SEX COMB BRISTLE NUMBER VARIATION IN

DROSOPHILA MELANOGASTER

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By

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ABSTRACT

The sex comb an array of specialized bristles on the foreleg, is a highly variable male trait of *Drosophila* that provides an ideal system for integrative studies of morphological evolution. Here, studies of the genetic and developmental architecture of sex comb bristle number variation in *Drosophila melanogaster* are described. Analysis of the response to twenty-four generations of divergent artificial selection indicated high genetic variance underlying this trait, and demonstrated a weak relationship with other, developmentally related non-sex bristle systems. I also present evidence showing bristle number is associated with mating success. Manipulation of diet in full-sib families confirmed that this trait is condition dependent, and that there is a genetic basis for condition dependence. Further partitioning of variance components using a half-sib mating design revealed a strong maternal, dominance and/or X chromosome effect on sex comb bristle number variation. Finally, sex comb bristle number was not correlated with comb orientation in wild type, High and Low artificial selection lines, or the mutant strain *bric à brac PR72*. Analysis of patterns of variation in comb orientation over ontogeny in these lines showed that this aspect of the sex comb phenotype is highly canalized. This body of work provides important insight into *D. melanogaster* sex comb evolvability, and represents a timely approach to bridging the gap between population genetics and development in studies of phenotypic evolution.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 PARADIGMS AND PROGRAMS IN THE STUDY OF MORPHOLOGICAL EVOLUTION

Variation is the defining feature of the living world, seen in different types of traits from morphology and physiology to life history and molecules. Much biological research aims to understand how and why there is variation in one type of trait or another. In evolutionary biology variation is a central topic, both conceptually and historically. Darwin (1859) noted the amazing diversity of forms in the living species he encountered during his travels, and it was in trying to explain morphological variation that the theory of evolution by natural selection was formulated. In the years since the field of morphological evolution has grown tremendously. Two main schools of thought emerged with progress and advances in understanding the genetic and developmental machinery underlying phenotypic variation. Each emphasized the importance of different factors in shaping the trajectory of evolutionary change, and different approaches and programs for its study.

The discovery that genes, located linearly on chromosomes, are the physical units of inheritance (Morgan *et al.* 1915), and the subsequent development of the statistical methods of population genetics (Fisher 1930) provided the stimulus for the modern synthesis of evolutionary biology. Under the

neo-Darwinian paradigm, phenotypic variation is generated by the joint effect of many genetic variants of small magnitude, and selection acting on this phenotypic variation is the ultimate driving force of evolutionary change. Thus an understanding of the genetic architecture and the nature of selective forces acting on a trait should allow us to reconstruct the past, and make predictions about the future evolution of a trait. This approach does work well to explain the evolution of adaptive traits, but is unable to sufficiently address other evolutionary phenomena such as the occurrence of non-adaptive traits (Gould and Lewontin 1979), or rapid changes in form (Eldredge and Gould 1972). Also, if new variation is generated by accumulation of existing genetic variants, how can the emergence of qualitatively new traits be explained (Muller and Wagner 1991)?

Developmental biologists attributed these explanatory deficits of neo-Darwinism to its neglect of internal processes that convert genotype to phenotype and influence what kind of morphological variation will arise from genetic variation. Change in certain directions of the morphospace may be constrained or limited (Alberch 1982; Maynard Smith *et al.* 1985) while there may be heightened potential for change in other directions (Arthur 2004; Brakefield 2006). This may be due to developmental, architectural or mechanical properties of cells, tissues and molecules and the interactions between them. The "constraint school" thus contrasts from the neo-Darwinian view that any morphological variation can be produced given abundant genetic variance, and emphasizes the

importance of internal factors over external pressures in shaping morphological change.

Traditionally there has been a gap between the fields of population genetics and developmental biology, but it is clear that integrative evo- devo studies are needed to reconstruct the past and make future predictions about how a trait evolves. Evolvability, the intrinsic potential of a system to produce heritable phenotypic variation, has long been studied only from a gene centric view i.e. by measuring levels of additive genetic variance (Houle 1992). By also looking at the variational properties of developmental systems (Salazar-Ciudad *et al.* 2003) we can gain insight into the developmental capacity of lineage to produce change. An integrative approach has the potential to shed light on broader issues in the field of evolution as well: much remains to be learned about the relative importance of and interplay between external and internal factors in shaping the trajectory of evolutionary change, and there is a search for general principals and patterns in the evolution of development.

1.2 Rapidly evolving male sex traits and sexual selection

Traits that evolve rapidly over relatively short time scales and show large variation between closely related species provide a powerful model for studies of morphological evolution. Across the animal kingdom male sexual traits represent some of the most elaborate, exaggerated and highly divergent traits seen in nature

(Civetta and Singh 1999; Singh and Kulathinal 2000). In *Drosophila* for instance, male genitalia are complex and diversify rapidly and are often the only morphological characteristics that enable differentiation between closely related species (Eberhard 1985). The morphology of internal structures like the testes and accessory glands is also highly variable between species (Patterson and Stone 1952) and there is extraordinary variation even at the cellular level in sperm structure and size among different species of *Drosophila* (Joly *et al.* 1995).

Darwin (1871) proposed that the rapid divergence of male sex traits is driven by differential reproductive success arising from competition for mates and fertilization, i.e. sexual selection. According to the female mate choice theory, the variation in male traits like ornamentation is driven by variation in female preferences for different forms of the trait. This could potentially lead to a "runaway" process of change (Fisher 1930). As female preference for the trait grows so will the male trait, leading to rapid evolutionary exaggeration and diversification of sexual traits until counter balanced by natural selection. Sexual selection can also occur through male – male competition for access to mates and can drive the rapid adaptive radiation of male traits like weaponry (Emlen 2008). Alternatively, Parker (1979) proposed that the differences in the reproductive interests of males and females leads to conflict between the sexes wherein they engage in a coevolutionary arms race to control reproduction. Such antagonistic co-evolution may be the driving force behind the rapid divergence of male

secondary sexual traits that are accompanied by changes in corresponding female traits (Chapman *et al.* 2003). To explain phenomena such as the evolution of new male biased genes, male's aggressive behaviour and maintenance of costly variations in males, Singh and Kulathinal (2005) proposed the male sex drive theory. Male sex drive results from the dominance of males over females in developing new strategies (structural, physiological, behavioural) in an effort to gain matings.

Male sex traits are of particular interest to evolutionary biologists for another reason: Divergent sexual selection driving rapid divergence in morphology and correlated changes in mating behaviour, when coupled with population isolation, could lead to the establishment of barriers to reproduction during the early stages of speciation (Civetta and Singh 1999). Evidence from a variety of taxa, including Hawaiian *Drosophila* (Carson 1997) and sticklebacks (Boughman 2001) supports the potential role of secondary sexual traits in speciation.

1.3. THE SEX COMB OF *DROSOPHILA*

The sex comb, an array of specialized bristles on the foreleg, is a highly variable, novel male trait of the *melanogaster* and *obscura* species groups of *Drosophila*. Females lack a sex comb, and in *D. melanogaster* it has been has been shown that the most distal row of transverse bristles on the first tarsus is homologous to the male sex comb (Tokunaga 1962). Sex comb

morphology has diverged very rapidly between species in terms of the location and orientation of rows and the number of bristles per row (Kopp and True 2002): For instance, in *D. eugracilis*, the sex comb consists of only two enlarged bristles, transversely oriented on the first tarsus whereas in *D. ficusphila*, the comb is longitudinal in orientation and extends along the entire length of the first and second tarsus, with 20 – 30 bristles in each row. All the species of the *melanogaster* sub group have a single, longitudinal row of 8–14 teeth while members of the *takahashii* subgroup have several transverse rows on the first, second and sometimes even third tarsus. *D. lucipennis* has lost its sex combs altogether. Within a species, the location and orientation of the comb is conserved but bristle number can be variable (Coyne 1985).

The male sex comb has been the subject of extensive population genetics and developmental analysis, but there has been limited communication between studies from the two fields. This is largely because the different fields have focused on different aspects of the trait. In the following section I review the existing literature and discuss the potential of the sex comb as a powerful system for integrative studies of morphological evolution.

1.3.1 Function and Sexual Selection

From observations of courtship and copulation in various *melanogaster* and *obscura* group species it appears that the exact function of the comb during

mating differs between species with differing morphology. In some species with large longitudinal comb covering the entire tarsus, males appear to grasp the female's abdomen with the sex comb, while in species with smaller transverse combs they appear to be used to spread the wings of the female apart during copulation (Speith 1952). In species of the *melanogaster* subgroup that have smaller longitudinal combs it appears that the comb makes very transient contact with the females body, and is thought to grasp the extruded genitalia during mounting, but is not used once final copulation position is obtained (Speith 1952; Coyne 1985). The importance of the sex comb for mating success has been further confirmed by experimentally removing the sex comb. Reduced ability to inseminate females was reported when the foreleg was amputated above, but not below, the sex comb in *D. pseudoobscura* and *D. persimilis* (Speith 1952) as well as in *D. mauritiana* and *D. simulans* (Coyne 1985). Amputation of the comb itself led to delayed copulation in *D. melanogaster* and *D. simulans*, but no other aspects of courtship were affected (Cook 1977). Ng and Kopp (2008) genetically ablated the sex comb by expressing the female specific isoform of *transformer* in the tarsal segments of the male leg. As a result sex comb teeth were modified into normal mechansensory bristles resembling those of other *Drosophila* lacking sex comb while other aspects of leg morphology remained the same. Direct observations and differences in insemination rate confirmed that loss of the comb reduces the ability of males to copulate with females, but does not affect

copulatory behaviour.

Sex comb bristle number has been shown to affect mating success and the direction of sexual selection on bristle number also appears to differ between species: Markow *et al.* (1996) found that mating male *D. simulans* in a natural population had significantly fewer sex comb teeth than males not found copulating. The opposite effect was seen in *D. bipectinata*, where there was an increase in the number of teeth in the second row of copulating males in a natural population (Polak *et al.* 2004). In *D. pseudoobscura* size did not appear to affect mating success (Markow *et al.* 1996). Most recently, Polak and Simmons (2009) assayed males form *D. bipectinata* with large and small combs for competitive fertilization ability and reported that sexual selection for increasing sex comb size in this species may be post-copulatory in nature.

It is still not clear if sex combs are a display trait whose size and shape is perceived by females to assess male quality. Alternatively, the sex comb could be a mechanical male sex drive trait used to grasp the female, and size could affect the efficiency of grasping. The large number of bristles on the female's abdomen, genitalia, and wings could serve as mechanosensory organs to perceive comb size and shape, or as an anchor if the comb is a purely mechanical trait (Coyne 1985; Ng and Kopp 2008). Detailed studies of comb morphology and correlated changes in male and female mating behaviour and morphology are needed to ascertain if the rapid evolution of sex comb morphology could be driven by changes in

female preferences, or by co-evolution between sex combs and female external genitalia (Ng and Kopp 2008).

1.3.2 GENETICS

Two different approaches have been employed in investigations of sex comb genetics. Developmental genetics studies aim to elucidate the molecular pathways involved in the specification and differentiation of sex combs i.e. they attempt to explain how a sex comb is made. These studies have uncovered a network consisting of positive and negative regulatory inputs from HOX, proximodistal identity and sex determination pathways (Barmina and Kopp 2007; Randsholt and Santamaria 2008). Quantitative geneticists have focused on attempting to understand how sex comb variation is produced. QTL mapping studies have been conducted to map chromosomal regions contributing to bristle number variation within and between different species of the *melanogaster* complex (Coyne 1985; True *et al.* 1997; Macdonald and Goldstein 1999; Nuzhdin and Reiwitch 2000; Kopp *et al.* 2003; Tatsuta and Takano-Shimizu 2006; Graze *et al.* 2007). Overall, these studies have revealed sex comb bristle number variation is controlled by multiple loci on different chromosomes, and that some of the same loci that are responsible for intra-specific variation can also contribute to inter-specific differences. Studies of developmental genetics have informed QTL mapping studies, allowing the identification of candidate genes within large chromosomal regions. Thus far researchers have been unable to identify causal

polymorphisms at the nucleotide level or confirm candidate gene associations in natural populations.

1.3.3 Development and pattern formation

Sex comb bristles arise from proneural clusters of cells from which a single cell, the sensory organ precursor, is selected to form the bristle and its associated components (Held 2002). Using *D. melanogaster* gynanders and mosaics Tokunaga (1962) showed that the comb forms from the most distal transverse row that then rotates almost 90 degrees to come to its final longitudinal orientation. Held *et al.* (2004) confirmed that rotation occurs between 16 and 24 hours after pupation. Detailed analysis of this process by live confocal imaging of *D. melanogaster* pupal legs has shed light on the underlying cellular dynamics (Atallah *et al.* 2009a). The sex comb bristle precursors are initially non-contiguous, eventually joining together in a single row through intercalation. It appears that most of the rotation to the final longitudinal orientation takes place after the comb is a single unit, as a result of cellular rearrangements as the leg itself becomes thinner and longer.

Phylogenetic analysis of sex comb evolution has shown that the transition between transverse and longitudinal sex combs has occurred several times in the *melanogaster* species group and in both directions (Kopp and True 2002). Comparison of comb development in different species has shown that two

different cellular mechanisms are employed in species with longitudinal combs (Atallah *et al.* 2009b; Tanaka *et al.* 2009). In species with the large longitudinal combs comb precursors arise in the final longitudinal orientation, while in others the comb originates in a transverse orientation and then rotates and aligns to form a single longitudinal row. The finding that different mechanisms give rise to similar morphologies, and that these mechanisms have evolved independently in two or more lineages shows that this complex developmental system can be quite plastic.

1.4 THESIS OVERVIEW

As a rapidly diverging, novel trait that is amenable to both population genetic and developmental analyses, the sex comb of *Drosophila* is a very attractive system for integrative studies of the evolution of form. Given its affect on male mating success in different species, bristle number appears to be an evolutionarily important component of the trait. Variation within a species is the substrate for selection and speciation, and here I have focused on bristle number variation within *D. melanogaster*, a model for population genetics and developmental biology. I utilize an integrative approach, exploring the genetic variation, response to environmental variation, and variation during development.

In Chapter 2, I begin with characterizing the genetic architecture of sex comb bristle number variation in *D. melanogaster* by analyzing the response to 24

generations of replicated divergent artificial selection. I also assessed correlated responses in developmentally related bristle traits to shed light on potential constraints on comb evolution, and assayed life history traits like mating success and fertility in selection lines. I further investigate the genetic architecture of sex comb bristle number variation in Chapter 3 by assessing the response to environmental manipulation. I conducted quantitative genetic analyses using full- and half-sibling families to test if sex comb bristle number is a condition dependent trait, and if condition itself harbours high genetic variance. Developmental analysis of the relationship between sex comb bristle number and orientation is presented in Chapter 4. I analyze the patterns of variation in comb orientation over ontogeny in genotypes with differing bristle numbers: High and Low sex comb bristle number lines and *bric à brac PR72*, a mutant strain with ectopic sex combs. Finally, in Chapter 5, I put together the results from these studies and discuss what they can tell us about *D. melanogaster* sex comb evolvability, and discuss avenues for further research.

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CHAPTER 2

VARIATION AND EVOLUTION OF MALE SEX COMBS IN *DROSOPHILA*: NATURE OF SELECTION RESPONSE AND THEORIES OF GENETIC VARIATION FOR SEXUAL TRAITS

This chapter has been published in Genetics. To gain insight into basic evolution and inheritance of sex comb bristle number in *Drosophila melanoagster* I assessed in this trait in 32 different geographical populations. I characterized the genetic architecture of sex comb bristle number variation in *D. melanogaster* by analyzing the response to 24 generations of replicated divergent artificial selection for bristle number. I also assessed correlated responses in developmentally related bristle traits to shed light on potential constraints on comb evolution, and assayed life history traits like mating success and fertility in the selection lines. I designed and performed all experiments and wrote the manuscript with input from Rama Singh.

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2.1 ABSTRACT

We investigated the genetic architecture of variation in male sex comb bristle number, a rapidly evolving secondary sexual character of *Drosophila.* Twenty-four generations of divergent artificial selection for sex comb bristle number in a heterogeneous population of *D. melanogaster* resulted in a significant response that was more pronounced in the direction of low bristle numbers. We observed a strong positive correlated response to selection in the corresponding female transverse bristle row. The correlated response in male abdominal and sternopleural bristle numbers, on the other hand, did not follow the same pattern as sex comb bristle number differences between selection lines. Relaxation-of-selection experiments along with mate choice and fecundity assays using the selection lines developed demonstrated the action of stabilizing selection on sex comb bristle number. Our results show (a) substantial genetic variation underlying sex comb bristle number variation, (b) a weak relationship between the sex comb and developmentally related, non-sex bristle systems, and (c) that sexual selection may be a driving force in sex comb evolution, indicating their potential to diversify rapidly during population differentiation and speciation. We discuss the implications of these results for theories of genetic variation in display and non-display male sex traits.

2.2 INTRODUCTION

Male secondary sexual traits are one of the most rapidly diverging morphological characters of higher animals (Eberhard 1985). Their evolution is thought to be driven by sexual selection (Darwin 1871) acting through female choice (Fisher 1930; Andersson 1994), male-male competition (Parker 1970; Emlen *et al.* 2001) and/or sexual antagonism (Parker 1979; Chapman *et al.* 2003). Directional sexual selection acting on variation in male sex traits within populations, at different rates or in opposite directions across populations, can result in rapid phenotypic divergence. This can contribute to the establishment of behavioural reproductive isolation (West Eberhard 1983; Civetta and Singh 1998; Boughman 2001). Such prezygotic barriers to mating may be more important in the early stages of species formation as compared to postzygotic isolating mechanisms like hybrid sterility (Turelli *et al.* 2001; Kirkpatrick and Ravigne 2002; Coyne and Orr 2004).

The male sex comb, an array of specialized bristles on the forelegs, is one such highly variable secondary sexual trait of the *melanogaster* and *obscura* species groups of Drosophila (Kopp and True 2002; Schawaroch 2002). Behavioral studies performed in these groups have suggested that the sex combs are involved in grasping the female's abdomen or in spreading her wings during mating and that their role may even vary between species depending on morphology (Speith 1952; Cook 1977; Coyne 1985). Markow *et al.* (1996) found

that *D. simulans* males captured from a natural population while mating had significantly fewer sex comb teeth than males found not mating. In *D. bipectinata,* on the other hand, mating males in similar natural conditions had a significantly increased number of sex comb teeth (Polak *et al.* 2004). Evidence that the number of sex comb teeth affects mating success in opposite directions in different species suggests that the high intra– and interspecific variation seen in sex comb bristle number (Coyne 1985; Kopp *et al.* 2003; Tatsuta and Takano-Shimizu 2006) may be driven by sexual selection.

Previous studies have investigated sex comb bristle number variation within and between species of the *melanogaster* complex using Quantitative Trait Loci (QTL) mapping and gene expression analyses (Coyne 1985; True *et al.* 1997; Macdonald and Goldstein 1999; Nuzhdin and Reiwitch 2000; Kopp *et al.* 2003; Tatsuta and Takano-Shimizu 2006, Graze *et al.* 2007). Despite a large amount of work in this field, there remains a gap in our basic understanding of sex comb bristle number inheritance and evolution. In the present study we have used artificial selection in combination with relaxation-of-selection tests, investigation of genetic correlations with other bristle traits and measurement of various fitness components in the lines developed. Together, these experiments provide insights into the evolutionary potential of sex comb bristle number to respond to selection and the mechanisms responsible for maintenance of variation in this trait.

