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MECHANICAL PROPERTIES OF DEMEMBRANATED FLIGHT MUSCLE FIBRES FROM A DRAGONFLY

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Summary

The mechanical properties of demembranated muscle fibres of synchronous flight muscle from a dragonfly *Libellula quadrimaculata*, asynchronous flight muscle from the giant waterbug *Lethocerus indicus* and synchronous psoas muscle from rabbit were compared in relaxed, active and rigor conditions. The properties were compared to the known structure and protein compositions of these muscles. We found that active tension of *L. indicus* flight muscles was stretch-activated (tension was low and was significantly increased following a rapid stretch of 1 % of muscle length), whereas both dragonfly flight muscle and rabbit psoas muscle were not (active tension was high and did not significantly increase following a rapid stretch of 1 %). Three different properties have been suggested to give rise to stretch activation in asynchronous muscles: (1) a matching of the helix periodicities of actin target sites to myosin crossbridge heads, (2) a special form of troponin subunit called troponin-H, and (3) the high resting stiffness of these muscles inducing strain in the thick filaments. Rabbit psoas muscle has none of these properties. Dragonfly flight muscles do not have the helix matching, but they do have a form of troponin-H and a high resting stiffness. It seems most likely that dragonfly flight muscles are not stretch-activated because they do not have the helix matching.

Introduction

Insect flight muscles can be synchronous or asynchronous. In insects that have synchronous flight muscles, the wingbeat frequency is controlled by the frequency of nervous excitation. In insects that have asynchronous flight muscles, wingbeat frequency is independent of the frequency of nervous excitation. Asynchronous flight muscles are stretch-activated (Jewell and Ruegg, 1966; Pringle, 1967): following calcium activation, both tension and ATPase activity show large maintained increases when the muscles are given a small maintained stretch. This increase in tension is delayed with respect to the change in length, and it is this delayed tension change that enables the flight muscles to power flight.

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In asynchronous muscles, stretch activation can be explained by an increase in the probability of attachment of myosin heads to actin target sites due to stretch (Thorson and White, 1969). Three different properties have been suggested to give rise to stretch activation (Table 1). The first property is a matching of the 4-start helical pitch (start is the number of helical tracks of myosin heads on the surface of the thick filament) of the myosin heads on the thick filament to that of the actin filament (38 nm, Wray, 1979*a*). Owing to this steric arrangement, a stretch of about 1 % of resting muscle length can greatly change the number of actin target sites for myosin heads (Deschereveskii, 1971; Wray, 1979*b*). The second property is the presence of a special form of troponin subunit, troponin-H (Bullard *et al.* 1988), the carboxyl end of which may extend and form an inflexible link, possibly with myosin, influencing crossbridge attachment. The third is strain on the myosin-containing filament (Thorson and White, 1969, 1983), arising from the high resting stiffness (White *et al.* 1979; White, 1983), resulting from a connection between the thick filament and the Z-line across the short I-band in these muscles. This strain may alter the rate constants within a crossbridge cycle, thereby maintaining tension at a high level following a stretch. Arthrin, a conjugate between actin and ubiquitin (Ball *et al.* 1987), which has only been found in asynchronous flight muscles (Bullard, 1983), probably does not have a major role in stretch-activation as it is only found in some asynchronous flight muscles such as those of the Diptera (flies) and Hemiptera (bugs) (B. Bullard, unpublished observations). It is not found in the synchronous flight muscles of dragonflies (B. Bullard, R. Cripps and M. Peckham, unpublished observations).

Vertebrate skeletal muscles are not stretch-activated as defined above, and although they do show a delayed tension increase following a stretch, the amplitude of this increase is small compared to the amplitude of the tension increase produced by Ca^{2+}-activation (Goodall, 1956; Lorand and Moos, 1956; Abbott and Steiger, 1977). In these muscles, unlike asynchronous flight muscle, the 3-start helical pitch of myosin heads on the thick filament is 42.5 nm and does not match that of the actin target sites (38 nm), arthrin and troponin-H are absent, and the resting stiffness is low (Table 1). The helical mismatch means that there is very little overall change in the number of available target sites on actin for myosin heads when the muscle is given a small stretch. In this case, the delayed tension response is probably caused by an effect of distortion on the attachment and detachment rates of myosin heads from actin and not by a change in the overall number of attached heads (Thorson and White, 1983).

