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Flightin maintains myofilament lattice organization required for optimal flight power and courtship song quality in *Drosophila*

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The indirect flight muscles (IFMs) of *Drosophila* and other insects with asynchronous flight muscles are characterized by a crystalline myofilament lattice structure. The high-order lattice regularity is considered an adaptation for enhanced power output, but supporting evidence for this claim is lacking. We show that IFMs from transgenic flies expressing flightin with a deletion of its poorly conserved N-terminal domain ($fln^{\Delta N62}$) have reduced inter-thick filament spacing and a less regular lattice. This resulted in a decrease in flight ability by 33% and in skinned fibre oscillatory power output by 57%, but had no effect on wingbeat frequency or frequency of maximum power output, suggesting that the underlying actomyosin kinetics is not affected and that the flight impairment arises from deficits in force transmission. Moreover, we show that $fln^{\Delta N62}$ males produced an abnormal courtship song characterized by a higher sine song frequency and a pulse song with longer pulses and longer inter-pulse intervals (IPIs), the latter implicated in male reproductive success. When presented with a choice, wild-type females chose control males over mutant males in 92% of the competition events. These results demonstrate that flightin N-terminal domain is required for optimal myofilament lattice regularity and IFM activity, enabling powered flight and courtship song production. As the courtship song is subject to female choice, we propose that the low amino acid sequence conservation of the N-terminal domain reflects its role in fine-tuning species-specific courtship songs.

1. Introduction

Organisms perform complex behaviours to survive and to attract mates of the opposite sex for reproduction. Morphological adaptations (and the underlying physiological processes) that confer advantages in attracting a mate may have no immediate benefit to survival. However, it is possible, as has been posited for the evolution of plumage in basal birds [1], that sexually selected traits may adapt to fulfil functions that directly impact survival. The preponderance of flying insects (roughly six out of every 10 animal species is a flying insect [2]) is testimony to the selective advantages that flight confers in increasing the survival odds, particularly as a means to escape predators, disperse and conquer new habitats. As such, the advent of powered flight was a key innovation that promoted diversification of arthropods and facilitated speciation of insects, the animal world's most diverse and abundant life form. In addition to its role in locomotion, wing movement in insects serves a variety of other roles, foremost among which is sound production for communication and courtship purposes. The wing tones are considered to have arisen as an incidental outcome of flight [3], as are flapping wing-induced sounds in birds that serve as

communicative signals [4]. Consistent with this proposal are studies showing that wing vibrations in *Drosophila* are brought about by contraction of the indirect flight muscles (IFMs) that power flight [5,6]. Species-specific acoustic signals facilitate pre-mating reproductive isolation and subsequent speciation in vertebrates and insects [7]. A speciation mechanism for *Drosophila* spp. is sexual selection *via* the male courtship song consisting of rhythmic pulses and, in some species, sinusoidal hums generated by small-amplitude wing vibrations. Each species produces a song with unique characteristics that plays a role in species-specific mating, reproductive isolation and female sexual stimulation [8]. For example, the inter-pulse interval (IPI) is highly variable and carries the most salient species-specific signal among *Drosophila* [9], whereas the pulse duty cycle (PDC) or pulse singing vigour [10] and sine song frequency [11] stimulate copulation of *Drosophila melanogaster* females.

Several species of flying insects, including *Drosophila* species, have evolved a highly crystalline thick and thin filament lattice structure that extends from one sarcomere to the next along the entire length of the IFM myofibril [12,13]. This 'long-range myofibrillar crystallinity' is predominant among insects with asynchronous IFMs and other skilled flyers that together comprise a majority of flying insects. This coincidence raises the question whether a highly regular myofilament lattice structure has evolved in response to selection for high muscle power output and skilful flight. The IFM exhibits additional attributes that have contributed to the evolutionary success of flying insects, including an actomyosin motor with exceptionally fast kinetics [14], an asynchronous mode of contraction that produces fast wingbeat frequencies (approx. 200 Hz in *D. melanogaster*) at a lower rate (approx. 5 Hz) of motor neuron activation [15], an enhanced stretch activation-shortening deactivation response at a relatively constant [Ca^{2+}] [16], and structural modifications of the sarcomere that amplify power output and maintain myofilament lattice integrity [17]. The extent to which these features contribute to species-specific differences in the courtship song is currently unknown. A recent study demonstrating that mutations in the myosin regulatory light chain manifest differently in song and flight raises the possibility that muscle structural proteins are subject to sexual selection [6].

Here, we focus on flightin to gain insight into the interplay of selection mechanisms shaping the evolution of the IFMs. Flightin is a myosin-binding protein that in *D. melanogaster* is expressed exclusively in the IFMs, where it has been shown to be essential for the proper determination of length and stiffness of thick filaments [18–20]. Genetic ablation of flightin expression (fln^0 flies) resulted in sarcomere degradation, fibre hypercontraction, and structurally and mechanically compromised IFMs unable to generate oscillatory power, rendering fln^0 flies flightless but viable [19,20]. The structural, physiological and flight phenotypes are fully rescued by a wild-type flightin transgene [21], further evidence of the strict flightin requirement for IFM function. By contrast, a truncated flightin transgene lacking the COOH-terminal 44 amino acids ($fln^{\Delta C44}$) only partially rescues the structural and mechanical defects evident in fln^0 IFMs and is unable to rescue flightlessness [22].

