

POWER OUTPUT OF GLYCERINATED BUMBLEBEE FLIGHT MUSCLE

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Summary

The properties of asynchronous insect flight muscle have been examined using a glycerol-extracted single-fibre preparation of dorsal longitudinal muscle from the bumblebees *Bombus lucorum* and *B. terrestris*. Chemical, mechanical and thermal conditions were controlled with the objective of maximizing power output. The problems arising from diffusion limitation were avoided through a combination of fibre paring and the use of an ATP backup system. Work and power output tended to increase with increasing oscillatory strain in the range 1–5%. Workloop shape, and hence work and power, varied with fibre extension; optimum extensions ranged from 4 to 12%. The mechanical performance of glycerinated bumblebee muscle fibres was strongly temperature-dependent, and rate processes (frequency, power) displayed higher thermal sensitivities than processes associated with tension development (work). The experimental conditions that maximized the power output were identified as: oscillatory strain $\epsilon=4\text{--}5\%$, extension $\epsilon_0=8\text{--}10\%$, oscillation frequency $f=50\text{Hz}$ and temperature $T=40^\circ\text{C}$. The maximum power output observed under these 'optimal' conditions was about 110 W kg^{-1} (muscle), demonstrating for the first time that glycerinated fibres are capable of producing the power predicted from free-flight studies to be required for flight: 100 W kg^{-1} .

Introduction

Insect flight muscle may be either synchronous, in which there is direct nervous stimulation of each muscle contraction, or asynchronous, in which the wingbeat frequency and the rate of nervous stimulation are independent. For an insect to fly, the mechanical power generated by the flight muscles must be sufficient to overcome the air resistance and internal friction of the flight motor. Based on theoretical models of muscle performance (Weis-Fogh and Alexander, 1977; Pennycuik and Rezende, 1984), Ellington (1985) has estimated that mechanical power outputs of 80 and 100 W kg^{-1} (muscle) should be attainable for synchronous and asynchronous muscle, respectively.

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Aerodynamic analyses of the power requirements for flight have yielded similar values (e.g. Casey, 1981; Ellington, 1984, 1991; Casey *et al.* 1985; Casey and Ellington, 1989).

Power output of the required magnitude has been obtained in experiments on live, isolated synchronous muscle (Josephson, 1985; Mizisin and Josephson, 1987; Stevenson and Josephson, 1990), but this is not the case for asynchronous muscle preparations. With live, isolated flight muscle from bumblebees and beetles, Machin and Pringle (1959) found maximum power outputs of 60 and 30 W kg⁻¹, respectively. Most of the work on asynchronous muscle, however, has used glycerol-extracted fibres from the giant waterbug *Lethocerus*. Membrane systems are osmotically ruptured by glycerol extraction, leaving only the myofibrils functioning in glycerinated fibres. Power outputs reported for this preparation are lower than those of live, isolated muscles: from 8–10 W kg⁻¹ (Pringle and Tregear, 1969; Steiger and Rüegg, 1969) to 43 W kg⁻¹ (Molloy, 1988). The major drawback to the glycerinated fibre preparation is that ATP requirements are met only by diffusion: if the rate of diffusion is too low, the core of the fibre suffers ATP depletion, and the fibre enters a 'high-tension state' characterized by low frequency characteristics and minimal work output (Jewell and Rüegg, 1966; Pringle, 1967). Attempts to maximize the power output of glycerinated fibres have been greatly hampered by diffusion limitation (e.g. Jewell and Rüegg, 1966; Pringle and Tregear, 1969; Molloy, 1988). Furthermore, as it is difficult to induce flight in captive waterbugs, little is known about their flight physiology or aerodynamics (Barber and Pringle, 1966). Their popularity for muscle studies is based on their large size and the regularity of their muscle structure (e.g. Pringle, 1977; Reedy and Garrett, 1977).

The purpose of this study was to determine whether glycerinated fibres are capable of producing the power predicted to be necessary for flight. Bumblebees were chosen as the experimental animal because a large body of data has been collected on their flight mechanics and energetics (e.g. Heinrich, 1979; Ellington, 1984; Dudley and Ellington, 1990*a,b*). Diffusion limitation was avoided through a combination of fibre paring and the use of an ATP backup system, allowing responses to be characterized over the range of thoracic temperatures observed *in vivo*: 20–40°C (Heinrich, 1979; Joos *et al.* 1991). A set of experimental conditions that maximized power production was identified and compared to the operating conditions *in vivo*.

Materials and methods

Experimental material

Queen and worker bumblebees of the species *Bombus lucorum* and *B. terrestris* (Hymenoptera: Apidae) were collected in the Cambridge University Botanic Garden. The results for the few (28) fibres taken from *B. terrestris* have been pooled with those from *B. lucorum* (95 fibres): note that they are closely allied species of similar size and wingbeat frequency (Alford, 1975). Insects were anaesthetized by cooling. Following removal of the head, abdomen and legs, the thorax was sagittally bisected with a scalpel. The dissected thoraces were immersed in glycerination solution and left at 4°C for 24h; the solution was changed after 1 and 6h. After 24h, the thoraces were stored in fresh

solution at -20°C . The composition of the glycerination solution was based on that of Molloy (1988): 50% (v/v) glycerol, 20mmol l^{-1} potassium phosphate buffer [a 1:2 (v/v) mixture of 1mol l^{-1} K_2HPO_4 : 1mol l^{-1} KH_2PO_4], 5mmol l^{-1} sodium azide, 2mmol l^{-1} dithiothreitol and 0.4% (v/v) Triton X-100, adjusted to pH7.0 with 1mol l^{-1} KOH. Muscle fibres were used between 2 and 42 days after glycerination.

Single glycerinated fibres were dissected from the dorsal longitudinal muscle (DLM) mass of the thorax, and pared down to approximately one-quarter of their original cross-sectional area. The fibre ends were crimped in T-clips (Ford *et al.* 1977) cut by hand from aluminium foil (Molloy, 1988). The dissection was performed in glycerination solution which was allowed to warm gradually from -20°C to room temperature. Throughout this paper, the term 'fibre', with respect to bumblebee experiments, refers to pared fibres.

Mechanical apparatus

The muscle rig was similar in design to those used by other workers (e.g. Griffiths *et al.* 1979; Cuminetti and Rossmann, 1980; Peckham *et al.* 1990). The fibre was suspended in a $100\ \mu\text{l}$ bath by slipping the T-clips over two electrolytically sharpened tungsten hooks. One hook was bonded to the beam of an undamped force transducer (Akers AE801, SensoNor) fitted to a brass housing and mounted on a three-way micromanipulator (Prior, 62899). The second hook was attached to the spindle of a servo-controlled mechanical vibrator (Ling Dynamic Systems model 101). A custom-built function generator controlled the movement of the mechanical vibrator. The output of the function generator was a desired number of cycles of a waveform of a specified type (sine or square), frequency, amplitude and offset. The oscillation amplitude (muscle fibre oscillatory strain, ϵ) and offset (extension, ϵ_0) were controlled by potentiometers as a percentage of the initial muscle length l_0 , which was similarly dialled into the function generator. The temperature of the Perspex incubation bath was detected by a sensor embedded in the bath block, and controlled with a Peltier effect heat pump in a feedback circuit.

The noise level of the amplified force transducer signal, at a sensitivity of $100\ \mu\text{NV}^{-1}$, was $2\ \mu\text{N}$. When the undamped transducer was loaded with a muscle fibre, resonance was observed at frequencies of 5kHz and 1kHz. The maximum excursion amplitude of the vibrator was 1mm peak-to-peak (p-p). A measure of vibrator performance is the rise time in response to a square-wave input; for a 0.4mm p-p square wave the 90% rise time was under 1ms, while a 1mm p-p (maximum) square wave elicited a 90% rise time of only 1.5ms. Measured values for frequency, oscillatory strain and extension were within 1% of the values set on the function generator. The accuracy of the temperature regulation was $\pm 0.5^{\circ}\text{C}$ with a precision of $\pm 0.3^{\circ}\text{C}$. Negligible overshoot occurred when the set temperature was changed. The maximum heating and cooling rates were $33^{\circ}\text{Cmin}^{-1}$ and $21^{\circ}\text{Cmin}^{-1}$, respectively.

