The Development and Evolution of Complex Patterns: The *Drosophila* Sex Comb as a Model System

By

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ABSTRACT

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One of the best-known structures in *Drosophila* is the sex comb, an arrangement of modified bristles on the tarsal forelegs of males. This complex, sexually-dimorphic trait shows striking variation among closely related species, although most other aspects of the tarsal bristle pattern have been conserved. I studied the development of the sex comb in the model organism *Drosophila melanogaster* and six related species. I confirmed that the *D. melanogaster* sex comb, although longitudinal in the adult, originates in a transverse orientation and rotates during development, and showed that this process occurs through male-specific convergent extension. However, in the species that I examined that have longitudinally-oriented sex combs that extend the full length of the tarsus, including *D. ficushila* and two species of the *montium* subgroup, the sex comb does not rotate, and instead forms from two longitudinal rows that converge during development. Another species of the *montium* subgroup, *D. nikananu*, has a sex comb that is convergently similar to *D. melanogaster*, but forms in a manner typical of its subgroup, showing that very similar combs can be formed through different processes. In all species, there is a strong correlation between the position of the sex comb and the
transverse bristle row on the foreleg tarsus just proximal to it. To test whether it is possible to violate this apparent constraint on development, I perturbed the expression of the leg patterning gene \textit{dachshund} to generate ectopic sex combs in \textit{D. melanogaster}. I found that while most patterns showed the same correlation, a few circumvent the constraint. I also demonstrated that the ectopic combs were formed non-autonomously and that overexpression of \textit{dachshund} can transform certain aspects of the sex comb phenotype to resemble the transverse bristles to which they are homologous.
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Chapter I: The sex comb as a complex system

1.1 Evolutionary developmental biology and the quest to understand phenotypic disparity

The diversity of the world's living species has fascinated scientists and philosophers since ancient times. The wide range of biological forms that exist in nature, and the variation in their lifestyles and ecology, has long proved challenging for taxonomists (Ridley, 1993). Yet as knowledge of biology has accelerated in recent decades, we have increasingly come to appreciate the commonalities that unite extant species separated by hundreds of millions of years of evolutionary time. A similar mechanism of inheritance and a standard genetic code are hallmarks of life as we know it (Hartl and Jones 2001). Metazoans from different phyla with divergent body plans have co-opted the same genes for generating segmentation, appendages, hearts, eyes and other structures (Gerhart and Kirschner 1997). While celebrating the discovery of these widespread or even universal features of life, we come up against a fundamental paradox (Wilkins 2002). If life is so similar at a basic genetic level, why is the natural world so phenotypically diverse?

It is instructive to recall at this point that the multitude of forms in nature occupy only a minute fraction of the available morphospace. For all its diversity, phenotypic variation is highly circumscribed. Features such as the basic body plan, for example, are usually conserved within a phylum (Raff 1996). It is often unclear why specific aspects of a phenotype have remained unchanged during evolution. A sampling of fruit fly species in the large genus *Drosophila* shows that the ancestral pattern of eight longitudinal rows of
bristles on their second legs strongly predominates (Held 1979). The reason why this aspect of morphology has been highly conserved is not immediately obvious.

Historically, two broad categories of explanations have been put forward to explain both the variation of natural forms and their conservation. One line of reasoning, developed by Darwin and Wallace in the 19th century and synthesized with Mendelian genetics by neo-Darwinists in the 20th century (Ridley 1993), focuses on external pressures. This school of thought emphasizes natural selection as the primary factor shaping evolution. The rare adaptive mutant thrives and leaves numerous viable offspring, while phenotypes that are not observed in nature are presumed to be maladaptive; although they may appear from time to time, they are quickly weeded out by selection, and hence fail to leave a record in life's history. While most biologists agree that selection plays a role in evolution, a position popularized by Gould and Lewontin (1979) has also emphasized factors internal to the development of the organism, including developmental constraints (Maynard Smith, et al. 1985) and self-organizing principles (Kaufmann 1993). Proponents of this view argue that many traits may be more likely to evolve than others due to an inherent property of the developmental program. These ideas have had a strong influence on the field of evolutionary developmental biology (Raff 1996), which formed a major component of evolutionary theory in the 19th century and is currently undergoing a renaissance (Wilkins 2002).

Most of what is known about development and genetics is limited to a handful of model organisms (Gerhart and Kirschner 1997). While our understanding is still limited even in
these cases, there are aspects of their biology that have been analyzed thoroughly enough that we may be able to make intelligent predictions about the internal changes that would be required for such features to evolve. Unfortunately, because these model organisms are separated by vast evolutionary distances, explaining why they are different is close to impossible. A finding that homologous genes (such as the Hox cluster) are used for a similar process (axial patterning) in organisms as different as the mouse and fruit fly is striking precisely because of the phenotypic dissimilarity of these organisms; a discovery that they are regulated differently, however, could be attributed to a vast number of causes.

What is required to understand the evolution of diversity at an ontogenetic level is a system of closely-related species that show divergence in a specific trait within a general pattern of conservation. Analysis is easier if these species include a model organism, since knowledge of the development of the model species can be used as a starting point, and tested reagents and protocols can often be applied to the entire system. In the past decade, some of the most high-profile studies of the developmental origin of variation have examined sexually dimorphic characters, which are known to change rapidly in evolution. In *Drosophila*, the analysis of male-specific abdominal pigmentation (Kopp et al. 2000; Couderc et al. 2002; Jeong et al. 2006) and wing spots (Gompel et al. 2005; Prud’homme et al. 2006) have yielded insight about how changes in gene expression and regulation in evolution can lead to new phenotypes.
One of the most prominent sexually dimorphic characters in *D. melanogaster* is the sex comb (known in technical parlance as the *ctenidium*), an arrangement of pigmented, curved, thickened bristles at the distal tip of the most proximal tarsal segment (referred to as the basitarsus or TS1) of the forelegs of males, often used by Drosophilists to sex flies (Figure 1.1 A,B). To understand this trait, it is necessary to know the basics of the *chaetotaxy* (bristle patterning) of the basitarsus (Hannah 1958). Like the transverse rows (which are present on the tarsi of forelegs and hindlegs but absent from the second legs of the mesothorax), the bristles that make up the sex comb (commonly referred to as *teeth*) form a contiguous structure, but in its longitudinal orientation (parallel to the proximodistal axis of the leg) the comb is approximately perpendicular to the transverse rows. In female *D. melanogaster* fruit flies (Figure 1.1 C), in lieu of the sex comb, there is an increased number of transverse rows, which continue to the tip of the segment. Fruit fly legs also have longitudinal rows (numbered in Figure 1.1B), where the bristles are noncontiguous, and curvilinear chemosensory bristles, which are found at precise positions on the tarsus (Held 2002). (The other classes of bristles, all of which are mechanosensory, are somewhat more variable in number and position). In addition to the sex comb, there are other, more subtle differences between males and females in the chaetotaxy of the foreleg basitarsus. Male fruit flies have more chemosensory bristles than females between longitudinal rows 5 and 6 (five instead of three) (Held 2002), and these bristles and the distal bristle of longitudinal row 5 bristles are further from the tip of the basitarsus than their female counterparts.
Figure 1.1. The first tarsal segment (TS1) on male and female legs in *D. melanogaster*. Sex combs are indicated with arrows and examples of transverse rows with arrowheads. (A) Scanning electron micrograph (SEM) of the first tarsal segment of the foreleg of a *D. melanogaster* male. (B) Diagram of the bristle pattern of the same segment (based on own analysis as well as Tokanaga (1962) and Held (2002)), with the region between the dashed lines being equivalent to the SEM in A. The pattern consists of mechanosensory bristles of the non-contiguous longitudinal rows (dark grey circles with no outline, with rows numbered according to convention (Tokanaga 1962; Held 2002)), contiguous transverse row bristles (small light grey circles), curvilinear chemosensory bristles (empty circles with light outline), a central bristle (asterisk) and sex comb teeth (light and dark grey circles with black outline). Note that most of the sex comb is homologous to a transverse row (light grey) while the distal tip is homologous to longitudinal rows 6 and 7 (dark grey). The campaniform sensilla are indicated with open circles with a dark outline. P= posterior, V=ventral, A=anterior and D=dorsal. (C) First tarsal segment of a female foreleg. There is no sex comb, and transverse rows continue to the end of the segment. Anterior is to the right and distal is down in all panels. Scale bars: 20 µm

Figure 1.2. The eyeless-Dominant (*ey*D) mutant phenotype. A fused segment of a male foreleg, equivalent to the first two tarsal segments in the wild-type, is shown. The position of the sex combs is indicated with an arrow and the distal transverse row with an arrowhead. Anterior is to the right and distal is down. Scale bar: 20 µm
As discussed more fully in Chapter IV, the structure and placement of sex combs varies widely among *D. melanogaster* and its closest relatives (see Figure 4.1) (Bock and Wheeler 1972; Lemeunier et al. 1986), despite the strong conservation of several other features of the tarsal bristle pattern, such as the number of longitudinal rows on second legs (Held 1979). While this fact, together with the importance of *Drosophila* as a model organism, suggests that the sex comb could be a promising candidate for studies of the developmental origin of diversity, it is the complexity and dynamic nature of the comb's development that inspired me to focus on this system for my thesis. In the following sections I will first discuss what is known about the development of the sex comb, and then explain why it is a complex system and what that implies about its evolutionary potential.

### 1.2 Early studies of sex comb ontogeny and the recent resurgence of interest in the system

The classic studies of sex comb lineage and rotation were carried out in Curt Stern's lab. Stern had previously worked on the development of bristles on the head and notum (discussed in his review, Stern 1968). Like earlier researchers such as Sturtevant (Sturtevant and Schultz 1931) and Goldschmidt (1938), he was fascinated by the concept of patterns and how they are controlled genetically and developmentally. A pattern, he reasoned, requires a pre-existing co-ordinate system to specify where specific structures (such as bristles) are to be located (Stern 1954, 1968). He called this system a "pre-pattern", a relatively neutral term that did not imply a specific physical mechanism. The one characteristic that would identify a gene involved in setting up pre-patterns was that
mutating it would be expected to have a non-cell-autonomous effect, a consequence of 
the fact that the gene would have to be coordinating processes in different physical 
locations.

Surprisingly, mutations in \textit{achaete} and \textit{scute}, two classic genes known since the early 
twentieth century to have a role in the bristle patterning of the thorax and head, were 
found to act cell-autonomously (Stern 1954, 1968). Stern thus expanded his model to 
include the concept of "competence". He surmised that these genes could not be affecting 
the prepattern itself, since mutating a prepattern gene would be expected to affect a 
level coordinate system. Instead, the genes determined whether the tissue was 
competent to respond to the invariant prepattern. From this came the idea of "cryptic 
prepatterns", latent patterns would have no physical effects in the organism of interest but 
could be used evolutionarily if the tissue evolved a competence to respond to them.

However, Stern still hoped to discover prepattern genes, and his lab searched for these 
meticulously on the \textit{Drosophila} foreleg (Stern 1968). Sex combs were particularly 
fascinating. Since they were present in males but not in females, it was possible to study 
their formation using gynanders, flies with both male and female tissue. In the process of 
looking for prepattern genes, Stern's lab made classic discoveries about the development 
of the \textit{D. melanogaster} sex comb. By using the gynanders to infer cell lineage, Tokanaga 
(1962) was able to show that the sex comb rotated during development. The proximal and 
medial regions of the comb formed from a distal transverse row, and about two of the 
most distal teeth were of the same lineage as longitudinal rows 6 and 7. During
development, the comb rotates almost 90 degrees to assume an orientation that is approximately longitudinal. This finding explained why the sex comb teeth point in a direction that is at a right angle to the other mechanosensory bristles (posteriorly instead of distally).

Stern and Hannah (1950) had previously demonstrated, using gynanders, that male tissue, within a specific distal region of the first tarsal segment (TS1) of the foreleg, generated teeth even on an otherwise female fly. This showed, in Stern's terminology, that the distal region of TS1 was prepatterned to form combs in both males and females, but that only male tissue was competent to respond to the prepattern. Female tissue in that region, on the other hand, formed transverse row bristles, showing that it was not "competent" to respond to the prepattern. Tokanaga (1962) showed that in cases where there was a combination of male teeth and female bristles (as a result of the prepatterned area having both male and female tissue), the sex comb teeth tended to rotate while the transverse row bristles did not. The association was not perfect, and the extent of the rotation was greater for larger sex combs; when the comb consisted of only two or three teeth, there sometimes was no rotation at all.

In another study, the existence of a "cryptic prepattern" was found by examining an \textit{engrailed} (\textit{en}) mutant where an ectopic sex comb was generated (Tokanaga, 1960). However, since only \textit{en} mutant tissue formed ectopic teeth, \textit{en} could not be responsible for organizing the prepattern. Instead, the mutation was thought to give the tissue the
competence to respond to the prepattern which was present in the wild-type but remained latent.

Stern’s lab continued their search for a prepattern (nonautonomous) mutant, finally succeeding with a dominant allele of *eyeless* (*ey<sup>D</sup>*) (Figure 1.2). Mutant males of this genotype have 27 to 48 teeth (Stern and Tokunaga, 1967), far exceeding the range of 8-13 in most wild-type strains (Hannah 1958), arranged in a number of rows at various degrees of rotation, while females possessed an increased number of transverse rows on TS1. In both sexes, the TS1-TS2 joint was abolished. Stern and Tokunaga’s finding that non-*ey<sup>D</sup>* regions of mosaic basitarsi also differentiated sex comb teeth demonstrated, according to their chain of reasoning, that this was a gene that affected the prepattern itself rather than simply the competence to respond to the prepattern.

Later generations of researchers also used bristles to analyze patterning, but the studies that yielded the greatest advances in our knowledge of the genetic control of bristle distribution focused on the notum, which contains macrochaetes, bristles which are much larger (and hence easier to follow in development) than the bristles on the leg (Held 2002). In modern molecular terms, the "competence" that Stern referred to was found to reside in shared cis-regulatory regions of the classic genes controlling bristle development, the proneural *achaete-scute* complex (Gómez-Skarmeta et al. 1995). The prepattern itself is controlled by upstream regulators, including *pannier* (Garcia-Garcia 1999) and the genes of the Iroquois complex (Gómez-Skarmeta et al. 1996; Leyns et al. 1996; Ikmi et al. 2008). Mutations in *achaete* and *scute* generally act autonomously.
because they interfere with specific enhancers that respond to particular combinations of upstream genes, which only exist at certain positions on the notum. It was also found that *achaete* and *scute* were first expressed in proneural clusters of cells (Cubas et al. 1991; Skeath and Carroll 1991). Every cell in the cluster is initially competent to form a sensory organ precursor (SOP) mother cell, which later divides into the four cells that made up the bristle and a glial cell that undergoes apoptosis. Once one cell has been selected, it inhibits neighbouring cells from becoming SOPs (Cubas et al. 1991; Parks et al. 1997; Castro et al. 2005; Renaud and Simpson 2001). This results in a regular spacing of bristles.

This model of bristle development, inferred by analyzing the macrochaetes on the notum, forms a basis for our understanding of how bristle patterns form on other structures, including the leg. Certain important differences, however, have been alleged. For example, it has been claimed that the bristles on the leg form from 8 proneural "stripes" rather than clusters (Held 2002), and that these stripes are delimited by expression of the gene *hairy* (Orenic et al. 1993). The stripes are said to be equivalent to the 8 longitudinal rows. It is important to note that this model was based on studies of prepupal imaginal leg discs, up to 6 hours after pupariation (hr AP). The authors were unable to stain older legs, due to the formation of the pupal cuticle. Only recently has this limitation been overcome by using the green fluorescent protein (GFP) (Ward et al. 2003) and modifying the antibody staining protocol (Mirth and Akam 2002). The current thesis mostly focuses on events that occur after 15 hr AP, the period when most of the SOPs of most of the
mechanosensory bristles on the tarsus are selected and divide. Figure 2.3 suggests that in this time range proneural clusters (as on the notum) rather than stripes form on the tarsus.

One of the complexities with studying bristle patterning on the leg is that many of the same genes that affect bristle arrangements on this structure also control its global patterning, including the number and identity of segments and compartments. The patterning of legs and other appendages in *Drosophila* (i.e. how position is specified along the anteroposterior, dorsoventral and proximodistal axes) has been an area of intense research over the past two decades. Bristle arrangements are often used as an indicator of changes in the global patterning. For example, mutations of *wingless*, a gene involved in setting up the ventral compartment in leg discs, can lead to the "dorsalization" of this compartment (Couso et al. 1993). The effect at the level of the bristle pattern will be for the ventral compartment to acquire dorsal bristles (i.e. longitudinal rows instead of transverse ones on the first leg). It is clear that the unit of organization that is being considered here is the compartment.

An example of a critical gene in proximodistal tarsal patterning with mutants displaying a sex comb phenotype is *bric à brac (bab)* (Godt et al. 1993; Couderc et al. 2002). Hypomorphic mutations of *bab* lead to the homeotic transformation of distal tarsal segments to TS1, with a graded phenotype: stronger mutations transform TS2-TS4 to a TS1 identity, while weaker ones only transform TS2. The effect of this is to generate transverse rows on first and third legs in both sexes and sex combs at the distal region of each segment in males. (An independent effect of mutating *bab* is to abolish the joints;
this phenotype is graded but affects only the distal segments in weaker mutants and both proximal and distal ones in stronger mutants). A somewhat similar phenotype is seen in gain-of-function mutations of dachshund (dac) (Docquier et al. 1997). The level of organization at which these genes are acting appears to be the segment, with the altered bristle pattern a consequence. However, a complicating factor is that at least two of the proximodistal patterning genes, Distal-less (Dll) and dac, are also known to play a role in the nervous system and to act on sensory organs (Angelini and Kaufman 2005). They therefore affect bristles both directly and indirectly, at two levels of organization. I will attempt to differentiate these two functions of dac in Chapter III.

The model developed by Orenic et al. (1993) for the formation of longitudinal rows did not explain how leg-specific bristles, such as those that form the transverse rows, are controlled genetically. Later studies provided evidence that Hox genes, including Sex combs reduced (Scr) (expressed on the first legs) and Ultrabithorax (Ubx) (expressed in the third legs) play a role in setting up these structures (Shroff et al. 2007). Scr has also been the primary focus of two recent studies of the sex comb and its diversity. Barmina and Kopp (2007) found that in species with very large, prominent combs on more than one tarsal segment, the gene was expressed on all comb-bearing segments in males. Another study supported the role of this gene in sex comb evolution at the segmental level and attempted to show how it was regulated (Randsholt and Santamaria 2008).

The resurgence of interest in the sex comb has also included quantitative trait loci (QTL) comparisons of both intraspecific and interspecific variation in sex comb tooth number
(True et al. 1997; Nuzhdin and Reiwitch 2000; Tatsuta and Takano-Shimizu 2006; Graze et al. 2007). What is still lacking, however, is a careful analysis of the process of sex comb development at the cellular level. This is the primary focus of the present work. I will discuss below how I will address this issue in the thesis, after describing more specifically why the sex comb can be considered a complex system.

1.3 The sex comb as a complex system

Complexity is defined in terms of not only the number of individual components of a system but also the number of possible interactions (Simon 1962). If all components were independent, the study of systems with thousands of components (and hence millions of interactions) would be close to impossible. In both the living and non-living worlds, however, components are organized into modules (Simon 1973; Larsen 1997) which display emergent behavior. This reduces the difficulty of the problem by allowing the analyst to consider the interactions of the modules without having to be concerned with how the individual components within each module behave.

A basic biological module is the cell, which exhibits a number of emergent behaviors (e.g. growth, division, apoptosis) that can be described without having to constantly refer to the lower levels of organization (organelles, cytoskeleton) that make these behaviors possible (although a study of these components can of course yield important insight into understanding this emergent behavior). One of the more complex cellular behaviors, intercalation, which will be shown to play a crucial role in sex comb development in this thesis, involves interactions between multiple cells. Blakenship et al. (2006) have
suggested that this process, when it occurs within an epithelium, can best be described by referring to a higher level of organization, a multicellular configuration which they refer to as the *rosette*. Rosettes are transient structures that only exist during intercalation, and are identified on the basis of the fact at a specific stage during the process all of cells meet at a single point. (An example of a 4-cell rosette is given in Figure 2.11).

Bristles are composed of four cells. As mentioned in the previous section, they originate from a single cell, known as a sensory organ precursor (SOP), which divides into five; one of these descendents later undergoes apoptosis. During and after the SOP stage but before the shaft has elongated, these structures are usually referred to as "presumptive" or "developing bristles". Regardless of the terminology, a crucial question for the analysis of bristle pattern development is the extent to which such structures behave as modules. Do they remain together and behave as a unit in their interactions with neighboring cells, or do they separate transiently and later rejoin? While this is to our knowledge the first time that tarsal bristle patterns have been studied using live imaging at a level of resolution sufficient to identify individual bristles, previous live imaging of the notum has shown that on this structure developing bristle cells remain connected.

At a higher level of organization, a similar question may be asked about the developing transverse rows: Do the presumptive bristles within this row remain connected? In what sense do they behave as a unit? The rotating sex comb may be analyzed from the same perspective. At one extreme, it is possible that during this rotation the developing sex comb teeth are entirely independent and move separately. The highest-order structure, in
this case, would be the developing sex comb tooth. At the other extreme, all of the teeth may rotate simultaneously at the same rate, suggesting that the whole comb could be considered a single unit. An intermediate position may be inferred if it is found that different regions of the comb, each consisting of more than one bristle, rotate at approximately the same rate.

The organization of the components of a system into higher order modules implies that these components are linked or coupled, although the strength of this coupling may vary from one system to another. According to some models, increased linkage leads to reduced evolvability (Gerhart and Kirschner 1997), which would be manifested in nature by decreased variation. Raff (1996) used such a model to explain the phylotypic stage of embryonic development: He proposed that the commonalities seen in vertebrates and echinoderms midway through development may be due to increased linkage of modules at this phase in development, which at an earlier period were unlinked and at later stages become more autonomous. In other words, Raff postulated that tight coupling at the phylotypic stage was the underlying reason for a developmental constraint. Constraints, discussed at the beginning of this chapter, are the flip side of evolvability; reduced evolvability may be a consequence of constraints.

The diversity of sex combs in nature, and the ability to generate synthetic sex comb patterns in the lab through mutation and transgenic technology, are two of the reasons why the study of sex combs could yield insight into evolvability. The plasticity of the system, and the range of patterns that can be generated, can be analyzed. An
understanding of the dynamics of comb development may help in the assessment of the extent to which components of the system are coupled, and whether this coupling has an effect on evolvability.

