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Failure to Launch:
Characterization of a Flightless Strain of *Drosophila hydei*

by

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ABSTRACT

Drosophila hydei is a fruit fly that last shared a common ancestor with the model organism *D. melanogaster* 60 million years ago. A domesticated strain, flightless *D. hydei*, is a feeder fly for amphibians, and breeders noticed that if cultures were exposed to high temperatures, some imagines of the next generation eclosed capable of flight. Heat shock proteins can correct misfolded proteins, masking a mutation which is revealed when heat shock challenges the heat shock protein system. In this case, heat shock results in a regain of function. This novel feature provided the impetus for this study. One of the most noticeable effects of developmental temperature on a fruit fly is on wing size, and insufficient wing area can inhibit flight. Wing loading was not conclusively different between flightless and wild type *D. hydei*. Thoracic muscle birefringence was diminished in flightless flies compared to wild type. This indicates that the thoracic muscle is abnormal and therefore inhibits flight. Short duration heat shock at multiple developmental stages did not yield flying flies. Long duration heat shock may provide better results. A potentially homologous locus in *D. melanogaster* is *Actn*. Further characterization of the muscles of flightless *D. hydei* would faciliate comparison.

INTRODUCTION

Charles Darwin begins *On the Origin of Species* with the chapter "Variation Under Domestication," noting that animals can show forms in captivity that would never survive in the wild. These variations can provide insight into the variation of the same organism in the wild. *Drosophila melanogaster*, with its thousands of alleles, is likely the best understood animal in the world, and hundreds of wild species have further expanded its context. However, Darwin's insight on domestication holds: domesticated varieties are viable, but monstrous, and they are unstudied. In that spirit, I am investigating a domesticated fruit fly with a form that would never survive in the wild. This flightless form of *Drosophila hydei* is a feeder for fish and amphibians, and Fred Gagnon at Magic Wings Butterfly Conservatory reported that when a greenhouse overheated, his stocks of flightless *D. hydei* showed flight ability in the next generation to eclose. Similar temperature-dependent flight has been reported in the same strain by other feeder insect breeders (Atchison, 2009). This effect could be attributable to the action of heat shock proteins. Hsp's comprise several families of phylogenetically conserved proteins that play important roles in the cell to protect against heat shock and also to support delicate proteins involved in transient processes such as signal transduction. Hsp's can therefore modify gene expression, but they can also interact with the genome in another way. Hsp90, the

best studied, binds to and stabilizes mutant proteins, often allowing normal function and therefore silencing the mutation. This process allows mutations to accumulate, and the mutations can be unmasked as a result of the additional stress of heat shock. Thus, new phenotypic variants can appear in a stressful situation (Rutherford and Lindquist, 1998). The reported temperature-based rescue of flightless *D. hydei*, indicating an interaction between temperature and flight ability, is the crux of my research. In order to understand a mutation that causes a heat-rescued flightless phenotype, we must understand the components of the flight system.

Phylogeny

D. hydei was discovered by R. R. Hyde and subsequently named after him by A. Sturtevant in 1921. The *Drosophila* genus is replete with species, containing over 1000; therefore, there are many taxonomic ranks defined: subgenus, group, subgroup, complex, species, and subspecies (Powell, 1997). *D. hydei* is in the *hydei* subgroup of the *repleta* group of the subgenus *Drosophila.* The *repleta* group consists primarily of cactophilic desert endemic species in northern Mexico and the southwestern United States, although *D. hydei* has become cosmopolitan (Markow, 1985l; Powell, 1997).The *repleta* group is phylogenetically distant from *D. melanogaster*, as found in whole genome sequencing of the member *D. mojavensis* (*Drosophila* 12 Genomes Consortium, 2007). The cladograms generated by this massive study confirmed that the branch leading to the *melanogaster* group and the branch leading to the *repleta* group

diverged at the base of the genus. The two species belong to the subgenera *Sophophora* and *Drosophila*, respectively, which diverged between 40 and 60 million years ago (Fig. 1, Powell, 1997).

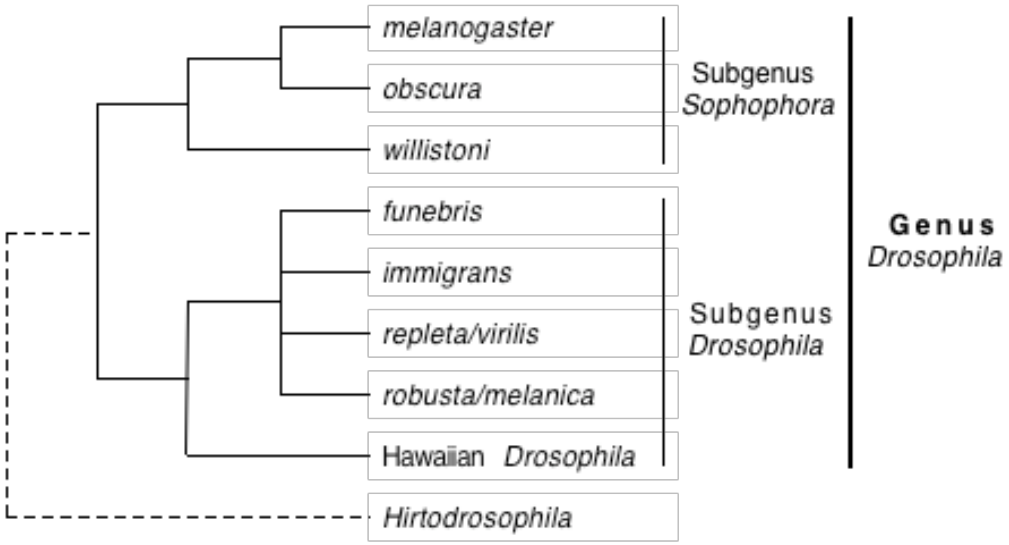

Figure 1: Phylogenetic Relationships of *Drosophila*. *Adapted from Powell, 1997.*

An older method of comparing the genetic material of organisms is to compare chromosome organization (Sturtevant, 1921; Powell, 1997). Specific methods include determining chromosome number by karyotyping and finding inversions by mapping loci and observing banding patterns of polytene chromosomes. A common chromosome arrangement is that found in *D. melanogaster*, where a female fly has two pairs of long chromosomes, one pair of short chromosomes, and one pair of dot chromosomes (Sturtevant, 1921). The *repleta* group displays five pairs of short chromosomes and one pair of dot chromosomes. The short chromosomes in *D. melanogaster* are its sex chromosomes, and therefore, the *repleta* organization suggests splitting of two

pairs of autosomes into four pairs of autosomes. H. Muller identified linkage groups consistently found across species, often referred to as Muller elements (Powell, 1997). In *D. melanogaster*, Muller Element A is the X chromosome, B and C are the arms of 2, D and E are the arms of 3, and F is the dot chromosome. In *D. hydei*, A is X, B is 4, C is 3, D is 5, E is 2, and F is 6. Because the conserved Muller elements are rearranged in a simple way between the species, the genetic loci in the two species have strong homology. Modern parsimony analysis suggests that *D. hydei* has the ancestral chromosome arrangement, while that of *D. melanogaster* arose via fusion (Powell, 1997). Nevertheless, in the 50 million years of divergence between the two species, many inversions and translocations have occurred, so the gene loci are far from identical (Spencer, 1949).

The Insect Flight Apparatus

The insect flight apparatus consists of two major mechanical structures: the wings themselves and the musculature to power the wings. The wings develop initially as invaginations that are present in newly hatched larvae and evaginate as the pupa forms. As the pupa grows, the wings grow larger and eventually must fold. After eclosion, the wings are straightened with hydraulic pressure between the two layers (Bodenstein, 1950). The development from a sac into a flat, stiff wing is unusual, and many hypotheses exist for the evolutionary origin of the insect wing. Étienne Geoffroy St. Hilaire suggested an analogy to membranous bird lungs or fish swim bladders (Rennie, 1831). One of the predominant contemporary theories is that adult wings are homologous to the hinged, beating

gills of mayfly nymphs and the other is that wings originated as nonmobile thoracic lobes (Dudley, 2000). Developmental defects in the wing can hinder a fly's flight ability; for example, selection for loss of veins in *D. ampelophila* (now known as *D. melanogaster*) can cause the wing to become structurally weak (Lutz, 1911). As will be discussed further, wing size is very important for flight ability.

The wing hinge could easily be studied as extensively as the wing itself. Even now, there is no consensus on the exact mechanism of articulation. Early work was performed by Boettiger and Furshpan (1952), who used CCl_4 anesthesia to generate muscle spasms and anaesthetic flight in Diptera. They found that the wings tended to stop in either an up or down position, and the wings could move between the two positions with the same attack angle changes observed during normal flight, leading them to formulate a theory that Diptera use this bistability or click mechanism during normal flight. However, Miyan and Ewing (1985) argued that normal flight did not resemble the click mechanism of wing motion during anesthesia, but in fact involved smooth transitions (cited in Dickinson and Tu, 1997). What is known is that the wing hinge is very dynamic and can be modified with small muscles in order to change the flight parameters. A full model of insect flight is far more complicated than simple up and down flapping of static airfoils.

A fly with fully developed wings would never take to the air without the power of the massive flight musculature system. In *Drosophila melanogaster*, the

flight muscle develops in the pupa, at the same time that the larval muscles are being histolyzed. The wing muscles grow in length, after 44 hours become striated, and are fully completed 34 hours later (Bodenstein, 1950). Insect muscle is in many ways very similar to vertebrate muscle. At the molecular level, muscle consists of many parallel fibers, which are divided into thick filaments made of myosin and thin filaments of actin (Elder, 1975). Perpendicular to the fiber, several types of bands appear. The parts of muscle were named before the molecular structure was known, and therefore many of the bands are illusory. The most obvious band is the Z-band, which divides each fiber into sarcomeres (Picken, 1960). Each sarcomere has an A-band, and between each A-band and Z-band is an I-band. During contraction, the A-band maintains its length, while the I-band often disappears. At the molecular level, the A-band represents the thick, fixed length myosin filaments. Actin filaments are interspersed between the myosin filaments and are held together in the Z-band. The I-band appears when the muscle is relaxed because there is a gap between the Z-band and the beginning of the myosin. One of the differences between vertebrate and insect muscle is in the myosin filaments. They have the same basic structure of two heavy chains and light chains, but the overall filament of insect muscle is thicker and has a higher density of myosin per unit length than vertebrate muscle (Tregear, 1975).

Muscle contraction is triggered by the release of bivalent calcium ions, which activates adenosine triphosphatase, and this calcium is released and subsequently sequestered by the sarcoplasmic reticulum (Elder, 1975). The power

produced by a muscle is limited by the quantity of mitochondria, while the rate at which a muscle can contract and relax is determined by the amount of sarcoplasmic reticulum, and both of these organelles take up a finite amount of space in the muscle cell. In the insects with the primitive flight muscle system, the orders Odonata, Orthoptera, and Lepidoptera, the flight muscles have a balance of sarcoplasmic reticulum and mitochondria. Ultrastructural analysis of derived insect flight muscle shows very regular bands of muscles separated by numerous mitochondria, with almost no sarcoplasmic reticulum. This is because advanced insects have evolved to have separate muscles for flight power and flight control. The control muscles have extensive sarcoplasmic reticulum and many fewer mitochondria. This physiological division of labor allows for greater efficiency. In the primitive state of flight musculature, the muscle contracts once per nerve impulse, and therefore the wing beats once per nerve impulse. These insects are often large, and therefore do not need to have extremely fast wing beats. This is analogous to the relationship between wing beat and body size in birds: an albatross has a much slower wing beat than a hummingbird. Thus, the extreme miniaturization of insects that occurs in higher orders requires faster wing beats. Advanced insect flight muscle is referred to as asynchronous, because more than one muscular contraction occurs per nerve impulse; for example, in the fly *Calliphora*, a 120 Hz wing beat can be driven by a 3 Hz nerve impulse (Dickinson and Tu, 1997). This uncoupling of nervous stimulation and muscle contraction is created by two opposing sets of muscle undergoing stretch activation. The

dorsoventral muscles and dorsal longitudinal muscles are indirect flight muscles, so called because they move the wings by deforming the thorax. Contraction of the dorsoventral muscles flattens and lengthens the thorax, which causes the wings to move up and also stretches the dorsal longitudinal muscles. When the dorsal longitudinal muscles are stretched enough, they are triggered to contract, which shortens and dorsoventrally expands the thorax. This causes the wings to move down and stretches the dorsoventral muscles, repeating the process. Stretch activation is created by cross bridges between muscle filaments. Researchers have even generated stretch activation in vertebrate muscle by generating cross bridges between thick and thin filaments (Dickinson and Tu, 1997).

Polarized light microscopy is a useful tool for evaluating the integrity of striated muscle, such as insect indirect flight muscle. Like phase microscopy, polarized light microscopy can greatly assist in the observation of translucent specimens. Polarized light reveals molecular anisotropy of a specimen; anisotropy means that the organization of the structure differs depending on the observed angle. A material such as striated muscle shows birefringence under polarized light, which is due to modulation of the speed of propagation of polarized light caused by differing indices of refraction of the components of the muscle (Dragomir *et al.*, 2007). The bands in the sarcomere of striated muscle are actually named for their optical properties. The A-band is anisotropic and the I-band is isotropic, although the I-band is only called isotropic because it shows weak birefringence, not because it is truly isotropic at a molecular level (Picken,

1960). The I-band nevertheless appears dark under polarized light (Patton, 1963). From an optical perspective, muscle is equivalent to a uniaxial anisotropic material, with its axis parallel to the long axis of the cell (Dragomir *et al.*, 2007). A disruption in the structural organization of striated muscle will cause a loss of the normal birefringence. This makes polarized light useful for quick analysis of muscle integrity, because staining or TEM preparation is not required. Chow *et al.* (2001) suggested polarized light as a method for evaluation of level of cardiac myocyte damage, because the more damaged the myocytes are, the less birefringence they exhibit. This allowed for detection of intermediate myocyte damage, which can normally be differentiated only with specialized stains. Kimura *et al.* (1986) used birefringence as a measure of posteclosional muscular degeneration in a *Drosophila* mutant and found that loss of muscle function was correlated to loss of birefringence, and that intermediate levels of muscle function were correlated to intermediate birefringence. Therefore, if the flightless flies show reduced levels of flight muscle birefringence compared to wild type, the flightless flies must have defective flight muscles.

