CHAPTER 3

Genetic interaction between sex determination pathway factor “double sex” and the position specifying factor “Sex combs reduced” during sex comb development.
3.1 Introduction:

Sexual dimorphism is common in the animal kingdom. Some of the prime examples of sexually dimorphic trait involve the elaborate peacock tail, male lion’s mane (Williams and Carroll, 2009), tails of birds of paradise (Diamond, 1986), horns of dung beetles (Moczek et al., 2006). Though there are exclusive descriptions and discussions of many of the sex limited traits, very few attempts have been made to understand the developmental basis of the appearance of these traits (Wilkins, 2004; Williams and Carroll, 2009). A molecular understanding of how these sex-specific traits develop has been elusive. To understand the development of any given sex specific trait the knowledge of both the sex-determination system and the genetic network that govern the development of the trait is required. Studies in diverse models including Drosophila reveal how sex determination system produces sex-limited expression and sex specific trait development. The emerging picture from these studies is that the major effectors of sex-determination systems act in combination with other non-sex-specific transcription factors to limit gene expression to a single sex and thereby promoting the development of such traits (Williams and Carroll, 2009). The sex specific abdominal pigmentation in males of Drosophila is a well known paradigm. The sex specific abdominal pigmentation resulted from a dimorphic expression of spatial regulator bric-a-brac (bab) which in turn is controlled by region and sex specific expression of Abdominal B (Abd-B) and female isoform encoded by doublesex (dsx) (Kopp et al., 2000; Williams et al., 2008). The goal in this chapter is to make an attempt to explore and elucidate
how candidate gene of somatic sex determination pathway integrates with the genetic networks that underlie the development of the sexually dimorphic trait - sex comb in *Drosophila*.

Sex comb is a male specific trait which is present on the prothoracic leg (T1) in the *Sophophora* group of *Drosophila*. As mentioned in the earlier chapters, it resides in a specific position - the 1st tarsal segment (ts1) of T1 in *D. melanogaster*. In species which have sex comb, it has been shown to provide better grasping during mating, and thereby increase the selective fitness of the male (Spieth, 1952; Markow et al., 1996; Polak et al., 2004). Earlier works (Barmina and Kopp, 2007; Randsholt and Santamaria, 2008) and the present study as well show a strong correlation between the expression modulations of SCR and the position, presence/absence of sex comb. The species which has sex comb has high expression of SCR in the region of sex comb formation. The sexual dimorphism of the sex comb has correlation with sexually dimorphic expression SCR as well which is seen in species with sex comb (Barmina and Kopp, 2007; Tanaka et al., 2011; the present study).

*doublesex* which controls the differentiation of most somatic tissues in *Drosophila* also has a crucial role to play in the development of sex comb. *dsxM* the male specific isoform, a product of alternative splicing, encoded by *dsx* determines the development of sex comb (Burtis and Baker, 1989; McKeown, 1992; Christiansen et al., 2002). In *dsx* null mutants, both males and females develop a vestigial sex comb: the bristles of the most distal TBR assume morphology intermediate between sex comb teeth and regular bristles, and
undergo a partial rotation (Hildreth 1965; Baker and Ridge, 1980). A similar intermediate sex comb develops in dominant \( dsx \) mutants (e.g., \( XX: dsx^{D}/+ \)), that express both male and female-specific \( dsx \) isoforms (Baker and Ridge 1980; Nagoshi and Baker, 1990). Over expression of \( dsxM \) results in the development of ectopic sex comb teeth (Jursnich and Burtis, 1993; Tanaka et al., 2011), whereas \( dsxF \) can reduce the number of sex comb teeth when expressed in males (Waterbury et al., 1999). Species primitively lacking sex comb (\( D. \) virilis, \( D. \) hydei) do not show the expression of \( dsx \) (Tanaka et al., 2011). Works of Cline and Meyer, (1996) showed the dependency on the ubiquitous expression of DsxM for the formation of sex comb in \( Drosophila \). Formation of proper sex comb with complete rotation and chitinization of bristles was not observed in \( dsx \) null clones (Baker and Ridge, 1980). Ectopic expression of \( dsxM \) has the potential to induce sex comb development in all the legs of male and female \( D. \) melanogaster (Jursnich and Burtis, 1993).

In the light of these previous findings, the candidate gene contributing from the somatic sex determining pathway is \textit{doublesex} (\textit{dsx}) and the spatial cue in the development of this trait is \textit{Sex combs reduced} (\textit{Scr}). The interaction between \textit{Scr} and splice variants of \textit{dsx} (\textit{dsxM} and \textit{dsxF}), and their role in bringing about a sexually dimorphic expression of \textit{SCR} during the development of sex comb was analyzed. The results are discussed in this chapter.
3.2 Materials and Methodology:

3.2.1 Fly stocks:

\[ dsx^1 p^P/\; TM3,Tb \text{ and } dsx^{136}/\; TM3,Tb \]

flies are gift of Kenneth C. Burtis. The pupae of \( dsx^1 p^P/\; TM3,Tb \) and \( dsx^{136}/\; TM3,Tb \) individuals are tubby in phenotype. \( dsx \) homozygote \( dsx^{136}/dsx^1,p^P \) was generated by crossing \( dsx^1 p^P/\; TM3,Tb \) and \( dsx^{136}/\; TM3,Tb \).

The \( dsx \) homozygote \( dsx^{136}/dsx^1,p^P \) are identified by the non tubby phenotype.