The present work analyzes the sex comb as a complex system using a variety of approaches. The remainder of the thesis is divided into four chapters. In Chapter II, I focus on wild-type sex comb development in the model organism, *D. melanogaster*, where it is possible to follow the process through live imaging. In Chapter III, I examine the leg patterning gene *dachshund* (which has mutants that display ectopic sex comb phenotypes) in an attempt to determine how perturbing the expression of this gene affects the *D. melanogaster* sex comb pattern. I use the resulting phenotypes to address questions of evolvability and constraints. In Chapter IV, I analyze the process of comb development in 6 non-model species, with the aim of comparing and contrasting the mechanisms of comb development in nature. Finally, in the last chapter, I return to the questions addressed in this introduction in light of my results, and also suggest possibilities for further research.
Chapter 2: The development of the D. melanogaster sex comb

2.1 Introduction

As I have discussed in detail in Chapter I, the Drosophila sex comb is an example of a relatively rapidly-evolving trait with the potential to become a model system for research in evolutionary developmental biology. It is in a sense surprising, therefore, that while other sexually dimorphic traits in Drosophila, such as abdominal pigmentation (Kopp et al. 2000; Couderc et al. 2002; Jeong et al. 2006) and wing spots (Gompel et al., 2005; Prud'homme et al. 2006), have been subjected to intense comparative developmental and genetic analysis, leading to publications in high-profile journals, understanding the development of the sex comb has been much more difficult, and recent studies (Barmina and Kopp 2007; Randsholt and Sanatamaria 2008) leave many questions unanswered, including the cellular mechanisms involved in comb development. The most likely explanation for this is the complexity of the system, discussed in Chapter I. While it is true that other examples of dimorphism may be generated as an outcome of a complex genetic circuit, the downstream events – the variation in the intensity of a specific pigment in wing spots or the abdomen – is hardly in question. In contrast, in the case of the sex comb, and the foreleg tarsal bristle pattern in general, we have only a limited appreciation of the developmental process at a basic, mechanistic level, even for the model organism D. melanogaster. The diversity of sensory organs and the intricate patterns they form on the tarsus (recall Figure 1.1), as well as the complex interactions among bristles that will be discussed later in this chapter, makes the problem even more challenging.
Tokanaga's (1962) finding that the comb achieves its final position after a period of rotation (see Figure 1.1) left the cellular mechanism as an open question. Without an understanding of the developmental process, it is difficult to examine the roles of specific genes in comb rotation. We are even less justified in speculating on the comparative evolution of the comb before its development is well-understood in the species that we are most familiar with.

The purpose of this chapter, therefore, is to lay the groundwork for other studies by analyzing the development of the sex comb in the model organism at the cellular level. Future chapters will offer a genetic perspective (Chapter III, which focuses on the leg patterning gene \textit{dachshund}) and examine the process of comb development in other species (Chaper IV).

I will start by outlining five scenarios that could conceivably account for the rotation of the comb (Figure 2.1). These scenarios may be broadly divided into two classes. The first class (I in Figure 2.1) envisages the active migration of the cells that make up the comb. The cells could remain contiguous throughout this process (Scenario Ia), with the entire structure migrating. This would require movement from a leading edge, as the developing comb navigated its way between the neighboring cells until it reached its final position. Alternatively (Scenario Ib), the presumptive sex comb teeth could be noncontiguous throughout the rotation (or possibly go through repeated transient periods of noncontiguity), migrating individually to their ultimate destination, where the comb would assemble. The second class of scenarios is based on the sex comb responding to
I. Presumptive sex comb tooth cells actively migrate to their new positions,
   a) The structure as a whole migrates, following a leading edge.
   b) Individual tooth sensory organs migrate independently. In order to intercalate separately between intervening cells, they would have to be noncontiguous during this process, at least transiently.

II. Changes in the local cellular environment of the sex comb lead to the rotation.
   a) Increased cell proliferation on one side of the comb pushes it into a more longitudinal orientation.
   b) Changes in in the relative dimensions of neighbouring cells causes the comb to change its orientation.
   c) The re-arrangement of neighbouring cells could be responsible for the rotation.

Figure 2.1. How the sex comb rotates: alternative hypotheses.
changes in its local environment which shift its position. For example, local mitosis could push the structure into a longitudinal orientation (Iia). Alternatively, the proximodistal lengthening of the cells surrounding the comb (IIb), or the re-arrangement of these cells through convergent extension in the same direction (IIc), could change the sex comb’s angle relative to a fixed axis. It is also possible, of course, that the rotation of the comb involves a combination of these processes.

2.2 Materials and Methods

2.2.1 Fly strains

The *ubi-DEcad::GFP* and *sqh::GFP* lines were generated by Oda and Tsukita (2000) and Royou et al. (2004) respectively, and I obtained copies from the Ulrich Tepass lab. A stock with GFP expressed under the control of the promoter of *Na pump α subunit (Atpα)* was obtained from FlyTrap (stock #1792). Standard genetic techniques were used to generate the *Sca-GAL4 UAS-mCD8::GFP* line. Unless otherwise noted, all other stocks used in this chapter and in Chapter III were obtained from the Bloomington Drosophila Stock Center at Indiana University.

2.2.2 Collecting and staging Drosophila pupae

Flies were grown in vials or bottles on yeast\molasses\cornmeal medium. White prepupae were collected, rinsed in water, sexed by determining the presence or absence of the male gonad, and allowed to age on agar plates at 25°C.
2.2.3 Live confocal imaging

Using a ZEISS laser scanning confocal microscope (LSM 510), Z-stacks, each composed of a series of images of the first tarsal segment at different focal depths, were generated at regular time intervals. In time lapse movies using the Sca-GAL4 UAS-mCD8::GFP line, the images were a composite of the Argon 488 channel and transmitted light captures using Nomarski optics, and a Z-interval of 2 micrometres was used. Only the Argon 488 channel was used with the Atpα::GFP and ubi-DEcad::GFP stock; the Z-interval for these stacks was 3 micrometres. 3D reconstructions of each stack were generating using the "Projection" tool on the LSM Image Browser software. All of the images of developing bristles patterns in live pupae shown in this chapter are 3D projections.

One difficulty with using the ubi-DEcad::GFP stock for live imaging was that the GFP was relatively weak, requiring an Argon laser excitation of at least 20%, and sometimes considerably higher (40% to 60%). This led to two problems: background from the pupal case which obscured data from lower slices in the 3D projections and rapid photobleaching of the GFP. The first problem could be dealt with by either manually processing each slice using the LSM Browser software to delete the background (which was clearly distinguishable as a non-specific haze) from the images before generating the projection (Figure 2.2 A-Q), or alternatively increasing the transparency of the projections (by using a lower opacity setting on the LSM Image Browser 3D projection tool) (Figure 2.2 R,S). Since the effect of increasing the transparency setting was to make the projections much fainter (Figure 2.2 R), I chose to delete the background manually in the projections generated for this chapter. Comparing projections using both methods
Figure 2.2. Removal of background from images and the generation of 3D projections. The raw data from the confocal microscope consisted of stacks of about 30 images (with a Z-interval of 3 micrometres) from each time period. Not all of these images contained data from the leg; some of them were made up entirely of background from the pupal case (these were not included in 3D projections), while deeper images showed progressively fainter slices of the tarsus (I-O), until the GFP was no longer detectable. It was necessary to image both above and below the region of interest because the focus changed moderately over time. There were a number of images which contained both background from the puparium (clearly identifiable as a non-specific haze) and data from the leg. Before generating a 3D projection, this background (outlined in red) was manually deleted from the image using the LSM Image Browser software, because otherwise it would block valuable data from slices below it. The image in P shows the projection before removal of the background, while Q is the projection after background removal. An alternative to removing the background was to generate a partially transparent projection (R), which is inevitably faint. Increasing the brightness (S) partly remedies this problem. The perfect correspondence between bristle positions in Q,R,S (arrows) shows that removal of the background had no effect on the relevant data. Scale bars: 10 µm.
(Figure 2.2 Q and R) shows that the size, shape and position of the cells and presumptive bristles was not changed at all by deleting the background.

The second problem, rapid photobleaching, was confounded by the fact that the strength of the GFP varied considerably, and I would often have to collect 10 or more pupae the day before the time-lapse with the hope that at least one of them would be suitable for my purposes (i.e. produce high-quality images at a laser excitation of 40% or less, and preferably closer to 20%). On a few occasions, I experimented with raising the excitation approximately halfway through the time-lapse if the brightness of the images had weakened considerably. Increasing the brightness and contrast of the images post-acquisition provided modest improvement (compare Figure 2.2 R and S).

2.2.4 Tissue labeling

In addition to live imaging, fixed pupal legs were stained with antibodies against the products of proneural genes and other proteins. Pupae were pre-dissected on ice in phosphate buffered solution (PBS) under a Leica MZ12 dissecting scope. The purpose of this initial dissection was to remove the pupa from its case and break open the cuticle by cutting the abdomen. The pupae were then fixed in 4% formaldehyde solution in PBS for a period previously determined for each species and age (see Table 2.1), rinsed 2X in PBS, and further dissected to expose the legs. In most cases, the pupal cuticle was not entirely removed from the legs; instead the cuticle was punctured at the base of the legs to allow access to the Phalloidin or antibody. Since the legs were not fully exposed, I used a relatively high concentration of antibody (see below for details) and long washes
(at least 30 minutes each after the fixation and at least an hour each after the primary and secondary antibodies).

After the second dissection, the pupae were washed 3 times in PBT (PBS with 0.3% TritonX-100) at room temperature then blocked at 4°C in PBTBS (PBT + 0.1% bovine serum albumin + 2% goat serum) for at least 4 hours. They were incubated in the primary antibody overnight at 4°C, washed 4 times in PBT at room temperature, blocked again in PBTBS for at least 4 hours at 4°C then incubated overnight in the secondary antibody at 4°C. This was followed by 4 washes of at least an hour each in PBT. The pupae were then transferred to PBS and the legs were dissected and mounted in Molecular Probes™ Prolong® Gold (Invitrogen) antifade reagent.

I obtained the guinea pig polyclonal anti-Senseless (GP55) antibody (Nolo et. al 2000) from Hugo J. Bellen at the Howard Hughes Medical Institute in Houston, Texas, and used it at a dilution of 1:500. The following primary monoclonal antibodies were all obtained from the Developmental Studies Hybridoma Bank, and used at the dilutions indicated: anti-DE-cadherin (DCAD-2; 1:2) (Oda et al. 1994); anti-Cut (2B10; 1:4) (Blochlinger et al. 1990); and anti-Dachshund (mABDAC2-3; 1:2) (Mardon et al. 1994). Secondary antibodies were conjugated with Alexa-488 (Invitrogen), Cy3 and Cy5 (Jackson Laboratories) and used at a dilution of 1:400.

Alexa 488-phalloidin and Alexa 546-phallodin (Invitrogen) were used to label F-actin.
Table 2.1: Length of fixation (in 4% formaldehyde at room temperature) used for dissected pupae labeled with antibodies (*D. melanogaster*). Most researchers have focused on imaginal and prepupal discs, in which short fixations (5 to 10 minutes) are preferable. As the prepupal leg develops, the pupal cuticle is deposited and longer fixations are required, reaching 45 minutes at 6 hours AP. I found that it was very difficult to stain legs between 6 and 14 hours AP, and that very long fixations are required for legs aged 15 hours AP. As the leg sheds the pupal cuticle (after 18 hours AP), shorter fixations can be used.

<table>
<thead>
<tr>
<th>Age (hours AP)</th>
<th>Fixation Time (hours)</th>
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<tbody>
<tr>
<td>6 – 7</td>
<td>0:45</td>
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<tr>
<td>15</td>
<td>1:45 – 2:00</td>
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<td>16</td>
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<td>18</td>
<td>1:45 – 2:00</td>
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<tr>
<td>20 or 21</td>
<td>1:45</td>
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<tr>
<td>22-24</td>
<td>1:30 – 1:45</td>
</tr>
<tr>
<td>28 or older</td>
<td>1:15 – 1:30</td>
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2.3 Results

2.3.1 Analysis of sex comb development

Obtaining high resolution images of developing tarsal bristle patterns was initially challenging. The development of larval and prepupal imaginal leg discs, up to about 6 hours after pupariation (hr AP), was well understood (Held, 2002), and staining protocols for such tissues were readily available. Researchers studying later stages in leg development had been challenged by the pupal cuticle, as Orenic et al. (1993) had discovered in their unsuccessful attempts to follow achaete expression during this period. Mirth and Akam (2002), in their study of joint development, used extended fixation times (20 minutes to 24 hours, depending on the antibody) followed by manual removal of the cuticle. I found that with all of the antibodies I used, fixing the tissue in 4% formaldehyde for 1:15 to 2:00 hours was sufficient for staining pupae 15 hours or older, with the longer end of the range necessary for 15 to 18 hr AP pupae (see Table 2.1). Puncturing the pupal cuticle allowed both the fixative and the antibodies to penetrate, and the main impediment to staining pupal legs (both in my own work and, apparently, that of previous researchers) had been a lack of knowledge regarding the appropriate fixation times. In contrast to Mirth and Akam (2002), I did not find that it was necessary to remove the cuticle entirely, an impossibility in any case for pupae younger than 18 hours. The cuticle sometimes fell off of older legs, but high-resolution images could be obtained regardless of its presence or absence. Using high concentrations of the primary antibody improved the quality of the images (see Section 2.2.4 for details).
Live imaging of sex comb development had been attempted unsuccessfully previously (Held et al. 2004). The authors endeavored to keep the pupae alive after removal of the pupal case, and found a 95% lethality rate. Acting on advice from colleagues experienced in the live imaging of embryos, I found that there was no need to remove the pupal case if the pupa was placed in 1-3 microlitres of halocarbon oil on a coverslip, with immersion oil between the coverslip and the objective. I used the $sca\text{-}GAL4$ driver, together with $UAS\text{-}mCD8\text{::}GFP$, to drive expression of GFP in sensory organ precursor cells (Rozowski and Akam 2002). Using a laser scanning confocal microscope (LSM 510), I took a stack of 35 images every 10 minutes. Due to the 3D structure of the leg, bristles from a slice at a specific imaging depth were often out of focus hours or even minutes later. I therefore used a technique referred to in the literature as “4D imaging” (da Silva and Vincent 2007): With the LSM Image Browser tool, I generated a 3D projection from each stack (see Materials and Methods).

A series of 8 representative 3D projections from one of the time lapse movies, spanning 12 hours, is shown in Figure 2.3. In the early images, the sensory organ precursor (SOP) cells have not yet been selected from the diffuse proneural clusters. As development proceeds, it becomes easier to identify individual developing bristles. However, bristles do not form from all proneural clusters: The GFP fades in some clusters (red arrowheads) before an SOP is selected. Note that these clusters are homologous to distal bristles in longitudinal row 7 (Figure 1.1 B) which are present in females but not in males. This suggests that a prepattern for these bristles exists in males but is repressed.
Figure 2.3. Formation and rotation of the sex comb (continued on the next page). A ventral view of the TS1 of a Sca-GAL4 UAS-mCD8::GFP pupal foreleg. At 15 hr AP (A) GFP is present in the region where the longitudinal rows (right) and sex comb (lower left) will form but notably absent in the proximal posterior region where the transverse rows will develop (upper left). Note that at this stage and an hour later (B), GFP highlights the proneural clusters, large groups of cells from which the sensory organ precursors (SOPs) will be selected, and this accounts for its diffuse distribution. By 17 hr AP (C) the sex comb SOPs have been selected and have started to divide. The presumptive teeth are not yet contiguous. The comb at this stage is approximately, but not quite, transverse; note that even in the earliest stages in which sex comb SOP’s can be identified, the anterior teeth (white arrows) are slightly distal to the posterior teeth (red arrows). Outside of the sex comb, some proneural clusters (red arrowheads in A-C), homologous to the distal bristles of longitudinal row 7 (see Figure 1.1 B) which are present in females but not in males, are visible early in development but not later; the GFP gradually faces and is not longer detectable at 20 hr AP (D). At this stage, the comb has clearly started to rotate; the central bristle is visible (white arrowhead); the cells that will form the longitudinal rows are distinguishable, and we see the first indications of the distal transverse row. Cont. on the next page.
Continuation of Figure 2.3. As the sex comb continues to rotate (E-H) the posterior portion (filled red arrow) moves in an anterior direction relative to the distal TR (filled white arrow). (F) 22 hr AP. (G) 23 hr AP. (H) 27 hr AP. Distal is in the direction of the yellow block arrow; anterior is perpendicular to this arrow and to the right. Scale bars: 10 µm
The sex comb, when it is first distinguishable (Figure 2.3 B,C), is approximately transverse, though a slight angle is evident close to the centre of the comb between the posterior (red arrow) and anterior (white arrow) developing teeth. At this stage, the presumptive teeth appear disconnected; in later slices (e.g. Figure 2.3D), they appear closer together. As development proceeds (Figure 2.3E-H), the comb rotates and the posterior, proximal region of the structure moves in an anterior direction relative to the distal transverse row.

Time lapse videos using this system showed how the sex comb and other features of the bristle pattern formed and changed during time, but the cellular mechanisms underlying these processes were still unclear. What was required was a GFP construct that would outline the membranes of all cells while highlighting the bristles, allowing me to follow bristle movement in the context of the overall cellular environment. One strain that was useful for observing cell division in the tarsus before and just after the formation of the sex comb (15 – 18 hr AP; Figure 2.4) was a GFP expressed under the control of the promoter of Na pump α subunit (Atpα). For detailed and quantitative studies of sex comb development, however, I found it easier to identify the bristles and cell outlines with the ubi-DEcad::GFP stock (Oda and Tsukita, 2000). Most of the results described in this chapter were obtained from 4D imaging of pupae from this stock, in addition to fixed samples stained with antibodies.
2.3.2 The presumptive sex comb teeth, and the transverse row bristles of each row, are initially separate and join into contiguous rows through cell intercalation

The presence of contiguous rows of bristles on the tarsus (such as the transverse rows and the sex comb) is considered a derived feature (Held, 2002). The ancestral condition is thought to resemble the second leg, where the tarsal bristles are all noncontiguous. From the perspective of bristle development, the presence of adjoining bristles is surprising. This is because the bristle forms from a single sensory organ precursor (SOP) cell which is selected from a larger proneural cluster (Cubas et al. 1991; Skeath and Carroll 1991), and the selected cell then inhibits other members of the cluster from becoming SOPs through Notch signaling (Parks et al. 1997; Castro et al. 2005) and the secreted molecule Scabrous (Renaud and Simpson 2001). In legs, this results in an approximately regular spacing of bristles as seen, for example, in the longitudinal rows. Held (2002) speculated that during the development of rows of contiguous bristles, individual bristle precursors might form separately and come together at a later stage. The present study confirms this hypothesis.

After division of the SOP cells, the presumptive sex comb teeth and transverse row bristles are separated by at least 1 cell diameter (Figure 2.5 A). As two developing teeth come closer together (white arrows in Figure 2.5), an intervening cell (outlined in red) gradually moves out of their path. Cells just distal to the comb (the lower cells outlined in blue, white and yellow), maintain their contacts with the two presumptive teeth and are apically constricted.
Figure 2.4. Cell division in the early TS1. Prior to approximately 18 hr AP, cell division is observed in the distal TS1. A distal foreleg TS1 is shown at 15:15 hrs AP (A) and 15:30 hrs AP (B). Cells enlarged in the apical domain (arrows in A) are about to undergo division. The products of 3 of these divisions are shown in B (arrows). Distal is in the direction of the yellow block arrow; anterior is perpendicular to this arrow and to the right. Scale bars: 10 µm.

Figure 2.5. Formation of the sex comb and a distal transverse row from noncontiguous precursors. The panels show the distal first tarsal segment (TS1) of a live *ubi-DEcad::GFP* pupa imaged at 17 hours after pupariation (hr AP) (A), 18:30 hr AP (B), 21 hr AP (C) and 23:30 hr AP (D). As presumptive sex comb teeth come together (white arrows), a cell between them (outlined in red, bottom right) is gradually displaced. Note that the outlined cells just distal to the these teeth remain in the same position relative to the comb, but the area of their apical surfaces decreases as the teeth come together. A similar process occurs in the region of the distal transverse row (yellow arrows), but in this case numerous cells (examples are outlined) “escape” between the presumptive bristles while they are coming together, always moving proximally. This is a consequence of the convergent extension that leads to the rotation of the comb. Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
A similar process occurs in the region of two presumptive transverse row bristles (Figure 2.5, yellow arrows). However, in this case another phenomenon is also observed: cells distal to the transverse row (the cells close to the yellow arrows outlined in red, blue, white and yellow) move proximally (up), “escaping” between the bristles as they join together. The reason for this is explained in Sections 2.3.5 and 2.3.7.

What is particularly interesting about the process by which sex comb teeth (and transverse row bristles) come together is that they line up instead of clumping together en masse. This suggests that if homophilic attraction is responsible for forming the contacts, the attraction is between specific faces of the cells.

2.3.3 Contiguous sex comb teeth or transverse row bristles form a barrier to cell movement

In Figure 2.5 C, at 21 hr AP, the sex comb is contiguous, and it is still contiguous 2.5 hours later in Figure 2.5 D. Live imaging of 12 pupal basitarsi demonstrated that this is a general phenomenon: once the teeth have joined into a contiguous row, they do not separate, even transiently. A similar conclusion was reached for the transverse rows.

Strong enrichment of DE-cadherin on the adjoining faces of contiguous bristle cells (Figure 2.6 A,B; see also the adjoining faces of contiguous sex comb teeth in Figure 4.8 A on page 128) suggests the presence of an increased number of adherens junctions between them, which may prevent them from separating even if the structure as a whole moves relative to surrounding cells.
Since cell-rearrangement occurs in a 2-dimensional epithelium (albeit on a curved 3-dimensional surface), an immediate corollary, verified in the 12 confocal movies mentioned above, is that after the sex comb or a transverse row is contiguous, it is impossible for neighbouring cells to pass between the presumptive bristles within a row, as the proximal three outlined cells do in Figure 2.5 before the bristles that make up the transverse row have joined together. The contiguous sex comb and transverse rows apparently form a barrier to cell movement. This result rules out Scenario IB in Figure 2.1. If the presumptive sex comb teeth remain together, individual teeth cannot migrate between neighbouring cells independently of the rest of the comb.

2.3.4 The distribution of the protein products of genes involved in regulating planar polarity, in the region of the sex comb, supports the hypothesis that active cell rearrangement could be occurring during comb rotation

In addition to apical-basal polarity, epithelia often show planar polarity. Certain genes in the frizzled pathway are known to have a role in regulating this polarity (Adler et al. 1997), including Flamingo (Fmi), a transmembrane cadherin (Usui et al. 1999; Das 2002). In the developing sex comb, Fmi was found to be enriched on the distal face of the socket cells of both transverse rows and the sex comb (white arrows in Figure 2.6 C). Within 2 to 3 cell diameters of the sex comb on either side of the structure, the distribution of Flamingo is similar to its distribution on the comb (yellow arrows). Note that the proximal part of the comb in Figure 2.6 C shows a high degree of rotation but the distal region is only weakly rotated; the orientation of the neighbouring cells, both proximally and distally, mimics that of the sex comb teeth that are adjacent to them. This
Figure 2.6. The localization of DE-cadherin and Flamingo in the region of the developing sex comb.
(A-C) The TS1 of the foreleg of a 28 hr AP pupa stained with antibodies against DE-cadherin (green) and Flamingo (Fmi) (red). The merge is shown in panel A, while B and C show each channel individually. Note the high concentration of DE-cadherin on the adjoining faces of the presumptive sex comb teeth (white arrows in B) and, to a lesser extent, the transverse row bristles (white arrowheads). Fmi shows the inverse pattern: white arrowheads in C mark the adjoining faces of transverse row bristles, where the concentration of Fmi is reduced, and white arrows show the distal or posterior faces of both the distal transverse row and the sex comb, where the protein is enriched. Note also that cells adjacent to the comb, within 2-4 cell diameters, (yellow arrows) show a polarized expression of Flamingo that mimics that of the rotating SC. In contrast, disconnected bristles (i.e. those that do not belong to the comb or the transverse rows) do not show any consistent bias in Fmi distribution (yellow arrowheads). Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
suggests that the rotation of the comb bears a relation to the events that are occurring in its cellular neighbourhood, rather than being due to the independent movement of the sex comb teeth (as was hypothesized in Class I of the scenarios in Figure 2.1).

