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Sex-, stage- and tissue-specific regulation by a mosquito hexamerin promoter

U. K. Jinwal,

Department of Neurobiology and Developmental Sciences, University of Arkansas for Medical Sciences, Little Rock, AR, USA

S. O. Zakharkin,

Section on Statistical Genetics, Department of Biostatistics, University of Alabama at Birmingham, Birmingham, AL, USA

O. V. Litvinova,

Department of Neurobiology and Developmental Sciences, University of Arkansas for Medical Sciences, Little Rock, AR, USA

S. Jain, and

Department of Neurobiology and Developmental Sciences, University of Arkansas for Medical Sciences, Little Rock, AR, USA

Helen Beneš

Abstract

A portion of the 5'-flanking region of the female-specific hexamerin gene, *Hex-1.2*, from the mosquito *Ochlerotatus atropalpus* was used to drive expression of the *luciferase* reporter gene in *Drosophila melanogaster*. The proximal 0.7 kb of 5'-flanking DNA were sufficient to partially repress reporter gene activity in males and to drive tissue- and stage-specific expression comparable with that of the endogenous *O. atropalpus Hex-1.2* gene. The *Drosophila* doublesex transcription factor (DSX), expressed in *Escherichia coli*, bound putative DSX sites of the *Hex-1.2* gene differentially *in vitro*. Blocking expression of the female isoform of the Doublesex transcription factor in transgenic female flies resulted in reduction of *luciferase* expression to levels comparable with those in males, suggesting that Doublesex could contribute to regulation of female-specific expression of the *O. atropalpus Hex-1.2* gene.

Keywords

hexamerin gene regulation; mosquito; fat body; gel-shift assays; sex specificity; doublesex

Introduction

Combating the worldwide spread of many deadly infectious diseases requires the development of novel strategies for control of insect-borne pathogens. Such strategies will most likely involve the use of molecular tools, including promoters targeting gene activity to particular developmental stages, tissues and/or gender of the disease vector. One of the species-specific methods for control of diseases vectored by insects is the sterile insect technique (SIT), which involves the release of sterilized males to mate with wild females (Tan, 2000). However, the

use of SIT is costly and labour-intensive. New approaches using transgenic insects have been proposed, such as the 'release of insects carrying a dominant lethal' (RIDL; reviewed in Alphey *et al.*, 2002), in which a dominant sex-specific lethal gene can be expressed to self-destruct the offspring from the mating of a released transgenic male population with wild females. For RIDL and other transgenetic sexing strategies (including more conventional SIT) to be effective, sex- and tissue-specific promoters are needed to drive expression of a repressible lethal gene. Furthermore, any transgenic approach to modify natural disease vector populations will require the introduction of selective advantages for the transgenic insects such as increased or enhanced reproductive fitness.

The insect fat body is the major biosynthetic organ, which maintains homeostasis of haemolymph proteins, lipids and carbohydrates and plays an important part in metabolism, development and reproduction (Candy, 1985; Haunerland & Shirk, 1995). Owing to its critical roles in reproduction and immunity, the fat body is an important tissue to target for transgenic approaches. Molecular analysis of fat body-specific genes of the fruit fly and mosquito suggests that their tissue-specific expression is governed by specific transcription factors, including C/EBP, GATA, BBF-2, HNF-4, and a fat body-specific repressor, AEF-1, binding to their cognate *cis*-acting DNA sequences (Garabedian *et al.*, 1986; Sofer & Martin, 1987; Abel *et al.*, 1992; Falb & Maniatis, 1992a,b; An & Wensink, 1995a,b; Beneš *et al.*, 1996; Kapitskaya *et al.*, 1998; Kokoza *et al.*, 2000; Martin *et al.*, 2001).

Hexamerins are insect storage proteins that are synthesized in the fat body (Telfer & Kunkel, 1991) and belong to a large arthropod protein family that includes haemocyanins and prophenol oxidases (reviewed in Haunerland, 1996 and Burmester *et al.*, 1998). Hexamerins or hexameric storage proteins of holometabolous insects are synthesized in the fat body, primarily at the end of larval development, secreted into the haemolymph and then taken up via receptor-mediated endocytosis into the larval fat body for storage in protein granules. These hexameric storage proteins, which are primarily rich in aromatic amino acids, are then utilized as an amino acid reserve during non-feeding periods of insect development or diapause. Diptera produce two immunologically distinct hexamerins typified by the larval serum proteins (LSPs), LSP-1 and LSP-2 of *Drosophila melanogaster* (reviewed in Burmester *et al.*, 1998). Very similar hexamerins are also expressed by the fat body of different species of mosquitoes (Zakharkin *et al.*, 1997; Korochkina *et al.*, 1997).

Previously, in our laboratory we identified putative female-specific regulatory elements in the 5'-flanking region of the hexamerin gene, *Hex-1.2*, of the aedine mosquito, *Ochlerotatus atropalpus* (Zakharkin *et al.*, 2001). These elements contain putative overlapping C/EBP and DSX (doublesex) transcription factor binding sites and are similar in structure to the female- and fat body-specific element from the 5'-flanking region of the *Yolk protein (Yp)* genes of the fruit fly, *Drosophila melanogaster* (An & Wensink, 1995a). As one of the downstream regulators of the *Drosophila* sex determination pathway (Burtis *et al.*, 1991), the *doublesex* locus encodes either male-specific DSX^M or female-specific DSX^F transcription factors that are produced through alternative RNA splicing (Burtis & Baker, 1989; Nagoshi & Baker, 1990). Action of the transformer (TRA) and transformer-2 (TRA-2) proteins is required to produce the female-specific DSX^F isoform (Inoue *et al.*, 1992). The two DSX proteins differ only in their C-termini and bind to the same DNA site in the *o-r* enhancer of the *yolk protein (Yp)* genes (An & Wensink, 1995a; Cho & Wensink, 1997). We hypothesized that female-specific activity of the mosquito *Hex-1.2* gene could be regulated similarly to the *Yp* genes in *D. melanogaster*, with Doublesex playing an important role.

In this study, we show that the putative DSX sites in the 5'-flanking region of *Hex-1.2* bind the *Drosophila* DSX protein and that in transgenic *Drosophila* a 738 bp promoter/enhancer sequence confers strong female-enhanced activity on a reporter gene. This female-enhanced

activity is fat body- and stage-specific, recapitulating in a distant dipteran insect the regulation that is observed for the mosquito *Hex-1.2* gene. In addition, using a null mutation (*transformer^l*) for one of the critical components of the *Drosophila* sex determination pathway, we show that the *Hex-1.2* DSX binding sequences are indeed functional in a heterologous dipteran insect.