2.3 Materials and Methods

All experiments were carried out at room temperature (22°C – 25°C) with flies reared on standard cornmeal-molasses-agar medium.

Derivation of base population

Thirty-two different lines of *D. melanogaster* were obtained and reared under uniform laboratory conditions for three to four generations (Supplementary Table 1). Adult males were anaesthetized on ice, and sex comb bristle number on both forelegs was counted in 30 males from each population under a light compound microscope. We used the mean of the left and right foreleg measurement as the sex comb bristle number score in all analyses. The absolute value of the numerical difference between the left and right foreleg measurement was used to calculate fluctuating asymmetry (FA) (Palmer and Strobeck 2003). To test for a relationship between degree of fluctuating asymmetry and size of the sex comb, Spearman rank correlation coefficients between FA and mean bristle number were calculated.

In order to obtain a genetically variable population, the six most extreme populations according to their mean sex comb bristle number (three with the highest population mean and three with the lowest, indicated in Supplementary Table 1) were crossed (Supplementary Figure 2.1). Approximately 40 male and 40 female offspring from each population cross were pooled and allowed to

interbreed for four generations to establish the base population from which we derived our replicate selection and control lines.

Artificial selection protocol

The selection experiment consisted of two replicates (designated 1 and 2) that each included one line selected for high sex comb bristle number (High), one line selected for low bristle number (Low) and one unselected control line (Control). Two hundred males from the base population were scored for sex comb bristle number and the highest scoring males, 10 for each replicate, were chosen as parents for the High lines. Similarly, the lowest scoring males, 10 for each replicate, were used as parents for the Low lines while the 10 males for each of the Control lines were chosen at random. Males for each line were mated with 10 randomly chosen females (Supplementary Figure 2.2).

Because of the low number of progeny obtained in generation 1 we scored varying numbers of males: 56 High 1, 54 High 2, 30 Control 1, 30 Control 2, 74 Low 1 and 46 Low 2 males. The 10 most extreme males from within each line were chosen as parents for the second generation. We revised the protocol and increased the number of randomly chosen females to 20. In each subsequent generation, 100 males from each line were scored and the most extreme 10 were selected. Control males and females were both chosen at random. Selection was continued in this manner for each of these lines for a total of 24 generations.

Realized heritability of male sex comb bristle number for each line was estimated by linear regression of cumulative selection response (mean sex comb bristle number for each generation added for each round of selection) on the cumulative selection differential (absolute value of parental mean minus generation mean, added to each other for each round of selection) (Falconer and Mackay 1996; Edwards *et al.* 2006). The first two generations of selection data were excluded because the method of selection was different. For each replicate, the coefficients of genetic (CV_G) and environmental (CV_E) variation were calculated as $CV_G = 100\sqrt{V_G}/\bar{x}$ and $CV_E = 100\sqrt{V_E}/\bar{x}$ (Houle 1992). V_G for each replicate was estimated as h^2V_P, where V_P was the average phenotypic variance of the respective Control line, and h^2 was calculated from divergence between the High and Low lines of that replicate. V_E was estimated as $V_P - V_G$. The mean ($\bar{x}$) was the mean sex comb bristle number of the respective Control line.

Relaxed selection protocol

Each of the four selection lines was divided into two sublines at generation 14. In the first subline, artificial selection was continued as described above. The second subline was maintained for 10 generations without further selection for sex comb bristle number. Sex comb bristle number was scored in 50 males in these relaxed sublines every alternate generation until the tenth generation after relaxation when 100 males were scored.

Correlated responses to selection

At generation 24, we scored bristle number in the most distal Transverse Bristle Row (TBR), the segment that corresponds to the male sex comb (Tokunaga 1962; Held *et al.* 2004), of both forelegs of 30 females from each line. We also scored bristle number in the fourth abdominal segment of 100 males, and in the left and right sternopleural plates of 30 males from each line at generation 24. The fifth abdominal segment is more commonly used as a measure of abdominal bristle number. The sternopleural and TBR score used was the mean of the left and right side measurement.

Within line fitness assays

We tested for the effect of sexual selection on sex comb bristle number by assessing differences in mating success associated with bristle number differences within the High 2, Control 2 and Low 2 lines. At generation 20, virgin males and females from each line were collected within 4 hours post eclosion using CO_2 anaesthesia and housed separately for 4 –5 days prior to mating assays. Based on their sex comb bristle number, males from within each line were divided into two classes, h (high scoring) and l (low scoring), and paired in such a way so as to maximize differences in sex comb bristle number. One male from each pair was marked with a notch at the base of either the right or left wing with forceps to allow for identification. We ensured that paired treatments within a set were

reciprocally marked for half the treatments. Of the 90 matings scored, 42 successful males had clipped wings and 48 were with non-clipped males. These differences are not statistically significant (χ^2=0.4, d.f. = 1, P = 0.52), confirming that notching had no significant effect on mating success. Each male pair was introduced into a vial with a female from their same line without anaesthesia. We recorded male courtship behaviours, including time spent in wing vibrations (in seconds) and the number of attempted copulations. Trials were terminated if a successful copulation did not occur within 15 minutes. A trial was retained for statistical analysis only if both males courted the females. If one male was not active or did not get a chance to court the female because copulation occurred too soon, the trial was discarded. Thirty successful trials were recorded for each line.

In order to assess the potential role of natural selection as a counteracting force in the selected lines, we assessed the number of progeny sired by males with different sex comb bristle numbers within each line at generation 22. As described above, males from High 2, Low 2 and Control 2 were collected as virgins, scored for bristle number and divided into two classes. Females were collected as virgins and aged for 4 –5 days. A single male was mated to three females in a vial for 4 days, after which the parents were discarded and progeny counts were made (on day 17). If any parent was found dead at day 5, the trial was discarded. In this manner, we assayed the number of progeny sired by 30 h and 30 l males from within each line in replicate 2.

2.4 RESULTS

Genetic variation in sex comb bristle number

The mean sex comb bristle numbers for each of the 32 different populations examined are presented in Supplementary Table 2.1. We detected significant differences in mean bristle number among these populations ($F_{31, 928}$ = 22.859, $P < 0.001$), indicating high intraspecific variation for sex comb bristle number among these lines of *D. melanogaster*.

Response to artificial selection for sex comb bristle number

Sex comb bristle number in *D. melanogaster* responded to divergent artificial selection, exhibiting significant differences in mean bristle number between lines after 24 generations of selection (Replicate 1: $F_{2,297}$ = 4986.3, $P <$ 0.001) (Replicate 2: $F_{2,297}$ = 3145.15, $P < 0.001$; Figure2.1). The phenotypic response was greater in the direction of decreased sex comb bristle number as seen in the estimates of heritability: $h^2 = 0.11 \pm 0.01$ ($P < 0.01$) and $h^2 = 0.07 \pm$ 0.02 ($P < 0.001$) for High 1 and High 2 lines respectively while Low 1 and Low 2 line heritability estimates were $h^2 = 0.21 \pm 0.02$ ($P < 0.001$) and $h^2 = 0.16 \pm 0.02$ ($P < 0.001$), respectively. Estimates of heritability derived from divergence between High and Low lines were also significant (Replicate 1: $h^2 = 0.15 \pm 0.01$, $P < 0.001$) (Replicate 2: $h^2 = 0.09 \pm 0.01$, $P < 0.001$). We note an increase in bristle number in Control 1 males (Figure 2.1) and this is likely due to random

genetic drift since the effective sample size is small and both males and females were chosen at random for the Control lines.

Stabilizing selection for sex comb bristle number

We compared mean sex comb bristle number of the relaxed sublines after 10 generations of relaxation with those of the paired source population before relaxation, at generation 14. Mean sex comb bristle number did not change significantly in both, High 1 relaxed subline ($t = 1.41$, $P = 0.96$) and Low 1 relaxed subline ($t = 1.96$, $P = 0.312$) after 10 generations of relaxation (Figure 2.1). Mean sex comb bristle number in the High 2 relaxed subline regressed towards control levels ($t = 9.67$, $P < 0.001$) while mean bristle number in Low 2 relaxed subline showed an increase in the direction of the controls ($t = 7.61$, $P < 0.001$) (Figure 1). This demonstrates the action of net stabilizing selection acting to maintain intermediate bristle numbers in these lines.

Correlated responses to selection

Significant differences in female last TBR bristle numbers were seen in both replicate 1 ($F_{2,87} = 251.3$, $P < 0.001$) and replicate 2 ($F_{2,87} = 220.14$, $P < 0.001$; Figures 2.2,2.3A). These differences followed the same pattern as sex comb bristle number differences between selection lines; both High lines exhibited significantly higher TBR bristle numbers than their respective Control lines, which had significantly more bristles than their respective Low lines (Tukey's HSD test, $P < 0.01$ for all comparisons). In contrast, the differences in

abdominal bristle number (replicate 1: $F_{2,297} = 30.33$, $P < 0.001$ and replicate 2: $F_{2,297} = 48.17$, $P < 0.001$)(Figure 2.3B) and sternopleural bristle number (replicate 1: $F_{2,87} = 14.7$, $P < 0.001$ and replicate 2: $F_{2,87} = 28.8$, $P < 0.001$)(Figure 3C) were not entirely consistent with the pattern of changes in sex comb bristle number. Mean abdominal bristle numbers of males from Low 1 and Low 2 lines were significantly *higher* than those of males from Control 1and Control 2 lines respectively (Tukey's HSD test, $P < 0.001$). Sternopleural bristle numbers did not change significantly from the respective Control lines in High 1 (Tukey's HSD test, $P = 0.12$) and Low 2 (Tukey's HSD test, $P = 0.52$) lines in response to selection for sex comb bristle number. All other comparisons of abdominal and sternopleural bristle numbers within replicates were statistically significant at $P < 0.01$ (Tukey's HSD test). These results show a strong positive correlated response to selection for sex comb bristle number in the homologous female TBRs but a weaker, inconsistent response in the abdominal and sternopleural bristles.

Sexual selection against extremely low sex comb bristle numbers

Females from High 2, Low 2 and Control 2 lines were given a choice between males that differed in sex comb bristle number from within their line to assess differences in mating success associated with differences in sex comb bristle number. Means of sex comb bristle numbers, time spent in wing vibration and numbers of attempted copulations of successful vs. unsuccessful males within each line were compared using a Wilcoxon paired sample test (Table 2.1). The

only comparison that showed a significant difference was mean sex comb bristle numbers of Low 2 males-successful males from the Low 2 line had more sex comb teeth than unsuccessful males (Table 2.1, Figure 2.4) (χ^2 = 19.2, d.f. = 1, P < 0.01). More l males were successful as compared to the h males in the High 2 (χ^2 = 1.2, d.f. = 1, P = 0.27) and Control 2 lines (χ^2 = 0.13, d.f. = 1, P = 0.71) (Figure 4), but these differences were not significant.

The number of progeny sired by males with different sex comb bristle numbers within High 2, Low 2 and Control 2 was assessed to uncover fecundity differences between these males (Figure 2.5). We detected a significant effect of line in assaying differences in fecundity ($F_{2,175}$ = 4.82, P = 0.009) and found that Low 2 males had significantly lower fertility than High 2 (Tukey's HSD test; P = 0.01) males. Within a line, however, multiple t tests showed no significant difference in the number of progeny sired by h and l males within High 2 (t = 1.69, P = 0.09), Low 2 (t = 1.98, P = 0.05) or Control 2(t = 1.89, P = 0.07)

2.5 Discussion

Genetic architecture of sex comb bristle number variation in *D. melanogaster*

The large differences in sex comb bristle number between the geographically widespread populations of *D. melanogaster* used in our study, coupled with the rapid, robust phenotypic response to artificial selection, show that there is substantial additive genetic variance underlying this trait. The

magnitude of the realized heritability, however, is relatively low (~0.1) when compared to other morphological traits such as abdominal bristle number (h^2~ 0.5; Clayton *et al* 1957) and body size (h^2~0.4; Robertson 1957) in Drosophila. The genetic and environmental coefficients of variation were 3.36 and 7.99 for replicate 1 and 2.96 and 7.87 for replicate 2 respectively. These values show that the low heritability of male sex comb bristle number is due to a higher proportion of environmental variance, rather than a lack of genetic variation, which is a typical feature of many secondary sexual traits (Alatalo *et al.* 1988; Pomiankowski and Moller 1995).

The response to selection was highly asymmetrical with both Low lines showing a greater per generation decrease in sex comb bristle number as compared to the increase in bristle number in the High lines. Such a response could have resulted from the action of selection (natural and/or sexual) operating along with the artificial selection applied. The regression of bristle numbers towards intermediate levels in both, High 2 and Low 2, on relaxation of selection shows that sex comb bristle number is under net stabilizing selection in these lines. The lack of a significant response to relaxation in Replicate 1 may be due to lower levels of genetic variation (Figure 2.1).

We measured correlated responses to selection for sex comb bristle number in other developmentally related mechanosensory bristle systems to assess the extent of genetic linkage between them. The last TBR of female is

homologous to the male sex comb (Tokunaga 1962; Held *et al.* 2004), and here we observed a strong indirect response to selection. Changes in male abdominal and sternopleural bristle numbers, on the other hand, were not entirely consistent with the pattern of differences seen in sex comb bristle number after 24 generations of selection. It appears that selection may have altered the frequency of a few loci affecting bristle number in general, but that there remain major loci affecting the sex comb system specifically which are not shared with other mechanosensory bristle systems. This weak relationship suggests reduced developmental constraint on the sex combs to evolve in concert with other, non-sex, bristle systems. The sex combs appear to be a sexual modification evolving relatively independently of related bristle systems, which could enable them to evolve rapidly, potentially with exaggeration.

Males from the Low 2 line appear to be unfit in comparison to males from both, High 2 and Control 2 lines. Low 2 males had lower fecundity than males from Control 2 and High 2, and within the Low 2 line, males with extremely low bristle numbers had reduced mating success. This could be due to the accumulation of deleterious alleles during the selection process. Sexual selection against small combs could also be responsible, maintaining higher frequencies of alleles for greater bristle numbers in the base population, and would explain the greater response in the downward direction when artificial selection was applied. Another interesting observation is that, although not significant, successful males

from within the Control 2 and High 2 lines had fewer sex comb bristles than unsuccessful males (Table 1, Figure 4). This is similar to the trend seen in natural populations of *D. simulans*, where mating males had significantly fewer sex comb teeth. Sexual selection appears to be an important driving force in sex comb evolution and it would be worthwhile to further investigate this in the *melanogaster* subgroup to help understand their potential role in species divergence.

Theories of genetic variation for male sexual traits

Sexual selection acting on male sex traits is expected to lead to rapid fixation of favourable alleles and depletion of heritable genetic variation in such traits (Borgia 1979; Taylor and Williams 1982). However, in contrast to this expectation, it has been shown that additive genetic variation is not only maintained, but is actually higher in male sex traits as compared to non-sex traits (Pomiankoski and Moller 1995). Different hypotheses have been proposed to explain this persistence of genetic variance in male traits but few studies have attempted to empirically test these. We find high levels of genetic variation underlying sex comb bristle number, allowing us to assess if, and how, current theories of genetic variation for sexual traits apply to this trait.

Pomiankowski and Moller (1995) have proposed that fitness increases exponentially as a sexually selected trait becomes exaggerated, which favours an

increase in phenotypic variance through the evolution of modifiers that can increase the number of genes and their effect on the trait. According to this hypothesis, the high additive genetic variance in male sex traits is a consequence of continual directional selection while traits subject to stabilizing selection should have reduced levels of genetic variation due to modifiers that restrict the number of loci and their effects. However, this explanation fails to consider that the exaggeration of a sexual trait does not continue indefinitely, and that after initial spread, most sexually selected traits are expected to be under stabilizing selection (Kirkpatrick and Ryan 1991; Andersson 1994). Indeed, sex comb bristle number shows only a limited increase in the High lines in spite of the strong artificial selection applied, and appears to be under stabilizing selection in the base population.

Alternatively, the Genic Capture Hypothesis (Rowe and Houle 1996) proposes that the expression of male sex traits is costly and condition dependent, and thus involves a large number of genes in the genome, which provides an inexhaustible source of variation in such traits. For this explanation to apply the secondary sexual trait must be condition dependent, but our results fail to find evidence to support this in the sex comb: A negative phenotypic correlation between size and degree of FA is expected for costly, condition dependent secondary sexual traits, since males of high condition should be able to simultaneously maximize size and minimize FA of sexual traits (Manning and

Hartley 1991; Moller and Pomiankowski 1993a; Tomkins and Simmons 2003). We did not detect a significant negative correlation in any of the 32 lines we tested (Supplementary Table 2.1). In a previous study, Polak *et al.* (2004) found a *positive* relationship between sex comb bristle number and FA in lab-reared populations of *D. bipectinata.*

Male secondary sexual traits can be classified into two types: costly, display traits subject to female choice, and traits not used for display but to coerce or drive females to mate, with or without females having any control (Singh and Kulathinal 2005). The absence of a negative relationship between FA and size suggests that the sex comb may not be a typical costly, condition dependent display trait (Moller and Cuervo 2003). Instead, it has been proposed that the combs help to grasp the female (Speith 1952), suggesting that males use them to control females during copulation. Singh and Kulathinal (2005) recently proposed the Male Sex Drive hypothesis, which offers a more general explanation for maintenance of variation in different types of male traits. Complementary to female choice, it is the concept that males are the sex that develops new strategies (morphological, physiological, behavioral) to mate and pass on offspring. This male drive to secure mates and reproduce leads to the recapture of any mutations affecting any male trait involved in sex and reproduction related functions. This selection driven continuous input of new mutations would compensate for loss of genetic variation.

From our findings of significant heritable genetic variation, weak relationship with other, non-sex bristle systems, and evidence of intra-specific sexual selection in *D. melanogaster*, we can conclude that the sex comb has the potential to diversify rapidly. The results presented here raise the possibility that sex comb bristles may not be a typical display trait and force us to think about the maintenance of genetic variation in display vs. non display traits. Our study lays the foundation for further work, providing experimental material to further analyze the genetic architecture, functional significance and evolutionary dynamics of the sex comb in Drosophila.

