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*Brief communication*

# 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*

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Sexually dimorphic characters have two-fold complexities in pattern formation as they have to get input from both somatic sex determination as well as the positional determining regulators. Sex comb development in *Drosophila* requires functions of the somatic sex-determining gene *doublesex* and the homeotic gene *Sex combs reduced*. Attempts have not been made to decipher the role of *dsx* in imparting sexually dimorphic expression of SCR and the differential function of sex-specific variants of *dsx* products in sex comb development. Our results in this study indicate that male-like pattern of SCR expression is independent of *dsx* function, and $dsx^F$ must be responsible for bringing about dimorphism in SCR expression, whereas $dsx^M$ function is required with *Scr* for the morphogenesis of sex comb.

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## 1. Introduction

Sex comb in *Drosophila* is a sexually dimorphic character limited to male flies. It is an evolutionarily recent and highly dynamic morphological trait. It has been shown to have selective advantage during mating (Spieth 1952; Markow *et al.* 1996; Polak *et al.* 2004), and hence variations in its morphology might have had critical evolutionary consequences. While its presence is confined to the Sophophora group of *Drosophila* (Lakovaara and Saura 1982; Lemeunier *et al.* 1986; Kopp and True 2002), within the group we see a vast divergence amongst different species with respect to the number and organization of the sex comb teeth (Lemeunier *et al.* 1986; Barmina and Kopp 2007; Atallah *et al.* 2009; Tanaka *et al.* 2009). Study of the developmental mechanisms controlling such sex-specific and evolutionarily diverse characters provides us a unique opportunity to understand the interplay between the positional regulatory cues and the somatic sex-determining pathway, which has resulted in sex-specific gene expression, and the development of a sexually dimorphic structure. Although there are exclusive descriptions and discussions of many of the sex-limited traits and their ecological and evolutionary implications, very few attempts have been made to understand the developmental basis of the appearance of these traits (Wilkins 2004; Williams and Carroll 2009). Recently fine molecular genetic dissection of the interactions between *bric a brac* regulatory sequences, *Abdominal-B* gene products and *double sex* (*dsx*) gene products have revealed an important genetic switch involved in evolution of abdominal pigmentation, a sexually dimorphic character in *Drosophila* (Kopp *et al.* 2000; Williams *et al.* 2008).

In *Drosophila melanogaster,* sex comb is represented by an array of 9–14 highly chitinized bristles arranged longitudinal to the axis of the first tarsal segment (ts1) of the prothoracic pair of legs (Tokunaga 1962). The positional specificity of sex comb to thoracic segment 1 (T1) along the anterior posterior body axis is provided by the homeotic selector gene *Sex combs reduced* (*Scr*) (Rogers *et al.* 1997; Hannah-Alava 1964; Kaufman 1980; Pattatucci *et al.* 1991; Shroff *et al.* 2007; Kopp 2011). Further, evolutionary origin and diversity of sex comb morphology amongst *Drosophila* species is regulated by the presence/absence or variation in expression domains of SCR within the tarsal segments of the developing prothoracic leg discs (Barmina and Kopp 2007; Randsholt and Santamaria 2008; Devi *et al.* 2013). The sexual dimorphism of the trait, by primary observation, also

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seems to have a correlation to SCR expression. As evidenced by previous works (Barmina and Kopp 2007; Tanaka *et al.* 2011) and the present analysis in the early pupal discs, SCR shows sexually dimorphic expression in the prothoracic leg discs.

*doublesex* in *D. melanogaster* has been shown to be a critical bifunctional gene that regulates both the male and female somatic sexual differentiation (Baker and Wolfner 1988; Burtis and Baker 1989; Burtis *et al.* 1991; Coschigano and Wensink 1993; Hutson and Bownes 2003; Williams *et al.* 2008). In general, loss of function *dsx* mutants (*dsx*) show intersex phenotypes, sex comb teeth, genitalia and improper formation of abdominal pigmentation in males, whereas they tend to show low level of yolk protein, which usually is seen only in females. On the contrary, the *dsx* females show incompletely formed sex-comb-like bristles, abdominal pigmentation and malformed genitalia (Baker and Ridge 1980; Bownes and Nothiger 1981). In wild type animals, *dsx* expression in the prothoracic leg is seen in spatially defined regions, the first tarsal segment, which is responsible for sex comb development (Tanaka *et al.* 2011). *dsx*-null clones in male foreleg fail to form proper sex comb with complete chitinization and rotation of bristles (Baker and Ridge 1980), while formation of male-specific sex comb is activated by the ubiquitous expression of the male-specific *dsx* product $Dsx^M$ (Jursnich and Burtis 1993; Waterbury *et al.* 1999). But, during the development of sex comb, as a somatic sex-determining gene, the nature of interaction that the *dsx* splice variants share with the spatial cue provider *Scr*, and their role in generation of dimorphic SCR expression is not completely understood.

