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RESEARCH ARTICLE

Very low force-generating ability and unusually high temperature dependency in hummingbird flight muscle fibers

Peter J. Reiser[1,*], Kenneth C. Welch, Jr[2,†], Raul K. Suarez[3] and Douglas L. Altshuler[2,4]

[1]Department of Oral Biology, Ohio State University, Columbus, OH, 43210, USA, [2]Department of Biology, University of California at Riverside, Riverside, CA, 92521, USA, [3]University of California at Santa Barbara, Santa Barbara, CA, 93106, USA and [4]Department of Zoology, University of British Columbia, Vancouver, BC, Canada, V6T 1Z4

*Author for correspondence (reiser.17@osu.edu)

†Present address: Department of Biological Sciences, University of Toronto Scarborough, Toronto, ON, Canada, M1C 1A4

SUMMARY

Hummingbird flight muscle is estimated to have among the highest mass-specific power output among vertebrates, based on aerodynamic models. However, little is known about the fundamental contractile properties of their remarkable flight muscles. We hypothesized that hummingbird pectoralis fibers generate relatively low force when activated in a tradeoff for high shortening speeds associated with the characteristic high wingbeat frequencies that are required for sustained hovering. Our objective was to measure maximal force-generating ability (maximal force/cross-sectional area, P_o/CSA) in single, skinned fibers from the pectoralis and supracoracoideus muscles, which power the wing downstroke and upstroke, respectively, in hummingbirds (*Calypte anna*) and in another similarly sized species, zebra finch (*Taeniopygia guttata*), which also has a very high wingbeat frequency during flight but does not perform a sustained hover. Mean P_o/CSA in hummingbird pectoralis fibers was very low – 1.6, 6.1 and 12.2 kN m^{-2}, at 10, 15 and 20°C, respectively. P_o/CSA in finch pectoralis fibers was also very low (for both species, ~5% of the reported P_o/CSA of chicken pectoralis fast fibers at 15°C). $Q_{10\text{-force}}$ (force generated at 20°C/force generated at 10°C) was very high for hummingbird and finch pectoralis fibers (mean=15.3 and 11.5, respectively) compared with rat slow and fast fibers (1.8 and 1.9, respectively). P_o/CSA in hummingbird leg fibers was much higher than in pectoralis fibers at each temperature, and the mean $Q_{10\text{-force}}$ was much lower. Thus, hummingbird and finch pectoralis fibers have an extremely low force-generating ability compared with other bird and mammalian limb fibers, and an extremely high temperature dependence of force generation. However, the extrapolated maximum force-generating ability of hummingbird pectoralis fibers *in vivo* (~48 kN m^{-2}) is substantially higher than the estimated requirements for hovering flight of *C. anna*. The unusually low P_o/CSA of hummingbird and zebra finch pectoralis fibers may reflect a constraint imposed by a need for extremely high contraction frequencies, especially during hummingbird hovering.

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INTRODUCTION

The pectoralis and supracoracoideus muscles of birds power the downstroke and upstroke, respectively, during flight. Among vertebrates, sustained hovering is unique to hummingbirds and demands one of the highest rates of mass-specific power output of any steady-state form of vertebrate locomotion [73–219 W kg^{-1} muscle, conservatively assuming perfect elastic storage of energy across stroke transitions (Altshuler et al., 2010a; Chai and Dudley, 1996; Wells, 1993)]. Investigators have, particularly recently, identified a number of dietary, behavioral, morphological and neuromuscular specializations in hummingbirds that appear to facilitate hovering flight and the production and modulation of the high power output it requires. Hummingbirds can sustain aerobic metabolism at some of the highest rates observed in vertebrates when hovering under benign conditions and can substantially increase metabolism to meet greater power output demands during hovering under more taxing conditions, such as at high elevation or in reduced density gas mixtures (Suarez, 1992; Chai and Dudley, 1996; Chai et al., 1996; Welch and Suarez, 2008). Several morphological and physiological specializations in hummingbird flight muscles support this elevated aerobic capacity. Like other small-bodied birds, flight muscles in hummingbirds are composed exclusively of fast-twitch, oxidative fibers (Rosser and George, 1986; Lundgren and Kiessling, 1988; Welch and Altshuler, 2009). In addition, hummingbird flight muscles have the highest capillary and mitochondrial densities of any vertebrate locomotor muscles (Mathieu-Costello et al., 1992a). All hummingbirds are nectarivorous and display both behavioral and physiological adaptations that result in an enhanced capacity for fuel use (Suarez et al., 1990; Welch et al., 2006; Welch and Suarez, 2007; Welch et al., 2007). Further, hummingbirds exhibit enhancement of physiological and biochemical pathways responsible for the oxidation of dietary carbohydrate (in a process recently named the ‘sugar oxidation cascade’) and endogenous fat stores (Suarez and Welch, 2009; Suarez, 1996; Suarez et al., 2009; Suarez et al., 2011). However, some of the same adaptations that serve sustained hovering, such as a high mitochondrial volume (Mathieu-Costello et al., 1992a), are likely to impact the magnitude of active

stress [force per unit of fiber cross-sectional area (CSA)] development by reducing the relative myofibrillar volume.

Significant progress has also been made in understanding the kinematics and aerodynamics of hummingbird hovering. The majority of force necessary to drive wingbeats during hovering is produced by flight muscles that undergo contractions at frequencies that are among the highest in vertebrate locomotory muscles. Wells reported very high power output of hummingbird flight muscles during hovering, based on an analysis of wing kinematic data (Wells, 1993). More recently, Warrick et al. employed a particle velocimetry analysis to study hovering in rufous hummingbirds (*Selsasphorus rufus*) and reported that both the upstroke and downstroke generate lift, but do so unequally (Warrick et al., 2005; Warrick et al., 2009). Several studies have employed reduced density air mixtures or the application of additional weight to uncover how hummingbirds modulate wingbeat kinematics to control lift production (e.g. Chai and Dudley, 1996; Chai et al., 1996; Altshuler and Dudley, 2003; Altshuler et al., 2010b). The results revealed that hummingbirds can increase wingbeat frequency, wing stroke amplitude or both parameters simultaneously to adjust mechanical power output.