As a brief aside, the use of polarized light to observe muscle has an interestingly striated history. It was many years after the observation and naming of the bands of muscle before the modern theory of overlapping fibers was formulated (Picken, 1960), and indeed, the observation of birefringence was powerful evidence for the presence of parallel strands of protein in muscle (Bozler and Cottrell, 1937). Birefringence remained the de facto method of observing

muscle; many researchers observed the variation of birefringence during muscle contraction (Bozler and Cottrell, 1937). For some time, birefringence was such a universally used technique that in a 1975 book about insect muscle, degeneration of muscle was measured using birefringence, but the birefringence measurement of "compensator azimuth" is not even explained (Finlayson, 1975). However, with the development of the electron microscope, biologists forgot light microscopy in the mad rush to examine the ultrastructure of every possible biological structure. The attrition of knowledge during this dark age yields an immortal excerpt from an extensive review article on insect muscle:

> Very early work on muscle contraction used light microscopy to measure changes in whole muscle birefringence during contraction, stretch, etc. These changes stem ultimately from changes in thick:thin filament arrangement, but we find these papers so far from our training and experience that we cannot interpret them from a modern point of view (Hooper *et al.*, 2008).

It is all too common for the physical underpinnings of a universally used technique to be categorized as common knowledge but then gradually forgotten.

Causes of Flightlessness in Insects

In the consideration of an unknown mutation, it is vital to consider how flight can be inhibited. In his 2000 treatise on insect flight, Dudley lists a number of potential reasons for flightlessness. A common cause of flightlessness is reduction or loss of the wing structure. Neurological pathways are rarely affected, but flight musculature may be insufficient or reduced. Biochemical pathways may fail to meet the strenuous metabolic demands of flight. Surprisingly, under some conditions, selective pressures can favor flightlessness: flightless forms can have

higher fecundity due to energy surpluses and therefore gain a competitive advantage in low stress environments.

Many flightless mutants have been isolated in *Drosophila melanogaster*. According to Koana and Hotta (1978), flightless mutations are useful for genetic dissection of the flight system, which in turn is a useful model for genetic dissection of environmental plasticity because loss of flight is relatively independent from the rest of the organism's body. The authors comment that many flightless mutants display an abnormal attitude of wing inclination, presumably caused by abnormal morphology of indirect flight muscle, and emphasize that fibrillar indirect flight muscle is independent from the tubular direct wing muscles used in wing adjustment and courtship rituals. They also found some mutants that cannot take off from a flat surface because they cannot jump but can fly after dropping off an elevated surface. They screened for X-linked flightless mutants and found that several alleles that caused complete loss of flight showed gross abnormalities in the ultrastructure of the indirect flight muscle, which is consistent with the complete loss of flight ability. Interestingly, they also found a temperature-dependent allele, where when raised at 24°C displayed impaired flight ability but when raised at 29° lost all flight ability.

This strain of *D. hydei* exhibits temperature-dependent flightlessness, so any interaction between temperature and the flight system must be considered. Wing area and body mass are both influenced by changes in developmental temperature, which could potentially interfere with successful flight. Flies with

wings intermediate in size between fully alate and fully apterous can be evaluated by considering their aerodynamic lift generation. Endogenous lift generated is determined by wing loading (body mass per wing area) and wing beat frequency. Wing loading is a parameter that can only be used for comparison with caution, because as mass is generally proportional to the cube of length and area is generally proportional to the square of length, in isometric scaling, wing loading itself is proportional to length (Gilchrist and Huey, 2004). Furthermore, because temperature affects both body mass and wing area, i.e. both the numerator and the denominator of wing loading, the relationship between wing loading and temperature is not straightforward.

Many studies have looked at the relationship between temperature and wing loading in *Drosophila*, and the general consensus is that lower temperature yields lower wing loading. One author postulates that wings develop at suboptimal size at temperatures other than the local mean (de Moed *et al.*, 1997), implying that a fly raised at too cold or too warm a temperature will have undersized wings; however, their results agree with many studies in concluding that wing size is inversely related to developmental temperature. According to the authors, wing size in *Drosophila* is dependent on both cell size and cell number, and environmental alteration of wing size can be caused by increase in either or both. Cold temperatures put strain on the flight system by limiting the rate of muscular contraction, so flies developing at cold temperatures eclose with larger wing areas and therefore lower wing loading (Frazier *et al.*, 2008). This study

found that flies raised at 28°C had almost no flight capability at 14°C, but flies raised at 15°C had much greater flight success at 14°C. The cold developmental temperature yielded flies with larger bodies, but the wings gained even more size than the body. In another study, Azevedo *et al.* (1998) stated that wing beat frequency increases with temperature; therefore, cold temperatures put pressure on the flight system. The authors examined *D. melanogaster* clines and found that wing:thorax size ratio increases with latitude (and corresponding reduction of temperature). They also found genetic predispositions toward larger wings in populations originating from colder climates. Gilchrist and Huey (2004) also compared clines, looking at wing loading in parallel clines of *D. subobscura* of the ancestral European populations and the recent colonists of South America. Noting that unless wing size is allometric, isometric growth to larger size will increase wing loading, they found that both European and South American *D. subobscura* displayed increased size but decreased wing loading when raised at low temperatures. However, while the ancestral European populations display lower wing loading for cooler clines, the South American populations display higher wing loading for cooler clines, suggesting that other forces drive selection for larger body size in the South American populations. The effect of developmental temperature on wing size is not limited to a single generation: Andersen *et al.* (2005) examined the effects of maternal temperature stress on offspring size and wing size, and found that maternal heat stress actually increased the centroid size of the wings of progeny. They hypothesized that

because increased wing size correlates to increased body size, which correlates to fitness, the stress on the mother increased the fitness of her progeny.

A fruit fly must achieve a wing beat frequency as high as 240 Hz to achieve flight (Maughan and Vigoreaux, 1999), so it is not surprising that a mutation could render a muscle incapable of producing the power output necessary for such rapid wing movement. There are many levels at which a muscular mutation can hinder the fly's ability to fly, due to the fractal-like nature of muscle. Maughan and Vigoreaux (1999) present the power generation of a fly in powers of ten: from the whole flight muscle system down to individual muscle fibers, single myofibrils, single myofilaments, and cross bridges between filaments. At the level of muscle subunits, power generation can be impaired either by some dysfunction in the subunit itself or a decreased total number of subunits. An insufficient amount of power generation at any level can propagate up to an overall inability to fly.

While temperature clearly has effects on the development of *Drosophila* wings, there is another mechanism that allows temperature to interact with phenotype. The heat shock protein (Hsp) system is a remarkable cellular defense system. It has been found in every organism so far tested, including Archaea, Eubacteria, plants, and animals (Lindquist and Craig, 1988). There are a number of Hsp's, ranging from Hsp22 to Hsp110. Many are present and active at normal organismal temperatures and overexpressed at stressful temperatures. Heat shock mRNA is transcribed within 4 minutes of heat shock, and heat shock proteins are

translated within 8-12 minutes of heat shock (Lindquist, 1980). Heat shock also causes a halt in the transcription and translation that was occurring, and also prevents mRNA transcript splicing and post-translational modifications (Pauli *et al.*, 1992). The three most important families of Hsp's are the small Hsp family, the Hsp70 family, and the Hsp90 family (Lindquist and Craig, 1988). The small Hsp family is unusual in that in organisms that have more than one (all eukaryotes except *Saccharomyces cerevisiae*), the proteins are more similar within the organism than between organisms. They are not well characterized, but they may protect inactive mRNA and are developmentally induced in specific levels in specific compartments. The Hsp70 family performs many housekeeping tasks, such as post-translational import of proteins into organelles. Many Hsp70 mechanisms involve using ATP to interfere with protein-protein interactions. Members of the Hsp90 family have 50% amino acid identity between eukaryotes and over 40% identity between eukaryotes and *Escherichia coli*. Hsp90 is commonly studied because it usually functions less in the simple maintenance of proteins and more in the support of delicate signaling proteins. However, Hsp90 reversibly binds to unstable proteins, regardless of sequence, so under conditions of environmental stress, Hsp90 must assist in housekeeping tasks, binding to partly denatured quotidian proteins. This can interfere with transduction pathways as well as the stabilization of mutant proteins. Hsp90 can silence mutations by stabilizing the gene product. A silenced mutation does not affect fitness, so it is not pushed out of the gene pool via selection, thus allowing mutations to

accumulate. Rutherford and Lindquist (1998) have described the accumulation of silenced mutations facilitated by Hsp90 as a genetic toolkit of phenotypic variation, or a capacitor of evolution. A mutation might fortuitously ameliorate environmental stress, and the authors suggested several examples of specific systems in *Drosophila* that would benefit from high amounts of latent variation: chemoreception, detoxification of larval food sources, and the immune system. Hsp90 is commonly studied because Hsp90 inhibitors seem to have potential in treating cancer. While Hsp90 is present as 1-2% of total proteins under normal conditions, in cancer cells, this figure can reach as high as 4-6%, due in part to the poor conditions in the cell (Li *et al.*, 2009).

Vision and Flight

The flightless *D. hydei* show light colored eyes, and eye color mutations in *Drosophila* can be associated with vision problems. Vision is very important to the flight system; a great deal of visual information is required to navigate successfully in a three-dimensional world. The fly retina contains two independent visual systems, the high sensitivity system and the high acuity system (Heisenberg, 1972). The high sensitivity system is specialized for low light situations and consists of retinula cells 1-6. The rhabdomeres of these cells are large, and the neural signals produced are sent to the lamina. The high acuity system is optimized for the best contrast and consists of retinula cells 7 and 8. In this case, the rhabdomeres are small and the neural signals are sent to the medulla. Because the signals from the two systems are processed differently, mutants can

show decreased function of one system without noticeable effects on the other. The visual system is used to control flight course and altitude (Heide and Gotz, 1996). The authors analyzed muscular response to visual cues in tethered flies. These visual cues consisted of striped patterns moved in the visual field of the fly, simulating the passing scenery a fly would encounter during flight. They found that several pairs of the direct wing muscles fired in response to the visual cues, changing the fly's course and altitude. Therefore, if the light eyes of the flightless *D. hydei* indicate a visual defect, the flies would most likely have an abnormal response to moving patterns.

Metabolic Capacity and Flight

Flight requires extremely high metabolic input, and insects have both unique challenges and unique solutions to these demands. To grasp an idea of the rate of metabolism required, consider that fresh muscle generally contains about 5 μmol of ATP per gram of tissue, while in flight as much as 2000 μmol/g is hydrolyzed per minute (Crabtree and Newsholme, 1975). In flies, the rate of ATP production can reach 2400 μmol/g/min. One of the biggest constraints on this extreme metabolic performance is the transport of fuel to the muscles. Unlike vertebrates, insects exhibit tidal circulation and an open circulatory system, so there can be wildly fluctuating levels of fuel concentration. Furthermore, diffusion distances can be long. This is compensated by a considerably higher concentration of fuel in hemolymph as compared to vertebrate blood. However, high concentrations of glucose would place osmotic pressure on the circulatory system,

and glucose has a relatively reactive aldehyde. The disaccharide trehalose is a very common fuel in insects, which has potentially adaptive advantages over glucose because replacing a monosaccharide with a disaccharide will halve the number of molecules present in the blood, and the reactive aldehyde is safely bound to the other sugar. The other primary demand of high metabolism is a steady supply of oxygen, and in this case, insects are more efficient than vertebrates. The diffusion of oxygen from the liquid phase is relatively slow, but insects do not use their circulatory system to transport oxygen. Instead, they have a system of cuticular tracheal tubes which provide oxygen directly to each cell. The rate of transfer of oxygen from a tracheole to a cell is much faster than it would be from hemoglobin to myoglobin, allowing insect muscle to use oxygen at a higher rate than vertebrate muscle.

Consequences of Flightlessness

The secondary use of the wings in *Drosophila* is courtship. Generally, a male initiates courtship of a female by approaching her, extending one wing, and vibrating it rapidly (Sturtevant, 1915). The song consists of two major components: buzzing sine song and amplitude-modulated pulse song (Ewing, 1977). The vibration is powered by the indirect flight muscles (Ewing, 1977) and modulated by the axillary and direct wing muscles (Ewing, 1979). Ewing described sine song as reduced power flight, as electrophysiology indicates that during sine song, muscle stimulations are less frequent than in either flight or pulse song. Furthermore, only a subset of the muscular units is recruited for sine

song. He also noted that each sound pulse in pulse song is caused by a single up and down motion of the wing, and that because the indirect flight muscles are used for this motion, the motion must be terminated to prevent oscillatory wing movement. He hypothesized that there must be a control muscle that initiates song and determines the inter-pulse interval, and later found that the axillary muscles behave in a manner consistent with the predicted control muscle. Tauber and Eberl (2003) extensively reviewed the literature on *Drosophila* song, noting that pulse song requires functioning mechanoreception for control. However, flight ability and song production ability are not fully coupled. Barnes *et al.* (1998) compared mating success between wild type flies and flies with wing-beat frequency mutations and found that the wing-beat frequency mutations had no significant effects on either courtship sound or mating success. The authors add that mutants exist with disrupted pulse song but normal sine song and normal wing-beat frequency. Tauber and Eberl (2003) also described several mutations that affect only one part of the wing muscle system. Alleles of *doublesex* eliminate sine song but not pulse song, while alleles of *fruitless* can eliminate pulse song or affect the mean inter-pulse interval. The gene *no-on-transientA* (*nonA*) was originally described as involved in the visual system, but the *dissonance* allele affects courtship song: flies with this allele show pulse song that begins normally but becomes polycyclic, suggesting a breakdown in the control mechanism. However, Ewing (1979) reported that *D. melanogaster* pulse songs often increase in amplitude as the song continues and that this can yield two or

three cycle pulses. This corresponds to the tendency for species with loud songs to have polycyclic pulses.

Research has also shown that females express most of the neuromuscular system required for sexually effective song production (Clyne and Miesenböck, 2008). Noting that mosaic flies required a male protocerebrum and thoracic ganglia to produce song, the researchers sought to stimulate female flies to produce song. Using a protocol that allowed the activation of nerves via light, they found that light activation of neurons expressing the gene *fruitless* produced song in both males and females. However, song production in the females required a higher energy density of light. Furthermore, while recordings of male song stimulate mating when played for a virgin female and a wingless male, who will otherwise not mate, recordings of forced female song did not stimulate such mating. Therefore, they developed female flies that expressed the male-specific protein product of *fruitless* in their neurons, which were found to respond to the same energy density of light as males and produce song that stimulated mating between a virgin female and a wingless male. They conclude that the females have the ability to produce the song, but their neurons lack the ability to initiate it. While female mice will display male behaviors when their pheromones are disrupted, female flies do not. However, both systems are different ways of producing sexually dimorphic behavior without encoding two entirely separate systems: switches at the top control the behavioral outcome.

