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Flight muscle properties and aerodynamic performance of *Drosophila* expressing a *flightin* transgene

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Summary

Flightin is a multiply phosphorylated, myosin-binding protein found specifically in indirect flight muscles (IFM) of *Drosophila.* A null mutation in the *flightin* gene (fln^0) compromises thick filament assembly and muscle integrity resulting in muscle degeneration and lost of flight ability. Using P-element-mediated transformation with the full-length *flightin* gene driven by the *Actin88F* promoter, we have achieved rescue of all fln^0-related ultrastructural and functional defects of the IFM. Transgenic $P\{fln^+\}fln^0$ 'rescued' flies have fewer thick filaments per myofbril than wild-type flies (782±13 *vs* 945±9) but have otherwise normal IFM. Transgenic $P\{fln^+\}fln^+$ 'tetraploid' flies have a normal number of thick filaments. The flightin protein levels in both transgenic strains are similar to wild type. By contrast, flightin levels are reduced in a myosin heavy chain tetraploid strain that produces excess myosin and excess thick filaments. These results suggest that regulation of flightin protein level is independent of gene copy number and that the number of thick filaments assembled per myofibril is influenced independently by myosin and flightin expression. We measured mechanical properties of IFM skinned fibers by sinusoidal analysis and found no significant differences in active viscoelastic properties of *flightin*-rescued and tetraploid transgenic flies *vs* wild type. The ability of the fln^+ transgene to overcome deficits in dynamic stiffness and power output in fln^0 suggest that the flightin protein contributes directly to fiber stiffness and stretch activation. However, flight parameters at maximum locomotor capacity, measured in a virtual reality flight simulator, are slightly compromised for both transgenic strains. $P\{fln^+\}fln^0$ and $P\{fln^+\}fln^+$ flies generated enough flight force to sustain hovering flight but showed reduced capability to produce forces in excess of hovering flight force. Both strains showed reductions in stroke frequency but only $P\{fln^+\}fln^+$ showed reductions in stroke amplitude. Muscle and aerodynamic efficiency are similar among the two transgenic strains and wild type. These results illustrate the importance of flightin in flight muscle development and function.

Key words: insect flight muscle, flightin, thick filaments, stretch activation.

Introduction

The myofibril is a multiprotein structure designed to produce and transmit contractile forces through the interaction of myosin-containing thick filaments and actin-containing thin filaments. In insect indirect flight muscles (IFM), these filaments are organized in a double hexagonal lattice and, as in other striated muscles, are stabilized laterally by structures at the M-line and Z-band. In particular, thick filaments are anchored at the center of the sarcomere through their association with unknown M-line proteins, and connected to the Z-band through projectin and kettin. Neither the composition of IFM thick filaments nor the nature of their assembly has been fully elucidated. In addition to myosin heavy chain (MHC) and its two associated (regulatory and essential) light chains, paramyosin/mini-paramyosin and flightin have been shown to be essential for normal thick filament structure and function (Arredondo et al., 2001; Liu et al., 2003; Reedy et al., 2000).

Electron microscopy studies have provided insight into the precise and ordered manner by which the myofilament lattice of *Drosophila* IFM is assembled throughout development (Reedy and Beall, 1993; Vigoreaux and Swank, 2004). Genetic approaches have been instrumental in elucidating the role of myofibrillar proteins on sarcomere assembly and muscle structure stability (for reviews see: Bernstein et al., 1993; Cripps, 2004; Vigoreaux, 2001). In particular, *Mhc* gene mutants have provided insight into the role of MHC protein domains in flight muscle development and function (for reviews see: Miller and Bernstein, 2004; Swank et al., 2000). Analysis of *flightin* gene mutants also have shown that flightin plays an essential role in thick filament formation and

flight muscle function (Reedy et al., 2000; Vigoreaux et al., 1998).

Drosophila flightin is a novel 20 kDa IFM-specific protein, that undergoes extensive phosphorylation during the late pupal stages of development and throughout the initial hours of adulthood, preceding the acquisition of flight (Vigoreaux and Perry, 1994; Vigoreaux et al., 1993). It has been shown that flightin is a component of the thick filament that, *in vitro*, binds the myosin rod (Ayer and Vigoreaux, 2003). A single amino acid substitution in the myosin rod (Glu 1554 to Lys, the Mhc^{13} allele) prevents the accumulation of flightin *in vivo* and its binding to MHC *in vitro* (Ayer and Vigoreaux, 2003; Kronert et al., 1995). Mhc^{13} flies are flightless and their IFM undergoes a time-dependent hypercontraction that is characterized by myosin proteolysis, thick filament instability and sarcomere degeneration (Kronert et al., 1995). A more recent study showed that a null mutation in the *flightin* (*fln*) gene, fln^0, leads to a remarkably similar phenotype as Mhc^{13} suggesting that the absence of flightin severely compromises IFM structure and function (Reedy et al., 2000). In addition, sarcomeres and thick filaments are longer than normal in IFM suggesting that flightin plays a key role in thick filament length determination. Mechanical analysis of skinned fibers from newly eclosed fln^0 and Mhc^{13} flies showed similar deficits in passive and dynamic stiffness, and a loss of the stretch activation response that resulted in no net positive work output (Henkin et al., 2004). Together with studies that showed flightin is distributed throughout the A-band of the sarcomere, these results suggest that flightin influences the viscoelastic properties of the thick filaments. To test the hypothesis that the ultrastructural and functional defects in fln^0 are attributed to the absence of flightin, we conducted the present study using genetic transformation of wild-type (fln^+/fln^+) and *flightin* null (fln^0/fln^0) *Drosophila* using a chimeric *Actin88F*-promoter–*fln* gene construct. We show that the transgene successfully rescued the ultrastructural and contractile defects engendered by fln^0 but the transgenic flies do not recover full flight competency. We also found that increasing the number of *flightin* genes to four has no adverse effect on IFM properties.

Materials and methods

Fly stocks

Drosophila melanogaster (Meigen) w^{1118}, an otherwise wild-type strain except for a *w* mutation, was obtained from the Bloomington Stock Center and used as host for generation of transgenic lines. Generation of the *flightin* null strain fln^0 has been described previously (Reedy et al., 2000). *w**; $T(2;3)ap^{Xa}$, ap^{Xa}/CyO; $TM3, Sb^1$ was used for linkage group analysis. Myosin over-expression strain *w;* $P\{w^+Mhc^+\}wm2$ was obtained from Sanford Bernstein.

Construction of transformation vector

To construct a transformation clone containing the *flightin* gene, we started with pW8-Act88F-3′Tm2, a *P-element* transformation vector derived from pW8/Tm2-35 (Miller et al., 1993) and obtained from Terese Tansey. This vector contains the *Actin88F* promoter region (extending from the *Xba*I site at –1420 to the G just 5′ of the initiation codon (Geyer and Fyrberg, 1986; Klemenz et al., 1987; Rubin and Spradling, 1983; Sanchez et al., 1983), part of the multiple cloning site from pW8, and *Tropomyosin* (*Tm2*) sequence from the 3′ untranslated region. Using *Kpn*I and *Pst*I restriction enzymes, the 3′-end of the *Tropomyosin* gene was excised from the vector and replaced with a 1.14 kb *Kpn*I–*Pst*I *flightin* genomic fragment obtained from a λ phage genomic library. This fragment extends from the *flightin* start codon to 0.55 kb past the translation stop codon. The λ phage library clone does not contain either the first untranslated exon or intron 1. This *Actin88F–fln* chimeric gene was excised from pW8 using *Eco*RI and *Pst*I restriction enzymes and subcloned into the *P-element* mediated transformation vector pCaSpeR (Flybase #FBmc0000168).