Results

Gel mobility shift assays for DSX binding

Previously, we identified three putative DSX binding sites in the 5' flanking region of the *O. atropalpus Hex-1.2* gene (Zakharkin *et al.*, 2001; Fig. 1). To determine if these putative sites are able to bind a cognate DSX transcription factor from a related species, we performed electrophoretic mobility shift assays (EMSA) with bacterially expressed *Drosophila* DSX proteins. An initial experiment was designed to demonstrate the specificity of *Escherichia coli*-expressed DSX^M and DSX^F proteins, using as a probe the DSX binding site from the *Drosophila Yp1* gene regulatory region (An & Wensink, 1995a). A shift in Yp1 probe was observed only with DSX^M- and DSX^F-containing *E. coli* extracts, but not with the control BL21 extracts (data not shown). DSX^F-containing extract (also called 'DSX^F protein') was used in all subsequent experiments, as DSX^F and DSX^M have identical DNA-binding domains and properties (Burtis *et al.*, 1991; Erdman & Burtis, 1993; Cho & Wensink, 1996; Erdman *et al.*, 1996).

A comparison of the binding patterns of Yp1, DSX-1, DSX-2 and DSX-3 oligonucleotide probes in the presence of varying amounts of DSX^F protein is shown in Fig. 2. The oligonucleotides containing putative DSX sites from the mosquito *Hex-1.2* gene were able to bind the DSX^F protein to varying degrees; retarded bands of the same mobility as ones obtained with the Yp1 probe were clearly recognizable in all reactions. This binding pattern was confirmed and quantified by competitive EMSA (Fig. 3A,B). DSX^F protein was incubated with a fixed amount of radio-labelled Yp1 probe and competed by increasing amounts of unlabelled (cold) Yp1, DSX-1, DSX-2, DSX-3 or non-specific probes. For the Yp1 control reaction, binding was significantly competed away by inclusion of a 250-fold molar excess of unlabelled homologous Yp1 probe (lane 3). Unlabelled DSX-1 and DSX-2 probes also performed well as competitors in 250-fold molar excess, competing away 55% (Fig. 3A,B, lane 6) and 43% (lane 10) of binding to the Yp1 probe, respectively. One thousand-fold molar excess of unlabelled DSX-1 was needed to compete away 90% (lane 8) of Yp1 binding. No significant competition was observed with DSX-3 (lanes 13–16) and in particular, the non-specific (lanes 17–20) unlabelled DNA probes. Hence, the efficiency of competition indicated that the DNA binding affinities of the DSX sites for DSX^F were in the following order: Yp1 >> DSX1 > DSX2 >> DSX3.

The *Hex-luc* promoter induces female-enhanced activity in *Drosophila*

To examine the function of the *Hex-1.2* DSX sites by a transgenic approach, we constructed a fusion gene, 0.7*Hex-luc*, containing positions −715 to +23 (relative to the transcription start site) of *Hex-1.2* (Fig. 1) and subcloned it into the *P*-element-based transformation vector, pCaSpeR, as described in the Experimental procedures. Using the *y^lw^{67c23}* strain (Beneš *et al.*, 1996), four independent transgenic lines were established. Each of the transgenic lines contained a single-copy *Hex-luc* insert, as determined by Southern blot analysis (data not shown); the chromosome of transgene insertion was identified by genetic crosses. Homozygous flies of one line, TR-6, were viable and fertile. Homozygous flies of two other lines, TR-4 and TR-10, were not viable; while TR-1 homozygotes were viable but sterile. More recently in another context, *Drosophila* transgenics with the same *Hex-1.2* mosquito DNA were obtained

without an excessive number of homozygous lethals; hence, there is nothing unusual about the mosquito DNA used in the *Hex-luc* construct.

To analyse differences in *Hex-luc* activity between genders, three independent transgenic lines (TR-4, TR-6 and TR-10) were assayed for luciferase activity. Individual late third-instar female and male larvae were collected and whole-animal extracts were assayed as described in Experimental procedures. Both homozygous and heterozygous flies of the TR-6 line were tested. The mean luciferase expression levels and the resulting female/male ratios in expression were different for each line, with ratios ranging from 2 to 4, reflecting different effects of chromosomal position on the transgene in each line (Table 1 and Fig. 4A). We determined that luciferase activity was clearly higher in females than in males using a series of pair-wise *t*-tests ($P < 0.001$). In order to confirm that the sex-enhanced luciferase activity was based on transcriptional, and not translational regulation, real-time RT-PCR analysis was performed to assess the levels of *luc* mRNA levels in male and female L3 larvae of TR-6 line. Results of real-time PCR analysis (Fig. 4B) indicated that female *luc* mRNA was transcribed at about a 4.6-fold higher level in female larvae than in males of the same age.

Expression of *Hex-luc* is tissue- and stage-specific

Hexamerins are insect storage proteins that are predominantly expressed in the fat body and occasionally in additional tissues, on a species-specific basis (Telfer & Kunkel, 1991). Hence, to determine if the *Hex-luc* transgene could elicit the expected fat body specificity in a heterologous dipteran insect (*Drosophila*), we assayed luciferase expression levels in fat bodies vs. the rest of the body (or carcass) in male and female late third-instar larvae. A typical, representative experiment is shown in Fig. 5(A); luciferase activity was almost exclusively confined to the fat body. Factorial ANOVA and a series of *posthoc* tests demonstrated that luciferase levels in male and female carcasses were not significantly different from each other ($P = 0.695$), while all others groups were significantly different ($P < 0.001$).

To determine the developmental profile of *Hex-luc* expression, we assayed whole-animal extracts of both males and females at different stages; results for the TR-4 line are shown in Fig. 5(B). The developmental profile of transgene expression was similar to that of the *Hex-1.2* gene in the mosquito (Zakharkin *et al.*, 1997), with peak levels found at the last larval and early pupal stages and some expression in adult flies. Differences in luciferase activity levels between genders at different stages of the life cycle were analysed using a series of pairwise *t*-tests. Starting from the last larval stage, the ratio of mean luciferase levels in females to mean luciferase levels in males was approximately 2 throughout development.