In the present study we have analysed the SCR expression pattern and the phenotypes in *dsx* mutant background. We have used two mutant alleles of *doublesex*: $dsx^{136}$ is a recessive allele, and it is a male-specific mutant. $dsx^{136}$ behaves as a wild type allele in XX; $dsx^{136}/dsx$ female, thus fully encoding the female function (Baker and Ridge 1980). XY; $dsx^{136}/dsx$ males are transformed into intersexes, since $dsx^{136}$ cannot make the male-specific product. $dsx^{1}$ makes an antimorph which could function as a dominant negative mutant (Hildreth 1965; Shirangi *et al.* 2006). In effect, it is a null allele wherein neither the male- nor the female-specific functional products are made and both chromosomally male and female flies get transformed into intersexes.

We found that the male-specific expression pattern of SCR is not dependent on the expression of the male-specific *dsx* product. The dimorphic expression of SCR seems to be rather achieved by the suppression of *Scr* at the distal region of the ts1 by $Dsx^F$ (*dsx* female-specific gene product). Further, male-specific ts1 expression pattern of SCR alone is insufficient for complete formation of sex comb. The function of $Dsx^M$ is needed along with *Scr* in order to facilitate the proper chitinization and rotation of the bristles. The interesting observations of our experiments are that *dsx* interacts with *Scr* at two levels, one through the $Dsx^F$ to maintain the dimorphic expression, and another through $Dsx^M$ to activate probable downstream effectors which might together with *Scr,* function to form the sex comb.

## 2. Materials and methods

### 2.1 *Fly strains*

The fly stocks $dsx^{1}$ $p^{P}$/ *TM3,Tb* and $dsx^{136}$ / *TM3,Tb* of *D. melanogaster* are gift of Kenneth C Burtis. To obtain the necessary genotypes, $dsx^{1}$ $p^{P}$/ *TM3,Tb* virgin females were crossed with $dsx^{136}$ / *TM3,Tb* males. The resulting $F_1$ flies were screened for $dsx^{136}$/ $dsx,^{1}$ $p^{P}$ XX and XY individuals. Flies with the genotype $dsx^{136}$/ $dsx^{1}$,$p^{p}$ were identified by their non-tubby phenotype.

### 2.2 *Immunostaining*

Non-tubby prepupae of the genotype $dsx^{136}$/ $dsx^{1}$, $p^{p}$ were selected. Zero hour pupae were collected from the culture bottles, sexed depending on the size of the developing gonad (Geigy 1931; Aboim 1945). The prepupae were placed on a moist tissue paper in a petridish and aged at 22±2°C for 3–4 h. The 3–4 h pupae were dissected for prothoracic imaginal discs in cold PBS.

The anti-SCR immunostaining was conducted as described by Devi *et al.* (2013), with mouse anti-SCR 6H4.1 (DSHB) at 1:60 dilution. A Biotinylated Goat anti-mouse IgG, at concentration of 1:1000 was used as secondary antibody, and streptavidin-horse radish peroxidase at a dilution of 1:500 was used as a signal amplifying enzyme complexin.

