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Role of the Drosophila Melanogaster Indirect Flight Muscles in Flight and Male Courtship Song: Studies on Flightin and Mydson Light Chain - 2

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ROLE OF THE *DROSOPHILA MELANOGASTER* INDIRECT FLIGHT MUSCLES IN FLIGHT AND MALE COURTSHIP SONG: STUDIES ON FLIGHTIN AND MYOSIN LIGHT CHAIN-2

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ABSTRACT

Complex behaviors using wings have facilitated the insect evolutionary success and diversification. The *Drosophila* indirect flight muscles (IFM) have evolved a highly ordered myofilament lattice structure and uses oscillatory contractions by pronounced stretch activation mechanism to drive the wings for high powered flight subject to natural selection. Moreover, the IFM is also utilized during small amplitude wing vibrations for species-specific male courtship song (sine and pulse), an important *Drosophila* mating behavior subject to sexual selection. Unlike flight, the contractile mechanism and contribution of any muscle gene in courtship song is not known. To gain insight into how separate selection regimes are manifested at the molecular level, we investigated the effect on flight and mating behaviors of mutations in two contractile proteins essential for IFM functions: an IFM-specific protein, flightin (FLN), known to be essential for structural and mechanical integrity of the IFM, and a ubiquitous muscle protein, myosin regulatory light chain (MLC2), known to enhance IFM stretch activation.

Comparison of FLN sequences across *Drosophila spp.*, reveal a dual nature with the N-terminal region (63 aa) evolving faster (dN/dS=0.4) than the rest of the protein (dN/dS=0.08). A deletion of the N-terminal region ($fln^{\Delta N62}$) resulted in reduced IFM fiber stiffness, oscillatory work and power output leading to a decreased flight ability (flight score: 2.8±0.1 vs 4.2±0.4 for fln^{+} rescued control) despite a normal wing beat frequency. Interestingly, the FLN N-terminal deletion reduced myofilament lattice spacing and order suggesting that this region is required to improve IFM lattice for enhancing power output and flight performance. Moreover, $fln^{\Delta N62}$ males sing the pulse song abnormally with a longer interpulse interval (IPI, 56±2.5 vs 37±0.7 ms for fln^{+}) and a reduced pulse duty cycle (PDC, 2.6±0.2 vs 7.3±0.2 % for fln^{+}) resulting in a 92% reduction in their courtship success. This suggested that FLN N-terminal region fine-tunes sexually selected song parameters in *D. melanogaster*, possibly explaining its hypervariability under positive selection. That FLN N-terminal region is not essential but required to optimize IFM functions of both flight and song, indicate that FLN could be an evolutionary innovation for IFM-driven behaviors, possibly through its role in lattice improvement.

Mutations of the highly conserved MLC2 [N-terminal 46 aa deletion (Ext), disruption of myosin light chain kinase phosphorylations (Phos), and the two mutations put together (Dual)] are known to impair or abolish flight through severe reductions in acto-myosin contractile kinetics and magnitude of the stretch activation response. Unlike FLN, these MLC2 mutations do not show a pleitropic effect on flight and song. Flight abolished Phos and Dual mutants are capable of singing suggesting that these mutations affect song minimally compared to flight. Moreover, unlike FLN, none of these mutations affect interpulse interval, the most critical sexually selected song parameter in *Drosophila*. Also, in contrary to the known additive effects of Ext and Phos in the Dual mutant on flight wing beat frequency, a subtractive effect on sine song frequency is found in this study. That mutations in MLC2 are manifested differently for song and flight suggest that stretch activation plays a minimal or no role in song production.

The results in this study suggest that the conserved regions of FLN and MLC2 are essential to support underlying IFM contractile structure and function necessary for flight, whereas the fast evolving FLN N-terminal region optimizes IFM's biological performance in flight and species-specific song possibly under positive selection regime.

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CHAPTER 1 INTRODUCTION

Overview

Structure and form of biological systems give function. Among them, muscle is the primary tool by which living systems act to produce diverse and complex behavioral outputs. The structure of muscle tissue is critical since some muscles have to produce force, and some bear force based on power and control needed for distinct behaviors. Hence, muscle is a highly structured biological material composed of ordered organization of thick and thin filaments composed of contractile proteins, and the connecting filaments that inter-connect them. This arrangement, in turn, follows a hierarchical organization into sarcomeres, myofibrils, fibers, and fiber bundles. In almost every muscle tissue system, the contractile proteins actin and myosin form the majority of the thin and thick filaments, respectively, which generate the force and power required for any behavior or movement. Even though the basic actin and myosin contractile functions are conserved throughout various muscle systems, yet, based on the power requirements of various performances, muscle tissues show great variations in acto-myosin contractile kinetics, myofibrillar structural, and fiber mechanical properties, as well as in types of accessory proteins. For example, the *Drosophila* indirect flight muscle (IFM)s have evolved one of the fastest myosin kinetics known, with a very high detachment rate of myosin motor domain from its actin target (cross-bridge detachment rate) compared to mammalian cardiac or skeletal muscle myosins (Figure 1-1) [2]. Also, at the myofibrillar level, there is a great diversity in the regularity of the myofilament

arrangement (lattice). For example, The *Drosophila* adult IFM myofibril shows highly regular myofilament lattice organization indicated by the higher order X-ray diffraction patterns compared to mammalian skeletal (Figure 1-2) or cardiac muscles [4]. At the whole fiber level as well, different muscles show diverse stiffness properties. For example, *Drosophila* IFM shows a higher passive stiffness compared to mammalian skeletal or cardiac muscles (Figure 1-3) [5].

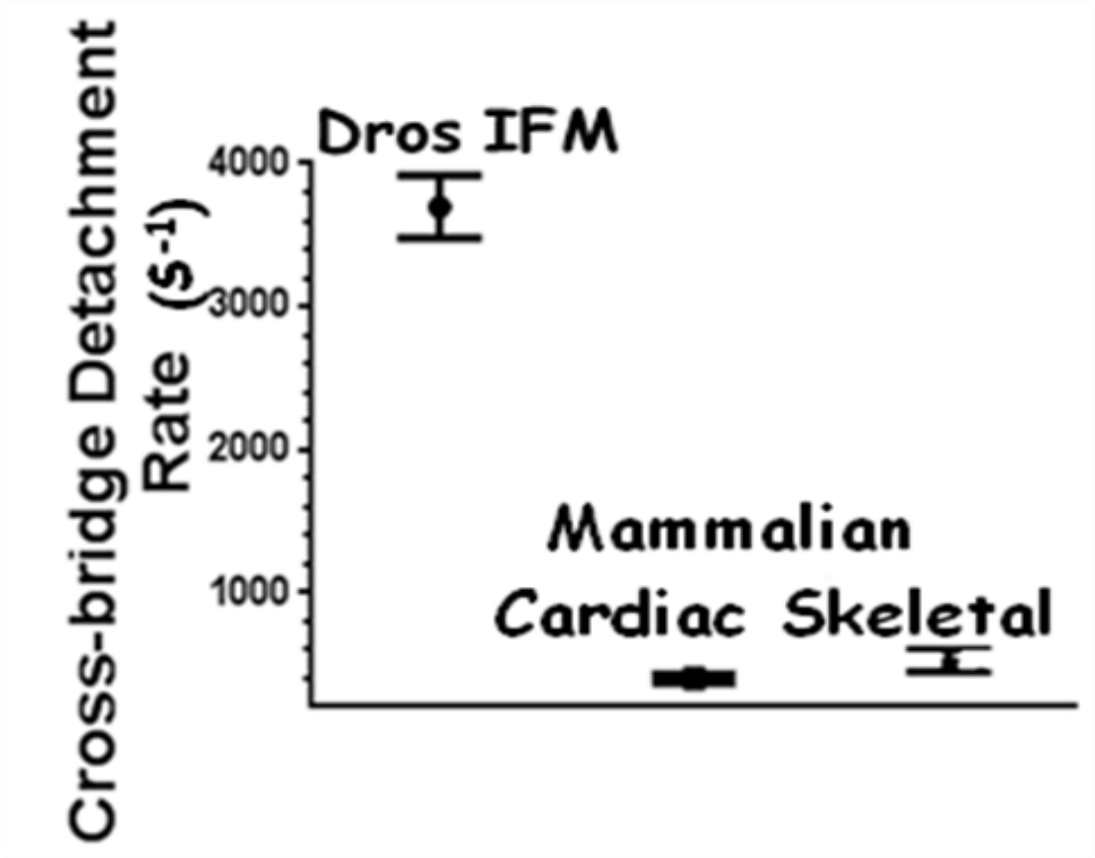

Figure 1-1. Cross-bridge detachment rate (in 1/seconds or s^{-1}) of *Drosophila* IFM, mammalian cardiac and mammalian skeletal muscles. The cross-bridge detachment rate shown here is the forward rate constant (k_{+2}) which characterizes the forward reaction of the work-absorbing actin-myosin-ATP isomerization associated with cross-bridge detachment [2]. *Drosophila* IFM has a much higher cross-bridge detachment rate indicative of a much faster myosin kinetics compared to mammalian cardiac or skeletal muscles. (Redrawn from [1,2])

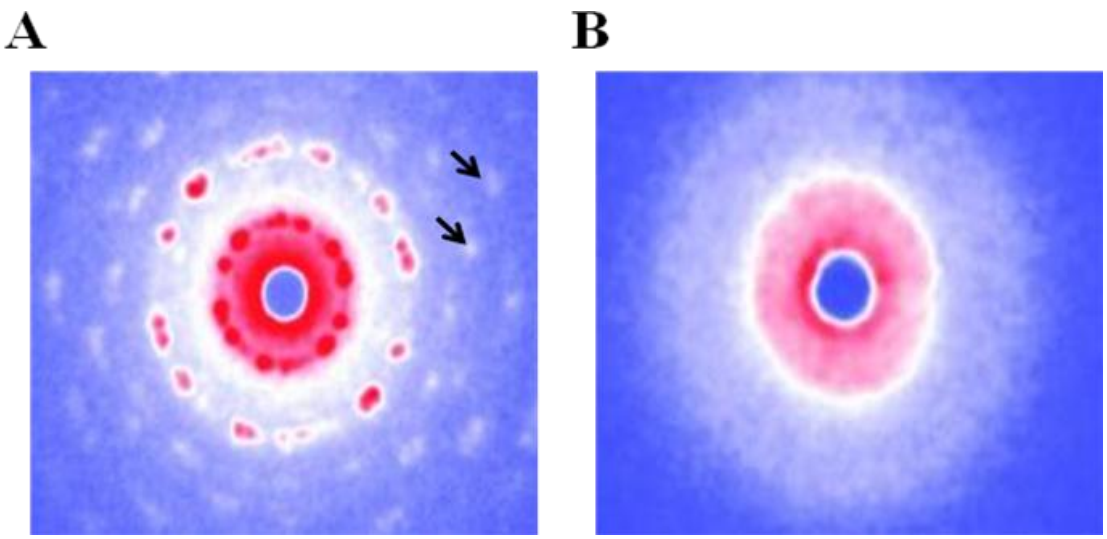

Figure 1-2. End-on X-ray microdiffraction patterns of adult *Drosophila* IFM myofibril (A) and rabbit psoas skeletal muscle myofibril (B). Arrows in (A) are examples of diffraction spots from a single myofibril of *Drosophila* IFM. No regular diffraction pattern indicated by spots could be seen in (B). Clearly, *Drosophila* IFM myofibril has more regular or crystalline myofilament organization than rabbit psoas skeletal muscle indicated by the regular and higher-order diffraction spots. (Redrawn (A) from [3], and (B) taken from [4]).

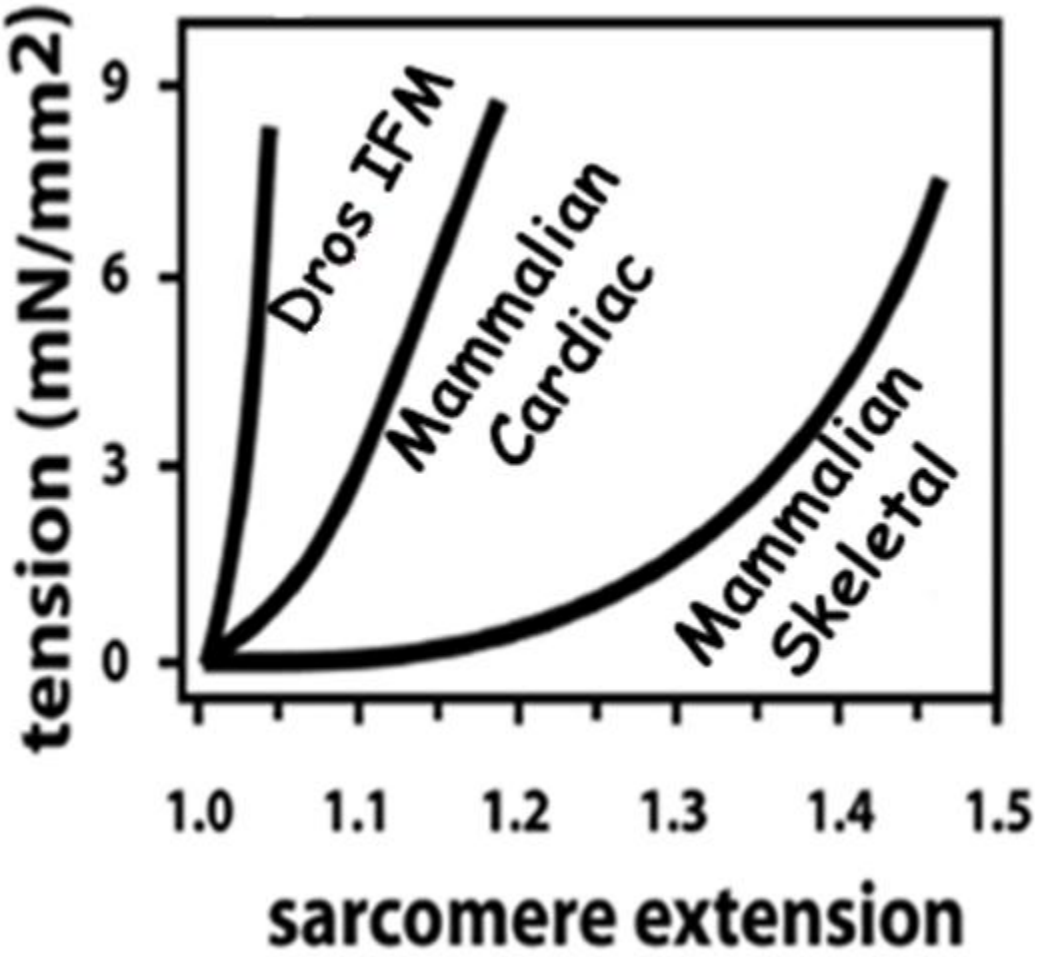

Figure 1-3. Tension (force per muscle fiber cross-sectional area) response (in milliNewton per millimeter square or mN/mm^2) vs sarcomere extension (due to stretch) curve of relaxed *Drosophila* IFM, mammalian cardiac and mammalian skeletal muscle fibers. Passive stiffness (=stress/strain i.e., tension/sarcomere extension) slope of *Drosophila* IFM is higher than that of mammalian cardiac or skeletal muscles. (Redrawn from [5]).

Moreover, accessory proteins that are associated with the myofilaments show great diversity based on functional requirements of the muscle. For example, flightin in *Drosophila* IFM [6,7] and vertebrate cardiac myosin binding protein C [8-10] are required to stabilize and stiffen the corresponding thick filaments. Whereas, an N-terminal extension (46 amino acids) of *Drosophila* IFM thick filament associated myosin regulatory light chain, not found in the vertebrate homolog [11], and is known to be required for IFM stretch activation response [12]. Diversities in accessory protein do not exclude the thin filaments as well. *Drosophila* IFM, in addition to normal tropomyosins, consists of two unique tropomyosins (TmH-33 and TmH-34), that are not present in the vertebrate thin filaments [13-15].

Therefore, to understand the diverse nature of complex behaviors actuated by the muscle systems, it is critical to understand the functions of these diverse accessory thick or thin filament associated proteins and their special properties. Moreover, it is the specialized adaptation of the basic contractile mechanism that creates this great diversity and versatility in muscle function. For example, even though all muscles show some level of stretch activation, the vertebrate cardiac and the insect flight muscles use oscillatory contraction modes in order to sustain power production via pronounced stretch activation mechanism, whereas the vertebrate skeletal muscles do not (reviewed in [5]). One of the fundamental aspects in muscle biology is how the versatility in function of the muscle tissue system emerged and how it evolved to enable the enormous diversity of muscle driven behaviors in nature. In particular, it is of notable interest to understand the

different innovations in muscle genes and their specific contributions to the different structural and mechanical properties of muscles.

Drosophila flight musculature has been a model for muscle research for decades [16], and has been used to elucidate functions of muscle genes from the molecular to the organismal level [17-19], especially with the numerous advances in tools for genetic manipulations [20,21] in this model system. There are two different types of muscles in the *Drosophila* thoracic musculature. The direct flight muscles (DFM), which are involved in the steering control of the wings during flight, are directly attached to the wing hinge and act as active springs rather than as force producing elements [22]. The indirect flight muscles (IFM), the major power producing muscles for flight, are attached to the thoracic exoskeleton, rather than directly to the wing hinge as the DFM. The IFM consist of two sets of muscles aligned transversely to one another. These muscle provide the power through oscillatory contractions driving the high frequency of wing flapping (~ 200 Hz) during flight, by alternately deforming the thoracic cuticle and setting it up as a resonant system with a frequency similar to the flight wing flapping frequency. The resonant cuticular movement drives the movement of the wings indirectly through the DFM that controls the wing kinematics. IFM achieves this oscillatory contraction myogenically since the contractions and nervous impulses are not in concert to each other at a 1:1 ratio (asynchronous mode), unlike the vertebrate skeletal muscle (synchronous mode) [23], where nervous system directly controls each contractile cycle through calcium activation of muscle regulatory units [24]. The oscillatory contraction of the

IFM, as well as vertebrate cardiac muscles, is powered by stretch activation where tension rises gradually, after a delay, in response to a stretch when fully activated by calcium through the neural drive. The stretch activation response is highly pronounced in the IFM compared to vertebrate cardiac or skeletal muscles (reviewed in [5]). Mutations in both *Drosophila* and humans that affect stretch activation response impair flight abilities in flies and cause cardiomyopathies in humans [16,17,19,25]. Hence, the *Drosophila* IFM allows us to test the maximum limits of what muscles can accomplish and to study the underlying factor and constraints that determine the limits of successful performances such as the high energy consuming, power requiring, and aerodynamically costly flight behavior.

Interestingly, like many other insects, *Drosophila* uses their flight muscles not only for flight, but also for other behaviors. The males generate a courtship song, as part of a mating ritual that is under sexual selection pressure and is highly variable across *Drosophila spp. D. melanogaster* males sing by generating low amplitude vibration of one wing to produce a temporally rhythmic pulse song and a sinusoidal humming sine song (reviewed in [26]). The IFM and the DFM are both neurally activated during courtship song [27,28], but their exact contributions to, and the contractile mechanism used for sound production is not known. It is known that wing movements for both pulse and sine songs are of much lower amplitude than flight wing movements [29], suggestive of much lower power requirement for singing. Therefore, the *Drosophila* IFM provides an excellent model system to understand the relative contributions of the various muscle

proteins for power generation and control during states of high (flight) and low (song) muscle mechanical power output. Moreover, numerous mutational genetics studies have been conducted throughout decades to understand the functions of contractile genes using *Drosophila* IFM as a model (reviewed in [19]). These muscle mutants present a goldmine for elucidating the role of the IFM in courtship song and, importantly, for establishing the extent to which genetic and physiological pathways are shared between these two distinct behaviors of flight and courtship.

The goal of this thesis is to study the contributions of specific contractile genes to the *Drosophila* IFM's unique structural and mechanical properties, and ultimately the behavioral outputs of flight and courtship song as a way to 1) understand how different muscle genes could be utilized by this tissue system for different functions, 2) gain insights into the contractile mechanism of courtship song in the flight musculature.

Evolution of Flight and Insect Diversification

More than half of all living species identified to date are insects making Insecta the most diverse class on Earth and arguably among the most successful metazoans in natural history [30,31]. The ability to fly is present in >70% of extant insect species, and is generally considered one of the main driving forces in the evolutionary success of insects [32]. Insects were the first to acquire flight abilities in evolution, about 90-170 million years before the earliest winged vertebrates [33], that possibly led to their enormous diversification. Along with an early start, flight acquisition gave insects the

opportunity for finding new niches and better habitats to colonize, by dispersing more easily. This dispersal possibly also facilitated in finding new food sources which increased their survival fitness. Similarly, powered flight allowed directionality in escape responses to avoid predators quickly, which would not have been so efficiently possible with simple quick jumps or leaps [34]. Therefore, flight increased the probability of survival fitness of the insects with all these factors, and ultimately got fixed in evolution under purifying selection forces. In some cases though, flight facilitated the aerial combats between males fighting for female mates under sexual selection [35]. But overall, flight is one of the major factors for insects success in survival and hence mostly subject to natural selection.

Although the precursors and origin of insects wings and hence flight is still debatable, there are two major alternative hypotheses based on fossil records, structure of current forms and molecular data: (i) wings derived from paranotal outgrowths of the thorax which could have facilitated gliding behavior giving rise to active flight [32], or (ii) wings evolved from aquatic gills from mayfly-like ancestors [36]. It has been proposed that gliding could have evolved many times in different taxa due to its simplistic way to develop a structure that can support flight without investing lot of energy [32]. A potential functionality for developing the gliding behavior is for avoiding predation by using gliding as an escape mechanism. The presence of the gliding behavior in different taxa possibly allows the different organisms to have controlled aerial descent even in the complete absence of wings, giving us clues that this type of behavior could be

precursor for flight [32]. Aerodynamic theory supports this as it postulates some form of gliding as the first step in the evolution of flight [37], with some evidences in wingless insect structures [38]. In contrast, molecular data and genetic signatures support the gill hypothesis that insect wings could have evolved from gill-like appendages, probably from a crustacean aquatic ancestor, having a common inherited gene expression pattern [39]. Water surface skimming and sailing performances by stoneflies support the hypothesis that insect wings evolved from articulated gill plates of aquatic ancestors through an intermediate semi-aquatic stage with small protowings and low muscle power output [40,41]. Whatever the origin of the wings and flight, it gave the insects an opportunity for wide foraging and dispersion. Aside from the acquisition of wings, striking modifications in muscle physiology represent key milestones in the evolutionary history of insects, like the emergence of asynchronous muscles with the ability to contract multiple times with every neural input [23]. It is certain; that flight muscles are precursors of flight and that this tissue system must have gone through an evolutionary trajectory to improve performance during flight among insects facilitating insect diversification, especially since variables affecting flight performance depend on the muscle themselves.

Evolution of Flight Muscles: Classifications

Since some of the variables that affect flight performance should ultimately depend on the flight muscles, the evolution of flight muscles is inter-twined to the

evolution of flight and insect diversification. It can be predicted that a high muscle mass/body mass ratio would be required for high power output that might be required for rapid escapes from predators [42], which would enhance the insect's fitness under natural selection, or for fighting with males in aerial combats for female mates under sexual selection [35]. But, a larger muscle mass could lead to heavier and larger body mass, which ultimately could increase travelling time of oxygen and sugars around the body, and the power required for takeoff will be proportionally greater. Therefore, miniaturization of insects opened to them many niches to diversify. But it posed a problem: too small a body size would force insects to flap their wings faster to stay in the air according to aerodynamic theory, not to mention the more air resistance that they would have to overcome. In this respect, flight muscle evolution, especially that of asynchronous muscles, gave way for insects to perform fast motor action for high frequency operations and higher power outputs economically in order to sustain efficient flight counter-balancing air resistance.

Insect flight muscles can be classified in various ways based on morphological characteristics, features of anatomical attachments, and physiological functions. On both morphological and physiological grounds, insect flight muscle is classified as synchronous, the ancestral type in which the rate of contraction matches the rate of motorneuron firing, or asynchronous, the derived form in which multiple muscle twitches can occur for every neuronal activation (reviewed in [23,43]). Asynchronous muscle is characterized by its distinctly circular myofibrils of large diameter (2-5 μm vs 0.05-0.1

μm in synchronous), and also by the scarcity of sarcoplasmic reticulum (SR). This is because the asynchronous muscles are mostly myogenic using stretch activation mechanism, and hence calcium does not have to diffuse as often to the myofibrils to activate and do not have to be taken back into the SR quickly during relaxation. Therefore, given less need for repeated diffusion of calcium to and from myoplasm to SR, a large and extensive SR network is not required for this type of muscles. This is relevant, in particular, since the muscle action potentials release calcium from the SR to a priming level in the myofibrils sufficient enough for stretch activation to take over. This leads the asynchronous muscles to contract in an oscillatory manner if they are connected to an appropriate resonant load like the wings or thorax of the insects. The absence of an extensive SR network allows the myofibrils to be easily dissociated, a feature that lead to the term 'fibrillar' being used to describe these muscles [44].

Asynchronous muscles are known to exist only in insects [43]. Using the above morphological criteria, Cullen [45] and Smith [46] used electron microscopy to examine the distribution of asynchronous muscle in insect orders. These studies indicated that asynchronous flight muscles represent a derived character, being derived possibly from synchronous muscle types. Based on Cullen's work, fibrillar asynchronous muscles have evolved 9 or 10 times overall with possible multiple independent origins [45,47], yet a remarkable innovation.

On the basis of anatomical attachments, insect flight muscles can be classified into direct and indirect flight muscles (Figure 1-4). The direct flight muscles (DFM)

insert directly on the base of a wing or on cuticular patches in the wing articulation that are in turn attached to the wings. The DFM lie ventral to the wings, contraction of which produces ventral movement of the wings and therefore are wing depressors. The indirect flight muscles (IFM) are comprised of the dorsal longitudinal muscles (DLM) that are wing depressors, and the dorsal ventral muscles (DVM) that are the wing elevators. The IFM induce wing movements by changing the position and shape of the tergum, the dorsal plate of the thorax [47,48].

Flight is a metabolically expensive, yet a voluntary controlled behavior which not only requires high mechanical power output from the flight muscles [49], but also demands control maneuverability. Another classification that could be made based on physiological output is power and control muscles. Power muscles can generally be distinguished from other muscles in the thorax like the control muscles from their pinkish-brown coloration which mostly comes from the high concentration of mitochondria [23]. This high concentration of mitochondria is required to support the high metabolic rates of the flight power muscles, since metabolism of flight muscles is mostly aerobic [50]. In addition to providing the power for driving the oscillation of the wings, insect flight systems include control muscles, contraction of which continuously adjusts wing stroke amplitude, stroke frequency, angle of attack for stable directed flight, and for flight turning or yaw movements [51,52]. For example, control muscles in locusts have been shown to control the twisting of the edges of the wings during upward and downward strokes [53].

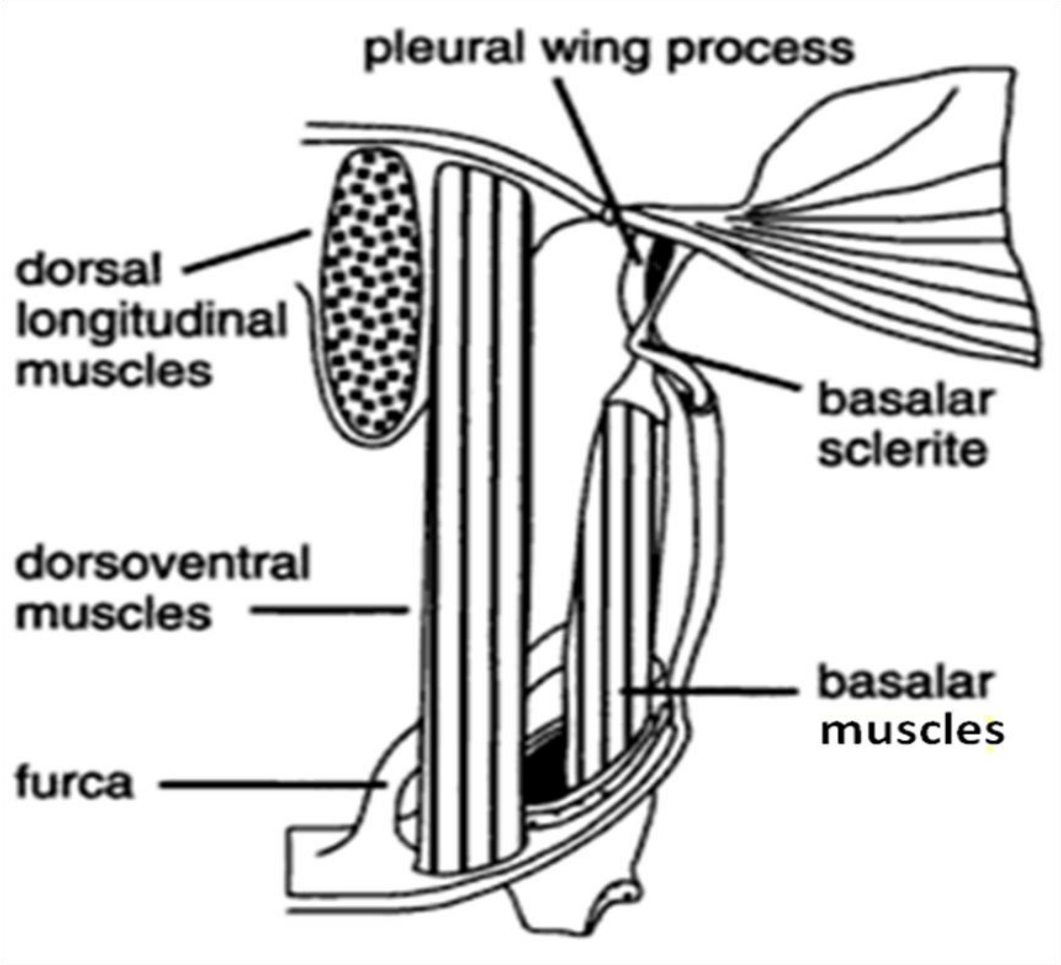

Figure 1-4. Direct and indirect flight muscles in a cross-section of a half-thorax (modified from [48]). The dorsal longitudinal and the dorsal ventral muscle constitute the indirect flight muscles, and the basalar muscles are part of the direct flight muscles.

Drosophila Adult Flight Musculature

There are two major types of adult musculature in *Drosophila*: the majority is made up of tubular muscles which include the leg, jump and the direct flight muscles [16], and the fibrillar muscles which are asynchronous and indirect and provide the power required for flight (Figure 1-5).

Tubular Muscles

The nomenclature of these muscles comes from their distinctive structural characteristic of rectangular myofibrils that are radially oriented surrounding a centrally located nucleus. The other critical features of this muscle are few mitochondria and abundant SR. The tergal depressor of trochanter (TDT), or jump muscle, is the largest in this group (Figure 1-5). This muscle gets activated by the giant fiber (GF) neural pathway through the tergotrochanteral motoroneuron (TTMn) (reviewed in [54]). The TDT initiates the escape response by powering the jump that starts the flight resonant system.

Fibrillar Muscles

Adult *Drosophila* fibrillar muscles include the major power generating indirect flight muscle (IFM). They can be divided into 12 fibers oriented dorsal longitudinally (DLMs) and 12 large and 2 small fibers oriented dorsal ventrally (DVMs). The thousands of myofibrils that constitute the fibers are circular with a ~1.8 μm diameter. In order to supply the high demand of metabolic energy for flight power, these flight muscle fibers are densely packed with mitochondria which have direct access to the myofibrils. The sarcomeres of the *Drosophila* fibrillar muscles are about 3.0-3.6 μm long with a very

short I-band (Figure 1-6) compared to tubular muscle sarcomeres. Moreover, the thick filaments occupy about 90% of the entire length of the sarcomere which allows for very little shortening. Also, the Z-bands of the fibrillar muscle sarcomeres are wider than tubular muscle which is indicative of their unique architecture [55,56]. The uniqueness in architecture can be seen in the myofibrillar cross sections as well, where a thick filament of the flight muscles is surrounded by six thin filaments with a thin to thick filament ratio of 3:1 [57,58] (Figure 1-7) as opposed to 2:1 in tubular muscle. Electron microscopic and live fly X-ray diffraction studies indicated that the flight muscle lattice arrangement of thick and thin filaments are highly regular and ordered compared to vertebrate skeletal muscle or other tubular flight muscles, possibly indicating an evolutionary advantage of having ordered lattice for enhancing flight power [4]. As shown in Figure 1-7, the IFM myofilament arrangement is a double hexagonal array of hollow thick filaments, each of which is surrounded by six thin filaments. This lattice arrangement has a highly regular spacing between consecutive thick filaments or, inter-planar distance ($d_{1,0}$), from which inter-thick filament spacing (center-to-center distance between thick filaments, Figure 4 oblique arrows) could be calculated. The flight muscle myofilament lattice spacing is generally measured using X-ray diffraction of live flies [59].

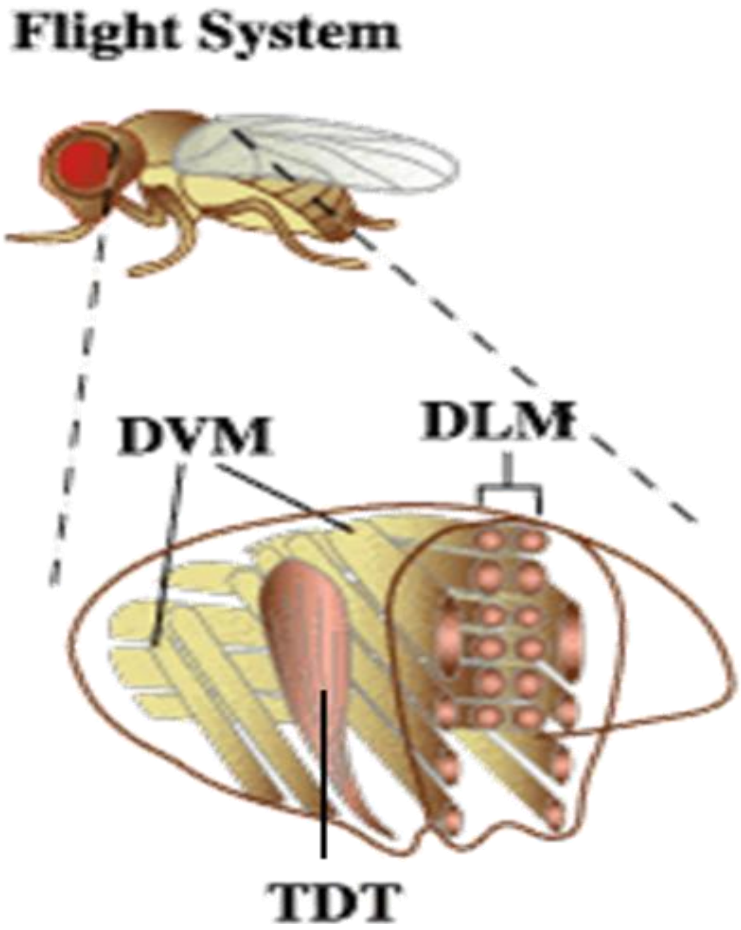

Figure 1-5. Adult *Drosophila* thoracic muscle system. There are 12 large and 2 (not shown here) small dorsal ventral muscle (DVM) fibers which are located towards the outside the thorax on both sides. The dorsal longitudinal muscle (DLM) fibers flank the mid-sagittal plane of the thorax. The tergal depressor of trochanter (TDT), interchangeably called TTM or "jump" muscle is also shown. Image taken from http://www.bumc.bu.edu/phys-biophys/people/faculty/moore/moore-laboratory/research/drosophilia/

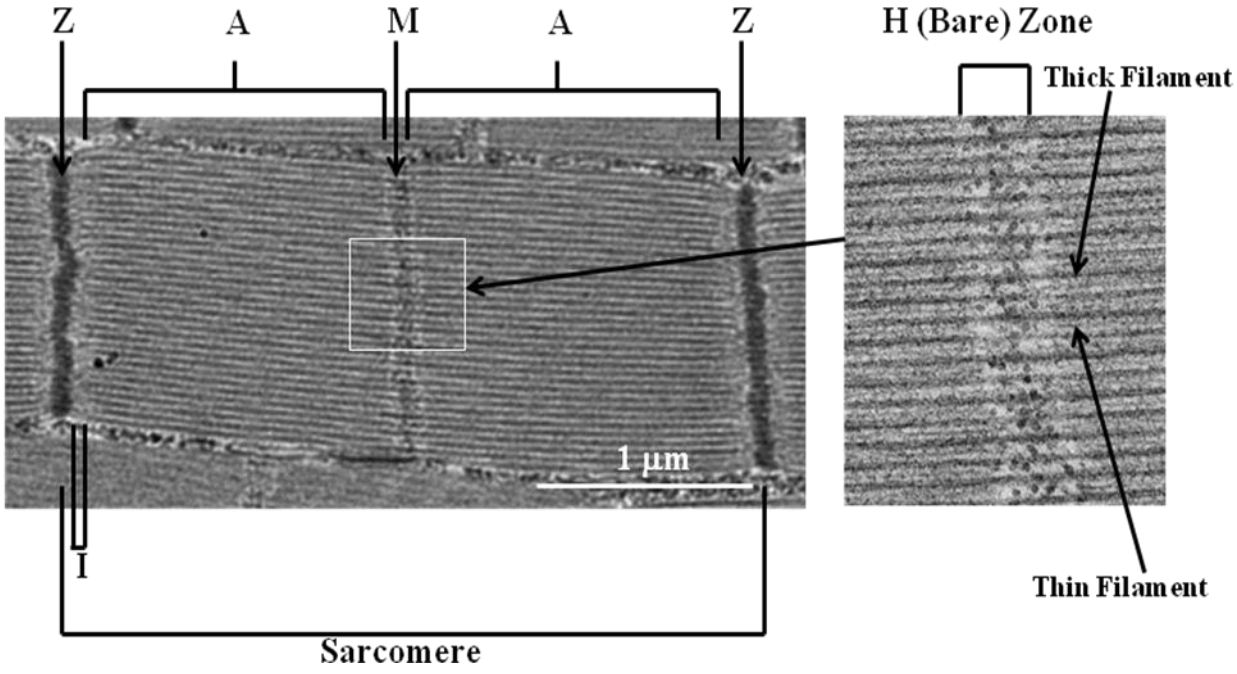

Figure 1-6. Electron micrograph of a *Drosophila* indirect flight muscle sarcomere which extends from Z-band (Z) to Z-band (Z) and is bisected by the M-line (M). The I-band (I), the thin filament-only region of the sarcomere, is very narrow. The A-band (A) is the overlap region between thick and thin filaments. The right panel is the zoomed part of the middle of the sarcomere, showing the bare zone (H zone) where there is no myosin head and no thin filament. Thick and thin filaments are also indicated.

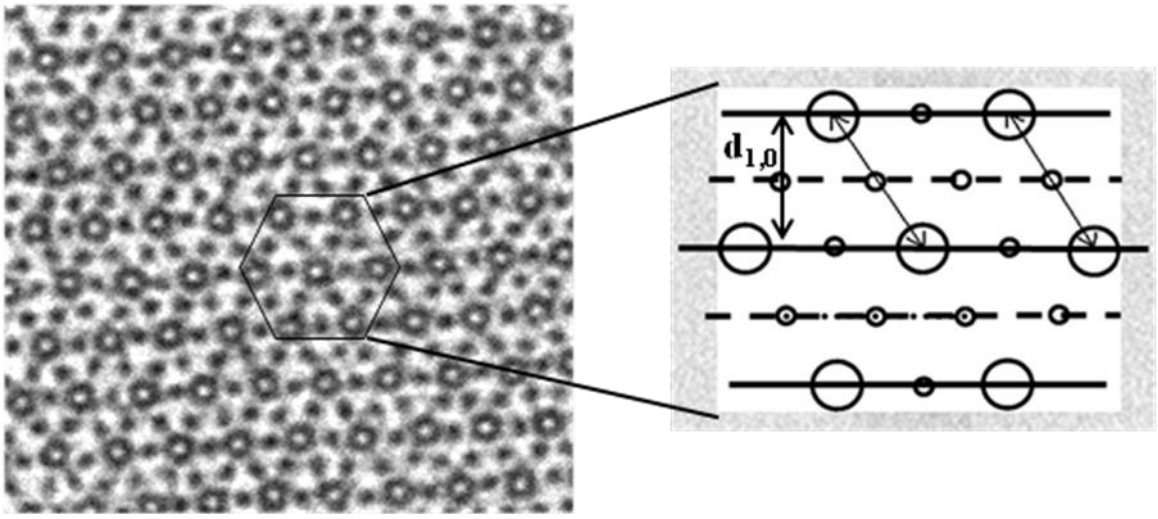

Figure 1-7. *Drosophila* indirect flight muscle myofibrillar cross-section electron micrograph image showing the double hexagonal array of hollow thick and filled thin filaments. The right panel shows a cartoon of the lattice arrangement where the bigger circles are each thick filaments and the smaller circles are each thin filaments. The $d_{1,0}$ lattice spacing is the distance between the consecutive thick filament planes (vertical two-headed arrow), from where the center to center spacing between thick filaments, or inter-thick filament spacing (angled two-headed arrows) could be retrieved.

Flight Muscle Adaptations

The flight muscles have gone through various adaptations to enhance flight performance. The evolution of asynchronous muscles in insects is probably one of the major adaptations of flight muscle throughout insect evolution.

Structural Adaptations: Lattice

Asynchronous flight muscles made hovering and various other aerodynamic feats easier to achieve and sustain for many flying insects. This type of muscle system enabled the wings to create pressure gradients that give added uplift to the insects [60]. Micro-X-ray analysis of frozen flight muscles of bumblebees showed that the myofibrils are extraordinarily symmetrical with successive sarcomeres in such a precise alignment [61] that the myofibrils are in effect giant protein crystals. Later, it was found out that this long-range myofilament lattice regularity is almost exclusive to asynchronous flight muscles [4]. In that study [4], there are very few exceptions, like the hummingbird hawkmoth, having synchronous flight muscles, yet the myofibrils showing higher order X-ray spot-like diffractions indicative of some local lattice register, but might not be for long-range. Since the asynchrony feature of flight muscles has evolved multiple times throughout insect evolution, it is easy to envision that the highly crystalline or regular lattice structure too has evolved independently multiple times. Bees (order Hymenoptera), flies (order Diptera), beetles (order Coleoptera), and true bugs (order Heteroptera) show similar regular hexagonal lattice array and long-range regularity. Iwamoto et al [4] found that the crystalline myofibrillar lattice structure is not only

exclusive to asynchronous muscles, but also restricted to only asynchronous "flight" muscles, suggesting that lattice regularity in muscles is required for flight. Moreover, Iwamoto et al [4] also found that skilled flyers have better registered or more crystalline myofibrillar structure than medium skilled or poor flyers. This indicated that the crystal nature of the myofilament lattice possibly is required for fast wing flapping and maneuvering for skilled flight. Although, it is definitely not clear how this lattice regularity could drive skilled flight. Some of the possibilities are that a well-registered or regular lattice enables force to be transmitted more efficiently along the length of the myofibrils and hence muscle fibers, leading to more efficient power output for skilled flying. Computational modeling studies have shown that sarcomeric geometry, in particular the arrangement of myofilaments in the lattice structure, could influence the coordinated cross-bridge binding and rate or amplitude of force development [62]. Therefore, it could be possible that the well registered asynchronous muscle lattice could allow better transmission of force along its filaments which in turn could lead to enhanced rate and amplitude of force development, and power output.

Mechanical and Physiological Adaptations

The hallmark of asynchronous muscle is pronounced stretch activation and its counterpart shortening deactivation [43]. This feature allows the muscle to produce force at constant strain after a stretch through a delayed rise in tension, and likewise, allows force to drop at constant strain after shortening (reviewed in [5,23]), the end result of

which is net positive work output. The underlying molecular mechanism of stretch activation remains elusive and is still under scrutiny. Several models have been proposed to explain stretch activation (reviewed in [5]) and recent evidence highlights that calcium activation plays an active part in the magnitude of stretch activation [1,63], with results differing in some way between species. More recent X-ray diffraction studies provide evidence that troponin-myosin bridges are key to the stretch activation response since these bridges could move tropomyosin away from the blocked state to enhance cross-bridge binding during stretch [64]. While all striated muscles exhibit stretch activation when stimulated experimentally, this mechanism is believed to be of physiological relevance only among muscles that power oscillatory systems, namely insect flight muscle and vertebrate cardiac muscle. A characteristic feature of stretch activated muscles is their high resting or passive stiffness which is contributed mostly by the connecting filaments (kettin and projectin in *Drosophila* IFM), the magnitude of which is proportional to the amplitude of stretch activation [5], and to a lesser degree by the thick or thin filament stiffness [reviewed in 65]. Stretch activated muscles operate at low strains (e.g., 3-5% for *Drosophila* IFM [67]), a condition almost forced by the nearly complete overlap of thick filaments and thin filaments due to the narrow I bands (Figure 1-3). The combination of these two factors, high stiffness and low strain, is probably interrelated and necessary for efficient force transmission conducive to oscillatory work output [5,23,66]. Moreover, the *Drosophila* IFM has the fastest known myosin kinetics,

in particular, a very fast cross-bridge detachment rate which could also be critical in the stretch activation response for fast oscillatory contraction cycles (Figure 1-1, [2]).

Interestingly, another model used to explain stretch activation is that asynchronous muscles have alternative troponin C (TnC) isoforms for calcium or stretch activation in the troponin-tropomyosin (Tn-Tm) complex; which is the major thin filament regulatory unit for striated muscle contraction. Indeed, in *Lethocerus* IFM, it was found that there are two TnC isforoms, one (F1) to regulate stretch activated tension and the other (F2) to regulate calcium activated tension [67] with a molar ratio of 5:1 (F1:F2) distributed on the thin filament [68]. Based on the findings of Agianan et al [67], it is suggested that even in low calcium concentration, regions of the thin filament containing F1 would more readily be able to transition to the open state and able to activate the cross-bridges, acting as a regulatory mechanism for stretch activation, while at higher calcium concentration, F2 is used for regulating thin filament activation and produce isometric tension.

The Role of Calcium in Flight

The *Drosophila* giant fiber neural system (GF) facilitates the signal transmission from the brain to the tergotrochanteral motoneuron (TTMn) and peripherally synapsing interneuron (PSI) in the thoracic ganglia to drive escape response and flight (reviewed in [54]). The neuronal firing releases calcium and subsequently controls IFM activation regulated through calcium-sensing units of Tn-Tm [24]. Acto-myosin cross-bridges start

to get recruited in the DLM, contraction of which stretches the DVM and therefore stretch activation takes over. Hence, calcium was regarded to have only a permissive role to maintain stretch activation during flight [69]. This notion was recently revised owing to the finding that calcium plays an active role in the IFM for modulating power during flight by both *in vitro* muscle mechanical [1] and *in vivo* [70] studies. Recently, Lehmann et al. [71] found that during flight maneuvering and turning movements in *Drosophila*, power adjustments occur through bilateral control of calcium levels between the muscles (both IFM and DFM) of the two thoracic segments. This further suggests that rather than differential recruitment of fibers, the calcium levels and gradients through the differential neural drive could modulate thin filament activation, and the number of cycling cross-bridges for power modulations.

Flight Muscle Thick Filaments

While paramyosin is the major constituent of large diameter thick filaments like those in molluscs (reviewed in [72]), myosin heavy chain (MHC) is the major constituent protein of most invertebrate thick filaments. The entire myosin molecule is a hexamer consisting of two MHC subunits, and four light chain subunits. The two light chains are the essential light chain (ELC or MLC1) and the regulatory light chain (RLC or MLC2). Moreover, there are other thick filament associated proteins, some which bind myosin. Paramyosin is one of them, which forms the wall of the hollow core of the thick filament around which myosin molecules assemble [73,74], and it is required for normal muscle

development and contractile function [75]. A spliced variant of it, miniparamyosin is suggested to maintain the pre-positioning of myosin heads through an interaction with the paramyosin scaffold [76]. Myofilin (20kDa) is another thick filament associated protein which is proposed to play a role in thick filament assembly [77]. The IFM thick filament contains a number of structural components that are likely to contribute to the stretch activation property [78], among which flightin and myosin regulatory light chain, have been studied previously and are described in detail in the following sections. In addition to the structural elements, the IFM thick filament and also thin filaments have a net negative electrostatic charge which has been known to influence myofilament lattice spacing and organization during myofibrillogenesis [79]. Moreover, along with the myosin's contractile function, the thick filament's mechanical properties, in particular stiffness, could play a significant role in contractility (reviewed in [65]). The mechanical properties of the filaments may influence how they align during myofibrillogenesis, and whether the resulting structure of the myofibril is a simple lattice or a superlattice [80]. Therefore, thick filament structural and mechanical properties play a significant role in overall muscle structure and contractile function.

Flightin

Gene Structure and Expression

Drosophila melanogaster flightin is a ~ 20kDa protein expressed exclusively in the adult IFM, as evidenced by its onset of expression from late pupal stages [81,82].

Flightin is encoded by a single gene located in the polytene region 76 D/E of the 3rd chromosome [82]. The first intron of the gene separates a small exon from the open reading frame and two small introns (66 bp and 62 bp) interrupt the coding region. There are two transcripts that originate due to alternative start sites that differ in their 5' non-coding region [82]. Alternative expression patterns of flightin are seen at the post-translational level with multiple isoelectric variants generated by differential phosphorylations [83]. By performing LC-MSMS, a cluster of seven phosphorylation sites were found in the flightin amino terminal region (Vigoreaux JO and Ballif BA unpublished data). The flightin gene from some insects, including the twelve sequenced *Drosophila* species (see Chapter 3), and some crustaceans reveal that the gene structure is similar among these species, with the middle coding region having highest sequence conservation. This conserved middle region is named WYR based on the most prevalent conserved amino acids present (Tryptophan, W; Tyrosine, Y; Arginine, R) (unpublished results [Soto-Adams F, Alvarez P, and Vigoreaux JO]).

Sequence Features

Flightin bears no sequence homology to any known protein or protein domains, and its presence is restricted to some arthropods (unpublished results [Soto-Adams F, Vigoreaux JO]). Although arthropods are a big group consisting of chelicerates, myriapods, crustaceans, and hexapods, flightin's absence in other phyla including vertebrates could possibly designate it to be a taxonomically restricted gene. Fulfilling the criteria of unique sequence and taxonomically restricted expression could possibly

assign flightin as a taxonomically restricted or an orphan gene with no known ancestral or related gene sequence as mentioned in [84].

There are interesting features in the flightin amino acid sequence that could be relevant to its function in *Drosophila* IFM. The N-terminal one-third of the protein is composed mostly of acidic residues whereas the rest of the sequence is composed mostly of basic residues possibly indicating that the N-terminal region could have a distinct function.

Relationship to IFM Mechanical and Structural Properties

Flightin is a hyper-phosphorylated protein that binds to the light meromyosin (LMM) region of the myosin rod [83,85]. It is distributed homogeneously throughout the A-band of the sarcomere except at the M-line and the edge of the A/I junction [86]. Flightin is known to be essential for thick filament assembly, sarcomere stability, and normal contractile activity of *Drosophila* IFM [7,83,85-88]. *Drosophila* with a null mutation in the flightin gene (fln^0), are viable but flightless due to age-dependent degeneration of their flight musculature and adult muscle hypercontraction [86]. fln^0 IFM thick filaments and sarcomeres from late stage pupa are, on average, ~30% longer than that in wild-type IFM [86] suggesting that flightin plays a major role in thick filament assembly during myofibrillogenesis. Also, fln^0 thick filaments are about 30-45% more compliant than normal [89] suggesting that flightin is required for normal thick filament stiffness. However, it is not clear how flightin's contribution to thick filament stiffness is related to its role in thick filament assembly process *in vivo*. As described in [90], one

likely possibility is that flightin binding to the LMM of an inner myosin molecule in the subfilament interacts with the S2 hinge region of a neighboring outer myosin reinforcing or "welding" them laterally giving stability and normal stiffness to the thick filament (see Figure 5-1 of Chapter 5). Moreover, more recent studies suggest that flightin binding restricts myosin molecule incorporation / dissociation during the assembly process [91], as originally proposed [86]. It is still not clear though how flightin i) stiffens the thick filament and also ii) maintains normal myosin incorporation during thick filament assembly. It is also not known if there is any relationship between these two functions of flightin at the molecular level. One hypothesis is that by laterally reinforcing adjacent myosin molecules in a subfilament, flightin stiffens the thick filament, which in turn stabilizes the thick filament by resisting abnormal myosin incorporation or dissociation leading to normal thick filament assembly. fln^0 sarcomeres are structurally compromised, since its fibers are unable to withstand contractile forces, resulting in sarcomere breakage and fiber hypercontraction [86,92]. These structural and functional abnormalities in fln^0 are fully rescued with the introduction of a full length normal fln^+ transgene [88]. Overall, the results show that flightin is an important protein for *Drosophila* IFM structure and function.

Muscle mechanical studies using small amplitude sinusoidal length perturbation analysis of skinned IFM fibers from three flightless mutants that affect flightin expression: (i) fln^0 , a flightin null mutant [86] (ii) Mhc^{13}, a myosin rod point mutant with reduced levels of flightin and (iii) Mhc^6, a second myosin rod point mutant with reduced

levels of phosphorylated flightin [93], revealed that normal expression levels and phophorylation of flightin is required for IFM stretch activation response. IFM fibers from all three mutants showed marked reductions in passive and dynamic viscoelastic properties that resulted in significant lower oscillatory work and power output. Passive tension and passive stiffness, important pre-requisites for stretch activation, were significantly reduced in fln^0 and Mhc^{13} but not in Mhc^6. Interestingly, fln^0 fibers could generate normal calcium activated tension under isometric conditions, suggesting that calcium activation of thin filament regulatory components and number of cross-bridge recruitment for force production was unaltered due to the absence of flightin. However, when subjected to sinusoidal length perturbations, the fln^0 fibers absorbed work instead of producing, resulting in no net positive work output, rendering the flies unable to beat their wings for flight. Since flightin's absence has no effect on isometric tension indicate the mutant fibers are capable of producing and transmitting normal level of force in isometric conditions only. Therefore, the reduced oscialltory work and power output could possibly arise due to defects in force production or transmission by the heads in small amplitude length perturbation conditions (non-isometric conditions). This possibly could arise due to the ultrastructural abnormalities in the absence of flightin. From these studies, it was concluded that flightin is a major contributor to myofilament stiffness, and to the *in vivo* stretch activation response for oscillatory power output in *Drosophila* IFM [7].

