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RESEARCH ARTICLE

Temperature gradients drive mechanical energy gradients in the flight muscle of *Manduca sexta*

N. T. George*, S. Sponberg and T. L. Daniel
University of Washington, Seattle, WA 98195, USA
*Author for correspondence (ntgeorge@u.washington.edu)

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SUMMARY

A temperature gradient throughout the dominant flight muscle (dorsolongitudinal muscle, DLM_1) of the hawkmoth *Manduca sexta*, together with temperature-dependent muscle contractile rates, demonstrates that significant spatial variation in power production is possible within a single muscle. Using *in situ* work-loop analyses under varying muscle temperatures and phases of activation, we show that regional differences in muscle temperature will induce a spatial gradient in the mechanical power output throughout the DLM_1. Indeed, we note that this power gradient spans from positive to negative values across the predicted temperature range. Warm ventral subunits produce positive power at their *in vivo* operating temperatures, and therefore act as motors. Concurrently, as muscle temperature decreases dorsally, the subunits produce approximately zero mechanical power output, acting as an elastic energy storage source, and negative power output, behaving as a damper. Adjusting the phase of activation further influences the temperature sensitivity of power output, significantly affecting the mechanical power output gradient that is expressed. Additionally, the separate subregions of the DLM_1 did not appear to employ significant physiological compensation for the temperature-induced differences in power output. Thus, although the components of a muscle are commonly thought to operate uniformly, a significant within-muscle temperature gradient has the potential to induce a mechanical power gradient, whereby subunits within a muscle operate with separate and distinct functional roles.

Key words: mechanical energy gradient, temperature gradient, insect flight muscle, work-loop.

INTRODUCTION

Metabolic heat production, a byproduct of muscle contraction, can lead to a core body temperature that is significantly higher than ambient temperature. Many organisms from large insects to mammals benefit from the enhanced muscle performance that arises from this elevated temperature. Specifically, the rates of force production and the magnitude of power produced by muscle significantly increase with an increase in temperature (Bennett, 1984; Josephson, 1984; Bennett, 1985; Rall and Woledge, 1990; Stevenson and Josephson, 1990; Swoap et al., 1993; Rome et al., 1999). Therefore, temperature-dependent changes in muscle activity can have important functional consequences for the performance of animal locomotion. Yet, the temperature of an animal's musculature does not necessarily need to be spatially uniform. Metabolic heat production paired with convective and radiative cooling to the surrounding environment can potentially create a temperature gradient even in a single muscle. For example, we previously showed that a significant dorso-ventral temperature gradient arises during tethered flight, with a temperature difference of ~6°C, throughout the dominant flight muscle of the hawkmoth *Manduca sexta* (George and Daniel, 2011). Because muscle contractile rates and power production are temperature dependent, functional heterogeneity may therefore occur within a single muscle. Thus, although it is clear that *in vivo* temperatures affect muscle function, the question remains, can an *in vivo* temperature gradient actually produce a mechanical and functional gradient that is not apparent if we consider the whole animal to operate at one uniform temperature?

Work-loop studies, where muscle is cyclically oscillated and periodically stimulated, provide a means to determine how various neural and mechanical determinants, including muscle strain, length and phase of activation (the timing of muscle stimulus relative to the strain cycle), influence mechanical power output across a range of operating conditions (Josephson, 1985; Stevenson and Josephson, 1990; Johnson and Johnston, 1991; Swoap et al., 1993; Full et al., 1998; Rome et al., 1999; Tu and Daniel, 2004b; Donley et al., 2007). Although these work-loop studies have elucidated how muscle mechanics affect animal locomotor performance, they have generally assumed that a given muscle has a spatially uniform temperature and thus generates a spatially uniform function under a given set of conditions. Despite growing evidence showing that functional heterogeneity may occur within regions of a muscle because of morphological or neurological differences [e.g. fiber type (Mu and Sanders, 2001; Wang and Kernell, 2001), segment strain (Pappas et al., 2002; Ahn et al., 2003; Higham et al., 2008; Higham and Biewener, 2008), motor recruitment (English et al., 1993; Holtermann et al., 2005; Wakeling, 2009) and neural activation (Sponberg et al., 2011)], the role of the physiological environment in which the muscle operates has generally not been considered. Given the notable Q_{10} of the physiological properties of muscle and the presence of thermal gradients, temperature itself likely produces significant functional differences within a single muscle. Thus, although muscles are classically thought to function solely as a motor, spring, brake or strut, in some cases they may actually concurrently operate with an array of functions as a consequence of an internal temperature gradient (Altringham et al., 1993; Full

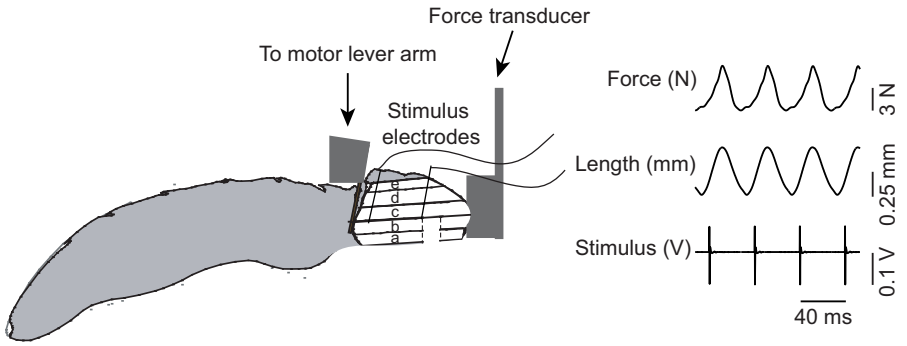

Fig. 1. *Manduca sexta* preparation (head, wings, legs and scales removed) for the work-loop studies and an example trace of the force, length and stimulus pattern. The thorax of *M. sexta* was fixed between an anterior grip attached to a force transducer and a posterior grip attached to a motor lever arm. A circumferential strip of cuticle ~2 mm wide was excised from the middle of the thorax. The dorsoventral muscles and leg muscles were then removed to isolate just the dorsolongitudinal muscle ($DLM_{1a–e}$) between the motor and force transducer. The DLM_1 was cyclically oscillated at 25 Hz by the motor lever arm with a strain amplitude of ~0.5 mm. At specific phases of each length cycle, the muscle was stimulated with 0.2 ms supramaximal stimuli. The force transducer then detected force output by the DLM_1. For these experiments, the DLM_1 subunits were either left intact or the dorsal (DLM_{1D}; depicted here) or ventral (DLM_{1V}) subunits were isolated. Figure adapted from Tu and Daniel (Tu and Daniel, 2004a).

et al., 1998; Dickinson et al., 2000). Using work-loop techniques conducted at different temperatures, we can examine how muscles respond to temperature gradients under *in vivo* stimulus and strain conditions.

