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# Bursicon, Metamorphosis and Development of Resilin in the Fruit Fly

David Paul Anthony Neff  
dneff@marshall.edu

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# **BURSICON, METAMORPHOSIS AND DEVELOPMENT OF RESILIN IN THE FRUIT FLY**

A Thesis submitted to the Graduate  
College of Marshall University

In partial fulfillment of  
the requirements for the degree  
of Master of Science in Biology

by  
David Paul Anthony Neff

Approved by

Simon Collier, Ph.D., Committee Chairperson  
Sasha Zill, Ph.D.  
Victor Fet, Ph.D.  
Michael Norton, Ph.D.

Marshall University  
May 2012

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## Abstract

Hovering flight is the primary form of locomotion in the numerous and diverse forms of winged insects. A specialized elastic protein, Resilin, contributes to flight mechanics in most described insect orders. Although many studies have inferred functions of Resilin in insect flight, we report the first direct evidence that Resilin is a necessary component in flight mechanics of the fruit fly. We have successfully knocked down Resilin synthesis with RNAi and have generated a new *resilin* deletion mutant *Df(2R)anhedra*<sup>1</sup>. By disturbing the formation of the Resilin rich tergopleural tendon, we have shown that Resilin functions in wing control. We also use RNAi based techniques to show that the Bursicon hormonal pathway which governs final stages of metamorphosis in flies does not play a necessary role in Resilin maturation. Bursicon may, via one of its subunits (Burs), play a previously unknown role in eclosion behaviour of *D. melanogaster*.

## **Background**

### Elasticity in locomotion

A minimum requirement for reproductive success in terrestrial animals is the ability to maintain stability under the force of gravity and to produce efficient locomotion. The study of these abilities is the science of organism level biomechanics. Animals have evolved a variety of structural adaptations enabling motion; these include closed system hydraulics as in the echinoderms (e.g. starfish), open system hydraulics as in cephalopod molluscs (e.g. squid), and wavelike muscular movement in the gastropod molluscs (e.g. snails). Locomotion in insects and terrestrial vertebrates is produced by muscle attached to skeleton. Despite the difference in arrangement of the insect exoskeleton and vertebrate endoskeleton, the basic mechanisms of motion can be described in simple terms as a combination of actuators (muscles) acting through a system of levers and springs (skeletal components). It is neuromuscular control that allows animals to move effectively but many elastic arrangements in skeleton augment and simplify this control. Examples include retraction of foot in walking/running in cockroach (Frazier, et.al. 1999, Neff,et.al., 2000), wing folding in damselfly (Gorb, 1999) and during other less strenuous activities as in head suspension during grazing in large mammals and claw retraction in cats (Vogel, 1998, Russell and Bryant, 2001). In the aforementioned examples, elastic elements work in parallel with a muscle and can oppose muscular action thereby serving as a “passive” antagonist. Resilient skeletal elements when working alongside muscle can also provide rapid release of power that has been stored by more gradual expenditure of energy as in the latch and release mechanism of the flea’s jump (Lyons, 2011) or that of a grasshopper (Sasha Zill

personal communication). These examples are analogous to the drawing of a bow (the strings of which have historically been made from collagenous sinews) in preparation for firing an arrow.

Elastic elements often act in series with muscular action to increase efficiency of locomotion. Energy storage is provided by the spring-like extension and recoil of ligaments, tendons, and the muscles themselves. In insects and vertebrates, muscles insert and act on the skeleton through tendons. In walking, the result of force transmission from muscle through skeleton is propulsion of the animal not only in the horizontal plane but also vertically, which leads to both kinetic energy and gravitational potential energy (gravitational potential is converted to kinetic energy as the mass of the animal approaches the ground). In running, a considerable amount of energy is stored when biological springs are stretched upon foot plant and then partly recovered prior to leg lifting (in swing). Similarly, in insect flight the down-stroke of the wing opposes the force of gravity; potential energy can be stored when raising the wing and stretching elastic tendons, ligaments, and otherwise deforming the body wall (with energy contribution from force of gravity). These elastic deformations can store energy for use in the next down-stroke (Weis-Fogh, 1960). The forces of gravity and inertia that muscular actions oppose during one part of the gait serve to passively stretch tendons and ligaments during another.

The skeletons of both vertebrates and insects are a combination of rigid and flexible components. In vertebrates, various degrees of mineralization in bones, tendons, and ligaments provide rigidity. These skeletal tissues appear histologically as highly ordered helically arranged proteins (collagen) sometimes interspersed with hydroxyapatite

(calcium and phosphate) crystals (Ottani, et.al., 2001). Composition of insect skeleton is discussed in more detail below. In comparison to the skeleton proper, tendons and ligaments are typically not mineralized (or, in insects, sclerotized) and can readily both store energy and dampen forces. For example, some ligaments that form the arch of the human foot (plantar calcaneo-navicular ligaments) are essential during the high impact foot plant of human running. As the ligaments of the arch stretch, they absorb the shock of foot plant while simultaneously storing potential energy returned at the next lift off. This storage mechanism also applies to the gastrocnemius tendon during running in turkey though some tendons in running birds are highly mineralized (Freeman and Silver, 2004). Elastic mechanisms can also serve to increase efficiency in flight (especially energy intensive hovering flight of insects and hummingbirds). In hovering flight, elastic dampening of the wing's up beat (non-power stroke) can replace the muscular energy that may otherwise be required to slow the wing (King, 2010). The energy stored in the stretching of tendons and muscle is regained during down beat. In this arrangement the tendons and muscles are known as series elastic elements which are believed to be important in hummingbird flight (Wells, 1993) . Some specialized tissues in vertebrates such as the neck ligament (ligamentum nuchae) in grazing animals and the yellow ligaments (ligamentum flava) in our vertebral columns are made of a mixture of collagen and another vertebrate protein, elastin (Chalmers, et.al., 1999 and Morocutti, et.al., 1991). These ligaments are generally much more extensible than those containing only collagen and readily release stored energy as they return to their un-stretched length. In the case of grazers, the highly elastic ligamentum nuchae allows them to graze indefinitely with head suspended and to raise their heads quickly

with little muscular effort. Another notable example of an elastic mechanism requiring little muscular energy is the elastic tendon responsible for the retraction of the cat claw. Estimation of the elastin content of these cat ligaments is apparently not in the literature but the anatomy suggests that this ligament, like others containing elastin, is more extensible than those containing only collagen as in the turkey gastrocnemius (Russell, 2001 and Freeman, 2004). Elastic recoil has been shown to oppose promotion in the foot (tarsus) of the cockroach *P. americana* (Neff, et.al., 2000).

In addition to providing energy storage (for power amplification and work recovery), elastic elements can serve as shock absorbers (dampeners) either in compression or extension, thereby mediating muscular forces as they act on the skeleton. The fruit fly tendon that is the focus of this work appears to serve as a moderator of force application as it intervenes between the steering muscles and skeleton at the wing hinge of derived dipterans such as the fruit fly (see Andersen and Weis Fogh (1964) for first description of Resilin in Diptera). Results presented here indicate that the bulk of this tendon (here called the tergo-pleural tendon of *D. melanogaster* or TP tendon), is composed of the elastic protein Resilin; its structure, function and development during metamorphosis are the objects that inspired this paper.

### Histology of insect exoskeleton

Diversity of insect cuticle is seen in skeletal adaptations that have evolved in myriad different species. In holometabolous metamorphic insects, each species has evolved to produce at least two different basic cuticular morphologies; one in response to larval and one to adult life habit. The basic structure of the cuticle includes a basal lamina which separates the epithelial cells from the hemolymph. These epithelial cells

are believed to synthesize and/or regulate secretion of all cuticular molecules including proteins, chitin polysaccharides, and crosslinking molecules. The cells are approximately cuboidal (or a hexagonal variant of cuboidal) and when they are secreting cuticle they are known to have actin supported micro-villous projections (Payre, 2004). It is in the vicinity of this apical border that the molecules of the cuticle are deposited and further chemically modified (Weis-Fogh, 1970). The layers of the cuticle (as generally accepted) are the procuticle, the water impermeable epicuticle and a wax layer, cuticulin (figure 1). It is the procuticle that defines the mechanical properties of the exoskeleton and where chitin, cuticular proteins, and the elastic protein Resilin is found. Procuticle is further divided into exo- and endocuticle (figure 1B). Most of the endocuticle is reabsorbed and reused when an insect molts, whereas the normally highly sclerotized (hardened) exocuticle is what we see as the molted skeleton or exuvium. In the insect exoskeleton, as many as 100 different (many closely related says Vincent, 2004) proteins in conjunction with the polymer sugar chitin (poly N-acetylglucosamine) combine in a fiber composite arrangement to provide the skeletal specializations necessary for locomotion (figure 2A-D). At least three factors determine the rigidity and flexibility of insect procuticular structures.

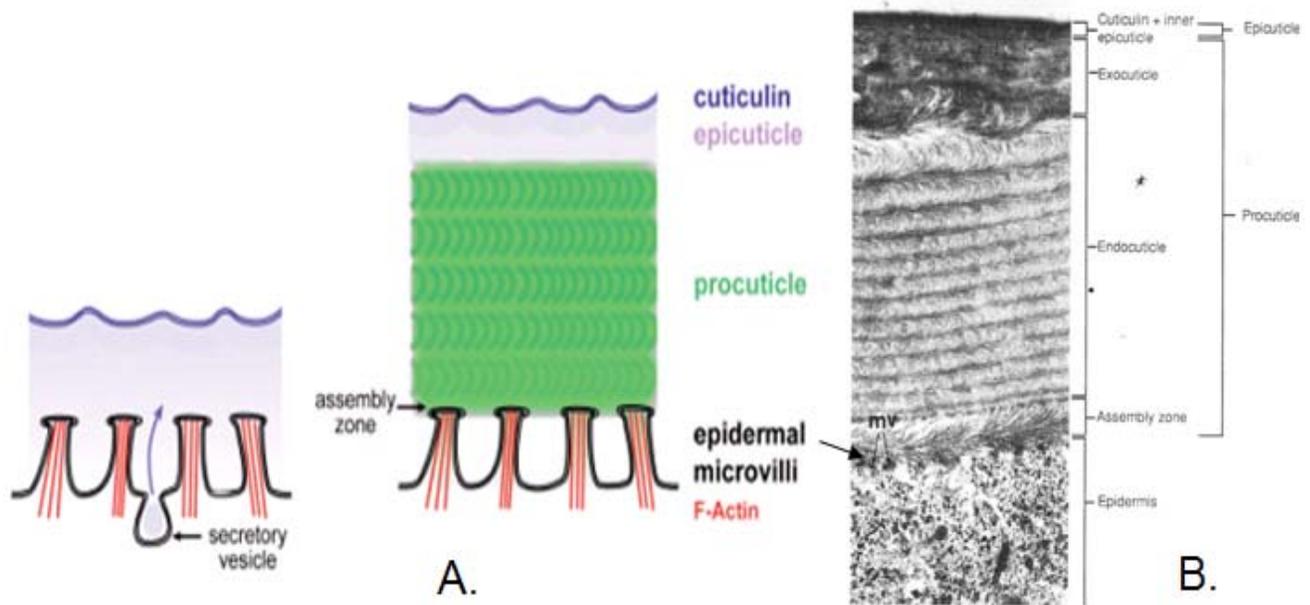


Figure 1. General structure of cuticle. A .Diagram of cuticular epithelium (epidermis)(Payre, 2004). Area above and surrounding the microvilli is believed to be location of deposition of chitin, cuticular proteins and the location of Resilin crosslinking (Weis-Fogh, 1970). Cuticulin is a poorly studied wax/protein layer, epicuticle is lipoprotein, fats, polyphenols, believed by Neville (1978) to have no chitin but shown by Fristrom to contain chitin (Fristrom and Fristrom, 1993). B. Transmission electron micrograph of *Drosophila pupal* cuticle from Fristrom(1993). Procuticle is divided into highly sclerotized exocuticle and less sclerotized endocuticle. According to Fristrom, this division no longer exists in adult *Drosophila*. However TEM of adult grasshopper cuticle (not shown) has endo- and exocuticle (Neville, 1998). The pupal cuticle seen in B. is 18 hours after pupal case formation, no scale available but entire thickness epithelium to epicuticle is ~50um.

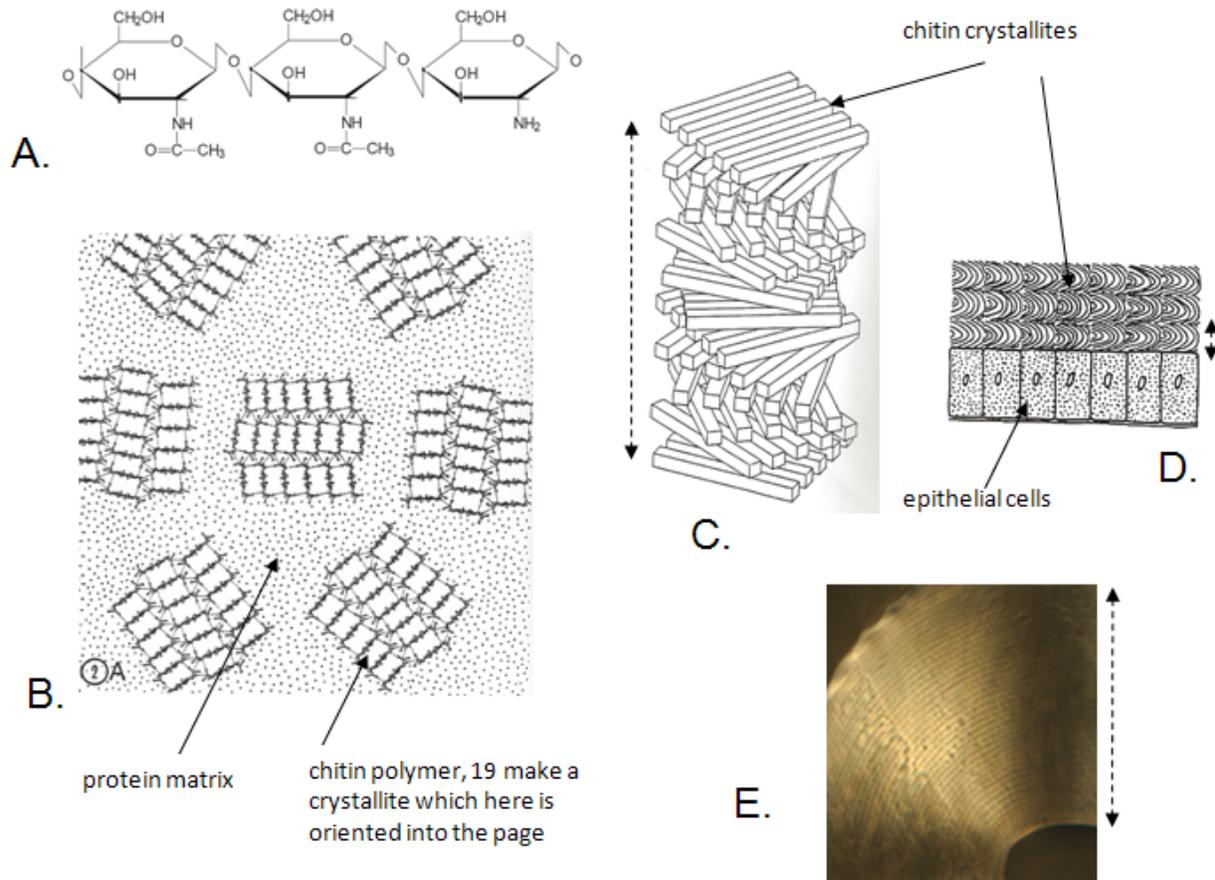


Figure 2. Molecular architecture of cuticle. A. A chitin polymer, this is 2 N-acetyl-D-glucosamine monomers with one glucosamine, chain oriented left to right (Klowden, 2007). B. 7 Chitin crystallite rods, each made of 19 chitin polymers, chains are oriented **into** page and are embedded in a protein matrix (Neville, 1998). C. Each bar is a chitin crystallite with the layers parallel to the surface of the insect. Each successive layer is slightly rotated relative to the last giving helical chitin architecture common in insect cuticle. The arcades (evidence of helical architecture) seen in D are formed of chitin molecules oriented roughly the same as in C (Neville, 1998). E (Neff/Zill unpublished) shows a frozen section of Resilin/chitin containing cuticle from cockroach leg (PLM image), each band visible is made of a series of layers of chitin crystallites as they rotate through 180 degrees. Each band is ~1 $\mu$ m thick for a total thickness of ~50 $\mu$ m. The protein that makes up the matrix in this tissue is Resilin. The double headed arrows are given for perspective: in C & D, arrow = one helical chitin layer (~1 $\mu$ m). E, arrow = 55 helical chitin layers (~50 $\mu$ m).

First, the orientation of chitin fibers that are present within the matrix of cuticular proteins are varied to alter and adapt the mechanical properties of the cuticle (figure 2B). Just as in a plywood, chitin fibers that are oriented parallel to the surface but at different angles relative to each other can provide resistance to forces from all directions (figure 2C and D). When sclerotized or hardened, this arrangement provides strength in the many load bearing plates (sclerites) of the thorax and legs. The same helical architecture can occur in un-sclerotized form which can be found in soft joint membranes and in some tissues helically arranged chitin is associated with Resilin (Neff,et.al., 2000, Frazier, et.al. 1999, figure 2E). In contrast to this helical layered arrangement, simple tendons (termed apodemes) typically have chitin fibers oriented along their length (somewhat like the arrangement of collagen in vertebrate tendon) so they can efficiently transfer muscular forces to their insertions.

The second determinant of cuticular stiffness is cuticular sclerotization and tanning (referring to the darkening that often accompanies the hardening). Crosslinks are formed between the non-Resilin proteins in cuticle (and possibly to chitin though evidence is thus far lacking (Vincent, 2004)) via the two chemical mechanisms known as B-sclerotization and quinone tanning, these chemistries are pictured in figure 3 (Klowden, 2007) and involve crosslinks formed through tyrosine and derivatives. The formation of crosslinks is regulated by epithelial cells as the compounds are actively transported to the cuticle from hemolymph. Both sclerotization pathways lead to crosslinking of cuticular proteins, likely via their lysine and histidine groups (Andersen, 1996). In a related synthetic pathway, production of melanin is controlled and

determines the degree of tanning (some lighter coloration can exist without melanin (Andersen, 1996). Sclerotization is governed by the peptide hormone Bursicon, which is secreted from the nervous system of the fly, is responsible for increased uptake of tyrosine by epithelial cells<sup>1</sup>. In preparation for Bursicon signal, the steroid hormone ecdysone apparently up-regulates enzymes responsible for conversion of tyrosine to chemically active crosslinkers such as dopamine (Klowden, 2007 and Honegger, et.al., 2008). In most insect forms, the exocuticle is more heavily sclerotized than the endocuticle (Neville, 1978 and 1998) and is shed by the animal at molting. However, Fristrom (1993) states, that in the adult fruit fly, there is no longer any differentiation between the two layers. His evidence is that, in the adult fly, the protein composition of the two layers is identical. Other adult insects (e.g. flour beetle) show persistent difference in adult morphology of these layers (Neville, 1966). Just after ecdysis in cockroaches and locusts, mature Resilin can be found both in the newly shed exuvium and in the cuticle of the newly formed or teneral adult (Andersen, 2004 and personal observation in cockroach exuvium). The significance of these layers to protein deposition in tissues of the fruitfly wing and wing hinge is unclear and should be clarified by future studies.

The third type of biochemical specialization that determines mechanical properties of cuticle is the presence of the protein Resilin. This protein occurs either in its pure form or complexed with chitin. An example of this chitin/ Resilin mix can be

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<sup>1</sup> Tyrosine is a necessary precursor to sclerotization pathways and is not synthesized by insects. This makes tyrosine useful in pulse chase experiments that track where and when tyrosine is incorporated into proteins or between proteins in sclerotization). The micrograph in Appendix figure A2 is from such an experiment.

seen in the section of cockroach tibiotarsal joint membrane in figure 2E. Wherever Resilin occurs, it gives the cuticle one or more characteristics that can be broadly be

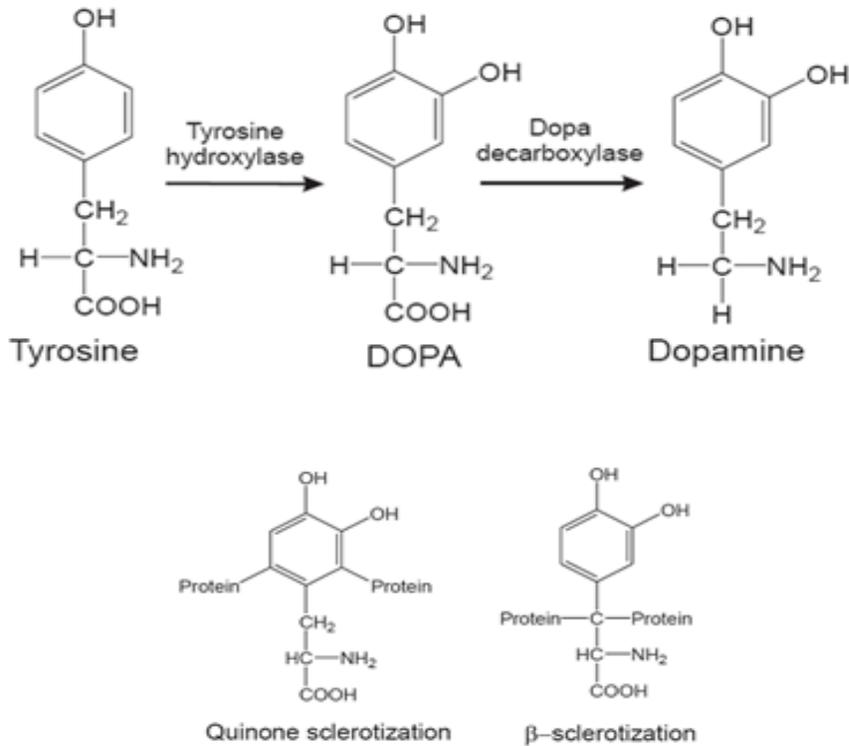
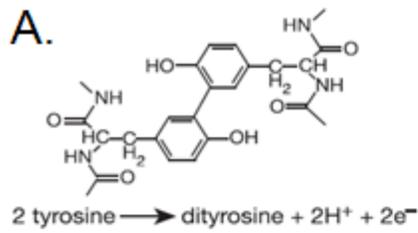


Figure 3. Chemistry of sclerotization. Precursors (top) and sclerotization products (protein crosslinks, bottom) common in hardened cuticle (Klowden, 2007). These are not the same type of tyrosine crosslinks seen in Resilin containing tissue. The precursors for these molecules are actively transported from the hemolymph by epithelial cells (Andersen, 1996).

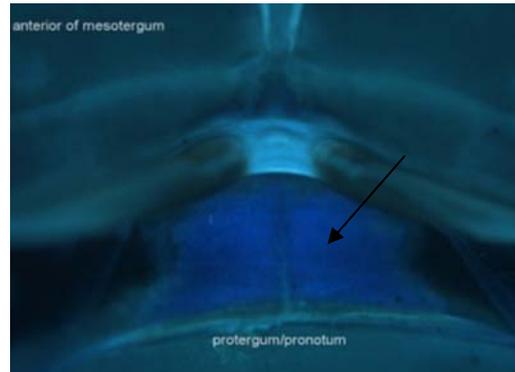
described as elasticity (see following text for some physical aspects of elasticity).

Tissues containing Resilin or Resilin/chitin composite are perhaps most notable for their apparent lack of other proteins and absence of sclerotization. These tissues appear

glass like and are always soft and flexible when hydrated. Instead of the mixture of DOPA (dihydroxyphenylalanine) and dopamine based crosslinks found in hardened cuticle, the polypeptide chains of Resilin are linked with di- and tri- tyrosine compounds that are the side chains of the peptide itself (Andersen, 1966). These crosslinks fluoresce brightly under UV illumination (as seen in figure 4) and serve as a molecular marker in our search for post-deposition modification of Resilin.



B.



C.

/gly-gly-arg-pro-ser-asp-ser-tyr-gly-ala-pro-gly-gly-gly-asn/ x 2

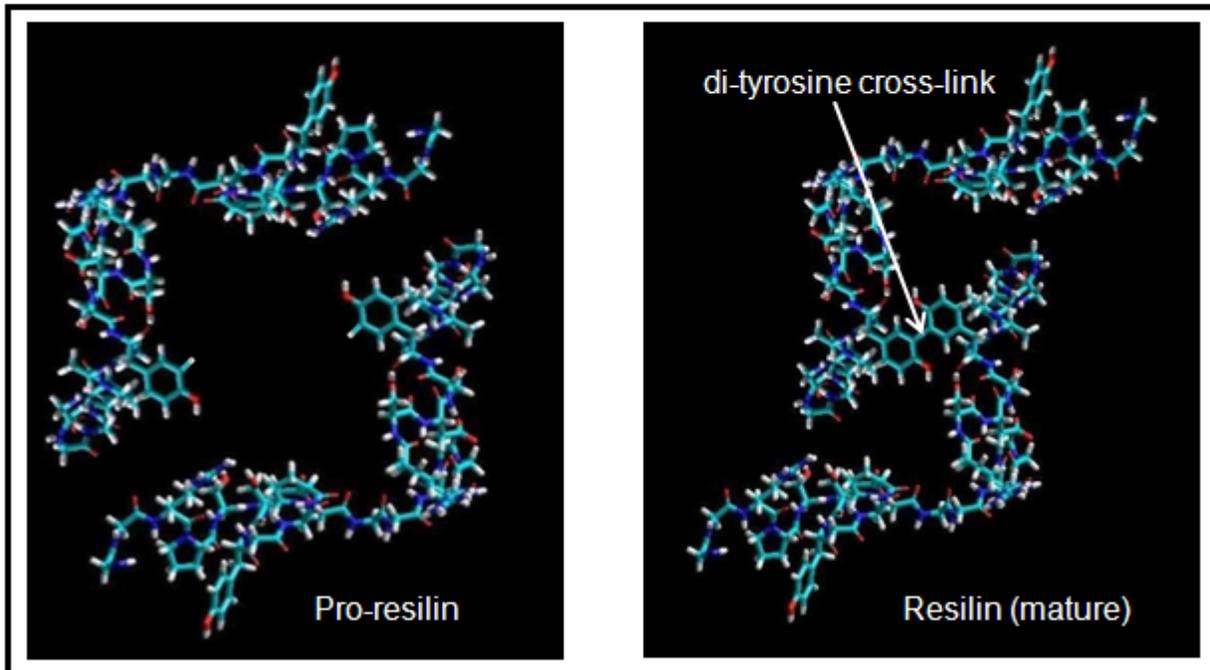


Figure 4. Crosslinks in resilin. A. Crosslinking chemistry that occurs in vitro and in vivo. The diagram is from Elvin et al., 2005 in which they crosslinked rec-1 resilin with UV light and a catalyst. B. Fluorescence of di-tyrosine in adult cockroach pronotum (head shield) joint membrane (arrow). C. Hyperchem model of rec-1 Resilin peptide(GGRPSDSYGAPGGGN) with di-tyrosine crosslink shown (arrow) and highly conserved sequence in red at top of C. This molecule was computer rendered at Marshall U. Norton Lab, MBIC; light blue; carbon, dark blue; nitrogen, red; oxygen, white; hydrogen.

## Resilin in biomechanics of insects

The elastic protein Resilin has been inferred to serve various functions in many orders of insects including noisemaking in cicada (Hemiptera) (Bennet-Clark, 1997), jumping in cricket (Orthoptera, unpublished observation), wing folding in beetles (Coleoptera) (Haas, et.al., 2000), and foot lifting in cockroach (Blattaria); see (Frazier, et.al., 1999 and Eldijk, 2011 for a more comprehensive review). Resilin plays a special role in insects; the insects as a group (clade) are defined largely by their ability to fly, and this mode of locomotion requires many skeletal specializations. Notable in the study of insect skeletal elasticity are the examples of Resilin found in their flight mechanisms. Resilin has been identified in wing associated structures of dragonfly (Odonata) and grasshopper (Orthoptera), and as mentioned above, occurs in the more complex flight mechanism of the true flies (Diptera) (Andersen and Weis-Fogh, 1964). An especially interesting case is that of the flea (Siphonaptera) (Bennet-Clark, 1967) in which loss of flight is a derived character. The flea uses the elastic storage capacity of pads of Resilin to slingshot itself into its remarkable high jump. These pads are derived from the same elastic wing base (the basalar sclerite and associated flexible connections) found to contain Resilin in grasshoppers and presumably a flying ancestor of the flea. Figure 5 shows family level diversity in some of the major orders of insects (Hexapoda); Resilin is present in all of these distantly related orders. Resilin is also present in the decapod crustaceans (Burrows, 2009), so the most parsimonious explanation would suggest a single evolutionary origin of Resilin well before the radiation of hexapod insects (presence of Resilin is a symplesiomorphic character of

insects). Insect flight as we know it has evolved in a context that includes prominent tendons and connectives rich in the protein Resilin.