Data acquisition

Direct-current voltage signals proportional to length and force were digitized with a Digital Storage Adaptor (Thurlby, DSA524), displayed on an oscilloscope, and transferred to a BBC Master microcomputer. The function generator settings and the

incubation bath temperature (as d.c. voltages) were read into the computer with a 12-bit, eight-channel a/d converter (CUBAN-12, Control Universal Ltd). Programs were written in BBC Hi-BASIC to control the transfer and storage of data, to plot force-length curves (workloops) and to calculate work and power from the area of the workloops.

Fibre mass estimation

Work and power were normalized for the mass of the fibre. A projection microscope produced an enlarged image of a fibre suspended vertically under slight tension in a vial of glycerination solution. The fibre was mounted so that it could be rotated through 180° about its longitudinal axis. Diameter measurements were made at 10° intervals at several points along the length and calibrated against a wire of known diameter. Areas were then calculated assuming that the cross section was an irregular polygon, and the fibre volume, exclusive of the T-clip sections, was determined as the product of length and mean cross-sectional area. Multiplication of the volume by a density of 1060 kg m⁻³ (Méndez and Keys, 1960) yielded the muscle fibre mass. The assumption made in this calculation is that the glycerinated fibre cross-sectional area is representative of that of a live fibre. To test this assumption, the diameters of 6–10 fibres in each of five bisected thoraces were measured *in situ* before and after glycerination; no significant difference ($P=0.06$) was detected. Although swelling of mechanically skinned fibres is well documented (Matsubara and Elliot, 1972; Godt and Maughan, 1977), chemical skinning using either detergents or glycerol seems to have little, if any, effect on fibre diameter (J. D. Altringham, personal communication; Rome, 1967; Altringham, 1981; Matsubara *et al.* 1985).

Given the small oscillatory strains (typically <12%) used in this study, changes in cross-sectional area during extension should be negligible. To verify this, single fibres were fixed in T-clips and diameter measurements were made before and after stretching to the point of breakage. The diameter after breakage was, on average, 97% (S.D.=4%, $N=40$) of the pre-stretch value. Experimental fibres were never stressed to this degree, and changes in cross-sectional area are therefore not significant under all conditions. For the 196 pared fibres on which diameter measurements were made, the mean diameter was 66 μm (S.D.=11 μm) and the mean length was 1.2mm (S.D.=0.2mm), giving a mean mass of 4.6 μm (S.D.=2.0 μm).

Fibre preparation and the standardized workloop (SWL)

Glycerinated asynchronous fibres are activated by stretch and by calcium ions, so the mounted fibre was initially submerged in a calcium-free 'relaxing' solution (composition: 15mmol l⁻¹ ATP, 8mmol l⁻¹ sodium creatine phosphate, 2mgml⁻¹ creatine kinase, 15mmol l⁻¹ MgCl₂, 20mmol l⁻¹ histidine, 6mmol l⁻¹ EGTA, ionic strength 120mmol l⁻¹, pCa8, pH7.0). The rest length l_0 was set by adjusting the force transducer manipulator such that the fibre was just taut (≤ 10 μN force). The rest length was measured with a graticule in the eyepiece of a binocular microscope, and the value was dialled into the function generator. The relaxing solution was then replaced with an 'activating' solution containing free calcium ions (composition as for the relaxing solution, but with the addition of 6mmol l⁻¹ CaEGTA, giving an ionic strength of

121 mmol l⁻¹ and a pCa of 4.7), and force changes were monitored for 1–3 min, after which experiments were initiated. For almost all fibres, this set-up protocol was followed by the collection of a standardized workloop (SWL) to measure fibre ‘quality’. The fibre was extended by 2% and isometric force changes were recorded for 3 min. The SWL was then acquired using the following settings: extension $\epsilon_o=2\%$, oscillatory strain $\epsilon=1\%$, frequency $f=15\text{Hz}$ and temperature $T=20^\circ\text{C}$.

Experimental procedures for quick stretch trials

Step length changes of 1%, lasting for approximately 60 ms, were applied to fibres held at an extension of 2%. Transient force responses were recorded at 5°C intervals from 20 to 40°C with the DSA and BBC microcomputer. A least-squares fitting program was used to model the data as a sum of up to four exponential processes. The program was written by N. J. Carter (Department of Biology, University of York) for QuickBasic 4.5 on a PC. File-handling and data-windowing routines were written by Dr J. E. Molloy (Department of Biology, University of York). Values for the rate constant of the relaxation phase τ_4 may be unreliable, since the duration of this fourth phase was restricted by the period of the step change (e.g. Molloy, 1988).

Experimental procedures for workloop trials

Early experiments led to a standard protocol in which the mechanical parameters of extension ϵ_o , oscillatory strain ϵ and frequency f were varied systematically. Following the collection of a SWL, a fibre was extended by a pre-determined amount (set values of $\epsilon_o=2, 4, 6, 8$ or 10%) and a reference loop was acquired ($\epsilon=1\%$, $f=15\text{Hz}$, $T=20^\circ\text{C}$ and $\epsilon_o=\text{set value}$). Using an oscillatory strain of 1%, workloops were then collected at 5 Hz intervals from 5 or 10 Hz to $f > f_{p,\text{max}}$, where $f_{p,\text{max}}$ is the frequency for maximum power production. This procedure was repeated at integral values of strain from 1% until the strain was too high for the set extension, and the fibre went slack for part of the length cycle (‘tailed’ workloop). A reference loop was collected after the frequency sweep at each strain value. One glycerinated fibre was used for each extension, so a complete set of measurements required five fibres from an individual bee.

Fibres used in workloop temperature trials were set up normally and the SWL was collected. At each temperature, workloops were collected at 5 Hz intervals from $f < f_{w,\text{max}}$ to $f > f_{p,\text{max}}$, where $f_{w,\text{max}}$ is the optimum frequency for work production. Specific combinations of oscillatory strain and extension (termed treatments) were chosen, and one fibre was used for each combination: $\epsilon=1\%$ with $\epsilon_o=2\%$ (referred to as low strain, low extension or LSLE), $\epsilon=1\%$ with $\epsilon_o=8\text{--}10\%$ (low strain, high extension or LSHE), and $\epsilon=4$ or 5% with $\epsilon_o=6\text{--}8\%$ (high strain, high extension or HSHE). The temperature was raised in 3°C steps from 20 to 41°C, and a 1 min equilibration period was imposed following each temperature increase. Trials were terminated if the workloops collapsed or the optimum frequencies were substantially lower after a temperature increase; these were signs of diffusion limitation (Jewell and Rüegg, 1966).

In maximum power trials, the standard set-up was used, but SWLs were not collected since this would have damaged the fibre. The temperature was raised from 20°C to 40–44°C, and the fibre was extended from the rest length by 2%. An oscillatory strain of

4–5% was selected, since this had previously been shown to maximize power. The frequency for maximum power production at an extension of 2% was determined quickly by scanning a range of frequencies and inspecting the workloop size; all subsequent workloops were collected at this frequency. Having set the temperature, oscillatory strain and frequency, workloops were acquired at progressively larger extensions until the fibre was overstretched, causing a reduction in power.

Work and power tended to decrease over the course of experiments. The extent of the reduction varied considerably among fibres and seemed to depend on a number of factors, including the oscillatory strain, frequency, extension, temperature and storage period. Under ‘severe’ conditions, i.e. high ϵ , ϵ_0 , f or T , the work and power decreased rapidly. Thus, to maximize the number of measurements that could be made on a single preparation, parameters were always tested in order of increasing severity. Trials were concluded if the work became very low (e.g. reference work <60% SWL work) as the result of a gradual decline in the performance of the preparation.