The dorsolongitudinal muscle (DLM_1) (*sensu* Kondoh and Obara, 1982), which is the dominant downstroke flight muscle of *M. sexta*, is an excellent system for a study of the functional consequences of a within-muscle temperature gradient. The DLM_1 consists of five separate muscle subunits – $DLM_{1a–e}$ – that are each ~1 mm thick (Fig. 1) (Eaton, 1988). Each of these separate subunits is innervated by a single motor neuron, four originating from the pterothoracic ganglion, while the fifth resides in the prothoracic ganglion with a long projection to DLM_{1e} (Kondoh and Obara, 1982; Eaton, 1988). Despite this potential for separate modulation, previous recordings indicate that all five separate subunits are activated nearly simultaneously by their respective motor neurons (George and Daniel, 2011). Furthermore, these muscle subunits each fire with a one-to-one relationship with the motor neurons: each action potential elicits only one muscle contraction (Kammer, 1968).

Because of these properties, the DLM_1 is commonly thought to operate uniformly as a power generator to indirectly depress the wings. However, *M. sexta*'s highly elevated core temperature during flight (~40–43°C maximum, ~15–25°C above ambient temperature) indicates that a significant temperature and functional gradient will exist throughout the flight musculature (McCrea and Heath, 1971; Heinrich and Casey, 1972). Although no attempt has yet been made to record a temperature gradient during free flight, the increased mechanical demands of free flight should lead to a temperature difference even greater than the ~6°C difference recorded during tethered flight (George and Daniel, 2011). The most ventral DLM_1 subunit likely operates near the maximum-recorded temperature (~40°C). Muscle temperature would then progressively decrease in the dorsal direction because of convective heat loss, with the superficial, dorsal-most subunit operating only slightly above ambient temperature (~25–30°C). Because the rate of muscle force generation depends strongly on temperature, this regional difference in temperature might induce significant mechanical differences across the muscle.

A potential gradient in mechanical power output would have important implications for the production, storage, dissipation and transmission of energy through the musculoskeletal system. Indeed, the warmer ventral subunits may act more as force generators, whereas the cooler dorsal subunits could act as springs, or even as damping elements. To test whether the power–temperature relationship of the DLM_1 would lead to regionally distinct functional roles, we conducted *in situ* work-loop studies on the DLM_1 at the *in vivo* phase of activation while varying muscle temperature from 25 to 40°C.

Regional specialization of the contractile machinery could compensate for the thermal gradients, providing a spatially uniform level of power output. Thus, we performed the same mechanical tests on isolated dorsal or ventral subunits of the DLM_1. If compensatory mechanisms maintain a uniform function across the DLM_1, then power output of dorsal *versus* ventral subunits would be comparable when each is operating at its respective *in vivo* temperatures (~25–30°C for dorsal subunits *versus* ~35–40°C for ventral subunits).

Lastly, it is a common property of muscle that altering the time at which activation occurs in relation to the strain cycle can lead to significant differences in power output (Josephson, 1985; Stevenson and Josephson, 1990; Tu and Daniel, 2004b). Recent evidence demonstrates that moths use neural feedback to actively modulate this phase of muscle activation during turning maneuvers (S.S. and T.L.D., unpublished). Therefore, in order to consider the role functional heterogeneity could play in controlling movement, it is important to examine how the phase of activation may alter the temperature sensitivity of mechanical power output. To test whether the power–temperature relationship itself depends on the phase of activation, we conducted additional temperature-controlled work-loops while varying the timing of muscle activation.

MATERIALS AND METHODS

Moths

Manduca sexta (L.) were obtained from a colony maintained by the Department of Biology at the University of Washington, Seattle, WA, USA. After eclosion, moths were kept in 24 h of light. Moths were used within 5 days of eclosion.

Experimental apparatus and muscle preparation

Work-loop methods were adapted from previous studies (Tu and Daniel, 2004b; George and Daniel, 2011). Moths were held at ~4°C for at least half an hour and up to 1 day prior to each experiment to

immobilize them for experimental preparation. We first weighed each moth and measured the resting length of its mesothorax with digital calipers. The head, prothorax, wings, legs and scales covering the thorax were then removed.

We conducted our work-loops using a semi-intact preparation, with the abdomen and tracheae undamaged. This allowed the DLM_1 to continue receiving a supply of oxygen and to remain viable throughout experiments. The DLM_1 was isolated between two grips: an anterior grip attached to a force transducer (Fort100, WPI, Sarasota, FL, USA) and a posterior grip mounted on a length driver (Model 305B Dual-Mode Lever Arm System, Aurora Scientific Inc., Aurora, ON, Canada) (Fig. 1). The length driver was tuned for the added mass of the grip plus the moth body until the system produced smooth length control. The anterior grip, a small brass block shaped to fit the anterior mesoscutum and first phragma, was secured to the mesothoracic cuticle with a mixture of cyanoacrylate and sodium bicarbonate powder. To facilitate adhesion, the dorsal aspect of the anterior half of the mesothoracic cuticle was first lightly scored. The posterior grip, consisting of two stainless steel needles (15 mm long, diameter of 0.68 mm) soldered to a small brass block, was inserted along the posterior face of the mesothorax and secured with a drop of cyanoacrylate. A strip of cuticle, ~2 mm wide, was then excised from around the mid-mesothoracic region. In addition, the antagonistic dorsoventral muscles along with the ventral aspect of the mesothorax just below DLM_{1a} were removed. This assured us that just the DLM_1 was mechanically isolated between the motor lever arm and force transducer. The force transducer was mounted to a micromanipulator, allowing us to adjust the length of the DLM_1 to the operating thorax length (0.98±0.02 of the rest length) (Tu and Daniel, 2004a). Two tungsten electrodes (~10 mm long), inserted through the posterior and anterior notum along the same longitudinal transect of the DLM_1, connected to a stimulator (PG4000 Digital Stimulator, Neuro Data Instruments Corp., East Stroudsburg, PA, USA) delivered 0.2 ms supramaximal stimuli (~600–900 mV) at 25 Hz (normal wingbeat frequency), consistent with prior studies (Tu and Daniel, 2004b; George and Daniel, 2011). Evoked potentials were recorded with a bipolar differential tungsten electrode inserted near the posterior grip and a common reference wire placed in the abdomen. The signal was amplified (×1000) with a differential AC amplifier (model 1800, A-M Systems, Sequim, WA, USA) and band-pass filtered (300–20 kHz).