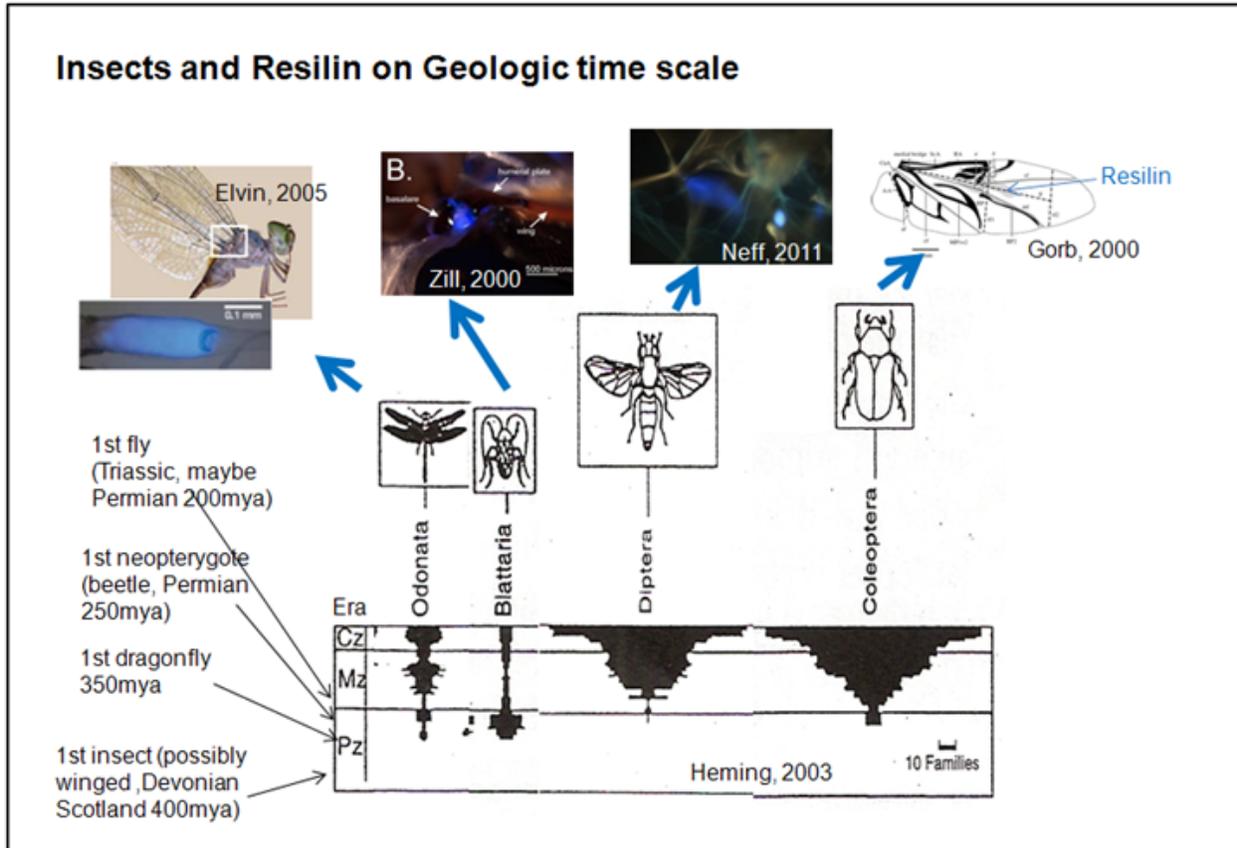


figure 5: Examples of Resilin in the flight mechanisms of 4 familiar insect orders. The most parsimonious explanation is that Resilin has been part of the insect wing/thorax since flight first evolved in the Paleozoic. Resilin's presence in crustaceans and other invertebrate clades imply that it is a shared ancestral character (symplesiomorphic) in flying insects.

In insects, muscles that power and control flight either insert onto the wing itself (direct flight muscles, DFM) or onto the thorax (indirect flight muscles, IFM). The dragonfly's downstroke is powered by direct flight muscles inserting on the bases of the wings, whereas the upstroke is an indirect mechanism (Simmons, 1977 Neville, 1960). In the fruitfly (Diptera), flight muscles transmit power to the wings through distortion of the thorax which behaves as a resonant, mechanically integrated "box". However, flight trajectory in flies is controlled by steering muscles (mostly DFM) that insert directly onto the wing base and associated sclerites (Wisser and Nachtigall, 1984). This arrangement is diagrammed in figure 6 (Dickinson, 2006).

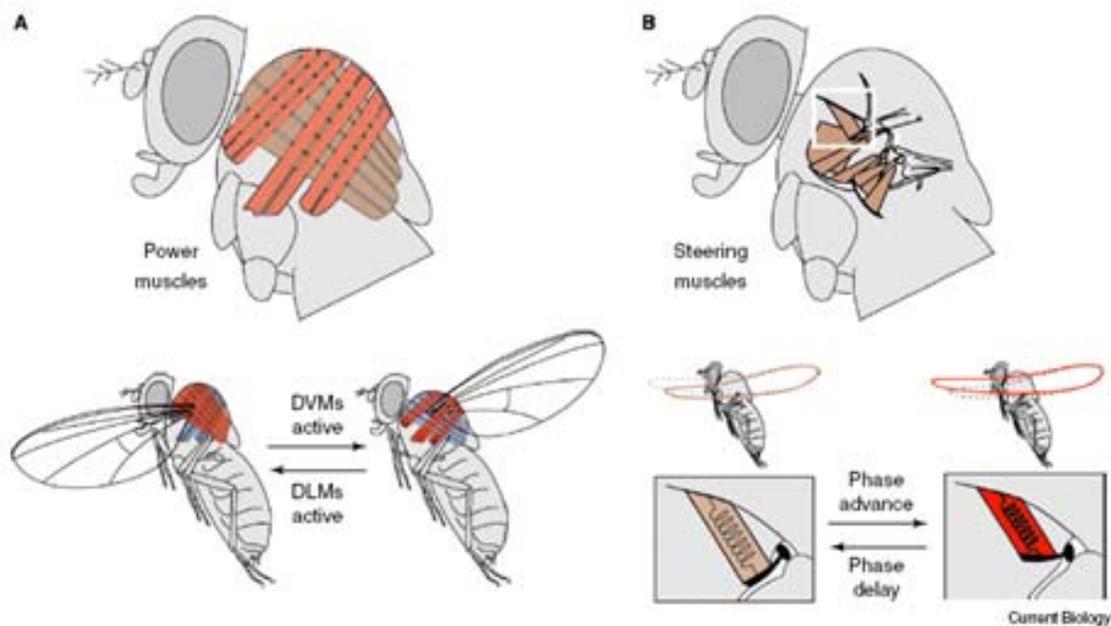


Figure 6. Flies use indirect flight muscles (IFM) to power wing beat via thoracic distortion (left) and direct flight muscles (DFM) including the tergopleural (TP) muscles to control wing trajectory. The basalar sclerite (black bar) and basalar muscles are shown at right (Dickinson, 2006).

The dragonfly (a primitive winged insect) is an archetype for directly driven pterygote flight mechanics. Its relatively low frequency wing beats (<100Hz) are governed directly by neurally driven contraction of muscle; that is, one contraction = one wing beat. These DFM acting on the wing are known as synchronous flight muscles. Flies, however, have wing beat frequencies up to or greater than 500 Hz (200Hz is typical of fruit flies (Weis-Fogh, 1972)). The wing beat frequency in flies is defined not only by neuromuscular control but by the resonant mechanics of the thorax, wings, and elastic elements of the exoskeleton (Wisser and Nachtigall, 1984 and Chapman, 1998). In flies, as stated above, IFM contractions supply the power for flight via the thorax. These muscles are known as asynchronous flight muscles because muscular activity is governed not by a one-to-one relationship between action potential and contraction but rather by stretch activation resulting from thoracic distortion (this type of contraction is also known as myogenic). Even this asynchronous force production is ultimately under neural control; the fly must start, stop and maintain asynchronous contraction by adjusting muscle chemistry with neural input (Dickinson, 1997).

In both dragonflies and dipterans special elastic tendons are found in series between direct flight muscles and the wing. It has been proposed that this arrangement contributes to the energetics of flight in both of these groups (Weis-Fogh, 1960 and Wisser and Nachtigall, 1984). Some of these tendons are made of the elastic protein Resilin. In both fly and dragonfly, the low elastic modulus and high efficiency (resilience) of Resilin allows these tendons to stretch and return the energy of that stretch without material fatigue or excessive heat production (King 2010).

Both anatomical position and morphology of the Resilin tendon of the 3rd subalar depressor (aka pleuro-subalar) muscle in the dragonfly make it reasonable to infer that it does store some energy during flight. The Resilin tendon in dragonflies forms the insertion of a small DFM on the posterior-proximal base of both fore and aft dragonfly wings. This 3rd subalar depressor muscle (Simmons, 1977 abbrev. pm3) supinates the wing allowing the animal to fly backward and hover (Neville, 1960 ). It does so via slow, steady isometric contraction; i.e. shortening of the 3rd subalar muscle leads to stretching of the average length of the Resilin tendon (during the upstroke) which leads to forces (defined by the Resilin 'spring' under tension) applied to the base of the wing causing supination. Interestingly, the larger more powerful subalar muscles which power the wing beat insert on the wing via relatively inextensible tendons more akin to a tough collagen tendon in their elastic properties. In 1960, Neville surgically cut the 3rd subalar tendon (he did not know at that time that it was made of a Resilin) of the 3rd subalar muscle and demonstrated that supination of the wing was deficient. In this however, he was unable to decouple the effects of complete muscle ablation and that of elimination of the Resilin tendon alone.

The presence of the tergopleural tendon (TP tendon) described in this study has been known in larger dipterans such as the blowfly for almost 50 years (Weis-Fogh and Andersen, 1964). They postulated a function similar to that of the dragonfly tendon discussed above. However, this and all other assertions (in the growing body of Resilin literature) as to the function of Resilin in energy storage and its importance to insect flight, though compelling, are inferential. In this study, we present direct evidence for the importance of Resilin to flight in fruit fly.

## Molecular properties of elastic proteins

Both compliance (extensibility and elastic modulus, box below and table 1) and resilience are properties of elastic structures that function in the locomotion of animals; molecular architectures must match function (Gosline, 2002). Elasticity is most often defined as the ability of a structure to readily and reversibly change shape (e.g. stretch and recoil of a tendon). Resilience is simply the measure of a structure's efficiency in storing and returning (as work, not as heat) the input of mechanical work that caused its elastic deformation. In animals, these attributes result from specialized arrangement of proteins in the skeleton. Notable among these proteins are collagen, elastin and the arthropod protein Resilin. Collagen, though evolutionarily ancient and in all major animal phyla,<sup>2</sup> and elastin are important mostly in terms of their contribution to vertebrate biomechanics. Vertebrate collagen type I predominates in tendon, ligament and bone so plays a greater role in energy storage during locomotion than other collagens (Ottani, 2001). Type I collagen is a highly ordered molecule with a repeating 3 amino acid motif (usually Glycine-Proline-Hydroxyproline or Glycine-Proline-Proline) that give it a distinctive higher order structure, three helical molecules in turn twisted into a second larger helix (Freeman, 2004) Unlike tissues in insect exoskeleton, if these collagenous tissues are damaged they can be renewed during the lifetime of an adult vertebrate.

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<sup>2</sup> Although collagen as a component in extracellular matrix is evolutionarily ancient (found in cnidarians and arthropods), the highly ordered super-molecular structures of tendon and ligamentous collagen evolved and functions efficiently in vertebrate animals (Boot-Handford, 2003)

Table 1 shows some material properties of collagen including its exceptional storage capacity to mass ratio when compared to steel, elastin, or Resilin (Gosline, 2002). There is evidence that collagen can store some energy entropically (Misof, 1997) (entropy is discussed below in context of elastin and Resilin) but it is believed to store most elastic potential energy in bond strain energy (Freeman, 2004) during changes in the higher order structure. The molecular bonds when deformed store energy and give collagen high resilience (90%) but they restrict extensibility to <1/10th that of Resilin and elastin (table 1). As discussed below, given the anatomy of insect wings and thorax, this degree of elongation is not sufficient to allow a functional wing beat.

	Resilin	Collagen	Elastin	Spring Steel
elastic modulus: stress/strain (Gpa)	.001 (Chalmers, 1999)	1.2	.0011	200
Resilience: Ein / Eout	92% (Weis-Fogh, 1961)	90%	90%	99%
energy storage capacity: (J/kg)	40-460 (from different insect tissue types, Weis-Fogh, 1960)	1000	95	115
extensibility: (max length/original length)	1.9	.13	1.5	.008

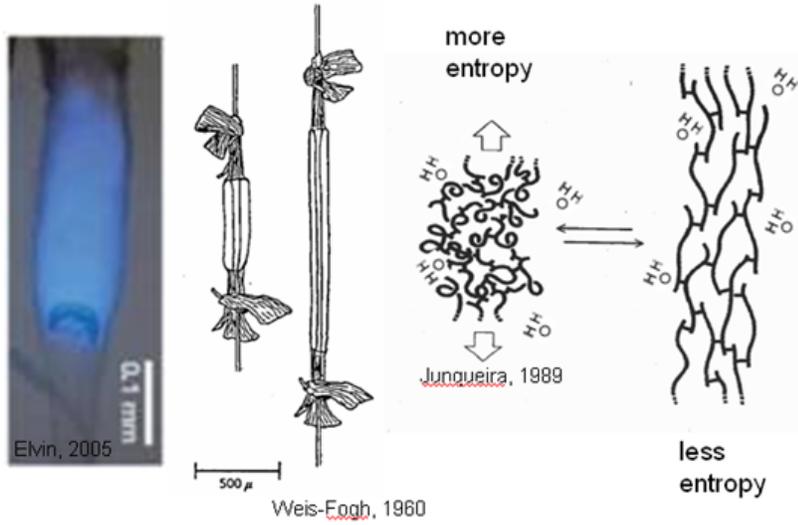
Table 1. Elastic properties of some materials discussed in the text.. Source of values, if not indicated, is Gosline et al. (2002)

In contrast to collagenous skeletal tissues in which energy is stored in bond strain via higher order supermolecular structure, Resilin and elastin are believed to act as true protein rubbers (Weis-Fogh, 1961) and, as such, have a different mechanism of energy storage. In their unstrained state, rubbers (including protein rubbers) are usually modeled as randomly coiled molecules that are crosslinked with each other with some regularity. They become uncoiled when strained (stretched) and are both resilient and extensible (figure 7A). Thermodynamic experiments with protein rubbers elastin (Gosline, 2002), Resilin (Elvin, 2005), and dragline spider silk (Gosline, 1984) have demonstrated that potential energy is stored through changes in the entropic state of these molecules. Essentially, an un-strained (relaxed) elastin or Resilin molecule has many degrees of freedom or possible conformations; it can be considered a liberated molecule<sup>3</sup>

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<sup>3</sup> Gosline (1984) studied the thermodynamic properties of dragline spider silk which does have a regular crystalline structure as seen by x-ray diffraction. He showed that this silk, when hydrated and strained, stores most of this strain energy entropically and concluded that dragline silk is composed of crystalline domains that constrain rubberlike domains with random coil configuration. A basic equation that he and Weis-Fogh (figure 7B) used for their experiments is:  $f = (\Delta E/\Delta L)_{T,V} + T(\Delta f/\Delta T)_{L,V}$  where it is assumed that the elastic material is of constant composition,  $f$  = recoil force,  $E$  = molecular internal bond energy,  $L$ =length,  $T$ =absolute temperature,  $V$ =volume. To summarize, total force of recoil ( $f$ ) equals force of recoil due to internal bond strain i.e.  $(\Delta E/\Delta L)$  plus force of recoil due to entropy changes i.e.  $T(\Delta f/\Delta T)$ .

A. **dragonfly tendon**



B.

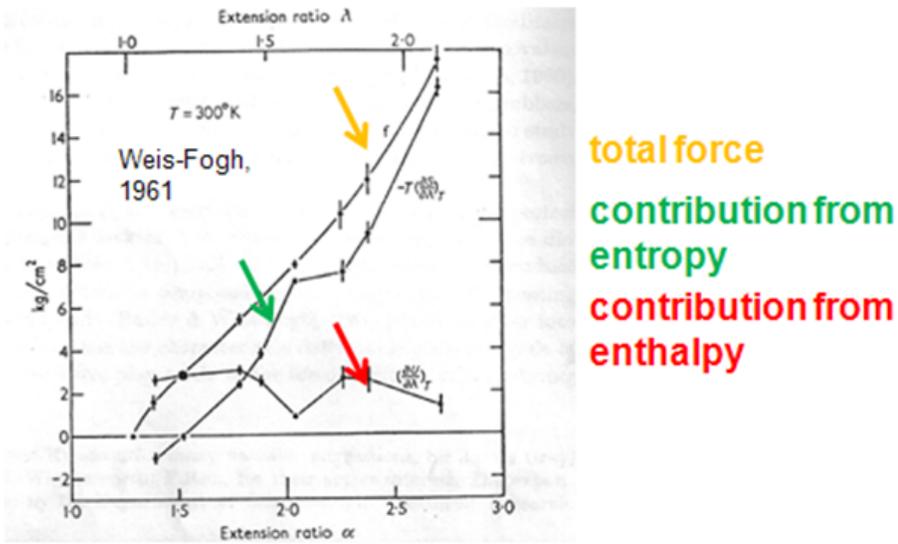


Figure 7. A, left shows experimental setup of Weis-Fogh in which he measured the elastic properties of dragonfly tendon made of nearly pure Resilin. At right is a diagram depicting a model of the entropy changes that occur when a true protein rubber (such as Resilin) is stretched. B shows data from Weis-Fogh's thermodynamic experiments on the dragonfly tendon – the raw data in these experiments are measurements of the relationships between force produced, absolute temperature and strain.

*Below are some relationships of physical properties discussed in the main text and in table 1. Although these properties apply to many different biomechanical geometries, it is most intuitively obvious to think of these terms as they apply to a tendon being pulled by muscular force:*

***stress** - force applied or returned / unit area (Pascal: 1 N/m<sup>2</sup> = 1Pascal or 1Pa)*

***strain** - change in length / original length (no units)*

***elastic or Young's modulus** - stress / strain (units here are also Pa)*

***elastic efficiency (resilience)** - energy out (recoil) / energy in (stretch) (no units)*

$$G_{total\ stored} = E_{internal} - T(S)$$

*T is the temperature (kelvin)*

*S is the entropy (joule per Kelvin, measure of conformational freedom or order)*

*E<sub>internal</sub> is energy stored bond strain (joule)*

*G<sub>total stored</sub> is total energy stored in an elastic tissue (joules, also Gibbs free energy)*

In all proteins, the peptide bonds have a partial double bond nature and cannot rotate, but, in the absence of secondary structure, the N-C $\alpha$  and C- C $\alpha$  bonds are not hindered by higher order structure and rotate freely. In Resilin (no secondary structure) as these peptide networks are stretched, the molecules straighten and become constrained (have less possible conformational states). When released, the chains again tend toward the disorder of higher entropy thereby returning mechanical energy. There are alternative hypotheses to the random coil mechanism (the sliding  $\beta$ -spiral, a series of proline dependent mobile  $\beta$ -turns, for example (Nairn, 2008)); all of these models for Resilin also involve gain and loss of stored energy primarily in terms of entropy changes. This thermal agitation based analysis of elastic proteins has been

carried out rigorously by Gosline et al. (1984) for spider silk and by Weis-Fogh for Resilin (1960). Their treatment of elastic rebound using force/distance curves and varying temperature allows accurate estimates for the entropy and internal energy contributions to energy storage. An excellent test object for these tests is Resilin in its purest known natural form from, dragonfly wing tendon (figure 7A). This tendon served as the control for the mechanical tests on recombinant crosslinked Resilin carried out by Elvin et al. in 2005. The preceding box (and footnote 3) shows some basic biomechanical relationships and quantities from which the formulae used in the aforementioned mechanical analyses of Resilin were derived. Figure 7B is data charted by Weis-Fogh (1961) which shows the relative contributions of enthalpy and entropy to the energy stored in dragonfly tendon Resilin. As postulated in the 1960s by Andersen and Weis-Fogh, unstrained Resilin is still generally believed to exist in random coil configuration.

As we can see in table 1, the properties described above give Resilin an efficiency of energy storage >90%. However, it is the extensibility of almost 200% (final length is 3x original length before failure) combined with high resilience of Resilin that has given the flying insects a molecular tool with which to modify and improve flight mechanics. Collagen has excellent resilience (when highly organized in tissues) but a tendon restricted to ~13% elongation would not allow for a workable wing stroke. King in 2010 showed that max % elongation (essentially strain) of the Resilin tendon in dragonfly (wing displacements within range of normal dragonfly wingstroke) is 160% in the hind wing and 250% in the forewing.

In mature elastin, molecules are crosslinked via lysine residues; this process has been described (Gacko, 2000) and includes cell directed assembly and inter-peptide linking of lysine residues by lysine oxidase in order to properly form the crosslinks. The crosslinks in elastin are called desmosine, isodesmosine, and lysinonorleucine (Gacko, 2000). Tropoelastin (un-crosslinked, immature elastin) variants are rich in short runs of proline and glycine, and valine residues. These sequences do not have as predictable a pattern of repeats as found in collagen but, as alluded to above, there is evidence for B-turns and B-spirals induced by the VPG repeats present in natural elastin and especially in elastin inspired engineered peptides. However, the presence and nature of higher order structure in natural elastin is still debated. (Eldijk, 2011)

Resilin is also rich in proline and the smallest amino acid glycine. Eldijk in his 2011 review states that Resilin is less likely to have any higher order structure of B-turns and B-spirals than elastin. In 2001, S.O. Andersen identified the first *resilin* gene in the fruit fly *Drosophila melanogaster* (Andersen, 2001 and Elvin, 2005). He did so by deducing genomic sequence from a partial digest of grasshopper Resilin (with basic logic alignment search tool or BLAST). A tyrosine-glycine-alanine-proline (YGAP) motif is conserved in all Resilins found to date (including mosquito and cockroach); two *Drosophila* peptide sequences from two different transcripts of the *resilin* gene are shown in figure 8. The YGAP sequence is part of a larger 15 a.a. conserved repeat found in fruit fly by Anderson and shown in figure 4. The first engineered *resilin* gene fragment to be cloned and translated (*rec-1 resilin*) is the same 15 amino acid peptide repeated 17 times in the sequence designed and published by Elvin in 2005. Elvin showed that this recombinant Resilin behaves much like that in natural dragonfly

tendon. The Hyperchem model pictured in figure 4 includes this conserved sequence including the crosslinks formed by tyrosine residues.



Figure 8. Peptides encoded by *resilin* transcripts. *Resilin* gene is in blue, the two protein products are Resilin-PB at top and Resilin-PA at bottom. Notice the tyrosine-glycine-alanine-proline containing repeats (additional glycine is present in all of these fruit fly repeats). YGAP is conserved from cockroach to mosquito to fruit fly. In the unprocessed protein, there is also an N-terminal export signal sequence. In Resilin-PA there is also a chitin binding domain called the Rebers Riddiford consensus sequence domain (Andersen, 2011) that is present in many cuticular proteins.

Since the early 1960s (Andersen, 1964) di-tyrosine (and tri-tyrosine) has been known to form the crosslinks in Resilin; Andersen reported that di- and tri-tyrosine is not found in non-Resilin containing cuticle. In a 2004 paper, he does report finding di- and tri-tyrosine molecules at low levels in some non-elastic sclerites (e.g. the pronotum) and at higher levels in the walls of the tracheae in locusts. Crosslinks occur with different frequencies in different insect tissues, but, in the locust, the wing hinge shows by far the most (12x the next highest level) of these crosslinks per mass of tissue (Andersen, 2004).<sup>4</sup> Di- and tri-tyrosines occur between about 25% of the available tyrosine residues (Andersen, 1966) in naturally occurring Resilin and were created in vitro at 21% when Elvin et al. used UV light and a metal catalyst to crosslink their bio-engineered recombinant protein, Rec-1 Resilin (Elvin, 2005). These percentages are consistent with Andersen's first rigorous estimate of the inter-link peptide mass (~5000Da) and with hypothetical calculations. In figure 8 we can see that both translational variants of Resilin are ~600 a.a. long and have MW of ~55kDa with ~38 tyrosine residues per molecule. If ~25% or 9 of 38 tyrosines per peptide were involved in crosslinks, we would expect the inter-link peptide runs to weigh about 1/10<sup>th</sup> of the 55kDa total MW or about 5.5 kDa.

These tyrosine based crosslinks are important to the work presented here; as subsequent figures will show, they fluoresce brightly when excited by UV light and

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<sup>4</sup> Tri-tyrosine/di-tyrosine ratio has been proposed as a good way to measure degree of cross-linking (Andersen, 2004). A problem arises in mixed composition tissues when we do not know what proportion of that tissue is Resilin. In this case, **absolute** quantity of di- and tri-tyrosine will not indicate **degree** of crosslinking. So, assuming that to get tri-tyrosines one would need a certain density of di-tyrosines as starting material, Andersen uses the ration of tri-/di-tyrosine to indicate degree of crosslinking - the greater the ratio, the greater the degree of crosslinking.

through this fluorescence indicate maturation of Resilin. Maturation is defined as pro-Resilin being crosslinked into mature Resilin (figure 4). Mechanism and timing of this crosslinking during metamorphosis are central themes in this paper.

The adaptive modifications of flight mechanics allowed by Resilin come in many forms including wing hinges, specialized tendons, and wing vein junctions. Proper function of these structures must include programmed developmental control of Resilin deposition and cross-linking during metamorphosis.

#### Holometabolous (endopterygote) metamorphosis

Presence of wings is the defining character of almost all adult insects. In holometabolous, metamorphic insects such as fruit fly, these wings appear as the animal changes from pupal juvenile to adult; fully formed wings and associated structures result from rapid (in the fruit fly <4days) and complete maturation (metamorphosis) of larval tissues. In insects, it is the exoskeleton (known as cuticle and functioning as both skeleton and integument) that forms the wings and the mechanical framework needed for muscle powered wing movement. The rapid and well defined period of these events in the thorax of the fly is the object for the present studies in development and function of wing associated elastic tissues. We also focus on the hormonal control of escape behaviour of the newly adult fly (teneral adult) from the pupal case. This behavior is called eclosion and occurs via inflation of the ptilinum and the movements needed to wriggle free of the pupal cuticle (exuvium) and pupal case (the ptilinum is an airbag like structure found on the head only in higher dipterans and is expanded by pumping with hemolymph, see figure 9).



Figure 9. At ~40 mins. prior to eclosion, the nearly mature fly (strictly speaking between pharate and teneral) expands its ptilinum as seen at points 1, 2 and 3 and finally opens the door to its pupal case at time point 4 (frame interval is 15 minutes). Fruitflies (like house flies) are highly derived dipterans known in various phylogenies as sub-order Cyclorapha because of the round door in their pupal case and as Schisophora because of the schism that the remainder of the ptilinum leaves in their head after eclosion.

The model animal for our experiments is the fruit fly *Drosophila melanogaster* (English translation for this binomial classification is a dew loving fly with a dark abdomen). *Drosophila* and all dipterans are holometabolous, which means that, after hatching from its egg case, the larval animal feeds and grows in size through a series of larval molts; in the case of fruit flies, 3 larval stages or instars (Chapman, 1998). Figure 10A shows that the third larval stage ends in formation of a pupal case within which the larva metamorphoses into adult form (0 hour APF pupa seen in figure 10B). Formation of the pupal case from the larval cuticle is known as puparium formation (**not** pupation which is the formation of the pupal cuticle within the case) and by convention is set as time zero for metamorphosis. In the remainder of this paper, APF (known in other references as time after white pre-pupa (WPP)) will refer to time after puparium formation. A basic time line of metamorphosis is shown in the text box below.

The timing of events during metamorphosis in the pupal case is summarized below, all times listed are APF (see above) and are from Fristrom and Fristrom (1993) unless otherwise noted.

T minus 6hours; burst of ecdysone in larva, signal for pupation

T 0 hours; clock starts with larva stopping, everting spiracles; trachea separate from new pupal tracheae and in dipterans, larval cuticle becomes the sclerotized puparium (new evidence indicates that Bursicon mediates puparium tanning (Loveall, 2010), wing discs evaginate (Kiger, 2007), dorsal and ventral surfaces assume their adult orientation

T 16 hours; pupal cuticle complete (called pupation, this cuticle will never serve the animal in the outside world)

T 18-27 hours; cell packing is remodeled in the wing epithelium, significant increase in proportion of hexagonal cells; cell polarity determined and wing hairs form (Doyle, et.al., 2008)

T 18-36hours; pupal cuticle intact but epidermal cells rearranging into form of adult tissues, including wing. Separation of pupal cuticle from pharate adult underlying is called pupal-adult apolysis (Bainbridge and Bownes, 1981 Vevarysta, 2011 calls it pupal-adult ecdysis)

T 36-80hours; possible absorption of pupal endocuticle into pharate adult; epithelial cells secrete adult cuticle, it takes shape beneath intact pupal cuticle; the adult procuticle in the wing has no helical lamellae and is quite thin as compared to sclerite cuticle

96>T>80 Resilin deposited (and likely crosslinked)

T 96 hours; increase of ecdysone primes cells to produce enzymes mediating sclerotization

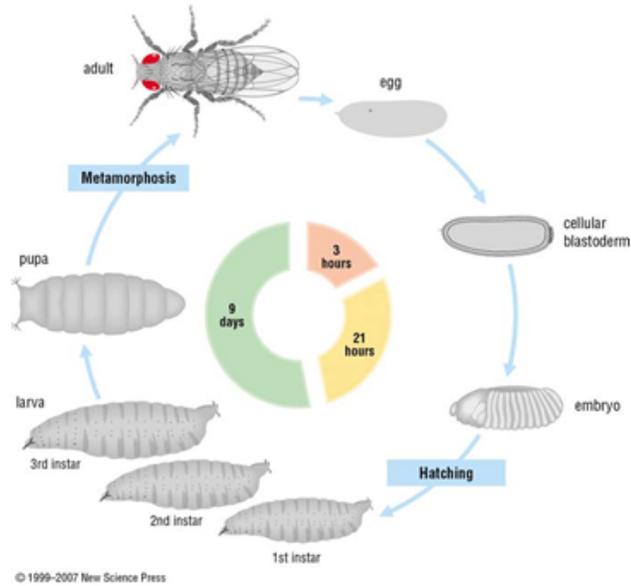
T 108 hours- just before adult emerges, a drop in ecdysone levels leads to EH, ETH, CCAP (and Bursicon) mediated adult/pupal cuticle separation (ecdysis) and emergence (eclosion) of adult fly

-eclosion from pupal case is performed via inflation of the ptilinum which pushes open the door of the pupal case (inflation begins 40 minutes pre-eclosion (Baker and Truman, 2002)

-wings unfold under influence of Bursicon via body contraction and resulting increase in hemolymph pressure – Bursicon induced sclerotization and wing remodeling proceeds and is complete within an hour or so (Kiger, 2007 and Davis, 2007)

A.

From *The Cell Cycle: Principles of Control* by David O Morgan



B.