Collectively, these studies have shown that the high power requirements and need for rapid and precise control characteristics of hovering flight in hummingbirds are associated with specialization of flight aerodynamics, kinematics and energetics as well as of the ultrastructure and morphology of flight muscles. However, fundamental contractile properties of muscle fibers that drive hovering in hummingbirds have not been examined. The primary goal of the present study was to measure the force-generating ability of single muscle fibers of hummingbird pectoralis muscle fibers, given their required high rates of contraction and relaxation during hovering and the reported trade-off between force and speed in muscles that operate at high frequencies (Rome et al., 1999; Rome, 2006). The results were compared with those from hummingbird leg muscle fibers, as well as pectoralis and leg muscle fibers from another avian species, the zebra finch, *Taeniopygia guttata*, a species of similar body size that does not perform sustained hovering but has a relatively high wingbeat frequency during flight [28–30 Hz during horizontal flight and rapid ascent (Tobalske et al., 2005)]. Furthermore, it is known that hummingbirds can enter torpor at night (Kruger et al., 1982; Hiebert, 1991; Hiebert, 1992; Hiebert, 1993; Calder, 1994; Powers et al., 2003), during which they can become relatively immobile when body temperature decreases to as low as ~10–13°C (Bartholomew et al., 1957; Hiebert, 1990). Force generation in striated muscle is known to be temperature dependent (Kuhn et al., 1979; Reiser and Lindley, 1990; Kawai, 2003; Ranatunga, 2010) (see also Discussion). Therefore, another goal was to measure the force-generating ability of hummingbird flight and leg muscles at lower temperatures, to determine whether entry into torpor might have functional consequences for flight and perching capabilities. The approach was to measure maximal force generation in isolated skinned muscle fibers during steady, maximal activation. We measured force generation from 10 to 20°C, and discuss forces extrapolated to higher temperature because skinned muscle fibers are not stable when activated at the normal body temperatures of birds.

MATERIALS AND METHODS

Animals

Adult male birds were used for this study. Anna's hummingbirds [*Calypte anna* (Lesson 1829), N=11; mean ± s.e.m. body mass: 4.7±0.1 g] were captured locally (Santa Barbara and Riverside, CA) and zebra finches (*Taeniopygia guttata* Reichenbach 1862, N=10; body mass: 14.7±0.6 g) were obtained from local vendors. Pectoralis strips (consisting of bundles of fibers, 1–2 mm thick) and the ankle extensor muscle group from the lower legs (with skin removed) were collected from both species immediately following euthanasia (carbon dioxide asphyxiation or overdose of ketamine/xylazine) and placed in cold storage solution ['relaxing' solution, containing 50% (v/v) glycerol (Reiser et al., 1985)]. Samples were shipped overnight with ice packs frozen at −20°C to Ohio State University for the single fiber force measurements. Samples were also obtained from one adult male house sparrow [*Passer domesticus* (Linnaeus 1758); body mass: 33.5 g], which was found shortly after death, presumably resulting from collision with a nearby building. The weather conditions included overcast sky and 4°C ambient temperature. The bird was quickly transported to the laboratory within ~10 min. Blood ran freely when the heart was excised. The pectoralis muscle, which had a normal appearance and was very pliable (rigor had not developed), was immediately isolated, placed in cold relaxing solution, and bundles were prepared. Samples of the fast-twitch tibialis anterior and slow-twitch soleus muscles were also prepared from 2-month-old male Sprague–Dawley rats (N=2; body mass: ~250 g), following anesthesia (ketamine/xylazine) and decapitation. All of the shipped and locally prepared muscle bundles were kept in storage solution at −20°C for up to 5 weeks. None of the hummingbird, finch, sparrow or rat fiber bundles were supported (i.e. tied to glass capillary tubes) at any time during shipment and/or storage in the present study. In two studies in this laboratory (P.J.R., unpublished), two sets of fibers (N=9 in both) from rabbit diaphragm were studied with or without support during storage, with all other conditions, methodology and apparatus being identical. The mean active stress with and without support during storage was 95.2 and 103.6 kN m^{-2}, respectively, and the difference was not statistically significant (t-test, P=0.195). We conclude from this that support during storage is not necessary. All of the procedures that were conducted on live animals were in accordance with protocols approved by the Institutional Animal Care and Use Committees of the University of California at Santa Barbara, University of California at Riverside and Ohio State University.

Force measurements

Fibers were isolated from the bundles in a Petri dish containing relaxing solution and using a dissection microscope, and were mounted in the experimental chamber. Individual hummingbird and finch leg muscles, from which fibers were isolated, were not identified. All of the fibers, except two sparrow pectoralis fibers, were studied within 5 weeks following euthanasia and force-generating ability did not change significantly over this time. The force-generating ability of two sparrow pectoralis fibers that were studied on day 50 was not different from the others in this group. The fibers were attached to a servo-controlled torque motor (model 322C, Aurora Scientific, Aurora, ON, Canada) on one end and to an isometric force transducer (model 406 for bird fibers and model 403 for rat fibers, Aurora Scientific) on the other end, as illustrated by Moss (Moss, 1979). The motor and transducer were mounted on three-way positioners. A U-shaped experimental chamber, which was placed on the stage of an upright Nikon Labophot microscope, housed the mounted servomotor and force transducer, and a central spring-loaded stainless steel insert, the latter with a coverglass floor for transillumination of the fiber. The insert had three wells that contained solutions for bathing the fibers. The temperature of the insert was monitored with a thermistor, which was embedded in one of the wells and was connected to a thermometer (model 43TD Tele-Thermometer, Yellow Springs Instruments, Yellow Springs,

OH, USA). Output from the thermometer was connected to a custom-built regulated power supply, which was, in turn, connected to three thermoelectric devices that cooled the chamber and central insert.

The mounted fibers were slowly stretched manually, by sliding the motor, until the fiber was set to just above slack length. The length of the portion of the fiber that was exposed to the bathing solution was determined by measuring the displacement of the experimental chamber when a microscope eyepiece reticle was aligned, first on one end, then on the other end, of the fiber. The chamber displacement was measured with a dial indicator (model 25-881, L. S. Starrett Co., Athol, MA, USA; resolution: 10 μm) that was in contact with the microscope stage. A camera, which was mounted on the top of the microscope, was used to capture images of the fibers in the experimental chamber, using brightfield illumination. An image of the central portion of the fiber was captured at this length and the sarcomere length at this fiber length (referred to as SL_{null}) was determined. There was a broad range of SL_{null} values between groups of fibers. A group in this study consisted of pectoralis, supracoracoideus (hummingbird only) or leg fibers from an individual species. The pectoralis fibers from each bird species had very short mean SL_{null} values (the mean for the three species ranged from 1.84 to 1.89 μm) and hummingbird leg fibers had a much longer mean value (2.45±0.08 μm). Several initial hummingbird pectoralis and supracoracoideus fibers were stretched to a slightly (5–10%) longer resting sarcomere length (SL_{rest}) and were activated, but they immediately tore. Force generation was successfully measured in three hummingbird pectoralis fibers at several sarcomere lengths, beginning with SL_{null} and followed by setting the fibers to longer sarcomere lengths prior to activation. On average, generated force increased by about twofold from ~1.9 to ~2.2 μm and precipitously fell with additional increases in sarcomere length in each of these fibers. Therefore, the fibers from hummingbird and finch pectoralis and hummingbird supracoracoideus were set to a resting sarcomere length (SL_{rest}), prior to activation, that was equal or very close to SL_{null} (Table 1) to prevent tearing during activation. SL_{rest} in rat fibers was typically set to a value that is closer to the SL corresponding to maximal force generation in mammalian fibers (2.2–2.5 μm). This resulted in force being measured at different SLs in different groups of fibers. Several experiments were conducted to evaluate the contribution of the variation in SL_{rest} to the differences in force-generating ability between groups of fibers (see Results).