The aim of this study was to investigate the mechanical properties of synchronous flight muscles from dragonfly (Wilson, 1968) and to correlate them with the presence or absence of the three properties outlined above which may be required for stretch activation: that is, the helix periodicity of myosin crossbridges and actin target sites, the presence of troponin-H and the resting stiffness (Table 1). These muscles have an extensive T-tubular system (Smith, 1966) and were expected to be fully activated by calcium. They were not expected to be stretch-activated. The ATPase activity was not measured in this paper, and the criterion for stretch

Table 1. *Sarcomere properties*

	Property							
	1 Filament helical properties			2	3	Stretch activation		
						Length activation of		
	A-filament pitch (nm)	Start*	I-filament pitch (nm)	Tn-H present?	Resting stiffness	Tension†	ATPase	Oscillatory work‡
Libellula quadrimaculata	36	4	38	Yes	Medium	No	–	No
Rabbit	42.5	3	38	No	Low	No	No	Yes
Lethocerus indicus	38	4	38	Yes	High	Yes	Yes	Yes

The three properties of the muscles refer to those discussed in the Introduction and Discussion, for comparison with the stretch activation parameters, maintained activation of tension and ATPase activity by small imposed stretch, and production of oscillatory work.

* Start, the number of helical tracks of myosin heads on the thick filament surface.

† Amplitude of tension after a stretch of active muscle more than doubles the steady-state tension.

‡ For length oscillations of more than 0.3 % peak-to-peak amplitudes.

Tn-H, troponin-H.

activation was that a small (1 % of muscle length) stretch produced a large change in active tension of more than double the steady-state tension.

Materials and methods

Dragonflies of the species *Libellula quadrimaculata* were captured from Skipwith Common, near York. To demembranate the synchronous flight muscle, each dragonfly was anaesthetised with diethyl ether, the head and abdomen were removed, and the thorax was cut into two halves along the dorsal midline, using scissors, and placed in a 50 % glycerol solution on ice. The solution contained: glycerol, 50 % (v/v); potassium phosphate buffer, 20 mmol l^{-1}; sodium azide, 1 mmol l^{-1}, dithiothreitol (DTT), 1 mmol l^{-1}; $MgCl_2$, 2 mmol l^{-1}; pH 7.0 (as described previously; Peckham *et al.* 1990). It was kept immersed in this solution for about an hour, on ice. The thorax was then transferred to fresh solution and stored at −20 °C. The fibres were used between 5 days and 4 weeks after demembranation.

Lethocerus indicus were obtained from Dr R. Sanit from Thailand and the preparation of the thoraces for demembranation of the asynchronous flight muscles was the same as that described previously (Peckham *et al.* 1990). The thoraces were stored in the same solution and under the same conditions as those for the dragonfly thoraces. Chemically skinned rabbit muscle fibres were prepared as described previously (Peckham and Irving, 1989). Both *L. indicus* and rabbit fibres were used between 5 days and 2 months after demembranation.

A short description of the experimental details is given below. All the procedures for fibre dissection, crimping in T-clips, mounting of the fibres on the experimental rig, the design of the rig, storage and analysis of experimental data, and the compositions of the experimental solutions were essentially as described earlier (Peckham *et al.* 1990). Solution compositions are repeated here (Table 2), together with the compositions of some other solutions used. Rest length for each

Table 2. *Solution compositions*

Constituent	A Relaxing	B Rigor	C Activating	D Activating	E Activating	F Pre-activating
KCl (mmol l^{-1})	12	50	12	–	12	–
$MgCl_2$ (mmol l^{-1})	14	12	14	14	5	14
EGTA (mmol l^{-1})	5	5	–	–	–	0.1
Ca^{2+}-EGTA (mmol l^{-1})	–	–	5	5	5	–
ATP (mmol l^{-1})	15	–	15	15	15	5
PO_4^{3-} (mmol l^{-1})	–	–	–	10	10	–
Histidine (mmol l^{-1})	–	20	20	–	–	20
Sodium creatine phosphate (mmol l^{-1})	–	–	–	–	8	–
Creatine kinase (mg ml^{-1})	–	–	–	–	1	–

