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The UAS-Gal4 System in *D. melanogaster*: An Insight into the Influence of microRNAs on the Developmental Pathways of the Wing

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The UAS-Gal4 System in *D. melanogaster*: An Insight into the Influence of microRNAs on the Developmental Pathways of the Wing

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Abstract

By examining genetic pathways in *D. melanogaster*, a better understanding of the homologous regulatory mechanisms in humans can be utilized to further enhance knowledge of the roles of microRNA within development. This study utilizes the UAS-Gal4 system in order to produce a mutant phenotype capable of being visually studied and analyzed, focusing on the developmental pathway of the wing in *D. melanogaster*. Dissections of the wandering third instar larvae yielded wing disc tissue expressing the downregulation of *loquacious* and *CG17386*.

Key Terms: UAS-Gal4, microRNA, *Drosophila melanogaster*, homologous, phenotype, wandering third instar larvae, wing disc, *loquacious, CG17386*

Dedication

Daddy, Bogie, Megan, Greyson, Brian, Shelby, Samantha, JT, Pops, Momma:

Thank you for your consistent support and love.

At every step of the way, you have been supportive and generous. I couldn't ask for a better family to have as a part of my life.

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Table of Contents

List of Illustrations and Figures

Illustrations

Figures

List of Abbreviations

USM	(The University of Southern Mississippi)
miRNA	(microRNA)
UAS	(Upstream Activation Sequence)
RBP	(RNA-binding protein)
HIPK	(Homeodomain Interacting Protein Kinase)
esg	(Escargot)
sna	(Snail)
loq	(Loquacious)

Introduction

The developmental pathway of the wing in *D. melanogaster* is a multistep process involving various genes and their products under temporal and spatial control. Regulation is enforced by modulating gene expression using proteins such as transcription factors and RBPs, which monitor transcription and post-translational modification respectively. By utilizing the UAS-Gal4 pathway to manipulate protein expression and produce mutant offspring, the effects of altering the level of expression of a particular gene can be examined and analyzed in order to determine the gene's role within the process of wing development. The diverse genes, proteins, and factors involved in the regulation and control of the development of the *D. melanogaster* wings may have effects on other body parts and processes through RNA silencing or post-transcriptional regulation of expression. This phenomenon can lead to a more concise understanding of the role each protein may have in the overall developmental progression. These insights may have medical implications linked to effectively diagnosing, treating, and developing cures for developmental disorders, diseases, and cancers, which may have a foundation in the improper functioning of miRNA.

RBPs are a prime example of units that have a central role as regulatory components of RNA within a cell, mostly through post-transcriptional control. They are largely dependent on expression and localization to determine their involvement in cellular processes, such as splicing and translation; therefore, altering the expression levels of RBPs causes significant large-scale changes in the post-transcriptional regulatory networks they mediate. By modifying the RBP used within the pathway, the network becomes inaccessible, disrupting the mechanisms for the natural expression of

genes and resulting in a mutant phenotype. Other factors that can be altered to produce mutant offspring, such as the genes coding for mRNA production, may lead to heterochronous deviation from the wild type, failure to properly transcribe proteins, inability to recognize specific chemical signals, and more.

This experiment investigates the mechanism of alterations in gene expression due to microRNA influence on development in *Drosophila melanogaster*, specifically in determining the mechanisms through which phenotypic deviations from the wild type wing anatomy are produced. The wings are part of a well-studied system that will allow loss of function phenotypes to be tied to specific developmental signaling pathways. Understanding the full process behind the growth and maturation of an organism allows for solutions to biological problems concerning mankind to be addressed more easily. These pressing medical concerns involving the functionality of miRNAs include cancer, muscular atrophies, and neurological disorders, the mechanisms of which involve pathways which we can gain insight into through the further investigation of the pathways involved in growth and development.

The UAS-GAL4 System

The UAS-Gal4 system allows for the definition of which cells are expressing a particular gene or its product. There are many model organisms such as *D. melanogaster* which have been genetically engineered to express the Gal4 gene in a particular section of tissue. These are known as Gal4 lines. Within these lines the Gal4 protein (a transcription factor) might be expressed only in a certain type of cell or in a specific appendage, such as a small section of the brain or a part of the wing. In this way Gal4 is a useful "system for targeted gene expression" (Duffy, 2002, p. 1).