Fiber CSA was calculated from width and depth measurements and assuming an ellipsoidal shape. Fiber width was measured from images as described above for measuring SL. Fiber depth was measured with the same camera, using a fiber optic lamp and two front surface mirrors in the experimental chamber, which allowed capturing a side-view of the fiber.

Solutions, contained in the wells of the chamber insert, for force measurements were prepared at each temperature, as described in Greaser et al. (Greaser et al., 1988). A separate set of solutions (pCa 4.0, pCa 9.0 and the HDTA-containing solution) was prepared for 10, 15 and 20°C. The same constituent concentrations, ionic strength (180 mmol l^{-1}) and pH (7.0) were specified for each temperature. All fibers were activated with pCa 4.0 (i.e. free $[Ca^{2+}]$=0.1 mmol l^{-1}) solution four times at each temperature and an average absolute force was calculated. Resting force, determined in pCa 9.0 solution, was subtracted from the total force to calculate the active force (P_o) generated. The fiber was briefly soaked in a solution with pCa 9.0, 0.05 mmol l^{-1} EGTA and 6.5 mmol l^{-1} HDTA prior to activation in the pCa 4.0 solution (Greaser et al., 1988). Mean active force at each temperature was normalized with fiber CSA (P_o/CSA). The relative force/pCa relationship at 20°C was determined, as described previously (Greaser et al., 1988), for three fibers from hummingbird and finch pectoralis and leg muscles and from sparrow pectoralis muscle to ensure that pCa 4.0 was maximally activating. This temperature was chosen because the low forces generated at submaximal levels of activation at lower temperatures were difficult to measure reliably. The fibers were isolated from one additional animal of each species. The results (shown in supplementary material Fig. S1) indicate that relative force was maximal at pCa 4.0 or at a slightly higher pCa value for each group of fibers. Results from previous studies (e.g. Metzger and Moss, 1990; Wahr et al., 1997; Danieli-Betto et al., 2000) have shown that pCa 4.0 or higher is maximally activating for rat fast and slow fibers.

Initial sets of hummingbird supracoracoideus and pectoralis fibers were studied at only 10°C. Subsequently, 10 fibers from hummingbird and finch pectoralis and leg muscles (i.e. 40 fibers total) were studied at each temperature and a complete set of data was obtained for every fiber – that is, four force measurements at each temperature. The average of the force generated by each fiber at each temperature was used for statistical analysis. The order in which temperature was varied, from 10, 15 and 20°C, or 20, 15 and 10°C, was alternated for each subsequent fiber. Measurements at higher temperatures were not attempted because skinned muscle fibers are not stable above ~20°C when activated in solutions with a calcium concentration that ensures maximal activation. The reason for the instability is not clear but it likely is related, at least in part, to the loss of the sarcolemma, which otherwise could provide structural support during episodes of high force generation. Five fast and five slow fibers from rat tibialis anterior and soleus, respectively, were also studied at each temperature. Five fibers from the pectoralis muscle of one adult

Table 1. Summary of force/cross-sectional area (P_o/CSA; kN m^{-2}), $Q_{10\text{-force}}$ (force generated at 20°C/force generated at 10°C), sarcomere length just beyond slack (SL_{null}, see Materials and methods) and resting sarcomere length (SL_{rest}) prior to activation in single muscle fibers

	Hummingbird		Finch		Rat		Sparrow
	Pectoralis (*N*=10)	Leg (*N*=10)	Pectoralis (*N*=10)	Leg (*N*=10)	Limb slow (*N*=5)	Limb fast (*N*=5)	Pectoralis (*N*=10)
P_o/CSA at 10°C	1.6±0.5	43.5±5.1	2.2±0.3	29.1±5.7	68.8±6.0	66.8±4.2	9.2±1.5
P_o/CSA at 15°C	6.1±1.4	74.7±8.8	9.6±1.2	52.6±5.4	98.5±8.3	88.2±3.8	
P_o/CSA at 20°C	12.2±2.3	94.2±10.5	22.2±2.3	79.3±6.4	123.1±7.3	122.0±5.3	
$Q_{10\text{-force}}$	15.3±4.5	2.2±0.1	11.5±1.3	3.6±0.5	1.8±0.1	1.9±0.1	
SL_{null} (μm)	1.84±0.02	2.45±0.08	1.87±0.04	1.98±0.07	2.27±0.05	2.32±0.03	1.89±0.05
SL_{rest} (μm)	1.89±0.03	2.56±0.04	1.87±0.04	2.20±0.05	2.53±0.03	2.53±0.02	2.30±0.05
CSA (μm^2)	637±36	754±52	1020±80	1763±139	3220±298	8134±588	1118±51
Absolute force at 10°C (μN)	1.0±0.3	32.1±3.7	2.3±0.4	55.1±13.9	217.8±17.6	538.4±24.3	10.1±1.5

All values are means ± s.e.m.

house sparrow were studied at only 10°C. A 'stability factor' was calculated for each fiber, as follows. The ratio of the force generated during the last (fourth) activation was divided by the force generated during the first activation at each temperature at which a fiber was studied. The average of the ratios (stability factor) for each temperature for a given fiber was calculated. The average of the stability factor for each group of fibers was then calculated. A stability factor greater or lesser than 1.00 indicates that, on average across all temperatures, the final force generated was greater or lesser, respectively, than the first force generated. For hummingbird pectoralis, supracoracoideus and leg fibers, the average stability factors were 1.11, 1.12 and 1.01, respectively. For finch pectoralis and leg fibers, the average stability factors were 1.09 and 1.01, respectively. For sparrow pectoralis fibers, the average stability factor was 1.12. For rat fast and slow fibers, the average stability factors were 1.00 and 0.98, respectively. It was concluded that all groups of fibers were stable during the measurements, with a small augmentation of force in the pectoralis and supracoracoideus fibers. The basis for the augmentation was not determined.

Slack (~15% of fiber length) was introduced in the fiber by rapid (completed in ~3 ms) movement of the motor when peak steady force was attained. The motor was maintained at this position for 90 s to allow sufficient time to return the fiber to the pCa 9.0 solution and complete relaxation (both usually complete within 10 s) before being re-extended. The slackening of the fiber dropped the force to a baseline (illustrated in Fig. 2). The drop in force was measured and was recorded as total force. An identical slackening step was performed when the fiber was fully relaxed, while bathed in the solution with pCa 9.0, to measure the resting force in the fiber. Active force was calculated by subtracting resting force (at pCa 9.0) from total force (at pCa 4.0). The fibers took up the induced slack very slowly (over several seconds) at 10°C. Attempts were made to measure the maximal shortening velocity from the force records (using the 'slack test'), but the very low signal-to-noise ratio in pectoralis fibers of all species precluded making reliable measurements. The same fibers took up the induced slack faster at 15°C, but it was still very difficult to measure shortening velocity accurately, for the same reason. Therefore, shortening velocity was not measured in this study.