2.6 Acknowledgments

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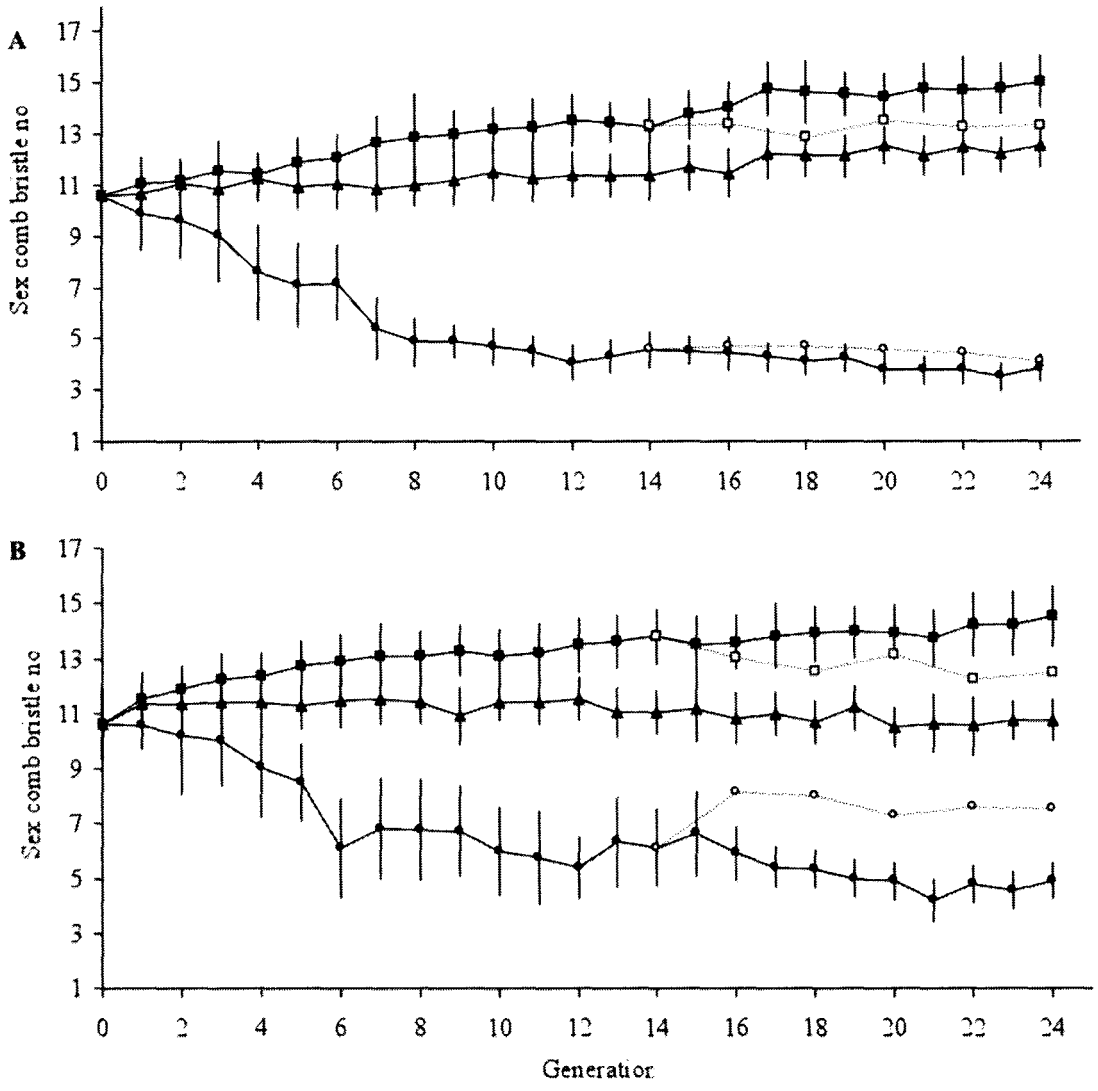


Figure 2.1: Response to artificial selection for male sex comb bristle number in *D. melanogaster*. Mean sex comb bristle numbers in High (square), Low (circle) and Control (triangle) lines over 24 generations in (A) replicate 1 and (B) replicate 2. Solid lines with black symbols indicate artificial selection lines and dashed lines with white symbols indicate relaxed sublines. Error bars are the standard deviation.

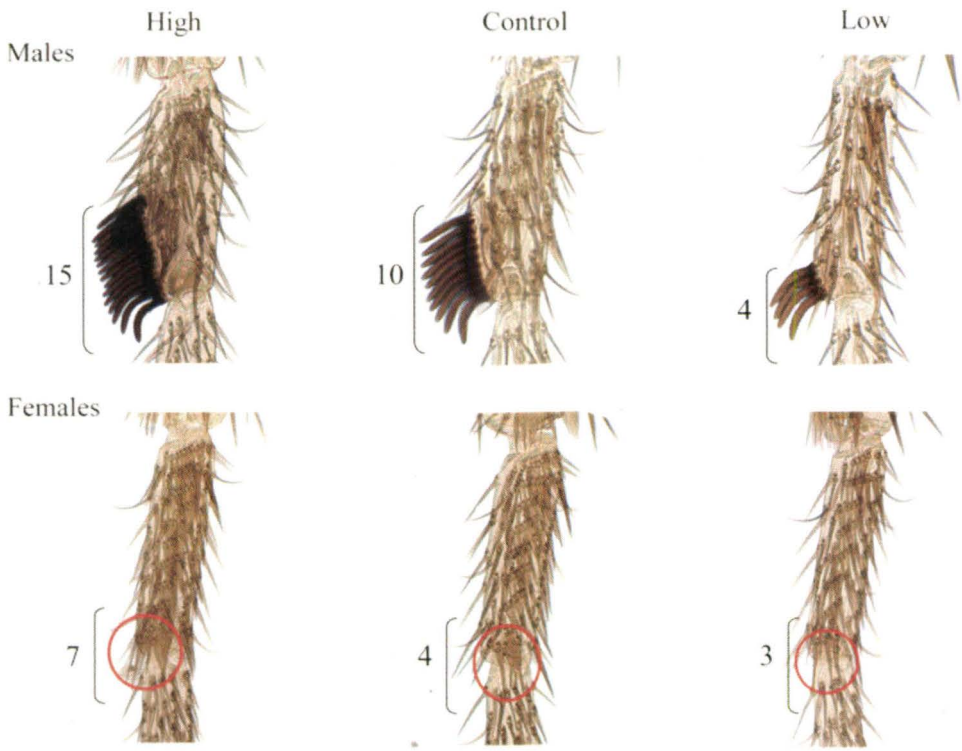


Figure 2.2: Forelegs of males (showing sex comb) and females (showing TBRs) from High, Control and Low lines of *D. melanogaster* after 24 generations of artificial selection. Bristle number of the foreleg shown is indicated in the lower left corner.

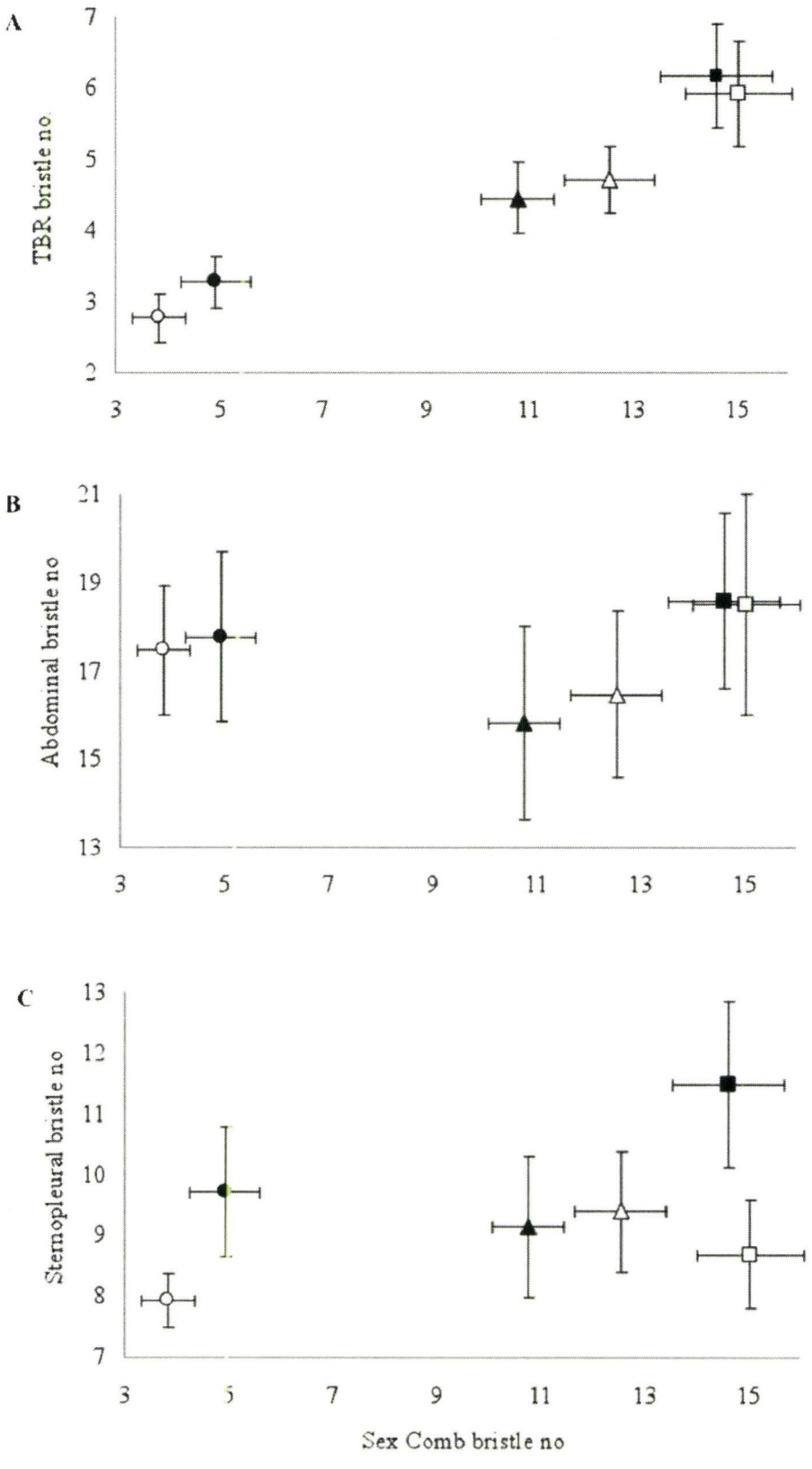

A
TBR bristle no
7
6
5
4
3
2
3
5
7
9
11
13
15
B
Abdominal bristle no
21
19
17
15
13
3
5
7
9
11
13
15
C
Sternopleural bristle no
13
12
11
10
9
8
7
3
5
7
9
11
13
15
Sex Comb bristle no

Figure 2.3: Correlated responses to divergent selection for sex comb bristle number. Mean sex comb bristle numbers are plotted against (A) Mean female last TBR bristle numbers (B) Mean male abdominal bristle numbers and (C) Mean male sternopleural bristle numbers of High (square), Low (circle) and Control (triangle) lines at generation 24 in replicate 1 (white) and replicate 2 (black). Error bars represent standard deviation.

Sex comb bristle no.

Mean (± SE)	15.91 (0.06)	13.01 (0.08)	13.7 (0.09)	10.63 (0.09)	5.45 (0.06)	3.31 (0.05)
Range	15 – 16.5	12 - 13.5	13 – 14.5	9.5 – 11.5	5 - 6	2.5 – 3.5

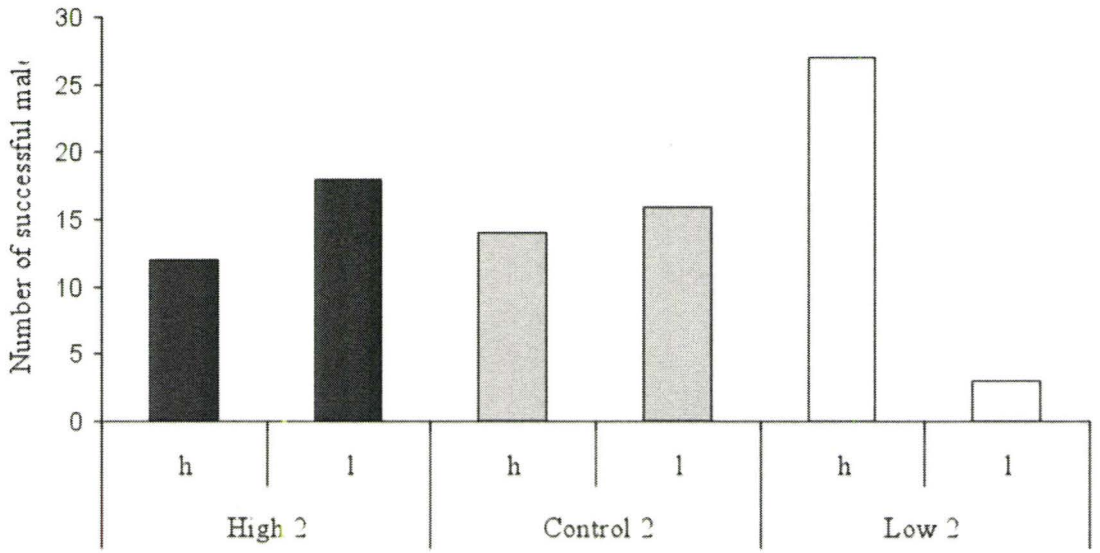


Figure 2.4: Numbers of h and l class males that were successful in mating trials conducted within each selection line. Mean (± SE) and range of sex comb bristle numbers of all (30) males from each class are indicated above bars.

Sex comb bristle no.						
Mean (+ SE)	15.26 (0.09)	12.6 (0.17)	12.6 (0.12)	10.81 (0.53)	5.43 (0.07)	3.21 (0.05)
Range	14.5 - 16.5	12 – 13.5	12 – 13.5	9 - 11.5	5 – 6.5	2.5 - 3.5

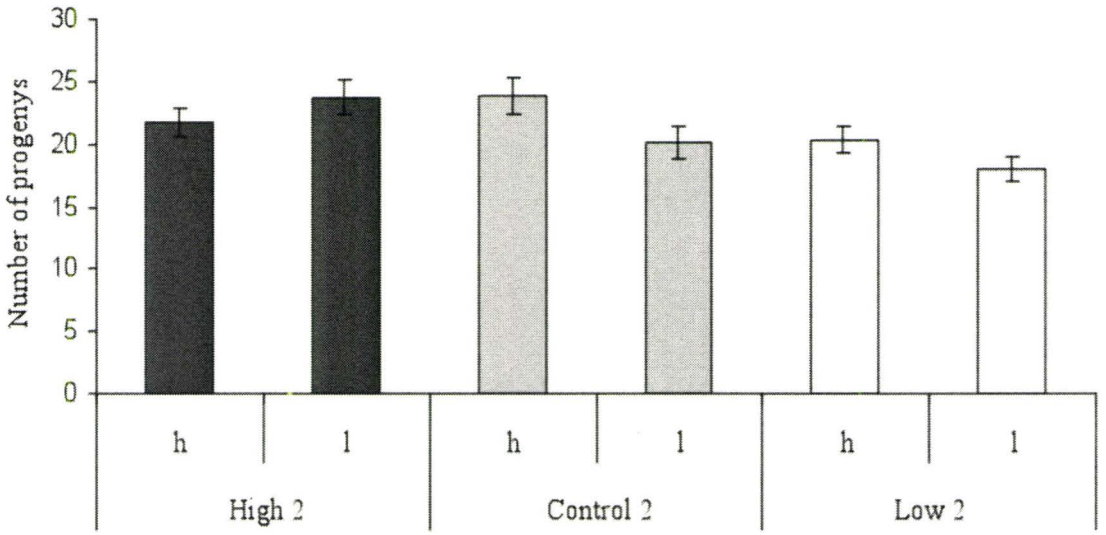
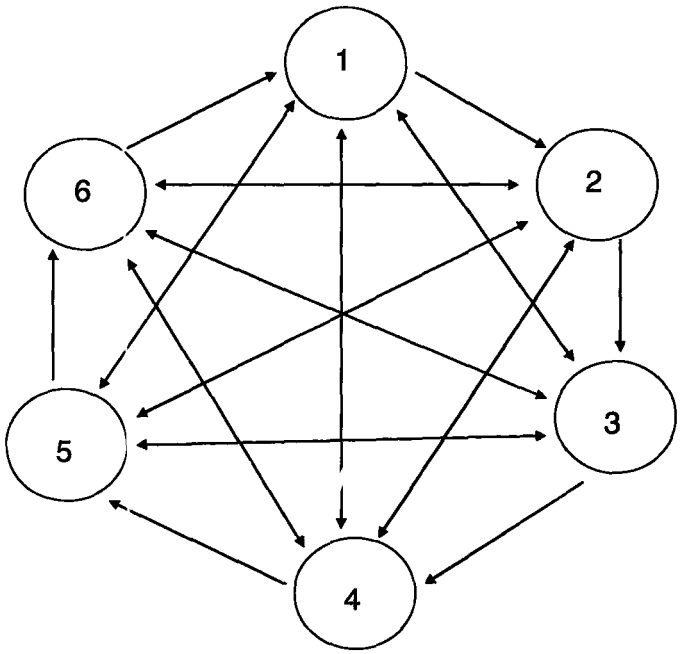


Figure 2.5: Mean number of progeny (± SE) sired by h and l class males from within each selection line. Mean (± SE) and range of sex comb bristle numbers of all (30) males from each class are indicated above bars.

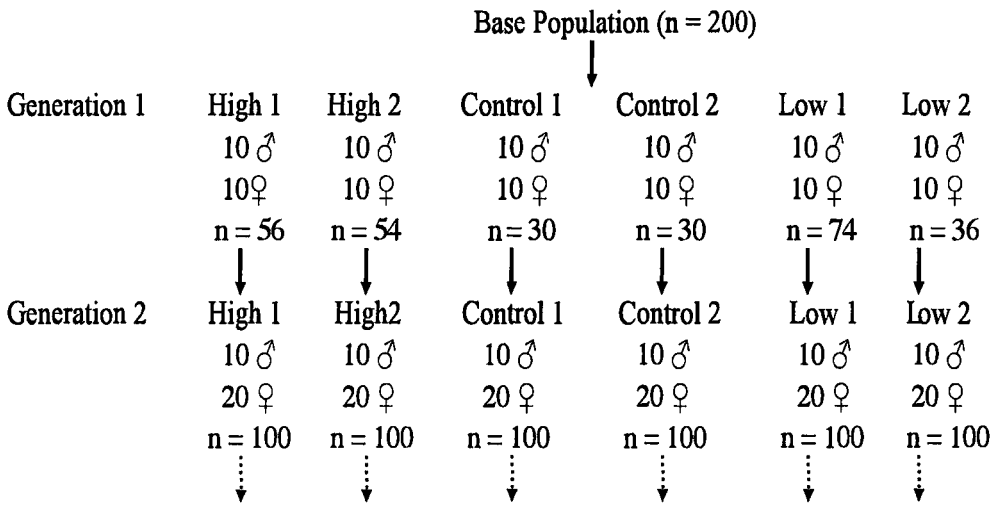
Table 2.1: Means (± SD) of sex comb bristle number, time spent in wing vibration and number of attempted copulations of successful and unsuccessful males from mating trials between h and l males from within High 2, Control 2 and Low 2 lines. Comparisons between successful and unsuccessful males were performed using a Wilcoxon paired sample test.

	Sex comb (bristle no.)			Time in wing vibration (secs)			Attempted copulations (no.)		
Line	Successful	Un-successful	P	Successful	Un-successful	P	Successful	Un-successful	P
High 2	14.15 (1.6)	14.78 (1.38)	t = 176, P = 0.24	25 (14.74)	20.76 (14.4)	t = 186.5, P = 0.36	2.8 (2.3)	2.8 (1.56)	t = 214, P = 0.87
Control 2	12.13 (1.3)	12.26 (1.62)	t = 231.5 , P = 0.98	14.8 (12.8)	9.46 (8.6)	t = 157.5, P = 0.13	1.6 (1.52)	1.46 (1.57)	t = 202, P = 0.75
Low 2	5.21 (0.71)	3.53 (0.73)	t = 50, P = 0**	14.8 (9.15)	19.06 (12.61)	t = 141, P = 0.06	2.63 (2.15)	2.93 (1.92)	t = 173, P = 0.35

** $P < 0.01$



Supplementary Figure 2.1: Crossing scheme for derivation of Base Population. Numbers correspond to populations indicated in Supplementary Table 2.1. The resulting hybrid offspring were pooled and interbred for four generations.



Supplementary Figure 2.2: Crossing scheme for artificial selection experiment.

