Insects exhibit a variety of sex determining mechanisms including male or female heterogamety and haplodiploidy. The primary signal that starts sex determination is processed by a cascade of genes ending with the conserved switch doublesex that controls sexual differentiation. Transformer is the doublesex splicing regulator and has been found in all examined insects, indicating its ancestral function as a sex-determining gene. Despite this conserved function, the variation in sex determining mechanisms has evolved.

Introduction

Sexual development, one of the most important and widespread developmental processes, essentially entails one simple choice: becoming male or female. Although this suggests a common underlying genetic mechanism, an astoundingly diverse array of pathways regulates sex determination. Sanchez [11**] reviewed current knowledge of sex determining mechanisms with a focus on primary signals. In flies (Diptera) the gene doublesex (dsx) acts as a conserved major switch at the bottom of the sex-determining cascade [11**,2,3]. The part of the sex-determining cascade where the primary signal is transmitted to dsx has, until recently, received less attention. Data from Hymenoptera enabled comparison of sex determination mechanisms at a wider level within the insect class. This has directed focus towards transformer (tra) as a central player in the evolution of sex determination in insects. In this review, we describe how tra translates different primary signals into one of two sex-specific pathways and consider how its function may serve as the key process around which insect sex determination mechanisms have evolved.

Drosophila sex determination: the reference

Insect sex determination has been extensively examined in Drosophila melanogaster [4–6] and has served as a reference for all other insects [7–9]. In Drosophila, the upstream genomic region of Sexlethal (Sxl) contains two promoters: Pearly and Pmaintenance, which is the late promoter. The primary signal is based on the concentration of X-linked signal elements (XSE) that activate the early Sxl promoter in diploid XX individuals only [10*] (see Figure 1). Transcription from the early promoter of Sxl yields a transcript that is spliced to encode a functional early SXL protein. This splice pattern depends on the use of the 5′ splice site from the early exon E1, whereas in later stages the 5′ splice site of late exon 2 is used [11]. It results in the default exclusion of exon 3— that contains in-frame stop codons— in the early transcript. This early protein enables the production of a functional late SXL protein, which further maintains female-specific Sxl splicing by auto regulation. SXL also directs cryptic splicing of tra by binding to a polypyrimidine tract in the first tra intron and forces the general splicing factor U2AF to use the female-specific 3′ splice site in exon 3 instead of the nonsex-specific 3′ splice site in exon 2. This tra transcript yields a functional TRA protein [12–14], which interacts with the nonsex-specific transformer2 protein (TRA2) [15] and binds to the dsx transcript in the middle of exon 4, called the dsx repeat element (dsxRE). This dsxRE contains six copies of the 13 nucleotide sequence TG(T/A) (T/A)(A)G(T/A)ATCAAC[16]. Located between repeat element five and six of the dsxRE is a purine-rich enhancer element (PRE) which is required for the specific binding of TRA2 to the dsxRE [17]. The binding of TRA/TRA2 to the dsxRE and PRE sites retains exon four in the dsx pre-mRNA resulting in female-specific splicing of dsx at the bottom of the cascade [18–20], generating a female-specific DSX protein.

In XY males the level of XSEs is insufficient for early Sxl transcription and no early SXL protein is synthesized, preventing the auto regulatory loop from establishing. As a result, Sxl pre-mRNA from the late promoter is male specifically spliced by default, yielding a truncated nonfunctional SXL protein. The absence of SXL leads to the ‘default’ splicing of the tra pre-mRNA and a nonfunctional TRA protein. Without TRA, dsx pre-mRNA is spliced by default generating a male-specific DSX protein.
The TRA/TRA2 complex also regulates female-specific splicing of *fruitless* (*fru*), which yields a nonfunctional FRU protein [21], while absence of TRA leads to male-specific *fru* splicing and a functional FRU protein. *Fru* is not part of the (morphological) sex determination pathway but seems conserved in insects [22,23] and reviewed in [24]. It is conserved in both gene structure and its function as a determiner of male sexual behavior.

**Conservation of sex-determining genes in insects**

There is a common pattern in insect sex-determining cascades: at the bottom is *dsx*, which has been identified for all examined dipteran [25–32] and hymenopteran insect species [33,34]. *DSX* has two characteristic domains: a DNA binding domain (DM or OD1) and an oligomerization domain (*dsx dimer* or OD2). Oliveira *et al.* [34] showed for several insect species that amino acid alignment of these domains followed the established phylogeny, suggesting their importance in sexual differentiation. Conservation of *dsx* is in agreement with Wilkins’ theory [35] stating that regulatory elements are recruited into sex-determining pathways, causing divergence towards the top, while *dsx* remains conserved at the bottom. However, as more and more sex-determining cascades are elucidated, it appears that conservation is not only at the level of *dsx*, but also at the regulation of its sex-specific splicing.