Force was measured in several fibers before and after being soaked in 10 μmol l^{-1} exogenous fast-type chicken troponin-C (TnC) for 35 min at 15°C to test whether the low force generation observed in some fibers was due to a possible loss of TnC during storage.

Estimation of myofibrillar volume

Relative myofibrillar area was determined from a set of 8×10 inch prints, made from a subset of the original 70 mm electron micrograph negatives generated from transverse sections of rufous hummingbird (*S. rufus*) pectoralis muscle analyzed by Mathieu-Costello et al. (Mathieu-Costello et al., 1992a). A detailed description of tissue sampling, preparation and how electron micrographs were obtained is given in Mathieu-Costello et al. (Mathieu-Costello et al., 1992a). Micrographs from hummingbirds (*S. rufus*; N=4; body mass: 3.2±0.1 g) were digitized at 600 dpi resolution. Three to 12 micrographs from non-overlapping areas of muscle from each bird were analyzed as follows. Myofibrils were carefully traced using the 'wand' tool in Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA). The traced area was copied, made entirely black and saved as a separate image at the same resolution. The selected area of myofibril was determined in pixels using the 'measurement' function within ImageJ, version 1.45s (National Institutes of Health, Bethesda, MD, USA). This process was repeated for all non-myofibrillar areas of the fiber (e.g. mitochondria, lipid droplets, sarcoplasmic reticulum). Relative myofibrillar area was calculated as the total myofibrillar area (in pixels) divided by the total area of myofibrillar area and non-myofibrillar area (in pixels). The values reported are means ± s.e.m., calculated from the average value for each individual.

Statistics

Fibers within a group were pooled from multiple animals, because there was very little variation between individual birds of the same species. The statistical significance of differences between mean values were evaluated with either a paired (comparing a single group of fibers at different temperatures) or unpaired (same temperature, different fiber groups) t-test. Significance was set at P-values less than or equal to 0.05. All of the values are reported as means ± s.e.m.

RESULTS

An image of a typical hummingbird pectoralis fiber, mounted in the experimental chamber, is shown in Fig. 1. The striation pattern in the majority of fibers was very clear and uniform, allowing for reliable sarcomere length measurements (Table 1). Fibers from hummingbird pectoralis and supracoracoideus muscles (10 fibers from both muscles) were initially studied at 10°C and the mean force/CSA generated in the pCa 4.0 solution was 3.2±1.1 and 3.8±1.4 kN m^{-2}, respectively.

Given the very low force-generating ability [maximal force (P_o)/CSA] in these two groups of fibers at 10°C, measurements were made at 10, 15 and 20°C in another group of hummingbird and finch pectoralis and leg muscle fibers (10 in each group, 40 total). Force records from one hummingbird pectoralis fiber are shown in Fig. 2. The mean P_o/CSA generated by hummingbird and finch pectoralis fibers at 10°C was again extremely low (Table 1) and did not differ between the two species (P=0.293). The P_o/CSA generated in pectoralis fibers at 15°C was significantly greater (~4-fold; P<0.001) in both species than the force generated at 10°C, and differed significantly between species (P<0.04). Forces generated at 20°C were even greater than at 15°C (~2-fold; P<0.00001) for both species, and differed between species (P<0.004). The mean $Q_{10\text{-force}}$ values (force at 20°C/force at 10°C) were very high (Table 1) for hummingbird and finch pectoralis fibers and did not differ significantly between species.

Leg muscle fibers from both species generated greater P_o/CSA at each temperature (Table 1), compared with pectoralis fibers in the same species (P<0.0002 for all comparisons). Hummingbird and finch leg fibers differed from each other only at 15°C (P<0.047). The mean $Q_{10\text{-force}}$ values for hummingbird and finch leg muscle fibers, from 10 to 20°C, were much lower than in pectoralis fibers (Table 1) and differed significantly (P<0.022) between species.

The relationship between P_o/CSA and temperature was linear between 10 and 20°C for each group of fibers (i.e. hummingbird and finch pectoralis and leg). The correlation coefficient of the regression of generated force with temperature was calculated for each fiber in each group. The mean of the coefficient for all of the fibers in a group varied from 0.98 to 0.99.

Five sparrow pectoralis fibers were studied at 10°C (Table 1). The maximal P_o/CSA was much greater (P<0.00003) than in the hummingbird and finch pectoralis fibers at 10°C. Ten rat limb muscle fibers (five fast fibers from the tibialis anterior and five slow from the soleus) were also studied at 10, 15 and 20°C (Table 1). Fiber type identification (either slow or fast) was based upon myosin

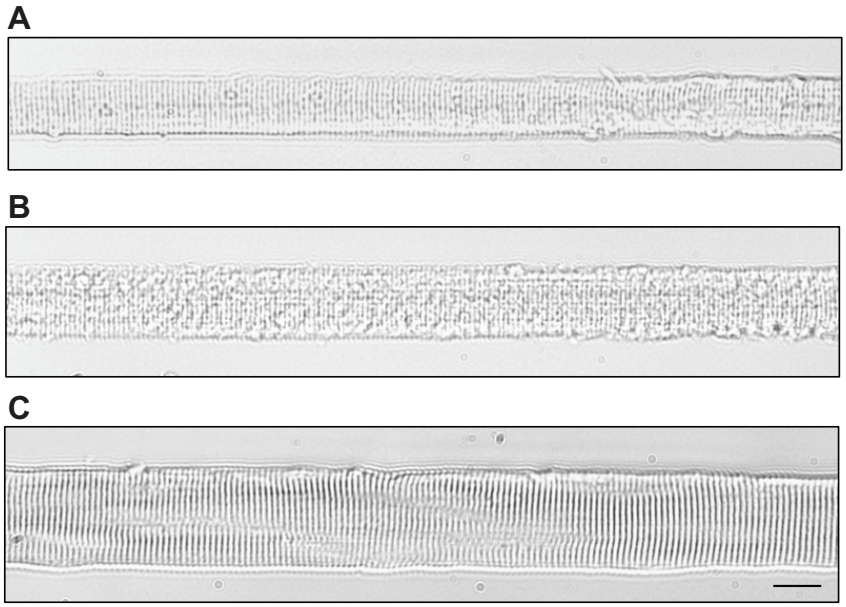

Fig. 1. Representative hummingbird pectoralis fiber (A), supracorcoideus fiber (B) and leg muscle fiber (C), each mounted in the experimental chamber (all at the same magnification). Scale bar, 25 μm.

heavy chain isoform composition, as determined by SDS-PAGE (not shown). The mean P_o/CSA at each temperature was very similar for both groups of fibers (Table 1). The mean $Q_{10\text{-force}}$ values for fast and slow fibers were very similar and both were much lower than for hummingbird and finch pectoralis fibers.