Supplementary Table 2.1: Mean (± SD) bristle number and fluctuating asymmetry (FA) in sex comb of males from geographically widespread populations of D. melanogaster. Relationship between size of comb and degree of fluctuating asymmetry is given as Spearman rank correlation coefficient (r_s). Populations are ranked roughly from south to north. Source code: a – Tucson Drosophila Stock Center, b – Bloomington Drosophila Stock Centre, c – Dr. J.R. David, d – Dr. P. Capy, e – Dr. S.V. Nuzhdin.

Strain	Bristle no.	FA	r_s	Source
14021-0231.16	10.58 (0.9)	1.02 (0.84)	0.098	Queensland, Australia (2002) (a)
K0 42	11.08 (1.21)	0.7 (0.7)	0.006	Kenya (2002) (c)
14021-0231.17	9.61 (0.76)	0.56 (0.62)	0.253	Eugella National Park, Australia (a)
ZS 53	11.06 (0.6)	0.83 (0.69)	-0.057	Sengwa, Zimbabwe (1993) (d)
ZH 12^3	11.61 (0.66)	0.70 (0.74)	0.122	Harare, Zimbabwe (1993) (d)
14021-0231.15	9.7 (0.7)	0.68 (0.8)	-0.182	Brazil (a)
3841^5	9.33 (1.8)	0.79 (0.97)	0.405	Bogota, Columbia (1962) (b)

Madibou 4[2]	11.71 (1.08)	1.047 (0.95)	0.033	Brazzaville, Congo (2000) (d)
Primus 20	11.28 (0.9)	0.8 (0.723)	0.262	Brazzaville, Congo (2000) (d)
Kronenbourg	11.11 (0.93)	0.891 (0.76)	0.022	Brazzaville, Congo (1989) (d)
Brazzaville	9.96 (1.01)	0.83 (0.79)	0.094	Brazzaville, Congo (1986) (c)
Primus 25	11 (0.83)	1.02 (0.65)	0.139	Brazzaville. Congo (2002) (d)
Madibou1	10.51 (1.1)	0.9 (0.81)	0.33*	Madibou, Congo (2000) (d)
Madibou 2	10.75 (0.67)	1.07 (0.85)	0.191	Madibou, Congo (2000) (d)
Loua	11.16 (0.83)	1.00 (0.78)	0.517**	Brazzaville, Congo (1989) (d)
14021-0231.35	10.88 (0.82)	1.1 (0.84)	0.257	St. Kitts, Carribean Sea (2005) (a)
14021-0231.25	10.96 (0.91)	0.73 (0.69)	-0.047	Oaxaca, Mexico (2003) (a)
14021-0231.28	10.68 (1.36)	0.96 (0.76)	0.338	Jalisco, Mexico (2004) (a)
14021-0231.27	10.2 (0.8)	1.06 (0.82)	0.024	Sinaloa, Mexico (2004) (a)
14021-0231.23	10.26 (0.9)	1.02 (0.87)	-0.166	Greece (2002) (a)
14021-0231.29	11.25 (0.86)	0.97(0.84)	0.001	New Mexico, USA (2004) (a)
4272[6]	9.2 (0.5)	0.5 (0.65)	0.481*	Illinois, USA (1997) (b)
Oregon R	10.73 (0.62)	1.06 (0.87)	-0.155	Oregon, USA (e)

Grandlieu	9.68 (0.83)	0.5 (0.62)	0.056	Bordeaux, France (2002) (d)
Colmar	9.75 (0.6)	1.03 (1.06)	-0.127	France (c)
Foissac	9.78 (0.6)	1.03 (0.85)	-0.104	France (c)
Besancon	10.1 (0.82)	0.63 (0.71)	-0.065	France (c)
Draviel	10.28 (0.92)	1.1 (0.75)	0.337	France (d)
Bordeaux	10.41 (0.72)	0.9 (0.66)	0.177	France (c)
Grandeferrade	10.85 (0.8)	0.9 (0.75)	0.238	France (c)
8522[1]	12.86 (0.61)	0.87 (0.68)	0.117	Berlin, Germany (2004) (b)
2b3[4]	9.6 (0.46)	1.06 (0.78)	0.23	Russia (e)

1,2,3 Populations with the three highest mean sex comb bristle numbers

4,5,6 Populations with the three lowest mean sex comb bristle numbers

*$P < 0.05$, ** $P < 0.01$*

CHAPTER 3

CONDITION DEPENDENCE AND NATURE OF GENETIC VARIATION FOR MALE SEX COMB BRISTLE NUMBER IN *DROSOPHILA MELANOGASTER*

This chapter is based on a manuscript that has been accepted for publication in Genetica pending minor revisions. I tested for and compared condition dependence of male sex comb bristle number with other *D. melanogaster* bristle systems by assessing the affect of diet manipulation in full-sibs. I also conducted a half-sib analysis to estimate genetic variance in condition and sex comb bristle number. I designed all experiments, analyzed the data and wrote the manuscript with input from R.S. Singh. Scott De Vito assisted in conducting the half-sib experiment.

Ahuja, A., De Vito, S. and Singh, R.S. 2010. Condition dependence and nature of genetic variation for male sex comb bristle number in *Drosophila melanogaster*. Genetica (submitted August 5th, 2010)(Manuscript ID: Gene1666).

3.1 ABSTRACT

We conducted quantitative genetic analyses of variation in male sex comb bristle number, a rapidly evolving secondary sexual character of *Drosophila.* First, in order to test for condition dependence, diet was manipulated in a set of ten *Drosophila melanogaster* full-sib families. We confirmed heightened condition dependent expression of sex comb bristle number and its female homologue (distal transverse row bristles) as compared to non-sex sternopleural bristles. Significant genotype by environment effects were detected for the sex traits, suggesting a genetic basis for condition dependence. Next, a half-sibling breeding design was used to estimate genetic variation for sex comb and sternopleural bristle number as well as residual mass, a commonly used condition index. We detected genetic variation with a strong possible dominance and/or maternal effect or X chromosome effect for sex comb bristle number. Heritability for condition was high compared to sex comb and sternopleural bristles, but CV_A value was lower than previously published estimates for other fitness related traits of *Drosophila.* Our results provide insight into the genetic architecture of sex comb bristle number variation in *D. melanogaster*, and we discuss their broader implications in the context of the genic capture mechanism for the maintenance of genetic variation.

3.2 INTRODUCTION

The sex comb, an array of specialized bristles on the foreleg, is a rapidly diverging male secondary sexual character in *Drosophila.* Sex combs are thought to be used by males to grasp a female's abdomen or spread her wings during mating, and have been shown to be important for mating to occur (Speith 1952; Ng and Kopp 2008). Sex comb morphology varies dramatically between closely related species, indicating that their evolution is driven by sexual selection (Kopp and True 2002). Indeed, sex comb bristle number exhibits high intra- and inter-specific variation (Coyne 1985), and has been shown to affect mating success in opposite directions in natural *D. simulans* (Markow *et al.* 1996) and *D. bipectinata* (Polak *et al.* 2004) populations. Recent evidence suggests that sexual selection for size in *D. bipectinata* may be post-copulatory in nature (Polak and Simmons 2009). In an earlier study we reported high intra-specific variation for sex comb bristle number within *D. melanogaster* populations, as well as a rapid response to artificial selection for extremely high and low bristle numbers, indicating high levels of additive genetic variation underlying this trait (Ahuja and Singh 2008). Furthermore, we noted that within the Low artificial selection line higher bristle number was associated with higher mating success. Here we follow up on this study and further investigate the genetic architecture of *D. melanogaster* sex comb bristle number variation.

Sexually selected traits are generally expected to be condition dependent

in their expression (Price *et al.*1993; Andersson 1994; Johnstone 1995), where condition is defined as the pool of resources available to an individual for allocation to competing life history traits. Condition dependent expression of male sex traits is a key element of good genes models of sexual selection. Because condition reflects genetic or environmental variance in ability to acquire resources, condition dependent traits may honestly signal an individual's phenotypic and/or genotypic quality (Zahavi 1975; Grafen 1990; Punzalan *et al.* 2008). We tested for condition dependence of the male sex comb by manipulating diet quality and quantity and assessing its effect on sex comb bristle number and length in a set of ten full-sib families. We also examined two related mechanosensory bristle traits: The most distal transverse row of females rotates and thickens to form the sex comb in males (Tokunaga 1962; Held *et al.* 2004). It exhibited a strong, positive correlated response to artificial selection for sex comb bristle number (Ahuja and Singh 2008) though the nature and direction of selection acting on the trait is unclear. On the other hand, the sternopleural bristles did not show a consistent correlated response to selection for sex comb bristle number, and were used as a non-sex control since they are thought to be evolving under weak stabilizing selection (Mackay 1985).

Condition dependence of sexually selected traits also has important implications for understanding the maintenance of genetic variation. Under the genic capture model, if trait values depend on condition, and condition itself

depends on many loci, then traits will inevitably capture and express the high genetic variance of condition (Rowe and Houle 1996; Tomkins *et al.* 2004). In our second experiment we estimated genetic variation using a nested full-sib, half-sib mating design. We partitioned the variance components and derived estimates of heritability and the coefficient of additive genetic variance (CV_A) for residual body mass (a commonly used condition index), sex comb and sternopleural bristle number. Overall, this set of experimental tests provides insight into the genetic architecture of sex comb bristle number variation in *D. melanogaster*. To our knowledge no previous study has investigated condition dependence of male sex traits or genetic variance in condition in *D. melanogaster*, which is otherwise a model system for studies of sexual selection and quantitative genetics. We draw on the wealth of *Drosophila* literature to discuss their implications in the context of the genic capture mechanism for maintenance of genetic variation.

3.2 Material and methods

Experiment 1: Full-sib analysis

An out-bred base population was established by crossing six geographic *D. melanogaster* populations that were highly divergent for sex comb bristle number (for details see Ahuja and Singh 2008). A single virgin male and female from this population were crossed to initiate ten such full sibling families. The lines were then maintained on standard laboratory cornmeal-molasses-agar diet (15.6g yeast, 7.8g agar, 54.6ml molasses, 78g cornmeal per liter of water) for

three generations before the condition experiment was started. The rich conditioning diet contained 70g yeast, 8g agar, 60ml molasses and 80g cornmeal per liter of water while the poor condition diet comprised 13g yeast, 8g agar, 15ml molasses and 20g cornmeal per litre. These diets have been modified from Imasheva *et al.* (1993) who reported a significant decrease in larval viability under poor feeding conditions. Hence, larval density cannot be strictly controlled and vials with rich diet were transferred every 48 hours while poor diet vials were transferred every 96 hours. In this manner, poor diet vials had low quality as well as less quantity of food per individual. Five virgin male – female pairs from each full sibling family were introduced into two rich and poor diet vials each. The resulting progeny were aged on the respective diet for 5 days and were stored at -20°C for subsequent morphological measurements. Thirty male and thirty female progeny from each full sibling family were scored for a total of 300 individuals of each sex in each diet.

Sex comb and sternopleural bristle number in males and distal TBR bristle number in females were counted under a light microscope. The length of the first tarsus in males and females was used as an index of body size, and along with male sex comb length, was measured using an ocular micrometer in arbitrary micrometer units (72 units = 1000μm). Sex comb length was measured as the length of the shortest straight line from the base of the most distal to the most proximal bristle. All characters were measured on both, left and right sides of the

fly and absolute trait sizes were calculated as the average of the left and right side score. Since measurements of continuous traits were performed twice, we calculated the average of the two measurements made on each side to obtain the score for that side. Repeatability values, calculated using the method of Bland and Altman (1996), were 1.09, 2.75 and 3.5 ocular units for male sex comb length, male tarsus length and female tarsus length respectively.

Data analysis was conducted using the Statisxl add on package in Microsoft Excel. The data were not normally distributed (Kolmogorov Smirnov one-sample tests; $P < 0.01$), and we were unable to transform to approximate a normal distribution. However given the large sample sizes, under the central limit theorem, parameter estimates are expected to be close to normally distributed and parametric tests were used. We also confirmed the validity of results obtained using non-parametric tests as indicated. Absolute trait sizes between treatments were compared using a *t*-test and repeated with a Mann-Whitney *U*-test. We measured strength of condition dependence by estimating magnitude of treatment effect. Standardized mean effect size was calculated for each trait using Hedge's g statistic, where $g = t\sqrt{(n_1 + n_2/ n_1 n_2)}$ and n_1 and n_2 represent sample size of each group (Nakagawa and Cuthill 2007).To correct for body size, a mixed model ANCOVA was conducted with tarsus length as covariate and family (genotype), treatment (environment) and their interaction (genotype by environment) as factors. We confirmed these results with a second, albeit less reliable, method to

correct for body size: Relative trait sizes (absolute trait size divided by tarsus length) between treatments were compared using a Mann-Whitney *U* test.

Experiment 2: Half-sib analysis

Thirty virgin males and 90 virgin females were randomly taken from the base population. They were aged for 5 days and each male was then crossed to three females in a standard diet vial supplemented with live yeast (ad libitium). Males were discarded after 48 hours and females were separated and transferred to petri dishes with standard medium for egg laying for 48 hours. Twenty larvae obtained from each female were transferred to individual vials. Flies collected from each individual vial were aged for four days and frozen for morphological measurements. Five male progeny of each dam from each half-sibling family were scored in this manner for a total of 450 individuals scored.

Condition is defined as the resources available for utilization, and we used body mass corrected for body size as a surrogate measure since residual mass is expected to be positively correlated with reserves such as fat or sugar (Kotiaho 1999). Body size was estimated from wing length, measured as the length of the longitudinal vein L3 from the intersection with the anterior cross vein to the tip of the wing. Each wing was measured twice and repeatability value for wing length was 1.3 ocular units and average of the left and ride side wing measurement was calculated as the score for each individual. Flies were individually weighed to the nearest 0.001µg on a Mettler UMT2 microbalance. Residuals from log

transformation of mass and wing length were used to correct for the fact that a unit of reserves may be more useful for larger individuals than smaller ones (Kotiaho 1999). The use of residual mass as a condition index carries several assumptions and caveats (Green 2001), and while we did not directly test these in our system, overall this surrogate has been shown to provide a reasonable estimate of condition in other taxa (Schulte Hostede 2005; Birkhead *et al.* 2006). Sex comb and sternopleural bristle numbers were scored as described previously. Data were analyzed with nested ANOVA with sire and dams (sires) as factors. Standard procedures were used for the calculations of heritability (Lynch and Walsh 1998). Coefficients of genetic variation were calculated as $CV_A = 100\sqrt{V_A}/x$, where V_A is the additive genetic variance and x is the mean of a trait (Houle 1992). Since residuals have a mean of zero, these were standardized by adding mean body mass for calculating CV_A of condition (Kotiaho *et al.* 2001, Birkhead *et al.* 2006).

3.3 RESULTS

Male sex comb shows condition dependence

Thirty flies from each full sib family from each diet regimen were scored for the bristle traits under study (Figure 3.1). For each trait, flies reared on the rich diet exhibited larger absolute trait sizes as compared to flies reared on the poor diet (Table 3.1, Figure 3.1). Not surprisingly, we detected strong correlations between

sex comb bristle number and sex comb length on each diet (Supplementary Figure 3.1). As seen by the magnitude of effect size of diet treatment (Table 3.1), female transverse bristles exhibited the strongest condition dependence, followed by sex comb length and bristle number and finally male sternopleurals. Partitioning of variance due to effects from various sources revealed that for all four traits effect of diet treatment persisted even after controlling for body size (Table 3.2). In addition we also detected a genotype effect as seen by the significant effect of family. Most interesting were the effects due to interaction between family and diet: Significant genotype x environment interaction was detected for male sex comb bristle number and length, as well as for female transverse bristles, but not for the non-sex male sternopleural bristle numbers. Results obtained using non-parametric tests (Supplementary Table 3.1) were qualitatively similar, confirming our findings.

Genetic variation in condition

Measurements of mean, heritability, and coefficient of additive genetic variance for each trait are presented in Figure 3.2. The effect of sire was highly significant for condition (P= 0.005,Table 3.3), indicating heritable additive genetic variance for this trait. Sire effect was marginally significant for sex comb bristle number (P= 0.07), but not significant for sternopleural bristle number variation (P= 0.11). Dam effect was significant only for the sex comb and sternopleural bristles (Table 3.3). Given the large dam component of variance, we

cannot rule out dominance or maternal effects, and only the sire component was used to estimate heritability. Estimates of heritability (0.45± 0.17) and CV_A (4.4) for condition derived from log transformed residuals were moderate (Figure 3.2). Log transformation, while providing a reasonable estimate of condition, introduces scaling effects that preclude comparisons between organisms of different sizes. Our values of CV_A derived from untransformed residuals (6.45) (ANOVA not shown) are in line with values estimated from residual mass in dung beetles (8.14) (Simmons and Kotiaho 2002), and zebra finch (5.70) (Birkhead *et al.* 2006). As expected, value of CV_A for sex comb bristle number estimated from half siblings (3.44) in this study is comparable to estimates from artificial selection lines (3.05) derived from the same base population (Ahuja and Singh 2008). However, value of sternopleural bristle number CV_A (4.49) derived in our study was lower than that reported in previous studies (8.39)(Houle 1992). Overall, condition exhibited the highest heritability, and value of CV_A was higher than that of the sex comb, and comparable to sternopleural bristle number (Figure 2).

3.4 DISCUSSION

Our data provide novel insights at several levels: First, we assess how the patterns of condition dependence detected for *D. melanogaster* mechanosensory bristles compare with theoretical predictions. Next, we focus on the genetics of condition and discuss our results in light of the genic capture mechanism for

maintenance of variation. Finally, we sum up the results from this study and previous work to put together a picture of the genetic architecture of sex comb bristle number variation in *D. melanogaster.*

Patterns of condition dependence

Individuals in higher condition are expected to have a larger pool of resources to allocate to costly, fitness enhancing male traits (Andersson 1982; Nur and Hasson 1984; Iwasa and Pomiankowski 1994). Sexually selected traits like the male sex comb should exhibit strong condition dependence since increased investment in such traits results in increased fitness (Andersson 1982; Nur and Hasson 1984). Furthermore, if the male sex comb is an indictor trait of genotypic quality, one expects that condition dependence would be heritable (Tomkins et al. 2004). Given the strong genetic correlation between the male sex comb and female transverse bristles (Ahuja and Singh 2008) we also expect to find strong condition dependence for this trait (Bonduriansky and Rowe 2005; Punzalan *et al.* 2008). Finally, traits under weak stabilizing selection are not predicted to exhibit strong condition dependence as allocation of more resources to such traits does not increase fitness (Schluter *et al.*1991). We predict that sternopleural bristle number exhibits weaker condition dependence as compared to the sex comb.

Size of both components of the male sex comb, bristle number and length, exhibited positive condition dependence (Table 3.1) and we also detected a

significant genotype by environment effect for this trait (Table 3.2). This suggests that different genotypes allocate resources to this trait at different rates and that condition dependence is heritable, although we cannot rule out the possibility that genotypes may differ in their overall genetic quality. We also detected significant condition dependence in the homologous female transverse bristle row and a significant genotype X environment effect (Table 3.1,3.2). This suggests that loci involved in condition dependence are being expressed in both sexes resulting in intersexual genetic correlation for condition dependence. Surprisingly, magnitude of effect of condition manipulation was stronger than that for the male sex comb, contrary to expectations if the evolution of condition dependence is driven primarily by sexual selection on males (Bonduriansky and Rowe 2005). Since the sex comb is a modification of the transverse rows and represents a later, sex – specific stage of development, this raises the possibility that condition dependence may be stronger in the early stages of development as compared to later stages. Finally, while absolute male sternopleural bristle number also exhibited significant condition dependence, magnitude of effect of condition manipulation was weaker than the male sex comb and female bristles and the effect of genotype X environment was not significant. Moreover, it appears that changes in sternopleural bristle number due to condition manipulation are largely accounted for by changes in body size (Table 3.2, Supplementary table 3.1). Overall, these findings are consistent with theoretical predictions.