Immediately after each experiment, the moth's thorax was carefully removed from the grips and placed in *M. sexta* saline (Lei et al., 2004). We then removed the remaining DLM_1 from the mesothorax. The DLM_1 was quickly blotted with a tissue and weighed to the nearest 0.1 mg.

Work-loops were conducted on three separate muscle preparations to test for possible regional differences in the power output sensitivity to temperature: (1) DLM_1 with all five subunits intact (intact DLM_1), (2) DLM_1 with the ventral subunits cut through, leaving just the dorsal subunits intact (DLM_{1D}; Fig. 1), and (3) DLM_1 with the dorsal subunits cut, leaving the ventral subunits intact (DLM_{1V}) (N=5 moths per subgroup). Given the constraints of muscle isolation and stimulation, we were unable to compare the power output of individual DLM_1 subunits. Instead, we were confined to resolve power output at the spatial scale of two to three subunits. However, the mean *in vivo* operating temperatures of the dorsal and ventral subunits would still be significantly different. Thus, comparing power output in response to temperature at this spatial scale would be sufficient to determine whether compensatory mechanisms exist.

To minimize the experiment duration and to preserve muscle tissue, we heated the DLM_1 sequentially rather than randomly. To determine whether the muscle significantly fatigued during the ~20 min test period, we did an additional set of 'control' work-loop tests in which we repeated the previous stimulation procedure and experiment duration but held the muscle temperature at 35°C (N=5 moths).

Muscle length and strain

The DLM_1 was electrically stimulated and sinusoidally lengthened for 2 s, with a peak-to-peak strain amplitude of ~0.5 mm (~±2.5% of the initial muscle length), a duty factor (calculated as the fraction of time spent shortening during the length cycle) of ~0.5, and a frequency of 25 Hz (see Tu and Daniel, 2004a). The DLM_1 undergoes a net shortening during flight; the mean *in vivo* operating length is 0.98±0.02 of the rest length (Tu and Daniel, 2004a). We measured the rest length of the thorax while the moth was immobilized from cold exposure, and then set the starting experimental length of the DLM_1 to the calculated operating length.

The limitations inherent with conducting *in situ* work-loops on *M. sexta* required that the actual length changes we were able to impose differed from the *in vivo* strain. The large inertial mass of the *M. sexta* body oscillating on the motor lever arm limited us to induce strains that were only a fraction (~50%) of the *in vivo* strain (Tu and Daniel, 2004a). The slight intra-animal variability in strain trajectory may have been because of unavoidable differences in body size, motor unit recruitment and the magnitude of muscle force output. Despite these complications with imposed strain, Tu and Daniel (Tu and Daniel, 2004b) found that the effect of strain amplitude on the magnitude of power output of the DLM_1 was relatively small and did not influence the shape of the power–phase curve. In addition, we found that the effect of temperature, phase and subunit was consistent across animals. Thus, we are confident that our data accurately reflect the mechanical and functional consequences of the temperature gradient.

Muscle temperature

To control muscle operating temperature, *M. sexta* saline in a flask seated on a heating apparatus was slowly dripped over the exposed DLM_1. The saline was gradually heated to elevate muscle temperature to 25, 30, 35 and 40°C. We chose these values to encompass the full range of the possible temperature gradient during flight; from the mean temperature of the dorsal-most subunit recorded during tethered flight (~25°C) to the maximum temperature recorded during free flight (~40°C) (Heinrich and Casey, 1972; George and Daniel, 2011). Muscle temperature was measured with a thermocouple embedded in a 30 gauge hypodermic probe (HYP-1, Omega Engineering Inc., Stamford, CT, USA). Experiments were performed once the muscle thermocouple settled on the target temperature. This method was sufficient to control muscle temperature to within 1°C over the course of each experimental trial.

Phase of activation

To test whether the power–temperature relationship for the DLM_1 or the subunits depended on the phase of activation, we repeated the 2 s series of work-loops, where the muscle was subject to controlled cyclic length changes, while monitoring force. We used four different phases of activation, ~0.18, 0.28, 0.46 (approximately *in vivo*) and 0.58, at each temperature while performing these work-loops (Tu and Daniel, 2004a). These phases were chosen to encompass a range surrounding the phase that produced peak positive power output (~0.36), as determined elsewhere (Tu and Daniel, 2004b). Here, phase of activation is calculated as the duration of time from the start of muscle lengthening to the peak of the evoked action potential divided by the cycle period.

Data acquisition

Force, muscle length and evoked potentials were recorded at 5000 Hz by a data acquisition system (NI USB-6229, National Instruments, Austin, TX, USA). Evoked potentials were analyzed using custom-designed peak detection software in MATLAB (The MathWorks, Natick, MA, USA) developed by M. S. Tu (University of Washington, Seattle, WA, USA).

Net work per cycle was calculated by integrating force output with respect to muscle length. We calculated the net work for 20 cycles per trial, starting with the tenth cycle. Mass-specific power output is the product of mean net work and cycle frequency (25 Hz) divided by the mass of the DLM_1.