Muscle fibers from which TnC is extracted or is spontaneously lost during storage generate less force compared with intact fibers (Nakayama et al., 1983; Moss et al., 1986). Therefore, force generation was measured (at 10°C) in three additional hummingbird fibers (two pectoralis and one supracoracoideus), before and after soaking the fibers in exogenous fast-type chicken TnC [16.7 μmol l^{-1} in pCa 9.0 solution, for 35 min (Davis et al., 2002)], to test whether the low force generation in these fibers was due to loss of TnC during storage. Mean force after the TnC soak was 0.5 kN m^{-2}. These fibers were in the low end of the range in the force-generating ability (P_o/CSA) of hummingbird pectoralis and supracoracoideus fibers, and force did not increase significantly after the TnC soak. This indicates that the low force-generating ability of hummingbird and finch pectoralis fibers was not due to loss of TnC during storage.

Given the very short SL_{null} in hummingbird pectoralis and supracoracoideus fibers and the very low P_o/CSA at 10°C, the mean P_o/CSA generated in each of the hummingbird fibers at 10°C was plotted against the sarcomere length (SL_{rest}) to which each fiber was set prior to force measurements. The results, shown in Fig. 3, indicate that P_o/CSA is not related to sarcomere length, over the range of SL_{rest} used in this study, for the supracoracoideus fibers and leg fibers (P=0.737 and 0.886, respectively). The relationship between P_o/CSA and sarcomere length in pectoralis fibers was marginally significant (P=0.049). However, most pectoralis and supracoracoideus fibers, when stretched beyond SL_{null}, tore when activated (see Materials and methods), so they could not be compared with leg fibers at the same sarcomere lengths.

The relative myofibrillar and mitochondrial areas of hummingbird pectoralis muscle were 67±1 and 27±1%, respectively, which is close to values for volume densities reported by Mathieu-Costello et al. (Mathieu-Costello et al., 1992a).

DISCUSSION

The results of this study reveal very low force-generating ability of hummingbird pectoralis and supracoracoideus muscle fibers. The results also demonstrate an exceedingly high temperature sensitivity of force generation in pectoralis fibers. Low force generation and relatively high temperature sensitivity are also characteristics of zebra finch pectoralis fibers. The P_o/CSA of hummingbird and finch pectoralis fibers is ~5% of that reported for chicken pectoralis fibers at 15°C (165 kN m^{-2}) (Reiser et al., 1996).

The observed mean $Q_{10\text{-force}}$ values of force generation of skinned hummingbird and finch pectoralis fibers (15.3 and 11.5, respectively) are much greater than the $Q_{10\text{-force}}$ of fibers from other vertebrate species (Medler, 2002) and are among the highest of studied biological processes (Dell et al., 2011). Tetanic force increases approximately threefold in mouse flexor digitorum brevis muscle fibers, from 10 to 20°C (Lännergren and Westerblad, 1987), and the $Q_{10\text{-force}}$ values of rat slow and fast fibers in the present study were 1.8–1.9 over the same temperature range. Human vastus lateralis fibers had a $Q_{10\text{-force}}$ of force generation of ~2.2 from 12 to 22°C in one study (Bottinelli et al., 1996). An approximate fivefold increase in force generated in rabbit psoas fibers, following a rapidly induced temperature jump of more than 30°C (from ~5 to ~36°C), has been reported (Bershitsky and Tsaturyan, 2002). $Q_{10\text{-force}}$ values for force generation in frog fibers are even lower [~1.2–1.4 (reviewed in Rall and Woledge, 1990; Tsaturyan et al., 1999)]. The mean P_o/CSA of adult chicken pectoralis fibers at 15°C (Reiser et al., 1992; Reiser et al., 1996) was approximately three times greater than at 10°C (P.J.R., unpublished). Thus, force generation of chicken pectoralis fibers is also very temperature dependent, but apparently not to the extent that it is in hummingbirds and finches. Therefore, the $Q_{10\text{-force}}$ of force generation in hummingbird and finch pectoralis fibers is unusually high, compared with other reported values.

Generally, muscle stiffness, interpreted as reflecting the number of attached crossbridges, increases much less with increasing temperature than does force generation, suggesting that the force generated per crossbridge increases with temperature (Ford et al., 1977; Kuhn et al., 1979; Goldman et al., 1987; Piazzesi et al., 2003; Decostre et al., 2005). However, Kawai and co-workers have proposed that force generated per crossbridge is independent of temperature and that the increase in muscle force with increased temperature is due to a shift in the crossbridge population (reviewed in Kawai, 2003). The mechanism underlying the steep dependence

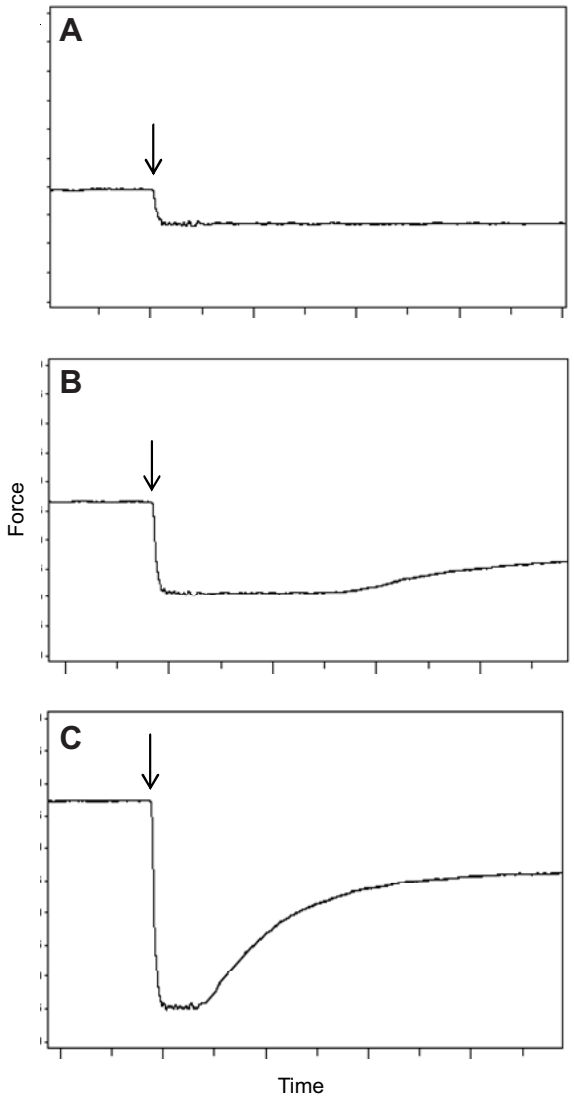

Fig. 2. Force records, in pCa 4.0 solution, from a hummingbird pectoralis fiber at (A) 10°C, (B) 15°C and (C) 20°C. Each mark on the horizontal axis in each panel is 50 ms. Force dropped to baseline ~100 ms after the beginning of each trace (arrow), coincident with movement of the motor to induce slack in the fiber. Active (generated) force in pCa 4.0 solution was calculated by subtracting the resting force, determined in pCa 9.0 solution, from the total force at each temperature. Active force, normalized with fiber cross-sectional area, was 4.7, 14.0 and 26.1 kN m^{-2} at 10, 15 and 20°C, respectively.