The presence of the gene alone has no (or negligible) effect on the organism. In order to activate Gal4 it must bind to a UAS promoter region which most cells do not have, leaving that gene transcriptionally silent. To resolve this issue, reporter (or responder) lines are used. Responder lines are strains of flies with the UAS region placed next to a specific gene of interest, where "to activate their transcription, responder lines are mated to flies expressing Gal4 in a particular pattern, termed the driver" (Duffy, 2002, p. 1). When a cross is made and offspring are produced, the "resulting progeny then express the responder in a transcriptional pattern that reflects the Gal4 pattern of the respective driver" (Duffy, 2002, p. 1). The cells that are producing Gal4 activate the UAS sequence to switch the gene on and begin transcribing the genetic code into a protein. Phenotypes resulting from the expression of that protein indicates the function of the protein encoded by the gene next to the UAS region.

This is known as the bipartite approach, where the responder line and driver originate from separate parental lines. The method was first used and detailed by Brand and Perrimon (Duffy, 2002). One of the major "strength[s] of the system arises from the ability to target expression of any responder in a variety of spatial and temporal fashions by mating it with distinct GAL4 drivers" (Duffy, 2002, p. 2).

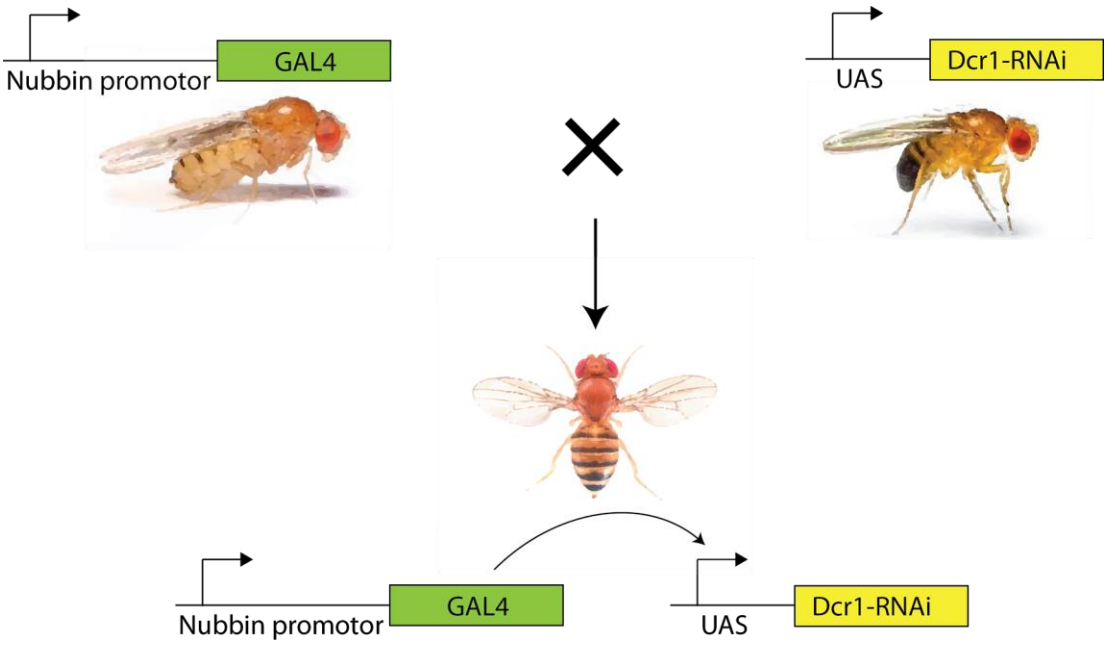

Illustration 1: UAS-GAL4 diagram

RNA-binding proteins (RBPs) and miRNAs

At the post-transcriptional level RBPs are capable of taking on the role of splicing, polyadenylation, transport, mRNA stability, localization, and translational control (Kechavarzi, 2014, p. 1). These molecules are important pieces of the developmental pathway because of their central role in affecting gene expression at the post-translational level. In some cases altering the efficacy or functionality of these proteins may affect the regulation of alternative cleavage mechanisms or regulation of splicing on transcripts, which can precede the development cancer (Kechavarzi, 2014, p. 2). This characteristic of RBPs is important in the formation of microRNAs, which form by folding back on themselves after being cleaved from an mRNA strand. Many other serious human ailments - such as muscular atrophies and neurological disorders - have been linked to mutations in RBPs or their binding sites, highlighting the importance of understanding their multifaceted role in developmental processes (Clery, 2011).

Kechavarzi asserts that since "RBPs exhibit a particularly high level of expression in some tissues suggests a need for extensive post-transcriptional control of gene expression in them" (2002, p. 2). He goes on to describe how cyclic cycles, such as spermatogenesis, require spatial and temporal expression of specific genes and that the dysregulation of the RBPs in control of their mediation may result in a mutant phenotype of these tissues (Kechavarzi, 2002). For noncyclic cycles such as wing growth, it is important to stop growth at the appropriate time to avoid the malformation of the wings and a subsequent state of flightlessness. Deregulation of mRNA by RBPs and miRNAs can result in the overproduction of certain proteins by impeding RNA silencing.