Figure 10 . A. Scheme of holometabolous (also known as endopteryogotic) metamorphosis of the fruitfly. The focus of the descriptions given in the text are the final developmental stages while the animal is in the pupal case and during emergence and wing unfolding (from web resource, <http://www.new-science-press.com/browse/cellcycle>). B. Example of a fly at 0hour APF (after puparium formation) also known as WPP (white pre-pupa).

The final stage of metamorphosis for all winged insects is the expansion and maturation of the wings. When the cuticle becomes sclerotized, cell rearrangement and changes in tissue shape are no longer possible and metamorphosis (by definition) ceases. In the widely accepted scheme, the protein hormone Bursicon (original gene name *batone*, Latin from Greek – hide , as in skin) is secreted from central and peripheral neurons shortly after the newly formed adult fly leaves the pupal case (eclosion). Bursicon apparently signals the final steps in maturation of the wing as well as sclerotization of other adult cuticle (Natzle, 2008). Many studies have shown that Bursicon signaling mutants show delayed wing development and poor cuticle sclerotization. The processes involving Bursicon are presumed by most authors to begin at eclosion and to progress for some time (minutes to hours) after eclosion or emergence from the pupal case. Wing unfolding and sclerotization are generally complete by 80 minutes post-eclosion (Peabody, 2009) after which epithelial cells are no longer found in the wing (Kiger, 2007). The mature wing is semi-rigid (selectively sclerotized and reinforced with veins), two layered, and (with the exception of the wing veins and peripheral sensory nerves) acellular; it is an airfoil made of cuticle (Murray, 1995).

The wing, like other fly appendages, arises from imaginal discs which are already present in the larval fly. These discs not only differentiate into the structures of wing but also form associated thoracic structures (e.g. wing hinge, Morata, 2001). This fact is of practical interest in that the selection of genetic drivers depends on knowledge of what genes are being promoted in which tissues and when. For example, the *MS1096-Gal4* driver line (presented in the methods section) is supposed to drive *UAS* associated genes only in the dorsal surface of the wing and in some of the thorax. Another driver

line we used known as *apterous-Gal4*, shows expression in the wing and wing hinge. We are currently mapping its expression pattern. Whether or not the TP tendon with the wing itself is affected by these driver lines depends upon which parts of the imaginal disk develop in concert and which do so independently.

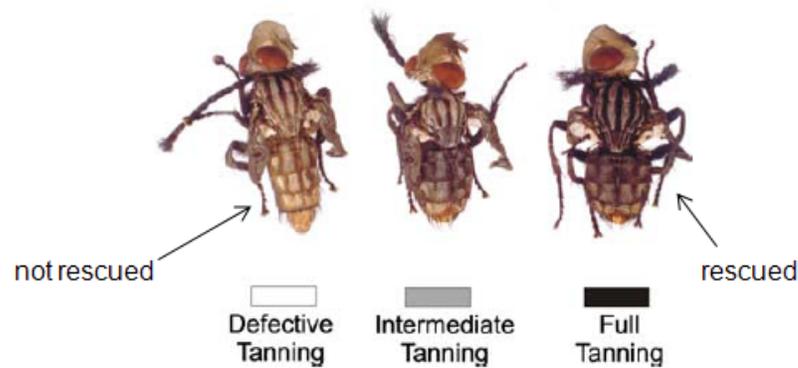
### The Bursicon hormonal pathway

The activity of Bursicon (then un-named) was first well described in the early 1960s by a series of experiments carried out in 3 large dipterans; 2 blowfly species and a fleshfly (Fraenkel and Hsiao, 1962). In these experiments, the post-eclosion environment was manipulated either by forcing the newly emerged flies to re-enter an empty puparium or by inhibiting centrally generated hormone signals via neck ligature (i.e. tying a human hair around the neck of a teneral adult fly, tantamount to decapitation (Cottrell, 1962)). They found that flies so treated did not properly sclerotize nor did their wings expand normally. When extracts of the hemolymph were taken from a normally maturing fly and injected into a decapitated fly, maturation proceeded as normal (minus the head of course). The hormone went unnamed until 1966 when Fraenkel, Hsiao, and Seligman (1966) isolated a 40kDa protein from teneral flies and named it Bursicon. In 2004, this protein was fully sequenced and cloned by Elizabeth Dewey (Dewey, 2004 and Luo, 2005). The MW of Bursicon (actually a heterodimer of Bursicon and Partner of Bursicon) is just over 30kD, Dewey used the classic neck ligature assay to cut off central governance and test the activity of her recombinant Bursicon dimer; in so doing, she observed the same sclerotization recovery more than 40 years after Frenkel's first observations.

By definition, eclosion from the pupal case and wing expansion are the heralds of sexual maturity in flies. Within hours of the start of the Bursicon program, flies are able to mate. From two aspects, the Bursicon signaling pathway in flies resembles anterior pituitary governance in sexually mature (or newly maturing) vertebrates.

First, like the vertebrate hormones LH and FSH, Bursicon (Burs) in combination with its structurally similar partner termed Partner of Bursicon (Pburs) is a heterodimeric cysteine knot protein (here called Burs-Pburs). Review of the proteomics source uniprot (<http://www.ebi.ac.uk/uniprot/>), suggests structural family relationship, not strict homology with vertebrate glycoproteins (figure 11B). In this family of dimers, both the secondary structure of each subunit and the tertiary structure of the dimer are defined largely by cysteine residues that have joined via di-sulfide linkage. In addition to LH and FSH, this family of proteins also includes transforming growth factor (TGF- $\beta$ ), platelet derived growth factor, and bone morphogenic protein. The genes for *burs*, *pburs*, and its G-protein coupled receptor *dLgr2* have recently been cloned and described (Dewey, 2004).

A.



B.

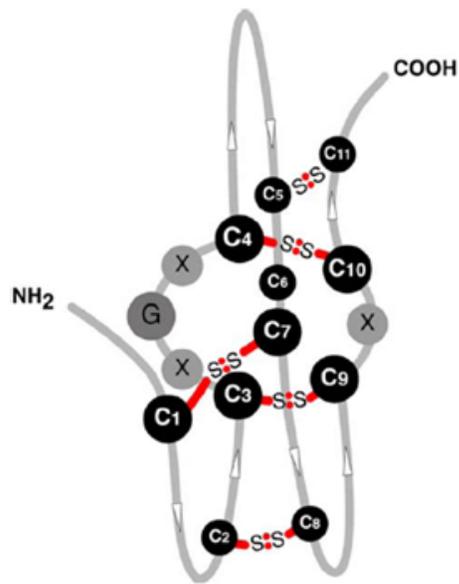


Fig. 2 Predicted cystine knot structure of BURS and PBURS. Adapted from Luo et al. (2005), Fig. 1b

Figure 11. A. Neck ligation assay by Mendive 2005; equivalent to decapitation which removes central (brain) control of maturation. Data not shown here indicate that rescue to full tanning is induced by injections of cAMP, recombinant Burs-Pburs, hemolymph of another teneral fly, or CNS extract, but not by Burs or Pburs subunits alone. Clearly, wing expansion is not rescued; even if behaviours were rescued, the flies could not properly gulp air.

B. Bursicon (Burs-Pburs) is first dimeric cysteine knot protein found in insects; it is homologous to human dimeric glycoproteins (from anterior pituitary) such as LH and FSH and TSH (Peabody, 2008) Shown is a molecular model of one subunit (from Luo, 2005). Di-sulfide bonds also participate in dimer formation.

The basic structure of the receptor for Burs-Pburs is also found in vertebrates as the LGR2 family. DLGR2 (Drosophila LGR2) is the receptor for Burs-Pburs (Mendive, 2005). Also known in the Drosophila community as Rickets (gene *rickets*), the DLGR2 protein is part of a large family of Leucine rich G-protein coupled receptors (LGR proteins) These include the anterior pituitary glycoprotein receptors (including those for LH and FSH) in vertebrates (Mendive 2005). LGRs have multiple transmembrane domains and use cAMP as second messenger; LGR2 is one among 8 classes of LGR. Mendive (2005) showed that both subunits of Burs-Pburs (produced and processed by the same cell) are necessary to activate the DLGR2 receptor and that the Burs-Pburs heterodimer shows no activity in other tested LGR (such as the human HLGR4). Despite these dissimilarities, at least some members of LGR family are probable homologues. Interestingly, in the neck ligature assay, cAMP alone in the absence of Burs-Pburs (and in absence of DLGR2 receptor) is capable of rescuing the sclerotization defects whereas Pburs or Burs alone (as monomers) cannot. (figure 11A, Mendive, 2005). Also, Truman and Baker (2002) have shown that in their experiments, cAMP does rescue sclerotization but does not rescue wing expansion, which implies that neuro-behavioral effects of Burs-Pburs act through a non-cAMP dependent mechanism.

The second similarity to vertebrate anterior pituitary activity is that of the centrally driven neuro-endocrine relationship between a CNS signal and the hormone source that leads to a rise in hormone levels throughout the animal via the circulation. Figure 12, shows a diagram of the human anterior pituitary at puberty in comparison with Burs-Pburs signaling in the newly eclosed fruitfly. Shown are the neural pathways driving the

maturation of the fly and the human. Both involve neural signaling that leads to increase in humoral (circulating) hormone levels and both ultimately have behavioural and non-neural, tissue specific effects.

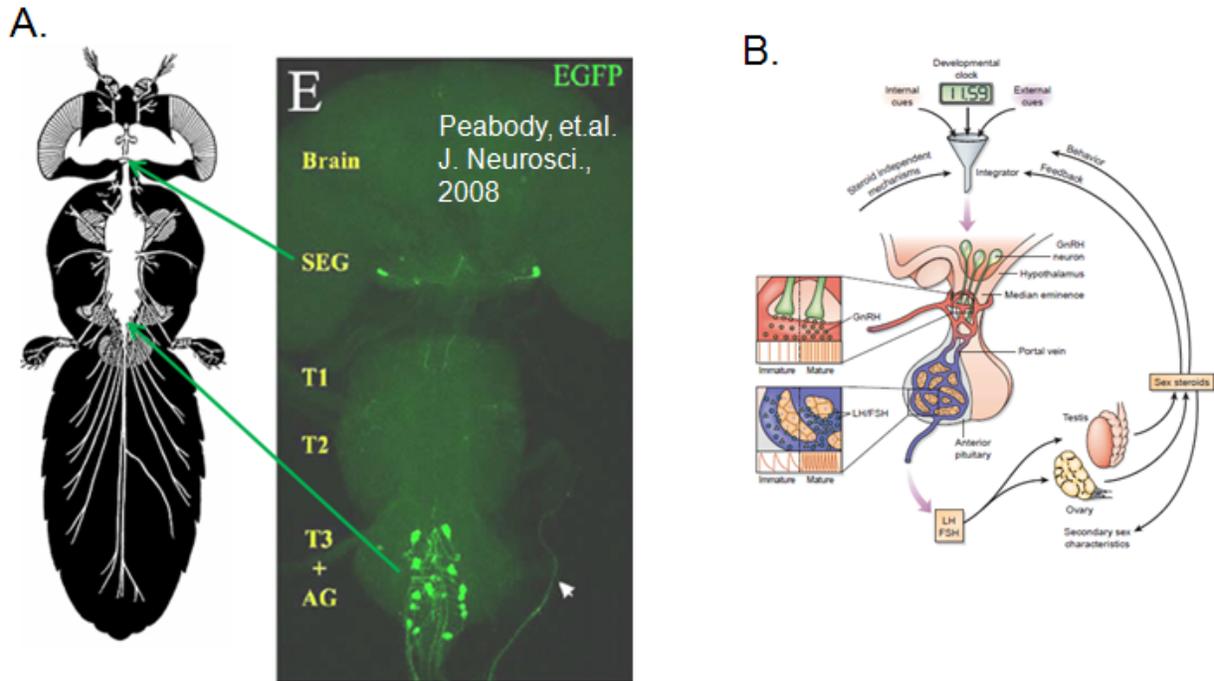


Figure 12. A. GFP (green fluorescent protein) under the Bursicon promoter shows the neurons that secrete and are responsive to Burs-Pburs. The subesophageal ganglion neurons (SEG) are believed to make neural connections with the cells in the abdominal ganglion and with the body wall muscles of the fly. The abdominal ganglion (AG) cells secrete Burs-Pburs into the hemolymph to induce sclerotization and then undergo apoptosis after the maturation program is complete (left Demerec, 1950 and right Peabody, 2008) B. These hormonal signals are reminiscent of activity in the peri-pubescent vertebrate anterior pituitary gland (Sisk, 2004). Burs-Pburs, and DLGR2 receptor are structural homologs to LH, FSH, and their human glycoprotein receptor.

Burs-Pburs is secreted from 2 different populations of cells in the fly (figure 12A), those of the sub-esophageal ganglion (SEG) and those of the abdominal ganglion (AG). Both populations of cells also produce crustacean cardioactive peptide or CCAP (for significance of CCAP see final diagram in figure 14B). At the time of eclosion, two neurons of the SEG produce measurable levels of Burs-Pburs. These cells have extensive connectives to the peripheral nervous system of the fly and are believed to drive the behavioural program that leads to wing expansion. These SEG neurons are also believed to synapse with the second population of Burs-Pburs positive cells found in the AG. These AG neurons are believed to secrete the majority of circulating hormone thereby remotely regulating the changes in cell function that lead to sclerotization, and selective apoptosis in at least 2 types of cells (Peabody, 2008). The Burs-Pburs reactive cells of the AG themselves undergo apoptosis after wing expansion. Also, the epithelial cells that make the cuticle of wing blade undergo an epithelial to mesenchyme transition under the influence of Burs-Pburs (Kiger, 2007). These epithelial cells remove themselves from the wing cuticle, become mobile, produce the extracellular matrix that will glue the top and bottom wing surfaces together and finally leave the wing blade via the wing hinge. Figure 13 shows a beautifully simple time course experiment of post-eclosion wings as the former epithelial cells dedifferentiate into mesenchyme (fibroblast like cells) and disappear from the wing blade (Kiger, 2007). It is possible that hemocytes also play a role in the final stages in wing development though Kiger et al. (2007) believe it unlikely. A well-orchestrated, programmed cell death (apoptosis) has been shown to be the likely fate of these cells

that were formerly wing epithelium (Natzle, 2008). Data presented in the results section here confirm that our knockdown of *ricketts* (gene *rk* codes for Burs-Pburs receptor) in the wing does interfere with this epithelial/mesenchyme transition.

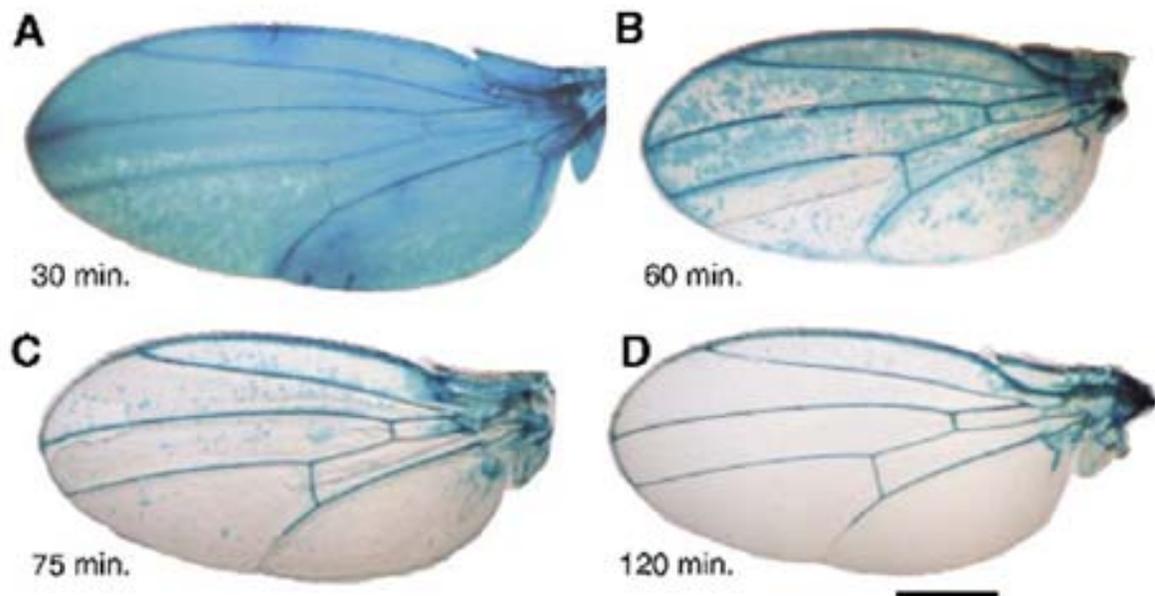


Figure 13. **Epithelial cells leave the wing during maturation.** These images by Kiger(2007) are fruitfly wings taken at the times indicated post-eclosion. Epithelial cells stain blue due to enzymatic label, notice that by 2 hours all cells have disappeared from the maturing wing. Scale bar = 400um.

The following scheme of the final stages of metamorphosis is based (unless otherwise indicated) on research by Davis, 2007 and Arakane, 2008. Final, non-Resilin, sclerotization reactions involve the products of DOPA de-carboxylase (Ddc) and Tyrosine Hydroxylase (TH); i.e. dopamine and related molecules (figure 14A). The referenced notes added to Davis' figure in 14B show that this pathway has not been completely resolved.

-Neural Inputs trigger ecdysone release from prothoracic gland (just dorsal to the brain in dipterans like *Drosophila*) Pupal hemolymph pressure which increases with increase in tissue volume within the inextensible puparium, may initiate pupal ecdysone burst (Dr. James Joy, personal communication). The steroid ecdysone is converted to its active product HE (hydroxyecdysone) the primary function of which during pupation is the induction of adult cuticular structures.

-Drop in Ecdysone triggers positive feedback loop that results in a surge of ecdysis triggering hormone (ETH) peripherally produced by non-neural cells and eclosion hormone (EH) centrally produced in brain neurons (Ewer, 1997 in lepidopterans and Baker, 1999 in flies)

-EH potentiates CCAP release which induces the final ecdysis and eclosion behaviours: increase in heart rate, gross movements in the puparium including ptilinum inflation and escape of the teneral adult fly

#### **ECLOSION**

-Davis (2007) also found CCAP to increase translation of tyrosine hydroxylase

-Burs-Pburs responsive neural pathways induce wing expansion behaviours including air gulping and abdominal (cibarium) pumping

-Burs-Pburs are released to hemolymph from AG neurons and via Rickets receptor induce epithelial to mesenchymal transition of the wing epithelium – cells leave the wing which results in a wing blade almost entirely acellular and sclerotized

-Burs-Pburs via Rickets receptor phosphorylate TH making it active – this leads to dopamine incorporation into cuticle known as sclerotization or tanning

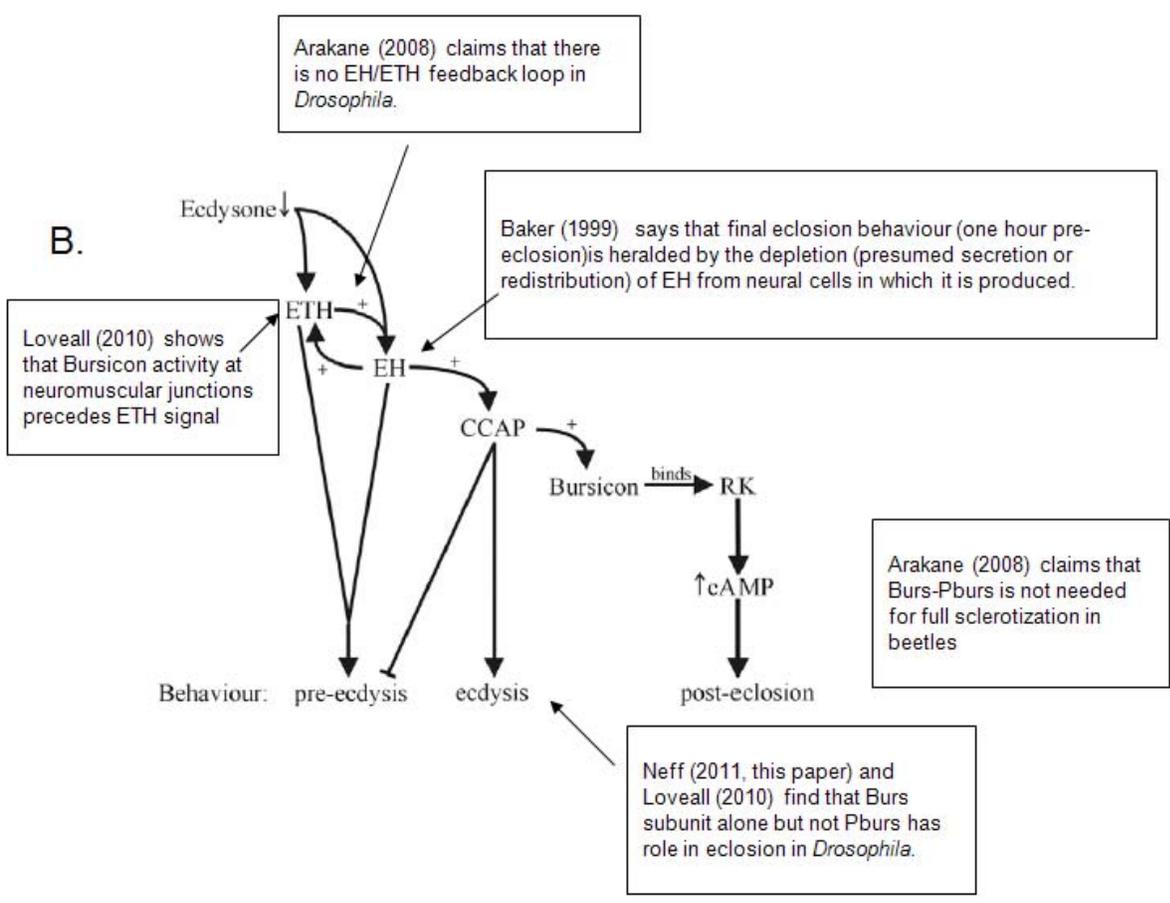


Figure 14. At top is a scheme of enzymatic production of sclerotization precursor dopamine. Bottom is a simplified view of the ecdysis, eclosion, and maturation cascade; both Adapted from Davis(2007).

## Deposition of Resilin during metamorphosis

*Drosophila* has been shown to express measurable Resilin mRNA during and only during pupal development (Elvin, 2005). A remarkably strong peak of expression can be seen in figure 15. However, these studies do not indicate absolute quantity, location of expression, or timing of modification of Resilin. It has been proposed that, in flies, Resilin processing (as that of other cuticle) is subject to hormonally mediated environmental cues. The genesis of my current thesis research (i.e. the interplay between Bursicon hormone signal and Resilin) can be traced to my reading of a paper on blowfly eclosion (Sabaratnam 1973). In nature, a blowfly adult emerges from the pupal case underground and digs upward to the surface to become a fully mature adult. Sabaratnam observed that newly emerged adult blowflies whose digging behavior was experimentally prolonged showed deficiency not only in the usual final signs of metamorphosis (wing expansion and cuticle darkening) but also in formation and crosslinking of the Resilin containing pleurotergal tendon in the thorax of the blowfly (in most subsequent literature and here the nomenclature tergopleural or TP is used). He implicated insufficient Bursicon signal in this deficiency. These data are at odds with assertions made in a study on the locust, *Schistocerca gregaria* by Coles (1966) who claims based on radioactive pulse chase experiments that only small amounts of pro-Resilin exist at any time. Coles' data and assertions by Andersen (1966) imply that pro-Resilin (soluble) is crosslinked to Resilin (insoluble) soon after protein export from epithelium. Experiments described in this thesis are designed to test Sabaratnam's, Andersen's and Cole's conclusions about the deposition of Resilin into elastic tissues.

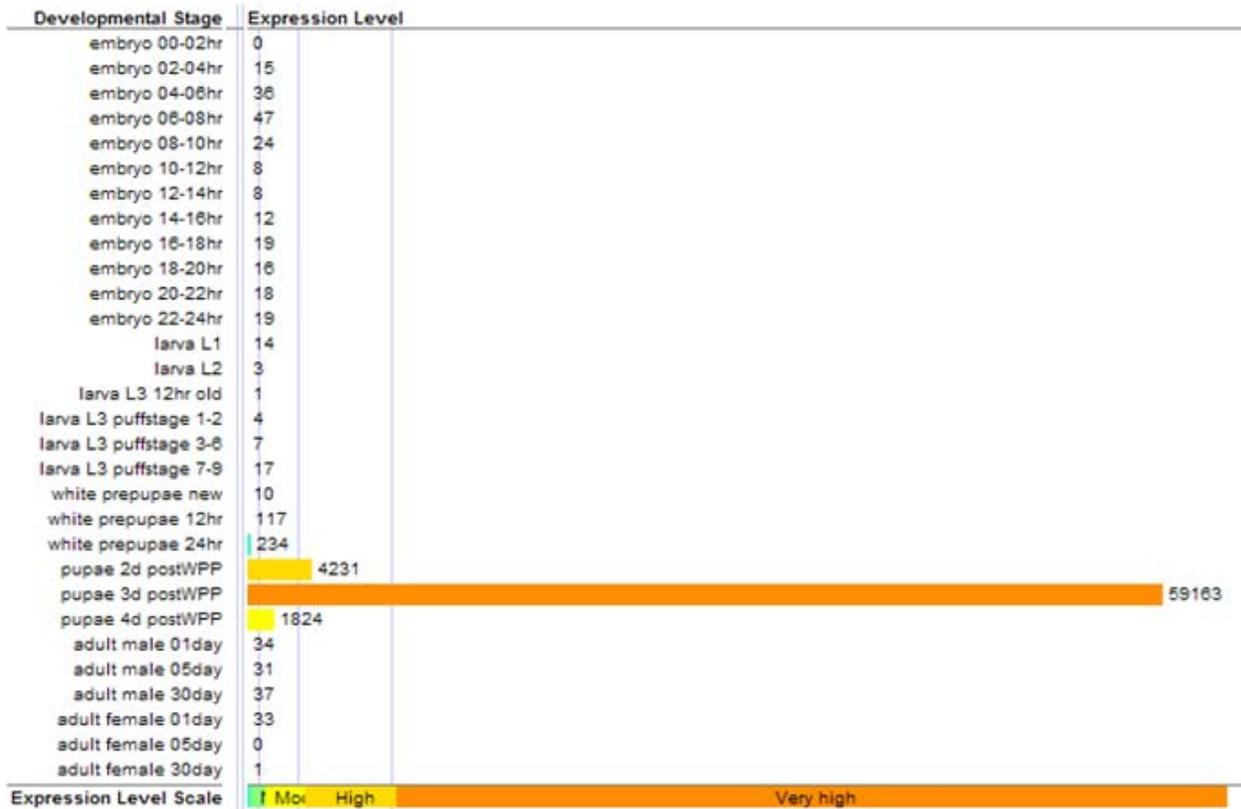
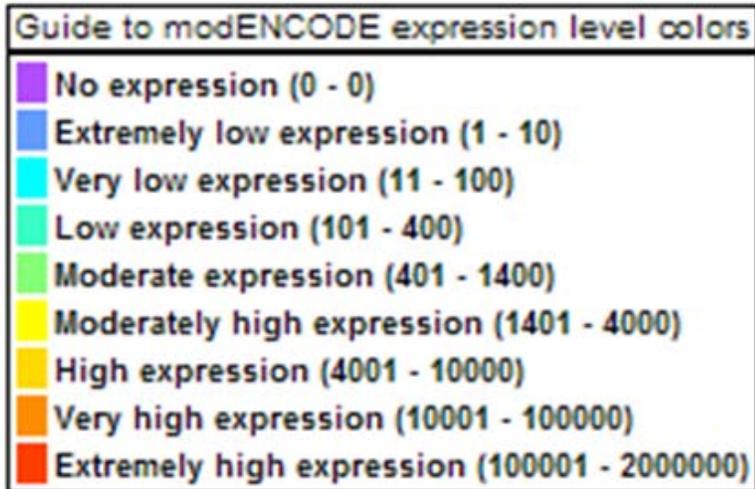
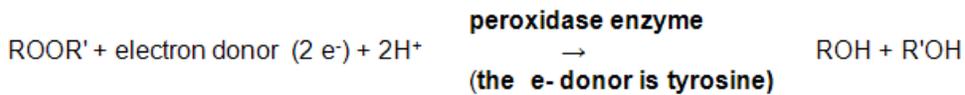
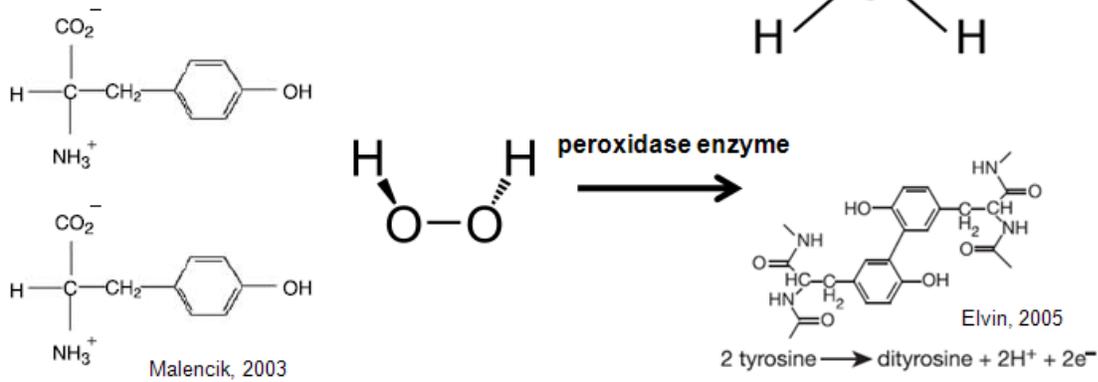


Figure 15. mRNA based expression level of the *resilin* gene shows a very high expression level between 72 and 96 hours APF.

(<http://flybase.org> and modEncode project model organism Encyclopedia of DNA elements, Celniker, 2009)

A.



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B.