Generation of transgenic lines

Transformation was performed as described elsewhere (Spradling and Rubin, 1982). The helper plasmid used was pUChsΔ2–3 (Flybase #0000938), which was obtained from Margarita Cervera. The pCasPeR plasmid vector and helper plasmid were amplified in XL1 Blue *E. coli* cells (Novagen, Madison, WI, USA) in LB broth and purified using Qiagen maxiprep kit (Qiagen, Valencia, CA, USA). The concentration of DNA injected was 0.39 mg ml^{-1} of pCasPeR and 0.171 mg ml^{-1} of Δ2–3. Transformants were identified by yellow or orange eye color in the G1 generation. Homozygous strains were produced from crosses of individuals with darker eye color that resulted in no *white* eye progeny. Each transgene was mapped to its resident chromosome using *w**; $T(2;3)ap^{Xa}$, ap^{Xa}/CyO; $TM3, Sb^1$ by standard crossing techniques. The transgene was crossed into the fln^0 strain using standard crossing techniques.

Gel electrophoresis and western blot analysis

Denaturing one-dimensional (1DE) gel electrophoresis was performed using the discontinuous buffer system (Laemmli, 1970) as described previously (Vigoreaux et al., 1991). Two-dimensional (2DE) gel electrophoresis was performed using the Protean IEF cell (BioRad Inc., Hercules, CA, USA). IEF strips (pH 4–7 gradient) were used for the first dimension and precast 12.5% gels were used for the second dimension. Separation in the first dimension was carried out using a three step protocol. The IEF strips were rehydrated for 12 h at 20°C. Step two involved a 2 h rapid volt ramp to 3500 V h^{-1} and step three focused the strips for 14 h or 50,000 volt hours. To prepare samples for electrophoresis, flies were placed in acetone for 1 h at room temperature followed by lyophilization in a speed vac. The thorax was dissected away from other body parts and homogenized in IEF sample buffer and spun down to remove the cuticle debris.

Western blots were performed as described in Vigoreaux et al. (1993) with an anti-flightin polyclonal antibody described

in Reedy et al. (2000). For developmental blots, pupae were staged according to Bainbridge and Bownes (1981) and homogenized in Laemmli sample buffer with 8 mol l^{-1} urea and protease inhibitors (Vigoreaux et al., 1991). Samples were run on a 12% SDS gel, blotted onto membrane and processed for antibody detection as described (Vigoreaux et al., 1993).

Protein expression assays

To determine relative expression levels, whole thoraces were homogenized as described above. Protein concentration was determined using the BioRad DC protein quantification kit and equal amounts of protein were loaded in individual lanes of a 12% SDS gel. After electrophoresis, proteins were transferred to Bio-Rad PVDF membrane and the membranes were blocked with Aqua Block (East Coast Biologics, Inc., North Berwick, ME, USA). The primary antibody was an anti-flightin polyclonal (Reedy et al., 2000) and Alexaflor 698 fluorescent antibodies (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. After staining and washing, the membranes were scanned on an Odyssey fluorescent scanner (LI-COR Biosciences, Lincoln, NE, USA) and analyzed with Phoretix 1D software (Nonlinear Dynamics, Durham, NC, USA) as follows. Each image was first converted from color to grayscale in Photoshop and opened as a new experiment. After automatic selection of lanes, the bands were manually selected and their borders adjusted based on the peak profile in the analysis window. Protein quantity was obtained from band volume after background subtraction.

Transmission electron microscopy

Fly thoraces were bisected and the separated halves were fixed for 2 h in 2.5% glutaraldehyde and 0.1% paraformaldehyde. After fixation samples were stored in 0.1 mol l^{-1} Millonigs phosphate buffer, pH 7.2. Samples were dehydrated through a series of ethanol from 35% through absolute for 10 min in each concentration. The final dehydration step was in propylene oxide 3× for 5 min each. Infiltration was performed with propylene oxide and Spurr's resin 3:1 for 30 min, 1:1 for 30 min, 1:3 for 45 min and 100% Spurr's resin for 45 min. Embedding was done in 100% Spurr's resin and polymerized for 24 h at 70°C. Semi-thin sections (1 μm) were cut with glass knives on a Reichert ultracut microtome, stained with methylene blue (azure II), and evaluated for areas of interest. Ultrathin sections (60–80 nm) were cut with a diamond knife, retrieved onto 150 mesh copper grids, contrasted with uranyl acetate (2% in 50% ethanol) and lead citrate, and examined with a JEOL 1210 TEM (JEOL USA, Inc., Peabody, MA, USA) operating at 60 kV.

Polarized light microscopy

Examination of IFM fiber morphology was done essentially as described previously (Nongthomba and Ramachandra, 1999). Whole flies were dehydrated through a series of 50, 70, 80, 90 and 100% ethanol for 1 h in each solution at room temperature. The flies were then placed in methyl salicylate for one hour at room temperature, fixed on slides using permount and viewed under polarized light. Pictures were taken with a digital camera and Magnafire imaging software (Oreko, Dulles, VA, USA).

Flight test and wing-beat-frequency analysis

Flight test analysis and wing-beat-frequency analysis were performed as described previously (Vigoreaux et al., 1998).

Sinusoidal analysis of skinned flight muscle fibers

The sinusoidal procedure was performed as described previously (Dickinson et al., 1997) using IFM fibers from the dorsolongitudinal muscle (DLM), with the exception that after being stretched to just taught, each fiber was stretched by 2% increments until the oscillatory work (B component) was maximized, defined as a <3% increase in B. The solutions used are described in Henkin et al. (2004).

In vivo *estimates of kinematic and muscle performance*

To evaluate muscle mechanical power output in an intact fly in flight, we used an improved version of a method described elsewhere (Lehmann and Dickinson, 1998; Lehmann and Dickinson, 1997). Here we present only a brief description of the experimental procedure and focus mainly on the differences from the previous studies. Female fruit flies were tethered and flown in a virtual reality flight arena in which stroke amplitude, stroke frequency, total force production and carbon dioxide release were measured simultaneously. The flies actively modulated the azimuth velocity of a vertical dark stripe displayed in the arena using the relative difference in stroke amplitude between the two beating wings (closed-loop feedback conditions). While flying in closed-loop, the animals typically modulate kinematic and respirometric parameters in response to the motion of an open-loop stripe grating (horizontal stripes) that were oscillated vertically around the fly with a sinusoidal velocity profile. We have shown previously that under those conditions, fruit flies may maximize their locomotor output allowing the evaluation of maximum locomotor capacity (Lehmann and Dickinson, 1997). We employed flow-through respirometry with a flow rate of 1000 ml min^{-1} and used a Li-cor 7000 gas analyzer to measure the rate of carbon dioxide release during flight. Compared with previous studies on flight energetics in *Drosophila*, the higher flow rate yielded better temporal resolution of the metabolic measures permitting a tighter correlation between flight force and carbon dioxide production measures. We estimated the temporal shift of the CO_2 signal, due to the delay of the connecting gas tubings and the wash-out characteristics of the respirometric chamber, by performing cross-correlation between the force and CO_2 signal. Since a previous study on flight energetics in *Drosophila* has shown a transient effect on flight parameters following take-off, we excluded the initial 5 s and the last 2 s of flight time within each flight sequence (Lehmann and Dickinson, 1997). The average flight time of the three tested fly lines (wild-type Canton S, *P{fln*$^{+}$*}fln*^{0}*, P{fln*$^{+}$*}fln*$^{+}$) was 1747±680, 1271±93 and

Fig. 1. The Act88F-fln transgene used in this study. The *Actin88F* promoter is pale grey, *flightin* exons are the dark grey boxes, and introns and the 3′UTR are represented as empty boxes. Bar, 100 bp.