Genic capture and the genetics of condition

For genic capture to operate, two main premises must be met: 1) The expression of male sex traits should be condition dependent, and 2) Condition should harbour high genetic variance. Condition dependence in a variety of male sex traits has been reported across a variety of taxa (Cotton *et al.* 2004; Boughman 2007), and the patterns of condition dependence detected for sex comb bristle number in this study are in line with this premise. Much less progress has been made in understanding the genetics of condition, largely due to the somewhat abstract nature of this concept. Condition carries several definitions in the literature and is commonly understood to be a trait summarizing the health and vigour of an individual such that it is closely associated with fitness (Iwasa and Pomiankowski 1994; Tomkins *et al.* 2004). Under Rowe and Houle's model (1996), it is defined more narrowly as the pool of resources available for utilization, akin to residual reproductive value or state in life history models. Since it is an internal property of an individual, condition can be manipulated experimentally by manipulating resource availability (by altering diet, for instance), but cannot be measured directly. Instead, different phenotypic measures of resource acquisition ability or standing resource pool are commonly used. Here we used residual mass as our condition index since mass corrected for body size is expected to approximate well the reserves of fat or sugar that can be converted into energy (Kotiaho *et al.* 2001, Simmons and Kotiaho 2002; Birkhead *et al.*

2006).

We detected a strong sire effect on condition with little residual variance and no significant dam effect (Table 3.3). These results exhibit the existence of a heritable component to condition. Theoretically, one expects high genetic variance for condition since any allele that affects the ability of an individual to acquire or utilize resources will also affect condition (Andersson 1982). To further investigate if condition harbours high levels of additive genetic variation, we can compare our genetic estimates for condition with those of other traits expected to exhibit high genetic variance in *Drosophila.* Comparison with other fitness related traits in *D. melanogaster* reveals that CV_A for condition (4.4) is lower than traits like late male mating ability (13.9)(Hughes 1995), male longevity (8.3)(Hughes 1995), female longevity (5.3)(Rose and Charlesworth 1981) and fecundity (11.9)(Houle 1992). However, it is important to remember that condition was measured very indirectly from residuals of morphological traits (which are known to exhibit lower CV_A), while all the other traits considered here were measured directly, in different units, and the interpretation of these comparisons is not entirely straight forward. Studies that have directly measured energy reserves (Blackenhorn and Hosken 2003) or resource acquisition efficiency (Gienapp and Merila 2010) have been faced with their own limitations and have not been able to unequivocally resolve the issue of genetic variance in condition. From our results we can conclude that condition harbours additive

genetic variance, but it is unclear whether these levels are sufficiently high as required under the genic capture model. Currently, condition is more useful as a heuristic concept (Blackenhorn and Hosken 2003), and a direct empirical measure such as a multivariate condition index (Bussiere *et al.* 2008) that allows for meaningful comparisons is needed.

Genetic architecture of sex comb bristle number variation

The sire component for sex comb bristle number variation was only marginally significant. At first glance this results suggests that there may be no additive genetic variance for this trait, in contradiction with high additive genetic variation detected from analysis of response to artificial selection (Ahuja and Singh 2008). However, further inspection of variance components of half-sib analysis reveals a very strong dam effect which is rendering the sire component less significant (Table 3.3). Several studies suggest that heritability estimates based on sires may have a significant downward bias if the X chromosome contributes strongly to male fitness variance (Cowley and Atchley 1988; Long and Rice 2007; Connallon 2010). Given the large X chromosome of *Drosophila*, this is a strong possibility in our study. Overall, taking the results from the half-sib analysis together with the rapid response to artificial selection (Ahuja and Singh 2008) and significant genotype effect detected in the full-sib analysis (Table 3.2), we conclude that there is genetic variation for sex comb bristle number with possibly a strong dominance or maternal effect or X chromosome

effect. Our findings of heritable condition dependence and genetic variance in condition are consistent with the operation of genic capture, but do not allow us to separate this from other mechanisms such as non-equilibrium populations, frequency-dependent selection etc. In particular, given the genetic correlation between the male sex comb and female transverse bristles (Ahuja and Singh 2008), it is possible that sexual antagonism is an important contributing factor. Much remains to be learned about the mechanisms maintaining variation for sex comb bristle number, and this is an exciting avenue for further research.

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Figure 3.1: Mean trait size of full-sibs reared on poor and rich diets. Each filled circle represents mean score of thirty flies, with each colour representing a full-sib family.

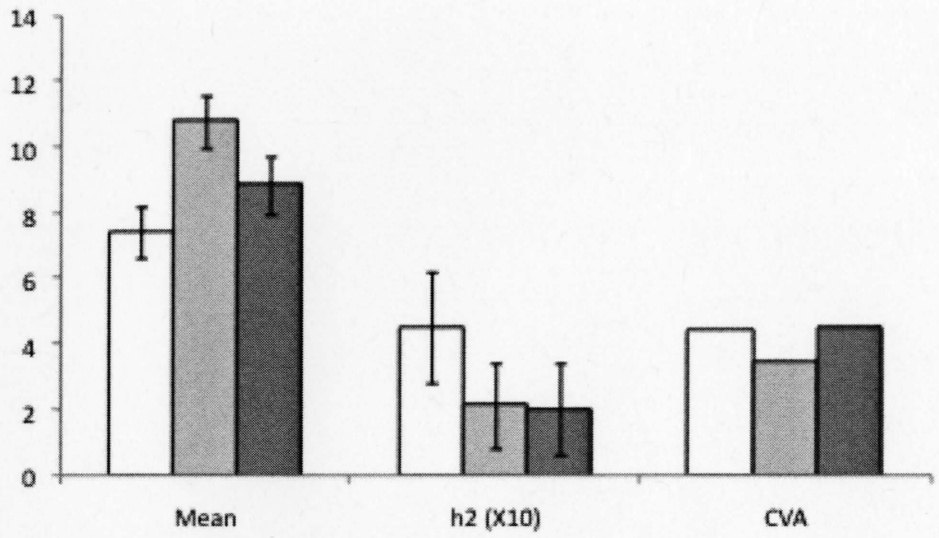


Figure 3.2. Mean (±SD), heritability (x10)(±SE) and coefficient of additive genetic variation for condition (white), sex comb bristle number (light gray) and sternopleural bristle number (dark gray).

Table 3.1: Comparison of mean (±SD) (n = 300) absolute size of bristle traits in flies reared under poor and rich diet regimens. Effect size of diet treatment represents standardized difference between the two means for each trait.

Trait	Poor Diet	Rich Diet	Comparison (*t*)	Effect size (*g*)(95% CI)
Sex comb bristle no.(♂)	10.69(0.77)	11.22(0.79)	7.9***	6.44 (8.08-4.8)
Sex comb length (um) (♂)	19.321 (1.31)	20.34 (1.42)	9.2***	7.5 (9.16-5.85)
Sternopleural bristle no. (♂)	8.82(0.86)	9.08(0.96)	3.7***	3.01 (4.62-1.41)
Transvere bristle row no.(♀)	3.7(0.42)	4.16(0.46)	13.2***	10.77 (12.48-9.05)

***$P < 0.001$

Table 3.2: Analysis of covariance examining the effect of diet, line, and their interaction on four bristle traits in 10 isofemale lines. Tarsus length was used as the covariate to control for body size variation.

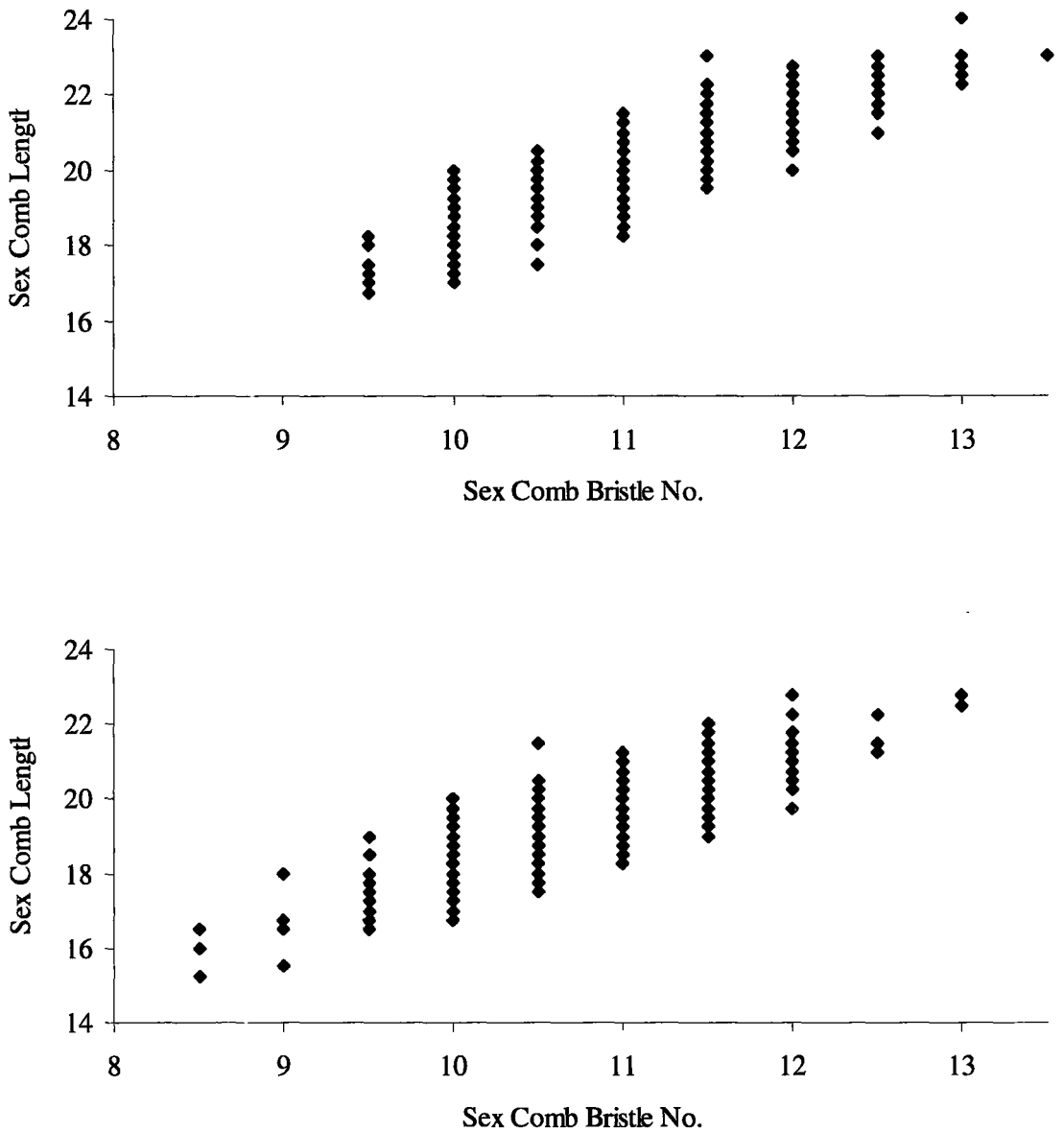
		Sex comb bristle no.		Sex comb length		Sternopleural bristle no.		Transverse row bristle no.	
Factor	df	MS	*F*	MS	*F*	MS	*F*	MS	*F*
Tarsus length	1	25.089	47.15***	168.948	127.54***	4.283	6.12*	0.874	6.14*
Diet treatment	1	21.734	40.84***	64.782	48.9***	5.597	8.00**	27.293	191.85***
Family	9	1.435	2.69**	7.689	5.8***	8.702	12.44***	0.396	2.78**
Treatment X Family	9	1.587	2.98**	3.901	2.94**	1.093	1.563	0.483	3.39***

*$P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$*

Table 3.3: Nested analysis of variance of three traits in thirty *D. melanogaster* half sib families

		Condition		Sex comb bristle no.		Sternopleural bristle no.	
Factor	df	MS	*F*	MS	*F*	MS	*F*
Sire	29	0.067	2.63**	1.417	1.57	1.921	1.44
Dam(Sire)	60	0.025	1.24	0.9	1.66**	1.33	2.23***
Sibs(Dam)	360	0.02		0.54		0.59	

**P < 0.05, ** P < 0.01, *** P < 0.0001*



Supplementary Figure 3.1: Relationship between sex comb bristle number and sex comb length in a) Rich (r_s=*0.862, P <0.001*) and b) Poor diet males (r_s=*0.829, P <0.001*).

Supplementary Table 3.1: Comparison of median (inter–quartile range) absolute and relative trait sizes in individuals reared on rich and poor diets (n = 300)

	Absolute Size			Relative Size		
Trait	Poor	Rich	Comparison (*U*)	Poor	Rich	Comparison (*U*)
Sex Comb bristle no. (♂)	10.5(10.0-11.0)	11.25(10.5-12.0)	61294***	21.21(20.0 - 22.19)	21.64(20.6-22.7)	52604***
Sex Comb length (♂)	19.5(18.5-20.25	20.5(19.5-21.25)	63054***	38.31(36.7-39.8)	39.43(37.5-40.98)	55406***
Sternopleural bristle no. (♂)	9.0(8.5-9.5)	9.0(8.5-9.5)	51917***	17.3(16.26-18.65)	17.5(16.12-18.76)	45612
Transverse bristle row no. (♀)	4.0(3.5-4.0)	4.0(4.0-4.0)	66713***	0.065(0.058-0.068)	0.068(0.066-0.074)	65668.5***

CHAPTER 4

DEVELOPMENTAL REGULATION OF SEX COMB ORIENTATION IN *DROSOPHILA MELANOGASTER* BRISTLE NUMBER GENOTYPES

This chapter is currently being prepared for submission for publication. Here I studied the developmental relationship between sex comb bristle number and orientation. I tested the hypothesis that bristle number is correlated with comb orientation in adults from lines with differing sex comb bristles numbers. I took advantage of the live imaging protocol established by Atallah *et al.* (2009a) to study comb rotation in developing *D. melanogaster* pupae and analyzed patterns of variation in comb orientation over ontogeny in each line.

I introgressed *ubi-DEcad::GFP* insert into selection lines and Didem Sarikaya performed *babPR72* crosses. Nicolas Malagon performed measurements of adult legs with assistance from Sheng Cheng. Nicolas Malagon and I jointly performed confocal imaging of pupal legs. I conducted measurements of comb orientation from confocal images with assistance from Sergio Muñoz. I analyzed the data and wrote the manuscript with input from Nicolas Malagon, Ellen Larsen and Rama Singh.

4.1 ABSTRACT

The male sex comb of *Drosophila* exhibits high inter-specific variation in orientation and bristle number, and bristle number varies within a species as well. Here we assessed changes in *Drosophila melanogaster* sex comb orientation in response to changes in bristle number using High and Low sex comb bristle number artificial selection lines, and mutant strain *babPR72* with ectopic sex combs on the second tarsus. We detected a lack of correlation between sex comb bristle number and orientation in adults from within each line. Between lines comb orientation was conserved on the first tarsus. Comb orientation during ontogeny was assessed via live imaging of pupal legs to gain insight into the developmental response to genetic perturbations. Comparisons with wild type revealed that different lines exhibit different intermediate sex comb shapes to eventually reach the same final orientation. Furthermore, variability in comb shape decreases over ontogeny providing support for the idea that sex comb orientation is a canalized trait. Ectopic sex combs on the second tarsus of *babPR72* did not show the same patterns as first tarsal segment combs highlighting the importance of local cellular dynamics and emergent properties of developmental systems in this process. These results provide insight into sex comb evolvability, and show that the underlying developmental architecture is an important influence in shaping the trajectory of phenotypic change in a trait.

4.2 INTRODUCTION

It is now well established that an integrative evo–devo approach is needed to gain a complete picture of trait evolvability, the inherent capacity of a system to produce heritable phenotypic variation (Kirschner and Gerhart 1998; Hendrikse *et al.* 2007). The standard approach in traditional population genetics studies has been the analysis of patterns of variation exhibited by adults, often in response to different types of genetic or environmental perturbations. Much has been learned about the genetic architecture underlying phenotypic variation in morphological traits from such analyses. Fewer studies have attempted to investigate the developmental architecture of trait variation, largely because the approaches and methods to tackle this issue are still being explored. One approach is to track a phenotype during development. Analysis of patterns of trait variation over ontogeny in response to perturbations can provide insight into how developmental processes structure the translation of genetic variation into phenotypic variation. Here we apply this approach in the sex comb (SC) of *Drosophila*, an emerging model system in evolutionary developmental biology (True 2008).

Sex combs are a row of specialized bristles on the male foreleg that have been shown to be important for mating (Ng and Kopp 2008). The SC exhibits high inter-specific variation in the location and orientation of the rows (Kopp and True 2006). Variation is also seen in bristle number, both within and between closely related species (Coyne 1985). Population genetic analyses of sex comb

bristle number variation have shown high genetic variation underlying this trait in *Drosophila melanogaster* (Ahuja and Singh 2008). Developmental studies, on the other hand, have focused on comb orientation (Atallah *et al.* 2009a,b; Tanaka *et al.* 2009). In *D. melanogaster* the SC is initially transverse in orientation, eventually rotating during pupal development to come to its final longitudinal orientation (Held 2004). Atallah *et al.* (2009a) showed that the wild type SC, with 9 – 11 bristles on the first tarsus (TS1), does not rotate as a single unit. Instead, different bristles rotate at different rates. The SC exhibits characteristic intermediate forms at 23, 28 and 50 hrs after pupation (AP) that are associated spatially and temporally with different cellular processes occurring in the developing pupal leg (Figure 4.1). Furthermore, they noted a reduction in variation in comb orientation over ontogeny.

Here we assessed changes in *Drosophila melanogaster* sex comb orientation in response to changes in bristle number using the following lines: High (12-16 bristles on TS1) and Low (4-6 bristles on TS1) SC bristle number lines were developed through artificial selection in a highly diverse population of *D. melanogaster* (Figure 4.2)(Ahuja and Singh 2008). We also studied the mutant strain *bric à brac (bab) PR72*. In this mutation, homeotic transformation of the second tarsus (TS2) to TS1 occurs (Godt *et al.* 1993) with formation of ectopic sex combs on TS2 (homozygotes 4–6 bristles; heterozygotes 3–5 bristles) and an increase in bristle number on TS1 of homozygotes (11–13 bristles) and

heterozygotes (10–12 bristles)(Figure 4.2). Thus, we chose genotypes that produce similar bristle number phenotypes, but the nature of the genetic perturbation is very different. Artificial selection represents phenotypic change through the accumulation of many genetic changes of small effect, while the mutant strain represents a single genetic change of large effect.

First we measured and compared sex comb orientation in adult males from each line. Next, comb orientation was tracked over ontogeny via live imaging of developing pupae to assess the developmental response to change in bristle number. We characterized the shape of the comb at three time points in each genotype and quantified how similar an individual's rotation is to the wild type. We also assessed patterns of variability in comb shape within and between genotypes. Overall, our results show that developmental mechanisms involved in comb rotation buffer the final phenotype in the face of genetic perturbation, indicating that comb orientation is a canalized trait. Our results provide insight into sex comb evolvability, and highlight the importance of underlying developmental architecture in shaping the trajectory of phenotypic change.