Other genes that show a polarized distribution in the embryo are known to have a role in cell intercalation during axis elongation, including myosin II (Bertet et al. 2004; Blankenship et al. 2006) and DE-cadherin (Blakenship et al. 2006). Using an sqh::GFP fusion stock (the sqh gene encodes myosin II regulatory light chain), Bertet et al. (2004) showed that during an intercalation event in the embryo involving 4 cells, myosin is strongly enriched at cell junctions that are reducing in size (such as the junction between cells c and d in Figure 2.10 D). A similar distribution is evident in multicellular rosettes (Blankenship et al. 2006), where the “unit of intercalation” is greater than four cells. A role for DE-cadherin in cell intercalation has also been suggested (Blankenship et al. 2006).

In addition to the enrichment of DE-cadherin on the adjoining faces of contiguous bristle socket cells, evident in Figure 2.6, at even earlier stages (Figure 2.7 A,B,E) the protein is enriched not only in the sex comb but also in the surrounding cells. The high concentration of DE-cadherin distal to the comb (white arrow in Figure 2.7 E) could be a consequence of the presence of a large number of cells that are constricted in their apical domains (recall Figure 2.5). However, the fact that it is also seen in places proximal to the comb where the cells' apical surface area is larger (yellow arrow in Figure 2.7 E) may indicate that there is cell rearrangement in this region. The strong enrichment of Sqh-GFP
Figure 2.7. The developing sex comb at 20 hr AP. An sqh::GFP pupa was stained with an antibody against DE-cadherin (red). (A-C) The sex comb (arrow) and a series of transverse rows (arrowheads) are visible. The sex comb teeth are contiguous, but many of the transverse row bristles are separated by one or more intervening cells. (D-F) A close-up of the region around the sex comb. The most proximal two teeth (arrows in D) show very little rotation relative to each other. The concentration of DE-cadherin is strong both proximal (yellow arrow) and distal (white arrow) to the comb. Note also the enrichment of Sqh::GFP on the proximal and distal faces of cells close to the sex comb (yellow arrows in F). This polarized pattern could be indicative of active cell rearrangement in this region. Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
on the proximal and distal faces of cells close to the comb in Figure 2.7F (yellow arrows), showing a polarized distribution, also supports this hypothesis, and suggests – if intercalation in the leg is similar to intercalation in the embryo – that these interfaces between proximal and distal cells (parallel to the anteroposterior axis) could be in the process of decreasing in length. This leads to the question of whether cell intercalation is in fact occurring in the region of the sex comb.

2.3.5 **Male-specific convergent extension leads to sex comb rotation**

During the early period of comb rotation (approximately 16 to 22 hr AP), a number of different cellular processes take place simultaneously. When observed through live imaging, cell division is apparent (Figure 2.4); this includes but is not limited to the sensory organ precursor cells that divide to form the four cells that make up the bristle and a glial cell (Gho et al. 1999; Reddy and Rodrigues 1999). The sex comb teeth and transverse row bristles converge to form contiguous rows, as described above. The shape of the leg changes dramatically, becoming thinner in the anteroposterior direction as a consequence of cell shape change (Fristrom and Fristrom 1993). It is very difficult, therefore, to analyze sex comb rotation during this period. I observed, however, that the rotation of the comb continues long after the first two of the three processes mentioned above stop. After 23 hr AP, I did not observe cell division in the live imaging videos of any of the 10 pupae I examined; in 80% of these pupae, the sex comb teeth were contiguous, and most of the teeth of the distal transverse row had also converged. I concluded that sex comb rotation is not dependent on either cell division or the convergence of teeth, and focused my quantitative analysis on the later part of the
rotation (looking specifically at the time period between 23 and 28 hr AP), in an effort to reduce extraneous variables to a minimum.

Comparing male and female legs during this time period proved insightful (Figure 2.8). Taking landmark bristles in the distal region of TS1 (Table 2.2; Figure 2.9 A,C), I found that the number of intervening cells in the proximodistal direction increased significantly in males, but not in females. Moreover, the proximodistal lengthening of the male tissue was specifically a feature of the distal part of the segment; the number of cells separating more proximal bristles did not change significantly (Figure 2.9 B). To arrive at a better understanding of what was occurring, I followed individual proximodistal rows of cells through time, using an analysis similar to that employed by Irvine and Wieschaus (1994) in studying embryonic germband extension (Figure 2.10). I found that, in a manner comparable to the convergent extension that occurs during germband elongation cells posterior and anterior to the outlined rows in Figure 2.10 intercalated between them, lengthening the rows. This suggested that a second process of intercalation, independent of the process that brings together the transverse row bristles and sex comb teeth, was acting to lengthen the distal TS1 in males.

The lengthening of the distal region of TS1 through cell intercalation occurs not only in the region of the sex comb, but also just proximal to another landmark, the distal ventral campaniform sensillum (red open arrow in Figure 2.8; yellow arrow in Figure 2.10), greatly increasing the number of cells between this sensory organ and the distal longitudinal row 5 bristle, with its neighboring chemosensory bristles (Figure 2.8 A,B;
Table 2.2: Proximodistal distances (in cell diameters) between landmark bristles at 2 time points in live pupae. Standard deviations are in brackets.

<table>
<thead>
<tr>
<th>Landmark bristles</th>
<th>N</th>
<th>Proximodistal distance (in cell diameters) at 23 hr AP (mean)</th>
<th>Proximodistal distance (in cell diameters) at 28 hr AP (mean)</th>
<th>P (paired Student’s t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior sex comb tooth (SCT) to distal row 7 bristle (males)</td>
<td>10</td>
<td>8.5 (1.18)</td>
<td>10.9 (1.73)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Campaniform sensillum to distal chemosensory bristle (males)</td>
<td>10</td>
<td>4.3 (1.34)</td>
<td>6.5 (1.78)</td>
<td>0.00001</td>
</tr>
<tr>
<td>Distal to medial row 7 bristles (males)</td>
<td>10</td>
<td>4.2 (1.62)</td>
<td>4.2 (1.23)</td>
<td>1</td>
</tr>
<tr>
<td>Distal chemosensory to row 6 bristle (males)</td>
<td>10</td>
<td>3.6 (0.97)</td>
<td>3.3 (1.49)</td>
<td>0.17</td>
</tr>
<tr>
<td>Distal row 7 bristles (females)</td>
<td>10</td>
<td>4 (0.67)</td>
<td>3.7 (0.48)</td>
<td>0.081</td>
</tr>
<tr>
<td>Sensillum to closest bristle (females)</td>
<td>10</td>
<td>2.8 (0.79)</td>
<td>2.8 (0.79)</td>
<td>Values unchanged</td>
</tr>
</tbody>
</table>
Figure 2.8. Convergent extension in the distal TS1. (See Figure 2.9 and the text for a quantitative analysis). (A,B) A ubi-DE-cad-GFP male foreleg TS1 at 23 hours AP (A) and 28 hours AP (B). Note that the number of cells in the proximodistal direction between two distal landmark sensory organs, the posterior sex comb tooth (white open arrow) and the distal longitudinal row 7 bristle (white filled arrow) has increased, as has the number of cells between the campaniform sensillum (red open arrow) and the closest chemosensory bristle (red filled arrow), but there is no evidence of extension in the more proximal region between the closed arrow (white or red) and the arrowhead of the same colour. The number of cells separating the posterior sex comb tooth and the campaniform sensillum have also decreased, indicative of convergence in this region. (C,D) A ubi-DE-cad-GFP female foreleg TS1 at 23 hr AP (A) and 28 hours AP (B). No extension or convergence is evident, even in the distal region of the segment. Scale bars: 10 µm.
Figure 2.9. Quantitative analysis of extension and sex comb tooth movement in the tarsus. (A-C) Comparison of the number of cells separating landmark bristles in the foreleg TS1 of 10 male (A,B) and female (C) pupae, at 23 hours AP and 28 hours AP. The bristles used in the analysis are labeled with red letters in the schematics in panels D and E. Convergent extension in these schematics is shown by two black arrows representing cell intercalation and a block arrow signifying tissue elongation; for a key to the other symbols, see the legend to Figure 1B. There is significant extension in the distal TS1 in males (A), but not in females (C). There is no evidence of extension in the medial male TS1 (B). (F) Comparative movement of two sex comb teeth (the most proximal bristle, anterior to (left of) the dotted line in panel D, and a bristle just posterior to (right of) this line) relative to the distal transverse row, in both the proximodistal and anteroposterior directions. Error bars show 95% confidence intervals. Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
Distal region of TS1 in the male foreleg

Mean cell number

Distal region of TS1 in the female foreleg

Mean cell number

Medial region of TS1 in the male foreleg

Mean cell number

Relative movement of SC teeth

Cell diameters
Figure 2.10. The rotation of the sex comb and its displacement relative to a distal transverse row. Images are shown from 3D projections of a time lapse movie of the distal TS1 (same approximate region and protocol as in Figure 2, different pupa) of a male ubi-DEcad::GFP pupal foreleg, at 23 hr AP (A), 25 hr AP (B) and 31 hr AP (C). Intercalation between cells (outlined in red and yellow and followed through time) leads to convergent extension. A proximal-distal line (dashed) shows the anterior extent of the contiguous distal TR (white arrowhead). The sex comb teeth in the region posterior to this line (left) move relative to the TR until the most posterior tooth (white arrow) is just anterior to the line. Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.

Figure 2.11. How cell intercalation can lead to rotation. Cells are labelled and followed from 26 hr AP (A), when cells b and c are in contact and the membrane (open arrow) is parallel to the anteroposterior direction through 26:20 hr AP (B) to 27:10 hr AP (C), when a and d are in contact and the membrane is parallel to the proximal-distal axis. Note the change in the angle between 2 of the sex comb teeth and the anteroposterior axis. The schematic in D interprets the intercalation in light of the model presented by Bertet et al. (2004) for germband extension. Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
Once again, the lengthening of tissue is limited to the distal tip of this segment; analysis of the relative positions of more proximal landmark bristles found no significant difference in the number of cells separating them in the proximal-distal direction. Indeed, even more noticeably than in the case of the sex comb, I found that during the period of 23 to 28 hr AP, the intercalation proximal to the sensillum is limited to the region adjacent to this organ, with most of the intercalation occurring between the sensillum and the cell just proximal to it (Figure 2.10).

One of the effects of the intercalation that takes place in the distal TS1 is to bring the sex comb and the distal ventral campaniform sensillum closer together. Between 23 and 28 hr AP, the number of cells separating the most anterior (and distal) sex comb tooth (red arrow in Figure 2.10) and the sensillum (yellow arrow in Figure 2.10) decreases from 5.075 to 2.65 (n=10; p<0.01, Wilcoxon signed-rank test). In the adult, the male distal ventral sensillum is adjacent to the most distal sex comb tooth (Figure 1.1 B).

The lengthening of tissue in one direction and its concomitant thinning in the perpendicular direction has been termed convergent extension, and is known to occur in a wide variety of systems in the animal kingdom (Wallingford et al. 2002). However, this is the first example that I am aware of in which convergent extension is sexually dimorphic.

To understand how convergent extension leads to comb rotation, it is important to recall the result highlighted in Section 2.3.3: Contiguous bristles, in the form of transverse rows
or sex combs, apparently form a barrier, isolating cells and their associated dynamics on either side of it. As cells proximal and anterior to the comb converge, those that move in a posterior direction can be replaced by more anterior cells, while those that move in an anterior direction can only be replaced by the cells that make up the sex comb (due to its function as a barrier), causing it to rotate (Figure 2.11). The overall decrease in the length of the interface between proximal and distal cells (parallel to the anteroposterior axis; white arrow in Figure 2.11 A) and the concomitant increase in the length of the interface between anterior and posterior cells (parallel to the proximodistal axis; white arrow in Figure 2.11 C), as is expected in convergent extension (Figure 2.11 D; Bertet et al. 2004; Blankenship et al. 2006), leads the sex comb to acquire a more longitudinal orientation.

It is still possible, however, that changes in cell length in one or more dimensions is contributing to the rotation of the sex comb. To determine whether cell rearrangement, cell length changes or both of these processes are responsible for the rotation, I carried out an analysis of these processes in the TS1 of 10 living forelegs (Figure 2.12), both proximal and distal to the comb. Due to changes in the shape of the leg, which flattens somewhat on the ventral surface during development, 3D distances were measured directly from the raw data using the LSM Image Browser "Orthogonal" tool (projections tended to underestimate the true distance). The results (Figure 2.12) show that a highly significant (p < 0.01, paired Student's t-test) increase in cell number in the proximodistal direction, and a decrease in cell number in the anteroposterior direction at the same level of significance, are responsible for the rotation (as would be expected due to convergent extension). Changes in cell length, on the other hand, were not significant.
Figure 2.12. Cell rearrangement, not a change in cell length, is responsible for the rotation of the sex comb. (A-B) Images of a sex comb at 23 hr AP (A) and 28 hr AP (B), with yellow lines drawn parallel to the anteroposterior axis (A-P) and red lines parallel to the proximodistal (P-D) axis. Measurements were taken for right legs on 10 pupae, comparing the number of cells separating the first and eighth SC tooth (counting from the distal end of the comb) in each of these directions, both proximal and distal to the comb (C and D). 3D measurements were also taken to calculate the mean length of these cells (E and F) in micrometres. Error bars show 95% confidence intervals. Scale bars: 10 µm
2.3.6 The significance of the sinusoidal shape of the sex comb during development

The transient sinusoidal shape of the comb during development was noted by Held et al. (2004), though he found it to be inconsistent and did not attribute any significance to it. My own analysis of 10 tarsi between 23 and 28 hr AP showed that in at least 8 of the 10 cases the comb went through a sinusoidal phase (it is possible, of course, that this also occurred in the other cases, though not during the period that I imaged). When the angle between individual teeth and the anteroposterior axis in the 3D projections is measured and plotted on a graph, (Figure 2.13), at 23 hrs AP (blue plot) the angle between medial teeth (centre) is considerably greater than that between either the proximal teeth (left) or the distal ones (right), as would be expected for a sinusoidally shaped comb. This pattern is still evident at 28 hrs AP (red plot), although at this stage the angle between adjoining proximal teeth is closer to the angle between the medial teeth (see also the shape of the comb in the 28 hr AP leg in Figure 2.12B, where the proximal medial regions of the comb are at approximately the same angle, while the distal comb is at much less of an angle).

This sinusoidal shape is a consequence of the process discussed in the last section. Note that we observed a slight initial angle (Figure 2.3 B,C) between 2 or 3 posterior distal teeth (homologous to a longitudinal row) and the presumptive teeth just anterior to them (homologous to a distal transverse row). As already explained using Figure 2.11, the effect of convergent extension in this context is to accentuate an angle that is already present. The result is that the medial part of the sex comb rotates before the remainder of
Figure 2.13. Rotation of the sex comb between 23 and 28 hr AP. Plots of the angle between adjacent sex comb teeth and the anteroposterior axis at 23 hr AP (blue plot) and 28 hr AP (red plot) for forelegs from 10 developing pupae. All legs had between 9 and 11 sex comb teeth. In legs with 9 teeth, T1 is the most proximal tooth and T9 is the most distal. In legs with 10 teeth, the most distal tooth was not considered, and in legs with 11 teeth, both the most proximal and most distal tooth were dropped from the analysis. Error bars show 95% confidence intervals.
the structure. The posterior \ proximal and anterior \ distal regions of the comb, which are approximately transverse at 16 – 17 hr AP, do not rotate at first. Indeed, the distal region does not complete its rotation until much later (see Figure 2.14, discussed below).

The strong increase in the angle between the proximal teeth in the period from 23 to 28 hrs AP, however, particularly the first two, shown in Figure 2.13, is surprising, in light of the much slower rotation of the distal teeth. This phenomenon is analyzed further in the next section.

2.3.7 Proximal sex comb teeth move in an anterior direction relative to the distal transverse row

It is possible to imagine the sex comb rotating while pivoted on its medial tooth. According to this model, the average tooth position along the anteroposterior axis would remain constant, with the variance decreasing. This, however, is not what we observe. Instead, the posterior region of the sex comb moves in an anterior direction in relation to the transverse rows (compare the positions of the red and white arrows in Figure 2.3 E-H; in Figure 2.10, follow the movement of the white arrow relative to the dashed line).

This result may be explained by noting that in addition to the sex comb, a second barrier is involved in the rotation. As discussed above, while the bristles that form the distal transverse row are converging into a contiguous formation, cells in the region between the transverse row and sex comb, which are undergoing convergent extension, rapidly escape between the converging bristles of the transverse row, always moving proximally
(outlined upper cells in Figure 2.5). Once the row is contiguous, however, these cells are caught in a bottleneck between two barriers (region left of the white dashed line in Figure 2.9 D and Figure 2.10 A-C), and must move around the transverse row. This leads to both a rapid rotation of the posterior proximal teeth (Figure 2.13, left region of both plots) as the cells proximal to this part of the comb escape the bottleneck, and to the anterior movement of the proximal comb relative to the transverse row. I compared the movement of the most posterior proximal tooth (labeled G in Figure 2.9 D), which is always within the bottleneck at 23 hr AP, with a tooth just outside the bottleneck at this stage (labeled H in Figure 2.9 D), over a five-hour period (23 to 28 hr AP). I found that the mean anterior movement of the former, relative to the distal transverse row, was 4 cell diameters, while the tooth outside the bottleneck moved only 1.25 cell diameters (Table 2.3; Figure 2.9 F). The most posterior tooth was also on average 2.55 cells closer to the distal transverse row in the proximodistal direction at 28 hr AP, in contrast to the tooth outside the bottleneck which actually moved slightly distally (0.5 cell diameters). This movement of the proximal teeth relative to this transverse row stops when the comb has moved beyond it and there are no longer any cells between the two structures along the proximodistal axis (Figure 2.10 C, Figure 2.14 A-D). Note that the final position of the most posterior sex comb tooth, just anterior to that of the most anterior contiguous transverse row bristle (Figure 2.14 D), is also evident in the chaetotaxy of the adult TS1 (Figure 1.1B). The comb, however, is not anterior to noncontiguous transverse row-like bristles (red arrowhead in Figure 2.14 A-D), which sometimes form anterior to the main row (and may be considered homologous to longitudinal row 7; see Figure 1.1B).
Table 2.3: Relative movement of sex comb teeth within and outside the bottleneck at two time points in live male pupae. Standard deviations are in brackets.

<table>
<thead>
<tr>
<th>Direction of movement</th>
<th>N</th>
<th>Distance (in cell diameters) at 23 hr AP (mean)</th>
<th>Distance (in cell diameters) at 28 hr AP</th>
<th>P (paired Student's t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximodistal</td>
<td>10</td>
<td>2.55 (1.07)</td>
<td>-0.55 (1.64)</td>
<td>0.002</td>
</tr>
<tr>
<td>Anteroposterior</td>
<td>10</td>
<td>4 (1.35)</td>
<td>1.25 (0.86)</td>
<td>0.0004</td>
</tr>
</tbody>
</table>
2.3.8 After 30 hr AP, most of the rotation occurs distally

The sinusoidal shape of the sex comb at approximately 23 hr AP, with 3 regions at different degrees of rotation, is replaced by a bimodal angular distribution at 28 – 30 hr AP, with a strongly rotated proximal half and a much weaker rotation of the distal half (see the red plot in Figure 2.13, and note also the shape of the comb in Figures 2.8 B and Figure 2.12 B). After the proximal region has moved beyond the distal transverse row, most of the rotation occurs in the distal part of the structure (Figure 2.14 A-D; compare also the plot in 2.14 E with the red plot in Figure 2.13). This is also the period when chemosensory bristle neuronal dendrites (red arrows in Figure 2.14 B,C) extend into the bristle shafts (not visible in ubi-DE-cad::GFP). In all other bristles on the leg, the neuron extends only to the base of the shaft (Held 2002).
Figure 2.14. The later phase of sex comb rotation. Most of the rotation after 30 hr AP occurs in the distal region of the comb (yellow arrows in A-D, angle with anteroposterior axis outlined in yellow). Note also that the position of the proximal teeth (white arrows in A-D, angle with anteroposterior axis outlined in white) relative to the distal TR (white arrowheads) does not change. The comb is anterior to all contiguous transverse bristles, but not the non-contiguous bristles (red arrowheads). Red arrows in B and C show the elongating chemosensory bristles. (A) 31 hours AP (same image as in Figure 2.10 C). (B) 35 hours AP. (C) 42 hours AP. (D) 52 hr AP (different pupa). (E) The mean angles between successive sex comb teeth and the anteroposterior axis in forelegs from 10 pupae ranging in age from 51 to 54 hr AP. Error bars show 95% confidence intervals. The numbering of the teeth was similar to Figure 2.13. In contrast to both the 23 hr AP and 28 hr AP plots in that figure, however, the angles formed by successive distal teeth (right) are at least equal to the angles subtended by proximal teeth (left), showing that most of the later rotation occurs distally. In panels A-C, anterior is to the right and distal is down. Scale bars: 10 µm.
2.4 Discussion

The rotation of the *Drosophila* sex comb is an example of a process that has long been inferred from indirect evidence but remained poorly understood. This is in a sense paradoxical, given the widespread use of *Drosophila* as a model organism and the fact that combs are utilized for sexing purposes on a daily basis by all Drosophilists. The curvature of the leg, which required the use of 4D confocal microscopy in my analysis, the background from the pupal case, the length of the process and the challenge that the pupal cuticle poses for antibody staining may explain why successful research on comb development has been limited. Having overcome these difficulties, however, I now believe that the sex comb is an excellent model system for analyzing cell behaviour.

Groups of interacting cells can behave as a unit and display emergent properties. This has recently been demonstrated in the embryo: During germ band extension, cells behave collectively as higher-order modules referred to as "rosettes" (Blankenship et al., 2006). In the developing tarsus, additional higher-order structures are formed, including configurations of bristles such as the transverse rows and the sex comb. The components of these structures (the bristles) can arguably be described as "weakly coupled" (as defined in Chapter I) because the developing sex comb teeth, for example, can rotate at different rates (Figures 2.13 and 2.14). However, they are still units in the sense that they do not physically separate once they are contiguous. As a consequence of this, they apparently display an emergent property, acting as barriers to cell movement (Section 2.3.3), which has an important influence on later development (Section 2.3.5 and 2.3.7).
Previous research (Tokanaga 1962; Held et al., 2004), which demonstrated that the comb rotated without specifying a mechanism, left us with numerous possible hypotheses to account for it: the direct migration of presumptive sex comb cells to new positions, local cell proliferation pushing presumptive sex comb teeth into a new orientation, global changes in leg shape. While all of these processes occur during the early period of the rotation (approximately 16 to 23 hr AP), we were able to rule out the first two by observing that the comb continued to rotate after those processes had ceased. This simplified our analysis considerably, and demonstrates that the system is experimentally tractable. However, the more complex earlier phase may be very interesting to researchers who want to study how multiple cellular processes work together in concert to shape form. It was recently discovered that germ band extension, long known to be a consequence of cell intercalation in the anterior region of the embryo (Irvine and Wieschaus 1994), is driven by oriented cell division in the posterior region (da Silva and Vincent 2007).