3.2.2 Staging and sexing of pupae:

Larvae were cultured at \( 22^{\circ}\pm2^{\circ} \) C on white wheat cream agar medium and fed with diluted yeast solution twice a day. The concentration of the yeast solution was increased as the larvae grew older. The larvae were staged till first, second, third instar and white prepupal stage (zero hour pupae). To obtain synchronised pupae, white pre-pupae were collected from culture bottles. Pupae were sexed depending on the presence and absence of the developing testis which is visible in the form of a bubble like structure in the lateral side of the larva in the posterior part in case of males (Geigy, 1931; Aboim, 1945). The zero hr pupae were then placed on a moist tissue paper in a petridish and aged at \( 22^{\circ}\pm2^{\circ} \) C for 3-4 hrs.
3.2.3 Pupal dissection:

3-4 hrs pupae were taken in a cavity block and washed with 1XPBS to remove the media sticking on them. Half of the pupae were cut off from the posterior end. Gut and fat bodies were carefully removed and the pupae were cut open laterally from both the sides using micro scissor. The dorsal wall of the pupae were removed carefully and fixed in 4% paraformaldehyde for 15 min at room temperature and washed twice with PBT (PBS+0.1% triton- X 100). The everting prothoracic leg discs were removed carefully with micro needle and forceps. 10-20 such discs of males and females were used for immunostaining.

3.2.4 Immunostaining:

Non tubby prepupae of the genotype dsx\textsuperscript{136}/dsx\textsuperscript{1},p\textsuperscript{0} were selected. Zero hour pupae were collected from the culture bottles, sexed depending on the size of the developing gonad (Geigy, 1931; Aboim, 1945), placed on a moist tissue paper in a petridish and aged at 22° ± 2C for 3-4 hrs. 10-20 pupae of XX and XY individuals of each genotype (dsx\textsuperscript{136}/dsx\textsuperscript{1},p\textsuperscript{0} and wild type) were dissected for prothoracic imaginal discs in cold Phosphate buffered saline (PBS), (Sodium chloride 130mM, Disodium hydrogen phosphate, 70mM, Sodium dihydrogen phosphate 30mM, pH 7.2). The prothoracic imaginal discs were fixed for 15 min in cold 4% paraformaldehyde (4% Paraformaldehyde was prepared by diluting 16% paraformaldehyde with PBS pH 7.2, prepared freshly before used) at room temperature (RT). Samples were washed for 10 min X 3 in PBT (PBS pH 7.2, with 0.1% triton X-100). Washes were given at RT. The samples were blocked
with 15% normal goat serum in PBT for 2hrs at RT. The discs were stained with anti-SCR antibody (DSHB) at 1:60 dilution (anti-SCR: 15% normal goat serum in PBT) and kept for incubation overnight at 4°C. The samples were washed for 2hrs (10 min X 3, 30 min X 3) with PBT. The imaginal discs were incubated with Biotinylated Goat anti-mouse IgG, which was used as secondary antibody, at a concentration of 1:1000 (Goat antimouse IgG: 15% normal goat serum in PBT) for 2hrs at RT.

The imaginal discs were washed with PBT for 2hrs (10min X 3, 30min X 3) at RT, and treated with streptavidin-horse radish peroxidase (Streptavidin-HRP) complex. It was used as a signal amplifying enzyme complexin, at a concentration of 1:500 (Streptavidin-HRP: 15% normal goat serum in PBT) for 2hrs at RT. Washes were given in PBT for 2hrs (10 min X 3, 30 min X 3) at RT. The HRP reaction was performed in staining solution containing DAB (250µg/200µl of PBT) and H₂O₂ (1%). Washes were conducted for 30 min (10min X 3) with PBT at RT.

**Mounting and observation:**

As described in Chapter 1, the prothoracic imaginal discs were mounted on a clean micro slide in 50% glycerol (PBS pH 7.2 with glycerol, 1:1). Maximum care was taken to avoid air bubble while placing the micro cover slip. The observation was done using a Leica bright field microscope at 10X, 20X and 40X. The staining pattern and the diagrammatic representation of the observation were noted down.
Imaging and image processing:

Imaging was performed on a Leica bright field microscope using DMRA2 camera and LAS software. The images were arranged and labeled using adobe photoshop software version-7.

3.2.5 PCR analysis of chromosome complement:

Zero hour non-tubby prepupae of the genotype $dsx^{136}/dsx^1,p^p$ were sexed on the basis of gonadal size. The male and female prepupae were allowed to grow in separate 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 $dsx^{136}/dsx^1,p^p$.

3.2.5.1 Genomic DNA extraction:

Genomic DNA was extracted as described in Vienna Drosophila RNAi Centre. The flies were homogenized with 250µl of solution A on ice and kept on ice for 15min at RT. It was incubated for 30 min at 70°C. 35µl of potassium acetate (8M) was added and shaken well. It was incubated again for 30min on ice and centrifuged for 15min at 13,000 rpm. The supernatant was transferred to a new tube and equal volume of phenol: chloroform: isoamyl (25:24:1) was added. It was centrifuged for 10 min at 13,000 rpm at RT. The aqueous upper layer was transferred to a new tube and equal volume of chloroform: isoamyl (24:1) was
added; vortexed and centrifused at 13,000rpm for 10min at RT. To the aqueous phase equal volume of isopropanol was added. Fibrous translucent precipitate of genomic DNA was observed. Centrifused for 5 min at 10,000 and the supernatant is pipetted out without losing the pallet. The pellet is washed with 70% ethanol. 2-3 such ethanol washes were given and final pellet was dried under vaccum. Over drying was avoided. The pellet was dissolved in 100 µl of TE.