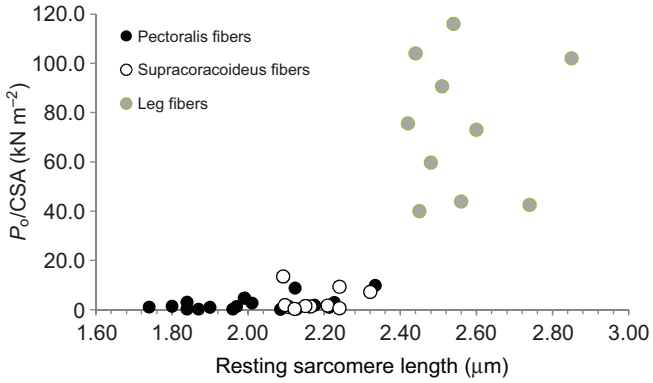

Fig. 3. Force-generating ability at 10°C and resting sarcomere length in all hummingbird pectoralis, supracoracoideus and leg muscle fibers. The coefficients of determination (R^2) were 0.2219, 0.0149 and 0.0028 for pectoralis, supracoracoideus and leg fibers, respectively.

of force generation on temperature in hummingbird and finch pectoralis fibers in not known. It is possible that an increase in temperature shifts attached crossbridges to a higher force-generating state that is not extensively populated at lower temperatures (Woledge et al., 2009). Regardless of whether the observed temperature sensitivity is due to greater force per crossbridge and/or a shift in the crossbridge population, the change(s) appear to be of far greater magnitude than in other studied muscles.

Contraction kinetics of the hummingbird and finch pectoralis muscle must be very fast to drive wingbeat frequencies of 40–50 and 25–30 Hz, respectively. It might be expected that a muscle that operates cyclically at high frequencies would generate relatively low peak twitch force, due to rapid calcium uptake to drive fast relaxation. The fibers in the present study were activated in a steady state, so the stresses generated during wingbeat twitches *in vivo* would presumably be even lower than the values in Table 1, at the same temperatures. The low force is explained partly by the high mitochondrial volume in these fibers (Mathieu-Costello et al., 1992a), as discussed below. In addition, it is possible that the same characteristics of the myosin that is expressed in the pectoralis of hummingbirds, and perhaps other small birds, which allow high rates of crossbridge cycling, are also associated with low force generation and a steep temperature dependence of force generation.

High wingbeat frequencies during hovering require that periods of force production are brief so that continued tension does not hamper muscle lengthening during the opposing stroke. Flight muscle (pectoralis and supracoracoideus) represents, on average, ~26% of body mass, based upon a comparison of 24 hummingbird species (Hartman, 1961; Altshuler et al., 2010a), whereas it is ~17% in most bird species (Greenewalt, 1962). It is possible that a greater muscle mass, and corresponding muscle CSA, are required to power hovering flight, as a compensation for the low force-generating ability, the latter possibly being required in exchange for high rates of contraction during hovering, consistent with a trade-off between force and speed in very fast muscles (Rome et al., 1999; Rome, 2006).

Mitochondrial volume, relative to total cell volume, is much greater (approximately one-third of cell volume) in rufous and ruby-throated hummingbird pectoralis and supracoracoideus muscle fibers (Grinyer and George, 1969; Mathieu-Costello et al., 1992a) than in fibers of other muscles [e.g. 4 and 6% in cat gracilis and soleus, respectively (Schwerzmann et al., 1989); 6% in rat soleus (Mathieu-Costello et al., 1992b)]. The enhanced oxidative capacity enabled, in part, by such high mitochondrial densities, permits the relatively high metabolic rates displayed by hovering hummingbirds (Suarez, 1992), facilitating high levels of sustained mechanical power output. However, finite fiber volumes mean that increased mitochondrial densities must be accompanied by lower densities of other fiber components. Relative myofibrillar volume in hummingbird pectoralis fibers is ~60% (Mathieu-Costello et al., 1992a), which is much lower than in rat soleus [90% (Mathieu-Costello et al., 1992b)]. From analysis of cross-sectional electron micrographs of hummingbird pectoralis, we calculated that myofibrillar CSA accounted for ~67% of total fiber CSA, a value

quite similar to relative volume. Using this value, and assuming that published values for relative myofibrillar volume in the rat are likewise similar to relative CSA (a reasonable assumption given that the former were estimated *via* point counts of transverse section micrographs), it is possible to calculate maximum isometric force per myofibrillar CSA (P_o/CSA_{my}) from our measurements. At 20°C, P_o/CSA_{my} values are 18.2 and 136.3 kN m^{-2} in the hummingbird pectoralis and rat hindlimb fast fibers, respectively, an ~7.5-fold difference. Further, assuming that relative myofibrillar CSA in hummingbird leg muscle fibers is similar to the value for rat soleus [90% (Mathieu-Costello et al., 1992b)], the calculated value for P_o/CSA_{my} in the hummingbird leg fibers is 105.3 kN m^{-2}, an almost sixfold greater value than in hummingbird pectoralis. Although the smaller relative myofibrillar CSA of hummingbird pectoralis fibers can partially account for the low P_o/CSA in hummingbird flight muscle fibers, it is not sufficient to fully account for the observed differences.

The mean thin filament length in hummingbird pectoralis fibers is short [0.88 μm; half of the thin filament length reported by Mathieu-Costello et al. (Mathieu-Costello et al., 1992a)]. Given the mean sarcomere length of 1.97 μm reported by Mathieu-Costello et al. (Mathieu-Costello et al., 1992a), we conclude that the thin filament length in their report likely represents the combined I-band length from two adjacent sarcomeres and the intervening Z-band length (I-Z-I length). Reported thin filament lengths in other vertebrate muscles are longer: they average ~1.31 μm in several bovine muscles, 1.16 μm in rabbit psoas muscle and 1.05 μm in chicken pectoralis muscle (Ringkob et al., 2004), and ~1.03 μm in frog leg and chicken pectoralis muscle [half of the I-Z-I length reported by Page and Huxley (Page and Huxley, 1963)]. The short thin filament length in hummingbird pectoralis fibers correlates well with the very short SL_{null} values in bird pectoralis fibers in the present study. Fibers with short thin filaments, and correspondingly short resting SL, would presumably be able to undergo relatively less shortening, as the maximal shortening of all sarcomeres in isolated muscle fibers would likely be limited by the ends of thick filaments colliding with Z discs. However, skeletal arrangements *in vivo* are likely to limit shortening before sarcomere lengths reach such low values. Therefore, it is likely that flight muscle fibers of hummingbirds and other small birds undergo relatively short length changes during each wingbeat, which is in agreement with *in vivo* strain trajectory estimates of ~11% in the rufous hummingbird (Tobalske et al., 2010), the lowest values reported for any flying bird.