Hex-luc expression in *tra* mutants

Activity of the *transformer* gene is required for female-specific splicing of *dsx* mRNA (Hoshijima *et al.*, 1991); in a fly carrying a null *transformer* mutation (*tra*¹) over a deficiency of the same locus, splicing produces only the default male *dsx* mRNA in both sexes. Similarly, in both male and female flies carrying dominant *dsx* mutations, only the male DSX isoform is produced in both sexes (Nagoshi & Baker, 1990). To confirm that *Hex-luc* transgene activity was under the control of the DSX transcription factor and dependent on sex-specific differential splicing of the *dsx* transcripts, we chose to use the *tra*¹ rather than any *dsx* mutation. Hexamerins and yolk proteins are similar storage proteins produced by the fat body and are likely to use similar regulatory networks. As both *tra* and *dsx* mutations have the same effect on yolk protein expression (Artyom Kopp, personal communication) and dominant *dsx* mutants are difficult to maintain in the laboratory, we found little advantage to working with *dsx* mutations in our experimental system. Hence, we placed the *Hex-luc* transgene in the context of *tra*¹ by genetic crossing of appropriate stocks (Fig. 6A). Among transgenic animals carrying the *Hex-luc* fusion gene, as judged by a red eye colour, we selected three groups: genetic males, genetic females,

and pseudomales. Genetic males were identified by their bar-shaped eyes, due to a dominantly marked duplication chromosome, *Dp(1;Y)B^S*, while pseudomales had a normal eye shape. Pseudomales were genetic females that carried the *tra¹* mutation uncovered by the *Df(3L)st-j7,Ki¹* deficiency, leading to a total absence of functional TRA protein and production of the male DSX^M isoform instead of the female-specific DSX^F. Individual adult animals at 1–2 days after eclosion were selected and whole-animal extracts assayed for firefly *luciferase* reporter gene activity as described in the Experimental procedures (Fig. 6B). Inactivation of the transformer protein, leading to production of the male DSX^M factor in genetic females (or pseudomales), resulted in reduction of *Hex-luc* transgene expression to levels more similar to those in males. One-way ANOVA and a series of *posthoc* tests confirmed that mean luciferase levels in pseudomales were not different from males ($P = 0.797$), while both were significantly different from females ($P < 0.001$ in both cases).

Discussion

Molecular tools for transgenic manipulation of numerous arthropod pests (of plants and animals) are needed to control the pests themselves as well as the diseases that they can spread. Such tools should include DNA sequences whose role in regulating gene activity can be reliably predicted in terms of sex, stage and tissue specificity. In particular, the insect fat body, as a central organ of intermediate metabolism, immune defence and reproduction, is an important tissue to target in such transgenic approaches. Accordingly, we have begun the characterization of a female, fat body-specific mosquito gene whose strongest activity is in the late larval stage but which continues at low levels uniquely in the adult female (Zakharkin *et al.*, 2001). In this study, analysis of transgenic 0.7*Hex-luc* flies shows that 738 bp of *Hex-1.2*, including its transcription start site, constitute a very strong promoter and enhancer whose activities are not only significantly enhanced in females of an heterologous dipteran species but are also negatively affected by a *tra* mutation, which abrogates DSX^F-regulated activity in female flies. Furthermore, we demonstrate that at least two DSX sites, previously proposed within the *Hex-1.2* 5'-flanking region (Zakharkin *et al.*, 2001), are able to bind the *Drosophila* DSX^F isoform with reasonably high affinity. These findings suggest that the mosquito *Hex-1.2* enhancer/promoter is a target of Doublesex regulation and likely to function sex specifically in other transgenic insects, given the extensive conservation of the *doublesex* or DM domain, a cysteine-rich DNA-binding motif first identified in the *Drosophila* DSX protein, genes among eukaryotes as distantly related as worms and vertebrates (Cline & Meyer, 1996; Raymond *et al.*, 1998; Kuhn *et al.*, 2000; Hodgkin, 2002; Scali *et al.*, 2005). Recently, characterization of sex-specific *doublesex* transcripts and the single *dsx* gene from the mosquito *Anopheles gambiae* showed that DSX isoforms of this mosquito possess the same domain structure as the *Drosophila* DSX proteins (Scali *et al.*, 2005). The presence of specific genomic regulatory elements in the *Agdsx* gene suggests that in this mosquito the TRA/TRA2 regulatory pathway is used to produce the female-specific DSX domain by a mechanism more similar to the splicing of the *Drosophila fruitless* transcript.

Using an *E. coli* extract containing highly expressed *Drosophila* DSX^F protein, we observed that the DSX-1 and DSX-2 sites (Fig. 1) from the *Hex-1.2* gene were able to bind DSX^F with a measurable (Fig. 3A,B) but somewhat weaker affinity than does the *Drosophila Yp1* regulatory element. We estimate from the quantification of competitive EMSA that the *Hex-1.2* DSX-1 site exhibits about 20-fold weaker affinity than the Yp1 site, and that the DSX-2 site is five times weaker than the DSX-1 site in binding DSX^F. These relative DSX^F binding strengths correspond in part to the extent of identity between *Hex-1.2* DSX sites and the proposed consensus for the *Drosophila* DSX factor (Erdman *et al.*, 1996). Six of the seven consensus positions are identical between both DSX-1 and DSX-2, and the *Drosophila* consensus with the divergent nucleotide being in the +1 or -1 position of the palindrome, respectively. Predictably, the DSX-3 site, which has significantly less homology with the

Drosophila DSX consensus binding site, showed little if any binding to the *Drosophila* DSX protein. Given the high conservation of the DNA binding region of the different Doublesex proteins as well as of their DNA response elements (Zarkower, 2002; Hodgkin, 2002; Suzuki *et al.*, 2003; Hediger *et al.*, 2004; Scali *et al.*, 2005), we maintain that there are at least two *bona fide* DSX sites in the *Hex-1.2* enhancer/promoter region. As the DSX-3 site is placed very near the 'TATA box', in a regulatory region that is usually highly conserved among TATA-box-containing genes, we suggest that this site may also be functional in *O. atropalpus*. It is possible that the homologous mosquito DSX protein will be found to have higher affinity for the DSX sites of the *O. atropalpus Hex-1.2* gene.