4.3 Materials and Methods

Measurement of adult sex comb orientation

High and Low sex comb bristle number lines were developed by artificial selection for 24 generations, following which lines were maintained by selecting every 4–5 generations (Ahuja and Singh, 2008). Wild type males came from the

outbred base population described in Ahuja and Singh (2008) and *babPR72* strain is described in Godt *et al.* (1993). All flies were reared on yeast-cornmeal-molasses medium. Adults from each line were treated in 2N sodium hydroxide for thirty minutes. This procedure digests the internal tissue of the leg allowing clear imaging and the outer chitin covering remains intact allowing for accurate measurement. Legs were dissected, mounted on slides, and imaged. Angle of the sex comb with respect to the joint was measured using the angle tool of Image J imaging software as illustrated in figure 4.2. Analysis of variance with each group as a factor was performed using the Statisxl add-on package in Microsoft Excel.

Confocal Imaging

The *ubi-DEcad::GFP* lines were generated by Oda and Tsukita (2000). In order to introgress the *ubi-DE::cadGFP* construct into the artificial selection lines, males from the *ubi–DE cad::GFP* line were scored for sex comb bristle number. Males with the lowest sex comb bristle number were crossed with females from the Low artificial selection line and highest scoring males with High line females. In this manner, male progeny at each generation were screened for green fluorescent protein (GFP), scored for sex comb bristle number, and backcrossed repeatedly to selection line females for 11 generations. Each line was then selfed for 4 generations to obtain individuals homozygous for GFP. During the course of the experiment each line was screened for sex comb bristle number and GFP at each generation to maintain *D. melanogaster* lines with high and low

sex comb bristle numbers that are homozygous for *ubi–DE cad::GFP*. Standard crossing techniques were used to introgress *ubi–DE cad::GFP* into *babpr72*.

White prepupae were collected, rinsed in water, sexed by determining the presence of male gonads and aged on agar plates at 25°C. Pupae were placed in 1-3 ul of halocarbon oil on a coverslip, with immersion oil between the coverslip and objective. Z- stacks composed of a series of images of the first tarsal segment at different focal depths were generated at 23, 28, and 50(±2) hrs after pupation using a Zeiss laser scanning confocal microscope (LSM 510; Munich, Germany). The Argon 488 channel was used with a Z–interval of 3 micrometres with an Argon excitation of at least 20%. Using the projection tool of LSM image browser software, we generated 3D reconstructions of each stack. Background from the pupal case that obscured data from lower slices in 3D projections was deleted manually from each slice using the LSM browser software as it was easily distinguished as a non-specific haze. Measurements of sex comb shape were performed as described in Atallah *et al.* (2009a). We measured the angles between successive pairs of adjoining teeth and the anteroposterior axis from 2D projections along the length of the developing combs of live pupae (see figure 4.3 to visualize how angles were measured).

Data Analysis

The difference in comb orientation of each individual from the mean wild

type shape at each time point was quantified. Since the number of bristles differed between wild type and individuals from different genetic backgrounds, we performed a "sliding window" analysis. The shorter comb was moved along the length of the longer comb, and the absolute difference of the angle between adjacent bristles was measured for each bristle pair. In this manner, we moved the entire comb along the length of the wild type comb. The average of the absolute difference of the angle between adjacent teeth over the length of the comb was calculated for each alignment. To get the most conservative estimate, the minimum deviation at each time point was recorded as the value for each individual at each time point. We tested the patterns of deviation within each genotype with a one way analysis of variance.

To compare variability in comb shape within each genotype we used a modified version of Levene's test (Van Valen 2005). For each bristle pair (in each genotype at each time point) we calculated the mean angle subtended with the anteroposterior axis. We then calculated the absolute difference of each original angle from the mean and divided by the mean. We calculated the mean of this new variable for each bristle pair (the more varying the sample, the higher the value). These means for each bristle pair were then tested for equality with an analysis of variance within each genotype.

To make comparisons between genotypes we had to control for bristle number and we used a subset of the data. Mean variability between individuals

from different genotypes with the same SC bristle numbers was compared using multiple *t*-tests with Bonferroni corrections.

4.4 RESULTS

Adult SC orientation

The angle of the sex comb with respect to the anteroposterior axis was measured in adult males from each line as shown in Figure 4.2. Within each group we did not detect a correlation between bristle number and adult angle of rotation on the first tarsal segment: Wild type (R^2 = 0.05, P = 0.53)(n = 10), High (R^2 = 0.59, P = 0.71)(n = 10), Low (R^2 = 0.05, P = 0.14)(n = 10), *babPR72* homozygous TS1 (R^2 = 0.173, P = 0.3)(n = 8), *babPR72* heterozygous TS1 (R^2 = 0.43, P = 0.03)(n = 9). Similarly, no correlation was detected on the second tarsal segment of *babPR72* homozygotes (R^2 = 0.003, P = 0.756)(n = 30) or *babPR72* heterozygotes (R^2 = 0.02,P = 0.37)(n = 35). Comparison between groups revealed a significant difference in final comb orientation ($F_{6,105}$ = 68.332***)(Figure 4.4). Post-hoc Tukey's Honestly Significant Difference (HSD) tests showed that the final SC angle of rotation of ectopic sex combs of *babPR72 TS2* homozygotes and heterozygotes differed significantly from the combs on the first tarsal segment ($P < 0.001$ for all comparisons).

SC orientation over ontogeny

Mean angle between adjacent bristles and the anteroposterior axis along

the length of the SC for each line at each time point is presented in Figure 4.5. Wild type combs exhibit a sinusoidal shape at 23hrs after pupation (AP) where the angle subtended by adjacent medial bristles is greater than that between either the adjoining proximal bristles or the distal bristles (Atallah *et al.* 2009a). At 28 hrs the angle between proximal bristles is closer to that of the medial bristles, while the distal region is still at much less of an angle. The comb has a bimodal shape. After this stage, most of the rotation appears to occur at the distal end such that the comb reaches its final longitudinal orientation by 50hrs AP. High line individuals exhibit patterns similar to the wild type. A sinusoidal shape is seen at 23hrs AP, by 28 hrs the proximal region also rotates and the distal end completes rotation by 50 hrs (Figure 4.3,4.5). The typical wild type sinusoidal shape is not seen on the first tarsal segment of *babPR72* homozygotes or heterozygotes at 23hrs AP. Instead a bimodal shape is seen at 23 hrs, continues at 28 hrs, and the final orientation is achieved by 50 hrs. The smaller sex combs of the Low line appear also do not exhibit the sinusoidal or bimodal shape and appear to rotate as a single unit, likely because of the small size of the comb. The ectopic *babPR72* combs on the second tarsal segment of also do not exhibit the stereotypical patterns seen in the wild type.

We quantified how much individual comb shape differs from mean wild type sex comb shape for each line at each time point. Mean deviation (95% confidence interval) of individuals from each genotype at each time point is

presented in figure 4.6. Analysis of Variance revealed a significant difference in mean individual deviation from wild type over time in the High line ($F_{2,42}$ = 28.527***). Post hoc Tukey's HSD tests showed that maximum deviation was at 23 hours, followed by 28 hours, and the least at 48 hours (Figure 4.6). A similar pattern was seen within the Low line ($F_{2,36}$ = 6.304**), with significantly higher deviation at 23 hours as compared to 28 and 48 hours. Maximum deviation from wild type for *babPR72* homozygotes TS1 ($F_{2,27}$ = 4.17**) and *babpr72* heterozygotes TS1 ($F_{2,27}$ = 3.972*) was seen at 23 hours and 28 hours AP respectively. In contrast, in the case of *babPR72* homozygotes TS2 ($F_{2,27}$ = 22.105***) and heterozygotes TS2 ($F_{2,27}$ = 18.223***) maximum mean deviation from wild type was at 48 hrs hours AP.

Patterns of variability

Variability of angle for each bristle pair was calculated at each time point and analyzed with ANOVA within each group followed by post hoc Tukey's HSD tests. A significant effect was detected in wild type ($F_{2,24}$ = 23.4***), High line ($F_{2,39}$ = 14.79***), Low line ($F_{2,12}$ = 13.669**), *babPR72* homozygotes TS1 ($F_{2,33}$ = 8.323**) and *babpr72* heterozygotes TS1 ($F_{2,30}$ = 4.93*). In general, there was a decrease in mean variability of the bristle pairs at 28 hours, followed by further reduction at 50 hours (Figure 6.7). On the other hand no significant change in variability over ontogeny was noted on the second tarsal segment of *babPR72* homozygotes TS2 ($F_{2,12}$ = 0.001) and heterozygotes TS2 ($F_{2,6}$ = 3.87). Pair wise

comparisons between legs from different lines with the same SC bristle number were performed at each time point. For individuals with 12 bristles, variability of *babPR72* homozygous mutant legs was greater at 48 hours as compared to the High line (Table 6.1). Similarly, variability was greater in *babPR72* homozygote 2TS at 48 hours as compared to Low line individuals with 5 bristles (Table 6.2).

4.5 Discussion

Species with large sex combs with high bristle numbers tend to have longitudinal sex combs, while combs in species with fewer bristles tend to be transverse in orientation, suggesting that these two aspects of the sex comb phenotype are correlated with each other (Lemunier *et al.* 1986). Within *D. melanoagaster*, data from Hannah-Alava (1958) suggests that degree of rotation of ectopic sex combs increases with increasing bristle number. However, contrary to theoretical predictions our data shows a lack of a correlation between the two components of the sex comb within different *D. melanogaster* genotypes, including high and low sex comb bristles number lines and ectopic sex combs. Furthermore, between lines comb orientation in adults is conserved in response to different types of genetic perturbations (with the exception of ectopic SC on the TS2 discussed below)(Figure 4.4). Thus, while sex comb orientation evolves rapidly between species, it is a stable phenotype within *D. melanoagster.*

Tracking comb orientation over ontogeny indicates that the stability of this

phenotype is maintained via buffering by underlying developmental processes i.e canalization (Waddington 1942). There are two scenarios that can explain how canalization acts to maintain the stability of the phenotype: minimize/prevent the production of variants (Wagner and Misof 1993), or restore deviants to wild type phenotype (Waddington 1952). Tracking comb orientation over ontogeny demonstrated that even though ultimately the same wild type phenotype was attained, different intermediate phenotypes were exhibited by different genotypes (Figure 4.6). It is interesting to note that a similar pattern is seen between species where combs with similar longitudinal orientation arise by different underlying mechanisms (Atallah *et al.* 2009b, Tanaka *et al.* 2009). Furthermore, within individual deviation from wild type was high at the earlier stages for each genotype, but reduced towards the later stages (Figure 5). This supports the latter scenario where variants are generated, but are eventually restored to the wild type trajectory. Further support for canalization of comb orientation comes from patterns of variation over ontogeny. In the absence of processes regulating morphogenesis phenotypic variance of a population is expected to increase over ontogeny (Zelditch *et al.* 2004). Reduction of variation among individuals over ontogeny on the first tarsus of each genotype shows that this process is internally regulated, such that variation within the population that was present early in ontogeny is removed and all individuals appear to be converging onto the targeted adult morphology.

Some insight into the nature of the underlying mechanisms that produce the patterns above can be gained from previous studies of this system. Atallah *et al.* (2009a) studied the cellular dynamics associated with the rotation of the sex comb and highlighted the importance of "self-organization" in this process. They show that comb rotation is a consequence of coordinated physical interactions between the bristles and higher order structures, like the contiguous rows of bristles that act as barriers, and the cellular environment of the developing tarsus (Figure 4.1). It is possible that gene induced variants generated in our study are corrected by physical forces and conditions. In such a complex, dynamic system our data provides clues where to focus further investigation. For instance, in *babPR72* the typical sinusoidal shape of the wild type is not seen at 23 hours AP. One hypothesis to explain this phenomenon is that there is variation in the developmental timing of this process such that the sinusoidal shape has already passed before 23 hours. Live imaging of pupal leg development in these lines to enable detailed analyses of the cellular dynamics that give rise to the different intermediate phenotypes is currently underway (Malagon *et al.* in prep).

The patterns seen on the second tarsal segment of *babPR72* warrant further explanation. In adults the combs from these lines are not rotated to the same extent as combs on the first tarsal segment. A breakdown of the canalizing mechanism appears to have occurred. In contrast with the observations on the first tarsal segment, within individual deviation from wild type *increased* over

ontogeny for the second tarsal segment in both the homozygous and heterozygous individuals. Moreover, no significant reduction in variability over ontogeny was detected. Three possible explanations could apply: First, the difference could be due to the fact that the relevant modifiers operating on the first tarsal segment do not operate in the second tarsal segment. Secondly, in light of Atallah *et al.* (2009a) the local cellular dynamics of the region could play an important role. Mutations in the *bric à brac* gene have been shown to also affect joint development and this could lead to major disruptions in the cellular processes like intercalation in the region. The differences in patterns of variation on the first and second tarsal segment of the same genotype further support the idea that SC orientation is not the sole outcome of a specific genetic architecture, but also of cell dynamics and physical constraints in a complex dynamic system.

Theoretically the degree of canalization is expected to differ between the artificial selection lines and *babPR72* mutant line. Artificial selection lines are functional in nature and are expected to be more canalized. Mutations, on the other hand, are expected to be deleterious and cause large scale disruptions in the developmental architecture of a trait. As a result they are expected to be less canalized and exhibit higher variance (Scharloo 1964, 1991). Our comparisons of legs from selection lines vs. mutants with the same bristle numbers revealed higher variability in mutants at 48 hours in some comparisons but not others. Given the small sample sizes and limited number of genotypes studied, further

work is needed to confirm these results.

Sex comb orientation appears to be a stable, highly canalized phenotype in *Drosophila melanogaster.* Canalization reduces the variability of traits and hence their capacity to respond to selection or to diverge by genetic drift (Gibson and Wagner 2000). In contrast, sex comb bristle number exhibited a robust response to artificial selection (Ahuja and Singh 2008). Lack of correlation between sex comb bristle umber and orientation detected in this study shows that even though comb orientation may be canalized, sex comb bristle number can evolve relatively independently of change/stasis in comb orientation.

This study contributes to our understanding of how developmental architecture influences trait variation. In addition to constraining or biasing the direction in which variation is produced (Alberch 1982; Brakefield 2006), developmental processes can also modulate the amount of variation produced (Waddington 19757). Canalization reflects the minimization of variation in the face of genetic or environmental insults and appears to be a ubiquitous phenomenon in disparate biological systems that is revealed through patterns such as the ontogenetic reduction of variance. As we better understand the mechanisms behind canalization, the modulation of variation by development will contribute increasingly to our understanding of morphological evolution.

4.6 References

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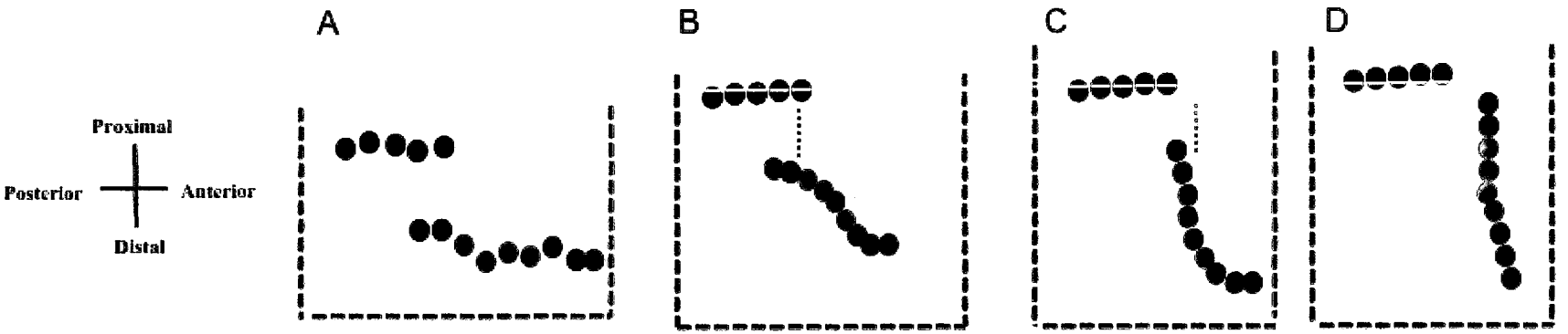

A
B
C
D
Proximal
Posterior
Anterior
Distal

Figure 4.1: Schematic representation of sex comb (SC) rotation drawn from data reported in Atallah *et al.* (2009a)(Ventral view). SC bristles are indicated with coloured circles and most distal transverse row (TR) bristles with black circles. During this period the developing leg progressively gets thinner and longer.

A) At approx. 17 hrs after pupation (AP) the presumptive SC and distal TR bristles are not yet contiguous. The SC is almost, but not quite transverse in orientation. The anterior bristles (blue circles) are slightly distal to the posterior bristles (red circles). Green circles represent most anterior section of the comb

B) By 23 hrs AP the SC and TR bristles are contiguous. There is no more cell division. During the period from 23–28hrs AP intercalation of cells in the regions just proximal to the SC (pink shaded region) and the distal ventral campaniform sensillum (blue shaded region) occurs. Atallah *et al.* (2009a) proposed that this process leads to rotation of medial part of the comb (blue circles): As the cells proximal and anterior to the comb converge those that move in an anterior direction can only be replaced by cells that make up the SC (the contiguous SC and TR form a barrier) causing it to rotate. The slight initial angle between posterior distal bristle (red) and the presumptive teeth just anterior to them (blue) gets accentuated due to this intercalation and the medial part of the SC rotates before the remainder of the structure.

C) At 28 hrs AP a strong increase in the angle between the most proximal teeth is seen i.e. the proximal region of the comb moves anterior relative to TR.

The bottleneck model attributes this particularly rapid rotation of the comb to a bottleneck between the SC and TR (left of the dashed red line in B and C). The cells in this region are not able to move proximally but are instead forced to move anteriorly leading to rapid rotation of the posterior proximal region of the comb. As the above processes continue, the sinusoidal shape of the SC at approximately 23 h AP, with 3 regions at different degrees of rotation is replaced by a bimodal "L" shape around 28–30 hrs AP. There is a strongly rotated proximal portion of the SC (red and blue) and a much weaker rotation of the distal portion (green circles).

D) The rapid movement of the proximal SC slows down when teeth have moved beyond distal TR and there is no longer a bottleneck. After the proximal region has moved beyond the distal transverse row most of the rotation occurs in the distal part of the structure and the comb is fully rotated around 50hrs AP. Atallah *et al.* (2009a) did not conduct a detailed analysis of the cellular processes occurring at this time.

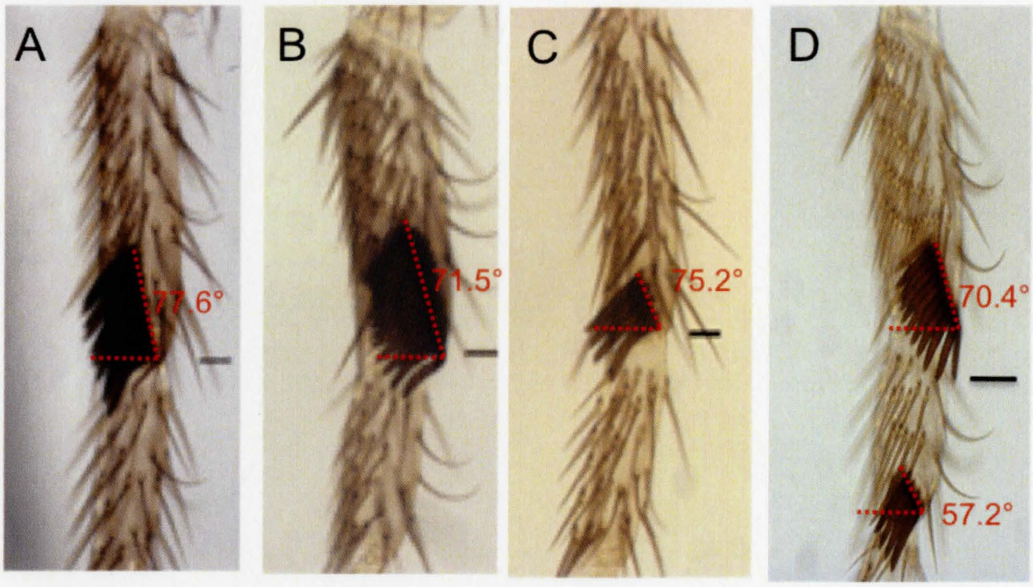


Figure 4.2**:** Forelegs of males showing sex comb from A) Wild type B) High line C) Low line and D) *babpr72* homozygotes. Dashed red lines illustrate measurement of final angle of rotation of the comb.