Solutions used:

a) Solution A: Contains Tris Hcl 0.1M (pH 9.0), Ethylenediamminetetraacetate (EDTA) 0.1M, Sodium dodecyl sulfate (SDS) 1%

b) Tris-EDTA pH 8 (TE): TE was prepared as explained in chapter1.

3.2.5.2 Quantification of genomic DNA:

Quantification of the genomic DNA was conducted based on the known marker concentration. The extracted genomic DNA and a known quantity of the marker were subjected to 0.8% agarose gel electrophoresis. The concentration of the marker band whose intensity matches with that of the genomic DNA was considered to quantify the concentration of the genomic DNA. The concentration of the marker band is calculated using the formula mentioned below.

\[
\frac{LB \times CM}{LM} = CB
\]

LB= length of the band in terms of kilo base pair.
LM= total length of the marker in kilobase pair.
CM= concentration of the marker loaded in terms of µg.
CB = concentration of the band in µg.

**Reagents required:**
Reagents for Quantification of genomic DNA are same as described below in Section 3.2.5.5

**3.2.5.3 Ethanol precipitation:**
1/10ʰ of 3M Sodium acetate was added to the total volume of the aqueous DNA solution. Twice or three times the volume of chilled 100% ethanol was added. It was mixed by inverting the tube and incubated at -20°C overnight. The sample was centrifuged for 30min at 12,000 rpm at 4°C. The supernatant was discarded and the pellet was washed with 70% ethanol, centrifuged for 5min at 13,000rpm at RT. It is repeated once more and the supernatant is discarded. 3-4 times similar washes were given with 70% ethanol. The pellet was dried completely of 70% ethanol, and dissolved in 50µl TE.

**3.2.5.4 PCR:**

PCR was conducted to confirm the XX or XY chromosomal complement of the mutant flies of the genotype $dsx^{136}/dsx^{1},p^{p}$. The amplification was done for the fertility gene $kl3$ situated on the non-pseudoautosomal region of the Y chromosome. 926 bp of the 1ˢᵗ exon of $kl3$ was amplified. An amplified product specifies XY chromosomal complement and an unamplified product specifies XX chromosomal complement of the mutant individuals used for the amplification. PCR was also conducted in wild type male and female individuals of *D. melanogaster* as a control for the experiment.
The thermal profile for the amplification of exon 1 of *kl3* in male and female individuals of wild type and *dsx*¹³⁶/*dsx*¹,pp of *D. melanogaster.*

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**Amplification cycles**

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**Final extension cycles**

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Zero hour non-tubby prepupae of the genotype *dsx*¹³⁶/*dsx*¹,pp were sexed depending on the size of the developing gonads. The male and the female individuals were allowed to grow in separate culture bottles. Genomic DNA was extracted for PCR amplification. 1 μl of 50ng of genomic DNA of XX and XY wild
type and $dsx^{136}/dsx^{1}, p$ individual of $D. melanogaster$ were used as templates for different PCR reactions. Forward primer (kl3F), 5’GATCCTACATCAATACAGCCAC 3’, and reverse primer (kl3R), 5’AGTACTGGTAGCTCAATGCG 3’ were directed against the exon 1 of male fertility gene $kl3$.

**Reagents for PCR:**

a) Genomic DNA 50ng

b) Buffer 10X 5µl

c) dNTP’s 10mM 3µl

d) Primer 100picomoles (each of kl3R and kl3F) stock solution (390 picomoles/µl)

Forward primer (kl3F): 1 µl of 100picomoles.

Reverse primer (kl3R): 1 µl of 100picomoles.

e) *Taq* polymerase 1 µl of 4 units.

38 µl of nuclease free water was used to make up the final volume to 50µl.

**3.2.5.5 Agarose gel electrophoresis:**

The required percentage of agarose gel was prepared by dissolving agarose in Tris-acetate-EDTA (TAE) buffer at 60°C. The agarose solution was cooled to 40°C and ethidium bromide solution was added at a concentration of 0.5µg/ml (Sambrook and Russell, 2001), and poured into the casting tray. The gel
was allowed to polymerize for 20-30 min at room temperature. 1µl each of the PCR amplified products were diluted to 10 µl with nuclease free water (Bangalore Genie), and Gel loading dye (6X, Bangalore Genie), consisting of Bromophenol blue and Xylene cyanol.

The samples mixed with the gel loading dye were loaded in the wells. Markers with known concentration and banding pattern were used for reference.

The electrophoresis was carried out at 60-80 volt for 40-60 min duration. The DNA in the gel was visualized with the aid of a transilluminator. The image of the gel was captured with the help of the camera attached to the transilluminator.

**Reagents for gel electrophoresis:**

a) TAE(1X): TAE (1X) contains 40mM tris acetate and 1mM EDTA. The 50X stock solution I comprises of 242g of Tris base, 57.1ml of glacial acetic acid, 100ml of 0.5 M EDTA (pH 8) in 1 litre of distilled water. The solution is autoclaved and stored at room temperature.

b) Ethidium bromide: Ethidium bromide stock is prepared by dissolving 10mg of it in 1ml of distilled water.

c) DNA marker: Phi X 174DNA/Hae III marker with 11 bands namely 1353 bp, 1078 bp, 872 bp, 603 bp, 310 bp, 281bp, 271 bp, 234 bp, 194 bp, 118 bp, 72 bp respectively was used.