### 2.3 *PCR*

Zero hour non-tubby prepupae of the genotype $dsx^{136}$/ $dsx^{1}$,$p^{p}$ were sexed on the basis of gonadal size and the male and the female prepupae were allowed to grow in separate culture vials. Five such replicates each with approximately 20 sexed prepupae were checked for each sex. The flies after eclosion were checked carefully for their phenotype and pigmentation development. Twenty-five flies from each category were used for PCR analysis in order to confirm the XX or XY chromosomal complement of the mutant flies of the genotype $dsx^{136}$/*dsx1,* $p^{p}$. Direct correlation was observed between the gonadal sexing, phenotype and the chromosome complement. PCR amplification was done for the fertility gene *kl3* situated on the non-pseudoautosomal region of the Y chromosome. Forward primer (kl3F) with sequence 5′ GATCCTACATCAATACAGCCAC 3′ and Reverse primer (kl3R) with sequence 5′AGTACTGGTAGCTCAATGCG 3′ were directed against the exon 1 of male fertility gene *kl3*. PCR

reactions were performed in a final volume of 50 μL containing 100 pmol/μL of the primer (kl3R, kl3F). Amplification was carried out by 35 cycle repeats of a thermo profile of 95°C for 45 s. (denaturation), 57°C for 30 s. (annealing) and 72°C for 45 s. (extension). An amplified product specifies XY chromosomal complement and absence of product specifies XX chromosomal complement of the mutant individuals used. PCR was also conducted with wild type male and female individuals of *D. melanogaster* as a control for the experiment.

## 3. Results and discussion

Development of the sex-specific and segment-specific trait sex comb should have input from both the spatial specifying cues