Male *Drosophila* courtship song has a number of acoustical features

interpreted by the females. In general, sine song, which does not have many identifying features, is considered to be priming – that is, its main purpose is to generate sexual excitement in the female. Pulse song has more features, varies more between species, and thus likely functions in species recognition (Tauber and Eberl, 2003). The inter-pulse interval of the pulse song is one of the most important and identifying song characters, and it oscillates as well – artificially produced song with constant inter-pulse interval does not garner a reaction from females. Early studies of *D. melanogaster* song used mechanically generated song (Bennet-Clark and Ewing, 1969). The authors admitted that their song generation device only simulates the acoustic component of mating song, not the near field effects of the wing vibration; therefore, it is less effective than genuine mating song. However, wingless male flies encouraged by simulated song were more successful at mating than wingless flies without acoustical enhancement. The authors compared the reactions of *D. melanogaster* and *D. simulans*, as the closely related species will hybridize in the lab and have courtship songs that differ only in pulse interval. They exposed both species to simulated song that varied in either pulse length or pulse interval, and found that while there was no difference in response to pulse length, females responded most favorably to song with their species-specific pulse interval. Given that flies will habituate to diurnal cycles either half or double the length of normal, they also generated songs with either halved or doubled pulse intervals, but the females did not respond favorably to these songs. They pointed out that although they varied pulse length, the pulses

had the same total energy, which implies they would generate the same amplitude of effect on the arista. Routtu *et al.* (2007) compared wing traits and song characters between allopatric populations of *D. montana*, finding a high level of divergence in both, including song traits known to be important for sexual selection and species recognition. However, the authors attributed the variation in wing characters to environmental factors and do not discuss a possible correlation between variation in wing and variation in song. In a similar vein, Sene *et al.* (1998) compared two song types in well-fed and poorly-fed males of *D. mercatorum* (a member of the *repleta* group). The well-fed males had larger wings, and between the two groups, there was a difference in the B song, related to female sexual stimulation, but little variation in the A song, related to species recognition.

There is a large amount of interspecific variation in female response to song. The females of some species are quite gracious in accepting a suitor, while others are less so. In many species, the female will spread her wings as a signal to the male that she will accept him, but in some, she leaves it to the male to spread her wings with his head (Sturtevant, 1921). Sturtevant noted that some males fail to mate because they are unable to spread the female's wings. In his survey of the mating habits of a few dozen species, he stated that *D. repleta*, *D. hydei*, and *D. virilis* spread their wings while *D. melanogaster* and *D. simulans* do not. However, Vuoristo *et al.* (1996) mentioned that studies have observed wing spreading in *D. melanogaster*. The females of some species of *Drosophila* prefer

specific male song traits and refuse to mate with closely related species, even though their songs are very similar to the males of their species. On the other hand, for some females, the stimulus of heterospecific song suffices to encourage heterospecific mating, indicating that these females prefer any song to silence (Tauber and Eberl, 2003). In some cases, the preferred song is an indication of a genetic benefit. Hoikkala *et al.* (1998) found that *D. montana* males with preferred songs imparted indirect benefit to the female, because their progeny were more likely to survive to adulthood. Interestingly, the asymmetry of the male wings did not correlate with their desirability, suggesting that wing asymmetry does not impact song quality. Asymmetry will be discussed later. In a followup study, Ritchie *et al.* (1998) noted that even though males with preferred songs father more vigorous offspring, this does not rule out the possibility that the males are preferred for characteristics other than song. *D. montana* females will rarely mate without a song, and they spread their wings to indicate their readiness to mate. Therefore, the authors analyzed female response to simulated mating song, with varied frequency and pulse length. They found that females did respond most favorably to the preferred song, even in the absence of males. The authors mention the song response of females of other species. For example, in *D. biauraria*, heterospecific song inhibits mating. Saarikettu *et al.* (2005) found ample variation of behavior between strains of *D. virilis*, yet the strains still interbreed in the lab. However, *D. virilis* is unusual among its relatives for being surprisingly unpicky in its mates; females are known to permit coitus before any

song is produced, a stark contrast to the demanding *D. montana*. However, in most species, flies will mate without song, though song decreases the length of courtship required.

Drosophila seem to be quite adept at distinguishing mating song from background noise. The wing spreading response of *D. montana* females was observed with exposure to mating song and varying loudness of background noise, at a frequency either overlapping or not overlapping the song (Samarra *et al.*, 2009). They found that the mating song was not masked by background noise of a different frequency, but if the background noise overlapped the frequency of the mating song and was 3 or more decibels louder, masking effects began to emerge. However, the percentage of females responding to the masked song was not zero, and *D. montana* very rarely exhibits wing spreading without hearing a conspecific mating song, so some of the females must have been able to detect the song. This suggests that the acoustic apparatus has some ability to filter out frequencies, even if the background noise is louder than the mating song. In the wild, *D. montana* might be exposed to conspecific or heterospecific biotic noise as well as abiotic noise. The authors provide the noise of a river as an example of an abiotic background noise that overlaps the frequency of the courtship song. However, masking could also be used by males to interfere with a competitor's attempts to mate with the same female. Perhaps counterintuitively, males exhibit less singing in the presence of another male (Tauber and Eberl, 2002). In situations with competition, males add a new type of song, referred to as a

rejection signal. In this study, the authors compared frequency and length of singing bouts between males alone with females and males with one female and one male with amputated wings. In the latter situation, the amputated male nevertheless vigorously courted the female. However, compared with the winged male allowed privacy, the winged male in the competitive setting sang less frequently and his song bursts were slightly shorter. Some evidence suggests that the reduction in the amount of singing is caused by physical interference between males. This raises the possibility that a female may not receive enough song to distinguish song traits such as the oscillation of the inter-pulse interval, generally thought to be important in species recognition. It is possible that female choice is actually exercised on shorter duration characters.

A study in the field cricket, *Teleogryllus oceanicus*, found that if selective pressure is high enough that almost all males become unable to produce courtship song, females can be forced to relinquish their previous discriminating tendencies (Zuk *et al.*, 2006). Crickets, unlike *Drosophila*, use song to locate mates, but song is also used for courtship, and under undisturbed conditions, female crickets will not mate without song. A Hawaiian population of field crickets has been parasitized by a fly, *Ormia ochracea*, which uses the chirping to find males and turn them into terminal incubators for their maggot offspring. A silent male phenotype emerged and spread to almost 90% of the males over the course of several years. Normal males express a file and scraper on their wings in order to produce sound through stridulation, but the mutant males showed a small file at

the wrong angle, much more similar to the female wing. However, females will not be able to find silent males, nor will they allow silent males to copulate. Both sexes adapted in order to allow mating to continue. First, silent males approached a speaker (simulating a calling male) closer than they would normally approach another male, increasing their chance of finding a female attracted by the song. Furthermore, females in populations with silent males allowed silent males to mount. As a corollary, if a *Drosophila* species in which females normally require male song for mating experienced a phenomenon that caused all males to become silent, the females would have to learn to accept silent males or else be unable to find a mate. Is it possible that a few hundred generations of flightlessness – and inability to sing – has made song less important to flightless *D. hydei*?

Sturtevant (1915) performed a number of mating tests using some of T.H. Morgan's mutant flies and concluded that there was no mate choice in *Drosophila*: a fertile female would mate with the first male who courted her while a fertile male would mate with the first female who accepted him. Subsequent studies have suggested that larger males have an advantage in mating, but this is not the case in every species. For example, the laboratory-born offspring of wild-caught *D. willistoni* display no correlation between body size and mating success, even when mating occurs in a chamber designed to simulate mass matings found in the wild (Basso da Silva and Valente, 2001). Partridge *et al.* (1987) found higher mating success in larger *D. melanogaster*, noting that large males courted more, sang more, sang more loudly, and moved more. A large component of their

advantage may come from the fact that they are better able to chase and catch females. Ewing (1964) supported the hypothesis that larger wings yield louder sound which yields more partners. Wing size was modified in three ways: developmental temperature, selection, and amputation. Flies reared at lower temperatures had wings nearly 30% larger and mated with more partners while spending less time vibrating their wings. Flies selected for larger wings had more partners than flies selected for smaller wings, even though the smaller winged flies spent more time vibrating, suggesting pleiotropic effects related to the selection for smaller wings. Finally, he found a linear relationship between percent of wing remaining and courtship success, which, when extrapolated, suggested that 80% of sexual stimulation was due to wing vibration.

D. hydei displays some mating traits that are quite different from those of *D. melanogaster* (Markow, 1985). The difference most significant to mating choice is that most female *D. hydei* will re-mate within 60 minutes or less, while *D. melanogaster* females will not mate again for five days after copulation. Furthermore, the first male to mate with a female does not have a significantly higher chance of fathering her offspring, because the sperm from successive matings mix in the female's sperm storage organs. Hypothetically, this situation should decrease the pressure of sexual selection on both sexes. The female does not need to be as cautious in choosing, because she is not as limited in mates as a *D. melanogaster* female. The male has less incentive to be the first male to mate with any given female, because that will not increase the number of progeny he

has with that female. Markow found that in *D. hydei*, male size does correlate with mating success. Both Sturtevant (1921) and Ewing and Miyan (1986) noted that courtship in *D. hydei* is extremely short. In Sturtevant's description of *D. hydei* courtship, he stated that they do not exhibit the vibration observed in *D. melanogaster* and other species. However, perhaps he did not detect the brief vibration during the brief courtship, because Ewing and Miyan recorded *D. hydei* mating song. They divided the song of the *repleta* group into A song and B song. The A song is produced at the beginning of a song and consists of just a few pulses with very short inter-pulse interval, while the B song consists of more pulses, more widely spaced. While B song can vary, A song is conserved, suggesting that A song might function in species recognition. The authors also noted that the beginning of courtship is the most logical place for a species recognition signal. They specifically pointed out that in the *hydei* subgroup, the A and B songs tend to run together. The short length of courtship potentially suggests that song quality is not an extremely important factor in *D. hydei* mating. They also stated that while the members of the *hydei* subgroup have similar song, they tend to be geographically isolated and therefore are under less pressure to evolve divergent songs. This is in contrast to, for example, *D. arizonensis* and *D. mojavensis*, which are closely related and create fertile hybrids in the laboratory, yet no fertile hybrids have been found in the wild, likely because the two species have divergent song inter-pulse interval. Because *D. hydei*'s songs resemble those of its closest relatives and its courtship is very brief, it is unlikely that it is one of

the *Drosophila* species whose females are extremely discriminating in their song choice.

Another potential consequence of flightlessness is the possibility of reduced selective pressure on the flight system. All laboratory raised flies are released from selection to some degree, but flight is a particularly good example of an ability that is vital to survival in the wild but nearly irrelevant in the lab. Flightless flies seem to have a harder time extricating themselves from the quicksand of liquefying medium, but they face no other peril. Therefore, many generations of flies bred in the lab could accumulate mutations, and a mutation in the wings might interfere less with a flightless fly's fitness than a mutation in any other part of the body. Fluctuating asymmetry has frequently been proposed as a phenotypic measure of genotypic quality, but not without controversy. Fluctuating asymmetry is precisely defined as asymmetry in a population with a mean asymmetry of zero, where all asymmetries are relatively small (Swaddle, 2003). It must be distinguished from directional asymmetry, where one side is consistently larger than the other, such as the mammalian heart, or antisymmetry, where either side might be bigger, such as the large claw in the males of fiddler crabs. In some taxa, stress and genetic degradation yield higher fluctuating asymmetry, while in others, mortality-inducing stress does not increase it. Fluctuating asymmetry must be measured and analyzed very carefully, and conclusions are difficult to generalize. Measurement error can easily be larger than fluctuating asymmetry, so all measurements should be repeated multiple times and sample sizes should be

large. It is not even fully accepted that low fluctuating asymmetry is adaptive in and of itself. For example, in the wasp *Trichogramma brassicae*, an increase of fluctuating asymmetry does not decrease fitness until it reaches a certain threshold. Swaddle suggests that fluctuating asymmetry may have a canalizing nature, i.e. it only appears under conditions of extreme developmental stress (either environmental or genetic). One argument against the significance of fluctuating asymmetry for sexual selection is the inconclusive evidence of whether or not the organisms can actually detect it, because it is often found to be on the order of 1%. In investigations of *D. melanogaster*, Carter *et al.* (2009) compared fluctuating asymmetry of inbred and outbred flies and found that offspring from a stock that had been in culture for 34 years showed higher fluctuating asymmetry for inbred flies, while a stock that had been in culture for 18 years did not show significant differences between inbred and outbred flies. The authors stated that other studies report higher fluctuating asymmetry in homozygous populations, and the older culture also had a smaller starting number of flies (200 versus 400), so the stock as a whole likely had a more limited gene pool, making the inbred flies particularly homozygous. The newer stock most likely started with a more varied gene pool, so its inbred offspring are still somewhat heterozygous. Clearly, fluctuating asymmetry is confounded by many complications, not least being the difficulty of measuring error. However, if it is indeed a reliable external measure of genetic or environmental stress, the flightless *D. hydei* might have more wing asymmetry than wild-type *D. hydei*.

However, there are two caveats: first, the flight system might be so highly conserved that mutations do not accumulate at an appreciable rate, and second, the wings may play a role in male attractiveness such that sexual selection will prevent accumulation of wing mutations. Furthermore, the length of time the culture has been in the lab and the number and variability of founding members of the culture would likely be the largest influence on fluctuating asymmetry.

Purpose

This project sought to determine the cause of a flightless phenotype in *Drosophila hydei* and the mechanism of its temperature-based rescue. These two goals are of course interrelated: determining the cause of flightlessness could suggest a likely developmental window for susceptibility to heat shock, while finding the proper timing of heat shock rescue could narrow the possibilities for the cause of flightlessness. Therefore, heat shock should be performed at a variety of developmental stages. There are many levels at which this mutation could be investigated; however, I concentrated on organismal level experiments and observations that could narrow the tissue level and molecular level explanations. I also considered potential causes for flightlessness and how they would be modified by temperature during development. The heat shock system is involved in the support of many cellular processes. It is important to broaden our knowledge of what effects heat shock can have on an organism. If a complex function is restored by heat shock, it may be an intriguing expansion of the evolutionary potential of heat shock or a window on an unusual sort of defect. I

endeavored to obtain flight-capable flies from the flightless strain and to determine what prevents the flightless flies from taking to the air.