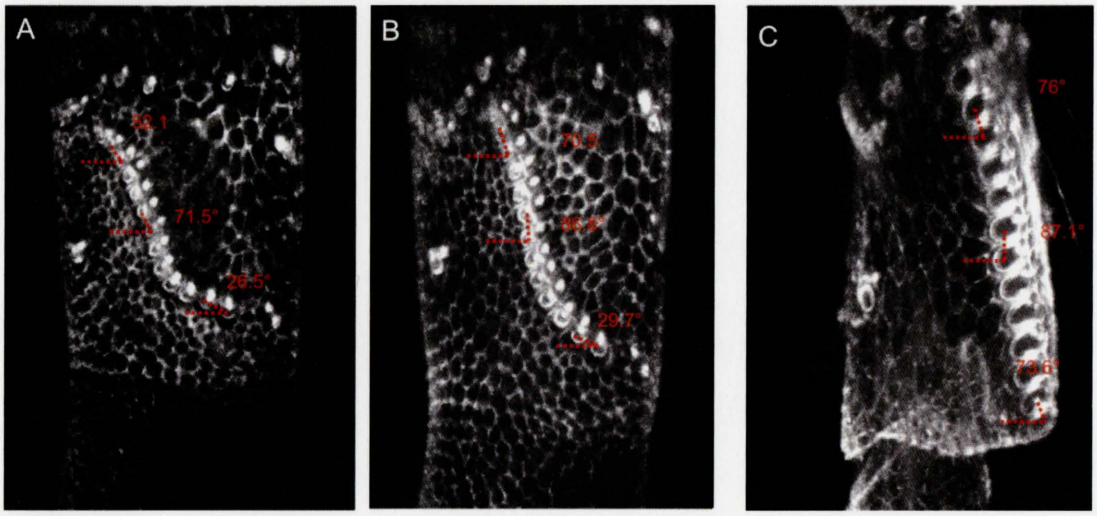


Figure 4.3: Confocal micrograph of developing pupal leg from High line individual with 14 bristles at A) 23 hrs AP B) 28hrs AP and C) 50hrs AP. Dashed red lines demonstrate how angle between consecutive bristle was measured.

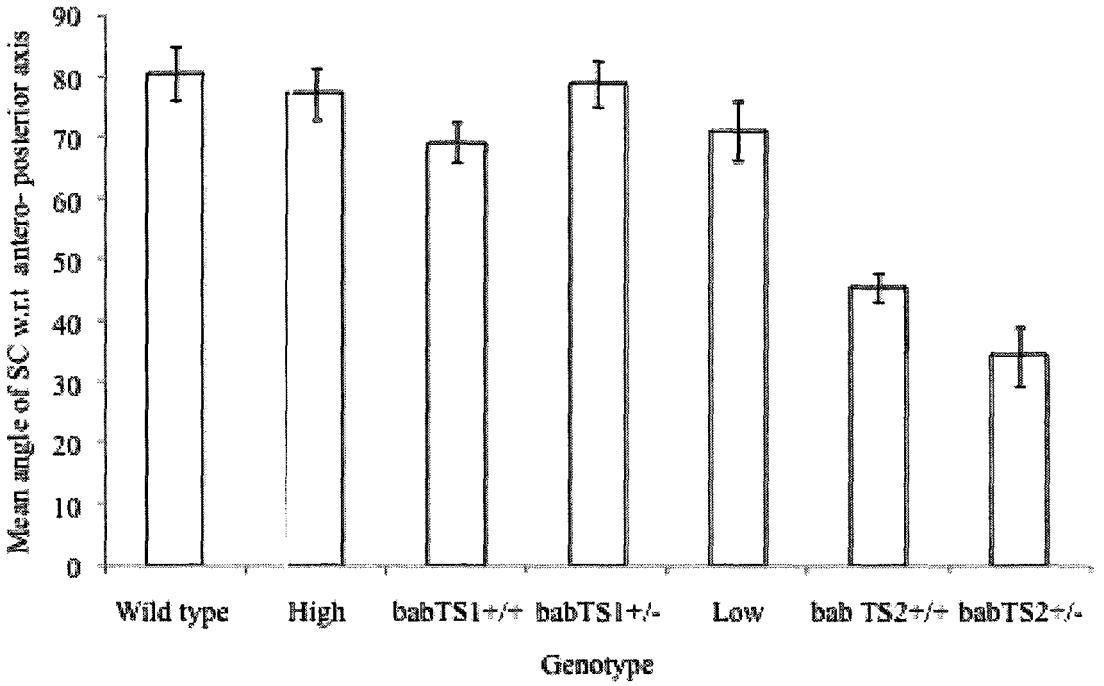
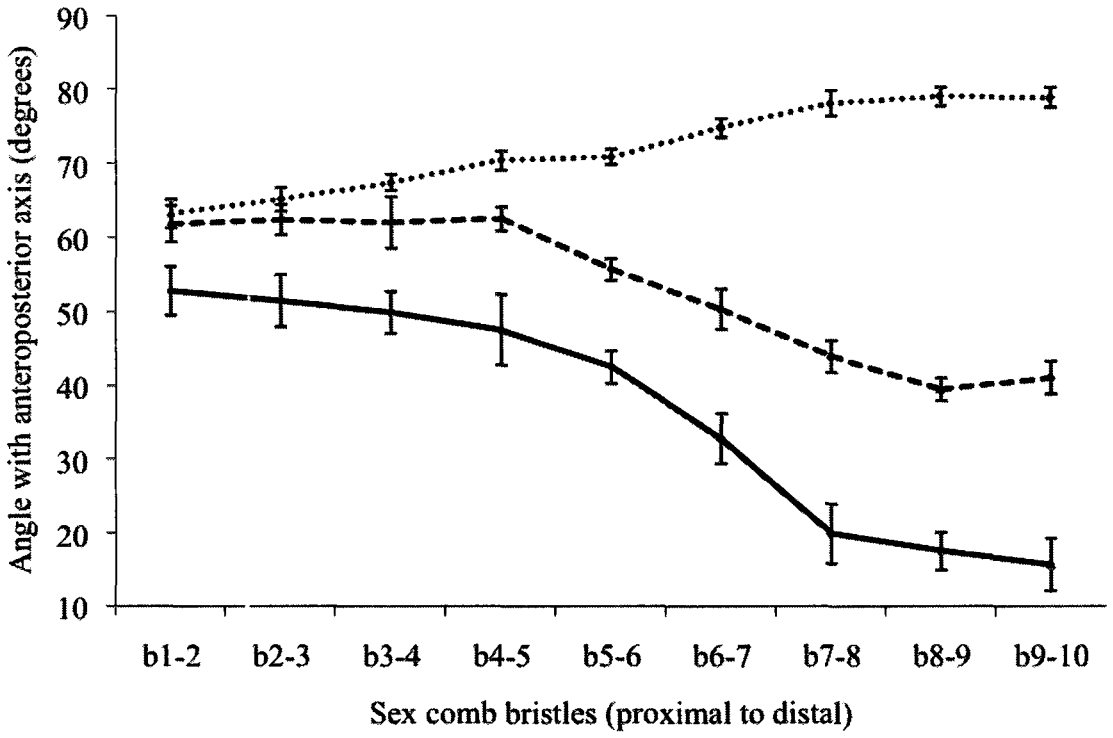


Figure 4.4: Final SC orienation in adult males from each genotype. Error bars represent 95% confidence intervals.


Angle with anteroposterior axis (degrees)
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30
20
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b1-2
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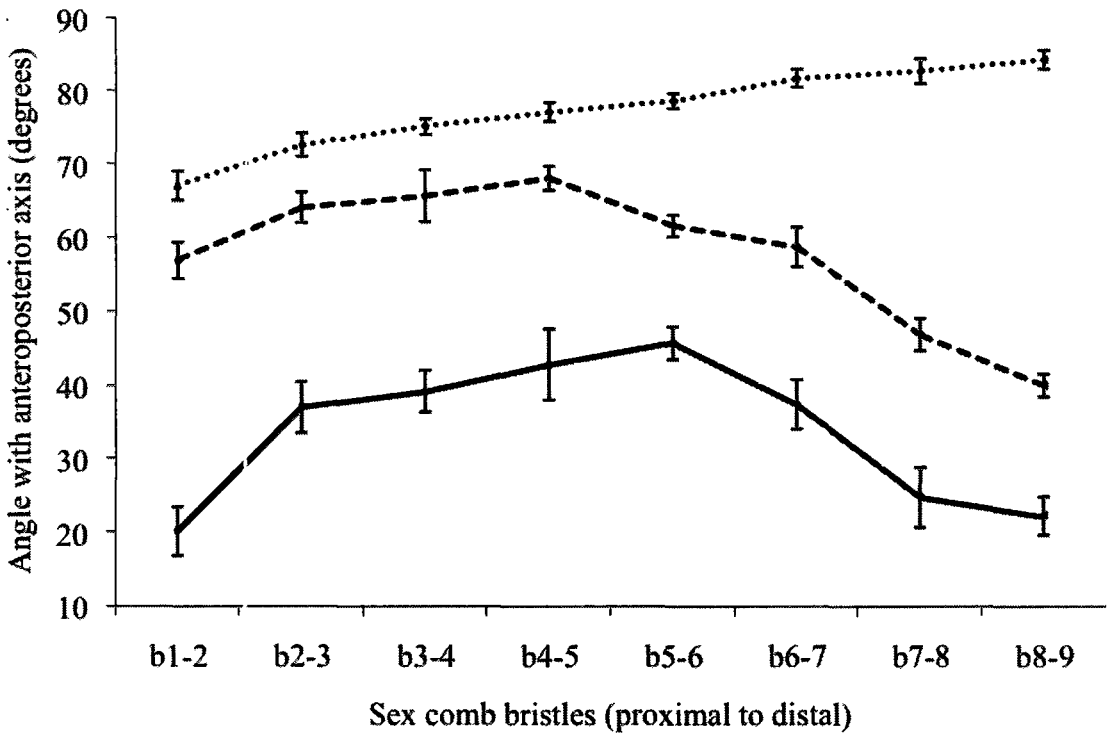
b3-4
b4-5
b5-6
b6-7
b7-8
b8-9
b9-10
Sex comb bristles (proximal to distal)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b6-7
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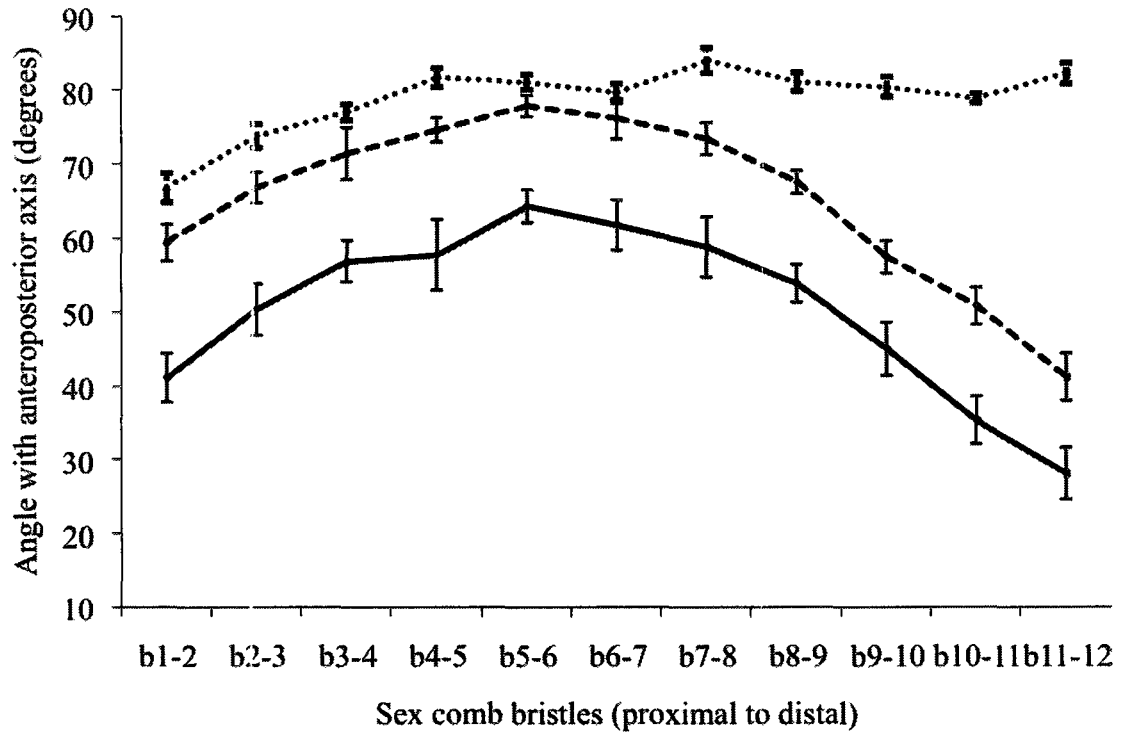
b8-9
Sex comb bristles (proximal to distal)


Angle with anteroposterior axis (degrees)
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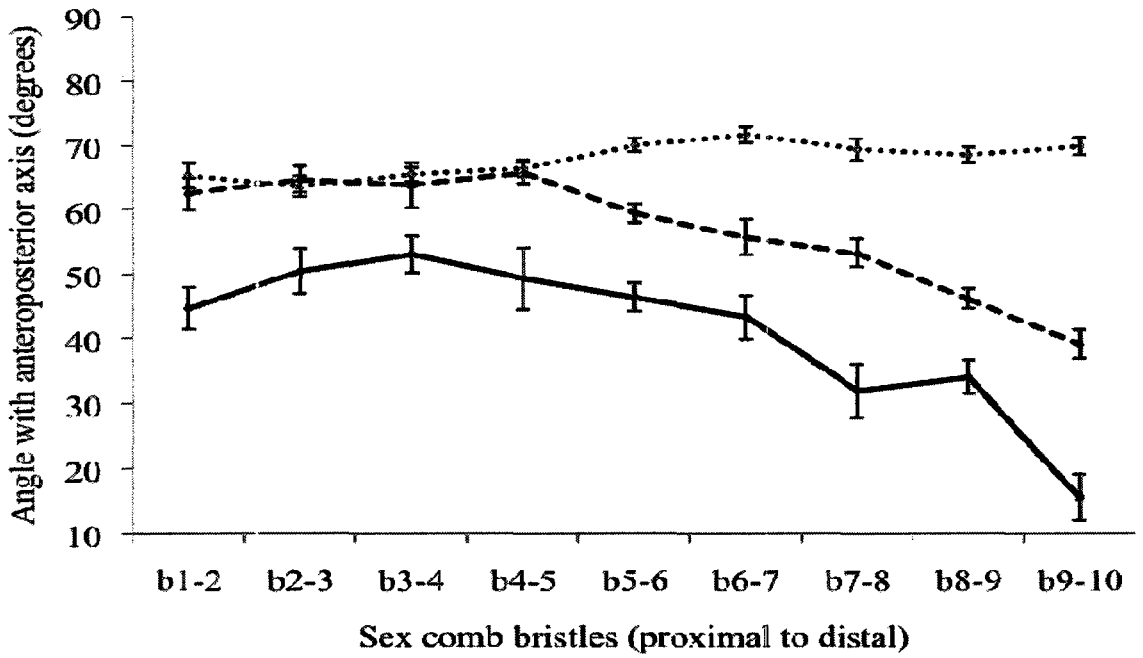
Sex comb bristles (proximal to distal)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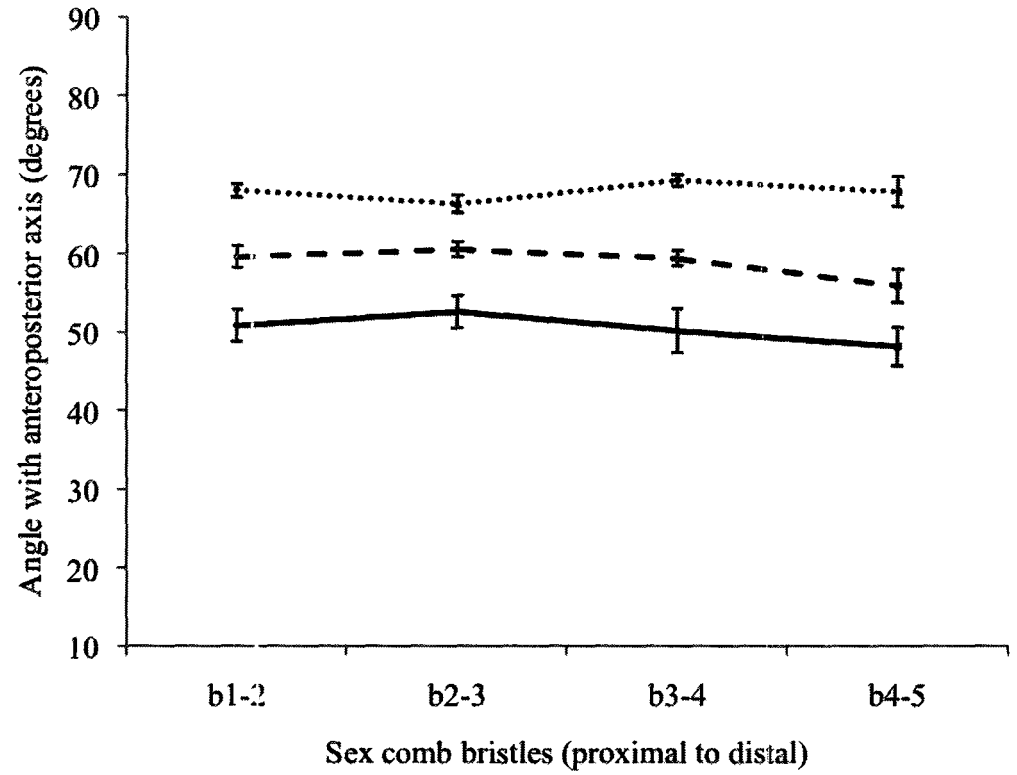
Sex comb bristles (proximal to distal)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Angle with anteroposterior axis (degrees)
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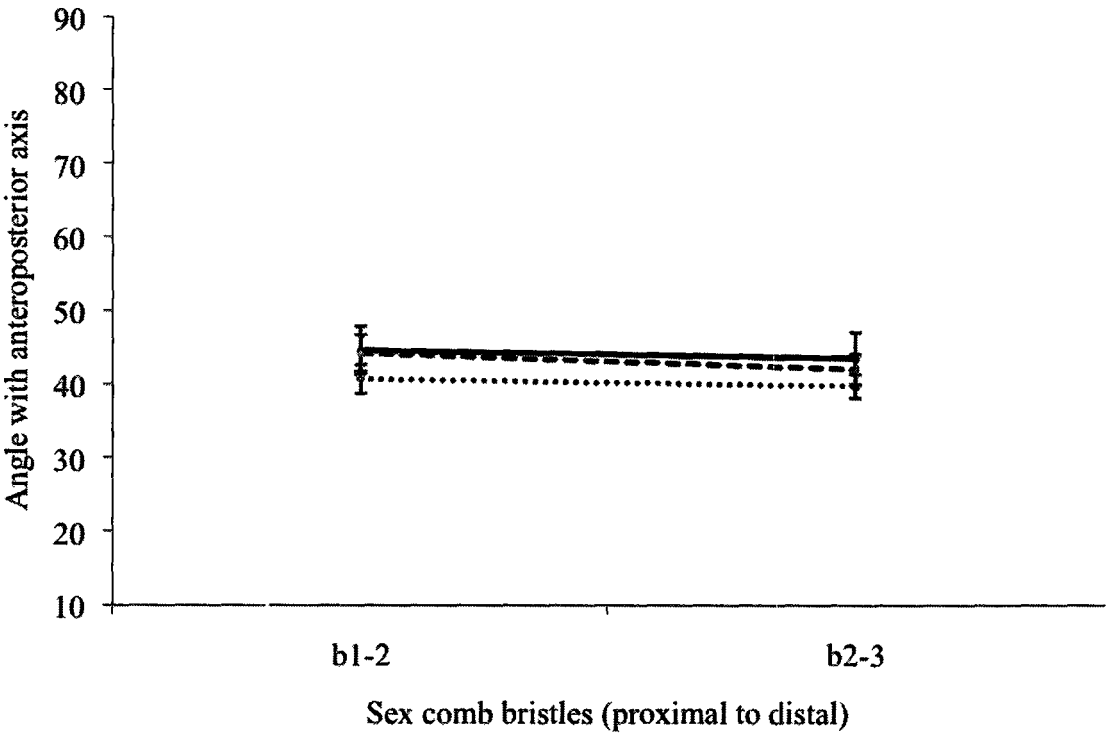
Sex comb bristles (proximal to distal)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Angle with anteroposterior axis
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Sex comb bristles (proximal to distal)