2.4.1 The evidence suggests that the sex comb, and possibly the anterior distal campaniformia sensillum, may induce convergent extension in more proximal cells

As I described in Section 2.3.4, recent research has linked genes involved in regulating planar cell polarity and genes encoding myosin II (Bertet et al. 2004; Blankenship et al. 2006) and DE-cadherin (Blankenship et al. 2006), to convergent extension. I have shown that during sex comb rotation, the planar polarity of cells within a few diameters of the sex comb differs strongly from more distant ones (Figures 2.6 and 2.7). The ability of cells to alter the planar polarity of their neighbours has been demonstrated in the non-
autonomous effects of *frizzled* mutations (Zheng et al. 1995). A variety of theoretical models seek to account for this behaviour (e.g. Amonlirdviman et al. 2005). Flamingo, whose distribution I examined in Figure 2.6 (see Section 2.3.4), is involved specifically in the regulation of planar polarity downstream of Frizzled.

When the results of our study are combined with the fact that ectopic combs can rotate (Chapter IV) and previous evidence showing that even a small number of sex comb teeth (as few as 5) can rotate almost 90° (Tokanaga 1962), the implication is that the teeth themselves are inducing more proximal cells to alter their planar polarity and undergo convergent extension. It would also appear that the distal anterior campaniform sensillum is having a similar effect on the cells just proximal to it. Precisely why these two structures should induce this behaviour in their cellular neighbourhood, and the signaling molecules that are involved, remains to be determined.

### 2.4.2 The final position of the sex comb may be developmentally constrained

The anteroposterior displacement of sex comb teeth relative to transverse row bristles is apparent in the drawings foreleg male tarsi by Bock and Wheeler (1972) in their guide to the *D. melanogaster* species group, as well as other guides (e.g. Lemeunier et al. 1986), but these sources do not mention or discuss this phenotype explicitly. The present study provides an explanation of this phenotype in terms of the bottleneck that is set up when convergent extension occurs between the posterior sex comb teeth and the distal transverse row. In Chapter III I test this hypothesis experimentally by generating ectopic combs and determining whether they are also displaced relative to the transverse rows.
However, at this point it is instructive to note that the data of previous researchers lend support to it. Tokanaga (1962) generated gynandric tarsi in which some of the sex comb teeth in males were replaced by female bristles. Although she does not comment on it, in all 13 of the figure panels she displays (Figs. 6 and 7 in Tokanaga 1962) the male sex comb teeth (which vary in number from 1 to 6) are just anterior to the most distal transverse row.

As we show in Chapter IV (Figure 4.18), for species from a variety of subgroups within this group, as well as those in the *D. obscura* species group (the other well-studied group within the *Sophophora* subgenus where males have sex combs), the anteroposterior comb position on TS1 can be reliably predicted if a transverse row is just proximal to it, as it can in *D. melanogaster* (Figure 2.1). Without a knowledge of the cell dynamics responsible for sex comb rotation and displacement, however, this positional bias, consistent across species, is not likely to be noticed. While there has been considerable discussion and debate regarding the existence of developmental bias or (in its stronger form), developmental constraints (e.g. Alberch 1982; Raff 1996; Brakefield 2006), most of the alleged examples have been based on the absence of evidence, without a strong developmental argument in support of why such a constraint should exist. Tests of such claims have tended to refute them. The absence of biramous appendages in insects, for example, was alleged to be the consequence of a developmental constraint; Dworkin et al. (2001) showed that biramous *Drosophila* antennae-leg combinations could be created relatively easily in insects, using a combination of only 2 mutations. What distinguishes the present study is that we have evidence for a developmental bias which we are not
likely to have predicted *a priori* from the observation of adult phenotypes, but which appears to be the logical outcome of the developmental process that generates the sex comb’s position and orientation. Chapters III and IV will shed further light on this issue.
Chapter 3

The generation of ectopic sex combs by manipulating expression of the leg patterning gene *dachshund*

3.1 Introduction

In the previous chapter, I analyzed the formation and rotation of the *D. melanogaster* sex comb from a developmental and cellular perspective. Historically, however, one of the prime motivations for studying the sex comb has been genetic. There are a large number of genes with mutants that display a sex comb phenotype (for a partial list of 19, see Nuzhdin and Reiwitch 2000), and both QTL analysis (Nuzhdin and Reiwitch 2000; Graze et al. 2007) and microarrays (Barmina et al. 2005; Graze et al. 2007) have been used in an attempt to identify additional candidates. Candidate genes include the Hox genes *Sex combs reduced (Scr)* (Barmina and Kopp 2007; Randsholdt and Santamaria 2008) and *Antennapedia (Antp)* (Duncan and Kaufman 1975), *Polycomb* group genes (Nuzhdin and Reiwitch 2000), genes in the sex determination pathway, including *Doublesex (Dsx)* (Jursnich and Burtis 1993), and a number of leg segmentation genes, including *bric à brac (bab)* (Godt et al. 1993; Couderc et al. 2002) and *dachshund (dac)* (Docquier et al. 1997; Randsholt and Santamaria 2008).

The sheer number and diversity of sex comb mutant phenotypes, however, combined with limited knowledge of sex comb development, has made interpretation of the precise roles of these genes difficult. It is not always clear whether a mutation has a cell-autonomous role in sex comb specification or whether the altered phenotype is an indirect by-product of global leg or segmental patterning. Specific markers for sex combs have
been hard to come by (Tatsuta and Takano-Shimizu 2006). The discussion of complexity in Chapter I, where modules or units of organization were described, is relevant in this context. A gene such as *bric à brac* (*bab*), for example, generates ectopic sex combs through a homeotic transformation of the bristle pattern of distal tarsal segments to TS1 (Godt et al. 1993), which then acquire sex combs as a consequence of their new identity. The relevant module, in this case, is the segment. Alternatively, a gene could be acting directly at the level of the bristle.

Although it has been known for over 45 years that the majority of the sex comb is homologous to a distal transverse row (Tokanaga 1962), an important unanswered question is how sex comb teeth, which are pigmented, thickened and rounded at the tips, are differentiated genetically from transverse row bristles, which lack pigmentation (see Figure 3.6C) and are both thin and pencil shaped (Figure 3.1). While it has been possible to determine that the product of a specific gene, *Pox neuro*, is necessary and sufficient to transform mechanosensory bristles into chemosensory ones (Nottebohm et al. 1992), the same cannot be said for the sex comb\transverse row dichotomy.

In the present study, I analyzed the effect on the bristle pattern of the *Drosophila* tarsus of both repressing and ectopically expressing the transcription factor *dachshund* (*dac*). A report of gain-of-function *dac* mutants with ectopic sex combs had suggested that this gene could have a role in shaping sex comb morphology (Docquier et al. 1997). When I analyzed the distribution of Dac, however, I found that the protein, while near-ubiquitous on TS1, was very specifically reduced in the sex comb (Figure 3.2; described in Section
Figure 3.1: Types of bristles on the Drosophila tarsus. The tip of the basitarsus is shown for a D. melanogaster male (A) and a D. nikananu female (B) (similar to a D. melanogaster female; compare with Figure 3.4 D). The sex comb, the most conspicuous feature on the male tarsus, consists of thick elongated bristles with a rounded tip (red arrow). Most transverse row bristles are pencil-shaped (yellow arrows), although the most anterior transverse row bristles (yellow arrowheads) are sometimes closer in shape to the needle-like longitudinal row bristles (white arrows). Curvilinear chemosensory bristles (red arrowheads) form easily identifiable landmarks. Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
3.3.1). This motivated me to seek to determine whether Dac might actually repress sex combs.

In addition to its function in retinal determination (Tavsanli et al. 2004), dac plays a crucial role in the development of the Drosophila medial leg, from the femur to the third tarsal segment (Mardon et al. 1994). In dac mutants, these segments are fused, shortened, and thickened. As a marker for the medial leg, dac expression is conserved in a great diversity of insects (for a review, see Kaufmann 2005). The role of dac in leg patterning was recently investigated in the milkweed bug Oncopeltus fasciatus (Angelini and Kaufman 2004). The injection of dac RNAi into O. fasciatus embryos led to the loss of the tibia, suggesting that its function (in addition to its expression pattern) is conserved between hemimetabolous and holometabolous insects.

Consistent with a function in neuronal development, dac is also expressed in the developing nervous system (Mardon et al. 1994). The expression of dac in sensory organs is conserved in other insects such as beetles (Prpic et al. 2001; Angelini and Kaufman 2005), and even in the myriapod Glomeris marginata (Prpic and Tautz 2003). The gene therefore has a dual function in the leg, in both overall patterning of the medial region and sensory organ development. It is not known which of these functions evolved first (Angelini and Kaufmann 2005).

A possible role for dac in sex comb diversity was surmised in light of the ability of gain-of-function dac mutations to generate sex combs on TS2 (Docquier et al. 1997). The
mutations were referred to as montium-like (Mil) because of the presence of a sex comb on each of the first two tarsal segments is typical in this species subgroup. This suggested that dac might actively promote sex comb development, and such a conclusion was reached in a recent study (Randsholt et al. 2008). Another study, however, found that dac expression on TS2 did not differ between D. melanogaster, which has a sex comb only on TS1, and D. ficusphila, which has a pronounced sex comb on each of the first two tarsal segments (Barmina and Kopp 2007).

The primary focus of these studies was to discover how the number of comb-bearing segments in a fruit fly species is determined genetically. The motivation for the present study, however, was different. Instead of looking at the segment as a module and focusing on the number of segments with sex combs, I was interested in the diversity of patterns that could be generated on a single segment by modifying the expression of a single gene (dac) in D. melanogaster. The relevant module, in this case, was the bristle. I sought to determine how, on the first tarsal segment, the up-regulation, or repression of dac expression, affected the sex comb pattern, and if this effect was cell-autonomous. I also analyzed the range of phenotypes that could be synthesized by generating dac mitotic clones, and the extent to which these phenotypes were consistent with the constraints of sex comb morphology that we observe in nature. I was interested in both the evolvability of sex combs (see the discussion in Section 1.3) and in observing if there was a developmental bias (discussed below) in the position in which they were generated.
I proposed in the previous chapter that the final position of the sex comb in *D. melanogaster* can be predicted if one knows the position of the transverse row just proximal to it. In the next chapter, I will extend this finding to other species, showing that it applies to flies from diverse taxa, despite the vast variation in sex comb morphology among these fruit flies. The discovery of a pattern in nature leads to the question of whether this pattern is an outcome of an internal developmental bias, or whether it is a consequence of an external force such as natural selection. I showed in the last chapter that the comb does not start out in its final position, and explained that its movement to a location anterior to the distal transverse row can be explained as an outcome of basic cellular processes within a physically constrained environment. It is possible, however, that it is relatively easy to generate adult combs in other positions using mutants or transgenics. Testing possible developmental constraints through genetic manipulation may be achieved through a "Designer Organism Approach" (Dworkin et al. 2001). Since sex comb patterns are remarkably diverse both in nature and in lab-generated synthetic genetic backgrounds, they are an ideal system for comparing natural variation to what can be produced using the genetic tools currently available. This allows us to test the evolvability and plasticity of the system before natural selection has weeded out the maladaptive phenotypes.

I show in this chapter that when *dac* is ectopically expressed in sex comb teeth it promotes two aspects of the transverse-row phenotype. While additional teeth may be generated ectopically by down-regulating the gene, these are not formed cell-autonomously. Most of the resulting legs adhere to the previously described constraint on
sex comb position observed in nature, and those that do not, exhibit an unusual, possibly maladaptive, phenotype.

3.2 Materials and Methods

3.2.1 Ectopic expression of dac

The TARGET system was used to ectopically express dac (McGuire et al. 2003) during specific periods in development. This was necessary because ectopic expression using a UAS-dac construct (a gift from Douglas Allen (Shen and Mardon 1997)) and a variety of GAL4 drivers (including Sca-GAL4 and rn-GAL4) was found to be lethal by the early pupal stage (the UAS-GAL4 system is discussed in Phelps et al. 1998). In order to repress the GAL4 in embryonic and early larval stages, a tub-GAL80ts construct was used. The temperature-sensitive GAL80 binds to GAL4 at the permissive temperature and prevents it from binding to the UAS (McGuire et al. 2003). The crosses (described in detail in the Results section) were carried out at 19°C, the permissive temperature for the GAL80, and at a later stage the progeny were moved to a 32°C water bath. At this temperature, the GAL80 is inactive and the GAL4 can bind to the UAS.

3.2.2 Mitotic clones

The FLP/FRT system was used to generate dac mutant clones through mitotic recombination (Thiodosiou and Xu 1998). I used both a hypomorphic allele (dacE462) (donated to the Bloomington Drosophila Stock Center by Jessica Treisman) and a null allele (dac4) (a gift from Graeme Mardon) together with the FRT 40A::GFP insertion on the second chromosome and a FLP recombinase insertion with a heat-shock promoter on the X chromosome. Larvae were heat-shocked for 1 hour at 37°C at 72 hours after hatching to induce mitotic recombination. Clones were marked by the absence of GFP.
**Tissue labeling** (antibody and Phalloidin staining) and **scanning electron microscopy** were carried out as described in Chapter II.

### 3.3 Results

#### 3.3.1 The expression pattern of *dac* in developing tarsal bristles varies according to the bristle type

On the scale of the developing leg as a whole, the expression of *dac* is generally correlated with its function as a patterning gene of the medial region of the appendage. It has been reported as absent from both the proximal portion of the leg (the coxa and trochanter) and the distal region (tarsal segments 3-5) (Mardon et al. 1994; Tetsuya 2004). Significantly, these regions of the leg are unaffected in *dac* mutants. One apparent discrepancy between the expression pattern and the phenotype, however, is that Dac has been reported to be absent in the third tarsal segment (TS3) (Kojima 2004), even though in mutants this segment is clearly affected (Mardon et al. 1994).

At a similar level of analysis, the *dac* expression pattern has been described as identical in *D. melanogaster* and *D. ficusphila* (Barmina and Kopp 2007), despite the strikingly different sex combs in these two species (compare Figure 1.1 A with Figure 4.7 A,B). For the authors of this paper, the relevant module or unit of organization (see my discussion in Section 1.3) was the segment: they were focusing on how the number of comb-bearing segments is regulated. Given the sex comb phenotype of *dac* mutants (Docquier et al. 1997), however, and the role of *dac* in sensory organ development, I was interested in
studying its distribution at a finer level of analysis, and determining how it was expressed in specific bristles.

In male pupae at 30 hours after pupariation (hr AP), when the developing bristle pattern bears a strong resemblance to the adult chaetotaxy, Dac (red) is near-ubiquitous in the nuclei of epithelial cells on the basitarsus (Figure 3.2 A-C). It is upregulated, however, in the socket cells of the transverse row (white arrowhead) and chemosensory bristles (yellow arrowheads). In the mechanosensory bristles of the longitudinal rows, the level of expression varies from slightly stronger than the surrounding cells in rows that are close to the transverse rows (blue arrowhead) to slightly weaker than the ambient expression in some of the bristles in rows on the dorsal side of the segment (not shown). Surprisingly, Dac is absent from both the shaft and socket cells of the sex comb. This absence is evident even when the detector gain on the confocal microscope used to capture the images is increased to maximum, leading to saturation in all surrounding cells (Figure 3.2 D).

The expression of dac in non-bristle cells on TS2 is limited to the proximal third of the segment (Figure 3.3). However, it is strongly expressed in all chemosensory bristles (arrowheads) on TS2, including distal ones. (Note that there are no transverse rows on TS2 in wild-type D. melanogaster flies). Dac is also detected, albeit at a reduced level and with considerable variability, in some of the bristles of more distal tarsal segments (arrowheads).
Figure 3.2: Dac expression in a 30 hr AP male basitarsus (TS1). In a 30 hr AP sca-GAL4 UAS-mCD8::GFP TS1 stained with antibodies against DE-cadherin (green in A and B) and Dac (red in all panels), Dac is reduced in the shaft and socket cells of sex comb teeth (white arrow), as well as the distal anterior ventral sensillum campaniformia (yellow arrow), but strongly expressed in chemosensory (yellow arrowheads) and transverse row bristles (white arrowhead). Mechanosensory bristles of the longitudinal rows show moderate expression (blue arrowhead). In panel C, GFP (green) is shown instead of DE-cadherin. The reduction of Dac in the sex comb is evident even when the “gain” setting on the laser scanning confocal microscope is increased to maximum in panel D. Note, however, that in this panel the presence of Dac in cells just above the subepithelial components of the developing sex comb teeth (weak in the other panels) is now evident. Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
Figure 3.3: Dac expression on distal tarsal segments in a 30 hr AP male. Segment 2 (top) to the proximal half of segment 5 (bottom) are shown. The pupa was stained for DE-cadherin (green in A and C) and Dac (red in all panels). The same images are shown in panels A,B and C,D, but in the latter the brightness and contrast of the red channel have been increased to show weak staining against Dac. The expression of Dac is relatively strong in the proximal quarter of TS2 (arrow) then drops sharply. It is strong in bristles on TS2 (arrowheads), however, and to a lesser extent, in bristles on more distal segments (the weak staining on TS3 bristles is not visible in panel B and only faintly observable when the brightness and contrast are increased in panel D). Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.

Figure 3.4 (next page): Dac expression on a 30 hr AP female TS1 (A-C). In a 30 hr AP sca-GAL4 UAS-mCD8::GFP female leg stained with antibodies against DE-cadherin (green in panel A; GFP is shown in panel C) and Dac (red), Dac is detected in the distal transverse row (arrows) which is homologous the male sex comb. Note, however, that it is reduced relative to its expression in more proximal transverse row bristles (white arrowhead). Dac is also strongly expressed in chemosensory bristles (yellow arrowhead). (D) A scanning electron micrograph (SEM) of a female TS1. The shape of more proximal transverse row bristles is pencil-like (upper inset, yellow arrows) while the distal transverse bristles (lower inset, red arrows) are larger and closer to the needle shape of the bristles of the longitudinal rows. Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
In females, the expression of Dac on the basitarsus generally mirrors the male pattern (Figure 3.4A-C). Expression in the transverse row bristles, however, is reduced somewhat in the distal three rows, and particularly the final row (arrows) which is homologous to the sex comb; however, even in this row it is elevated relative to its expression in surrounding non-bristle cells. It is notable that the morphology of the bristles of the last basitarsal transverse row in females is somewhat different from that of typical transverse row bristles (Figure 3.4D): they are slightly longer than the bristles on the other rows and more needle-shaped (right inset) than pencil-shaped (compare with the more proximal row in the left inset).

In order to determine the temporal dynamics of Dac expression in the region of the developing sex comb, I stained late prepupal leg discs (6 hr AP) (Figure 3.5 A,B). In the ventral anterior portion of the TS1 of male prothoracic leg discs, where the future comb forms (white arrows), we observed no evidence of reduced Dac expression. In forelegs from 15 and 16 hr AP male pupae (Figure 3.5 C-F), the approximate time when the comb sensory organ precursor (SOP) cells are dividing and the comb begins its rotation, we found that Dac was reduced in the cells of the presumptive sex comb teeth (white arrows), though not to the same extent as in the 30 hr AP legs. I was not able to obtain useful data on Dac distribution from pupal legs between 8 and 14 hrs AP. (The strong adhesion between the pupal cuticle and the underlying leg tissue at this stage makes antibody staining very difficult.) It is clear that a reduction in the expression level of Dac in the sex comb has started by 16 hrs AP and becomes more pronounced thereafter.
Figure 3.5: Dac expression in young male foreleg 
basitarsi. Arrows show the area of the presumptive 
sex comb teeth. Pupae were stained for Dac (red) 
(A,B) In a 6 hr AP sca-GAL4 UAS-mCD8::GFP 
pupal foreleg, Dac is not reduced in the distal TS1 where the sex comb will later form. A slight 
reduction in Dac is evident in the distal TS1 of a 15 hr AP pupal foreleg (C,D), and is more distinct in 
the 16 hr AP basitarsus in panels E and F, where the sex comb has already started to rotate. Anterior is to 
the right and distal is down in all panels. Scale bars: 10 µm.
3.3.2 The ectopic expression of \( dac \) in sex comb teeth transforms their shape to resemble transverse row bristles

As discussed in Materials and Methods, the TARGET system (McGuire et al. 2003) was used to ectopically express \( dac \) at specific times during development. I crossed \( tub-GAL80^{ts}; UAS-dac / TM6B \) males to virgin females from the \( sca-GAL4 UAS-mCD8::GFP \) stock. As discussed in Chapter II, \( sca-GAL4 \) drives expression of the UAS construct in sensory organ precursor (SOP) cells and their descendents (see Figure 2.3). The presence of the gene encoding the green fluorescent protein in the \( UAS-mCD8::GFP \) construct allowed me to determine, using a fluorescent dissecting scope, whether the GAL4 was active. I found that if the flies were moved from 19°C to 32°C to inactivate the GAL80 (and hence allow the \( sca-GAL4 \) to bind to the UAS constructs) during the wandering larval stage, it was at least 8 hours, and often two or three times that time, before the GFP fluorescence was observable. In a number of cases, I did not observe fluorescence in the animals even in the pharate adult stage; in these situations, no transformations were observed, and the leg phenotype was essentially wild-type. The reason for this was not clear; presumably, the GAL80 was still active and continued to repress the GAL4. However, when the flies were moved to 32°C at earlier stages (prior to the wandering larval stage), fluorescence was eventually observable in all animals.

In cases where the animals were moved to 32°C during the wandering larval stage and GFP fluorescence was observable within 24 hours, two aspects of the sex comb bristle phenotype were altered (Figure 3.6 A): the teeth became thinner relative to the other bristles on the leg, and they lost their characteristic rounded tip. Pigmentation, however,
Figure 3.6: The effect of ectopically expressing dac in sensory organ precursor cells and their descendents. (A) Ectopic expression of dac in a tub-GAL80ts/scaGAL4 UAS-mCD8::GFP; UAS-dac/+ fly (transferred from 19°C to 32°C during the wandering larval stage) causes sex comb teeth (white and red arrows) to lose their characteristic rounded tips and (proximally, white arrow) the thickened shafts that distinguish them from other bristles. Chemosensory bristles (arrowheads in all three panels) are shorter and no longer curvilinear. (B) Transferring the flies to 32°C prior to the larval stage does not significantly increase the severity of the sex comb phenotype, but the chemosensory bristles (arrowheads) are now vestigial. (C) Wild-type D. melanogaster sex comb. Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
the third defining trait of sex comb teeth, was not completely lost. The extent to which the sex comb teeth were modified varied considerably from one tooth to another and from pupa to pupa. Note, for example, that in Figure 4.6 A the distal teeth are thicker than the proximal ones, although all teeth are pointed at the tip. It is possible for the teeth to have a pointed tip (red arrow in Figure 3.6 A) even if they retain the approximate thickness of the wild-type (Figure 3.6 C), suggesting that a lower Dac threshold is required to transform the tip than to affect the thickness of the shaft. Another noticeable effect of overexpressing *dac* was that the sex comb was sometimes (as in Figure 3.6 A) but not always (Figure 3.6 B) split into two separate structures. Transferring the flies to 32°C at earlier stages did not greatly increase or otherwise alter the severity of the phenotype (Figure 3.6B). In no case did the sex comb teeth lose their pigmentation. Furthermore, in all cases the sex comb was at least partially rotated.