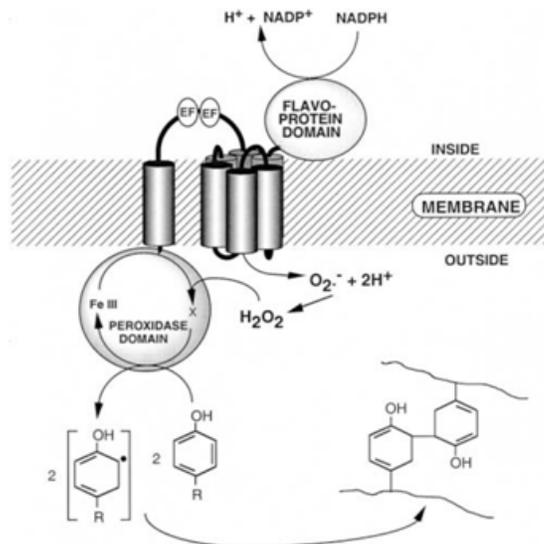


Figure 9. Proposed topology model for Duox. See text for details.

Figure 16 A. scheme of di-tyrosine formation via peroxidation (adapted from Elvin, 2005 and Malencik, 2003) B. Model of Duox peroxidase activity from Edens, 2001. This model is not compatible with the TEM image in figure Appendix1. If all Resilin molecules were crosslinked at the epithelial cell microvillous border, there would be no single Resilin molecules outside the assembly zone.

Although Bursicon is the “master hormone” regulating cuticle maturation, there are other molecules (such as TH and Ddc) that drive the terminal chemical reactions of sclerotization and Resilin crosslinking. Since the 1960s, the enzyme involved in crosslinking Resilin has been assumed to be a peroxidase that works via a reaction similar to that shown in figure 16A (Elvin, 2005, Malencik, 2003, and Edens, 2001). Some experiments described in the results section (following) were designed to determine if the product of a peroxidase gene in fruit fly was responsible for the final chemistry of Resilin crosslinking. Others’ work that we discovered after beginning our experiments led us to a second peroxidase enzyme (Duox or dual oxidase in Edens 2001, and Anh, 2011). Edens’ research focuses on *C. elegans* but he does present a scheme of Resilin crosslinking that we are using as our working model (figure 16B). Other than a brief reference to Resilin in Anh (2011) little work attempting to correlate Duox activity with Resilin maturation has been published since 2001.

In addition to the attempts to shed light on the processes of metamorphosis mentioned above, the work described in this paper adds to the literature of biomechanics with a thorough description of the form and function of the TP Resilin tendon in the wing hinge of fruit fly.

## Results and Discussion

### Resilin and the anatomy of the *D. melanogaster* wing hinge

Through dissection and micrographic documentation we show anatomy of an elastic tendon in the fruit fly that was first described in Diptera in the 1960s (Andersen and Weis-Fogh 1964). Although incomplete, our results are the most thorough treatment of this tendon to date. Mike Dickinson has spent most of his very productive career studying the mechanics and physiology of flight in flies (usually house flies and blowflies which are 10s to 100s of times larger than fruit flies). In a 1997 paper, he and Michael Tu described the wing hinge of dipterans as “...the most complicated joint in the animal kingdom.” My attempts to place our results into broader anatomical context have been frustrated by the overwhelming complexity of the wing hinge and the small size of *D. melanogaster*. However basic anatomy of the fruit fly wing hinge centering on the tergopleural tendon is presented here; I believe that the description here of the TP tendon is the most thorough currently available. There were no notable differences in anatomy between male and female flies but all specimens shown here are female unless otherwise indicated. Wild type flies are used unless noted and are of strain OregonR.

Figure 17A shows a sagittally split and cleared mount of a wild type (OreR) adult fruitfly as viewed from lateral aspect (outside of fly). The wing (mesothoracic or front wing) is labeled as is the specialized rear wing derivative (metathoracic) known as the haltere. In dipterans (two winged insects) the haltere serves as a sensory organ which gives neural input during flight.

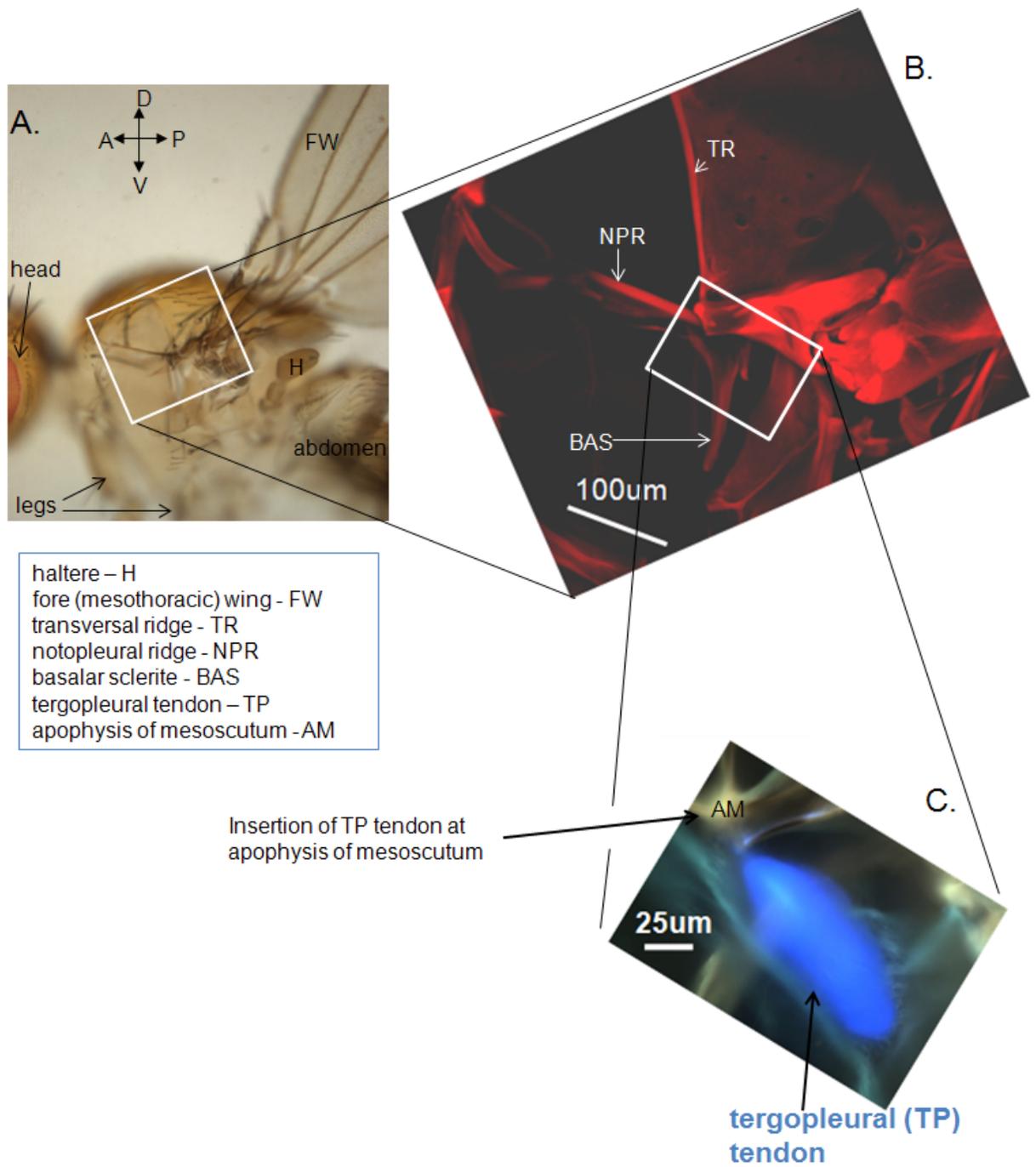


Figure 17. Thorax of *D. melanogaster* and wing hinge anatomy in relationship to the tergopleural (TP) tendon. A. Brightfield image of sagittal split. B. Confocal reconstruction viewed from inside of animal. This preparation is slightly distorted due to the wing being forced dorsally upon mounting. C. Fluorescence micrograph of TP tendon.

The very same mount is shown (enlarged) in figure 17B. This image is a reconstruction of multiple optical sections taken from inside of the split fly with a laser scanning confocal microscope. The red in this image represents autofluorescent emissions from the sclerotized pro-cuticle of the fly's thorax. Notable in 17B are ridges of thickened exoskeleton known in dipterans as the transversal ridge (TR, running D-V) and the notopleural ridge (NPR, running A-P). Thickenings (ribs in engineering terminology) of this type give rigidity to fly thorax (Ewing and Miyan, 1985). Similar ridges in cockroach leg have been shown to concentrate forces at mechanosensory structures during walking (Flannigan, 1998, Zill, 2000,)<sup>5</sup>

In *D. melanogaster*, the NPR and TR follow the wall of the thorax and converge just anterior to the wing's main articulation with the thorax. This main articulation involves the base of the radial vein of wing with the axillary sclerites of the thorax. Here the ridges merge and become an internal projection of cuticle known as the apophysis of the mesoscutum. This apophysis serves as the insertion point of the tergopleural tendon as shown in figure 17C.

Figure 18 shows the same fluorescent micrographs in the context of exoskeletal function during flight. As mentioned in the background section, muscle in the fly thorax can be divided into asynchronous (myogenic) and synchronous types. The 3 basalar

---

<sup>5</sup> In this study confocal image data from the cockroach were analyzed by engineers from CWRU with a technique called finite element analysis (FEA). Briefly, fluorescent emissions mapped in 3D are used to create a computer based model to which material properties are assigned. Virtual forces can be applied to this model and resultant strains mapped.

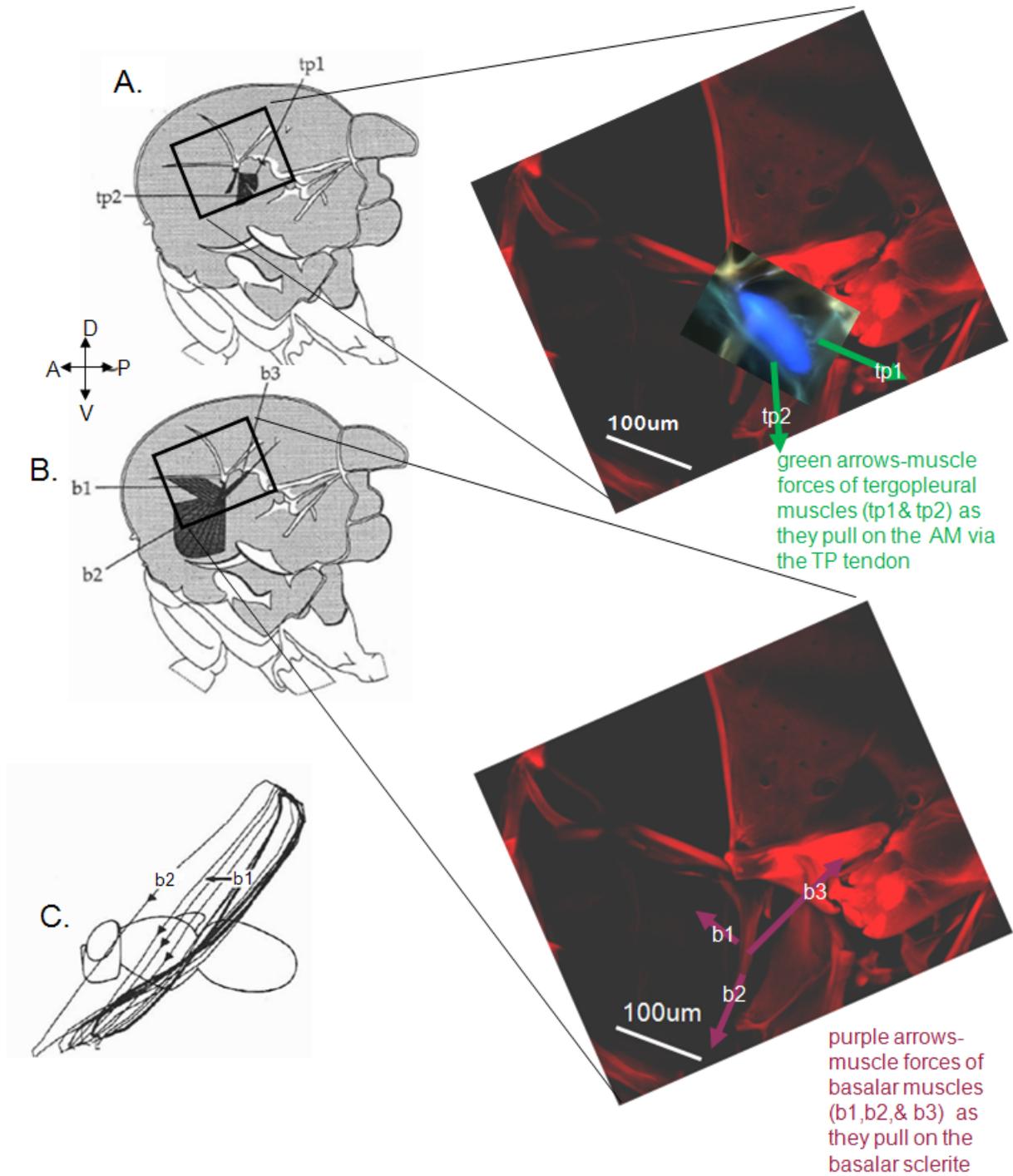


Figure 18. Anatomy and function of the tergoleural (A.) and basalar (B. and C.) muscles in flies. Drawings at left are blowflies from Dickinson (1997). Micrographs at right are *D. melanogaster*. Confocal reconstruction viewed from inside of animal.

and 2 tergopleural muscles shown as arrows in figure 18 are all synchronous, that is their contractions are elicited one for one by neural inputs.

There is a further division among the control muscles in the fly. Direct control muscles have traditionally been considered those that insert directly onto one of 3 axillary sclerites (aka pterale I, II, III). These sclerites make contact with the wing itself, specifically, the base of the radial wing vein (Ewing and Miyan, 1985). Due to their profound effect on flight control, Dickinson and Tu (1996) also designate the muscles (b1, b2, and b3 in figure 18) of the basalar sclerite (BAS) to be direct flight muscles despite the fact that the BAS does not make direct contact with the wing base. The basalar sclerite and its associated muscles have been shown to be critical components in dipteran flight control (Dickinson and Tu, 1997).

The tergopleural muscles are known as either indirect (Dickinson, 1997) or direct (Wisser and Nachtingall, 1984) depending on the author (both of these studies were done with blowflies). It seems that (in context of function) the line between direct and indirect steering muscles is not always clear given the complexity of the wing hinge and the potential for interplay between the dozens of sclerites (with connective tissues) and control muscles at the joint (Dickinson and Tu report 31 pairs of control muscles). The tergopleural muscles as with most other steering muscles in flies are each innervated by a single large axon from the thoracic ganglion (Heide, 1983).

The most thoroughly described of the control muscles in flies are the basalar muscles. Briefly the function of these is as shown in figure 18B and C. They pull on the basalar sclerite, b1 and b2 pulls the sclerite rostrally and b3 pulls it caudally. Dickinson (1996) showed with electrophysiological techniques during tethered flight that when the

basalar muscles b1 and b2 are firing they change wing trajectory as shown in figure 18C. Alteration of firing patterns in b2 and b1 can change both the amplitude and phase of left and right wing independently; among other effects, Dickinson showed that during turning, b1 on the outside wing fires ahead of b1 on the inside wing. b3 is positioned to oppose or moderate these changes but its activity was not recorded in these experiments.

Two tergopleural muscles insert via the tendon (TP tendon) on a large apophysis of the mesoscutum just anterior to the wing hinge (see confocal reconstruction of thorax in figure 18A). The gross anatomical arrangement of the TP tendon suggests that it acts to combine the forces of the TP muscles into a single force acting on this apophysis. In 1964, Weis-Fogh and Andersen (1964, pages 15-16) reported that an irregularly shaped tendon (later and here called the TP tendon) exists in the blowfly *Calliphora* and went further to say that the anatomy of this tendon “..left no doubt...” as to its function. They inferred from its anatomy that the TP tendon is held under tension by the TP muscles and that the elasticity of the tendon allows it to stretch and recoil (while transmitting a TP muscle defined average force) during high frequency wing movements. This arrangement allows the synchronous (slow) TP muscles (which are unable to contract at the >200Hz of the wing beat) to modify, via the TP tendon, the high frequency forces transmitted to the thorax and wings (by the asynchronous, fast, power muscles). After literature search I found that the only other function suggested for the TP muscles and tendon is presented by Dickinson and Tu (1997). They suggest that the TP muscles may stiffen the thorax by drawing together the sclerites on either side of the episternal cleft (see figure 19). We have considered the possibility that the TP

tendon serves as a center for force detection. If the TP tendon were somehow innervated by proprioceptive neurons it could serve as an integration center for the forces of the steering muscles (including the TP and BA muscles) as they act on the wing hinge. Also possible (and maybe more likely as there is precedent for mechanoreceptors in sclerites of the insect flight mechanism) is that sclerites of the wing hinge, mechanically coupled to the TP tendon, detect deformation via mechanoreceptors. There are stretch receptors in the wing hinge of locusts and moths (Frye, 2001) and deformation sensitive receptors (campaniform sensilla) in the wing veins of *Drosophila* (Dickinson, 1987). Clearly, the TP muscles and tendon are well positioned for controlling flight in the fruit fly but their role is currently unknown. However, evidence that they are essential to flight in *D. melanogaster* is presented later.

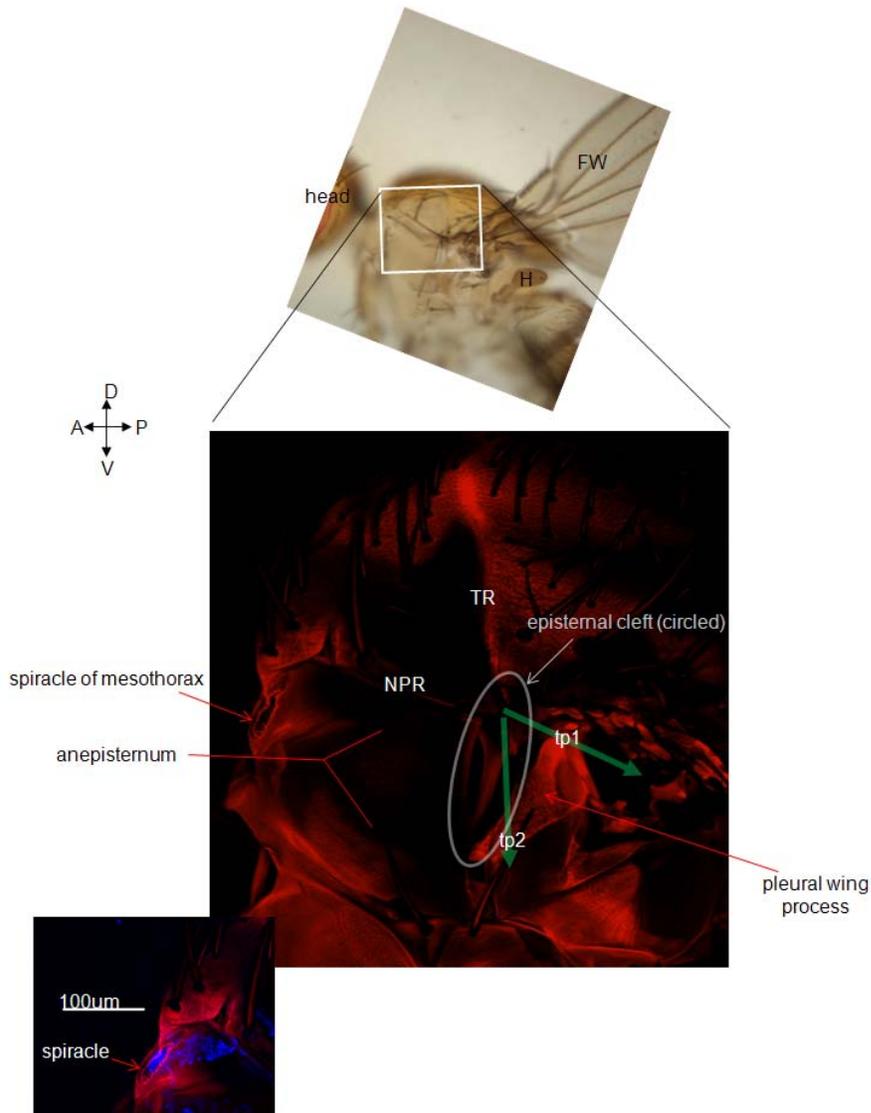
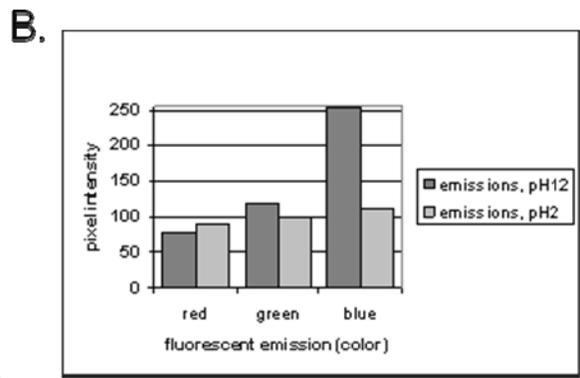
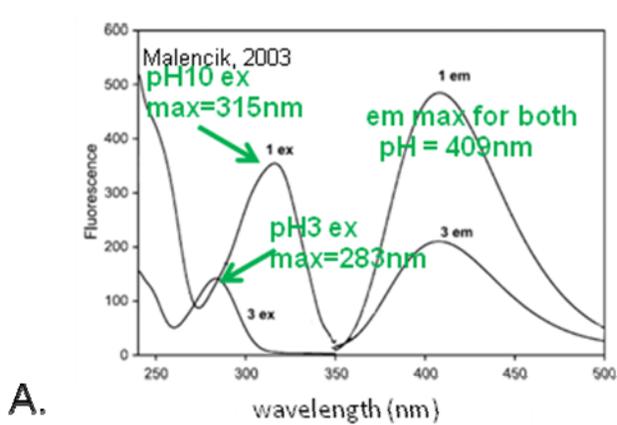


Figure 19. Proposed anatomical function (by Tu and Dickinson, 1997) of TP muscles in closing the episternal cleft (grey ellipse). The basalar sclerite can be seen through the cleft. Confocal reconstruction viewed from outside of animal. In this mount the pleural wing process and episternal cleft are somewhat distorted by downward/inward displacement of wing by the coverglass. At lower left are blue emissions showing presumptive tracheae as they originate at the spiracle and enter the thorax; the scale bar applies to both the inset and main fluorescent image.

### Simple photochemical tests for Resilin

In the initial description of the di- and tri- tyrosine crosslinks in Resilin (Andersen, 1966) it was noted that these molecules showed bright and spectrally well defined blue fluorescence. Additionally, Andersen reported that the absorption spectrum of Resilin's crosslinks shifted with change in pH. This observation led us to attempt to induce this shift in whole mount tissues suspected to contain Resilin. A robust and reversible pH dependent shift of Resilin in situ was used to confirm presence of Resilin in the cockroach leg (Neff et.al., 2000). At near neutral pH the absorption maximum for di-tyrosine is 315nm while its emission maximum is 409nm. A strong mercury peak at 365nm is generated by most available fluorescence microscope sources (the high pressure Hg lamp and variations on this design). Although not perfectly aligned with the 315nm abs. max. of di-tyrosine, these lamps have proven to be very practical in exciting Resilin. The typical emission filter found in the UV filter cube of epi-fluorescent microscopes is a 420 long pass filter which also proves sufficient to collect the bright blue emissions of crosslinked Resilin. The light source and filters used for the micrographs of the TP tendon are similar to those described above (details in methods section).

Figure 20 shows 2 chemical induced shifts in the fluorescence of the TP tendon that can be used to confirm presence of Resilin (Malencik, 2003). These changes were completely reversible (not shown). In figure 21A, we see that at high pH the TP tendon



**C.**

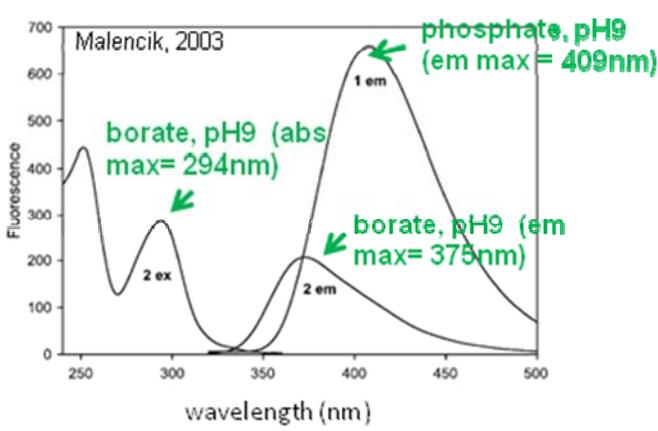


Figure 20. Simple chemical test for presence of Resilin in situ. These changes in optical properties are completely reversible. A. effects of pH shift. In TP tendon (bottom) and spectra of di-tyrosine in solution (top, Malencik, 2003) B. Blue fluorescence is diminished while red and green emissions are stable. C. Bottom, in TP tendon, borate ion shifts both excitation and emission spectra toward UV and away from peaks of usual microscope filter/lamp combinations. Top, di-tyrosine in solution of borate vs phosphate buffers (Malencik, 2003)

emits strongly in the blue wavelengths while at pH2, this fluorescence is much diminished. This change results not only from a decrease in emission peak height (also called decrease in quantum efficiency - figure 20A top) but also from a shift in excitation wavelength away from the typical UV microscope excitation band around 350-360nm. In figure 20B we can see that while the blue emissions of di- and tri-tyrosine are quenched, the weaker red and green light emitted from the TP tendon (region analyzed indicated by grey circles in A.) are essentially unaffected by pH shift. It is the singly deprotonated form of di-tyrosine that emits blue light when stimulated with UV; the fully protonated form (pH2) absorbs and emits relatively weakly (Malencik, 2003).

Figure 20C shows a more specific test in which Resilin's fluorescence is quenched by borate ion at pH9 but not by phosphate at the same pH. Notice that in both tests, the broad spectrum fluorescence of the sclerotized cuticle in the background (the ridges and the apophysis) is notably quenched by neither borate nor low pH (protonation). The detailed mechanisms for the quenching are beyond the scope of this paper but have been described by Malencik (2003).

### Bursicon and the maturation of pro-Resilin to Resilin

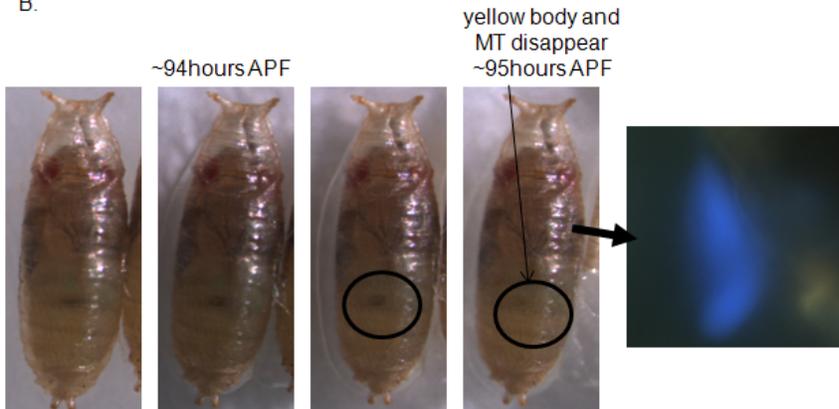
As mentioned in the background above, conflicting assertions about the processing of Resilin in flies during deposition and after eclosion inspired us to study the effects of Bursicon-Pburs on developing Resilin tissues. To this end, we used a variety of *Drosophila* mutants chosen for their potential to display phenotypes of abnormal activity in the Bursicon-Pburs hormonal pathway. However, before testing various genetic manipulations, we tested the hypothesis that Bursicon-Pburs signals Resilin maturation at eclosion (as implied by Sabaratnam, 1973) by dissecting and observing

the TP tendon of late pupal flies that had not yet undergone the Bursicon-Pburs pulse at eclosion; these were selected at ~85-100 hours after puparium formation. Figure 21C shows one such fly and its TP tendon (inset).

A.



B.



C.

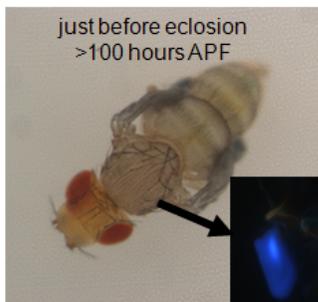


Figure 21 Development of TP tendon at different time points APF. Staging was done using observable sign posts of metamorphosis (appearance of abdominal bristles in A or disappearance of yellow body/malpighian tubules (MT) in B; Bainbridge and Bownes, 1981). The pupal flies at stage shown in the final panel of each row were removed from puparium and dissected. A. Pharate adult 88 hour APF with TP tendon (dim appearance here may be dissection artifact), B. Pharate adult 95hour APF with TP tendon and C. Pharate adult just prior to eclosion with apparently well formed and crosslinked tendon, notice that the ptilinum has begun to expand rostrally from the fly's head and that the non-Resilin cuticle of head, thorax and abdomen

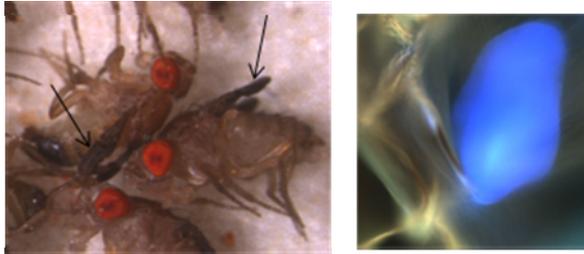
The fly was removed from the puparium at time >100 hours APF (after puparium formation). TP tendons in these flies consistently appear well formed and crosslinked so we rejected the supposition that the post eclosion pulse of Bursicon-Pburs induces Resilin maturation. We were also able to see a crosslinked tendon in a fly 88hours APF figure 21A and at ~95hours APF in 21B. Flies at these time points APF were still quite soft, which made dissection difficult, but the TP tendon can be seen in flies from both of these time points. The tendon in figure 21A (fly 88hours APF) does appear to be incompletely developed (lacks bright blue emissions) and although data in figure 15 shows a peak of *resilin mRNA* expression at ~72hours APF, protein translation and crosslinking are not necessarily coincident in time with transcription; these events (evident in fluorescence of TP tendon) could occur later. Interestingly, although the pharate adult (uneclosed) fly in figure 21C showed a very brightly fluorescing TP tendon, it and others at similar stage show less fluorescence in skeletal structures outside the tendon indicating that, as suspected, Bursicon-Pburs induced sclerotization was incomplete.

