Dev Biol 2008;52:837–56.

4. Cho S, Huang ZY, Zhang J. Sex-specific splicing of the honeybee doublesex gene reveals 300 million years of evolution at the bottom of the insect sex-determination pathway. Genetics 2007;177:1733–41.

5. Oliveira DCSG, Wilken JH, Verhulst EC, et al. Identification and characterization of the doublesex gene of Nasonia. Insect Mol Biol 2009;18:315–24.

6. Ohbayashi F, Suzuki MG, Mita K, et al. A homologue of the Drosophila doublesex gene is transcribed into sex-specific mRNA isoforms in the silkworm, Bombyx mori. Comp Biochem Phys B 2001;128:145–58.

7. Suzuki MG, Ohbayashi F, Mita K, et al. The mechanism of sex-specific splicing at the doublesex gene is different between Drosophila melanogaster and Bombyx mori. Insect Mol Biol 2001;31:1201–11.

8. Shukla JN, Nagaraju J. Doublesex: a conserved downstream gene controlled by diverse upstream regulators. J Genet 2010;89:941–56.

9. Sugimoto TN, Fujii T, Kiyukawa T, et al. Expression of a doublesex homologue is altered in sexual mosaics of Ostrinia scapularis moths infected with Wolbachia. Insect Mol Biol 2010;20:847–54.

10. Kijimoto T, Moczek AP, Andrews J. Diversification of doublesex function underlies morph-, sex-, and species-specific development of beetle horns. Proc Natl Acad Sci USA 2012;109:20526–31.

11. Ito Y, Horigai A, Nakata M, et al. The role of doublesex in the evolution of exaggerated horns in the Japanese rhinoceros beetle. EMBO Rep 2013;14:561–7.

12. Shukla JN, Palli SR. Doublesex target genes in the red flour beetle, Tribolium castaneum. Sci Rep 2012;2.

13. Salvemini M, Mauro U, Lombardo F, et al. Genomic organization and splicing evolution of the doublesex gene, a Drosophila regulator of sexual differentiation, in the dengue and yellow fever mosquito Aedes aegypti. BMC Evol Biol 2011;11.

14. Dübendorfer A, Hediger M, Burghardt G, et al. Musca domestica, a window on the evolution of sex-determining mechanisms in insects. Int J Dev Biol 2002;46:75–9.

15. Pane A, Salvemini M, Delli Bovi P, et al. The transformer gene in Ceratitis capitata provides a genetic basis for selecting and remembering the sexual fate. Development 2002;129:3715–25.

16. Saccone G, Salvemini M, Polito LC. The transformer gene of Ceratitis capitata: a paradigm for a conserved epigenetic master regulator of sex determination in insects. Genetica 2011;139:99–111.

17. Scal C, Catteruccia F, Li Q, et al. Identification of sex-specific transcripts of the Anopheles gambiae doublesex gene. J Exp Biol 2005;208:3701–9.

18. Ruiz MF, Stefani RN, Mascarenhas KO, et al. The gene doublesex of the fruit fly Anastrepha obliqua (Diptera, Tephritidae). Genetics 2005;171:849–54.

19. Ruiz MF, Elrín-López JM, Stefani RN, et al. The gene doublesex of Anastrepha fruit flies (Diptera, Tephritidae) and its evolution in insects. Dev Genes Evol 2007;217:725–31.

20. Permpoon R, Aketarawong N, Thanaphum S. Isolation and characterization of doublesex homologues in the Bactrocera species: B. dorsalis (Hendel) and B. correcta (Bezzi) and their putative promoter regulatory regions. Genetica 2011;139:113–27.

21. Chen SL, Dai SM, Lu KH, et al. Female-specific doublesex dsRNA interrupts yolk protein gene expression and reproductive ability in oriental fruit fly, Bactrocera dorsalis (Hendel). Insect Biochem Mol Biol 2008;38:155–65.

22. Lagos D, Ruiz MF, Sánchez L, et al. Isolation and characterization of the Bactrocera oleae genes orthologous to the sex determining Sex-lethal and doublesex genes of Drosophila melanogaster. Gene 2005;348:111–21.

23. Shearmur DCA, Frommer M. The Bactrocera tryoni homologue of the Drosophila melanogaster sex-determination gene doublesex. Insect Mol Biol 1998;7:355–6.

24. Burtis KC, Baker BS. Drosophila doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell 1989;56:997–1010.

25. Concha C, Li F, Scott MJ. Conservation and sex-specific splicing of the doublesex gene in the economically important pest species Lucilia cuprina. J Genet 2010;89:279–85.

26. Sievert V, Kuhn S, Traut W. Expression of the sex determining cascade genes Sex-lethal and doublesex in the phorid fly Megastasis scalaris. Genome 1997;40:211–4.

27. Kuhn S, Sievert V, Traut W. The sex-determining doublesex gene in the fly Megastasis scalaris: conserved structure and sex-specific splicing. Genome 2000;43:1011–20.