By testing the function of these mosquito DSX binding sites *in vivo* in transgenic *Drosophila* carrying the 0.7*Hex-luc* gene, we found that they could account for the significantly (two- to six-fold) higher reporter gene activity in female larvae, pupae and adult flies. The differences between the two sexes were entirely reproducible although influenced to some degree by transgene position effects. Despite considerable animal-to-animal variation in reporter gene activity (as reflected in the large SDs; Figs 4 and 5), the differences between male and female activities were statistically significant in all three lines examined. However, in none of the transgenic *Drosophila* lines did we obtain complete repression of the transgene in male larvae or adults, as occurs for the *Hex-1.2* gene in male *O. atropalpus*. Other regulatory sequences, upstream, downstream or both, of the tested 738 bp enhancer/promoter may be necessary for full repression in males. Alternatively, one or more coactivators and/or corepressors are lacking in the heterologous fruit fly. In a previous analysis (Zakharkin *et al.*, 2001), we found no potential regulatory factor binding sites downstream of the *Hex-1.2* transcription start site. Hence, we are currently exploring the impact of DNA sequences located further upstream of the transcription start site.

Further confirmation that the DSX sites of the *O. atropalpus Hex-1.2* gene are functional and at least partially determine sex-specific gene activity in a heterologous dipteran species came from our experiments with the *transformer* null mutant *tra*¹. When only the default male isoform, DSX^M, was produced in genetic females, there was no enhancement of reporter gene expression. This observation suggests that female larvae, pupae and adult flies need the DSX^F isoform to express the transgene at a higher level. However, it is still possible that other TRA targets, regulators similar to Doublesex and Fruitless (Heinrichs *et al.*, 1998; Dulac, 2005) but as yet undiscovered, may bring about sex-specific activity of the mosquito *Hex-1.2* gene. Future experiments in which the *Hex-1.2* DSX sites are mutated and tested in transgenic *Drosophila* and mosquitoes should directly demonstrate if these DSX-binding sites are indeed essential for female-specific activity of *Hex-1.2* in *O. atropalpus*.

This study also demonstrates that the 738 bp of mosquito DNA tested are sufficient to confer the same tissue and similar stage specificity in *Drosophila* as in the mosquito but not complete sex specificity. The weak activity of the reporter gene detected in carcasses could be due to technical constraints: it is extremely difficult to remove all of the fat body cells; even a few cells remaining in the carcass could result in significant luciferase activity. Alternatively, the transgene could be expressed in tissues other than fat body, although we consider this possibility quite unlikely, given the results of our previous studies of hexamerin expression in mosquitoes (Zakharkin *et al.*, 1997; Korochkina *et al.*, 1997). Somewhat surprisingly, the relative level of reporter gene activity in 1–3-day and 7–10 day-old adult transgenic *Drosophila* was much higher than in adult female *Ochlerotatus* mosquitoes. The luciferase level in adult fruit flies was about half of the peak level in L3 larvae, while in adult female mosquitoes the *Hex-1.2* mRNA level was only about 1/100 of the larval peak (Zakharkin *et al.*, 2001). This may be due to differences between fruit flies and mosquitoes in gene regulation that may operate at the transcriptional or post-transcriptional levels. However, most studies of the use of heterologous DNA sequences in transgenic *Drosophila* have shown conservation of

tissue-specific regulatory elements (from the first by Mitsialis & Kafatos, 1985 to a more recent one by Kokoza *et al.*, 2001). Alternatively, some additional sequences, beyond what we have tested, might be necessary to achieve more restricted stage specificity in *Drosophila*.

Previous studies (Falb & Maniatis, 1992a,b; Attardo *et al.*, 2005), including those in our laboratory (Beneš *et al.*, 1996), have defined a limited number of transcription factors and their DNA response elements that are sufficient for targeting gene activity to the dipteran insect fat body. The fat body-specific regulatory unit was proposed to consist of at least one positive element binding a bZIP protein such as C/EBP and a negative element binding a repressor such as AEF-1, which restricts gene activity to the fat body (Falb & Maniatis, 1992a; An & Wensink, 1995b). Each of the *Hex-1.2* DSX sites tested in this study is associated with one or more C/EBP sites whose specific roles will need to be tested in future experiments. Also critical to establishing tissue specificity may be binding sites for the GATA factor, which is highly conserved among vertebrates and invertebrates and harbours a very conserved DNA-binding domain associated with other more variable domains that allow interaction with numerous transcriptional activators and repressors (reviewed in Attardo *et al.*, 2005). The roles of two putative GATA sites located in close proximity to the sex-specific DSX-C/EBP regulatory module will also be examined to fully understand the integration of sex- and fat body-specific activity of the *Hex-1.2* gene.

In conclusion, we have demonstrated that 738 bp of *Ochlerotatus* mosquito DNA from the 5'-flanking region of the *Hex-1.2* gene are capable of directing stage- and fat body-specific and sex-enhanced gene activity in a heterologous dipteran insect. To date, few target genes regulated by DSX have been identified in *Drosophila*, mosquitoes and other insects. The *Ochlerotatus Hex-1.2* gene may be the first example for mosquito species. Further studies are now in progress to isolate a culicine mosquito homologue of the DSX factor and to test the *Hex-1.2* regulatory sequences in transgenic mosquitoes.

Experimental procedures

Animals and genetic manipulations

Fruit flies, *Drosophila melanogaster*, were reared as described previously (Beneš *et al.*, 1996). *y¹w^{67c23}* flies were a kind gift of Jean-Antoine Lepesant. *f¹/Dp(1;Y)B^S; Df(3L)st-j7,Ki¹/TM6B, Tb¹* (BL-5416) and *w^a/Y; tra¹/TM2* (BL-675) flies were obtained from the Bloomington Drosophila Stock Center (Indiana University, Bloomington, IN, USA) and used to generate other lines as needed. Pseudomale, male and female flies carrying a *Hex-luc* transgene and the *transformer* mutation, *tra¹*, were generated by crossing *y¹w^{67c23}/Dp(1;Y)B^S; Sp/CyO; Df(3L)st-j7,Ki¹/TM6B, Tb¹* males with *y¹w^{67c23}/y¹w^{67c23}; P{0.7Hex-luc}/P{0.7Hex-luc}; tra¹/TM3, Sb* females. Among the progeny, true males were selected by eye shape from expression of the *Bar* gene (*B^S*) and pseudomales as non-Stubble, non-Tubby flies carrying the *Ki* dominant bristle marker; true females were identified as non-Kinked, non-Bar, Tubby flies.