**Transformer**

After the initial identification in *Ceratitis capitata* [36], also in other insect species (*Anastrepha* sp., *Bactrocera oleae*, *Lucilia cuprina*, *Musca domestica*, *Apis mellifera* and *Nasonia vitripennis*), *dsx* splicing regulator genes have been identified that all appear to be *D. melanogaster tra* orthologs [37,38,39,40,41,42]. A *tra* ortholog has not (yet) been identified in Lepidoptera, perhaps because of the strong sequence divergence that characterizes *tra* evolution. In *Bombyx mori* no *tra* ortholog has been found based on the lack of *dsxRE* or PRE binding sites on *Bmdsx* and the presumed default mode of female-specific splicing [43,44]. However, *dsxRE/PRE* binding sites have only been identified in dipterans based on homology to *Drosophila* and are probably so diverged that recognition of these sites in other orders is difficult. Cho *et al.* [33] reported the absence of *dsxRE/PRE* binding sites in the hymenopteran *A. mellifera* and suggested that *Amdsx* follows default female-specific splicing, similar to *B. mori*. 

Figure 1

The sex determination cascade in *Drosophila melanogaster*. Boxes with numbers indicate transcripts with exon number and relative exon size. Dark gray transcripts are full length and yield a functional protein. Light gray transcripts contain early in-frame stop codons and give truncated nonfunctional proteins. Transcripts are designated by their gene name in italic. Proteins are designated by their capital gene name. Superscript F and M stand for female-specific transcript or protein or male-specific transcript or protein, respectively. *pSxl* indicates *Sxl* transcript from the early promoter, *pαSxl* indicates *Sxl* from the late promoter, *DSX* directs primarily male morphology but also interacts a little with FRU to direct male behavior, which is indicated by a smaller arrow. The gray bottom half of the sex-determining cascade shows the conserved part of the cascade.
Nevertheless, a functional tra ortholog has recently been found in A. mellifera, termed feminizer, which is functionally and structurally similar to tra [39,45]. Interestingly, a comparison between dsx of hymenopterans A. mellifera and N. vitripennis revealed putative dsxRE/PRE binding sites that indeed have severely diverged from those of Diptera [23]. Similar dsxRE/PRE binding sites have been identified in the Nasonia fruitless gene [23] and in Netra [42]. Hence, the illustrious feminizing factor on the W chromosome in B. mori [46], may be an unconfirmed ortholog of tra that also functions as active feminizing factor. In the mosquito Anopheles gambiae and the phorid fly Megaselia scalaris, only dsx has been found to date, but tra is surmised to be the regulating splice factor of Agdsx and Msdsx since dsxRE/PRE binding sites have been identified [31,27].

The functional importance of these dsx splicing regulators in female development has been shown by RNA interference (RNAi) in early embryos, which resulted in male-specific dsx splicing [36,37,38,45,40,41,42]. The subsequent transformation of otherwise female offspring was not always complete and resulted in intersexes with various stages of masculinization, while male development remained unaffected. Although these tra genes differ largely in their nucleotide and amino acid composition, their function as the sex-specific splicing regulator of dsx appears conserved [9]. Strikingly, a conserved pattern of tra regulation in all insect species is the sex-specific alternative splicing that produces transcripts in males that contain early in-frame stop codons and yield no protein. Only the female-specific splicing of tra pre-mRNA yields a full-length transcript and leads to TRA protein production. This active TRA protein directs female specific splicing of dsx, implying a functional conservation in insect sex determination.

Tra regulation of dsx apparently constitutes the axis of insect sex determination. It likely acquired its function in the early ancestors of the insects, as tra orthologs are found throughout the insect class including Diptera, Hymenoptera and Coleoptera (Figure 2), but apparently has no sex determining function in the crustacean Daphnia [48]. The large sequence divergence indicates that tra conservation is predominantly at the functional and less at the structural level. This becomes apparent when TRA protein sequences are compared among species. Comparison of the insect classification to a phylogeny based on the TRA protein sequence reveals that its evolution has followed species divergence confirming that conservation lies in function rather than sequence (Figure 3). Strikingly, alignment of TRA orthologs shows that only the proline and Arg/Ser rich regions are conserved throughout the examined insect species, reflecting their function as
splice factor. One additional domain is conserved in Hymenoptera only, a second domain is conserved in all species except Drosophila [48] and a third domain is conserved in all Diptera (Figure 3). The second domain may function in tra auto regulation that is absent in D. melanogaster and replaced by Sxl. The other two domains are apparently not involved in tra splicing but may have other unknown functions.