To ensure that *dac* was in fact being ectopically expressed in sex comb tooth socket cells, I dissected and stained *tub-GAL80^ts* / *scaGAL4 UAS-mCD8::GFP; UAS-dac / +* pupae at 28-32 hrs AP with Phalloidin (to label F-actin) and an antibody against Dac (Figure 3.7). Dac was strongly expressed in the socket cells of some of the presumptive teeth (white arrows), while in other cases the ectopic expression was much weaker (yellow arrow). It is likely that this variation in the extent of ectopic expression is responsible for the considerable variability in the degree of tooth transformation in the adult flies. There were also cases of pupae where there was little or no ectopic expression (not shown).
Figure 3.7: The ectopic expression of dachshund in a pupal foreleg basitarsus. Phalloidin was used to label F-actin (green). Note the presence of Dac (red) in the presumptive teeth of the split sex comb (white and yellow arrows, with the latter showing weaker expression) of the 28 hr AP sca-GAL4 UASmCD8::GFP / tubGAL80ts; UAS-dac / + pupa (transferred from 19°C to 32°C 16 hrs prior to pupariation). Although the effect is not as obvious as in Figure 4.6, it is still possible to discern that the structure of the teeth does not differ markedly from the transverse row bristles (arrowhead); compare with the Phalloidin staining in Figure 3.9. Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
3.3.3 The effect of ectopically expressing \textit{dac} using the \textit{rn-GAL4} driver is strongly dependent on the developmental stage when the \textit{GAL4} is derepressed

Using the \textit{sca-GAL4} driver, I had ectopically expressed \textit{dac} in all bristle precursor cells and their descendents. I was interested in determining the effect of ectopically expressing \textit{dac} more extensively. The \textit{da-GAL4} driver was used to express the gene ubiquitously, but I found that the pupae all died even when this was carried out in conjunction with the \textit{tub-GAL80^{ts}} and the flies were not moved from 19°C to 32°C until just before pupariation. A similar result was obtained using \textit{bab-GAL4}. However, when the \textit{rotund-GAL4 (rn-GAL4)} driver was used together with \textit{tub-GAL80^{ts}} system, the flies survived to the pharate adult stage, and some of them eclosed. The \textit{rn-GAL4} drives the gene with the UAS promoter in the distal region of TS1 and all of TS2 to TS4 (Barmina et al. 2005).

I crossed \textit{rn-GAL4 / TM3 Ser Act-GFP} males to \textit{tub-GAL80^{ts}; UAS-dac / TM6B} virgins at 19°C. The desired progeny (\textit{tub-GAL80^{ts}/+; rn-GAL4 / UAS-dac}) were recognizable by the absence of both GFP and the \textit{Tubby} phenotype, both of which were apparent at larval to adult stages of development. The resulting phenotypes of these progeny may be divided into 3 classes, depending on the stage of the larvae when they were moved to 32°C. A relatively mild phenotype was observed when the larvae were transferred during the late third instar (Figure 3.8A). All five tarsal segments were intact, and the sex comb teeth, despite a slight disruption in their organization on TS1, retained their characteristic shape. A few ectopic sex comb teeth were sometimes visible on TS2 (black arrow in Figure 3.8A).
Figure 3.8: The effect of ectopically expressing dac using the rn-GAL4 driver. Three classes of phenotypes are shown. In A, all five tarsal segments are present (inset), and the effect on the sex comb pattern of TS1 is minor, although the thickened bristles at the distal tip of TS2 (arrow) are sex-comb-like in appearance. Chemosensory bristles are not affected (black arrowheads in all panels). This fly was transferred to 32°C less than 20 hours before pupariation (late third instar). In B, only 3 tarsal segments remain and the number of teeth is reduced, with the sex comb only slightly rotated. Note the presence of a single transverse row bristle between contiguous sex comb teeth (red arrow on TS1) and the unrotated sex-comb-like bristles on TS2 (black arrow shows one with a relatively strong sex comb phenotype (thicker, rounded at the tip), blue arrow shows a bristle with a weaker sex comb phenotype). The transfer from 19°C to 32°C in this case occurred 30 hours prior to pupariation, just before the middle of the third instar. The phenotype in C and D (same leg), on the other hand, was generally seen in cases where the flies were transferred to 32°C early in the third instar or during second instar. Tarsal segments 2-4 have been abolished. The sex comb has been replaced by a distal transverse row that is parallel and not displaced relative to the preceding row, despite the presence of at least one tooth-like bristle (red arrow). Chemosensory bristles extend to the tip of the segment. Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
When the transfer to 32°C occurred just before the middle of the third instar (Figure 3.8 B), one or more of the distal tarsal segments were often missing. Ectopic transverse rows were apparent on TS2 and sometimes TS3. The most distal of these rows often had sex-comb-like bristles that were not rotated. The extent to which these bristles displayed the full sex comb phenotype (pigmented, thickened and curved at the tip) varied (arrows on TS2 in Figure 3.8B point to sex comb teeth with differing degrees of modification).

On the first tarsal segment, the number of sex comb teeth was frequently reduced. The teeth varied considerably in thickness. Sometimes the teeth were joined together with unmodified bristles in contiguous rows, a phenotype reminiscent of sex combs in the D. takahashii and D. ananassae species subgroups (see Figure 4.3 A,B and Figure 4.18 B,C). However, a phenotype was also seen that is never observed in nature: sex comb teeth with intervening transverse row bristles (red arrow in Figure 3.8 B). In wild-type flies, regardless of the species, the sex comb teeth are always anterior to unmodified transverse row bristles.

The generation of additional teeth on distal tarsal segments (in this case, TS2 and TS3) by over-expressing dac is reminiscent of dachshund Montium-like (dac<sup>MTl</sup>) a neomorphic mutation that leads to distal sex combs (Docquier et al. 1997). The name refers to the typical montium species subgroup sex combs which are present on both TS1 and TS2.

When the larvae were transferred to 32°C well before the middle of the third instar (Figure 3.8 C,D), the forelegs were drastically altered. Segments 2-4 were lost and TS1,
in males, was altered so that instead of a sex comb, transverse rows extended to the tip of the segment, resembling the chaetotaxy of wild-type females. Note, however, that another aspect of the male bristle pattern – the 5 chemosensory bristles between longitudinal rows 5 and 6 (black arrowheads) – was retained (females only have 3) (Held 2002). Significantly, the distal chemosensory bristles extended to the tip of the segment (black arrowheads; compare with panel A). In chapter II, I showed that in males the distal chemosensory bristle starts in this position but moves in a proximal direction as a consequence of convergent extension. The presence of chemosensory bristles extending to the distal end of TS1, in addition to the unrotated sex comb that is not displaced relative to the transverse rows, suggests that the early overexpression of dac has blocked convergent extension.

3.3.4 The downregulation of dac is not sufficient to autonomously induce the formation of sex comb teeth, although ectopic teeth are generated non-autonomously

Given that dac is not expressed in the sex comb, I wanted to know if repressing dac was sufficient to induce additional teeth. I used the FLP-FRT system to generate hypomorphic and null dac mitotic clones through FLP-mediated somatic recombination. The clones were generated by crossing males from either w; dac<sup>E462</sup> P<sup>neoFRT40A</sup>/ CyO Act-GFP or w; dac<sup>4</sup> P<sup>neoFRT40A</sup>/ CyO Act-GFP to hsFLP;P<sup>neoFRT40A</sup>GFP virgins, and heat-shocking the larval progeny for one hour at 37°C. The dac<sup>E462</sup> allele is hypomorphic, and the dac<sup>4</sup> allele (Mardon et al. 1994) is a null. In the experiments discussed below, the larvae were heat-shocked at 72 hours after hatching (hr A.H), the middle of the third
instar, a time when rapid cell division is occurring in the imaginal leg discs and hence an optimal period for inducing mitotic clones.

A great diversity of sex comb phenotypes was observed in heat-shocked progeny from both of these crosses. Within both hypomorphic (Figure 3.9) and null (Figure 3.10) clones, identified by the absence of GFP (yellow arrowheads in both figures), bristles were found that did not display the modifications typical of sex comb teeth. This refutes the hypothesis that the reduction of Dac is sufficient to autonomously induce the formation of sex comb teeth. However, many legs showed multiple rows of teeth (Figure 3.9; Figure 3.11), and the total number of teeth on TS1 in many cases greatly exceeded the wild-type average of 10. Interestingly, although ectopic teeth were found outside of the clones, Dac was always absent from their shaft and socket cells (yellow arrows in Figure 3.9 and 3.10). This paradoxical result implies that the repression of dac indirectly resulted in the increase of sex comb teeth on TS1, and that dac was then reduced in these teeth by a secondary process.

3.3.5 Despite the range of phenotypes observed when dac was downregulated, the vast majority of sex comb teeth were still anterior to adjacent or proximal transverse row bristles

Examples of adult basitarsi produced as an outcome of generating the dac hypomorphic and null clones (out of a total of 54 legs from 29 individuals that were analyzed) are shown in Figure 3.11. As observed in the examples in Section 3.3.2 in which dac was expressed ectopically, there are some cases, such as Figure 3.11 B, where sex comb teeth
Figure 3.9: The effect of generating hypomorphic dac clones. The FLP/FRT system was used to generate dac^{E462} hypomorphic clones (marked by the absence of GFP). A male 28 hr AP distal pupal foreleg basitarsus is shown, stained with Phallodin to label F-actin (white in A,B and D) and an antibody against Dac (red in D and E). Two rows of sex comb teeth are apparent (arrows), with a total of at least 17 teeth visible. Dac expression (D and E) is reduced in all teeth; however, this is not a direct consequence of generating a clone (yellow arrowhead in all panels) but a secondary effect. Note that both rows show an anterior displacement relative to the TR. Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
**Figure 3.10: The nonautonomy of dac null clones** (the $dac^4$ allele was used). The leg was dissected at 32AP, and the absence of GFP (green, A) marks a null clone. Note the expression of Dac (red, B) and the structure of the developing leg with its additional rows of SC teeth, shown with Phalloidin staining (white, C). Within a null clone, however, bristles are not necessarily transformed into teeth (arrowheads). Instead, we often find that the additional teeth are generated non-autonomously (arrow, C), with Dac being reduced in these teeth by a secondary process (B). This figure shows the dorsal surface of the leg, in contrast to previous figures showing the ventral surface. Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
Figure 3.11: The affect on sex comb patterning of generating *dac* mitotic clones using the FLP/FRT system with the *dac*^{462} hypomorphic allele. While most of the SC patterns generated did not resemble those of extant species, note the interesting similarity between the pattern in C and *D. biarmipes* (Figure 4.5). In D, the most posterior tooth of the most proximal SC row (white arrow in all panels) is not quite anterior to the most anterior contiguous bristle of the TR row just proximal to it (white arrowhead in all panels), although the medial tooth in this configuration (blue arrow in all panels) is still anterior to the medial tooth of this row (blue arrowhead in all panels). Yellow arrowheads show noncontiguous transverse row bristles. Anterior is to the right in all panels and distal is down. Scale bars: 20 µm.
and transverse row bristles join together in contiguous rows, a phenotype not seen in wild-type *D. melanogaster* but observed in species of the *takahashii* (see Figure 4.3 A,B) and *ananassae* (see Figure 4.18 B,C) subgroups. Note that the teeth are anterior to the transverse row bristles, as observed in wild-type flies of these species. There are also examples of two partially rotated combs (Figure 3.11 C), a phenotype reminiscent of *D. biarmipes*.

Despite the apparently random arrangement of teeth on some legs, in approximately 80% of the cases (43 out the 54 legs), when a transverse row was just proximal or adjacent to a row of sex comb teeth, the most posterior tooth was just anterior to most anterior contiguous transverse row bristle. These cases thus conformed to the positional constraint that we have described in the other chapters. In some of the cases that violate this constraint, we observe a phenotype never seen in the wild-type comb of any well-characterized species: a row of teeth folded over against itself. Consider the example of the proximal row of teeth in Figure 3.11 D. The most proximal three teeth are rotated in the direction that we observe in wild-type cases, with the most posterior teeth being more proximal than the anterior ones. The rest of the row, however, being composed of the two teeth just distal to them, appears to have folded back in the opposite direction, instead of rotating clockwise to move beyond the transverse row proximal to it. The distal comb shows a similar phenotype.
3.4 Discussion

3.4.1 The dual role of dac on the leg may explain the paradoxical sex comb phenotypes of dac mutant and transgenic flies

While upregulating dac expression on the basitarsus did not lead to ectopic sex comb teeth, ectopic teeth were observed on TS2, and sometimes TS3, when dac was ectopically expressed on these segments using the rn-GAL4 driver at a specific time during development (middle of the third instar). While this result was surprising in light of our finding that dac actually represses two aspects of the sex comb phenotype, and that overexpression prior to the third instar in the distal TS1 abolishes the sex comb entirely, it is consistent with the phenotype of the gain of function dac^{MtlTS} mutation (Docquier et al. 1997). How can we explain the paradoxical effect of overexpressing dac?

In the cases where ectopic sex combs were produced on TS2 and TS3, they formed on each segment at the distal end of a series of transverse rows, and were sometimes contiguous with transverse row bristles. This phenotype is reminiscent of bric à brac (bab) mutants, where both ectopic transverse rows and sex comb teeth appear on TS2 and, in strong mutants, TS3 and TS4, with the teeth always forming at the distal tip of the segment (Godt et al. 1993; Couderc et al. 2002). It is relevant in this regard that dac has been shown to repress bab during the third larval instar (Galindo et al. 2002). Overexpression of dac on distal tarsal segments would be expected to reduce bab expression and possibly generate a bab mutant phenotype.
From this we can conclude that a likely explanation for the paradoxical \textit{dac} ectopic expression results is based on the dual role of \textit{dac} in both sensory organ development and leg patterning. At the level of bristle development, \textit{dac} represses at least two aspects of the sex comb phenotype. Since the gene has a function in global leg patterning, however, ectopic expression of \textit{dac} may induce a homeotic transformation of the bristle pattern of distal segments to the basitarsus, in a similar manner to a \textit{bab} mutant (Godt et al. 1993; Couderc et al. 2002). When these segments acquire a TS1 identity, they form both transverse rows and – distally, as on TS1 – sex comb teeth.

These two functions of \textit{dac} appear to be spatially and temporally separated, a developmental strategy that allows the parsimonious use of the same gene in more than one context. As I showed in Chapter II, the selection of sex comb teeth from proneural clusters and the division of the sensory organ precursor cells occurs between 16 and 20 hr AP. To transform the sex comb teeth to a transverse row bristle phenotype, it was necessary to overexpress \textit{dac} specifically in sensory organ precursor cells (using the \textit{sca-GAL4} driver) and transfer the flies to 32°C at least a few hours before pupariation (in the wandering larval stage). Given the delay of 8-24 hours between the transfer and derepression of the \textit{sca-GAL4}, this allowed the overexpression to occur before the bristle precursor cells were specified. On the other hand, the transformation of TS2 to a TS1 identity using the \textit{rn-GAL4} driver (Figure 3.8 B) required a much earlier transfer of the flies, at a time close to the middle of the 3\textsuperscript{rd} larval instar, a sensitive period for global proximodistal leg patterning (Kojima 2004).
Another paradox was encountered when *dac* mitotic clones were generated. The fact that Dac is reduced in presumptive sex comb teeth in a very specific manner might suggest the hypothesis that the removal of Dac would allow additional teeth cell-autonomously. Surprisingly, my clonal analysis showed that although ectopic rows of teeth were often formed, this effect is indirect: the teeth are generated outside of both null and hypomorphic clones. Given that legs that formed numerous ectopic teeth were often widened (e.g. Figure 3.11 B) it is possible that the ectopic teeth were a consequence of enlarging the region that is competent to form sex combs.

### 3.4.2 The effect of overexpression of *dac* on the rotation of the sex comb

Since Dac is strongly reduced in sex comb teeth in wild-type flies, it is not surprising at first glance that ectopically expressing *dac* in the distal region of TS1 would reduce the size of the sex comb (Figure 3.8 B-D). However, the absence of *dac* in the wild-type sex comb is very specific, being limited to the socket and shaft cells. Ectopically expressing *dac* in these structures, as we have seen with the *scaGAL4* driver, transforms the shape of the teeth but does not eliminate the pigmentation or rotation.

However, when I overexpressed *dac* throughout the distal TS1 at an earlier stage, I found that it was possible to reduce the size of the sex comb and even abolish the comb completely (Figure 3.8 C,D). This also led to chemosensory bristles extending to the tip of the TS1, an indication that the convergent extension in the distal tarsus, which causes these bristles to move proximally (Chapter II) had been prevented. Mechanistically, this result may have been achieved by repressing *rotund*, which has a role early in the third
instar (Tetsuya 2004) and is repressed by Dac. rotund mutants abolish the sex comb (St. Pierre et al. 2002). Regardless of the precise mechanism, the results suggest that the rotation of the comb is strongly coupled to the displacement of the distal chemosensory bristles, a result that lends further support to the model of comb rotation developed in Chapter II (see Figure 2.10, which shows that both of these processes occur through convergent extension).

3.4.3 The sex comb as a model system for testing developmental constraints

The diversity of sex combs in nature is paralleled by the variety of comb phenotypes – in a single species – that can be generated in the lab. The other two major chapters in this thesis focus on how the wild-type patterns form during development, and how sex comb diversity on the foreleg tarsus is generated from a bristle pattern that - on other legs, in females, and even other parts of the foreleg – is remarkably conserved. This chapter takes a reciprocal approach: instead of taking the diversity for granted and determining how it is generated, I attempted to analyze the range of diversity that can be formed by modifying the expression of a single gene. Specifically, I considered the question of how these phenotypes conform to – or differ from – a constraint observed in nature.

Despite the diversity of sex comb phenotypes, a result of the non-autonomous effects of downregulating dac expression, in 80% of the cases, rows of teeth were found to be consistently just anterior to a contiguous transverse row just proximal to it them. However, I also observed an unusual phenotype in cases that violated this regularity: Sex combs that have apparently folded back onto themselves. In wild-type D. melanogaster,
sex comb teeth move in an anterior direction until they have passed beyond the neighbouring transverse row bristles. There is nothing that theoretically prevents them from moving in the opposite direction (i.e. counter-clockwise in the orientation of the images in this thesis), but we do not observe this in any wild-type species in any of the known groups.

This result illustrates that in the lab a constraint on form observed in nature may sometimes be partially overcome (the combs were still in an anterior position to the transverse rows, they just had not moved completely beyond them) by the generation of a new phenotype. It is possible that this phenotype is selectively deleterious. However, before making a final judgement call as to whether this is the case, it will be necessary to arrive at a better characterization of the comb of *D. pinnitarsus* (Bock 1976) and its close relatives. The sex combs of these species have been described as "bushy" but it is not clear if any of them fold back on themselves.
Chapter IV: Sex comb development in non-model species of the *melanogaster* group

4.1 Introduction

The resurgence of evolutionary developmental biology in the last two decades has generated increased interest in understanding the developmental origin of trait differences between model organisms and their closest relatives (see, for example, Mills et al. 2007). Given the extent to which current knowledge of genetics, development and cell biology is indebted to the study of *Drosophila melanogaster*, it is not surprising that comparative studies of other Drosophilids are becoming more frequent (Stern 1998; Wittkopp et al. 2003; Gompel et al. 2005). However, many of the differences between species in the family *Drosophilidae*, such as trichome patterns on larvae (Succena et al. 2003; McGregor et al. 2007), are subtle. It is often very difficult for the non-expert to distinguish between females of closely related species. The more salient distinctive traits are all male-specific, with abdominal shape and pigmentation, wing spots, and sex combs being the most prominent.

Of these traits, sex combs are arguably the most complex and variable. The late Dipteran taxonomist Lachaise referred to the radiation in sex comb morphology as the "most remarkable changes in Drosophilids world-wide" (Lachaise and Chassagnard 2002). Furthermore, combs have diverged remarkably in species that are very closely related to *D. melanogaster*, facilitating analysis and comparisons with the model organism (Bock and Wheeler 1972; Kopp and True 2002a). The greatest diversity is found within the *D. melanogaster* species group of the *Sophophora* subgenus, the approximately 150 species most closely related to the model organism.
This diversity has made a discrete classification of interspecific differences in sex comb structure difficult. As a perusal of sex comb descriptions in the classic works on *Drosophila* taxonomy shows (e.g. Bock and Wheeler 1972), combs may be classified according to the number of comb-bearing tarsal segments on the foreleg, the comb orientation, the extent to which the teeth are "modified" or different from other bristles, the number of rows of teeth on a segment, and the length of each row (in terms of either the number of teeth or the distance that the row extends along the segment).

The number of comb-bearing segments (Table 4.1; Figure 4.1 A-C) is highly variable and has generated considerable interest as to its genetic basis (Barmina and Kopp 2007; Randsholdt and Santamaria 2008). In many cases (such as the species of the *D. montium* subgroup) it is very obvious how many segments bear combs. Sometimes, however, the situation is less clear. For example, the flies of the species *D. biarmipes* may or may not have a single tooth on the second tarsal segment (TS2) (Figure 4.5 D). This can cause certain problems when optimizing sex comb traits onto a phylogenetic tree, a process that requires the identification of discrete character states.

There are no known wild-type examples of species with more than three tarsal segments with sex comb teeth, although mutations in *D. melanogaster* genes such as *bric à brac* (*bab*) sometimes produce sex comb teeth on four segments (Godt et al. 1993; Couderc et al. 2002). In all wild-type species, teeth are found on distal segments only if they are also present on proximal ones. This is also the situation for many *D. melanogaster* mutants,
Figure 4.1: Sex comb diversity. Three of the differences between sex combs (arrows) are shown. (A-C) The number of comb-bearing segments varies, but never exceeds 3 in wild-type flies. Note that distal segments have fewer teeth than proximal ones. (D-F) Sex comb orientation, the primary focus of this chapter. When the angle between the teeth and the anteroposterior axis is comparable to that of the transverse rows, the combs are referred to as “transverse” (D). The term “oblique” or “partially rotated” is often used to describe situations where the sex comb is parallel to neither axis (E), while in cases where the comb runs the length of the proximodistal axis it is referred to as “longitudinal” (F). (G-H) Teeth may be “strongly modified”: dark and considerably thicker than the transverse row bristles (G) or only “weakly modified” (H). Anterior is to the right and distal is down in all panels. Scale bars: 20 µm.
Comb-bearing segments

D. melanogaster  D. takahashii  D. phaeopleura

D. pallidosa  D. pseudoobscura  D. serrata

Orientation

D. suzukii  D. pallidosa

Tooth modification
although it has been possible to transgenically generate teeth on a fourth tarsal segment without having teeth on intervening ones (Dworkin 2004).