MATERIALS AND METHODS

Culturing and Anesthesia

I maintained four stock cultures: flightless *D. hydei* (Black Jungle Terrarium Supply) starting in June 2009, golden flightless *D. hydei* (flycultures.net) starting in July 2009, "Turkish glider" weakly flying *D. melanogaster* (Black Jungle Terrarium Supply) starting in July 2009, and flying wild type *D. hydei* (UCSD stock center) starting in January 2010. The flies were kept in 100 mL plastic vials with instant Drosophila medium (Black Jungle Terrarium Supply), with the manufacturer's recipe adapted for smaller vials: 30 mL of medium and 45 mL of distilled water. Baker's yeast was added dry as food and Excelsior (shaved aspen wood, Black Jungle Terrarium Supply) was added for climbing and surface area. Experimental cultures were kept in an environmental chamber at 25°C and 50% relative humidity. Stock cultures were kept in an environmental chamber at 16-18°C. Subcultures were made when crowding became apparent. To reduce loss of flighted individuals, a square of nylon netting was secured over the top of a vial with a rubber band, and a small hole was cut in the netting at the edge of the vial. This bottleneck reduced potential escape routes for the flies. Furthermore, the plug could be replaced and removed from the vial with the netting in place.

Flies were sexed after CO_2 anesthesia under a stereo dissecting

microscope. Sex was determined solely by observation of external genitalia. *D. hydei* has delayed maturation compared to *D. melanogaster*, with females maturing at 3 days and males maturing at 9 days (Markow, 1985). Therefore, for experiments requiring virgins, virgins were collected at 24 to 48 hour intervals and kept in separate vials. However, the flies' mating behavior is very sensitive to environmental perturbations, as CO_2 exposure, cold anesthesia, and mechanical shocks can all delay mating (Barron, 1999). In this species, however, the delayed maturation forces a lengthy recovery time, so that by the time males are sexually mature, all lingering effects of anesthesia should be negligible. All mating trials were performed a minimum of 72 hours after CO_2 anesthesia.

Flight Ability

Qualitative flight analyses were performed in several ways. An entire vial of flies was tested by gently shaking the flies into the relatively large arena of a large plastic storage box partially covered with its lid. Flies walking up the sides were occasionally discouraged by tapping the box down onto a surface, and the population was watched for flight. To analyze flight after sexing under CO_2 anesthesia, flies were placed in separate fresh vials and allowed at least 2d to recover. The absence of pupae on the walls of the vial allowed for good visualization of the flies inside. Tapping the vial on a pad knocked the flies to the bottom, and those that could would quickly fly up to the top. Flies could also be tested individually or in small groups. Flies were placed in a small bucket which was partially covered and chased with a paintbrush for 5 minutes or until they

flew. Other methods were also employed to attempt to differentiate between flight ability and flight willingness. To test whether the flies could fly when the requirement of jumping was removed, flies were placed on the tip of an elevated strip of paper and air was blown over them both orally and with an aquarium pump. To test whether predation would encourage reluctant but capable flyers to take to wing, flies were placed in a tank with live myrmeleontid larvae (antlions), a predator that traps ants and other terrestrial prey in a pit it creates in sandy soil.

Wing Loading

Wing loading is a simple quotient of two values: gravitational force exerted on the fly and total wing area. Gravitational force is the product of gravitational acceleration and mass. Therefore, the relevant parameters are the fly's mass and wing area. Flies were first tested for flight ability. A comparison was performed between flightless strain *D. hydei* that tested incapable of flight and wild type *D. hydei* that tested capable of flight. After flight testing, the flies were anesthetized with FlyNap (Carolina Biological Supply) and massed to 0.01 mg precision using a Mettler balance. FlyNap was used to immobilize the flies quickly and fully without adding mass. Cold anesthesia requires at least 10 minutes to take effect and may increase condensation on the fly. Massing the fly in a microfuge tube or on weigh paper can lead to tare error on the order of the mass of the fly. Therefore, to reduce error, flies were placed directly on the pan of the balance. After massing, flies were transferred to separate, numbered microfuge tubes. Each fly was then re-anesthetized with CO_2 and both wings were

removed using dissecting forceps. The wing pair was placed on a slide, maintaining in vivo orientation, and mounted in a glycerin-ethanol mixture. Each wing was imaged in brightfield at 32x magnification, using μScope (PixeLINK) and a CCD mounted on the microscope. Each image was processed with Photoshop (Adobe) to remove the background using the magnetic lasso tool. The proximal region of the wing was often damaged during removal, so the images were standardized by inscribing a line beginning at the humeral cross-vein and tangent to the third posterior cell (C, Fig. 2) and removing the portion proximal to that line. The area of each wing was measured in ImageJ (NIH). The length (L, Fig. 2) was measured along the third longitudinal vein from the anterior cross-vein (Ferris, 1950) to the distal tip (after de Moed *et al.*, 1997). Wing loading was then calculated in Excel (Microsoft).

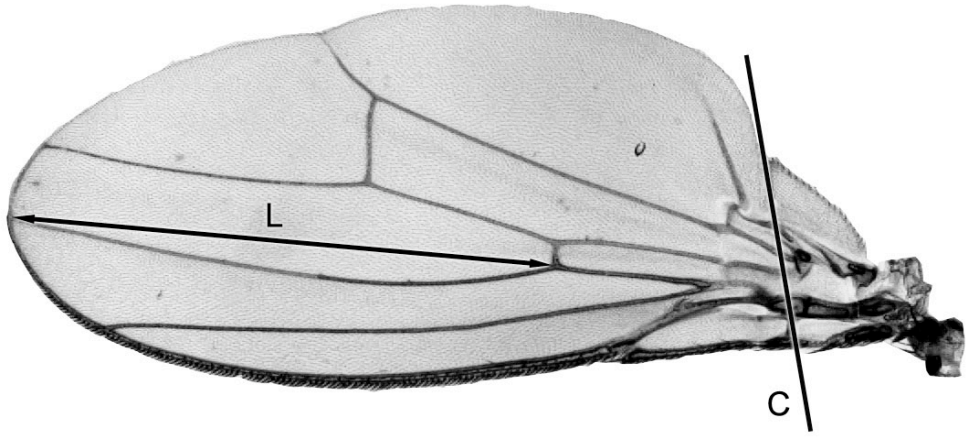

Figure 2: Length and Area on the *D. hydei* Wing. In preparation for measurement, the background was removed and the portion of the wing proximal to the line tangent to the third posterior cell and intersecting the humeral cross-vein (C). The length was measured along the third longitudinal vein from the anterior cross-vein to the distal tip (L).

Mating

Two types of mating trial were performed. Mating trials were performed in a 2 cm diameter plastic vial, and a cotton ball was pushed down to within 4 cm of the bottom, to restrict the flies' movement. Mating behavior was observed for a single pair of flies, either same strain or between strains. Specifically, male approach, genital licking, attempted copulation, time to mating, and duration of mating was noted. Single females were also given a choice between a male of her strain and an age-matched male of the other strain. Time and duration of all courtship attempts were noted. Flies were observed until coitus finished or until 30 minutes elapsed, whichever came first.

Virgin golden flightless and dark bodied flightless flies were mated to determine whether the flightless allele was complementary. Four pairs of 10 day old virgin wild type female and flightless male were mated, and the reciprocal cross was also performed. The P_0 flies were allowed to oviposit for 13 days and then removed. F_1 from both crosses were sexed every 24-48 hours, and some were transferred to fresh bottles while some were discarded. F_2 from both crosses were separated into fresh bottles by both sex and eye color and observed for flight after several days of recovery. χ^2 tests were performed, with a null hypothesis of 1:1 male:female ratio and 3:1 dark eyed:light eyed ratio.

Heat Shock

In order to heat shock many larval stages at once, I placed 6 pairs of mature, flightless *D. hydei* into each of several vials and allowed them to oviposit

for 8 days, then cleared the adults. This yielded cultures with an age range between eggs and third instar larvae. The next day, half of the vials were heat shocked in a 37°C water bath for 85 minutes, while the other half were placed in a beaker of water to the same depth as the water bath, and water was dripped onto the vial plug to simulate the humidity of the water bath. Offspring were collected every 24-48 hours for 16 days, flight tested, and sexed. χ^2 tests were performed, with a null hypothesis of 1:1 male:female ratio.

Muscles

The least invasive way to compare the flight muscle of the flightless and wild type strains would be to compare birefringence, which is bright for normal muscle and dim for abnormal muscle. Adult flies, especially in strains that express dark pigment, have opaque cuticle, foreclosing the possibility of observing thoracic muscle in an intact fly. However, immediately after eclosion, the cuticle of the thorax is not fully cross-linked, leaving the fly somewhat translucent. Even in a fly with a fully tanned cuticle, the legs are sufficiently thin that leg muscle is highly visible under polarized light, to the point that the bright colors distract from the more subtle birefringence of the thoracic muscles. Therefore, for optimal observation of thoracic muscle, freshly eclosed flies were anesthetized with CO_2 and legs were removed, then the flies were preserved in either glycerin and ethanol or mineral oil. The preserved flies were then observed dorsally and laterally under a polarized light microscope. A polarizing microscope has a rotating stage, and the appearance of the birefringence changes as the stage is

rotated. The degree of birefringence was judged by comparing the maximum birefringence and the minimum birefringence, which occurs at a 45° offset from the maximum. An image was captured of minimum and maximum birefringence using the CCD mounted on the polarizing microscope. The appearance of thoracic musculature in wild type and flightless flies was compared. Photoshop (Adobe) was used to create an optical difference between the overlapped minimum and maximum birefringence images from the same fly.

Homology

In order to seek potential homologous genes in *D. melanogaster*, alleles expressing a flightless phenotype were retrieved from FlyBase. “Results Analysis/Refinement” was used to find the alleles that matched the anatomy most likely involved. The allele descriptions were compared to the observed behavior of the flightless *D. hydei* strain, and a list of the most similar alleles was compiled.

RESULTS AND DISCUSSION

Each potential cause of flightlessness, some of which are outlined in Figure 3, will predict certain changes in behavior and structure. These potential correlates of flightlessness in turn guide my observations of the flies. For instance, most of the flightless flies can jump, which suggests that flight initiation is not the

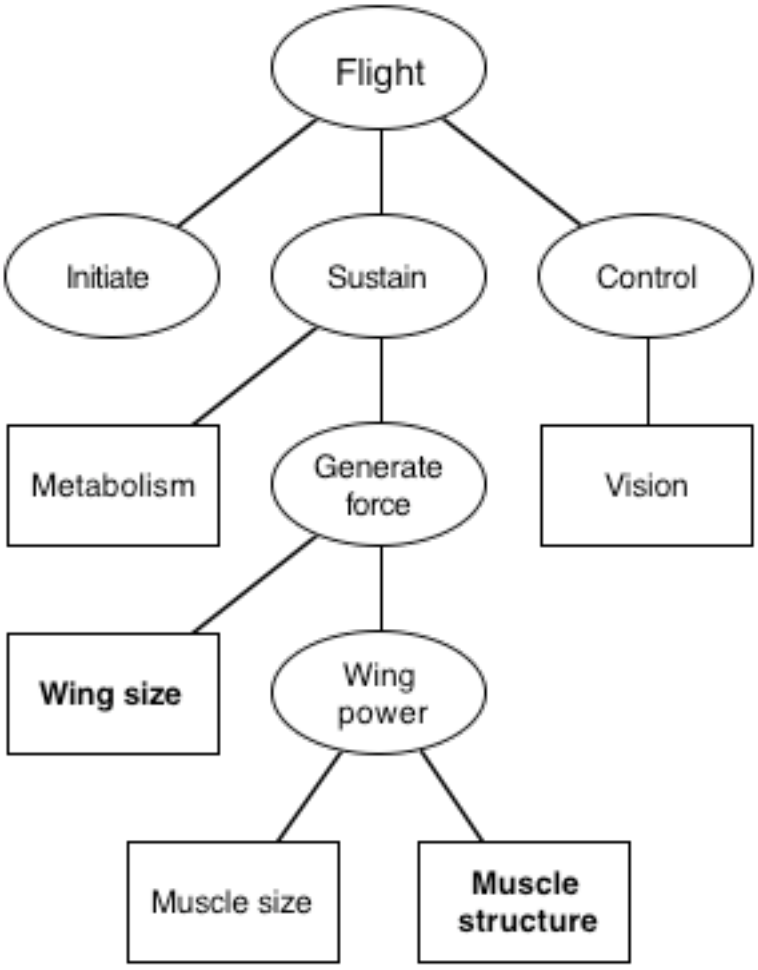

Figure 3: Major Components of the Insect Flight System

problem. However, some flies do seem uncoordinated and may not land on their feet after a jump. All individuals of the flightless strain seem to have more difficulty righting themselves after a fall; while wild type flies are rarely on their back for more than a second, the flightless flies can struggle for 15 seconds or longer. The flightless flies spend a lot of time walking and grooming; they do not seem to be less active than the flying flies. However, due to the truly enormous metabolic demands of flight, this activity does not rule out metabolic impairment of the flightless flies. Adequate lift generation cannot be judged from mere observation; so both insufficient wing area and muscle size are possible causes of flightlessness. Flies grounded by wing loading would still be expected vainly to beat the air, but the flightless flies exhibit no wing vibration whatsoever. They do not move their wings any faster than a slow up and down motion. Wing vibration is powered by the indirect flight muscles, while slow wing movements are powered by the direct flight muscles. This suggests a potential impairment of metabolism or indirect flight muscle. Some of the flightless flies also hold their wings slightly above the normal resting position, a phenotype that is common in flies with muscle defects (Koana and Hotta, 1978). The wild type flies have deep red eyes, whereas the flightless flies – both golden bodied and dark bodied – have bright orange eyes, similar to the vermillion mutant of *D. melanogaster*. Such a mutation was noted in *D. hydei* in the 1940s (Spencer, 1949). The eye color could be pleiotropically related to the flightless mutation or entirely separate. If the apparent absence of brown pigment interferes with the flies' vision, it could also

impair their flight ability. The flightless flies seem to exhibit a normal tendency to explore their environments visually, but it is difficult to separate vision from gravitropism, chemotaxis, phototaxis, and other stimuli that are likely to affect how flies move through their environment. Flies that were unwilling to fly due to defects in the visual system should exhibit normal wing vibration when electrically stimulated.

Flight

No formal method of flight testing found flying flies; however, a few flying flies from a flightless genotypic background did appear. I cultured the flightless stock for nearly 11 months, during which time I estimate extremely conservatively that I bred 250 flies a each week, or over 10,000 flies in total. Out of all of these flies, I observed no more than two dozen flights. Breeding the even smaller number of flying flies I managed to catch yielded only flightless offspring. Even more perplexing were the multiple instances of observing a fly take a short flight but then land and blend back in with its innumerable walking brethren and sistren. I have also caught these one-time flyers and encouraged them to fly by chasing them with a brush, without being able to elicit a second flight. This behavior could be explained by a small metabolic store that is quickly depleted and cannot be replenished quickly or indirect flight muscles that barely function and are further damaged by use in flight. While the vast majority of the flightless genotype *D. hydei* showed a flightless phenotype, there are additional ways to probe the function of parts of the flight system. The method described by

Boettiger and Furshpan (1952) of exposing flies to CCl_4 induces flight in flight-capable flies. If the anaesthetic functions by interfacing directly with the muscle and bypassing the nerves, it could induce flight in flies with nervous dysfunctions. Another way to bypass the nervous system would be direct electrical stimulation of the flight muscles. The flight inducing nature of CCl_4 would force a hesitant fly to beat its wings, thus proving that it was physiologically able to fly. However, the wild type strain frequently exhibited wing vibration at the beginning of CO_2 anaesthesia. Because none of the flightless flies observed showed this behavior under CO_2, it is unlikely that there is a widespread phenotype in which the fly can fly but refuses to because it cannot see where it is going.