More recently, truncation of the 44 amino acids from the flightin COOH-terminus ($fln^{\Delta C44}$) abolished flight even with some partial rescue in IFM structural and mechanical properties, compared to that in complete absence of flightin [94]. $fln^{\Delta C44}$ IFM fibers generated significantly reduced oscillatory work and power output with reduced underlying cross-bridge kinetics compared to fln^{+} rescued control null fibers. This suggested that the partial rescue in $fln^{\Delta C44}$ sarcomere structure was not sufficient enough for myofibrillar stability and normal contractile kinetics. Since $fln^{\Delta C44}$ IFM sarcomeric structure is not normal enough for flight, the marked reduction in cross-bridge kinetics could be due to the sarcomeric structural aberrations like abnormalities in M- and Z-lines, and A-band breaks. Moreover, adult $fln^{\Delta C44}$ myofilament lattice is highly disordered [94], indicating that the COOH-terminal region is required for normal lattice organization. From this study, it can be concluded that flightin COOH-terminal region is required for IFM's sarcomeric and myofibrillar structural stability, that in turn, is required for normal cross-bridge behavior during oscillatory contractions.

Relationship to Myofilament Lattice Stability

The mutational studies discussed above do indicate that flightin is an important structural component of the IFM thick filaments contributing to the overall myofilament and sarcomeric stability, which gets portrayed in the whole fiber mechanical behavior [86,89,94]. These studies also indicate that flightin is essential for proper IFM development and function. Previously it was observed that phosphorylation patterns of

flightin get affected by unlinked mutations in either thick or thin filaments [95]. This shows that flightin expression pattern is sensitive to either thick or thin filament mutations suggesting that it could play a role in inter-filament interactions. *Drosophila* heterozygous for a genetic deficiency spanning the flightin gene, $Df(3L)fln^{1}$, show a 20% reduction in flightin expression which impairs flight ability and causes slight defects in the myofibrillar structure [87]. These myofibrils have an intact normal central core, but the peripheral myofilaments are loosely organized. These loose myofilaments get washed away on treatment with non-ionic detergent suggesting that they were not optimally connected to the core lattice [87]. This finding suggests that flightin is essential for maintaining the overall myofilament lattice integrity, either by inter-filament interaction, or by stabilizing the thick filaments. Moreover, as discussed above, flightin COOH-terminal region is shown to be required for normal lattice order as evidenced by X-ray diffraction on live $fln^{\Delta C44}$ flies. Electron microscopy revealed that $fln^{\Delta C44}$ myofibrils have frequent breaks in the lattice and are less regular in shape than normal. All of these indicate that flightin contributes to maintaining normal myofilament lattice order and stability.

Myosin Light Chain 2

Gene Structure and Expression

The *Drosophila* myosin regulatory light chain (DMLC2) is encoded by a single gene (*Dmlc2*) located in the region 99E1-3 on the chromosome 3 right arm. Two

transcripts differing in polyadenylation sites are encoded by the gene, both of which code for a ~ 20kDa protein. The DMLC2 is homologous to vertebrate MLC2s [11], except that the DMLC2 has an additional 46 amino acid N-terminal extension. This extension is characterized by a stretch of basic amino acids towards the N-terminus followed by a proline-alanine rich sequence, similar to the extension found in vertebrate MLC1. Moreover, this unique N-terminal extension pushes the myosin light chain kinase phophorylation sites (2 Serines) to residue 66 and 67 in DMLC2 compared to residues 18 and 19 for vertebrate smooth muscle and residues 11 and 12 for vertebrate skeletal muscle, respectively. Comparison of DMLC2 with vertebrate MLC2s revealed three conserved regions [11]. This comparison confirmed DMLC2 as a member of the troponin C super family [96] due to the presence of an EF-hand calcium binding motif surrounding residue 80.

Molecular Function in IFM

There has been a substantial amount of work done to understand the function of the unique N-terminal extension and the conserved phosphorylation sites (Serines 66 and 67). Mutations of the DMLC2 are known to have a large effect on stretch activation response, myosin kinetics and flight performance [12], but have no major effect on calcium activation response of muscle fibers [97-99]. Two such mutations in the DMLC2, have been extensively characterized for their roles in IFM structure, cross-bridge kinetics, stretch activation response and power output for maximal wing flapping

frequency and flight performance [12,97-101]. X-ray diffraction of IFM in living *Drosophila* at rest and electron microscopic studies showed that truncation of the 46 amino acids N-terminal extension (D*mlc*2$^{\Delta 2\text{-}46}$ or Ext) move the myosin heads towards the thick filament backbone away from their actin target zones [101] as evidenced by an increase in myosin mass associated with thick filaments (Figure 1-8). These studies also showed that alanine substitutions of the two myosin light chain kinase phosphorylation sites (D*mlc*2^{S66A,S67A} or Phos) increased the spread of the axial distribution of the myosin heads along the thick filament indicating that the heads are less oriented towards the actin target and are spread at larger angles (Figure 1-8). Moreover, in a dual mutant *Drosophila* having both the above single mutations (D*mlc*2$^{\Delta 2\text{-}46;S66A,67A}$ or Dual), the results are additive with the myosin heads moving further away and less oriented towards the actin target zones compared to the single mutations [101] (Figure 1-8). This indicated that both the N-terminal extension and the normal DMLC2 phosphorylation are required to pre-position (alignment and orientation) the myosin heads towards actin target zone to increase their probability of strong binding. Small amplitude length perturbations of skinned IFM fibers by sinusoidal analysis revealed that all the mutations attenuated the stretch activation response [12], concomitant with the structural data with myosin heads moving away from and/or less oriented towards actin target zones. The Phos and the Ext mutants do not show any major IFM structural abnormality [12,97,99] suggesting that the myosin positional and contractile defects to be direct effect of the mutations, and not due to other structural damage. However, the Dual mutant showed slight but significant

peripheral myofibril defects [101]. The movement of the myosin heads away from the thin filament and towards the thick filament backbone [101] reduced cross-bridge kinetics in the mutants leading to decreased number of strongly bound cross-bridges. This, in turn, significantly attenuated muscle fiber oscillatory work and power output [80]. These structural and mechanical effects were reflected in the whole fly where the Ext mutant was flight impaired and the Phos mutant was almost flightless with large reductions in wing beat frequency compared to control flies [12]. In accord with the structural and mechanical data, the Dual mutant showed an additive effect of the single mutations, with the flies completely unable to beat their wings for flight. This indicated further that both the DMLC2 N-terminal extension and the phoshorylation sites are required for stretch activation response of the IFM to maximize power output for fulfilling flight requirements. The findings led to a proposed model for stretch activation. Given the similarity with the vertebrate MLC1 N-terminal extension [102,103], the DMLC2 N-terminal extension could act as a short tether to the thin filament [97] as has been shown for vertebrate MLC1. Upon stretch, this tether could bring the myosin heads in close proximity to their actin target zones. Additionally, structural data from X-ray diffraction of live flies suggested that the DMLC2 phosphorylations could stiffen the myosin head so as to orient it optimally towards actin target [101]. It was suggested that these two effects thereby could additively cause delayed activation by increasing the number of strongly bound active cross-bridges.

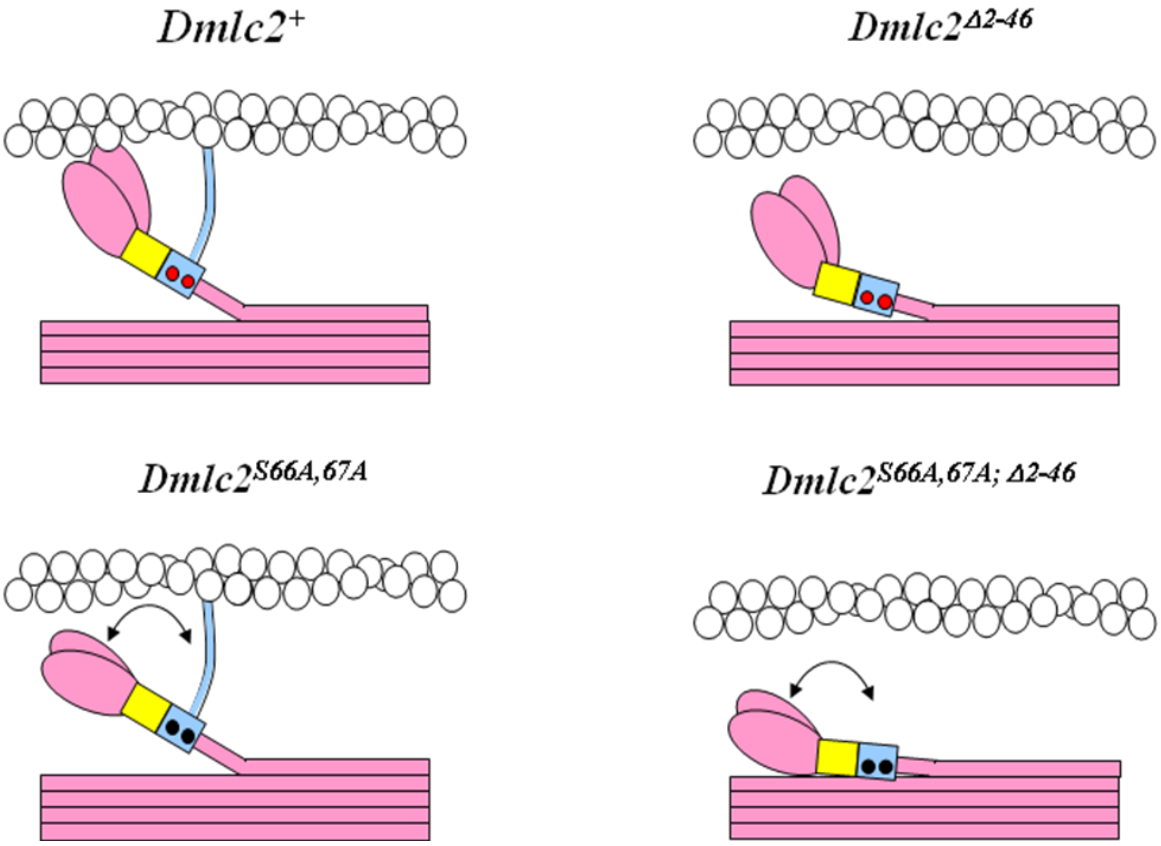

Figure 1-8. Schematic illustration of the proposed model for interactions of the DMLC2 with the thin filament and effects on myosin head positions. When the phosphorylation sites (red filled circles) and N-terminal extension are present (*Dmlc2*$^{+}$ or control) the myosin head is held in close proximity to the thin filament and in a relatively restricted angular range (axially), favorable for acto-myosin interaction. When only the extension is removed (*Dmlc2*$^{\Delta 2\text{-}46}$) the head maintains its angular orientation but are further from the thin filament and closer to the thick filament backbone. When phosphorylation is prevented (*Dmlc2*^{S66A,S67A}), as shown by filled black circles, the angular orientation of the head changes even though the extension, acting as a tether to the thin filament, keeps the

head in close proximity to the thin filament. When both phosphorylation and the extension are removed in the dual mutant (*Dmlc2*$^{\Delta 2\text{-}46;S66A,S67A}$), the range of myosin head angular movements increases and proximity to the thin filament decreases, so that acto-myosin interaction is decreased. (Picture modified from [101]).

Drosophila Male Courtship Song

Survival and reproduction are two of the major goals of living organisms. As discussed above, insect flight is subject to purifying natural selection since it increased the survival fitness of the insects that led to their speciation and diversification [104]. Yet, the stronger force is regarded to be sexual selection which drives speciation stronger and at a faster time scale than natural selection due to its direct effect in reproductive isolation (inter-specific sexual selection), and then subsequent selection pressure within species (intra-specific sexual selection) (reviewed in [105]). Among the different sexually selected traits and behaviors, courtship behaviors (pre-copulatory behaviors) are one of the major factors for pre-mating isolation, con-specific mating and subsequent speciation. Moreover, courtship behaviors arising due to intra-specific sexual selection through female mate choice and male-male competition could drive intra-specific diversity and subsequence emergence of varieties of strains that could be geographically isolated [105]. *Drosophila spp.* is not an exception, where depending on species, both sexes engage in elaborate courtship rituals with most of the behavioral aspects having evolutionary implications (reviewed in [106,107]). Among sexually selected courtship behaviors in

nature, acoustic communication or courtship song is one of the major ones among different species, including frogs, birds or insects (reviewed in [108]). Males of most *Drosophila* species also generate a courtship song for species recognition and con-specific mating as well as female stimulation [106]. This trait of courtship song is highly species-specific and has been shown to be used for both species recognition (inter-specific sexual selection) and female male choice (intra-specific sexual selection) facilitating *Drosophila* speciation (reviewed in [106,107]). *Drosophila* male courtship song is an important part of a structured courtship ritual. For example, *D. melanogaster* males (Figure 1-9) engage in sequential steps of courtship ritual which includes: the male chasing the female and orienting towards her, then tapping with the foreleg, followed by courtship singing by unilateral wing extension, licking, and curling the abdomen to attempt copulation. Finally the receptive female generally spreads its two wings to allow the male to mount and copulate, the final outcome of a successful courtship.

As mentioned before, *Drosophila* male courtship song is species-specific with great variability. For example, in some *Drosophila* lineages like *willistoni* species group, there is higher courtship song type and number variability than in some others like *melanogaster* and v*irilis* species groups [106]. The diversity in male courtship song among *Drosophila* is such that it is difficult to find any particular song parameter variation to be indicative of any pattern across lineages [106,109]. The *D. melanogaster* male courtship song consists of rhythmic pulses called pulse song, and sinusoidal hums called sine song (details of song types and parameters reviewed in [110], see Figure 2-1

of Chapter 2). These songs are generated by small amplitude wing vibrations using the thoracic musculature (reviewed in [26]). Each *Drosophila* species within the *melanogaster* subgroup have unique song characteristics including varying carrier frequencies and temporal patterns of the song waveforms [111]. This variability in song characteristics, in particular, facilitates con-specific (same species) mating, reproductive isolation and female sexual stimulation. For example, the interpulse interval (IPI) of the pulse song is highly variable and carries the most salient species-specific signal throughout *Drosophila*, specifically in the *melanogaster* subgroup [112] facilitating pre-mating isolation and con-specific mating. Other parameters like pulse singing vigor and sine song frequency is known to stimulate *D. melanogaster* females [113] implicated in mate choice. Recently, it has been shown that male's choice of the courtship song structure depends on the proximity of the female to the male [114]. This study showed that the courting males dynamically adjust the relative proportions of the song components, pulse song or sine song, by assessing female locomotion and position. In particular, the male sings more pulses than sines when females are moving fast or further away, possibly to communicate con-specific signals. The male shifts to a singing mode of equal proportions of pulse song and sine song as the female moves closer. This indicates possibly that the pulse song evolved as a species-recognition system in *Drosophila* whereas sine song has a more stimulatory role. Pulse song is present in all singing species of *Drosophila* whereas sine song is mostly restricted to the *melanogaster* subgroup

(reviewed in [110]). Ubiquitous presence of pulse song supports the statement above that pulse song could be critical for con-specific mating and inter-specific sexual selection.

Many genes have been shown to be influencing courtship song (reviewed in [115-117]), among which some are highly variable and shown to be under positive selection with fast evolutionary rates across *Drosophila* (*nonA* gene: [118,119]; *period* gene: [120,121]). This is not surprising, since most fast evolving genes in *Drosophila* are involved in adaptive functions like reproduction (pre-mating or post-mating) that should be under sexual selection [122-124]. Moreover, it is also known that *Drosophila* genes that are involved in speciation process by acting in reproductive isolation under sexual selection are typically very rapidly evolving (reviewed in [125]).

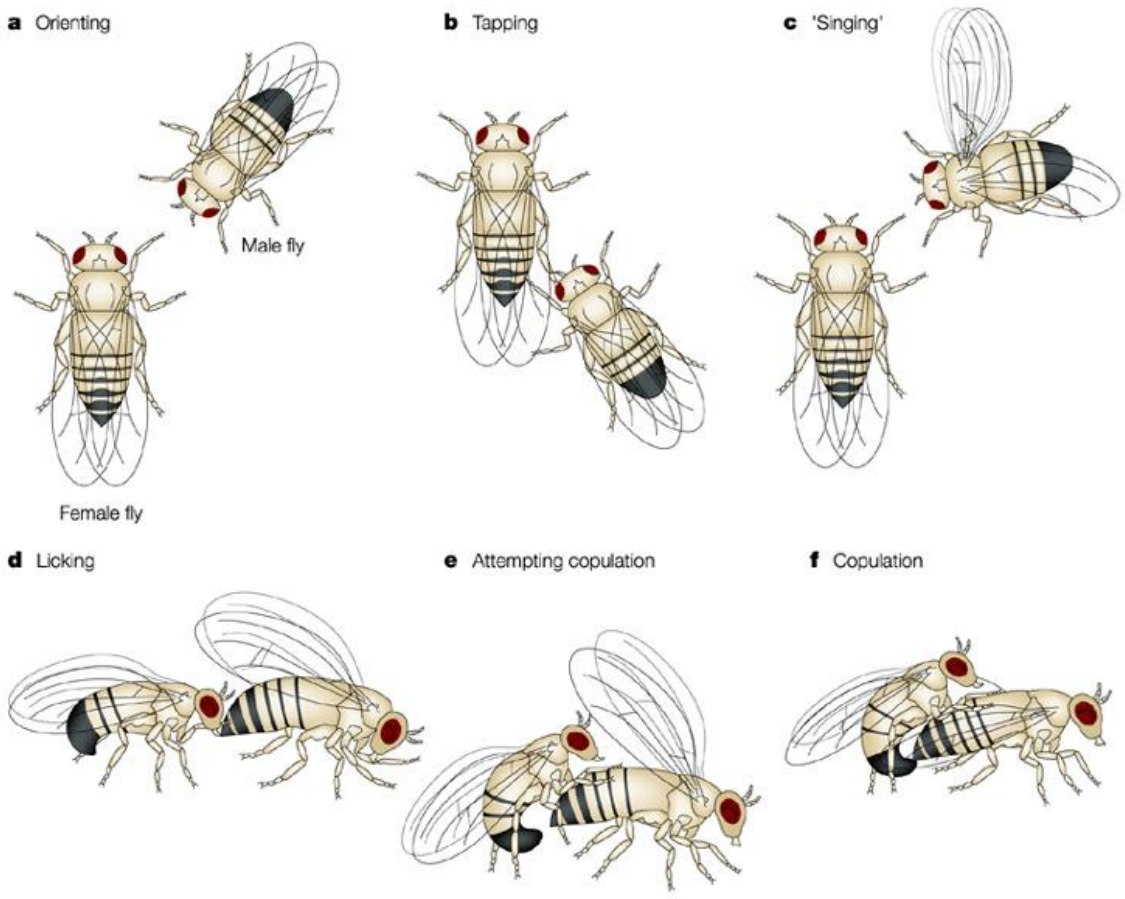

Figure 1-9. Courtship ritual of *Drosophila melanogaster*. A male chases the female and then orient himself towards her (a), then taps with its foreleg to transfer male pheromones (b). This is followed by small amplitude single wing vibration for generating courtship song to stimulate the females (c) followed by licking the female genitalia with its proboscis (d) for further stimulation. Then the male tries to copulate by curling its abdomen (e). Thereafter, if the female is responsive to the male's trial, it spreads both wings to allow the male to mount and copulate. (Figure taken from [116])

Role of Flight Musculature in Courtship Song

Along with the emergence of wings, the flight muscles are obviously the other major innovation for flight to evolve and hence this tissue system requires specializations for high power output. Interestingly, in nature, the same flight muscle system is commonly used for different functions with specific requirements other than flight by many insects, some of which require much less power and potentially different mechanisms. For example, the honeybee antagonistic flight muscles contract simultaneously for warmup behavior and alternately for flight [126], whereas different firing patterns from same motor neurons can cause stridulations or flight in crickets [127]. *Drosophila* flight musculature (both IFM and DFM) also gets neurally activated during both pulse and sine courtship song generation [27,28] as evidenced by electrical recordings. However, the contractile mechanism utilized and the contributions of the different muscle types or genes to courtship song production are not known. Ewing [27] showed that muscle potentials in the IFM during pulse song are related to subsequent and not the preceding sound pulses. IFM gets stimulated at a much lower frequency during sine song compared to flight or pulse song, with some motor units in the thorax remaining inactive. *In vivo* power output during wing vibrations for courtship song has not been measured yet, as has been done in flight [49]. Given, the lower frequency and amplitude of wing beats, and slower neuronal firing rate during sine sing compared to that of flight or pulse song, it is possible that sine song production probably does not require much power. Ewing [28] also recorded muscle potentials in the DFM during both

pulse and sine song. In this study, electrophysiological recordings revealed that the motor units of axillary muscles fire during the interpulse interval in the pulse song. Also, it was found that the basalar muscles fire almost synchronously with each up-stroke wing movements during both pulse and sine song, similar to that shown in flight, and that the sternobasalar muscles' motorunits also fire on each up-stroke but only during pulse song and not sine song.

Even though the DFM is activated during singing, it is less likely that it could be the major power generator for wing movements for this behavior. This could be because it has been previously shown that the control DFM muscles act as springs, rather than force producing elements. In fact, some of the DFM muscle like the b1 basalar muscle has been shown to produce net negative work during its contraction [22], although this study was on blowfly *Calliphora*, not *Drosophila*. The b1 basalar muscle along with the sternobasalar (SB) muscles has been shown to be continuously neurally activated during *Drosophila* flight with a frequency equal to or slightly below one spike per wing beat cycle [128], with the SB muscles having lower activity. But, during courtship song, not all DFMs are active; with the SB muscles completely inactive during the sine song. Whereas, all the IFM motor units are active during *Drosophila* courtship song [27]. Therefore, relatively speaking, it could be possible that DFM is not the major power generator during singing. Most likely, the major contractile unit for courtship song is the IFM.

Therefore, studies spanning several decades examining a large number of IFM mutant and transgenic strains present a golden opportunity for elucidating the role of the IFM in courtship song and, importantly, for establishing the extent to which genetic and physiological pathways are shared between these two distinct behaviors.

This thesis is the first attempt to understand the role of muscle genes in courtship song and to understand the relationship of flight and song in the *Drosophila* IFM. Therefore, to introduce this novel research aspect to the *Drosophila* muscle community, detailed courtship song and behavioral analyses methodology is described in Chapter 2. Chapter 3 describes the function of flightin, specifically its N-terminal region in IFM structure, mechanics and behavioral outputs. Chapter 4 describes the effects of myosin regulatory light chain mutations on *Drosophila* courtship song behavior and compared to their effects on flight. Chapter 5 concludes this thesis with the major findings from previous chapters, interpretations, broader perspectives, and future goals of this work.

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CHAPTER 2 JOURNAL ARTICLE

Courtship Song Analysis of *Drosophila* Muscle Mutants

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ABSTRACT

As part of the mating ritual, males of *Drosophila* species produce species-specific courtship songs through wing vibrations generated by the thoracic musculature. While previous studies have shown that indirect flight muscles (IFM) are neurally activated during courtship song production, the precise role of these muscles in song production has not been investigated. Fortunately, IFM mutants abound in *Drosophila melanogaster* and studies spanning several decades have shed light on the role of muscle proteins in IFM-powered flight. Analysis of courtship songs in these mutants offers the opportunity to uncover the role of the IFM in a behavior distinct than flight and subject to different evolutionary selection regimes. Here, we describe protocols for the recording and analysis of courtship behavior and mating song of *D. melanogaster* muscle transgenic and mutant strains. To record faint acoustic signal of courtship songs, an insulated mating compartment was used inside a recording device (INSECTAVOX) equipped with a modified electret microphone, a low-noise power supply, and noise filters. Songs recorded in the INSECTAVOX are digitized using Goldwave, whose several features enable extraction of critical song parameters, including carrier frequencies for pulse song and sine song. We demonstrate the utility of this approach by showing that deletion of the N-terminal region of the myosin regulatory light chain, a mutation known to decrease wing beat frequency and flight power, affects courtship song parameters.

1. INTRODUCTION

Animals rely on acoustic communication to convey information about their physiology, ecology, and behavior. Acoustic communication has evolved to provide conspecific signals to prospective mates [1-3] and as mechanisms for premating reproductive isolation and subsequent speciation [4]. Insects produce a variety of acoustic signals, most notably the "mating song" that is a key component of the courtship ritual in many species. For example, *Drosophila* males produce a courtship song through wing vibrations, a behavior that is under sexual selection and plays a major role in species recognition and speciation [5-12]. Thus, studies of the genes involved and the mechanisms responsible for *Drosophila* male courtship song production will uncover the molecular basis of sexual selection and speciation, and provide information about the physiological basis of acoustic communication.

Electrical recording of the *Drosophila melanogaster* (*D. melanogaster*) indirect flight muscles (IFM), the major power producing muscles for flight, revealed that these muscles are neurally activated during the courtship song [13]. However, the precise role of the IFM in courtship song production has not been examined. Additionally, many genes that affect courtship song have been identified through mutational and quantitative analyses [14, 15], but none of them are muscle protein genes. Interestingly, none of the genes identified through classical genetic approaches are among the candidates identified by quantitative genetic approaches [14] suggesting that our understanding of the genetic basis of mating song is still in its

infancy.

The *D. melanogaster* IFM has been studied extensively for its role in flight and, in general, as a model system for muscle function, development, and disease [for reviews, see 16-21]. These studies, spanning several decades, have examined a large number of IFM mutant and transgenic strains many of which have been analyzed in great detail, from the molecular to the organismal level [20, 22, 23, and 24]. These mutants thus present a golden opportunity for elucidating the role of the IFM in courtship song and, importantly, for establishing the extent to which genetic and physiological pathways are shared between the two distinct behaviors of flight and courtship. As an example of this approach, we show here that deletion of the N-terminal extension of the myosin regulatory light chain, a mutation that alters myosin kinetics and impairs flight performance [25, 26], affects the courtship song.

D. melanogaster readily engages in courtship behavior and mating in standard laboratory settings. Specialized instrumentation has been devised to capture the acoustic signals generated by males during courtship [e.g. 27]. Here we describe detailed methodology for setting up courtship assays that include recording the male courtship song of *D. melanogaster*. We present an analysis of the spectra generated from these recordings with an emphasis on identifying song parameters, including frequencies that may reveal the contributions of muscle genes to this important behavior.

D. melanogaster Male Courtship Song

Drosophila males produce a courtship song that is part of an elaborate, species-specific mating ritual [28-30]. A sequence of behaviors that culminate in copulation has been documented for *D. melanogaster*. A male that perceives the presence of a female prospective mate (through visual and other cues) reacts by chasing and orienting itself towards the female. This is followed by tapping the female with its foreleg, vibrating one wing to produce a song (see below), licking of the female genitalia, and grabbing the female body with its foreleg to attempt copulation. If the female is receptive, she will spread both wings to allow the male to mount [30]. The acoustic signal produced by the wing vibrations is critical for stimulating the female, and therefore, for courtship success [9].

To produce the song, a *D. melanogaster* male extends one wing, normally the one nearest to the female head, about 90^{o} to its body axes and generates small amplitude vibrations [13, 28, and 29]. There are two types of songs, pulse song and sine song [Fig. 2-1; 28, 29, and 31]. The pulse song is characterized by a train of pulses [32], each of which is a strongly modified tone consisting of one to three cycles [33] occurring at intervals of about 35 ms [34, 35]. The amplitude of the pulse song is about twice as high as that of the sine song [12]. The pulse song is characterized by several components (Table 2-1 and Fig. 2-1), some which play a defining role in species recognition and mating [28, 34, and 36]. For example, in *D. melanogaster* the interpulse interval (IPI) is an important parameter in mate recognition [36]. The sine

song is characterized by a continuous sinusoidal hum with a fundamental carrier frequency, along with presence of higher-order harmonics [37]. The duration of the sine song burst, like its carrier frequency, is influenced by temperature and can vary within a range of 125-250 ms [35]. The sine song has been proposed to be essential for female stimulation [7, 8]. The range of intrapulse frequency (IPF) and sine song frequency (SSF) produced by a wild-type male is broad: 200 Hz to 400 Hz [37] and 130 Hz to 185 Hz [35], respectively.

Theoretical Basis of Song Recording and Analysis

The ability to rear *Drosophila* in the lab has facilitated the study of courtship song, which would otherwise require sophisticated equipment to capture insect calls in the wild [38]. *Drosophila* courtship songs have been routinely recorded using a custom-made instrument known as an INSECTAVOX. A detailed description of the design of the INSECTAVOX is provided elsewhere [27]; some of the essential features are summarized here. The INSECTAVOX is a recording box encased in metal plates at least ¼ inch thick to dampen environmental noise and stray 60 Hz electronic noise. The flies are placed inside a sound insulated chamber that allows recording of the low amplitude song produced by the vibrating wings. The chamber has a clear window on top with a magnifying glass, to allow direct visualization of flies, and is equipped with an internal light source and a modified electret condenser microphone that is highly sensitive to particle velocity, the major component of wing beat vibrations [28].

The INSECTAVOX consists of a microphone compartment, an electronics

compartment, and a compartment for the transformer and battery. The design places two walls separating the microphone compartment from the transformer to shield acoustic recordings from electronic and magnetic interference. Power to the microphone is fed by a low noise 9 volt battery to avoid interference that would result from DC power. The microphone output passes through a large capacitor and an attenuator before reaching a high gain and low noise pre-amplifier. The circuit branches in two directions preceded by operational amplifiers; one an audio output (e.g., headphones) and the other an instrument output (e.g., computer).

The analog signal from the microphone is digitized with Goldwave (v5.58) on a computer with an A/D converter sound card. After recording, the song files are stored in uncompressed PCM "wav" file format. This is preferable than compressed formats like MP3. In this process of digital sampling, the A/D converter samples the instantaneous voltage amplitude of an input signal at a particular sampling rate. The precision of the digitization process depends on the rate at which amplitude measurements are made (the sampling rate or sampling frequency), and the number of bits used to represent each amplitude measurement (the sample size). The higher the sampling rate, the wider the frequency response of the recording. The upper frequency limit is slightly less than half of the sampling rate. A sampling rate of 44.1 kHz with 16 bits of resolution and a bit rate of 705.5 kbps per channel are adequate for good reproduction of up to 20 kHz frequency signal.

2. MATERIALS

2.1. Fly stocks

The wild-type *D. melanogaster* stock is a laboratory strain of Oregon R. The generation of the two transgenic strains studied here has been previously described: the rescued myosin regulatory light chain (MLC2) null, D*mlc2*$^{+}$ [39] used here as the control strain, and the truncated N-terminal extension of myosin regulatory light chain D*mlc2*$^{\Delta 2\text{-}46}$ [40], used here as the mutant strain. The flies were fed standard corn meal food (see http://stockcenter.ucsd.edu/info/food_cornmeal.php for ingredients and recipe).

2.2. Courtship song assay

2.2.1. Fly rearing and collecting for song assay

(i) 25^{o}C incubator.

(ii) 20^{o}C, 65% humidity environmental room with 12:12 light:dark cycles.

(iii) 25 × 95 mm polypropylene fly vials (Fisher Scientific).

(iv) Six oz. square bottom polypropylene bottles (Genesse Scientific).

(v) Agar and yeast (SciMart).

(vi) Propionic acid (Fisher).

(vii) Custom-made aspirator: An aspirator is a mouth suction device used for the transfer of flies into the mating chamber. It is made of a 10-15 cm. long, 1 ml graduated disposable pipette attached to a 20 inch long aquarium airline tubing, which in turn is fitted into the wide end of a plastic pipette tip (250 μl wide-bore tip without aerosol barrier). A small piece of fine fabric (e.g., a polyester

silk-screen fabric) two times wider than the wide end of the pipette tip is placed over the pipette tip wide end before tightly fitting the tip into the tubing.

2.2.2. Sound proofing of song assay set up

(i) One inch thick soft foam (Michaels Store, http://www.michaels.com/).

(ii) Anechoic foam mat (Aircraft Spruce & Specialty Co.)

2.2.3. Courtship song recording apparatus

(i) Mating chamber: small plexiglass chamber (1cm diameter × 4mm height) with a nylon mesh bottom and a small sealable side entry hole to insert flies [41].

(ii) Filter paper (Whatman, ashless, grade 42).

(iii) Kimwipes (Kimberly-Clark).

(iv) 70% Ethanol (Pharmco-AAPER).

(v) INSECTAVOX [27].

2.2.4. Analysis of courtship song

(i) Computer (Intel® Core™ 2; 2.66 GHz processor with 1.99 GB RAM and a 32 bit operating system).

(ii) Audio lead to connect INSECTAVOX to a computer.

(iii) Goldwave v5.58 software [42].

2.3. Courtship behavior assay (optional)

(i) 65X SD camcorder (Samsung).

(ii) Tripod (Vanguard).

3. DESCRIPTION OF METHOD

3.1. Fly rearing and collection conditions for courtship song behavioral assay

Flies are raised in standard corn meal food at 22^{o}C and 70% humidity in a room with 12:12 light:dark cycles. Disturbance of the circadian rhythm may affect the courtship song and should be avoided [37, 41, 43, and 44]. Virgin males and females are collected using CO_2; however, subsequent use of CO_2 should be avoided and other forms of immobilization, such as aspiration, should be used [45]. Males are aged 3-5 days after eclosion (see 4.1), the typical age at which they reach full sexual maturity [46]. Females are aged for approximately 24 hrs after eclosion. Virgin females 24 hrs old or less, while not receptive to copulation, stimulate the males to produce more songs (see 4.2). After collecting, the males and females are kept in separate vials in small groups of 5 to 10 [37]. To nullify any grouping effect, the males are aspirated into single vials and kept isolated for about 24 hrs before testing. The isolation also helps to increase the amount of song production [8, 37, 45 and 46]. On the day of testing, a male and a female are aspirated into the mating chamber for courtship song assay. Aspiration avoids any residual effects of CO_2 (or other forms of immobilization) on mating behavior.

3.2 Courtship song assay

3.2.1. Fly strains used

When examining the effect of mutations on mating song, careful consideration must be given to the choice of a control strain to minimize the effect of within-strain

variability in some of the song parameters. For example, for a P-element mediated imprecise excision mutant, a precise excision strain provides the best control [30]. In the case of an experimental male generated by the GAL4-UAS system, uni-transgenic flies (Gal4/+ and UAS/+ heterozygotes) must be tested for each assay [30]. The muscle mutant strain used here expresses a mutant DMLC2 transgene in a DMLC2 null background (D*mlc*2$^{\Delta 2\text{-}46}$). As a control, we used a strain with a wild-type DMLC2 transgene in the same DMLC2 null background (D*mlc*2^{+}). For transgenics expressing mini-white (*w*+), as is the case here, testing for courtship behavior in dark or dim light is preferable as *w*+ has effects on visual system and courtship behavior [30, 47 and 48]. Flies expressing mutations in *ebony* should be avoided as they produce pulse song with different frequency and less sine song [49].

3.2.2. Instrumentation and room set up

The courtship song recording is carried out in an INSECTAVOX [27], a custom-made instrument equipped with a particle velocity sensitive microphone that gives a high signal to noise ratio (see 4.3). Certain precautions are taken to reduce noise interference. The song assay is best performed in an anechoic room. Here, a quiet basement room away from the elevators was selected to minimize vibrations. Electrostatic and magnetic interference from sources like central heating/air conditioning units should also be avoided. The room should be devoid of pumps and refrigerators. The INSECTAVOX is placed inside an anechoic foam wall with soft foam placed underneath it as a further barrier to vibrations (see 4.4 and 4.5). Courtship song assay are

done at 20^{O}C and 65% humidity.

3.2.3. Courtship song recording and video

Recordings are best done between 7:00 AM and 9:00 AM shortly after flies are first exposed to light [45]. The mating chamber is rinsed with 70% ethanol to remove residual odor cues. Wet filter paper is put inside the INSECTAVOX chamber to maintain humidity during the experiment [9]. Using an aspirator, one male fly is introduced in the dried mating chamber first and allowed to recover alone for 2-3 minutes. The female fly is then introduced in the chamber, the entry way is plugged with cork and the chamber placed inside the INSECTAVOX as quickly as possible as males may begin to sing instantly. Throughout the sound recording, the room is not occupied and is kept dark except for the small and relatively cool light source inside the INSECTAVOX (see 4.6). A camcorder mounted on a tripod is used to obtain video documentation of the courtship ritual. Videos are synchronized to audio and used to confirm that the sounds recorded correspond to wing vibrations. The synchronization is done in a PC using Windows Movie Maker software.

To record the song, select “New Sound” from the menu in Goldwave and then select “mono” for the number of channels, “44100” or 44.1 kHz for sampling rate and “30 mins” for initial file length. Mono provides a larger window to examine the song oscillogram and also produces a smaller file size. Mating sessions are recorded for at least five minutes but extend for as long as 30 minutes.

3.2.4. Courtship song analysis

Table 2-1 lists all the characteristic parameters of the courtship song, their corresponding abbreviations and definitions. All of the parameters can be measured manually from Goldwave.

3.2.4.1. The recorded song file is opened in Goldwave v5.58.

3.2.4.2. Use the automatic offset feature to scan and correct the balance of the song waveform until the peaks and the troughs are approximately of the same height relative to the zero line.

3.2.4.3. Individual pulse song and sine song trains are detected manually by listening to the sound while examining the waveform of the recorded oscillogram [see 4.7 and Supplementary Data]. A pulse song is identified by the characteristic sound of a train of pulses, a sound easily distinguished from background noises (e.g., walking, wing flicks) and the humming sound of sine song [35]. The beginning pulse in a train is considered to be the first peak that exceeds three times the absolute amplitude of the background sound level that precedes it [46]. Similarly, the signal at which the amplitude either decreases to the level of the background noise or stops completely is taken as the end of the pulse. A sine song is identified as a tracing with a constant sinusoidal waveform lasting at least 175 ms and accompanied by a humming sound of constant frequency. The sine song has lower amplitude than the pulse song.

3.2.4.4. We select for analysis pulse trains and sine song bursts (Fig. 2-1) from the beginning, middle and end of a song oscillogram that add up to at least 500 pulses. Each

train must consist of at least three pulses [50]. Oscillograms with less than 40 pulses are excluded [51].

3.2.4.5. Pulse Song: A description of the pulse song parameters and their calculations follows (Table 2-1 and Fig. 2-1):

(i) Cycles per pulse (CPP): A pulse is defined as a voltage signal with a distinct repetitive frequency [37; see 4.8]. To determine CPP, identify the beginning and end of the pulse (as defined above), count the number of "zero crossings" from the beginning to the end of the pulse and divide by two [46; but see 4.9]. Fig. 2-1 shows the zero crossings counted per pulse represented by "c" markings. Fig. 2-2A shows one example of CPP values calculated for two consecutive pulses produced by wild type OR (CPP= 3, 3), D*mlc*2^{+} (CPP= 2, 2.5) and D*mlc*$2^{\Delta 2\text{-}46}$ (CPP= 4, 4.5) males. The difference between the mutant and controls is visually evident.

(ii) Pulse length (PL): PL is defined as the time span from the beginning to the end of a pulse. Normally for a wild type *D. melanogaster* male (Canton S), the duration of one pulse is about 10 ms [35]. In the example shown here in Fig. 2-2A, both pulses produced by an OR male have PL values of 14 ms, comparable to PL value of 12 ms and 9 ms pulses produced by a D*mlc*2^{+} male. In contrast, the D*mlc*$2^{\Delta 2\text{-}46}$ male produces noticeably longer pulses of 17 and 20 ms (Fig. 2-2A).

(iii) Intrapulse frequency (IPF): IPF is the carrier frequency of a pulse train. IPF can vary broadly within and among flies with a normal range of 200 Hz to 400 Hz in wild type *D. melanogaster* flies [35, 37]. It is calculated manually by dividing CPP by PL

[Fig. 2-1]. IPF also can be obtained automatically in Goldwave, by selecting the entire pulse for Fast Fourier Transform (FFT), which converts the time varying waveform into a frequency spectrum with amplitude (decibels) in the y-axis. The frequency of the highest peak in the FFT spectrum is noted as the carrier frequency or IPF (see 4.10). Fig. 2-2 shows examples of IPFs for OR, D*mlc*2^{+} and D*mlc*$2^{\Delta 2\text{-}46}$. IPF for the mutant is in the same range as the wild-type and rescued control due to its proportional increase in CPP and PL.

(iv) Interpulse interval (IPI): IPI is the time interval between the start of two consecutive pulses and is calculated directly from the waveform tracing by measuring the distance between equivalent peaks in two successive pulses (Fig. 2-1). At 25^{o}C, the modal IPI of *D. melanogaster* is 35 ms [28, 34, and 37]. Mean IPI can be affected by temperature [31, 45 and 52]. Since pulses occur in trains which themselves have a broad range of length, it is important to set minimum and maximum cut-off values for IPI to determine the starts and ends of trains. The minimum and maximum cut-off values used for IPI are assigned somewhat arbitrarily at 15 ms and 80 ms (100 ms in [37]), respectively, based on empirical examination of many song files. Silence or noise between two pulses exceeding the maximum cut-off is considered a break between trains (i.e., intertrain interval) and the two pulses are considered to be in different trains [Fig. 2-1; 35, 37, 46 and 50]. Mean of means of pulse trains for IPI is calculated with the above cut-offs [50]. The IPI also cycles in a sinusoidal fashion by increasing and then decreasing to the starting value over a span of 50-60 s in *D.*

melanogaster. This is commonly referred to as the Kyriacou and Hall (K&H) cycle [43, 44 and 53]. Calculating the K&H cycle in 10 s time bins reduces the variability in the mean IPI [54]. The example shown in Fig. 2A indicates that truncating the myosin regulatory light chain (D*mlc*$2^{\Delta 2\text{-}46}$) has little to no effect on IPI.

(v) Pulse number (PN): Number of pulses are manually counted from each train of pulses and averaged over one song oscillogram (Fig. 2-1).

(vi) Pulse train length (PTL): Refers to the time interval from the start of the first pulse to the end of the last pulse in a train (Fig. 2-1). The IPI maximum cut-off criterion (80 ms in the examples provided here) is used to decide if two consecutive pulses are in the same or different trains. Fig. 2-1 show two successive pulses separated by 84 ms and therefore assigned to different trains, PTL1 and PTL2.

(vii) Pulse amplitude (PA): PA measurements are attempts to quantify a song's loudness [51]. This is calculated by measuring the waveform levels or states of the pulse in a pulse train. Amplitude is a relative measurement where a baseline value would need to be determined. The amplitude scale in the waveform tracing in Goldwave is unitless and maps the sound states to a linear range of -1 to +1, where zero is silence. With Goldwave it is possible to measure sounds up to 0 dB (– infinity dB is silence), or they can be measured in percentage scale where 100% is the maximum amplitude [42]. Amplitude can also be measured in millivolts. PA is influenced by the proximity of the male fly to the microphone and thus is difficult to measure reliably. Hence, absolute PA is not an accurate measure of pulse song loudness. The amplitude ratio, described next, provides a

more reliable indicator of song loudness. To determine PA, select the cycle with the highest amplitude within a pulse and measure the full height from peak to trough (shaded area in Fig 2-1, middle panel).

(viii) Amplitude ratio (AMP-RT): This parameter is a measure of the relative amplitude (loudness) of the sine song to the pulse song. Similar to PA, sine amplitude (SA) is measured from peak to trough. AMP-RT is then calculated by dividing SA by PA (Fig. 2-1). Events at the transition between a sine song and a pulse song should be chosen for calculation of AMP-RT, as indicated in Figure 2-1, as events separated in time may have occurred at different distances from the microphone [55]. The example in Fig. 2-2 shows that the AMP-RT of D*mlc*2$^{\Delta 2\text{-}46}$ (AMP-RT= 0.7) is greater than that of D*mlc*2^{+} (AMP-RT= 0.125) and the wild type strain (AMP-RT= 0.5), most likely resulting from a louder sine song.

(ix) PAUSE: This is the time interval between the end of one pulse and the start of the following pulse in a train, corresponding to a period of silence (Fig. 2-1). As seen in Fig. 2-2A, N-terminal truncation of the myosin regulatory light chain shortens PAUSE by 50%.

3.2.4.6. Sine Song: The sine song consists of a humming sound that oscillates sinusoidally and usually precedes the pulse song [Fig. 2-1; 7 and 8]. What follows is a description of sine song parameters and their calculations (Table 2-1 and Fig. 2-1):

(i) Sine song frequency (SSF): To measure SSF, first the sine song trains are identified manually by simultaneously listening to the soundtrack and examining the waveform

recording and using the method of "zero crossing rate" of the voltage signal [35, 50]. The portion of the recorded waveform with a constant rate and lasting a minimum of 175 ms is denoted as a sine song train [37]. This minimum time limitation is set to discard false positives [see 4.11; and 37]. For wild-type males, an additional criterion for scoring sine song is that the frequency is between 100 and 200 Hz [37]. After the sine song train is detected, the SSF is estimated by manually counting the number of cycles per second. The cycles are counted by noting the number of zero line crossings for a given time interval, then dividing by the time using 150-250 msec sample segments [34, 50]. To verify the fundamental frequency of the sine train, the entire train is selected and FFT spectrum is created automatically in Goldwave (see 4.12). FFT analysis reliably identifies the fundamental frequency among songs with variable frequency content (see 4.12) or with a noisy signal. Fig. 2-2B shows examples of SSFs, where the value for the mutant is considerably higher than that of the rescued control.

(ii) Sine song burst duration (SDUR): The beginning and end of a sine song are established from changes in the waveform as sine song are generally preceded and followed by a pulse. Examination of the waveform with playback audio provides further confirmation of the beginning and the end. Sine songs of short duration (see above) or low amplitude are not considered for SDUR calculation.

(iii) Sine amplitude (SA): SA is determined from peak to trough, as described above for PA.

3.2.5. Software options

Goldwave (v5.58) [42] contains many features that facilitate recording and analysis of courtship song, including real time graphic visuals of oscillograms. The oscillogram pictures are clear, allowing selection of waveform and easy retrieval of song parameters. Carrier frequencies are retrieved automatically on selected images by clicking on the "spectrum filter" option. The program also provides filters for noise reduction to clean up unwanted sounds (hiss, hums, pops or clicks) from the oscillogram. Noise filtering can reduce the overall sound quality and alter the waveform of the pulse and sine song, which may result in inaccurate song parameter values. We avoid noise filtering by recording songs in a noise-free environment following the conditions outlined in 3.2.2. Application of noise filters, if necessary, should be reported to ensure reproducibility.

Goldwave supports a variety of audio file formats, including WAV and MP3, and has user friendly file format conversion features and multiple undo levels. Goldwave is available only for Windows PC.

Any sound analysis software that provides clear waveforms of courtship songs and generates FFT spectrum can be used for analysis. Two other programs that have been used for analyzing *Drosophila* male courtship are Raven and Lifesong. Raven can perform similar functions as Goldwave but is more expensive. It produces crisp oscillograms with good resolution and has the ability to generate spectrograms [56]. Compressed file formats like MP3 are not well supported resulting in poor sound quality.

Raven can run in Windows, Linux, and MAC operating systems. Examples of its use in song analysis can be found in [57, 58]. Lifesong [46] and Lifesong X were specifically developed for use in *Drosophila* courtship behavior and song analysis. Lifesong X is a MAC operating system X-based program that can be obtained from Brandeis University (http://lifesong.bio.brandeis.edu/). An example of its use is found in reference [59].

4. TROUBLESHOOTING / NOTES:

4.1. Aging flies for 5-9 days have been reported to increase the amount of courtship song produced by mutant strains (e.g., *fruitless*) that normally produce less song [37]. *Drosophila* species other than *D. melanogaster* may require longer time to reach sexual maturity. For example, males of the obscura group species, specifically *D. persimilis* and *D. pseudoobscura*, should be aged for 8 days before testing [45].

4.2. D. melanogaster males perform several behaviors as part of the courtship ritual, the courtship song being an important one [29]. Virgin females aged 24 hrs or less have not reached sexual maturity and will not copulate, resulting in longer bouts of courtship behavior. If the experimental design requires copulation as an endpoint, then virgin females aged 3-5 days should be used.

4.3. An alternative approach for eliminating background noise would be to use two microphones, one for recording the song signal and the noise, and the other for recording the ambient noise only. This setup minimizes the need for sound insulation [60].

4.4. The INSECTAVOX gain should be set initially at 50%. If the noise level is too high, the gain level should be adjusted at a lower setting. If the noise persists, the external wiring of the INSECTAVOX should be checked for loose connections. Loose wiring may result in static or "pop" sounds, overdriving the microphone setup. Use of an additional external amplifier besides the in-built amplifier in the INSECTAVOX is not necessary and could lead to noise amplification.

4.5. Soundproofing can be achieved by noise reduction or absorption. Soft objects tend to absorb sound, and hard objects tend to reflect sound. To eliminate noise interference from a source inside the recording room, soundproofing the INSECTAVOX (or the object generating the noise) is often sufficient. This can be achieved by lining or encasing the INSECTAVOX in soundproof foam. A less cost-effective solution would be soundproofing the room if the noise source is outside the recording room. Methods for sound proofing a room are: barrier methods where the walls are lined with a thick, solid substance to reflect the external noise away from the room; absorbing methods where the walls are lined with a thick, soft substance to dampen and absorb noise. Egg carton shaped foam tiles can be used for this purpose.

4.6. Temperature increase due to the small light source at the microphone surface inside the INSECTAVOX was reported as less than 1^{o}C during a 30 minute session [27].

4.7. Fly walking or falling can have similar waveform tracing as a pulse train, but will invariably have different sound and frequency. A program that detects the signal peak amplitude and time that each pulse occurs has been described [35]. Sine song can be

detected by a program which measures the "zero crossing rate" of the waveform per unit time [37].

4.8. There can be three types of pulses based on number of cycles present: "normal pulses have ≤ 3 CPP and have a smooth amplitude envelope; "polycyclic" pulses have greater than 3 CPPs and are without severe intrapulse oscillation breaks; "broken" pulses have severe breaks in intrapulse oscillation denoted by their FFT spectrum showing multiple frequency peaks [37].

4.9. We followed the established approach of including the first and last "zero crossings" in calculating the CPP as this permits comparison of new results with published data. The correct number of cycles per pulse is obtained by subtracting one from the number of zero crossings ("c" markings in Fig. 2-1) divided by 2.

4.10. The pulse FFT spectrum refers to pulse attributes like the nature of the cycles within the pulse, IPF and a number of subjective qualities like general shape and symmetry of the pulse envelope [37]. Mutants such as *dissonance* and *cacophony* have been shown to have multiple peaks in their pulse FFT spectrum, interpreted as broken cycles due to a failure to control pulse wing beats [37]. Peaks in the spectrum with an amplitude of less than 20% of the highest amplitude spectral peak are not included in the analysis [37].

4.11. A high rate of false positives has been documented among short segments (< 150 ms) of waveform that generally fit sine song criteria [35].

4.12. Histogram plots of individual male SSF sometimes revealed events at frequencies

between 200 Hz and 250 Hz, i.e., within the range of the flight wing beat. These events are generally of low amplitude and often occur at the beginning and/or end of the main sine song event [35].

5. CONCLUSIONS

Techniques to document and quantify courtship song and mating behavior add a new dimension to the study of *D. melanogaster* flight muscle mutants and contribute to understanding the functional dichotomy of the IFM. Among *Drosophila* adult tissues, the IFM is one of the largest contributors to total body mass and a major consumer of metabolic energy. The energetic cost is justified given the IFM's contribution to behavioral strategies that affect individual fitness and life history traits, namely reproductive effort (mating song), and foraging and territorial behavior (flight). The well-established genetics of the IFM can be put to good use for dissecting the contributions of muscle proteins to the evolution of mating and flight, and to examine the trade- offs between two essential and distinct behaviors. The example presented here demonstrates that a mutation in the myosin regulatory light chain affects mating song parameters in addition to its well established effects on flight mechanics. Protocols such as the ones described here can be combined with other experimental paradigms (e.g., mate competition) to validate the effect of muscle mutations on mate selection and reproductive isolation.

6. SUPPLEMENTARY DATA

Supplementary files 1, 2 and 3 are representative male courtship song audio recordings with corresponding waveforms from wild type (OR), rescued null control (D*mlc2*$^{+}$) and the mutant (D*mlc2*$^{\Delta 2\text{-}46}$) strains and is linked to the online version of this paper http://www.sciencedirect.com/science/article/pii/S1046202311001721 . The original audios are 2.6 s (OR), 2.4 s (D*mlc2*$^{+}$) and 3.1 s (D*mlc2*$^{\Delta 2\text{-}46}$). The recordings were done at a sampling rate of 44.1 kHz with 16 bits of resolution and a bit rate of 705.5 kbps per channel.

FIGURE LEGENDS

Figure 2-1: Example of male courtship song of wild type *D. melanogaster* Oregon R strain. The top oscillogram shows one sine song burst and two pulse trains (PTL1 and PTL2) with PN equal to 15 and 13, respectively. The arrow indicates the beginning of the sine song. The asterisks mark two successive pulses assigned to separate trains because the interval separating them is greater than 80 msec. The middle panel is an expanded view of the section of the oscillogram marking the end of a sine burst and the beginning of a pulse train. The first two pulses of the first train are expanded on the bottom panel and the different parameters extracted from this information are indicated. The "c" markings in the bottom panel indicate zero-crossing points of the waveform within a pulse. See Table 2-1 for a list of abbreviations.

Figure 2-2: Comparison of pulse song (A) and sine song (B) parameters from the wild type strain (OR), the rescued myosin regulatory light chain strain (D*mlc2*$^{+}$), and the mutant strain expressing truncated N-terminal myosin regulatory light chain extension (D*mlc2*$^{\Delta 2\text{-}46}$). Evident differences are found in this example between the control rescued null and the mutant with regard to the following parameters: SSF, SA, PA, AMP-RT, PL, CPP, and PAUSE. See Table 2-1 for a list of abbreviations.