The mean thick filament length in hummingbird pectoralis fibers is 1.45 μm (Mathieu-Costello et al., 1992a). Therefore, an upper limit of the maximal velocity of shortening during flight ($V_{max\text{-}flight}$) can be estimated from the SL_{null} values, the mean thick filament length (which would limit the extent of shortening) and the wingbeat frequency, which is ~43 Hz in *C. anna* (Altshuler et al., 2010b). Assuming that SL shortens from 1.84 to 1.45 μm during the first half of each wingbeat (i.e. over ~12 ms), the upper limit of $V_{max\text{-}flight}$ would be ~18 sarcomere (or fiber) lengths per second. If the SL in pectoralis fibers prior to shortening was longer, due to contraction of the antagonist supracoracoideus muscle, then the upper limit of $V_{max\text{-}flight}$ would be proportionately higher. This very high $V_{max\text{-}flight}$ could contribute to high power output (power=force × velocity) to drive hovering even if P_o/CSA at body temperatures during hovering is relatively low.

Normal (non-torpor) body temperature is ~40°C in *C. anna* (Bartholomew et al., 1957; Lasiewski, 1964) and ~41°C in *T. guttata* (McCue et al., 2011). P_o/CSA at 40°C can be predicted from the mean P_o/CSA values in hummingbird and finch pectoralis fibers at 10, 15 and 20°C reported here. Force and temperature were linearly regressed and the equation of the line was used to extrapolate what the force-generating ability would be at normal body temperature, assuming a linear relationship over the entire temperature range. The $Q_{10\text{-}force}$ value from 10 to 20°C could not be used to extrapolate force generation at a higher temperature because the temperature dependence of the $Q_{10\text{-}force}$ in these fibers is not known (Fig. 4). However, this extrapolation should be viewed with caution, as force typically increases less as temperature increases further (Rall and Woledge, 1990; Piazzesi et al., 2003). With the stated linearity assumption, the value obtained is ~33 kN m^{-2} for *C. anna* and ~63 kN m^{-2} for *T. guttata*. Therefore, it is likely that at normal body temperature, *T. guttata* pectoralis fibers generate significantly more force than do pectoralis fibers of *C. anna*, with the additional assumption that the relationship between maximum isometric force *in vitro* and peak isotonic force *in vivo* is assumed to be similar in the two species. Both values are still very low compared with those recorded in mammalian muscle fibers studied at much lower temperatures (~120 kN m^{-2} in rat slow and fast fibers at 15°C, in this study) (Medler, 2002). Calculated force for hummingbird pectoralis fibers at 30°C is ~23 kN m^{-2}, which is almost identical to that reported for slow fibers from red muscle in skipjack tuna (*Katsuwonus pelamis*; 24 kN m^{-2} at 25°C) and kawakawa (*Euthynuus affinis*; 25 kN m^{-2} at 30°C) (Johnston and Brill, 1984). Therefore, very low force generation is a characteristic that can be shared by very different fiber types in vertebrates.

Roots et al. reported that intact fiber bundles from rat brevis muscle generate 187 kN m^{-2} of maximum stress at 20°C (Roots et al., 2007). We did not correct our P_o/CSA measurements for the swelling of single muscle fibers that occurs when they are skinned. The correction factor for swelling is 1.44, due to a 20% increase in fiber diameter (Godt and Maughan, 1981). Therefore, multiplying the mean force generated by rat slow and fast fibers at 20°C (Table 1) by 1.44 yields values of 177 and 176 kN m^{-2}, which are very similar to the value reported by Roots et al. (Roots et al., 2007). Correction of the estimated value for *C. anna* pectoralis fibers in Fig. 4 yields an estimated maximal stress of 47.5 kN m^{-2} at 40°C. The estimated stress in *C. anna* pectoralis fibers at 40°C (~48 kN m^{-2}) is, however, much lower than the reported isometric tetanic stress (P_o/CSA) in other avian species. Reported P_o/CSA is 122 kN m^{-2} (39°C) in starling pectoralis (Biewener et al., 1992), 167 kN m^{-2} (40°C) in

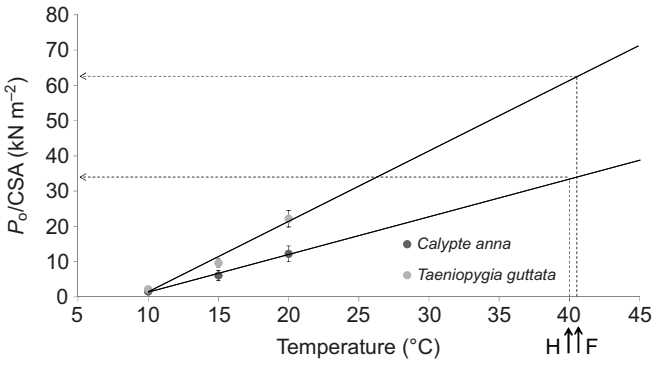

Fig. 4. Prediction of force-generating ability of hummingbird (H, *Calypte anna*) and zebra finch (F, *Taeniopygia guttata*) pectoralis fibers at normal body temperatures. Arrows on horizontal axis indicate normal body temperatures for these two species. Dashed lines trace the extrapolation of force generated to body temperature.

zebra finch pectoralis (Ellerby and Askew, 2007), 131 and ~200 kN m^{-2} (both at 40°C) in quail pectoralis (Askew and Marsh, 2001; Johnston, 1985), 220 kN m^{-2} (40°C) in budgerigar pectoralis (Ellerby and Askew, 2007) and 215 kN m^{-2} (40°C) in cockatiel pectoralis (Morris and Askew, 2010). Although our measurements were made using a very different preparation (skinned fibers) and under very different conditions (much lower temperatures) than those reported for other species (whole muscles or living fiber bundles at 40°C), it appears that the maximal isometric stress generated in hummingbird pectoralis is significantly lower than that of other bird species and possibly reflects, at least in part, a tradeoff of force generation for speed (Rome et al., 1999).

There are no measurements available for the stress of whole hummingbird pectoralis major muscles, but we have estimated this value based on previously published data. The aerodynamic power required for hovering in *C. anna* is estimated to be 107 W kg^{-1} of flight muscle based on the morphology, kinematics and fluid properties from nine individuals (D.L.A., unpublished data) when combined with equations presented in Ellington (Ellington, 1984). The mass, velocity and displacement of the muscle during the downstroke were calculated using morphological measurements and wingbeat frequencies (mean=43 Hz) of *C. anna* (Altshuler et al., 2010a; Altshuler et al., 2010b) and strain measurements for *S. rufus* (Tobalske et al., 2010). These values were used to solve for the average force (0.417 N) produced by one pectoralis major muscle during shortening in hovering flight. This value was normalized to stress using measurements of the CSA of the muscle in three individuals (D.L.A., unpublished data). This produced estimates for the average stress required from each pectoralis major during hovering ranging from 10.4 to 16.7 kN m^{-2} depending upon the CSA (range: 25–40 mm^2) along the muscle length. It is expected that the maximum stress that the muscle is capable of producing is considerably higher to account for more mechanically demanding modes of flight in hummingbirds, such as flight in low density air (Chai and Dudley, 1995). Our estimate of average stress during flight is 22–35% of our estimate of the isometric tetanic stress at 40°C. This is slightly higher than estimates by others from measurements in other species: 10–18% in budgerigar pectoralis, 11–23% in zebra finch pectoralis (Ellerby and Askew, 2007) and 13% in cockatiel pectoralis (Morris and Askew, 2010). Peak stresses generated during flight are reported to be higher – ~28 and 29% of peak isometric stress in starling pectoralis (Biewener et al., 1992) and pigeon pectoralis (Dial and Biewener, 1993), respectively. We cannot estimate peak stress in the hummingbird pectoralis during flight; therefore, we cannot directly compare the hummingbird pectoralis with the latter reported values. Nevertheless, hummingbird pectoralis appears to operate during hovering in a manner that is fundamentally similar to flight muscle in other bird species, but at much lower stress levels and with an exceptionally high temperature sensitivity.