Results

Quality of glycerinated fibres

SWLs ($\epsilon=1\%$, $\epsilon_0=2\%$, $f=15\text{Hz}$, $T=20^\circ\text{C}$) were collected for 123 fibres from 25 bees. The SWL work is a linear function of the isometric stress σ_0 at the same extension (Fig. 1), and the slope of the line provides an index of fibre quality. The work per unit mass of fibre W^* done during cyclic contractions is:

$$W^* = \sigma_c \epsilon / \rho,$$

where ρ is the mass density of the fibre (e.g. Pennycuick and Rezende, 1984; Ellington, 1985, 1991). Stress varies throughout the cycle, and the cyclic stress σ_c is a suitably weighted mean value for the difference in stress between the shortening and lengthening phases; in practice, it is calculated from W^* , ϵ and ρ . The slope of Fig. 1 is therefore:

$$\text{slope} = \frac{W^*}{\sigma_0} = \frac{\sigma_c}{\sigma_0} \cdot \frac{\epsilon}{\rho},$$

and is proportional to the ratio of cyclic and isometric stresses for a given oscillatory strain and density. The greater the slope, the larger the proportion of the isometric stress realized in cyclic contractions, and hence the ‘better’ the fibres. By calculating slopes for subsets of interest, comparisons can be made in terms of fibre quality.

Lines were fitted to the data using reduced major axis (RMA) analysis in preference to linear regression because the relative errors in work and isometric stress were likely to be of similar magnitude. Slopes were judged to be significantly different when 95% confidence intervals did not overlap.

RMA lines were initially calculated for the 11 individual bees from which at least five fibres had been used. No significant differences were observed, which justified the pooling of results from different bees. Fibres from queen and worker bumblebees did not differ in performance (Table 1). When fibres were used within 48h of glycerination their performance was better than average (Table 1), but otherwise, the length of storage (3–42

days at -20°C) had no significant effect. Fibres from over-wintered queen bees caught in May were significantly poorer in quality than those from new queens caught in August and September, while fibres from the first generation of worker bees captured in May were significantly better than average (Table 1).

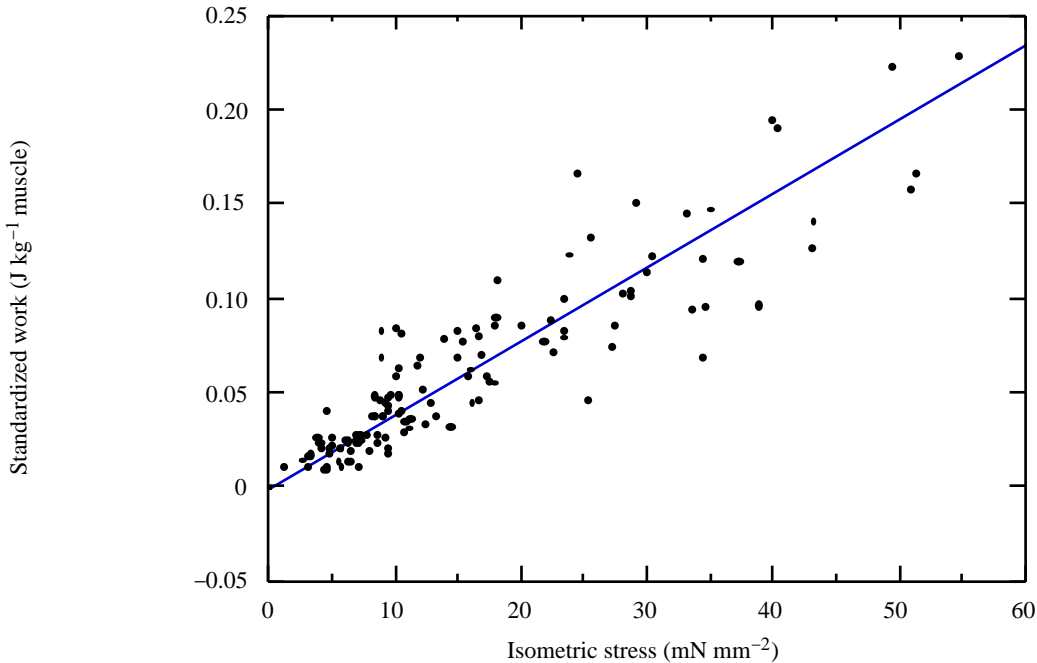


Fig. 1. Fibre quality data from the standardized workloop. SWL work during cyclic contraction is plotted against the isometric stress at an extension ϵ_0 of 2%. The reduced major axis (RMA) line through the pooled data is shown. The y-intercept of the line does not differ significantly from zero, and the line is drawn through the origin for convenience.

Table 1. Slopes and 95% confidence intervals of RMA lines for the SWL data

Data set	N	Slope	95% confidence interval for slope
Pooled	123	0.0039	0.0036, 0.0043
Workers	64	0.0039	0.0035, 0.0044
Queens	59	0.0039	0.0034, 0.0044
1–2 days storage	19	0.0062	0.0048, 0.0080*
Collection date			
May queens	17	0.0033	0.0029, 0.0037†
Aug./Sept. queens	20	0.0048	0.0038, 0.0062†
May workers	11	0.0068	0.0044, 0.011*

* marks a subset which is significantly different from the pooled, and † marks subsets which are significantly different from each other.

Note that the confidence intervals for RMA slopes are asymmetrical.

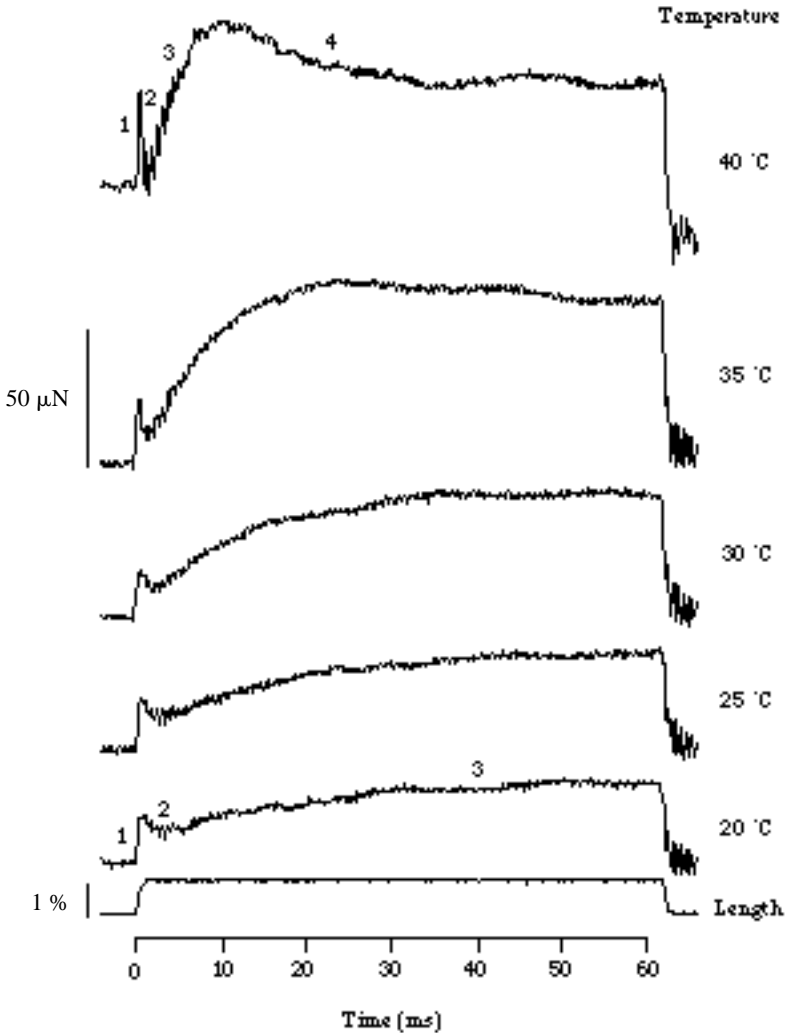


Fig. 2. The effect of increasing temperature on the tension response to a 1% step change in length for a fibre from a worker bee. The phases of the response are indicated for two temperatures (20 and 40°C): (1) a tension increase coincident with length change, (2) a rapid tension fall, (3) a delayed increase in tension and (4) a decay of tension to a new steady-state level. The force scale bar applies to all five traces.