1247±83 s (mean ± S.D., *N*=11, 15, 11), respectively. The ambient temperature was similar in all experiments, approximately 23.9±1.0°C (*t*-test, *P*>0.05).

To derive muscle efficiency in the flying animal, we estimated the power requirements for flapping flight according to a set of equations and parameters published previously (Lehmann and Dickinson, 1998; Lehmann and Dickinson, 1997). Assuming 100% elastic energy storage within an entire flapping cycle, muscle efficiency is given by the ratio between the sum of induced power and profile power requirements for flight, and metabolic power due to ATP conversion. It is difficult to derive exact values for profile power because this measure critically depends on the drag coefficient of the flapping wings that varies with wing kinematics. Previous studies derived drag coefficient from Reynolds number assuming that a decrease in wing flapping velocity is correlated with an increase in drag coefficient (Lehmann and Dickinson, 1997). By contrast, here we estimated drag coefficient from lift coefficient values employed by the tethered animal during flight. The latter coefficient can be calculated from wing velocity and force measurements using conventional aerodynamic theory (Ellington, 1984; Lehmann and Dickinson, 1998). Subsequently, the drag coefficient in the flying animal was derived from lift (C_L) and drag coefficient (C_D) polars measured in a dynamically scaled 3D *Drosophila* robotic wing using the following equations:

$$C_L = 0.225 + 1.58 \sin (2.13\alpha - 7.29) \quad (1)$$

$$C_D = 1.92 + 1.55 \cos (2.04\alpha - 9.82) \quad (2)$$

in which α is the morphological angle of attack of the flapping wing with respect to the oncoming flow (Dickinson et al., 1999).

Results

The wild-type flightin *transgene restores flight and structural IFM defects engendered by* fln^0

We constructed a *P-element* transformation vector that consisted of the *Actin88F* (*Act88F*) promoter and the coding region of the *flightin* gene (Fig. 1). The *Act88F* fragment extends from –1420 to +628 and had been previously shown to drive transgenic expression of other contractile protein genes in IFM (Cripps et al., 1999). The *flightin* fragment extends from +1 to +1136 and includes 455 base pairs of 3′-noncoding DNA (Fig. 1).

We generated 11 independent transgenic lines, two on the *X* chromosome, two on the second chromosome, five on the third chromosome, and an additional two that have not been mapped. Three of these strains were crossed to *fln^0* and tested for flight. We focused on the strain with the best flight score, a transgenic line with an *X* chromosome insertion, *P{Act88F-fln$^+$}; fln^{0}e-2* (hereby referred to as *P{fln$^+$}0.2*). Table 1 summarizes the results of flight test of normal and *P{fln$^+$}0.2* transgenic flies. Note the significant improvement in flight performance of the rescued strain *vs fln^0*. However, flight ability is not fully restored.

A characteristic feature of *fln^0* IFM is that fibers hypercontract, resulting in detachment of one or both ends of the fiber from the cuticle and 'bunching' of the muscle mass (Reedy et al., 2000). We inspected IFM fibers of the *P{fln$^+$}0.2* rescued lines by polarized light microscopy and determined their morphology to be normal (not shown). A more-detailed analysis of sarcomere structure was conducted by electron microscopy. On longitudinal sections sarcomeres appear normal, with well-defined Z-bands and clearly depicted A-bands and I-bands (Fig. 2). Sarcomere length is uniform and similar to sarcomere length in wild-type flies (Table 1 and Fig. 2). On cross sections, myofibrils from *P{fln$^+$}0.2* rescued flies are circular with well-defined diameter, and show the normal double hexagonal array of interdigitated thick filaments and thin filaments (Fig. 2). However, there is a decreased number of thick filaments per myofibril (Table 1). Wild-type myofibrils had an average of 945 thick filaments per myofibril, while *P{fln$^+$}0.2* had significantly fewer with 782, a decrease of about 17%. The number of myofibrils per area was no different in the transgenic *vs* the control (not shown).

Table 1. *Flight and muscle properties of normal, mutant and transgenic flies*

Genotype	Flight index (0–6)	Thick filaments per sarcomere	Sarcomere length (μm)†
+/+	5.4±0.1 (*N*=30)	945±9 (*N*=48)	3.2±0.03 (*N*=87)
P{fln$^+$}0.2	3.2±0.2* (*N*=30)	782±13* (*N*=67)	3.4±0.03 (*N*=87)
P{fln$^+$}2.2	5.6±0.1 (*N*=30)	943±16 (*N*=10)	3.5±0.03 (*N*=16)
fln^0	0 (*N*=30)	ND	2.3±0.09* (*N*=64)

*Denotes a significant difference (*P*<0.05) from wild type; †all measurements from 2–5-day-old adults. All values are means ± S.E.M.

While the majority of the myofibrils appear normal, we did observe occasional myofibril defects not commonly seen in IFM of wild-type flies (Fig. 2I and 2J). Broken down sarcomeres with partially torn Z-bands and missing M-lines are seen, as well as myofibrils with fractures that suggest a faultily assembled lattice.

The reduced number of thick filaments and the reduced flight ability could result from incorrect timing of gene expression, incorrect timing of phosphorylation, and/or insufficient *flightin* expression. We tested all three of these possibilities by conducting western blot analysis of flightin accumulation and phosphorylation throughout pupal development and in adults. We first looked at the developmental expression of the *flightin* transgene. In wild-type flies, flightin begins to accumulate at pupal stage P8 (~60 h after pupation and ~22 h after initial myofibrils appear at 25°C; Vigoreaux and Swank, 2004) and continues to accumulate at increasing levels into adulthood (Vigoreaux et al., 1993). An almost identical profile is seen in *P{fln⁺}0.2* transgenic flies except that expression starts at the P7 stage, or 2–8 h before expression of the endogenous protein normally begins (Fig. 3). This earlier onset of expression is not unexpected given that the *Act88F* promoter is activated during myoblast fusion, at ~16 h after puparium formation (Fernandes et al., 1991).