The marker was heated at 65°C for 5 min and rapidly chilled on ice before use.
3.2.6 Imaging and image processing of adult flies:

Imaging for the morphological observations of XX and XY individuals of \( dsx \) mutant \( (dsx^{136}/dsx^1,p^0) \) of \( D. \ melanogaster \) was performed on a Leica stereosome attached with a sony digital camera. The images were arranged and labeled using adobe photoshop software version-7.
3.3 RESULTS:

In order to analyze the role of dsx in bringing about sexually dimorphic expression of SCR and also to assay the role of sex specific variants of dsx in the context of sex comb development, SCR expression was studied in dsx mutant background (dsx\textsuperscript{136}/dsx\textsuperscript{1},p\textsuperscript{p}). dsx\textsuperscript{136} is a recessive allele of dsx that affects only male and transforms them into intersexes, but has no effect in chromosomally female flies (Baker and Ridge, 1980). dsx\textsuperscript{1} is a point mutation allele. It is a single-base deletion which leads to a frameshift, retaining positions 1-135 of the protein intact (Shirangi et al., 2006). dsx\textsuperscript{1} acts as a null allele and affects both male and female individuals. It encodes a non-functional protein in both males and females which functions as an antimorph. XX individual of the genotype dsx\textsuperscript{136}/dsx\textsuperscript{1},p\textsuperscript{p} will have a functional single copy of dsxF. dsx\textsuperscript{136}/dsx\textsuperscript{1},p\textsuperscript{p} individuals of the genetic composition XY will not have functional product of dsx as both the copies of dsx (dsx\textsuperscript{1} and dsx\textsuperscript{136}) are affected in males.

Chromosomal composition of dsx\textsuperscript{136}/dsx\textsuperscript{1},p\textsuperscript{p} individuals with different phenotype was confirmed by observing the presence of one of the male fertility gene-kl3. kl3 (CG17629) is located on the non-pseudoautosomal region of the long arm of Y chromosome. It encodes a polypeptide which forms the structural component of sperm (Goldstein et al., 1982; Charlesworth, 2001). Deficiency for kl3 are correlated with the absence of specific structures from the axoneme of flagella—i.e., the outer dynein arms of the peripheral doublets, which are required for sperm motility (Goldstein et al., 1982). 926 bp of the first exon of the kl3 gene
was amplified. An amplified product implies the chromosome composition XY, where as the absence of amplification denotes XX complement (fig 3.1).

3.3.1 Analysis of the chromosomal complement of mutant individuals \((dsx^{136}/dsx^1,p^p)\) of \(D.\ melanogaster\):

Because the mutants \((dsx^{136}/dsx^1,p^p)\), show an intermediate phenotype it was important to get a confirmation of the chromosomal sex of these mutant individuals before proceeding with further experimentation. The chromosomal complement of the given mutant was confirmed by analyzing its DNA for presence of Y chromosome specific gene kl3. PCR amplification was done for an exonic fragment from this gene. Genomic DNA of wild type and \(dsx^{136}/dsx^1,p^p\) (male and female), was isolated. 926 bp of 1st exon of the fertility gene \(kl3\), located on the non-pseudoautosomal region of Y chromosome was amplified using specific primers by PCR. The amplified product corresponds to the presence of Y chromosome and absence of amplification corresponds to the absence of Y chromosome. An amplified product of the 926 bp fragment was observed in the XY; \(dsx^{136}/dsx^1,p^p\) individuals and wild type males (fig 3.1, lane2 and lane 3), whereas such amplified product could not be seen in the XX individuals of \(dsx^{136}/dsx^1,p^p\) and wild type females (fig 3.1, lane 4 and lane 5).

Such analysis was done with Forward primer (kl3F) with sequence 5’GATCCTACATCAATACAGCCAC3’, and reverse primer (kl3R) with sequence 5’AGTACTGGTAGCTCAATGCG 3’. They were directed against the 926bp fragment of exon 1 of male fertility gene \(kl3\). PCR reaction was performed in a final volume of 50µl containing 100pmol/ µl of the primer (kl3F, kl3R).
Amplification was carried out for 30 cycle repeats of a thermal profile of 95°C for 45° seconds (denaturation), 57°C for 30 seconds (annealing) and 72°C for 45 seconds (extension). PCR conducted with the wild type male and female *D. melanogaster* act as a control for the experiment.