All solutions had a pH of 7.0.

muscle type was defined as that at which the muscle length was just taut in relaxing solution. The instantaneous stiffness (if any) and tension (if any) of each fibre type were measured in relaxing (ATP present, but no calcium), activating (ATP and calcium present) and rigor (no ATP, no calcium) solutions. Instantaneous stiffness was estimated from the slope at the steepest part of the curve of a plot of tension against length during a ramp change in length (350 μs in duration), sampled at a high rate. The delayed tension response to a step increase in length of 1 % in activating solution was analysed as the sum of three exponentials, using the curve-fitting program DISCRETE (Provencher, 1976*a*,*b*). Phase 2 was the initial fall in tension following the stretch (amplitude $A2$, rate constant $r2$), phase 3 was the secondary rise in tension (amplitude $A3$, rate constant $r3$) and phase 4 was the final fall in tension (amplitude $A4$, rate constant $r4$). The exponential fits to the data were used to compare quantitatively the responses for each of the three species. For sinusoidal analysis, the length signal to drive the motor was derived from a transfer function analyser (Solatron type JM 1600), which also served for analysis of the resulting tension and of the motor output. The tension signals were corrected for this measured performance of the motor. The data are presented in the form of Nyquist plots.

The delayed tension response was recorded for fibres in an activating solution containing 10 mmol l^{-1} inorganic phosphate, since, in fibres from both dragonfly and rabbit, tension is lower and better maintained in the presence of phosphate than in its absence. Dragonfly and rabbit fibres were both washed in a pre-activating solution (Table 2) before activation. The pre-activating solution contains a lower concentration of EGTA (0.1 mmol l^{-1}) which buffers calcium less well than the relaxing solution (5 mmol l^{-1} EGTA). When activating solution (containing Ca^{2+}-EGTA) is added, the increase in free [Ca^{2+}] is fast, allowing uniform activation of the fibre (Moisescu, 1976).

Tensions and stiffnesses were normalised for the number of thick (A or myosin-containing) filaments in each preparation. In experiments using *L. indicus* muscle, whole fibres were always used (mean A-filament content per fibre of 0.59×10^6, Peckham *et al.* 1990). For rabbit muscle, single fibres were used and at the end of each experiment the fibres were fixed and sectioned for electron microscopy. The number of thick filaments per fibre was estimated by measuring the total area of the preparation from transverse sections, from the number of A-filaments per μm^2 and from the percentage myofibrillar content. The A-filament content per rabbit fibre was found to vary from 3.1×10^6 to 4.6×10^6. This number is greater than that of *L. indicus* even though the diameters of the skinned fibres are similar because the area occupied by mitochondria is much greater in *L. indicus*. For dragonfly, fibres were too small (about 20 μm in diameter) to use singly, so bundles of 3–5 fibres were used. At the end of each experiment, the fibres were fixed and sectioned for electron microscopy. The A-filament content was estimated from light and electron micrographs in the same way as described for the leg muscles of *L. indicus* (Peckham *et al.* 1990). The mean A-filament content of 11 dragonfly fibre preparations was $0.8\times10^6\pm0.1\times10^6$.

Results

In dragonfly flight muscle, there was a large rise in tension when demembranated fibres were activated by calcium (Table 3). The active tension was similar in magnitude to that of demembranated rabbit psoas fibres but about 7–8 times greater than that seen in the demembranated asynchronous flight muscle fibres from *L. indicus*. After subtraction of the relaxed stiffness, the active stiffness of dragonfly flight muscle was about 60 % of that of rabbit psoas muscle, but about four times greater than that of *L. indicus* flight muscle (Table 3).