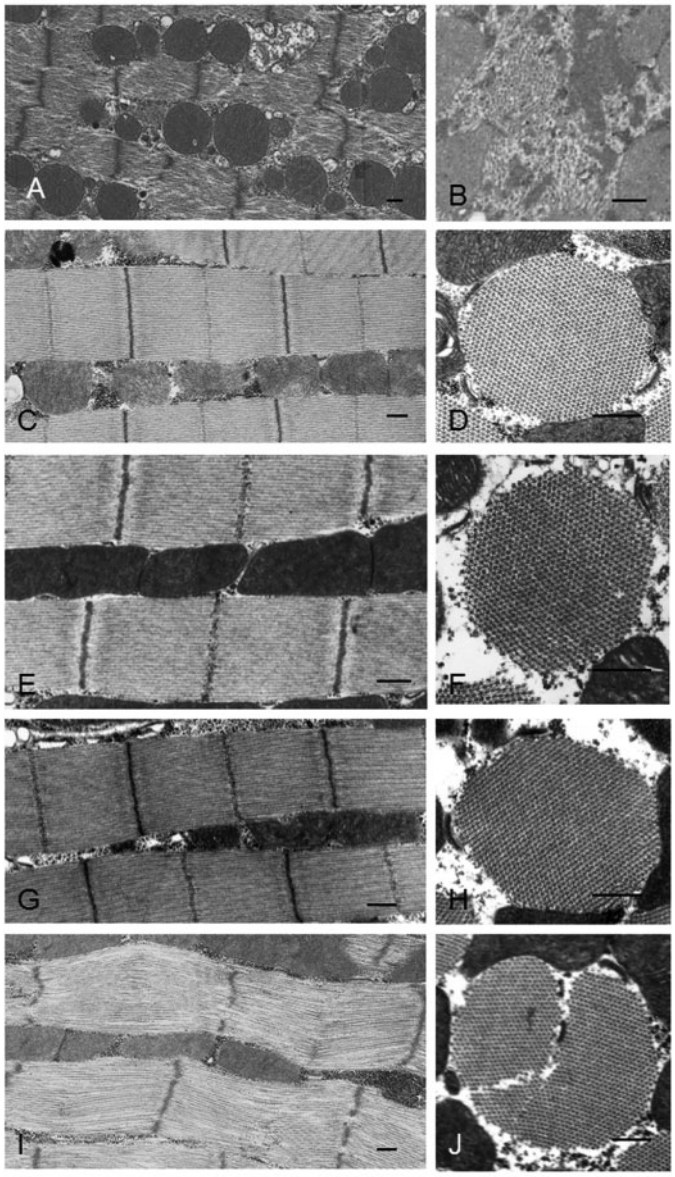


Fig. 2. Electron microscopy of flight muscle from *fln⁰*, wild-type and transgenic flies. All pictures are from 2–5-day-old adult IFM. (A,B), *fln⁰*; (C,D), wild-type; (E,F), *P{fln⁺}0.2*; (G,H), *P{fln⁺}2.2*; (I and J), *P{fln⁺}0.2*. A, C, E, G, I are longitudinal sections; B, D, F, H, and J are cross sections. Note that the highly disrupted sarcomere structure in *fln⁰* (in A,B) is no longer evident in the rescued line. However, there are fewer thick filaments per myofibril cross-sectional area. G and H, flies expressing four copies of *fln⁺* have normal IFM. I and J, are examples of occasional defects seen in *P{fln⁺}0.2* flies. Bar, 0.5 µm.

Phosphorylation of flightin begins during late stages of pupal development, culminating in nine phosphovariants in mature adults (Vigoreaux and Perry, 1994). We conducted 2DE analysis to determine if the premature expression of *flightin* in *P{fln⁺}0.2* results in premature phosphorylation, as is seen in some IFM mutants (Vigoreaux, 1994). Fig. 4 shows the pattern of phosphovariant accumulation in *P{fln⁺}0.2* is undistinguishable from that in wild-type flies from stage P15 through adult.

Next we determined if levels of *flightin* expression are different in transgenic and wild-type strains. The relative abundance of flightin was estimated by western blot analysis after normalization to total thoracic protein (see Materials and methods). The rescued line showed a small reduction in flightin levels (Fig. 5).

Normal fiber mechanics in wild-type flightin *transgenic lines*

The increased compliance of IFM fibers devoid of flightin results in loss of power output, most likely due to internal absorption of a large amount of the actomyosin generated work (Table 2) (Henkin et al., 2004). There is no statistically significant difference in the dynamic stiffness of fibers from *P{fln⁺}0.2* rescued flies and those of wild-type flies. The

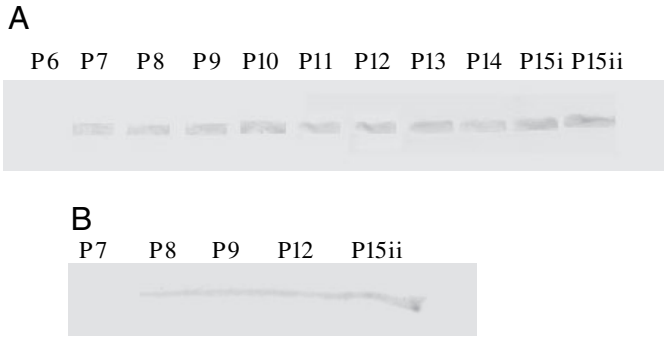


Fig. 3. Western blot of flightin levels throughout pupal stages of development. (A) *P{fln⁺}0.2* (B) wild type. Pupa were staged by visual inspection using standard criteria (Bainbridge and Bownes, 1981). Accumulation of flightin in wild type begins at stage P8 (Vigoreaux et al., 1993), approx 2–8 h after accumulation in *P{fln⁺}0.2*-rescued flies.

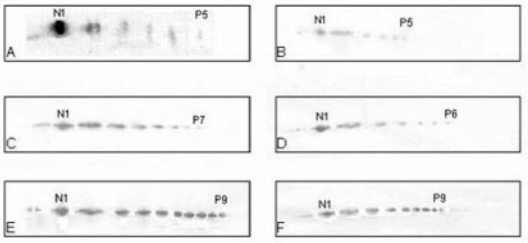


Fig. 4. Western blot analysis of 2DE reveal normal temporal phosphorylation of flightin in transgenic line. (A,C,E) wild type; (B,D,F) *P{fln⁺}0.2*. A and B, P15 pupa; C and D, <1 h post-eclosion; E and F, 2–4-day-old adult. For all blots, the basic end is to the left. 2D gel analysis was followed by immunoblot analysis using anti-flightin polyclonal antibody. N1 and N2 are the non-phosphorylated isoelectic variants while P1 through P9 are phosphorylated variants (see Vigoreaux and Perry, 1994). For each of the developmental stages, *P{fln⁺}0.2* transgenic flies have a nearly identical phosphorylation pattern when compared to the wild type.

complex modulus (an index of dynamic stiffness) is composed of two components, the elastic modulus (E_e), and the viscous modulus (E_v). E_e is a measure of fiber compliance and in *P{fln⁺}0.2*-rescued flies E_e is statistically the same as wild type at the frequency at which maximum power generation occurs (f_{max}) (Table 2). Fig. 6A is a plot of E_e *vs* frequency at maximal Ca^{2+} activation (pCa 5). Note that *P{fln⁺}0.2* produces a normal triphasic response, indicative of restoration of wild-type function. E_v is a measure of the work produced (negative values) and absorbed (positive values) by the fiber. Fig. 6B demonstrates that E_v values for *P{fln⁺}0.2* are nearly identical