Statistical analysis

Given the length of the experimental trials, different sets of five animals were used for the intact DLM_1 and for each of the two subgroup conditions. The experimental design was balanced for each of these trials. Results and statistical tests for each experimental condition are reported as means across individuals. A one-way ANOVA was employed to determine how power output depends on both temperature and phase of activation and whether these dependencies differ among subunit groups. A Tukey–Kramer honestly significant difference (HSD) test was then used to isolate specific differences between the means at each temperature and phase of activation. Non-parametric Wilcoxon tests gave similar results. Data are represented as means ± s.e.m. unless otherwise noted.

RESULTS

Operating conditions

Mean (±s.d.) body mass of the 15 moths used in these work-loop trials was 2.59±0.25 g. Mean (±s.d.) resting length of the mesothorax was 10.19±0.39 mm. Mean (±s.d.) thorax operating length was 9.91±2.74 mm. Neither moth size nor mass differed significantly among the three groups tested (ANOVA, $P>0.1$). The mean (±s.d.) peak-to-peak strain amplitude imposed on the DLM_1 was 0.48±0.04 mm. This strain amplitude did vary slightly (~8%) between individuals because of the constraints of our *in situ* preparation, including the large mass of *M. sexta* on the motor lever arm, slight flexibility of the force transducer and individual variation in activation dynamics. The four phases of activation used in these preparations had mean (±s.d.) values of 0.18±0.01, 0.28±0.02, 0.46±0.01 and 0.58±0.01. Mean (±s.d.) muscle mass of intact DLM_1, DLM_{1D} and DLM_{1V} after being dissected out of the thorax was 0.193±0.010, 0.169±0.023 and 0.112±0.019 g, respectively. Mean muscle mass of the two isolated subunit groups sum to a value greater than the mean muscle mass of intact DLM_1 because we were not able to isolate DLM_{1D} and DLM_{1V} precisely at the subunit level while the animal was mounted in the work-loop apparatus (portions of the middle subunit were occasionally included in both groups).

Effect of temperature on power output at the *in vivo* phase of activation

We analyzed power output and its temperature dependence in intact DLM_1 at the *in vivo* phase of activation (~0.46) (Tu and Daniel, 2004a). Muscles were cyclically lengthened and stimulated at muscle temperatures of 25, 30, 35 and 40°C. The mean value for power output of intact DLM_1 (~60 W kg^{-1}) at the *in vivo* phase of activation at 35°C is consistent with the power output measured in a prior work-loop study on *M. sexta* (Tu and Daniel, 2004b).

We observed a strong temperature dependence of net work. The DLM_1 produced negative, approximately zero and positive work-loops across the range of temperatures we predict to simultaneously occur within this muscle during sustained flight (25–40°C) (George and Daniel, 2011) (Fig. 2). For all individuals tested, the power output of intact DLM_1 (at the approximate *in vivo* phase of activation) was greatest at either 35 or 40°C, and significantly decreased as temperature decreased. There were statistically significant differences in power output between each separate temperature point, with the exception of one individual from 35 to 40°C (Tukey–Kramer HSD, $P<0.0001$; $P\approx0.1$ for 35–40°C). With each moth, power output transitioned from positive to negative between 35 and 30°C (Fig. 3 and Table 1). Mean power output of intact DLM_1 decreased from 60.53±3.40 W kg^{-1} at 35°C, to –74.66±4.41 W kg^{-1} at 30°C. Assuming a linear relationship between these temperatures indicates that the transition temperature for positive to negative power output occurs at ~33°C. Because power output at the lower temperatures was negative we cannot calculate the Q_{10} of power output for these trials.

Although the coefficient of variation across the 20 work-loop cycles recorded for each condition was small (0.18), there was significant variability in individual performance (ANOVA, *F*-

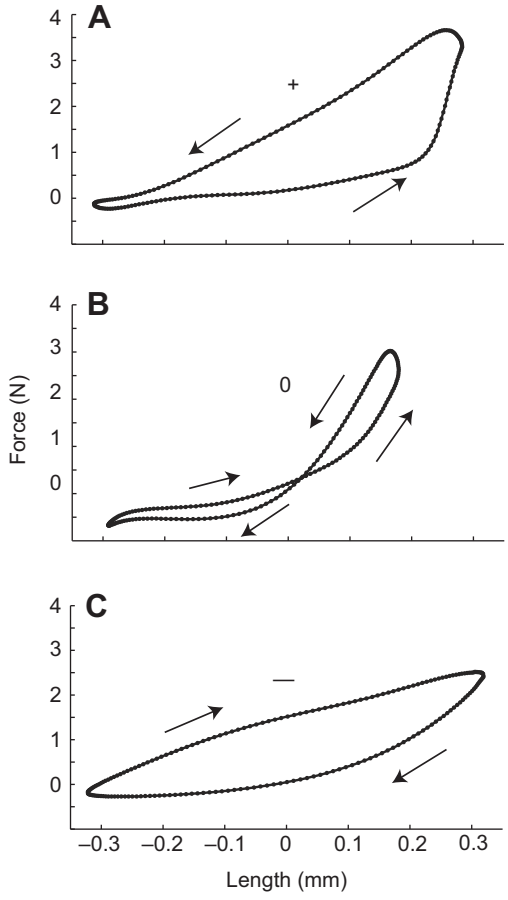

Fig. 2. Example positive, approximately zero and negative work-loops at (A) 35°C, (B) 30°C and (C) 25°C. Net work is calculated as the area within each loop. The counter-clockwise direction of work-loop A is indicative of positive work, or energy production. The clockwise direction of work-loop C is indicative of negative work, resulting in energy absorption. Net work became significantly more negative as temperature decreased. +, 0 or – indicate net energy generation, storage and return, or dissipation, respectively.