All three muscle types showed a delayed tension increase following a rapid stretch, of 1 % of rest length, in activating solution (phase 3, Fig. 1). The tension transient that followed the stretch was analysed as the sum of three exponentials (see Materials and methods). This analysis showed that the amplitudes of phase 3, ($A3$, Table 4), roughly equivalent to the amplitude of the delayed tension increase, were similar for the three types of muscle (all at rest length, or unstretched, when activated). However, this amplitude was almost double the steady-state active tension in *L. indicus* flight muscle, but only 29 % of the steady-state active tension in rabbit psoas muscle, and only 18 % of that in dragonfly flight muscle. Furthermore, when active *L. indicus* flight muscle is pre-stretched by 1 %, the amplitude of phase 3 following a rapid stretch of 1 % was about double that of the unstretched muscle (Fig. 1). A small pre-stretch did not alter the amplitude of phase 3 for fibres from rabbit psoas or dragonfly flight muscle. The amplitudes of phase 2, roughly equivalent to the amplitude of the initial tension recovery ($A2$, Table 4; phase 2, Fig. 1), were similar in magnitude for dragonfly flight muscle and rabbit psoas muscle, but much smaller in *L. indicus* flight muscle. In contrast, the amplitudes of phase 4, roughly equivalent to the amplitude of the final tension relaxation ($A4$, Table 4; phase 4, Fig. 1), were similar in magnitude in dragonfly flight muscle and *L. indicus* flight muscle, but much less than the amplitude of phase 4 in rabbit psoas muscle.

The rate constant for phase 3 of the tension response was about twice as great following a stretch for dragonfly flight muscle ($r3$, Table 4) than for *L. indicus*

Table 3. *Steady-state tensions and stiffnesses*

	Libellula quadrimaculata	Rabbit	*Lethocerus indicus*
Steady-state tension (pN per A-filament)			
Active	245±27 (11)	214±15 (5)	30±3 (9)
Rigor	46±5 (9)	84±9 (5)	72±10 (11)
Stiffness (pN per A-filament per % length change)			
Relaxed	22±4 (11)	3.5±1.0 (5)	98±9 (12)
Active	213±33 (11)	329±48 (5)	147±17 (9)
Rigor	230±47 (10)	318±49 (5)	581±71 (11)

Values are given as mean ± standard error of the mean, with the number of fibres tested in brackets.