A comparison of flightin sequences from 12 *Drosophila* species revealed that the N-terminal region (amino acids 1–63 in *D. melanogaster*), just before the conserved WYR domain [19], shows higher sequence variability (approx. 22% identity) than the rest of the protein (more than 76% identity) (electronic supplementary material, figure S1) [23,24]. Based on this observation and on the dual role of the IFM in generating wing movements for flight and for the courtship song, we hypothesized that the N-terminal region represents an independent domain evolving under different constraints from those of the rest of the protein. To test this hypothesis, and to understand the function of the flightin N-terminal region, we generated and characterized a transgenic line ($fln^{\Delta N62}$) expressing flightin devoid of the 62 N-terminal amino acids. The findings show that the N-terminal region, while not essential for IFM function, is required for the structural regularity of the myofilament lattice that underscores optimal mechanics for flight and for tuning of the male courtship song.

2. Material and methods

Detailed experimental procedures are found in the electronic supplementary material, Experimental Procedures (SEP) Methods 1–11.

(a) Flight performance

Flight tests and wingbeat frequency analysis of 2–3 days-old flies were performed as previously described [25].

(b) Courtship behaviour recording and analysis

Behavioural experiments and analysis were performed as described in [6] and electronic supplementary material, Method 5. Courtship parameters measured include: sine song frequency (SSF), intrapulse frequency (IPF), cycles per pulse (CPP), pulse length (PL), inter-pulse interval (IPI), pulse duty cycle (PDC), courtship index (CI) and wing extension index (WEI).

(c) Transmission electron microscopy

Thoraces from 2–3 days-old flies were bisected, fixed, dehydrated, infiltrated, embedded, sectioned, imaged and analysed as described in [21] and electronic supplementary material, Methods 6–8.

(d) Single muscle fibre mechanics by sinusoidal analysis

Preparation of muscle fibres from 2–3 days-old flies, solutions used, mechanical measurements and curve fitting were carried out as in previous studies [22,26] with some modifications as described in electronic supplementary material, Method 9.

(e) Statistical analysis

All values are mean $\pm$ s.e. Detailed statistical analyses are described in electronic supplementary material, Method 11.

3. Results

(a) $fln^{\Delta N62}$ flies are flight compromised

$fln^{\Delta N62}$ flies (see electronic supplementary material, Results for the fly strain generation) express a lower molecular weight flightin (electronic supplementary material, figure S2*a*,*b*) and are capable of flight, indicating that an N-terminal truncated flightin is sufficient to restore flight ability of fln^0 flies. Compared with fln^+ rescued null control flies, $fln^{\Delta N62}$ flies