We continued our investigation by checking TP tendon development in a classic mutant fly called *rk[4]*. This mutant is deficient in the Bursicon-Pburs receptor now known as Rickets or DLGR2. We used fly lines called *rk[4]* and *rk[1]*. The mutant shown in figure 22 is *rk[4]* which is homozygous for a point mutation in the *rk* gene (left arm of chromosome 2). *Rk[4]* flies do show delayed tanning and some leg phenotypes but most obvious phenotypes are the unexpanded wings seen in fig 22A (black arrow); this will be referred to as a classic *rk* phenotype, in this case severe. Apparently, this wing phenotype is primarily due to a behavioral deficit as these flies do not gulp air nor

contract their abdomens as do wild type flies (Loveall, 2010 and <http://flybase.org/reports/FBaI0014577.html>).

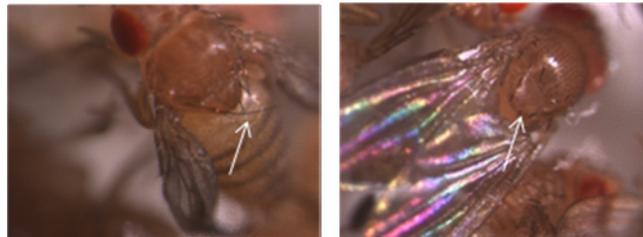
Figure 22B. shows results from attempt to knock down Bursicon signaling at three different points in the hormonal pathway. As discussed in the background section, active Bursicon-Pburs is a heterodimer of Bursicon and Pburs (partner of Bursicon). Most of the literature shows that there is no activity of one subunit without the other (Mendive, 2005). Using double stranded interfering RNA (RNAi) against *burs*, *pburs*, and the *rk* receptor transcripts, we knocked down the main components of the Bursicon-Pburs - DLGR2 pathway. In figure 22B top, two things are notable. First, a close look at the bristles (macrochaetes) on thoraces of these flies will reveal that flies inheriting the driver chromosome (expressing *Gal4* under Tubulin promoter needed to drive the *UAS* RNAi ubiquitously in offspring) show wild type, long, tapered, curving bristles while the offspring receiving the *TM3-Sb* balancer chromosome (not expressing *Gal4* therefore not driving RNAi) have bristles with the phenotype known as stubble. Notice that the wings of the flies receiving the driver chromosome show classic *rk* phenotype while those receiving the balancer appear as wild type. The *rk* wing phenotype strength was not measured and is similar in *burs*, *pburs*, and *rk* RNAi flies so only one example is shown here. Results of differential effects of *burs*, *pburs*, and *rk* RNAi knock downs are discussed later. Figure 22B bottom 3 rows shows that despite RNAi knock down of *burs*, *pburs*, and *rk*, the TP tendon shows seemingly normal morphology and crosslinking in adult flies; offspring receiving the balancer chromosome appeared identical (in TP tendon development) to those with the driver. Additionally, no

A. *rickets[4]* (homozygous mutant at *rickets* gene for DLGR2 receptor)



B.

3<sup>rd</sup> chromosome inherited  
driver                      balancer



MALES	x	FEMALES		
<i>UAS-bursRNAi</i>	x	<i>tubulin-Gal4/TM3 Sb</i>	only one fly enclosed from this cross, none enclosed at 25 or 30deg	18deg
<i>UAS-pbursRNAi</i>	x	<i>tubulin-Gal4/TM3 Sb</i>		18deg
<i>UAS-ricketsRNAi</i>	x	<i>tubulin-Gal4/TM3 Sb</i>		28deg

Figure 22. A. Bursicon mutants *rk[4]* (left) and *rk[1]* (not shown) demonstrate normal TP tendon despite severe wing phenotype (black arrows). B. RNAi knockdowns of molecular actors in Bursicon pathway have no obvious effect on TP tendon development. Each box showing TP tendon is 110umx110um. White arrows indicate wild type bristles (driver chromosome) at left top and stubble bristles (balancer) at top right.

difference in the TP tendon was noted in flies that were raised at low (18deg C) vs. medium (25deg C) or high (28 or 30deg C) temperatures (results of temp. series not shown). This temperature series is designed to test the strength of RNAi expression as driven by *Gal4*. Offspring of Bursicon-Pburs pathway RNAi knock downs raised at higher temperatures did show (results shown in next section) stronger *rk* wing phenotypes but showed no appreciable difference in TP tendon morphology; this further supports the conclusion that Resilin deposition and maturation proceeds without influence of the Bursicon-Pburs signal cascade. The note at top left in figure 22B. indicates a finding that is discussed in a later section.

Figure 23 shows results from tests of a *Gal4* driver (*MS1096*) that drives *UAS* only in the wing. In this cross, we knocked down *rk* (*dlgr2*) in the wing and found perturbation of the epithelial to mesenchyme transition (EMT) that normally leads to the departure of wing epithelial cells from the wing after eclosion (figure 13); this perturbation can be appreciated in the cells (brown clumps) left behind in the wrinkled wing of the male offspring shown in figure 23, left. These results also show the expected temperature dependence of *Gal4-UAS* driven expression system. The EMT defect is much more severe in flies raised at 28deg C. Interestingly, the classic *rk[4]* homozygous mutant shows the same deranged EMT phenotype. Natzle (2008) in a study of *rk* mutant flies claims that those cells left in the wing (i.e.undergoing no EMT) do still receive a non-DLGR2 (*rk* coded receptor) mediated signal to undergo apoptosis (as is believed to also occur in the wild type). Notice that the TP tendons in these flies

appear as wild type.<sup>6</sup>

*UAS-rickets* RNAi x *MS1096-Gal4*

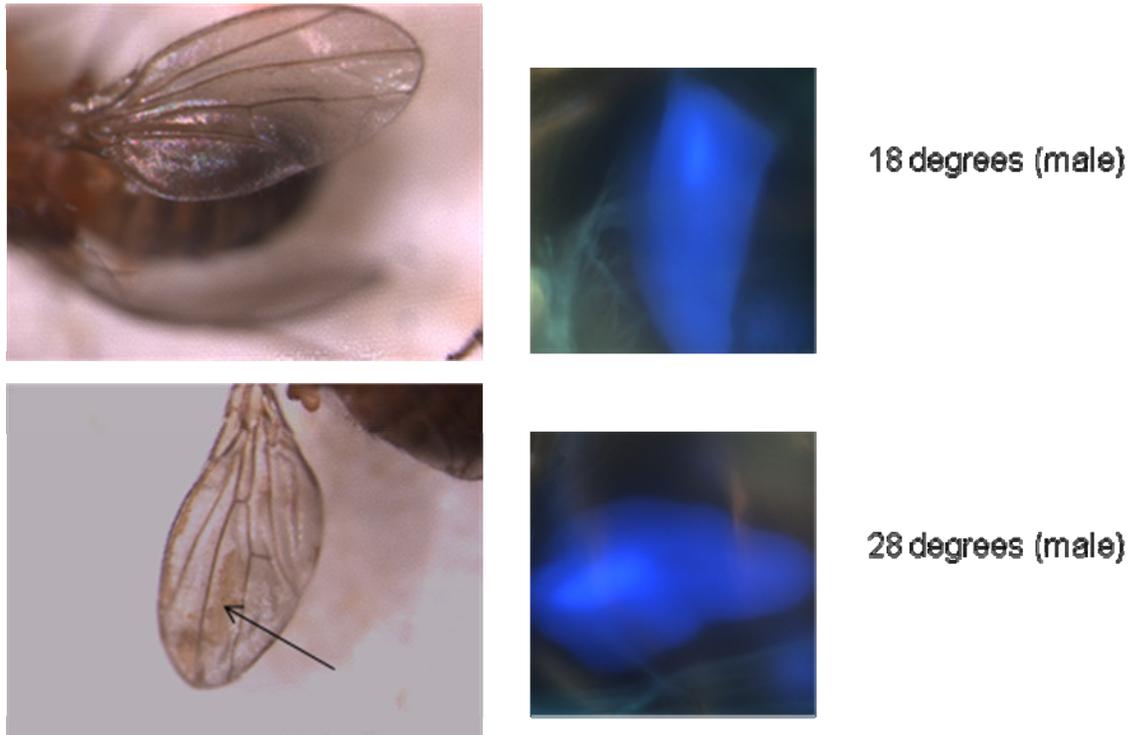


Figure 23. Results of knockdown of *rk* gene (receptor DLGR2) in the wing of *Drosophila*. EMT is disrupted and knockdown is temperature dependent. TP tendon formation is unaffected, each box showing TP tendon (at right) is 110umx110um.

<sup>6</sup> This EMT suppression was somewhat less severe in females from these crosses. Gender in flies is determined by the X:autosomal chromosome ratio. Unlike in vertebrates in which the Y determines gender and one of the female Xs is completely switched off, in female flies both X chromosomes are active; thus, each female fly has heterozygous contribution from 2 Xs and without dosage compensation would receive 2 times X chromosomal gene products as its male counterpart. However, although both X chromosomes are active in female flies, they are each transcriptionally suppressed to  $\sim\frac{1}{2}$  the level of the single X chromosome in males. *MS1096* is an X linked driver chromosome and has no balancer chromosome. Females are homogametic and heterozygous for X and male flies by definition are hemizygous for X. So although each offspring (male and female) receive one copy of *MS1096*, this copy (and thereby *Gal4*) in males is expressed at double the rate as in females.

From this data, we can reasonably conclude that in flies, Resilin rich tissues are fully formed (or nearly so) at the time of eclosion; i.e. the teneral adult fly has a seemingly mature TP tendon. In contrast with this finding, Neville(1963) found that in the locust (hemimetabolous insects undergoing incomplete metamorphosis) most of the mass of adult solid cuticle is added after final eclosion. More specifically, he found that the locust does add significant mass to their Resilin rich tissues after their final moult and that they show daily, post eclosion growth layers in Resilin tissues within which are found different degrees of crosslink density based on circadian cues of light and temperature. This phenomenon is seen in the daily growth bands in the locust wing hinge (Neville, 1967). Furthermore, Andersen (2004) showed that fully mature adult locusts had 4-5 times as much dityrosine/mg of tissue in their wing hinge when compared to teneral adult locusts. In locusts, Resilin deposition continues post final ecdysis and this Resilin contains more crosslinks than that deposited before this final ecdysis. It took locusts up to 5 weeks post eclosion to reach Andersen's definition of full maturity. Andersen's study suggests that more careful measurements through time post ecdysis/eclosion of di- and tri-tyrosine content in the TP tendon would show increase in crosslinking that is not appreciable with the microscopic techniques used in this current study. Alternatively, and I believe more likely, is that in the holometabolous flies elastic cuticular tissues such as the TP tendon are nearly fully formed in the pharate (figure 21C) adult and that this along with the final program of wing unfolding and non-Resilin cuticular sclerotization allows these animals to fly within an hour of eclosion.

## Interfering with Resilin synthesis

In an attempt to knock down the *resilin* transcript thereby disrupting formation of the TP tendon, we initially used *UAS-resilin*RNAi with *Gal4* expressed under a dorsal wing disk promoter (*MS1096-Gal4* mentioned above). Offspring of this cross resulted in seemingly normal tendon formation (figure 24A) leading us to conclude that the precursors of the TP tendon have origin other than the dorsal wing disk.

We proceeded to try driver lines in which *Gal4* is expressed ubiquitously throughout the fly and throughout developmental time (*Gal4* in these fly lines is co-expressed with constitutive intracellular proteins). The lines we tried drove *Gal4* under either *actin* promoters or *tubulin* promoter, these crosses all had similar effects on the TP tendon. Figure 24B shows the effect of *tubulin* promoter driven *Gal4 UAS-resilin* RNAi knock down of *resilin* transcript production. Only the flies receiving the driver chromosome (as in 22B, those with wild type bristles) show deficient TP tendon formation. There does not appear to be any temperature dependence associated with this knock down. Figure 24C. shows the same genetic cross with male and female roles reversed (male is driver and female is *UAS* line); results are the same in this and all other *UAS-resilin* RNAi x *tubulin Gal4/TM3-Sb* crosses we performed (not shown).

To demonstrate the invariability of *resilin* knock down by these ubiquitous driver lines, *actin-Gal4/CyO* x *UAS-resilin* RNAi results are shown in Figure 25. At top in A. are TP tendons from 8 flies which inherited the balancer chromosome (with curly winged phenotype) and no TP tendon derangement; at bottom in B are tendons from 8 flies (of the same cross) which inherited the driver chromosome. The TP tendon is much reduced in the RNAi expressing offspring (B. 8 panels at right). Also, at bottom

left, the flies with poorly formed TP tendon are shown to have downward pointing or anedral wings.

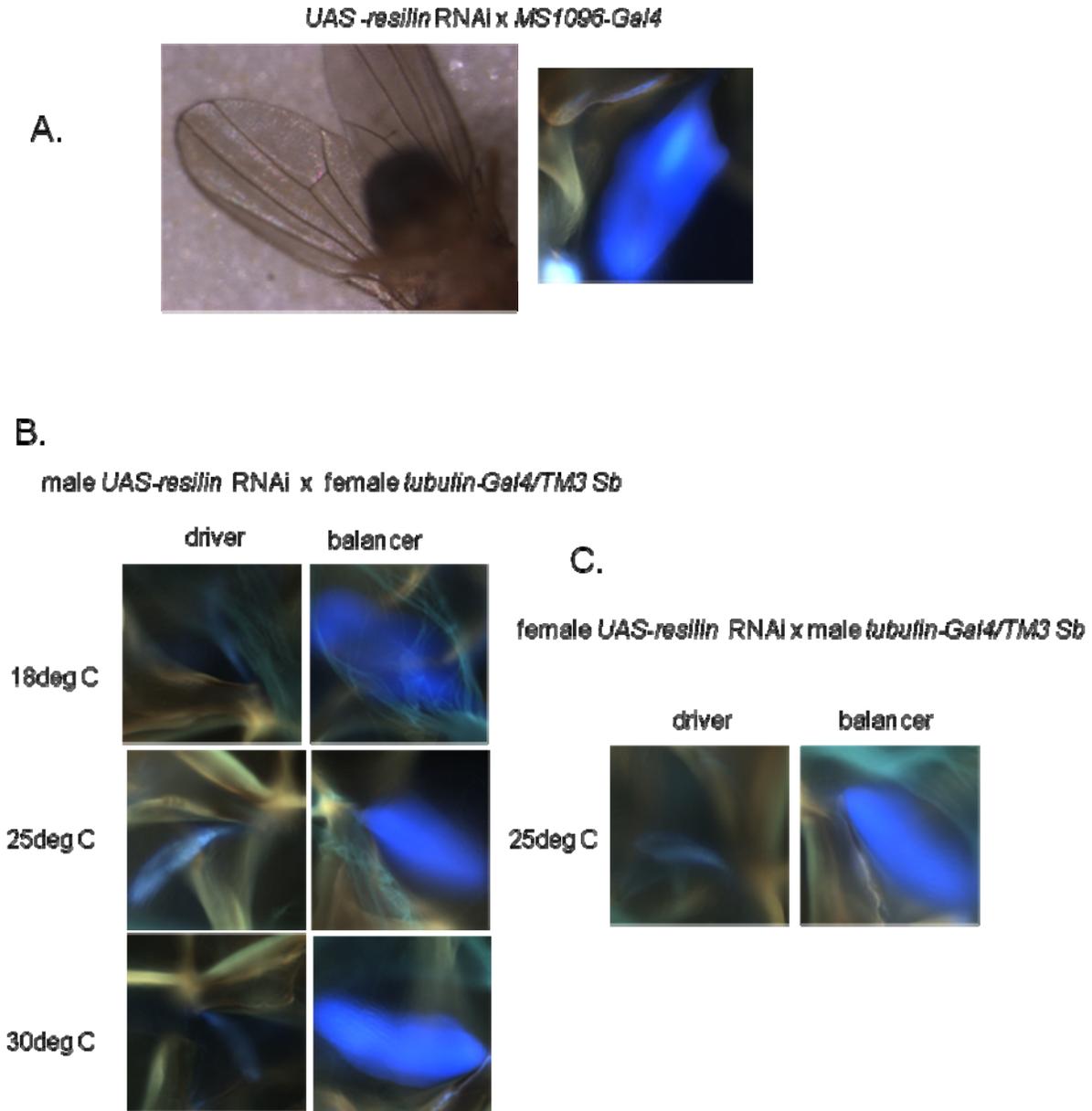
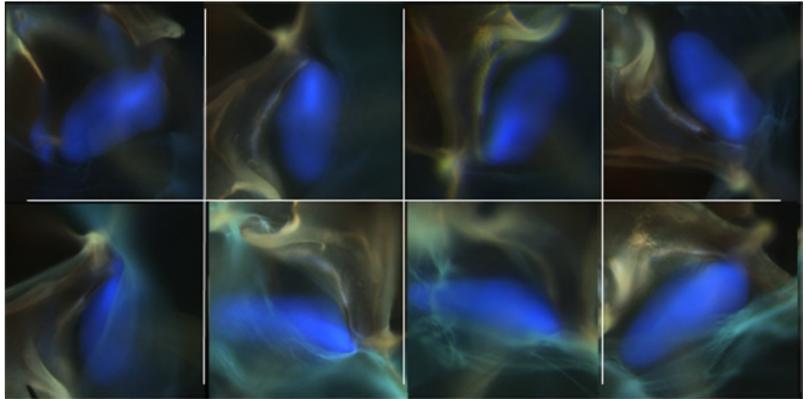


Figure 24. A. Attempted knockdown of Resilin production by *MS1096-Gal4* in which *Gal4* is expressed therefore *UAS* driven only in the dorsal wing disk. In B. we see successful knockdown of Resilin production by driver line *tubulin-Gal4*. C. is a similar cross to that in B. with male and female parents roles in *Gal4-UAS* system reversed. Results in C. are essentially the same as those in B. There does not seem to be temperature dependence of Resilin knockdown with *Gal4-UAS* system. All images with TP tendon are 110umx110um.

male *UAS-resilin* RNAi x female *actin5c-Gal4* / *CyO*

A.

balancer  
chromosome  
(*CyO*)



B.

driver  
chromosome  
(*actin5c-Gal4*)

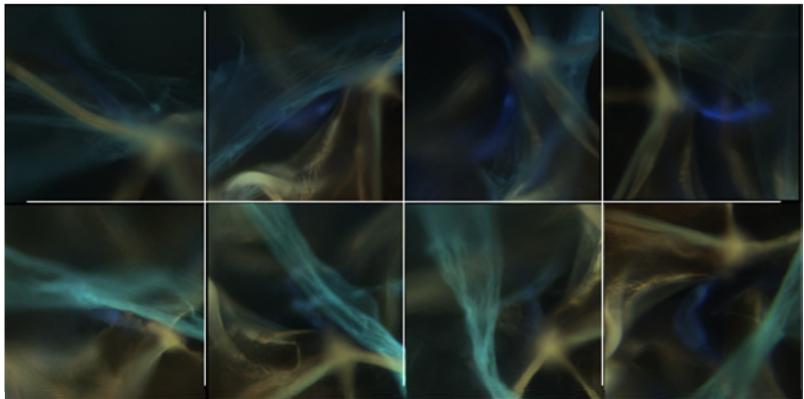


Figure 25. Results of knockdown of Resilin production in the wing of *Drosophila* by *Gal4* under *actin* promoter driving *UAS-resilin* RNAi – flies and tendons shown are male offspring. Notice the curly wing phenotype (*CyO* balancer chromosome) at top left and the anedral wing phenotype (see figure 27) resulting from TP tendon Resilin knockdown at bottom left. Each TP tendon image is 145umx145um.

We coined the term “anhedral” in reference to fruit flies when Simon Collier successfully disrupted Resilin formation in the TP tendon by making a stable homozygous viable mutant that lacks the *resilin* gene (figure 26).

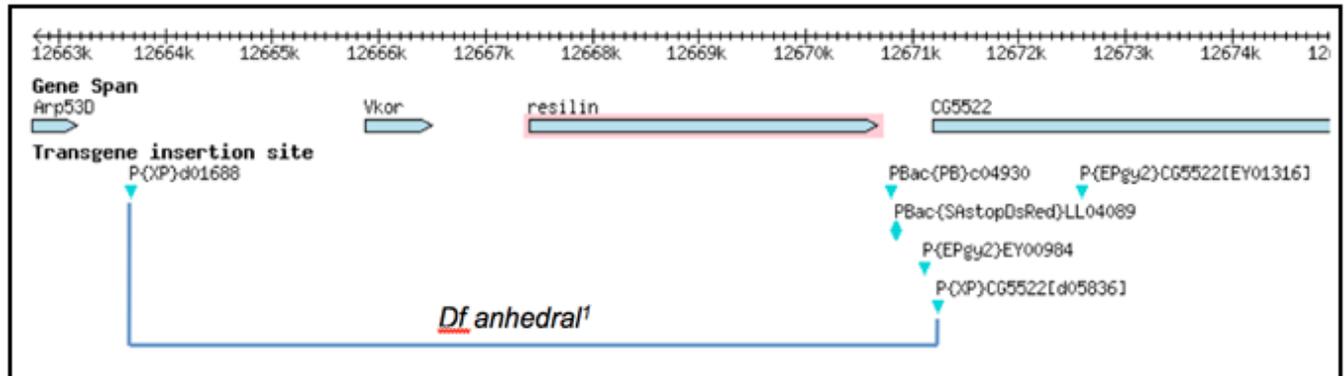


Figure 26 Generation of a *resilin* mutant chromosome.

"Since no *resilin* mutant existed, we generated a small (~7.5kb) deletion spanning the *resilin* gene by FLP/FRT recombination using the Exelixis transposon stocks *P{XP}d01688* and *P{XP}d05836* (Parks, et. al. 2004). The deletion removes the entire *resilin* gene, plus the Vitamin-K epoxide reductase gene (*Vkor*) and one transcript of gene *CG5522*. The *resilin* deletion is homozygous viable and has no visible phenotype besides the ‘held-down’ wing phenotype also observed in *resilin* RNAi knockdown flies. For this reason, we have named the deletion *Df(2R)anhedral<sup>1</sup>* (anhedral is an aviation term for downward-sloping wings). Homozygous *anhedral<sup>1</sup>* flies have dramatically reduced fluorescence in the wing hinge, compatible with loss of mature Resilin protein. Hemizygous *anhedral<sup>1</sup>* flies (figure 27B) have levels of wing hinge fluorescence within the normal range and hold their wings normally suggesting that the deletion has no dominant effects. "

(Simon Collier, 2011)

The diagram at the top of this figure shows the part of the right arm of the 2<sup>nd</sup> chromosome where the *resilin* gene is located (in all *D. melanogaster* to our knowledge excepting our new mutant line). These flies are now known as *Df(2R)anhedral<sup>1</sup>* (figure 27) and they, along with the *resilin*RNAi knock downs described above show the anhedral wing phenotype seen in figure 27A. Interestingly, this wing phenotype takes some time (hours) to develop after the mutant flies eclose. Upon eclosion the wings appear wild type (held flat against the flies' backs) but with time the wings droop to the anhedral conformation. The cause for this transformation may be mechanical displacement of the wing due to hardening of wing hinge sclerites in the mutant teneral adult. Alternatively, the flies may be actively holding their wings against their backs during cuticle maturation until proper skeletal and nervous development obviate the need for actively maintained wild type wing position; the anhedral flies may simply become acclimated or fatigued and abandon the wild type posture; improperly formed TP tendons cannot passively prevent the wings from drooping.

A.

OreR (wild type)  
live fly with dihedral  
wing posture

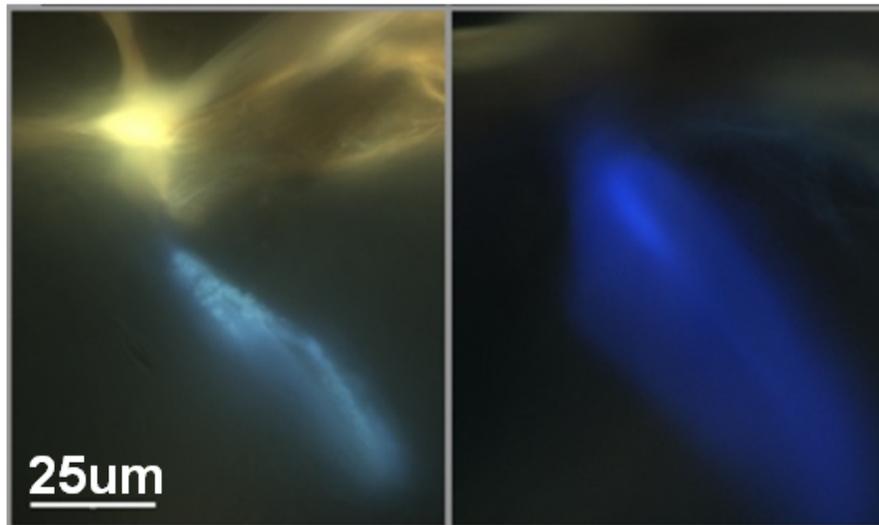


*Df(2R)anhedral<sup>1</sup>*  
live fly with anhedral  
wings posture



[www.bvmjets.com/](http://www.bvmjets.com/)

B.



*Df(2R)anhedral<sup>1</sup>*  
homozygous

*Df(2R)anhedral<sup>1</sup>* hemizygous

Figure 27. Results of a new deletion mutant of resilin gene knocking out *resilin* production in the TP tendon of *Drosophila*. A. shows the grossly apparent wing phenotype of *Df(2R)anhedral<sup>1</sup>* bottom as compared to wild type (*OreR*) top. B. Shows the appearance of the TP tendon in the homozygotic mutant left and the hemizygote at right. Scale bar applies to both images.

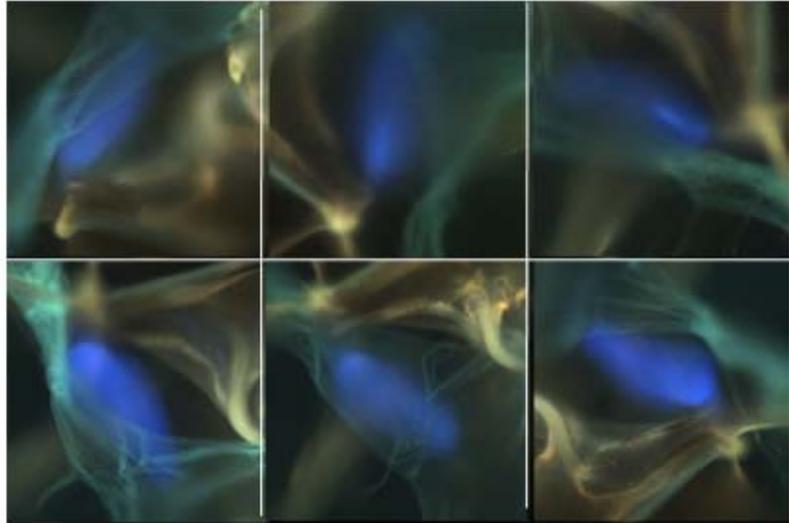
The TP tendon in these *Df(2R)anhedral*<sup>1</sup> flies is much diminished but not completely absent. A high magnification image of an *anhedral* tendon (Fig 27B, left) shows what appears to be an incompletely filled tendon sheath with unevenly dispersed fluorescent accumulations. Wild type tendons are generally quite homogeneous (Figure 17C). We crossed these *Df(2R)anhedral*<sup>1</sup> with *CyO* balanced flies and maintain this cross in parallel with our homozygous stocks in order to maintain the *anhedral* chromosome. TP tendons appear normal in these hemizygous flies (Figure 27B right).<sup>7</sup>

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<sup>7</sup> When maintained as a homozygous (for chromosome 2) stock, *Df(2R)anhedral*<sup>1</sup> at first had very low fecundity, we almost lost the homozygous line; with successive generations, the mutants retained their *anhedral* phenotype while becoming more reproductively fit. After a few generations, we are now able to easily maintain the mutant chromosome in a homozygous stock.

A.

*Df(2R)Exel7145*



B.

*Df(2R)anhedral<sup>1</sup>*

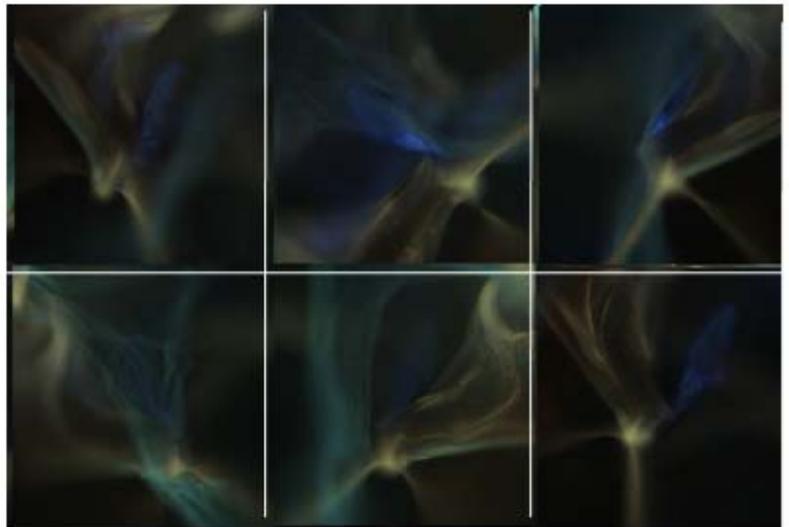


Figure 28. Results of knockdown of *resilin* production in the wing of *Drosophila* by deletion of the *resilin* gene. A shows TP tendons from a heterozygous *resilin* deletion mutant. B shows TP tendons from the new homozygous viable Resilin deletion made in the Collier lab. Tendons from 6 flies are shown from each strain. Individual images are each 145umx145um.