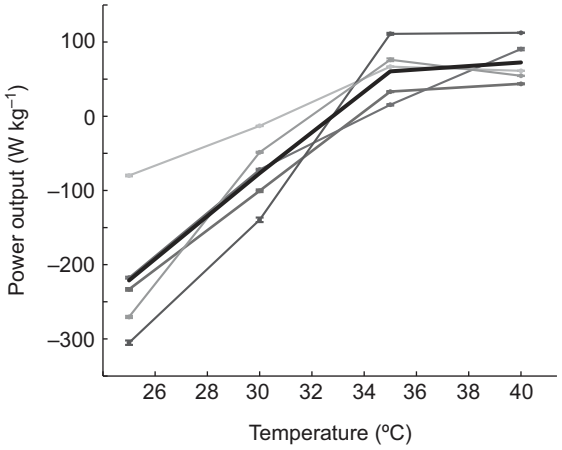

Fig. 3. Temperature significantly affected power output in intact DLM_1. Mass-specific power output is plotted as a function of temperature at the approximately *in vivo* phase of activation (0.46) for intact DLM_1. Each individual of the intact DLM_1 preparation is represented as a separate gray line. There were statistically significant differences in power output between each temperature point, except for one individual between 35 and 40°C (Tukey–Kramer HSD, P<0.0001). The thick black line superimposed over the individual data represents the mean power output of all moths. Power output was positive between 40 and 35°C and negative between 30 and 25°C. Values are reported as means ± s.e.m. (N=5 moths, 20 cycles per trial).

ratio=36.4, P<0.0001; Fig. 3). However, the effect of temperature was greater than the effect of the individual on power output (mean difference of ~294 W kg^{-1} between 25 and 40°C *versus* a mean maximum individual difference of ~129 W kg^{-1}; F-ratio ~29-fold greater for effect of temperature *versus* individual).

The results from our work-loop study were not confounded by a decline in performance during the experiment due to muscle fatigue. Routine muscle stimulation and contraction over a 20 min period in our fatigue controls did not produce significantly detectable changes in power output (a difference of ~5 W kg^{-1}, from 38.33±1.79 to 43.31±1.84 W kg^{-1}; ANOVA, P>0.1).

Subunit differences

Work-loops conducted on isolated dorsal (DLM_{1D}) and ventral (DLM_{1V}) subunits at the approximate *in vivo* phase of activation showed significant differences in power output between the two isolated subgroups, even when considering the effect of temperature (ANOVA, P<0.0001). In addition, the relationship between power output and temperature differed for DLM_{1D} and DLM_{1V} (ANOVA temperature–subgroup interaction, F-ratio=54.5, P<0.0001).

Table 1. Mean power output (W kg^{-1}) for the *in vivo* phase of activation (0.46) at 25, 30, 35 and 40ºC for the three *M. sexta* preparations

Temperature (ºC)	Intact DLM_1	DLM_{1D}	DLM_{1V}
25	–221.26±7.77	–125.80±4.62	–209.02±8.43
30	–74.66±4.41	–37.05±2.54	–63.72±2.98
35	60.53±3.40	32.42±3.29	26.72±1.62
40	72.50±2.57	62.14±4.99	87.50±2.75

DLM_1, dorsolongitudinal muscle.
Preparations: (1) intact DLM_1 composed of all five subunits, (2) the dorsal subunits (DLM_{1D}), and (3) the ventral subunits (DLM_{1V}).
N=5 moths per group, 20 cycles per trial. Values are reported as means ± s.e.m.

Compared with the ventral subunit group, DLM_{1D} had a shallower mean power–temperature curve, with higher power output at 25°C and lower power output at 40°C (Fig. 4). However, regardless of the increase in performance at cooler temperatures, DLM_{1D} would still fail to yield power output comparable to the ventral subunits at their warmer *in vivo* operating temperatures. At 30°C, DLM_{1D} produced approximately –37 W kg^{-1}, yielding ~27 W kg^{-1} more than DLM_{1V} at the same temperature. This increase in power output of the dorsal subunits could only account for 22–42% of the rise in power output afforded by even a 5–10°C increase in ventral subunit temperature (~64–124 W kg^{-1} more; Table 1). Furthermore, temperature had a more dominant effect on power output than did subgroup (mean difference of 242 W kg^{-1} between 25 and 40°C *versus* a mean difference of 35 W kg^{-1} between DLM_{1D} and DLM_{1V}; F-ratio ~22-fold greater for the effect of temperature *versus* subgroup).

The small mechanical power differences between the dorsal and ventral groups at a fixed temperature are overwhelmed by the very strong regional temperature-dependent power differences. DLM_{1D}, at the *in vivo* phase of activation and the predicted operating temperatures for the dorsal subunits (~25–30°C), always produced net negative power. In contrast, DLM_{1V}, predicted to operate at the *in vivo* ventral subunit temperatures of ~35–40°C, yielded positive mechanical power output. Thus, we do not find sufficient regional specialization in mechanical performance to negate the diversity in power output that will result from subunit-specific *in vivo* operating temperatures.

Effect of phase of activation on the power–temperature relationship

Because of the temperature-induced gradient in the mechanical power output of the DLM_1, we investigated the potential for neural

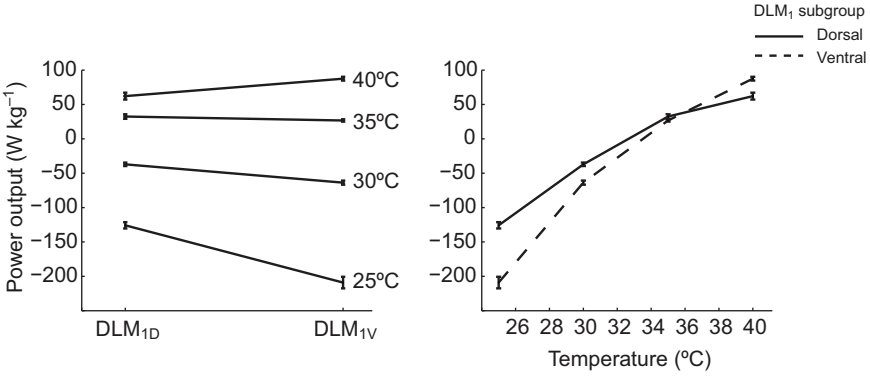

Fig. 4. Mechanical power output of the dorsal subunits (DLM_{1D}) compared with the ventral subunits (DLM_{1V}) of *M. sexta* flight muscle at the approximately *in vivo* phase of activation. Mean power output is plotted as a function of temperature for DLM_{1D} and DLM_{1V}. Although the two subunit groups had significantly different responses to temperature, this difference was not functionally significant. Mean power output for the two groups remained positive between 40 and 35°C and negative between 30 and 25°C. Values are reported as means ± s.e.m. (N=5 moths per group, 20 cycles per trial).