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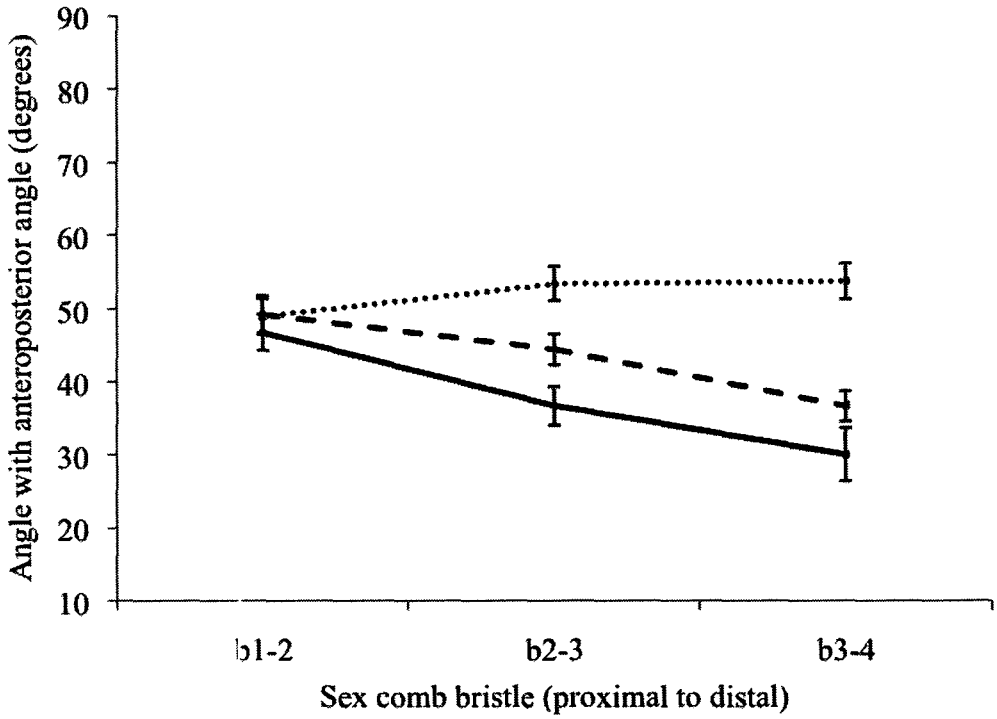

Figure 4.5: Graphical representation of sex comb orientation during rotation. Angle between adjacent sex comb bristles and anteroposterior axis at 23 (solid line), 28 (dashed line) and 50 (±2) (dotted line) hrs after pupation (AP). Error bars represent standard deviation.

(A) Wild type (n=10)(reproduced from Atallah *et al.* 2009a). All legs had between 9 and 11 SC bristles. Following the method of Atallah *et al.* (2009a) in legs with 10 teeth the most distal tooth was dropped to enable graphical representation. In legs with 11 teeth both the most proximal and most distal tooth were dropped

(B) High line (n = 15). All legs had between 12 – 16 bristles. In legs with 13 bristles the most proximal tooth was dropped, 14 bristles the most proximal and most distal, in 15 the two most distal and most proximal and in 16 bristles the two most proximal and distal.

(C) *babPR72* TS1 (homozygous)(n=11).All legs had between 10-13 bristles. In legs with 11 bristles more proximal tooth was dropped, most prximal and most distal for 12 bristles and most proximal and two most distal for 13.

(D) *babPR72* TS1 (heterozygous)(n=10). All legs had between 10- 12 bristles. Most proximal bristle was dropped for 11 bristles and proximal and distal for 12 bristles.

(E) Low line (n=11). All legs had between 5 and 6 bristels. The most proximal bristle was dropped in legs with 6 bristles

(F) *babPR72* TS2 (homozygous)(n=9). All legs had between 4 and 6 bristles. The most distal bristle was dropped in 5 bristle legs and most proximal and distal in 6 bristle legs

(G) *babPR72* TS2 (heterozygous)(n=10). All legs had between 3 and 4 bristles. The most proximal bristle was dropped in legs with 4 bristles

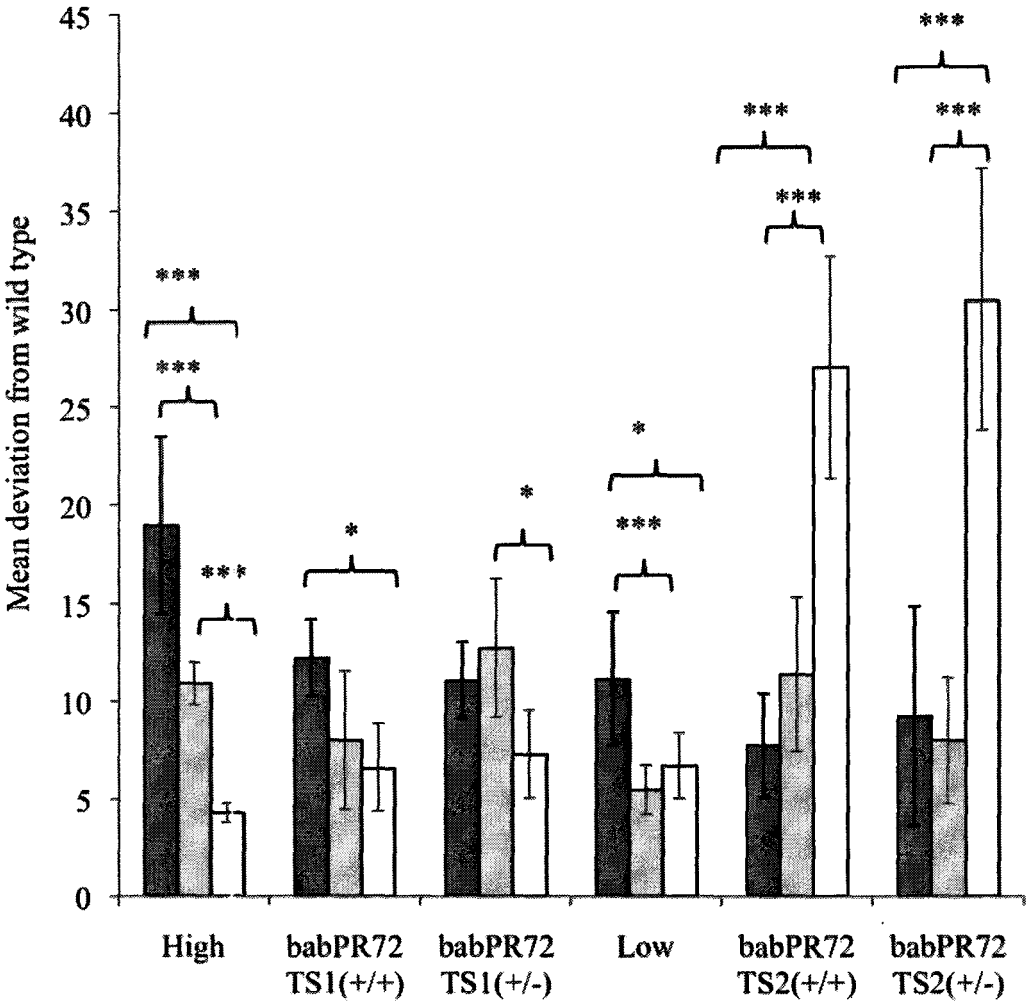

Figure 4.6: Mean deviation of individual sex comb shape from mean wild type shape at 23 (black), 28 (grey) and 50 (±2) (white) hrs AP in each genotype.

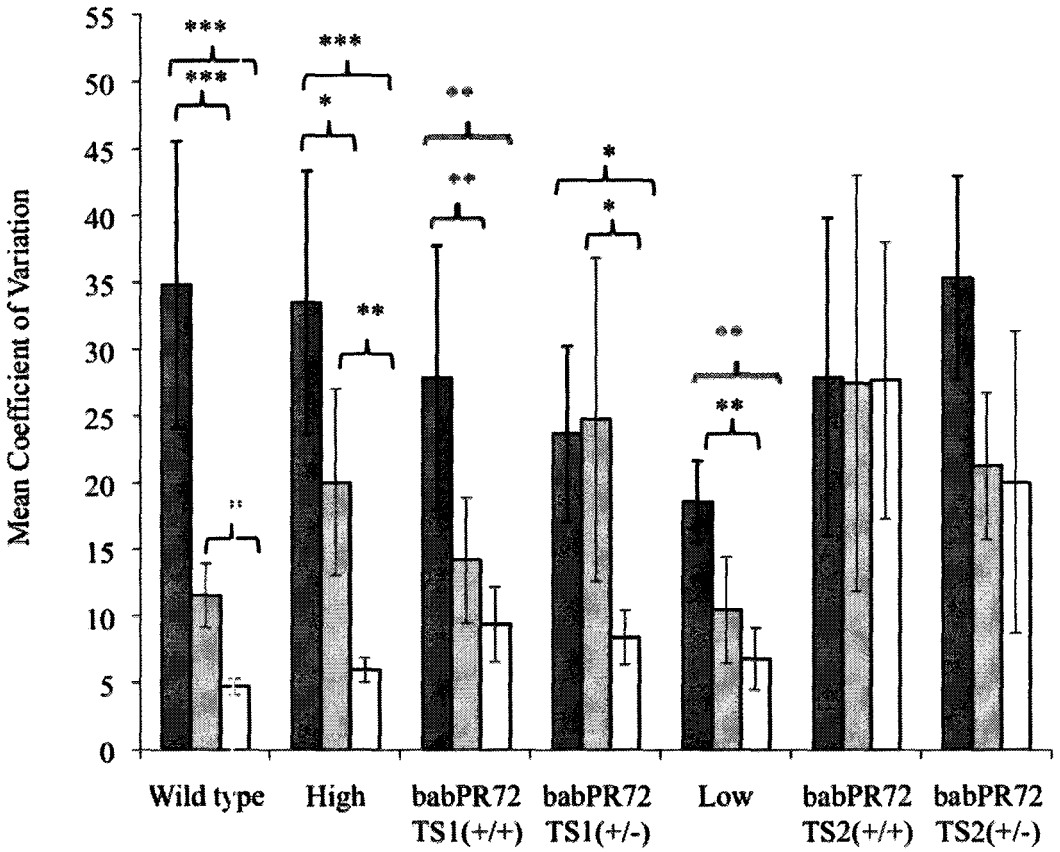

Figure 4.7: Mean variability of angle between successive bristles at 23 (black), 28 (grey) and 50 (±2) (white) hrs AP in each genotype. Error bars represent 95% confidence interval.

Table 4.1: Pair wise comparisons of variability between High and *babPR72* with the same bristle numbers.

Time	High (13)(n=6)	*babPR72* TS1 (13)(+/+)(n=3)	*t*	High (12)(n=5)	*babPR72* TS1 (12)(+/+)(n=4)	*t*	High (12)(n=5)	*babPR72* TS1 (12)(+/-)(n=3)	*t*
23	22.8(14.2)	16.5(12.54)	1.15	24.7(9)	26.7(20.1)	0.21	24.7(9)	21.7(13.7)	0.71
28	12.9(9.7)	18.6(17.54)	0.99	9.2(5.5)	13.8(7.1)	1.71	9.2(5.5)	13.9(8)	1.61
48	6.5(2.2)	6.8(4.4)	0.18	6.5(2.5)	12.5(5.5)	3.25**	6.5(2.5)	6(2.5)	0.44

Table 4.2: Pair wise comparisons of variability between Low and *babPR72* with the same bristle numbers.

Low (5)(n=6)	*babPR72* (TS2)(+/+) (5)(n=3)	*t*	Low (6)(n=5)	*babPR72* (TS2)(+/+) (6)(n=4)	*t*
13.8(4)	17.3(6.9)	0.85	21.44(4.54)	25.6(15.8)	0.56
8.2(1.8)	17.9(8.7)	2.17	8.6(5.4)	21.28(20.8)	1.30
5.8(1.8)	13.4(2.1)	5.35**	7.39(4.78)	26.1(18.2)	2.22

CHAPTER 5

GENERAL CONCLUSION

5.1 SUMMARY

The broad goal of this thesis was to study variation in sex comb bristle number in *Drosophila melanogaster*. This included measuring the response to artificial selection, susceptibility to environmental perturbation, and developmental analysis of ontogenetic trajectory. The preceding chapters make a significant contribution to the understanding of the genetic and developmental architecture of this trait and shed light on its evolutionary potential (i.e. evolvability).

Chapter 2 presents the results of twenty-four generations of divergent artificial selection for sex comb bristle number in a heterogeneous base population. The rapid, robust response to artificial selection, along with evidence of intra-specific variation for bristle number in different geographical *D. melanogaster* populations indicates high heritable genetic variance underlying this trait. We also observed lack of a consistent correlated response to artificial selection for sex comb bristle number in other developmentally related, non-sex mechanosensory bristle systems. These results suggest a decoupling of developmental relationship between sexual and non-sexual bristle systems.

We further explored the genetic architecture of *D. melanogaster* sex comb

bristle number in terms of their susceptibility to environmental (condition) perturbation in Chapter 3. Sex comb bristle number and length responded to condition manipulation and showed increase in individuals of high condition. Further partitioning of variance components revealed a strong maternal effect and/or dominance effect on sex comb bristle number variation. Our findings of heritable condition dependence and genetic variance in condition were consistent with the operation of genic capture for the maintenance of genetic variation in this trait, but did not allow us to separate this from other mechanisms such as non-equilibrium populations, frequency-dependent selection and sexual antagonism.

Chapter 4 presents the results of developmental analysis of the relationship between sex comb bristle number and orientation. Analysis of patterns of variation over ontogeny in wild type, high and low sex comb bristle number selection lines, and the mutant strain *bric à brac PR72* showed that sex comb orientation is a highly canalized trait. Sex comb orientation was conserved between these genotypes with differing bristle numbers. Even within each line, sex comb bristle number and orientation were not correlated with each other. These results show that these two aspects of the SC are not coupled, and that bristle number can change relatively independently of change/stasis in comb orientation.

D. melanogaster sex comb bristle number harbours high genetic variance and the significant response to environmental manipulation demonstrates

condition-dependent developmental plasticity. Furthermore, bristle number appears to be decoupled from other developmentally related non-sex bristle systems, and even from another aspect of the same trait, orientation. Finally, there is evidence that sex comb bristle number affects mating success. Thus, taken together these results show that sex comb bristle number has the potential to change rapidly and contribute to sexual selection and speciation. This body of work highlights the importance of considering a holistic view of evolvability, and represents an example and a timely approach to bridging the gap between population genetics and development in the study of phenotypic evolution.

5.2 Future Directions

This work firmly establishes the utility and power of the sex comb as an important model in the maturing field of evolutionary developmental biology (True 2008) and lays the foundation for further research using this system. One of the most important questions that remain to be answered conclusively is the function of the sex comb and nature of sexual selection acting on this trait (Ng and Kopp 2008). Mating assays of selection lines provided evidence that bristle number is associated with mating success and condition dependence suggests that this trait can signal male quality. However it is still unclear in D. melanoagster if sex comb bristle number is a display trait subject to female choice, or if it serves a mechanical function in stimulating or grasping a female. Recently a new experimental chamber for studying behaviour in *Drosophila* has been developed

that allows for high-resolution digital imaging (Simon and Dickinson 2010). Direct observation of the sex combs during courtship and mating in the high and low sex comb bristle number lines, as well as species with differing comb morphologies will help to address this issue.

The artificial selection lines developed here can serve as an important tool for the identification of candidate genes for sex comb bristle number variation in *D. melanogaster*. Currently, the thrust in the field is to use a combination of techniques – traditional QTL mapping along with new and established genomic technologies such as microarrays (Wayne and McIntyre 2002) or high throughput sequencing (Miles and Wayne 2008) can help identify loci that contribute to differences in bristle number between these lines. Once identified, comparative genomic analysis with other sequenced *Drosophila* species (Stark *et al.* 2007) with varying comb morphologies can address such questions as do the same genes contribute to intra- and inter- specific variation in sex comb bristle number and do the genes contributing to sex comb bristle number variation evolve rapidly or show high divergence between closely related species? The divergence and convergence in sex comb morphologies between species provides a model system to pursue studies of evolution of development and rates of speciation (Gould, 1977).

To understand how trait variation is generated, genetic differences must be placed in the context of defined cellular behaviours such as proliferation, cell

death, differentiation etc. (Parichy 2005). As mentioned in chapter 4, live imaging of the cellular dynamics involved in comb rotation from 23–48hrs after pupation (AP) in the developing pupal legs of the High and Low lines is currently underway (Malagon *et al.* in prep). Unfortunately live imaging of *ubiDEcad::GFP* pupal legs to visualize proneural clusters at early stages is not feasible. One alternative is staining of prepupal discs at 6 hrs AP, and possibly 7 hrs AP with antibodies that allow visualization of proneural clusters. This will help to answer questions such as do more cells differentiate into proneural clusters in High line pupae, or do fewer proneural clusters die off? Is there a polarity to loss or gain of bristles and/or proneural clusters in these lines? Such studies will help to understand how development can define and delimit the response to artificial selection and contribute to the mechanistic understanding of sex comb bristle number determination.

Overall, the sex comb system has the potential to be one of the first systems in which we are able to accomplish the ultimate goal of combining a detailed knowledge of selection with a genotype-phenotype map (Lewontin 1974; Houle 2010), and promises to be an increasingly important model in evolutionary biology.

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