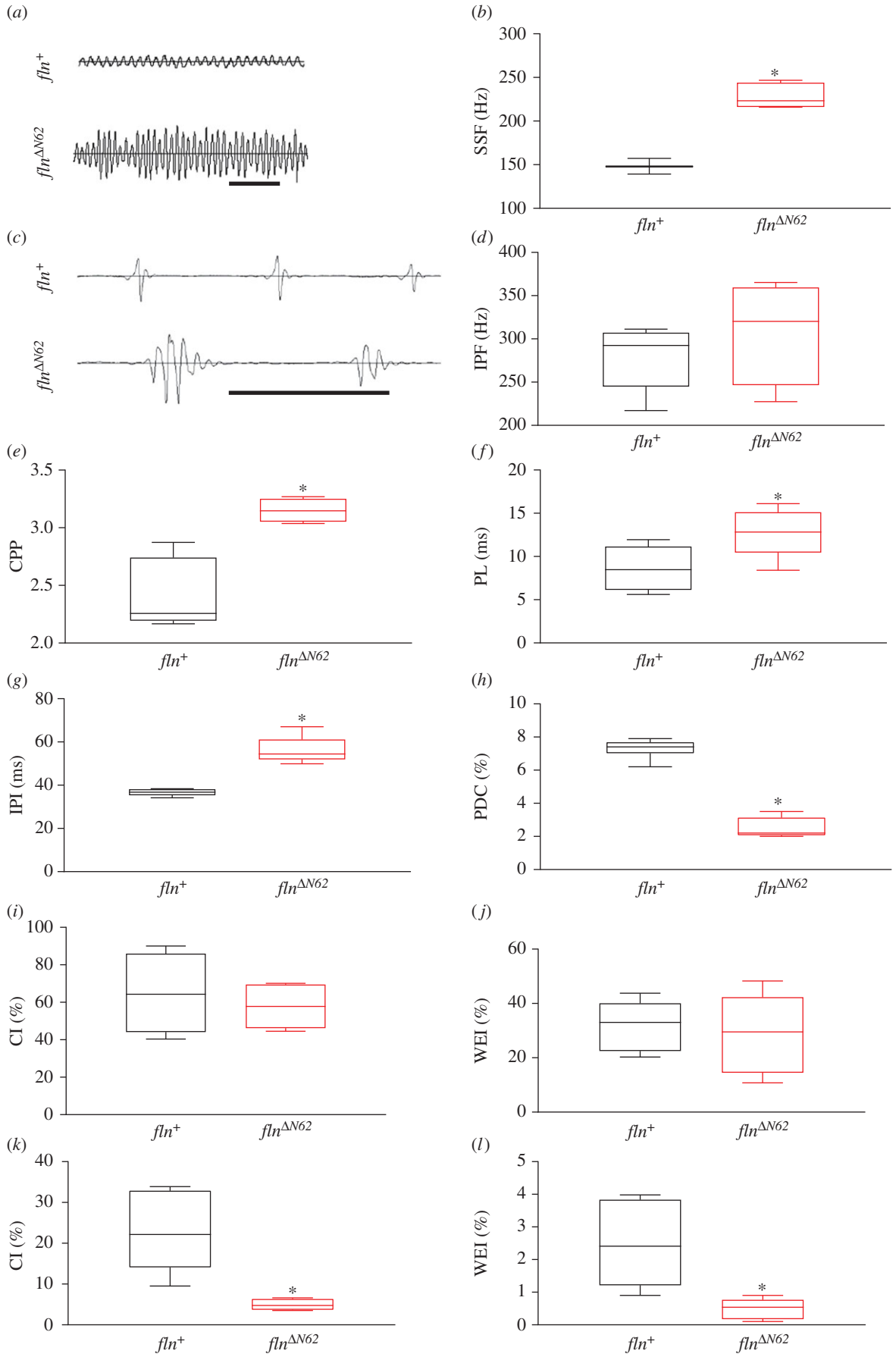

Figure 1. (*Caption overleaf.*)

show an approximately 33% decrease in flight ability (flight score: 2.8 ± 0.1 ($n = 66$) versus 4.2 ± 0.4 for fln^{+} ($n = 35$); $p < 0.05$). 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% versus 95% for fln^{+}, or produce a wingbeat (90% versus 100% for fln^{+}). The flight impairment is not due to a change in wingbeat frequency (195 ± 4 Hz, $fln^{\Delta N62}$ ($n = 45$) versus 198 ± 2 Hz, fln^{+} ($n = 25$)).

Figure 1. (*Overleaf.*) $fln^{\Delta N62}$ males sing an abnormal courtship song and are outcompeted for female choice. Courtship sine song (*a* and *b*) and pulse song (*c* through *h*) of fln^{+} and $fln^{\Delta N62}$ males (scale bar (thick lines below the oscillograms) = 50 ms). Compared to the fln^{+} control males, $fln^{\Delta N62}$ males produce a higher-frequency sine song, SSF (*b*), a pulse song with similar frequency, IPF (*d*) but with more cycles per pulse, CPP (*e*), longer pulse length, PL (*f*), longer inter-pulse interval, IPI (*g*), and reduced pulse duty cycle (PDC: the ratio of pulse song duration to the total time of recording including song plus silence [6]) (*h*). The mean value of each song parameter is the mean of average values for each fly song for *N* number of fly song recordings. $N = 7-8$ thirty-minute fly song recordings. *Significant difference ($p < 0.05$) from fln^{+} control. Courtship behaviour during single pair mating (*i* and *j*) and competition mating between fln^{+} and $fln^{\Delta N62}$ males (*k* and *l*). fln^{+} and $fln^{\Delta N62}$ males have a similar courtship index (CI) and wing extension index (WEI) when paired singly with a wild-type (Oregon R strain) female. When competing with fln^{+} males, $fln^{\Delta N62}$ males have a significantly reduced courtship index and wing extension index. Courtship index is the total time duration of courtship behaviour by a male divided by the total time of video recording, or until courtship success; wing extension index is the total time duration of wing extension to produce the courtship song by a male divided by the total time of the video recording, or until courtship success [6]. Both indices are expressed as percentages. $N = 25$ and 10 for mating competition assays and single pair mating assays, respectively. *Significant difference ($p < 0.05$) from fln^{+} control.

(b) $fln^{\Delta N62}$ males produce abnormal courtship song

Courtship song recordings from single male–female pairs revealed that flightin null male flies (fln^{0}) produce no song. Singing is restored by the N-terminal-deleted flightin ($fln^{\Delta N62}$) and by the full-length flightin (fln^{+}; electronic supplementary material, figure S3 and Audios S1 and S2). Closer examination of the oscillograms revealed that the N-terminal deletion affects the sine song as well as the pulse song (figure 1*a*,*c*). The frequency of the sine song (SSF) produced by $fln^{\Delta N62}$ is significantly higher than that produced by control males (228 ± 6 Hz versus 148 ± 5 Hz for fln^{+}, $p < 0.05$; figure 1*b*). By contrast, the intrapulse frequency (IPF) is not affected by the mutation (figure 1*d*). However, other aspects of the pulse song produced by $fln^{\Delta N62}$ males are abnormal, including a greater number of cycles per pulse (CPP, 3.9 ± 0.4 versus 2.4 ± 0.1 for fln^{+}; $p < 0.05$; figure 1*e*), longer pulse length (PL, 12.8 ± 1.5 ms versus 8.6 ± 1.2 ms for fln^{+}; $p < 0.05$; figure 1*f*), longer inter-pulse intervals (IPI; 56.1 ± 2.5 ms versus 36.7 ± 0.7 ms for fln^{+}; $p < 0.05$; figure 1*g*) and lower pulse duty cycle (PDC, $2.5 \pm 0.4\%$ versus $7.3 \pm 0.4\%$ for fln^{+}; $p < 0.05$; figure 1*h*). In addition, pulse songs produced by $fln^{\Delta N62}$ males are characterized by a wider range of IPIs than pulse songs produced by fln^{+} males (figure 2), indicating that $fln^{\Delta N62}$ males are unable to maintain the proper timing of their pulses across song trains.