The orientation of the teeth on the leg is one of the most salient differences between species (Figure 4.1 D-F). Rows of teeth that are parallel to the anteroposterior axis have been labeled "transverse" while those that align with the proximodistal axis are called "longitudinal". However, intermediate phenotypes abound. The angle of the teeth may vary even within a single row. It is important to note that much of the literature refers to non-transverse teeth as "rotated", a term which implies a developmental origin, although the process of comb development in a non-model organism has not previously been investigated.

All sex comb teeth are (by definition) dark at the tips, but the degree of pigmentation, the curvature at the tip (another distinguishing feature), and the difference in size between the teeth and other mechanosensory bristles on the legs varies widely (Figure 4.1 G-H). These features are often correlated and are sometimes lumped together under the heading "modification". In cases where the teeth are weakly modified, it may be difficult to distinguish them from other bristles, particularly in scanning electron micrographs (SEMs) where the colour is not apparent. On a single leg, there is often a "modification gradient", with anterior and distal teeth being more radically transformed (and larger) than proximal and posterior ones; the latter may seamlessly blend with the transverse rows. A similar gradient is often apparent in *D. melanogaster* mutants or transgenics. A curious, almost bimodal distinction between a set of smaller proximal teeth and a larger
group of distal ones is apparent on the TS1 of many *D. montium* species (Figure 4.12). Curiously, the teeth on TS2 are almost always the same size.

There are other differences in sex comb structure between species which have not been optimized onto a phylogeny but are worthy of mention. The number of rows of teeth on a segment varies widely. *D. biarmipes* (Figure 4.5) and *D. bipectinata* (Figure 4.19 A) may be considered to have two sex comb rows on TS1, although in *D. biarmipes* we occasionally find an "orphan tooth" adjacent to these rows (Figure 4.5A). In the *D. takahashii* species subgroup (Figure 4.3) and the *D. ananassae* complex of the *D. ananassae* subgroup (Figure 4.1 D; Figure 4.18 B,C), slightly rotated transverse rows appear to be modified, or partially modified, into sex comb teeth, with the number of modified rows varying from one individual to another.

In species with only a single row of teeth, the length of the row may be a distinguishing feature. Within the *D. montium* subgroup, the number of teeth varies greatly from one species to another, with *D. serrata* having approximately twice as many teeth on either segment as *D. vulcana* (see Figure 4.12), although the former shows more intraspecific variation. In both of these cases, however, and in most species of this subgroup, the teeth extend the full length of each segment. (We refer to this below as the "extended sex comb phenotype"). In contrast, the sex comb of *D. nikananu* is limited to approximately the distal third of TS1, and is composed of only 7-11 teeth. This, combined with the absence of a sex comb on TS2, makes this species (and the other 3 within its complex) highly
atypical for its subgroup. The *D. nikananu* sex comb has been accurately described as convergently similar to *D. melanogaster* (Lemeunier et al. 1986; Schawaroch 2002).

My aim in this chapter is to analyze the process of sex comb formation in 2 dimensional space (albeit on a 3-dimensional surface). Sex comb orientation and how it develops will be a primary focus. As such, the level of analysis will differ from recent studies which have focused on the number of comb-bearing segments (Barmina and Kopp 2007; Randsholdt and Santamaria 2008). Such studies have tried to quantify the concentration of gene products on a segment as whole, and compare across segments. I will instead be looking at the finer detail of the structure of the developing pattern within a segment at different points in time, using the relevant gene products as markers. Since the choice of representative species will have an obvious bearing on the results, I will devote the following section to a brief outline of the taxonomy of the fruit fly species that display sex combs.

### 4.1.1 The classification of comb-bearing species

The taxonomy of *Drosophilidae* shows the inherent limitations of Linnean binomial classification. Historical contingencies in classification have led to the erection of huge genera, with the result that a name such as "*Drosophila ficusphila*" gives us no indication as to how this species is thought to be related to the more than 1250 other species in its genus (Bock 1976). To facilitate matters (see Table 4.1), there are four taxonomic ranks between the genus and the species: the subgenus, species group, species subgroup and complex (Ashburner et al. 2005). While this is an undeniable aid to classification,
Table 4.1 Taxonomy of comb-bearing species. Not all lower-level taxa are shown. S = Number of comb-bearing tarsal segments on the foreleg. * Considered a genus by some authorities.

<table>
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<th>Genus:</th>
<th>Species group</th>
<th>Species subgroup</th>
<th>Complex</th>
<th>Representative species</th>
<th>S</th>
<th>Sex comb structure</th>
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<td></td>
<td></td>
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<td>3</td>
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<td></td>
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<td></td>
<td></td>
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<td></td>
<td>large, oblique</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>D. suzukii</td>
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<td></td>
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<td>2</td>
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<td></td>
<td>0-2</td>
<td>One oblique row on each segment</td>
</tr>
<tr>
<td>Fima</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>Usually transverse or oblique, occasionally longitudinal</td>
</tr>
<tr>
<td>Lordiphosa</td>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td>2</td>
<td>extended</td>
</tr>
<tr>
<td></td>
<td>Lordiphosa</td>
<td>denticeps</td>
<td></td>
<td></td>
<td>0</td>
<td>short, distal transverse rows</td>
</tr>
</tbody>
</table>
confusing these ranks is a common error. It is important to remember, for example, that while *D. melanogaster* is obviously a member of the *Drosophila* genus, it is not a member of the *Drosophila* subgenus (which includes *D. virilis*), belonging instead to *Sophophora* at that taxonomic level. The species group and subgroup are commonly confused, even occasionally in the published literature. The *D. melanogaster* group is made of at least 160 species (Toda 1991), with great diversity in their sex comb morphology. The *D. melanogaster* subgroup, on the other hand, is composed of only 9 species (Ashburner et al. 2005), with very similar sex combs.

It is sometimes claimed that the presence of sex combs is a synapomorphy of fruit flies in the *Sophophora* subgenus (Lachaise and Chassagnard 2002). However, weakly modified sex-comb-like bristles are found in the *denticeps* species group of the *Lordiphosa* genus (Zhang 1993). Furthermore, when the *nipponica* subgroup, at one time placed within the *melanogaster* group in *Sophophora* (Bock 1980), was synonymized with the *miki* group in the genus *Lordiphosa* (Okada 1984; see Table 4.1), three species with conspicuous longitudinal combs were transferred to another genus.

Within *Sophophora*, the *obscura*, *dentissima*, *fima* and *melanogaster* species groups have comb-bearing flies, while combs are absent in the New World *saltans* and *willistoni* species groups (Table 4.1). Short, oblique sex combs, of 5-8 teeth on each of the first two tarsal segments, is the norm for the *obscura* group (Lakovaara and Saura 1982). The *dentissima* group was considered a subgroup within the *D. melanogaster* group by Bock and Wheeler (1972), but it was elevated to group status by Tsacas (1980a) who also
redefined it (Tsacas 1980b). The strong similarity between the sex combs of these species (elongated and longitudinal) and the typical pattern of the montium subgroup of the melanogaster group may be one reason why Bock (1980) suggested that dentissima is a montium derivative.

Species of the fima group have combs with a transverse or oblique (i.e. not parallel to anteroposterior axis but not quite longitudinal), and occasionally longitudinal (D. akai – see Burla 1954) orientation (Lachaise and Chassagnard 2002). One of the most interesting phenotypes is in the D. dyula or "tarsus-modified" complex (Tsacas and Lachaise 1981; Lachaise and Chassagnard 2002). On each of the first two tarsal comb-bearing segments, the tarsus has an outgrowth or extension just proximal to the comb. While this is highly unusual, it is not quite unique to this taxon, as Lachaise and Chassaganard (2002) claim. It is in fact reminiscient of the situation in D. ficusphila (Figure 4.7). In both cases, the extension is proportionately longer on the second tarsal segment than on the first, although a significant difference is that the D. ficusphila sex comb is longitudinal rather than transverse, as in the case of the D. dyula complex. It is possible that this tarsal modification is a consequence of convergent extension, which the sex comb may induce (Chapter II). A developmental analysis of species in the dyula complex (and the later stages in the development of the D. ficusphila tarsus (the focus of our analysis in this chapter is on the earlier stages and how the sex comb forms, before the extension of the tarsus is complete)) should be a high priority for future studies.
It is the *melanogaster* group, however, which has the greatest diversity in sex comb morphology (Lachaise and Chassagnard 2002): combs on one, two or three segments, or in a few cases secondarily absent (e.g. *D. lucipennis* and *D. flavohirta*), representing the full spectrum of orientations from transverse to longitudinal, and varying in the total number teeth on a leg from 0 to well over 50 (*D. serrata*). Nearly all of the patterns found in other species groups are represented in the *melanogaster* group. This diversity, and the fact that the species are closely related to a model organism, are the reasons why I chose to focus on this group in the present study.

### 4.1.2 Phylogenetic issues

It is important to remember that most of the world's organisms were originally classified by taxonomists in the pre-cladistic age when acknowledged "experts" could create taxonomic groups and populate them with species using whatever criteria and methodology they thought appropriate. Traditional classification is at best an intelligent guess as to true evolutionary relationships. It is perhaps a tribute to the intuition of the experts, however, that the numerous attempts in recent years to derive molecular phylogenies of the *D. melanogaster* species group generally agree that the majority of the more than ten recognized subgroups (Ashburner et al. 2005) are monophyletic. The major exception is the *suzukii* subgroup.

Relationships within and between the subgroups are far more controversial. It has been recognized for 50 years that there are three major lineages within the *D. melanogaster* group: the *D. ananassae* subgroup, the *D. montium* subgroup, and the "Oriental" lineage,
a generic term that covers all other subgroups. In the last decade, considerable debate has raged over the precise relationships between these lineages (Figure 4.2 A). Recent molecular phylogenies, which have used far more data (close to 10,000 bps) than previous ones have provided strong evidence that the ananassae subgroup is basal and the D. montium subgroup is a sister-lineage to the Oriental taxa (Kopp 2006).

For the purposes of this study, the most important phylogenetic controversies are the ones that have a direct bearing on the history of sex comb evolution. For example, the origin of elongated, longitudinal combs that extend the full lengths of the segments they occupy, referred to in this chapter as the "extended sex comb phenotype", is an issue of interest given the large number of species that display it (Figure 4.2B). With a single exception, the precise relationships among the D. montium species are not particularly important, since almost all of these species show the extended phenotype (Bock and Wheeler 1972). It is only when we consider the D. nikananu complex, where the sex comb differs strikingly from the pattern on the vast majority of D. montium species (Lemeunier et al. 1986), that the position of a taxon within this subgroup, and whether its unusual sex comb (which does not extend the full length of the segment it occupies) is a derived or basal condition, demands our attention. The phylogenies to date strongly support the view that D. nikananu is nested deep within the D. montium group, and that its exceptional comb is an apomorph (derived) character (Schawaroch 2002; Barmina and Kopp 2007).

Within the Oriental lineage, species of the D. ficusphila (Bock et al., 1972; Bock 1980) subgroup have comb structures that closely resemble the predominant pattern in the D.
Oriental species groups

montium group

ananassae group

Oriental species groups

montium group

ananassae group

montium group

Figure 4.2: Controversial issues in the phylogeny of the melanogaster group. (A) Three hypotheses of the evolution of the major lineages. *These studies concluded that the montium and Oriental lineages are paraphyletic, but they still place the ananassae subgroup as an outgroup. (B) The evolution of the extended sex comb phenotype (1=gain, 0=loss). It was almost certainly lost in the small D. nikananu complex, but whether it evolved once in the ancestor of D. ficusphila and the montium subgroup, or convergently in the two lineages, is an open question.
montium subgroup (with a few subtle differences, discussed in Section 4.3.2). It is indeed the only subgroup within this lineage with longitudinal combs that extend the full length of the tarsal segments they occupy. Furthermore, as we will show in this study, comb development in *D. ficushila* is similar to *D. vulcana* and *D. serrata* in the *D. montium* group and differs from the process in the other Oriental species that we have examined. Most studies have shown that *D. ficushila* occupies a position close to the base of the Oriental lineage (Schawaroch 2002; Kopp and True 2002a,b; Barmina and Kopp 2007, but see Yang 2004 for an alternative placement), making it possible (if the latter is indeed a sister-clade to the *D. montium* group) that evolution of this type of comb occurred only once (Figure 4.2B).

In this chapter, I present the results of my analysis of sex comb formation and subsequent development in species with radically different comb morphologies. I show that the comb originates from transverse rows in two Oriental species that are closely related to *D. melanogaster*. In species with combs that extend the full length of first two tarsal segments, however, including *D. ficushila* and two species of the *montium* subgroup, I demonstrate that the sex comb forms from longitudinal rows. There are therefore at least two ways of forming longitudinal combs, and these two methods may produce combs that are strikingly similar in position, size and orientation. I discuss the implications of this finding in light of an apparent constraint on sex comb position.
4.2 Materials and Methods

4.2.1 Fly strains

All fly stocks were obtained from the Tucson Drosophila Stock Center. In some cases, it was necessary to combine different strains from the same species to obtain viable cultures. Consequently, only the species number (not the strain) is given in the list in Table 4.2.

Table 4.2: List of species analyzed in this chapter with their stock numbers.

<table>
<thead>
<tr>
<th>Species</th>
<th>Tucson Stock Center Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. biarmipes</em></td>
<td>14023-0361</td>
</tr>
<tr>
<td><em>D. ficusphila</em></td>
<td>14025-0441</td>
</tr>
<tr>
<td><em>D. nikananu</em></td>
<td>14028-0601</td>
</tr>
<tr>
<td><em>D. serrata</em></td>
<td>14028-0681</td>
</tr>
<tr>
<td><em>D. takahashii</em></td>
<td>14022-0311</td>
</tr>
<tr>
<td><em>D. vulcana</em></td>
<td>14028-0711</td>
</tr>
</tbody>
</table>

4.2.2 Tissue labeling

White prepupae of the appropriate species were collected and aged at 25°C. Antibody staining and Phalloidin labeling were carried out as described in Chapter II, except that fixation time was predetermined on the basis of both the species and the age of the dissected pupa, and varied from 1.25 to 2.75 hours.

4.2.3 Confocal microscopy

A ZEISS laser scanning confocal microscope (LSM510) was used to capture images. For each mounted leg, a Z-stack of confocal slices was generated using the LSM software, with an interval of 1µm between each slice. A 3D projection was then generated from each stack using the Projection tool on the LSM Image Browser Version 3,5,0,376. The
"transparent" setting was used with the threshold value set at 0, the ramp at 100, and the opacity at either 50 or 100 percent.

### 4.2.4 Scanning electron microscopy

Adult flies were left in 70% ethanol for at least a week prior to dissection. To dehydrate the tissue, the flies were washed twice for at least 5 minutes in ethanol dilutions of increasing concentration (70% ethanol, 95% ethanol and 100% ethanol). They were air-dried and mounted individually on stainless steel stubs, with glue from double-sided adhesive tape holding them down. An effort was made to ensure that one of the two forelegs was pointing upward. Conductive paint was applied to the stub so that it made contact with the tissue. After gold-plating using a Baltec SCD 050 sputter coater, the legs were viewed on a Hitachi S2500 scanning electron microscope.
4.3 Results

In my analysis of sex comb development, I used Phalloidin staining and antibodies against Senseless, Cut, DE-cadherin and Dac. In older pupae, Phalloidin labeling of F-actin can give superb images of the structure of the leg including the position and morphology of the bristles (see Figure 4.7 C,D). In younger legs, however, prior to the elongation of the bristle shaft, Phalloidin is not as useful for studying chaetotaxy. Senseless distribution, on the other hand, is more useful for analyzing very early stages in bristle development. This is because expression of senseless, a proneural gene in the same pathway as achaete-scute (Nolo et al. 2000) is strong in sensory organ precursors but is not persistent in older legs, since it disappears during the division of the sensory organ precursors (SOPs) (Nolo et al. 2000). The persistence of Cut in SOP daughter nuclei (Blochlinger et al. 1993) makes it a very informative marker for identifying the cells that will form bristles (and other external sensory organs). However, since it is present in all of the progeny of each SOP mother cell, in legs stained against this protein it is often unclear whether two nuclei belong to the same bristle or to different ones, making it difficult to distinguish the position of one bristle from another.

This problem can be circumvented by using DE-cadherin as a marker, which highlights both the bristles and the surrounding cells. Enrichment of this protein in the cell lineage that forms the neuron, sheath and glial cells (Le Borgne et al. 2002) makes it possible to distinguish presumptive bristles in legs stained against DE-cadherin from the first SOP division until late in development. In the apical domain where expression is strongest,
each cell in the lineage has a distinctive shape. Just prior to the division of the pIIIb cell to form the neuron and sheath cells, for example, the shaft cell forms a distinctive crescent within the socket cell, with the pIIIb cell protruding into the centre of this crescent. The developing bristle, as a whole, therefore, forms a recognizable structure. It is also possible to distinguish between the two main bristle types (mechanosensory and chemosensory). This is because chemosensory bristles have five neurons, and at 40X magnification the aggregate of the five cell bodies of the neurons form large puncta (e.g. white arrowheads in Figure 4.8A), just below the epithelium, which are approximately 5X the area of the equivalent puncta formed by mechanosensory bristles (e.g. yellow arrows in Figure 4.8A). Furthermore, at later stages, when the shaft has started to elongate, the dendrites of four of the chemosensory neurons extend into the shaft of the bristle (Held 2002), providing further confirmation of the bristle type. In live imaging using the ubi-DEcad::GFP stock (Chapter II), the neuronal elongation in a chemosensory bristle can be watched in real-time (follow the red arrows in Figure 2.14 B,C).

All of the images that show Phalloidin and antibody staining are 3D projections of confocal stacks (see Materials and Methods for a discussion of how this was carried out). Most of what appears in the projections is at the surface, where the concentration of F-actin and the antibodies I used is usually strongest. However, occasionally subsurface features are prominent. As mentioned above, neurons feature prominently in stainings against DE-cadherin, and the neuron descends below the surface to a cell body. The bright puncta visible in bristles (including the large ones in chemosensory bristles, discussed above) are subepithelial.
The species that I analyzed were chosen as representatives of the full range of sex comb structure and orientation. *D. takahashii* (Figure 4.3), at one end of the extreme, has short rows of comparatively inconspicuous teeth at the distal end of the first two tarsal segments which are approximately parallel to the unmodified transverse bristles rows. *D. biarmipes* (Figure 4.5) has teeth that are considerably larger and more distinguished compared to the transverse row bristles, and the two rows that they form on TS1 are at an oblique angle, neither transverse nor longitudinal. I studied three species with extended longitudinal combs: *D. ficupshila* (Figure 4.7) from the Oriental lineage, and *D. vulcana* (Figure 4.12 A,B) and *D. serrata* (Figure 4.12 C,D) from the *montium* subgroup, the largest within the *D. melanogaster* group (Lemeunier et al. 1986). Finally *D. nikananu* (Figure 4.16), despite being a *montium* species, has a comb that is strikingly different from the typical extended phenotype, and is in fact convergently similar to *D. melanogaster* (Lemeunier et al. 1986; Schawaroch 2002).

### 4.3.1 Transverse and oblique sex combs

*D. takahashii*

The *D. takahashii* sex combs (Figure 4.3 A,B) are largely representative of their subgroup, widely believed to be monophyletic (Schawaroch 2002; Yang et al. 2004; Lewis et al. 2005). Combs are found in the distal region of the first two tarsal segments (Bock 1980), and are only slightly rotated. The sex comb teeth are combined with unmodified transverse row bristles in single rows, with the former only slightly larger and more curved at the tip than the latter, making it very difficult to distinguish the two in SEMs.
Figure 4.3: The *D. takahashii* sex comb pattern. (A) Scanning electron micrograph (SEM) of the first two tarsal segments (TS1 and TS2) of a *D. takahashii* adult male foreleg. (B) Image of the same region in another adult in transmitted light. (C-E) TS1 and TS2 of a pupal foreleg at 26 hours after pupariation (hr AP), triple stained with Phalloidin (green in C) and antibodies against Dachshund (Dac) (red in all three panels) and DE-cadherin (green in E). The sex comb (SC) teeth (arrows) are always anterior to the transverse row bristles (arrowheads). Anterior is to the right and distal is down in all panels. Scale bars: 20 μm.
(Figure 4.3 A). In transmitted light the telltale pigmentation of the teeth helps them to stand out (Figure 4.3 B). This pattern – shared rows of teeth and transverse row bristles – resembles the typical tarsal chaetotaxy of the ananassae complex of the ananassae subgroup (compare with D. phaeopleura and D. pallidosa in Figure 4.1 C,D).

Within a row that contains both teeth and transverse row bristles, the former are always anterior to the latter (Figure 4.3 A,B). This can also be seen through antibody staining of pupal legs. We found previously in D. melanogaster (Chapter III) that protein product of the leg patterning gene dac is absent in the shaft and socket cells of sex comb teeth, though dac is strongly expressed in the transverse row bristles. In a 26 hour D. takahashii pupal foreleg, a time when the pattern is well established and resembles the adult, triple staining with Phallodin (Figure 4.3 C) and antibodies against both DEcadherin (Figure 4.3 E) and Dac (Figure 4.3 C-E) reveals transverse rows of developing bristles with elongated shafts (arrows in Figure 4.3 C) along TS1. Proximally, dac shows strong expression in all the bristles that make up these rows (arrowheads). Dac is absent, however, in the anterior bristles of the two most distal rows (arrows). Comparison with the adult shows that these are the bristles that form the sex comb teeth. Note that dac is expressed in the posterior bristles of these rows (distal arrowheads), which in the adult have a transverse row bristle morphology.

As in D. melanogaster, we find that in a much younger 16 hr AP leg (Figure 4.4 A-C) the developing rows of presumptive bristles are not yet contiguous. Even before they fully come together, however, the presumptive sex comb teeth (using the absence of Dac as a
Figure 4.4: The development of the *D. takahashii* tarsal bristle pattern. Pupae were stained with antibodies against *DE*-cadherin (green in all panels) and Cut (red in A-C) or Dac (red in D-F). (A-C) Putative sex com teeth (white arrows) and transverse row bristles (arrowheads) are initially noncontiguous. The foreleg of a 16 hr AP *D. takahashii* pupa is shown. While none of the rows are entirely contiguous, development of the distal rows on TS1 (likely to consist largely of SC teeth – see Figure 4.3) has progressed further than the proximal ones. (D-F) Sex comb teeth are anterior to transverse row bristles even in young pupae. The distal TS1 and TS2 of an 18 hr AP pupal foreleg is shown. Reduced expression of Dac (red) marks the sex comb teeth (arrows) which even at this early stage are anterior to the transverse row bristles (arrowheads). Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
marker) are already anterior to the presumptive transverse row bristles of the same row (Figure 4.4 D-F). Assuming that the cell dynamics in the D. takahashi male foreleg tarsus are similar to D. melanogaster, this finding may help to explain why the comb rotates very little in this species. I noted in Chapter II (Section 2.3.7) that rotation of the proximal region of the comb is associated with anterior movement of the proximal sex comb relative to the transverse row bristles, as cells between the two structures move out of the bottleneck. This movement stops when the entire comb is anterior to the row proximal to it. In D. takahashii, however, there is never any proximodistal overlap between the comb and the adjacent proximal transverse row bristles. The rapid rotation that brings the proximal comb in D. melanogaster to a position anterior to the adjacent transverse row, therefore, would not be expected to occur.