**Doublesex**

Dsx belongs to a class of DM domain containing genes that are conserved outside the insect class, and regulates sex determination in both vertebrates [43,49–53] and invertebrates [54–56]. Tra, on the other hand, has been identified as a dsx splicing factor in insects only [48]. A comparison of sex-determining cascades in different insect groups reveals that diversity essentially starts at the level of regulation of tra splicing and that tra acts as receptor for various primary signals. These signals are very diverse and include X-chromosome dose [10*], a male determining factor on the Y chromosome and/or autosome [36**,57], and a feminizing factor on the W chromosome [47] in diploids, as well as complementary sex determination and genomic imprinting sex determination in haplodiploids [42**,58,59**]. Therefore, the conserved part of the insect sex-determining cascade must be extended to include tra, and from an evolutionary perspective, tra appears to serve as the gene around which flexibility in sex determination is manifested.

**Evolution of tra regulation...**

In all tra (or fem) containing insects except D. melanogaster, female specific splicing of tra involves an auto regulatory loop, in which the TRA protein is required for female
specific splicing of \( tra \) pre-mRNA [36**,60,40,38,45,41*,42**]. Maternal input of \( tra \) mRNA or protein into the eggs has been demonstrated for all examined species except \( D. melanogaster \) and \( A. mellifera \) and has been surmised to start \( tra \) auto regulation. In the haplodiploid \( N. vitripennis \) it has been shown for the first time that sufficient levels of maternally provided \( tra \) mRNA in eggs are required for female development. Knockdown of \( tra \) in mothers leads to a diminished amount of maternally provided \( tra \) mRNA in eggs and results in diploid males [42**]. In \( Drosophila, Sxl \) has been recruited upstream of \( tra \) and is female specifically regulated through its own auto regulatory loop. However, Siera and Cline [61*] showed that \( tra \) auto regulation may also be ancestral in \( Drosophila \) since a positive feedback loop of \( tra \) still operates through \( Sxl \), which in turn regulates \( tra \) splicing. \( Tra \) regulation by X chromosome dose may occur outside the Drosophilidae but most likely in the absence of \( Sxl \). How this is accomplished remains to be investigated. In \( A. mellifera \) a duplication of \( fem \) has been recruited into the sex-determining cascade and initiates female-specific splicing of \( fem \) transcripts [39]. Overall, the maternal provision of \( tra \) to eggs appears to be an ancestral regulatory mechanism, as all deviations from this system are of recent origin.

Two intriguing questions are how variations in \( tra \) regulation can account for the large variety in sex determining mechanisms in insects and how turnovers in signals and genes controlling \( tra \) can occur during evolution. A comparison between diploid and haplodiploid sex determination is particularly illustrative as a ‘flipover’ of \( tra \) regulation may lie at the basis of the difference between these two modes of sex determination.

**in diploid insects**

The principle of \( tra \) regulation in diploid insects is that the paternally inherited genome inhibits female splicing of \( tra \) in a variety of ways. A diverse array of primary signals directly or indirectly regulates sex-specific splicing of \( tra \). A common theme in a number of dipteran insects is a masculinizing (M) factor on the Y chromosome that is transmitted through males only. M actively blocks the transcription or translation of \( tra \), preventing the auto-regulatory loop from establishing in ways that are not yet well understood [36**,40,41*]. Thus, the paternally inherited \( M \) factor actively inhibits female development in XX individuals. In \( Drosophila \) the presence of twice as much X signal elements in XX animals directs female-specific transcription of \( Sxl \) and starts the female-specific path of the sex-determining cascade [10*].

A special case is Lepidoptera in which females are the heterogametic sex (ZW females, ZZ males) [46*]. As only females contribute a W chromosome containing a feminizing factor, males passively promote male development. In their theoretical treatise on the evolution of sex determination, Pomiankowski et al. [62] inferred how, based upon initial allelic variation for \( dsx (dsx^M) \): masculinizing factor and \( dsx^f \): feminizing factor) conversion to \( tra \) regulation can evolve. Assuming that \( TRA \) splices only \( dsx^f \) into a female form but not \( dsx^M \), a mutation creating a stop codon in the \( tra \) exon 2 (\( tra^S \)) would be favourable for \( tra^S/tra^M \) males, as no female DSX is produced. Simulations showed that this could eventually lead to elimination of \( dsx^M \) and to evolution of female heterogamy for \( tra^S/tra^* \) [62].

Two general rules emerge from comparing the different primary signals of diploid insects. First, a paternally derived genome is always necessary for male development and second, actively or passively, it always prevents the activation of \( tra \) (or \( Sxl \)).