28. Hediger M, Burghardt G, Siegenthaler C, et al. Sex determination in Drosophila melanogaster and Musca domestica converges at the level of the terminal regulator doublesex. Dev Genes Evol 2010;220:29–42.

29. Greverink E, Beukeboom LW. Phylogenetic distribution and evolutionary dynamics of the sex determination genes doublesex and transformer in insects. Sex Dev 2014;8:38–49.

30. Bopp D, Saccone G, Beye M. Sex determination in insects: variations on a common theme. Sex Dev 2014;8:20–8.

31. Scott MJ, Pimsler ML, Tarone AM. Sex determination mechanisms in the Calliphoridae (blow flies). Sex Dev 2014;8:29–37.

32. Nagaraju J, Gopinath G, Sharma V, et al. Lepidopteran sex determination: a cascade of surprises. Sex Dev 2014;8:104–12.

33. Hildreth PE. Doublesex, a recessive gene that transforms both males and females of Drosophila into intersexes. Genetics 1965;51:659.

34. Baker BS, Wolfnier MF. A molecular analysis of doublesex, a bifunctional gene that controls both male and female sexual differentiation in Drosophila melanogaster. Genes Dev 1988;2:477–89.

35. Chen MM, Bockmann V, Gopinath G, Sharma V, et al. Sex determination in the Calliphoridae (blow flies). Sex Dev 2014;8:29–37.

36. Nagaraju J, Gopinath G, Sharma V, et al. Lepidopteran sex determination: a cascade of surprises. Sex Dev 2014;8:104–12.

37. Raymond CS, Shamu CE, Shen MM, et al. Evidence for evolutionary conservation of sex-determining genes. Nature 1998;391:691–5.

38. Raymond CS, Kettlewell H, Janssen J-P. The function of Dmrt genes in vertebrate development: It is not just about sex. Dev Biol 2007;310:1–9.

39. Kopp A. Dmrt genes in the development and evolution of sexual dimorphism. Trends Genet 2012;28:175–84.
41. Bellefroid EJ, Leclère L, Saulnier A, et al. Expanding roles for the evolutionarily conserved Dmrt sex transcriptional regulators during embryogenesis. Cell Mol Life Sci 2013;70:3829–45.
42. Erdman SE, Chen HJ, Burtis KC. Functional and genetic characterization of the oligomerization and DNA binding properties of the Drosophila doublesex proteins. Genetics 1996;144:1639–52.
43. An W, Cho S, Ishii H, et al. Sex-specific and non-sex-specific oligomerization domains in both of the doublesex transcription factors from Drosophila melanogaster. Mol Cell Biol 1996;16:3106–11.
44. Zhu L, Wilken J, Phillips NB, et al. Drosophila intersex. Dev Biol 2005;288:528–44.
50. Garrett-Engele CM, Siegal ML, Manoli DS, et al. Doublesex and mab-3 regulate the A/P organizer to direct sex determination. Dev Dyn 2012;241:1076–90.
51. Waterbury JA, Jackson LL, Schedl P. Analysis of the doublesex gene in Drosophila melanogaster: role on sexual differentiation and behavior and dependence on intersex. Genetics 1999;152:1653–67.
52. Siegal ML, Baker BS. Functional conservation and divergence of intersex, a gene required for female sexual development in Drosophila, is expressed in both sexes and functions together with doublesex to regulate terminal differentiation. Development 2002;129:4661–75.
53. Arunkumar KP, Nagaraju J. Drosophila intersex orthologue in the silkworm, Bombyx mori and related species. Genetica 2011;139:141–7.
54. Li H, Baker BS. Her, a gene required for sexual differentiation in Drosophila, encodes a zinc finger protein with characteristics of ZFY-like proteins and is expressed independently of the sex determination hierarchy. Development 1998;125:225–35.
55. Barmina O, Kopp A. Sex-specific expression of a HOX gene associated with rapid morphological evolution. Dev Biol 2007;311:277–86.
56. Barmina O, Gonzalo M, McIntyre LM, et al. Sex- and segment-specific modulation of gene expression profiles in Drosophila. Dev Biol 2005;288:528–44.
57. Tanaka K, Barmina O, Sanders LE, et al. Evolution of sex-specific traits through changes in HOX-dependent doublesex expression. PLoS Biol 2011;9:e1001131.
58. Devi TR, Shyamala B. Male- and female-specific variants of doublesex gene products have different roles to play towards regulation of Sex combs reduced expression and sex comb morphogenesis in Drosophila. J Biosci (Bangalore) 2013;38:455–60.
78. Shepherd B, Garabedian MJ, Hung M-C, et al. Developmental control of Drosophila yolk protein 1 gene by cis-acting DNA elements. *Cold Spring Harbor Symp Quant Biol* 1985;50:521–6.

79. Garabedian MJ, Shepherd BM, Wensink PC. A tissue-specific transcription enhancer from the *Drosophila* yolk protein 1 gene. *Cell* 1986;45:859–67.