Fusion gene constructs

Portions of the 5'-flanking regions of the *Hex-1.2* gene were isolated and subcloned as described previously (Zakharkin *et al.*, 2001; GenBank accession number AF430247). First, a 1.3Hex-luc (*hexamerin/luciferase*) fusion gene construct was created by insertion of a 1.3 kb *MluI/XhoI* DNA fragment of the *Hex-1.2* 5'-flanking region into the promoterless pGL2-Basic vector (Promega, Madison, WI, USA). Then, a 0.7Hex-luc fusion gene construct, containing positions -715 to +23 (relative to the transcription start site) of the *Hex-1.2* gene, was derived from it by *KpnI/NsiI* digestion and ligation. All constructs were verified by DNA sequencing. To

generate transgenic flies, a 3.9 kb *XhoI/BamHI* DNA fragment of 0.7*Hex-luc* was subcloned into the *Drosophila* *P*-element transformation vector, pCaSpeR (Pirrotta, 1988).

Generation of *Drosophila* transformants

P-element transformation was performed according to Rubin & Spradling (1982), using *y¹w^{67c23}* host flies as previously described (Beneš *et al.*, 1996). Surviving adults were individually backcrossed to the parental *y¹w^{67c23}* line; stable transformants were selected by eye colour. Transgenes were localized to specific chromosomes by genetic crosses using dominantly marked balancer chromosomes according to standard protocols. Transgene copy number was determined by genomic Southern blot analysis for each transformed line; lines with multiple *P*-element insertions were discarded.

Luciferase activity assay

Individual animals were collected at different stages and either frozen or assayed immediately. TR-4, TR-6 and TR-10 lines were used to assay sex-enhanced activity in transgenic larvae. For analysis of tissue-specific gene expression, fat bodies of third-instar TR-4 larvae were separated from the carcasses by dissection (Beneš *et al.*, 1996) and immediately frozen in liquid nitrogen. Samples were homogenized in lysis buffer from the Luciferase Reporter Gene Assay System (Promega) after adding a protease inhibitor cocktail at the manufacturer's recommended concentration (Roche, Indianapolis, IA, USA) and aprotinin (Sigma, St Louis, MO, USA) at 1 µg/ml to reduce protein degradation. Luciferase activity was measured using the Victor Light 1420 Luminescence counter (Perkin Elmer, Shelton, CT, USA). For each sample, relative light units were normalized to protein concentration measured using the Protein Assay Reagent (Pierce, Rockford, IL, USA) detected with a Multiskan Ascent spectrophotometer (Labsystems, Madison, WI, USA).

Real-time PCR

Total RNA was isolated from five pooled, frozen male or female larvae using the TRIzol reagent according to the manufacturer's specifications (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA from each sample was treated with 1 unit of RNase-free deoxyribonuclease I (Amplification Grade, Invitrogen) according to the manufacturer's protocol. First-strand cDNA synthesis was carried out on 1 µg of DNase-treated RNA using a poly dT20 primer and SuperScript II reverse transcriptase (Invitrogen). Real-time quantitative PCR (q-PCR) amplification reactions were performed using the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). Primers were as follows: Luciferase Forward: 5'-TACAACACCCCAACATCTTCG-3'; Luciferase Reverse: 5'-TCCACAAACACAACCTCCTCC-3'; S3 ribosomal protein Forward: 5'-GCTGAATTGAACGAGTTCCT-3'; S3 ribosomal protein Reverse: 5'-ACCTTCTCGGCGTACAGCTC-3'. Samples were analysed using the DNA Engine Opticon 2 Detection System (MJ Research, Waltham, MA, USA) with Opticon 2 Monitor software. The *ribosomal protein S3* transcript was used as an internal control to normalize RNA. Amplifications were performed as follows: initial incubation at 50 °C for 2 min, denaturation at 95 °C for 2 min, followed by 34 cycles of 94 °C, 15 s; 56 °C, 15 s; 72 °C, 15 s. Fluorescence was recorded after the 72 °C/15 s step of each cycle. The specificity of each reaction was determined after completion of PCR cycling by analysis of the melting-point dissociation curve generated for temperatures from 60 to 95 °C at 0.5 °C/s, and by visual inspection of DNA bands resolved by agarose gel electrophoresis. The optimal threshold cycle value (C_T) was determined at the 0.05 fluorescence value, using the auto scale function provided by the analysis software, and was identified using regions of amplification curves that met the criterion of a two-fold increase in fluorescence per cycle. A validation experiment (data not shown) with eight-fold serial dilutions of cDNA, confirmed that the amplification efficiencies of luciferase

and *S3 ribosomal protein* cDNAs were comparable and that data could be analysed using the comparative $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001) to calculate differences in transcript concentrations between samples (Fig. 4B).

Electrophoretic mobility shift assay

Expression of DSX proteins—To express the male- and female-specific *Drosophila*, DSX^M and DSX^F, proteins, we obtained the plasmids pT7-7 Dx^{fe} and Dx^{me} as a generous gift from Pieter Wensink (Brandeis University, Waltham, MA, USA) through Gyunghee Lee (University of Tennessee-Knoxville, Knoxville, TN, USA). Protein expression was performed according to Burtis *et al.* (1991) with some modifications. DSX protein expression was induced in 15 ml of *E. coli* BL-21 AI cells (Invitrogen) at mid-log phase with 0.002% arabinose. After 2.5 h incubation at 37 °C, cells were harvested by centrifugation, washed with phosphate-buffered saline, resuspended in 1.5 ml of Buffer L [20 mM Tris-HCl (pH 8.0), 1 mM EDTA and 10% sucrose] and frozen in liquid N₂. Cells were thawed at 0 °C, and lysozyme added to 0.2 mg/ml. After 45 min at 0 °C, the cells were twice frozen in liquid N₂ and thawed at 0 °C. The lysate was centrifuged at 19630×g at 4 °C for 20 min; the pellet was resuspended in 3 ml of buffer Z-50 (Burtis *et al.*, 1991), aliquoted and stored at –80 °C. Control protein extracts from untransformed *E. coli* BL-21AI cells were prepared similarly. Protein concentrations were determined using the Bradford reagent (Sigma). Expression of the *Drosophila* DSX proteins within the bacterial lysates was confirmed by standard SDS–polyacrylamide gel electrophoresis and Western blotting (data not shown).