Figure 28 show comparison of the TP tendon in the previously described heterozygous mutant (balanced for 2<sup>nd</sup> chromosome) known as *Df(2R)Exel7145* with those from our new homozygous mutant *Df(2R)anhedral<sup>1</sup>*. The TP tendon is clearly malformed in our mutants (figure 28B) while it looks essentially normal in the mutants heterozygous for *resilin* deficiency (figure 28A). The presence of fluorescent material within the TP tendon in flies with apparently no *resilin* gene (figure 28B) implies that this tendon while rich in Resilin may be composed of a mixture of proteins. These other proteins may also be crosslinked with di- and tri-tyrosine molecules instead of the dopamine derivatives that tan most exoskeleton (Anh, 2011).

Neither the offspring of RNAi crosses yielding diminished synthesis of Resilin nor those of *Df(2R)anhedral<sup>1</sup>* mutant parents seemed affected in terms of viability or reproductive success (results not shown). *Anhedral* phenotype flies did show inability to quickly right themselves from lying on their backs and, as will be shown in the next section, were unable to fly (although they did seem to be excellent long distance jumpers as are wild type flies). These findings suggest that Resilin is quite specific to particular skeletal structures such as the TP tendon. If one were to globally prevent synthesis of the protein Elastin in a vertebrate, along with vertebral skeletal defects (of the ligamentum flava), the results would likely include severe arterial disease.

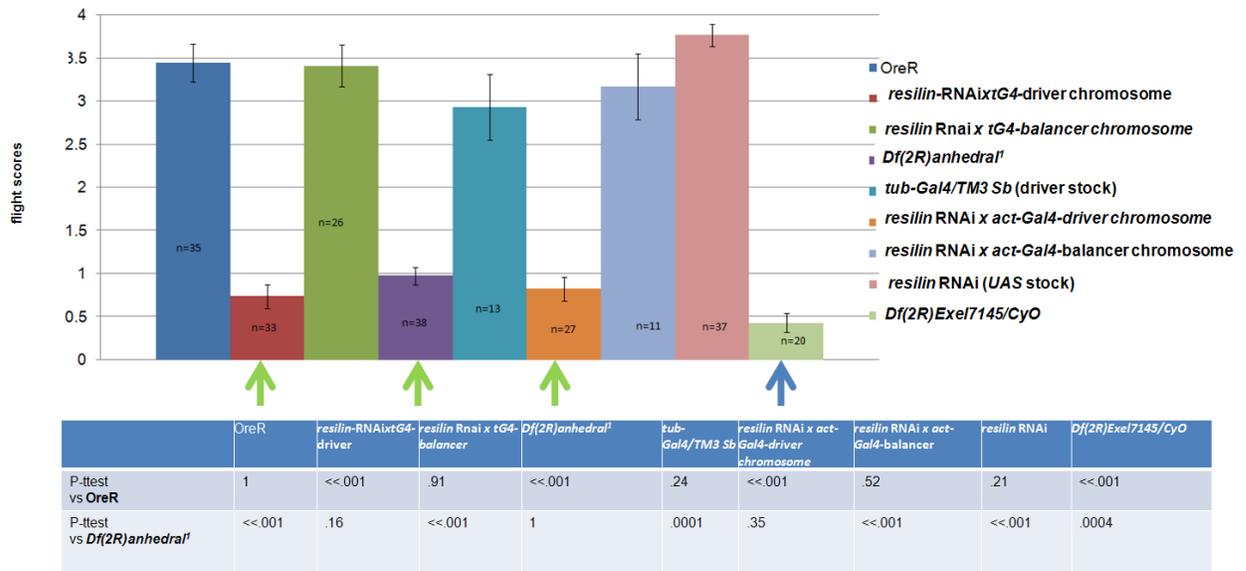
## Resilin's essential role in *Drosophila* flight

To quantitatively test the flight ability of flies deficient in Resilin we used the apparatus depicted in figure Appendix1. Ranking in these flight tests ranged from 0 (fell straight to the ground) to 4 (flew away without hitting the ground). Results of these tests are given in Fig 29 as mean of flight score with bars indication +/- 1SE. Green arrows indicate flies with the anhedral phenotype whether RNAi knock downs or *Df(2R)anhedral<sup>1</sup>* mutants. The blue arrow indicate flies of Curly wing phenotype from a stock balanced and heterozygous for a *resilin* deficiency (*Df(2R)Exel7145*) on chromosome 2. The TP tendon in these flies appears completely normal, their inability to fly is apparently a result of the curly wing phenotype. To our knowledge, this simple assay yields the first direct (not inferred) evidence of Resilin's essential role in insect flight.

Figure 29B shows our attempt to test the effects of the anhedral phenotype on the trajectory of the wing during flight. We immobilized wild type (left) and anhedral flies (right) and imaged a fluorescent spot on the left wing tip. A wing tip trajectory very similar to that described by Dickinson (2006) is seen in the OreR fly (figure 29B, left). Many trials yielded only one anhedral fly (*Df(2R)anhedral<sup>1</sup>*) that even attempted to fly. The wing trajectory of this fly can be seen in figure 29B right and is clearly different than the wild type. In fact, it appears that this anhedral fly did not complete a full wing beat but instead simply moved its wing forward then returned it along the same path. The bright white spot indicates that this fly's wing was stationary during most of the exposure time. If we are to learn anything about how the TP tendon controls wing trajectory in

flight we must develop a method to produce mutant offspring that will attempt to fly but still have a deficient TP tendon; that is, we need a less severe functional Resilin deficiency. To this end, we have had preliminary success knocking down transcription of a peroxidase enzyme gene as described below.

A.



B.

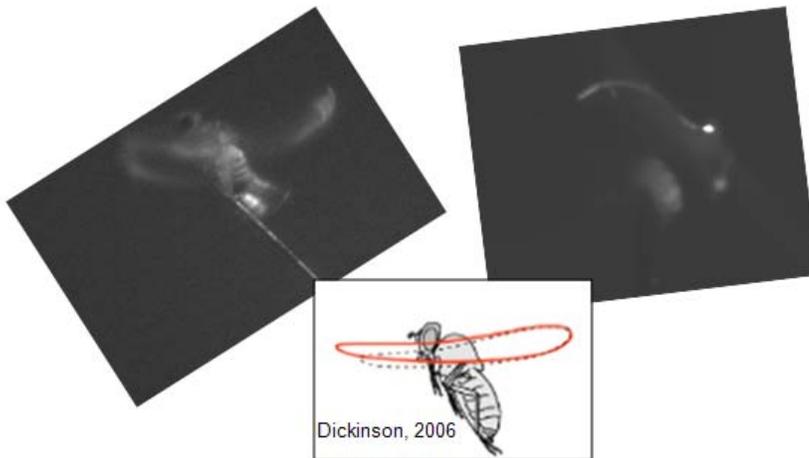


Figure 29 A. Results of flight tests in *D. melanogaster* of flies with the anhedral phenotype (green arrows) vs those with wild type wing posture and normal TP tendon. Data presented as mean +/- 1SE. probability/significance values using two sample t-test assuming unequal SD are shown at bottom. B. Images of tethered flies showing wing tip trajectory. Left is wild type (OreR), center bottom is diagram of normal wing trajectory in a generalized dipteran (Dickinson, 2006) and at right is a *Df(2R)anhedral*<sup>1</sup> fly which has poorly formed TP tendon.

## Attempt to alter crosslinking of Resilin by knocking down peroxidase enzyme genes

All available Resilin literature that addresses biochemistry of crosslinking implicates peroxidase activity in the formation of di- and tri-tyrosine molecules (Coles, 1966 Elvin, 2005 Edens, 2001 and many others). Figure 16 shows the enzyme catalyzed reaction (A) and hypothetical schematic (B) of crosslinking. This model prompted us to attempt to knock down 2 different peroxidase enzymes using RNAi driven by the *actin* and *tubulin-Gal4* (constitutive expression) driver lines as well as the *apterous-Gal4* wing driver line. We first tried to knock down the gene for the protein known simply in *Drosophila* as Peroxidase (*pxd*). The results of all of these crosses with *actin* and *tubulin-Gal4* drivers was lethality prior to puparium formation. Lethality of *pxd* gene mutants has been reported (<http://flybase.org/reports/FBgn0004577.html>). We then tried to affect TP tendon formation by knocking down another enzyme's gene with peroxidase activity known as Duox (gene *dual oxidase*). This enzyme is considered a homologue to NADPH and has been shown to be involved in wing maturation via control of tyrosine based tanning (Anh, 2011). Additionally, Edens (2001) has modeled Duox as a transmembrane protein with peroxidase activity (figure 16B). They suppose that its extracellular domain has peroxidase activity capable of using tyrosine and hydrogen peroxide as substrates yielding water and di-tyrosine as product. Figure 30 shows the results of Duox (gene *dual oxidase*) down as well as the knock down of transcripts for a protein called Mol (gene *moladietz*) that is suspected to act as a Duox maturation factor (<http://flybase.org/reports/FBgn0086711.html> and Xie, 2010). In the *dual oxidase* RNAi knock down we see that the TP tendon seems to occupy a near wild type volume but shown very little florescence indicative of di- and tri-

tyrosine crosslinks. In the *moladietz* RNAi knock down (middle) we see no TP tendon at all. Furthermore, we see in the both images other wing hinge connectives that are presumed to be elastic (Andersen Weis-Fogh, 1964) and may contain Resilin. They appear quite normal as compared to wild type morphology seen at bottom of figure 30. These results suggest that *apterous-Gal4* is promoted in the tissues of the wing disc that form the TP tendon but is not in those that form the connectives in the wall of the thorax at the wing hinge. We have thus far not tested these apparent Resilin crosslinking deficient flies to determine if they can fly normally. However, these results are promising in their potential to both selectively derange parts of the wing hinge mechanism and in that I believe that they are the first evidence of specific enzyme activity in crosslinking Resilin.

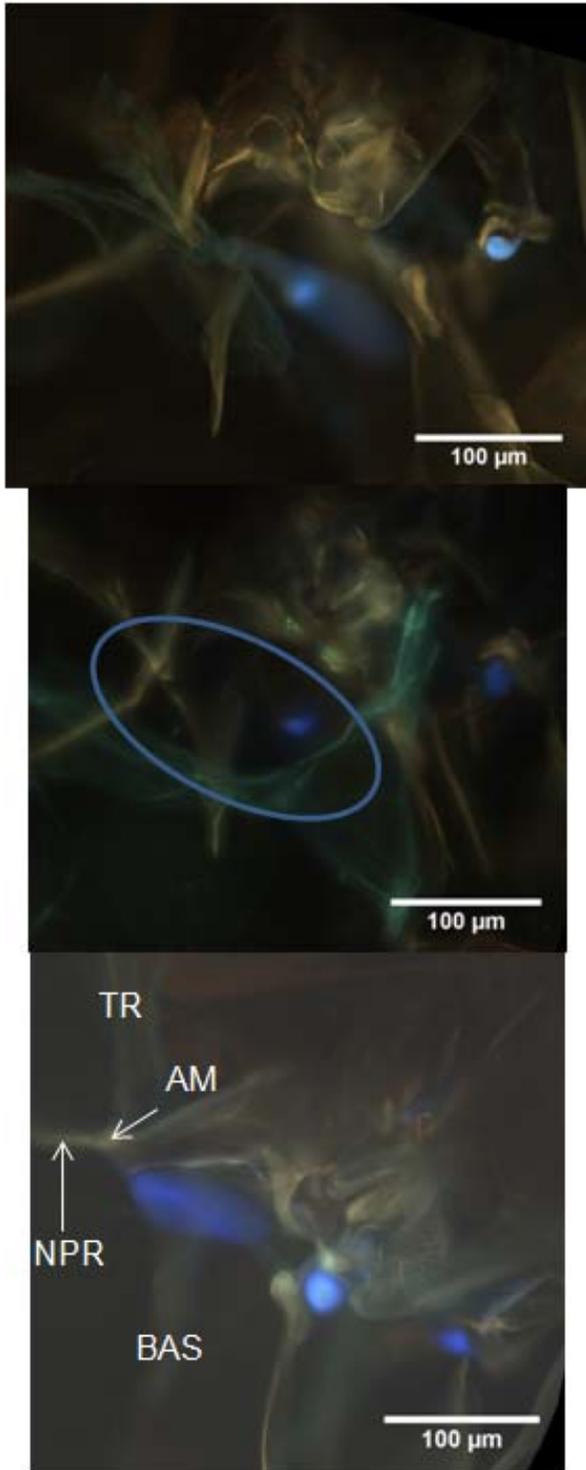


Figure 30. Top is offspring of *apterous-Gal4 x UAS-duox RNAi*; bright spot in middle of TP tendon is another cuticular structure out of focal plane behind tendon. Middle is offspring of *apterous-Gal4 x UAS-moladietz RNAi* both raised at 30 degrees. No TP tendon is visible here.

Bottom shows wild type morphology \*of the TP tendon and two other putative Resilin rich elastic connectives in the wing hinge. Andersen and Weis-Fogh (1964) mention that there are additional Resilin tendons between subalar sclerite and second axillary sclerite (pterale II) and between subalar sclerite and third axillary (pterale III).

\*This fly with wild type appearing tendon actually resulted from unsuccessful late pupal knockdown by *peroxidase RNAi*.

## Progress toward imaging the TP tendon at molecular resolution

One of the main motivations for this project has been my desire to reproduce, in the fruit fly, TEM investigations published by Weis-Fogh in 1970. In figure Appendix2, we can see that individual Resilin molecules are exported from epithelial cells into the tendon of a teneral locust. Their approximate length of 100-200nm is consistent with the size of the protein products of both known *Drosophila resilin* gene transcripts (~600 amino acids; <http://flybase.org/reports/FBgn0034157.html> and <http://www.tulane.edu/~biochem/med/second.htm> ). These micrographs were recorded more than 30 years before the first *resilin* gene sequence had been described (Andersen and Ardel, 2001). We prepared both wild type and anhedral phenotype flies for TEM investigations. In figure 31A are semithin sections resulting from our approach to the plane of the TP tendon. At left are the more anterior sections, the full range from left to right is ~150-200um. Notice that the T and NP ridges (double black arrows) converge to form the apophysis of the mesoscutum (single black arrow) onto which the TP tendon (white arrows) inserts. The TP tendon in section appears very much like the dragonfly tendon described by Weis-Fogh in 1960 (figure 7A; he described the dragonfly tendon as hollow and sausage like). In figure 31B at far right the TP tendon (white arrow) appears as a hollow cylinder of Resilin (lavender) with light pink stained connectives to the tergopleural muscles (stain is toluidine blue/basic fuschin, EMS)<sup>8</sup>. The TP tendon likely grows from the thoracic wall as an invagination of epithelium during metamorphosis. We are currently attempting use of more specific *Gal4* driver

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<sup>8</sup> Andersen (1964) reports that in hydrated Resilin tissues, toluidine blue (basic dye with colored cations) leaves resilin sapphire blue while basic fuschin, he reports, is 'useless'. Tissues shown in figure 31 are not hydrated but rather are dehydrated and embedded in epoxy resin; colors resulting from our staining reactions do not match those of Andersen's. We have not investigated the specificity of tol blue or basic fuschin to Resilin in epoxy.

lines to locate the tissue in the imaginal wing disk (nascent adult wing and wing hinge tissues) that eventually differentiate into the TP tendon. We also have prepared in-situ probes that will help us track *resilin* mRNA production.

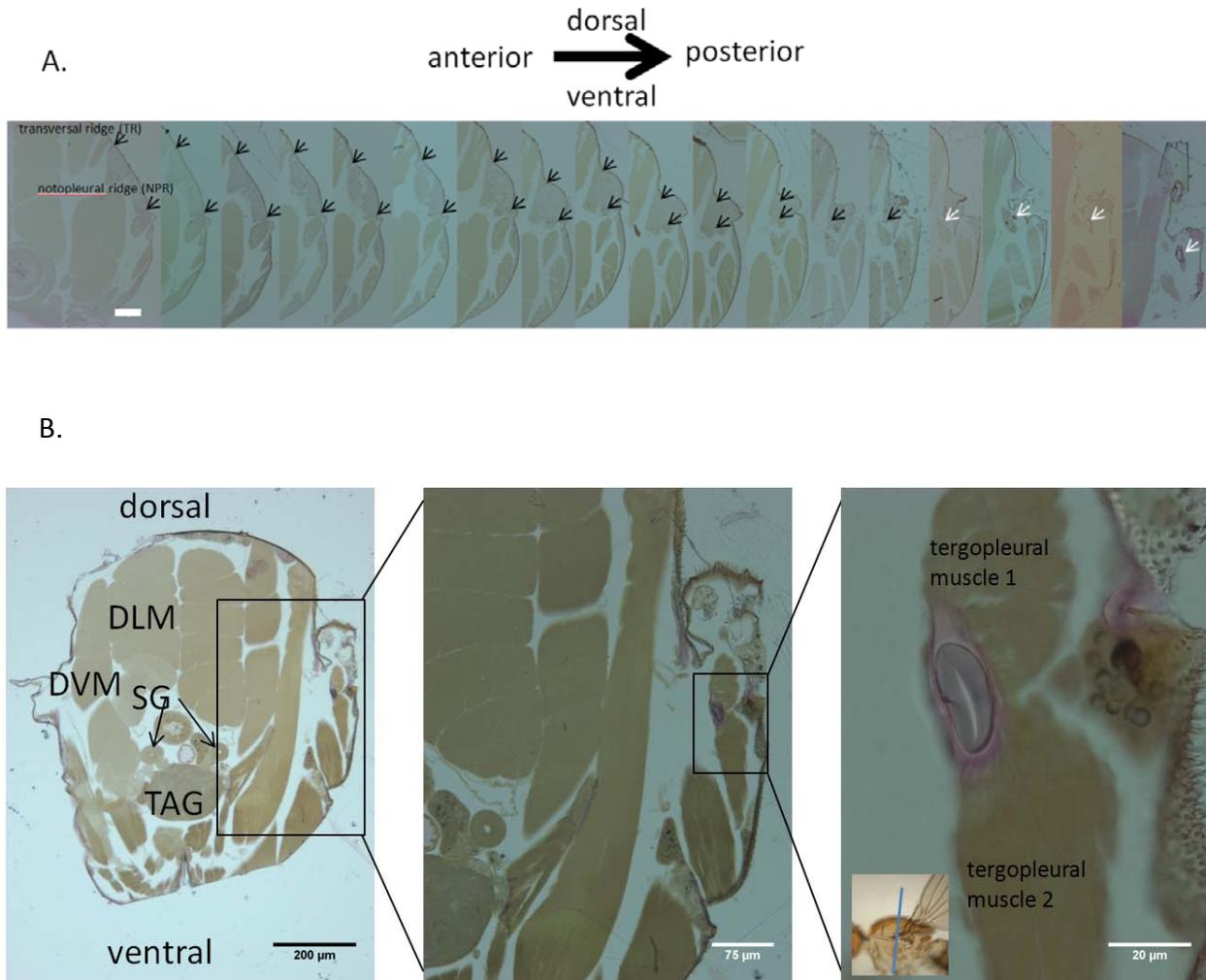


Figure 31. A. 18 semi-thin sections approaching the TP tendon from anterior of fly thorax. The scale bar at left represents 75um; full range from left to right is ~150-200um

B. Semi-thin (~1um) sections from the approximate intended final plane of section; transverse plane through the TP tendon taken from location of blue line in inset.

DLM (dorso-longitudinal muscles) DVM (dorso-ventral muscles), SG (salivary glands), CR (crop), TAG (thoracico-abdominal ganglion)

## Bursicon-Pburs hormonal pathway and eclosion

As described in the background section, the Bursicon hormonal pathway carries the presumed essential signal needed to promote the cellular and behavioral activity that completes metamorphosis; EMT of wing epithelium, apoptosis of these cells and the CCAP/Bursicon producing cells of the abdominal ganglion, the air gulping/abdominal contractions that serve to expand the wings, and cuticular sclerotization. Most current literature does not link the Bursicon pathway with initiation of ecdysis and eclosion (Peabody , 2009 and personal communication with Nathan Peabody, GSA conference 2011). While collecting data, I noted wing phenotypes typical of Bursicon-Pbursicon and Rickets derangements and I noted eclosion rates, but I do not report any data regarding degree of sclerotization; the results presented here do show the classic *rk* or *bursicon* mutant wing phenotype (figures 33 and 36). An observation made early in the series of experiments intended to test the relationship between Bursicon signal and Resilin maturation led us to the hypothesis that the Bursicon subunit alone, but neither its partner Pburs nor their receptor Rickets (DLGR2), has notable effects on eclosion behavior. The following experiments were performed to test this hypothesis.

Figure 32 shows the crosses that were used in these experiments. In all of these crosses, the female parents carried both the *Gal4* driver chromosome and a balancer chromosome (its homologous chromosome) that made obvious which of the offspring transcribed the RNAi intended to knock down parts of the Bursicon pathway. The driver lines used here are heterozygous with one of the homologs (the driver) having a ubiquitously activated promoter, in this case taken from either the *actin5C* gene or the *tubulin* gene. The homologous chromosome to the driver (the balancer)

has mutations that lead to a dominant phenotype that is easily recognized; in this case either tubby body (*TM6-Tb*) or stubble bristles (*TM3-Sb*). Any fly with the mutant phenotype does not transcribe the knock down RNAi of interest.

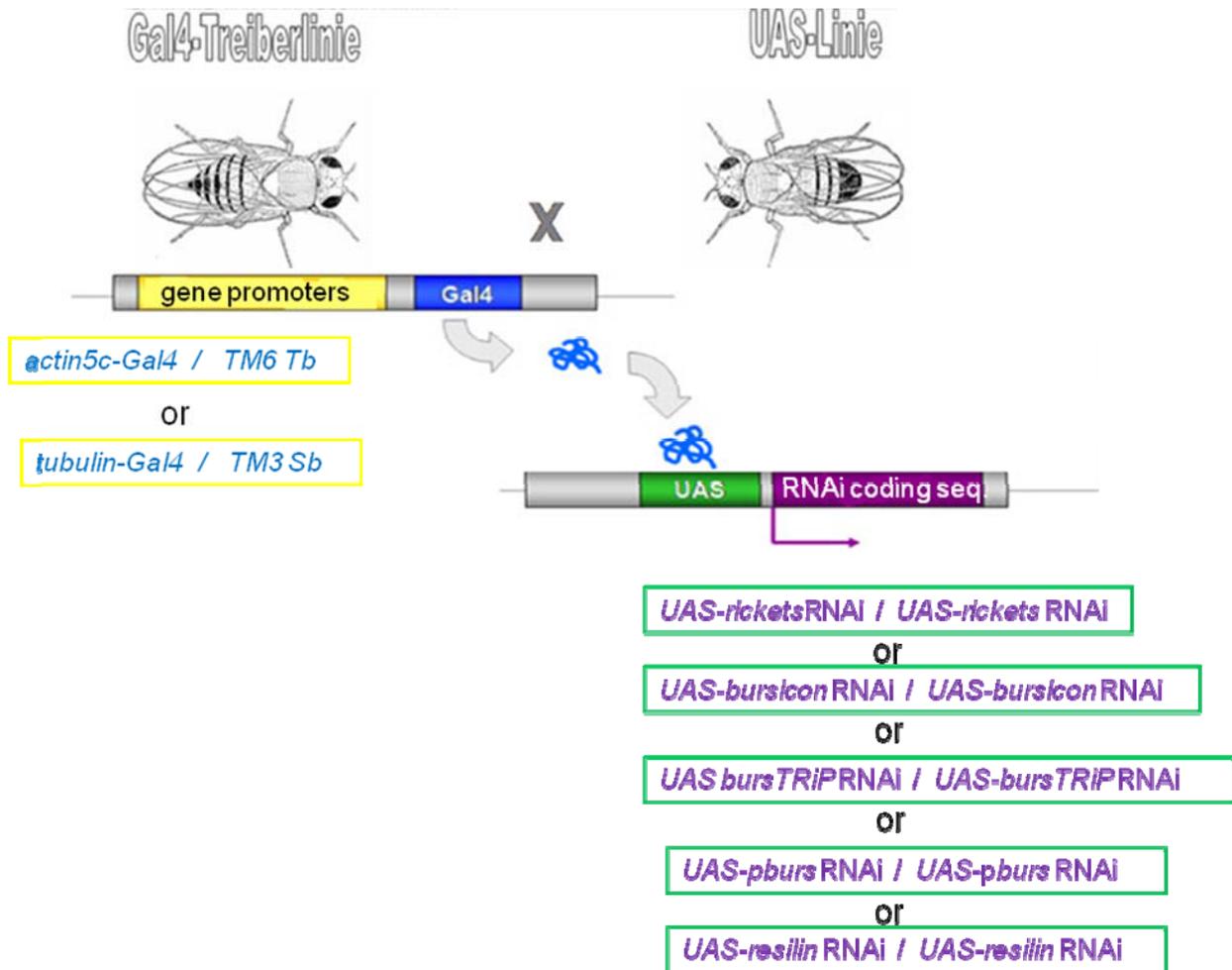


Figure 32. Diagram describing the crosses carried out to test the Bursicon signaling pathway. Each box represents a pair of homologous chromosomes. Each offspring inherits only one of these. In the case of the driver line (above) either the driver chromosome (e.g. *actin5c-Gal4*) or the *TM6-Tb* balancer chromosome (e.g. Tubby aka Tb). The *UAS* RNAi fly lines at right are all homozygous for the chromosome bearing the RNAi coding sequences for knockdown of either *rickets*, *bursicon*, or *partner of bursicon*. Each offspring inherits one copy of this *UAS* RNAi chromosome. Thus, RNAi expression is dependent on the presence of the driver chromosome. The *UAS resilin* RNAi line serves as a control as neither have we seen deleterious effects on eclosion when Resilin is knocked down nor is Resilin believed to interact with hormonal signaling in any way.

In yeast, *UAS* is part of a promoter and *Gal4* is inducible by galactose.

figure is adapted from <http://www.mpibpc.mpg.de/inform/MpiNews/cientif/jahrg10/4.04/fig3.html>

The fact that we have 2 unique *burs*RNAi lines with different genetic backgrounds (*bursicon* RNAi and *burs* TRiP RNAi) is simple good fortune as we requested these lines before we began our experiments. The *bursicon* RNAi, *pburs* RNAi and *rk* RNAi lines were all made in flies of approximately the same genetic background while the *burs* TRiP line is from a different source and genetic background.

Figure 33 shows results from the initial *burs*, *pburs*, *rk* knock down experiment we performed. These graphs are raw data showing the number of flies in these crosses that eclosed as a function of which chromosome they inherited (driver or balancer). The two *bursicon* RNAi knock downs showed many more instances of death in the pupal case as seen in the very low percentage eclosion (0% and 11% *burs* TRiP and *burs* RNAi respectively). The images inset into the graphs show the wing phenotypes of flies that inherited the driver chromosome and eclosed. The wing phenotypes seen in the various crosses were somewhat variable but not reproducibly stronger in any particular RNAi knock down. In this experiment no flies from the *UAS- bursTRiP* RNAi x *tubulin Gal4/ TM3-Sb* cross emerged so only unclosed flies are shown (top right). Subsequent repetition of this experiment showed that 64% was an unusually low eclosion percentage for the *rk* knock down flies (top left). We repeated these types of crosses 6 times and at 3 temperatures for each. Figures 34-36 show the results from these experiments. No difference was seen between the *Gal4* offspring promoted by *actin* or *tubulin* promoters so these crosses were pooled. Figures 34-36 show ratios of eclosion success calculated as follows;

**eclosion rate = flies eclosed / (flies eclosed + flies dead in pupal case)**

Results from each brood were calculated then pooled with data from other broods as described below.