One average twenty-two base pair miRNA molecule can affect the stability of hundreds of diverse mRNAs by causing instability, cleaving transcripts, and suppressing production of a large number of proteins (Meyer, 2004). These mechanisms are involved in RNA silencing, which cancels the production of a protein by interfering with the mRNA transcript and preventing translation. MicroRNAs accomplish this by binding with and destabilizing the mRNA molecule, tagging it for degradation instead of translation. The silencing of the transcript is accomplished by cleavage of the strand into two pieces, destabilization through shortening of the polyA tail, or less efficient translation. A result of this process is the downregulation of the gene directly downstream from the UAS.

Basic Structure and Development of the Fly Wing

A fly wing begins as "the wing imaginal disc, formed from the embryonic ectoderm by an invagination at the intersection of a dorsal/ventral stripe of Wingless with an anterior-posterior stripe of Decapentaplegic" (Brody, 1995). The cells comprising the

wing at this early embryonic stage originate from the posterior two-thirds of the abdomen. As the embryonic disc invaginates to form the ventral furrow, a few cells expressing the gene *aristaless* (a homeobox protein) become fated to be the most distal cells of the wings. Evagination is accompanied by an alteration in morphology and has recently been associated with cell rearrangement and division, driven by actin filaments. Other proteins like Distal-less and Vestigial are "required for pattern formation along the proximal-distal axis in the adult" (Brody, 1995). The second step of wing formation involves the proteins Escargot and Snail in the initial specification of the cells of the wing disc. In the absences of *esg* and *sna*, the wing primordium is converted into epidermis, inhibiting the formation of the wing blade. The disc is structured into three separate axes mediated by genes which assign polarity to the cells. Some of these genes are *engrailed, hedgehog,* and *dishevelled* - all three of which exhibit a gradient of expression to establish an anterior-posterior axis - along with the aforementioned *decapentaplegic, distal-less* (establishes the proximal-distal axis), *aristaless* (works with *distal-less*), *vestigial* (establishes the dorso-ventral axis), and *wingless* (structures the sensory hairs on the wing edges).

The adult fly's final act of morphogenesis is the maturation of the wings. Before the fly emerges from the pupae (a process called eclosion), "the epithelia within the folded wing begin to delaminate from the cuticle and that delamination is complete when the wing has fully expanded" (Brody, 1995). The cells then lose contact with each other, separating and migrating towards the thorax from the wing. Each cell compacts after severing their connections with neighboring cells, becoming round. These newly round cells begin changing shape again and elongating into spindles that associate with similar

cells as they extend thin cytoplasmic filaments. Migration of these units to the hinge and body of the fly leaves behind molecular components that are used to bond the dorsal and ventral cuticle surfaces.