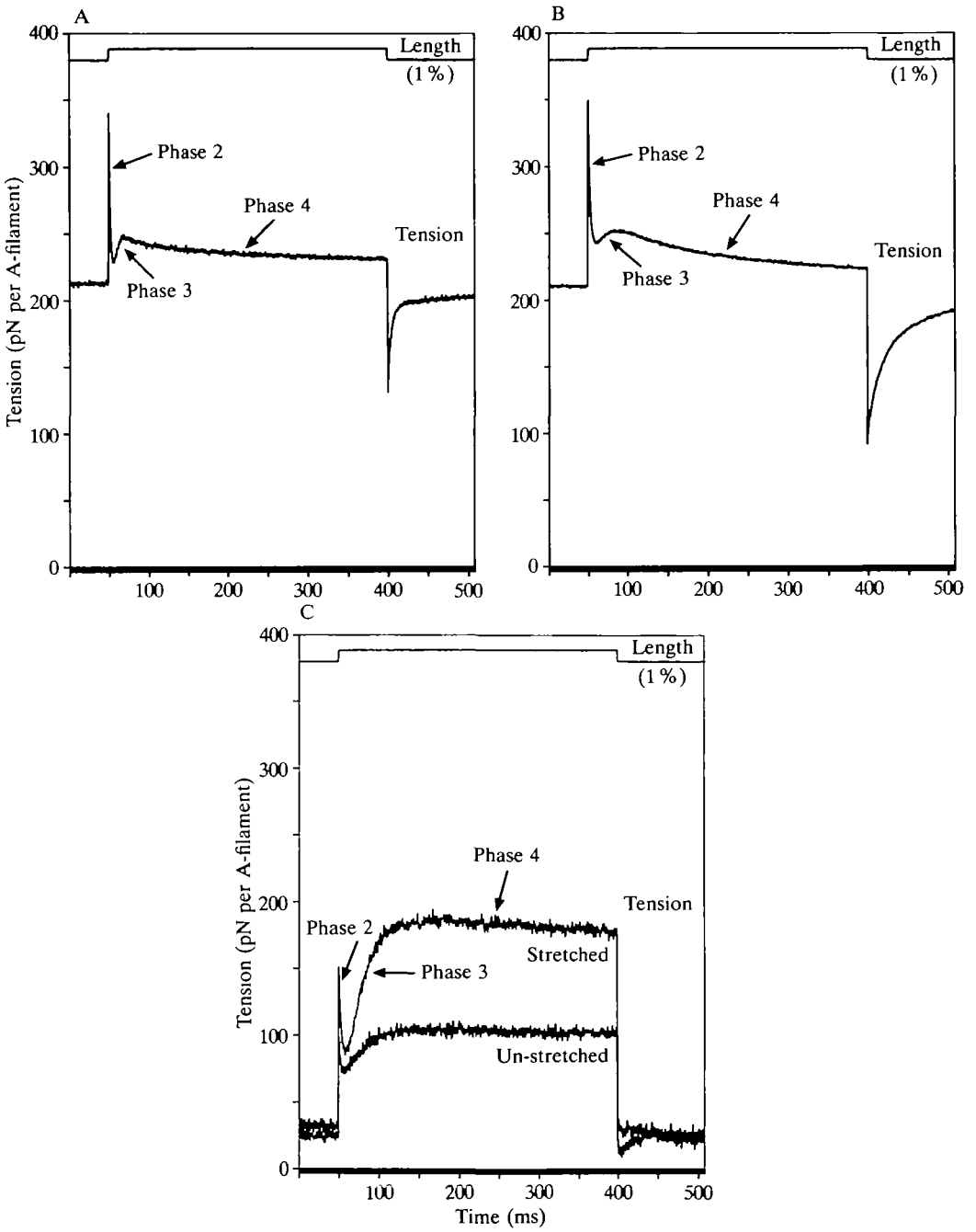

Fig. 1. Comparison of the tension responses in activating solution containing 10 mmol l^{-1} phosphate (Table 2) to a small rapid stretch of 1 % of the muscle length for demembranated fibres from dragonfly flight muscle (A), rabbit psoas muscle (B) and *Lethocerus indicus* flight muscle (C). The response for *L. indicus* is shown for a fibre activated at rest length (unstretched) and activated after a 1.5 % pre-stretch (stretched). All the responses are shown for the same time base and for the same tension scale.

Table 4. *Rate constants and amplitudes*

Variable	*Libellula quadrimaculata*	Rabbit	*Lethocerus indicus*
$A0$	15±3 (7)	38±6 (5)	48±8 (6)
$A2$	150±45 (5)	170±27 (5)	39±4 (6)
$A3$	−45±13 (7)	−63±12 (5)	−56±9 (6)
$A4$	15±3 (7)	122±19 (5)	13±2 (6)
$r2$	677±151 (5)	264±12 (5)	207±23 (6)
$r3$	136±8 (7)	66±16 (5)	53±4 (6)
$r4$	10±2 (7)	6±1 (5)	9±1 (4)

The tension response to a 1% stretch was fitted by the equation: $A_0+A_2e^{-r_2t}+A_3e^{-r_3t}+A_4e^{-r_4t}$, where A_0–A_4 are amplitudes (pN per A-filament) and r_1–r_4 are rate constants (s^{-1}). These are only analytical solutions to fit the curves, and are not intended to imply any mechanism (Thorson and White, 1983).

Values are given as mean±standard error, with the number of fibres tested in brackets.

flight muscle and for rabbit psoas muscle. The rate constant for phase 2 ($r2$, Table 4) following the stretch was greatest for dragonfly flight muscle, and similar in magnitude for rabbit psoas and *L. indicus* flight muscles.

The tension responses following the subsequent step decrease in length (1%) were monotonic relaxations in dragonfly and rabbit muscles (Fig. 1). There was a small delayed decrease of tension for *L. indicus* flight muscle following small amplitudes of length decrease, but for larger amplitudes this delayed decrease in tension was absent (see also White and Thorson, 1972).