Double-stranded DNA probes were produced from oligonucleotides specifically designed for central placement of a putative or known DSX binding site, shown as underlined letters. The following were used as probes for the putative DSX sites of the *Hex-1.2* gene, with each site numbered according to the female-specific element (or FSE) in which it is located (Fig. 1): DSX-1, 5'-TGCAACTTGCAACTTTGTGCTCTGTGTTAT-3'; DSX-2, 5'-CGGGTGAATATACTATGTTTCAATTTATAA-3'; and DSX-3, 5'-AGTGAGATCGAGAACATTTTTGCATATATA-3'. The dsxA binding site of the *Yp1* gene and a non-specific DNA (Cho & Wensink, 1997) were used as positive and negative controls, respectively: 5'-TCGACACAACACTACAATGTTGCAATCAGCTAGCC-3' (sense strand of dsxA) and 5'-GTTACCCGATGGATACTTAATAACC-3' (non-specific sense strand). These DNA probes were end-labelled using a DNA 5'-End-Labeling System (Promega) and [γ -³²P]-ATP (Perkin). Unincorporated nucleotides were removed with Quick-Spin columns (Qiagen, Santa Clara, CA, USA). Protein-DNA binding reactions were performed with 0–500 ng of DSX protein extract mixed with 2 fmol of ³²P-labelled, double-stranded probes in 40 μ l Z-50 Buffer for 40 min at room temperature. For the competition assay (Fig. 3A), unlabelled 0–1000-fold molar excess of double-stranded oligonucleotide competitors were preincubated with DSX protein extract in Z-50 buffer for 20 min on ice, followed by the addition of labelled *Yp1* probe and incubation for another 30 min at room temperature. The DNA–protein complexes were resolved on 6% non-denaturing polyacrylamide gels prerun for 1 h in Tris–glycine buffer (25 mM Tris-HCl; 190 mM glycine; 1 mM EDTA, pH 8.3) at 10 V/cm and 4 °C. The dried gels were exposed to a phosphor screen, scanned with a Personal Molecular Imager FX (Bio-Rad, Hercules, CA, USA). Quantification was performed with Quantity-One software from Bio-Rad: to determine the probe fraction bound, radioactivity associated with specific DNA-DSX complexes was divided by the sum of radioactivity present in the shifted and unshifted oligonucleotide probe.

Statistical analysis

Statistical analysis was performed using the SPSS-10 (SPSS Inc., Chicago, IL, USA) and SAS 9 (SAS Institute Inc., Cary, NC, USA) software packages. The sample distribution in each group was tested for normality using a one-sample Kolmogorov–Smirnov test. Pairwise

comparisons of sample groups were done using independent samples Welch *t*-tests, which do not have an equal variance assumption. Luciferase levels were compared (in multiple groups) using either one-way ANOVA (for analysis of *tra* mutants) or factorial ANOVA (for analysis of tissue specificity). Individual groups were contrasted using Tamhane *posthoc* tests, which do not assume equal variance. *P*-values below 0.05 were considered significant. The validation experiment and calculation of the SD of real time PCR data were performed according to the Applied Biosystems (Applied Biosystems, Foster City, CA, USA) 'Guide to performing relative quantification of gene expression using real-time quantitative PCR' (available online at <http://www.appliedbiosystems.com/support/apptech/>).

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References

- Abel T, Bhatt R, Maniatis T. A *Drosophila* CREB/ATF transcriptional activator binds to both fat body- and liver-specific regulatory elements. *Gen Dev* 1992;6:466–480.
- Alphey L, Beard CB, Billingsley P, Coetzee M, Crisanti A, Curtis C, et al. Malaria control with genetically manipulated insect vectors. *Science* 2002;298:119–121. [PubMed: 12364786]
- An W, Wensink PC. Three protein binding sites form an enhancer that regulates sex- and fat body-specific transcription of *Drosophila* yolk protein genes. *EMBO J* 1995a;14:1221–1230. [PubMed: 7720712]
- An W, Wensink PC. Integrating sex- and tissue-specific regulation within a single *Drosophila* enhancer. *Genes Dev* 1995b;9:256–266. [PubMed: 7851798]
- Attardo GM, Hansen IA, Raikhel AS. Nutritional regulation of vitellogenesis in mosquitoes: implications for anautogeny. *Insect Biochem Mol Biol* 2005;35:661–675. [PubMed: 15894184]
- Beneš H, Neal KC, Willis RL, Gadde D, Castleberry AB, Korochkina SE. Overlapping Lsp-2 gene sequences target expression to both the larval and adult *Drosophila* fat body. *Insect Mol Biol* 1996;5:39–49. [PubMed: 8630534]
- Burmester T, Massey HC Jr, Zakharkin SO, Beneš H. The evolution of hexamerins and the phylogeny of insects. *J Mol Evol* 1998;47:93–108. [PubMed: 9664700]
- 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. [PubMed: 2493994]
- Burtis KC, Coschigano KT, Baker BS, Wensink PC. The doublesex proteins of *Drosophila melanogaster* bind directly to a sex-specific yolk protein gene enhancer. *EMBO J* 1991;10:2577–2582. [PubMed: 1907913]
- Candy, DJ. Intermediary metabolism. In: Kerkurt, GA.; Gilbert, LI., editors. *Comprehensive Insect Physiology, Biochemistry and Pharmacology*. 10. Pergamon Press; Oxford: 1985. p. 1-41.
- Cho S, Wensink PC. Purification and physical properties of the male and female Doublesex proteins of *Drosophila*. *Proc Natl Acad Sci USA* 1996;93:2043–2047. [PubMed: 8700882]
- Cho S, Wensink PC. DNA binding by the male and female Doublesex proteins of *Drosophila melanogaster*. *J Biol Chem* 1997;272:3185–3189. [PubMed: 9013552]
- Cline TW, Meyer BJ. Vive la difference: males vs females in flies vs worms. *Annu Rev Genet* 1996;30:637–702. [PubMed: 8982468]
- Dulac C. Sex and the single splice. *Cell* 2005;121:664–666. [PubMed: 15935752]
- Erdman SE, Burtis KC. The *Drosophila doublesex* proteins share a novel zinc finger related DNA binding domain. *EMBO J* 1993;12:527–535. [PubMed: 8440242]