Post-eclosion, a sudden and coordinated group death of the epithelial sheets of the wings occurs. The protein Homeodomain interacting protein kinase (HIPK) is required "for collective death of the wing epithelium" which, "in this context defines an abrupt group behavior… at the tissue level… causing wholesale loss of intervein cells and coordinated elimination of the entire layer of epithelium" (Brody, 1995). This behavior is in stark contrast to most *in vivo* models, where a single cell surrounded by neighbors sporadically initiates apoptosis. As this occurs growth proceeds uniformly throughout the wing disc, some of the gene products are expressed in a gradient. This is possible due to the effects of mechanical forces on the cells, as well as regulation and inhibition by growth and transcription factors. For instance, as growth is induced in the center of the disc the peripheral regions undergo tangential stretching. The peripheral regions compress the center of the disc as growth continues, causing growth to be inhibited at the center. The larger the disc, the stronger the compressional forces become and the stronger the inhibiting effect upon the growth, stretching, and elongation of the wing. According to Brody (1995), "growth ceases when the growth factors can no longer overcome this inhibition [of the center of the disc]." Cellular rearrangement occurs throughout the entire wing, contributing to the extension and thinning of these flight appendages.

Hydraulic pressure may also have a role in the evagination and elongation of the wings and other appendages. It is assumed that hemolymph is moved throughout the body of the prepupae, pulsing in synchronization with the blood cells. Lack of proper

hydraulic pressure might be a plausible "reason for the less than optimal evagination of discs seen during in vitro culture" (Brody, 1995). Insects possess wing hearts in order to minimize the probability of this issue. These two bilateral muscular pumps are located in the thorax and ensure the regular flow of hemolymph through the wings. Other than this structure, *D. melanogaster* possess one tubular heart in an open circulatory system.

Wing hearts are important to the development of the wing because of the role they play in the maturation of the wings. As the cuticles of the intervein region bond to become the flexible wing blade, the vein region forms tubes lined with living cells from the cuticles there, which form structures through which the hemolymph may circulate in the adult specimen (Brody, 1995). The wing heart draws the hemolymph out of the wings post-eclosion, acting as a suction pump shortly after wing unfolding. These small muscular pumps are deemed essential to wing formation and are thought to be a crucial step in the development of wings, and therefore flight, in *D. melanogaster*.

Methods

In order to propagate the genetic lines, every week the flies were relocated into a clean food tube. Adult specimens from the Gal4 line (*nubbin*) were examined twice or more per week in order to collect virgin female *D. melanogaster*. This was done by anesthetizing the flies with carbon dioxide and visually distinguishing the virgin females from the inseminated ones and the males using a dissecting microscope. After replacing the non-virgin and male flies into the original container (using a paintbrush to sweep them gently inside) or disposing of them in ethanol, we isolated the virgins in an appropriately labelled tube.

As two or three virgin females were gathered in the same container, an equal number of males from one of the various Gal4 lines were placed into the container with the non-inseminated females in order to produce a cross. Offspring from the cross were collected after two or three weeks and examined to determine the phenotype with respect to the wings. The wings were detached, mounted, and photographed to document the cross and its resulting phenotype.

Dissections were performed on wandering third instar larvae from the *loquacious* and *CG17386* genetic cross lines to find and remove the wing discs. Pieces of somatic tissue from the body wall were left attached to the sample to facilitate the visual identification of the wing discs, which are almost transparent and attached to the body wall. Samples were preserved in an Eppendorf tube in a solution of PSBTx (1 tab/200 mL) in a refrigerator at 4°C. 15 pairs of wing discs were collected from each line.

Results

A screening of various fly lines provided multiple genes which produced a visible mutant phenotype of the wings. Genes *loquacious* and *CG17386* were chosen at random from those responder lines which produced visible wing mutations. Flies from the F1 generation of the *loquacious* cross produced either severe defects or no visible defects at all (Figures 1, 2, and 3). Malformed wings seem to have an overproduction of hairs, veins, or both. Hairs on the wings varied in thickness and length compared to wild-type structures but polarity was indeterminable. Wings presenting deformities were variably notched (see Figure 3).