Fig 3.1: PCR-amplified DNA of 926bp of 1st exon of *kl3* gene from *dsx*¹³⁶/*dsx¹*,p⁰ mutant and wild type in *D. melanogaster*. *kl3* is one of the male fertility gene located on the non-pseudoautosomal region of Y-chromosome which encode dynein heavy chains of the outer arm of the sperm tail axoneme. The 926 bp amplicon of exon 1 of *kl3* was amplified using the primer *kl3F/*kl3R. DNA marker (Lane 1), PCR-amplified *kl3* from XY *dsx*¹³⁶/*dsx¹*,p⁰ mutant (Lane 2), male wild type (Lane 3), XX *dsx*¹³⁶/*dsx¹*,p⁰ mutant (Lane 4), and female wild type (Lane 5). An amplified product of the gene can be seen in XY *dsx*¹³⁶/*dsx¹*,p⁰ and male wild type which specifies the XY chromosomal compliment of the individual. XX *dsx*¹³⁶/*dsx¹*,p⁰ individuals and female wild type show the absence of the amplification which denotes the XX compliment.
3.3.2: Morphological observations of XX and XY individuals of dsx mutant in a background of dsx\textsuperscript{136}/dsx\textsuperscript{1},\textit{p}\textsuperscript{p} of \textit{D. melanogaster}:

Sexual dimorphism is strikingly evident in \textit{D. melanogaster} adult. The three striking regions which show sexual dimorphism in \textit{D. melanogaster} are (1) abdominal pigmentation (2) the structure of the terminal abdominal segments and genitalia and (3) the bristle pattern in ts1.

![Morphological phenotypes of abdominal pigmentation phenotype of wild type and dsx\textsuperscript{136}/dsx\textsuperscript{1},\textit{p}\textsuperscript{p} mutant of \textit{D. melanogaster}. Abdominal pigmentation of (a) wild type male, (b) XX dsx\textsuperscript{136}/dsx\textsuperscript{1},\textit{p}\textsuperscript{p} mutant, and (c) XY dsx\textsuperscript{136}/dsx\textsuperscript{1},\textit{p}\textsuperscript{p} mutant in \textit{D. melanogaster}. \textit{D. melanogaster} is sexually dimorphic for abdominal pigmentation, in wild type males the posterior segments of the abdomen are dark whereas in wild type females the posterior segments are lightly pigmented (fig not shown). In XY: dsx\textsuperscript{136}/dsx\textsuperscript{1},\textit{p}\textsuperscript{p} mutant individuals, where both the copies of \textit{dsxM} are defective the posterior segment of the abdomen is relatively darker than the wild type female abdomen but comparatively lighter than the abdominal pigmentation in wild type males. The abdominal pigmentation of XX: dsx\textsuperscript{136}/dsx\textsuperscript{1},\textit{p}\textsuperscript{p} mutant is similar with wild type female.](image)

In wild type males the fifth and sixth dorsal segments of the abdomen (fig 3.2a) are uniformly darkly pigmented in the male, but yellowish over the anterior portions and darkly pigmented along their posterior margins in the females. In XY: dsx\textsuperscript{136}/dsx\textsuperscript{1},\textit{p}\textsuperscript{p} individuals the abdominal pigmentation is not as intense as the wild type XY individuals (fig 3.2c). The intensity of abdominal pigmentation seems
Fig 3.3: Morphological phenotypes of abdominal tip of \( dsx^{136}/dsx^{1},p^p \) mutant and wild type \( D. \ melanogaster \). The abdominal tip of (b) XX \( dsx^{136}/dsx^{1},p^p \) mutant shows no remarkable difference from that of the wild type female (figure not shown), whereas the abdominal tip of (c) XY \( dsx^{136}/dsx^{1},p^p \) mutant shows a different phenotype from that of (a) wild type male.

Fig 3.4: Morphological features of tarsal segments of the prothoracic legs of \( dsx^{136}/dsx^{1},p^p \) mutant and wild type \( D. \ melanogaster \). First tarsal segment (ts1) in (a) wild type male shows the presence of sex comb (SC). The most distal TBR undergo rotation by 90° which forms the sex comb. First tarsal segment in (b) XX: \( dsx^{136}/dsx^{1},p^p \) mutant shows only the presence of TBR without sex comb which show no remarkable difference from ts1 of wild type female (fig not shown). The first tarsal segment of (c) XY: \( dsx^{136}/dsx^{1},p^p \) mutant individuals with defective copies of \( dsx^M \) shows subtle transformation of few TBR into sex comb like bristle (SCLB). The SCLB are little more blunt and thicker in comparison with the TBR, slightly rotated in comparison with the dark and thick bristles of sex comb.
to be intermediate between the XY wild type individuals and XX wild type individuals. The abdominal pigmentation of the XX: \(dsx^{136}/dsx^1,p^0\) (fig 3.2b) does not show remarkable difference from that of the XX wild type individuals (fig not shown).

In XY wild type the abdominal tip is round (fig 3.3a) shows a genital plate whereas in XX wild type the abdominal tip is pointed with egg guide. The abdominal tip of XX: \(dsx^{136}/dsx^1,p^0\) (fig 3.3b) is similar to that of XX: wild type individuals (figure not shown). Abdominal tip of XY: \(dsx^{136}/dsx^1,p^0\) (fig 3.3c) is strikingly different from that of XY wild type. It is partially transformed towards female wild type like. The tip is not as blunt as wild type male and there are very weak symptoms of formation of a genital plate.