- Erdman SE, Chen HJ, Burtis KC. Functional and genetic characterization of the oligomerization and DNA binding properties of the *Drosophila doublesex* proteins. *Genetics* 1996;144:1639–1652. [PubMed: 8978051]
- Falb D, Maniatis T. *Drosophila* transcriptional repressor protein that binds specifically to negative control elements in fat body enhancers. *Mol Cell Biol* 1992a;12:4093–4103. [PubMed: 1508206]
- Falb D, Maniatis T. A conserved regulatory unit implicated in tissue-specific gene expression in *Drosophila* and man. *Genes Dev* 1992b;6:454–465. [PubMed: 1547943]
- Garabedian MJ, Shepherd BM, Wensink PC. A tissue-specific transcription enhancer from the *Drosophila* yolk protein 1 gene. *Cell* 1986;45:859–867. [PubMed: 2423251]
- Haunerland NH. Insect storage proteins: gene families and receptors. *Insect Biochem Mol Biol* 1996;26:755–765. [PubMed: 9014325]
- Haunerland NH, Shirk PD. Regional and functional differentiation in the insect fat body. *Annu Rev Entomol* 1995;40:121–145.
- Hediger M, Burghardt G, Siegenthaler C, Buser N, Hilfiker-Kleiner D, Dübendorfer, Bopp D. Sex determination in *Drosophila melanogaster* and *Musca domestica* converges at the level of the terminal regulator *doublesex*. *Dev Genes Evol* 2004;214:29–42. [PubMed: 14673649]
- Heinrichs V, Ryner LC, Baker BS. Regulation of sex-specific selection of fruitless 5' splice sites by transformer and transformer-2. *Mol Cell Biol* 1998;18:450–458. [PubMed: 9418892]
- Hodgkin J. The remarkable ubiquity of DM domain factors as regulators of sexual phenotype: ancestry or aptitude. *Genes Dev* 2002;16:2322–2326. [PubMed: 12231620]
- Hoshijima K, Inoue K, Higuchi I, Sakamoto H, Shimura Y. Control of doublesex alternative splicing by transformer and transformer-2 in *Drosophila*. *Science* 1991;252:833–836. [PubMed: 1902987]
- Inoue K, Hoshijima K, Higuchi I, Sakamoto H, Shimura Y. Binding of the *Drosophila* transformer and transformer-2 proteins to the regulatory elements of *doublesex* primary transcript for sex-specific RNA processing. *Proc Natl Acad Sci USA* 1992;89:8092–8096. [PubMed: 1518835]
- Kapitskaya MZ, Dittmer NT, Deutsch KW, Cho WL, Taylor DG, Leff T, Raikhel AS. Three isoforms of a hepatocyte nuclear factor-4 transcription factor with tissue- and stage-specific expression in the adult mosquito. *J Biol Chem* 1998;273:29801–29810. [PubMed: 9792695]
- Kokoza V, Ahmed A, Cho WL, Jasinskiene N, James AA, Raikhel A. Engineering blood meal-activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. *Proc Natl Acad Sci USA* 2000;97:9144–9149. [PubMed: 10908672]
- Kokoza VA, Martin D, Mienaltowski MJ, Ahmed A, Christina MM, Raikhel AS. Transcriptional regulation of the mosquito vitellogenin gene via a blood meal-triggered cascade. *Gene* 2001;274:47–65. [PubMed: 11674997]
- Korochkina SE, Gordadze AV, Zakharkin SO, Beneš H. Differential accumulation and tissue distribution of mosquito hexamerins during metamorphosis. *Insect Biochem Mol Biol* 1997;27:813–824. [PubMed: 9474778]
- Kuhn S, Sievert V, Traut W. The sex-determining gene *doublesex* in the fly *Megaselia scalaris*: conserved structure and sex-specific splicing. *Genome* 2000;43:1011–1020. [PubMed: 11195332]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2001;25:402–408. [PubMed: 11846609]
- Martin D, Piulachs MD, Raikhel AS. A novel GATA factor transcriptionally represses *yolk protein* precursor genes in the mosquito *Aedes aegypti* via interaction with the CtBP corepressor. *Mol Cell Biol* 2001;21:164–174. [PubMed: 11113191]
- Mitsialis SA, Kafatos FC. Regulatory elements controlling chorion gene expression are conserved between flies and moths. *Nature* 1985;317:453–456. [PubMed: 2413366]
- Nagoshi RN, Baker BS. Regulation of sex-specific RNA splicing at the *Drosophila* doublesex gene: *cis*-acting mutations in exon sequences alter sex-specific RNA splicing patterns. *Genes Dev* 1990;4:89–97. [PubMed: 2155161]
- Pirrotta V. Vectors for *P*-mediated transformation in *Drosophila*. *Biotechnology* 1988;10:437–456. [PubMed: 2850048]
- Raymond CS, Shamu CE, Shen MM, Seifert KJ, Hirsch B, Hodgkin J, Zarkower D. Evidence for evolutionary conservation of sex-determining genes. *Nature* 1998;391:691–695. [PubMed: 9490411]

- Rubin GM, Spradling AC. Genetic transformation of *Drosophila* with transposable element vectors. *Science* 1982;218:348–353. [PubMed: 6289436]
- Scali C, Catteruccia F, Li Q, Crisanti A. Identification of sex-specific transcripts of the *Anopheles gambiae doublesex* gene. *J Exp Biol* 2005;208:3701–3709. [PubMed: 16169947]
- Sofer W, Martin PF. Analysis of *alcohol dehydrogenase* gene expression in *Drosophila*. *Annu Rev Genet* 1987;21:203–205. [PubMed: 3327463]
- Suzuki MG, Funaguma S, Kanda T, Tamura T, Shimada T. Analysis of the biological functions of a *doublesex* homologue in *Bombyx mori*. *Dev Genes Evol* 2003;213:345–354. [PubMed: 12733073]
- Tan, KH. Pulau Penang. Penerbit Universiti Sains; Wallingford, UK: 2000. Area-wide Control of Fruit Flies and Other Pests.
- Telfer WH, Kunkel JG. The function and evolution of insect storage hexamers. *Annu Rev Entomol* 1991;36:205–228. [PubMed: 2006868]
- Zakharkin SO, Gordadze AV, Korochkina SE, Mathiopoulos KD, della Torre A, Beneš H. Molecular cloning and expression of an hexamerin gene from the malaria mosquito, *Anopheles gambiae*. *Eur J Biochem* 1997;246:719–726. [PubMed: 9219531]
- Zakharkin SO, Headley VV, Kumar NK, Buck NA, Wheeler DE, Beneš H. Female-specific expression of a hexamerin gene in larvae of an autogenous mosquito. *Eur J Biochem* 2001;268:5713–5722. [PubMed: 11722555]
- Zarkower D. Invertebrates may not be so different after all. *Novartis Found Symp* 2002;244:115–126. [PubMed: 11990787] Discussion 126–135(203–116): 253–117

Figure 1.