Quick stretches

The transient response of glycerinated bumblebee fibres at 20°C and 25°C to step length changes of 1% generally displayed only three of the four phases which can be observed (Fig. 2). The force initially rose simultaneously with the applied length change (phase 1). This increase was followed by a rapid fall (phase 2) and a subsequent delayed tension rise (phase 3). Normally, the delayed tension increase is succeeded by a slow relaxation (phase 4), as in the responses for 30°C and higher, but this phase was not

Table 2. Rate constants for the exponential processes in the response to a step length change

T (°C)	N	r_2 (s ⁻¹)	r_3 (s ⁻¹)	r_4 (s ⁻¹)
20	6	719±320	42±15	–
25	7	664±369	56±15	–
30	7	1174±412	66±21	5.3±4.3
35	6	1675±198	115±21	39±24
40	3	1695±785	180±66	126±24

N gives the number of different fibres included in the analysis.

Results for queens and workers have been pooled.

Phase 1 is not exponential and hence no r_1 value is given.

Mean ± S.D.

observed in the responses at lower temperatures because the period of the applied length change was too short. The rate constants for phases 3 and 4 (r_3 and r_4) increased with temperature, while r_2 showed a less well-defined thermal sensitivity (Table 2). The relationship between $\log(r_3)$ and temperature was significant; the slope was 0.030, with a 95% confidence interval (C.I.) of ± 0.009 .

Mechanical parameters ϵ , ϵ_o , f

Although work and power varied consistently with oscillatory strain, extension and frequency, these trends were confounded by the high degree of fibre-to-fibre variation. The reduction in work and power frequently observed over the course of an experiment also obscured underlying trends and may have resulted from a variety of sources, including damage and irreversible stretching of passive elastic components in the fibre. To overcome the latter, the extension was adjusted between workloops to maintain a constant isometric tension. Compensation for fibre-to-fibre variation and damage was accomplished by normalizing work and power values by the standardized work and power (SWL values), allowing for any decline present.

Once normalization for fibre-to-fibre variation and damage had been performed, mean values for the pooled data were calculated. Despite the attempts to normalize the data, the standard deviations were larger than the differences between groups, and therefore the normalized data were examined on a fibre-by-fibre basis. Work and power results for fibres from a single individual are shown in Fig. 3. The oscillatory strain that most commonly maximized work and power was $\epsilon=4\%$ (of 39 fibres, the optimum strain was 4% for work in 22 fibres, and for power in 25). The results for extension were not as clear: extensions of 4, 6 and 8% were optimal equally often. Fibres from queen and worker bees did not differ in the optimum strain, but a similar comparison could not be made for extension because of the variability of this parameter. The effect of extension on workloop shape is displayed in Fig. 4. If the extension was too low, the workloop was tailed (slack during part of the length-change cycle) or bottom-pointed. Overextension produced long, thin workloops which were pointed at the top.

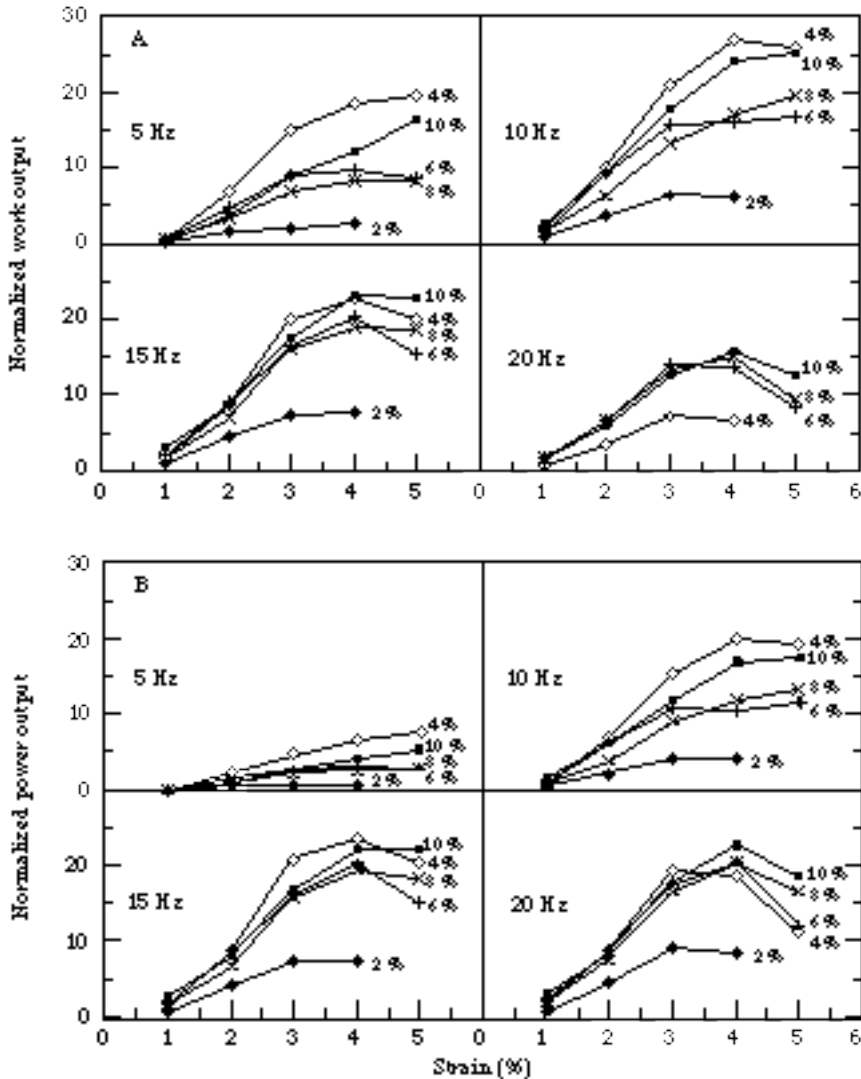


Fig. 3. (A) Work and (B) power output at 20°C as a function of oscillatory strain and extension for five fibres from a single worker bee. The symbols identify the extension at which the trial was performed. One fibre was used for each extension. Values have been normalized and corrected for the effect of damage as described in the text.

The optimum frequencies for work and power were noted for each fibre at every oscillatory strain. The frequency of maximum work $f_{w,max}$ (mean 10.4Hz, S.D.=3.0Hz) was significantly lower (paired t -test on all data, $t=-16.801$, $P=0.0001$, $N=176$) than the frequency for maximum power $f_{p,max}$ (mean 15.3Hz, S.D.=5.0Hz).

Temperature

Work and power were expressed as percentages of the maximum values for a fibre, as this tended to reduce fibre-to-fibre variation. Fibres rapidly became damaged at high

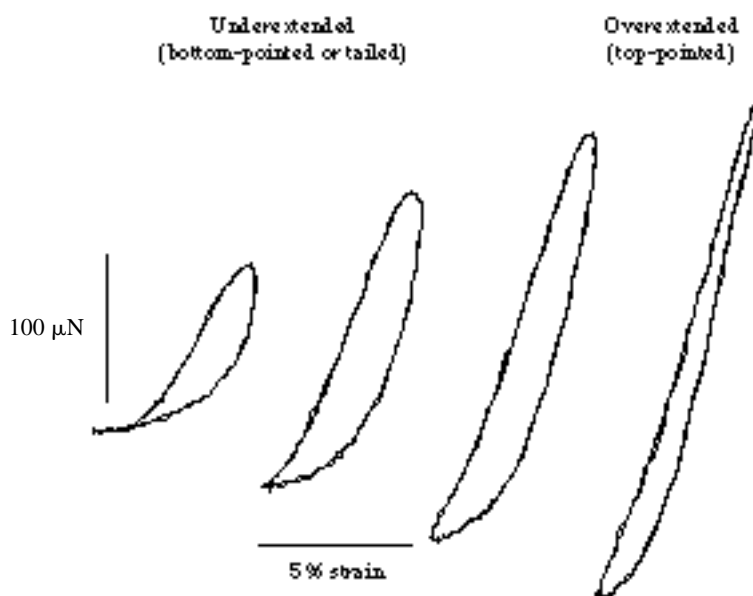


Fig. 4. The effect of extension on workloop shape for a single fibre from a worker bee. All four workloops were collected at 40°C, 52Hz and 5% oscillatory strain. The extensions were 4, 7, 10 and 14% from left to right.

temperatures, making the monitoring of fibre performance very difficult. Therefore, no attempt has been made to correct work and power values for the effects of damage. Maximum power P_{\max} , the frequency of maximum power $f_{p,\max}$ and the frequency of maximum work $f_{w,\max}$ increased with temperature in all three treatments; low strain and extension (LSLE), low strain and high extension (LSHE), and high strain and extension (HSHE) (Fig. 5). The maximum work W_{\max} also tended to increase with temperature in the LSLE trials. In the following analyses, W_{\max} , P_{\max} , $f_{w,\max}$ and $f_{p,\max}$ are considered.