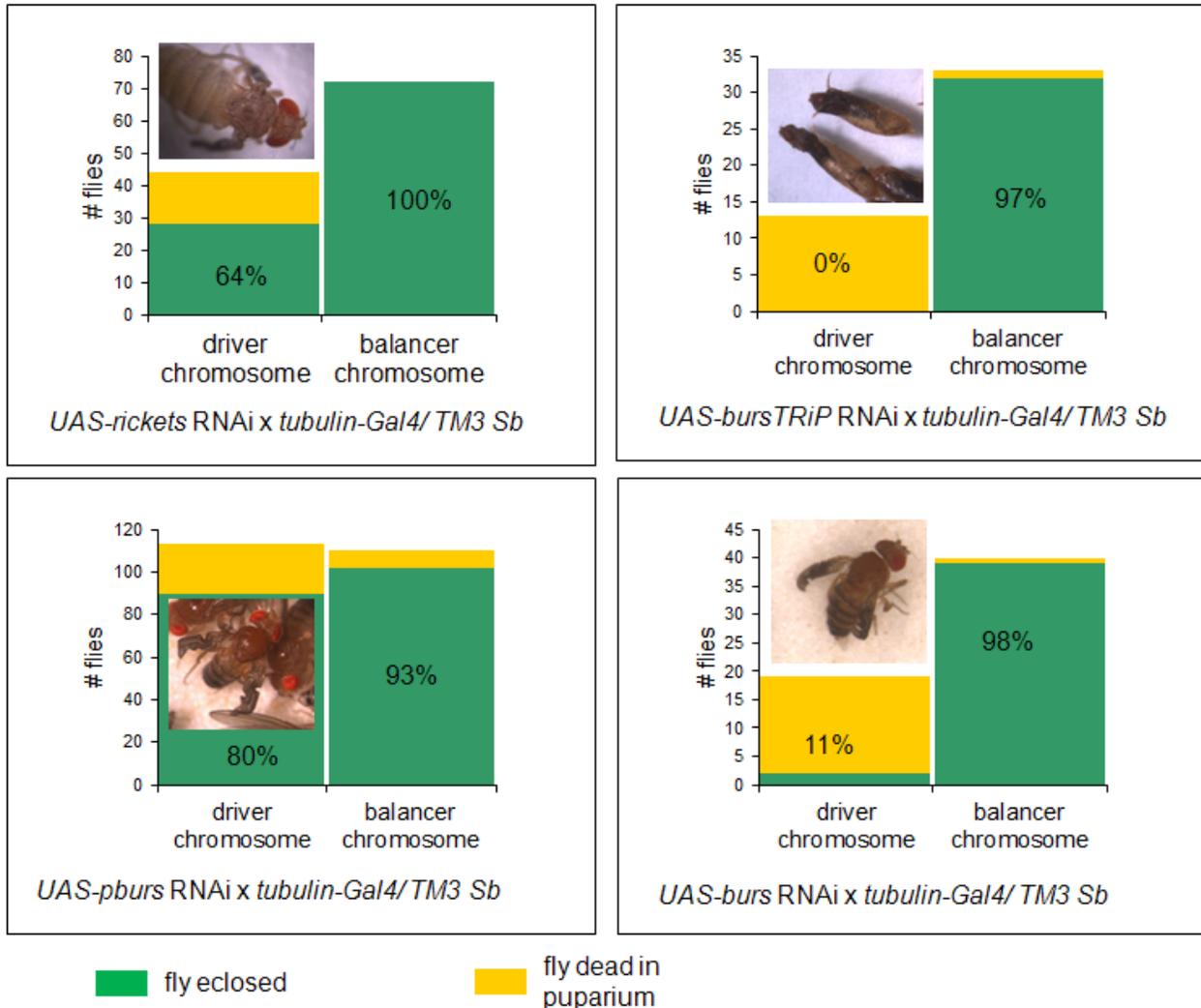


Figure 33. This is an example of the raw data collected in the Bursicon pathway knockdown experiments. Notice that only the two *UAS RNAi* lines (*burs RNAi* and *bursTRiP RNAi*) that knock down the Bursicon subunit have <15% successful eclosion. Images of flies are representative of those that inherited the driver chromosome and eclosed. None eclosed in the *bursTRiP* cross so flies dead in puparium are shown. These crosses were raised at 25deg C. Percentages shown are

**flies eclosed / (flies eclosed + flies dead in pupal case) x 100**

In figure 34, data taken at various rearing temperatures were pooled and effects of *Gal4* expression were tested by comparing eclosion in offspring inheriting the driver vs balancer chromosome. Clearly, when either *burs* RNAi or *burs* TRiP RNAi is driven, the rate of eclosion is significantly lowered while the rates of eclosion for other driven RNAi knock downs are similar to those seen when the balancer chromosome is inherited. The enormous error bar seen in the *burs* TRiP data is a result of one of the crosses from which emerged a total of 4 flies (from only 4 pupal cases). One of these empty cases had the driver phenotype (i.e. not tubby). As there were no dead pupae seen in this cross, this was scored a 100% eclosion. Three of the other crosses combined to arrive at the mean plotted with this bar were scored 0% eclosion, thus the large SD. Over 200 flies were categorized to arrive at this *burs* TRiP data point, but ratio of eclosion to failure in each brood was used as the metric for eclosion success so data was not weighted regarding # of flies. Figure 35 displays data testing the effects of rearing temperature on eclosion rate. In the *UAS-Gal4* system, higher rearing temperatures often lead to stronger *Gal4* driven phenotypes (see figure 23 for another example of this effect). Only the flies in which the Bursicon subunit transcript was knocked down show notable difference in eclosion rate as a function of temperature. Notice also that the *rk[4]* mutant (for *rk* gene (aka *dlgr2*)) here and in the next figure is used as a non-RNAi control. OreR may have been a preferable control but while maintaining *rk[4]* stocks for the past 2 years I have never noticed any indication of failure to eclose at higher or lower rates than wild type.

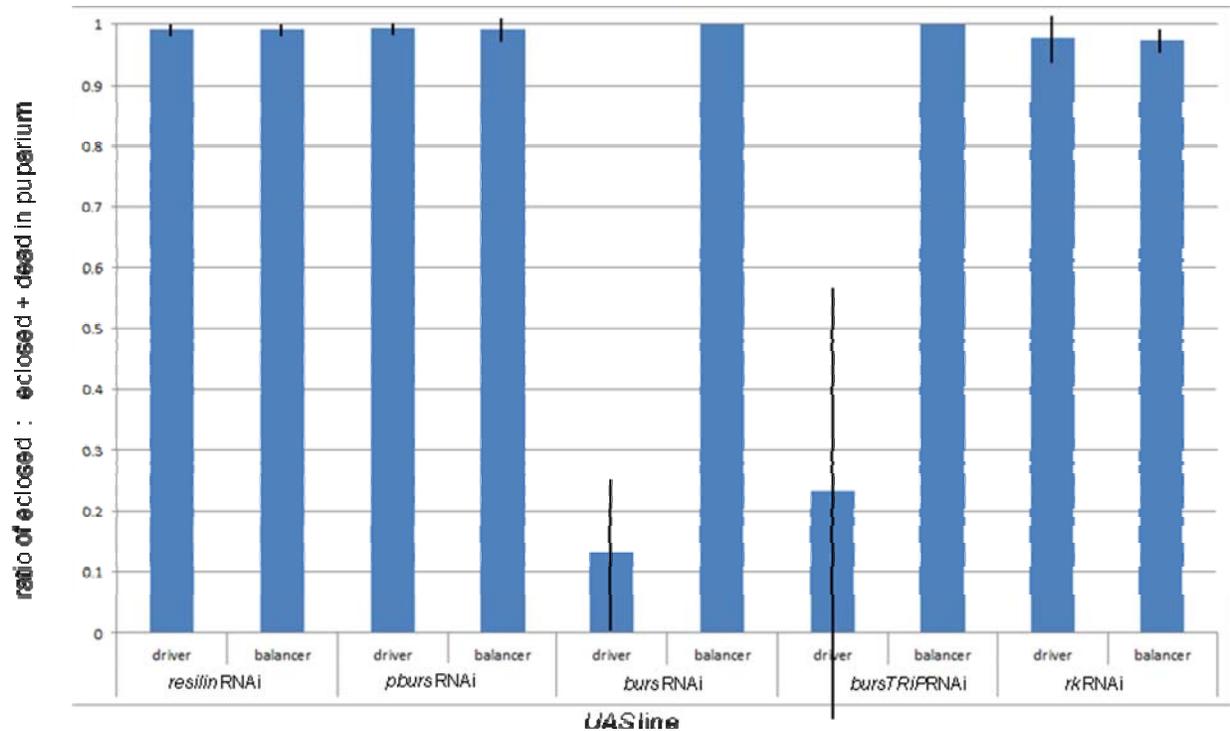


Figure 34. Comparison of offspring that either inherited the *Gal4* driver chromosome or the balancer chromosome in Bursicon pathway knockdown experiments. Only the flies in which the Bursicon subunit itself was knocked down show significant difference in eclosion rate as a function of inheriting driver vs balancer chromosome. These are combined results are from both the *tubulin-Gal4* and the *actin5c-Gal4* drivers.

Data reflecting different rearing temperatures was pooled (18deg C, 25deg C, and natural light/temp). Displayed as mean +/- one SD.

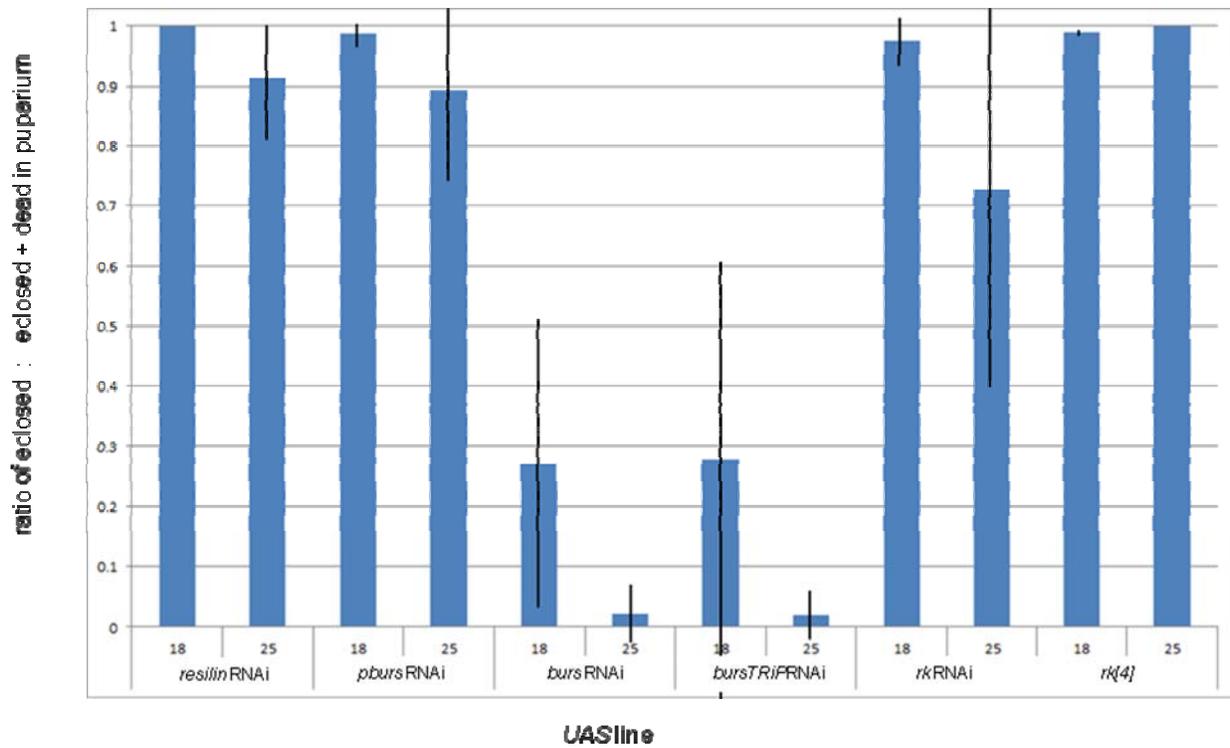
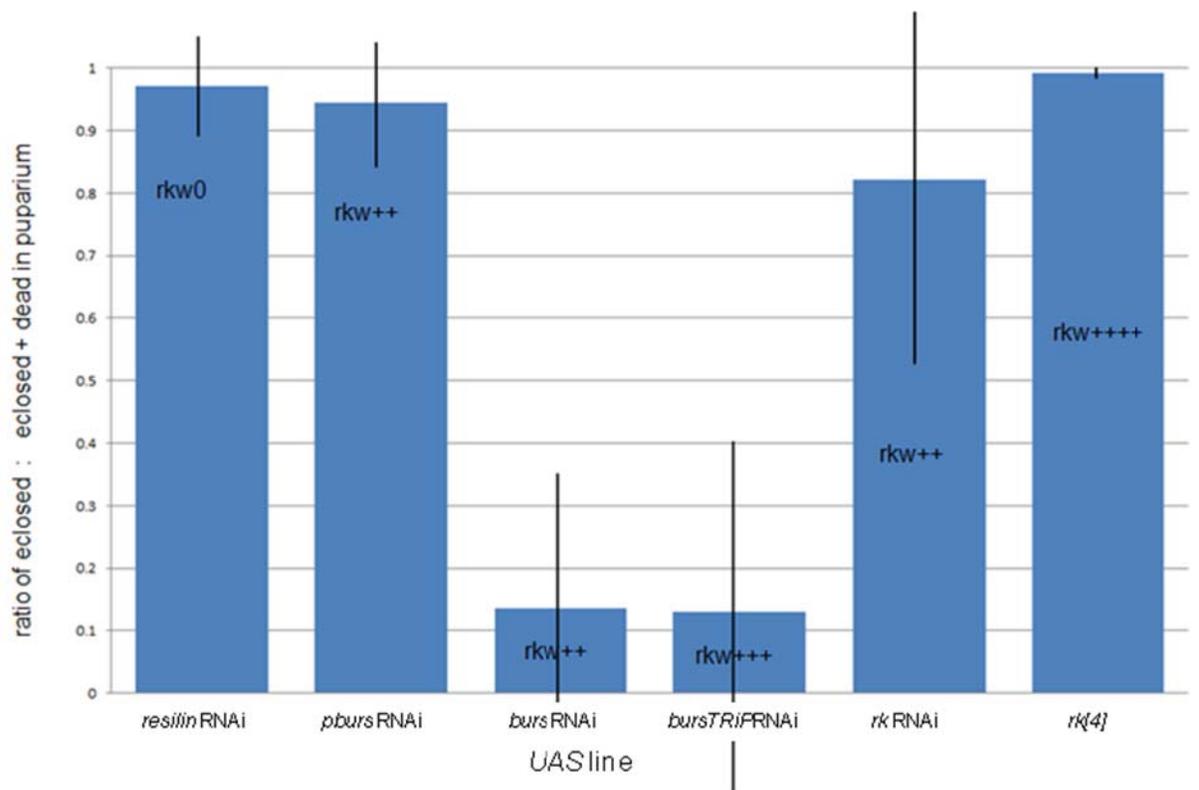


Figure 35. Comparison of different rearing temperatures in only those flies that inherited the *Gal4* driver chromosome. These are combined results from experiments using the *tubulin-Gal4* and the *actin5c-Gal4* drivers. Rearing temperatures are 18deg C and 25deg C. Displayed as mean +/- one SD.

Figure 36 is data pooled from all driver lines and from flies raised at all temperatures. The differences in the mean eclosion rates are statistically significant when either of the *bursicon* knock downs is compared to any of the other knock downs (*rk*, *resilin*, *pburs*, or *rk[4]* mutant). The two *bursicon*RNAi lines are not significantly different from each other. Neither are the non-*bursicon* subunit knock down rates different from one another. Included in this graph are rankings of unexpanded wing phenotypes ranked from *rkw0* through *rkw++++*. Each bar in this graph represents hundreds of flies and the wing phenotypes were often quite variable even within a single cross so this ranking is a crude estimate based on notes taken while flies were counted. The exception to this variability is the classic rickets *rk[4]* mutant which always gave offspring with a *rkw++++* ranking; although the mutant *rk[4]* has the most severe of wing phenotypes, eclosion rate is very high.

These RNAi crosses support our initial observation that the Bursicon subunit (but not *Pburs* or *Rickets*) may play an as yet undefined role in eclosion behaviour. Truman and Baker (2002) divide the final stages of metamorphosis into three; pre-ecdysis, ecdysis (both pre-eclosion), and expansion phases (post-eclosion). That Bursicon-*Pburs* and *Rickets* are involved in the expansion phase is very well supported (Baker and Truman, 2002, Peabody and White, 2009, Loveall, 2010) and is shown in our results by the wing phenotypes seen in all Bursicon, *Pburs*, and *Rickets* related crosses but not in the *resilin*RNAi crosses.



UAS line	<i>resilin</i> RNAi	<i>pburs</i> RNAi	<i>burs</i> RNAi	<i>bursTRiP</i> RNAi	<i>rk</i> RNAi	<i>rk[4]</i> (not RNAi)
P-ttest vs <i>resilin</i> RNAi	1	.52	<<.001	<<.001	.08	.46
P-ttest vs <i>burs</i> RNAi	<<.001	<<.001	1	.94	<<.001	<<.001

Figure 36. Data pooled from crosses raised at 18deg C, 25deg C, 28deg C and 30deg C and driven by *tubulin-Gal4* and *actin5c-Gal4*. Included on the bars are semi-quantitative rankings of the severity of the classic *rk* phenotype as described in the box at right.

Displayed as mean +/- one SD. At bottom are probability/significance values using two sample t-test assuming unequal variance

However, in this study we are interested in the pre-ecdysis phase which ends in eclosion. Davis (2007) states that CCAP, ET, and ETH, all control ecdysis and eclosion behavior whereas Bursicon signal governs post-eclosion maturation and refers to Bursicon as the “post-eclosion hormone”. Peabody (2009) shows no measurable immunoreactivity to Bursicon subunit in the hemolymph of the fly until after eclosion and subsequent perching in preparation for wing expansion. However, our data imply and there are indications in the literature that Bursicon signal acts before the expansion phase; that is, the Bursicon subunit is a control factor in eclosion. Below is a summary of the relevant interplay between the Bursicon-Pburs signaling pathway that imply a special and undefined role for the Bursicon subunit at eclosion.

Baker and Truman (2002) found that there are several pre-eclosion developmental periods (windows) during which behavior can be altered by removing central governance via neck ligation (aka decapitation). If ligated very early, prior to 3 hours pre-eclosion, all CNS (EH, CCAP, and Bursicon) signal is blocked and flies do not attempt to eclose.

Flies that are neck ligated during a very late window (50 mins. pre-eclosion through eclosion, similar stage to the fly seen in figure 21C) attempt eclosion immediately but neither show tanning nor wing expansion behavior; this is the same outcome that occurs if flies are decapitated just after eclosion and the same outcome seen in unligated *rk[4]* mutants.

During an intermediate window that spans about 3 hours to 1 hour pre-eclosion, flies that are neck ligated do attempt to eclose, and many do so more quickly than

normal (within minutes). Those that attempt eclosion quickly after ligation show wing expansion behaviours immediately after leaving the puparium. This wing expansion behaviour also occurs in neck ligated *rickets* mutant flies. The precocious eclosion event in ligated flies during this intermediate window implies that there is a centrally generated signal (removed by decapitation) that suppresses eclosion until the appropriate time. Additionally, in wild type unligated flies, there is a minutes-long delay between eclosion and wing expansion (flies normally find a perch before expanding wings) that is not seen in flies decapitated during the intermediate window. These observations imply that a central signal delaying wing expansion behavior is also eliminated in the ligated flies. The nature of these descending signals is not clear (Baker and Truman, 2002) but SEG generated Bursicon subunit is one possibility.

Work by Loveall et.al. (2010) expands the role of Bursicon to include pre-eclosion signaling. Their findings include both the pre-eclosion localization of Bursicon subunit alone (not Pburs) to neuromuscular junction (NMJ) at the body wall and the failure to eclose of certain *rk* mutants. The latter finding was attributed to poorly formed “rickets” legs preventing escape which was not observed in our experiments.

Loveall et.al. (2010) also reported *Gal4* driven *bursicon*RNAi results much like ours (failure to eclose) and showed that there was very little body wall NMJ Bursicon immunoreactivity in the *bursicon*RNAi knock down flies. The Loveall work did not test Pburs in this way and their *rk* RNAi knock downs (unlike ours) led to nearly 100% lethality before pupal stage. In Loveall’s experiments, when *rk* RNAi larvae were rescued by hand and a special grape juice agar media was provided the larvae did develop but they saw a variety of lethal pupal deformations that we did not see in our *rk*

RNAi knock downs.

The expression of only the Bursicon subunit in neurons pre-eclosion is supported by another study (Luo, 2005) that demonstrated Bursicon and PBurs are not always co-expressed. Luo (2005) showed that in 3<sup>rd</sup> instar *D. melanogaster* (larvae) Bursicon and Pbars were coexpressed in some but not all of the neurons of the sub-esophageal (SEG) and abdominal ganglion (AG). Bursicon expression without Pbars was seen in some cells while Pbars was apparently not expressed without Bursicon (Luo, 2005 and Peabody, 2008). To this point, there is no molecular evidence that Burs and Pbars work as anything but a heterodimer, however the results just cited (Loveall et.al., 2010 and Luo, 2005) as well as those from this report suggest further research into this possibility.

Recent work using RNAi knock down of *rk*, *burs* and *pburs* in the flour beetle pupae (via injection of double stranded RNAi into late larval beetles) has shown that they do work as a heterodimer (Burs-Pbars) via the Rickets receptor as in the fruit fly (Arakane, 2008). Arakane also showed that knockdown of the Bursicon subunit alone (not Pbars or Rickets) led to weaker pre-eclosion behaviours (A-P contractions) in the flour beetle thus suggesting a role for non-heterodimeric form of Bursicon. There is great diversity of function of these hormonal cascades in insects of various orders.

It seems that reshuffling of the chromosomal location of *bursicon* and *pburs* genes may reflect their differential function in different insect orders. The *bursicon* and *pburs* genes are on different chromosomes in *Drosophila* but nearly adjacent to each other in honey bee (Hymenoptera) and beetle (Coleoptera). In mosquito (Diptera), transcripts of these two genes have been shown to be spliced together by what is

known as trans-splicing (contrast with cis-splicing or intron removal (Arakane, 2010)).

Arakane (2010) and Baker and Truman (2002) have suggested that plasticity of the neural mechanisms that govern ecdysis and eclosion have increased as insect eclosion behaviours have diversified. Truman and Baker (2002) propose that the primitive character in holometabolous insects is that sclerotization and unfolding occur immediately upon release from the puparium (this is what is described above when he decapitated the very late pupae). The more derived insects (i.e. dipterans) have developed neuro-endocrine driven strategies to delay tanning and expansion. We believe this central control is necessary because of the dipterans specialized behaviours during pupation.

Early work in eclosion and maturation in flies is exemplified by the Sabaratnam (1973) experiments in which he delayed maturation of the blowfly by artificially maintaining its digging behaviour. Blowfly larvae dig some distance into the ground before pupa formation and must dig out to complete maturation. Sabaratnam put newly eclosed flies in a tube full of dirt and repeatedly inverted it confusing the fly's response to gravity. He then reported deficient Resilin tendon formation due to Bursicon insufficiency (there was only one protein entity known as Bursicon in 1973). His paper (and those that gave conflicting accounts of Resilin maturation) provided the motivation and structure for the experiments reported in this thesis.

## Conclusions

1. We have more fully described the anatomy of the TP tendon in *Drosophila* which possibly because of its small size has to this point not been well characterized.

Furthermore, through manipulation of transcription (RNAi) and the *Drosophila* genome (*Df(2R)anhedral<sup>1</sup>*) we have shown that the TP tendon is largely composed of protein translated from the *resilin* gene first identified by S.O. Andersen and Ardel (2001).

Additional experiments could show with more detail the interaction of Resilin with surrounding molecules/tissues (e.g. is there differential expression of Resilin transcripts with and without chitin binding domain?). Also, further work should be done regarding the anatomy of the TP tendon and associated sclerites and elastic connectives.

Description of this functional anatomy is incomplete.

2. Through results from flight testing of our *resilin* knock downs, we present the first direct evidence of the necessary role of Resilin in flight mechanism of an insect (*D. melanogaster*).

Future work should include better definition of the TP tendon's role in flight control, energy storage and other behaviors (maintenance of wing posture during sclerotization and mating song). Other Resilin containing connectives in the wing hinge should be included in future descriptions.

3. We have shown that the post-eclosion pulse of Bursicon-Pburs signal that acts through the DLGR2 (*Rickets*) receptor is not necessary for complete maturation (crosslinking) of the TP tendon in the adult fruit fly.

Through further experimentation with TEM and light microscopy (including specific probes against mRNA and protein epitopes) we hope will show in more detail the timing and location of the epithelium driven deposition and crosslinking of Resilin.

Molecular cues of origin other than the Bursicon pathway will likely be found.

Additionally, using cultured insect cells such as S2 fly cells it may be possible to create biotechnologically designed protein sheets with programmed molecular structure.

4. In RNAi knock downs of *duox* and *moladietz* (Duox maturation factor), we have shown (preliminarily) that the peroxidase activity of the Duox enzyme plays a critical role in forming the di- and tri-tyrosine crosslinks in Resilin.

We hope to create fly lines in which the TP tendon will retain partial wild type form and function. In studying these flies histologically and testing their proficiency in flight we hope to more specifically define the role of Resilin in fruit fly behaviour.

5. In testing the knock down effects of Bursicon pathway components, we have added to the small but growing body of evidence that supports a role for the Bursicon subunit in eclosion that is independent of its widely accepted role (with Pburs and Rickets) in driving post eclosion wing changes and sclerotization.

In addition to its role as a neuropeptide hormone (and likely neurotransmitter), Bursicon signal has been implicated in transcriptional regulation as it has been shown to regulate components of chromatin remodeling complexes in house flies (An, 2009). If future studies are able to find molecular activity of the Bursicon subunit as a monomer or homodimer this would establish a new role for the structurally highly conserved cysteine knot family of neurohormonal peptides.

## Methods

### Rearing *D. melanogaster* stocks

Short descriptions of the genes and fly strains used in this work and their source are listed in table appendix table A1. Flies were raised in incubators that were kept constant at the temperatures mentioned throughout this report (18, 25, 28, or 30deg C). There was no control of light/dark cycle during this study. Despite this I did noticed that most flies tend to eclose in the late morning and that fly populations in different vials reared in the same incubator show widely varying times of high eclosion rate (e.g. best collection times could range from 8:30AM-2PM). I believe this variability could be minimized with a more regular light/dark cycle.

Below are approximate generation times at the temperatures indicated. (Ashburner, 2005). Generation time (equivalent to the time it takes between introduction of a fly cross to a vial and final maturation of adult progeny) was also quite variable.

temp	generation time
18 deg C	16 days
25deg C	8.5 days
30deg C	11 days

Below are durations of pupation reported by Bainbridge and Bownes (table 4, 1981). P1 is 0 hour APF, P15 is eclosion)

*Table 4. Durations of period from P1–P15 inclusive at four temperatures, estimated from the literature on timing of metamorphosis*

Temperature (°C)	Factor	Estimated durations (h)	
		Female	Male
25	× 1 (reported)	(our conditions) 98 (calculated)	(our conditions) 102 (calculated)
18	× 2.07	203*	211*
22	× 1.31	128	133
29	× 0.79	77*	80*

\* These eclosion times are in rough agreement with our observations of eclosion at these temperatures.

Fliea were raised in plastic vials and bottles with breathable stoppers or cotton balls; media (recipie in box below) was poured into these and solidified as it cooled.

Just before flies were introduced to vials/bottles, granular dry yeast was added to the surface of the media to help the adults with mechanical food uptake prior to hatching of young larvae the larvae improve access to media by tearing it up with their mouthparts.

**Fly food (solid agar media)**

heat in large pot until boiling stirring often

1.5L distilled water

18g agar

30g brewers yeast

120g cornmeal

225ml molasses

### Dissection of the *D. melanogaster* wing hinge

Flies were anesthetized with CO<sub>2</sub> and categorized by phenotype (e.g. balancer or driver, wild type or anhedral were selected). To facilitate isolation of the thorax whole flies were placed in plastic dishes in PBS and held for 5-10 minutes in -20deg freezer or 20 mins in 4deg fridge; when removed from the cold, flies are inanimate for at least 5 minutes. PBS was aspirated from the dish and a #21 scalpel blade was used in a rocking motion (chef's knife style) to remove both head and abdomen. For support while sectioning, thorax samples were embedded in 4% agar gel that had been melted in a microwave oven. The thoraces of newly prepared flies were placed in the warm agar and put in the fridge. When the agar had solidified, freshly broken razor blades were used to cut away the lateral most part of the thorax along the lines indicated in figure Appendix3. These sagittal wing hinge slabs were placed between two coverslips with PBS pH 12 unless otherwise indicated. For confocal reconstructions Hoyers media was used, see text box for recipe. For tests confirming presence of Resilin PBS at pH 2, pH12, pH9 or sodium borate at pH9 were used. NaOH and HCl were used to adjust pH. To prevent thorax sections from being crushed between coverslips, small pieces of breakable razor blade were used as spacers.

Hoyers media (1:1 dilution with water) was used as a clearing agent in preparing the sagittal splits for confocal reconstructions seen in figures 17-19 and the brightfield image seen in figures 17, 19, and inset of 31. This treatment allowed clear visualization of the thoracic wall, wing and wing hinge sclerites without the disruption that results from mechanically removing the muscle and other soft tissues. When a fly is properly cleared, the soft tissues completely disappear as viewed with transmitted light.

The settings on the Leica SP5 TCII confocal microscope for fluorescent reconstructions were as follows:

- excitation was from 405nm diode laser with transmission AOTF set at ~10% and from argon gas laser set at 30% power with transmission AOTF for 488nm line set at 33%

- two emission channels were collected, one centered/passing at 430/20nm and the other at 640/30nm.

In these Hoyers cleared preps, the blue channel (405nm ex 430/20nm em) showed bright emissions from some soft tissues especially what appeared to be tracheae in the thorax (figure 19, bottom left in blue). The 640/30nm emission channel collected emissions specifically from sclerotized cuticle which appears red in these images.

**Hoyers media (clearing agent)**

30g gum arabic in 50ml distilled water –dissolve with stirring overnight  
200g chloral hydrate added gradually to dissolve  
20 grams glycerol

-centrifuge 30 mins at ~10000g, take supernatant and filter through glass filter  
-can be diluted with water (we diluted 1:1)

**Sodium Borate monobasic (.5M after Malencik, 2003)**

.6 g boric acid  
20ml distilled water  
.66ml 5M NaOH

Imaging the TP tendon and simple biochemical tests for Resilin

All fluorescent TP tendon images shown were taken with 20x or 40x air objectives on a BX51 Olympus microscope with UV long pass cube of following specs: excitation 360-370nm, beam splitter 400nm long pass, emission 420nm long pass. The images were captured with a Spot RT3 (Diagnostic Instruments, MI, USA) color camera. Color balance was set such that images looked very much as the samples appeared through the eyepieces. Illumination intensity and exposure for all TP tendon images were nominally the same but as these experiments were done over a 2 year period, light source intensity can not be guaranteed due to aging of the Hg arc based bulb (EXFO X-cite120, Lumen dynamics, Ontario, CAN).

For comparisons of di-tyrosine intensity under influence of pH or presence of borate ion, solutions were changed by concurrently wicking and replacement of solutions between coverslips then imaging after 5-10 minute equilibration with the epi-fluorescence setup mentioned above. Solutions were pH adjusted with HCL (38%) or 5M NaOH. Reversal of fluorescence quenching after returning basic pH or removing borate was also confirmed (not shown).

Semi-thin sections

To record normal anatomy of the wing hinge and TP tendon fly strains used are adult OreR females at least 2 days post eclosion from the pupal case.