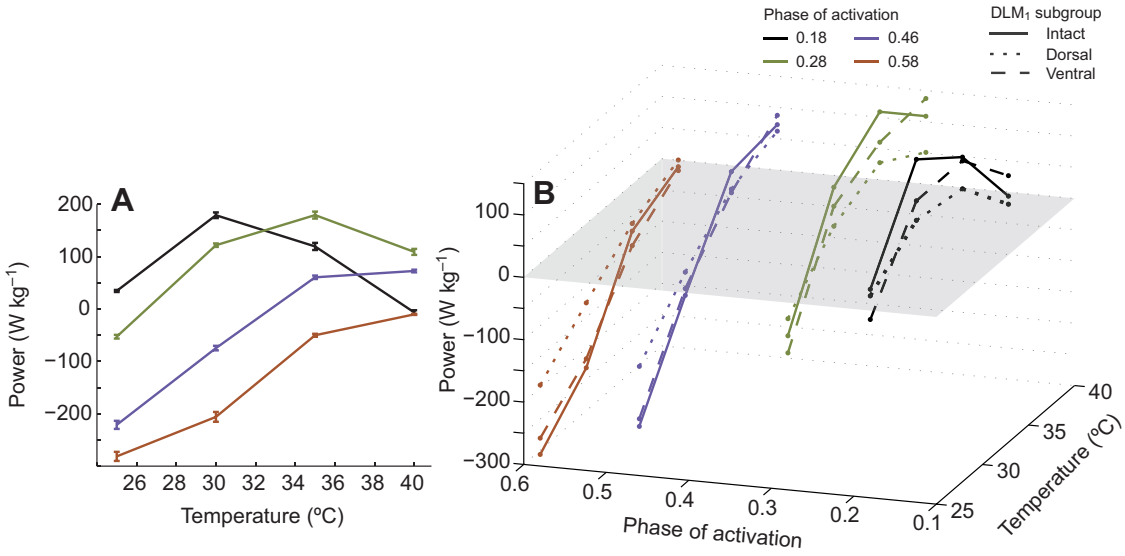

Fig. 5. The effect of phase of activation on power output in the presence of a temperature gradient. (A) Mass-specific power output for intact DLM_1 as a function of temperature for each phase of activation (0.18, 0.28, 0.46 and 0.58). Shifting the phase of activation results in a significantly different power–temperature relationship. Early phases of activation (0.18–0.28) produced peak power at intermediate temperatures (30 or 35°C). Conversely, later phases of activation (0.46–0.58) resulted in monotonically increasing power output with temperature. (B) Mass-specific power output as a function of temperature and the phase of activation. In the presence of a temperature gradient, adjusting the phase of activation represents a neuronal mechanism by which the organism could modify the functional gradient. The gray plane indicates the transition point between positive and negative power output. At approximately the *in vivo* phase, positive power was produced only between 40 and 35°C. However, advancing the phase to ~0.28 increased the temperature range that produced positive power to 40–30°C. Values are reported as means ± s.e.m. (*N*=5 moths per group, 20 cycles per trial).

modulation to actively regulate the activity of the DLM_1. Work-loops of intact DLM_1 at four different phases of activation (0.18, 0.28, 0.46 and 0.58) show that phase and temperature interact to affect the mechanical power output of this muscle (Fig. 5). For each phase of activation, a change in temperature from 25 to 40°C significantly affected power output, with all but one pairwise increase in temperature being statistically different (ANOVA, $P<0.0001$; Tukey–Kramer HSD, $P<0.0001$). Interestingly, phase of activation also affected the actual relationship between power output and temperature (ANOVA temperature–phase interaction, F-ratio=219.8, $P<0.0001$). Early phases of activation (0.18 and 0.28) produced peak power at intermediate temperatures (30 or 35°C), whereas later phases of activation (0.46 and 0.58) led to monotonically increasing power throughout the temperature range. As a consequence, maximum power output occurred at sequentially warmer temperatures as phase increased; for a phase of 0.18 mean maximum power output occurred at 30°C, for a phase of 0.28 it occurred at 35°C, and for phases of 0.46 and 0.58 it occurred at 40°C (Fig. 5A and Table 2).

As mentioned above, the *in vivo* phase of activation (~0.46) yielded positive power only at 35 and 40°C. Notably, advancing muscle activation to an earlier phase (e.g. 0.28) actually extended the temperature range across which positive power output was produced to 30–40°C. Conversely, a later phase (e.g. 0.58) resulted in negative power output across all temperatures (Table 2). The *in vivo* phase was submaximal for power production at all temperatures, consistent with earlier single temperature results (Tu and Daniel, 2004b).

As with a phase of activation of 0.46, the two subunit groups, DLM_{1D} and DLM_{1V}, had different power–temperature relationships for the additional phases of 0.18, 0.28 and 0.58 (ANOVA temperature–subgroup interaction, F-ratio=21.5–81.6, $P<0.0001$) (Fig. 5B). Overall, at each phase of activation, DLM_{1D} and DLM_{1V} had power–temperature curves that were similar in shape to each other and to that of the intact DLM_1 group (i.e. either power monotonically increased with temperature or maximum power occurred at some intermediate temperature). However, at each phase, DLM_{1D} maintained a shallower power–temperature curve than DLM_{1V}, with comparatively higher power produced at cooler temperatures and lower power produced at warmer temperatures. Furthermore, the temperature point at which the power–temperature curves for DLM_{1D} and DLM_{1V} crossed was itself phase dependent. For early phases of activation (0.18–0.28), power output of DLM_{1D} and DLM_{1V} crossed between 25 and 30°C, whereas for a phase of 0.46, power crossed at 35°C, and for a phase of 0.58, based on the trajectories of the power–temperature curves, we predict power to

Table 2. Mean power output (W kg^{-1}) for intact DLM_1 across the temperature gradient for all phases of activation (0.18–0.58)

Temperature (ºC)	0.18	0.28	0.46	0.58
25	34.37±2.00	–52.93±3.57	–221.26±7.77	–281.43±8.67
30	179.02±5.46	121.76±3.36	–74.66±4.41	–206.04±9.41
35	119.40±6.77	179.28±6.71	60.53±3.40	–50.16±2.88
40	–6.59±4.17	109.01±5.70	72.50±2.57	–10.24±1.09

N=5 moths per group, 20 cycles per trial. Values are reported as means ± s.e.m.

cross at a temperature greater than 40°C. However, the differences in power output between DLM_{1D} and DLM_{1V} were still relatively small compared with the effect of temperature (*F*-ratio ~5- to 8-fold greater for the effect of temperature *versus* subgroup). The difference in mean power output between the two subunit groups was never more than 40% of the total change in power caused by temperature. In addition, despite these differences in magnitude, the actual sign of power output, and therefore the functional role, did not change in either subunit group (with the exception of a phase of 0.18 at 25°C, where power output only differed by ~37 W kg^{-1}).