Regression lines were calculated for $\log(W_{\max})$ and $\log(P_{\max})$ versus temperature (Fig. 6). In the LSLE treatment, W_{\max} increased with temperature (slope=0.010, 95% C.I.=±0.005). However, as the conditions became more severe, W_{\max} first failed to increase (LSHE: slope=-0.003, 95% C.I.=±0.003), then declined (HSHE: slope=-0.013, 95% C.I.=±0.011), as the temperature was raised. These changes were probably caused by damage effects becoming increasingly important. P_{\max} increased with temperature in all three treatments (Fig. 6 illustrates this for the LSLE treatment) and the slopes did not differ significantly. The increase in P_{\max} with temperature in all treatments, despite decreases in W_{\max} in some trials, emphasized the importance of frequency changes.

Linear regression was performed on $\log(f_{p,\max})$ (Fig. 7) and $\log(f_{w,\max})$ versus temperature. Both $f_{w,\max}$ and $f_{p,\max}$ increased with temperature in all three treatments. All 95% confidence intervals for slopes overlapped, indicating that the temperature sensitivity was not affected by oscillatory strain or extension. Significant differences were found to exist among the y-intercepts ($F=41.88$, $P<0.0005$), and the Tukey test was

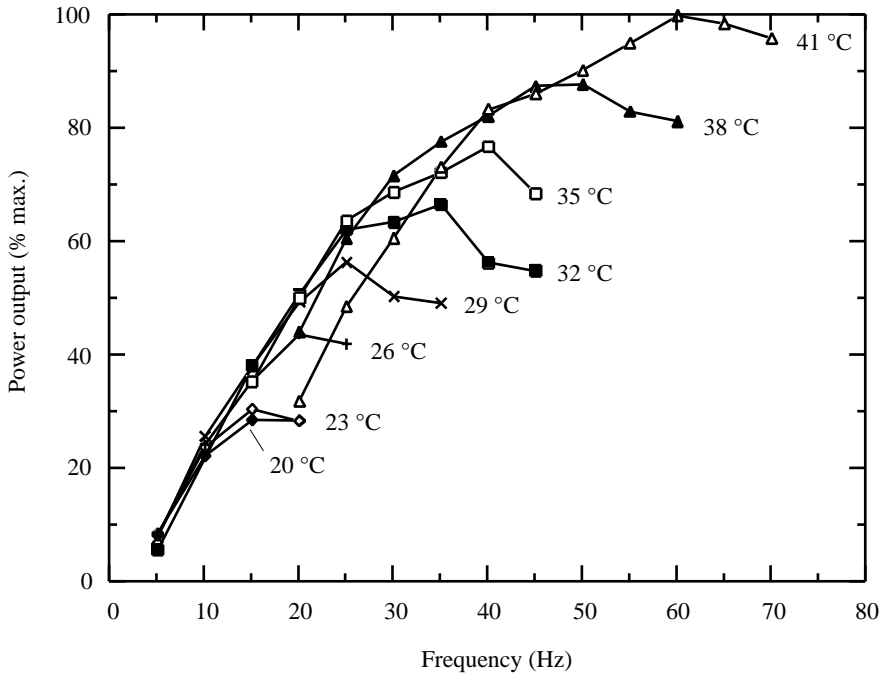


Fig. 5. The effect of temperature on the power output of a fibre from a queen bee. The fibre was oscillated at an oscillatory strain of 1% and an extension of 2%. Power is expressed as a percentage of the maximum that the fibre produced.

used for multiple comparisons (Zar, 1984). In all three treatments, the y -intercept of the $f_{p,max}$ regression (LSLE 0.70, LSHE 0.54, HSHE 0.57) was significantly greater than that of $f_{w,max}$ (LSLE 0.65, LSHE 0.53, HSHE 0.37).

No significant differences were observed between queens and workers in the LSLE temperature sensitivity of W_{max} and P_{max} . In similar comparisons for $\log(f_{w,max})$ and $\log(f_{p,max})$ versus temperature, the slopes of the regressions did not differ, but the y -intercepts for workers ($f_{w,max}$ 0.83, $f_{p,max}$ 0.79) were significantly greater than those for queens ($f_{w,max}$ 0.39, $f_{p,max}$ 0.57). No significant differences were observed for the LSHE and HSHE treatments.

To summarize the effect of temperature on the performance of glycerinated fibre preparations, Table 3 presents Q_{10} values compiled from means for the LSLE and quick stretch data. The R_{10} value is given for maximum work; R_{10} is analogous to Q_{10} but is used for parameters which are not rates (Bennett, 1984). Q_{10} and R_{10} values apply over the temperature range 20–40°C, as no significant variation was observed within this range.

Maximum power

Power output (for $\epsilon=4-5\%$, $T=40^\circ\text{C}$, $f = f_{p,max}$) was dependent on fibre extension, with the maximum occurring at $\epsilon_0=8-12\%$. Queen and worker fibres did not differ in the

extension at which power was maximized. The power produced by fibres from workers was significantly greater than that from queens ($t=2.537$, $P=0.022$, $N=18$), although the groups did not differ in work output. However, the oscillation frequency for workers, on average 54.4Hz (s.d.=6.5Hz, $N=10$), was significantly higher ($t=2.987$, $P=0.009$) than

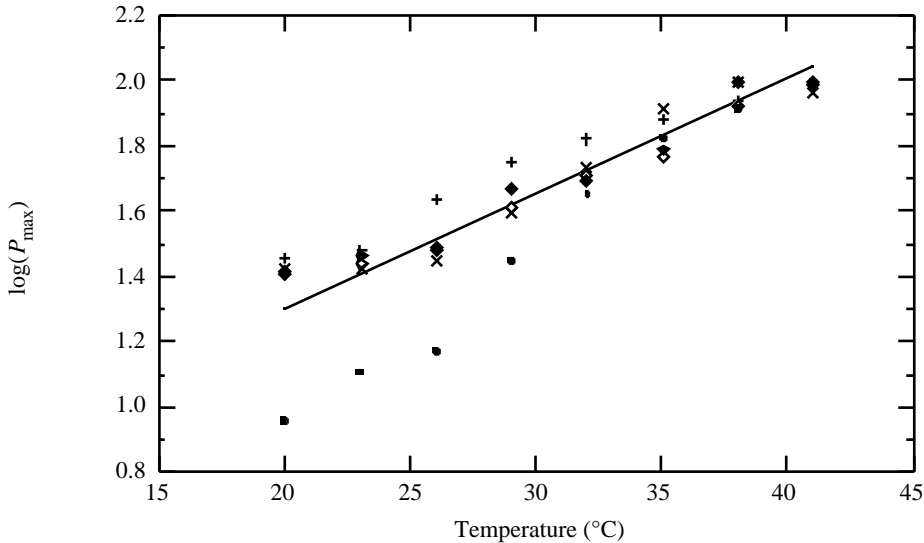


Fig. 6. The effect of temperature on P_{\max} illustrated by the LSLE data ($\epsilon=1\%$, $\epsilon_0=2\%$). Values are from five fibres. Regression statistics are y-intercept 0.61, slope 0.035, 95% C.I. ± 0.006 .