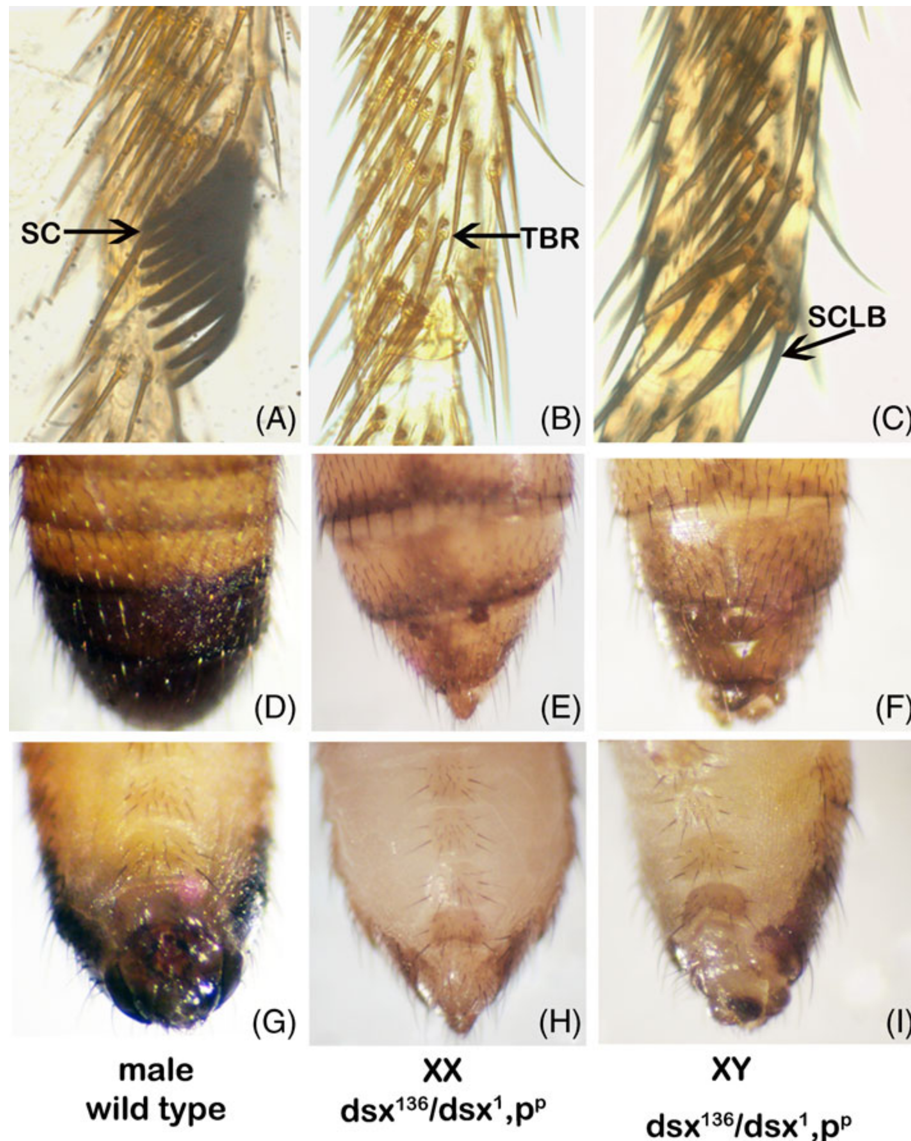


**Figure 1.** Morphological analysis of tarsal segment of the prothoracic leg, pigmentation phenotype and abdominal tip of $dsx^{136}/dsx^{1},p^{p}$ mutant and wild type *D. melanogaster*. First tarsal segment (ts1) in (**a**) wild type male showing sex comb, (**b**) XX $dsx^{136}/dsx^{1},p^{p}$ mutant showing the transverse bristle rows (TBRs), (**c**) XY $dsx^{136}/dsx^{1},p^{p}$ mutant showing subtle transformation of distal TBR into sex comb like bristle (SCLB), (**d**) abdominal pigmentation of wild type male, (**e**) XX $dsx^{136}/dsx^{1},p^{p}$ mutant, and (**f**) XY $dsx^{136}/dsx^{1},p^{p}$ mutant. The ventral view of the abdominal tip of (**h**) XX $dsx^{136}/dsx^{1},p^{p}$ mutant showing no remarkable difference from that of the wild type female (figure not shown), (**i**) the abdominal tip of XY $dsx^{136}/dsx^{1},p^{p}$ mutant showing partial transformation of male genitalia, and (**g**) abdominal tip of wild type male.

and the somatic sex-determining factors. Earlier works point towards *Scr* being the segmental component (Hannah-Alava 1964; Barmina and Kopp 2007; Shroff *et al.* 2007), whereas the recent work of Tanaka *et al.* (2011) has revealed complementary interaction between Dsx and SCR in sex comb formation. In the present study, we see that the dimorphism in SCR expression is clear as early as 3–4 h pupal prothoracic leg discs. Expression of SCR was analysed in the prothoracic discs of $dsx^{136}/dsx^{1},p^{p}$; chromosomally XX and XY pupae.

The first tarsal segment, pigmentation phenotypes and the abdominal tip of the wild type male and female, as well as the two mutant flies analysed are presented in figure 1a–i. The phenotype of the female mutant fly with genotype XX; $dsx^{136}$ / $dsx,^{1}p^{p}$ is similar to that of wild type female (figure 1b, e and h). We do not see any indications of transformation of the transverse bristles towards sex comb like teeth/thickenings. The phenotype of the adult of the genotype XY; $dsx\ ^{136}/dsx,\ ^{1}p^{p}$ is shown in figure 1c, f and i. There is a partial transformation of the genitalia; the abdominal pigmentation pattern is intermediate between male and female wild type individuals. But, regarding the prothoracic legs, we do not see noticeable transformation of the transverse bristles on the first tarsal segment into structures of sex comb. There are very subtle indications of slight thickening of few transverse row bristles (figure 1c). The chromosomal composition of the pupae was confirmed by assaying their genomic DNA for PCR amplification of a Y-chromosome specific gene *kl3* (CG17629) (Goldstein *et al.* 1982; Charlesworth 2001). An amplified product confirms the chromosome complement of XY, whereas the absence of amplification denotes XX complement.

### 3.1 *Sexually dimorphic expression of Sex combs reduced is through repression of Scr by female-specific product of doublesex*

In the male prothoracic leg disc, SCR expression extends till the distal tip of the first tarsal segment (ts1), which is actually the sex comb forming domain, whereas in the female leg disc, the expression is not seen at the distal part of the first tarsal segment (figure 2a and b). Interestingly, the prothoracic discs from XX; $dsx^{136}/dsx^{1},p^{p}$ pupae, of 3–4 h APF stage, which is chromosomally a female, when subjected to immunostaining with Anti-SCR antibody, showed expression of SCR similar to that seen in the wild type male disc of *D. melanogaster* (figure 2c and b ). The expression in these discs is strong in the sex comb forming domain, unlike that seen in that of the wild type female (figure 2a). As mentioned earlier, $dsx^{136}$ is an allele of *dsx*, which makes a fully functional female product in a chromosomally female (XX) individual. Considering that $dsx^{1}$ is a null allele, the XX; $dsx^{136}/dsx^{1},p^{p}$ individual will be heterozygous for the $Dsx^{F}$ product. With a deficiency of one copy of female-specific *dsx* gene product, the SCR expression domain in the prothoracic disc (figure 2c) shows a marked difference from that of the wild type female (figure 2a), and in this case it simulates the wild type male pattern (figure 2b).