In wild type XY individual male the ts1 shows the presence of transverse bristle rows and sex comb (fig 3.4a). As previously mentioned in chapter 1, sex comb bristles are highly chitinized and blunt which makes them dramatically different from the TBR. In XX wild type the chaetotaxy of ts1 shows only the TBR. In XX: \(dsx^{136}/dsx^1,p^0\) (fig 3.4b), the arrangement of bristles is similar with XX wild type female (figure not shown). In XY: \(dsx^{136}/dsx^1,p^0\) (fig 3.4c), the last row of TBR shows subtle transformation into sex comb like bristles (SCLB). The SCLBs are slightly thicker compared to the normal TBRs and also shows slight rotation in their orientation of arrangement.
3.3.3 SCR expression in prothoracic leg discs of wild type and \textit{dsx} mutant (\textit{dsx}^{136}/\textit{dsx}^{1},p^p) of \textit{D. melanogaster}:

Fig 3.5: \textit{Scr} expression pattern in prothoracic leg discs of wild type and \textit{dsx}^{136}/\textit{dsx}^{1},p^p mutant of \textit{D. melanogaster}. 3-4 hours pupae were dissected for prothoracic leg discs and were stained with anti-SCR antibody. SCR could be detected in the tibia (ti) and the first tarsal segment (ts1). Arrow indicates the distal portion of the ts1. (a) wild type female, SCR could not be detected in the distal tip of ts1 whereas SCR expression extends to the distal end of ts1 in (b) wild type male \textit{D. melanogaster}. (c) XX: \textit{dsx}^{136}/\textit{dsx}^{1},p^p individuals with expression of SCR depicting a similar profile with that wild type male individuals. There is no noticeable change in the SCR profile of XX: \textit{dsx}^{136}/\textit{dsx}^{1},p^p from that of (d) XY: \textit{dsx}^{136}/\textit{dsx}^{1},p^p individuals and male wild type individuals. SCR expression extends till the distal end of ts1 in all these cases. Scale bar is 0.1mm and same in all the images displayed.
The expression pattern of SCR which is a key genetic regulator in the development of sex comb was analyzed in the prothoracic leg discs of 3-4hrs pupae in wild type and \( dsx \) mutants (\( dsx^{136}/dsx^1,p^0 \)) of \( D. \ melanogaster \). The prepupae were sexed by gonadal size as explained earlier. The expression pattern was analyzed both in the XX and the XY individuals. The expression was studied by immunostaining. SCR expression could be localized in the tibia (ti) and the ts1 in both female and male individuals of wild type \( D. \ melanogaster \) (fig 3.5a, b). The SCR profile depicts sexually dimorphic expression between male and the female individuals in ts1 of prothoracic pupal leg discs. In males the expression of SCR extends till the distal margin of ts1 whereas such extension in the expression of SCR in the ts1 could not be witnessed in females T1 leg discs. In \( dsx^{136}/dsx^1,p^0 \), SCR could be witnessed in the ti and ts1. No sexually dimorphic expression of SCR was evident in the XY: \( dsx^{136}/dsx^1,p^0 \) and XX: \( dsx^{136}/dsx^1,p^0 \) (fig 3.5 c, d), T1 leg discs.

Interesting both XY: \( dsx^{136}/dsx^1,p^0 \) and XX: \( dsx^{136}/dsx^1,p^0 \) individuals despite the defect in \( dsx \) and their chromosome compliment showed a male like pattern of SCR expression.
3.4 Discussion:

Sex comb a sexually dimorphic trait which shows its presence only in the male individuals of some of the species of *Drosophila* also shows a spatial specific development. The ts1 of the prothoracic leg in male has gained the potential of developing sex comb in *D. melanogaster*. Data from earlier works (Kopp *et al.*, 2000; Williams and Carroll, 2009; Foronda *et al*; 2012), on the development of sexually dimorphic traits illustrates the existence of spatial specific genetic regulators playing a key role in the genetic pathway of development of such traits. Spatial specific regulators like *bric-a-brac* and *Abdominal B* in the development of sexually dimorphic abdominal pigmentation (as discussed in chapter 1) and *Abd-B* in the development of abdominal segment can be cited as a good example. The abdominal seventh segment is absent in *Drosophila* male but present in female individuals. The spatial regulator *Abd-B* regulates the transcription of the sex determining gene *dsx*. The male splice product of *dsx* controls *extramacrochetae* and *spaghetti-squash* expression which in turn extrudes the precursor of seventh abdominal segment during early pupae. In the light of these earlier findings and sex comb being a spatial specific trait the input from spatial specific regulator/s is expected to interact with somatic sex determining regulators during the development of sex comb.

As discussed in detail in chapter 1, sex comb development in the T1 leg is promoted by the *Hox* gene *Scr* (LeMotte *et al*., 1989; Rogers *et al*., 1997). In homozygous *Scr* null clones and in trans-heterozygous combinations of strong
hypomorphic mutations there is complete loss of sex comb (Lewis et al., 1980; Struhl, 1982; Pattatucci et al., 1991). A strong reduction in the number of sex comb teeth is seen in weaker hypomorphic combinations and flies heterozygous for Scr nulls or deficiencies (Kaufman et al., 1980; Lewis et al., 1980; Pattatucci et al., 1991). An increase in the number of sex comb teeth is seen in Scr duplications (Capdevila et al., 1986; Kennison and Russel 1987; Boube et al., 1997; Shroff et al., 2007). Ectopic expression of Scr which are caused due to gain-of-function mutations in meso and meta thoracic leg are sufficient to induce ectopic sex combs in these legs (Hannah-Alava, 1964; Kaufman et al., 1980; Kennison and Tamkun, 1988; Pattatucci and Kaufman 1991). Work of Barmina and Kopp, (2007), showed that ectopic expression of Scr in the second tarsal segment of the T1 leg in male D. melanogaster has gained the potential to develop ectopic sex comb in this segment. Scr was found to exhibit considerable expression pattern plasticity which is correlated with comb number variation in the Sophophoran species (Barmina and Kopp, 2007; Randsholt and Santamaria, 2008). These findings illustrate the key role played by Scr in the development of sex comb as the spatial cue provider. As stated earlier sexually dimorphic expression of SCR can be seen in the distal tip of ts1 in D. melanogaster: in males the expression extends till the distal end of ts1 whereas in females SCR expression do not reach the distal tip. Regulation of this sexually dimorphic expression of SCR is an exciting question as yet.