(c) Flightin N-terminal region truncation 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 behaviour and mating success. In single pair mating assays, $fln^{\Delta N62}$ males were able to copulate at the same frequency, and perform the courtship ritual with the same efficiency as control fln^{+} males as determined by the CI and WEI (figure 1*i*,*j*; electronic supplementary material, videos S1 and S2). By contrast, $fln^{\Delta N62}$ male courtship efficiency decreases markedly in the presence of an fln^{+} male. $fln^{\Delta N62}$ males display a more than 75% reduction in CI (0.05 ± 0.01 versus 0.20 ± 0.05 for fln^{+}, $p < 0.05$) and WEI (0.005 ± 0.001 versus 0.025 ± 0.007 for fln^{+}, $p < 0.05$; figure 1*k*,*l* and electronic supplementary material, video S3). When presented with a choice of $fln^{\Delta N62}$ and fln^{+} males, wild-type (OR) females chose the fln^{+} male 92% of the times, indicating female preference towards the fln^{+} control male. Additionally, the female moved away and displayed aggressive rejection behaviours (kicking with hind and mid legs) towards the $fln^{\Delta N62}$ male (electronic supplementary material, video S3).

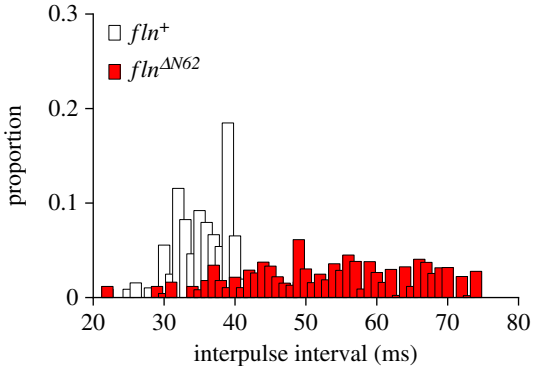

Figure 2. Distribution of inter-pulse interval (IPI) of fln^{+} (white bars) and $fln^{\Delta N62}$ (red bars) male pulse songs. Each bar represents the frequency at which IPIs occur among different fly songs. $N = 7-8$ thirty-minute fly song recordings.

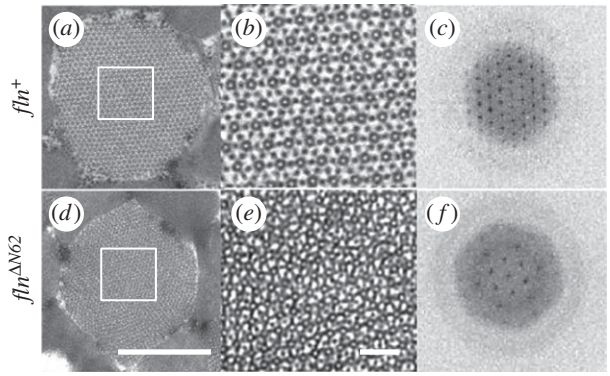

Figure 3. Reduced myofilament lattice organization in $fln^{\Delta N62}$ IFM fibres. Transmission electron microscopy images of cross-sections of IFM from fln^{+} (*a* and *b*) and $fln^{\Delta N62}$ (*d* and *e*) transgenic fly lines. Note that myofibrils show the characteristic cylindrical shape of normal IFM, and have similar diameters. Regions within white boxes in (*a* and *d*) are magnified in (*b* and *e*), respectively. Panel (*e*) shows a more compact and less ordered hexagonal lattice than (*b*). This is reflected in the Fourier power spectra (*c* and *f*) from the images (*b* and *e*), respectively. Scale bars = 1 µm (for *a* and *d*) and 0.1 µm (for *b* and *e*).

(d) Flightin N-terminal region truncation affects sarcomere structure and myofilament lattice properties

Sarcomeric defects in fln^{0} IFMs [19] are completely reversed by reintroducing the full-length flightin transgene (fln^{+}) ([21], and electronic supplementary material, figures S4A,B and 3*a*–*c*). Expression of an N-terminal truncated flightin also results in substantial improvement in sarcomere structure, but the rescue is not complete. Sarcomeres in $fln^{\Delta N62}$

Table 1. IFM myofibril and sarcomere characteristics from control and mutant flightin strains. Values are mean ± s.e. Numbers of sarcomeres, myofibrils and fibre cross-sections are shown in parenthesis. For each line, electron microscopy images from two flies were analysed.

strain	sarcomere length (μm)	myofibril cross-sectional area (μm^2)	thick filaments per myofibril cross-section (#)	myofibril area per 100 μm^2 fibre 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)

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

Table 2. IFM myofilament lattice properties from Fourier transforms of electron microscopy images. Values are mean ± s.e. Numbers of myofibril cross-sections analysed are shown in parenthesis. For each line, EM images from two flies were analysed.

strain	$d_{1,0}$ (nm)	inter-thick filament spacing (nm)	resolution (nm)	1,0 half-width (pixels)	1,0 peak intensity, $I_{1,0}$ ($\log_{10}$)(arbitrary units)
fln^+	43.0 ± 0.3 (56)	49.7 ± 0.4 (56)	13.6 ± 0.3 (56)	12.7 ± 0.9 (14)	1.78 ± 0.02 (14)
$fln^{\Delta N62}$	38.2 ± 1.2* (42)	44.1 ± 1.3* (42)	18.2 ± 1.0* (42)	19.1 ± 1* (13)	1.72 ± 0.01* (13)