Schematic diagram of the *Hex-1.2* 5'-flanking region in the *0.7Hex-luc* fusion gene. The *Hex-luc* gene consists of 0.74 kb from the *Hex-1.2* 5'-flanking region, isolated using the indicated *Nsi*I and *Xho*I sites and fused to the luciferase reporter gene as described in the Experimental procedures. The major (+1) transcription start site and the 'TATA' box are shown. The positions of the putative DSX and C/EBP binding sites, which may comprise female-specific elements (FSEs), are indicated; the positions of protein binding sites are not to scale. Numbers beneath the DSX sites are placed at the 0 positions of the DSX DNA-binding consensus sequences (Erdman *et al.*, 1996).

Figure 2.

Putative sequences from *Hex-1.2* bind *Drosophila* DSX^F. *E. coli* extracts (0, 12.5, 25, 125, 250 and 500 ng) containing expressed DSX^F protein were incubated with Yp1 (lanes 1–6), DSX-1 (lanes 7–12), DSX-2 (lanes 13–18) and DSX-3 (lanes 19–24) DNA probes and examined by EMSA. Positions of DNA-protein complexes are indicated with solid (specific) or open (non-specific) arrows. *Unbound DNA probe.

Figure 3.

DSX-binding sites from the *Hex-1.2* gene differ in affinity for *Drosophila* DSX^F. (A) Different affinities of *Hex-1.2* DSX-binding sites. *E. coli*-expressed DSX^F protein (25 ng) was incubated with 2 fmol of ^{32}P -labelled Yp1 probe in the absence (lane 1) and presence of 50–500-fold molar excess of unlabelled (cold) Yp1 (lanes 2–4), or 50–1000-fold molar excess of unlabelled DSX-1 (lanes 5–8), DSX-2 (lanes 9–12), DSX-3 (lanes 13–16) and non-specific DNA (lanes 17–20). EMSA was performed as described in Experimental procedures. Positions of specific DNA-protein complexes are indicated with solid arrows. *Indicates unbound DNA probe. (B) Quantification of DSX-1, DSX-2 and DSX-3 affinities. Radioactivity associated with the protein-DNA complexes in (A) was quantified by phosphorimager analysis and used to

determine the fraction bound. Each bar (corresponding to the gel lanes, left to right, of A) represents the percentage of binding relative to the control reaction without unlabelled competitor (lane 1, A).

Figure 4.

Sex-specific regulation of *Hex-luc* in *Drosophila* larvae. (A) Female-enhanced expression in third-instar larvae. Normalized firefly luciferase activity (RLU/ μ g protein; mean $\pm$ SD) was measured for individual male or female larvae from three transgenic lines, TR-4 ($n = 40$ /group), TR-6 ($n = 40$ /group) and TR-10 ($n = 50$ /group). Untransformed larvae of the same age from the host strain, y^1w^{67c23} , served as controls. F, females; M, males; hom, homozygous flies; het, heterozygous flies. (B) Transcriptional activity of *Hex-luc* in male and female larvae. Total RNA was isolated from pools of five sexed L3 larvae of the TR-6 line and cDNA synthesized from equal amounts of DNase-treated total RNA; real-time PCRs were performed in triplicate. Data were normalized relative to independent real-time PCR analysis of *ribosomal protein S3* (*S3P*) cDNA levels in the same samples. The difference between male and female *luc* mRNA levels was calculated by the comparative method and $\Delta\Delta C_T$ was statistically significant ($P = 4.5 \times 10^{-5}$).

Figure 5.

Conservation of mosquito promoter specificity in *Drosophila*. (A) Tissue-specific expression of the *Hex-luc* transgene. Extracts were prepared from individual L3 larvae of the TR-4 transgenic line and assayed for protein concentration and luciferase activity (mean $\pm$ SD; $n = 20$). FB F, female fat body; CAR F, female carcass; FB M, male fat body; CAR M, male carcass. (B) Developmental profile of *Hex-luc* expression. Extracts were prepared from individual animals of the TR-4 line at each indicated stage; normalized firefly luciferase activity (RLU/ μ g protein) was measured for each sample, and mean values ($\pm$ SD) determined for each group ($n = 20$). L2, second-instar larvae; L3, third-instar larvae; P, 0–2-h pupae; A3, 1–3-day old adults; A10, 7–10-day old adults; F, females; M, males; control, untransformed larvae, y^1w^{67c23} , of the L3 stage.

Figure 6.

Modification of female-enhanced *Hex-luc* activity by a *tra^l* mutation. (A) Genetic cross to test the effect of the *tra^l* mutation. Parental lines were constructed from the TR-6 line and appropriate stocks obtained from the Bloomington *Drosophila* Stock Center. The three indicated progeny from the cross were selected using dominant phenotypic markers. (B) Sex-specific *Hex-luc* activity in a *tra^l* mutant. Luciferase activities (mean $\pm$ SD) were determined for adult flies (1–2 days old) of each genotype (50 flies/group) shown in (A); untransformed adult male, *y^lw^{67c23}* flies.

Table 1
Sex-enhanced luciferase activity in transgenic *Drosophila* larvae

Group	n	Mean	SD	Ratio (F/M)	P-value
Controls	20	4.1	0.7		
TR-4 females	40	4320.5	2314.2	2.15	3.50×10^{-7} *
TR-4 males	40	2014.1	936.8		
TR-6 homozygous females	40	16440.9	8979.9	4.33	8.70×10^{-13} *
TR-6 homozygous males	40	3794.2	2136.7		
TR-6 heterozygous females	40	11597.9	8507.3	3.37	1.08×10^{-6} *
TR-6 heterozygous males	40	3442.8	1765.2		
TR-10 females	50	5012.4	2598.3	2.06	2.53×10^{-6} *
TR-10 males	50	2435.4	1098.6		

Normalized firefly luciferase activity (RLU/ μ g protein) was measured in transgenic *Hex-luc* larvae; the host *y¹67c23* strain was used as a control. *n*, number of samples in the group; SD, SD; ratio, ratio of the female: male means of normalized luciferase activity. *P*-values are given for pair-wise right-tailed *t*-tests comparing mean luciferase levels in males and females.

Asterisks (*) indicate significant differences at the nominal α -level = 0.05.