Development and the evolution of many sexually dimorphic traits require genetic cues from regulators of sex determination pathway. In many organisms
including *Drosophila*, the genetic input from the sex determining pathway is provided by *dsx*, in the development of such traits. The *dsx* gene of *D. melanogaster* undergoes differential splicing to give male-specific and female-specific polypeptides. The primary transcript of the *dsx* gene is alternatively spliced in males and females to yield sex-specific mRNAs which encode male-specific and female-specific polypeptides *DsxM* and *DsxF* respectively (Burtis and Baker, 1989). Some of the prominent examples where *dsx* has been shown to play a crucial role in the development of sexually dimorphic traits are abdominal pigmentation, yolk protein (Bownes and Nothiger, 1981; Hutson and Bownes, 2003), and genitalia development in *Drosophila* (Keisman and Baker, 2001). Yolk protein genes *yp-1* and *yp-2* are the best characterized targets for *dsx* regulations. These genes are regulated sex specifically in the adult fat body by *dsxM* and *dsxF*. Both forms of *dsx* bind to the same sites within the fat body enhancer (FBE) that lies directly between the two yolk protein genes *yp-1* and *yp-2* (Erdman and Burtis, 1993; An and Wensink, 1995) with opposite regulatory effects on *yp-1* transcription where in *DsxF* activates and *DsxM* represses (Burtis *et al.*, 1991; Coschigano and Wensink, 1993). *dsx* interacts with *Abd-B* and a variety of signaling pathways to control sexually dimorphic development of the *Drosophila* genitalia (Keisman and Baker, 2001; Sanchez *et al.*, 2001; Ahmad and Baker, 2002; Christiansen *et al.*, 2002). The mutant *doublesex*, when homozygous in either chromosomally male or female individuals, causes them to develop into intersexes (Hildreth, 1965). The most strikingly evident dimorphic characters of wild type males and females develop intermediatively to an intersex
individual (Hildreth, 1965). The intensity of pigmentation in the fifth and the sixth dorsal segments of the abdomen (tergites) will be between that of male and female pigmentation. The genitalia are not fully formed.

The bristles of the t1 resemble neither the male nor the female wild type individuals. Development of ectopic sex comb teeth is visible when there is over expression of \( dsxM \) (Jursnich and Burtis 1993; Tanaka et al., 2011), whereas \( dsxF \) can reduce the number of sex comb teeth when expressed in males (Waterbury et al., 1999). These earlier findings show that \( dsx \) plays a critical role during the development of sex comb. Works of Tanaka et al., (2011), have demonstrated that there is interaction of \( dsx \) and \( Scr \) during the development of sex comb. Their study have shown that \( Scr \) activate \( dsx \) expression in the presumptive sex comb region, \( dsxM \) up-regulates \( Scr \), so that both genes become expressed at high levels in this region in males but not in females as the splice variant of \( dsx \) in female is \( dsxF \) and not \( dsxM \). The present study has attempted to see if/ how there is a role played by \( dsx \) in bringing about the dimorphic expression of \( Scr \), and the differential requirement of \( dsxM \) and \( dsxF \) for two separate functions during the development of sex comb. The results are discussed.

3.4.1 Sexually dimorphic expression of SCR is under the regulation of \( dsxF \):

As described earlier (fig 3.5a,b) the SCR profile in males and females \( D. melanogaster \) show a dimorphic expression. The males show an extended expression till the distal tip of ts1, and in females the expression does not extend
till the distal tip of ts1 (fig 3.5a, b). The T1 imaginal discs of the genotype XX: $dsx^{136}/dsx^1,p^0$ individuals subjected for Anti-SCR antibody staining, showed SCR expression pattern similar with the SCR expression pattern of the wild type males (XY) individuals (fig 3.5c, b). The SCR expression could be seen in the ti and the ts1. The expression extends till the distal portion of ts1. The expression pattern is remarkably different from wild type female (fig 3.5a): wherein the expression does not extend till the distal end of ts1. As described earlier $dsx^{136}$ is male specific mutant allele, and $dsx^1$ affects both male and female equally. Female individual with the genetic composition $dsx^{136}/dsx^1,p^0$, will have a single copy of a functional $dsxF$ against two functional copies as in a wild type female. From the present study, it seems like in wild type female, with two functional copies of $dsxF$, the SCR expression is suppressed in the distal portion of the ts1. In the female mutant background with a single functional copy of $dsxF$, the suppression of $Scr$ is inhibited and the SCR expression extends till the distal end of the ts1.