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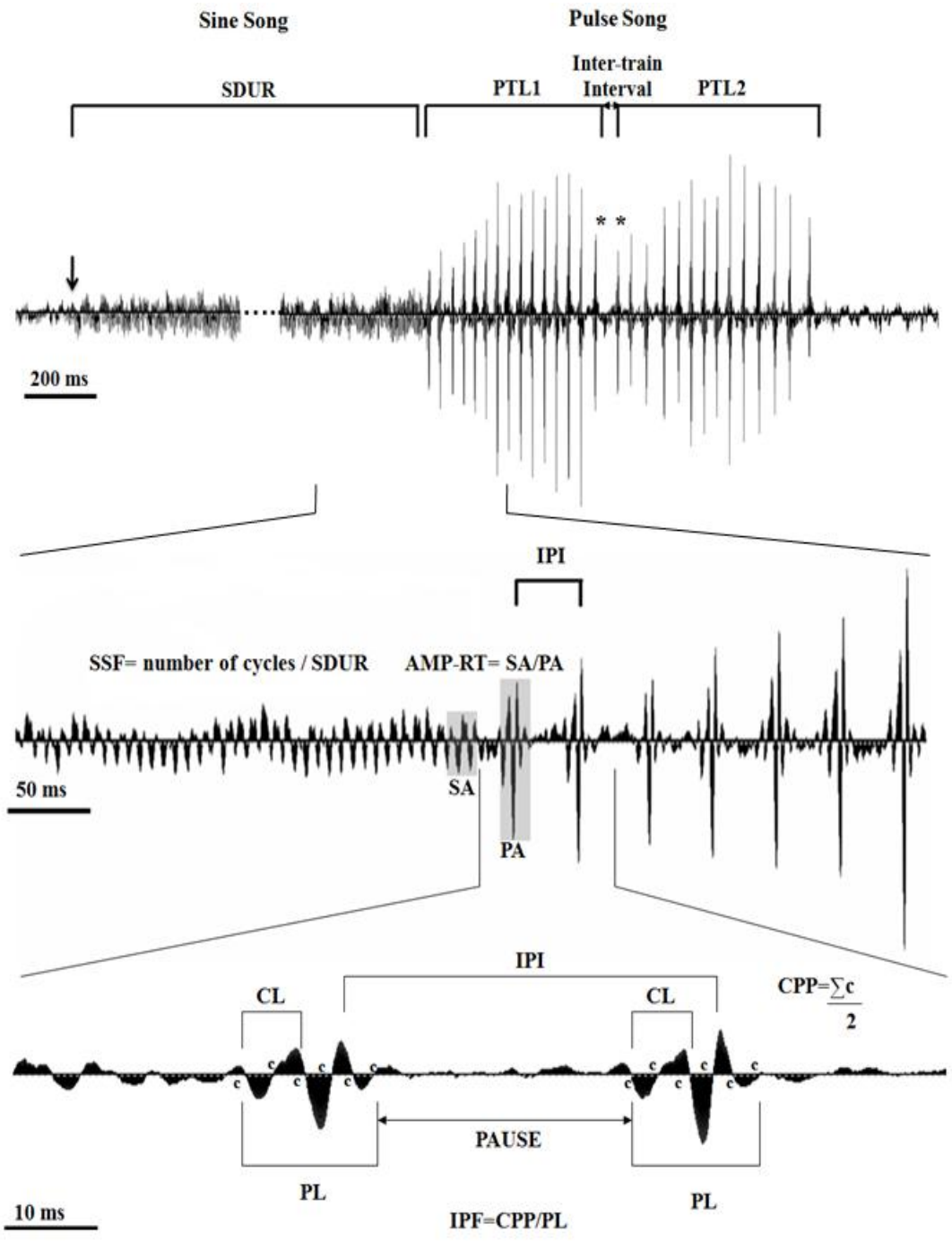

Figure 2-1. *Drosophila melanogaster* male courtship song

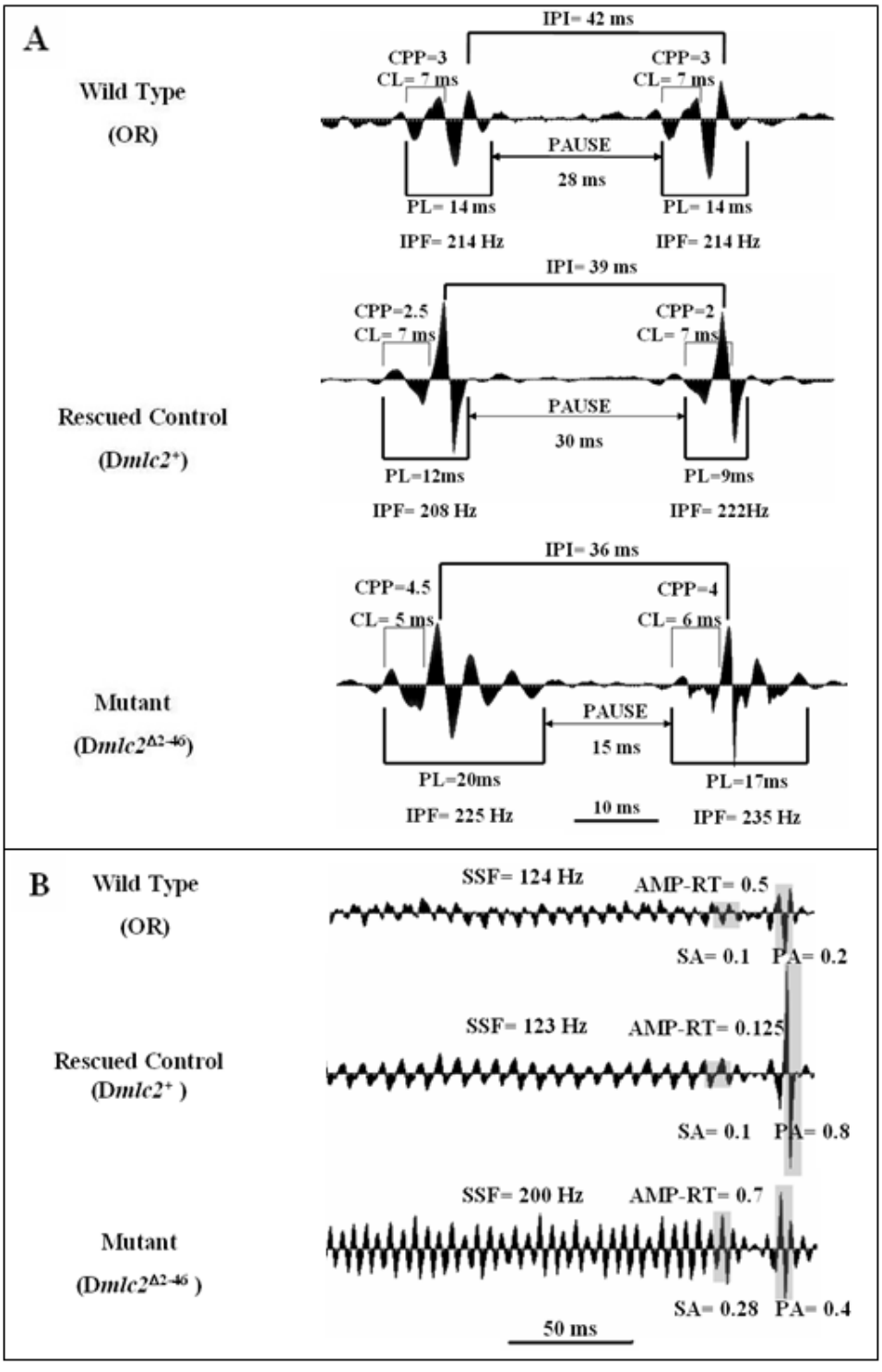

Figure 2-2. Pulse and Sine song of *D. melanogaster*

Table 2-1. Courtship song parameters

Parameter	Abbreviation	Description/Definition
Sine song burst duration	SDUR	Time duration (ms) of a sine song burst.
Sine song frequency	SSF	Carrier frequency (Hz) of a sine song burst; equivalent to the number of sinusoidal cycles divided by SDUR.
Sine amplitude	SA	Measure of the loudness (mV, dB, or arbitrary units) of sine song.
Pulse amplitude	PA	Measure of the loudness (mV, dB, or arbitrary units) of pulse song.
Amplitude ratio	AMP-RT	SA/PA.
Pulse duty cycle	PDC	Equivalent to the ratio of pulse song to the total time of recording (song + silence).
Pulse train length	PTL	Time duration (ms) of a pulse song train.
Pulse number	PN	Number of pulses in a pulse song train.

Pulse length	PL	Time duration (ms) of a pulse.
Cycles per pulse	CPP	Number of zero crossings by the pulse waveform divided by two.
Intrapulse frequency	IPF	Carrier frequency (Hz) of a pulse; equivalent to CPP/PL.
Cycle length	CL	Time duration (ms) of one cycle in a pulse.
PAUSE		Time duration (ms) between the end of one pulse and start of the next pulse in a train.
Interpulse interval	IPI	Time duration (ms) between the equivalent peaks of two consecutive pulses in a train; equivalent to PL+PAUSE.
Kyriacou and Hall cycle	K&H Cycle	Period (ms) of the IPI cycle.

Fast Fourier Transform	FFT	Frequency spectrum profile of a pulse song and sine song indicating carrier frequencies (IPF and SSF, respectively) and additional harmonics, if present.

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CHAPTER 3 JOURNAL ARTICLE

An N-terminal Deletion of the Orphan Gene Flightin Reduces Myofilament Lattice Spacing: Implications for Flight and Mating Behaviors in *Drosophila*

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ABSTRACT

As with most flying insects, the asynchronous, indirect flight muscles (IFM) of *Drosophila* are characterized by their remarkable crystalline myofilament lattice structure that has evolved for powering skilled flight to survive. Also the IFM, gets neurally stimulated for male courtship song, a sexually selected pre-mating behavior for reproduction. It is not known how physiologically and genetically IFM generates two distinct behaviors under separate evolutionary schemes. Flightin, a 20kDa orphan myosin-binding protein that in *Drosophila* is exclusively expressed in the IFM, is required for muscle structural integrity and flight. The flightin amino (N)-terminal sequence (~63 aa in *D. melanogaster*) is highly variable, unlike the rest of the *Drosophila* protein. Given the fast evolutionary trajectory and functional contribution of orphan genes in novel adaptive species-specific traits, here we explore the hypothesis that the flightin N-terminal region is highly variable to optimize the two distinct IFM driven behavioral performances. We investigated the function of the flightin N-terminal region by creating transgenic *Drosophila* expressing a truncated flightin missing first 62 aa ($fln^{\Delta N62}$). By electron microscopy, fourier image analyses, muscle fiber mechanics and behavioral studies, we show that the flightin N-terminal region is not essential for IFM function, but critically required for maintaining myofilament lattice spacing and crystallinity for optimal force transmission required for skilled flight. Moreover, we found that the maintenance of the lattice structure by flightin N-terminal region is required for tuning the muscle for sexually selected timely rhythms of courtship song, notably interpulse interval (IPI) implicated in male reproductive success. Together these

results suggest that flightin N-terminal region is required for optimizing the biological performance of IFM in both flight and song. This signifies the importance of orphan genes like flightin in the diversification of flying insects and to be incorporated in versatile systems like *Drosophila* IFM to fulfill its adaptive functions.

AUTHOR SUMMARY

Structural form gives function in biological systems. Elucidating the function of motor proteins critical for optimizing muscle structural properties will not only help us to understand contractile mechanism and evolution of muscle-driven complex behaviors, but also develops our knowledge in the pursuit for creating biologically-inspired materials for the future. The majority of flying insects, by far the major species in our planet, has evolved to have a highly regular muscle lattice structure for their asynchronous mode of flight muscle contractions for powering flight to survive. Moreover, among them in the *Drosophila spp.*, a more derived group, the asynchronous indirect flight muscles (IFM) are further utilized for producing male courtship song by wing vibrations, a reproductive behavior distinct from flight. Here we show that a highly variable potentially fast evolving region of an orphan gene flightin optimizes the *Drosophila* IFM lattice regularity required for normal stiffness and power output which optimizes both flight and species-specific courtship song parameters, explaining its hypervariability possibly due to evolutionary positive selection. This work signifies the importance of orphan genes to be incorporated in tissue systems for their versatile functions as in IFM. This work also reinforces the importance of thick filament

associated proteins like flightin in filament stiffness and myofilament lattice regularity for optimal performance of muscel tissue systems.

Blurb

Structural, physiological and behavioral studies reveal a possible fast evolving domain of an orphan gene flightin, as an evolutionary innovation for dictating *Drosophila* indirect flight muscle structural regularity for optimizing flight and courtship behaviors.

INTRODUCTION

Understanding how complex behaviors for distinct organismal needs are manifested mechanistically and at the molecular level is fundamental to biology. Organisms perform complex behaviors that could be subject to opposing evolutionary selection pressures (natural and sexual selection) [1,2]. Moreover, to fulfill species-specific behavioral requirements, molecular innovations like the "orphan" or taxonomically restricted genes with species-specific functions have evolved [3,4]. Therefore, it is important to understand these molecular innovations and functions by which distinct behaviors could be generated for species-specific survival and reproduction.

Flight is one of the major innovations in species survival and diversification in the history of life subject to natural selection. The evolution of flight has facilitated the survival and diversification of insect species including *Drosophila*, which has involved integration of metabolic, morphological and complex behavioral adaptive evolutions [5,6]. Skilled

flying insects including *Drosophila spp.* have been shown to have evolved a crystalline, highly regular thick and thin filament lattice organization in the asynchronous indirect flight muscles (IFM) responsible for powering flight [7-9]. With the fastest kinetics of any acto-myosin cross-bridge cycle [10], the *Drosophila* asynchronous IFM twitching is synchronized to high wing beat frequencies of ~200 Hz, even with a much lower rate (~ 5Hz) of motor neuron activation [11,12]. The IFM fulfills this by using the mechanism of stretch activation and shortening deactivation at a relatively constant [Ca^{2+}] [13]. The IFM consists of two antagonistic sets of muscles, the dorsal longitudinal and dorsal ventral muscles (DLM and DVM), connected to the thoracic exoskeleton rather than the wing hinge [14,15]. These muscles function together to create a reciprocally activating resonant thoracic box [16,17] driving the large sweeping motion of the wings [18-20]. This impressive system has evolved multiple times in insects and is correlated with adaptive radiation of body miniaturization [20].

For reproductive behaviors, species-specific acoustic communication signals are critically important [21-29], facilitating pre-mating reproductive isolation and subsequent speciation [30] of both vertebrates and insects. *Drosophila spp.* speciation has occurred through various mechanisms [6]. One of them is sexual isolation via male courtship song consisting of rhythmic pulses and sinusoidal hums generated by small amplitude wing vibrations using the thoracic musculature [31-35]. Each *Drosophila* species has unique song characteristics [35-37]. Courtship song is immensely variable across *Drosophila spp.* from very high frequency (>6 kHz) pulses of the exotic Hawaiian *grimshawi* subgroup [38] to low frequency sine songs (~150 Hz) of some *melanogaster* subgroup

members. This variability in song characteristics facilitates con-specific mating, reproductive isolation and female sexual stimulation. For example, the interpulse interval (IPI) of the pulse song is highly variable carrying the most salient species-specific signal throughout *Drosophila* [35,39,40], whereas pulse singing vigor [40] and sine song frequency [41,42] stimulates *D. melanogaster* females.

The common tissue system involved in both these complex behaviors of flight to survive and courtship song in *Drosophila* is the IFM. Along with the IFM's role in flight (see above), it is neurally activated during male singing [43], indicating that it is involved in courtship song generation. Although *Drosophila* male courtship song has been an ideal system for extensive studies on the neuronal basis of complex behaviors [44-51], how the song generation is controlled by the IFM and its constituent motor proteins has not been previously attempted. In addition, none of the genes affecting courtship song identified through classical genetic approaches are among the ones identified by quantitative genetic approaches [37], indicating that our understanding of genetic basis of courtship song has still a long way to go.

Therefore it is not known how genetically and physiologically the same tissue system (IFM) generates high power for flight and wing vibrations for courtship song. As a step towards understanding this, the questions that arise are: What genetic evolutionary innovations IFM has utilized to fulfill its dual functionality? How do the innovations in the genetic level modulate the structural order and mechanical properties of the IFM to optimize its functions in flight and song? In nature, muscle tissues from different species have been previously shown to have dual functionality with distinct mechanisms. For

example, honeybee antagonistic flight muscles contract simultaneously for warmup behavior and alternately for flight [52], whereas different firing patterns from same motor neurons can cause stridulations or flight in crickets [53]. Given the roles of orphan genes in species-specific functions and evolution [4,54], here we investigated the role of flightin, an orphan gene [54] that in *Drosophila* is expressed exclusively in the IFM [55], in muscle structure, function and IFM driven complex behaviors (flight and courtship song).

Flightin is a ~20 kDa (182 amino acids) protein binding the myosin rod as shown *in vitro* [56] and as suggested by *in vivo* studies [57-60]. Genetic ablation of flightin expression causes >30% longer and 30-45% more compliant thick filaments than normal [61]. These result in severe sarcomere degradation, fiber hypercontraction, and structurally and mechanically compromised IFM unable to generate force and power, rendering the fln^0 flies flightless but viable [59,62]. The extreme hypercontractions due to compromised IFM structural and mechanical properties with flightin's absence or reduced expression [57,59,62,63] suggests that flightin is crucial for normal thick filament assembly during development and for myofilament lattice integrity, manifested from the filament to the whole fiber level. All of the structural, physiological and flightless phenotypes are completely rescued by a full length flightin transgene under an IFM-specific Act88F promoter [64] indicating that flightin is essential for IFM structure and function. Moreover, truncation of the flightin COOH-terminus also abolishes flight even with partial rescue in IFM structural and mechanical properties compared to that in complete absence of flightin [65].

Comparative flightin sequence analysis of 12 *Drosophila* species encompassing about 42 million years of evolution, reveals a dual organization (Figure 3-1). The flightin N-terminal region (amino acids 1-63 in *D. melanogaster*) is highly variable having only 20-23% identity (14 identical positions out of 61 or 71), which is in stark contrast to the rest of the protein with about 79% identity (93 identical positions out of 118). Moreover, the flightin N-terminal region appears to be under distinct selection pressure with potential signatures of positive selection and a higher average rate of evolution compared to the rest of the gene (Figure S3-1). The variability in the N-terminal region is intriguing given that no other muscle genes have been reported to be fast evolving or having putative signatures of positive selection. It is known that most genes involved in reproductive processes are fast evolving [66-68] and given the extensive variability in courtship song among Drosophilids, it is not unreasonable for genes that determine muscle contractility to be under positive or sexual selection. This led to our hypothesis that IFM utilizes flightin as an evolutionary innovation i.e., the flightin N-terminal region is evolving fast due to positive selection to fine-tune the IFM for species-specific courtship song generation, whereas the conserved rest of the protein is under purifying selection to fulfill the essential function of flight. To test our hypothesis, we generated a new transgenic line expressing flightin with the 62 N-terminal amino acids removed (highlighted region of Figure 3-1) and characterized the line from the level of myofilament structure to whole organismal behavioral performance. The findings show that flightin N-terminal region is not essential for IFM function, but required to optimize the muscle structural lattice and biological performance of IFM for flight and song. This

could possibly suggest a dual selection pressure on the flightin gene used as an evolutionary innovation for IFM function.

RESULTS

Generation of *Drosophila* Transgenic Lines Expressing an N-terminal Truncated Flightin

To investigate the functional properties of the N-terminal region of flightin, we generated transgenic lines expressing a flightin construct missing amino acids 2 through 63 (Figure 3-1). Five lines with independent second chromosome insertions were tested for their flight ability and wing beat frequency. All of the lines behaved similarly to full length flightin rescued control null strain, fln^{+} flies [64] indicating that the mutated transgene does not have a dominant negative effect (data not shown). We next crossed each transgenic to fln^{0} to generate lines expressing an N-terminal truncated flightin in the absence of endogenous flightin (see Materials & Methods). The lines were found not to differ significantly from each other in protein expression and flight performance (data not shown). Two lines, $fln^{\Delta N62A}$ and $fln^{\Delta N62B}$, were chosen for this study and the data combined (herein referred to as $fln^{\Delta N62}$) when the muscle structural and mechanical analyses (described below) showed they did not differ from one another.

The mutant flightin construct codes for a 120 amino acid protein with a predicted molecular mass of 14,381 Da, compared to 20,656 Da for the full-length flightin [55]. One dimensional SDS-PAGE (Figure 3-2A) and western blot analysis (Figure 3-2B) of proteins extracted from skinned IFM fibers from $fln^{\Delta N62}$ and fln^{+} flies

show that the truncated flightin is expressed and incorporated in the myofibril. As predicted from its theoretical molecular mass, the truncated protein migrates further (~15 kDa) than full-length flightin, which typically migrates at ~26 kDa [55]. The N-terminal truncated flightin is recognized by an anti-flightin polyclonal antibody, albeit not as strongly as the full length flightin (Figure 3-2B). This is not unexpected given that the truncation removed ~1/3 of the flightin sequence.

fln$^{\Delta N62}$ Flies are Flight Compromised

fln$^{\Delta N62}$ flies are capable of flight indicating that an N-terminal truncated flightin is sufficient to restore flight ability of *fln*^{0} flies [59]. Compared to *fln*$^{+}$, *fln*$^{\Delta N62}$ flies show a ~33% decrease in flight ability (flight score: 2.8±0.1 vs 4.2±0.4 for *fln*$^{+}$; Table 3-1). That the mutant flightin is not capable of fully restoring flight ability is also evident in the decreased proportion of flies that are able to fly, 82% vs 95% for *fln*$^{+}$. The flight impairment is not due to a change in wing beat frequency as it was found to be similar between *fln*$^{\Delta N62}$ and *fln*$^{+}$ flies (195 ± 4 Hz vs 198 ± 2 Hz, respectively). However, 10% of the *fln*$^{\Delta N62}$ flies tested did not produce a wing beat, while 100% of *fln*$^{+}$ flies did (Table 3-1).

fln$^{\Delta N62}$ Males Produce Abnormal Courtship Song Characteristics.

Single male-female pair courtship song recordings revealed that flightin mutant male flies produce no song (*fln*^{0}) or an abnormal song (*fln*$^{\Delta N62}$) when compared to control, flightin null rescued male flies (*fln*$^{+}$; Figure 3-3 and Audios S3-1 and S3-2). Closer examination of the oscillograms revealed that the N-terminal deletion affects the sine

song as well as the pulse song (Figure 3-4A,B). The frequency of the sine song produced by $fln^{\Delta N62}$ is significantly higher than that produced by control males (228 ± 5.5 Hz vs 148 ± 5.2 Hz for fln^{+}; Figures 3-4C). In contrast, the intrapulse frequency is not affected by the mutation (Figure 3-4D). However, other aspects of the pulse song produced by $fln^{\Delta N62}$ males are abnormal including a greater number of cycles per pulse (3.9 ± 0.4 vs 2.4 ± 0.1 for fln^{+}; Figure 3-4E), longer pulse length (12.8 ± 1.5 ms vs 8.6 ± 1.2 ms for fln^{+}; Figure 3-4F), longer average interpulse intervals (56.1 ± 2.5 ms vs 36.7 ± 0.7 ms for fln^{+}; Figure 3-4G), and lower pulse duty cycle (2.5 ± 0.4 % vs 7.3 ± 0.4 % for fln^{+}; Figure 3-4H). In addition, pulse songs produced by $fln^{\Delta N62}$ males are characterized by a wider range of interpulse intervals (IPI) than pulse songs produced by fln^{+} males (Figure 3-5), indicating that $fln^{\Delta N62}$ males are unable to maintain the proper timing of their pulses across trains.

Mutant Flightin Reduces Mating Competitiveness in Males

We performed mating assays to determine if the song abnormalities observed in the $fln^{\Delta N62}$ males affect their courtship behavior and mating success. In single pair mating assays (see Materials and Methods), $fln^{\Delta N62}$ males were able to perform the courtship ritual with the same efficiency as control fln^{+} males, as determined by the courtship index and wing extension index (Figure 3-6A,B and Videos S3-1 and S3-2). In contrast, $fln^{\Delta N62}$ male courtship efficiency decreases markedly in the presence of a fln^{+} male in mating competition assays (see Materials and Methods). When presented with a choice of $fln^{\Delta N62}$ and fln^{+} males, a wild-type (OR) female chose the fln^{+} male 92% of the time. The female

moved away and displayed aggressive rejection behaviors towards the $fln^{\Delta N62}$ male such as kicking with hind and mid legs (Video S3-3). The reduced courtship success of $fln^{\Delta N62}$ males may result from a significant (~75%) reduction in the courtship index (0.049 ± 0.01 vs 0.2 ± 0.05 for fln^{+}) and wing extension index (0.005 ± 0.001 vs 0.025 ± 0.007 for fln^{+}; Figure 3-6C,D and Video S3-3).

Mutant Flightin Affects Sarcomere Structure and Myofilament Lattice Properties

IFM sarcomeres in adult fln^{0} flies are highly disrupted, with no discernible Z bands or M lines [59]. All sarcomeric defects are completely reversed by re-introducing the full-length flightin transgene (fln^{+}) [65, and Figures 3-7A,B and 3-8A-C]. Expression of an N-terminal truncated flightin also results in substantial improvement in sarcomere structure, but the rescue is not complete. Sarcomeres in $fln^{\Delta N62}$ IFM are ~13% shorter than in fln^{+} (2.86 ± 0.01 μm vs 3.30 ± 0.01 μm, respectively; Figure 3-7A vs 3-7C; Table 3-2). They are also characterized by the absence of the H-zone and a narrower M-line that shows occasional gaps (Figure 3-7B vs 3-7D). The average cross sectional area of the myofibril is similar compared to fln^{+} (Table 3-2). However, $fln^{\Delta N62}$ myofibril cross-sections are characterized by an ~11% increase in the number of thick filaments (903 ± 25 vs 810 ± 18 for fln^{+}; Table 3-2). The myofilament lattice organization appears to be more compact and less regular than that of fln^{+}, which shows the characteristic double hexagonal arrays of evenly spaced thick and thin filaments typical of wild-type IFM (Figure 3-8B vs 3-8E). The myofibrillar area per fiber cross-section is higher in the $fln^{\Delta N62}$ fibers compared to fln^{+} fibers (45 ± 1 % vs 39 ± 2 % for fln^{+}; Table 3-2).

To quantify the difference in myofilament lattice structure, we conducted digital two-dimensional fourier transform of the cross section EM images. The corresponding power spectra (Figure 3-8C,F) show reflections that are of lower intensity in the mutant than in the control. Harmonics of the lattice reflections in the fourier power spectrum that are clearly resolved in fln^{+} are absent in $fln^{\Delta N62}$ (see also Table 3-3). By indexing the reflections to those of an hexagonal lattice, we obtained $d_{1,0}$ and calculated the inter-thick filament distance (see Materials & Methods, Method MS2 and MS3). The results, summarized in Table 3-3, show that the flightin mutation decreases $d_{1,0}$ and inter-thick filament spacing by ~ 11%. Moreover, the standard errors of means are three to four times higher in the mutant suggestive of greater heterogeneity in the myofilament lattice.

To gain further insight into the regularity of myofilament lattice, we measured the peak intensity and the half width of the 1,0 plane spots. $fln^{\Delta N62}$ myofilament lattice fourier spectrum 1,0 spots were of lower intensities with wider half-width compared to control fln^{+} (Table 3-3). Altogether, the myofilament lattice structural organization and order are reduced in the $fln^{\Delta N62}$ myofibrils compared to that of fln^{+}.

Mutant Flightin Affects Mechanical Performance of IFM Fibers

Deletion of the flightin N-terminal region resulted in a nearly 50% reduction in passive and active isometric tension and more than 60% reduction in rigor tension (Table 3-4). The elastic modulus for $fln^{\Delta N62}$ fibers in relaxed and rigor conditions were decreased at all frequencies tested compared to fln^{+}(Figure 3-9A,B). The viscous modulus for $fln^{\Delta N62}$ fibers was decreased at frequencies between 40 Hz and 650 Hz in relaxed

condition (Figure 3-9C), whereas decreased at all frequencies tested in rigor condition (Figure 3-9D), compared to *fln*$^{+/-}$. In addition, compared to *fln*$^{+}$ fibers, a slightly larger proportion of the *fln*$^{\Delta N62}$ fibers were unable to withstand tension in rigor conditions (20% vs 13% for *fln*$^{+}$), resulting in tearing or complete breakage of the fiber. The mutant fibers exhibited significantly lower net rigor yield strength before onset of breakage compared to that withstood by control fibers (1.6 ± 0.1 kN/m^2 vs 5.3 ± 0.4 kN/m^2 for *fln*$^{+}$; Table 3-4).

To gain insight into the structural flaws that may underlie fiber failure in rigor, we examined fibers by electron microscopy to compare the structure of sarcomeres from normal and mutant fibers at and away from the breakage site. The breakage site sarcomeres in *fln*$^{\Delta N62}$ fibers exhibited greater distortions in the Z bands and M lines than breakage site sarcomeres in control fibers (Figure S3-4). Additionally, thick filaments in the mutant sarcomeres tended to buckle, a feature not seen in control sarcomeres. These features are unique to the breakage site as they were not detected in mutant or control sarcomeres away from the breakage site (Figure S3-4).

At maximal calcium activation (pCa 4.5), the *fln*$^{\Delta N62}$ fibers had a smaller elastic moduli at all frequencies tested and a smaller viscous moduli from 75-280 Hz (Figure 3-10A,B). Figures 3-10C,D show that *fln*$^{\Delta N62}$ fibers had reduced maximum oscillatory work (0.2±0.02 Joules/m^3 vs 0.45±0.05 Joules/m^3 for *fln*$^{+/-}$) and power (38±4.6 Watts/m^3 vs 89±9.5 Watts/m^3 for *fln*$^{+/-}$) output, while the corresponding frequencies of maximum work

and power were similar (171±8 Hz, 205±7 Hz for $fln^{\Delta N62}$, and 179±8 Hz, 217±7 Hz for fln^{+}, respectively).

DISCUSSION

This study demonstrates that a hypervariable N-terminal region of flightin, a taxonomically restricted (orphan) thick filament associated protein, is required for optimal myofilament lattice organization typical of asynchronous muscles, which in turn dictates i) muscle fiber mechanical performance to generate optimal power output for normal flight independent of myosin kinetics, and ii) timely rhythmic contractions to produce species-specific male courtship song. Moreover, to our knowledge this is the first study where the role of a muscle gene on courtship song generation has been investigated, opening up a new area of study. The findings exemplify how biological tissue systems like the *Drosophila* IFM might incorporate species-specific orphan protein or protein domains that could possibly be under distinct selection regimes, in order to perform complex behavioral needs like flight and courtship song. As will be discussed below, we hypothesize that, in order to fulfill IFM's need to perform two distinct behaviors, the orphan gene flightin is under dual selection pressure for it to be used as a behavioral innovation. We also discuss the possible evolutionary trajectory of the flightin N-terminus in relationship with its function for flight and song behaviors based on the implications of the findings in this study.

Flightin N-terminus is Not Essential for Myosin Binding, Basic IFM Structure and Flight

The N-terminal region of flightin is not essential for flightin expression or incorporation into the thick filaments of the sarcomere (Figure 3-2). The reduced intensity of the lower molecular mass N-terminal truncated flightin band compared to that of full length flightin band in the western blot (Figure 3-2B) is possibly due to 34% truncation of the protein resulting in lower affinity of the antibody, but not due to lower functional expression. Previously, a flightin deficiency heterozygote mutant [*Df(3L)fln1*] showed ~20% reduction in flightin expression due to presence of only a single copy of the gene [69]. IFM structural and mechanical properties are different between these two lines, with the $fln^{\Delta N62}$ fibers showing much improved myofilament organization, reduced peak power output without change in myosin kinetics unlike that of *Df(3L)fln1* fibers which show disorganized myofilaments, unchanged peak power output with increased myosin kinetics. Also, the indices of the flight ability of the two lines are different. If the flightin N-terminal region truncation phenotypes are result of lower functional expression rather than the truncation *per se*, the $fln^{\Delta N62}$ line phenotype should have been similar to that of the *Df(3L)fln1*, which is not the case. Hence, it is a fair conclusion that the N-terminal truncation of flightin does not reduce protein expression.

Previously, it has been shown that the COOH-terminal truncated flightin was expressed and incorporated in the sarcomeric thick filaments in a fln^{0} background [$fln^{\Delta C44}$: 65], suggesting that both N-terminus and COOH-terminus of flightin do not possess critical amino acid sequences for binding the myosin rod. Together, this suggests

that the overlap sequence (amino acids 64-137 in *D. melanogaster*) of the two mutants is the site for thick filament incorporation of flightin.

$fln^{\Delta N62}$ is the first flightin mutation where flight is not abolished (Table 3-1) most likely due to a substantial structural rescue (Figures 3-7 and 3-8) , suggesting that the N-terminus is not an essential region for flightin's basic role in structure and stretch activated contractile function for flight. Previously, fln^{0} flies, created by genetic ablation, were unable to fly due to severely disrupted sarcomeres having Z-band breakdown and complete loss of M-line [59]. Moreover, $fln^{\Delta C44}$ flies were also unable to fly with only partial rescue of the fln^{0} aberrant structural phenotypes [65]. Although $fln^{\Delta N62}$ muscle ultrastructure has subtle aberrations compared to fln^{+} (discussed below: Figures 3-7 and 3-8, Tables 3-2 and 3-3), the overall muscle integrity and sarcomere stability is greatly improved over that of fln^{0} (1.5-3 days old) and $fln^{\Delta C44}$. This structural rescue is evident in the ability of $fln^{\Delta N62}$ flies to generate wing beat frequency similar to fln^{+} control flies (Table 3-1) suggestive of similar myosin kinetics (Figures 3-10C-D dotted lines) and hence they fly albeit with some impairment (Table 3-1).

Flightin N-terminus is Required to Maintain Sarcomere Geometry

During development, IFM thin filaments have been shown to stop growing once the H-zone is reached [70], consistent with the dynamic model of IFM filament assembly [71]. Hence in this study, a faint to no discernible H-zone in the mutant (Figure 3-7D) could indicate that it is the thick filaments that have grown into the H-zone area driving thin filament growth as well, since thick-to-thin filament interaction possibly through

myosin heads is required for defining filament length [72]. Additionally, the data also indicate that the overlap of the anti-parallel thick filament rod-only region in the sarcomere is shorter in the mutant, leading to a possibly very short H-zone that we are not been able to detect (Figure 3-7D). Alternatively, since the mutant sarcomeres are shorter compared to the control (Table 3-2, Figure 3-7), the thin filaments could have grown into the H-zone in the mutant independent of the thick filaments. A wavy, inconsistent M-line (Figure 3-7D) along with no detectable H-zone indicates that the thick filaments are not in optimal alignment in the middle of the mutant sarcomere and that the flightin N-terminus maintains the normal inter-filament overlap. An alternative possibility is that the flightin N-terminus interacts with an M-line component. Hence, without this interaction in the mutant, the M-lines would be wavy and inconsistent causing misaligned, less stable thick filaments affecting their length. However since flightin has been shown to be excluded from the M-line region and the bare zone [73], we consider this possibility unlikely.

Assuming no change from a normal IFM fiber length in the $fln^{\Delta N62}$ thorax, the shorter sarcomeric length (Figure 3-7 and Table 3-2) indicates a lesser number of myosin molecules (≡ number of myosin heads) per sarcomeric unit in the mutant (since IFM sarcomere length is determined primarily by thick filament length). But image analysis revealed that $fln^{\Delta N62}$ have ~11% greater number of thick filaments (≡ number of myosin heads) per myofibril cross-section (Table 3-2) which is comparable to the extent at which sarcomere length is reduced (13%, Table 3-2). This attractive correlation indicates that flightin N-terminus has no effect on the incorporation of the final number of possible

heads or myosin motors per sarcomeric unit, but is involved in the nature of the thick filament assembly process in the developing muscle. Sarcomere shortening and greater thick filament number per myofibril cross-section (Figure 3-7, 3-8 and Table 3-2) of the mutant and their correlation suggest that flightin N-terminus maintains the normal geometry of the sarcomere by influencing the nature of the thick filament assembly. This interpretation agrees well with the previous findings that flightin regulates thick filament assembly process [59,73].

Flightin N-terminus Maintains Normal IFM Myofilament Lattice Spacing and Regularity Possibly by Facilitating Inter-Filament Electrostatic Interaction

How does flightin N-terminus regulate normal filament assembly and sarcomeric geometry? We found here that flightin N-terminus establishes or maintains $d_{1,0}$ as suggested by their ~11% decrease in the mutant (Figures 3-8B,C and E,F, Table 3-3). The inter-thick filament spacing value for the control fln^{+} obtained here is 11.56% smaller than previously reported from *in vivo* X-ray diffraction measurements of live flies (56.2 ± 0.1 nm in [65] vs 49.7±0.4 nm, Table 3-3). This is due to lattice shrinking by dehydration steps performed for electron microcopy sample preparation (see Materials and Methods). There is also a greater lattice spacing heterogeneity in the mutant compared to control (SEM of $d_{1,0}$ and inter-thick filament spacing in Table 3-3) indicating further that the filaments are not in optimal alignment. Moreover, the reduced lattice regularity in the mutant (Table 3-3 resolution and sharpness, also see Materials and Methods) indicates further that the myofilament lattice spacing is heterogeneous with the regularity (or crystallinity) being compromised. We hypothesize that by maintaining

normal lattice spacing and regularity, the flightin N-terminus regulates thick filament assembly process and hence overall sarcomere geometry.

Thick and thin filaments are known to polymerize separately at the start of the sarcomerogenesis process, before they incorporate into a structural lattice that aligns them into an ordered double hexagonal array [74,75]. As an exception to other muscle genes, flightin expression [57] and its phosphorylation pattern [60] is highly affected by mutations in genes coding for proteins in both filament types, indicating it possibly interacts with both filaments. Moreover, the flightin N-terminal region (63 aa) is highly acidic (Asp/Glu rich) compared to the rest of the protein which is highly basic (Arg/Lys rich). Additionally, the flightin N-terminus (63 aa) is predicted to be an intrinsically disordered region compared to rest of the protein (Figure S3-3) indicating that potentially this region could be extending out of the thick filament backbone surface with a high negative charge for an estimated 27 nm maximal length (theoretical bond length calculation: Cα-N= 0.145 nm × 63 amino acids + Cα-C= 0.153 × 63 amino acids + C-N= 0.133 × 62 amino acids) sufficient enough to reach thin filament surface (thick filament surface-to-thin filament surface distance ~ 18 nm, [76]). We propose a structural model (Figure 3-11), where the flightin N-terminus containing acidic residues with high negative charge (pI 3.78; [55]) could be extending out from the thick filament backbone and enhancing the charge distribution field of the thick filaments, and possibly interacting with the corresponding thin filament through electrostatic repulsive force (since actin is negatively charged). This inter-filament electrostatic interaction could potentially maintain normal lattice spacing between thick and thin filaments (and hence inter-thick

filament spacing). This in turn could regulate the normal nature of thick filament assembly and the overall geometry of the sarcomere. The highly acidic nature and marked similarity of the extreme N-terminal end of flightin with actin [55] supports the above model in that electrostatic charges of similar amino acids repel each other strongly. Taken together, these suggest that flightin N-terminus is important for maintaining normal IFM lattice spacing and regularity (crystallinity) for optimal sarcomere geometry, possibly by facilitating inter-filament electrostatic interaction.

Flightin N-terminus Maintains Normal IFM Myofilament Lattice Stiffness for Optimal Force Transmission

Previous measurements on fln^0 fibers indicated that the reduction of filament stiffness [61] could possibly lower the rate of force development [62]. Here, we observe that without the flightin N-terminal region, reduced lattice spacing and lack of lattice regularity compromises uniform and timely transmission of force either radially across or longitudinally through myofibrils causing a marked reduction in maximum work and power production at single IFM fiber level (Figures 3-10C,D), affecting normal flight (Table 3-1). The question arises as to how abnormal sarcomeric geometry and lattice irregularity causes compromised underlying fiber performance? The effect of flightin N-terminus truncation on myofilament lattice organization is reflected physiologically in single IFM fibers in that a proportionally similar (~50-60%) reduction in passive, active and rigor isometric tensions (Table 3-4) and viscoelastic properties (Figures 3-9 and 3-10A,B) is observed. Since the mutation affects isometric tension and moduli by similar levels regardless of whether cross-bridges are strongly attached (active and rigor

conditions) or weakly attached (passive condition) without affecting myosin kinetics (Figures 3-10C,D dotted lines), the mutation has minimal effect on the number or stiffness of cross-bridges and is possibly affecting some passive structural element(s). Since *Drosophila* IFM thick filaments are reported to be about 17 times stiffer than the more easily stretched connecting filaments [77,78] and hence their contribution to stiffness is difficult to retrieve from passive measurements, a reduced passive moduli in the $fln^{\Delta N62}$ (Figure 5A-B) reflects mechanical properties of the passive elements (connecting filaments) being compromised. During mechanics experiments, before passive measurements, to maximize work production in active condition, the mutant fibers, albeit not significantly, were stretched further (~16%) compared to control fibers (27.8 ± 2.7 % vs 23.9 ± 1.3 % stretch for control) from initial length at just taut. This additional stretch could compensate for the shorter (~13%) length of the mutant sarcomere (Table 3-2) and the connecting filaments should be completely unfolded to sustain passive tension. Moreover, flightin homologue from giant water bug *Lethocerus,* has been shown to be absent from the A-I junction region of the sarcomere where the connecting filaments are located [79]. Additionally, in the null mutant $Act88F^{KM88}$ lacking sarcomeric Z-discs and an organized connecting filament, flightin is found to be normally present in the sarcomeric A-band [59], indicating that flightin (or its N-terminus) do not interact with the connecting filaments for its primary location and function. Hence, the marked reduction in passive tension and moduli reflects a compromised or missing passive structural element other than the connecting filaments. We propose that the flightin N-terminus is the missing element which enhances the

passive stiffness of the IFM myofilament lattice by maintaining normal myofilament lattice spacing and regularity, possibly by enhancing the normal inter-filament electrostatic interactions, as described above in the structural model (Figure 3-11).

Moreover, the active mutant fibers have a heterogenous population of heads with possible broad range of cycling kinetics, as seen in the broader range of the frequencies of maximum work and maximum power than control fibers (boxed regions in figures 3-10C,D). This is most likely due to the lack of lattice regularity and heterogeneous lattice spacing in the mutant (Figure 3-8E,F). The broader range of myosin kinetics is indicative of lesser cooperativity between myosin heads and thin filament regulatory units, which could lead to abnormal transmission of force production as have been demonstrated by computer modeling studies [80-83].

In rigor condition, the slightly greater number of $fln^{\Delta N62}$ fibers break compared to control (see Results), reminiscent of fln^{0} fibers [62], but without any *in vivo* IFM hypercontraction characteristics of fln^{0} [59]. Electron microscopy revealed that the overall sarcomeric underlying lattice structure is weaker in the mutant torn-zone fibers as seen by the wavy Z-line and the M-line (Figure S3-4D arrows) and buckling up of A-bands (Figure S3-4D circles), compared to the few torn-zone fibers of the control (Figure S3-4B). The net rigor yield strength of the mutant fibers is 50% less than control fibers before breakage (Table 3-4) indicating that the mutant fibers are incapable of either generating enough force or transmitting force optimally. Thus, it is evident that the flightin N-terminus contributes to the myofilament lattice organization possibly facilitating in an inter-filament interaction, to optimize the viscoelastic mechanical

performance of the underlying structure to transmit force efficiently in order to produce sufficient work and power for normal flight.

Flightin N-terminus is required to Fine-tune the IFM for Species-Specific Pulse Song for Male Reproductive Success

Observation from sequence analysis indicates that flightin N-terminus could be evolving faster than the rest of the protein across *Drosophila* with putative positively selected sites (Figure S3-1), unlike the pattern seen in some other IFM genes for thick filament proteins like myosin regulatory light chain, myofilin, and paramyosin (Figure S3-2). What explains the flightin N-terminal sequence high variability?

Similar to flight, the flightin N-terminus is not an essential protein region for courtship song generation, since the $fln^{\Delta N62}$ male is capable of producing both pulse and sine songs (Figure 3-3 bottom panel, Audio S3-2). Moreover, we show here for the first time that IFM is required for both pulse and sine song since singing is completely abolished in males of the IFM-specific mutant, fln^{0} (Figure 3-3 top panel), and is completely rescued in fln^{+} (Figure 3-3 middle panel, Audio S3-1).

Interestingly, we find that flightin N-terminus is required for generating species-specific *D. melanogaster* male courtship song parameters (namely interpulse interval, cycles per pulse, pulse length, pulse duty cycle and sine song frequency) during mating ritual (Figures 3-4,3-5) reducing the mutant males' courtship behavioral success (Figure 3-6). Ewing (1977) [43] had shown that during pulse song, muscle potentials in IFM motor units are functionally related to a subsequent sound pulse with a 1:1 ratio, which is indicative of a more Ca^{2+} activated dependency for start of a pulse contraction. The

reduced lattice organization and stiffness and a potentially heterogeneous cross-bridge population (Figure 3-8, Table 3-3) in the mutant could lead to a slower rate of force development and decay due to lower mechanical cooperativity among cross-bridges and between thin filament's Ca^{2+} sensing regulatory units. This interpretation is in agreement with computer modeling studies which predicts that reduced myofilament lattice organization [83], and reduced lattice stiffness [82] contribute to reduced dynamics of force production and decay due to lower cooperativity between myosin motors and also between thin filament regulatory units. Thus, in the mutant, after a Ca^{2+} spike just before a pulse, slower rate of force development could delay the precisely timed start of a pulse contraction leading to a longer interpulse interval or IPI (Table S3-3, Figures 3-4B,G). Once the pulse starts, $fln^{\Delta N62}$ male song possibly has a lesser dampening effect due to more compliant lattice and reduced cross-bridge cooperativity, causing greater CPP and PL (Table S3-3, Figures 3-4B,E,F). Moreover, due to the lattice compliance and possibly lesser dampening effect, song energy in the mutant could not be maintained to a confined narrow band leading to a much broader distribution of IPI than control (Figure 3-5). Also, the greater mean IPI and its broader distribution in the mutant than the control (Figures 3-4B,G and 3-5) are due to the truncated mutation of flightin, not due to natural variations, since the IPI effects seen here are greater than the shifts seen in natural variations in the longitudinal study by Turner and Miller 2012 [84]. $fln^{\Delta N62}$ males were also not able to sustain their pulse singing for long due to reduced mechanical properties of IFM (Figures 3-9,3-10, Table 3-4) as reflected in their reduced PDC (Table S1, Figure 3-4H), which is taken as a measure of the male's singing vigor and quality by the female [40]. This

possibly contributes to the mutant male's lower reproductive success (Figures 3-6C,D, Video S3-3) compared to control.

The higher sine song frequency (SSF) in the mutant (Figures 3-4A,C) is surprising to find, in contrast to similar flight wing beat frequency (Table 3-1) and IPF (Figure 3-4D). This suggests that different populations of cross-bridges could be utilized for sine song, flight and pulse song. Power requirement for normal sine singing should be lower than that of flight or pulse, given lower wing beat amplitude, frequency and motor neuron firing rate for this behavior [43]. Potentially this can be fulfilled by low force producing Ca^{2+} activated cross-bridge subsets undergoing force generating conformational changes while remaining attached throughout the cycle. Motor neuron firing rate in direct flight muscles (DFM) during sine song has 1:1 correlation with wing movements [85] tempting us to speculate that sine could be generated entirely by the DFM contractions. But our finding that sine song is abolished in an IFM-specific mutant, fln^{0} (Figure 3-3 top panel) argues against this possibility. Previously, it was shown that IFM projectin muscle mutant, bent(D)+ [86] and paramyosin mutant, pmS18A [87] had increased flight wing beat frequency even with reduced muscle stiffness and power output. It was proposed to be an over-compensatory mechanism by the fly to increase the optimum resonant frequency of power output of the flight system to bring it in line with that of the myofilaments [88]. In addition, the thoracic box movement of flies has been shown to synchronously modulate with sine song sound modulations [43]. We propose here that the higher SSF in the mutant is due to similar over-tuning of resonant frequency of the thoracic box to compensate for low stiffness and power of the IFM lattice.

With these song parameters being affected in the $fln^{\Delta N62}$, the mutant males have a lower courtship success (both for female preference and for performing courtship behavior: Figure 3-6C,D, Video S3-3), in a more natural competitive situation [89] with the control fln^{+}, than in single pair mating (Figure 3-6A,B, Videos S3-1 and S3-2). Evidence of wild type female rejecting the mutant's courtship song and mounting attempts (Video S3-3) in close proximity, is further suggestive of lower female preference for the aberrant mutant song. The flightin N-terminal region is specifically required to fine-tune the IFM lattice structure for dictating the critical song parameters important for species recognition (IPI: [90]) and female receptivity (PDC: [40]) in *D. melanogaster*, possibly explaining its hypervariability in *Drosophila*. Experimental evidence showed that courtship song frequencies are not important for species recognition, female mate choice in *D. melanoagster* [91]. Hence, no effect of flightin N-terminus on the carrier frequency of pulses (Figure 3-4D) in addition to its effect on IPI and PDC suggests that flightin N-terminus could be required in *Drosophila* to to optimize muscle structural and mechanical properties for species-specific sexually selected courtship song signals.

Flightin N-terminus Effect on Myofilament Lattice Structure: Evolutionary Adaptation by IFM for Biological Performance

Flightin is an "orphan" [54], to our knowledge the only known IFM-specific protein in *Drosophila melanogaster* having no sequence homology to any known protein domain, and is taxonomically restricted to hexapods and crustaceans [Soto-Adams FN et al. 2013, unpublished data]. Orphan genes are regarded to be important for lineage or

species-specific adaptive functions and play critical role in speciation process [3,4,54]. In this study, we show that the flightin N-terminus dictates myofilament lattice regularity fulfilling the IFM's behavioral functions of flight to survive and courtship song to reproduce, respectively.

The flightin N-terminus (63 aa) is similar in length to another thick filament extension of 46 amino acids found in *Drosophila* myosin regulatory light chain (RLC), proposed to reach out and act as a tether on the thin filament to fulfill the stretch activation response for flight [92-95]. Given the similar possibility of the flightin N-terminal region to extend out of the thick filament backbone with high negative charge based on its amino acid composition, a comparison of the effect of this region to that of the RLC N-terminal extension could potentially lead us to the specific functional contribution of this region. Intriguingly, the functional effects on IFM underlying structure, mechanics and whole organismal flight seen by truncating these two regions from RLC and flightin are similar. Both mutations do not have a drastic effect on IFM sarcomeric structure and mechanical properties and do not abolish flight. But there are some critical differences in their effects that are almost exactly opposite to each other. RLC N-terminal truncation reduces fP_{max} (frequency of maximum power) slowing down myosin kinetics, which in turn lowers fly wing beat frequency and flight ability, but without any major effect on myofilament lattice organization, maximum work, maximum power and active viscoelastic moduli [95]. In contrast, flightin N-terminus truncation has no effect on myosin kinetics, fP_{max}, fly wing beat frequency, but causes reduced inter-thick filament spacing and lattice regularity, lowering work and power output and

compromised active viscoelastic moduli, suggestive of this region's specific role in optimizing lattice organization, rather than the fast contractile kinetics known to power flight muscles [10] as in the case of the RLC N-terminal extension. Importantly, this indicates that the flightin N-terminal region is really one of the critical links in the IFM for better registered and crystalline lattices, which has been shown to be an indispensable requirement for asynchronous flight muscles in *Drosophila* and other flying insects [7-9]. Additionally, the lattice organizational defects of the mutant also influence courtship song properties which are subject to distinct and opposing selection pressure (sexual selection) than flight subject to natural selection ([96], reviewed in [1],[97]). This reflects on the versatility of IFM in using the N-terminal sequence of the "orphan" flightin as one of the potential evolutionary innovation for asynchronous high-power producing oscillatory contractions as well as rhythmic wing vibrations for male courtship song. Given the importance of flightin N-terminus in optimizing myofilament lattice spacing and crystallinity, this highly variable region is possibly under adaptive positive selection for fine-tuning lattice structural features in order to fulfill species-specific flight muscle biological performance.

Yet, the evolutionary conundrum is that how a highly variable region of an orphan gene does influences distinct behaviors of the tissue system that are under opposing selection pressures? Flight is one of the most energy consuming, high power requiring and aerodynamically costly behaviors, with the flight muscles of skilled flying insects having highly regular lattice organization of thick and thin filaments. On the other hand, courtship singing in *D. melanogaster* possibly requires much less power than flight.

Given mechanical power is proportional to cubed product of wing stroke frequency and amplitude, this is due to much smaller ($1/4^{th}$) wing beat amplitude [98] with only one wing usage at a time [33] during singing, loading only half of the thoracic musculature [discussed in 98]. Given that sexual selection generally acts at a faster time scale [99-101] than natural selection, it is easy to envisage how flightin N-terminus may have evolved fast under sexual selection for a less physiologically demanding behavior i.e., species-specific courtship song, and in the process fortuitously improved IFM myofilament lattice regularity for enhancing power output of the more physiologically demanding flight behavior in *Drosophila*. This proposal, if true, agrees well with the orphan gene evolutionary model [54] of a fast evolution of the gene due to strong positive selection followed by slow evolution due to functional conservation.

In summary, the flightin N-terminal region optimizes myofilament lattice spacing and regularity, sarcomeric geometry, enabling normal force transmission to power flight and fine-tunes the sexually selected temporal rhythms of the male love song. This study exemplifies the importance of thick filament associated proteins in promoting normal myofilament lattice structure, muscle function and whole organismal behavior. Furthermore, this work emphasizes the versatility and adaptability of IFM as a system to be able to incorporate "orphan" genes like flightin in its repertoire and to utilize for its functional needs. Evidences of adaptive functional evolution acting on flightin N-terminus sequence found in this study will be interesting to pursue further by creating transgenic lines expressing chimeric flightin with its N-terminal region from a different species than *D. melanogaster*, and testing structural and behavioral outcomes. Moreover,

to our knowledge, the transgenic system ($fln^{\Delta N62}$) created in this study is the first IFM-specific mutant to date having flight impairment without affecting acto-myosin cross-bridge kinetics but with subtle myofilament lattice disorder. We anticipate that this system will prove valuable for understanding further the link between muscle lattice structural order and contractile function.

MATERIALS AND METHODS

Sequence Analysis

Flightin and its orthologous amino acid sequences from twelve *Drosophila* species (*D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. pseudoobscura*, *D. persimilis*, *D. willistoni*, *D. virilis*, *D. mojavensis*, *D. grimshawi*) were retrieved from flybase (http://www.flybase.org). For a list of annotated symbols and flybase ID of the sequences see Table S3-1A. Amino acid sequences were aligned using ClustalW.

Drosophila Strains

Drosophila melanogaster w^{1118}, and $w^*; T(2;3)\ ap^{Xa}, ap^{Xa}$ / $CyO; TM3, Sb^1$ (used for linkage group analysis) were obtained from the Bloomington Stock Center (Bloomington, IN). w^{1118} was used as host for generating the transgenic strains. $w^{1118}; P\{w^+, Act88Ffln^+\}; fln^o, e$, the transgenic strain expressing the wild-type flightin gene in a fln^0 background [64], was used as the control line and henceforth will be referred to as

fln^{+}. The flightin null mutant line (*fln*^{0}) used here was previously made [59]. All fly lines were maintained in a constant temperature and humidity (21±1°C, 70%) environmental room on a 12:12 light:dark cycle.