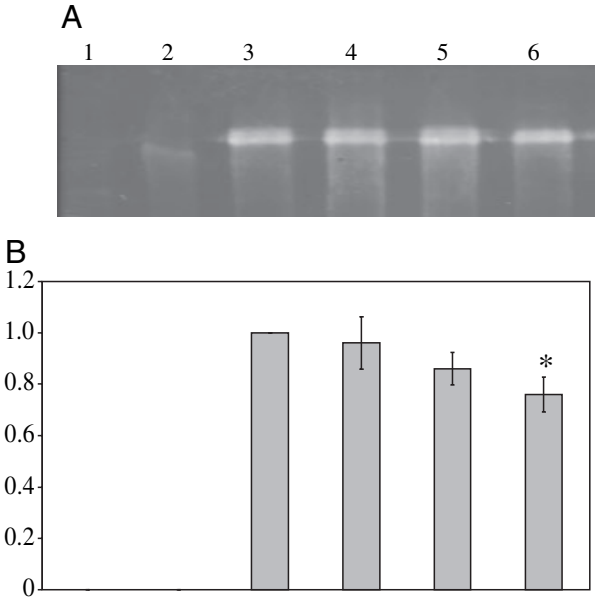


Fig. 5. Quantification of flightin in normal and transgenic IFM. (A) Western blot of 12% SDS-PAGE probed with an anti-flightin specific antibody. Lane 1, fln^0; lane 2, molecular weight marker; lane 3, wild type; lane 4, *P{fln⁺}2.2*; lane 5, *P{fln⁺}0.2*; lane 6, *w; P{w⁺Mhc⁺}wm2*. (B) Relative intensity of the flightin bands in A. (*P<0.05 *vs* wt, N=3). Each lane was loaded with 0.15 μg of protein.

to wild type and the value at the frequency at which maximum power occurs is not statistically different from wild type (Table 2). Power production by *P{fln⁺}0.2* fibers is lower than power production by wild-type fibers but the differences are not statistically significant (Fig. 6C and Table 2).

Increased flightin *gene copy number does not have an effect on muscle structure or contractile properties*

We studied one line of wild-type flies that had been transformed with *P{fln⁺}* (w^{1118}, *P{w⁺, Act88F-fln⁺};fln⁺*, abbreviated *P{fln⁺}2.2*) to determine if increasing gene copy number has an effect on flightin protein levels and IFM properties. Flies that carry four copies of the *flightin* gene have normal flight ability (Table 1). The myofibrillar structure is also normal (Fig. 2) with regular sarcomere length and a normal number of thick filaments per myofibril (Table 1). Despite the doubling in *flightin* gene copy number, *P{fln⁺}2.2* flies do not exhibit an increase in flightin protein accumulation relative to wild-type flies, but a significant increase relative to *P{fln⁺}0.2* rescued flies (Fig. 5). In all respects, *P{fln⁺}2.2* are more similar to wild-type flies than are *P{fln⁺}0.2* flies.

Unlike flightin protein levels, expression of MHC protein increases with doubling of gene copy number. Thus, flies carrying four copies of the *Mhc⁺* gene express 2–4 times more myosin than normal diploid flies and also have an over-abundance of thick filaments (Cripps et al., 1994). We determined the expression levels of flightin in the *Drosophila* line *w; P{w⁺Mhc⁺}wm2* and found that it was not significantly different from *P{fln⁺}0.2* but significantly less than wild type. Sinusoidal analysis of skinned *P{fln⁺}2.2* fibers revealed that their mechanical properties are on par with wild-type fibers (Table 2 and Fig. 6).

Muscle power output in vivo *decreases in* flightin *transgenic lines*

While flying in the virtual reality flight simulator, flight performance of the *Drosophila* transgenic rescued line *P{fln⁺}0.2* and the multi-gene copy line *P{fln⁺}2.2* is significantly reduced during maximum locomotor capacity compared with wild-type flies. Although all flies generate enough flight force to sustain hovering flight, Table 3 shows that the reduced capability of the transgenic lines to produce flight force in excess of hovering flight force appears to be due to a reduction in both stroke amplitude (*P{fln⁺}2.2*) and frequency (*P{fln⁺}0.2* and *P{fln⁺}2.2*), whereas muscle and aerodynamic efficiency appear to be widely similar in the three lines.

Aerodynamic flight force reduction amounts to 15% in *P{fln⁺}0.2* and 24% in *P{fln⁺}2.2* compared with wild type that is correlated with a significant reduction in both stroke amplitude of approximately 5 and 13 degrees, and stroke frequency of ~23 and ~9 Hz, respectively. As a consequence, the cost of generating lift (induced power) decreases significantly by ~31 and ~36% in the two transgenic lines compared with the control animals. Similar results were obtained for profile power, the cost to overcome the drag on

Table 2. *Mechanical parameters from IFM skinned fiber sinusoidal analysis*

Genotype	*N*	f_{max} (Hz)	Power output (W m^{-3})	E_v (kN m^{-3})	E_e (kN m^{-3})	Dynamic stiffness (kN m^{-3})
+/+	10	131±6	46±7	–95±15	441±40	453±40
P{fln$^+$}0.2	8	113±8	39±7	–88±15	419±61	428±62
P{fln$^+$}2.2	4	130±15	34±5	–73±74	362±42	374±38
fln^0 †	14	77±38	–4±3	–3±5	56±14	57±15

Values are means ± S.E.M. at the frequency at which maximum power production occurred (f_{max}) at maximum Ca^{2+} activation (pCa 5). *N*, number of fibers tested. E_v, viscous modulus, E_e, elastic modulus. All fibers from 2–5-day-old flies except *fln^0*, †from Henkin et al. (2004). Oscillatory work is proportional to $-E_v$.

Table 3. *Morphometrics and flight parameters at maximum locomotor capacity of wild-type* Drosophila *Canton S and the two* flightin *lines* P{fln$^+$}0.2 *and* P{fln$^+$}2.2