**in haplodiploid insects**

A number of insect groups, including thrips (Thysanoptera), beetles (Coleoptera) and all Hymenoptera, have haplodiploid sex determination: males are haploid, develop from unfertilized eggs and only inherit a maternal genome, whereas females are diploid, develop from fertilized eggs and inherit a paternal and a maternal genome. It is therefore impossible for the paternally inherited genome to have a masculinizing effect as in diploids. Instead, the paternal genome must have acquired a complete reversal in sex determining function, that is by feminizing rather than masculinizing.

Until recently, knowledge about primary signals in haplodiploid species was limited to complementary sex determination (csd) in which gender is determined by the allelic state of the complementary sex determiner (csd) gene. Although csd has been inferred for more than 60 hymenopterans [63], the \( csd \) gene has been characterized in the honey bee only [58]. Females are heterozygous and males hemizygous at this locus, but the biochemical details of CSD function are not yet completely known [39*,45] . Interestingly, the csd primary signal can also be based on multiple loci (ml-csd) [64]. However, in all csd cases the paternally contributed genome provides the second csd allele that is required for female development.

In another hymenopteran, \( Nasonia \), csd has been ruled out as the primary signal [65]. In \( Nasonia \) female-specific \( tra \) is maternally provided to eggs. In embryos from fertilized eggs early zygotic expression of \( tra \) is higher than in embryos from unfertilized eggs, which initiates an auto regulatory loop of \( tra \) and results in female \( dsx \) splicing [42**]. In embryos from unfertilized eggs no early zygotic expression of \( tra \) occurs and the auto regulatory loop does not establish, leading to male-specific \( tra \) and \( dsx \) splicing. The difference in zygotic \( tra \) expression cannot be explained by masculinizing factors. Instead, the \( tra \) gene, or a trans acting factor that influences \( tra \) expression, on the maternal genome is rendered inactive by maternal
imprinting. In an unfertilized egg, only this maternally imprinted gene is present that prevents tra transcription and precludes the auto regulatory loop. In a fertilized egg, both a maternal and a paternal genome are present. The paternal set has an active, nonsilenced gene so that tra will be transcribed enabling the maternally provided tra mRNA to start auto regulation of tra, eventually leading to female development [42**]. A mutant stain of N. cinctipes that produces gynandromorphs and females from haploid unfertilized eggs [66,67] may be explained by incomplete imprinting in the maternal germ line.

The honeybee and Nasonia results indicate that, in contrast to diploids, the paternally inherited chromatin is always necessary for female development in haplodiploids and, actively or passively, promotes the activation of tra.

Conclusions and outlook
Much has been learned about sex determination in mammals [68] and plants [69–71], but comparative work on a variety of insect species has been particularly fruitful for understanding how sex determination regulation evolves. Twenty-five years ago Nöthiger and Steinmann-Zwicky [5] proposed that sex determination in all insects is based on a single principle. We can conclude that these authors were partly correct. The sex-specific regulation of dsx splicing by tra appears to constitute a conserved gene axis in all insects. Clearly, the central gene around which diversity evolves is not Sxl, as was suggested from studies in Drosophila, but tra. A striking example of the central role of tra in the evolution of insect sex determination is the complete reversal in the paternal regulation of tra upon the separation of Hymenoptera and Diptera.

Although Drosophila has XX–XY sex determination, its processing of the primary signal and regulation of tra is different from all other flies with this mode of sex determination. A number of other insect groups likely rely on sex chromosome dose as primary signal, such as species with XX–XO sex determination (e.g. grasshoppers (Orthoptera)), ZO–ZZ sex determination (e.g. some Lepidoptera [46**]) and paternal X chromosome inactivation (e.g. coccids (Homoptera) and Sciarid flies [1**]). Whether and how tra regulation occurs in these groups remains an interesting unanswered question. In general, a broader taxonomic screen of how primary signals are processed by tra would be worthwhile as our current knowledge is virtually restricted to Diptera and Hymenoptera. Exploiting next generation sequencing technology will greatly expedite such an endeavor.

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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

Developmental mechanisms, patterning and evolution


In this paper it has been demonstrated for the first time that the transformer gene of Ceratitis is able to autoregulate, differently to the Drosophila orthologue. A transient depletion of maternal and zygotic Cctra mRNA by RNAi, causes complete masculinization of XX individuals and a permanent shift of the Cctra splicing into the male mode.


This study identified a tra homolog (fem) in honeybees and shows that cd is not an ancestral gene but arose from a duplication of fem.


First study to show that the F factor in M. domestica is indeed tra, by carefully studying two mutant strains for F and relating findings to tra function.


This study showed for the first time that imprinting may lie at the basis of sex determination in a hymenopteran without cd.


Comprehensive review about sex determination in Lepidoptera and Trichoptera that share a female-heterogametic sex chromosome system.