Solutions:

Aldehyde fixative consists of 9ml of a 4% paraformaldehyde solution mixed with 1ml of a 50% glutaraldehyde solution. Final concentration 3.6% formaldehyde and 5% glutaraldehyde in .1M cacodylate buffer

OsO<sub>4</sub> post fixative: 2% weight/volume OsO<sub>4</sub> crystals (EMS PA, USA) dissolved in .1M cacodylate buffer

Cacodylic acid buffer: .2M sodium cacodylate stock (EMS)

Propylene oxide (Sigma, USA)

Graded ethanol series

Epoxy embedding mixture (Epon-812 substitute kit, EMS)

-combined while stirring gently: 47g Epon-812 substitute, 21g DDSA, 32g NMA, and 1.4ml DMP-30 mixed with stir bar 30 minutes . Refrigerate until use.

Stain for semithin sections is filtered stain: toluidine blue/basic fuchin (EMS)

Fixation and dehydration:

Adult flies were anesthetized with CO<sub>2</sub> and females of proper phenotype(wild type or anhedral) were selected. To facilitate isolation of the thorax whole flies were placed in plastic dishes in PBS and chilled as described above. PBS was aspirated from the dish and a #21 scalpel blade was used in a rocking motion to remove both head and abdomen. The thoraces were treated as follows:

-placed immediately after dissection in aldehyde fixative on ice; 12 hours

-rinse 2x .2M NaCacodylate buffer

-postfixed in 2% OsO<sub>4</sub> for 2 hours on ice

-rinse 3x .2M cacodylate buffer (stored over night in final rinse)

-graded ethanol series with 15 minutes in each 50%-75%-80%-95%-100%-100%-100%

-directly from 100% ethanol to propylene oxide 2x 15 minutes each

Embedding:

Samples were embedded in Epon epoxy as follows:

-transfer thoraces from propylene oxide to 2:1 propylene oxide:Epon 1 hour on rocker

-transfer to 1:2 propylene oxide:Epon overnight on rocker

-transfer to 100% Epon (allow Epon to warm before opening), 5 hours. Transfer to 2<sup>nd</sup> 100% Epon, overnight on rocker.

-poured out thoraces and Epon onto parafilm, dropped them into Beem capsules (Polysciences PA, USA) and filled the capsules to 2/3 full with Epon, before proceeding, samples were oriented so that resulting sections would be in the transverse plane of the thorax. Finally, sample labels were inserted.

-capsules into 45 deg. oven 10 hours

-capsules into 60 deg. oven over weekend

Sectioning and staining:

Glass knives were made on a LKB 7800 knife maker. Plastic troughs (EMS) for floatation fluid were glued to the knives with fingernail polish. Knives used for ultrathin sections were made no more than 24 hours before sectioning (no ultrathin sections shown). Knives more than 24 hours old were used for block trimming and semi-thin sections.

Either a Reichert-Jung ultramicrotome (model Ultracut E), or a Sorvall MT5000 was used for semi- thin sections. Semi-thin sections were ~1 um thick. They were picked from the glass knife trough, placed on glass slides by floating them on a drop of water followed by evaporation of the water on a hotplate (set on 2 of 10, cooler than boiling point). Slides were removed from heat, placed on bench top and immediately covered in filtered toluidine blue/basic fuchin stain (ElectronMicroscopySciences #14950, see appendix). Less background staining and precipitate were noticed when stain was applied to slides on the benchtop vs those stained on the hotplate. After 5-10 minutes , slides were rinsed by successive, repeated immersion in 2 large volumes (>500ml) of water (i.e. pre-rinse followed by final rinse). Coverslips were applied using Gelmount (Biomedica, CA, USA) as an aqueous mounting medium. At this step we should have used permount as this is refractive index matched with the epoxy section and glass but gelmount gave acceptable results.

The most important challenge that slowed our work initially concerned orientation in A/P axis (i.e. finding the TP tendon). Figure 31 shows clearly that this problem has been overcome. In orienting ourselves to collect these sections, we were able to view the fly within the epoxy embedding media by placing a small piece of coverslip on the trimmed epoxy surface with a water drop intervening to prevent reflection from the epoxy. This created a clear window into the interior of the block such that we could see about where along the A-P axis of thorax we were cutting. The nearly serial semi-thin sections seen in figure 31A allowed us to approach and section the TP tendon with confidence.

#### Collecting and crossing flies

Flies used in the crosses described in the results section are listed in table1 in the appendix.

Staging pupae was done by selecting what are known as white pre-pupae (as shown in figure 10B). This method proved to be a somewhat unreliable because time to complete metamorphosis is quite variable in individual flies within any cross. A more reliable method involves staging the flies based on well described characters seen during metamorphosis. Figure 21 shows two such characters. These are 2 different pupae (top vs bottom) imaged through time from left to right at 30 min intervals. Top row shows abdominal bristles becoming visible at ~85 hours APF while the bottom row shows yellow body and malpighian tubules disappearing at~95 hours APF (Bainbridge and Bownes, 1981).

To raise offspring that express both the driver chromosome and the *UAS RNAi* chromosome of choice, virgin (newly eclosed) females were selected to cross with male

flies. These were selected based on their unsclerotized, pliable abdomen and visible black spot (presumed gut) within. Females (usually with *Gal4* driver) were crossed with males (usually *UAS RNAi* line) in vials of food prepared as described above. These flies were left to breed and lay eggs for 48 hours then transferred to a second vial. This was repeated once more giving 3 vials of offspring from each cross; this allowed us to collect and compare offspring that grew at different temperatures.

Figure 32 shows the basic scheme of the *Gal4-UAS* system and most of the crosses (*UAS-Gal4* combinations) used. *Gal4* is a transcription activator and *UAS* an enhancer sequence that in our crosses is positioned to drive RNAi. The system is native to yeast and is widely used in controlling expression of genes in fruit flies. To determine which offspring inherited the *Gal4* driver chromosome and which inherited the balancer, we looked for the balancer phenotype in the offspring. Figure A2 shows results of a cross with the mother passing on either TM3-*Stubble* balancer / the *tubulin-Gal4* driver chromosome to the progeny. Here, bristle form is used to differentiate which chromosome was inherited.

In the case of the *actin5c-Gal4* driven crosses, tubby looking pupal cases were used to separate flies that inherited the driver vs the balancer chromosome.

### Flight tests

Flight tests were performed using progeny from the crosses indicated in figure 29A.

The results include data from 2 different testing sessions. Offspring were tested a minimum of 36 hours after eclosion. No flies were more than one week old.

Care was taken to collect and test male and female progeny separately. We found no difference in their flight performance so pooled male and female results. To avoid the confounding factor of CO<sub>2</sub> anesthesia, we collected flies by aspiration into cotton plugged glass Pasteur pipettes and stored them until testing in the same pipettes (stored no more than 20 minutes).

We transferred the flies (approximately 6 at a time) into the test setup by gentle blowing; i.e. we moved the flies out of the pipettes through a small hole in a lid over a plugged funnel with no neck (figure Appendix1). This funnel was suspended 60cm above a target of concentric circles which were centered under the outlet of the funnel and had the diameters as shown in figure Appendix1. The plug was removed from the funnel bottom and the funnel was tapped repeatedly with an unsharpened pencil to knock the flies through the funnel hole. Flies were ranked from 0-3 based on where they fell on the target or given a rank of 4 if they flew without landing. This flight assay is based on one used to test flight in neuro-deficient mutants (Park, 2006 and Pesah, 2004). However, we were unable to reliably repeat the methods described in these papers and we believe that our test is a much more repeatable and generally improved assay. Data are presented as mean +/- 1 SE.

In attempts to track wing trajectory in tethered flies. We glue a fine wire to the abdomen of anesthetized flies with Krazyglue (cyanoacrylate) and decorate their wing tips with a spot of fluorescein powder dissolved in gelmount mounting media. The flies were illuminated with blue light from a Hg arc light source. A digital video camera (Scion) with macro lens was placed so its optical axis was at 90 degrees to the illumination axis, a long pass filter was placed in front of the camera lens to block blue light from the illuminator. Green emissions of fluorescein provides sufficient contrast for us to track the wing tip. We used long exposure times (~1 second) which cannot provide frame by frame wing tip location but do give an integrated view of 200 or so wing beat cycles (assuming ~200Hz frequency). This appears as a streak of light indicating wing tip trajectory. Flies were induced to attempt flight by blowing at their heads.

### Eclosion assays

Flies were crossed as described above. At 8, 11, or 16 days (25deg C, 28deg C, or 18deg C respectively) after parents were introduced, vials were checked for newly emerged flies. Subsequently, vials were checked once per day. Flies of each phenotype (balancer or driver) that had eclosed were noted and removed. Flies continued to eclose for a period of 4-5 days after the first fly eclosed which represents the variability of developmental period (all eggs were laid during a 48 hour period). About 48 hours after the last fly eclosed, flies dead in puparium were counted. This condition was obvious in that these pupae were quite black (rotten) with the exception of the eyes. For *tubulin-Gal4/TM3-Sb* crosses the pupae were pulled from the puparium to check for driver/balancer phenotype (i.e. bristle form). For the *act5c Gal4/TM6-Tb* crosses the phenotype was obvious in that the pupal case was either wild type or much shorter than wild type (tubby).

### Statistics

In both the flight test assay and the eclosion assay, data was compared using a variation of the two sample t-test statistic. This test (similar to Welch's t-test) accounts for disparity in the standard deviations of the two populations being compared. Microsoft Excel program was used to run this test. As can be seen in the respective figures each population was compared and tested sequentially against OreR and *Df(2R)anhedral<sup>1</sup>* in figure 29 or against resilin RNAi (non-Bursicon pathway control) and bursRNAi in figure 36. Probability of the observed difference in compared means resulting from chance sampling event (and not a measurable difference between the groups compared) is listed below the graphs (P-value).

## Appendix

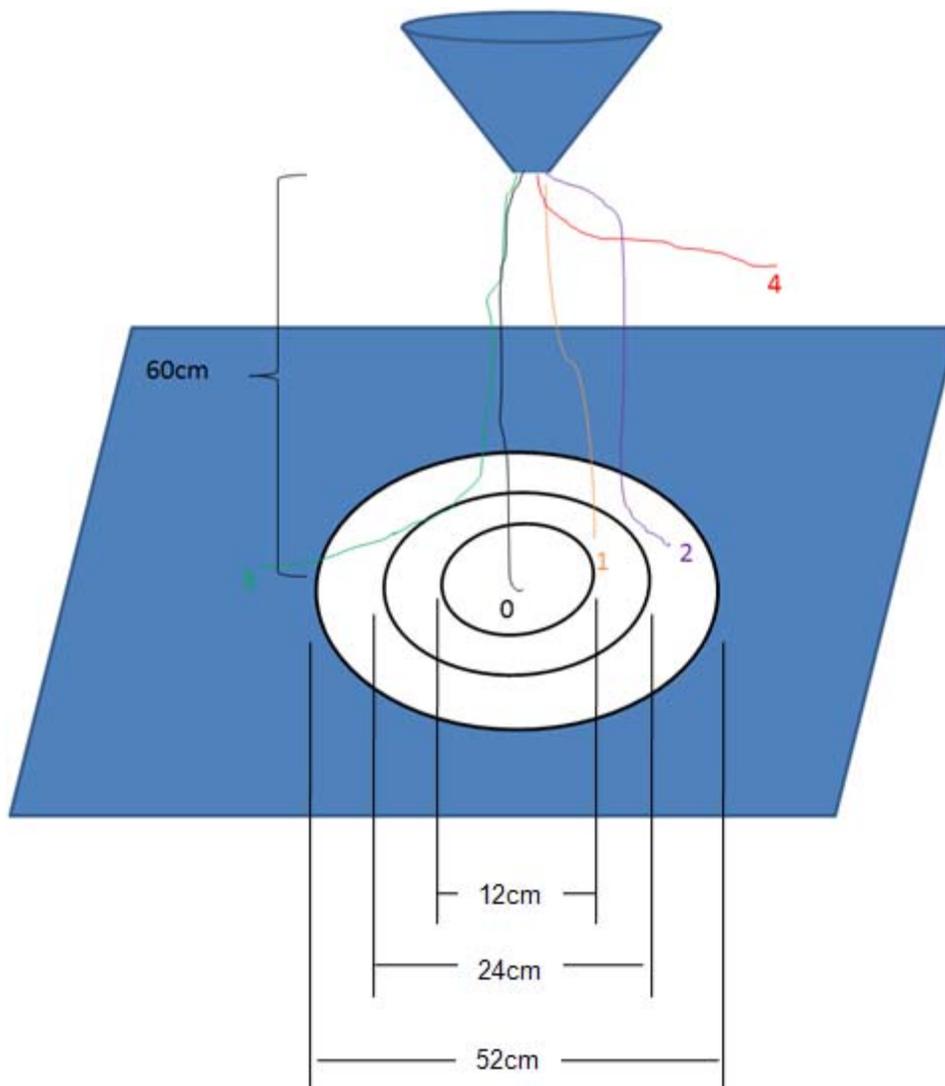


Figure Appendix 1. The flight test apparatus used here is a variation (in our hands much improved) of that used by and reported in Park (2006) and Pesah (2004).

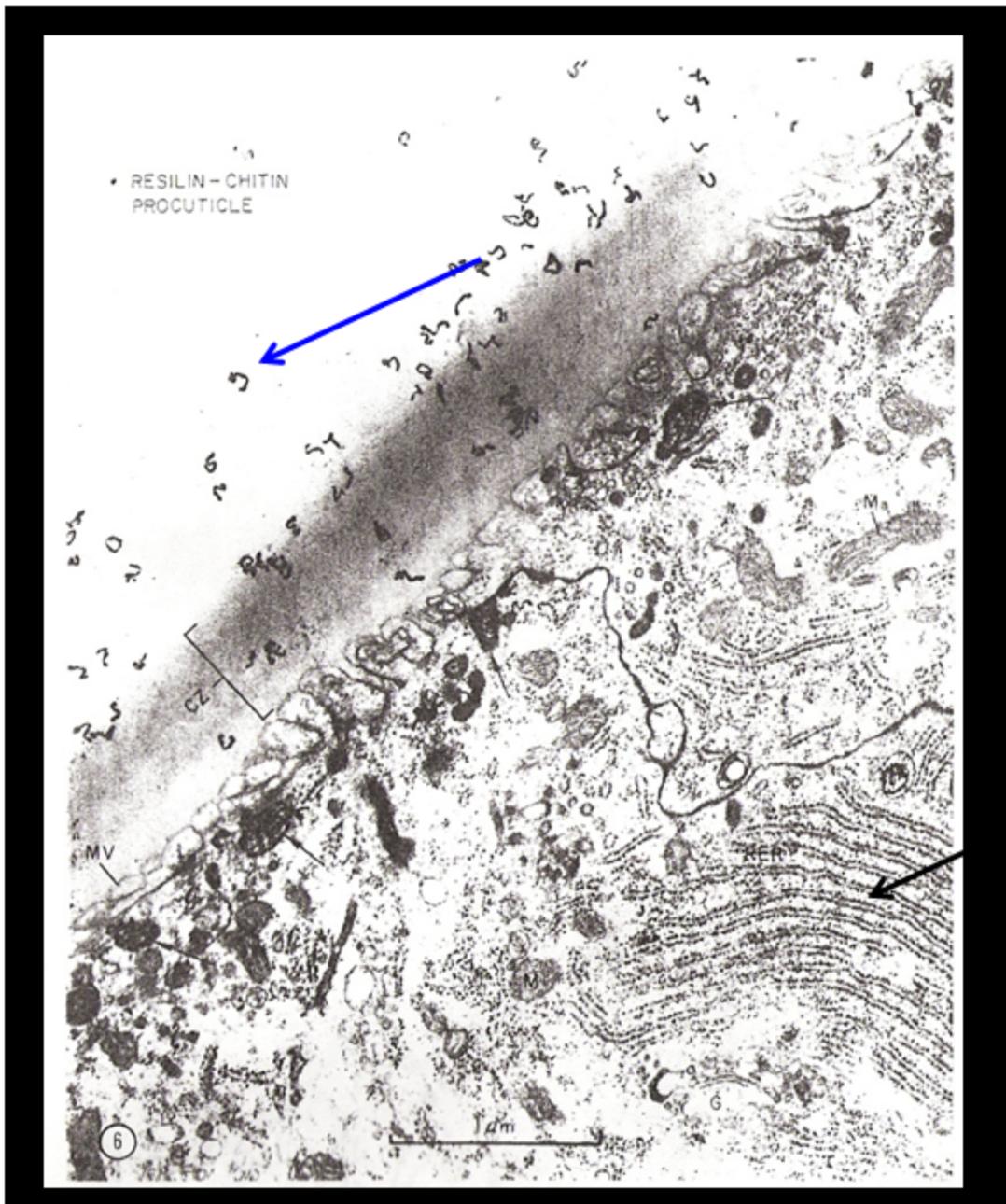


Figure Appendix 2. Autoradiograph/TEM of newly emerged adult grasshopper pre-alar arm. Electron dense (dark) regions are labeled with radioactive tyrosine, black arrow points to radiodense rough endoplasmic reticulum, blue arrow points to uncrosslinked resilin in procuticle. This image was taken after a 3 minute hot pulse followed by a 13 minutes cold chase of labeled tyrosine. MV-microvillous border of epithelium CZ-chitin zone (assembly zone) From Weis-Fogh (1970).

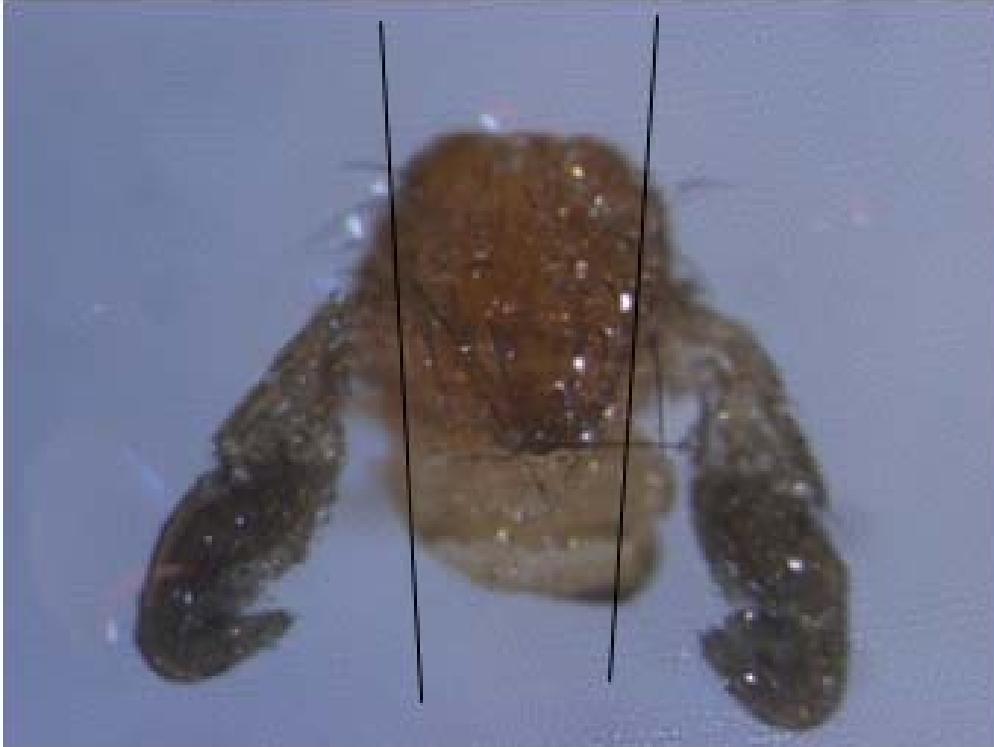


Figure Appendix 3. A rk4 fly embedded in agarose ready for sagittal split as indicated by lines. All fly lines were cut in similar fashion.

protein name (used in this paper)	ID (curated gene #) / name of gene	function discussed in this paper	gene location chromosome and arm	
Peroxidase (Pxd)	cg3477 / <i>peroxidase</i>	unknown-ubiquitous knockdown is lethal	3R	
Dual oxidase (Duox)	cg3131 / <i>dual oxidase</i>	di-tyrosine crosslinker	2R	
Duox maturation factor	cg4482 / <i>mol</i> or <i>duox maturation factor</i> or <i>NIP</i>	activator of Duox	2L	
Resilin	cg15920 / <i>resilin</i>	elastic protein	2R	
Rickets or DLGR2	cg8930 / <i>rickets</i> or <i>DLGR2</i>	hormone receptor	2L	
Partner of Bursicon	cg15284 / <i>partner of bursicon</i>	hormone subunit	2L	
Bursicon	cg13419 / <i>bursicon</i>	hormone subunit	3R	
<b>wild type fly stocks</b>	<b>source</b>			
Oregon R	Bloomington-5 Oregon R-C	wild type for control		
<b>mutant fly stocks</b>	<b>Source Stock center # B-Bloomington V-Vienna</b>	<b>flybase genotype or description</b>	<b>purpose in this paper</b>	<b>homozygous or balancer phenotype</b>
<i>Df(2R)anhedral<sup>1</sup></i>	Collier lab	<i>Df(2R)anhedral</i>	new homozygous viable mutant-deletion of <i>resilin</i> gene	homozygous
<i>Df(2R)resilin/Cy</i>	B-7887	w1118; <i>Df(2R)Exel7145/CyO</i>	homozygous lethal mutant of <i>resilin</i> gene	curly winged adults
<i>rickets4 - rk4</i>	B-3590	<i>Df(1)RK4/FM7k/Dp(1;Y)y+, y1</i>	defective Rk receptor	homozygous
<i>rickets1 - rk1</i>	B-3589	<i>rk[1]cn[1]bw[1]</i>	defective Rk receptor	homozygous
<i>ms1096 Gal4</i>	B-8696	w1118 P{GawB}BxMS1096	driver of UAS in dorsal wing blade	homozygous
<i>tubulin Gal4/TM3-Sb</i>	B-5138	y1 w <sup>+</sup> ; P{tubP-GAL4}LL7/TM3, TM3-Sb1	drive UAS ubiquitously under <i>tubulin</i> promoter	stubble thoracic bristles
<i>actin5c Gal4/Tb</i>	B-3954	y <sup>+</sup> w <sup>+</sup> ; P{Act5C-GAL4}17bFO1/TM6B, Tb <sup>+</sup>	drive UAS ubiquitously under <i>actin</i> promoter	tubby larvae and adults
<i>actin5c Gal4/Cy</i>	B-25374	y <sup>+</sup> w <sup>+</sup> ; P{Act5C-GAL4-w}E1/CyO	drive UAS ubiquitously under <i>actin</i> promoter	curly winged adults
<i>apterous Gal4 / Cy</i>	B-3041	y <sup>+</sup> w <sup>+</sup> <sup>1118</sup> ; P{GawB}ap <sup>md544</sup> /CyO	drive UAS in wing and wing hinge	curly winged adults
UAS <i>rickets</i> RNAi	V-105360kk	phiC31 RNAi Library	knockdown of DLGR2 when driven with <i>Gal4</i> line	homozygous
UAS <i>pburs</i> RNAi	V-102690kk	phiC31 RNAi Library	knockdown of Pburs when driven with <i>Gal4</i> line	homozygous
UAS <i>bursicon</i> RNAi	V-102204kk	phiC31 RNAi Library	knockdown of Burs when driven with <i>Gal4</i> line	homozygous
UAS <i>bursicon</i> TRiP RNAi	B-26719	y1 v1; P{TRiP.JF02260}attP2	knockdown of Burs when driven with <i>Gal4</i> line	homozygous
UAS <i>peroxidase(2)</i> RNAi	V-48587gd	P-Element RNAi Library	knockdown of Peroxidase when driven with <i>Gal4</i> line	homozygous
UAS <i>peroxidase(3)</i> RNAi	V-48588gd	P-Element RNAi Library	knockdown of Peroxidase when driven with <i>Gal4</i> line	homozygous
UAS <i>duox</i> RNAi			knockdown of Duox when driven with <i>Gal4</i> line	
UAS <i>mol</i> RNAi			knockdown of Duox maturation factor when driven with <i>Gal4</i> line	

Table Appendix1. Genes and fly stocks discussed and used in experiments

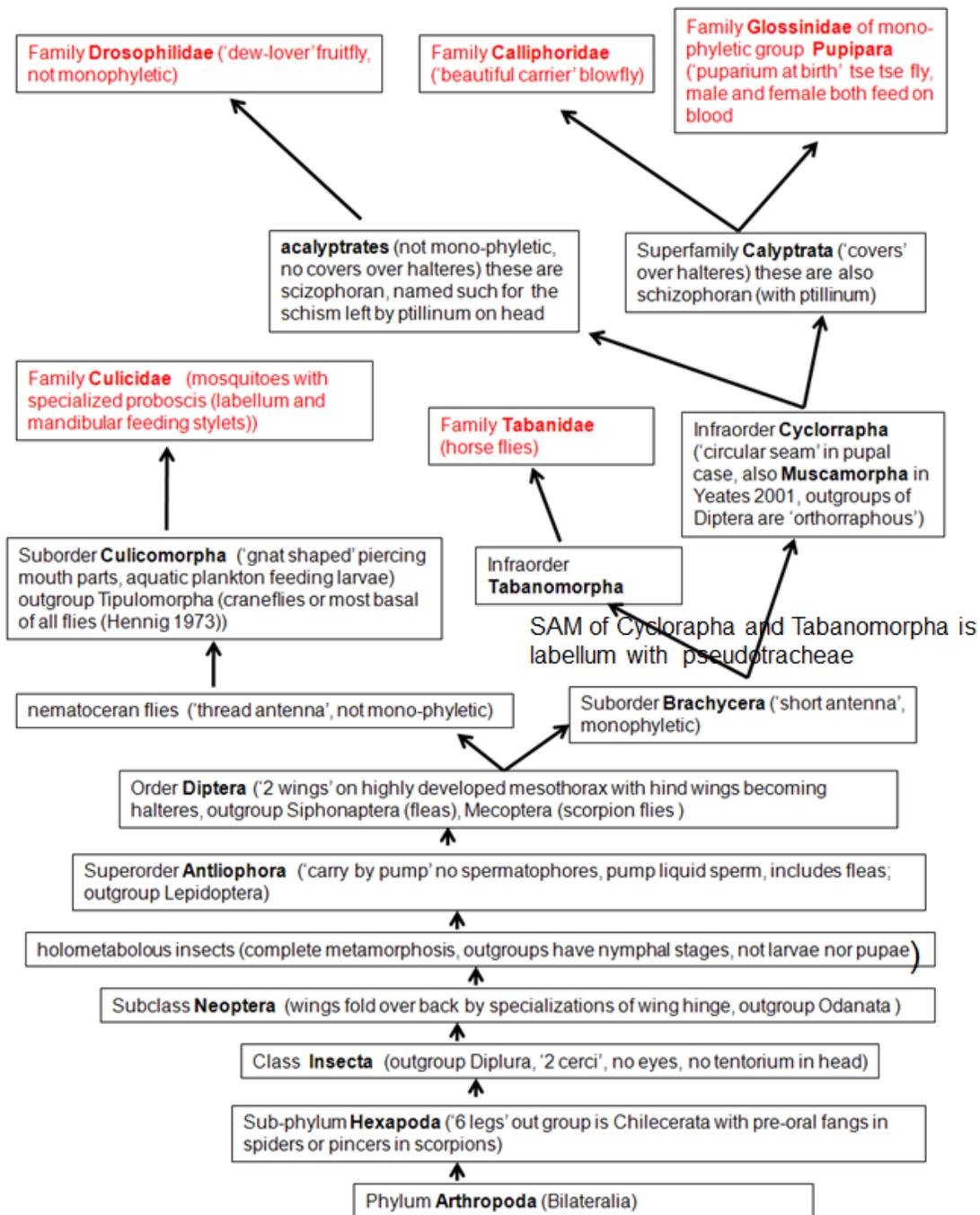


Figure Appendix4. Phylogeny of some dipterans. The name Cyclorapha refers to the round door of the pupal case from which higher dipterans emerge (eclose) using their ptilinum. Notice also that the fleas and flies are both members of the Antliophora. Fleas have adapted the Resilin in the wing hinge of a flying ancestor to use as a power amplifier during jumping. References used in arranging this phylogeny are on the following page.

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## **Epoxy Tissue Stain (E.T.S.)**

<http://www.emsdiasum.com/microscopy/>

EMS Catalog #[14950](#)

Biological specimens embedded in epoxy need to be examined by light microscopy, either for orientation prior to further ultra-thin sectioning, or for light microscopy studies.

Many histological stains fail to react with the specimen in the epoxy media. Using our E.T.S., sections of tissue embedded in epoxy can be stained by a very simple method. This polychromatic staining solution can be used for staining sections of epoxy-embedded specimens prepared for electron microscopy examination, e.g.

[glutaraldehyde](#) and [osmium](#) fixation.

The most common application is for sections (0.5 - 1.5µm thick) cut in the ultramicrotome with either glass or diamond knives. The sections are placed on a glass microscope slide and dried on a hot plate. A drop of E.T.S. is added to cover the section on the warmed slide, and the slide is put back on the hot plate until a silver rim is formed. Any excess stain is removed with distilled water from a squeeze bottle, the slide is allowed to dry, and the sections are covered with a cover-slip.

The intensity of the stain can be controlled by altering the time that the sections remain on the hot plate. For darker results, allow the stain to dry completely. If sections are over-stained, some of the stain can be removed by washing the section with absolute alcohol.

### **Other methods can be used prior to embedding:**

En-block, where the specimen is fixed in the usual manner with glutaraldehyde and osmium, rinsed in buffer, and stained for 15 minutes with E.T.S. in a capped vial at 65°C. The specimen is allowed to return to room temperature (10-15 minutes), then dehydrated in ethanol and embedded in epoxy resin.

After sectioning off a preliminary block, a secondary staining yields a superior polychromatic coloration for light microscopy. Also, there are no adverse results or artifacts or ultra-structural changes caused by the stain at the E.M. level. The only trace of E.T.S. staining is a slight, bluish coloration of thin sections.

A better reaction of this polychromatic stain can be obtained when tissue is fixed with glutaraldehyde alone and not post-fixed with osmium.

### **Reference:**

Spurlock, B. O. et al, American Journal of Clinical Pathology, Vol 46 #2, 252 (1966)

Toluidine Blue and Basic Fuchsin. For staining semi thin sections of epoxy embedded tissues.

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