Construction of the Transformation Vector

The N-terminal 62 amino acids deletion was engineered in a P-element transformation vector pCaSpeR (Flybase ID: FBmc0000168) containing the full-length flightin gene and the actin *Act88F* promoter [64] by using primers:

Forward: 5' TTTTTGGTACCATGAAAGCACCGCCGCCTCCG 3' and

Reverse: 5' GCACTAGCTGCAGAACCCCTCATACCTGCCG 3' with underlined bases representing KpnI and PstI restriction enzyme sites in the forward and reverse primer sequences, respectively. The forward primer was designed to amplify from the 189th base of the coding sequence of the flightin gene with ATG start site and KpnI site in overhangs so as to delete the 62 amino acids after Methionine (see Figure 3-2). The reverse primer was the same as designed for the 3' end of the 1.14 kb flightin genomic fragment previously cloned into pCaSpeR [64]. The 1.14 kb flightin gene was excised from the vector and replaced by the 954 bp flightin N-terminal deleted fragment using KpnI and PstI restriction endonucleases. The same aforementioned primers were used for sequencing verification of the N-terminal deleted construct.

Generation of the *P{fln*$^{\Delta N62}$*}* Strains

Microinjection of the transformation vector into *w*^{1118} host strain was carried out by Genetic Services, Inc., Sudbury, MA. Linkage group was determined by standard

crosses to *w*; T(2;3) ap^{Xa}, ap^{Xa} / CyO; TM3, Sb^{1}*. Five parental strains were created in a *fln^{+}* background, each with a second chromosome insertion, and were subsequently crossed into the flightin null background (*fln^{0}*) [59] to generate homozygous transgenic strains with no endogenous flightin expression. All the transformed strains have the genotype *w^{1118}*; *P{w^{+}, Act88Ffln$^{\Delta N62}$}*; *fln^{0}*, *e* and herein will be referred to as *fln$^{\Delta N62X}$* where X is a letter from A through E (Table S3-2). Expression of the transgene was confirmed by RT- PCR analysis via RNA isolated from 30 two-day old flies (data not shown), using the forward and reverse primers described in the previous section. Based on protein expression of the transgene and flight ability, two lines, *fln$^{\Delta N62A}$* and *fln$^{\Delta N62B}$* were selected for subsequent analyses.

Gel Electrophoresis and Western Blot Analysis

One dimensional gel electrophoresis and western blot analysis were done as previously described [64], with the following modifications. IFM fibers from three flies, each from control, *fln^{0}* and N-terminal deletion lines were dissected in skinning solution (pCa 8.0; 20 mM N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES), 10 mM DTT, 5 mM EGTA, 1 mM Mg^{2+}, 5 mM MgATP, 0.25 mM P_i, protease inhibitor cocktail (Roche; Indianapolis, IN), ionic strength of 175 mEq adjusted with sodium methane sulfate, pH 7.0, 50% w/v glycerol, and 0.25% v/v Triton X-100.)and incubated in the same skinning solution overnight at -20^{o}C. The following morning the fibers were collected by a brief spin on a table top microfuge, the skinning solution was removed and the fibers rinsed five times for 3 minutes each in relaxing solution (pCa 8, 20 mM BES, 20 mM CP, 450 U/mL CPK, 1 mM DTT, 5 mM EGTA, 1 mM Mg^{2+}, 12 mM MgATP, 2

mM P_i, protease inhibitor cocktail, 200 mEq ionic strength, pH 7.0) to remove the glycerol and Triton X-100 completely. The fibers were then dissolved in SDS gel sample buffer (62.5 mM Tris-HCL, 100 mM DTT, 4% w/v SDS, and 20% w/v Glycerol with protease inhibitor cocktail). 10 μL of each sample was loaded per lane of 10-20% gradient SDS-PAGE gels (Criterion Bio-Rad, Catalogue # 567-1114) in duplicate; one gel was stained with Krypton (Pierce, Rockford, IL) infrared protein stain and the other gel was blotted onto PVDF membrane (0.2 μm pore size, Bio-Rad Catalog # 162-0174) at 65V for 1 hr using a Tris-Glycine buffer (National Diagnostics, Atlanta, GA). For krypton staining, the gel was fixed with 50% v/v ethanol, 15% v/v acetic acid, stained overnight, destained with 5% v/v acetic acid, 0.1% v/v Tween-20 for 5 mins, and scanned in an Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE). For western blots, PVDF membranes were blocked using a 1:1 Aquablock-PBS solution (Aquablock: East Coast Biologics, North Berwick, ME) and incubated with a 1:3000 dilution of anti-flightin polyclonal antibody [59]. After primary antibody incubation, the membrane was washed two times for 5 mins each and then three times for 15 mins each with PBST (1X PBS with 0.1% Tween-20). The membrane was then incubated for one hour in a 1:7500 dilution of secondary antibody, Alexafluor 680 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA). The membrane was washed again with PBST two times for 5 mins each, and then three times for 10 mins each, and a final wash with 1X PBS (without Tween-20) two times for 20 mins each. The blot was allowed to dry overnight in the dark and then scanned in an Odyssey Imaging System.

Flight Performance

Flight tests and wing-beat frequency analysis were performed as previously described [69].

Courtship Song Recording and Analysis

Virgin males and females were anesthetized and collected using CO_2; however CO_2 was not used for any subsequent process. Males were aspirated gently into single vials with standard cornmeal food and kept isolated for 24 hrs before testing to nullify grouping effect and to increase amount of song production [41,102-104]. Males aged 3 days and females aged 24 hrs or less were used for courtship song assays to stimulate the males to produce more song. A male and a female were aspirated into a small plexiglass mating chamber (1cm diameter × 4 mm height) and placed inside an INSECTAVOX [105] for song recording for upto 30 mins. For details, see (Chapter 2 or [106]).

The recorded songs were digitized and analyzed using Goldwave v5.58 [107], Table S3-3 lists the song parameters studied here. Representative song oscillograms were generated with Audacity 2.0 [http://audacity.sourceforge.net/]. For additional details of courtship song analysis, see (Chapter 2 or [106]).

Single Pair Mating Assay

Three to five day old virgin males and females were used. Each assay consisted of one male of a transgenic strain and one wild type (Oregon R) female introduced into a plexiglass mating chamber (1.7 cm diameter × 5 mm height). The courtship activities were video recorded until successful copulation, or longer (30-50 mins) in the absence of copulation, using a 65X SD camcorder (Samsung) mounted on a tripod. The assays were

done under room light at 22^{o}C temperature and 70% humidity. From the videos, courtship index (CI) and wing extension index (WEI) were calculated for each male as described in [34]. Briefly, CI is the fraction of the total recording time the male displayed courtship behaviors (orienting, chasing, tapping, licking, singing, copulation attempts), and WEI is the fraction of the total recording time the male extends and vibrates a wing.

Courtship Competition Assay

Courtship competition mating assays were performed exactly similarly as in single pair mating assays described above, but with only two transgenic males of different genotypes. Each assay consisted of two males of different transgenic strains and one wild type (Oregon R) female. To distinguish the males, one of them was marked on its thorax with a neon-yellow acrylic paint using a fine point paintbrush. The marking was done 24 hours before testing to allow the fly full recovery from CO_2. The strain of the marked male was alternated between trials to avoid a marking effect on female selection.

Transmission Electron Microscopy

Fly thoraces were bisected, fixed, dehydrated, infiltrated, embedded, and sectioned as previously described [64], and imaged at 8000x magnification, 1.426 nm pixel size.

Images were analyzed using NIH ImageJ [108] and Metamorph Software (Molecular Devices, LLC, California, USA). Values reported for the myofibril area and myofibril area per 100 μm^2 fiber cross-section (Table 3-2) were measured using Metamorph software. All other measurements (in Tables 3-2 and 3-3) were performed in

ImageJ. The distance between the thick filament planes, $d_{1,0}$ [109], was quantified using two-dimensional fast Fourier transform power spectra from cross sectional images of the myofibrils that were divided into boxes of 512 × 512 or 1024 × 1024 pixels (for detailed protocol and validation of this method see Method MS2 and MS3, respectively). The theoretical resolution of an image is equal to twice the pixel size of the image. Each pixel in the FFT is inversely proportional to the real space pixel in the corresponding image multiplied by FFT dimensions. Distances in FFT space are inversely related to distances in real space. Thus, $d_{1,0}$ in real space can be obtained by multiplying the FFT space distance in number of pixels with value of pixel size in FFT space and then calculating the inverse of it. Or, in other words, distance in FFT corresponds to one half of real space in pixels. Hence, the total number of pixels in the FFT multiplied by the distance per pixel in the original image should be constant. Using this correlation, $d_{1,0}$ was measured from the center to the 1^{st} order reflection spots in the FFT. The inter-thick filament spacing was calculated as $2/\sqrt{3}$ multiplied by $d_{1,0}$ [109].

Structural Regularity: This was quantified by processing the following structural informations:

i) Resolutions to which the filaments in the myofibrillar lattice diffract by measuring the distance of the farthest away spot visible from the center of the fourier transform.

ii) Sharpness by which the filaments across the myofibrillar lattice diffract by measuring the quality of the intensity peaks ($\log_{10}$ peak and half-width) at the reflection plane (1,0). The FFT of the original myofibrillar cross-sectional

images were transformed to polar coordinates in Image J and the intensity profile across the reflection planes was plotted. The sharpness of the intensity profile is a measure of the $\log_{10}$ peak and half width of the intensities.

Single Muscle Fiber Mechanics by Sinusoidal Analysis

Solutions for muscle fiber mechanics were prepared according to a computer program that solves the ionic equilibria [110]. Concentrations are expressed in mmol/L. Unless listed otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Skinning solution was same as the one used for the skinning fibers for gel electrophoresis. Storage solution was skinning solution without Triton X-100. Activating solution was pCa 4.5, 20 BES, 20 CP, 450 U/mL CPK, 1 DTT, 5 EGTA, 1 Mg^{2+}, 12 MgATP, 2 P_i, 200 mEq ionic strength, pH 7.0. Relaxing solution was the same as activating solution except pCa adjusted to 8.0. Rigor solution was like activating solution without CP, CPK and MgATP. Dextran T-500 (Pharmacosmos, Holbaek, Denmark) was added to activating, relaxing and rigor solutions to a final concentration of 4% w/v to compress the myofilament lattice spacing to near *in vivo* values [95].

Fiber preparation, mechanical measurements and curve fitting were carried out as in previous studies [65,95] with the following modifications. Briefly, fibers from 2-3 days old female flies were mounted in Dextran-free relaxing solution, then activated, and shortened until slack. After 5 minutes fibers were re-stretched and then sequentially stretched in 3% increments until oscillatory work reached a stable maximum, as measured by sinusoidal length-perturbation analysis. Fibers were then washed in

Dextran-free relaxing solution, then returned to relaxing solution with 4% w/v Dextran T-500, and finally placed in rigor solution.

Statistical Analysis

All values are mean ± SE. Statistical analyses were performed using SPSS (v.20.0, SPSS, Chicago, IL) and Matlab, with values considered significant at $p<0.05$. Student's *t*-test was used to examine differences between fln^{+} and $fln^{\Delta N62}$ for most variables except for the elastic modulus-, viscous modulus-, work-, and power-frequency relationships. For these measurements, we applied a linear mixed model using frequency as the repeated measure, followed by Fischer's LSD pairwise comparisons between the two groups at each frequency. For statistical analysis on courtship song data, the average value of each song parameter was calculated for each fly; hence the number of statistical samples is the number of flies.

FIGURE LEGENDS

Figure 3-1. Flightin sequence alignment reveals two conservation patterns. Clustal W alignment of flightin amino acid sequences from 12 *Drosophila* species of the sub-genus Drosophila and Sophophora reveals differences in sequence conservation, with the N-terminal region (63 aa in *D. melanogaster*) having much lower conservation compared to the rest of the protein. Identities are marked by asterisks (*). Colon (:) indicates residues at that position are very similar based on their properties, and dot (.) indicates residues at that position are more or less similar. The region deleted in this study (shaded grey) is amino acids 2 through 63 (*D. melanogaster* numbering).

Figure 3-2. *fln*$^{\Delta N62}$ expresses flightin of lower molecular mass. One dimensional SDS-PAGE (A) and western blot (B) of IFM skinned fibers from control (*fln*$^{+}$) and mutant (*fln*$^{\Delta N62}$ and *fln*^{0}) flightin strains. The double arrowhead showing band of ~15 kDa only in the *fln*$^{\Delta N62}$ sample in the gel stained with Krypton (A) and a corresponding band of similar size as detected by western blot with an anti-flightin polyclonal antibody (B), respectively. The band detected in the *fln*$^{+}$ sample corresponds to full length flightin.

Figure 3-3. *fln*$^{\Delta N62}$ males can sing. Representative male courtship song oscillograms of flightin null (*fln*^{0}) mutant (top panel), control *fln*$^{+}$ (middle panel) and *fln*$^{\Delta N62}$ (bottom panel) males. *fln*^{0} males were unable to produce courtship song, while *fln*$^{+}$ control males and *fln*$^{\Delta N62}$ mutant males produce sine song and pulse song. Scale bar represents 500 ms.

Figure 3-4. *fln*$^{\Delta N62}$ males sing an abnormal courtship song. Courtship sine song (A) and pulse song (B) of *fln*$^{+}$ and *fln*$^{\Delta N62}$ males (scale bar = 50 ms). *fln*$^{\Delta N62}$ males produce a higher frequency sine song (C) and impaired pulse song with longer cycles per pulse (E), longer pulse length (F), longer interpulse interval (G), and reduced pulse duty cycle (H) but similar intrapulse frequency (D), compared to the *fln*$^{+}$ control males. N= 7-8 thirty minute fly song recordings. * Significant difference ($p<0.05$) from *fln*$^{+}$ control.

Figure 3-5. *fln*$^{\Delta N62}$ pulse song has a broader interpulse interval distribution. Distribution of interpulse interval (IPI) of *fln*$^{+}$ (open) and *fln*$^{\Delta N62}$ (filled) male pulse songs. Each bar represents the frequency at which IPIs occur among different fly songs. N= 7-8 thirty minute fly song recordings.

Figure 3-6. Courtship behavior of *fln*$^{\Delta N62}$ males. Courtship behavior during single pair mating (A and B) and competition mating between *fln*$^{+}$ and *fln*$^{\Delta N62}$ *males* (C and D). *fln*$^{+}$

and $fln^{\Delta N62}$ males have similar courtship index (A) and wing extension index (B) when paired singly with a wild type (Oregon R strain) female. When competing with fln^{+}males, $fln^{\Delta N62}$ males have significantly reduced courtship index (C) and wing extension index (D). Courtship index = total time duration of courtship behavior by a male / total time of video recording or until courtship success; Wing extension index= total time duration of wing extension to produce courtship song by a male / total time of video recording or until courtship success [34,106]. N=25 and 10 for mating competition assays and single pair mating assays, respectively. *Significant difference ($p<0.05$) from fln^{+} control.

Figure 3-7. **Sarcomeric structure of $fln^{\Delta N62}$ IFM fibers.** Transmission electron microscopy images of longitudinal sections of IFM from fln^{+} (A and B) and $fln^{\Delta N62}$ (C and D) transgenic fly lines. Note that the sarcomere in $fln^{\Delta N62}$ is shorter than the sarcomere in the control strain. B and D are magnified views of the boxed regions in A and C, respectively. The mutant sarcomere lacks an H zone and the M line is often interrupted by gaps (arrow). Note also that the M line is narrower and not as straight compared to the control. Scale bar = 1μm for A-D.

Figure 3-8. **$fln^{\Delta N62}$ IFM fibers have reduced myofilament lattice organization.** Transmission electron microscopy images of cross-sections of IFM from fln^{+} (A and B) and $fln^{\Delta N62}$ (D and E) transgenic fly lines. Note that myofibrils show the characteristic cylindrical shape of normal IFM, and have similar diameters. Region within white boxes in A and D are magnified in B and E, respectively. (E) shows a more compact and less ordered hexagonal lattice than (B). This is reflected in the power spectra (C and F) from

the fourier transform of B and E, respectively. Scale bars = 1μm (for A and D) and 0.1μm (for B and E).

Figure 3-9. ***fln*$^{\Delta N62}$ IFM fibers have reduced stiffness and viscous properties.** Elastic and viscous moduli of skinned IFM fibers from *fln*$^{+}$ (open circles) and *fln*$^{\Delta N62}$(filled squares) in relaxing (A and B) and rigor (C and D) solutions. Horizontal lines below asterisks denote frequency range through which measured values are significantly different between *fln*$^{+}$ and *fln*$^{\Delta N62}$ ($p<0.05$).

Figure 3-10. ***fln*$^{\Delta N62}$ IFM fibers have reduced power output at nearly normal frequency.** Elastic modulus (A), viscous modulus (B), work (C), and power (D) for active IFM fibers from *fln*$^{+}$ (open circles) and *fln*$^{\Delta N62}$ (filled squares) strains. Lines below asterisks denote frequency ranges where measured values are significantly different between *fln*$^{+}$ and *fln*$^{\Delta N62}$ ($p<0.05$). Vertical dashed lines in C and D represent frequency of maximum oscillatory work and power, occurring at 171 ± 8 Hz and 205 ± 7 Hz for *fln*$^{\Delta N62}$ compared to 179 ± 8 Hz and 217 ± 7 Hz for *fln*$^{+}$. The frequencies of maximum oscillatory work and power are not significantly different between control and mutant strains. Boxes in (C) and (D) possibly indicate a broader range of the frequencies of maximum oscillatory work and power respectively in the mutant fibers compared to that of control.

Figure 3-11. Structural model of flightin N-terminus function. Cross-sectional schematic of a thick filament (hollow circle) and a thin filament (closed circle), both having negative charges (-) on their surface. For simplicity, only two flightin N-terminus (yellow floppy string) having acidic residues with high negative charge (pI:3.78)

extending out of the thick filament is shown. The flightin N-terminal region is proposed here to extend out enhancing the charge field of the thick filament and is involved in an electrostatic repulsive force with negatively charged thin filament surface. The flightin N-terminus could maintain myofilament lattice spacing by the electrostatic interaction with thin filament surface leading to a normal sarcomeric organization and geometry.

Table 3-1. Flight properties of control and mutant flightin strains.

Strain	Flight score (0-6)	Able to fly (%)	Wing beat frequency (Hz)	Able to beat wings (%)
fln^{+}	4.2±0.4 (35)	95	198±2 (25)	100
fln$^{\Delta N62}$	2.8±0.1* (66)	82	195±4 (45)	90

Values are mean ± SE. Number of flies analyzed is shown in parenthesis. * Significant difference ($p<0.05$) from *fln*$^{+}$ control.

Table 3-2. Structural characteristics of IFM from control and mutant flightin strains from electron microscopy images.

Strain	Sarcomere length (μm)	Myofibril cross-sectional area (μm^2)	Thick filaments per myofibril cross-section (#)	Myofibril area per 100 μm^2 fiber cross-section (%)
fln^+	3.30±0.01 (316)	2.18±0.05 (99)	810±18 (46)	39±2 (17)
$fln^{\Delta N62}$	2.86±0.01* (1086)	2.03±0.04 (91)	903±25* (48)	45±1* (19)

Values are mean ± SE. Number of sarcomeres or myofibril cross-sections are shown in parenthesis. For the myofibril area / fiber cross-section, number in parenthesis indicate number of fiber cross-sections of 100 μm^2 analyzed. For each line, electron microscopy images from two flies were analyzed.

* Significant difference ($p<0.05$) from fln^+ control.

Table 3-3. Structural characteristics of IFM from control and mutant flightin strains from fast fourier transforms of electron microscopy images.

Strain	$d_{1,0}$ (nm)	Inter-thick filament spacing (nm)	Resolution (nm)	1,0 Peak Intensity ($I_{1,0}$)	1,0 Half-width (pixels)
fln^{+}	43.04±0.33 (56)	49.7±0.4 (56)	13.6±0.3 (56)	1.78±0.02 (14)	12.7±0.9 (14)
$fln^{\Delta N62}$	38.17±1.15* (42)	44.1±1.3* (42)	18.2±1.0* (42)	1.72±0.01* (13)	19.1±1* (13)

Values are mean ± SE. Number of myofibril cross-sections analyzed is shown in parenthesis. For each line, EM images from two flies were analyzed. * Significant difference ($p<0.05$) from fln^{+} control.

Table 3-4. Isometric tension measurements from skinned IFM fibers.

Line	Relaxed tension (kN/m^2)	Net active tension (kN/m^2)	Net rigor tension (kN/m^2)	Net rigor yield strength (kN/m^2)
fln^+	1.7±0.3 (15)	1.5±0.2 (15)	3.1±0.4 (11)	5.3±0.4 (2)
$fln^{\Delta N62}$	0.9±0.1* (15)	0.8±0.1* (15)	1.1±0.2* (8)	1.6±0.1* (3)

Values are mean ± SE. Numbers in parentheses indicate number of fibers analyzed. Net active (pCa4.5) and net rigor (pCa4.5) values represent tension increase from relaxed (pCa8.0) condition.

Net rigor yield strength = Total maximal tension withstood before fiber starts tearing minus relaxed tension

* Significant difference ($p<0.05$) from fln^+ control.

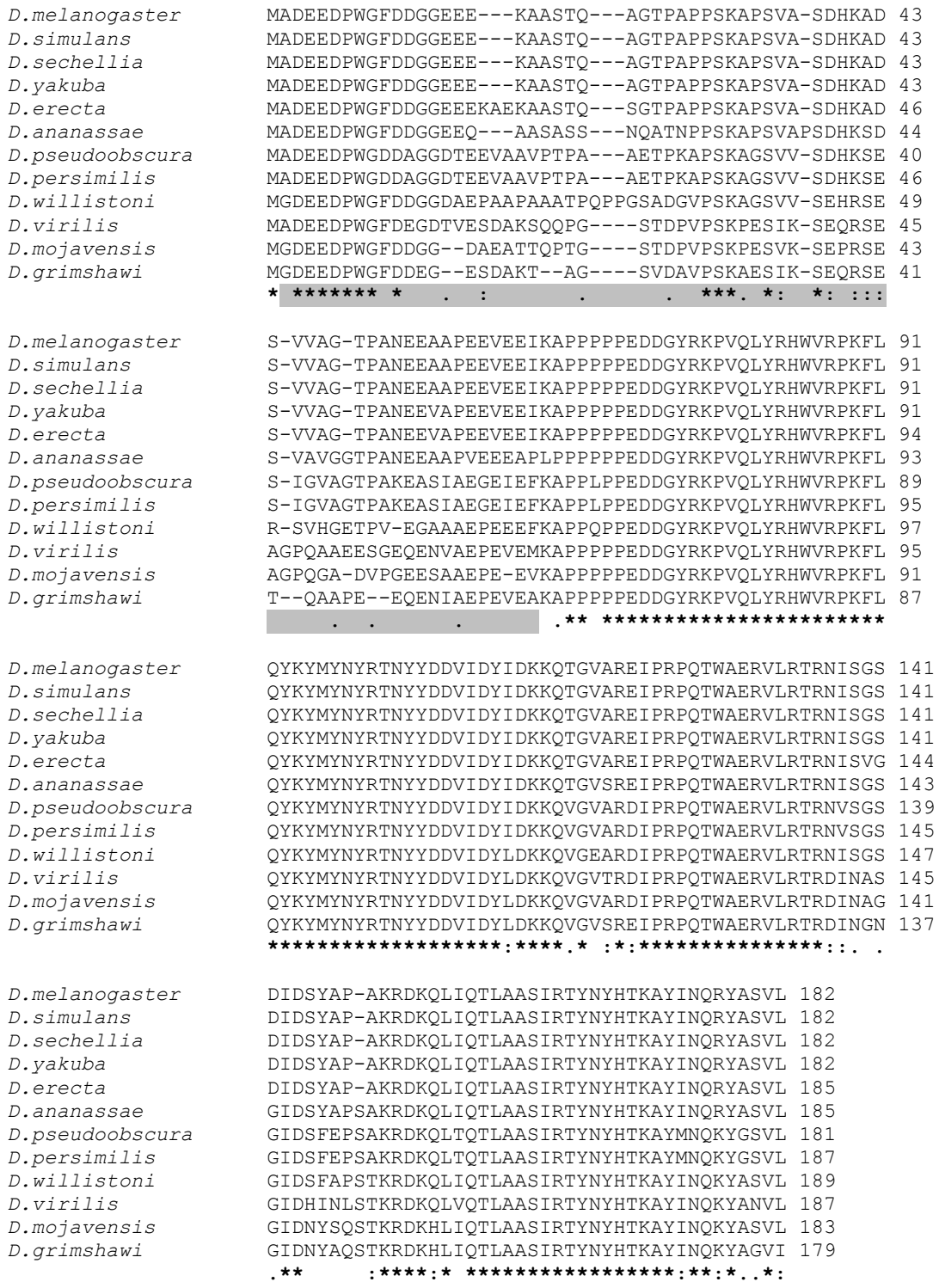

Figure 3-1. Flightin sequence alignment.

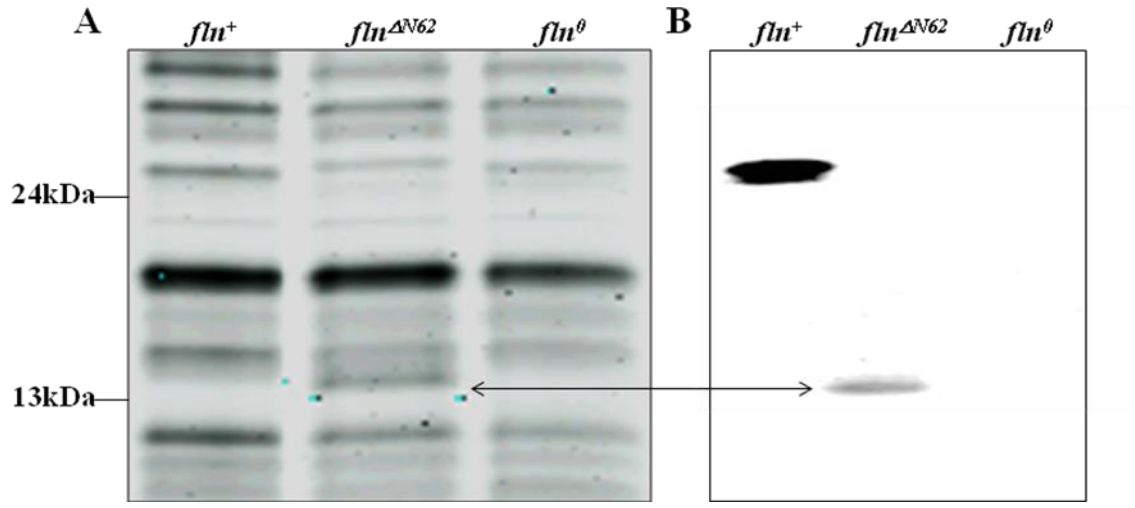

Figure 3-2. Flightin expression in IFM of Drosophila strains.

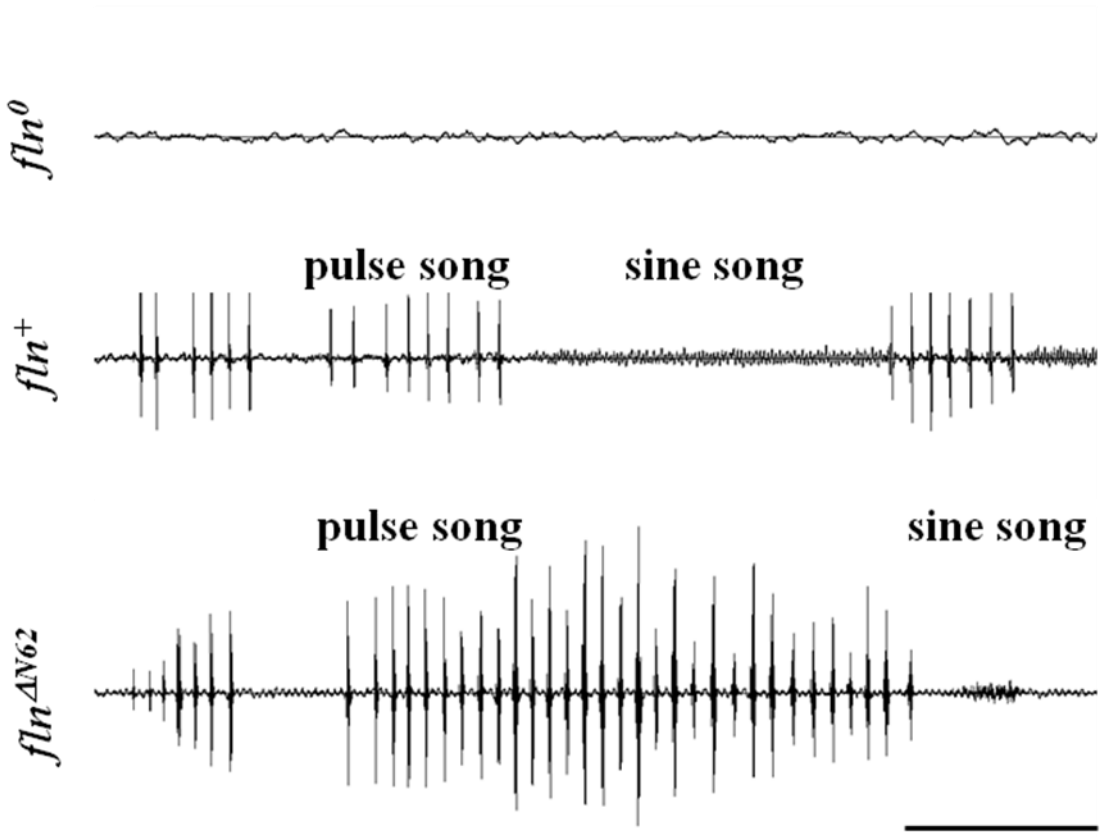

Figure 3-3. Male courtship song oscillogram samples.

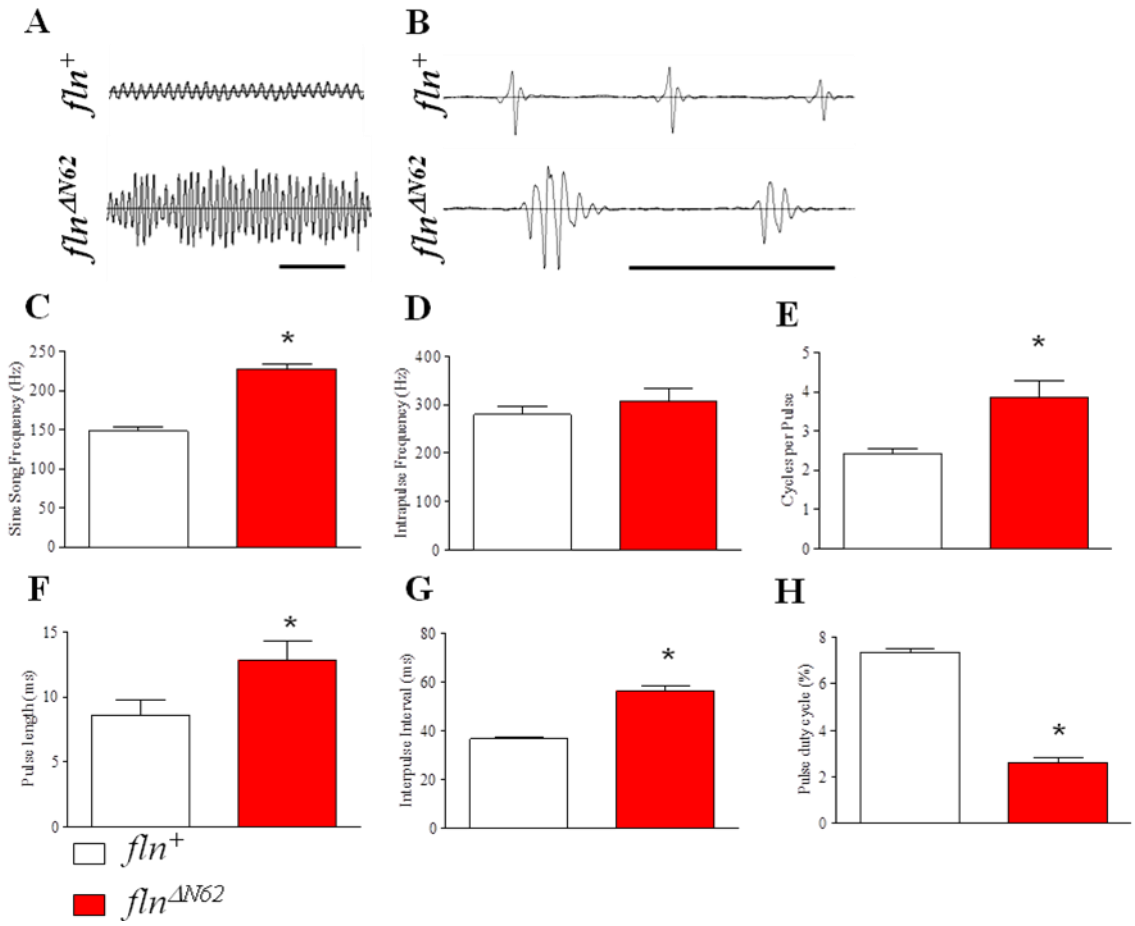

Figure 3-4. Courtship song properties of transgenic strains.

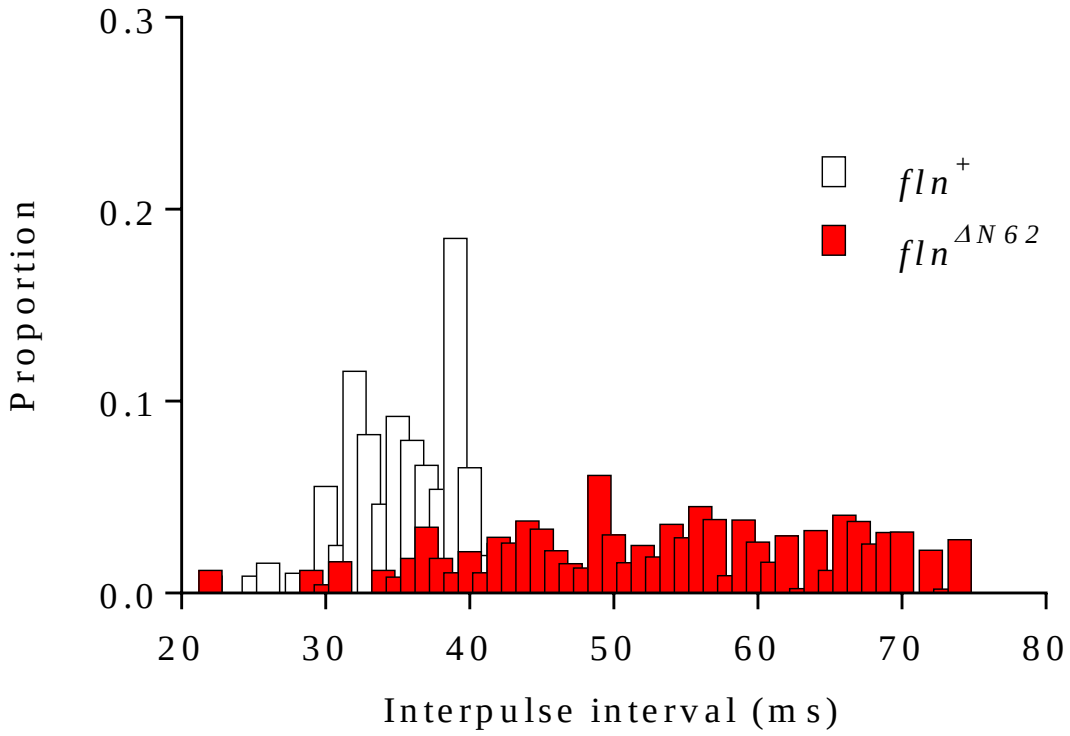

Figure 3-5. Pulse song interpulse interval distribution.

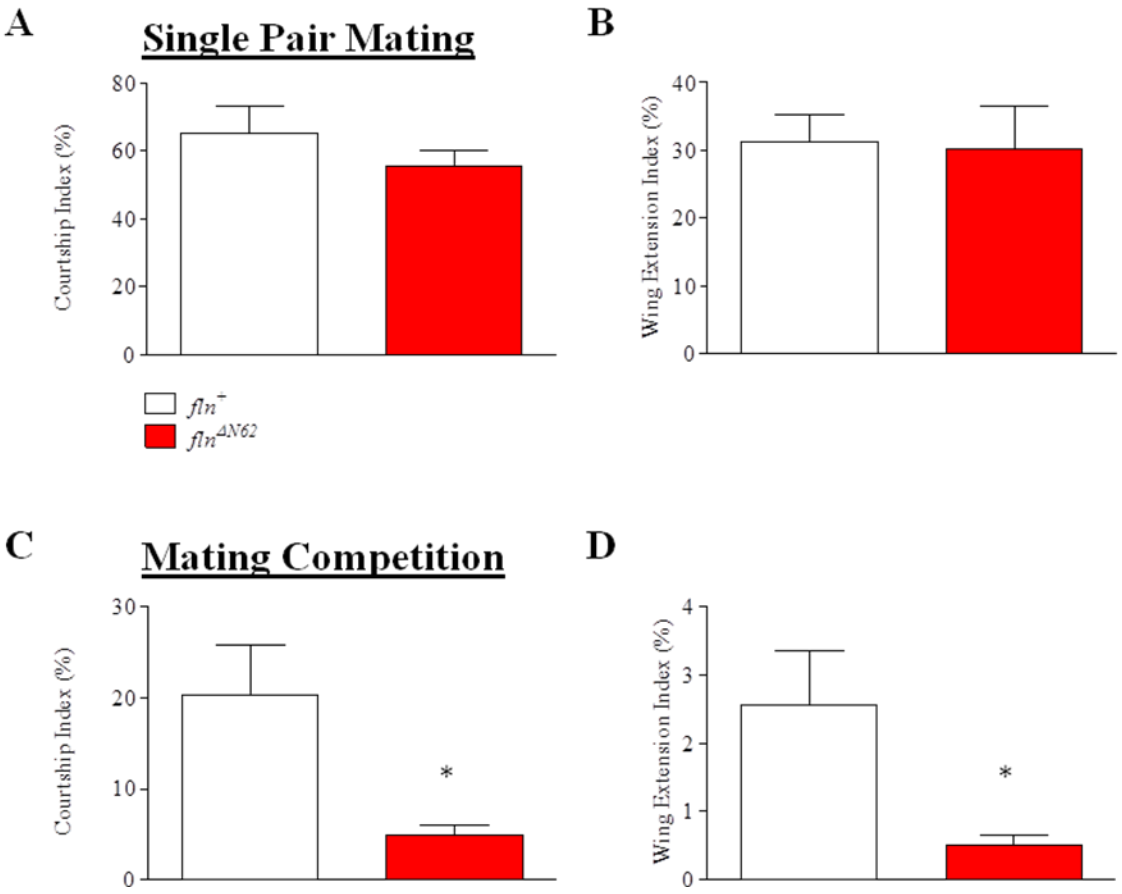

Figure 3-6. Male courtship behavioral properties of transgenic strains.

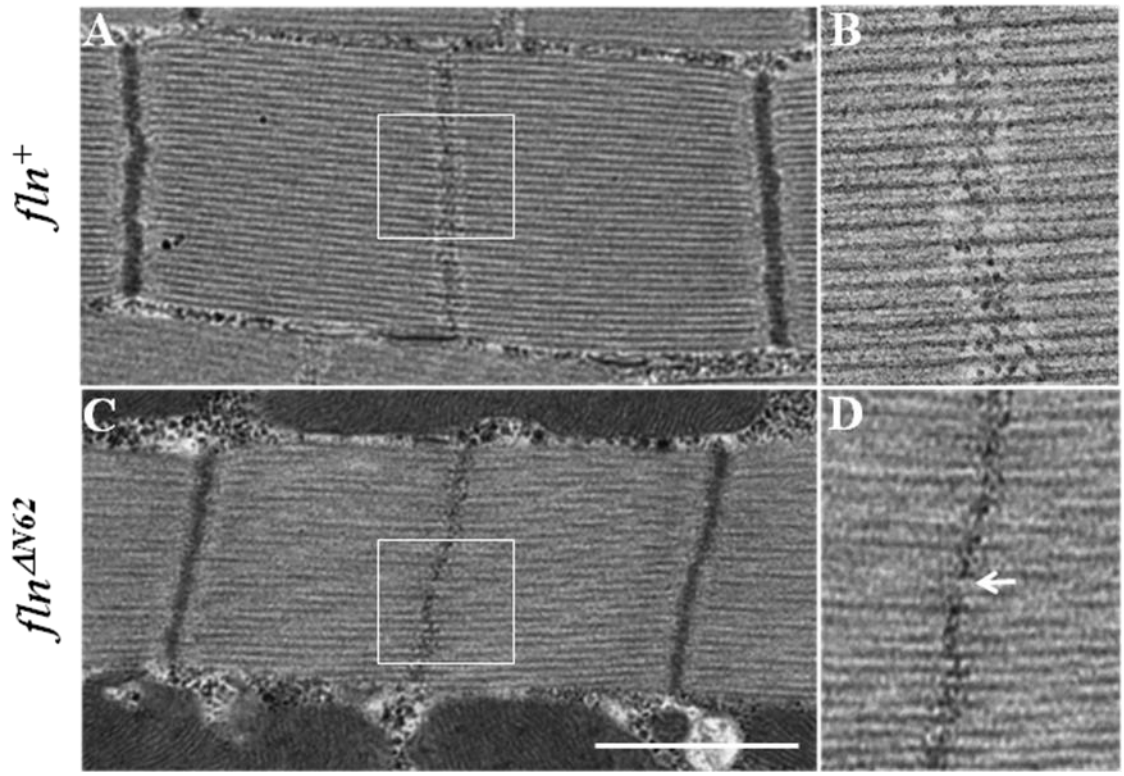

Figure 3-7. Transmission electron microscopy of *Drosophila* IFM sarcomeres.

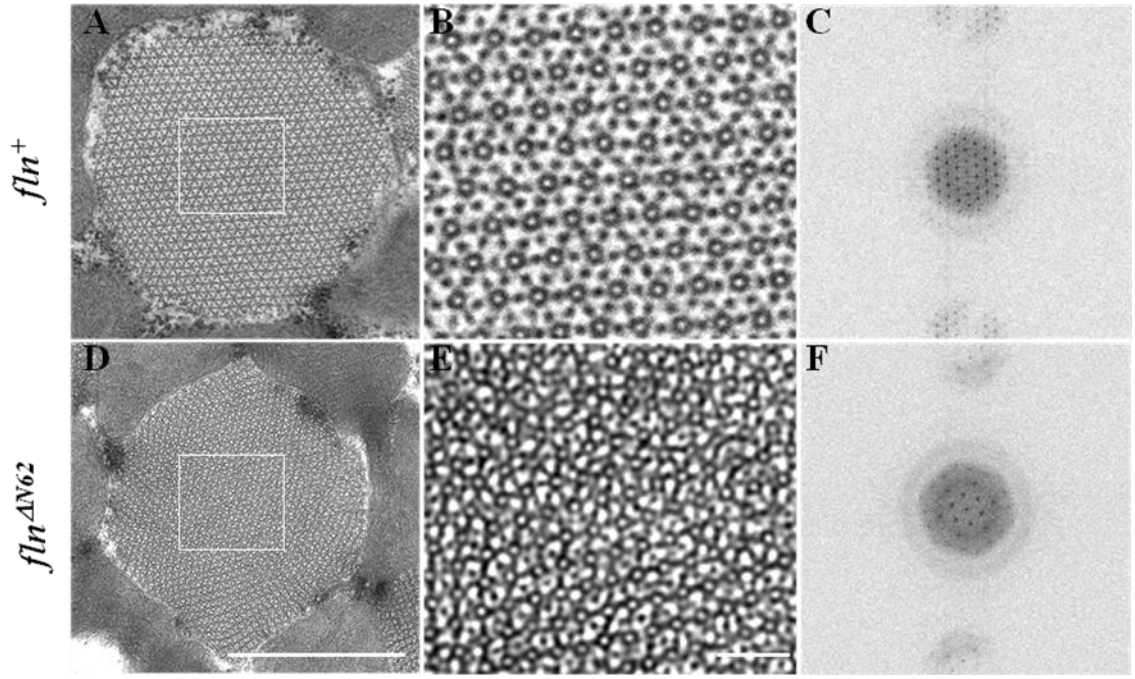

Figure 3-8. Transmission electron microscopy and Fourier transforms of *Drosophila* IFM cross sections.

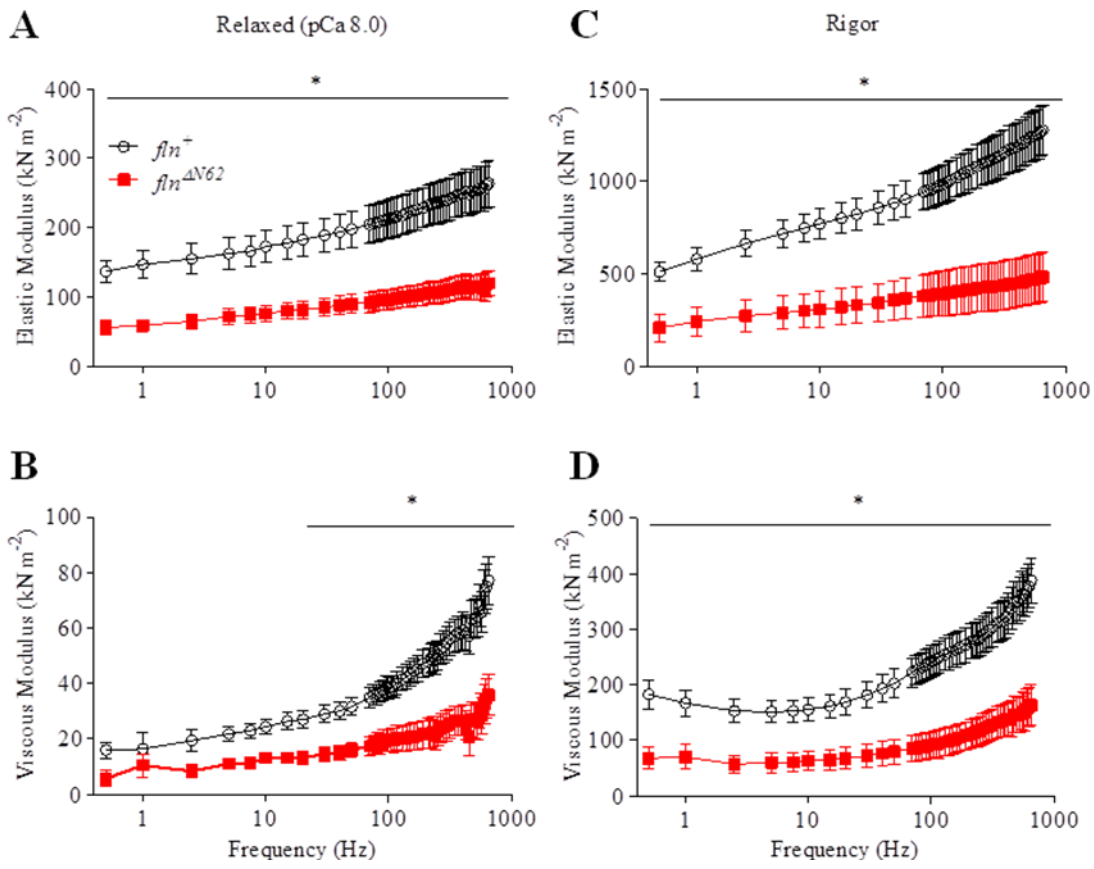

Figure 3-9. Mechanical parameters of IFM fibers in relaxed and rigor conditions.

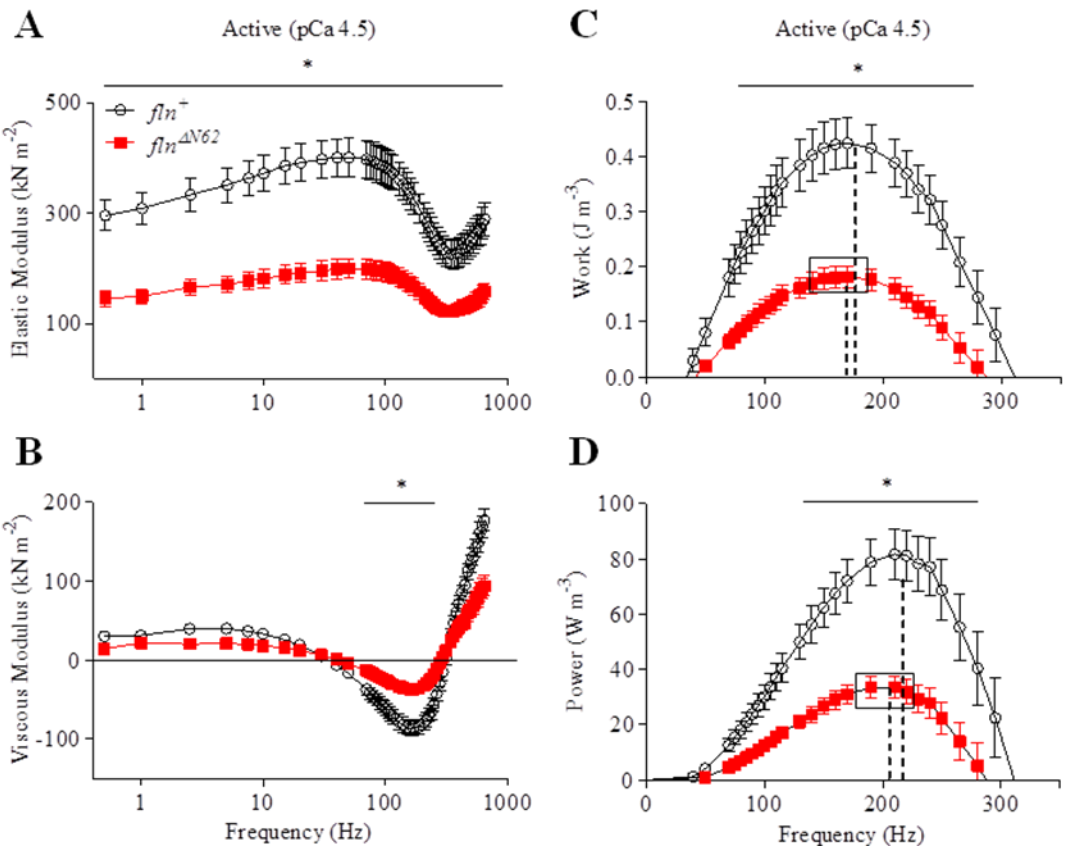

Figure 3-10. Mechanical parameters of IFM fibers: active condition.

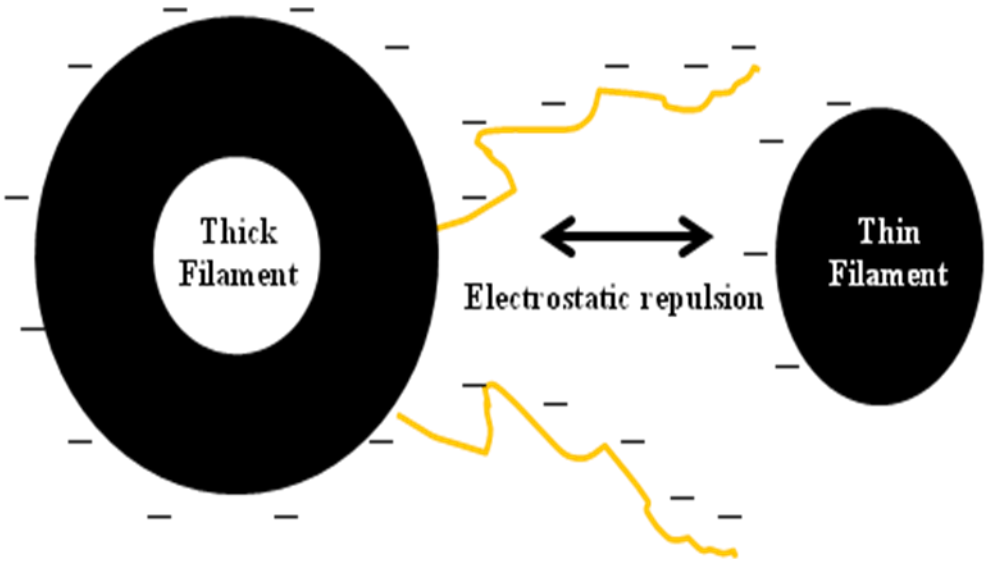

Figure 3-11. Structural model of flightin N-terminus function.

SUPPORTING INFORMATION

Method MS1. Sequence Analysis.

Sequence analysis for putative positively selected sites and average rate of evolution were performed on codon sequences of 12 *Drosophila* species retrieved from flybase (see Materials and Methods), leading to *D. melanogaster* using the Selecton server [111,112] with a combined mechanistic and empirical codon (MEC) evolutionary model [113] and M8a null model [114] which do not allow for positive selection. Average rate of evolution was calculated for each region of the protein sequence in *D. melanogaster* taking evolutionary rate of individual amino acid positions. Coding sequences in *D. melanoagster* and orthologs in other eleven *Drosophila* species for myosin regulatory light chain, myofilin, and paramyosin were retrieved from flybase (http://www.flybase.org/). For a list of annotated symbols and flybase ID of the sequences, see Table S3-1B-D.

Protein primary sequence disorder prediction and net charge calculation were performed in PONDR VL-XT server [115-117].