Sinusoidal oscillations over a restricted range of frequencies at full calcium activation (pCa 4.5) produced positive work loops in both rabbit psoas and *L. indicus* flight muscle but not in dragonfly flight muscle for amplitudes greater than 0.3% (data not shown). In an effort to find conditions in which work loops might be produced in dragonfly flight muscle, the conditions of the activating solution were altered in various ways: an ATP regeneration system was added (Table 2), orthophosphate concentration was varied between 0 and 10 mmol l^{-1}, temperature was reduced from 15 to 10°C, and the calcium concentration was reduced to give sub-maximal activating conditions. The amplitude of the oscillations was varied between 0.3 and 1% but no small-signal analysis at very low amplitudes (<0.3%) was made.

At a reduced calcium concentration (pCa 5.79) and in the presence of an ATP regeneration system, one dragonfly fibre preparation, out of six, nearly produced positive work (see Nyquist plot, Fig. 2). Both *L. indicus* flight muscle and rabbit psoas muscle produced positive work under these conditions (Fig. 2). Rabbit psoas muscle produced greatest work output during sinusoidal oscillations at pCa 6.02, in the presence of inorganic phosphate and an ATP regeneration system. In comparison, in the presence of phosphate, *L. indicus* flight muscle produced greatest work output at pCa 4.5 (maximal calcium concentration) and an ATP regeneration system was not required.

The relaxed stiffness of dragonfly flight muscle was higher than that of rabbit psoas but lower than that of *L. indicus* flight muscle (Table 3). The stiffness may be related to the length of the I-band at body length (White, 1967). The I-band is shortest in *L. indicus* flight muscle and longest in rabbit psoas. Rigor stiffnesses and tensions were generally similar for all the muscle types, except that rigor tension and stiffness was slightly lower for dragonfly flight muscle and rigor stiffness was highest in *L. indicus* flight muscle even after subtraction of the relaxed stiffness.

Discussion

Dragonfly synchronous flight muscle is not stretch-activated as is *L. indicus* flight muscle: steady-state tension is high in the presence of calcium, length changes do not significantly increase steady-state tension, and the muscle is unable to perform oscillatory work for length oscillations of greater than 0.3 % in amplitude. However, this muscle does have a high relaxed stiffness which is intermediate between that of *L. indicus* asynchronous flight muscle and that of rabbit psoas.

At resting lengths, the higher tension and stiffness, normalised for A-filament content, of active dragonfly flight muscle compared to *L. indicus* flight muscle is not due to a difference in A-filament length such that there are more crossbridges in parallel, because the number of crossbridges is the same (Table 5). The higher active tension and stiffness of dragonfly flight muscle is therefore more likely to be due to a greater number of attached crossbridges at rest length in this muscle. However, stretching *L. indicus* flight muscle does give comparable tensions to those of dragonfly flight muscle (Fig. 1), and the stiffness of the muscle also increases (White, 1983), confirming the idea that stretching *L. indicus* flight muscle increases the number of crossbridges attached at any instant. The lengths of the A-filaments in dragonfly flight muscle and rabbit psoas are different, however. Assuming in these two muscles that all the crossbridges contribute equally to tension, the tension per crossbridge of dragonfly flight muscle (0.8 pN) is of the same order of magnitude as that of rabbit psoas muscle (1.4 pN), when fully activated.

By comparing the presence of stretch-activation with the presence of each of the different properties suggested to give rise to it (Table 1), the property most important for stretch-activation can be determined. Of the three different species described, only the flight muscle of *Lethocerus indicus* is stretch-activated. It has

Table 5. *Sarcomere dimensions*

	A-filament length (μm)	Number of crossbridges per half sarcomere
Libellula quadrimaculata	2.4	315
Rabbit	1.6	155
Lethocerus indicus	2.4	315