XY individuals with the genetic composition $dsx^{136}/dsx^1,p^0$ (fig 3.5d) also show a similar SCR profile as with the wild type male (fig 3.5b) individuals. XY mutant background has both the copies of $dsxM$ defective as $dsx^{136}$ is male specific mutant and $dsx^1$ is a mutant allele in both XX and XY individuals. In the allelic combination $dsx^{136}/dsx^1,p^0$, the XY individuals will be completely lacking the male specific variant of $dsx$. The SCR expression in the prothoracic discs of XY; $dsx^{136}/dsx^1,p^0$ implies that $dsxM$ has no role to play with the expression of SCR. Since chromosomally it is male individual $dsxF$ will not be made, and the flies will be null for $dsx$ function. Thus, the male like SCR expression could be default
pattern and it might be independent of \textit{dsx}. The male like expression in SCR in XX ($dsx^{136}/dsx^{1},p^{p}$) strongly suggest that, there is a dose dependency, and \textit{dsx} female function is needed in two copies for repression of \textit{Scr} in the distal part of the ts1.

\textbf{3.4.2 The sexually dimorphic trait sex comb is a product of interaction between \textit{dsx}M and \textit{Scr}:

The ts1, abdominal pigmentation phenotype and the abdominal tip of the male and female wild type and $dsx^{136}/dsx^{1},p^{p}$ are depicted in fig 3.4, 3.2 and 3.3. XX: $dsx^{136}/dsx^{1},p^{p}$ shows similar phenotype of these morphological structures with wild type females. The phenotypes of XX: $dsx^{136}/dsx^{1},p^{p}$ are shown in fig 3.2b, 3.3b, and 3.4b, the arrangement of bristles in ts1 (fig 3.4b), is similar with that of wild type female, there is no indication of transformation of the bristles towards the sex comb phenotype. The SCR expression in these mutants (fig 3.5c), depicts male like pattern (3.5b) of expression with an extended expression till the distal tip of ts1.

In \textit{Sophophoran} group there exist a correlation between the plasticity of SCR expression and number of sex comb present (Barmina and Kopp 2007;Randsholt and Santamaria, 2008). There also exists a correlation between presence of sex comb and SCR expression in the distal end of ts1 in male against negligible expression of SCR in the distal tip of ts1 and absence of sex comb in females in \textit{D. melanogaster} (Barmina \textit{et al.}, 2007). Interestingly the male like pattern of SCR expression in XX: $dsx^{136}/dsx^{1},p^{p}$ is not able to activate the development of sex comb structure.
The chaetotaxy in the ts1, abdominal pigmentation, and abdominal tip of XY: $dsx^{136}/dsx^1,p^\alpha$ displays the characteristic of intersex (fig 3.4c, 3.2c, 3.3c). The abdominal pigmentation (fig 3.2c) is not as intense as the wild type individuals. The genitalia has been transformed to intermediate state (fig 3.3c). The arrangement of the bristle in the ts1 (fig 3.4c) resembles neither the male (fig 3.4a) nor the female individuals. The last row of the TBRs though slightly thickened does not show chitinization comparable to sex comb. Slight rotation of the TBRs through 10°-15° could be observed. Transformation of the last row of the TBR into the sex comb is not evident. The analysis made on SCR expression with $dsx$ mutant background reveals fascinating points, that the male like extended expression pattern of SCR in XX: $dsx^{136}/dsx^1,p^\alpha$ (fig 3.5c), and XY: $dsx^{136}/dsx^1,p^\alpha$ (fig 3.5d) is not sufficient enough to induce the morphogenesis of sex comb. The male SCR expression pattern is not dependent on $dsxM$. But the function of $dsxM$ together with SCR is required for the complete transformation of the bristles into sex comb. The development of sex comb requires input from $dsxM$. Findings of Burtis and Jursnich, 1993, have demonstrated that there was thickening and transformation of the bristles into sex comb like bristles when there is ubiquitous expression of $dsxM$, the extend of transformation was more prominent in distal part of tarsal segment in SCR expression domain. As described, earlier work of Tanaka et al., (2011), has revealed that there exists a feedback loop for expression of SCR and Dsx in the tarsal segment which forms a crucial event for the morphogenesis of sex comb. The findings of the present study gives an intriguing result: $dsx$ is not needed for the male like pattern of SCR
expression in early stage of development at 3-4 hrs APF, whereas the feedback loop as described by Tanaka et al.,(2011) probably is initiated in 24hrs APF. Since Scr alone in the absence of dsxM is not able to induce sex comb morphogenesis, there might be downstream targets of dsxM and Scr which cooperate with each other in shaping the morphogenesis of sex comb. There is mounting evidence of dsx regulating the development of the sexually dimorphic traits in Drosophila as described earlier. During the event of development of such dimorphic structures, the male specific isoform (dsxM) promotes the development of male specific structures and represses female-specific structures, while the female-specific isoform (dsxF) promotes female-specific and represses male-specific structures (Baker and Ridge, 1980; Jursnich and Burtis, 1993; Li and Baker 1998; Waterbury et al.1999). Findings from the present study demonstrate an interesting role of dsxF during the development of sex comb. DsxF suppressed the expression of Scr in the distal portion of ts1 in female thus results in the dimorphic expression of SCR between male and female individuals in species with sex comb. The female SCR pattern which is resulted due to suppression of SCR by DsxF indirectly affects the development of sex comb. In the complete absence of dsxF in males, with a male like SCR expression pattern in ts1, is not able to induce the formation of sex comb, which strongly suggest that sex comb morphogenesis requires dsxM which in combination with SCR gain the potential to transform the TBRs into sex comb in the ts1 of prothoracic leg of Sophophoran male flies. Thus the two different sex specific variants have got two separate functions to play during sex comb morphogenesis.