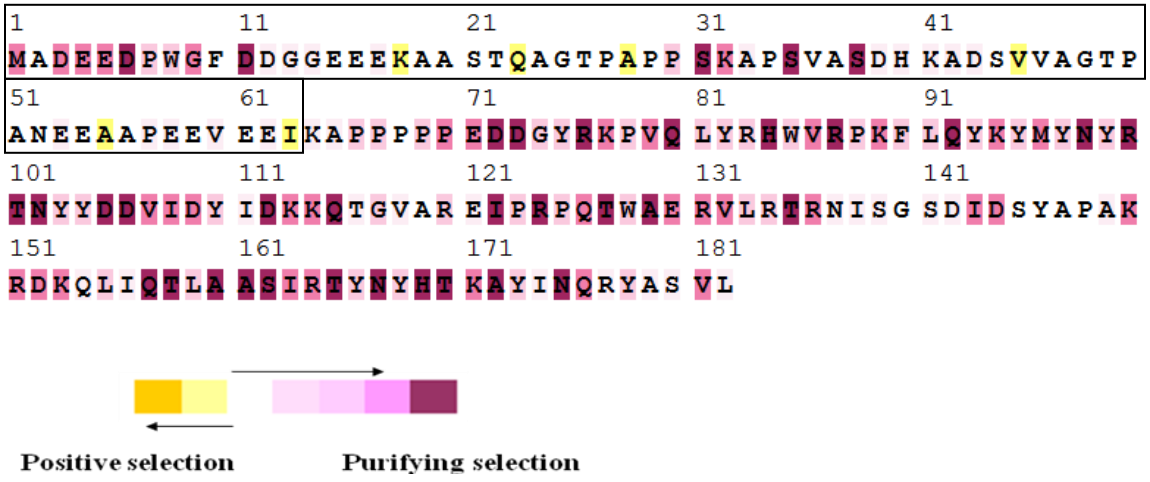

Figure S3-1. Selection regimes acting on flightin amino acid sequence.

A

```
1               11              21              31              41
MADEKKKVKK KKTKEEGGTS ETASEAASEA ATPAPAATPA PAASATGSKR
51              61              71              81              91
ASGGSRGSRK SKRAGSSVFS VFSQKQIAEF KEAFQLMDIA NDKELDAMLG
101             111             121             131             141
EASGPINFTQ LLTLFANRMA TSGANDEDEV VIAAFKTFDN DGLIDGDKFR
151             161             171             181             191
EMLMNFGDKF TMKEVDDAYD QMVIDDKNQI DTAALIEMLT GKGEEEEEEA
A
```

B

```
1               11              21              31              41
MFKNHLEMIG RNESPSKKAK FWQSYIRSLK GSEDIRAHEA PRASRPYSSY
51              61              71              81              91
LDSPSYRSIY DEPATANERV QSSGYRYLPV SRDTYGYSPR AIYDHHYSRT
101             111             121             131             141
IPANYDAEKA WNDHLKRMQE IERRYPSRYG LYLRDKPLTP NSLVPLEYEP
151             161
EDKLLAELNK GKDIKDIF
```

C

Figure S3-2. Selection regimes acting on myosin regulatory light chain (A), myofilin (B) and paramyosin (C) amino acid sequence.

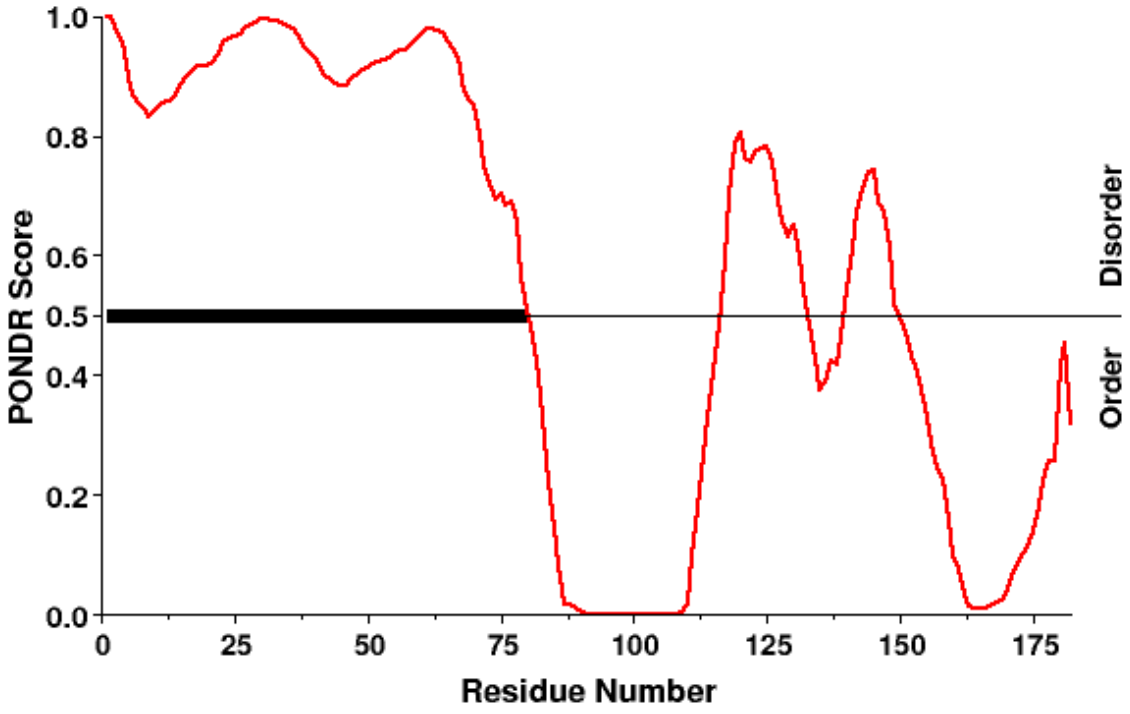

Figure S3-3. Structural disorder prediction of *D. melanogaster* flightin amino acid sequence.

Method MS2. Procedure for Fourier processing of EM Myofibrillar Cross-sections to Quantify Myofilament Lattice Spacing and Regularity.

FFT processing of EM cross-sectional images:

All cross section images with same magnification were selected without contrast enhancement, brightness modification and/or changing image size. Full cross-section of a single myofibril was selected (Figure MS2A left image) and copied to a new image with 512×512 or 1024×1024 pixel size to make sure only myofilaments are included in the image as shown below in the snapshot (Figure MS2A right image).

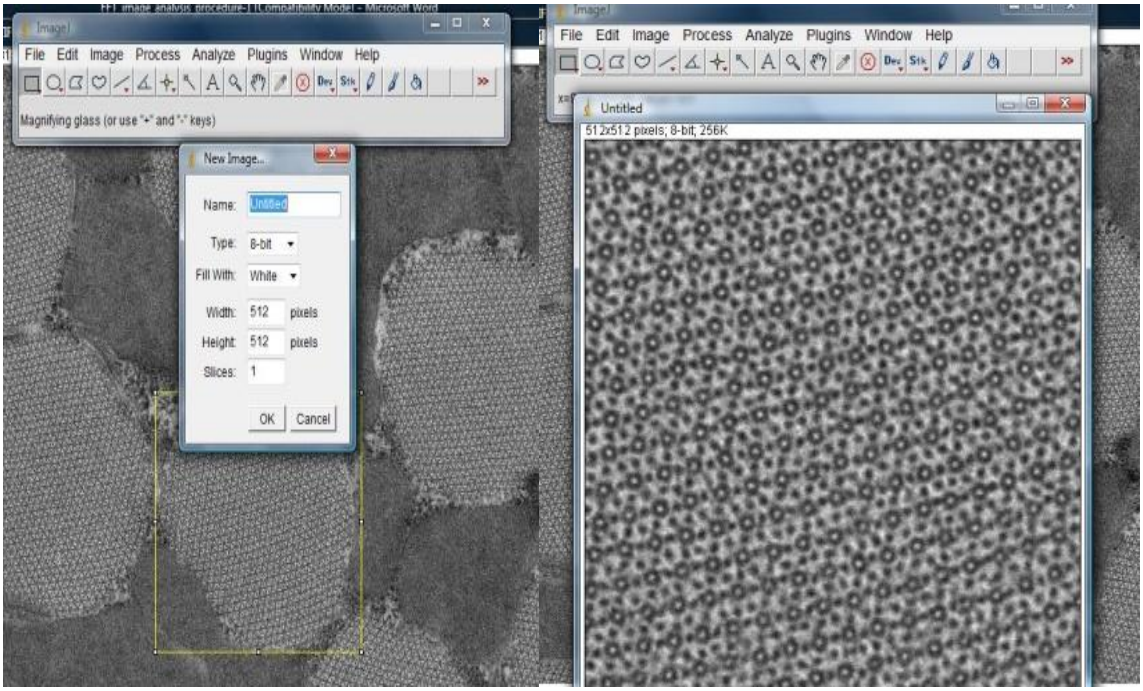

Figure MS2A

ImageJ FFT option was used to process the fourier transform of the image as shown below (Figure MS2B).

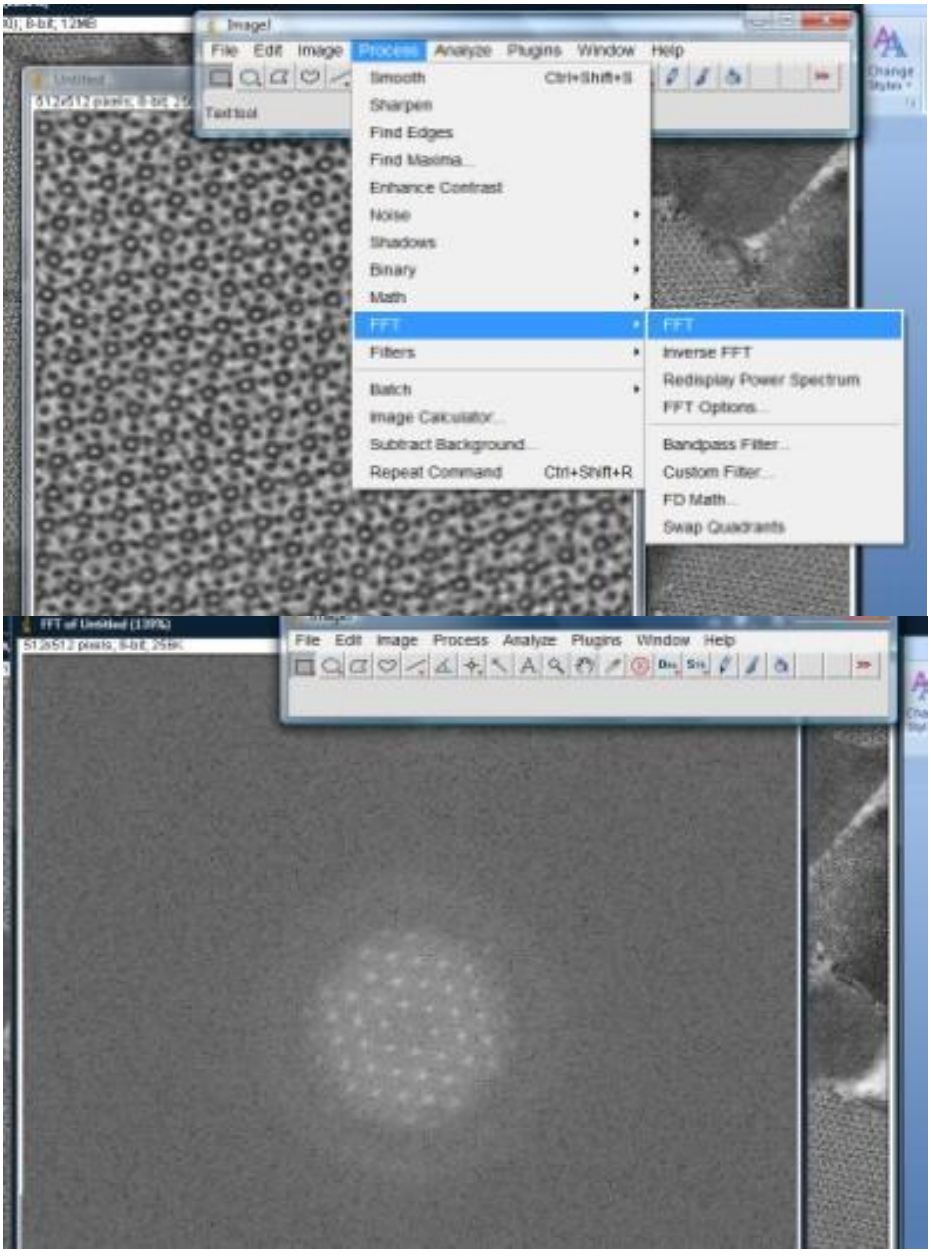

Figure MS2B

FFT analysis to quantify inter-filament distance measurements:

Pixel size (eg.: 14.26Å) in the original image EM image was noted down. FFT image was scaled (yellow horizontal line in figure MS2C) arbitrarily as shown below in the snapshot (eg.: 512 pixels in length in Figure MS2C).

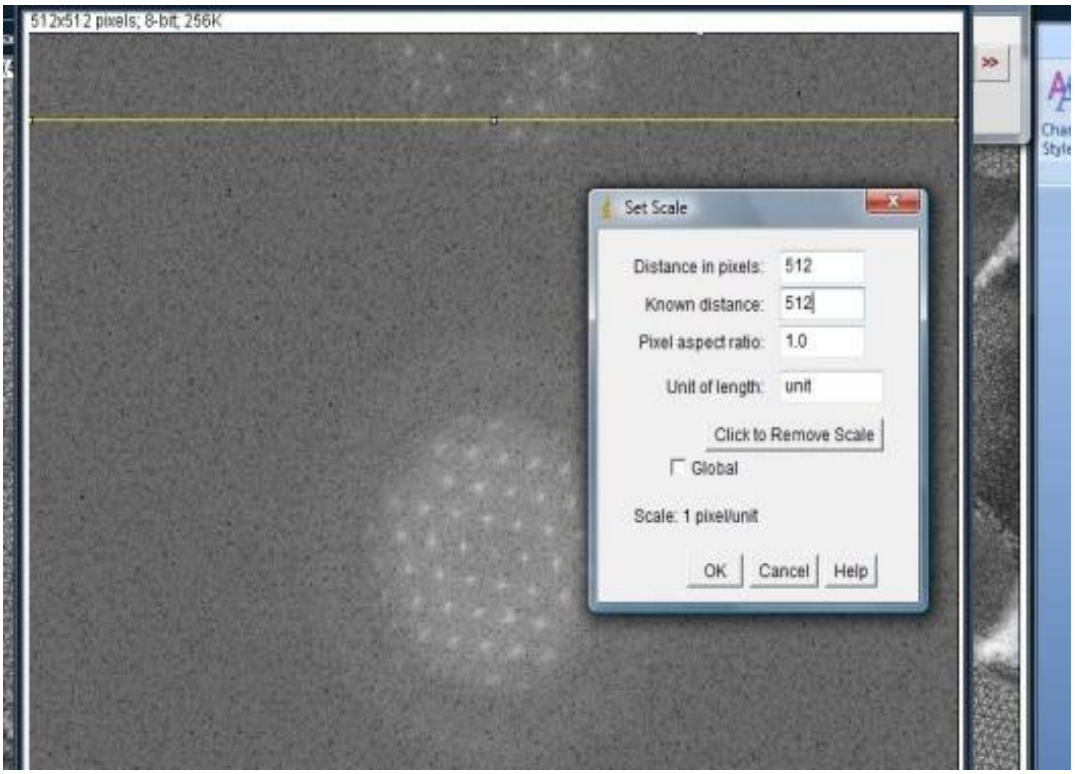

Figure MS2C

The distance from the center to the 1st order reflection in the FFT was measured. For accuracy, the distance in pixels on a drawn line (passing through the center) was measured (Figure MS2D) and was divided by the number of inter-spot distances included

in the line. In this example (figure MS2D), the length of the line is 114.242 pixels and the number of spots passing through the line is seven.

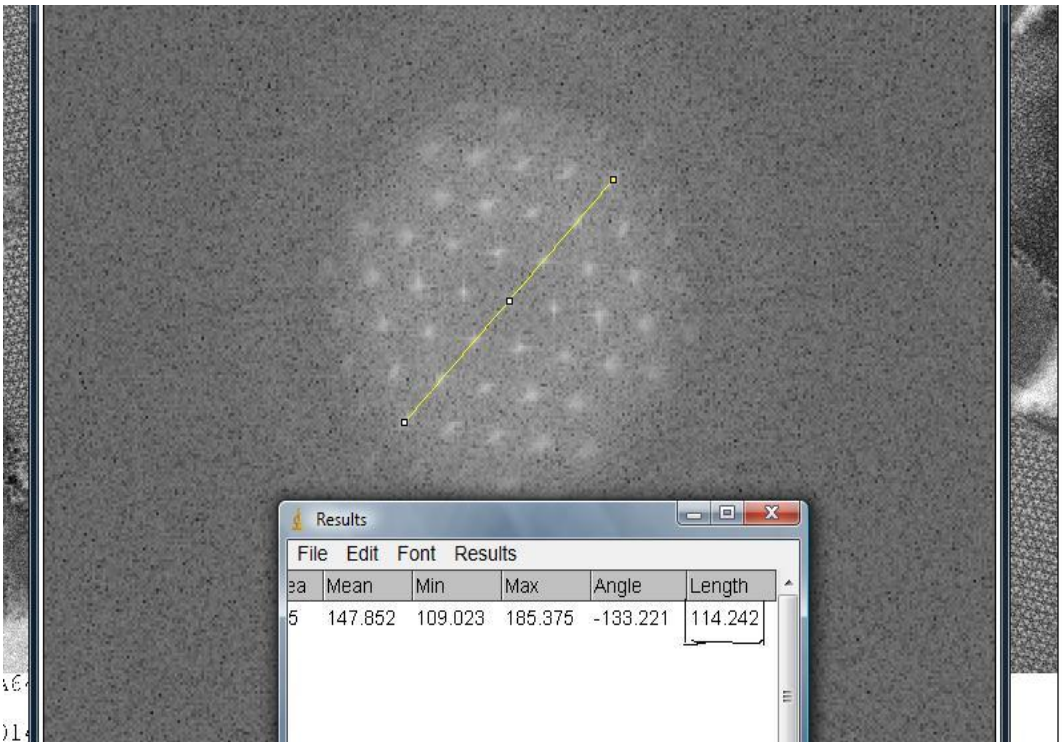

Figure MS2D

There is a strict correlation between real and fourier space. Total number of pixels of FFT × distance / pixel (from the original image) should be constant. Therefore, 512 × 14.26 Å = 19.04 × inter-filament distance ($d_{1,0}$).

Example: $d_{1,0}$ = 512 × 14.26 Å / 19.04 = 383.46 Å or 38.35 nm. Therefore, inter-thick filament distance = $2/\sqrt{3} \times d_{1,0}$ = 38.35 × $2/\sqrt{3}$ = 44.28 nm.

Order or regularity of the lattice as a measure of resolution of the fourier power spectrum and the sharpness of the 1,0 FFT spot intensities:

Resolution:

FFT image was scaled. A line was drawn connecting as many spots as can be seen across both sides of the center of the FFT (Figure MS2E). The distance in pixels of the line was measured and divided by 2 (eg. 138.593/2 spots= 69.3 pixel resolution in Fourier space). Resolution of the myofilament lattice was calculated as the total number of pixels of FFT × distance per pixel (from the original image) divided by pixel resolution in fourier space. In this example, 512 × 1.426 nm / 69.3 = 10.54 nm resolution.

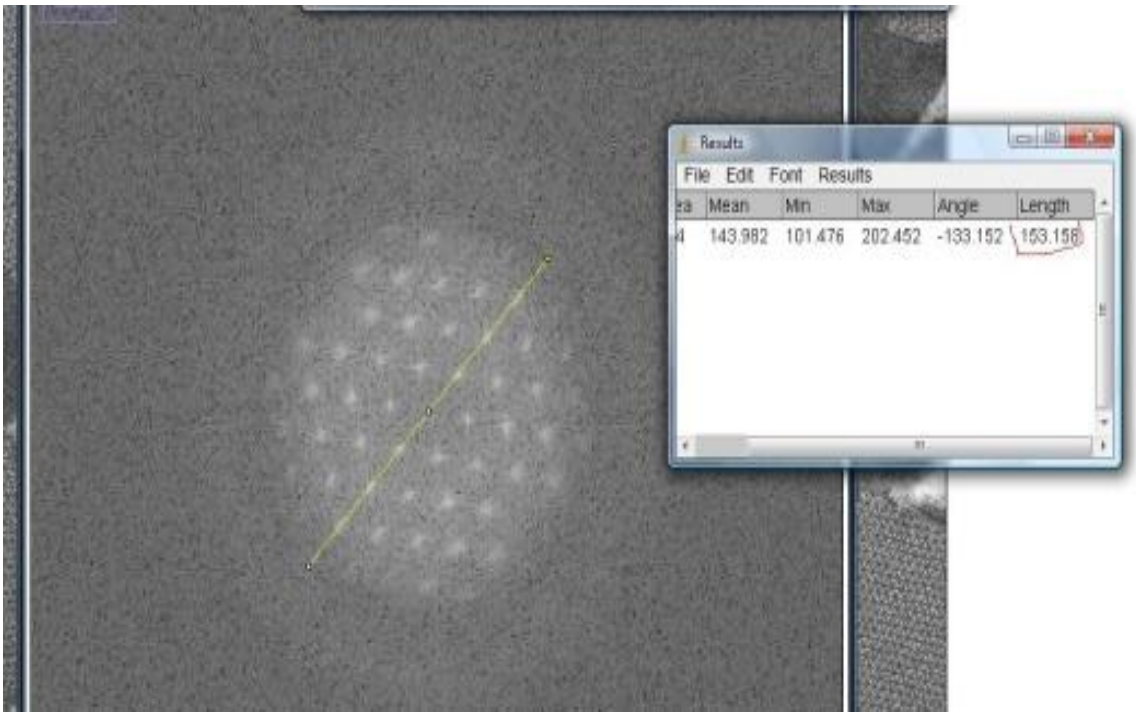

Figure MS2E

Sharpness:

FFT images were transformed from Cartesian coordinate to polar coordinate using “Polar Transform” plugin in ImageJ.

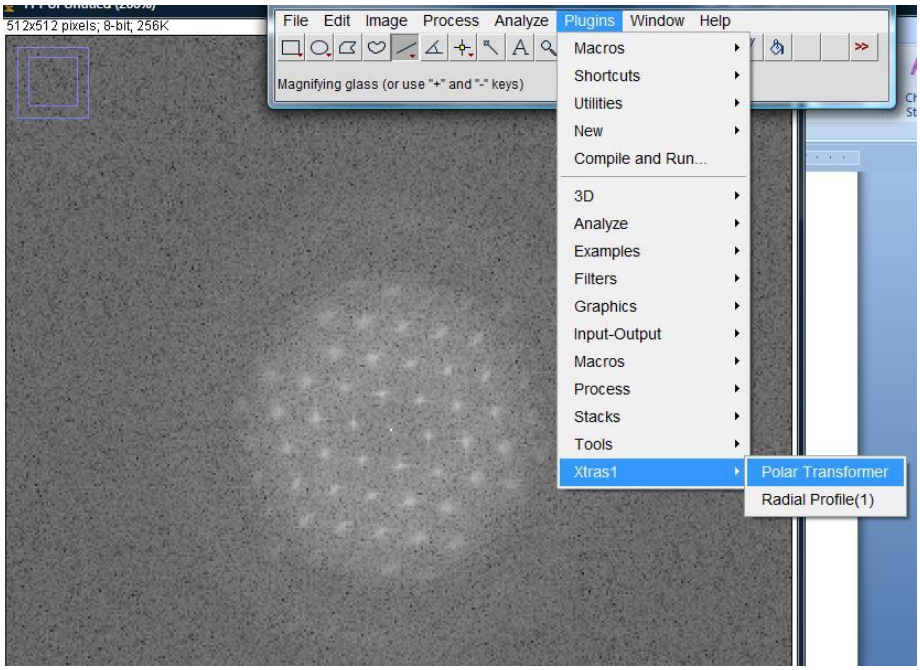

Figure MS2F

A line along the (1,0) spots were drawn and intensity profile was plotted.

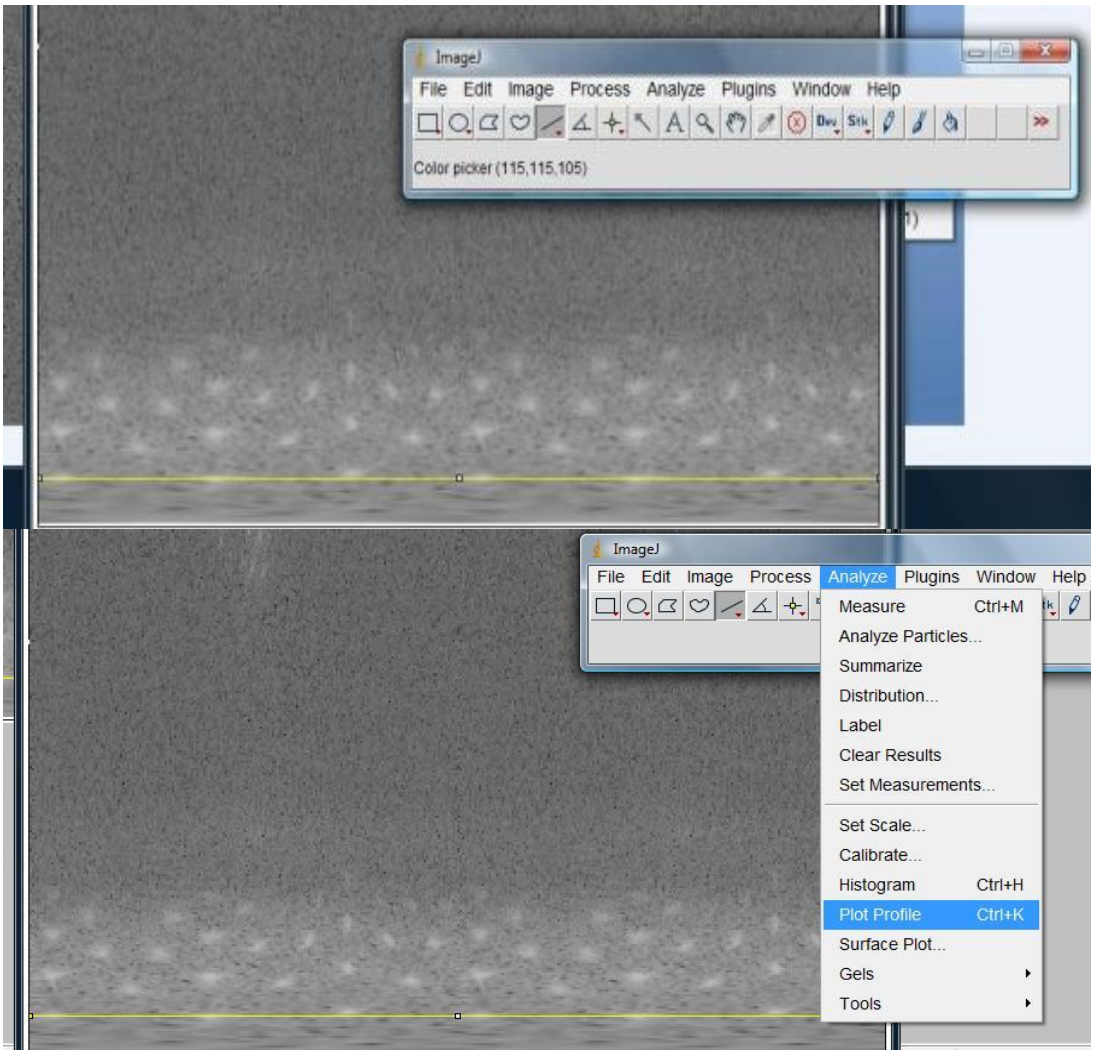

Figure MS2G

From the intensity profile plot, the log of peak height of the spot intensities and the width at half maximum of the intensity peaks were measured from a baseline as shown in Figure MS2H (the double headed arrows), for each of the 6 spots in the 1,0 reflection plane and then averaged.

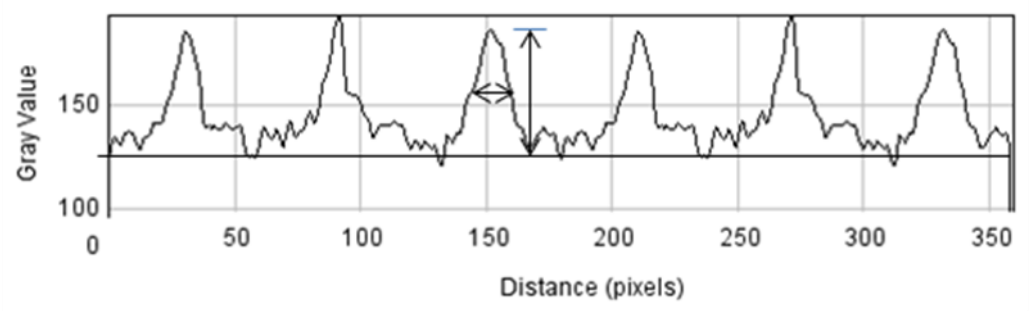

Figure MS2H

The log of peak height and the width at the half maximum of the intensities are a measure of the spot sharpness and provide an estimate of the regularity of the lattice. Lower peak intensities and broader half width will indicate more variability in the spacing between lattice planes across the cross section of the myofibril.

Method S3. Validation of the EM Fourier Power Spectrum Analysis to Measure Myofilament Lattice Spacing.

To validate the EM fourier power spectrum analysis to measure lattice spacing, we used the following strategies:

i) Pre-setting $d_{1,0}$ values on EM images, we measured the lattice spacing values by fourier analysis and compared with the pre-set values.

ii) Measurement of the $d_{1,0}$ spacing values by fourier analysis on EM images of myofibril cross-sections of the M-line region, and comparing it with that of the A-band region myofibrillar fourier analysis.

iii) Measurement of $d_{1,0}$ values by fourier analysis on EM images of unskinned myofibril cross-sections from flies of different ages [118] and comparing with that of the previously reported values by in vivo X-ray diffraction measurements [118].

iv) Pre-setting $d_{1,0}$ values on model myofibrils, we measured the lattice spacing values by fourier analysis and compared with the pre-set values.

Validation on real myofibrillar EM images:

i) To validate if the distance between the center and the 1,0 spots in the FFT of EM myofibril images are $d_{1,0}$ spacings [109], the $d_{1,0}$ values in the *fln*$^+$ control cross-sectional images were pre-set in the EM images to 45nm, 50nm and 55nm. The FFT measurements were done and correlated with the pre-set values as shown below in Table MS3A.

Table MS3A. FFT measurement validation by pre-set $d_{1,0}$ values in the EM myofibrillar images of the control *fln*$^+$ strain. All values mean±SEM, number in parenthesis indicate number of image measurements carried out.

Strain	Pre-set $d_{1,0}$ (nm)	FFT inter-spot distance measured (nm)
fln$^+$	45	44.67±0.2 (30)
fln$^+$	50	49.49±0.2 (30)
fln$^+$	55	55.01±0.2 (30)

Results in Table MS3A indicates that the FFT measurements were very similar to the pre-set $d_{1,0}$ values in the images.

ii) The method was further validated on M-line regions of the *fln*$^{+}$ myofibril cross-sections (Figure MS3A). The $d_{1,0}$ spacing values were 43.7±0.78 nm (5), where the value is mean±SEM and number in parenthesis indicate number of measurements performed. This value is similar to the control line $d_{1,0}$ value calculated on cross-section at the A-band region with both thick and thin filaments (compare with Table 3-3), and are similarly ~11% smaller compared to previously reported in vivo values in X-ray diffraction measurements of live flies [65]. This is due to lattice shrinking by dehydration steps performed for electron microcopy sample preparation (see Materials and Methods, and [64]).

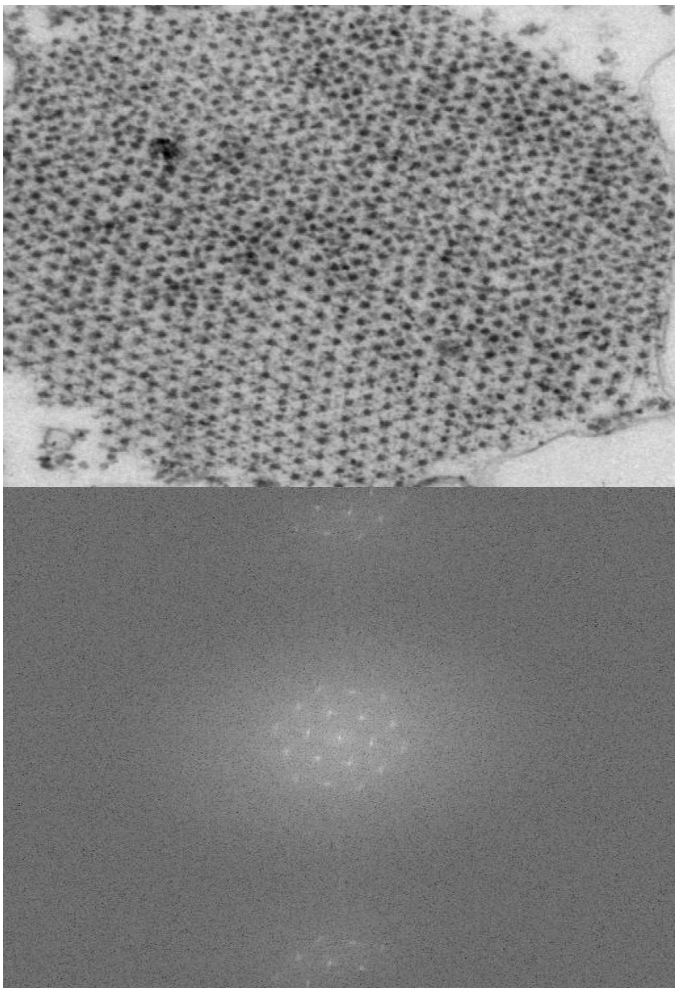

Figure MS3A

iii) To further validate the novel FFT analysis, using the young, median aged and old fly IFM myofibrillar cross-sectional images taken in Miller MS et al. Biophys J (2008) [118], the $d_{1,0}$ spacings were measured and corresponding inter-thick filament spacings were calculated by multiplying $d_{1,0}$ with $2/\sqrt{3}$. The analysis result is shown below in Table MS3B.

Table MS3B. FFT analysis on myofibrillar cross-sectional EM images from flies of different ages as used for in vivo X-ray diffraction experiments done in Miller MS et al Biophys J (2008) [118]. All values are mean±SEM, numbers in parenthesis indicate number of myofibrillar cross-sections analyzed. * $p<0.05$ vs Young and Median aged flies.

Fly age	$d_{1,0}$ (nm)	Inter-thick filament spacing (nm)	Inter-thick filament spacing of live flies (nm) [118]
Young (1-3 days)	43.51±0.37 (22)	50.24±0.43 (22)	55.63±0.12 (19)
Median (7 weeks)	42.69±0.22 (75)	49.29±0.25 (75)	55.54±0.43 (8)
Old (8 weeks)	46.84±0.51* (20)	54.08±0.60* (20)	57.41±0.45 * (13)

The inter-thick filament spacing in our FFT measurements (Table MS3B) are ~ 6-11% smaller compared to in vivo X-ray diffraction measurements [118] due to lattice shrinkage by dehydration steps during EM preparation. The lattice spacing in the myofibrils of old (8 weeks) flies are significantly greater compared to that of young or median aged flies (Table MS3B), which is in accordance with the in vivo X-ray diffraction data [118]. This is a further validation of the FFT analysis for measuring $d_{1,0}$ lattice spacing.

iv) Validation on myofilament lattice models:

To validate the findings of the FFT analysis on real EM myofibrillar cross-section images, model myofibrils were created. Figure MS3B shows a model myofibril with the double hexagonal lattice of hollow thick and filled thin filaments (MyAc model), and its corresponding FFT spectrum whose brightness and contrast adjusted for clarity (not adjusted in real image FFTs, Table 3-3 data).

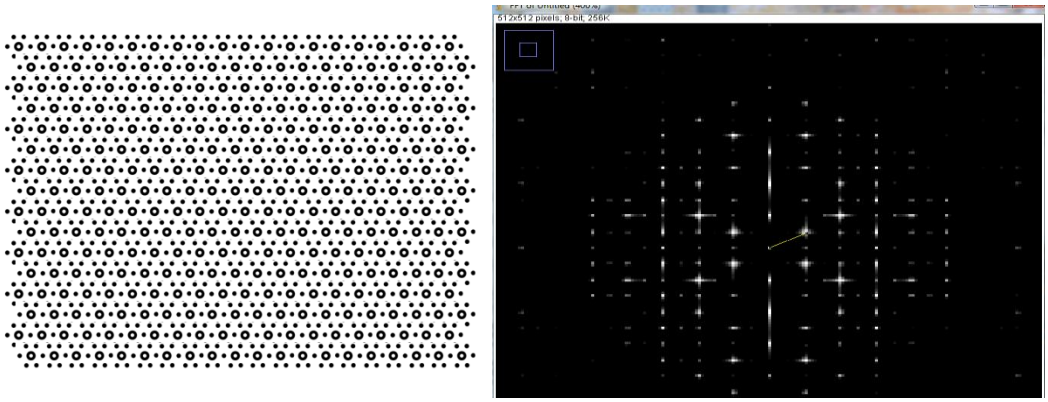

Figure MS3B. Myofibril cross-section model and corresponding FFT (brightness and contrast adjusted).

Figure MS3C shows a model myofibril with only hollow thick filaments (M model) and its corresponding FFT spectrum (brightness and contrast adjusted).

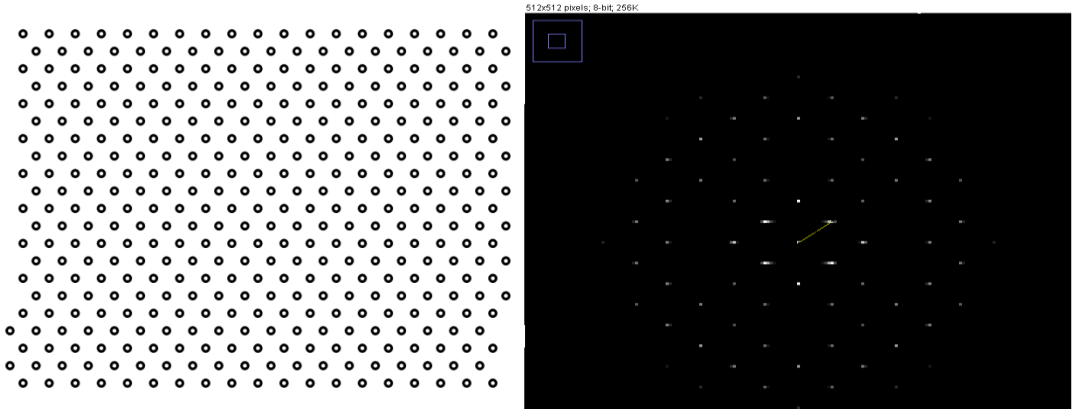

Figure MS3C. Only hollow thick filament cross-section model and corresponding FFT (brightness and contrast adjusted).

Both Figures MS3B and C have similar FFT spectrum spot patterns after brightness and contrast adjusting. This indicates that the FFT spots are representative of the thick filament planes in the myofilament lattice of the model.

Table MS3C. FFT measurement validation by pre-set $d_{1,0}$ values on myofibillar models. All values are mean±SEM, number in parenthesis indicate number of measurements carried out.

Model	Pre-set $d_{1,0}$ (nm)	FFT inter-spot distance measured (nm)
MyAc	45	44.75±0.06 (30)
MyAc	50	49.59±0.05 (31)
MyAc	55	54.70±0.08 (30)
M	45	44.98±0.02 (30)
M	50	49.86±0.05 (31)
M	55	55.08±0.08 (30)

Table MS3C shows the FFT analyzed values for the different myofibrillar models (Figure MS3B and C), where the measured values are similar to the pre-set $d_{1,0}$ spacing values in each model tested. Moreover, there was no significant difference between FFT analyzed values of the myofibril models and the real myofibril cross-section of *fln*$^{+}$ control strain (compare Table MS3C vs MS3A).

Overall, real and model myofibril FFT analyses using pre-set $d_{1,0}$ spacing values indicated that the FFT spots of the real myofibril cross-sections (Figure 3-4C and F) are representative of the thick filament (1,0) planar diffraction and subsequent harmonics of

it. This further indicated that the distance from the center of the fourier space to the 1,0 reflection spots is $d_{1,0}$ spacing in the myofilament lattice real space.

Method MS4. Transmission electron microscopy on fibers torn in rigor

Individual skinned muscle fibers with aluminum T-clips on both ends that tore in rigor during fiber mechanics experiments were removed from the strain gauge and motor after the completion of the mechanics protocol (see Materials and Methods), fixed overnight in Karnovsky's fixative (2.5% v/v glutaraldehyde, 1% v/v paraformaldehyde in 0.1M Cacodylate buffer), embedded in a small block of agarose (for ease of handling and visualizing single fibers), and prepared for imaging like the bisected fly thoraces as previously described [64]. Images were at 8000x magnification, 1.426 nm pixel size.

Figure S3-1. Putative positive selection in flightin N-terminal region. Evolutionary selective forces acting on individual amino acid positions in the *D. melanogaster* (reference species) flightin sequence (aa positions denoted by numbers) using 12 *Drosophila* flightin coding sequences as query in the Selecton server ([111,112], and see Method MS1). The rate of evolution (dN/dS) i.e. the ratio of the rate of non-synonymous (amino acid altering) to synonymous (silent) substitutions of each amino acid position of *D. melanogaster* flightin was retrieved. The *D. melanogaster* flightin N-terminus sequence (boxed region) has signatures of positive selection sites (dN/dS>1) compared to rest of the protein which is under purifying selections (dN/dS≤1). The average evolutionary rate (average dN/dS) of the lineage leading *D. melanogaster* flightin N-terminal region is 0.4 compared to 0.08 of the rest of the protein.

Figure S3-2. No evidence of positive selection on some other IFM genes. Evolutionary selective forces acting on individual amino acid positions in the *D. melanogaster* (reference species) myosin regulatory light chain (A), myofilin (B) and paramyosin (C) sequences (some sequence positions denoted by numbers) using respective coding sequences of 12 *Drosophila* species as query in the Selecton server ([111,112], and see Method MS1).

Figure S3-3. Flightin N-terminal sequence predicted to be disordered. PONDR VL-XT server [115-117] prediction of structural disorder of *D. melanogaster* flightin amino acid primary sequence. Flightin sequence residue number is shown in the x-axis and the PONDR prediction score for disorder is in y-axis. The cutoff score is 0.5 above which the amino acid sequence is predicted to be disordered. Flightin N-terminal region is predicted to be highly disordered compared to the rest of the protein.

Figure S3-4. **Electron microscopy of fibers torn during rigor measurements.** Transmission electron microscopy images showing longitudinal sections of fln^{+} (A and B) and $fln^{\Delta N62}$ (C and D) IFM fibers following sinusoidal length perturbation analysis. Shown are representative sarcomeres from two fibers that tore when placed in rigor solution. (A and C) sarcomeres away from the torn zone and (B and D) sarcomeres close to the torn zone. Note that Z bands in (D) are thin and perforated (black arrows), the M line is nearly vanished (white arrow) and thick filaments appear to buckle (circles). These features are unique to mutant sarcomeres in the torn zone. Scale bars represent 1μm (A-D).

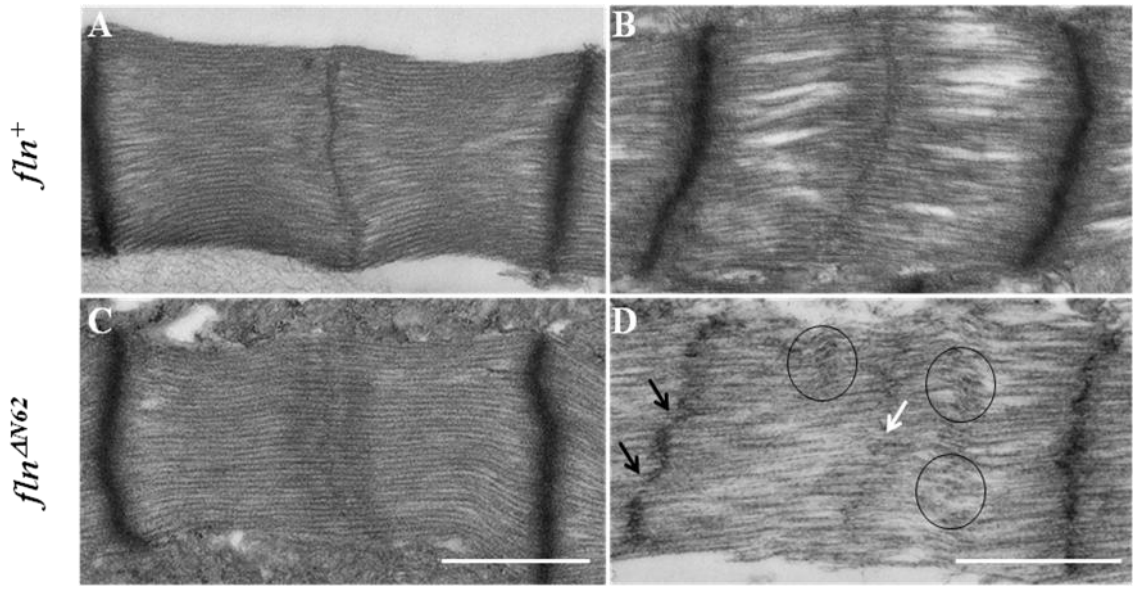

Figure S3-4. Electron microscopy showing longitudinal sections of IFM fibers torn in rigor.

Table S3-1A. Annotated symbol and flybase ID of flightin and corresponding orthologous sequences used in this study.

Gene	Species	Annotated Symbol	Flybase ID
flightin (fln)	*D. melanogaster*	CG7445	FBgn0005633
flightin (fln)	*D. simulans*	GD12234	FBgn0183970
flightin (fln)	*D. sechellia*	GM14859	FBgn0169780
flightin (fln)	*D. erecta*	GG13353	FBgn0105625
flightin (fln)	*D. yakuba*	GE22446	FBgn0067972
flightin (fln)	*D. ananassae*	GF10833	FBgn0087873
flightin (fln)	*D. pseudoobscura*	GA22938	FBgn0244340
flightin (fln)	*D. persimilis*	GL25050	FBgn0162637
flightin (fln)	*D. willistoni*	GK18981	FBgn0220979
flightin (fln)	*D. virilis*	GJ11502	FBgn0198760
flightin (fln)	*D. mojavensis*	GI13378	FBgn0136135
flightin (fln)	*D. grimshawi*	GH14726	FBgn0122202

Table S3-1B. Annotated symbol and flybase ID of myosin regulatory light chain and corresponding orthologous sequences used in this study.

Gene	Species	Annotated Symbol	Flybase ID
Myosin regulatory light chain (mlc2)	*D. melanogaster*	CG2184	FBgn0002773
Myosin regulatory light chain (mlc2)	*D. simulans*	GD17257	FBgn0188819
Myosin regulatory light chain (mlc2)	*D. sechellia*	GM12174	FBgn0167114
Myosin regulatory light chain (mlc2)	*D. erecta*	GG11956	FBgn0104251
Myosin regulatory light chain (mlc2)	*D. yakuba*	GE23405	FBgn0068125
Myosin regulatory light chain (mlc2)	*D. ananassae*	GF16196	FBgn0093218
Myosin regulatory light chain (mlc2)	*D. pseudoobscura*	GA15288	FBgn0075311
Myosin regulatory light chain (mlc2)	*D. persimilis*	GL14063	FBgn0151668
Myosin regulatory light chain (mlc2)	*D. willistoni*	GK13145	FBgn0215154
Myosin regulatory light chain (mlc2)	*D. virilis*	GJ10371	FBgn0197655
Myosin regulatory light chain (mlc2)	*D. mojavensis*	GI23377	FBgn0146103
Myosin regulatory light chain (mlc2)	*D. grimshawi*	GH18385	FBgn0125853

Table S3-1C. Annotated symbol and flybase ID of myofilin and corresponding orthologous sequences used in this study.

Gene	Species	Annotated Symbol	Flybase ID
myofilin (Mf)	*D. melanogaster*	CG6803	FBgn0038294
myofilin (Mf)	*D. simulans*	GD20380	FBgn0191853
myofilin (Mf)	*D. sechellia*	GM25804	FBgn0180660
myofilin (Mf)	*D. erecta*	GG20880	FBgn0113064
myofilin (Mf)	*D. yakuba*	GE26409	FBgn0068265
myofilin (Mf)	*D. ananassae*	GF17159	FBgn0094177
myofilin (Mf)	*D. pseudoobscura*	GA19873	FBgn0079869
myofilin (Mf)	*D. persimilis*	GL21688	FBgn0159281
myofilin (Mf)	*D. willistoni*	GK13979	FBgn0215985
myofilin (Mf)	*D. virilis*	GJ23223	FBgn0210325
myofilin (Mf)	*D. mojavensis*	GI23663	FBgn0146389
myofilin (Mf)	*D. grimshawi*	GH14549	FBgn0122025

Table S3-1D. Annotated symbol and flybase ID of paramyosin and corresponding orthologous sequences used in this study.

Gene	Species	Annotated Symbol	Flybase ID
Paramyosin (Prm)	*D. melanogaster*	CG5939	FBgn0003149
Paramyosin (Prm)	*D. simulans*	GD12965	FBgn0184689
Paramyosin (Prm)	*D. sechellia*	GM24918	FBgn0179780
Paramyosin (Prm)	*D. erecta*	GG15062	FBgn0107316
Paramyosin (Prm)	*D. yakuba*	GE21285	FBgn0238553
Paramyosin (Prm)	*D. ananassae*	GF10148	FBgn0087189
Paramyosin (Prm)	*D. pseudoobscura*	GA19246	FBgn0079243
Paramyosin (Prm)	*D. persimilis*	GL10286	FBgn0147896
Paramyosin (Prm)	*D. willistoni*	GK17471	FBgn0219470
Paramyosin (Prm)	*D. virilis*	GJ12302	FBgn0020071
Paramyosin (Prm)	*D. mojavensis*	GI12410	FBgn0135167
Paramyosin (Prm)	*D. grimshawi*	GH16141	FBgn0123612

Table S3-2. Flightin N-terminal truncation parental lines in wild type (+) background and corresponding daughter lines in *fln^{0}* background

Parental Lines (code)	Genotype	Daughter Lines	Genotype
fln$^{\Delta N62A}$ /+	*w^{1118};P{w^{+}, Act88Ffln$^{\Delta N62}$}; +, e*	*fln$^{\Delta N62A}$/fln^{0}* (fln-ndl4.26)	*w^{1118};P{w^{+}, Act88Ffln$^{\Delta N62}$}; fln^{0}, e*
fln$^{\Delta N62B}$ /+	*w^{1118};P{w^{+}, Act88Ffln$^{\Delta N62}$}; +, e*	*fln$^{\Delta N62B}$/fln^{0}* (fln-ndl5.21)	*w^{1118};P{w^{+}, Act88Ffln$^{\Delta N62}$}; fln^{0}, e*
fln$^{\Delta N62C}$ /+	*w^{1118};P{w^{+}, Act88Ffln$^{\Delta N62}$}; +, e*	*fln$^{\Delta N62C}$/fln^{0}* (fln-ndl4.27)	*w^{1118};P{w^{+}, Act88Ffln$^{\Delta N62}$}; fln^{0}, e*
fln$^{\Delta N62D}$ /+	*w^{1118};P{w^{+}, Act88Ffln$^{\Delta N62}$}; +, e*	*fln$^{\Delta N62D}$/fln^{0}* (fln-ndl7.14)	*w^{1118};P{w^{+}, Act88Ffln$^{\Delta N62}$}; fln^{0}, e*
fln$^{\Delta N62E}$ /+	*w^{1118};P{w^{+}, Act88Ffln$^{\Delta N62}$}; +, e*	*fln$^{\Delta N62E}$/fln^{0}* (fln-ndl8.21)	*w^{1118};P{w^{+}, Act88Ffln$^{\Delta N62}$}; fln^{0}, e*

Table S3-3. Courtship song parameters used in this study (see also Chapter 2 or [106])

Parameter	Abbreviation	Description
Sine song frequency	SSF	Carrier frequency (Hz) of sine song
Pulse duty cycle	PDC	Equivalent to the ratio of the length of pulse song to the total time of recording
Pulse length	PL	Time duration (ms) of a pulse
Cycles per pulse	CPP	Number of zero crossings by the pulse waveform divided by two
Intrapulse frequency	IPF	Carrier frequency (Hz) of a pulse
Interpulse interval	IPI	Time duration (ms) between the equivalent peaks of two consecutive pulses in a train

Audio S3-1. Male courtship song (sine and pulse) sample of *fln*$^{+}$ male (Figure 3-3 middle panel) in the presence of a wild type (Oregon R) strain female mate. File can be downloaded from the following weblink:

https://www.researchgate.net/publication/236123369_Audio_S3-1?ev=prf_pub

Audio S3-2. Male courtship song (sine and pulse) sample of *fln*$^{\Delta N62}$ male (Figure 3-3 bottom panel) in the presence of a wild type (Oregon R) strain female mate. File can be downloaded from the following weblink:

https://www.researchgate.net/publication/236123733_Audio_S3-2?ev=prf_pub

Video S3-1. Male courtship success of *fln*$^{+}$ male with wild type (Oregon R strain) female mate in a single pair mating assay (see Materials and Methods). File can be downloaded from the following weblink:

https://www.researchgate.net/publication/236123738_Video_S3-1?ev=prf_pub

Video S3-2. *fln*$^{\Delta N62}$ male gets courtship success for wild type (Oregon R strain) female mate in a single pair mating assay (see Materials and Methods). File can be downloaded from the following weblink:

https://www.researchgate.net/publication/236123741_Video_S3-2?ev=prf_pub

Video S3-3. *fln*$^{\Delta N62}$ male gets outcompeted by *fln*$^{+}$ control male for wild type (Oregon R strain) female mate choice. File can be downloaded from the following weblink:

https://www.researchgate.net/publication/236123745_Video_S3-3?ev=prf_pub

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AUTHOR CONTRIBUTIONS

The author(s) have made the following declarations about their contributions: Conceived and designed all experiments: SC JOV. Designed and supervised the fiber mechanics experiments: BCWT. Performed the experiments: SC VLF HV MR. Analyzed the data: SC BCWT. Wrote the paper: SC JOV.

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COMPETING INTEREST

No conflicts of interest, financial or otherwise, are declared by the author(s).