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

IFMs are approximately 13% shorter than those in fln^+ (table 1; electronic supplementary material, figure S4*a* versus S4*c*) and are characterized by the absence of the H-zone and a narrower M-line that shows occasional gaps (electronic supplementary material, figure S4*b* versus S4*d*). The average cross-sectional area of the myofibril is similar compared with fln^+ (table 1). However, $fln^{\Delta N62}$ myofibrils contain approximately 11% more thick filaments (903 ± 25 versus 810 ± 18 for fln^+, $p < 0.05$; table 1). The myofilament lattice organization appears 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*b* versus *e*). The myofibrillar area per fibre cross-section is higher in $fln^{\Delta N62}$ fibres compared with fln^+ fibres (45 ± 1% versus 39 ± 2% for fln^+; $p < 0.05$; table 1).

To quantify the difference in myofilament lattice organization, we conducted digital two-dimensional Fourier transforms of the EM cross-section images. The corresponding power spectra (figure 3*c*,*f*) show reflections that are of lower intensity in the mutant than in the control. Harmonics of the lattice reflections in the Fourier power spectra clearly resolved in fln^+ are absent in $fln^{\Delta N62}$ (table 2). By indexing the reflections using a hexagonal lattice, we obtained $d_{1,0}$ and calculated the inter-thick filament distance (electronic supplementary material, Methods 7 and 8). This analysis showed that the flightin N-terminal truncation decreased $d_{1,0}$ and the inter-thick filament spacing by approximately 11%. Moreover, the standard errors of means are three to four times higher in the mutant, suggestive of greater heterogeneity in the myofilament lattice (table 2).

To gain further insight into the regularity of the myofilament lattice, we measured the peak intensity and the peak half-width for the spots in the 1,0 plane. The $fln^{\Delta N62}$ myofilament lattice diffraction spots had significantly larger peak half-widths and lower peak intensities compared with control fln^+ (table 2), indicating that the mutation has compromised lattice regularity. The large difference in peak half-widths, together with the differences in interfilament distance, indicates that the myofilament lattice structural organization and order are reduced in the $fln^{\Delta N62}$ myofibrils compared with those of fln^+.

(e) Flightin N-terminal region truncation affects mechanical performance of indirect flight muscle fibres

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 rigour tension (electronic supplementary material, table S1). The elastic modulus for $fln^{\Delta N62}$ fibres in relaxed and rigour conditions were decreased at all frequencies tested compared to fln^+ (figure 4*a*,*b*). The viscous modulus for $fln^{\Delta N62}$ fibres was decreased at frequencies between 40 Hz and 650 Hz in the relaxed condition (figure 4*c*), and at all frequencies tested in rigour condition (figure 4*d*), compared with fln^+. In addition, compared with fln^+ fibres, a significantly larger proportion of the $fln^{\Delta N62}$ fibres were unable to withstand tension in rigour conditions (20% versus 13% for fln^+), resulting in tearing or complete breakage of the fibre, when allowed to remain in rigour solution for longer times.

To gain insight into the structural flaws that may underlie fibre failure in rigour, we examined the failed (torn) fibres by electron microscopy (electronic supplementary material, Method 10). The breakage site in $fln^{\Delta N62}$ fibres exhibited greater distortions in the Z-bands and M-lines than the breakage site in control fibres (see electronic supplementary material, figure S5*b*,*d*). Additionally, thick filaments in the mutant sarcomeres tended to buckle, a feature not seen in control sarcomeres. These features are unique to the breakage sites as they were not detected away from the breakage sites in either control or mutant fibres (see electronic supplementary material, figure S5*a*,*c*).

At maximal calcium activation (pCa 4.5), the $fln^{\Delta N62}$ fibres had a lower elastic modulus at all frequencies tested (figure 4*e*) and a lower viscous modulus between 75 and 280 Hz