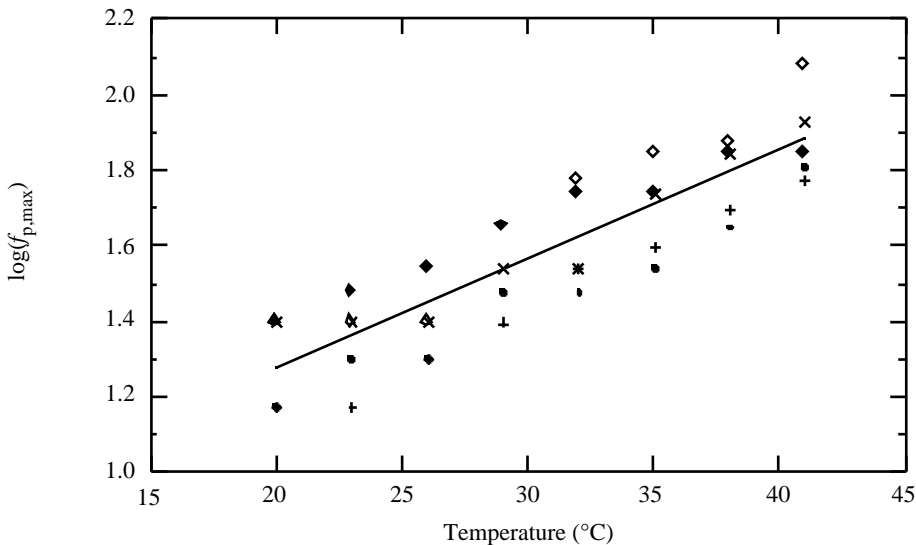


Fig. 7. The effect of temperature on $f_{p,\max}$ illustrated by the LSLE data ($\epsilon=1\%$, $\epsilon_0=2\%$). Regression statistics are y-intercept 0.70, slope 0.029, 95% C.I. ± 0.005 .

Table 3. Q_{10} and R_{10} values for the LSLE and quick stretch data

Variable	Q_{10} or R_{10}
W_{\max}	1.26
P_{\max}	2.24
$f_{w,\max}$	1.70
$f_{p,\max}$	1.95
r_3	1.99

that for queens, at 41.5Hz (S.D.=11.7Hz, $N=8$); note that this 'optimal' frequency was chosen on the basis of workloop size. The greatest work and power outputs observed in all glycerinated fibre experiments are listed in Table 4, and Fig. 8 shows the workloop corresponding to the highest work and power in Table 4.

Three of the entries in Table 4 include a second harmonic of the fundamental frequency in the length oscillations; the amplitude was 20% of that for the fundamental, with a zero phase shift. The discovery of a second harmonic for muscle length changes *in vivo*, and its effect on work and power in similar workloop experiments, are described in a companion paper (Gilmour and Ellington, 1993). Inclusion of the second harmonic in the driving signal generally decreases the work and power, but notable exceptions to this trend were found. Indeed, the two highest power outputs in Table 4 were obtained with second harmonics.

Discussion

Variability in the performance of single fibres

One of the most noticeable characteristics of the results was the variation in the performance of different glycerinated fibres. Even under identical conditions, power outputs could diverge by more than fivefold. A high degree of variability has been noted by other workers using large-amplitude oscillations (Pringle and Tregear, 1969; Lund

Table 4. *The ten highest power outputs observed*

Queen/ worker, Q/W	Strain (%)	Extension (%)	Frequency (Hz)	T (°C)	Second harmonic (%)	Work output (Jkg ⁻¹)	Power output (Wkg ⁻¹)
W	5	9	50	40	20	2.33*	116.0
W	5	13	60	40	20	1.90	113.6
W	4.8	5.4	54	40	0	1.75	94.1
W	5	12	100	40	0	0.75	74.1
W	4.7	10	47	40	20	1.58	73.8
W	5	6	45	44	0	1.43	65.0
Q	4.5	10	50	40	0	1.26	62.0
W	4	8	46	40	0	1.27	57.1
W	5	8	65	44	0	0.87	56.5
W	5	8	60	41	0	0.82	49.1

* marks the highest work value ever obtained.

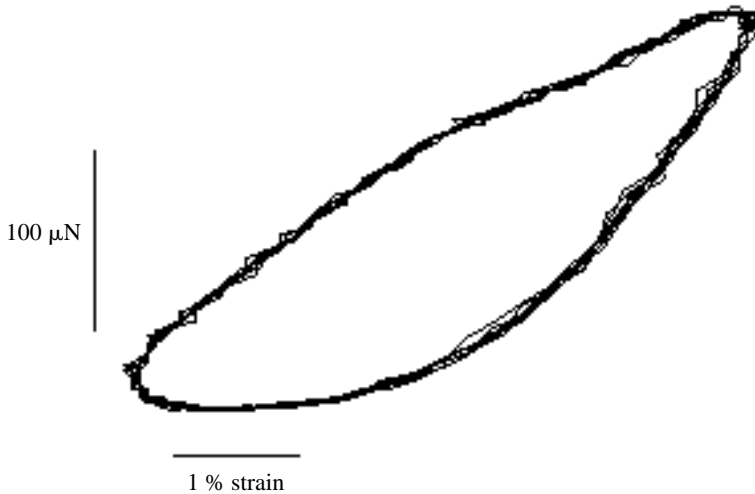


Fig. 8. The workloop corresponding to the highest work and power values ever observed (see Table 4).

et al. 1988; Molloy, 1988) and is likely to arise from a number of sources. The quality of the flight muscle varies according to factors such as the age of the bumblebee (reviewed by Rockstein and Miquel, 1973; Finlayson, 1975; Kammer and Heinrich, 1978). For example, fibres taken from queens captured in May were less satisfactory than those taken from August or September queens (Table 1). May queens are those that were reared the previous summer, have overwintered successfully, and are in the process of establishing a new nest. Only newly emerged queens are available in August and September (Alford, 1975). Similarly, all of the workers captured in May must have been only a few weeks old (Alford, 1975), and fibres from these bees performed better than average (Table 1). The process of glycerination, including the length of the storage period, introduces a second source of variability. The performance of glycerinated material tends to deteriorate over long storage periods (Pringle and Tregear, 1969; Abbott, 1973; Clarke and Tregear, 1980). Freshly glycerinated muscle (<48h storage here) is capable of producing work at higher oscillatory strains than older material (Pringle and Tregear, 1969) and displays a greater cyclic stress:isometric stress ratio (Table 1). The most important source of fibre-to-fibre variation is probably the dissection and preparation of the single, pared fibre; this is supported by the similarity of inter- and intra-bee fibre variation. Severely damaged fibres were detected by their very small standardized workloop and were discarded immediately. The variable performance of individual fibres seems inevitable, given the number of possible sources for its introduction. In studies such as this, which aim to relate the performance of muscle preparations to the *in vivo* muscle operation, the variation due to glycerination and fibre preparation is a disadvantage.

The gradual decline in work and power during the course of an experiment was probably a result of damage, which may be divided into irreversible stretching of passive elastic components in the fibre (Lund *et al.* 1988) and disruption of the sarcomere organization. The effect of irreversible stretching is to reduce the level of activation at a

given extension (Lund *et al.* 1988). Partial compensation can be achieved by increasing the extension to return the resting tension to a set level. However, increasing the extension tends to intensify disruption of the sarcomeres, and a decline in work output is the eventual outcome. Overstretching can cause the detachment from the Z-line of actin filaments, which may then interfere with tension development (Trombitás and Tigy-Sebes, 1984, 1985). Fibres occasionally entered a condition characterized by high resting tensions and greatly reduced work and power; however, they did not exhibit the sudden rise in mean tension which typifies the high-tension state associated with ATP diffusion limitation (Jewell and Rüegg, 1966). When this condition was observed, the results were excluded from the data set.