D. biarmipes

From the perspective of comb orientation, D. melanogaster and D. takahashii represent two extremes, with the sex comb of the former rotating almost 90°, and the latter having combs that rotate only slightly more than their associated transverse rows. D. biarmipes, a species in the paraphyletic D. suzukii subgroup, has a sex comb with an intermediate orientation: two rows of oblique sex comb teeth on TS1. In the strain that I used, the distal sex comb row has 3 to 5 teeth and the proximal row 4 to 7 teeth. On any given leg, the number of teeth in the proximal row is always equal to or (more commonly) greater than the number in the distal row. An orphan tooth is sometimes found between the two rows (Figure 4.5 A). In contrast to D. takahashii, it is generally easy to distinguish between the rows of sex comb teeth and the transverse rows (although a bristle is
Figure 4.5: The *D. biarmipes* sex comb. (A-B) SEMs of the ventral TS1. Note that in both of these legs, the rows of sex comb teeth are just anterior to the transverse rows. In panel A there is an orphan tooth (arrow) between the rows of teeth. (C) Dorsal view of another leg. (D) Ventral view of TS1 and TS2 in transmitted light. The single tooth on TS2 (arrow) is not always present. Distal is down in all panels and anterior is to the right in panels A, B and D. In panel C, where the basitarsus is viewed from an anterior perspective, dorsal is to the right. Scale bars: 20 µm.
sometimes apparent at the posterior end of the proximal row of teeth that is only partially modified into a sex comb tooth (Figure 4.5 A)). Note, however, that there are transverse rows posterior to the sex comb and distal to the region where the first sex comb begins (Figure 4.5 A). This contrasts with the *D. melanogaster* pattern, where the region just posterior to the comb, from where the comb begins to the end of the segment, is free of transverse rows (though a single "central bristle" is always present; Figure 1.1 A,B).

I found that both rows of teeth are initially almost transverse and approximately parallel to the more proximal transverse rows (Figure 4.6 A, white arrows). They rotate simultaneously and move in an anterior direction relative to these rows (Figure 4.6 A-D). In their final orientation, the entire rows of teeth are anterior to the transverse rows (see the adult in Fig 4.5 A,B,D). As in *D. melanogaster* and *D. takahashii*, Dac expression is greatly reduced in all sex comb teeth (Figure 4.6 C,D).

In contrast to *D. takahashii*, the transverse rows that are posterior and adjacent to the proximal row of teeth (yellow arrowheads in Figure 4.6 B,C) do not join together with these teeth into a contiguous row. The distance between the structures may prevent this, and it appears the teeth meet the row proximal to it (white arrowhead) first. These rows are comparable to the central bristle in *D. melanogaster* (see Figure 1.1 A,B; it is also possible to discern the central bristle to the left of the sex comb in Figure 2.14 A-D), which originates from the same lineage as the sex comb (Tokanaga 1962) but does not rotate.
Figure 4.6: Sex comb development in *D. biarmipes*. Pupae were stained for DE-cadherin (green) and the third pupa (the basitarsus in C) was also stained for Dac (red in D). Two rows of sex comb teeth form on TS1. At 20 hr AP (A) the teeth in both rows (white arrows) are disconnected and approximately parallel to the transverse rows. The combs rotate simultaneously, continuing to rotate after the teeth in each have come together (compare B, at 28 hr AP, and C, at 30 hr AP) and moving in an anterior direction relative to the transverse rows (dashed line; the white arrowhead shows the most anterior bristle of the transverse row just proximal to the most proximal row of sex comb teeth). Transverse row bristles posterior (but not proximal) to the sex comb teeth are visible in B-D and marked with yellow arrowheads. Dac is strongly reduced in all teeth (D). Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
4.3.2 Species with extended longitudinal combs

Probably the most noticeable (and visually compelling) sex comb morphology is the longitudinal comb that extends the full length of the first two tarsal segments. It is best known as the prevalent sex comb phenotype of the *montium* species subgroup, the largest within the *melanogaster* group, and is typical of all 79 *montium* species with the exception of the small *D. nikananu* complex. The pattern is by no means unique to this taxon, however. In the Oriental *melanogaster* group lineage, it is found in the small *ficusphila* subgroup (Kikkawa and Peng 1938; Bock and Wheeler 1972) Outside of the *melanogaster* group, it is characteristic of the *D. dentissima* group (Bock and Wheeler 1972; Tsacas 1980a; Lachaise and Chassagnard 2001) and is even found outside of *Drosophila* in the *nipponica* species group of the *Lordiphosa* genus (Okada 1956).

On the basis of what we know about sex comb development in *D. melanogaster* (Chapter II) and the species discussed earlier in this chapter, it would be reasonable to hypothesize that each of these extended combs might form from a transverse row that rotated during metamorphosis. Researchers have typically referred to combs in *D. ficusphila* and the *montium* species as rotated (Kopp and True 2002a), and in some studies they have been assigned the same character state as *D. melanogaster* when optimizing comb orientation onto a phylogeny (Kopp and True 2002b). However, the length of some of the combs, exceeding 30 teeth on TS1 in *D. serrata* (Bock and Wheeler 1972; Figure 4.12 D) and approaching 50 teeth on the same segment in *D. kilimanjarica* (Lachaise and Chassagnard 2001), is problematic for such a model. Transverse rows of more than 15 bristles are extremely rare, if they exist. If the aggregate length of the cells that formed
the teeth were even half the length before they rotated that they are in the adult, they would form a ring around the entire tarsal segment. Furthermore, my analysis of comb rotation in *D. melanogaster* (Chapter II) has demonstrated the importance of convergent extension in this process, which causes bristles close to the distal tip to move proximally. If a similar process were responsible for the sex comb orientation of a species with a comb extending the full length of a segment, bristles initially proximal to the sex comb would have to move to the tibia. It is unlikely, therefore, that the *D. ficusphila* sex comb forms from the rotation of a single transverse row, although it is possible that a number of different transverse rows rotate simultaneously and align.

*D. ficusphila*

*D. ficusphila* belongs to a small subgroup consisting of only 3 known species (Bock 1980; Lemeunier et al., 1986). As discussed previously, this subgroup is unique among Oriental taxa in that the longitudinal sex combs extend the full length of the first two tarsal segments (Figure 4.7), and in this respect strongly resemble the typical *D. montium* pattern (Bock 1980). However, unlike the *D. montium* species, *D. ficusphila* has additional noncontiguous sex comb teeth (red arrows in Figure 4.7), approximately evenly spaced, posterior and roughly parallel to the main contiguous rows. They are sharper at the tip, and slightly thinner, than the other teeth (Bock and Wheeler, 1972).

There is also another row of bristles, just posterior to the row of noncontiguous teeth, which appear unique to this subgroup (yellow arrows in Figure 4.7). Their curvilinear shape might suggest that they are chemosensory, but my analysis of their structure using
Figure 4.7: The distinctive *D. ficusphila* male tarsal bristle pattern. Key elements of the pattern include the main sex combs (white arrows) the additional noncontiguous teeth (red arrows) and the curved noncontiguous row of bristles just posterior to them (yellow arrows). (A-B) SEMs of the *D. ficusphila* tarsus, showing all five segments (A) and just the first two (B). (C) 3D projection of a confocal image stack of a 44 hr AP pupal *D. ficuspila* foreleg TS1, stained with Phalloidin. (D) TS2-TS5 of the same leg. Distal is down in all panels and anterior is to the right in A-C. In D, dorsal is to the right. Scale bars: 20 µm.
confocal microscopy (yellow arrows in Figure 4.8) shows that they do not have the additional neurons (visible as enlarged puncta in DE-cadherin; arrowheads in Figure 4.8 A) associated with chemosensory bristles, nor do their neurons extend beyond the base of the shaft. Given that the microtrichia (just visible in Figure 4.7 A,B, to the left of the yellow arrows) end just posterior to this row, it is possible that these bristles are homologous to longitudinal row 8, with their exceptional shape and prominence being an innovation in this taxon. The unusual chaetotaxy of D. ficusphila actually facilitates the analysis of the development of its bristle pattern, since the additional sex comb teeth and sometimes the curvilinear bristles are apparent in samples stained with antibodies against proneural genes relatively early in pupal development (notice the additional teeth (yellow arrows) in Figure 4.9, and the complete pattern in each of the panels in Figure 4.10), providing informative landmarks.

Three stages in D. ficusphila comb development are shown in Figure 4.10. Although the first two examples are from pupae of the same age (27 hr AP), it is clear (see the explanation below) that the first pupa (Figure 4.10 A-C), is at a somewhat earlier stage in the process of comb assembly than the second (Figure 4.10 D-F), which more closely resembles the 30 hr AP pupa (Figure 4.10 G-I). This third example is the easiest to interpret in light of the adult bristle pattern. The main contiguous sex combs, extending longitudinally the entire length of the segments they occupy, are readily identified. The additional teeth and the bristle row posterior to them are also apparent. Dissecting and staining D. ficusphila pupae 3 hours earlier, however, revealed a very different structure for the main sex combs. The two examples of 27 hr AP pupae are evidence of a dynamic
Figure 4.8: The characteristic pattern on the *D. ficusphila* foreleg basitarsus during pupal development, as revealed by staining for DE-cadherin (A) and F-actin (B) in a 36 hr AP pupa. (A) Note that the unusual curvilinear bristles (yellow arrows in both panels) that form posterior to the noncontiguous teeth (red arrows in both panels) differ in structure from chemosensory bristles (white arrowheads; see text for details). (B) The protrusion of bristle shafts is evident. The structural differences between the main comb (white arrow in both panels) and the additional teeth (red arrows) are already apparent. Anterior is to the right (dorsal to the far right) and distal is down in all panels. Scale bars: 10 µm.
Figure 4.9: Early development of the *D. ficusphila* sex comb. The first tarsal segment of a 15 hr AP pupal foreleg is shown, stained with antibodies against Senseless (green in A and B) and Dachshund (red in B and C). The precursor cells that will make up the primary comb (white arrow) are arranged in two jagged longitudinal rows, with the anterior row (right) forming an arc that approximates the shape of the adult comb. Note also the additional teeth (yellow arrows). We do not observe reduced expression of Dac in comb precursors at this early stage. Anterior is to the right (dorsal to the far right) and distal is down in all panels. Scale bars: 10 µm.
**Figure 4.10: Sex comb formation in* D. ficusphila.** The first two tarsal segments of male pupal forelegs are shown, stained with antibodies against DE-cadherin (green in A,B,D,E,G,H) and Cut (red in A,C,D,F,G,I). The inset in each image shows a region of the developing major sex comb (arrowheads) on TS1. Examples of the presumptive additional SC teeth, unique to this subgroup, are labeled with arrows. (A-C) A foreleg from a 27 hr AP pupa. The major comb on each segment consists of two longitudinal rows, which have already started to converge. Relative to the major comb, the additional sex comb teeth are already positioned in an orientation comparable to that in the adult. (D-F) Another 27 hr AP pupa, slightly further along in development. (G-I) By 30 hr AP, the presumptive main comb is contiguous. Anterior is to the right (dorsal to the far right) and distal is down in all panels. Scale bars: 10 µm.
process in which many or all of the teeth are not yet contiguous. The first pupa (Figure 4.10 A-C) shows two rows of presumptive teeth, approximately parallel, extending in a proximodistal (longitudinal) direction along both segments. In the second example, most of the presumptive teeth cells on TS2 are aligned, but many regions of the TS1 comb are not, with a number of cells appearing slightly off the main axis of the comb, almost as if they are jostling into position.

Exactly how the comb assembles in *D. ficushila* is impossible to determine in the absence of live imaging with a fluorescent marker, which is currently not available in this non-model species. However, it is clear from Figure 4.10 and earlier images (including Figure 4.9) that the comb forms from two longitudinal rows which intercalate and align slightly before 30 hr AP. The self-assembly of presumptive comb teeth is reminiscent of the situation between 15 and 20 hr AP in *D. melanogaster* (Chapter II), when the future teeth come together into an aligned row (Figure 2.5) by intercalating between intervening cells (a similar process, beginning and ending slightly later, leads to the formation of the transverse rows). However, in *D. melanogaster*, following these events, the comb is not yet longitudinal. There is a long process of rotation, described in detail in Chapter II, most of which occurs after the presumptive sex comb is contiguous. In striking contrast, there is no evidence of such a process in *D. ficushila*: once the comb has assembled, it is already longitudinal.

Consistent with the other species that we have examined, the absence of Dac in shaft and socket cells at 30 AP in *D. ficushila* distinguishes presumptive sex comb teeth on TS1
Figure 4.11: The expression of *dac* in the first two segments of a *D. ficusphila* foreleg tarsus at 30 hr AP. The leg shown in the upper panels differs from the sample in the lower panels. Both were stained with antibodies against *DE*-cadherin (green) and Dac (red) (A-B). On TS1, Dac is near-ubiquitous but strongly reduced in both the primary comb (white arrow) and the additional teeth (yellow arrows). (C-D) On TS2, the expression of Dac in the sex comb is similar, but overall expression also drops sharply in the distal region of the segment, as expected (Kojima 2004). Note, however, that it is expressed strongly in a chemosensory bristle just distal to this abrupt drop (white arrowhead). Anterior is to the right (dorsal to the far right) and distal is down in all panels. Scale bars: 10 µm.
(Figure 4.11, white arrow), since the protein is near-ubiquitous elsewhere on the segment. Even the additional teeth which are unique to *D. ficusphila* (yellow arrowheads in Figure 4.11) lack Dac, while the unusual bristles just posterior to them display the protein. Dac is not ubiquitous on TS2; close to the distal tip of the segment, expression fades rapidly. However, in the proximal 2/3 of TS2 where it is normally present, it is absent in all sex comb teeth. Interestingly, *dac* is expressed in presumptive chemosensory bristle cells beyond the distal limit of its expression in other epidermal cells (white arrowhead), which is consistent with my results for *D. melanogaster* (Figure 3.3).

**The montium subgroup**

The largest subgroup within the *melanogaster* group, *montium* species – with the exception of the *nikananu* complex – have elongated, distinctive sex combs on the first two tarsal segments of male forelegs. While the combs almost always extend the entire distance from the proximal to distal tip of these segments, the exact number of teeth varies widely from one species to another. I chose to focus on *D. vulcana* (Figure 4.12 A-B), which has approximately 19 teeth on TS1 and 14 teeth on TS2 (Bock and Wheeler, 1972), as a representative of the typical pattern of this taxon, but I also carried out a more limited analysis of *D. serrata*, a species with denser combs and close to double the number of teeth (Figure 4.12 C-D).

Despite the similarities to *D. ficusphila*, the *montium* species do not possess the additional sex comb teeth characteristic of the former. Another interesting feature of the typical *montium* pattern which is not characteristic of *D. ficusphila* is a gradient in sex
Figure 4.12: Sex combs of the montium species group. (A-B) SEMs of the first two tarsal segments of male forelegs of *D. vulcana* (A,B) and *D. serrata* (C,D). Yellow arrows show the distal teeth on TS1 that are off-axis relative to the other teeth, and also sharper at the tips. Red arrows show chemosensory bristles. Distal is down in all panels and anterior is to the right in panels A and C. In panels B and D, which show an anterior view, dorsal is to the right. Scale bars: 20 µm.
comb tooth size on TS1. Distal TS1 teeth in *D. vulcana* are much larger than proximal ones (Bock, 1972; Figure 4.12 B). In *D. serrata*, the change is abrupt, giving the appearance of a bimodal distribution of small and large teeth (Figure 4.12 D). Furthermore, the most distal 2-4 teeth on this segment differ in both morphology and orientation from the rest of the comb, with sharper tips and an orientation that is at an angle to the neighbouring teeth (Bock and Wheeler 1972; yellow arrowheads in Figure 4.12). Curiously, on TS2 all teeth are approximately the same size, although the most distal tooth sometimes has a sharper point.

As was found for the main *D. ficusphila* sex combs, the combs on each of the most proximal two tarsal segments in *D. vulcana* start as two parallel longitudinal rows (Figure 4.13). By 24 hr AP (Figure 4.13D), the presumptive teeth in the distal half of TS1 form a single row, but they are still separated by at least one cell diameter. More proximally on this segment, two rows are still apparent.

The reduced density of presumptive sex comb teeth in the distal region of TS1 (lower half of the insets in Figure 4.13 C,D) may explain why these bristles are larger in the adult (Figure 4.12 B). They have more room to grow. They also develop earlier, since at 18 hr AP (Figure 4.13 A,B) more distal than proximal cells have divided to form the characteristic crescent shape of bristle precursors when viewed in DE-cadherin. This situation may be contrasted with TS2 (Figure 4.13 B), where the presumptive teeth (arrows), regardless of their proximodistal co-ordinates, are at approximately the same stage in development.
Figure 4.13: Sex comb development in *D. vulcana*. The panels show regions of the foreleg tarsi of pupae of increasing age. The pupae were stained with Phalloidin (white in G,H) and antibodies against DE-cadherin (green in A-E) and Cut (red in A,B) or Dac (red in F,G,I). In 18 hr AP (A,B), and 20 hr AP (C) pupal forelegs, two longitudinal rows are apparent in the area where the sex comb teeth (white and yellow arrows) develop, on both TS1 (A,C) and TS2 (B,C) (three different pupae). White arrowheads in A show chemosensory bristles, recognizable by the enlarged DE-caderin puncta. Note that the two most distal teeth (yellow arrows in A,B,C,D,E,H) are initially separated from the others by approximately 3 cell diameters and come together (C,D) before they join with the rest of the comb (E). By 24 hr AP (D), a single noncontiguous longitudinal row is apparent in the distal half of TS1, but proximally there are still two rows (inset). A single contiguous row is apparent by 30 AP (E,F). The expression of Dac is reduced in all SC teeth (F, I), even at 48 hr AP (G-I). Anterior is to the right (dorsal to the far right) and distal is down in all panels. Scale bars: 10 µm.
The increased spacing of developing teeth in the distal basitarsus reaches its peak close to the tip of the segment where the last two teeth (yellow arrows in Figure 4.13 A,C,D and E) are separated from the others by at least three cell diameters at 18 and 20 hr AP (Figure 4.13 A,C). These teeth join together before they become part of the rest of the comb (Figure 4.13 D) which may explain why they are off-axis in the adult (Figure 4.12 B).

An important question is the identity of the two longitudinal rows that come together to form the sex comb in these species. The presence of four chemosensory bristles just anterior to them even at an early stage in development (Figure 4.13 A, white arrowheads) is revealing, since these bristles (which are numerically increased in males relative to females) are found between rows 5 and 6 in *D. melanogaster* (Figure 1.1 B). It is hence likely that the two rows that form the comb in *D. vulcana* (and the two rows that form the main sex comb in *D. ficusphila*) are homologous to longitudinal rows 6 and 7 in the model organism. It should be noted that 2-3 bristles at the distal tip of the *D. melanogaster* comb are homologous to rows 6 and 7 (Figure 1.1 B; Tokanaga 1962). Whereas only about one fifth of the comb forms from row 6 and 7 bristles in the model organism, the entire structure appears to be made up of these rows in *D. vulcana*.

Evidence for the longitudinal orientation of the *D. vulcana* sex comb even in the stage when it is composed of two rows of noncontiguous teeth is also evident from the distribution of Flamingo (Fmi) (Figure 4.14). In Chapter II, I showed that within 2 or 3 cell diameters of the developing sex comb, Fmi is strongly expressed on cell surfaces
Figure 4.14: Planar polarity in the region of the developing *D. vulcana* sex comb. The proximal half of the basitarsus of a 24 hr AP pupal foreleg is shown, stained with antibodies against DE-cadherin (green in A, B and D) and Flamingo (red in A, B and C). In B, the brightness of the green channel has been reduced to show the strong expression of DE-cadherin in the area of the developing comb. The arrangement of the presumptive sex comb teeth (white arrows) shows that two non-contiguous longitudinal rows are present. Even at this early stage, however, Flamingo is strongly expressed on the anterior and posterior surfaces of cells just posterior to the developing comb (yellow arrows) i.e. longitudinally. Recall (Chapter II) that the expression of Fmi on equivalent cells in *D. melanogaster* (Figure 2.6) was an indicator of the extent of sex comb rotation. Anterior is to the right and distal is down in all panels. Scale bars: 20 µm.
parallel to the orientation of the presumptive sex comb teeth i.e. proximal and distal surfaces next to transverse teeth and anterior and posterior surfaces next to longitudinal teeth (Figure 2.6). In *D. vulcana*, Fmi is found on anterior and posterior surfaces of cells just posterior to the developing comb (yellow arrows in Figure 4.14 B,C), even while the comb consists of 2 rows of nonadjoining teeth (white arrows), suggesting that the structure is longitudinal at this stage.

The *D. serrata* sex comb (Figure 4.15) forms in a manner similar to *D. vulcana*. Note the enrichment of DE-cadherin in the region of the developing comb (recall Section 2.3.4 and Figure 2.7), and the increased width of the area of the enriched protein in the proximal half of TS1, indicative of the presence of two rows at 22 hr AP (Figure 4.15 A,B, left inset) in this part of the segment (in contrast to a single row in the distal half of the segment (right inset in Figure 4.15 A,B) where the teeth are larger in the adult (Figure 4.12 C,D)). By 31 hr AP (Figure 4.15D), contiguous sex combs have formed on both TS1 and TS2. The bristle shafts start to elongate a few hours later (Figure 4.15 E to F).

**D. nikananu: a species with a sex comb convergently similar to D. melanogaster**

In striking contrast to the typical *D. montium* pattern, *D. nikananu*, a species in a small complex of the same name within the subgroup, has a single short sex comb on TS1 limited to the distal tip of the segment (Figure 4.16). The comb consists of between 7 to 11 teeth and its position, size, and presence on only one segment is reminiscent of the *D. melanogaster* group (Lemeunier et al. 1986; Lachaise and Chassagnard 2002) The resemblance is almost certainly through convergent evolution (Bock 1980; Schawaroch
Figure 4.15: Sex comb development in *D. serrata*. (A-C) Formation of the comb. The first two tarsal segments of a 22 hr AP leg are shown, stained with antibodies against DE-cadherin (green) and Cut (red). In the first tarsal segment, the region showing elevated DE-cadherin expression is broader proximally than distally, and this is correlated with increased expression in the region of the sex comb (see Figure 2.7 B,E). The presumptive teeth are more numerous than in *D. vulcana*, as expected. In the proximal region of the first tarsal segment (left inset in A,B), the two rows are still visible in places (arrows), while distally (right inset in A,B), only one row is apparent, though the presumptive teeth (arrows) are still separated by intervening cells. (D-F) Later development of the comb. Phalloidin staining was used to label F-actin (white). By 31 hr AP (D), the sex combs (yellow arrows) are contiguous on both of the first two tarsal segments. The bristle shafts are just starting to protrude by 40 hr AP (E) and have elongated by 50 hr AP (F). Anterior is to the right and distal is down in all panels. Scale bars: 10 µm.
Figure 4.16: The *D. nikananu* sex comb. (A-B) SEMs of the *D. nikananu* TS1. (C) An image of the first two tarsal segments of *D. nikananu* in transmitted light. Sex comb teeth are only found on the distal tip of the segment and are absent on TS2. The pattern is very similar to *D. melanogaster*, but note that the transverse rows continue to the end of the first segment. (D-E) A 30 hr AP pupal foreleg TS1, stained with antibodies against DE-cadherin (green) and Cut (red). The pattern is almost identical to the adults in A-C. Distal is down in all panels and anterior is to the right in all panels except B (anterior view) where dorsal is to the right. Scale bars: 20 µm.
2002), since *D. nikananu* is deeply embedded in the *montium* subgroup in all phylogenies that have considered the taxon.