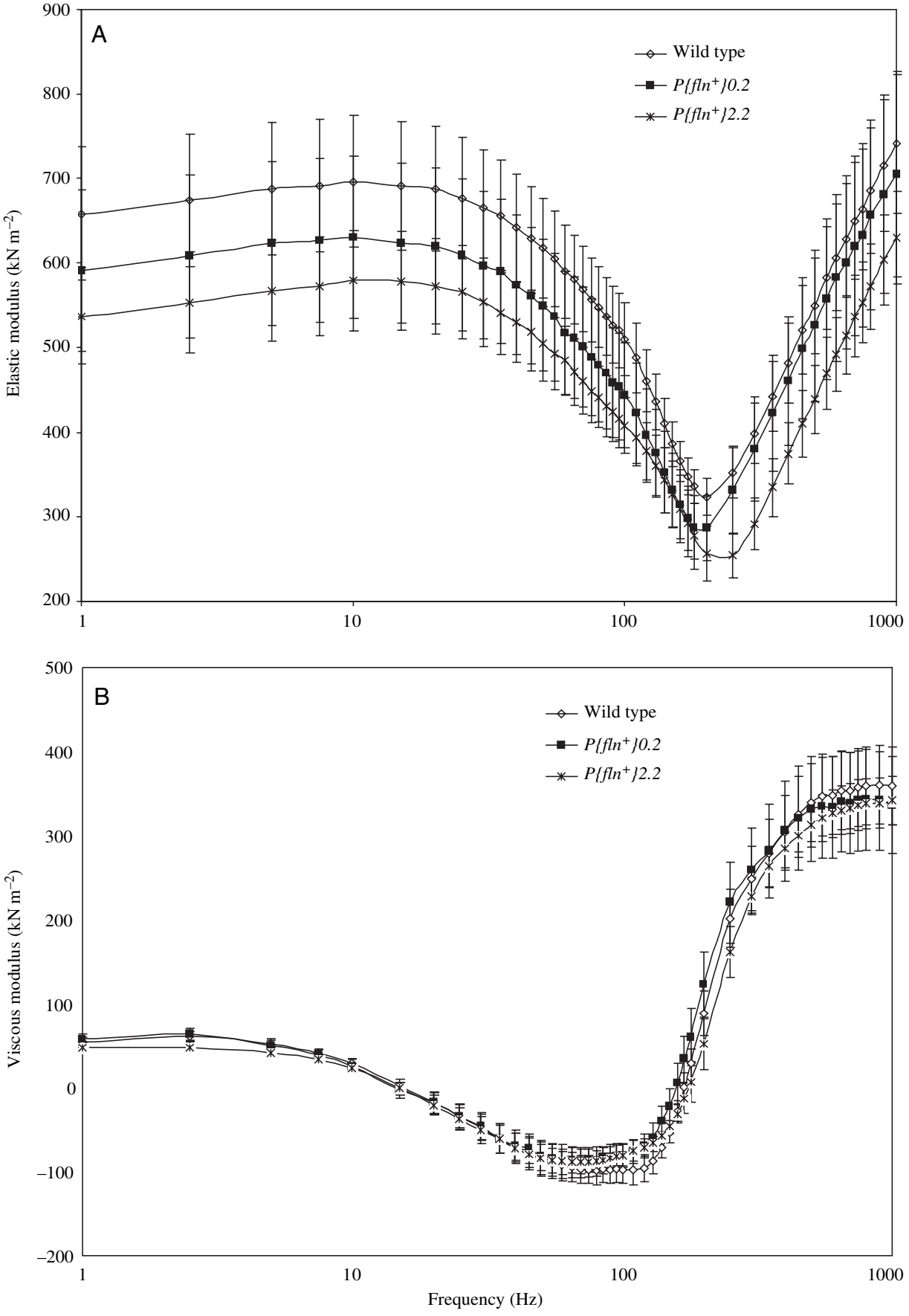
	(1) *wt* (*N*=10)	(2) P*[fln$^+$]*0.2 (*N*=11)	(3) P*[fln$^+$]*2.2 (*N*=15)	Statistics
Body mass (mg)	1.10±0.15	0.88±0.09	1.01±0.11	(1,2)‡, (1,3)NS, (2,3)†
Wing length (mm)	2.41±0.16	2.42±0.10	2.45±0.11	(1,2)NS, (1,3)NS, (2,3)NS
Wing area (mm^2)	2.00±0.13	1.87±0.10	1.94±0.11	(1,2)†, (1,3)NS, (2,3)NS
Stroke amplitude (deg.)	176±7.01	171±7.90	163±10.0	(1,2)NS, (1,3)‡, (2,3)*
Stroke frequency (Hz)	221±12.0	198±15.8	212±13.6	(1,2)‡, (1,3)*, (2,3)*
Maximum flight force (μN)	14.8±2.50	9.69±1.33	10.2±1.37	(1,2)‡, (1,3)‡, (2,3)NS
Normalized force	1.36±0.24	1.15±0.23	1.03±0.11	(1,2)*, (1,3)‡, (2,3)NS
P_{ind} (W kg^{-1})	33.8±8.06	23.3±5.98	21.5±3.17	(1,2)†, (1,3)‡, (2,3)NS
P_{pro} (W kg^{-1})	64.7±14.0	47.2±10.4	43.9±6.30	(1,2)†, (1,3)‡, (2,3)NS
P_{mech} (W kg^{-1})	98.5±22.0	70.4±16.1	65.3±8.91	(1,2)†, (1,3)‡, (2,3)NS
P_{MR} (W kg^{-1})	1030±241	759±134	742±88.4	(1,2)†, (1,3)‡, (2,3)NS
Muscle efficiency (%)	9.76±1.24	9.43±1.28	8.89±1.04	(1,2)NS, (1,3)*, (2,3)NS
Aerodynamic efficiency (%)	26.8±0.93	25.7±1.81	25.7±1.85	(1,2)NS, (1,3)*, (2,3)NS

The values were obtained while the fly was maximizing flight force production in a virtual reality flight arena in response to visual lift stimuli. The values represent the 1% maximum values measured in each flight sequence. Normalized flight force is the ratio between maximum flight force and the body mass of the animal. Muscle efficiency is the ratio between muscle mechanical power (P_{mech}) and metabolic power (P_{MR}) that we calculated from the carbon dioxide release during flight. Muscle efficiency was estimated assuming 100% elastic energy storage in the thoracic flight motor. Aerodynamic efficiency is the aerodynamic loss during force production and equal to the ratio between the minimum power requirements for flight (Rankine–Froude power) and the sum of induced (P_{ind}) and profile power requirements (P_{pro}). All power values are given in flight muscle mass specific units. We calculated the significances between the measures performing two-tailed *t*-test on the means. All values are given as means ± S.D. *P<0.05; †P<0.01; ‡P<0.001; NS, not significant. Body mass was estimated at the end of the experiment.

the moving wings (Table 3). Muscle mechanical power output in the behaving flies, given as the sum of induced and profile power, decreases in the transgenic lines by approximately 29% (*P{fln$^+$}0.2*) and 34% (*P{fln$^+$}2.2*) compared with wild type. A similar trend was observed at the single fiber level. Power output by *P{fln$^+$}0.2* and *P{fln$^+$}2.2* fibers was decreased by 15% and 26%, respectively, compared with wild type (Table 2). However, these differences were not statistically significant.

The reduction in muscle performance is consistent with a reduction in metabolic power, yielding constant values of muscle efficiency ranging from 8.89% in *P{fln$^+$}2.2* to 9.76% in wild type. The modification in *flightin* expression did not alter aerodynamic efficiency of force production between the lines that ranges from 25.7% to 26.8% indicating that the cost of flight force production due to wing flapping did not change among the three lines.

We did not find any significant differences in maximum flight force production, muscle mechanical power output, metabolic power and the two efficiency estimates between *P{fln$^+$}0.2* and *P{fln$^+$}2.2*. Interestingly, the two transgenic lines generated maximum flight force using different combinations of stroke amplitude and stroke frequency. It was shown previously that force production in *Drosophila* linearly depends on wing velocity, given by the product of amplitude and frequency (Lehmann and Dickinson, 1998). Although mean wing velocity at the center of wing area is similar in both transgenic *Drosophila* lines of approximately 1.97±0.1 (*P{fln$^+$}0.2*) and 2.01±0.2 m s^{-1} (*P{fln$^+$}2.2*), stroke amplitude was significantly higher in (*P{fln$^+$}0.2*) line compared with *P{fln$^+$}2.2* whereas stroke frequency was approximately 6% higher in *P{fln$^+$}2.2* compared with (*P{fln$^+$}0.2*). The latter is consistent with results at the fiber level where *fmax* is 13% higher in *P{fln$^+$}2.2* than in *P{fln$^+$}0.2*. The kinematic