DISCUSSION

Our results show that a temperature gradient leads to a mechanical functional gradient within the DLM_1: as muscle temperature decreased from 40 to 25°C, power output of the DLM_1 transitioned from positive values to considerably negative ones – leading to regions in which a single muscle may act more as an actuator, more spring-like or more like a damping element. Although a small difference existed in the temperature dependence of power output between the two subunit groups (DLM_{1D} and DLM_{1V}), these differences were not sufficient to account for the large variation in mechanical power output that resulted from the temperature gradient predicted within the DLM_1 *in vivo* (10–15°C). The combined evidence suggests that a functional gradient will follow the temperature gradient, with individual subunits of the DLM_1 contributing separately to energy production, storage and/or absorption. In the discussion below, we expand upon our observations of a mechanical energy gradient and explore its implications for the coordinated muscle movement required for animal locomotion.

Temperature gradients within flight power muscles translate to functional gradients

Recent studies have revealed that muscles perform diverse functions to meet the fundamental demands of locomotion, including energy production, storage, absorption and transmission (Marsh and Olsen, 1994; Tu and Dickinson, 1994; Roberts et al., 1997; Biewener et al., 1998; Full et al., 1998; Swank and Rome, 2001; Sponberg et al., 2011). Thus, muscles may act as actuators or may behave more like springs, struts or even damping elements. However, these studies assumed spatially uniform temperatures within muscle, such that the contractile properties of the whole muscular unit follow from a spatially uniform mechanical behavior.

Temperature has a significant effect on muscle performance. Organisms across a range of habitats and locomotor modes experience increased contractile rates (i.e. activation and relaxation), and therefore increased mechanical power output, as muscle temperature increases (Josephson, 1984; Bennett, 1985; Rall and Woledge, 1990; Swoap et al., 1993; Rome at al., 1999; Donley at al., 2007). For example, a 20°C increase in muscle temperature yielded ~70 and 111 W kg^{-1} more specific mechanical power output in moths and lizards, respectively (Stevenson and Josephson, 1990; Swoap et al., 1993). Given this strong temperature dependence, the spatial pattern of temperature within a muscle may be a key factor determining overall muscle performance.

Our work-loop study revealed that power output of the DLM_1 was indeed highly temperature dependent. Notably, across the temperature range that we expect to occur in the DLM_1 of *M. sexta* (~15°C, from 40 to 25°C), power output at the *in vivo* phase of activation transitioned from positive to highly negative values, with a mean difference of ~294 W kg^{-1}. Even a more conservative estimate of a temperature difference of 10°C (from 40 to 30°C) resulted in a shift from positive to negative power output with a mean difference of ~147 W kg^{-1} (Fig. 3; Table 1). Thus, without significant physiological differentiation among the subunits of the DLM_1, ventral subunits will operate more as power producers while dorsal subunits, being significantly cooler, will operate as energy absorbers.

Differences in physiology across the muscle do not compensate for temperature gradients

Despite the presence of a temperature gradient, the DLM_1 could have a spatially uniform mechanical power output if appropriate physiological mechanisms could compensate for the local temperature. Spatially uniform power output throughout the DLM_1 could be accomplished by both extrinsic factors (i.e. neural activation) and intrinsic factors (i.e. fiber contractile dynamics).

Although each of the five DLM_1 subunits are separately innervated by neurons in the IIN_{1C} nerve (Kondoh and Obara, 1982; Eaton, 1988), allowing the potential for individual activation, they are effectively activated simultaneously (difference of only ~0.22 ms or ~0.6% of the wingbeat cycle) (George and Daniel, 2011). Thus, moths do not appear to utilize dorso-ventral phase adjustments in muscle activation to yield increased power output in cooler dorsal subunits.

In addition, regional differences in the intrinsic properties of the muscle fibers comprising the DLM_1 could offset the consequences of temperature gradients. Prior studies have already demonstrated variation in both fiber type and contractile properties (i.e. activation and relaxation rates) across and within muscle (Swank et al., 1997; Mu and Sanders, 2001; Swank and Rome, 2001; Wang and Kernell, 2001), though not specifically within *M. sexta* flight muscle. Not surprisingly, varying the rate of muscle activation and relaxation greatly influences the magnitude of power produced in oscillatory movement (Rome and Swank, 1992; Josephson, 1993; Swoap et al., 1993; Swank and Rome, 2001). For example, a 20% decrease in twitch activation time associated with cold acclimation resulted in up to a 2.5-fold increase in power production in fish muscle (Swank and Rome, 2001). Because of these observations, we must determine local responses to the temperature of subunits within a muscle before we can interpret the functional consequences of a temperature gradient.

Despite the small variation in the power–temperature relationship between ventral and dorsal DLM_1 subunits, this difference fails to be functionally significant. At the predicted *in vivo* operating temperatures for the dorsal subunits, 25 and 30°C, DLM_{1D} did produce higher power output than DLM_{1V}. However, this increase in performance was still significantly below the power produced by the ventral subunits at their predicted *in vivo* temperatures of 35 and 40°C (Table 1). More importantly, the power produced by DLM_{1D} remained negative at 25 and 30°C, whereas DLM_{1V} produced positive power at 35 and 40°C. Thus, regional specialization of the DLM_1 subunits does not appear to compensate for the large temperature dependence in such a way as to produce uniform mechanical power output.