The main difficulty of individual flight testing was losing flies, both during the flight test and while retrieving test subjects from the population vial. It would have been possible to obtain more accurate figures for flight ability with an improved method of flight testing. Dillon and Frazier (2006) coated the lower half of their flight chamber with fluon, a substance flies cannot walk over. Therefore, the only route to the attractant at the top is by air. Some of the studies carried out their flight tests at slightly elevated temperatures, whereas my flight tests were carried out at the variable room temperatures of the laboratory. The rate of invertebrate muscular contraction is directly related to temperature, so elevated temperature could help a fly with weak muscles take off. However, flies that can only fly at elevated temperatures generally show attempted flight, including wing flapping, at lower temperatures (Frazier *et al.*, 2008), and I never observed wing

flapping in the flightless *D. hydei*.

Wing Loading

I compared the square of wing length to wing area (Fig. 4) in order to compensate

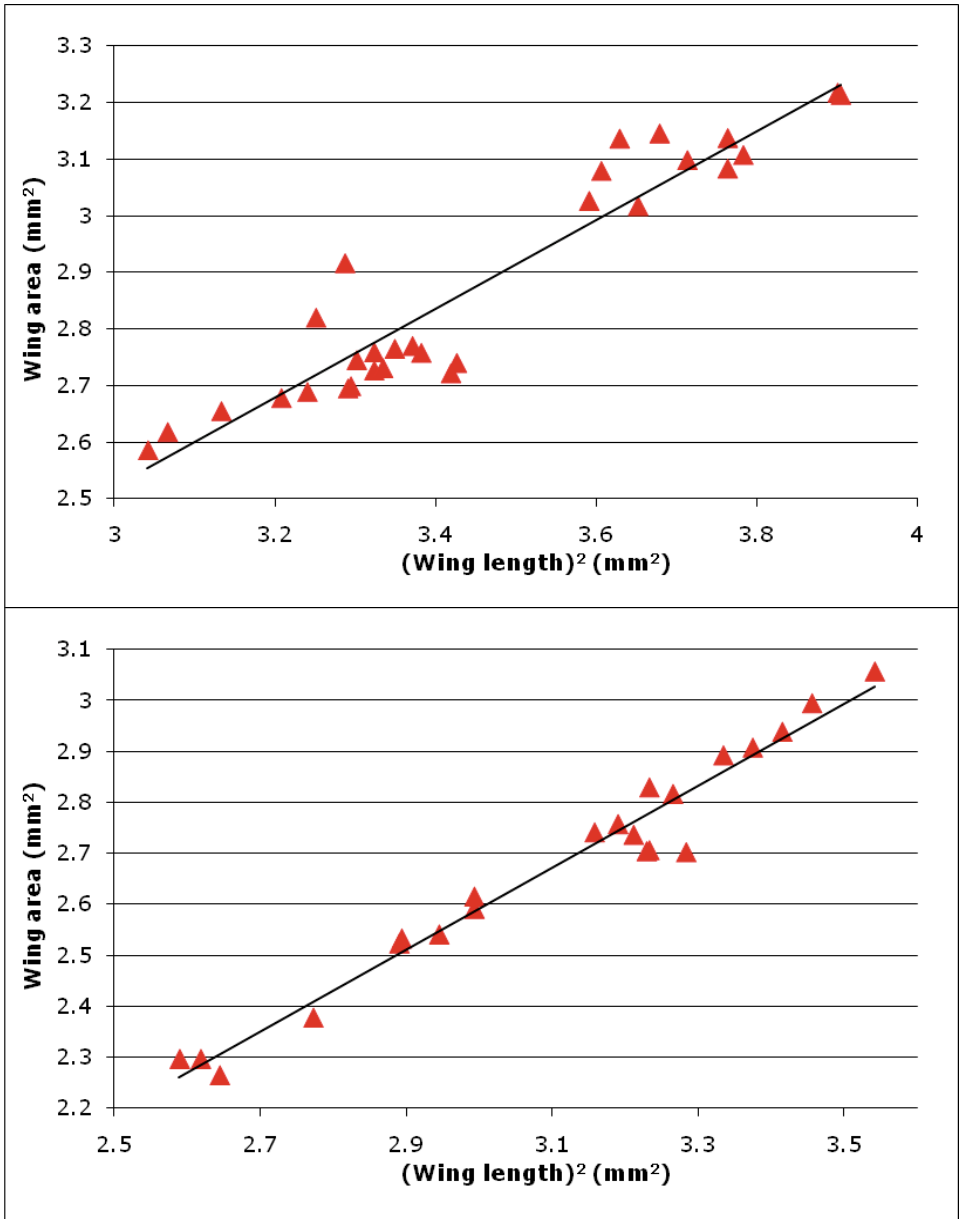

Figure 4: Length-Area Relationship in *D. hydei* Wings. The square of wing length (see Fig. 2) was compared to the total wing area for wild type (top) and flightless (bottom) *D. hydei*. For wild type, the trendline equation is y = 0.7857x + 0.1634. For flightless, the trendline equation is y = 0.8039x + 0.1791.

for areas of wings that were damaged during excision. The trend line allowed interpolation of area from length of damaged wings (n = 2 for flightless individuals). If one wing was damaged, the area of the undamaged wing was doubled to give a total wing area; otherwise, the sum of both wing areas was used. The wing loading for flightless and wild type *D. hydei* is presented in Figure 5. The groups were divided by sex because male and female flies tend to have different wing loading (Frazier *et al.*, 2008). For both sexes, the flightless flies (open shapes, n = 15) had lower average wing loading (indicated by circles) than

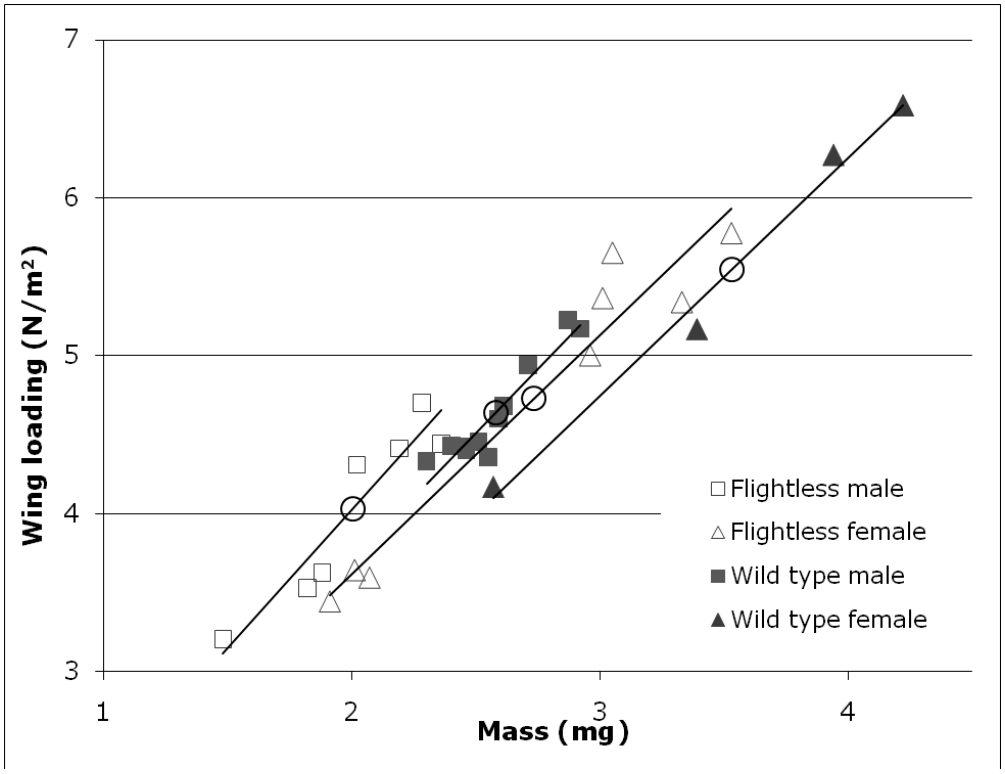

Figure 5: Wing Loading of Flightless and Wild Type *D. hydei*. Wing loading is shown as a function of mass for male (squares) and female (triangles) flightless (open shapes) and wild type (closed shapes) *D. hydei*. Average wing loading and mass is indicated for each group with a circle. While the flightless flies show a lower average wing loading, they have a higher wing loading per mass.

the wild type flies (filled shapes, n = 15). For males, the average wing loading was 4.03 N/m^2 for flightless and 4.64 N/m^2 for wild type, while in females, the average wing loading was 4.73 N/m^2 for flightless and 5.55 N/m^2 for wild type. However, the wild type flies had higher body mass, and for both sexes, the flightless flies had higher wing loading per unit mass (as indicated by the trend lines). The difference between the strains is 0.3-0.4 N/m^2 at the same mass. Frazier *et al.* (2008) compared the flight ability and wing loading of *Drosophila melanogaster* raised at different temperatures. The temperature groups had differential flight ability and differed in wing loading by as little as 0.3 N/m^2. However, these results cannot be compared precisely. Most importantly, *D. hydei* weighs considerably more than *D. melanogaster* – my *D. hydei* ranged from 1.5-4.5 mg while the *D. melanogaster* in Frazier *et al.* ranged from 0.7-1.6 mg – so the same difference in wing loading has different aerodynamic implications. Furthermore, Frazier *et al.* were using wing loading to confirm the cause of induced flightlessness, not to predict the cause of existing flightlessness. If I had bred flying *D. hydei* from the flightless line, then a difference in wing loading would be more meaningful. Therefore, it is unlikely that the flightless phenotype is caused by increased wing loading. More experiments could support or refute wing loading as a potential limiting factor. The wing loading of the variously abled F_2 offspring could be tested. These flies have different combinations of the genetic material of both strains and could help provide information on whether or not the difference in wing loading between the two strains is genuine. Other

parameters that affect wing loading and lift generation could be modulated, such as air density or gravitational acceleration. Air density is lower on mountain peaks, which is the habitat of some *Drosophila*.

Mating and Crosses

The most important observation was that I did not see or hear any courtship song, either by wild type or flightless males. If courtship is minimal, the female has fewer cues to use as the basis for choosing between mates. Furthermore, this would also place the flightless males at less of a disadvantage. The difference between "can't sing" and "didn't sing" is irrelevant if both end in mating. Another surprising observation was that wt × wt and fl × wt matings tended to exhibit a longer time to mating than wt × fl matings; that is, the mutant males successfully mated sooner. A flightless male and a wild type male were offered first to a wild type female and then to a flightless female. No overt competition was observed; in both cases the female summarily mated with the male of her strain. However, some features of *D. hydei* could help explain the observed behavior. The females will remate, sometimes within 10 minutes of the previous mating. This means that a female does not have to be very cautious in choosing her mate, because she will have others. Markow (1985) found that the sperm from successive matings mix in the spermatheca and are used equally in producing offspring; that is, there is no particular incentive for males to be the first to mate with any given female. Therefore, there is reduced pressure for males to compete over females, and reduced incentive for females to be choosy. The

ideal experimental situation would be to allow a female to choose between males that were genetically identical except for flight ability, but a step in that direction would be to provide flightless and flying F_2 males with matched eye color. The test performed showed that in both cases, the female mated with the male with the same eye color, and because no mating song was exhibited, the visual cue was more likely to have influenced choice. The primary methodological difficulty with this testing was that in the 10 days necessary to mature virgin males, the food vials tended to succumb to overgrowth by microorganisms. Many of the research teams that recorded mating song used a small chamber with netting at the top. It is possible that I did not detect mating song because the plastic tube and cotton ball muffled the sound of wing vibration from the wild type males, although the vibration should be easily visible. However, it would be much more rigorous to use a netting topped mating chamber and make video recordings of the mating. This would allow repeated viewings, more accurate measurement of time to mating and duration of copulation, and viewing in slow motion, where mating behavior would be easier to resolve. Furthermore, recordings of any song could be played back for females in a variety of situations.

The F_1 from the golden × dark and dark × golden crosses showed dark bodies, light eyes, and flightlessness. The F_2 were approximately ¼ golden and continued to be flightless. This indicates that the two strains have noncomplementary flightless mutations.

The F_1 from the wt × fl and fl × wt crosses showed dark eyes and a high

level of flight ability. No more than 1 in 10 of F_1 were flightless. The sex ratios of both F_1 crosses were not significantly different from the expected even ratio (Table 1). F_2 from both crosses yielded four phenotypes: dark eyed flying, dark eyed flightless, light eyed flying, and light eyed flightless.

Table 1: Sex of Offspring of Flightless (fl) and Wild Type (wt) *D. hydei* Crosses

Culture	Female	Male	p
wt x fl	135	160	0.1
fl x wt	135	136	0.95

Table 2: Sex and Eye Color of Offspring of Flightless (fl) and Wild Type (wt) *D. hydei* F2 Crosses

Culture	Female	Male	p	Dark eyes	Light eyes	p
wt x fl F2	86	97	0.4	131	52	0.3
fl x wt F2	87	126	0.008	141	72	0.01

However, the sex and eye color ratios were not always as expected (Table 2). The fl × wt F_2 showed significantly more males than expected ($p = 0.008$) and significantly fewer dark eyed flies than expected ($p = 0.01$). An uneven sex ratio could indicate that one sex had reduced viability, which could indicate that the mutation was sex linked. This could be confirmed by determining sex ratios and survival in the larval and pupal stages. It would be informative to compare survival from egg to imago in both strains. Certain X-chromosome inversions in *obscura* group species lead to defective Y-bearing sperm, thus generating a population strongly biased towards females (Powell, 1997). This would lead to reduced numbers of male eggs laid, which would manifest in a reduced number of male larvae immediately after hatching. Selective larval or pupal abortion of one sex would be indicated by an even sex ratio of larvae that gradually became more

skewed. *Drosophila* can be sexed as larvae and pupae (Cooper, 1950), so these trends could be studied.