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CHAPTER 4 JOURNAL ARTICLE

Differential Effects of *Drosophila* Flight Muscle Myosin Regulatory Light Chain Mutations on Flight and Male Courtship Song: Evidence of Distinct Contractile Mechanisms for Flight Muscle Driven Behaviors

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ABSTRACT

The *Drosophila* asynchronous indirect flight muscles (IFM) is a versatile machine driving the high power requiring flight behavior necessary for survival, and is utilized during the male courtship song enhancing reproductive success. IFM powers flight by the stretch activation mechanism, but its contractile mechanism and the role of muscle genes in song production is not known. The function of thick filament associated protein, myosin regulatory light chain (Dmlc2) has been studied extensively for its role in stretch activation and flight using the following mutants: D*mlc*2$^{\Delta 2\text{-}46}$ (Ext) with the N-terminal extension truncation, D*mlc*2^{S66A,S67A} (Phos) with disruption of myosin light chain kinase phosphorylation sites, and D*mlc*2$^{\Delta 2\text{-}46;S66A,S67A}$ (Dual) with both the above mutations. These mutants are known to have an IFM compromised in stretch activation response and myosin kinetics leading to reduced wing beat frequency and flight performance. By performing behavioral assays and analyzing the courtship song characteristics, this study aims to elucidate if these *Dmlc2* mutations affect courtship song as a way to understand the contractile mechanism of IFM during singing. Results show that *Dmlc2* mutations do not have a pleiotropic effect on flight and song. Flightless mutants Phos and Dual are capable of both pulse and sine singing suggesting that these mutations affect song minimally compared to flight. Pulse song is least affected with none of these mutations affecting interpulse interval (IPI), the most critical sexually selected pulse song parameter in *Drosophila*, especially in the *melanogaster* subgroup, as well as the intrapulse frequency (IPF) compared to rescued control null, D*mlc*2^{+} (Control). Also, sine song frequency (SSF) was higher in the Ext and Phos mutants compared to Control but have a

subtractive effect in the Dual mutant male which sings with a normal SSF. This is the opposite of the known additive effects of Ext and Phos in the Dual mutant on flight wing beat frequency, suggesting a possible distinct population of myosin cross-bridges used for sine song compared to flight. That mutations in Dmlc2 are manifested differently for song and flight suggest that stretch activation plays a minimal or no role in song production.

INTRODUCTION

One of the fundamental interests in muscle biology is to understand what contractile mechanisms are utilized for power modulations in order to perform distinct power requiring behaviors used for different contexts by the same musculature. The ability to fly, present in a majority of insect species including *Drosophila*, is generally considered one of the main driving forces in the evolutionary success of insects subject to natural selection and subsequent speciation [1,2]. Flight is an aerodynamically costly behavior requiring high mechanical power output provided by its flight musculature [3]. Species-specific acoustic communication signals are also critically important [4-12] for facilitating pre-mating reproductive isolation under sexual selection and subsequent speciation [13] of both vertebrates and insects. For example, in *Drosophila*, males generate species-specific courtship songs of rhythmic pulses and sinusoidal hums generated by small amplitude wing vibrations [14-18] using the thoracic flight musculature that gets neurally activated during singing [19,20]. The elevated power from the flight musculature for wing flapping during flight in *Drosophila* has evolved to

overcome the high aerodynamic drag associated with lift production at relatively low Reynolds numbers (*Drosophila*, *Re*=134, [21]), that facilitated in the evolution of exceptionally high wing flapping frequencies of up to 1000 Hz [22]. Given that *Drosophila* males remain grounded while beating one wing at a time during courtship song (reviewed in [16]) with an amplitude that is $1/4^{th}$ of that during normal flight [23], suggests that song production requires much less power than flight. However, it is not known what contractile mechanism is utilized or the contribution of muscle genes in the flight musculature during courtship song production. Undertstanding the role of muscle genes for song and flight, will pave the way to understand the contractile mechanism of muscle tissue systems to modulate power separate behaviors.

Drosophila uses their thoracic asynchronous indirect flight muscles (IFM) to generate the high wing flapping frequencies of ~200Hz, even with a much lower rate (~ 5Hz) of motor neuron activation [24, 25]. The IFM fulfills this myogenically by using the mechanism of stretch activation and shortening deactivation at a relatively constant $[Ca^{2+}]$ [22]. The IFM accomplishes this with the help of its two antagonistic sets of muscles, the dorsal longitudinal and dorsal ventral muscles (DLM and DVM), connected to the thoracic exoskeleton rather than the wing hinge [26]. These muscles function together to create a reciprocally activating resonant thoracic box [27,28] driving the large sweeping motion of the wings during flight [29,30].

Courtship song carrier frequencies have broad distributions with the *D. melanogaster* pulse song frequency (IPF, Table S4-1) ranging from 200-400 Hz [31], and the sine song frequency (SSF, Table S4-1) ranging from 130-185 Hz [32], whereas the

wing beat frequency during flight also varies from 180-240 Hz. Even though the IFM, the main power generating muscles for flight, has been shown to be neurally activated [19] and directly involved [Chapter 3] during song, it is not known how the different and broad frequency ranges of wing beats for the two behaviors are controlled in the same system. In contrast, *Drosophila* mating does not occur aerially and therefore the males do not need to overcome drag forces for lift production indicating that courtship singing by the wing might require much less muscle mechanical power than flight. Therefore, IFM provides an excellent system to understand the contractile mechanisms of behavioral outputs with separate power requirements, especially with song being much distinct from flight from the point of view of ecology, evolution, physiology and aerodynamics.

In order understand how muscle genes are being utilized for the two behaviors in the IFM, and the contractile mechanism during singing, we tested for the effect on courtship song generation of mutations of the highly conserved [33] *Drosophila* thick filament protein myosin regulatory light chain (Dmlc2) (Figure 4-1). These mutants did not have any major IFM structural abnormality [34,36,39] which enable us to understand the influence of Dmlc2 on courtship song, rather than being masked by the effect of any underlying structural defect. The mutations are known to have a large effect on stretch activation, myosin kinetics and flight performance [39], without having any major effect on calcium activated isometric tension [34-36]. Two such mutations in the *Dmlc2*, have been extensively characterized for their roles in IFM structure, cross-bridge kinetics, stretch activation response and power output for maximal wing flapping frequency and flight performance [34-38]. Truncation of the 46 amino acids N-terminal extension

(D*mlc*2$^{\Delta 2\text{-}46}$ or Ext) results in attenuation of stretch activation response with myosin heads moving away from actin target zones. The alanine substitutions of two myosin light chain kinase phosphorylation sites (D*mlc*2^{S66A,S67A} or Phos) results in even further attenuation of stretch activation response compared to the Ext mutant, with myosin heads less oriented towards actin target zones for strong binding [38,39]. This movement of the myosin heads away from the thin filament and towards the thick filament backbone due to these individual mutations [38] lowered cross-bridge kinetics in the mutants leading to reduced number of strongly bound cross-bridges. These further led to significantly lowering of fly wing beat frequency, work and power output [39]. The myosin head positional and mechanical effects were reflected in the whole fly where the Ext mutant was flight impaired and the Phos mutant was almost flightless compared to control flies [39]. The single mutations, when put together had an additive effect in the dual mutant (D*mlc*2$^{\Delta 2\text{-}46;S66A,67A}$ or Dual) *Drosophila* which carry both of the above mutations. In the Dual mutant, there was a much further impairment in normal myosin head pre-position towards actin target along with a marked decrease in maximum power output and no detectable wing beats for flying [39]. This indicated that both the Dmlc2 N-terminal extension and the phoshorylation sites are required for stretch activation response of the IFM to maximize power output for fulfilling flight requirement.

The above findings on the Dmlc2 mutations further led to a model for stretch activation. Similar to the N-terminal extension of the vertebrate essential light chain [40,41], the Dmlc2 N-terminal extension could act as a short tether to the thin filament [34] and upon stretch could bring the myosin heads in close proximity to their actin target

zones. Structural data drawn from X-ray diffraction of live flies suggested that the Dmlc2 phosphorylations could stiffen the myosin head so as to orient it optimally to increase the probability of its strong binding to the actin target [38,39]. Hence, it was suggested that these two (alignment and orientation) effects thereby could additively enhance stretch activation response by increasing the number of strongly bound active cross-bridges upon stretch.

These studies indicated that the Dmlc2 N-terminal extension and phosphorylations affect myosin motor position and function to enhance stretch activation for fulfilling high power requirement for flight [39]. Since IFM is required for courtship song, and given that courtship song potentially requires much less power than flight, we hypothesized that mutations of the *Dmlc2* will affect courtship singing to a lesser extent than flight. In other words, we hypothesized that flight abolished Dmlc2 mutants will be able to generate wing beats for courtship singing. To test this hypothesis, we recorded male courtship songs of the afore-mentioned Ext, Phos and Dual mutants, analyzed the song parameters (see Table S4-1), and compared the with those of a full length *Dmlc2* control rescued null (D*mlc*2^{+} or Control) male. Moreover, we tested if there is any song abnormality that has any biological significance, that is, if it affects the mutant males' mating success in competition with control males for wild type female mate. Here we report that the *Dmlc2* mutations do not have a major effect on courtship song parameters, unlike effect on flight mechanics. We find here that the mutants which are unable beat their wings for flying completely due to severely reduced stretch activation response of the IFM fibers, are able to generate courtship song. We also find evidence of a

subtractive effect of the *Dmlc2* single mutations (Ext and Phos) in the Dual mutant during sine song, contrary to their additive effects seen during flight. We conclude with implications of a separate contractile mechanism for singing based on our findings that Dmlc2 mutations affecting stretch activation have minimal to no effect on courtship song parameters.

RESULTS

Dmlc2 Mutant Males are Flight Compromised and Unable to Generate Normal Wing Beat Frequency

Miller et al. [39] had shown that the females of the single mutants (Ext, Phos) and the Dual mutant are flight impaired and flightless, respectively, compared to the Control females with reduced or no wing beat frequencies (Table S4-2). Since this study focuses on understanding whether these mutations affect male courtship song, we first tested the mutant males' wing beat frequency during flight. No significant difference was found in comparing the tethered wing beat frequencies of the males (Table 4-1) with the females (Table S4-2). This suggests that similar to the finding on females [39], the single mutant (Ext, Phos) males are flight impaired and show reduced wing beat frequencies compared to the Control males, with Phos mutation having a larger effect than Ext mutation. As with the females, the male Dual mutant is unable to generate wing beats for flying.

Flight Compromised *Dmlc2* Mutant Males are Capable of Generating Courtship Song

All the mutants (Ext, Phos and Dual) are capable of generating both courtship pulse and sine songs as shown by representative oscillograms (Figure 4-2) and the song audio clips [Audio S4-1, S4-2, S4-3, and S4-4].

***Dmlc2* Single Mutations increase Sine Song frequency**

During sine song, the Ext mutant has an abnormally high SSF (215±4.6 Hz) followed by Phos (176±4.0 Hz) compared to the Control male (131±0.7 Hz). Interestingly, even with the abnormal sine song in the single mutants, the Dual mutant sings a sine song with a similar SSF (137±1.7 Hz) as the Control male indicating that the single mutations are masking each other's effect (Figure 4-3A). There was no significant difference in sine song burst duration (SDUR, Figure 4-3B) between the mutants and the control, suggesting that the single mutations do not affect the sine singing vigor. The amplitudes of the sine songs of the control and the mutants are not important as differences could arise due to position of the singing male fly in the mating arena and the distance from the microphone set-up.

***Dmlc2* Mutations have Minimal Effect on Pulse Song**

Only the Ext mutant male shows longer cycles per pulse (CPP, Figure 4-4A) and a concomitant increase in pulse length (PL, Figure 4-4B) compared to Control and other mutants. In contrast to the reduced or abolished flight wing beat frequencies in the *Dmlc2*

mutant males (Table 4-1), the carrier frequency of the pulse song or intrapulse frequency (IPF, Figure 4-4C) is similar in all three mutants compared to control. The Phos mutant shows slightly lower IPF than Ext or Dual mutants, but not compared to the Control (Figure 4-4C). None of the *Dmlc2* mutations have an effect on inter-pulse interval (IPI, Figure 4-4D), one of the salient parameters under sexual selection in the *melanogaster* subgroup [42-44].

Interestingly, the Dual mutant could not sustain pulse singing as long as the Control and the single mutants, reflected by its lower (~ 85%) pulse duty cycle (PDC, Table S4-1) (Figure 4-4E). The amplitude ratio of sine to pulse song (AMP-RT, Table S4-1) is higher for the single (Ext and Phos) mutants as well as the Dual mutant compared to Control male (Figure 4-4F) indicating either the mutants sing with a louder sine song or a softer pulse song.

The Courtship Song Aberrations affect Male Courtship Success

We tested males in single pair mating assays with wild type (OR) females and found that all the mutant males are successful at courtship, albeit showing much reduced courtship performance compared to the Control line (Figure 4-6). Both courtship index (CI) and wing extension index (WEI) of all the mutants, especially the Ext mutant, in particular showed severe reduction compared to Control line. To understand if the subtle song aberrations found in the mutants affect their courtship success in competition with a Control male for an OR female, we performed courtship competition assays and calculated the female preference index (FPI), courtship index (CI) and wing extension

index (WEI) (see Materials and Methods for details). All the mutant males were outcompeted by the Control male for female preference (Video S4-1, S4-2, S4-3, Figure 4-5A) due to lower courtship performance, indicated by a lower CI and WEI for the Ext male and Dual male, and lower WEI for the Phos male compared to the Control male respectively (Figure 4-5B,C). The Ext mutant gets outcompeted for female preference (Video S4-4, Figure 3-5A) by both Phos and Dual mutants due to its lower CI and (Figure 4-5B,C). The Dual mutant males, which show the least song aberrations compared to other mutants based on the number of parameters affected (Figure 4-4E,F), were able to outcompete the Ext mutant males for female preference (Video S4-5, Figure 4-5A) due to their higher CI and WEI (Figure 4-5 B,C). There is no female preference between the Dual and the Phos mutant males (Figure 4-5A), most likely as a result of their similar CI and WEI in the courtship competition assays (Figure 4-5B,C).

DISCUSSION

We show strong evidence here that muscle genes can be utilized differently possibly via distinct contractile mechanisms for separate behaviors that shaped the evolution of a species through natural and sexual selection. The *Dmlc2* mutations used here had been extensively studied for their effect on IFM structure, muscle mechanical properties and whole organismal flight performance for more than a decade [34, 36-39]. Hence, these mutants present a great opportunity for elucidating the role of the IFM in courtship song and, importantly, for establishing the extent to which genetic and physiological pathways are shared between the two distinct behaviors of flight and

courtship. This opens up a new functional area of study, that is, to understand the function of IFM muscle genes in courtship song mechanics, contractile function and its correlation to that of flight mechanics.

***Dmlc2* N-terminal Extension and Phoshorylation Sites are Minimally Utilized for Courtship Song: Evidence of Distinct Genetic Control for Singing in the IFM**

The Ext mutant male is flight impaired with reduced wing beat frequency, whereas the Phos and Dual mutants are flightless (Tables 4-1, S4-2), Yet, our finding that all the *Dmlc2* mutants are capable of singing both pulse and sine songs (Figure 4-2) indicate that these mutations have minimal effect on courtship song generation compared to flight. Given that the *Dmlc2* mutations do not affect the underlying IFM structure from the sarcomere to the myofibril level [34,36], yet affecting flight and courtship song differently, suggests that the IFM is used differentially for these behaviors, and that the major use of *Dmlc2* in the IFM is in flight mechanics. The finding that the mutations do not have any major effect on the important traits of pulse song, notably intrapulse frequency (IPF; Figure 4-4C) and interpulse interval (IPI; Figure 4-4D), suggests the minimal effect compared to the drastic effect on flight. Albeit, it is evident that the pulse song vigor given by the traits of pulse duty cycle or PDC [44] and amplitude ratio or AMP-RT [45] are somewhat affected, notably the lower PDC of the Dual mutant compared to all other lines. This reduced pulse singing could be due to some locomotory defects in the mutants, especially the Dual mutant (data not shown) as the *Dmlc2* mutations are ubiquitous, and not only IFM-specific [34,36,38,46]. This is due to the reason that courtship song can be affected by any part of the entire sequential mating

ritual (for details of the sequential ritual see Chapter 1, [17]). The results on the parameters of the pulse song (CPP, PL, IPF, IPI, Table S4-1, and Figure 4-4) indicate that the mutations, whether single or dual, have no major effect on them, comparing to the marked and major effect on flight. This opens up the possibility that IFM could use muscle genes differentially for flight and song, two behaviors under competing selection regimes. Previously, unannotated flightless mutants with severely reduced or abolished wing beat frequency have been shown to have no major effect on courtship song production [47]. That study speculated that the mutations affected physiological control systems (neuronal or muscular) that are not common elements shared by the wing movements of flight and male courtship song. But it is not known what gene(s) are affected in those mutants, as well as whether the mutations affected neuronal or muscular sytem and to what extent. To our knowledge, this is the first study on the role of specific, known, and well characterized (for flight) muscle gene mutations of IFM in courtship song and its comparison with the effect on flight mechanics. Our results indicate that the *Dmlc2* mutations have differential effects on flight and courtship song. This minimal effect of *Dmlc2* mutations on courtship song compared to that in flight, possibly explains the gene's high conservation across *Drosophila* (see Chapter 3 Supporting Information Figure S3-2A) under natural selection for the basic contractile function for flight behavior. Moreover, the N-terminal extension of the *Drosophila* Dmlc2 is not present in the vertebrate homolog [33]. Our finding that the N-terminal truncated Ext mutant can produce courtship song even with impairment in flight and wing beat frequency, indicate that this Dmlc2 extension is specifically an innovation for IFM for flight mechanics and

function. This study is the first evidence of possible dichotomy in the IFM's genetic control for flight and courtship.

Evidence of Distinct Mechanism and Acto-Myosin Cross-Bridges used for Flight and Pulse Song

Our finding that, in contrast to the reduced or abolished wing beat frequencies in the *Dmlc2* mutants, the carrier frequency of the pulse or intrapulse frequency (IPF, Table S4-1, Figure 4-4C) is similar in all three mutants compared to control possibly indicates that contractile mechanism other than stretch activation could be utilized for pulse song. Moreover, the Ext and Phos mutations render the movement of myosin heads away and less oriented from the thin filament target zones [38,39] reducing the myosin kinetics and wing beat frequency impairing flight. In the Dual mutant, these single mutations have an additive effect in further reducing the cross-bridge kinetics and wing beat frequency of the fly [39]. Given these mutations do not affect the IPF of pulse song (Figure 4-4C), could potentially indicate that pulse singing utilizes a distinct population of acto-myosin cross-bridges which gets minimally affected by the *Dmlc2* mutations and do not move the myosin heads towards the thick filament backbone away from actin target. But this possibility is less likely since the mutations are ubiquitoes and have been shown to affect pre-position of the entire IFM ensemble of myosin heads [38,39]. Alternatively, the myosin target zones on the thin filament for contractile function for pulse song production could be distinct from flight. In this case, the pre-position of the same population of myosin heads used for stretch activation might not be comparatively far

away from the actin targets used for song, even though the mutations severely affect their pre-positions to bind actin targets for flight.

In addition, none of the *Dmlc2* mutations have an effect on the interval between successive pulses (IPI, Table S4-1, Figure 4-4D) indicating that start of pulses do not depend on the stretch activation mechanism, and may possibly "entirely" be driven by calcium activation. Ewing 1977 [19] had shown that muscle potentials during pulse song are more closely spaced than during sine song or flight with i) the activities of all the IFM motor units are correlated with the timing of sound pulses, and that ii) muscle potentials in the IFM are functionally related to the subsequent but not the preceding sound pulses during the pulse song. This potentially indicated that pulses are initiated entirely in a calcium-activated manner which agrees well with the interpretation of our data. By performing *in vitro* muscle fiber mechanical studies at an *in vivo* myofilament lattice condition using osmotic compression by 4% Dextran T-500, it was found that the calcium activated isometric tensions of all the *Dmlc2* mutant fibers were normal [Miller et al 2013 by personal communication] indicative of a normal calcium activated response. Therefore, since calcium activation of the *Dmlc2* mutant IFM fibers are normal, no change in IPF and IPI of the *Dmlc2* mutant males compared to Control further supports the interpretation that pulse song could potentially be generated by entirely calcium activation without utilizing stretch activation.

Among the mutants, only the Ext mutant shows a greater number of cycles per pulse (CPP) and a longer pulse length (PL) compared to Control male (Figure 4-4A,B). Calcium sensitivity has been shown to be slightly decreased in the Ext mutant IFM fibers

[37]. Assuming that contraction during pulse singing is driven entirely by calcium activation (as discussed above), the lower calcium sensitivity of Ext mutant fibers explains the greater CPP and longer PL in its pulse song. This is since lower calcium sensitivity can reduce the cooperativity between myosin heads and thin filament regulatory units slowing down rate of contractile force development and decay, as demonstrated by computer modeling studies [48-51]. Overall the minimal effect of Dmlc2 mutations on pulse song suggests that pulses are initiated and driven by "entirely" calcium activated manner possibly using distinct actin target zones for myosin binding which is least affected by the mutations during singing.

Sine Song could be driven by Stretch Activation using Cross-Bridge Population Distinct from Flight

The ability to generate sine song (Figure 4-2) with normal durations (Figure 4-3B) by all the *Dmlc2* mutants, even with major or complete impairment in flight performance or wing beat frequency (Tables 4-1, S4-1) suggests that sine song too could be driven by a mechanism other than stretch activation, using mode of cross-bridge function (possible distinct actin targets) distinct from flight, as discussed in case of pulse song. The *Dmlc2* single mutants (Ext and Phos) that show aberrant stretch activation, have a higher sine song frequency (SSF; Figure 4-3A) compared to control. Given the finding that these mutations do affect SSF, suggest that stretch activation may play some role in sine song production. Moreover, in contrast to the additive effects of the single mutations (Ext and Phos) on the Dual mutant on cross-bridge kinetics, wing beat

frequency and power output during flight (Tables 4-1, S4-1, [39]), sine song frequency (SSF, Table S4-2) data suggests a rather subtractive effect (Figure 4-3A), indicating a distinct cross-bridge population for sine singing.

Ewing (1977) [19] had shown that the *Drosophila* thorax shows small oscillatory movements that modulate synchronously with sine song sound modulations, suggesting that some thoracic resonant properties are being used during sine song, similar to that during flight but at a smaller magnitude. During stretch activation of IFM for flight, *in vivo* muscle strain amplitude is ~ 3.5% of resting muscle length [52]. The wing stroke amplitude during courtship song is much lower than that in flight [23]. Given that wing stroke amplitude correlates well with both force [53,54] and power output [55] of the flight system, it is thus reasonable to suppose that if potentially low power-requiring sine song is driven by stretch activation, then the strain amplitude of IFM during sine song must be lower than 3.5% that occurs during flight. Therefore, sine song generation might need very small oscillatory contraction using stretch activation compared to flight as was observed in the throacic movements by Ewing (1977) [19]. It could be possible for the IFM to use the resonant frequency of the thoracic flight system via smaller magnitude of stretch activation during sine song.

Our results show that the *Dmlc2* mutations which affect stretch activation response in flight additively, do affect SSF as well, but in a subtractive way (Figure 4-3A). This indicates that even though with the possible use of stretch activation, a different population of cross-bridges behaving differently is being utilized for sine song. If SSF would have been driven entirely by calcium activation, we might not have seen any effect

on SSF due to the *Dmlc2* mutations as seen in similar IPF of pulse song of the mutants (Figure 4-4C). Therefore, it is likely that sine song is driven by stretch activation using the resonant frequency of the thoracic flight system, albeit using different cross-bridge populations compared to flight. Important thing to note here is that the *Dmlc2* mutations are not IFM-specific but are present in all muscles of the fly. Therefore, the higher SSF seen in the Ext and the Phos mutant males could be an effect of the direct flight muscles (DFM) enhancing the frequency of the wing beats and amplitude ratio of sine to pulse song (AMP-RT, Table S4-2, Figure 4-4F). This could happen in response to a lower power generating IFM due to the mutations in order to bring back the resonant frequency of the thoracic box by changing the stiffness and shape of it, as has been shown previously [56]. In case of the Ext and Phos mutants whose IFM's are compromised in the ability to maximize power generation [39], the DFM might be over-compensating and hence increasing the SSF compared to Control, similar to projectin mutant previously described [36]. But this possibility is less likely since in this case, the Dual mutant's SSF would have been higher as well due to the much reduced power output of the Dual mutant's IFM [39]. Clearly, this is not the case, since the Dual mutant sings a normal sine song with normal SSF (Figure 4-3A).

Alternatively, it has been shown that the N-terminal extension in the vertebrate myosin essential light chain, which is similar to that of the Dmlc2, acts as a tether to actin to give an internal load slowing down cross-bridge kinetics [57]. Hence, if in the distinct cross-bridges used for sine song, the Dmlc2 N-terminal extension has a similar effect, then Ext mutant's SSF will be increased due to the extension's truncation, as seen in

figure 4-4A. The Phos mutant is expected to have a reduced effect (Figure 4-4A) since it contains the Dmlc2 N-terminal extension. In the possible distinct cross-bridges used for sine song, the two single mutations are probably interacting to mask each other's effect, as a result of which the Dual mutant could sing sine song with normal SSF (Figure 4-4A). This subtractive effect is another indication that sine song is driven by cross-bridge population distinct from flight, where the *Dmlc2* mutations have almost an opposite effect. Alternatively, the same populations of cross-bridges are used for sine song and flight, but the actin target zones differ. But this alternative is less likely since we observe an exactly subtractive effect of the single mutations (Ext and Phos) in the Dual mutant's sine song frequency, rather than an additive effect of the mutations on cross-bridges utilized for flight. Overall, our data indicate that sine song is driven by stretch activation at a smaller magnitude and possibly using cross-bridge population in the IFM distinct from flight.

Normal Pulse Duty Cycle and Sine Song Frequency is required for Female Preference and Male Courtship Success

Even though the *Dmlc2* mutants have no major defect in courtship song, the mutant males do not perform well in single pair matings with wild type OR females showing differences in their courtship index and wing extension index compared to Control males (Figure 4-6). This is most likely due to locomotor defects since the mutations are ubiquitous which could affect courtship rituals other than song and lower courtship index [17]. Therefore, it is difficult to figure out the abnormalities of courtship

song parameters that influence mating competitiveness and female preference of the mutants. Nevertheless, in the courtship competition assays two significant patterns emerge which are as follows:

1) All the mutants are outcompeted for female preference by the Control male, including the Dual mutant male which shows the least abnormal courtship song. This indicates that the lower pulse duty cycle (PDC) of the Dual mutant (Figure 4-4E) reduces its mating competitiveness, and female preference against a Control male (Figure 4-5A-C), since higher PDC has been shown to be involved in female stimulation [44], especially at longer distances between male and the female [58].

2) Among the *Dmlc2* mutations, Phos mutation had the greatest effect on flight. In contrary, the Ext mutation has the greatest effect in both courtship song and courtship competition assays (Figures 4-3,4-4) reducing mating competitiveness and female preference against all other lines (Figure 4-5A-C). The most notable song abnormality in the Ext mutant is the much higher sine song frequency (SSF) compared to Control and other mutant lines (Figure 4-3A), suggesting that this SSF aberration could possibly have a large effect in copulatory priming of females and its mating success, as has been shown previously [59-61]. SSF abnormality of Ext mutant is the most likely cause for the lack of mating success since at closer distances between the male and the female sine song plays a critical role to stimulate females [58].

Possible Model of Contractile Mechanism during Courtship song in the IFM

Based on our data on the effects of *Dmlc2* mutations in courtship song and observations from others' work, we interpret here that i) pulse song is most likely driven by "entirely" calcium activation, unlike flight, ii) sine song is most likely driven by stretch activation but at a smaller magnitude than that during flight, iii) IFM possibly utilizes actin target zones for myosin binding or uses population of cross-bridges for both pulse and sine songs that are distinct from flight.

Pulse song carrier frequencies are of broad range with the IPF ranging from 200-400 Hz, whereas the wing beat frequency during flight ranges from 180-240 Hz. One of the conundram is how IFM controls wing beats of differing frequencies for separate behaviors. Wang et al. (2011) [62] has shown that varying [Ca^{2+}] *in vitro* not only modulates IFM power, but also increasing [Ca^{2+}] can increase cross-bridge kinetics. This gives a clue that possibly calcium modulation is one of the key mechanism by which distinct cross-bridge kinetics could be fulfilled for differential frequencies of wing beats. Additionally, at the start of *Drosophila* flight, a synchronous burst of muscle potentials occur in the IFM motor units [63] after which the firing rate slows down with stretch activation taking over. This indicates that myoplasmic synchronous burst of calcium release plays a major role in the start of flight. Interestingly, the time interval between the starter jump and the first recorded flight wing beat is ~ 12 ms which is similar to ~ 16 ms interval between a muscle potential and a sound pulse [19], suggesting that start of a pulse could be driven entirely by calcium activation.

Another plausible alternative is that the nervous system could be differentially recruiting IFM muscle fibers for modulating power for courtship song generation. IFM could accomplish this by sequentially recruiting few motor units (motor neuron and muscle fiber that it innervates), since courtship song potentially requires much less power than flight (as discussed above). There is evidence that flight muscles of insects are innervated by only fast axons [64], suggesting that in a mononeuronal system like *Drosophila* IFM, different impulse patterns and rate of neuronal firing most likely be modulating myoplasmic calcium levels, rather than differential fiber recruitment. During flight, calcium was regarded to have only a permissive role to maintain stretch activation [27]. But this notion is revived now due to the finding that calcium plays an active role in the IFM for modulating power during flight by both *in vitro* muscle mechanical [62] and *in vivo* [65] studies. Recently, Lehman et al. (2013) [66] found that during flight maneuvering and turning movements, IFM power adjustments occur through bilateral control of calcium levels between the two thoracic segments. This further suggests that rather than differential recruitment of fibers, the calcium levels and gradients through the differential neural drive could modulate thin filament activation, and number of cycling cross-bridges for power modulations in order to perform distinct power requiring behaviors. Therefore, courtship song, in particular pulse song, potentially requiring the minimum power range by the IFM compared to flight, could be modulated by the nervous system through calcium levels and activation.

Additionally, in nature, muscle tissues from different species have been previously shown to have multiple functions with distinct mechanisms. For example, pre-

flight thermogenic (warm-up) behaviors of honeybees, bumblebees and other larger insects have been accomplished by antagonistic IFM contracting simultaneously using entirely calcium activated isometric tetanic contractions driven by faster neural drive, and alternately for flight using stretch activation while the neural drive slows down [67]. Another example of modulations under nervous system control is the different firing patterns from same motor neurons that can cause stridulations or flight in crickets [68]. Therefore, our findings of minimal effect of Dmlc2 mutations in pulse song, notably IPF and IPI (Figure 4-4), lead us to favor the model that courtship song, pulse song in particular, could be driven by contractile mechanism other than stretch activation, possibly "entirely" by calcium activation.

For sine song, it is suggested that the carrier frequency, SSF (~ 150-160 Hz) is near to the resonant frequency of the entire thoracic flight system [19]. Ewing (1977) [19] discussed that during *Drosophila* flight termination, the wing beat frequency probably goes down to the level of the flight thoracic system's resonant frequency of ~ 150-160 Hz from ~ 200-250 Hz (during flight), similar to what is known in *Muscina* [63]. Therefore, it could be possible for the IFM to use the resonant frequency of the thoracic flight system via smaller magnitude of stretch activation during sine song. This model is supported by the finding that during sine song, the intervals between muscle potentials are consistently greater than in flight or pulse, and that muscle potentials are not in synchrony with wing beats for sine song cycles [19], indicating that the stretch activation and resonant frequency of the thoracic system is being used. Alternatively, since sine song amplitude is 25% of that of pulse song [31,45], and given calcium's active role in

modulating IFM power in flight [62,65,66], a lower calcium spike facilitated by lower rate of muscle potentials in the IFM might be sufficient enough for sine song generation. In the latter case, sine song can be fulfilled by the IFM through entirely calcium activation with the cross-bridges ratcheting back and forth of the same actin target as shown in *Lethocerus* isometrically actively contracting IFM [69]. But this possibility is less likely, based on our finding that the SSF gets affected due to the *Dmlc2* mutations (Figure 4-3A) indicating some level of stretch activation must be used for sine song.

Thirdly, it is known that only 7-23 % of all the myosin heads available are used during stretch activation in *Lethocerus* IFM [69] indicating that there are other head populations that can be available for contractile function. These other myosin head population could be readly used for distinct behavioral requirements like courtship song. Interestingly, the *Drosophila* IFM consists of two isoforms of troponin C (TnC), one postulated to be stretch activated (DmTnC4 or TnC4; symbol: CG12408; flybase ID: FBgn0033027) and other to be calcium activated (DmTnC1 or TnC41C; symbol: CG2981; flybase ID: FBgn0013348) [70], similar to the F1 and F2 isoforms in *Lethocerus* flight muscles at a molar ratio of ~ 10:1 respectively in the same myofibril [71,72]. The presence of both stretch-sensing and calcium-sensing TnCs in the same muscle potentially indicates an evolutionary advantage of this hybrid expression pattern for IFM's fulfillment of dual contractile behavioral needs. Albeit, the contractile mechanism of courtship song is not clear, our data indicate that IFM could potentially utilize an entirely calcium activated mechanism for pulse song. This could possibly be accomplished by recruiting a subset of myosin heads through these calcium-activated

TnC isoforms (DmTnC1 or TnC41C) instead of stretch activation, potentially using actin target zones for myosin binding distinct from flight. For sine song, a subset of myosin and a small subset of the stretch activated TnC isoforms could be utilized for the possible low amplitude strains and power for singing.

CONCLUSION

Execution of muscle-driven behaviors with diverse power requirements demand muscle activation in proper sequence and precise timing, changes in either of which could result in altered behavioral output [73]. Here we show evidence that *Drosophila* IFM is a versatile machine using muscle genes distinctly for flight and courtship song, behaviors with possibly distinct power requirements and under separate selection regimes. In particular, we show that mutations of *Drosophila* myosin regulatory light chain (*Dmlc2*) known to markedly reduce stretch activation of IFM, rendering flies incapable to generate enough power for normal flight, can sing male courtship song with no major aberrations. To our knowledge, this is the first study to understand the function of muscle genes on courtship song, a behavior distinct from flight, in the IFM, opening a new area of study. Our findings have interesting implications which are as follows: 1) Muscle genes in the IFM could be separately utilized for distinct functions of flight and courtship song indicating that these genes could be under specific or dual selection regimes. 2) Contractile mechanism for flight and courtship song in the IFM could be distinct and that mechanism other than stretch activation is used during pulse singing. 3) To fulfill flight and song, two distinct power requiring behaviors, the IFM might be using entirely calcium activated mechanism (at least for pulse song) under nervous system control,

rather than stretch activation. 4) For courtship song behavior, IFM might be using a population of heads distinct from flight to fulfill its dual functionality, pointing us towards the reason of the need for both calcium sensing and stretch sensing troponin C isoforms in the thin filament of IFM. Therefore, in future, it will be interesting to understand in greater detail the contractile mechanism of courtship song using live physiological experiments on behaving fly as well as using the powerful *Drosophila* genetic tools to understand the function of muscle genes, troponin C isoforms in particular, on song and flight behaviors. This will enable us to understand how a complex muscle tissue system evolve genetically and physiolgically to carry out important multiple functions.

MATERIALS AND METHODS

Drosophila Lines Used

The wild type *D. melanogaster* stock is a laboratory strain of Oregon R (OR). The generation of the following transgenic strains used in this study has been previously described (Figure 4-1): one with the rescued myosin regulatory light chain (D*mlc2*$^{+}$) or Control [46], one with the truncated N-terminal extension of myosin regulatory light chain (D*mlc2*$^{\Delta 2\text{-}46}$) or Ext [36], one with the disrupted myosin light chain kinase phosphorylation sites (D*mlc2*^{S66A,S67A}) or Phos [34], and one with both the phosphorylation and the N-terminal truncation mutations (D*mlc2*$^{\Delta 2\text{-}46;\ S66A,S67A}$) or Dual

mutant [38]. The mutant proteins are expressed for each the above-mentioned lines. The flies were raised in standard corn meal food.

(see http://stockcenter.ucsd.edu/info/food_cornmeal.php for ingredients and recipe)

Flight Performance

Flight tests and wing-beat frequency analysis were performed as previously described [74].

Courtship Song Recording

Flies of all strains were reared at 22^{o}C and 70% humidity in a room with 12:12 hr light:dark cycles. Virgin males and females were collected using CO_2; however CO_2 was not used for any subsequent process. Males were aspirated into single vials and kept isolated for 24 hrs before testing so as to nullify any grouping effect and to increase amount of song production [41, 75]. Males aged 3 days and females aged 24 hrs or less were used for courtship song assays to stimulate the males to produce more songs. A male and a female were aspirated into a small plexiglass mating chamber (1cm diameter × 4 mm height) and placed inside an INSECTAVOX [76] for song recording for 30 mins duration. For details, see [77].

Courtship Song Analysis

The recorded songs from the INSECTAVOX were directly digitized using Goldwave v5.58 software [78]. The digitized waveform of the recorded songs were then

logged and analyzed in Goldwave v5.58 manually to extract the courtship song parameters, which are listed in Table S4-2. For details of courtship song analysis procedure, see [77]. The average value of each song parameter was calculated for each fly for statistics; hence the number of statistical samples is the number of flies.

Courtship Competition Assay

3-5 day old virgin males and females were used. 24 hrs before testing, the males were anesthetized with CO2 and one of them was marked on its thorax with a neon-orange paint using a fine point paintbrush. Two males (one marked and one unmarked) of different transgenic strains and one wild type female were introduced into a rectangular mating chamber (1.3 cm length × 1 cm width × 4 mm height) and courtship activities were video recorded for 30-50 mins using a 65X SD camcorder (Samsung) mounted on a tripod (Vanguard). The assays were done under light at 22^{o}C temperature and 70% humidity. The competition videos were observed and the strain of the male that succeded in courting and and copulating with the wild type female was noted. Female preference index (FPI) was calculated as the relative advantage of the mutant male over the Control male (i.e., the excess copulations with the mutant male divided by the total number of copulations, [79]. Courtship index (CI) and wing extension index (WEI) were also calculated for each male as described in [17] to note the strain of the male that outcompeted the other in performing the courtship rituals.

Single Pair Mating Assay

Three to five day old virgin males and females were used. Each assay consisted of one male of a transgenic strain and one wild type (Oregon R) female introduced into a plexiglass mating chamber (1.7 cm diameter × 5 mm height). The courtship activities were video recorded until successful copulation, or longer (30-50 mins) in the absence of copulation, using a 65X SD camcorder (Samsung) mounted on a tripod. The assays were done under room light at 22^{o}C temperature and 70% humidity. From the videos, courtship index (CI) and wing extension index (WEI) were calculated for each male as described in [17]. Briefly, CI is the fraction of the total recording time the male displayed courtship behaviors (orienting, chasing, tapping, licking, singing, copulation attempts), and WEI is the fraction of the total recording time the male extends and vibrates a wing.

Statistical Analysis

All values are mean ± SE. Statistical analyses were performed using SPSS (v.20.0, SPSS, Chicago, IL), with values considered significant at $p<0.05$. One-way ANOVA followed by a post-hoc test by Fischer's LSD pairwise comparisons between any two groups was used to examine differences between the Ext, Phos, Dual and Control for all variables. For statistical analysis on courtship song data, the average value of each song parameter was calculated for each fly; hence the number of statistical samples is the number of flies.

FIGURE LEGENDS

Figure 4-1. Schematic representation of expressed myosin regulatory light chain proteins of Drosophila indirect flight muscles (DMLC2) by the transgenic strains used. All representations are aligned with N-terminus to the left and C-terminus to the right. $DMLC2^{+}$ = full length *Dmlc2* rescued control null or control; $DMLC2^{\Delta 2\text{-}46}$ = truncated N-terminal extension; $DMLC^{S66A,S67A}$ = disrupted phosphorylation sites, and $DMLC2^{\Delta 2\text{-}46;S66A,S67A}$ = truncated N-terminal extension and disrupted phosphorylation sites. S = serine, A = alanine. The transgenic flies expressing the proteins shown here are $Dmlc2^{+}$, $Dmlc2^{\Delta 2\text{-}46}$, $Dmlc2^{S66A,S67A}$ and $Dmlc2^{\Delta 2\text{-}46;S66A,S67A}$ and will be denoted as Control, Ext, Phos and Dual lines respectively. The figure is modified from Miller et al. 2011 [39].

Figure 4-2. Courtship song oscillogram samples of control and mutant lines. Courtship song samples from transgenic males (A) $Dmlc2^{+}$ (Control) or , (B) $Dmlc2^{\Delta 2\text{-}46}$ (Ext) or, (C) $Dmlc2^{S66A,S67A}$ (Phos) , and (D) $Dmlc2^{\Delta 2\text{-}46;S66A,S67A}$ (Dual) . In all cases, male courtship song was induced by providing wild type (Oregon R) virgin female *D. melanogaster* (WT). Both sine and pulse components of the song are shown in each of the panels (A-D). Song recording was done at 22^{o}C and 70% humidity in a dark room with the only light source in the song recording chamber inside the INSECTAVOX [76,77]. The samples here were retrieved from Audacity software (http://audacity.sourceforge.net/).

Figure 4-3. Mutations of *Dmlc2* affect sine song frequency. Representative sine song oscillograms from Control, Ext, Phos and Dual males are shown here (top to bottom panels, respectively). (A) Compared to Control (blue), Sine song frequency is significantly higher in Ext (red) and Phos (green) mutants but similar for the Dual mutant (yellow). Also note that Ext mutant sings with a significantly higher sine song frequency compared to Ext and Dual mutants. (B) Sine song burst duration (SDUR, Table S4-1) is similar for all the lines. See Materials and Methods, Table S1 for details and retrieval method of the sine song parameters. n = 7-8 males for each line. **** ($p<0.0001$), Ext^{Δ} ($p<0.0001$) and $Phos^{\Delta}$ ($p<0.0001$) indicate significant difference from Control, Ext, and Phos respectively. Error bars indicate SEM.

Figure 4-4. Pulse song parameters are minimally affected by mutations in Dmlc2. Representative pulse song oscillograms from Control, Ext, Phos and Dual males shown here (top to bottom panels, respectively). All mutant males sing with similar cycles per pulse (CPP, Table S4-1) (A), and pulse length (PL, Table S4-1) (B) compared to Control males, except the Ext mutant which sings with a greater CPP and longer PL. All the mutant males sing the pulse song with normal carrier frequency (IPF, Table S4-1), with only the Phos mutants' IPF is slightly reduced compared to the Ext or Dual mutant but not compared to Control (C). None of the mutations affect interpulse interval (D). The Dual mutant has significantly reduced pulse duty cycle compared to Control, Ext, and Phos. (F) Amplitude ratio (AMP-RT) of consecutive sine to pulse song is significantly higher in individual (Ext, Phos) and Dual mutants compared to Control. See Materials and Methods, Table S1 for details and retrieval method of the pulse song parameters. n =

7-8 males for each line. * ($p<0.05$), *** ($p<0.001$), **** ($p<0.0001$) indicate significant differences from Control. Ext ($p<0.05$) and Phos ($p<0.05$) indicate significant difference from Ext and Phos mutants respectively. Error bars indicate SEM.

Figure 4-5. *Dmlc2* mutations induced courtship song aberrations affect female preference and male courtship vigor. (A) Female preference index is the relative advantage of a male of specific genotype over a male of a different genotype, i.e., the excess number of copulations with a male of specific genotype divided by the total number of copulations [79]; Ext (red), Phos (green) and Dual (yellow) males were outcompeted by the Control (blue) male for female preference respectively. In competition between mutants, Phos and Dual mutants individually outcompeted the Ext mutant. There is no female preference between the Phos and Dual mutants (index= 0). (B-C) Male courtship vigor in competitive mating situation was calculated via. courtship index (CI) and wing extension index (WEI). Ext and Dual mutants had significantly reduced CI and WEI but Phos mutant had only significantly reduced WEI compared to Control. In competition between mutants, Phos and Dual mutants have significantly higher CI and WEI compared to the Ext mutant while there is no difference between Phos and Dual. n = 20-30 for each mating competition group. * ($p<0.05$) indicate significant difference from Control. Ext ($p<0.05$) and Ext^{Δ} ($p<0.0001$) indicate significant differences from Ext mutant. No error bars in (A), (B-C) error bars indicate SEM.

Figure 4-6. *Dmlc2* mutants show reduced behavioral performance with wild type female in single pair mating assays. Male courtship vigor in competitive mating situation was calculated via. courtship index (CI) and wing extension index (WEI). Ext and Phos mutants had significantly reduced CI, and Dual mutant's CI is marginally reduced compared to Control ($p=0.054$) compared to Control (A). All Ext, Phos, and Dual mutants had significantly reduced WEI compared to Control (B). The Ext mutant, in particular, had the greatest reduction in CI and WEI compared to Control. n = 4-6 for each mating competition group. * ($p<0.05$) indicate significant difference from Control. Ext ($p<0.05$) indicate significant differences from Ext mutant. No error bars in (A), (B-C) error bars indicate SEM.

Table 4-1. Summary of male tethered flight wing beat frequency

Line	**Wing beat frequency (Hz)**
Dmlc2^{+} (Control)	196 ± 2 (10)
Dmlc2$^{\Delta 2\text{-}46}$ (Ext)	170 ± 3* (10)
Dmlc2^{S66A,S67A} (Phos)	168 ± 7* (10)
Dmlc2$^{\Delta 2\text{-}46;S66A,S67A}$ (Dual)	0 ± 0*§ (10)

All values are mean ± SEM. Numbers in parenthesis indicate number of flies tested. Temperature = 22ºC.

*Significant difference from *Dmlc2*$^{+}$.

§Significant difference from *Dmlc2*$^{\Delta 2\text{-}46}$ and *Dmlc2*$^{\Delta 2\text{-}46;S66A,S67A}$.

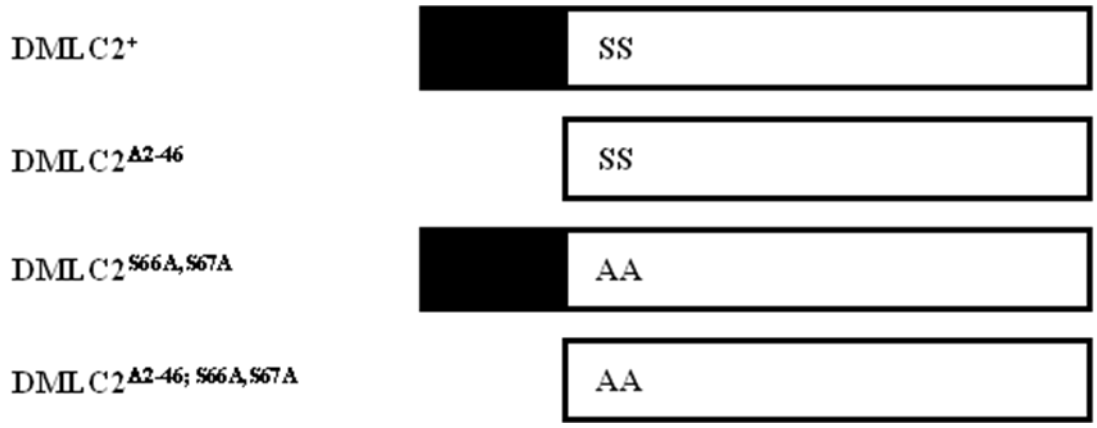

Figure 4-1. Schematic of Dmlc2 protein variants expressed by lines used in this study.

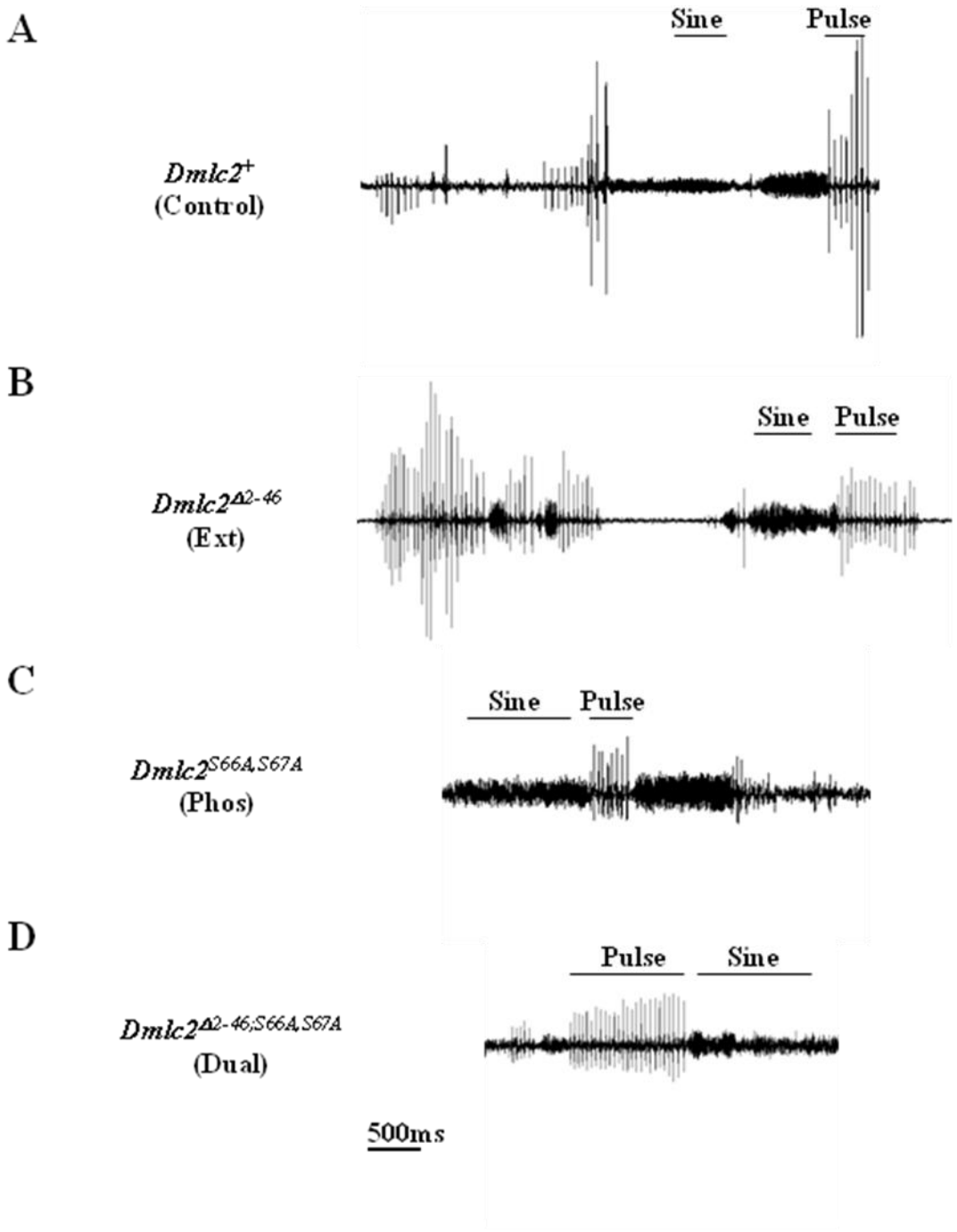

Figure 4-2. Representative male courtship song oscillograms of the control and the mutant lines.

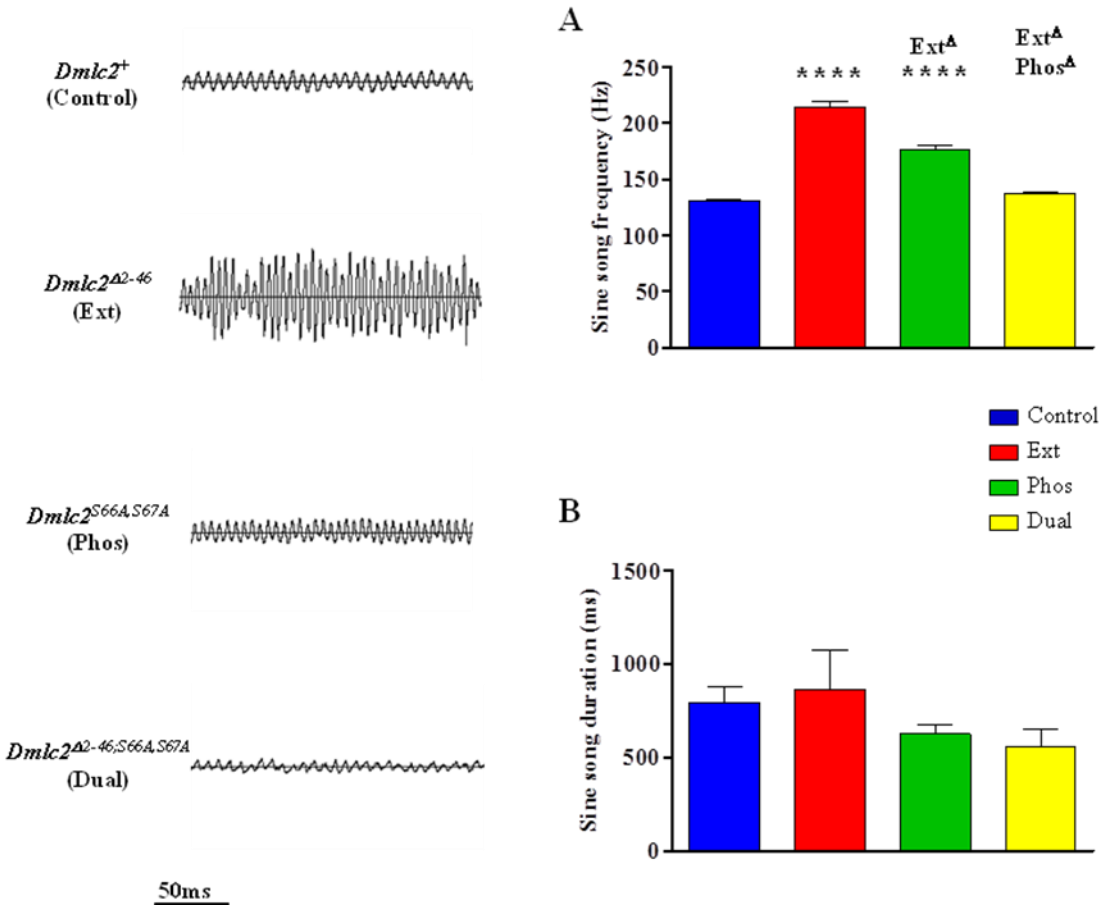

Figure 4-3. Representative sine song oscillograms, and sine song parameters of mutants and control lines.