Further, in pupae with XY; $dsx\ ^{136}/dsx^{1},\ p^{p}$ genotype, the prothoracic leg discs showed SCR expression in the first tarsal segment similar to that of the wild type male (figure 2d and b). $dsx^{136}$ allele is defective only in males and is complemented by the dominant male specific product expressing alleles, $dsx^{D}$ and $dsx^{Mas}$ (Baker and Ridge 1980; Baker and Wolfner 1988). Thus, in allelic combination $dsx^{136}/\ dsx^{1},\ p^{p}$, the male will be

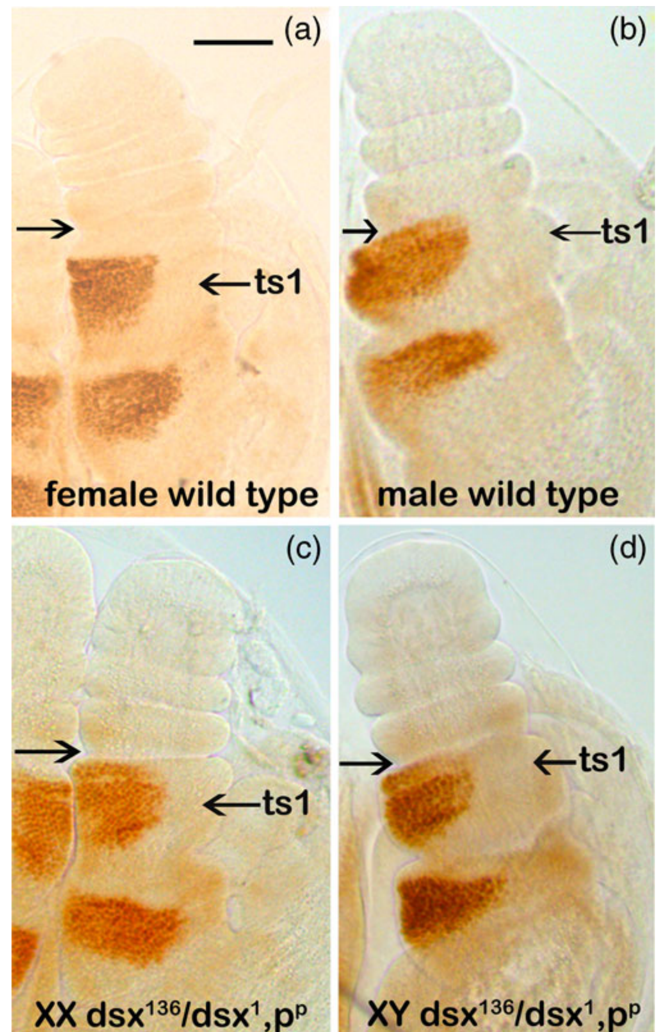


**Figure 2.** SCR expression pattern in prothoracic leg discs of wild type *D. melanogaster* and $dsx^{136}/dsx^{1},p^{p}$ mutant as demonstrated by anti-SCR antibody staining. Arrow indicates the distal edge of the ts1: (**a**) wild type female and (**b**) wild type male. SCR could not be detected in the distal tip of ts1 in female discs, whereas in males SCR expression extends till the distal tip of ts1. In (**c**) XX $dsx^{136}/dsx^{1},p^{p}$ individuals the expression of SCR is interestingly similar with that of wild type male individuals. SCR profile of (**d**) XY $dsx^{136}/dsx^{1},p^{p}$ individuals, which is similar to that of wild type male. Scale bar is 0.1mm.