Implications for the role of different subunits in flight

Whereas the DLM_1 is generally assumed to solely produce power, driving the downstroke of the wing, we have demonstrated that muscle function in the DLM_1 may actually be systematically and heterogeneously distributed (Fig. 6). Warm ventral subunits, operating at ~35–40°C, will produce positive power and drive wing depression. However, the dorsal subunits, operating at ~25–30°C, will produce negative power and function more as energy absorbers,

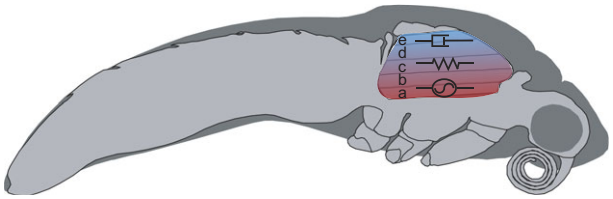

Fig. 6. A schematic representation of a temperature-induced functional gradient. Proceeding from the ventral subunits to the dorsal subunits, the symbols represent a motor, a spring and a dashpot. Our work-loop studies demonstrated that a temperature difference of 10–15°C throughout the $DLM_{1a–e}$ would lead to a significant gradient in mechanical power output. Warm ventral subunits operating around 40–35°C would produce positive power and operate as a motor. Proceeding dorsally, mechanical power output of the DLM_1 subunits would decrease significantly with the decline in muscle temperature. Thus, more dorsal subunits, operating around 35–25°C, would produce close to zero or negative mechanical power, and function more as an elastic energy storage source or more as a damper, respectively. Therefore, although the DLM_1 is commonly thought to function solely as a motor, a temperature gradient throughout the muscle will likely result in a spatial gradient of functional performance.

possibly contributing to the stability of the system. Interestingly, this transition in power output necessarily implies that at some midpoint in the DLM_1, muscle would generate little or no mechanical power, possibly providing a more spring-like behavior. This is not to say that the subunits could not all contribute to energy storage. Rather, we point out that a region of zero mechanical power output may still be functionally important.

Several studies suggest that elastic energy storage plays a crucial role in insect flight, allowing insects to reduce the inertial power costs of accelerating the wings (Alexander and Bennet-Clark, 1977; Ellington, 1984; Dickinson and Lighton, 1995; Wu and Sun, 2005). Although resilin, a rubber-like protein within cuticular structures, is presumed to be the main site of this energy storage (Jensen and Weis-Fogh, 1962; Haas et al., 2000; Gosline et al., 2002), a recent study found that thick filaments in asynchronous muscle deform elastically (Dickinson et al., 2005), indicating that a portion of energy storage could occur within the myofilaments themselves. We suggest that a temperature gradient, because of its effect on contraction dynamics within a single muscle, provides an additional mechanism for elastic energy storage to be utilized. The cross-bridges of the cooler dorsal subunits, with their reduced cycling rates, would remain on average more attached to thin filaments, forming a locked spring lattice. Elastic energy could be stored in the cross-bridges and in the axial portions of the myofilament lattice as well. The stored mechanical energy could then be released during the next part of the wingbeat cycle, acting in concert with the antagonistic dorsoventral upstroke muscles to elevate the wings. Cross-bridges and the filament lattice could therefore contribute to the total energy storage needed for flight.

Implications for locomotor control

The timing of muscle activation relative to the length cycle significantly affects mechanical power output (Josephson 1985; Josephson, 1999; Tu and Daniel, 2004b). Several organisms use different phases of activation to enable functional diversity across different muscles (Tu and Dickinson, 1994; Altringham and Ellerby, 1999; Dickinson et al., 2000; Ahn and Full, 2002). In moths, this dependence goes further, affording the organism a mechanism by which the nervous system can rapidly modulate muscle power output by adjusting the phase of the DLM_1 in response to sensory feedback from visual stimuli (S.S. and T.L.D., unpublished). Given this possibility of sensory feedback control, the phase of activation could additionally regulate the extent to which muscle function is diversified by influencing the relationship between power and temperature. During sustained flight, the DLM_1 of *M. sexta* activate just before shortening (phase of ~0.49) (Tu and Daniel, 2004a). At this approximate phase of activation, a 10°C difference (40–30°C) across the DLM_1 led to positive power output at 40 and 35°C but negative power output at 30°C. Delaying the phase to ~0.58 essentially led to negative power production at all temperatures. However, advancing the phase to ~0.28 actually increased the effective temperature range that yielded positive power output to 40–30°C. Thus, advancing the phase of activation from that during steady-state flight may generate additional mechanical power output by enabling a larger percentage of the DLM_1 to function as a motor.

Given an inherent temperature gradient, the ability of the nervous system to control the phase of activation may in fact enhance the roles that the power muscles can play in neuromechanical control of locomotion. Surprisingly, operation at submaximal levels of power output may occur among flying, swimming and terrestrial organisms (Josephson, 1997; Tu and Daniel, 2004b) (S.S. and T.L.D., unpublished). Perhaps this energy conservation leaves reserves available to the organism for use in locomotor control or in extreme behaviors such as escape maneuvers. With a temperature gradient in place, an organism could simply shift the phase of activation to increase the proportion of muscle acting as a motor rather than as an energy storage source or damper. Thus, given a fixed temperature gradient, an organism could modulate the phase of activation and thereby diversify the functional gradients accessible, effectively increasing the performance range.

CONCLUSIONS

A spatial gradient in the mechanical power output driven by a temperature gradient reveals a considerable spatial gradient in the functional consequences of simultaneous muscle activation in a single muscle. Our work-loop analyses show that the separate subunits of the DLM_1 concurrently operate as a power generator, an elastic energy storage source or an energy absorber. Thus, the common assumption that the individual components of a muscle operate uniformly to command a single function should be re-evaluated in the context of measured spatial profiles of temperature. In addition to our current study, a growing body of evidence indicates that a single muscle can exhibit functional heterogeneity driven by morphological, neurological and/or physiological differences (Mu and Sanders, 2001; Ahn and Full, 2002; Ahn et al., 2003; Higham et al., 2008; Wakeling, 2009). The subunits of the DLM_1 may function synergistically during flight, but this function may include elastic energy storage and damping in addition to the canonical view of power production. Although it has not been extensively studied in other organisms, significant temperature gradients in any locomotor muscle would necessarily imply a gradient in the functional roles played by regions within a single muscle.

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