The *CG17386* cross presented abnormalities of hair thickness, hair overgrowth, and vein production (Figure 4). Wing cells appear to be absent. Notching, if present, is not obvious due to the unnatural folded character of the mutant wing (Figure 5). A wild-type wing demonstrates the thinner character of the hairs, establishes normal polarity of the hairs of the wing, displays natural wing cell shape, details proper vein placement, and provides a wild-type comparison for the degree of notching and folding of the two mutant lines (Figure 6).

Discussion and Conclusion

The effects of microRNAs on the development of animals are important regulating events which monitor the degradation of mRNAs within cells. These small non-coding RNA fragments regulate many aspects of cellular growth and division such as control of the cell cycle, differentiation and tissue induction (such as hematopoeisis), as well as many other critical processes. Effects of microRNAs on tissues can be investigated and visualized using the UAS-Gal4 system with *Drosophila melanogaster* fruit flies. Virgin female flies from the *nubbin* line carrying the Gal4 transcription factor were mated with males from various responder lines. The resulting offspring were screened for visible mutations of the wing tissue in mature adult flies.

Genes *loq* and *CG17386* were chosen at random to be investigated. There has been no previous research to indicate their functionality or influence in microRNA regulation within the development or maturation of the wings in *D. melanogaster*. The deformities presented by flies from both lines seem to indicate dysregulation in the developmental processes critical to the correct formation and functioning of the wing related to hair production, vein production, and formation of the wing cells.

Larval dissections of third instar wandering larvae from both lines yielded 15 pairs of wing discs to be analyzed. Research regarding the miRNA activity in the wing tissue is ongoing. It is predicted that miRNA regulation disruption within the developing wing discs of the larvae will be shown to have a direct impact on cell movement and tissue induction during the growth of the organism.

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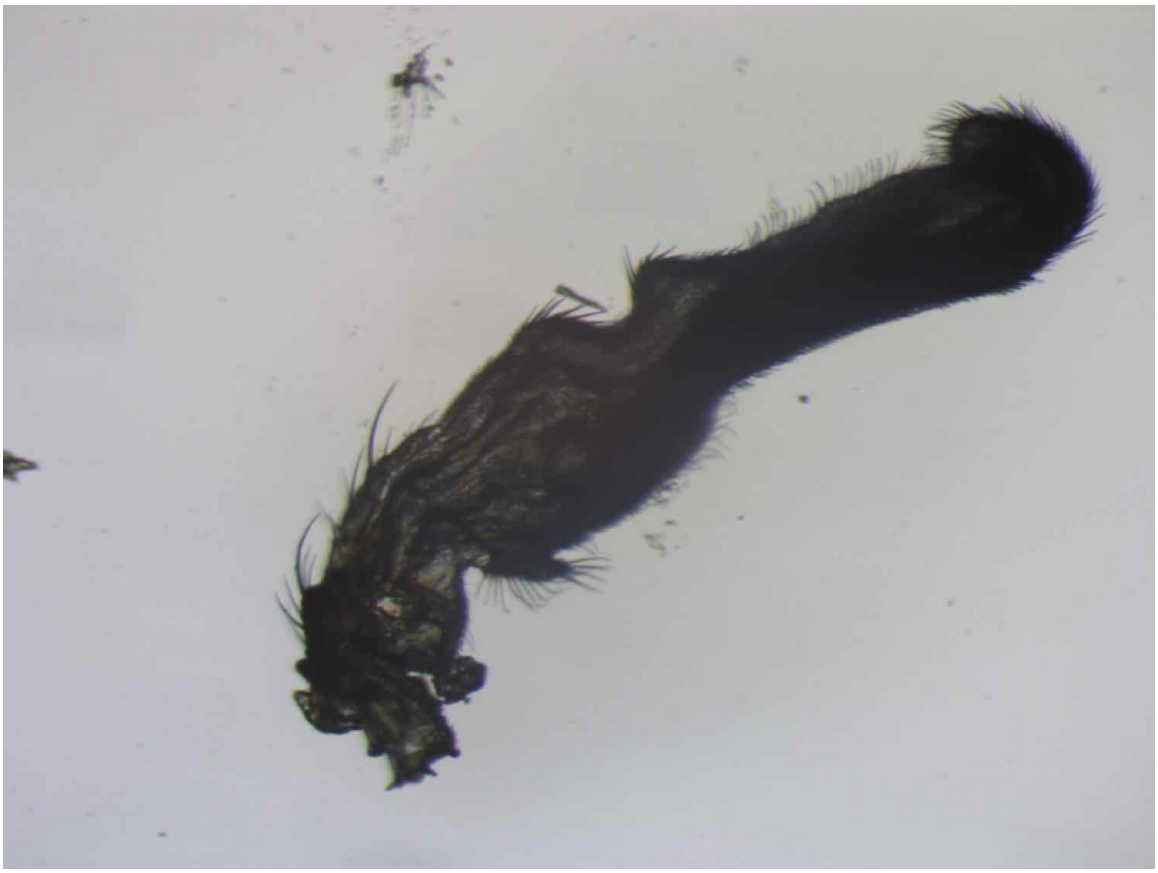