Heat Shock

During the heat shock, many of the larvae crawled out of the medium, above the water level of the water bath. No flies were observed to fly from either the control or the heat shock vials. Clearly, the experimental setup did not yield the same results that have been observed. It is possible that the heat shock needed to be earlier in the life cycle. Larvae and pupae were heat shocked, but the vials were cleared 24 hours before heat shock, and it is possible that the sensitivity window could be within the first 24 hours of embryonic development. Andersen *et al.* (2005) found that heat shocking females before they laid eggs affects the wing size of their offspring, so the sensitivity could be in the mothers before the eggs were laid. It is also possible that the heat shock would need to be of a longer duration. Previous observations of eclosion of flying individuals of this strain were in the context of weather conditions or overheated greenhouses, both of which could cause a heat shock of 10 or more hours.

Two of the heat shocked vials eclosed significantly more females than males (Table 3 ; p = 0.0006 and p = 0.01). If the mutation is on the X chromosome, it would be expected to affect the males

Table 3: Sex of Heat Shocked *D. hydei*

Culture	Female	Male	p
2/8 1 (heat shock)	84	45	0.0006
2/8 2 (heat shock)	8	10	0.6
2/16 1 (control)	141	123	0.3
2/16 2 (heat shock)	100	98	0.9
2/16 3 (control)	163	163	1
2/16 4 (heat shock)	141	101	0.01

more, because they only have one copy of the allele. If this result were repeated, it would support the localization of the mutation to the X chromosome. However, because the flightless phenotype appears in over 99% of the flies, it is likely that the population is fully homozygous for the mutant allele, and therefore males and females would have equivalent genetic backgrounds.

Some flies from all vials showed a consistent pattern of wing malformation. There was either a fluid-filled bubble or a crumpled circular blister between the anterior and posterior cross-veins, only on one wing, and usually on the left wing. Because this occurred in both control and experimental cultures, it is likely a genetic defect present in the strain.

Muscle

The polarized light technique showed clear, bright birefringence in the thorax of wild type flies, particularly prominent in a dorsal view (Fig. 6). The dorsal birefringence is clearly from large muscles, so it must correspond to the indirect flight power muscles; specifically, the dorsal longitudinal muscles. In the wild type fly, peak birefringence occurs when the anterior-posterior axis of the fly is lined up between the polarizing planes of the cross polarized filters (Fig. 6, top), and very little birefringence is visible when the fly is aligned with the polarizing planes (Fig. 6, bottom). This is because nearly all of the muscle fibers are parallel to its body axis. In contrast, while some birefringence was detectable in the flightless flies (Fig. 7), the magnitude of difference between minimum and maximum was much smaller compared to wild type (Fig. 8). Reduced

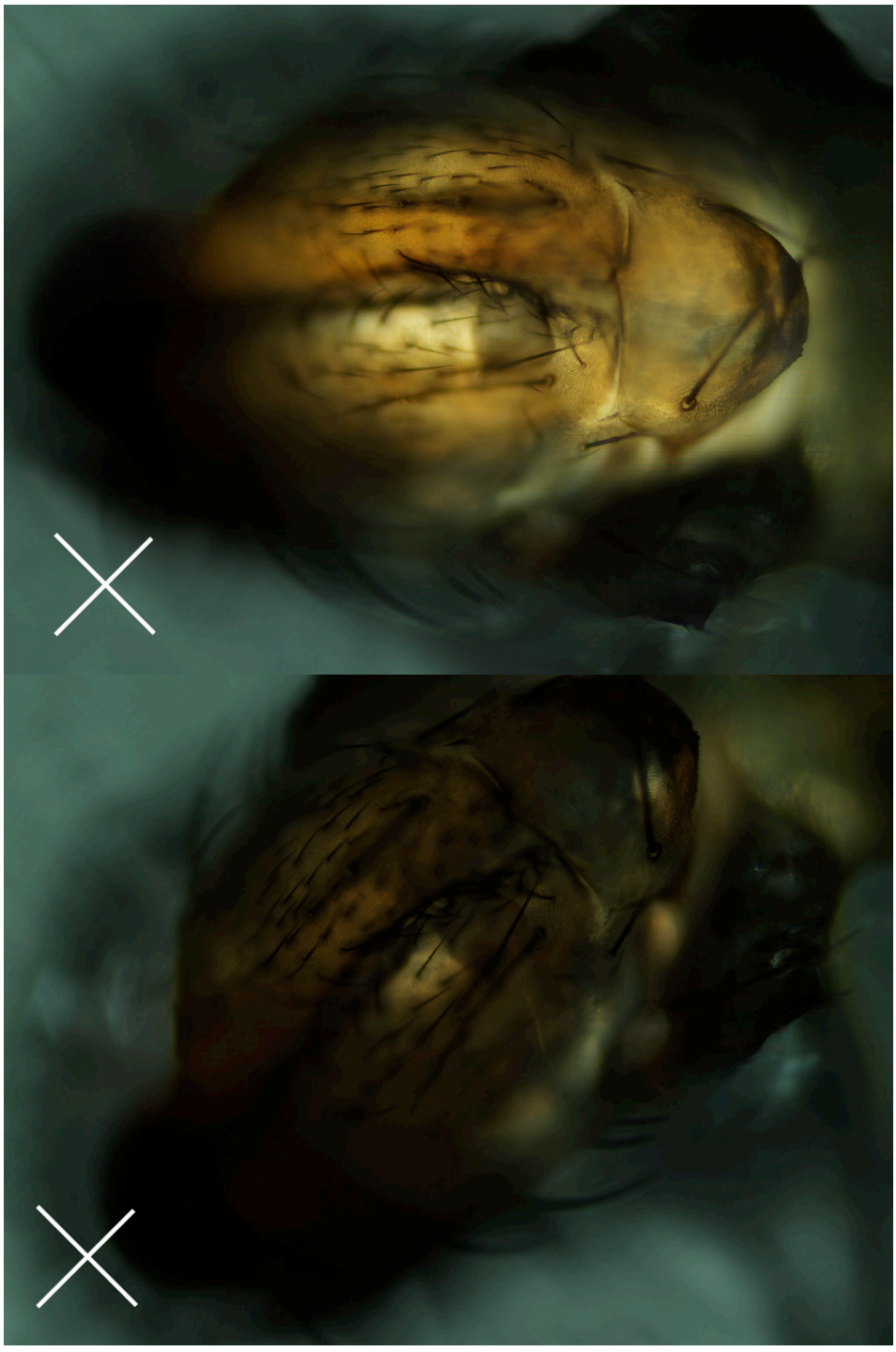

Figure 6: Birefringence of Wild Type *D. hydei* Thoracic Muscle. When aligned 45° relative to the planes of the polarizing filters (indicated by the ×), a dorsal view of a wild type fly reveals strong birefringence (top). When aligned parallel to the polarizing planes, the birefringence is negligible (bottom).

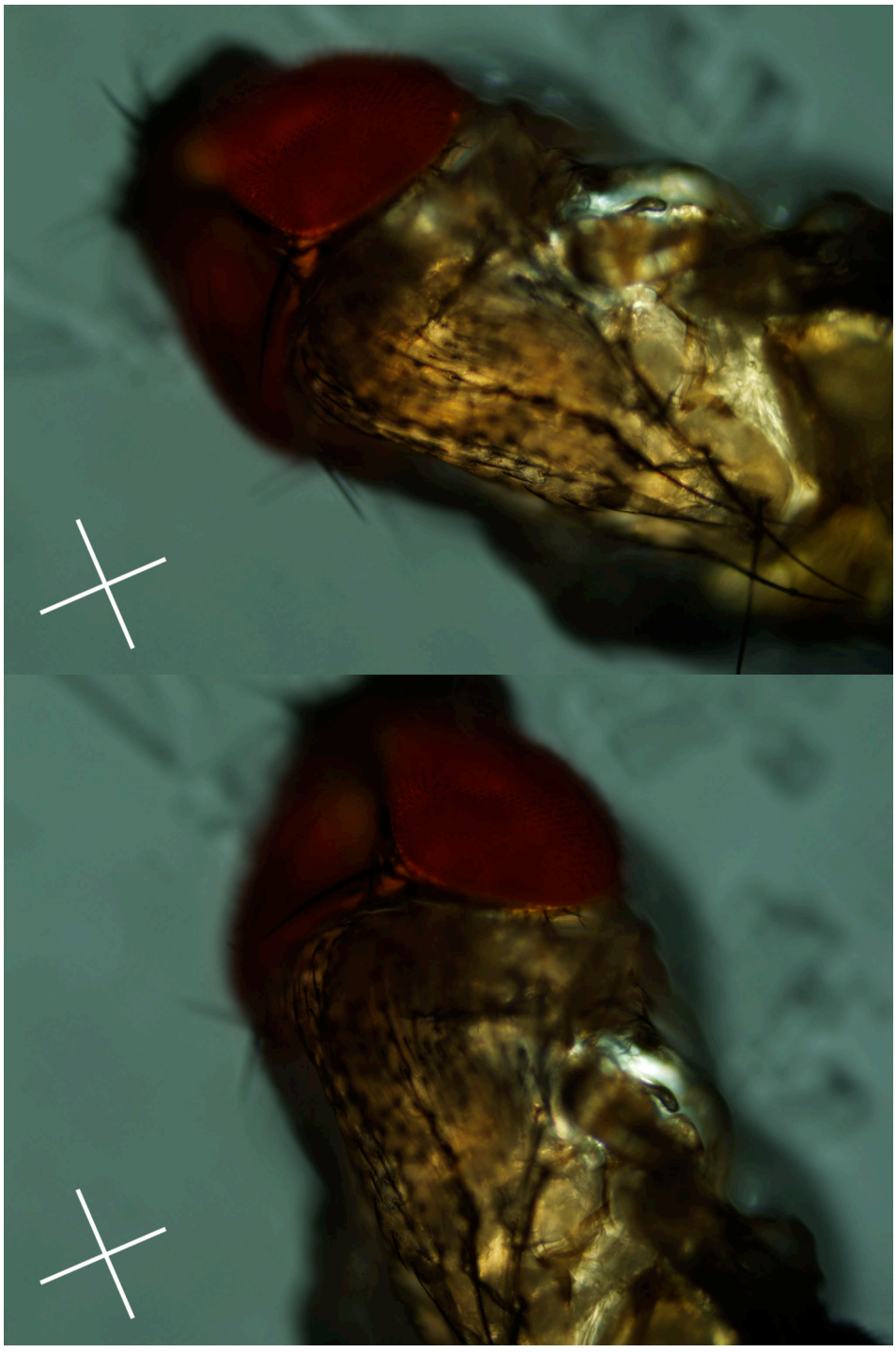

Figure 7: Birefringence of Flightless *D. hydei* Thoracic Muscle. When aligned 45° relative to the planes of the polarizing filters (indicated by the ×), a dorsal view of a flightless fly reveals weak birefringence (top). When aligned parallel to the polarizing planes, some birefringence is still visible (bottom).

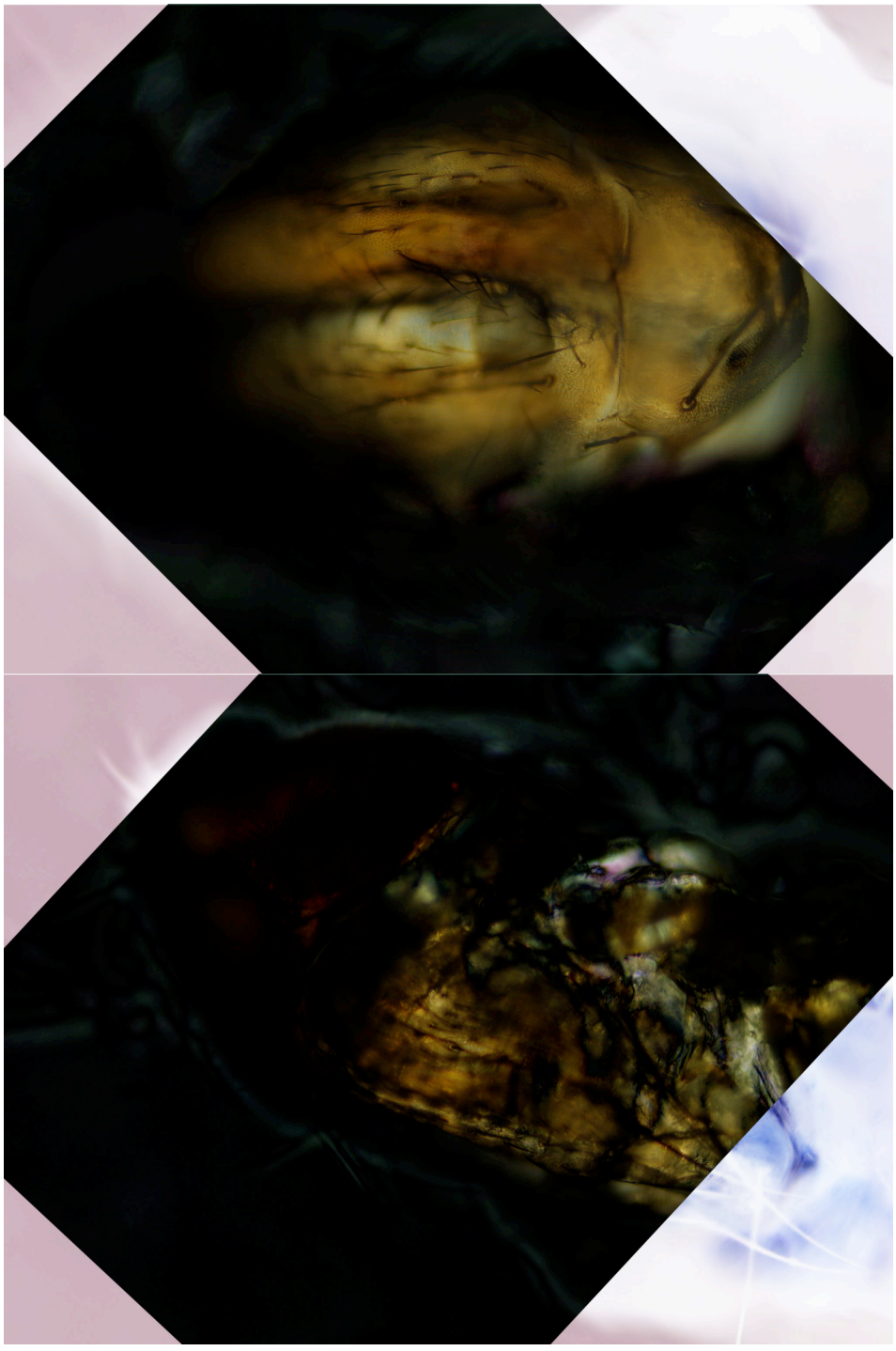

Figure 8: Amount of Birefringence in Wild Type and Flightless *D. hydei* Thoracic Muscle. The images of Figs 6 and 7 were overlaid and Photoshop (Adobe) was used to find the optical difference. The wild type fly (top) shows a greater difference of birefringence than the flightless fly (bottom).

birefringence when the fly's body axis is lined up between the polarizing planes suggests that the muscle fibers are not properly lined up with the body axis. These results were consistent in the six flightless individuals observed. This suggests that the indirect flight muscles, or more specifically the dorsal longitudinal muscles, of the flightless flies are defective. Defects significant enough to reduce birefringence in either set of indirect flight muscles would be sufficient to prevent powered flight. The data gathered suggest that defective flight muscles prevent flight in this flightless strain of *D. hydei*. In the pupa of *D. melanogaster*, the flight muscles grow for nearly 2 days before showing striation (Bodenstein, 1950). It is possible that the muscles never develop properly, but it is also possible that they develop properly but degenerate later in the pupal stage; therefore, different ages of pupae could be dissected to observe the muscle. The stage at which the muscles first appear abnormal could be a clue as to when the potential heat shock window would be. I hypothesized that the individuals that displayed a single, non-repeated flight could be explained by degeneration of the flight muscle. However, several of the observed flightless flies, all of which showed abnormal thoracic muscle birefringence, were so young that their wings were still folded, and it is unlikely that a fly would use its indirect flight muscles before its wings were unfolded.