A
Wild type
P{fln+}0.2
P{fln+}2.2
Elastic modulus (kN m−2)
900
800
700
600
500
400
300
200
1
10
100
1000
B
Wild type
P{fln+}0.2
P{fln+}2.2
Viscous modulus (kN m−2)
500
400
300
200
100
0
−100
−200
1
10
100
1000
Frequency (Hz)

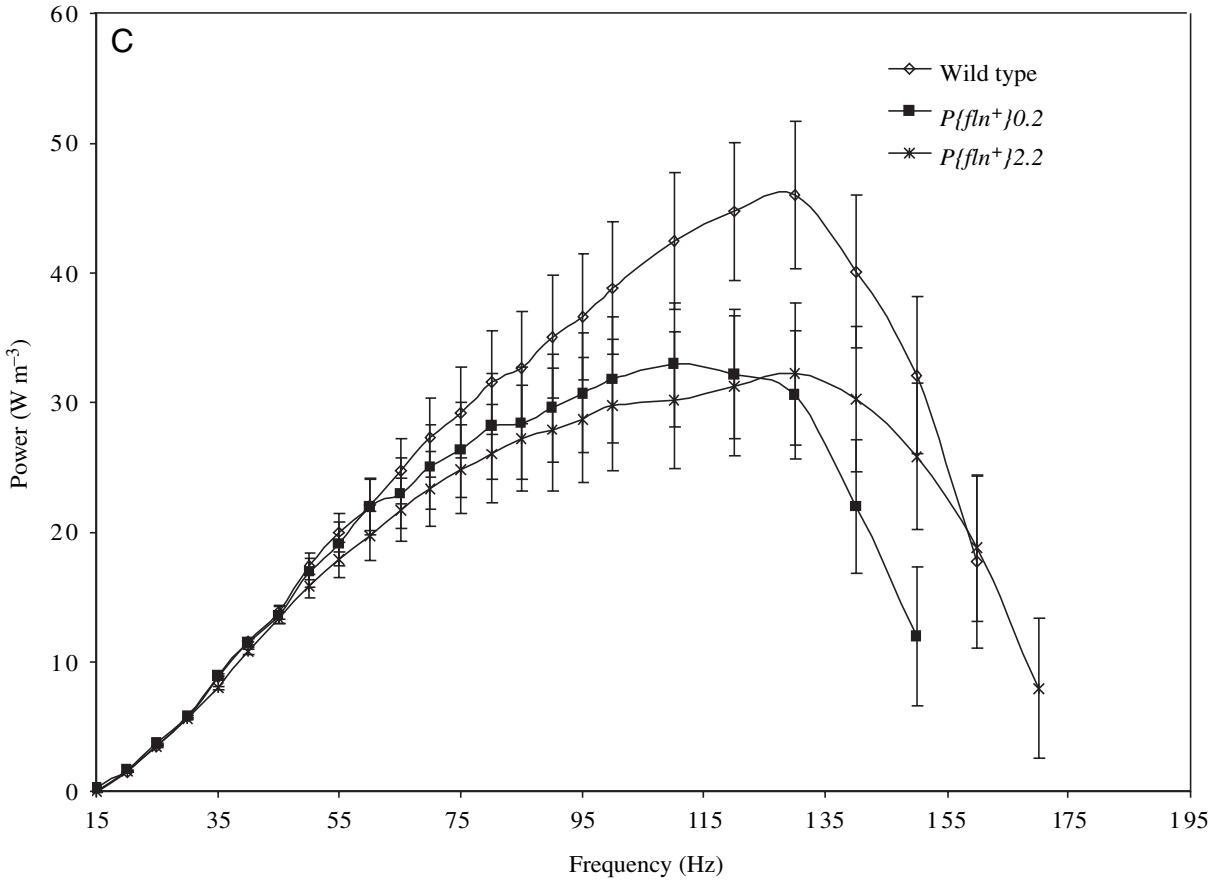


Fig. 6. Mechanical parameters of maximally Ca^{2+} (pCa 5.0) activated IFM fibers. (A) Elastic moduli as a function of frequency, (B) viscous moduli as a function of frequency, and (C) is power produced as a function of frequency. Values are mean ± S.E.M. for wild type, *P{fln⁺}0.2* and *P{fln⁺}2.2*. There is no significant difference among the three *Drosophila* lines for any of the parameters.

differences are accompanied by small but notably significant differences in body mass (body mass *P{fln⁺}0.2*=0.88 mg, *P{fln⁺}2.2*=1.01 mg) and a small but significant reduction in wing area between *P{fln⁺}0.2* (1.87 mm^2) and wild-type flies (2.00 mm^2; Table 3).

Discussion

The ability of the *fln⁺* transgene to restore the major ultrastructural and contractile defects engendered by *fln⁰* is further evidence that *flightin* is an essential protein for flight muscle development and function in *Drosophila*. The rescued strain *P{fln⁺}0.2* exhibits none of the sarcomeric defects that are characteristic of *fln⁰*. These include longer than normal sarcomeres in developing pupal IFM that become disrupted and shortened after eclosion (Reedy et al., 2000). More importantly, fiber hypercontraction is suppressed and *fln⁰* flies in the presence of the *fln⁺* transgene regain their flight ability. Unlike skinned fibers from *fln⁰* (Henkin et al., 2004), fibers from *P{fln⁺}0.2* are not functionally compromised by deficits in passive and dynamic viscoelastic properties. Altogether, these new results demonstrate that the absence of flightin alone accounts for the extreme loss of muscle function in *fln⁰* and provide further evidence that flightin is a chief contributor to myofilament stiffness and an important determinant of stretch activation in the IFM.

One remarkable feature of rescued *P{fln⁺}0.2* IFM is the decreased number of thick filaments per sarcomere. This decrease is not a result of *flightin* under-expression given that nearly normal levels of the protein are found in the mature IFM. Furthermore, a mutation that results in *flightin* under-expression shows a distinctly different phenotype (see below). A more plausible explanation is that the slightly premature expression of *flightin* during pupal development of *P{fln⁺}0.2* interferes with some aspect of thick filament assembly. The *Act88F* promoter, which drives expression of the *flightin* transgene, has been shown to be activated very early in IFM development (~16 h after puparium formation; Fernandes et al., 1991). While this study did not examine the timing of transcriptional activation of the *Act88F–fln* transgene, it is evident from western blot analysis (Fig. 3) that the transgene-encoded flightin protein begins to accumulate earlier than the endogenous gene-encoded flightin in wild-type flies (pupal stage P7 *vs* P8, respectively). The temporal program of flightin phosphorylation, however, seems to be unaffected by the untimely expression (Fig. 4).

In a previous study we had shown that *Drosophila* heterozygous for a deficiency that encompasses the *flightin* gene, *Df(3L)fln*^{1}, showed an ~20% reduction in flightin (Vigoreaux et al., 1998). This resulted in myofibrillar defects, evident as peripheral disassociation of the thick and thin filaments, as well as altered fiber kinetics and attenuated flight (Vigoreaux et al., 1998). Thus, while both *P{fln*$^{+}$*}0.2* and *Df(3L)fln*^{1} retain an intact myofibril core that is ~80% the diameter of the intact myofibril, *Df(3L)fln*^{1} exhibited loosely organized peripheral myofilaments while *P{fln*$^{+}$*}0.2* exhibited an ~17% reduction in the number of myofilaments. A second difference is that fiber power output is reduced by ~15% in *P{fln*$^{+}$*}0.2* but unchanged in *Df(3L)fln*^{1} that instead exhibited an increase in *fmax*. Altogether, these studies suggest that the premature accumulation of flightin in *P{fln*$^{+}$*}0.2* is the most likely explanation for the reduced number of thick filaments and that unphosphorylated flightin participates in the process by which the number of thick filaments is determined during sarcomerogenesis. One possibility is that 'premature' flightin binds monomeric myosin and prevents its incorporation into a growing polymer, perhaps by interfering with the electrostatic interactions between myosin rod coiled coils that are required for assembly (Atkinson and Stewart, 1991; McLachlan and Karn, 1982). Future studies will investigate this possibility.