Figure 1. Severely deformed *loq* mutant wing

Figure 2. Normal *loq* mutant wing

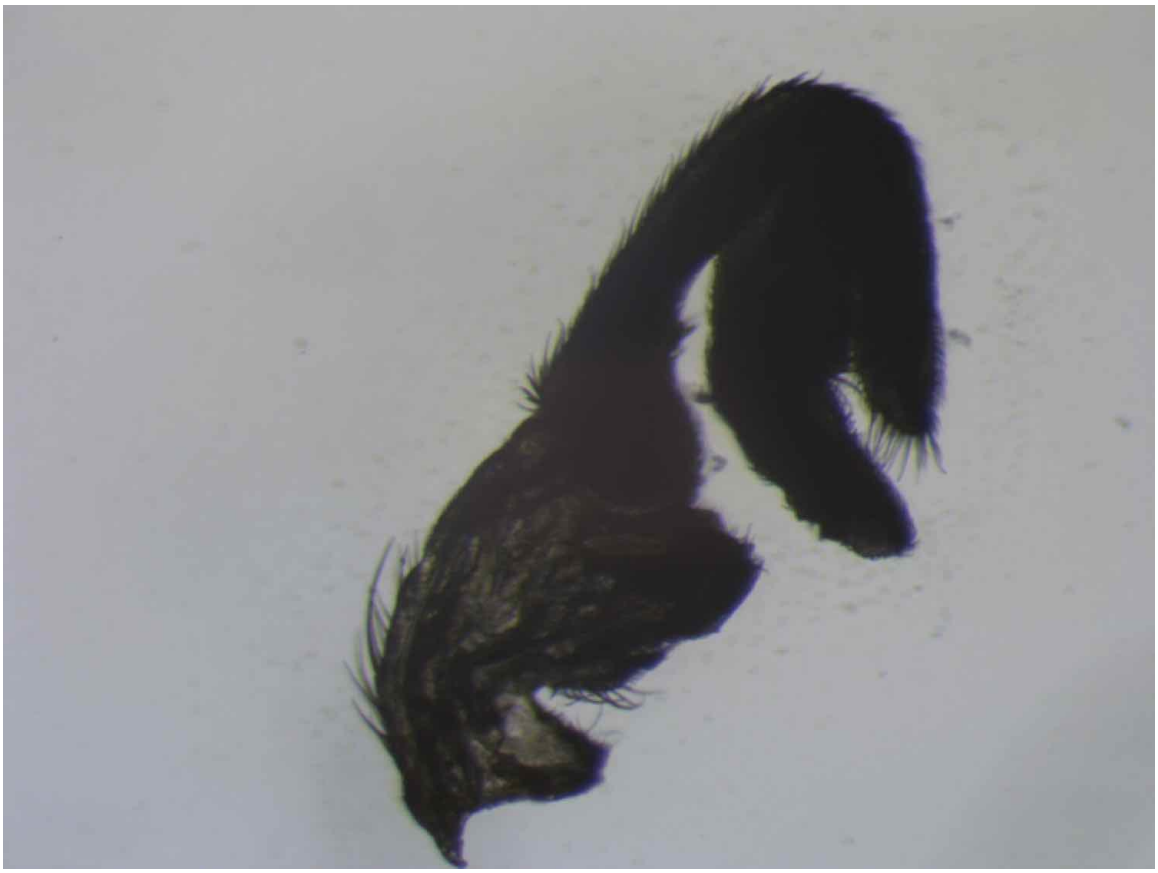

Figure 3. Notched *loq* mutant wing