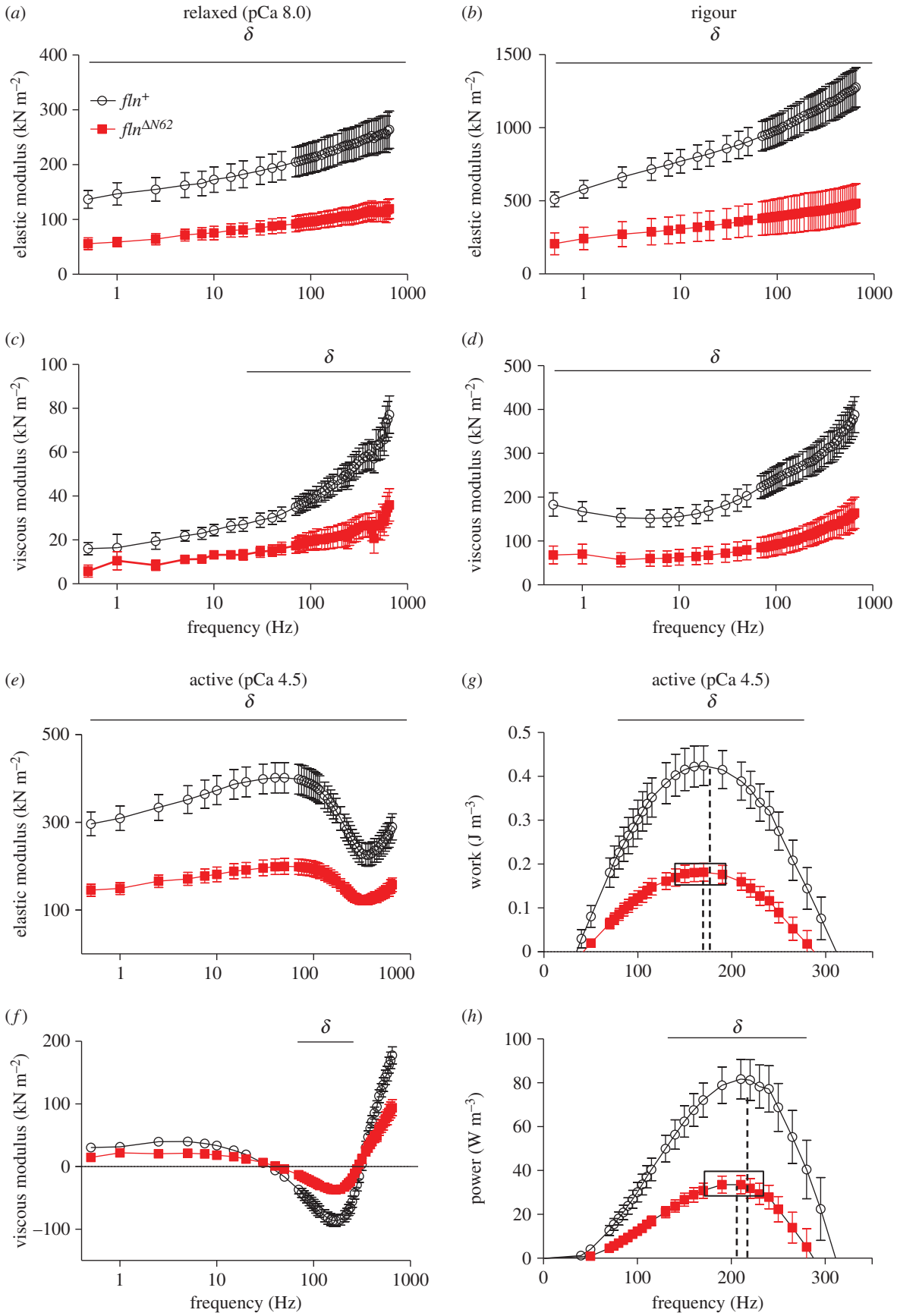

Figure 4. *fln*$^{\Delta N62}$ IFM fibres have reduced elastic and viscous moduli and reduced oscillatory work and power output. Elastic and viscous moduli of skinned IFM fibres from *fln*$^{+}$ (open black circles) and *fln*$^{\Delta N62}$ (filled red squares) in relaxing (*a* and *c*) and rigour (*b* and *d*) solutions. Horizontal lines below δ symbols denote the frequency range through which measured values are significantly different between *fln*$^{+}$ and *fln*$^{\Delta N62}$ ($p < 0.05$). Elastic modulus (*e*), viscous modulus (*f*), work output (*g*) and power output (*h*) for active IFM fibres from *fln*$^{+}$ (open circles) and *fln*$^{\Delta N62}$ (filled squares) strains are shown. Lines below delta (δ) denote frequency ranges where measured values are significantly different between *fln*$^{+}$ and *fln*$^{\Delta N62}$ ($p < 0.05$). Vertical dashed lines in (*g* and *h*) represent the frequency of maximum oscillatory work and power, occurring at 171 $\pm$ 8 Hz and 205 $\pm$ 7 Hz for *fln*$^{\Delta N62}$ compared to 179 $\pm$ 8 Hz and 217 $\pm$ 7 Hz for *fln*$^{+}$. The frequencies of maximum oscillatory work and power are not significantly different between control and mutant strains. Note the broader range of frequencies of maximum oscillatory work and power in the mutant fibres compared with that of control (boxes in (*g* and *h*), respectively).

(figure 4*f*). Figure 4*g*,*h* respectively show that $fln^{\Delta N62}$ fibres had reduced maximum oscillatory work output (0.20 ± 0.02 J m^{-3} versus 0.45 ± 0.05 J m^{-3} for fln^{+}; $p < 0.05$) and oscillatory power output (38 ± 5 W m^{-3} versus 89 ± 10 W m^{-3} for fln^{+}; $p < 0.05$), 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).

4. Discussion

This study provides evidence that myofibrillar proteins contribute to tonal aspects of the courtship song and demonstrates the requirement of the flightin N-terminal domain for myofilament lattice regularity, a defining feature of asynchronous flight muscles. The lattice structure disorganization resulting from the flightin mutation compromises flight ability and courtship success, two behaviours that underpin evolutionary processes driving insect speciation.