There is one other example where timing of expression adversely affects IFM development. Transgenic flies that express an *Act88F* promoter-mini-paramyosin chimeric gene in their IFM show subtle developmental defects that are compounded in the adult working muscle (Arredondo et al., 2001). Unlike *flightin*, mini-paramyosin that is under *Act88F* promoter regulation is over-expressed (Arredondo et al., 2001).

The reduced myofibrillar diameter in *P{fln*$^{+}$*}0.2* does not appear to have deleterious effect on fiber mechanics and flight parameters. Dynamic stiffness and power output from skinned *P{fln*$^{+}$*}0.2* fibers were more similar to wild type than the corresponding values from *P{fln*$^{+}$*}2.2*, despite the fact that the latter had the normal number of thick filaments per sarcomere. Likewise, normalized force and mechanical power measured in the flight arena for *P{fln*$^{+}$*}0.2* are more similar to wild-type values than *P{fln*$^{+}$*}2.2* values are, as are muscle and aerodynamic efficiency. The differences between any of the above parameters for *P{fln*$^{+}$*}0.2* and *P{fln*$^{+}$*}2.2* are not statistically significant. However, the observation that all values follow a similar trend suggest that the presence of extra copies of the *flightin* gene, while restoring the quota of thick filaments, has a moderately unfavorable effect on flight muscle function.

Measurements in the flight arena also revealed large differences between wild type and the two transgenic strains, differences that were not evident on the mechanical analysis of skinned single IFM fibers. One interpretation, as already surmised, is that *P{fln*$^{+}$*}0.2* is not fully rescued while *P{fln*$^{+}$*}2.2* exhibits detrimental effects of tetraploidy. It is not uncommon for transgenic strains not to perform to the same level as wild-type strains. For example, the wild-type *Mhc* gene can rescue the flightlessness imposed by the amorphic *Mhc*^{10} allele but transgenic flies are not fully flighted (Cripps et al., 1994). Similarly, the wild-type *Tropomyosin* (*Tm2*) gene was able to rescue the flightless behavior and IFM contractile defects engendered by the *Tm2* deletion allele *TmI*^{C10}, but performance fell short of that of wild-type flies (Kreuz et al., 1996). Thus under-performance of transgenic animals appears to be a general feature in *Drosophila* perhaps as a result of the random genomic integration of the *P-element*-shuttled transgene.

A second interpretation is that genetic differences among the strains, more so than the ability of the transgene to rescue the mutant phenotype, accounts for the variability. Allele differences among genes that directly or indirectly affect flight behavior cannot be completely ruled out even among strains that were derived from a common parental strain because their generation required different outcrosses. For example, the *fln*^{0}-carrying chromosome is marked by an *ebony* allele, a mutation that exhibits a variety of locomotor rhythm anomalies although none that is known to affect flight. Yet a third interpretation is that the differences in the flight arena reflect the contributions of muscles other than the DLM, in particular those of the opposing set of IFM, the dorsoventral muscles (DVM). It is assumed that the DVM have the same contractile properties (and *flightin* expression) as the DLM but this has not been experimentally tested given the greater difficulty of isolating DVM fibers. One important difference is that the DLM develops from a scaffold of larval muscles while the DVM develops *de novo* by fusion of imaginal myoblasts (Fernandes et al., 1991; for review see: Vigoreaux and Swank, 2004). The different developmental pathways of DLM and DVM may impose distinct regulatory constraints on the expression of the *Act88F–fln* transgene resulting in greater functional differences among these two fiber types.

IFM has been shown to be very sensitive to expression levels for a variety of its constituent proteins (for review see Vigoreaux and Swank, 2004). As mentioned earlier, over-expression of mini-paramyosin resulted in flight defects (Arredondo et al., 2001). Myofibril assembly occurred normally, and myofibrils in young adults were relatively normal, but as flies aged, degeneration occurred so that by 10 days into adulthood, there was considerable myofibrillar degeneration that translated into severe flight impairment. Over-expression of a heat shock-*sanpodo* (*spdo*) transgene, the *Drosophila Tropomodulin* homolog, during mid-to-late pupal stages caused shorter than normal thin filaments in IFM and flight impairment (Mardahl-Dumesnil and Fowler, 2001).

Mhc tetraploidy (*P{w*$^{+}$*Mhc*$^{+}$*}wm2*) resulted in a twofold increase in myosin expression, excess and loosely associated thick filaments residing in the myofibrillar peripheries, and a severe flight defect (Cripps et al., 1994). Some of the peripheral thick filaments also appeared to have a smaller diameter than those in the center of the myofibril. Given the excess thick filaments, it was surprising to find that flightin levels were lower in *P{w*$^{+}$*Mhc*$^{+}$*}wm2* than in wild type. One possible explanation for this observation is that the excess myosin outcompetes flightin for myosin binding during polymerization and

unassembled flightin is rapidly degraded. This scenario is consistent with our proposal above that 'premature' flightin binds and 'hijacks' monomeric myosin, resulting in less thick filaments polymerized. A second possibility is that the absence of thin filaments and of a well formed lattice in the myofibril periphery creates an environment where flightin is unstable. The absence of thin filaments is known to affect accumulation of flightin phosphovariants (Vigoreaux, 1994). It is interesting to note that the amorphous myofibril periphery in *P{w*$^{+}$*Mhc*$^{+}$*}wm2* is not unlike that seen in *Df(3L)fln*^{1} heterozygotes.

In contrast to Mhc, but similar to *P{fln*$^{+}$*}2.2*, tetraploidy of *Act88F* does not result in loss of flight ability (Hiromi et al., 1986). Because the study relied only on a simple flight test, it is not possible to establish if excess actin genes affected IFM function in ways that are not evident in the flight test. A recent study showed that copy number polymorphism is rather common in 'normal' humans (Sebat et al., 2004). While some of the polymorphisms may be associated with susceptibility to health problems, others may effect no phenotype. Hence expression of particular proteins in humans, like in flies, is influenced by gene copy number while expression of other proteins is not.

In summary, our results show that relative levels of flightin accumulation in the IFM are not strictly dictated by gene copy number, as has been demonstrated for other myofibrillar proteins. Instead, regulation of flightin levels appears to be tightly dependant on the process of thick filament and myofibril assembly, perhaps dictated by the availability of myosin binding sites and/or the integrity of the myofibrillar lattice. The results bring a new dimension to our understanding of myofibril assembly as they underscore the need to understand the role of protein interactions in addition to gene regulatory mechanisms. Proper regulation of flightin levels is essential for normal myofibrillogenesis and flight muscle function. Transgenic studies such as the one described here will continue to be pursued to further define the functional roles of flightin in muscle development and contraction.

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