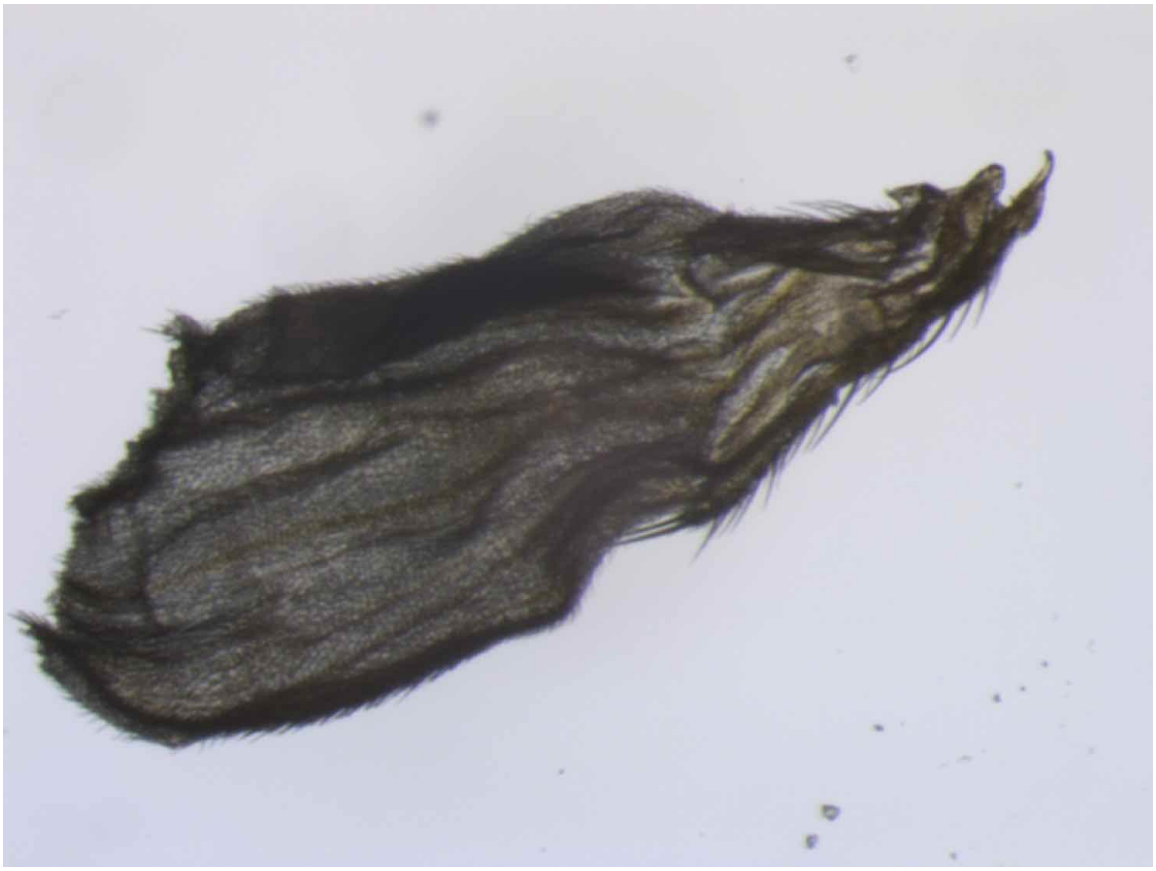

Figure 4. *CG17386* mutant wing

Figure 5. *CG17386* mutant fly

Figure 6. Normal wild-type wing