As discussed, it is not clear that courtship song is an important selective factor in the mating of *D. hydei*, as wing vibration was not observed in wild type *D. hydei* mating. However, defective indirect flight muscles will preclude the

production of mating song.

Homology

Due to the phylogenetic distance between *D. hydei* and *D. melanogaster* – 40-60 million years of divergence – it is highly unlikely that there is a perfect homologue in *D. melanogaster* for the *D. hydei* flightless mutant, with the same locus, mutation, and effects. However, consideration of *D. melanogaster* genes that cause similar problems suggests potential mechanisms for flightlessness. I searched FlyBase for *D. melanogaster* alleles involving the indirect flight muscles that showed a flightless phenotype. Because the flightless phenotype is rescued by crossing with wild type flies, I excluded alleles that were dominant or semidominant, as well as homozygous lethal. I also excluded in vitro constructs. The remaining similar alleles are summarized in Table 4. Several recessive alleles were ruled out as potential homologues due to the nature of their effects. Both $Amph^{26}$ and $wupA^{hdp\text{-}2}$ were ruled out because they exhibit larvae with impaired movement, while flightless *D. hydei* larvae seem equally active as wild type larvae. $fliI^{1}$ and $fliI^{2}$ show some flight ability at low temperature and lose it at high temperature, the opposite of the expected behavior of flightless *D. hydei*. fln^{0} shows muscle that is normal at eclosion but becomes abnormal within 2 days; the muscle abnormalities seen were present immediately post-eclosion. $park^{\Delta 21}$ shows several characteristics not present in flightless *D. hydei*: drooping wings, sterility, hypoactivity, and short lifespan. $Pink1^{B9}$ shows wings down and a crushed thorax, while the only abnormal wing position in flightless *D. hydei* wings is up and the

Table 4: Flightless alleles of *D. melanogaster* , involving the indirect flight muscle, not dominant or in vitro constructs

Gene	**Alleles**	**Key Features**	**Possible**
Actn	1, 2, 3, 4	Muscle detaches from cuticle; $Actn^{1}/Actn^{4}$ abnormal jumping/flight at 22°, rescued at 29°	
Amph	26	Sluggish larvae, neuromuscular defects	No
fldA	1	disrupted myofibrils, major proteins present, t-tubules fragmented, external nervous stimulation fails	
fliI	1,2	weak flight at 17°, flightless at 22°; muscle structure abnormal	No
fliI	3, 5, 8	abnormal Z-bands, myofilament fraying	
fln	0	muscle normal at eclosion, becomes hypercontracted at 2 days	No
flw	1, 6	flw^{1}: IFMs reduced; flw^{1}/flw^{6}: IFMs absent; flw^{6}: 99% male lethal	
flw	2	reduced jumping, 30% jumpless, 95% abnormal wing position	
ifm(2)RU2	1	most raised wings, thin DLMs, disorganized DVMs, some with normal wings weak flight	
park	Δ21	drooping wings, sterile, hypoactive, short lived	No
Pink1	B9	wings down, crushed thorax	No
up	1, 101, int-1, int-3	vertical wing position, filament arrangement in IFMs absent, jump ability gradually lost, sometimes indented thorax	No
wupA	hdp-2	larvae move abnormally, muscles crumpled	No

thorax appears normal. The *1*, *101*, *int-1*, and *int-3* alleles of *up* show a vertical wing position, no organization of muscle filaments, loss of jump ability, and sometimes an indented thorax. Flightless *D. hydei* do not have vertical wings, degenerating jump ability, or indented thoraces. All these alleles can be ruled out as the cause of the flightless phenotype in *D. hydei*, but some alleles cannot be ruled out with the information presented.

Several of the alleles show characteristics that suggest that they could be homologues of the *D. hydei* flightless mutation and lack characteristics that rule out homology. In several cases, this is merely because so little is known about the *D. melanogaster* mutation that nothing rules out homology. fld^{A} shows disrupted myofibrils, failure to contract with artificial nervous stimulation, but presence of all normal proteins. Alleles of *fliI* show abnormal Z-bands and myofilament fraying. These could be better compared to flightless *D. hydei* once ultrastructure and components of the muscle are known. *flw* is a possible locus, because flw^{1} shows reduced indirect flight muscles; however, several of the other alleles show severe phenotypes. flw^{6} is 99% male lethal, and flw^{1}/flw^{6} shows a complete absence of indirect flight muscles. flw^{2} affects jumping ability, and 95% of the flies have an abnormal wing position. In contrast, the flightless *D. hydei* can jump, and only limited numbers of the flies have a mildly abnormal wing position.

The allele $ifm(2)RU2^{1}$ was characterized in a study screening for indirect flight muscle mutations on the second chromosome (Nongthomba and

Ramachandra, 1999). Flies homozygous for *ifm(2)RU2*^{1} show incompletely penetrant raised wings, thinning dorsal longitudinal muscles, and disorganized dorsoventral muscles. Some of the flies with normal wings show weak flight ability. However, while the dorsal longitudinal muscles of these *D. melanogaster* show birefringence through most of the muscle, the DLMs of the flightless *D. hydei* have reduced birefringence. The concentration of the muscle damage to the dorsoventral muscles suggests that this allele is not homologous. No subsequent work has been performed in order to determine more characteristics of *ifm(2)RU2*.

Another *D. melanogaster* gene that is a possible homologue is *Actn*. This gene, localized on the X chromosome (Homyk and Sheppard, 1977), encodes α-actinin, a relative of the structure stabilizing proteins spectrin and dystrophin – responsible for muscular dystrophy (Fyrberg *et al.*, 1990). The authors found that mutations in this gene yield invariably lethal or flightless phenotypes, although the phenotypes only affect muscles. α-actinin is a 100 kDa protein that functions as an antiparallel homodimer that is approximately 7 nm by 48 nm. It consists of three domains: the N-terminal actin binding domain, 4 central repeats that promote dimerization, and a C-terminal EF-hand-like domain that may facilitate Ca^{++} binding. α-actinin binds stably to actin in muscles, where it is not sensitive to Ca^{++}; but it binds dynamically to actin microfilaments in the cytoplasm, at which point, it is sensitive to Ca^{++}. α-actinin in muscle is localized in the Z-bands, where it binds to the actin filaments. In *Drosophila*, mutant muscles show disrupted Z-bands and faulty attachment to epithelial tendon cells, the cells that attach muscle

to the body wall (Fyrberg *et al.*, 1990). *Actn*^{1} is flightless and not temperature dependent (Homyk and Sheppard, 1977). *Actn*^{4} is flightless and pupal lethal when raised at 29° C. *Actn*^{1}/*Actn*^{4} shows a flightless phenotype at 22° but is rescued when raised at 29° C (Homyk *et al.* 1980). *Actn*^{1} shows normal levels of α-actinin, while *Actn*^{4} has extremely reduced amounts of the protein. *Actn*^{1} is a point mutation that changes glycine to serine in the actin binding domain. *Actn*^{4} is a point mutation in an intron, which eliminates the 3′ portion of the mRNA. The protein is not translated at high levels. Despite the lack of α-actinin, the indirect flight muscles of *Actn*^{4} flies appear relatively normal, despite being paralyzed. This suggests that α-actinin does not function to create the muscle structure but rather to stabilize it (Roulier *et al.*, 1992). Further support for this hypothesis is that lengthening the dimer by splicing an additional central repeat from α-spectrin does not interfere with wild type function. Therefore, the length of α-actinin does not set any critical distance in the muscle, which suggests that the crosslinking between actin filaments is formed by other proteins (Dubreil and Wang, 2000).

Defective *Actn* could explain the phenotype seen in the flightless *D. hydei*. Sufficient loss of α-actinin would inhibit muscle contraction, so the flies would be unable to vibrate their wings. The indirect flight muscle would be abnormal in organization and thus show reduced optical birefringence. The defective muscle phenotype would be much more apparent in the thorax than in the legs. *Actn* is located on the X chromosome in *D. melanogaster*, and if it were still on the X chromosome in *D. hydei*, it could explain the populations with lower male

viability. Most compelling, of course, is the temperature-based rescue of *Actn*^{1}/*Actn*^{4} heterozygotes. Homyk *et al.* (1980) reported this behavior in a large screen for behavioral mutants, and unfortunately, as far as I can tell, they did not follow up on it. The result is even more surprising considering that *Actn*^{4} confers a pupal lethal phenotype at high temperature. *Actn*^{4} homozygotes have low levels of α-actinin, and *Actn*^{1} will restore some amount of albeit mutant protein, which must explain the complementation of the lethal phenotype. Temperature increases the rate of muscular contraction, so perhaps temperature makes the forming muscles in the pupa more active, but because the muscles are weak due to lack of α-actinin stabilization, activity leads to degeneration and death. As mentioned previously, heat shock prevents splicing (Pauli *et al.*, 1992), and the *Actn*^{4} protein is absent due to a splicing error (Roulier *et al.*, 1992). Perhaps the mutant *Actn*^{4} mRNA is preserved in the cell and then translated without splicing, leading to a harmful protein. But why would the combination of an essentially null mutation and a poorly functioning mutation yield a flying fly at high temperature? Hypothetically, an Hsp might completely block the actin binding site by binding to the mutant serine in the *Actn*^{1} α-actinin, and as an Hsp is on the same order of size as an α-actinin monomer, this would further prevent the α-actinin from properly binding to the actin it must stabilize. Heat shock would call the Hsp to support other proteins, freeing the α-actinin. However, this would not explain why the temperature-based rescue does not occur in the homozygote. Another conjecture is that the polarity of the serine causes the α-actinin to form hydrogen

bonds with other molecules, causing an aggregation. Hsp70 specializes in disrupting protein-protein interactions, and the increased level of Hsp70 expressed after heat shock could dissociate these α-actinin aggregates. The *Actn*^{1} homozygote might have too much α-actinin for Hsp70 to deaggregate, but the heterozygote might have just enough α-actinin to do its job. Of course, these examples are speculative, and many other interactions, both Hsp-moderated and not, could be the basis of the phenotypic change. Geldanamycin is a Hsp inhibitor used in heat shock studies because it simulates the action of heat shock (Rutherford and Lindquist, 1998). If both heat shock and geldanamycin treatment were shown to rescue the flightless phenotype, I would conclude that the temperature-based rescue was mediated by the heat shock protein response and not some other developmental effect of elevated temperature. Methods for testing the α-actinin of flightless *D. hydei* include immunofluorescent localization in dissected muscles and sequencing the *Actn* gene.

Searching for a homologous gene on FlyBase had its difficulties, but some of these difficulties were meaningful. For example, FlyBase does not have a phenotypic class to separate flightless flies into the subclasses of flies that vibrate their wings but cannot fly and flies that cannot vibrate their wings. In the present study, this is a very important distinction, but all scientific observation is shaped by what one is expecting. Most of the gene descriptions do not specify the diagnostic features I would need in order to compare that gene to the flightless flies, because each study is looking at the mutations for a different reason.

Furthermore, due to translocations, a homologous gene might not be in the same locus or even on the same chromosome in the two species.

Surveying the breadth of mutations that can cause flight-inhibiting defects in *Drosophila* indirect flight muscle provides a to-do list of more observations to make on the flightless *D. hydei*. Sectioning and TEM would be the most important, as this would reveal the nature of the disorganization of the muscle and determine whether or not the muscle is properly attached to the wall. Separation of the component proteins and gel electrophoresis would allow analysis of the quantity of proteins of various molecular weights. Many of the flightless *D. melanogaster* mutations showed missing indirect flight muscle proteins. The molecular weight of a missing or reduced protein could suggest whether it was a primary structural protein or one of the smaller, but still important, proteins.

To some extent, it is futile to speculate about the mechanism of the heat shock rescue until the heat shock rescue has actually been effected, especially since heat shock can have such a plethora of effects. To complicate matters further, the presence of impaired indirect flight muscles does not rule out the presence of other flight-inhibiting factors, which could be pleiotropic or completely separate. The behavior observed can most likely be fully explained by defective indirect flight muscles; nonetheless, the source of the flightless *D. hydei* is not fully known, and because the strain was not generated in a lab, it could carry mutations in multiple genes. The reciprocal crosses between a dark eyed flying fly and a light eyed flightless fly produced F_2 with light eyed flying flies

and dark eyed flightless flies, which suggests that there are at least two independent mutations in the flightless strain. More information about the defects in the muscle would make it considerably easier to determine the mechanism of the heat shock rescue. If the muscles are not properly attached to the cuticle, this could be due to insufficient quantities of a protein necessary for creating the attachment, and heat shock could upregulate the protein and allow the muscle to attach properly. If the muscle is attached properly but degenerated for another reason, the same mechanism could be true for a protein that, for example, organizes the Z-bands but does not function in attaching the muscle to the body wall. This mechanism could also upregulate a major flight protein if one were deficient.