The relationship between P_o/CSA and temperature may have actual biological relevance in hummingbirds, as these animals enter torpor at night under a variety of circumstances, as a means of reducing metabolic rate and achieving a net energy savings over normothermia (Hiebert, 1991; Hiebert, 1992; Hiebert, 1993; Calder, 1994; Powers et al., 2003). Hummingbird body temperature during torpor usually does not fall below the 18–20°C range (Kruger et al., 1982), but body temperatures during torpor as low as 9°C in *C. anna* (Bartholomew et al., 1957) and 13°C in *Selaphorus rufous* (Hiebert, 1990) have been reported. Bartholomew (Bartholomew et al., 1957) provided descriptions of two hummingbirds escaping torpor. The birds were relatively immobile as body temperature rose from a low temperature during torpor, even up to a body temperature of ~22°C. Both birds displayed normal activity when body temperature reached 35°C. The observed relationship between temperature and P_o/CSA in hummingbirds is consistent with Bartholomew's observation of immobility at low body temperatures and suggests that flight muscle power production is insufficient to enable flight at body temperatures only several degrees below normothermia. Facultative hypothermia has been reported in some sparrow and finch species (Steen, 1958; McKechnie and Lovegrove, 2002; McKechnie and Lovegrove, 2003; Dolby et al., 2004), but the extent of body temperature depression appears to be much less than in hummingbirds (3–5°C). It is unknown whether such temperature decreases are likely to be associated with substantial declines in muscle mechanical performance in finches and sparrows.

In light of the striking temperature dependence of P_o/CSA in hummingbird pectoralis fibers, the much lower temperature dependence of P_o/CSA in leg muscle fibers stands out. Leg muscle fibers in both bird species generated much greater P_o/CSA than did pectoralis or supracoracoideus muscle fibers. P_o/CSA in bird leg muscle fibers was more similar to that in rat leg muscle fibers, compared with flight muscle fibers, and the force $Q_{10\text{-force}}$ was similar among bird and rat leg muscle fibers. While hummingbird pectoralis fibers have evolved to operate at exceptionally high contraction cycle rates, and may be relatively weak as a consequence, the same is not true of hummingbird leg fibers. Data are not available on the typical or maximal contraction operating frequencies of hummingbird legs, but it is clear when handling hummingbirds that they cannot move their legs or digits anywhere as fast as their wings. In the absence of selection for high contraction cycling frequencies, the need to effectively grip twigs and remain perched even when body temperature is low seem well matched by high force-producing capacities and lower temperature sensitivity characteristic of most vertebrate muscle fibers. Additionally, the relatively higher P_o/CSA values allow for sufficient force with smaller leg muscle mass, thus reducing the payload during hovering in hummingbirds. Leg muscle mass constitutes a very small fraction (~1%) of total body mass in hummingbirds, compared with other birds, in many of which it is at least an order of magnitude greater (Hartman, 1961). Unlike in the pectoralis, leg muscles are composed of a heterogeneous mix of fiber types in both birds. Hummingbird leg muscles are comprised exclusively of oxidative fibers [fast oxidative glycolytic (FOG) and slow oxidative (SO)], whereas fast glycolytic (FG) fibers are also present in finch gastrocnemius (Welch and Altshuler, 2009). It is possible that only SO or FG fibers from the leg muscles were examined in this study, and that this explains the observed differences in P_o/CSA and its temperature dependence. Fiber type identities were not confirmed. However, 86% of the hummingbird leg muscle fibers are FOG (Welch and Altshuler, 2009) and it, therefore, seems unlikely that all hummingbird leg fibers examined were SO fibers.

A limitation of this study is that the sarcomere length to which fibers were set differed between groups of fibers. The analysis of the data suggests that the differences in sarcomere lengths between groups of fibers can explain only a small amount of the large differences in P_o/CSA between some of the fiber groups. For example, although many of the initially studied hummingbird pectoralis and supracoracoideus fibers tore when they were activated at sarcomere lengths beyond SL_{null}, three pectoralis fibers were successfully studied at ~1.9 and 2.2 μm and force generation approximately doubled over this range. However, the greater force in these three fibers at the longer sarcomere length was still less than 15% of the mean force generated by rat limb fibers at the same

temperature. Therefore, although differences in sarcomere length among groups of fibers contributed to the differences in P_o/CSA, this could explain only a small amount of the observed differences in force-generating ability. The very short SL_{null} values, the apparently limited sarcomere length range over which force is generated, and the high frequency of contractions during hovering suggest that hummingbird pectoralis fibers perform work very rapidly over a relatively short distance, in contrast to limb muscles of most other organisms that undergo a larger amount of shortening during isotonic contractions.

The maximal velocity of shortening in frog muscle fibers is greater very early during a tetanic contraction compared with later phases of a tetanus (Josephson and Edman, 1998). Josephson and Edman (Josephson and Edman, 1998) wrote that this is consistent with the 1957 Huxley model of muscle contraction (Huxley, 1957), because rapidly attaching crossbridges, at the beginning of a tetanus, are not yet pulled beyond their equilibrium position, at which time they would begin to impede shortening. This might be relevant to muscles contracting at high frequencies, such as hummingbird pectoralis and supracoracoideus, which presumably undergo very brief twitch-like contractions (a complete wingbeat cycle duration in *C. anna* is ~24 ms). This, in turn, could ensure maximal power output from these muscles as an inherent consequence of their brief contractions at very high frequencies.

In summary, our results show that muscle fibers that power hovering in hummingbirds generate very low P_o/CSA, perhaps as a requisite trade-off of force for contraction speed, as reported for other fast contracting muscles (Rome et al., 1999), and have an exceedingly high temperature dependency, with respect to force generation, in contrast to most other studied vertebrate muscle fibers. Hummingbird leg muscle fibers, in contrast, have a much greater force-generating ability and lower temperature dependency, which support the need to perch at all temperatures.

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AUTHOR CONTRIBUTIONS

All of the authors developed the concepts or approach, performed experiments or data analysis, and prepared or edited the manuscript prior to submission.

COMPETING INTERESTS

No competing interests declared.

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