80. Luo SD, Shi GW, Baker BS. Direct targets of the *Drosophila* melanogaster DSXF protein and the evolution of sexual development. *Development* 2011;138:2761–71.

81. Keisman EL, Baker BS. The *Drosophila* sex determination hierarchy modulates wingless and decapentaplegic signaling to deploy dachshund sex-specifically in the genital imaginal disc. Development 2001;128:1643–56.

82. Sánchez L, Gorfinikel N, Guerrero I. Sex determination genes control the development of the *Drosophila* genital disc, modulating the response to Hedgehog, Wingless and Decapentaplegic signals. *Development* 2001;128:1033–43.

83. Shukla JN, Nagaraju J. Two female-specific DSX proteins are encoded by the sex-specific transcripts of *dsx*, and are required for female sexual differentiation in two wild silkmoth species, *Antheraea assauna* and *Antheraea mylitta* (*Lepidoptera, Saturniidae*). *Insect Biochem Mol Biol* 2010;40:672–82.

84. Shukla JN, Jadhav S, Nagaraju J. Novel female-specific splice form of *dsx* in the silkworm, Bombyx mori. *Genetica* 2011;139:23–31.

85. Duan J, Xu H, Guo H, et al. New insights into the genomic organization and splicing of the *doublesex* gene, a terminal regulator of sexual differentiation in the silkworm Bombyx mori. *PLoS One* 2013;8:e79703.

86. Gotoh H, Cornette R, Koshikawa S, et al. Juvenile hormone regulates extreme mandible growth in male Stag beetles. *PLoS One* 2011;6:e21139.

87. Loehlin DW, Carroll SB. Evolutionary biology: sex, lies and butterflies. *Nature* 2014;507:172–3.

88. Hempel LU, Oliver B. Sex-specific *doublesexM* expression in subsets of Drosophila somatic gonad cells. *BMC Dev Biol* 2007;7:113.

89. Lee G, Hall JC, Park JH. Doublesex gene expression in the central nervous system of *Drosophila melanogaster*. *J Neurogenet* 2002;16:229–48.

90. Sanders LE, Arbeitman MN. Doublesex establishes sexual dimorphism in the *Drosophila* central nervous system in an isoform-dependent manner by directing cell number. *Dev Biol* 2008;320:378–90.

91. Rideout EJ, Dornan AJ, Neville MC, et al. Control of sexual differentiation and behavior by the *doublesex* gene in *Drosophila melanogaster*. *Nat Neurosci* 2010;13:458–66.

92. Mellert DJ, Knapp J-M, Manoli DS, et al. Midline crossing by gustatory receptor neuron axons is regulated by fruitless, *doublesex* and the *Roundabout* receptors. *Development* 2010;137:323–32.

93. Zhou C, Pan Y, Robinett Carmen C, et al. Central brain neurons expressing *doublesex* regulate female receptivity in *Drosophila*. *Neuron* 2014;83:149–63.

94. Rezával C, Nojima T, Neville Megan C, et al. Sexually dimorphic octopaminergic neurons modulate female post-mating behaviors in *Drosophila*. *Curr Biol* 2014;24:725–30.

95. Dauwalder B. The roles of fruitless and *doublesex* in the control of male courtship. *Int Rev Neurobiol* 2011, 87–105.

96. Robinett CC, Vaughan AG, Knapp J-M, et al. Sex and the single cell. II. There is a time and place for sex. *PLoS Biol* 2010;8:e1000365.

97. Permpoon R, Thanaphum S. Isolation and characterization of oligomerization domain I and II coding regions of *doublesex* genes in agricultural fruit flies (*Diptera: Tephritidae*). *Eur J Entomol* 2010;107.

98. Hughes AL. Runaway evolution of the male-specific exon of the *doublesex* gene in *Diptera*. *Gene* 2013;472:1–6.

99. Sobrinho IS, de Brito RA. Positive and purifying selection influence the evolution of *doublesex* in the *Anastrepha fraterculus* species group. *PLoS One* 2012;7:e33446-e.

100. Rogers WA, Salomone JR, Tacy DJ, et al. Recurrent modification of a conserved cis-regulatory element underlies fruit fly pigmentation diversity. *PLoS Genet* 2013;9:e1003740.

101. Gompel N, Carroll SB. Genetic mechanisms and constraints governing the evolution of correlated traits in drosophilid flies. *Nature* 2003;424:991–5.

102. Jeong S, Rokas A, Carroll SB. Regulation of body pigmentation by the Abdominal-B Hox protein and its gain and loss in *Drosophila* evolution. *Cell* 2006;125:1387–99.

103. Clough E, Oliver B. Genomics of sex determination in *Drosophila*. *Brief Funct Genomics* 2012;11:387–94.

104. Verhulst EC, Beukeboom LW, van de Zande L. Maternal control of haplodiploid sex determination in the wasp *Nasonia*. *Science* 2010;328:620–3.