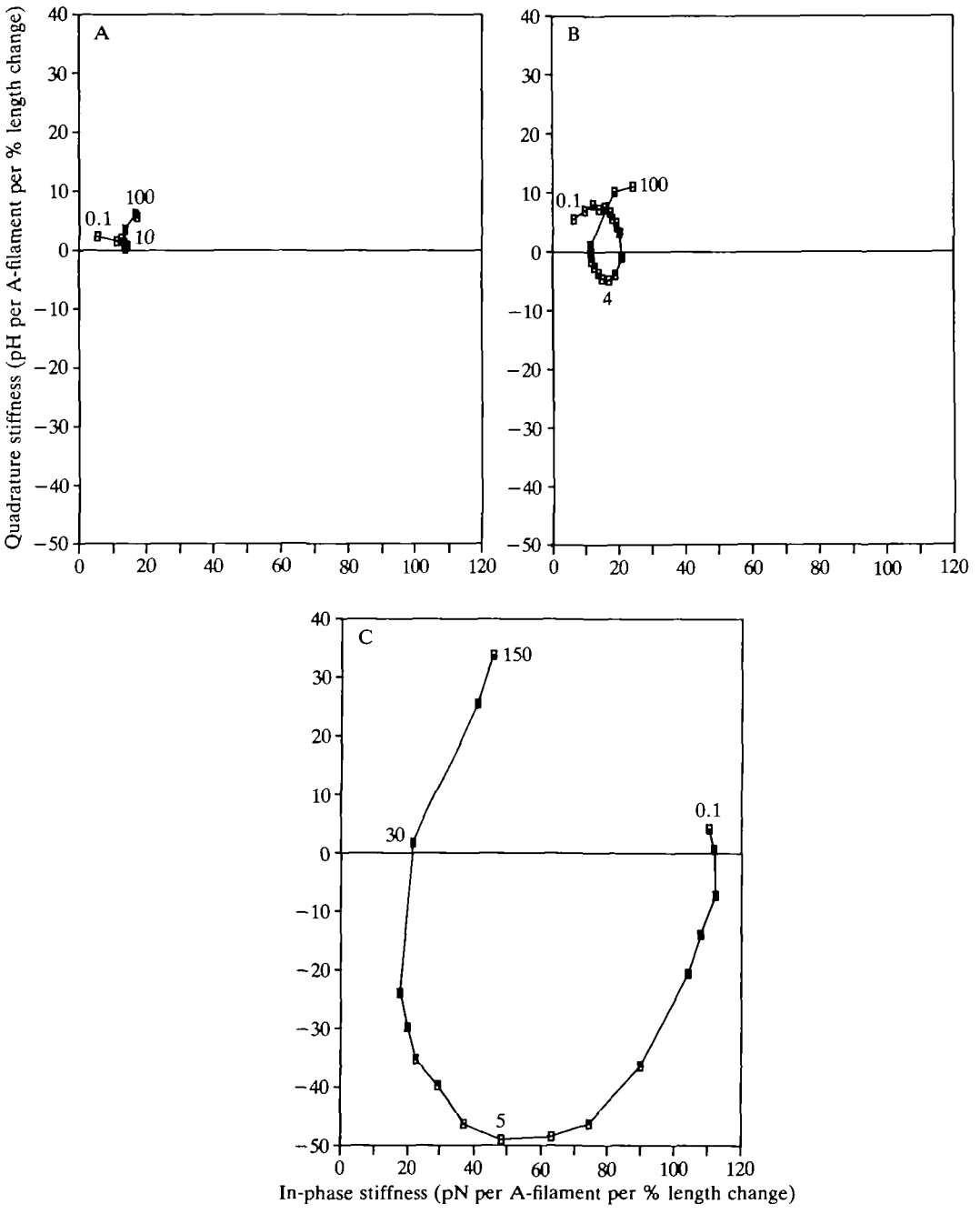

Fig. 2

all three properties: (1) helix matching, (2) Tn-H and (3) high stiffness present in the active muscle and possibly giving rise to strain on the thick filament. Rabbit psoas muscle is not stretch-activated and has none of these three properties