Conclusions

Humans have long been fascinated by flight, as evidenced by often envious admiration of bird flight and never-ending attempts at human-powered flight. We are surrounded by animals that fly, but the effortless gliding of a bird on the wing belies the complexity that underlies flight. This is also why maintenance of aircraft is far more stringent than maintenance of land vehicles. A stalled engine is far more disastrous in the air than on the ground, and it requires more sophisticated technology to generate lift than to cause wheels to turn. Likewise, the insect flight system has many components that can be defective. I focused on adequate wing area and proper flight muscle. I found that compared to wild type, flightless *D. hydei* have slightly lower average wing loading, but

slightly higher wing loading per mass. It is difficult to state conclusively whether or not this difference is sufficient to inhibit flight. Polarized light microscopy showed that wild type flies show high birefringence of the thoracic muscle, but the corresponding muscle of flightless flies shows much reduced birefringence. This indicates that the indirect flight muscle of flightless flies is disorganized and therefore dysfunctional. Abnormal indirect flight muscle is sufficient to prevent flight in an insect, so this is likely responsible for the flightless phenotype. The *Actn* gene of *D. melanogaster* may be homologous to the mutation in flightless *D. hydei*. More data should be gathered regarding the muscle; I did not successfully rescue the mutation with heat shock, so it would be vital to resume this line of investigation. Furthermore, histology would reveal whether or not the muscles are well-attached to the cuticle, TEM would allow analysis of the ultrastructural integrity of the muscle, and gel electrophoresis could determine whether important proteins are missing. If the evidence continues to support a defect in *Actn*, immunofluorescent localization of α-actinin would indicate whether it is present in the proper amount and location.

I began this project out of curiosity about how heat shock could possibly rescue a mutation, and my curiosity thus far remains unsatisfied, though at least I now have enough information to speculate as to how heat shock could interact with a particular defect and at least momentarily correct it. I found that the indirect flight muscles of these flies appear abnormal, but the relationship between muscle function and temperature remains to be determined. It still seems

unexpected that a system best known for revealing hidden mutations could instead correct one. Elucidation of this process will either reveal a new nuance of the heat shock system or show an unusual type of defect. I have played *hydei*-and-go-seek with hypotheses, so my project has exemplified the nature of science. Every organism is far more complicated than we can imagine, and every fact we learn raises more questions. Scientific inquiry, not unlike a fresh vial of fruit flies, often yields far more than we expected to reap. But until more avenues are pursued and the mechanism of heat rescue is ascertained, the flightless *D. hydei* are doomed to remain earthbound frog bait.

LITERATURE CITED

Andersen D. H., C. Pertoldi, V. Scali, and V. Loeschcke. 2005. Heat stress and age induced maternal effects on wing size and shape in parthenogenetic *Drosophila mercatorum*. J. Evol. Biol. 18:884-892.

Atchison J. 2009. *D. hydei sturtevant*. Available at http://www.livefoodcultures.com. Accessed 5 May 2010.

Azevedo R. B. R., A. C. James, J. McCabe, and L. Partridge. 1998. Latitudinal variation of wing:thorax size ratio and wing-aspect ratio in *Drosophila melanogaster*. Evolution 52:1353-1362.

Barnes P. T., L. Sullivan, and A. Villella. 1998. Wing-beat frequency mutants and courtship behavior in *Drosophila melanogaster* males. Behav. Genet. 28:137-151.

Barron A. B. 2000. Anaesthetising *Drosophila* for behavioural studies. J. Insect Physiol. 46:439-442.

Basso da Silva L., and V. L. S. Valente. 2001. Body size and mating success in *Drosophila willistoni* are uncorrelated under laboratory conditions. J. Genet. 80:77-81.

Bennet-Clark H. C., and A. W. Ewing. 1969. Pulse interval as a critical parameter in the courtship song of *Drosophila melanogaster*. Anim. Behav. 17:755-759.

Bodenstein D. 1950. The postembryonic development of *Drosophila*. Pp. 275-367 *in* M. Demerec, ed. Biology of *Drosophila*. John Wiley and Sons, New York.

Boettiger E. G., and E. Furshpan. 1952. The mechanics of flight movements in Diptera. Biol. Bull. 102:200-211.

Bozler E., and C. L. Cottrell. 1937. The birefringence of muscle and its variation during contraction. J. Cell. Comp. Physiol. 10:165-182.

Carter A. J. R., T. M. Weier, and D. Houle. 2009. The effect of inbreeding on fluctuating asymmetry of wing veins in two laboratory strains of *Drosophila melanogaster*. Heredity 102:563-572.

Chow L. H., D. R. Boughner, J. Dunmore-Buyze, H. Finlay, and J. G. Pickering. 2001. Enhanced detection of cardiac myocyte damage by polarized light microscopy: use in a model of coxsackievirus B3-induced myocarditis. Cardiovasc. Pathol. 10:83-86.

Clyne J. D., and G. Miesenböck. 2008. Sex-specific control and tuning of the pattern generator for courtship song in *Drosophila*. Cell 133:354-363.

Cooper K. 1950. Normal spermatogenesis in *Drosophila*. Pp. 1-61 *in* M. Demerec, ed. Biology of *Drosophila*. John Wiley and Sons, New York.

Crabtree B., and E. A. Newsholme. 1975. Comparative aspects of fuel utilization and metabolism by muscle. Pp. 405-500 *in* P. N. R. Usherwood, ed. Insect Muscle. Academic Press, New York.

Darwin C. 1859. On the origin of species. John Murray, London.

de Moed G. H., G. de Jong, and W. Scharloo. 1997. The phenotypic plasticity of wing size in *Drosophila melanogaster*: the cellular basis of its genetic variation. Heredity 79:260.

Dickinson M. H., and M. S. Tu. 1997. The function of Dipteran flight muscle. Comp. Biochem. Physiol. A 116:223-238.

Dillon M. E., and M. R. Frazier. 2006. *Drosophila melanogaster* locomotion in cold thin air. J. Exp. Biol. 209:364-371.

Dragomir N. M., X. M. Goh, C. L. Curl, L. M. D. Delbridge, and A. Roberts. 2007. Quantitative polarized phase microscopy for birefringence imaging. Opt. Express 15:17690-17698.

Drosophila 12 Genomes Consortium. 2007. Evolution of genes and genomes on the *Drosophila* phylogeny. Nature 450:203-218.

Dubreuil R. R., and P. Wang. 2000. Genetic analysis of the requirements for α-actinin function. J. Muscle Res. Cell. Motil. 21:705-713.

Dudley R. 2000. The biomechanics of insect flight: form, function, evolution. Princeton Univ. Press, Princeton, NJ.

Elder H. Y. 1975. Muscle structure. Pp. 1-74 *in* P. N. R. Usherwood, ed. Insect Muscle. Academic Press, New York.

Ewing A. W. 1964. The influence of wing area on the courtship behaviour of *Drosophila melanogaster*. Anim. Behav. 12:316-320.

—— 1977. The neuromuscular basis of courtship song in *Drosophila*: the role of the indirect flight muscles. J. Comp. Physiol. A 199:249-265.

—— 1979. The neuromuscular basis of courtship song in *Drosophila*: the role of the direct and axillary wing muscles. J. Comp. Physiol. A 130:87-93.

Ewing A. W., and J. A. Miyan. 1986. Sexual selection, sexual isolation and the evolution of song in the *Drosophila repleta* group of species. Anim. Behav. 34:421-429.

Ferris G. F. 1950. External morphology of the adult. Pp. 368-419 *in* M. Demerec, ed. Biology of *Drosophila*. John Wiley and Sons, New York.

Finlayson L. H. 1975. Development and degeneration. Pp. 75-149 *in* P. N. R. Usherwood, ed. Insect Muscle. Academic Press, New York.

Frazier M. R., J. F. Harrison, S. D. Kirkton, and S. P. Roberts. 2008. Cold rearing improves cold-flight performance in *Drosophila* via changes in wing morphology. J. Exp. Biol. 211:2116-2122.

Fyrberg E., M. Kelly, E. Ball, C. Fyrberg, and M. C. Reedy. 1990. Molecular genetics of *Drosophila* alpha-actinin: mutant alleles disrupt Z disc integrity and muscle insertions. J. Cell Biol. 110:1999-2011.

Gilchrist G. W., and R. B. Huey. 2004. Plastic and genetic variation in wing loading as a function of temperature within and among parallel clines in *Drosophila subobscura*. Integr. Comp. Biol. 44:461-470.

Heide G., and K. G. Gotz. 1996. Optomotor control of course and altitude in *Drosophila melanogaster* is correlated with distinct activities of at least three pairs of flight steering muscles. J. Exp. Biol. 199:1711-1726.

Heisenberg M. 1972. Comparative behavioral studies on two visual mutants of *Drosophila*. J. Comp. Physiol. A 80:119-136.

Hoikkala A., J. Aspi, and L. Suvanto. 1998. Male courtship song frequency as an indicator of male genetic quality in an insect species, *Drosophila montana*. Proc. R. Soc. Lond. B 265:503-508.

Homyk T., and D. E. Sheppard. 1977. Behavioral mutants of *Drosophila melanogaster*. I. isolation and mapping of mutations which decrease flight ability. Genetics 87:95-104.

Homyk T., J. Szidonya, and D. T. Suzuki. 1980. Behavioral mutants of *Drosophila melanogaster* . Mol. Gen. Genet. 177.

Hooper S. L., K. H. Hobbs, and J. B. Thuma. 2008. Invertebrate muscles: thin and thick filament structure; molecular basis of contraction and its regulation, catch and asynchronous muscle. Prog. Neurobiol. 86:72-127.

Kimura K., T. Shimozawa, and T. Tanimura. 1986. Muscle degeneration in the posteclosional development of a *Drosophila* mutant, abnormal proboscis extension reflex C (*aperC*). Dev. Biol. 117:194-203.

Koana T., and Y. Hotta. 1978. Isolation and characterization of flightless mutants in *Drosophila melanogaster* . J. Embryol. Exp. Morphol. 45:123-143.

Li Y., T. Zhang, S. J. Schwartz, and D. Sun. 2009. New developments in Hsp90 inhibitors as anti-cancer therapeutics: mechanisms, clinical perspective and more potential. Drug Resistance Updates 12:17-27.

Lindquist S. 1980. Varying patterns of protein synthesis in *Drosophila* during heat shock: implications for regulation. Dev. Biol. 77:463-479.

Lindquist S., and E. A. Craig. 1988. The heat-shock proteins. Annu. Rev. Genet.

22:631-677.

Lutz F. E. 1911. Experiments with *Drosophila ampelophila* concerning evolution. The Carnegie Institution of Washington, Washington, DC.

Markow T. A. 1985. A comparative investigation of the mating system of *Drosophila hydei*. Anim. Behav. 33:775-781.

Matsuo Y. 2000. Evolutionary change of codon usage for the histone gene family in *Drosophila melanogaster* and *Drosophila hydei*. Mol. Phylogenet. Evol. 15:283-291.

Maughan D. W., and J. O. Vigoreaux. 1999. An integrated view of insect flight muscle: genes, motor molecules, and motion. News Physiol. Sci. 14:87-92.

Nongthomba U., and N. B. Ramachandra. 1999. A direct screen identifies new flight muscle mutants on the *Drosophila* second chromosome. Genetics 153:261-274.

Partridge L., A. W. Ewing, and A. Chandler. 1987. Male size and mating success in *Drosophila melanogaster*: the roles of male and female behaviour. Anim. Behav. 35:555-562.

Patton R. L. 1963. Muscles and nerves. Pp. 117-144 *in* Introductory Insect Physiology. W. B. Saunders Company, Philadelphia.

Pauli D., A. Arrigo, and A. Tissières. 1992. Heat shock response in *Drosophila*. Cell. Mol. Life Sci. 48:623-629.

Picken L. 1960. The organization of cells and other organisms. Oxford Univ. Press, London.

Powell J. R. 1997. Progress and prospects in evolutionary biology: the *Drosophila* model. Oxford Univ. Press, New York.

Rennie J. 1831. Introduction. Pp. iii-lx *in* G. Montagu, ed. Ornithological Dictionary of British Birds. Hurst, Chance, and Co., London.

Ritchie M. G., R. M. Townhill, and A. Hoikkala. 1998. Female preference for fly song: playback experiments confirm the targets of sexual selection. Anim. Behav. 56:713-717.

Roulier E. M., C. Fyrberg, and E. Fyrberg. 1992. Perturbations of *Drosophila* α-actinin cause muscle paralysis, weakness, and atrophy but do not confer obvious nonmuscle phenotypes. J. Cell. Biol. 116:911-922.

Routtu J., D. Mazzi, K. Van der Linde, P. Mirol, R. K. Butlin, and A. Hoikkala. 2007. The extent of variation in male song, wing and genital characters among allopatric *Drosophila montana* populations. J. Evol. Biol. 20:1591-1601.

Rutherford S. L., and S. Lindquist. 1998. Hsp90 as a capacitor for morphological evolution. Nature 396:336-342.

Saarikettu M., J. O. Liimatainen, and A. Hoikkala. 2005. Intraspecific variation in mating behaviour does not cause sexual isolation between *Drosophila virilis* strains. Anim. Behav. 70:417-426.

Samarra F. I. P., K. Klappert, H. Brumm, and P. J. O. Miller. 2009. Background noise constrains communication: acoustic masking of courtship song in the fruit fly *Drosophila montana*. Behaviour 146:1635-1648.

Sene F., R. Tidon-Sklorz, and M. H. Manfrin. 1998. Influence of the wing size of *Drosophila mercatorum* males on the courtship sound. Rev. Bras. Biol. 58:175-180.

Spencer W. P. 1949. Gene homologies and the mutants of *Drosophila hydei*. Pp. 23-44 *in* G. L. Jepsen, E. Mayr and G. G. Simpson, eds. Genetics, Paleontology, and Evolution. Princeton Univ. Press, Princeton, NJ.

Sturtevant A. H. 1915. Experiments on sex recognition and the problem of sexual selection in *Drosophila*. J. Anim. Behav. 5:351-366.

—— 1921. The North American species of *Drosophila*. The Carnegie Institution of Washington, Washington, DC.

Swaddle J. P. 2003. Fluctuating asymmetry, animal behavior, and evolution. Pp. 169-205 *in* P. J. B. Slater, J. S. Rosenblatt, C. T. Snowdon and T. J. Roper, eds. Advances in the study of behavior. Academic Press, San Diego, CA.

Tauber E., and D. F. Eberl. 2002. The effect of male competition on the courtship song of *Drosophila melanogaster*. J. Insect Behav. 15:109-120.

—— 2003. Acoustic communication in *Drosophila*. Behav. Processes 64:197-210.

Tregear R. T. 1975. Biophysics of fibrillar flight muscle. Pp. 357-403 *in* P. N. R. Usherwood, ed. Insect Muscle. Academic Press, New York.

Vuoristo M., E. Isoherranen, and A. Hoikkala. 1996. Female wing spreading as acceptance signal in the *Drosophila virilis* group of species. J. Insect Behav. 9:505-516.

Zuk M., J. T. Rotenberry, and R. M. Tinghitella. 2006. Silent night: adaptive disappearance of a sexual signal in a parasitized population of field crickets. Biology Letters 2:521-524.