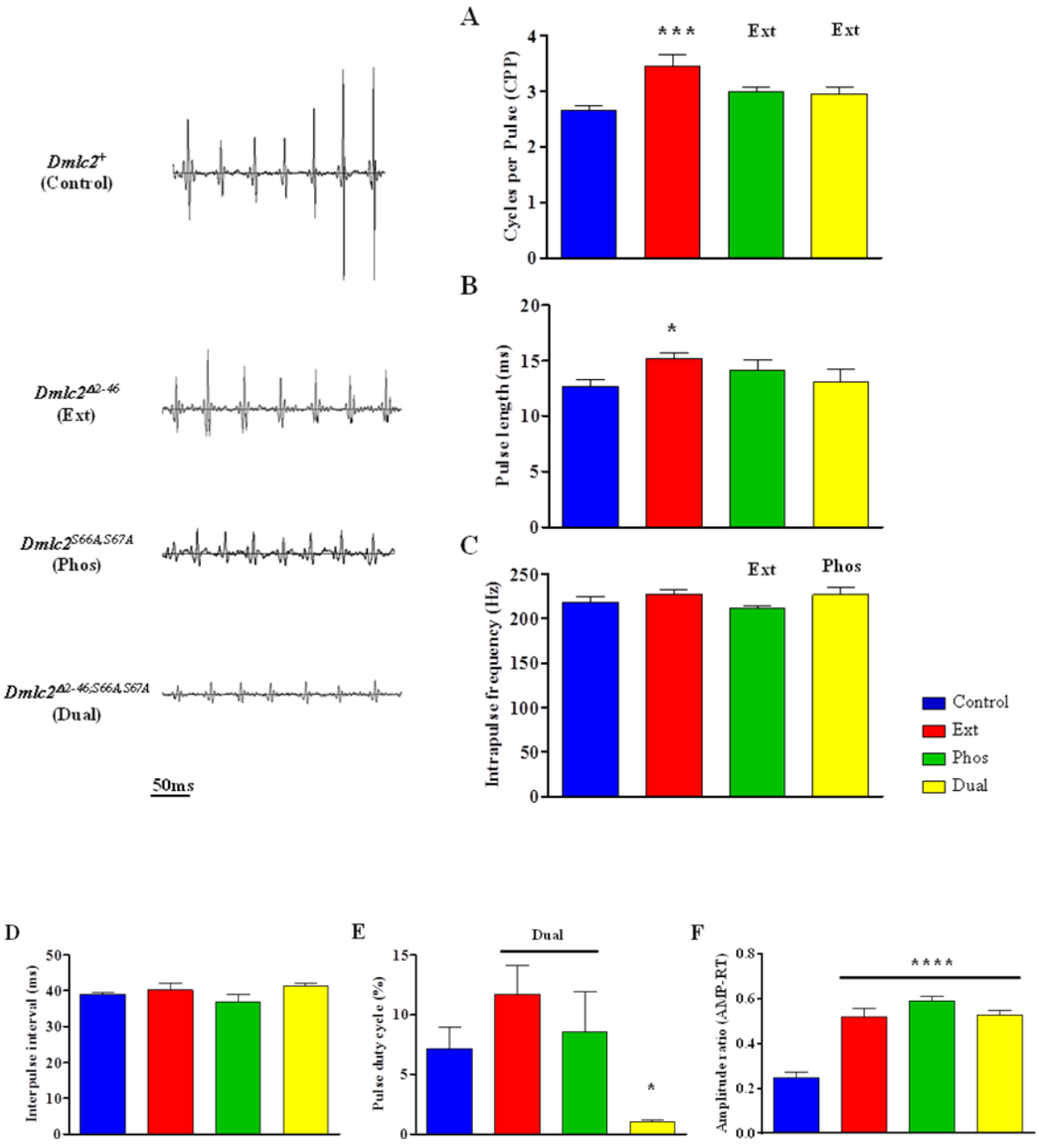

Figure 4-4. Representative pulse song oscillograms, and pulse song parameters of mutants and control lines.

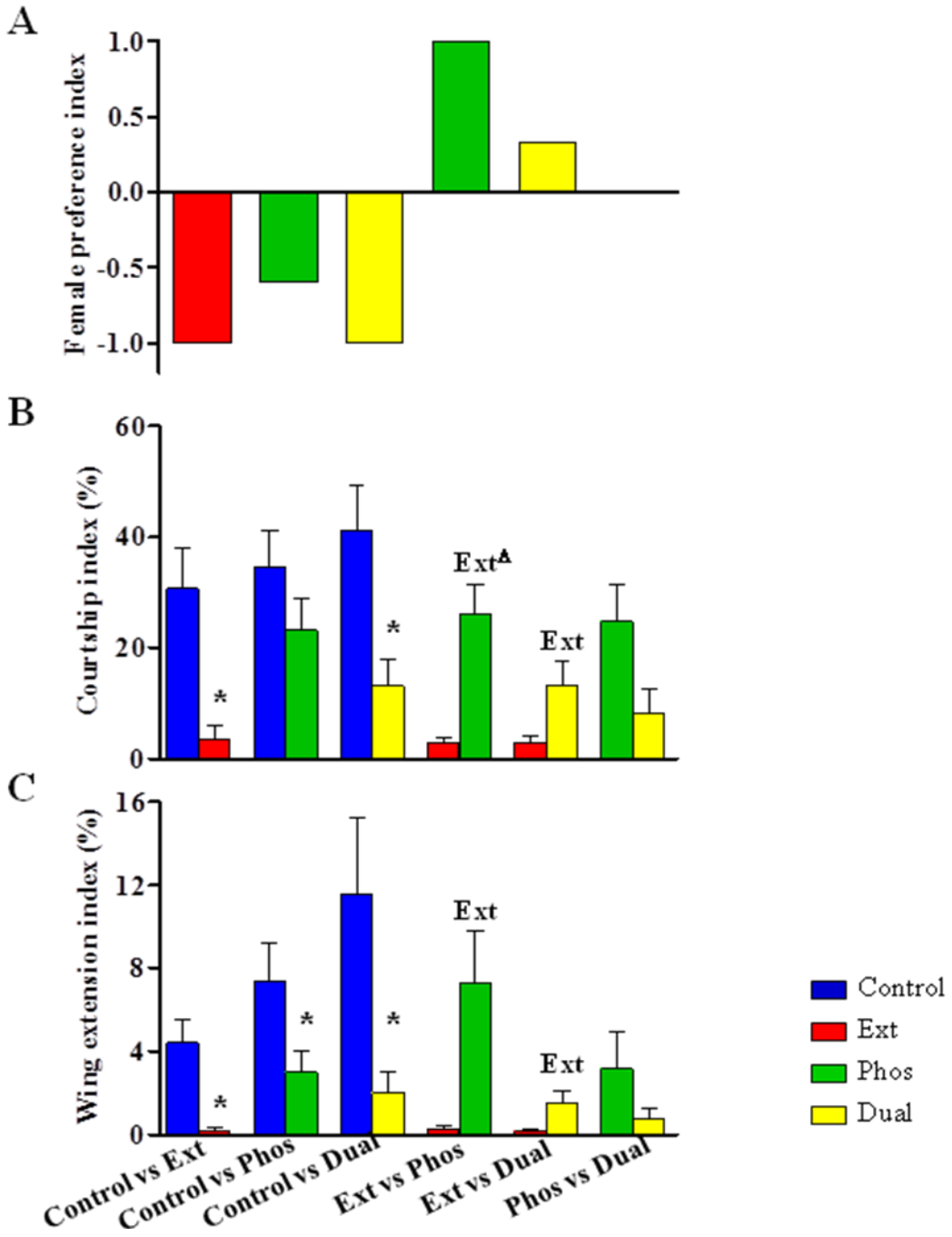

Figure 4-5. Courtship competition assay: Female preference and courtship behavioral performance of the mutants and the control for female mate.

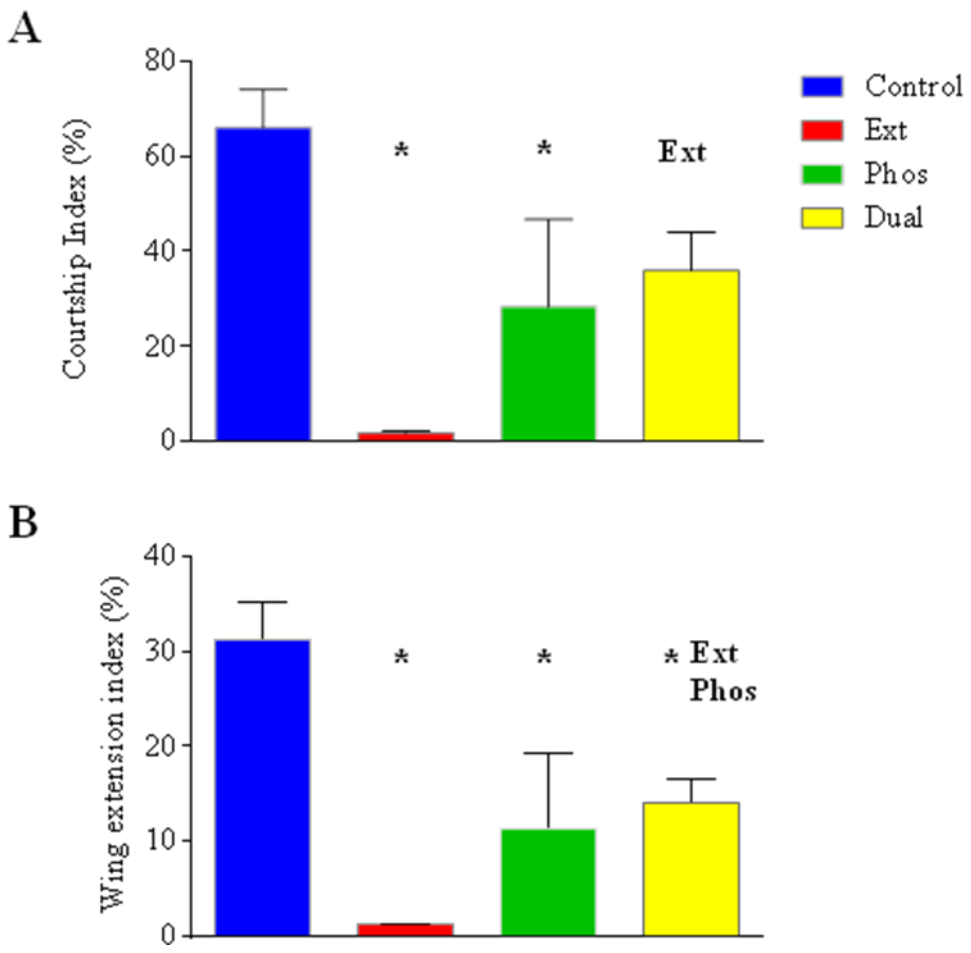

Figure 4-6. Single pair courtship assay: courtship behavioral performance (courtship and wing extension indices) of the mutant males with wild type female.

SUPPORTING INFORMATION

Table S4-1: Courtship song parameters

Parameter	Abbreviation	Description
Sine song burst duration	SDUR	Time duration (ms) of a sine song burst
Sine song frequency	SSF	Carrier frequency (Hz) of sine song
Amplitude ratio	AMP-RT	Ratio of amplitudes of sine song to pulse song
Pulse duty cycle	PDC	Equivalent to the ratio of the length of pulse song to the total time of recording
Pulse length	PL	Time duration (ms) of a pulse
Cycles per pulse	CPP	Number of zero crossings by the pulse waveform divided by two
Intrapulse frequency	IPF	Carrier frequency (Hz) of a pulse
Interpulse interval	IPI	Time duration (ms) between the equivalent peaks of two consecutive pulses in a train

Table S4-2. Summary of female flight characteristics (taken from [39])

Line	Flight index (0-6)	Able to fly (%)	Wing beat frequency (Hz)	Able to beat wings (%)
Dmlc2^{+}	5.1 ± 0.1 (60)	100	202 ± 3 (52)	100
Dmlc2$^{\Delta 2\text{-}46}$	4.6 ± 0.2* (60)	98	165 ± 2* (44)	100
Dmlc2^{S66A,S67A}	0.1 ± 0.1* (53)	10	158 ± 3* (11)	20
Dmlc2$^{\Delta 2\text{-}46;S66A,S67A}$	0.0 ± 0.0* (55)	0	0 ± 0*§ (30)	0

All values are mean ± SEM. Numbers in parenthesis indicate number of flies tested. Temperature = 22ºC.

*Significant difference from *Dmlc2*$^{+}$.

§Significant difference from *Dmlc2*$^{\Delta 2\text{-}46}$ and *Dmlc2*$^{\Delta 2\text{-}46;S66A,S67A}$.

Audio S4-1. Male courtship song (sine and pulse) sample of *Dmlc2*$^{+}$ or Control male (Figure 4-2A) in the presence of a wild type (Oregon R) female mate. File can be downloaded from the following weblink:

https://www.researchgate.net/publication/236123747_Audio_S4-1?ev=prf_pub

Audio S4-2. Male courtship song (sine and pulse) sample of *Dmlc2*$^{\Delta 2\text{-}46}$ or Ext male (Figure 4-2B) in the presence of a wild type (Oregon R) female mate. File can be downloaded from the following weblink:

https://www.researchgate.net/publication/236123748_Audio_S4-2?ev=prf_pub

Audio S4-3. Male courtship song (sine and pulse) sample of *Dmlc2*^{S6A6,67A} or Phos male (Figure 4-2C) in the presence of a wild type (Oregon R) female mate. File can be downloaded from the following weblink:

https://www.researchgate.net/publication/236123750_Audio_S4-3?ev=prf_pub

Audio S4-4. Male courtship song (sine and pulse) sample of *Dmlc2*$^{\Delta 2\text{-}46;S66,67A}$ or Dual male (Figure 4-2D) in the presence of a wild type (Oregon R) female mate. File can be downloaded from the following weblink:

https://www.researchgate.net/publication/236123752_Audio_S4-4?ev=prf_pub

Video S4-1. Courtship success of Control male over Ext male for wild type (Oregon R) female mate in a courtship competition assay (see Materials and Methods). File can be downloaded from the following weblink:

https://www.researchgate.net/publication/236123754_Video_S4-1?ev=prf_pub

Video S4-2. Courtship success of Control male over Phos male for wild type (Oregon R) female mate in a courtship competition assay (see Materials and Methods). File can be downloaded from the following weblink:

https://www.researchgate.net/publication/236123756_Video_S4-2?ev=prf_pub

Video S4-3. Courtship success of Control male over Dual male for wild type (Oregon R) female mate in a courtship competition assay (see Materials and Methods). File can be downloaded from the following weblink:

https://www.researchgate.net/publication/236123758_Video_S4-3?ev=prf_pub

Video S4-4. Courtship success of Phos male over Ext male for wild type (Oregon R) female mate in a courtship competition assay (see Materials and Methods). File can be downloaded from the following weblink:

https://www.researchgate.net/publication/236123761_Video_S4-4?ev=prf_pub

Video S4-5. Courtship success of Dual male over Ext male for wild type (Oregon R) female mate in a courtship competition assay (see Materials and Methods). File can be downloaded from the following weblink:

https://www.researchgate.net/publication/236123763_Video_S4-5?ev=prf_pub

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AUTHOR CONTRIBUTIONS

The author(s) have made the following declarations about their contributions: Conceived and designed all experiments: SC JOV. Performed the experiments: SC (most) HV (in part), VF (in part). Analyzed the data: SC. Wrote the paper: SC JOV.

FINANCIAL DISCLOSURE

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COMPETING INTEREST

No conflicts of interest, financial or otherwise, are declared by the author(s).

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CHAPTER 5 PERSPECTIVE

To Sing or To Fly: A View of Two Flight Muscle Genes in *Drosophila* Courtship Song and Flight Behaviors

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SUMMARY

Flight and male courtship song are the two important biological performances of *Drosophila* indirect flight muscles (IFM) for flies to enhance survival and reproduction, subject to natural and sexual selection, respectively. Deletion of a fast evolving N-terminal region of a *Drosophila* IFM-specific protein, flightin (FLN), impairs both flight ability and sexually selected courtship song parameters reducing biological performance. In contrast, deletion of a similar N-terminal extension of a ubiquitous muscle protein, *Drosophila* myosin regulatory light chain (MLC2), impairs flight ability and courtship song, but with no effect on sexually selected song parameters. Moreover, the conserved sequence of FLN is essential for the underlying IFM sarcomeric structure, and therefore both for flight and song. Contrary to this, the conserved phosphorylation sites of MLC2 are not essential for the underlying structure, but are required for enhancement of IFM contractile kinetics and stretch activation response for flight, with minimal effect on courtship song. These findings suggest differential utilization of muscle genes or gene sequences by the IFM for flight and courtship song, possibly explaining different selection pressures acting at the molecular level. Moreover, the finding that MLC2 mutations significantly affecting stretch activation mechanism of IFM during flight do not have a large effect on courtship song, possibly indicate distinct contractile mechanism utilized for courtship song generation. Therefore, these results refine our understanding of the versatility of IFM to be a power generator for flight and a sound generator for song, potentially by using distinct contractile mechanisms.

Drosophila indirect flight muscles (IFM) are the engines generating the power required for flight behavior necessary for the survival of the species, subject to natural selection (see Chapter 1). IFM drives the high power requiring flight behavior using pronounced stretch activation response. For this purpose, it has adapted the features of fast myosin kinetics [1], high passive stiffness [2], and a highly regular myofilament lattice with long-range crystallinity [3,4]. Throughout decades, *Drosophila* IFM has been a subject of study to understand flight mechanics and the role of the constituent proteins in the structural and mechanical adaptations of IFM [5-9]. But the thoracic musculature including IFM is not used solely for flight. It was known that male *Drosophila* uses their thoracic musculature for generating species-specific courtship song by unilateral wing vibration for con-specific mating and female stimulation, a behavior for reproduction subject to both inter- and intra-specific sexual selection (see Chapter 2). To our knowledge this thesis (in particular Chapters 3 and 4) is the first study which shows that the IFM is possibly the major unit for courtship song generation and that it is required for normal species-specific courtship song in *D. melanogaster*. These findings raise the possibility that IFM could be under dual selection pressure for performing flight and song behaviors. It also raises the possibility that the IFM muscle genes could be under distinct evolutionary pressures based on their contributing role for flight or song. Therefore, our studies of the two thick filament associated proteins, flightin (FLN) which in *Drosophila* is an exclusively IFM-specific protein, and a ubiquitous muscle protein myosin regulatory light chain (MLC2) on flight and song performance, gives us important clues on how the muscle genes are evolving to fulfill IFM's dual behavioral needs.

FLN has a dual conservation pattern (Chapter 3) with its N-terminal region (63 aa) highly variable across *Drosophila spp.* compared to the well conserved rest of the protein. This could suggest that FLN could be under dual selection pressure and that the N-terminal region is possibly evolving fast to fine-tune species-specific courtship song in *D. melanogaster*, whereas the rest of the protein is under purifying selection for maintaining underlying IFM sarcomeric structure and flight, an hypothesis that we tested in this work (Chapter 3). In contrast, the entire sequence of the *Drosophila* MLC2, despite its N-terminal extension not present in vertebrate homologs, is highly conserved across *Drosophila spp.* (Chapter 4), suggesting that its sequence is under purifying selection, possibly for its function in flight. We tested several mutations of FLN and MLC2 genes for their effect on flight and song behaviors in order to understand if the sequence conservation patterns explain the functional involvement of these genes and gene sequences in these two behaviors.

MUTATIONS OF FLN AND MLC2 AFFECT FLIGHT AND SONG DIFFERENTLY

Table 5-1 shows a summary of all the FLN and MLC2 mutants tested for flight and song in this study and some from previous studies. Based on these results, it is clear that FLN has a pleiotropic effect on flight and song. In absence of FLN (fln^{0}), the IFM structure gets disrupted abolishing the ability to fly and sing, that is completely rescued by the full length FLN rescued control line, indicating that IFM is directly involved and is indispensable in generating both behaviors. fln^{5STA}, fln^{3SA}, and fln^{2TSA} lines carry

mutations in the conserved C-terminal region of FLN [10] and $fln^{\Delta C44}$ is a deletion of the C-terminal region [11]. All of these FLN C-terminal mutations have severely disrupted IFM sarcomeric structure, and as a result are completely flightless [10,11] and unable to generate courtship song. These results indicate that the middle and C-terminal region of FLN are essential for IFM's underlying sarcomeric structure critical for IFM's function, possibly explaining the high amino acid conservation. A similar pleiotropic effect is seen in the FLN N-terminal truncated mutant ($fln^{\Delta N62}$), which is both flight and song capable. These results indicate that FLN is a critical gene in the IFM for its both flight and singing functions. The finding that the N-terminal region of FLN is not essential for flight or song, whereas the middle and C-terminal regions are, suggests dual functionality possibly due to dual selection pressures.

In contrast, none of the MLC2 mutations, except the Ext mutant, show a pleiotropic effect on flight and song. In particular, the Phos and the Dual mutant, albeit completely flightless, are capable of generating courtship song. This dichotomy reflects that IFM could potentially utilize distinct protein or protein domains differently for the two behaviors. It could be possible that distinct evolutionary selection could be forcing muscle genes to be involved in either of two behaviors, enabling IFM an efficient route for biological performance.

COMPARISON BETWEEN FLN AND MLC2 N-TERMINAL REGIONS: EVOLUTONARY IMPLICATIONS

Table 5-2 shows the courtship song parameters (for details on song parameters see [12]) of FLN and MLC2 mutants used in this study that are capable of singing. Pulse song parameters include interpulse interval (IPI), intrapulse frequency (IPF), and pulse duty cycle (PDC), among which IPI is the most salient feature for con-specific mating and partly female stimulation, whereas PDC is shown to be involved in only female stimulation, both parameters being under sexual selection (reviewed in [12]). Sine song frequency (SSF) is also known to stimulate females (reviewed in [12]). Results of the table 5-2 reflect the pattern that FLN N-terminal region is required for normal IPI, the most important sexually selected parameter, whereas none of the MLC2 regions have any role in IPI. To understand if the FLN N-terminal region's effect on IPI is specific or not, here we compare the effects of a similar N-terminal region in MLC2. The N-terminal region of FLN has a similar length to that of MLC2 (63 aa vs 46 aa for MLC2 N-terminal region). Moreover, FLN is IFM-specific in *Drosophila* and has no vertebrate homolog. Similarly, MLC2 N-terminal region is also unique to *Drosophila* and not present in the vertebrate regulatory light chain. The MLC2 N-terminal region has been postulated to be extending out of the thick filament backbone ([13]). Similarly, in this study, the FLN N-terminal region is postulated to be extending out of the thick filament backbone (see Chapter 3), based on its amino acid composition. Deletion of each region ($fln^{\Delta N62}$ vs Ext mutants) do not have any major effect on the underlying IFM sarcomeric organization, as

seen by electron micrographs ([14], Chapter 3). Moreover, both $fln^{\Delta N62}$ and Ext mutants are capable of flying and singing, with some impairment compared to their respective controls (Table 5-1,5-2). Therefore, these two N-terminal regions of FLN and MLC2 are similar in some aspects in their contribution to IFM structure and function. Comparing the song parameters of the FLN N-terminal deletion mutant and the MLC2 N-terminal deletion mutants (either single: Ext, or not: Dual) (Table 5-2), reveal the pattern that both mutations affect SSF, and PDC, but only the FLN N-terminal region affects the salient feature of IPI, required for con-specific mating and female preference. From this particular analysis, it could possibly be concluded that the FLN N-terminal region's effect on IPI is specifically due to the mutation. Therefore, it is possible that FLN N-terminal region started to evolve fast under sexual selection in *Drosophila* for fine-tuning species-specific IPI, and later in evolution acquired conserved sites under purifying selection that enhanced flight performance This could be tested further by creating transgenic flies with natural variation in the N-terminal region of FLN and testing song and flight properties, similar to the ongoing approach described in Appendix 1. Overall, our data suggest that FLN could be a key evolutionary innovation for the IFM that could be under dual selection pressure for enhancing flight and song performance. Therefore, based on previous knowledge and this study, in the next section, we attempt to understand the molecular function of the different regions of FLN (N-Terminal, middle, and C-terminal), that have different conservation patterns, in the IFM thick filament.

Table 5-1. Flight and courtship song abilities of mutant and transgenic lines

Line	Flight index (0-6)	Wing beat frequency (Hz)	Courtship pulse song	Courtship sine song
fln^{+} (FLN control)	4.2±0.4 (35)	198±2 (25)	Y	Y
fln^{0}	0*	0*	N	N
fln^{5STA}	0*	0*	N	N
fln^{3SA}	0*	0*	N	N
fln^{2TSA}	0*	0*	N	N
fln$^{\Delta C44}$	0*	0*	N	N
fln$^{\Delta N62}$	2.8±0.1* (66)	195±4 (45)	Y	Y
Dmlc2^{+} (MLC2 control)	5.1 ± 0.1 (60)	202 ± 3 (52)	Y	Y
Dmlc2$^{\Delta 2\text{-}46}$ (Ext)	4.6 ± 0.2$^{\delta}$ (60)	165 ± 2$^{\delta}$ (44)	Y	Y
Dmlc2^{S66A,67A} (Phos)	0$^{\delta}$ (53)	158 ± 3$^{\delta}$ (11)	Y	Y
Dmlc2$^{\Delta 2\text{-}46;S66A,67A}$ (Dual)	0$^{\delta}$ (55)	0$^{\delta}$ (30)	Y	Y

Values are mean ± SE. Numbers in parentheses indicate number of flies analyzed

* Significant difference ($p<0.05$) from fln^{+} control.

δ Significant difference ($p<0/05$) from D$mlc2^{+}$ control

Y Capable of courtship song

N Unable to generate courtship song

Flight index and wing beat frequency data of fln^{0} taken from [9].

Flight index and wing beat frequency data of fln^{5STA}, fln^{3SA}, fln^{2TSA} taken from [5]

Flight index and wing beat frequency data of $fln^{\Delta C44}$ taken from [6].

Flight index and wing beat frequency data of $Dmlc2^{+}$, $Dmlc2^{\Delta 2\text{-}46}$, $Dmlc2^{S66A,67A}$, $Dmlc2^{\Delta 2\text{-}46;S66A,67A}$ taken from [10].

Table 5-2. Important courtship song parameters of singing mutant and transgenic lines

Line	Interpulse interval (IPI), ms	Intrapulse frequency (IPF), Hz	Pulse duty cycle (PDC), %	Sine song frequency (SSF), Hz
fln^{+} (FLN control)	37 ± 0.7 (7)	279 ± 17 (7)	7.4 ± 0.2 (7)	148 ± 5 (7)
fln$^{\Delta N62}$	56 ± 3.0* (10)	306 ± 26 (10)	2.6 ± 0.2* (10)	228 ± 5* (10)
Dmlc2^{+} (MLC2 control)	39 ± 0.5 (7)	219 ± 6 (7)	7.2 ± 1.8 (7)	131 ± 1 (7)
Dmlc2$^{\Delta 2\text{-}46}$ (Ext)	40 ± 1.8 (7)	227 ± 5 (7)	11.7 ± 2.4 (7)	215 ± 5$^{\delta}$ (7)
Dmlc2^{S66A,67A} (Phos)	37 ± 2.2 (7)	212 ± 3 (7)	8.6 ± 3.4 (7)	176 ± 4$^{\delta}$ (7)
Dmlc2$^{\Delta 2\text{-}46;S66A,67A}$ (Dual)	41 ± 0.6 (7)	227 ± 8 (7)	1.1 ± 0.1$^{\delta}$ (7)	137 ± 2 (7)

Values are mean ± SE. Numbers in parentheses indicate number of flies analyzed

* Significant difference ($p<0.05$) from *fln*$^{+}$ control.

δ Significant difference ($p<0/05$) from D*mlc*2^{+} control

FACTS AND PERSPECTIVES ON FLN MOLECULAR FUNCTION

FLN has been shown to be required for the stiffness, structural integrity and normal length determination of IFM thick filament by both *in vitro* native filament [17] and *in vivo* mutagenesis [10,15,18] studies. In the absence of FLN, late stage pupal IFM sarcomeres are longer with compromised integrity [15] indicating that FLN plays a key role in maintaining the normal number of myosin incorporation during development determining filament length and stability. This is further supported by the recent finding suggesting that FLN regulates thick filament assembly kinetics by reducing both association and dissociation rates of myosin molecules in the thick filament [19]. Given that FLN increases thick filament stiffness [17,18], one possible way it can regulate thick filament assembly kinetics (length determination) is by enhancement of the packing of incorporated myosins which could stiffen the filament. This could lead to increased stability of the filament during development as the filament grows and FLN decorates it. This possibility is supported by the observation that in the *weeP26* flies devoid of FLN and with GFP exonic insertion into myosin heavy chain (MHC) gene, assembled filaments are less stable with diffusion of MHC molecules along the filament [19]. Moreover, study on native filaments devoid of FLN suggests too that FLN enhances thick filament stability and stiffness while regulating their length [17].

Role of the FLN C-terminal region (44 amino acids)

Skinned IFM fibers from transgenic flies expressing FLN with its C-terminal region truncated, ($fln^{\Delta C44}$: [11]) show proportionally similar (50-60%) reduction in

relaxed (weakly bound cross-bridges), active, and rigor (strongly bound cross-bridges) elastic modulus and isometric tension (Figures 1,2 of Appendix 3) in fiber mechanics experiments. This is indicative of lower passive stiffness due to some compromised passive structural element(s), rather than due to any cross-bridge dependent effect. Since FLN is mostly restricted to the core of the A-band [15], it is possible that the C-terminal region is required for normal thick filament stiffness. Important to note here that this result is in contrast to the unchanged relaxed and rigor moduli of the $fln^{\Delta C44}$ IFM fibers compared to control, observed in a previous study indicating that FLN C-terminal region does not play a role in fiber passive stiffness [11]. This disagreement could possibly arise due to the difference in experimental conditions, with Tanner et al [11] using skinned (swollen) fibers without 4% Dextran T-500 in solution, whereas this study uses lattice compression by dextran (4%) to mimic *in vivo* conditions, as has been done before [16]. There has been substantial evidence that fiber mechanical properties like stiffness, power output and frequency of maximum power output differ from 0% to 4% dextran data (14,16,20). One limitation of this interpretation is that it is not clear how dextran could influence stiffness properties of the lattice, in addition to its effect on lattice spacing. One way to test this is to measure the mutant fiber stiffness at different dextran concentrations, in order to understand the effect of lattice compression on lattice stiffness.

Moreover, the $fln^{\Delta C44}$ sarcomeric structure and myofibrillar organization is highly disorganized compared to control, reflected in abnormal sarcomeric Z and M-lines, A-band breaks and high lattice disorder [11]. Although, X-ray diffraction estimates a small

(0.7%) decrease in inter-thick filament spacing that could reduce cross-bridge kinetics [11], yet the $I_{2,0}/I_{1,0}$ intensity ratio (for details of this parameter see [21]) in the mutant is greatly increased (67%) [11], indicating that the mutant myosin heads are moving away from thick filament backbone towards thin filament target sites increasing the probability of strongly bound cross-bridges and increased kinetics. Therefore, this myosin head movement towards thin filament could not only potentially mask the mutation's effect on comparatively smaller lattice shrinking (reduction in inter-thick filament spacing), but also could enhance cross-bridge kinetics. Instead, a lowering of the underlying cross-bridge kinetics and power output is observed in the $fln^{\Delta C44}$ fibers ([11], Figure 2 of Appendix 3). Thus, it is likely that the structural instability of the $fln^{\Delta C44}$ sarcomere (A-band, Z and M-line aberrations) could be the root cause of lower cross-bridge kinetics and power output, rendering the flies completely flightless. Also, due to this abnormal passive structural elements (unstable Z-line, M-line and A-band) in the $fln^{\Delta C44}$ sarcomere, fiber passive stiffness could be compromised, which is what we observe in this study (Figure 1 of Appendix 3). The sarcomeric A-band instability in the $fln^{\Delta C44}$ fibers could result from lower thick filament stiffness, given FLN is known to stiffen the thick filaments (17,18). This, in turn, potentially could lower the stiffness of the sarcomeric unit and the fiber in relaxed conditions. Therefore, our mechanics data of $fln^{\Delta C44}$ fibers with lattice compression using dextran, showing reduced fiber stiffness, in particular resting (passive) stiffness, could possibly arise due to decreased thick filament stiffness,

and better matches whole fly flightlessness and gives a clearer understanding of the *in vivo* role of the FLN C-terminal region.

Interestingly, unlike in the complete absence of FLN, the C-terminal truncation does not have a major effect on sarcomere length, with a slight shortening effect [11]. This might indicate that the FLN C-terminal region do not play a major role in sarcomeric or thick filament length determination, but most likely is solely required for thick filament stiffness. Inspite of the above interpretation, it is still possible that this region could affect thick filament length determination during development. This could be possible if due to the lack of stability of the thick filaments as a result of the truncation, the thick filaments could lose myosin molecules through age-dependent degeneration during development and the sarcomere length could get shortened. This possibility could be tested by investigating on the sarcomeric structure and length of the truncated mutant at different developmental stages of the fly before adulthood.

Role of the FLN middle region (75 amino acids)

Both N-terminal (63 aa) and C-terminal (44 aa) truncated FLN variants are expressed normally in the skinned IFM fibers of $fln^{\Delta N62}$ (Chapter 3) and $fln^{\Delta C44}$ flies [11], respectively. This indicates that the common middle region (75 aa) is essential for FLN incorporation into the thick filament of the IFM sarcomeres. Biochemically though, it is not tested whether the middle region of the FLN protein binds the LMM region of the myosin rod, as shown with full length FLN [22]. Therefore, it is not certain that truncated versions of FLN bind to myosin rod with normal affinity and at the normal location.

Nevertheless, it is safe to envisage that the FLN middle region is the most likely possible region to directly interact with myosin. Immuno-EMs could be performed on the $fln^{\Delta N62}$ and $fln^{\Delta C44}$ IFM sarcomeres to understand the localization of expression of the truncated variants and compare to that in the control.

Since in absence of FLN expression and binding to myosin, the thick filaments are longer and less stiff [15,17,18], the middle region of FLN could be involved in not only binding myosin rod, but could also be involved in both length determination and stiffness enhancement of the thick filament. In the absence of FLN C-terminal region, the sarcomeres and hence the thick filaments are not longer but are structurally less stable [11] with lower fiber passive stiffness (Appendix 3) indicative of lower thick filament stiffness (as discussed above). Therefore, it could be possible that both middle and C-terminal region of FLN play an important role in stiffening the thick filament, whereas the myosin binding by the middle region is critical for the underlying stability of the filaments that could facilitate in normal myosin incorporation kinetics and length determination. We propose here that the thick filament stabilizing and stiffening effect of the FLN middle and C-terminal region could be through their role in packing the myosin molecules in the filament during assembly process. As suggested previously [15], FLN middle and C-terminal region could interact inter-molecularly with the hinge-LMM junction of the myosin rod welding the myosins together in the subfilament, stabilizing the thick filament during and after assembly.

Role of the FLN N-terminal region (63 amino acids)

The study described in Chapter 3 in this thesis is the first mutational study on the FLN N-terminal region whose amino acid composition is distinct from the rest of the protein giving it a theoretical high net negative charge (vs. net positive charge of the rest of the protein, see Chapter 3). The N-terminal region is also predicted to be disordered compared to the rest of the protein (Figure S3-3 of Chapter 3). Additionally, a cluster of phosphorylation sites (7 sites) have been found in the FLN N-terminal region (Vigoreaux JO and Ballif BA unpublished data). Our mechanics experimental results of the FLN N-terminal truncated mutant ($fln^{\Delta N62}$) skinned IFM fibers reveal that FLN N-terminal region is required for normal fiber stiffness, in particular passive stiffness, through its effect on some passive structural element, without any major effect on myosin motor function (Figures 3-9,3-10 of Chapter 3). Electron micrographs reveal that the mutant sarcomeres have no discernible bare zone and the M-line is thinner and wavy compared to control. Moreover, cross-sections of myofibrils reveal more number of thick filaments incorporated, with the myofilament lattice spacing being reduced. Importantly, due to the truncated mutation, the lattice regularity is highly compromised indicating that the lattice spacing is heterogeneous across cross-section of the mutant myofibrils. Since, FLN N-terminal region is predicted to be disordered with high negative charge; it could potentially extend out of the thick filament backbone and is possibly expanding the electrostatic field of the thick filament surface. It is known that the myofilament surface charge and inter-filament electrostatic interactions influence in maintaining lattice

spacing possibly through aligning the thick and thin filaments [23]. Therefore, the truncation of the FLN N-terminal region could potentially reduce the electrostatic interaction between the myofilaments (possibly between thick and thin filaments), which compromise the alignment of the filaments during myofibril assembly. This explains the lower lattice spacing and regularity that was observed in the mutant myofilament arrangement. We propose that the FLN N-terminal region, by maintaining the lattice spacing through the proposed electrostatic inter-filament interaction, facilitates in maintaining the normal number of thick filament incorporation in the myofibrils which could maintain normal lattice regularity. This abnormal thick filament incorporation and irregular A-band lattice, in turn, could affect normal M-line formation, which is observed in our data. This abnormal M-line formation (a secondary effect of the mutation) could lower the stiffness, stability and the symmetry of the mutant sarcomere A-band, as shown previously [24], that could reduce the passive stiffness of the sarcomere and ultimately the mutant fiber, as have been observed (Figure 3-9 of Chapter 3). Therefore, according to this proposal, FLN N-terminal region's function is to maintain lattice spacing that lead to normal thick filament incorporation per sarcomere and overall lattice regularity, but does not directly affect thick filament stiffness property *per se*. If this proposal is true, then it adds a distinctly important knowledge about the function of FLN, that is, FLN not only could stabilize thick filaments with its middle and C-terminal region, but also with its N-terminal region it regulates inter-filament interaction during assembly, influencing myofibrillar organization. This inter-filament interaction proposal gains support from findings that unlinked mutations on both thick and thin filament influences FLN

expressional pattern [25]. This proposal could be tested further by investigating the myofilament lattice and sarcomeric arrangement at different developmental stages in the $fln^{\Delta N62}$ line and comparing to that in the control. This approach could possibly elucidate the relationship of A-band lattice organization and M-line formation during myofilament assembly, and give us clues about the role of the N-terminal region. Moreover, it would be informative to test if expression levels of M-line proteins like Obscurin are reduced in the mutant fibers, which could potentially explain M-line abnormality, as an additional support of this model. Interestingly, the $fln^{\Delta N62}$ mutant sarcomeres are ~13% shorter which is proportionally similar to the greater number of thick filament incorporation (Table 3-2 of Chapter 3). This sarcomere shortening could be a compensatory mechanism in the IFM during development in order to maintain normal number of myosin heads per sarcomeric unit.

Alternatively to the above proposal, the reduction in relaxed fiber elastic modulus due to FLN N-terminal truncation (Figure 3-9 of Chapter 3) could indeed be due to the direct influence of the FLN N-terminal region on thick filament stiffness. The plausible reasoning is as follows. It is known that roughly 50% of FLN is phosphorylated whereas the rest ~ 50% is the unphosphorylated form in adult flies [26]. Therefore, it could be possible that the phosphorylating kinase(s) might have reduced access to the N-terminal region of FLN bound to the inner ("i", see Figure 5-1A) myosin in a subfilament of the thick filament, whereas the N-terminal region of FLN bound to the outer ("o", see Figure 5-1A) myosin on the surface of the thick filament is easily accessible to kinases

and is phosphorylated. It is also known that phosphorylation of proteins could facilitate in the protein structural transition from an disordered to an ordered form or *vice versa* which are functionally significant processes like in smooth muscle myosin regulatory light chain phosphorylation domain and phospholamban protein respectively [27,28]. Thus, N-terminal regions of 50% of FLN, that are bound to the inner myosin of the subfilament might have a different conformation and function than that of the other 50% of FLN bound to the outer myosin. In this case, the unphosphorylated N-terminal region of the inner FLN population could possibly contribute in the packing of the myosin molecules enhancing the stability of the filament, similar to and in concert with the possible function of middle and C-terminal regions described above. This possibility could lower thick filament stiffness due to the FLN N-terminal truncation, at an extent similar to the effect of FLN C-terminal truncation, which indeed what we observe (Appendix 3 Figure 1). Also, the underlying cross-bridge kinetics, fiber power output (Appendix 3 Figure 2) and sarcomeric structural organization (Chapter 3 Figure 3-7, [11]) are more compromised in the $fln^{\Delta C44}$ line than $fln^{\Delta N62}$ line, indicating that the N-terminal region could possibly play a lesser role in thick filament stiffness and stability than the C-terminal region. Moreover, computational simulation studies indicate that a less stiff thick filament could potentially increase cross-bridge kinetics [29,30]. Thus, theoretically, a less stiff thick filament in the $fln^{\Delta N62}$ fibers could lead to increased cross-bridge kinetics. Additonally, smaller inter-thick filament lattice spacing in the IFM has been shown to reduce cross-bridge kinetics [31]. Therefore, normal underlying cross-bridge kinetics in the $fln^{\Delta N62}$ line (Chapter 3 Figure 3-10) could be due to the masking

(compensatory) effect of less stiff thick filament and reduction in lattice spacing. In other words, the truncation effects of the N-terminal regions of the two FLN population bound to outer and inner myosins in the subfilament respectively, compensate for each other.

Another related possibility is that FLN N-terminal region function is specifically restricted towards the tip of growing thick filaments, where it provides stability during assembly. It has been shown that FLN progressively binds growing thick filaments tips during thick filament assembly [19]. Therefore, it is possible that due to the N-terminal truncation, the filaments are less stable towards the growing tip, and hence undergoes progressive age-dependent degeneration from the ends, leading to shorter sarcomeres in the $fln^{\Delta N62}$ fibers, as observed (Table 3-2 of Chapter 3). This thick filament end instability could hamper the formation of normal M-line rendering the entire A-band region of the sarcomere compromised. There is no evidence though that FLN N-terminal region could specifically act in the growing thick filament tips, hence making this model weaker. Thus, it would be informative to know the developmental progression of the mutant sarcomeres.

Possible model of functions of the FLN regions

In an attempt to combine our knowledge about FLN function in order to understand the three different regions' roles in the thick filament; we propose the most likely model (Figure 5-1). Figure 5-1A top panel shows the cross section of the insect IFM thick filament consisting of twelve subfilaments surrounding a paramyosin (pm) core. Each subfilament consists of two myosin molecules, one outer facing the thin

filament, and one inner. Each circle represents a myosin rod region. The bottom panel shows a zoomed subfilament with an outer and an inner myosin and the FLN N-terminal region (red) is shown only for simplicity. We propose two populations of the FLN N-terminal region: the N-terminal region of FLN bound to the inner myosin does not get phosphorylated and has different conformation compared to that of the FLN bound to the outer myosin which extends out and gets phosphorylated (P). We also propose that the FLN middle region binds myosin at the LMM region. Similar to previous suggestion [15], we propose that FLN middle region bound to the inner myosin together with the N-terminal region and C-terminal region enhances thick filament stiffness by interacting with the hinge-LMM junction region of the outer myosin of the same subfilament (Figure 5-1B). This interaction increases the packing of the myosin molecules in the subfilament and enhances stiffness. The stiffness enhancement stabilizes the thick filament during assembly which facilitates in restricting abnormal myosin association or dissociation along the growing filament, thus maintaining the assembly dynamics for normal thick filament length determination. The N-terminal region of FLN bound to the outer myosin, with its high negative charge, phosphorylations and disordered structure, extends out of the thick filament backbone (Figure 5-1A,B) and thus enhances the electrostatic field of the thick filament surface charge. This enhancement of charge field in turn facilitates the inter-filament electrostatic interaction for maintenance of proper and homogenenous lattice spacing between the myofilaments. We propose that this maintenance of normal lattice spacing during myofibrillogenesis resists abnormal thick filament incorporation so as to maintain the lattice regularity and geometry.

Our and previous findings and interpretations strongly indicate that FLN is composed of possibly three independent functional domains (N-terminal, middle, and C-terminal regions) acting in concert to maintain thick filament stiffness, normal length and also the myofilament lattice spacing and regularity.

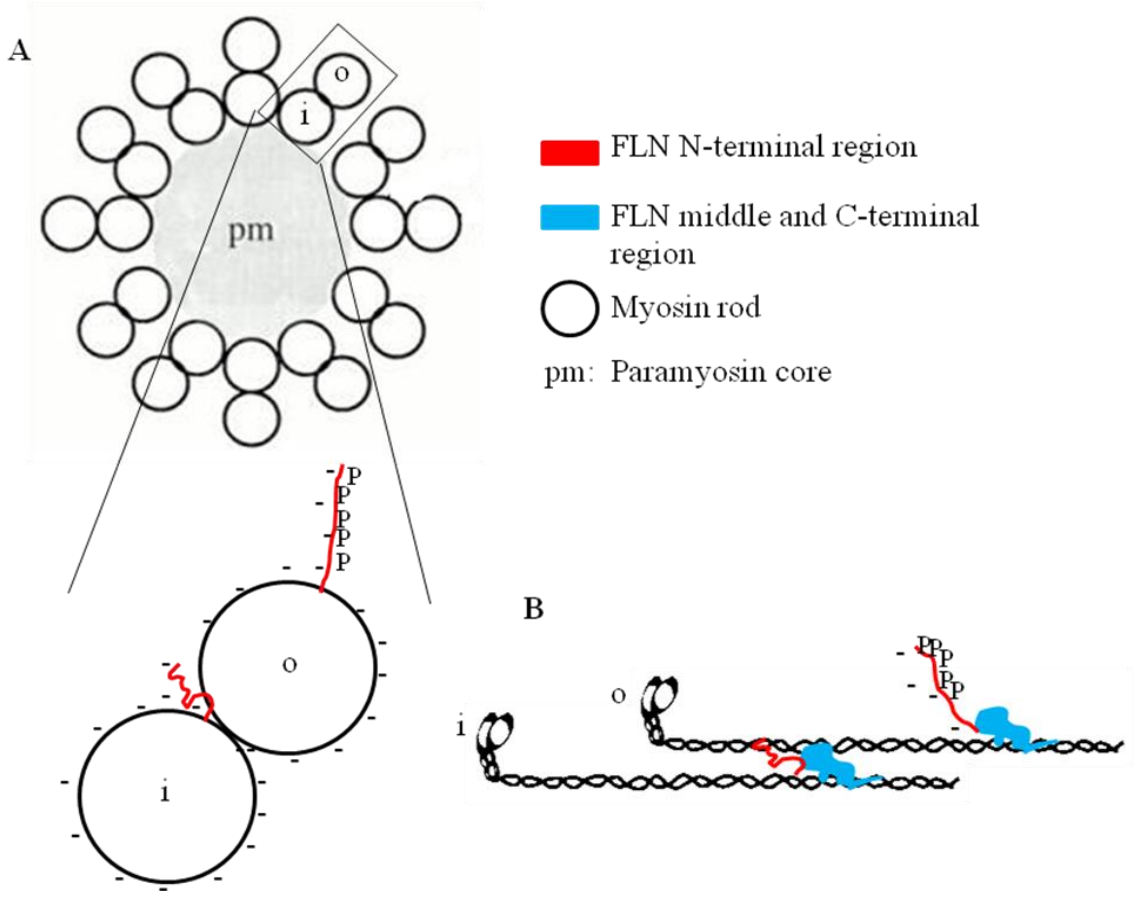

Figure 5-1. Possible model for FLN molecular function in thick filament. The top panel of (A) (modified from [15]) shows schematic cross-section of an insect flight muscle thick filament according to Beinbrech et al [32,33]. Each circle represents the rod region of a myosin molecule. There are twelve subfilaments surrounding a paramyosin (pm) core, with each subfilament consisting of two myosin molecules, an inner (denoted by "i") myosin and an outer (denoted by "o") myosin facing the thin filament. The bottom panel figure in (A) shows the zoomed view of a subfilament with an inner (i) and outer (o) myosin rod (boxed pair). We propose that the N-terminal region (red) of FLN binding

the inner myosin does not get phosphorylated and has a different conformation, compared to the N-terminal region of FLN binding the outer myosin which is hyperphosphorylated (P; only 5 phosphorylations shown for simplicity) and extends out of the thick filament backbone with high negative charge. According to this model, FLN middle region binds the myosin rod at the LMM region. FLN along with the N-terminal region (red) binding the inner (i) myosin shown as a longitudinal view in (B), interacts with the hinge-LMM junction of its outer (o) myosin partner in the subfilament so as to laterally "weld" and stabilize the two myosins together. These interactions stabilize and stiffen the subfilament. This in turn stiffens the thick filament which facilitates in maintaining normal myosin incorporation and thick filament length determination during development. The thick filament and thin filament surfaces are negatively charged [23]. Charges on thick filament surface are denoted by "-" and drawn in a simplistic manner (A). The N-terminal region (red) of FLN binding to the outer ("o") myosin (B), being predicted to be disordered with high negative charge and hyperphosphorylated (P), extends out of the thick filament backbone enhancing the electrostatic field of the thick filament. This enhancement of thick filament surface charge field maintains the thick-to-thin filament lattice spacing by electrostatic interactions aligning the thick and thin filaments (not shown in figure). The maintenance of lattice spacing and alignment of myofilaments influences in maintaining proper number of thick filament incorporation into the myofibril so as to maintain the geometry of each sarcomeric unit and the regularity of the myofilament lattice. For simplicity, myosin heads are not shown in (A). (Figure not drawn to scale or volume)

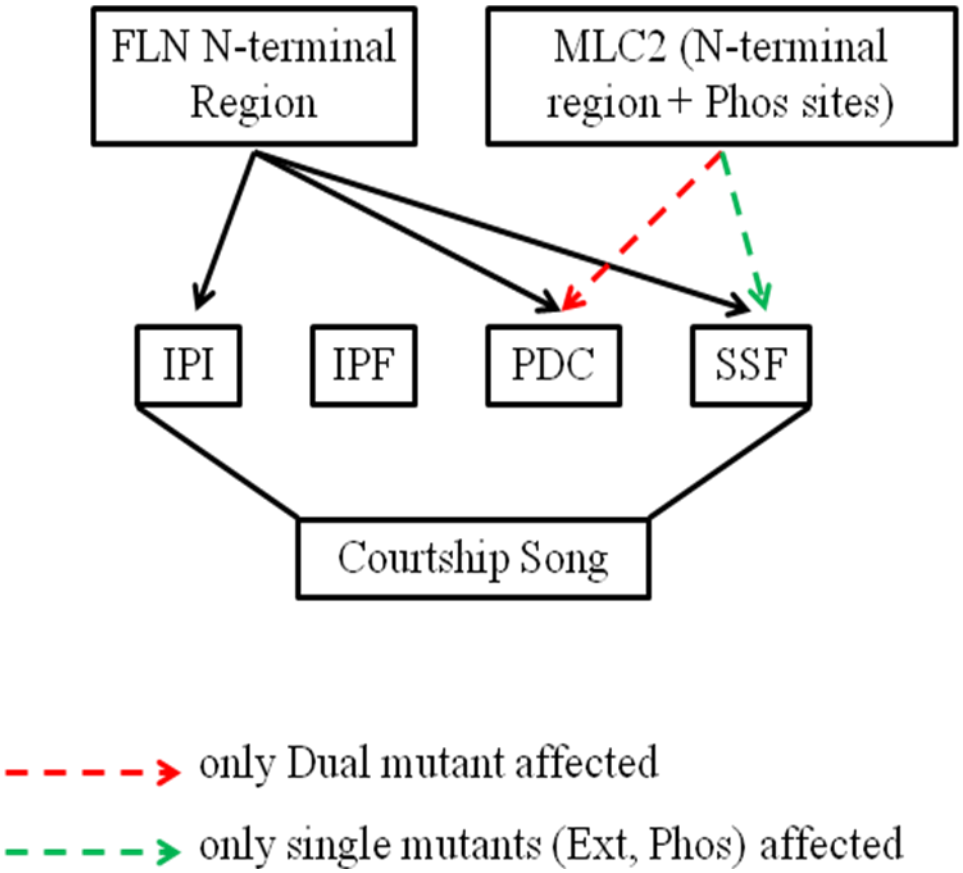

Figure 5-2. A schematic showing the effect of FLN N-terminal region and MLC2 regions on courtship song parameters. For details of parameters, see [12]. Arrows indicate a role in the parameter. For example, the FLN N-terminal region has a role in IPI, PDC and SSF. Red broken arrow denotes only the Dual MLC2 mutant gets affected in the PDC parameter. The green broken arrow indicates that only the single (Ext and Phos) MLC2 mutants get affected in the SSF parameter, but not the Dual mutant.

DROSOPHILA IFM: TRULY NATURE'S "VERSATILE" ENGINE

This study opens a new functional dimension of the *Drosophila* IFM; that of a new role in male courtship song besides its well-studied role in flight. Previous knowledge and this study show that the two thick filament proteins, FLN and MLC2 affect IFM structural and mechanical properties, and whole organismal flight (Chapter 3 and 4) and song behaviors differently (Figure 5-2). It is becoming clearer that the FLN middle and C-terminal regions are possibly under purifying selection to be essential for the underlying sarcomeric structure and stability of the IFM that is necessary for its function, whereas the variable N-terminal region is not essential but optimizes flight performance and sexually selected song features by maintaining lattice regularity and stiffness. In contrast, the ubiquitous MLC2 N-terminal extension and the phosphorylation sites regulate the fast myosin kinetics required for stretch activation response for enhancing whole organismal flight performance and wing beat frequency (Figure 5-2), with minimal effect on courtship song parameters (Figure 5-2). All these indicate that muscle genes in the IFM could be under distinct selection pressures for IFM's dual functional requirements. The minimal effect on courtship song seen in the MLC2 mutants that affect stretch activation, further suggest that distinct contractile mechanisms could be employed by the IFM for these two functions. Therefore, from muscle genes to mechanism, IFM may have evolved to be a versatile and multitasking engine which could drive wing beats for flight and song required for the organismal survival and reproductive fitness.

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APPENDIX 1

Functional Characterization of a *Drosophila* expressing a Chimeric Flightin: Implications on Muscle Lattice, Flight, and Male Courtship Song

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BACKGROUND AND GOAL

Flightin sequence analysis across 12 *Drosophila* revealed a highly variable N-terminal region (amino acids 1 through 63 in *D. melanogaster*) compared to the rest of the protein that exhibits ~70% conservation (Figure 3-1, Chapter 3). Given that most muscle structural genes are highly conserved, the hypervariability of the flightin N-terminal region is notable and could possibly indicate that either the region is under positive selection to fine-tune an adaptive function of the indirect flight muscles (IFM), or evolving by random genetic drift. Our findings that flightin N-terminal region has a critical role in myofilament lattice spacing, flight, and species-specific courtship song (Chapter 3) indicate that the region has an important functional role. The finding that the flightin N-terminal region is required for normal species-specific male courtship song parameters (Figures 3-4, 3-5 of Chapter 3) and courtship success (Figure 3-6 of Chapter 3), raise the possibility that the flightin N-terminal region is under sexual selection to fine-tune species-specific courtship song across *Drosophila*. To test this hypothesis, we created chimeric flightin transgenic lines that express a flightin whose N-terminal sequence (amino acids 1 through 67) derives from *D. virilis* and amino acids 68 through 182 derives from *D. melanogaster*.