We found that the *D. nikananu* comb forms in a similar manner to other species in the *montium* subgroup. At 18 hr AP, the region of small cells and elevated DE-cadherin expression, typical of the developing sex comb, is limited in this species to a distal portion of TS1 (Figure 4.17A,B; compare with *D. serrata* in Figure 4.15 A,B). Within this area, the developing comb at this stage is composed of two noncontiguous longitudinal rows. As the comb develops, a single row is increasingly apparent (Figure 4.17 C), particularly in the distal region. This row is initially noncontiguous, but the presumptive teeth gradually come together (Figure 4.17 C,D). Unlike *D. melanogaster*, where the sex comb must rotate and move relative to the transverse rows to achieve its final position, the *D. nikananu* comb is in an anterior position relative to the transverse rows long before the teeth have come together. Hence it is possible to form a comb of a comparable size, orientation and position to *D. melanogaster* from a different bristle lineage, and without rotation.

### 4.3.3 The predictable position of the sex comb

In *D. takahashii* (Figure 4.3 B) and *D. biarmipes* (Figure 4.5 A,B,D) sex comb teeth were always found anterior to the transverse row bristles just proximal to them. In *D. ficusphila* (Figure 4.7 A-C) and the species of the *montium* group (Figure 4.12 A,C), the sex comb was also anterior to the transverse rows, albeit for a different reason. We examined the adults of 11 other species from both the *melanogaster* and *obscura* groups
Figure 4.17: Sex comb development in *D. nikananu*. The *D. nikananu* sex comb, convergently similar to *D. melanogaster*’s, forms in a manner typical of other species of the *montium* subgroup. Pupae were stained with antibodies against DE-cadherin (green in A to C) and Cut (red in A) or Dac (red in D and E). F-actin was labelled with Phalloidin in D. The distal half of a foreleg TS1 is shown in all panels. (A,B) In the distal basitarsus of an 18 hr AP leg, the presumptive sex comb teeth (arrows) are arranged in two parallel longitudinal rows. (C) By 22 hr AP only one row is visible in the distal region of the comb (arrows), though not all the presumptive teeth are contiguous. (D,E) In a 24 hr AP pupa, the sex comb teeth are almost (though not quite) contiguous. The most proximal and distal teeth are marked with arrows. Note that even before the teeth have come together, the comb is already in an anterior position relative to the transverse row bristles (arrowheads in C to E). As in other species, Dac is strongly reduced in the region of the developing teeth. Anterior is to the right (dorsal to the far right) and distal is down in all panels. Scale bars: 10 µm.
(Figure 4.18) and found that on TS1 (more distal tarsal segments do not usually have transverse rows) the position of the most posterior sex comb tooth was easy to predict on the basis of the most anterior contiguous transverse row bristle, even in a hybrid fly (*D. bipectinata* male crossed to a *D. melerkotliana* female, Figure 4.19). A review of the images of sex combs in classic monographs (Bock and Wheeler 1972; Bock 1976) confirms this correlation for species where the sex comb does not extend the full length of the tarsus, including the *fima* group species (Tsacas and Lachaise 1981; Lachaise and Chassagnard 2002). In species showing the extended sex comb phenotype (such as the *dentissima* group (Lachaise and Chassagnard 2001)) the comb is also just anterior to the transverse rows, though of course none of these rows are proximal to the comb on a given segment.

4.4 Discussion

4.4.1 Comb rotation

A case has previously been made (Lachaise and Chassagnard 2002) for a correlation between comb rotation and comb length. This claim must be re-assessed, however, since my results show that in the case of the longest known combs, those displaying the extended phenotype, the sex comb does not form from transverse rows that rotate. In these flies, the length of the comb may be an outcome of the fact that more teeth can be packed into two longitudinal rows than a single transverse row.

All else being equal, the rotation may be greater in combs with more teeth due the synergistic effect of contiguous teeth held together by adherens junctions connecting
Figure 4.18: Sex comb teeth occupy an anterior position relative to transverse row bristles. When a row of sex comb teeth is in close proximity to a row of transverse row bristles, the most posterior sex comb tooth (white arrow) is usually just anterior to the most anterior transverse row bristle (white arrowhead). Occasionally we find one or more apparent transverse row bristles that are anterior to this tooth (yellow arrowhead in D), but closer examination generally reveals that these anterior bristles are non-contiguous (potentially allowing cells to escape during convergent extension and hence not creating a bottleneck) and should probably be categorized as belonging to longitudinal rows 6 and 7. Even in these cases, the medial tooth in the sex comb row (blue arrow in D) is still anterior to the medial bristle of the transverse row (blue arrowhead). Anterior is to the right in all panels and distal is down. Scale bars: 20 µm.
Figure 4.18 (continued)
Figure 4.19. Even hybrids show the anterior positional bias of sex comb teeth relative to transverse row bristles. Arrows are as in Figure 4.18, except that red arrows and arrowheads are used in lieu of white ones. Anterior is to the right in all panels and distal is down. Scale bars: 20 µm.
specific cell faces (see Figure 2.6 on page 36, and note the localization of DE-cadherin on
the adjoining faces of contiguous sex comb teeth in Figure 4.8 A on page 128), where the
rotation of one tooth will eventually pull neighbouring adjoining teeth. However, in
Chapter II I noted the importance of comb position relative to other bristles on the leg. I
found that the proximal region of the comb, which prior to the distal transverse row
bristles coming together is approximately parallel to this row, rotates rapidly after this
event as it moves in an anterior direction relative to the transverse row. I showed that the
rotation of this part of the comb and the anterior movement stops when there are no
longer any cells between the two structures in the proximodistal direction.

In this chapter, I showed that a similar sex comb movement occurred in *D. biarmipes*:
early in development, both rows of sex comb teeth overlap with the distal transverse row
in their anteroposterior co-ordinates. They move in an anterior direction as they rotate
and in the adult there is no longer an overlap. In *D. takahashii*, on the other hand, the
limited rotation of the comb may be a consequence of the fact that there does not appear
to be any overlap between the rows of sex comb teeth and the transverse row bristles just
proximal to each row.

However, in *D. takahashii*, and the species of the *anananassae* subgroup, although the
sex comb teeth are always anterior to the adjacent transverse row bristles (see Figure
4.18), they are not necessarily anterior to more proximal transverse rows. One might ask
why they do not move beyond these rows as well. It is important to note that while in
wild-type *D. melanogaster* the sex comb does not make contact with the transverse row
bristles, the norm in these species with transverse sex combs is for the teeth and unmodified bristles to be connected in tandem. It is possible that the unmodified bristles, which do not rotate, anchor the rows of sex comb teeth in place, preventing further anterior movement. In the previous chapter, I discussed examples in transgenic *D. melanogaster* flies (e.g. Figure 3.11 B) where this anchoring may have limited the rotation of a region of the sex comb.

### 4.4.2 Interpreting evolutionary change is only possible in a developmental context

It has long been recognized that evolution, in a basic, mechanistic sense, must be the outcome of changes in developmental programs. In most systems, however, our understanding of comparative development is severely limited, and some species are known only from museum holotypes. When morphological characters are used to construct a phylogeny, the choice of traits is largely biased in favour of the adult phenotype, and the family Drosophilidae is no exception (Ashburner 2005). The 217 character traits used in Gimaldi's (1990) pivotal revision of the family, for example, were all based on adult specimens. While molecular sequences are now used more frequently for phylogenetic reconstruction, one of the key purposes of constructing the phylogeny is usually to interpret morphological evolution by optimizing adult traits onto the derived tree.

Without an understanding of how these adult features form during development, however, our interpretation of their evolution may be inaccurate. The sex comb is an excellent example of this. In both the *D. melanogaster* species subgroup, and the *D. melanogaster* species subgroup, and the
montium subgroup, combs are approximately longitudinal in their orientation. The process by which they achieve this orientation, however, contrasts sharply between these two lineages. Since evolution requires a change in the underlying process, any hypothesis about the pattern of evolution must take these developmental differences into account.

Given that *D. melanogaster* is the model organism, researchers were able to infer long ago (Tokanaga 1962), using the techniques available in this system (in this case, mosaic analysis), that the comb undergoes rotation. Based on this knowledge, longitudinal combs in any species have been classified as "rotated". This shows the danger of extrapolating from a system with which we are familiar to less-familiar ones. While some extrapolation is inevitable in biology – we cannot analyze every process in every conceivable system - inferences in these situations must be qualified, especially as the phylogenetic distance between systems increases (in a more closely related species to *D. melanogaster* such as *D. biarmipes*, the combs do indeed rotate).

The resurgence in evolutionary developmental biology is a relatively recent phenomenon, and as more comparative work is carried out, the ability to follow the evolution of developmental mechanisms may revise our understanding of how we should classify adult traits. We may find that it is necessary to qualify Dobzhansky's (1964, 1973) celebrated quote ("Nothing in biology makes sense except in the light of evolution") with a corollary: "Evolution (at the morphological level) only makes sense in the light of development."
4.4.3 Evolutionary bias may be the outcome of cellular processes and physical constraints

The celebrated paleontologist Stephen J. Gould was one of the earliest prominent proponents of the claim that developmental processes may constrain evolution, and the issue has been intensely debated ever since. In a joint paper with Lewontin, Gould critiqued alleged excesses of the "adaptationist program" and the view that any given trait was the outcome of selection (Gould and Lewontin 1979). He suggested that many aspects of morphology may be a byproduct of evolutionary history and the machinery of ontogeny. Over a decade later, some analysts claimed that in response to this article evolutionists had gone too far in the opposite direction, invoking constraints of a plethora of types all too frequently (Antonovics and Tienderen 1991). In many cases, a resolution of the debate has been hampered by the limited understanding of the comparative ontogeny of the system. For example, the question of whether the evolution of complex sexually dimorphic modifications in the fourth abdominal sternite in sepsid flies was constrained prior to its occurrence in certain lineages (Eberhard 2001; Wagner and Muller 2002) could be more fully addressed if the developmental changes required to generate this novelty were understood.

Two recent studies on the relative scaling of traits in buttlerflies (Beldade et al. 2002; Frankino et al. 2005) have favored the adaptationist view, providing evidence that selection may be the reason why some correlations are absent in nature. Another study on the scaling of teeth in rodents (Kavanagh et al. 2007) analyzed the developmental mechanism of tooth formation and concluded that ontogeny may be a powerful predictor
of evolutionary change, although a role for an external force (ecology) was also inferred. The constraints that could operate in evolving allometries, however, where the bias may be due to a genetic correlation between morphological features, may be different in nature from constraints that arise when cells interact in a complex system of physical barriers. The *Drosophila* sex comb, dynamic in both ontogeny and evolution, is an excellent system for determining how developmental bias may be a direct outcome of the cellular mechanisms that generate both the adult phenotype (through development) and morphological disparity (through the tweaking of initial conditions).

Across lineages, the position of the sex comb teeth relative to the transverse row bristles appears to be constrained. The fascinating result of the present study, however, is that there may be more than one reason for this constraint: the rotation and displacement of the comb after its formation parallel to the transverse rows, or the comb originating from longitudinal rows 6 and 7, which by definition are anterior to these rows (Figure 1.1 B). In some species, such as *D. melanogaster*, about 80% of the comb forms from a transverse row, and only 2 bristles are homologous to rows 6 and 7 (Figure 1.1 B). Species with the extended sex comb phenotype, however, show the opposite extreme: the entire comb forms from rows 6 and 7. It is interesting that *D. melanogaster* males appear to be missing several bristles from these longitudinal rows that are normally present in females of the same species. Recall from Chapter II that several proneural clusters (red arrowheads in Figure 2.3), which are apparently present in males early in development (Figure 2.3 A-C), are repressed and never form bristles.
While we have presented a model to explain why transverse teeth are rotated and displaced until they are anterior to the transverse rows, it remains an open question why combs do not form from longitudinal rows other than 6 and 7. Future research will be necessary to determine what makes this region competent to form teeth.
Chapter V: Summary and Conclusions

As a relatively rapidly evolving trait in a taxon that includes a model organism, the *Drosophila* foreleg tarsus is arguably unique. The presence of a hierarchy of modules (from lower to higher levels: cells, bristles, configurations of bristles, tarsal segments; see Figure 1.1 B on page 5) of discrete types (in the bristle category, transverse row, longitudinal row, chemosensory bristles and sex comb teeth) that interact with one another (e.g. the "self-assembly" of transverse rows and the sex comb from individual units) makes this a truly complex system. Other systems, such as wing spots in *Drosophila* (Gompel et al. 2005), may be described as complex in the sense that the precise outlines of the spots vary both intraspecifically and interspecifically, but there is as yet no evidence that the spots are composed of modules at a higher level of organization than the cell. If wing spots are a complex system, it is at the level of the morphogen gradients that pattern them, rather than the physical features of the spots themselves. The prothoracic tarsus is seen to be complex at both levels.

In the previous paragraph, I referred to the tarsus rather than the sex comb. One of the conclusions of this thesis is that it is impossible to understand the evolution of the sex comb without considering other features on the tarsus. The same process of convergent extension involved in the rotation of the comb also leads to the male-specific proximal movement of the anterior chemosensory bristles and the bristles of longitudinal row 5 relative to the TS1-TS2 joint (Figure 2.10). As proposed in Chapter II, the final position of the comb relative to the distal transverse row can be explained by a model that considers the physical barriers that are an emergent property of both structures becoming
irreversibly contiguous in a 2-dimensional epithelium (and, possibly, an attraction between specific bristles on these structures).

Tarsi lacking sex combs, however (the tarsi of male midlegs and hindlegs, and all three legs on females), while complex, do not show the rapid evolution associated with their comb-bearing counterparts. It is this evolutionary dynamism within a pattern of stasis that makes this system a treasure trove of evo-devo insight. This is seen most dramatically by comparing the modifications on the first two comb-bearing tarsal segments on a fly such as *D. ficusphila* with the more distal, unmodified comb-free segments (Figure 4.7).

In Chapter I, I mentioned the possibility that modules such as bristles may be linked into higher-order structures during development. The "coupling of modules" has become a catch-phrase of evolutionary developmental biology, but in most cases the term is used metaphorically. In a structure such the sex comb, however, the linkage is both metaphorical and literal; the developing sex comb teeth become physically connected after a certain stage, and this linkage implies that throughout their later development they are only partially autonomous. Movement of one part of the comb will eventually lead to the movement of other parts of the structure. Even so, the coupling may be described as "weak", since not all bristles rotate at the same rate. Weakly coupled systems are considered highly evolvable, and this evolvability is clearly borne out in the variation in sex comb structure observed in nature.
The evolvability of the comb was further analyzed in Chapter III, where I looked at the range of phenotypes that could be produced by perturbing the expression of the leg patterning gene, *dachshund*. Some natural patterns, such as the two oblique combs on a single segment observed in *D. biarmipes*, and the linkage of combs and transverse rows in contiguous formations (not observed in wild-type *D. melanogaster* but present in numerous other species in the *D. takahashii* and *D. ananassae* subgroups) were easily phenocopied. Other patterns were not: the extended sex comb phenotype observed in *D. ficusphila*, the species of the *montium* subgroup and other taxa, was never seen in my ectopic combs. It is possible, of course, that this phenotype can easily be produced by perturbing another gene, but I have not seen it in any reports of sex comb mutants in *D. melanogaster*. A reason for this is suggested in Chapter IV: the extended phenotype is formed through a different mechanism from that which leads to the *D. melanogaster* comb, namely, the convergence of two longitudinal rows rather than the rotation of a transverse row.

In a clade with extended sex combs (the *D. montium* species subgroup), however, an offshoot – *D. nikananu* and the other species in its complex – have evolved a short comb limited to the distal region of the basitarsus, resembling the *D. melanogaster* comb, but using the same mechanism that produces the extended comb. It is no longer possible to assume that all longitudinally oriented sex combs are rotated. The finding highlights the importance of considering development in following the process of evolutionary change. As the comparative ontogeny of more non-model organisms is better understood, this will be increasingly feasible.
It is my hope that the present work will help lay a foundation for further studies. The possibilities for future research are almost endless. With the resurgence of interest in the sex comb (Graze et al. 2007; Randsholt and Sanatamaria 2008), it is important to focus efforts on the most promising channels of inquiry, and I believe that my work may have helped to identify these.

**Cellular and developmental questions**

This thesis has established a protocol for the live imaging and visualization of the cellular events that take place on *Drosophila* legs. The research on the development of the sex comb in the model organism (Chapter II) focused on legs with about 10 sex comb teeth, the approximate mean for this species (Hannah 1958). It has recently been shown, however, that through selection alone it is possible to generate lines with (on average) as low as 4, and as many as 15, teeth per leg (Ahuja and Singh 2008). Investigating the development of the sex comb in these extreme cases could help reveal how ontogeny changes in response to selection.

Through basic genetic techniques, genotypes can be synthesized with the most promising cellular markers (such as *ubi-DE-cad::GFP*) in a mutant background. The live imaging of mutants with multiple combs on the same segment, for example, can yield greater insight into how combs interact with each other, and the transverse rows. Since there are many mutants that show combs at varying degrees of rotation, it is possible to test how
the initial conditions (e.g. the initial position of rows of teeth relative to each other and the transverse bristle rows) influence the final pattern (the ultimate extent of rotation, the final position of the sex comb). One example of a mutant where such a study could prove fruitful is the \textit{eyeless-Dominant} (\textit{ey}^{D}) allele (Figure 1.2).

Understanding how the transverse row bristles and sex comb teeth join together into contiguous structures should be a research program of the highest priority. The links that are formed between adjoining faces of developing bristle socket cells, visible in the high concentration of \textit{DE-cadherin} between these faces (see Figure 2.6), are highly specific, with the anterior and posterior faces of these bristle sockets forming connections with one another but not with the proximal or distal faces. If this were not the case, clumps of bristles, instead of rows, would form. The unusual phenotype observed in Chapter III (Figure 3.11 D), where the comb has apparently folded back on itself, should be analyzed to determine if this represents a breakdown of this process. It is possible that filipodia are involved in bringing the developing bristles together, and assays should be carried out to determine if these are present. The dynamic rearrangement of adherens junctions and the molecular and cytoskeletal machinery involved has been analyzed carefully in the embryo (Harris and Peifer 2007), and similar studies can be carried out in the leg. Since intercalation in the tarsus takes longer than the equivalent process in the embryo, it may be possible in such a study to analyze these events at a higher temporal resolution.

Recent findings that link planar polarity with cell intercalation (Bertet et al. 2004; Blakenship et al. 2006) provide clues as to possible mechanisms that could lead to the
convergent extension in the distal tarsus. The fact that ectopic sex combs can rotate, and 
the very precise distribution of Flamingo in cells within a few a diameters of the sex comb 
that mirrors its distribution on distal faces of comb socket cells (Figure 2.6; Figure 4.14), 
suggests that the sex comb may be inducing changes in the polarity of neighbouring cells. 
Further studies are warranted on the distribution of genes in the frizzled pathway in the 
vicinity of the sex comb and in sex comb mutants, and on the effects on comb rotation of 
modifying the expression of these genes.

Paradoxically, despite the numerous genes that show sex-comb phenotypes when 
mutated, little is known about how these phenotypes arise, and whether the effects are 
cell-autonomous. In Chapter III, I showed that both the upregulation and downregulation 
of a single gene, the transcription factor dac, may generate ectopic sex comb teeth, but 
that both of these effects are indirect; when upregulated specifically in sensory organs, 
dac represses certain aspects of the sex comb phenotype, changing the shape of teeth to 
resemble transverse row bristles. It is important that more of the sex-comb-modifying 
mutants are subjected to a rigorous analysis. In the past, the focus has been primarily on 
early periods. Most of the studies of leg patterning described in a detailed review 
(Tetsuya 2004) looked at either larval and prepupal leg discs (prior to 6 hr AP) or the 
adult phenotype. This leaves a huge gap in development from 6 hr AP through 
metamorphosis, a process that occurs over a 100 hr period. While it is logical to look at 
imaginal discs if the purpose of the study is to analyze leg development at the level of the 
segment, to understand the origin of finer features such as leg bristle patterns it is 
necessary to look later. The present work, by identifying the period in development when
sex comb precursor cells are selected from proneural clusters, divide and form shafts, will facilitate future endeavours.

**Evolutionary questions**

Since the turn of the century, phylogenies of various groups of *Drosophilidae* have multiplied, with a particular focus on the *D. melanogaster* species group. However, there is still little information on the phylogenetic positions of certain clades of comb-bearing species, including the *dentissima* and *fima* species groups. A phylogeny with representatives from all taxa that have species with sex combs, including the *denticeps* and *miki* groups within the genus *Lordiphosa* might help to resolve questions such as the morphology of the earliest sex combs.

Of the 12 *Drosophila* genomes that have been sequenced (Stark et al. 2007), there are 8 that bear sex combs: the five sequenced genomes of the *D. melanogaster* species subgroup as well as *D. ananassae, D. pseudoobscura* and *D. persimilis*. The non-model species of the *D. melanogaster* subgroup show variation in mean number of teeth and have been used in QTL studies (True et al. 1997; Graze et al. 2007), but the orientation and placement of the combs is similar to *D. melanogaster*. Analyzing the genome of *D. ananassae* (Figure 4.18 B), which has a series of transverse sex combs on the first three foreleg tarsal segments and *D. pseudoobscura* (Figure 4.1 E), which has one oblique comb on each of the first two tarsal segments of the first legs, could yield important insights. For example, we could look for binding sites of upstream regulators in 5' and 3' regions of *dachshund* or other genes known to influence sex comb patterning. The sex
comb of *D. persimilis* is similar to that of *D. pseudoobscura*, to which it is closely related. Unfortunately, no species with the extended sex comb phenotype has been sequenced.

For my analysis of sex comb development in non-model organisms, I relied on specimens that were dissected and fixed at specific times in development. An understanding of the precise dynamics of comb formation in these organisms at a higher level of temporal resolution could be gleaned from live imaging, which would be possible if these species could be transformed with the green fluorescent protein (GFP), under the control of a relevant promoter. Developing protocols for the transformation of non-model *Drosophilids* is on the agenda of the *Drosophila 2007 White Paper* (http://flybase.bio.indiana.edu/static_pages/news/whitepapers/DrosBoardWP2007.pdf.) Understanding the function of candidate genes in these organisms might also be achieved by using RNAi in cultured legs. Comparisons of the disruption of homologous genes in, for example, *D. melanogaster* and *D. ficusphila*, which form their combs through different mechanisms, would be insightful.

In the 1980's and early 1990's, researchers hoping that developmental genetics would lead to an understanding of how differences between species are generated were faced instead with the discovery of the wide conservation of many basic pathways and processes (Gerhart and Kirschner 1997; Wilkins 2002). It was only later that results came in which started to give us the first hints about how ontogenetic differences might lead to evolutionary divergence (e.g. Stern 1998). In the current quest to understand the origin of
species in all their morphological complexity, it is hoped that this work has demonstrated
the utility and power of the *Drosophila* sex comb as a model system.
REFERENCES


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