completely lacking the male-specific product of *dsx*. The SCR expression in the discs in XY; $dsx^{136}/dsx^{1}$, $p^{p}$ pupae implies that the male-specific pattern of SCR expression does not depend on the male-specific *dsx* product. Added to the point, since this is a chromosomally male individual, $dsx^{F}$ will not be made, and in effect, these flies are null for *dsx* function. Thus, the SCR expression seen in the distal region could be a default pattern independent of *dsx* function. The sexual dimorphism seen in the SCR expression in the first tarsal segment of the prothoracic discs is probably due to the repression of its expression at the distal region, as seen in the female wild type discs by the female-specific function of the *dsx*. The male-like expression of SCR in XX; $dsx^{136}/dsx,^{1}p^{p}$, which has only one copy of Dsx $^{F}$, strongly suggests that there might be a dose dependency and *dsx* female function is needed in two copies for repression of *Scr* in the distal part of the first tarsal segment.

### 3.2 *Development of sex comb requires a combinatorial activity of male-specific product of dsx and the homeotic selector gene Sex combs reduced*

As observed in female mutant fly with genotype XX; $dsx^{136}$ / $dsx,^{1}p^{p}$ (figure 1b, e and h) and in male of the genotype XY; $dsx\ ^{136}/dsx,\ ^{1}p^{p}$ (figure 1c, f and i), there is no transformation of the TBRs into sex comb. But as far as the expression of SCR in the tarsal segment of the prothoracic discs of the pupae is concerned, as we have already discussed, in both these genotypes, the pattern is similar to that of the wild type male disc with a strong expression in the sex comb forming region (figure 2c and b). Increased expression level of SCR has a positive correlation with presence of sex comb, as shown by earlier workers (Barmina and Kopp 2007; Randsholt and Santamaria 2008; Devi *et al.* 2013). Considering the sexual dimorphism in the sex comb trait and the SCR expression in prothoracic leg disc of *D. melanogaster*, we can see a similar correlation between the enhanced SCR expression at the distal ts1 and the sex comb formation in males, as against the reduced SCR expression and absence of sex comb in females. Nevertheless, the male-like distally extended SCR expression *per se* in the above individuals (XX; $dsx\ ^{136}/dsx^{1},p^{p}$ and XY; $dsx\ ^{136}/dsx^{1},\ p^{p}$) is not able to activate the formation of the sex comb structure.

Our above mutant analysis reveals that the extended ts1 expression of SCR seen in XX; $dsx\ ^{136}/dsx^{1},\ p^{p}$ and XY; $dsx\ ^{136}/dsx^{1},\ p^{p}$ is obtained in the absence of male-specific function of *dsx*, but this distal ts1 expression of SCR is not sufficient to activate the sex comb morphogenesis. $dsx^{M}$ is not needed for male-like pattern of expression of SCR, but $dsx^{M}$ function is needed together with that of *Scr* to bring about complete transformation of TBRs into Sex comb. There might be downstream targets of $Dsx^{M}$ and *Scr* which cooperate with each other as effector genes in driving the morphogenetic changes of comb formation. Although Tanaka *et al.* (2011) have proposed a feedback loop between Scr and Dsx expression in ts1 (as seen in 24 h APF stage), our results demonstrates that the early expression of SCR (1-4hrs APF) does not require *dsx* function. Studies have shown that $Dsx^{F}$ and $Dsx^{M}$ products in general act to repress each other's somatic functions (Baker and Ridge 1980; Jursnich and Burtis 1993; Li and Baker 1998; Waterbury *et al.* 1999). Noticeably our present study shows that $Dsx^{F}$ is not directly repressing the $Dsx^{M}$ function towards sex comb formation, but it seems to be involved in repressing the expression of *Scr* in the distal region of first tarsal segment, resulting in a female-specific pattern of *Scr* expression. Thereby, it indirectly affects the sex comb development. The fact that, in the complete absence of $Dsx^{F}$ function (genotype XY; $dsx\ ^{136}/dsx^{1}\ p^{p}$), in spite of the availability of *Scr* product (figure 2d), there was no significant transformation of bristles into sex comb strongly suggests that sex comb morphogenesis is not a default pathway that is merely suppressed by $Dsx^{F}$ in females, but $Dsx^{M}$ function is actively required in combination with the *Scr* function, to achieve the sex comb transformation of the transverse row of bristles as seen in the prothoracic legs of wild type male flies.

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