Transgenic male fly expressing the flightin N-terminal truncated flightin ($fln^{\Delta N62}$) sings an abnormal courtship song with aberrations in species-specific, sexually selected parameters (Chapter 3). The most notable aberrations are longer interpulse interval (IPI), greater cycles per pulse (CPP), longer pulse length (PL) and a higher sine song frequency (SSF) (Table S3-3, Figure 3-4 in Chapter 3) of which the IPI is a highly variable trait

carrying the most salient species-specific signal throughout *Drosophila*, whereas normal SSF is required to stimulate females [1-3]. Based on our hypothesis that the flightin N-terminal region is under sexual selection to fine-tune species-specific courtship song parameters, we selected *D. virilis* which show the most divergent IPI (parameter most affected in $fln^{\Delta N62}$ male song) from *D. melanogaster* and produces no sine song [4-7], to create a transgenic line expressing a chimeric flightin. Table 1 show the different song parameters and tethered wing beat frequency of the two species. The *D. virilis* pulse song IPI and the PL values are same (both ~ 19.7 ms) indicating that there is no pause between two consecutive pulses, the most divergent from that of the *D. melanogaster* pulse song structure across *Drosophila*. Moreover, among the 12 *Drosophila* sequences (Figure 3-1 of Chapter 3), *D. virilis* shows the least flightin N-terminal region amino acid sequence similarity (~ 43% identity, Figure 1) with that of *D. melanogaster*, which led us to choose *D. virilis* flightin N-terminal region (67 aa) sequence to create the chimeric flightin transgenic. Based on our results on the $fln^{\Delta N62}$ male, we hypothesize that the *D. virilis* flightin N-terminal sequence will rescue the $fln^{\Delta N62}$ song abnormalities of IPI, CPP and PL and SSF to *D. melanogaster* type and enhance mating competitiveness and success. We do not expect the transgenic male expressing the chimeric flightin with flightin N-terminal region from *D. virilis*, to sing *virilis*-type of song, especially the IPI, due to the following reasons:

1) We know that entire flightin N-terminal region is required for fine-tuning species-specific song in *D. melangaster* (Chapter 3), but whether the entire or specific

sequence or some specific amino acids are the functional parts of this region for this effect on song is not known. Moreover, it is not known what role some other features like predicted disorder, high negative charge of the flightin N-terminal region (discussed in Chapter 3) play in maintaining species-specific song. Therefore, since in the chimeric flightin transgenic, we swap the entire N-terminal sequence which introduces the whole region but with changes in some amino acids keeping the predicted disorder and high negative charge intact, we expect the song changes that are observed in the $fln^{\Delta N62}$ male to be rescued.

2) *Drosophila* male courtship song is a polygenic behavior [8]. Therefore, we do not expect the inter-specific swap of the flightin N-terminal sequence to change the song completely to *virilis*-type, rather rescue the song to *melanogaster*-like. This will indicate whether the flightin N-terminal region is under positive selection in order to facilitate in the fine-tuning of critical courtship song parameters required for reproductive success.

MATERIALS AND METHODS

Drosophila Strains

Drosophila melanogaster w^{1118}, and *w*; T(2;3)* ap^{Xa}, ap^{Xa} */ CyO; TM3,* Sb^{1} (used for linkage group analysis) were obtained from the Bloomington Stock Center (Bloomington, IN). w^{1118} was used as host for generating the transgenic strains. w^{1118}*;* $P\{w^{+}, Act88Ffln^{+}\}$*;* fln^{o}*, e*, the transgenic strain expressing the wild-type flightin gene in

a *fln*^{0} background [9], was used as the control line and henceforth will be referred to as *fln*$^{+}$. The flightin null mutant line (*fln*^{0}) used here was previously made [10]. Wild type *D. virilis* flies were obtained from the Drosophila Species Stock Center, UCSD (Stock Number 15010-1051.00). All fly lines were maintained in a constant temperature and humidity (21±1°C, 70%) environmental room on a 12:12 light:dark cycle.

Construction of the Transformation Vector (flnVirNChcas)

The flightin N-terminal 67 amino acids (201 bp) from *D. virilis* was engineered into a P-element transformation vector pCaSpeR (Flybase ID: FBmc0000168) containing the 954 bp flightin gene with its N-terminal region deleted and the actin *Act88F* promoter (see Materials and Methods of Chapter 3) by using the following approach:

Using the following primers, the *D. virilis* flightin N-terminal region (201 bp, amino acids 1 through 67) was amplified.

Forward1: 5' TTTTTGGTACCATGGCGGACGAAGAAGATCCTTGG 3'

Reverse1: 5' TTCTGGCGGAGGCGGCGGTGCTTTCATTTCAACCTCAGG 3'

Nucleotides shown in red in the reverse primer is the *D. melanogaster* sequence corresponding to amino acids 64 through 71. The underlined bases represent a KpnI restriction enzyme site in the forward primer. The above primers were designed to amplify 236 bp fragment comprising the *D. virilis* flightin N-terminal region along with 24 bp of the *D. melanogaster* tail sequence (from *D. melanogaster* flightin 64th to 71st amino acid position). The amplified PCR product (236 bp fragment) was used as template for a nested PCR reaction in order to include an AgeI restriction site (the only Age I site

which is not present in the 201 bp N-terminal flightin region of *D. virilis* nor in the pCaSpeR vector containing *Act88F* promoter sequence) present in the *D. melanogaster* flightin sequence at the 3'end of the product. The Forward1 primer was used as the forward primer with the reverse primer as follows:

Reverse2: 5' CTGCACCGGTTTCCTGTAACCATCGTCTTCTGGCGGAGG 3'

with the underlined bases representing AgeI restriction enzyme site. The resulting 263 bp fragment was TA-cloned into the pGEM-T Easy vector (Promega A1360). The pCaSpeR vector containing 954 bp N-terminal truncated flightin gene with the actin *Act88F* promoter sequence (see Chapter 3), and the pGEM-T Easy vector containing the above-mentioned 263 bp fragment, both were digested with KpnI and AgeI restriction endonucleases. This prepared the pCaSpeR vector and pops-out the 263 bp fragment, both with KpnI and PstI digestions, for directional subcloning. Then the 263 bp fragment was subcloned into the pCaSpeR vector. The Forward1 with either Reverse1 or Reverse2 or the only reverse primer (Reverse) having a PstI site (mentioned in Chapter 3) were used for sequencing verifications of the N-terminal chimeric flightin construct. Figure 2 shows a diagram of the transformation vector (flnVirNChcas) indicating the KpnI, AgeI and PstI sites, along with the P_{Act88f} promoter and the flnVirNCh gene. Figure 3 shows the sequence verification of the chimeric flightin construct showing the junction area with the *D. virilis* N-terminal sequence ending at nucleotide position 201 (amino acid 67th position) after which the *D. melanogaster* sequence starts from the nucleotide position 202 (amino acid 68th position i.e., 64th aa position of *D. melanoagster* flightin sequence).

Generation of the *P{fln^{VirN67}}* Strains

Microinjection of the transformation vector into w^{1118} host strain was carried out by Genetic Services, Inc., Sudbury, MA. Linkage group was determined by standard crosses to *w*; T(2;3) ap^{Xa}, ap^{Xa} / CyO; TM3, Sb^{1}*. Five parental strains were created in a *fln^{+}* background, four of them with a second chromosome insertion and one of them with an X chromosome insertion, and were subsequently crossed into the flightin null background (*fln^{0}*) [8] to generate homozygous transgenic strains with no endogenous flightin expression. All the second chromosome transformed strains have the genotype w^{1118}; *P{w^{+}, Act88Ffln^{VirN67}}*; *fln^{0}*, *e* and herein will be referred to as *fln^{VirN67X}* where X is a letter or a number to denote individual lines. The X chromosome transformed strain has the genotype w^{1118}, *P{w^{+}, Act88Ffln^{VirN67}}*; + ; *fln^{0}*, *e*. All the parental (*fln^{+}* background) and the daughter (*fln^{0}*) lines with corresponding genotypes are listed in Table 2. Expression of the transgene was confirmed by RT- PCR analysis via RNA isolated from 30 two-day old flies (data not shown), using the Forward1 and Reverse primers described in the previous section. We will report here the preliminary results of three of the transgenic lines (2nd chromosome insertions), *fln$^{VirN67:4.41}$*, *fln$^{VirN67:5.26}$*, *fln$^{VirN67:47.30}$* (Table 2) namely expressing the chimeric flightin without endogenous flightin expression.

Flight Performance

Flight tests and wing-beat frequency analysis were performed as previously described [3].

Courtship Song Assays

Male courtship song assays are performed as described in Chapter 2.

Statistical Analysis

All values are mean ± SE. One-way ANOVA was performed using SPSS (v.20.0, SPSS, Chicago, IL) followed by Fischer's LSD pairwise comparisons as a post-hoc test, with values considered significant at $p<0.05$.

PRELIMINARY RESULTS AND INTERPRETATIONS

Chimeric Flightin Rescues Flight Ability Completely

Our preliminary flight tests show that the parental lines (Table 2) i.e., the $fln^{VirN67X}$ flies in a wild type (+) background had similar flight capability as *D. melanogaster* (OR) wild type flies (data not shown) indicative of no dominant negative effect by the transgene. $fln^{VirN67X}$ flies in a fln^{0} background were similar in flight capability among themselves as well as compared to fln^{+} full length rescued null control line and *D. virilis* wild type flies (Table 3). This indicates that the chimeric flightin protein is able to rescue completely the flight impairment of the fln^{0} flies (Table 3-1 of Chapter 3) as seen by a similar flight score between the $fln^{VirN67X}$ and fln^{+} lines (Table 3), compared to only partial rescue by the $fln^{\Delta N62}$ flies (Table 3-1 of Chapter 3). This suggests that the flightin N-terminal region's amino acid variance between *D.*

melanogaster and *D. virilis* have no effect on flight performance. Moreover, the chimeric flightin mutant and the control lines have similar flight abilities as the wild type *D. virilis* flies.

Chimeric Flightin Mutant Males are Able to Sing Courtship Song

Preliminary courtship song assays (for methods see Chapter 2 and Chapter 3) reveal that fln^{VirN67} males are capable of generating courtship song (Figure 4) to attract wild type *D. melanogaster* (Oregon R) female mate.

ONGOING AND FUTURE WORK

Ongoing and future work will be focused on characterizing the chimeric flightin transgenic lines by extending the tethered wing beat frequency analysis and comparing to fln^{+}, $fln^{\Delta N62}$, and *D. virilis*. Moreover, we will perform courtship song assays to analyze the effect of the chimeric fligthin on song parameters in comparison to those of fln^{+}, $fln^{\Delta N62}$, and *D. virilis*. We will also perform courtship competition assays between i) fln^{VirN67} male and fln^{+} male for OR female, ii) fln^{VirN67} male and fln^{+} male for *D. virilis* female, and iii) fln^{VirN67} male and *D. virilis* male for *D. virilis* female. This will enable us to understand if the chimeric flightin transgenic line sings a more virilis-type of song and whether the flightin N-terminal region is indeed under sexual selection or not. Along with that, we will perform, muscle structural and mechanical studies on the chimeric flightin

line. Hence, by taking an inter-disciplinary approach from muscle structural to behavioral output in characterizing the fly lines, we hope to understand further the function of the flightin N-terminal region and the reason for its low amino acid conservation.

FIGURE LENGENDS

Figure 1. *D. melanogaster* and *D. virilis* flightin amino acid sequence alignment. Clustal W alignment of flightin amino acid sequences from *D. melanogaster and D. virilis* reveals differences in sequence conservation, with the N-terminal region (63 aa in *D. melanogaster* and 67 aa in *D. virilis*) denoted by the box region, having much lower conservation (~ 43% identity) compared to the rest of the protein (~ 87% identity). Identities are marked by asterisks (*). Colon (:) indicates residues at that position are very similar based on their properties, and dot (.) indicates residues at that position are more or less similar. The region swapped in this study (boxed region) is amino acids 2 through 63 as per *D. melanogaster* numbering with amino acids 2 through 67 as per *D. virilis* numbering.

Figure 2. Diagram of the flnVirNChcas P-element transformation vector. The P_{Act88f} is the Actin88F promoter ending in the green arrow head (3' end), and flnVirNCh is the DNA cloned (see Figure 3). P-element ends are shown in brown. Also, the ampicillin resistance marker *pUC8r* and the eye-color marker *white* gene are shown. The corresponding coordinates in basepairs (bp) in parenthesis for each segment, and the important restriction enzyme sites are also shown.

Figure 3. Schematic of the sequence verification of the chimeric flightin (flnVirNCh) DNA used for cloning into the transformation vector. The first 201 bp segment is from *D. virilis* flightin N-terminal region (67 aa) in blue and the rest of the sequence is from *D. melanogaster* with the entire DNA to be of 1155 bp size. In 2 and In3 indicate the 2nd and 3rd intron sequences, and 3' UTR is the untranslated region seuqnece present in *D. melanogaster* flightin gene. The junction of the two species sequence in the chimeric flightin DNA is zoomed in the bottom panel in which the N-terminal *D. virilis* part of the seuquence ending at 201st position of the DNA, from where *D. melanogaster* part of the sequence starts (202nd position). Corresponding amino acids of codons in the junction are shown.

Figure 4. Example of courtship song oscillogram generated by the *fln*$^{VirN677.30}$ male for a wild type *D. melanogaster* (OR) female showing that it is capable of singing both sine and pulse songs. Scale bar = 1 second.

Table 1. Tethered wing beat frequency and male courtship song properties of *D. melanogaster* (Oregon R strain) and *D. virilis* species

Species	Tethered Wing beat frequency, Hz	Mean Interpulse interval (IPI), ms	Mean Intrapulse frequency (IPF), Hz	Cycles per pulse (CPP), #	Pulse length (PL), Ms	Mean sine song frequency (SSF), Hz
D. melanogaster	219±3.3 (10)	34	280	2	3	130
D. virilis	143±3.0* (10)	19.7	273	5.4	19.7	NA

Values are mean ± SE. Number of flies analyzed are shown in parenthesis. Wing beat frequency data is courtesy Panos Lekkas. Courtship song parameters are retrieved from [4-7].

* Significant difference ($p<0.05$) between strains.

Table 2. Chimeric flightin parental lines in wild type (+) background and corresponding daughter lines in *fln^{0}* background

Parental Lines	Genotype	Daughter Lines	Genotype
fln$^{VirN672.26}$/+	*w^{1118}*;*P{w^{+}, Act88Ffln^{VirN67}}*; +, *e*	*fln^{VirN67A}/fln^{0}*	*w^{1118}*;*P{w^{+}, Act88Ffln^{VirN67}}*; *fln^{0}*, *e*
fln$^{VirN672.27}$/+	*w^{1118}*, *P{w^{+}, Act88Ffln^{VirN67}}*; +; +, *e*	*fln^{VirN67B}/fln^{0}*	*w^{1118}*,*P{w^{+}, Act88Ffln^{VirN67}}*; +; *fln^{0}*, *e*
fln$^{VirN674.37}$/+	*w^{1118}*;*P{w^{+}, Act88Ffln^{VirN67}}*; +, *e*	*fln$^{VirN674.41}$/fln^{0}*	*w^{1118}*;*P{w^{+}, Act88Ffln^{VirN67}}*; *fln^{0}*, *e*
fln$^{VirN675.21}$/+	*w^{1118}*;*P{w^{+}, Act88Ffln^{VirN67}}*; +, *e*	*fln$^{VirN675.26}$/fln^{0}*	*w^{1118}*;*P{w^{+}, Act88Ffln^{VirN67}}*; *fln^{0}*, *e*
fln$^{VirN676.53}$/+	*w^{1118}*;*P{w^{+}, Act88Ffln^{VirN67}}*; +, *e*	*fln$^{VirN677.30}$/fln^{0}*	*w^{1118}*;*P{w^{+}, Act88Ffln^{VirN67}}*; *fln^{0}*, *e*

Table 3. Flight properties of control and mutant flightin chimeric strains

Strain	Flight score (0-6)
fln^{+}	3.9±0.3 (15)
$fln^{VirN674.41}$	4.5±0.2 (23)
$fln^{VirN675.26}$	3.8±0.3 (16)
$fln^{VirN677.30}$	4.1±0.2 (30)
D. virilis	4.5±0.3 (9)

Values are mean ± SE. Number of flies analyzed is shown in parenthesis.

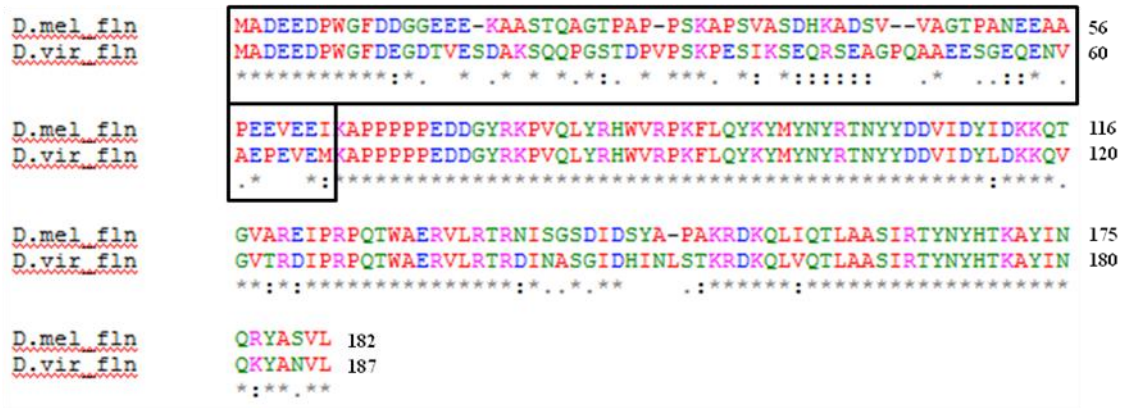

Figure 1. Sequence alignment of flightin amino acids from *D. melanogaster* (D.mel_fln) and *D. virilis* (D.vir_fln) with the highly variable N-terminal region shown in the boxed area.

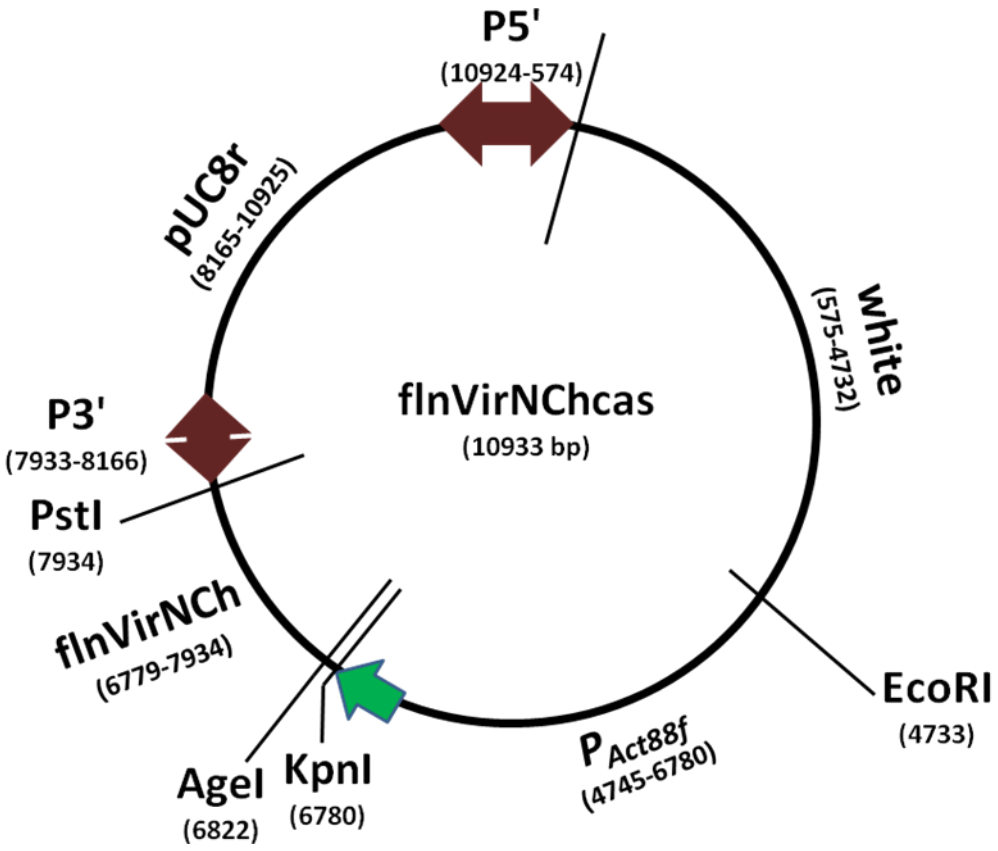

Figure 2. Diagram of the flnVirNChcas transformation vector with important restriction enzyme sites. .

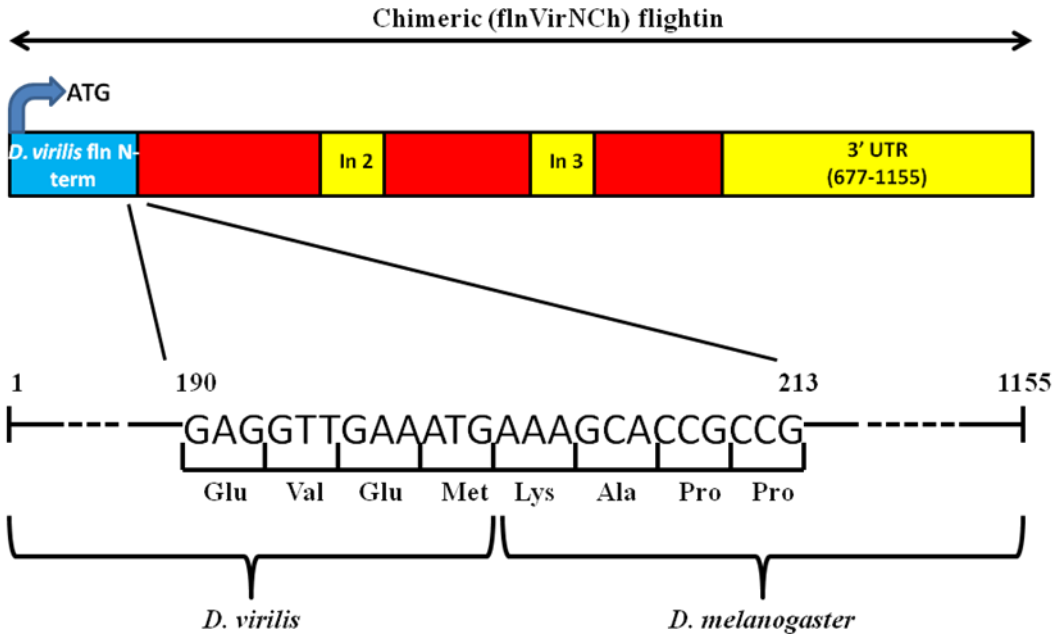

Figure 3. Schematic of sequence verification of the chimeric flightin (flnvirNCh) DNA.

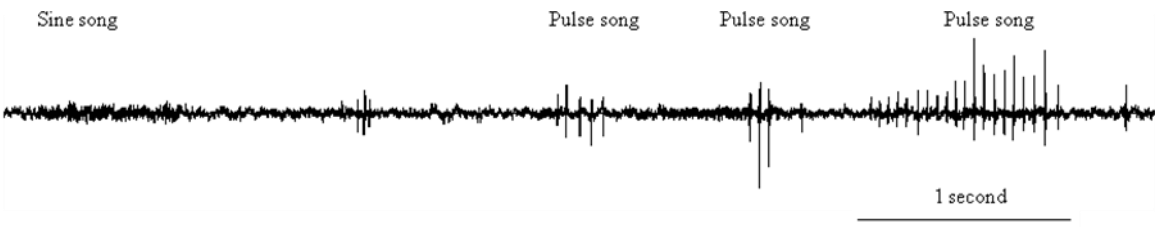

Figure 4. Male courtship song snapshot of *fln*$^{virN677.30}$ male.

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APPENDIX 2

Flight and Mating Behavior of a Dual Heterozygote *Drosophila* expressing Flightin NH2-Terminal and COOH-Terminal Truncated Proteins

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BACKGROUND AND GOAL

Muscle contraction is driven by the cyclic interaction between the myosin heads in the thick filament and the actin targets in thin filament. With the numerous advances in tools for genetic manipulations [1,2], *D. melanogaster* indirect flight muscles (IFM) has been a model for muscle research for decades [3], and has been used to elucidate functions of muscle genes from the molecular to the organismal level [4,5,6]. By using the thoracic musculature, *Drosophila* fly for survival (foraging, escaping predators etc), and the males sing by vibrating one wing to attract the females for con-specific mating and reproduction. The mechanism of stretch activation [7,8] is utilized in the *Drosophila* IFM along with exceptionally fast myosin cycling kinetics to power flight [9], a behavior subject to natural selection. Moreover, IFM gets neurally activated and is utilized during the male courtship song generation [10,11], which is an important component of a complex behavioral ritual under sexual selection [12].

Flightin is a ~20 kDa (182 amino acids) thick filament associated, myosin rod binding protein [13,14] that in *Drosophila* is exclusively expressed in the IFM [15]. It is essential for the structural and mechanical integrity of the IFM, and for flight [16-18]. Moreover, flightin null mutant ($fln^{0/0}$) males cannot sing (Chapter 3) suggesting that the IFM is directly involved in song generation since this mutation is IFM-specific. A comparison of the flightin amino acid sequences from twelve *Drosophila* species revealed a tripartite organization [19]: a hypervariable amino (N) terminus region (amino acids 1 through 63; *D. melanogaster* numbering) with only about 20% identity, a highly conserved middle region (amino acids 64 through 137) with ~ 92% identity, and a

somewhat conserved carboxy (C) terminus region (amino acids 138 through 182) with ~ 59% identity. The differential conservation of these three regions of flightin suggests that they are separate protein domains under distinct evolutionary selection regimes [19], and possibly with distinct functions like flight and courtship song. Our previous findings suggest that there could be substantial dichotomy in the type of muscle genes and gene regions being used for flight and courtship song in the IFM (Chapters 3 and 4). For example, N-terminal extension (46 aa) and the two critical phosphorylation sites (Serines 66 and 67) of myosin regulatory light chain (Dmlc2), a thick filament associated protein, do not have a major effect in courtship song generation while having a large effect in IFM power enhancement for maximal flight (Chapter 4, [20]). In contrary, the highly variable N-terminal region (63 aa) of flightin is not essential but required for both fine-tuning species-specific courtship song enhancing courtship success, and optimizing flight performance (Chapter 3), as evidenced in a transgenic fly expressing N-terminal truncated flightin ($fln^{\Delta N62/\Delta N62}$). On the other hand, the more conserved C-terminal region (44 aa) of flightin is essential for basic IFM structural integrity, contractile function, as seen in a mutant expressing C-terminal truncated flightin ($fln^{\Delta C44/\Delta C44}$) that are completely unable to fly [19] or sing (data not shown) due to major muscle structural defects. Therefore, our data suggest that one ubiquitous thick filament protein (Dmlc2) is being used by the IFM to specifically maximize flight behavior subject to natural selection, whereas, an IFM-specific thick filament protein (flightin) is possibly under dual selection pressure: being used for both basic and optimizing functions for the two behaviors through conserved (C-terminal) and highly variable (N-terminal) regions, respectively.

The pleitropic effect of flightin on both flight and song, and that it's two terminal regions (N-terminal and C-terminal) showing separate conservation patterns and behavioral effects, indicate that flightin is under dual evolutionary selection regimes and probably incorporated by the IFM in its repertoire as an evolutionary innovation to fulfill its two distinct behavioral needs. As a step towards testing this further, we hypothesized that the two truncated variants of the flightin gene, i.e., the N-terminal truncated gene and the C-terminal truncated gene (denoted from now on as *ΔN62* and *ΔC44* respectively), when expressed together having the common well-conserved middle region (74 aa), will genetically complement each other to fully rescue maximal flight performance and species-specific courtship song to enhance male mating success. This hypothesis is derived since flightin could possibly function as a dimer *in vivo* binding myosin rod facilitating the genetic complementation between *ΔN62* and *ΔC44*, given that zeelin 2, a flightin homologue in *Lethocerus* has been shown to form filaments *in vitro* at low ionic strength solutions [21] indicative of dimerization capacity. Therefore, this strategy will elucidate: i) the extent of the role of the N-terminal and C-terminal regions of flightin in flight and courtship song as a way to understand the possible dual selection, ii) any genetic preference for IFM to incorporate more a specific truncated variant, iii) possible genetic interaction between the two truncated variants, and iv) since previous mutational genetics studies ([19], Chapter 3) suggested that the middle region is required for thick filament incorporation of flightin, this strategy will help us to understand if the two variants having the common middle region, genetically compensate for each other. For this purpose, we created a dual heterozygote line expressing both the flightin N-terminus

(63 aa) truncated and C-terminus (44 aa) truncated proteins, $fln^{\Delta N62/\Delta C44}$, to test its flight abilities and male courtship success rates.

Previously, $fln^{\Delta C44/\Delta C44}$ flies were found to be completely flightless [19], and cannot produce a mating song, while the $fln^{\Delta N62/\Delta N62}$ flies can fly (Chapter 3, flight score: 2.82±0.1 vs 4.2±0.36 for fln^{+}) and sing, but with some impairments compared to the rescued control null ($fln^{+/+}$) strain (Chapter 3). Our goal is to understand that the two truncated flightin proteins, if co-expressed in the dual heterozygote ($fln^{\Delta N62/\Delta C44}$), could complement each other. Both truncated flightin proteins in the two homozygote transgenic lines ($fln^{\Delta N62/\Delta N62}$, $fln^{\Delta C44/\Delta C44}$) were expressed normally in the IFM (Chapter 3, [19]) suggesting that the N-terminal and C-terminal regions are not required for normal expression and incorporation of flightin into thick filaments. We tested the flight properties (flight score and the tethered wing beat frequency) of the $fln^{\Delta N62/\Delta C44}$ flies, compared them with several homozygote and heterozygote control lines, and currently testing for courtship song and courtship success rates in mating competition situations.

PRELIMINARY RESULTS AND INTERPRETATIONS

The $fln^{\Delta N62/\Delta C44}$ line is able to fly but only as good as the $fln^{\Delta N62/\Delta N62}$ line (flight score: 2.89±0.13 vs 2.82±0.1 or 0 for $fln^{\Delta N62/\Delta N62}$ or $fln^{\Delta C44/\Delta C44}$ respectively, Figure 1), indicating that a single copy of the *ΔN62* gene is able to rescue the flightlessness of the $fln^{\Delta C44/\Delta C44}$, but could not completely rescue compared to the $fln^{+/+}$ control (flight score: 4.14±0.37) line. This also indicates that the effect on the flight ability is independent of

copy number of the *ΔN62* gene. This is further supported by similar flight abilities of the $fln^{\Delta N62/0}$ to the $fln^{\Delta N62/\Delta C44}$ or the $fln^{\Delta N62/\Delta N62}$ lines (Figure 1). Moreover, the dual heterozygote $fln^{\Delta N62/\Delta C44}$ line's flight ability is similar to but not reduced than $fln^{\Delta N62/\Delta N62}$ line (Figure 1) indicating that the *ΔN62* allele is dominant over *ΔC44* allele. In other words, the above results suggest that flightin C-terminal region seems to be more involved in facilitating flight ability than the N-terminal region.

$fln^{\Delta N62/\Delta N62}$ line's flight impairment is completely rescued by a single full length flightin gene in the $fln^{\Delta N62/+}$ line (Figure 1), suggesting that full length flightin (+) is dominant over the *ΔN62* gene. In contrary, flight abolishment seen in $fln^{\Delta C44/\Delta C44}$ is only partially rescued by a single full length flightin gene in the $fln^{\Delta C44/+}$ line (Figure 1), suggesting that full length flightin (+) is only partially dominant over the *ΔC44* gene. This indicates that the presence of two copies of the flightin C-terminal region (in $fln^{\Delta N62/+}$) facilitates complete rescue of flight, whereas the presence of two copies of the N-terminal region (in $fln^{\Delta C44/+}$) could only partially rescue. From these data, we can infer that at least one copy of the flightin C-terminal region is necessary for flight whereas two copies are essential for maximal flight levels. On the other hand, the N-terminal region is neither essential nor necessary for flight, but is required for normal flight where only one copy of it will be sufficient. This further indicates that flightin C-terminal region is probably more critical for imparting maximal flight performance than the N-terminal region in the IFM.

Does the *ΔN62* and *ΔC44* complement each other in the dual heterozygote $fln^{\Delta N62/\Delta C44}$?

The wing beat frequency of the dual heterozygote $fln^{\Delta N62/\Delta C44}$ line is slightly, but significantly reduced compared to $fln^{\Delta N62/\Delta N62}$ or $fln^{+/+}$ lines (180.8±2.7 Hz vs 195.0±0.6 Hz or 197.8±0.4 Hz for $fln^{\Delta N62/\Delta N62}$ or $fln^{+/+}$ respectively, Figure 2). Our finding that the $fln^{\Delta N62/\Delta C44}$ could not achieve maximal flight performance or maximal wing beat frequency similar to $fln^{+/+}$, suggest that the two truncated flightin genes do not genetically complement each other, otherwise a full rescue could have been observed. As discussed above, one of the ways that the *ΔN62* and *ΔC44* genes could complement is the molecular situation where flightin functions as a dimer, in case of which the two copies of middle region from the two truncated proteins could have been binding the myosin rod (possibly at the same location). Data suggestive of non-complementation indicate that flightin does not function as a dimer. Given that $fln^{\Delta N62/\Delta C44}$ flies could only fly as good as the $fln^{\Delta N62/\Delta N62}$ line but with a lower wing beat frequency, suggest that the flightin protein with the C-terminal region but without the N-terminal region (product of *ΔN62*) could be incorporating into the thick filament more than protein with the N-terminal region and the without C-terminal region (product of *ΔC44*). This again indicates that the C-terminal region is more critical for flightin function in the IFM for flight.

Overall, the data on flight properties suggest that a) the two truncated mutant flightin proteins are unable to genetically complement each other indicating flightin does not function as a dimer, b) the C-terminal region is essential for flight, whereas the N-

terminal region is required for maximal flight performance d) the N-terminal and C-terminal regions of flightin have distinct conservation patterns potentially due to distinct selection regimes for separate IFM functions.

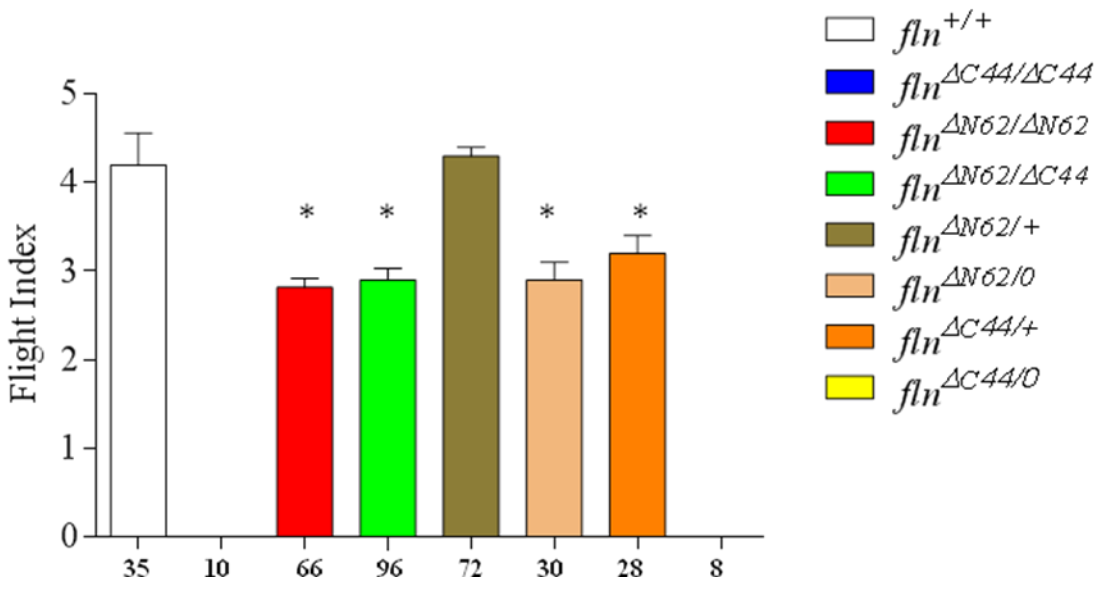

Figure 1. Flight properties of the transgenic lines tested in this study. Numbers below the bars represent numbers of flies tested. $fln^{\Delta C44/\Delta C44}$ has a flight index of zero (data from [19]). N=8 for $fln^{\Delta C44/0}$ with flight index of zero. One-way ANOVA: * $p<0.05$ vs fln^{+}, $fln^{\Delta C44/\Delta C44}$, $fln^{\Delta N62/+}$, and $fln^{\Delta C44/0}$

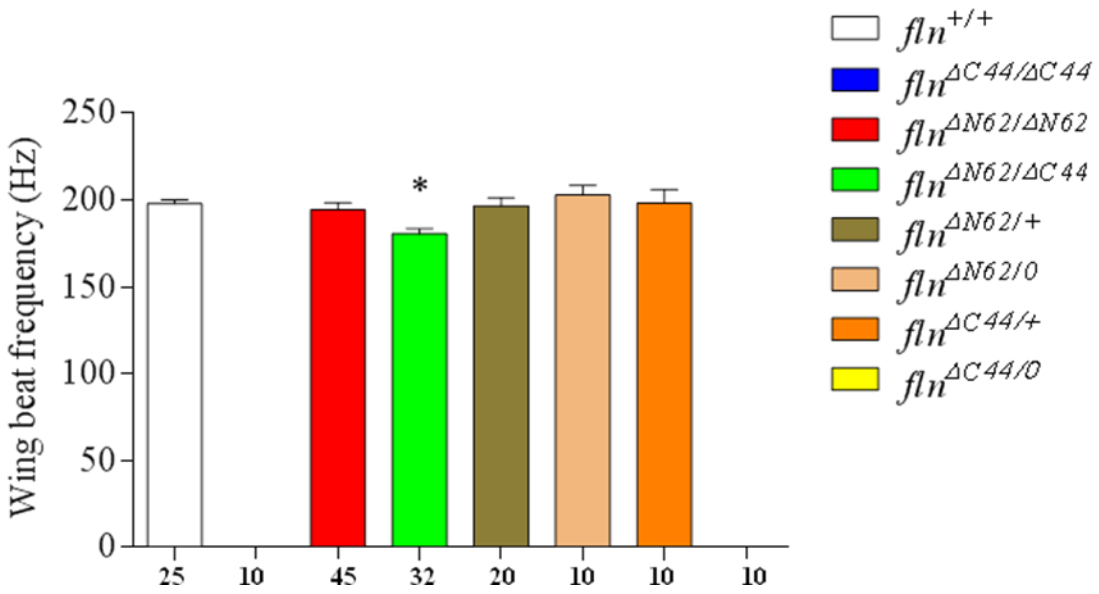

Figure 2. Tethered wing beat frequency of the transgenic lines tested in this study. Numbers below the bars represent numbers of flies tested. $fln^{\Delta C44/\Delta C44}$ has no wing beat (data from [19]). N=10 for $fln^{\Delta C44/0}$ with no wing beat. One-way ANOVA: * $p<0.05$ vs all lines.

ONGOING AND FUTURE WORK

Our preliminary work on flight properties of the dual heterozygote line and the control lines indicate that the flightin C-terminal region is more essentially used by the IFM than the N-terminal, for optimal flight performance which possibly explains the region's higher conservation pattern. Therefore, we will be testing for courtship song and courtship behavioral outcomes of this line to understand the effect these two regions of flightin have in IFM-driven courtship behavior, distinct from flight. Future studies will focus on a) testing the flight properties of the $fln^{+/0}$ line which has only one copy of a full length flightin gene to understand further the effect of flightin gene copy number on function, b) quantifying the relative expression levels of the flightin N-terminal truncated and C-terminal truncated proteins in the $fln^{\Delta N62/\Delta C44}$ line, b) $fln^{\Delta N62/\Delta C44}$ courtship song recording and analysis, c) mating competition between the $fln^{\Delta N62/\Delta C44}$ and the homozygote lines for wild type female mate. This inter-disciplinary approach of understanding the level of protein expression and the extent of the role of flightin regions in flight, male courtship song and mating success will elucidate the functional significance of distinct conservation patterns of flightin sequence. This study re-emphasizes the importance and requirement of thick filament associated muscle proteins in the muscle structural and functional integrity, physiology and behavior of the whole organism.

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APPENDIX 3

Skinned Muscle Fiber Mechanics by Sinusoidal Analysis and Isometric Tension Measurements

In chapter 3, all fiber mechanics experiments were done in solutions containing 4% T-500 dextran in order to osmotically compress the myofilament lattice spacing to bring it to *in vivo* spacing, as had been previously done [1]. As described in Chapter 3 Materials and Methods, skinned fiber mechanics by sinusoidal analysis was performed on $fln^{\Delta C44}$ line [2] IFM fibers. Moreover, fiber mechanics without 4% T-500 dextran was performed on all three transgenic and mutant line IFMs, namely fln^{+}, $fln^{\Delta N62}$, and $fln^{\Delta C44}$.

In 4% T-500 dextran solutions, the active, relaxed and rigor viscoelastic moduli of $fln^{\Delta C44}$ fibers were reduced at a similar extent as $fln^{\Delta N62}$ compared to fln^{+} fibers (Figure 1, 2A-B). The maximum work and power output of the $fln^{\Delta C44}$ fibers were severely reduced than both the fln^{+} and $fln^{\Delta N62}$ fibers, with a greatly reduced frequency of maximum work and power indicative of much reduced underlying cross-bridge kinetics (Figure 2C-D). Isoemtric tension for $fln^{\Delta C44}$ fibers were reduced compared to fln^{+} control fibers (Table 1). Data patterns without dextran were similar to with dextran, except that the frequencies of maximum work and maximum power were slightly reduced for all lines (Figure 3).

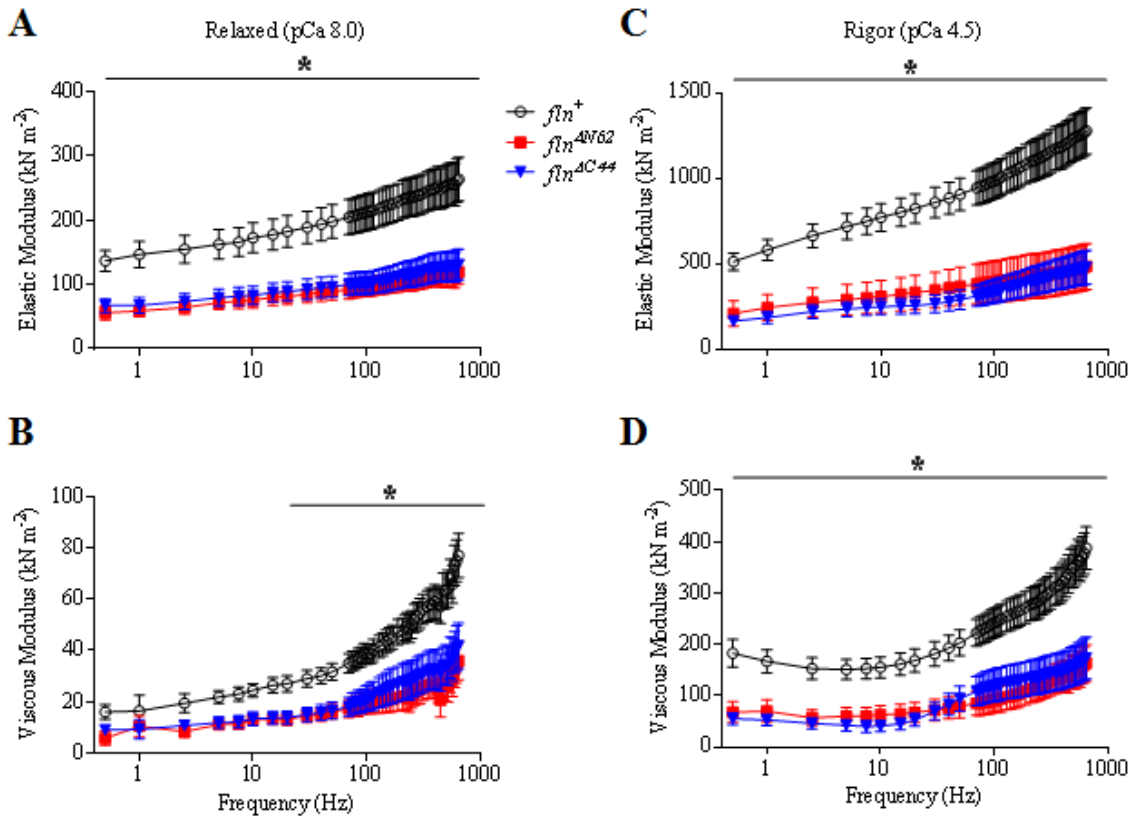

Figure 1. *fln*$^{\Delta N62}$ and *fln*$^{\Delta C44}$ IFM fibers have reduced stiffness and viscous properties. Elastic and viscous moduli of skinned IFM fibers from *fln*$^{+}$ (open circles), *fln*$^{\Delta N62}$(filled red squares), and *fln*$^{\Delta C44}$ (filled blue triangles) in relaxing (A and B) and rigor (C and D) solutions. Horizontal lines below asterisks denote frequency range through which measured values are significantly different between *fln*$^{\Delta N62}$ *or fln*$^{\Delta C44}$ *and fln*$^{+}$ ($p<0.05$).

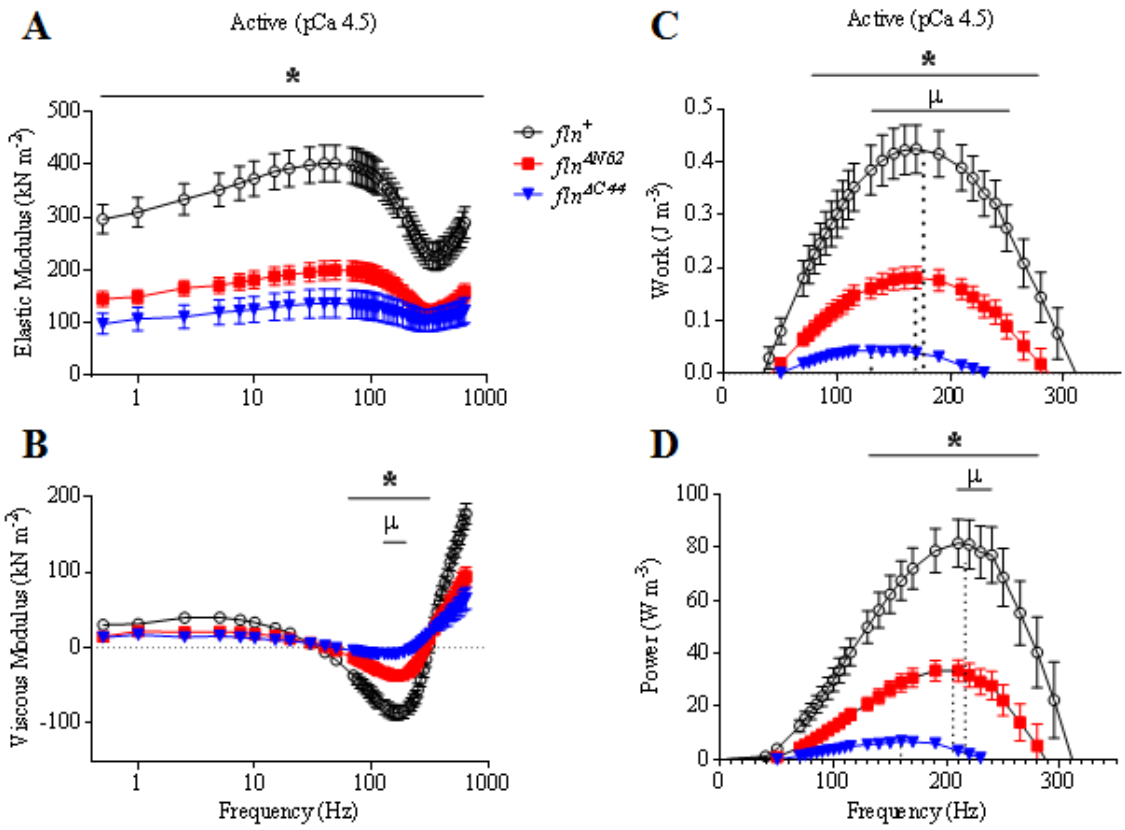

Figure 2. *fln*$^{\Delta N62}$ and *fln*$^{\Delta C44}$ IFM fibers have reduced work power output. *fln*$^{\Delta C44}$ IFM fibers generate maximum work and maximum power at much lower frequency than control *fln*$^{+}$ or *fln*$^{\Delta N62}$. Elastic modulus (A), viscous modulus (B), work (C), and power (D) for active IFM fibers from *fln*$^{+}$ (open circles) and *fln*$^{\Delta N62}$ (filled red squares), and *fln*$^{\Delta C44}$ (filled blue triangles) strains. Lines below asterisks denote frequency ranges where measured values are significantly different between *fln*$^{+}$ and *fln*$^{\Delta N62}$ or *fln*$^{\Delta C44}$ ($p<0.05$). Lines below "μ" denote frequency ranges where measured values are significantly different between *fln*$^{\Delta N62}$ and *fln*$^{\Delta C44}$ ($p<0.05$). Vertical dashed lines in C and D represent corresponding frequency of maximum oscillatory work and power output. The

frequencies of maximum oscillatory work and power are significantly different between $fln^{\Delta C44}$ and fln^{+} or $fln^{\Delta N62}$.

Table 1. Isometric tension measurements from skinned IFM fibers.

Line	Relaxed tension (kN/m^2)	Net active tension (kN/m^2)	Net rigor tension (kN/m^2)	Net rigor yield strength (kN/m^2)
fln^{+}	1.7±0.3 (15)	1.5±0.2 (15)	3.1±0.4 (11)	5.3±0.4 (2)
$fln^{\Delta N62}$	0.9±0.1* (15)	0.8±0.1* (15)	1.1±0.2* (8)	1.6±0.1* (3)
$fln^{\Delta C44}$	0.67±0.12* (4)	0.74±0.2* (4)	1.38±0.43* (4)	2.5±0.5* (2)

Values are mean ± SE. Numbers in parentheses indicate number of fibers analyzed. Developed active (pCa4.5) or developed rigor (pCa4.5) values represent tension increase from relaxed (pCa8.0) condition. Net rigor yield strength = Total maximal tension withstood before fiber starts tearing – relaxed tension. * Significant difference ($p<0.05$) from fln^{+} control.

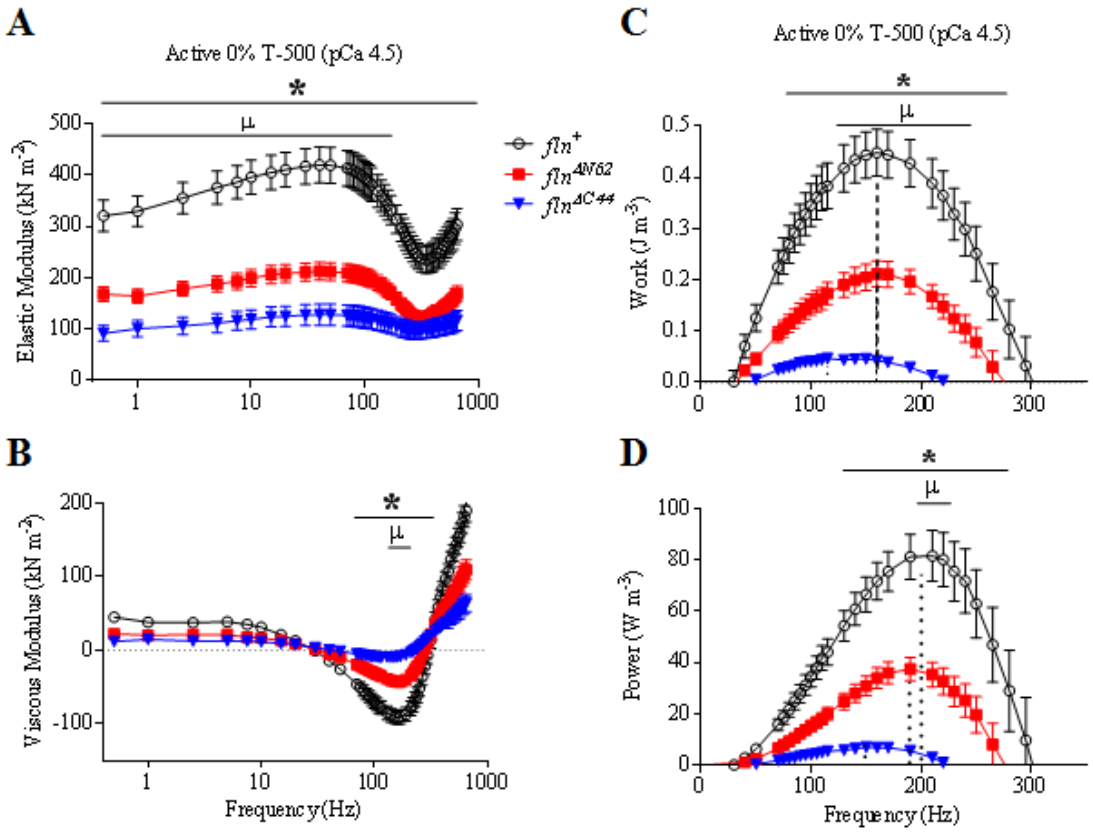

Figure 3. *fln*$^{\Delta N62}$ and *fln*$^{\Delta C44}$ IFM fibers have reduced work power output at 0% T-500 Dextran active solutions. *fln*$^{\Delta C44}$ IFM fibers generate maximum work and maximum power at much lower frequency than control *fln*$^{+}$ or *fln*$^{\Delta N62}$. Elastic modulus (A), viscous modulus (B), work (C), and power (D) for active IFM fibers from *fln*$^{+}$ (open circles) and *fln*$^{\Delta N62}$ (filled red squares), and *fln*$^{\Delta C44}$ (filled blue triangles) strains. Lines below asterisks denote frequency ranges where measured values are significantly different between *fln*$^{+}$ and *fln*$^{\Delta N62}$ or *fln*$^{\Delta C44}$ ($p<0.05$). Lines below "μ" denote frequency ranges where measured values are significantly different between *fln*$^{\Delta N62}$ and *fln*$^{\Delta C44}$ ($p<0.05$). Vertical dashed lines in C and D represent corresponding frequency of maximum oscillatory work and

power output. The frequencies of maximum oscillatory work and power are significantly different between $fln^{\Delta C44}$ and fln^{+} or $fln^{\Delta N62}$.

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