A number of chemical parameters have been shown to affect the performance of glycerinated *Lethocerus* fibres (Jewell and Rüegg, 1966; Rüegg and Tregear, 1966; vom Brocke, 1966; Schädler, 1967; Rüegg and Stumpf, 1969b; Steiger and Rüegg, 1969; Rüegg *et al.* 1971; Schädler *et al.* 1971; Abbott, 1972, 1973; White and Thorson, 1972; Pybus and Tregear, 1975; Griffiths *et al.* 1979; Loxdale, 1980; Wilson and White, 1983; Molloy, 1988; Marcussen and Kawai, 1990). In the incubation solutions used in this study, chemical concentrations were generally those that have been shown to optimize fibre performance for *Lethocerus*. Inorganic phosphate (P_i) was not included in the incubation solutions. Millimolar levels of P_i have been found nearly to double the frequency of maximum work for high-amplitude oscillations of *Lethocerus* fibres (Rüegg *et al.* 1971; White and Thorson, 1972; Pybus and Tregear, 1975), but at the cost of lowered isometric tension (Rüegg *et al.* 1971; Marcussen and Kawai, 1990) and a 40% reduction in work (Rüegg *et al.* 1971; Pybus and Tregear, 1975). The rate constant of the delayed tension component in the response to a step length change may be increased in the presence of P_i by 2–7 times (Rüegg *et al.* 1971; Peckham *et al.* 1990). Incubation solutions containing 20mmol l^{-1} P_i were used in preliminary trials (compositions as in White and Thorson, 1972), but positive workloops could not be obtained, so P_i was not included in the final solutions.

P_i has also been found to affect the form of the tension response to a step length change (White and Thorson, 1972). In the absence of P_i , the delayed tension momentarily rises to a high value to produce the 'phosphate-starvation transient' (PST). A PST and the short duration of the applied length change may account for the failure to observe phase 4 in the 20–25°C tension responses reported here; the resemblance to the response for a glycerinated *Bombus terrestris* fibre is striking (Fig. 1C of White *et al.* 1979). A value of approximately 60 s^{-1} can be estimated for r_3 (the delayed tension component) at 15°C from Fig. 6 of White *et al.* (1979). This rate is similar to the r_3 value of 55.3 s^{-1} measured for worker *B. lucorum* fibres at 20°C. Although the higher temperature might have been expected to increase the rate constant, the length step utilized by White *et al.* (1979) was 0.2%, compared with the 1% applied to *B. lucorum* fibres, and increased step amplitudes have been found to slow rate processes (Rüegg *et al.* 1984). Molloy (1988) reported values for r_3 of 135 s^{-1} and 202 s^{-1} at 15 and 20°C, respectively, for 1% stretches of *B. terrestris* fibres. No explanation for this large discrepancy in r_3 values under near-identical conditions has been found.

Optimum extension and oscillatory strain

Work and power were greatly affected by extension (Fig. 3). When the extension was too low or too high for the oscillatory strain, the shape of the workloop was altered, causing its area to decrease (Fig. 4). Within these limits, the force difference between the lengthening and shortening phases tended to increase with extension, resulting in greater work. An exact comparison between glycerinated fibre and *in vivo* extension is difficult; factors such as damage and shearing in the T-clip mount influence the optimum extension. Also, it is likely that mean tension, rather than extension, is adjusted *in vivo* (Pringle and Tregear, 1969; Pringle, 1974), and the tension at a given extension in glycerinated fibres was highly variable. Pringle and Tregear (1969) found a corresponding, variable dependence of high-amplitude work on extension in *Lethocerus* fibres. The mean tension in the muscle during flight is greater than the resting tension (McEnroe, 1952; Esch and Bastian, 1968; Esch and Goller, 1991), and the glycerinated fibre correlate is that high extension is required to maximize power.

In contrast to the difficulty of defining an average optimum extension, the oscillatory strain which maximized work and power was clearly 4–5% (Fig. 3). Positive workloops were occasionally obtained at oscillatory strains of 6–8%, but these were invariably bottom-pointed or tailed, and increasing the extension did not improve the shape. It is interesting that an oscillatory strain of 4% was found to maximize the power output of preparations of live bumblebee muscle (Machin and Pringle, 1959). *In vivo* oscillatory strains in bumblebee DLM, in contrast, have been measured as only 2% (Böttiger and Furshpan, 1954; Gilmour and Ellington, 1993). However, the T-clip probably did not hold all the myofibrils in the glycerinated fibre evenly, so the mean oscillatory strain in the fibre may have been lower than the applied strain. It is therefore conceivable that the optimal oscillatory strain *in vivo* is less than the 4–5% measured *in vitro*. Optimal oscillatory strains for glycerinated *Lethocerus* fibres range from 3 to 9% (R. T. Tregear, unpublished observations; Pringle and Tregear, 1969; Rüegg and Stumpf, 1969a; Molloy, 1988), but neither the *in vivo* oscillatory strain nor the optimum oscillatory strain for live muscle preparations has been reported for *Lethocerus*.

Optimum temperature and frequency

The thoracic temperature T_{th} of a freely flying bumblebee is normally between 35 and 40°C (Krogh and Zeuthen, 1941; Heinrich, 1972a,b,c, 1975, 1979; Joos *et al.* 1991). This elevated temperature is a product of muscle inefficiencies (Josephson, 1981; Kammer, 1981; Casey, 1988) and is also required for flight: bumblebees are unable to fly if T_{th} is less than 30°C (Krogh and Zeuthen, 1941). Although aspects of insect thermoregulation have been reviewed many times (e.g. Heinrich, 1974, 1981; Kammer and Heinrich, 1978; Casey, 1988, 1989), data on the mechanical responses of insect muscle to temperature are rather more limited (reviewed by Josephson, 1981).

In agreement with other work (see Josephson, 1981), the mechanical performance of glycerinated bumblebee fibres was strongly temperature-dependent, with rate processes (frequency, power) having higher thermal sensitivities than processes associated with tension development (work) (Table 3). The apparent temperature-independence or

negative thermal dependence of work in some trials was caused by damage rather than by diffusion limitation, since frequency parameters, which are greatly reduced in the high-tension state (Jewell and Rüegg, 1966), always showed a positive thermal dependence. In their experiments on live moth flight muscle, Stevenson and Josephson (1990) used a correction factor to estimate work and power in the absence of fatigue. The temperature coefficients they report for work and power are strikingly similar to those of glycerinated bumblebee fibres, despite the damage problems associated with the latter.

The increase in power with temperature was the outcome of increases in both the maximum work and the optimal frequency. Stevenson and Josephson (1990) also found that changes in both work and frequency contributed to raising the power. The increased work in their experiments was attributed to two sources; a higher optimum oscillatory strain, and a greater force difference between the lengthening and shortening half-cycles of the workloop. The relative contributions of the two sources were uncertain. In the bumblebee experiments, the oscillatory strain was held constant, and thus the positive thermal sensitivity of work must be due to increases in the force difference. Given that length changes follow the function: $\text{length} = X_o \sin(\omega t)$, where ω is the angular frequency ($2\pi f$) and X_o is the amplitude, then force changes may be described to a first approximation by: $\text{tension} = F_o \sin(\omega t + \theta)$, where F_o is the force amplitude and θ is the phase delay between force and length changes. The force difference could be increased through alterations in F_o or θ , or both. When the workloop shape was inspected, increases in both were indeed observed; the loop became both taller and more open as the temperature was raised. The apparent increase in θ might be due to a reduction in the higher harmonic content of the tension response, resulting in a more elliptical workloop of greater area; temperature increases in the range 5–15°C were found to reduce greatly the harmonic content for low-amplitude oscillations of *Lethocerus* fibres (Cuminetti and Rossmanith, 1980). The increase in F_o was probably due to a positive thermal dependence in tension development, as this exhibits a positive, but low, thermal sensitivity in synchronous muscle (Josephson, 1981). Also, increases in isometric tension with temperature have been noted for glycerinated *Lethocerus* fibres (Abbott and Mannherz, 1970; Kuhn *et al.* 1979), and work tends to rise with extension or isometric tension (Fig. 3).