(a) Muscle structure and mechanics

When subjected to contractile forces, sarcomeres devoid of flightin irreversibly collapse and cease to display the characteristic structural features of sarcomeres [19,27]. By contrast, sarcomeres in $fln^{\Delta N62}$ can not only withstand contractile forces but also produce enough power to elicit a wingbeat at normal frequency, demonstrating that the N-terminal domain is not required for flightin's most essential function as a structural protein that contributes to the stability and integrity of the sarcomere. A prominent difference in high-level sarcomere structure between $fln^{\Delta N62}$ and fln^{+} is the appearance of the H-zone and the M-line (electronic supplementary material, figure S4*b*,*d*). Flightin is largely excluded from the H-zone and its absence from adult fln^{0} sarcomeres results in complete loss of the M-line [19]. Thus, the N-terminal domain may confer structural and mechanical properties to the thick filament to properly scaffold assembly of the M-line. Recently, Gasek *et al.* [28] showed that the persistence length of $fln^{\Delta N62}$ thick filaments is only about 30% the persistence length of fln^{+} thick filaments. A reduction in $fln^{\Delta N62}$ thick filament stiffness, manifest as a greater bending propensity, is not accompanied by differences in filament length [28]. From these studies, we conclude that biomechanical properties of thick filaments are fundamentally important in defining some features of sarcomere architecture. Additionally, the reduction in fibre elastic modulus and the propensity of $fln^{\Delta N62}$ sarcomeres to tear and thick filaments to buckle under rigour conditions are further manifestations of thick filaments with compromised mechanics. In summary, the results presented here, together with the study of Gasek *et al.* [28], illustrate how filament compliance scales up and influences bulk properties of muscle and animal locomotory performance (reviewed in [29]).

This study also shows that the N-terminal domain of flightin is essential for myofilament lattice regularity and proper spacing between thick filaments ($d_{1,0}$). The approximately 11% compression in lattice spacing (table 2) accompanies a significant decrease in fibre viscous and elastic modulus (figure 4*a*–*f*) and a greater than 50% drop in oscillatory work and power output (figure 4*g*,*h*), with resultant deficits in flight ability. While we did not measure cross-bridge kinetics directly, the frequency of the wingbeats and the frequencies of maximum work and power output are not different between $fln^{\Delta N62}$ and fln^{+}, suggesting the underlying actomyosin kinetics is not markedly impaired. Thus, the compromised flight ability of $fln^{\Delta N62}$ flies is likely to arise from deficits in force generation (electronic supplementary material, table S1) or force transmission owing to more compliant fibres.

A defining feature of $fln^{\Delta N62}$ skinned fibres is their ability to sustain maximum work and maximum power over a broader range of frequencies than fln^{+} fibres (boxed regions in figure 4*g*,*h*). This raises the possibility that fine-tuning of actomyosin kinetics is compromised in $fln^{\Delta N62}$ fibres as a result of changes in lattice geometry that could make it harder for myosin to find a binding site on actin. This would consequently affect cooperativity in myosin binding and thin filament activation [30,31].

The reduction in lattice spacing and regularity could arise from errors in myofibrillogenesis. Other features observed in the adult mutant flight muscle also point towards a role for the flightin N-terminal domain in myofibrillogenesis. These include shorter sarcomere length (electronic supplementary material, figure S4), greater number of thick filaments per myofibril and a larger myofibrillar area per fibre cross-section compared with age-matched control flies.

While the mechanism by which the flightin N-terminal domain influences myofilament lattice spacing is unknown, it is likely to be dominated by electrostatic forces. In our model, the thick filament backbone is fringed by flightin N-terminal domains, in a manner similar to that described for neurofilament subunit C-terminal sidearms projecting away from the neurofilament core [32]. The N-terminal domain is characterized by a high negative charge (pI 3.78) and multiple phosphorylation sites (electronic supplementary material, figure S1), attributes that are conserved across *Drosophila* species despite the poor conservation of the amino acid sequence [24]. As flightin resides on the surface of the thick filament backbone [33] that also carries a net negative charge [34], fringing N-terminal domains expand the charge distribution field's contribution to radial forces that define and stabilize inter-thick filament distances.

A comparison of the results of this study with those that examined the properties of $fln^{\Delta C44}$ [22] provides important insight into the structure–function relation in flightin. Upon skinning, flightin remained associated with $fln^{\Delta N62}$ and $fln^{\Delta C44}$ muscle fibres, indicating that neither the N-terminal domain nor the C-terminal domain is required for myosin binding [18]. The only shared sequence between the N-terminal truncated flightin and the C-terminal truncated flightin is the central region extending from amino acid 63 to 138. This region is largely occupied by WYR, a domain that comprises the most conserved sequence in flightin and whose origin coincides with the ancestor of Pancrustacea more than 500 MYA [23]. Because interaction with myosin is essential for flightin stability and function [19,35], it is likely that the major myosin-binding site resides entirely within the WYR domain. With this critical function assigned to the WYR domain, the flightin sequences outside WYR are less constrained to evolve in support of taxon-specific needs. Indeed, the three domains show drastically different degrees of sequence conservation, suggesting that they are subject to different selective forces. Comparison of flightins from 12 sequenced *Drosophila* species [36] reveals amino acid identity is 22% for the N-terminal domain, 93% for the WYR domain and 60% for the C-terminal domain. On expanded

phylogenies, the differences are even greater [23]. It seems appropriate, then, that the N-terminal domain reflects lower conservation given its contributing role to thick filament stiffness, as persistence length is known to range broadly among thick filaments from different muscle types [29].