Fig. 2. Sinusoidal analysis using the transfer function analyser (see Materials and methods) of the tension response of a single preparation of demembranated fibres from *Libellula quadrimaculata* flight (A), rabbit psoas (B) and *Lethocerus indicus* (C) flight muscle to an imposed length oscillation presented as a Nyquist plot using the same scales for each species. The numbers by the side of the data points show the frequency of the imposed length oscillation (in Hz). The amplitude of the length oscillation was 1 % peak-to-peak for both dragonfly and rabbit, and 0.5 % for *L. indicus* muscle. The conditions were: for dragonfly, activating solution containing 10 mmol l^{-1} phosphate and an ATP regeneration system (E, Table 2), pCa 5.79: for rabbit, activating solution containing 10 mmol l^{-1} phosphate and an ATP regeneration system (E, Table 2), pCa 6.02: for *L. indicus*, activating solution containing 10 mmol l^{-1} phosphate, pCa 4.5 (D, Table 2).

Dragonfly flight muscle is not stretch-activated, but it does have a substantial resting stiffness, which, like that of *L. indicus* flight muscle, does not decrease on activation (White *et al.* 1979). It also contains a protein that is probably Tn-H (Peckham *et al.* 1991) because it shares two epitopes with the hydrophobic region of the Tn-H found in *L. indicus* flight muscle and it has a similar relative molecular mass (Bullard *et al.* 1988). However, dragonfly flight muscle does not have helix matching.

From this comparison it seems most likely that matching of the helix pitch of the myosin heads on the thick filaments to that of the actin target sites on the thin filaments is the property most important for stretch activation (Table 1). Dragonfly flight muscle is probably not stretch-activated because it does not have this helix matching. It is possible that filament matching alone does not give rise to stretch-activation in *L. indicus* flight muscle, but that a combination of helix matching, high resting stiffness and the presence of Tn-H are all required. However, either high resting stiffness and/or the presence of Tn-H are not, in themselves, sufficient to cause stretch activation. Previous modelling (Thorson and White, 1983) showed that maintained stretch-activation (in this case, maintained ATPase activity) could be produced either by strain on the thick filaments or by a displacement model, arising from the helix matching. The data presented here tend to support the displacement model for stretch-activation.

One might think that the absence of oscillatory work in the demembranated flight muscle of the dragonfly, at amplitudes of 0.3 % or greater, is somewhat surprising. Other demembranated synchronous muscles from the cicada (Aidley and White, 1969) and rabbit (Goodall, 1956; Abbott and Steiger, 1977, and this study) are able to produce net positive oscillatory work at these amplitudes when calcium-activated. Oscillatory work might be expected because there is a delayed tension increase in response to a step increase in length. However, the response to a step decrease in length shows no delayed decrease in tension, and a large tension relaxation (positive viscosity) over the relevant time course. Net positive oscillatory work is prevented by this viscous response to a step decrease in length, which dampens the negative viscous effect of the delayed tension increase in response to a step increase in length. In rabbit psoas muscle, the dampening effect in response

to a step decrease in length at the relevant time constant is reduced, allowing positive oscillatory work to be done in response to sinusoidal input. The amplitudes used here to look for oscillatory work are of similar size to those used to power flight in the dragonflies. There is no reason why the ability of dragonfly flight muscles to produce oscillatory work during flight should be a property of the myofibrils, and hence present in demembranated fibres at these amplitudes. However, as we did not explore small-signal analysis at smaller amplitudes (less than 0.3%), we cannot say that, at such small amplitudes, oscillatory work would not be seen in demembranated dragonfly flight muscle.

In the live dragonfly, flight is probably driven by oscillatory work produced by the flight muscle in response to phasic nervous stimulation, as in other species with synchronous flight muscles. Isolated intact synchronous muscles can produce net positive oscillatory work by cyclic nervous stimulation of a muscle undergoing cyclic changes of length, in which the timing of the nervous input to the muscle in each cycle is such as to cause tension to be delayed with respect to the length changes (Stephenson and Josephson, 1990; Altringham and Johnston, 1990*a*,*b*; Curtin and Woledge, 1989). Such work loops have been reproduced in the isolated intact dragonfly flight muscle (J. E. Molloy and M. Peckham, unpublished results).

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