The effects of temperature on frequency may be measured more reliably than on work or power because frequency characteristics exhibit relatively low fibre-to-fibre variation and little response to damage. Temperature coefficients of 2–3 have been measured for the optimal oscillation frequency of *Lethocerus* fibres (Jewell and Rüegg, 1966; Pringle and Tregear, 1969; Steiger and Rüegg, 1969; Schädler *et al.* 1971; Pybus and Tregear, 1975; Molloy, 1988). The Q_{10} of 2 measured here for bumblebee fibres is in good agreement with the *Lethocerus* results and also with the Q_{10} of 1.7 for live beetle muscle (Machin *et al.* 1962). A live synchronous muscle displayed a Q_{10} of 1.70 in the range 20–30°C, but this declined to 1.03 at flight temperatures (Stevenson and Josephson, 1990). In contrast, no reduction in thermal sensitivity at high temperatures has been noted for asynchronous muscle (Table 3; Machin *et al.* 1962; Pringle and Tregear, 1969; Steiger and Rüegg, 1969; Molloy, 1988). Similarly, the thermal sensitivity of moth muscle work and power tended to decrease as the temperature increased (Stevenson and Josephson, 1990) and, again, this trend is absent from the bumblebee data if the effects of

damage are discounted (Table 3). Bennett (1984) summarized information on temperature responses for muscle from a variety of taxa; thermal sensitivity was usually reduced at high temperatures. Although the data are too scarce to be conclusive, the thermal properties of the two types of insect flight muscle appear to differ.

The frequencies which maximized work $f_{w,max}$ and power $f_{p,max}$ for bumblebee fibres at 20°C were substantially higher than those for *Lethocerus*. At an oscillatory strain of 1%, mean optimal frequencies with bumblebee fibres were 12.1Hz (s.d.=3Hz, $N=39$) and 19.6Hz (s.d.=5.4Hz, $N=39$) for work and power, respectively. Comparable values for *Lethocerus* fibres are $f_{w,max}=2-5$ Hz and $f_{p,max}=4-9$ Hz (Pringle and Tregear, 1969; Steiger and Rüegg, 1969; White and Thorson, 1972; Molloy, 1988). The discrepancy is due to the very different wingbeat frequencies f_{WB} of the two insects: about 40Hz for *Lethocerus* (Molloy, 1988) compared with 150–200Hz for *Bombus* (Heinrich, 1979). The mean $f_{p,max}$ rose to 80Hz (s.d.=24Hz, $N=5$) at 40°C in the LSLE treatment. Despite its thermal sensitivity, $f_{p,max}$ at flight temperatures did not approach f_{WB} . This contrasts with certain results for *Lethocerus*. Molloy (1988) found $f_{p,max}$ 40Hz for *L. griseus* fibres at 40°C, and $f_{WB}=44$ Hz in this species at $T_{th}=41$ °C. Similarly, $f_{p,max}$ was 20Hz at 35°C for *L. maximus* fibres (Steiger and Rüegg, 1969), and f_{WB} was 21–25Hz (Barber and Pringle, 1966). Inorganic phosphate was not included in the incubation solution in any of these experiments, and therefore its absence does not explain why $f_{p,max}$ approaches f_{WB} at flight temperature for *Lethocerus* fibres and not for bumblebee fibres. Paring may damage the myofilaments, and it is possible, albeit unlikely, that rate processes are reduced in consequence. In support of this hypothesis, optimal frequencies were lower under more severe conditions. However, experiments by Thorson and White (1975) on live and glycerinated *Lethocerus* fibres demonstrated that paring had no effect on frequency characteristics. An alternative explanation is that the incubation solutions that are satisfactory for *Lethocerus* do not adequately mimic the intracellular environment of bumblebee fibres. The absence of inorganic phosphate in bumblebee trials could, by this theory, be a critical factor. It is interesting that the maximum work measured (2.33 J kg^{-1} , Table 4) is approximately twice the predicted maximum from free-flight studies (C. P. Ellington, A. J. Cooper and P. A. Northcott, in preparation). Addition of inorganic phosphate to the incubation solution would be expected to double $f_{p,max}$ while halving the work (Rüegg *et al.* 1971; Schädler *et al.* 1971; White and Thorson, 1972; Pybus and Tregear, 1975), giving the same power output. The differences between *in vitro* and *in vivo* could thus be eliminated. However, positive workloops could not be obtained for bumblebee fibres when inorganic phosphate was added to the incubation solutions. The intracellular environment of skeletal muscle has been characterized for only a few insect species (reviewed by Djamgoz, 1987), and further studies are clearly needed before more satisfactory incubation solutions can be developed.

The changes with temperature in the tension transient response to a quick stretch resembled those reported for *Lethocerus* fibres (Abbott and Steiger, 1977; Kuhn *et al.* 1979). The thermal sensitivity of the delayed tension component r_3 was similar to that of the optimal frequencies, and linear regression slopes did not differ significantly. This is not unexpected, as the two parameters are related, and for linear systems the relationship

may be expressed exactly: $r_3=2 f_{w,max}$ (Pringle, 1978). The Q_{10} of r_3 similarly showed no tendency to decline at high temperatures. The rate constant of the relaxation phase r_4 increased with temperature, but the relationship was not quantified because the data were few and unreliable. In contrast, r_2 did not increase consistently with temperature. Owing to the timebase on which the transients were collected, however, only gross changes in r_2 with temperature could be detected.

Maximum mechanical power

The combined results of the mechanical parameters and temperature trials enabled 'optimum' experimental conditions to be defined, under which mechanical power is maximized. The optimum oscillatory strain is 4–5% (Fig. 3). The extension varies between fibres, but is generally 8–10%. Provided that damage and diffusion limitation can be avoided, the temperature should be about 40°C (Fig. 5), giving $f_{p,max}=40\text{--}50\text{Hz}$ for high oscillatory strain. Under these conditions, glycerinated bumblebee fibres produced up to 110 W kg^{-1} (muscle) mechanical power (Table 4). However, only 2 of over 200 fibres yielded power of this magnitude, and these experiments included a second harmonic in the driving signal (Gilmour and Ellington, 1993). Other workers have attempted to determine the maximum power of glycerinated *Lethocerus* fibres. Pringle and Tregear (1969) obtained a maximum of 7.6 W kg^{-1} ($\epsilon=7\text{--}8\%$, ϵ_0 unknown, $f=2\text{Hz}$, $T=20^\circ\text{C}$); power was determined at $f_{w,max}$ rather than $f_{p,max}$ so the potential for somewhat higher values existed. Although the measurements of Steiger and Rüegg (1969) were made at the wingbeat frequency (20–25Hz) and at temperatures close to flight values (35°C), the low oscillatory strain (2–3%) limited the maximum power to 10 W kg^{-1} . The power of 43 W kg^{-1} reported by Molloy (1988) was measured at 7% oscillatory strain, but only 30°C ($f=15\text{Hz}$); diffusion limitation occurred at higher temperatures. The maximum power determined in this study may be close to the limit of which glycerinated fibres are capable and, for the first time, is consistent with the power requirements for flight (C. P. Ellington, A. J. Cooper and P. A. Northcott, in preparation).

Queen-worker differences

The order of magnitude size difference between queen and worker bumblebees affects their flight behaviour. For example, wingbeat frequency tends to decrease with increasing size (Young and Casey, 1989; C. P. Ellington, A. J. Cooper and P. A. Northcott, in preparation), and there are indications that stress difference (the force per unit area in contraction minus that in relaxation) may scale positively with body mass (Casey and Ellington, 1989). Muscle oscillatory strain does not seem to be correlated with size (Casey and Ellington, 1989). If size-related differences result from scaling effects on myofibrillar function, aspects of glycerinated fibre operation should be correlated with the mass of the bee from which the fibre was taken. This expectation was met to some extent. Optimal strain did not differ between queen and worker fibres. In several experiments, worker fibres were found to have higher frequency characteristics than queen fibres: $f_{p,max}$ at $\epsilon=1\%$ and $T=20^\circ\text{C}$, $f_{p,max}$ and $f_{w,max}$ in LSLE temperature trials, $f_{p,max}$ in maximum power trials, and r_3 at $T=20^\circ\text{C}$. Nevertheless, stress difference displayed no tendency to scale with mass. An example is seen in the maximum power

trials: at equal oscillatory strains, the work outputs of queen and worker fibres did not differ significantly. However, the effects of glycerination and damage in increasing fibre-to-fibre variation are likely to mask any size-related differences in force production.

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