Compared with $fln^{\Delta N62}$, deletion of the C-terminal domain had a marked impact on cross-bridge cycling kinetics, and consequent changes in frequency of the wingbeats (eradicated) and frequency of maximum power output (reduced by approximately 30%), a small but significant decrease in inter-thick filament spacing and a more pronounced effect on high-level sarcomere structure, including the absence of or dispersed M-lines and occasional defects in Z-bands [22]. Contrary to deletion of the N-terminal domain, deletion of the C-terminal domain affects thick filament length but not stiffness (persistence length [28]) nor passive skinned fibre stiffness (elastic modulus [22]). These results reveal a clear demarcation of functional roles between the N-terminal and the C-terminal domains and confirm that the N-terminal domain has a prominent role in maintaining thick filament stiffness and myofilament lattice geometry.

(b) Courtship song

Flightin joins the myosin regulatory light chain [6] as muscle proteins shown to influence courtship song qualities important for reproductive success. Unlike the myosin regulatory light chain that is expressed in all adult muscle, flightin expression in *Drosophila* is confined to the IFM [18]. We show here that fln^{0} males are mute, underscoring the essential contribution of the IFM to song production. More recent studies have confirmed this role, particularly as it relates to song intensity modulation in response to female distance, i.e. amplitude modulation with distance, or AMD [37].

Previous studies have shown the importance of the IPI in species recognition [9] and the PDC in female receptivity [10]. By contrast, courtship song frequencies do not appear to play a significant role in either species recognition or female mate choice in *D. melanogaster* [38]. In the light of our findings that deletion of the flightin N-terminal domain significantly decreases the PDC, increases the IPI and results in a broader range of IPIs than expected from natural variation [39], we conclude that the N-terminal domain fulfils a functional role in courtship song definition that is just as important as its role in flight power mechanics. The limited courtship success of $fln^{\Delta N62}$ males when competing with fln^{+} males for female access, as evident by lower CI (time courting) and WEI (time singing) (figure 1*k*,*l*), further supports this conclusion. Moreover, we did not see aggressive behaviour from the fln^{+} males towards the $fln^{\Delta N62}$ males that could have affected the mutant males' pre-mating behaviours and chances for copulation. In addition, very often in courtship competition assays, but not in single pair mating, the female exhibited high locomotory activity during and after a successful mounting attempt by the $fln^{\Delta N62}$ male, after which the female engaged in courtship behaviour and ultimately copulated with a fln^{+} control male. Studies have shown that *Drosophila* virgin females are highly mobile as a strategy to stimulate increased courtship behaviour by the male. By post-copulation, the female tends to be less mobile [40,41]. Our observation of high female mobility during and after successful $fln^{\Delta N62}$ male mounting could be indicative of the wild-type female's attempts to entice the fln^{+} male, as evidenced by her preference towards him compared with the lack of receptivity towards the $fln^{\Delta N62}$ male. This also explains the higher CI and WEI of the fln^{+} males during courtship competition assays.

A possible explanation for why a mutation in a muscle contractile protein may influence courtship behaviour can be found in the aforementioned interplay between visual cues and song production. *Drosophila* males adeptly integrate visual information related to their distance to a female to modulate the amplitude of their song [37]. While the relation between muscle power output and the amplitude of the pulse song remains unknown, our previous analysis of myosin regulatory light chain mutants suggests that the range of song amplitudes is limited by how much power the muscle can produce. Specifically, the dual myosin light chain mutant exhibits a reduction in maximum power output [26] and an increase in the amplitude ratio of sine song to pulse song, most probably as the result of a decrease in amplitude of the pulse song [6]. Similarly, we predict that the reduced power output in $fln^{\Delta N62}$ flies compromises the range of pulse song amplitudes and hence their ability to attract females at a distance, particularly in the presence of a competing male whose range of song amplitudes is not diminished. Sensing that the competing male is able to use the PDC or the AMD to its advantage, $fln^{\Delta N62}$ males may choose to disengage altogether from the mate pursuit. No such disengagement is evident in the absence of a competing male, as reflected by the similarity in CI and WEI values between $fln^{\Delta N62}$ and fln^{+} males in single pair mating assays (figure 1*i*,*j*). Thus, it is possible that male sensory processing in a courtship situation extends beyond modulation of the song to include locomotory responses.

The apparent faster rate of evolution of the N-terminal domain is reminiscent of that observed in genes involved in sex and reproduction, many of which are subject to positive selection [36]. Our previous finding that all six putative positive selected sites in flightin reside within the N-terminal domain [24] provides additional evidence that sexual selection (in this case, pre-copulatory female choice of song attributes) contributes at least in part to the evolution of flightin, and by extension to evolution of the flight machinery. This is consistent with studies showing that courtship song signals respond rapidly to selection and may contribute to a faster rate of speciation, particularly among species-rich taxa such as insects [42]. Thus, flightin provides an example of how natural selection and sexual selection act in concert to select traits that are equally beneficial for survival (enhanced flight power) and mating success (pulse song characteristics). Alternatively, as sexual selection acts on timescales generally shorter than natural selection, our results open up the possibility that variations in proteins that enhance flight performance could have arisen as unintended consequences of sexual selection. Females that judge male fitness on the strength of their pulse song power attributes may be aiding in the selection of skilful flyers with higher regularity of the myofilament lattice.

Data accessibility. All datasets supporting this article have been uploaded in Dryad repository [43] and also as part of the electronic supplementary material here.

Authors' contributions. S.C. and J.O.V. conceived and designed the experiments. S.C., V.L.F., H.V., M.R. and B.C.W.T. performed the experiments. S.C., B.C.W.T., T.R. and J.O.V. analysed the data. S.C. and J.O.V. wrote the paper.

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