



Fiber type homogeneity of the flight musculature in small birds

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ABSTRACT

Studies of medium- and large-bodied avian species have suggested that variation in flight muscle composition is related to differences in flight behavior. For example, slow-twitch or tonic fibers are generally found only in the flight muscles of non-volant or soaring/gliding birds. However, we know comparatively little about fiber composition of the muscles of the smallest birds. Here we describe the fiber composition of muscles from the wings, shoulders, and legs of two small avian species, which also display very high wingbeat frequencies: Anna's hummingbirds (*Calypte anna*) and zebra finches (*Taeniopygia guttata*). All flight muscles examined in both species contained exclusively fast oxidative glycolytic (FOG) fibers. These unique results suggest that fast oxidative fibers are both necessary and sufficient for the full range of flight behaviors in these small-bodied birds. Like all other studied birds, the zebra finch gastrocnemius, a tarsometatarsal extensor, contained a mixture of FOG (27.1%), slow oxidative (SO, 12.7%), and fast glycolytic (FG, 60.2%) fibers. By contrast, the hummingbird gastrocnemius lacked FG fibers (85.5% FOG, 14.5% SO), which may reflect the reduced role of the hindlimb during take-off. We further hypothesize that thermogenic requirements constrain fiber type heterogeneity in these small endothermic vertebrates.

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1. Introduction

Most vertebrate skeletal muscles are composed of a diverse assemblage of fiber types (Bottinelli and Reggiani, 2000; Rosser and George, 1986a; Rosser et al., 1996; Schiaffino and Reggiani, 1994). Such diversity of fiber types is indicative of the variety of functions skeletal muscles perform (Bottinelli and Reggiani, 2000). Three general types of twitch fibers, associated with specific subsets of mechanical function, are recognized as components of avian musculature throughout the literature. Slow oxidative fibers (SO) exhibit relatively slow contraction velocities and produce comparatively less force, but are resistant to fatigue and are well suited for slow, repetitive movements or periods of sustained isometric contraction, such as during maintenance of posture. Fast glycolytic (FG) fibers are noted for their rapid contraction dynamics and high level of force generation, while being relatively susceptible to fatigue. These fibers are suited for burst activities requiring great power production. Fast oxidative-glycolytic (FOG) fibers display contraction velocities and capacities for force generation that are intermediate between the other two types and are also relatively fatigue resistant. These motor elements are therefore useful during relatively high frequency activities conducted for longer durations (Bottinelli and Reggiani, 2000). An additional fast-twitch fiber type recognized as being relatively fatigue resistant and containing myosin isoforms distinct from those found in FG and FOG fibers is noted in mammalian skeletal muscle (Schiaffino and Reggiani,

1994). However, this fiber type has not been found in avian muscles (Rosser et al., 1996).

Avian flight musculature has long been of interest to physiologists and morphologists because of the great and diverse functional demands flight behavior places on this locomotory machinery. The pectoralis major powers the downstroke of the avian wingbeat, and the enormous power requirements associated with flight are reflected, at a gross level, in the relatively large size of this single muscle in volant species (Greenewalt, 1962). A great deal of previous work has investigated the diversity of fiber composition of this muscle in relation to the diversity of flight behaviors demonstrated across bird species (Kovacs and Meyers, 2000; Lundgren and Kiessling, 1988; Rosser and George, 1986a; Rosser et al., 1996; Tobalske, 2001; Torrella et al., 1999). Such studies have, for example, generally found that smaller-bodied birds that rely heavily upon powered, flapping flight have pectoralis muscles predominantly containing FOG fibers (e.g. Lundgren and Kiessling, 1988; Rosser and George, 1986a). In contrast, non-volant bird species have pectoralis muscles with comparatively higher concentrations of SO and FG fibers (Rosser and George, 1985; Rosser et al., 1987) and birds that glide or soar have pectoralis muscles with relatively higher numbers of SO fibers (Meyers and Stakebake, 2005; Rosser and George, 1986a,b; Rosser et al., 1994).

There are comparatively fewer studies of the other muscles acting on the wings of birds. Interestingly, even in birds that have pectoralis muscles with homogenous fiber compositions, various wing muscles may contain a diversity of fiber types (e.g. Geyikoglu and Ozkaral, 2000; Kovacs and Meyers, 2000; Marquez et al., 2006; Torrella et al., 1999). Such diversity of fiber composition indicates the potentially

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diverse roles these appendicular muscles might play during flight (Dial, 1992).

The fiber type composition of the wing musculature of the smallest birds is largely unknown. Wingbeat frequencies generally scale inversely with body mass, and the very smallest birds, hummingbirds, display a unique form of flight characterized by the highest wingbeat frequencies (Greenewalt, 1962). High wingbeat frequencies require high contraction/relaxation cycling in both power producing, as well as control elements of the flight machinery. Such extremely high operating frequencies, and the potentially long durations over which contraction cycling is sustained, would lead to the prediction that FOG fibers predominate in the wing muscles of these small birds. Previous studies confirm this prediction specifically with respect to the pectoralis and supracoracoideus muscles of hummingbirds (Grinyer and George, 1969; Lasiewski et al., 1965; Mathieu-Costello et al., 1992; Rosser and George, 1986a). However, it is not known how specific muscles in the wing contribute to the control of wing motion and accordingly, we are unable to predict the requirements for force generation, operating frequency, and fatigue resistance in any of the wing muscles. Data pertaining to the fiber composition of these muscles will be beneficial as we gain understanding of their potentially variable functions.

Here, we examine the fiber type composition of the pectoralis, supracoracoideus, and several wing muscles in the Anna's hummingbird (*Calypte anna*) and zebra finch (*Taeniopygia guttata*). These two species are the smallest in mass (<15 g) and display the highest wingbeat frequencies (>25 Hz) (Ellerby and Askew, 2007; Greenewalt, 1962) for which a survey of muscles other than the pectoralis and supracoracoideus has been reported. In addition to shoulder and wing muscles, we examined the fiber composition of the gastrocnemius in both taxa. All three major fiber types have been found in the gastrocnemius muscles of all other birds examined (Marquez et al., 2006; Olson and Olson, 2001; Torrella et al., 1999; Velotto and Crasto, 2004; Viscor et al., 1992; Wada et al., 1999). We anticipated finding the three major fiber types in the hummingbird and zebra finch gastrocnemius and thus chose this muscle for examination in part because we expected it would serve as a positive control with respect to the identification of the three major fiber types. Further, this muscle serves a decidedly different locomotory function compared to the flight muscles and provides information on the nature of fiber composition in each bird species as it relates to other locomotor functions.

2. Materials and methods

2.1. Specimens used in this study

Five adult male Anna's hummingbirds (*C. anna*) and 3 adult male zebra finches (*T. guttata*) were used in this study. Individual birds died during surgery or were euthanized via carbon dioxide asphyxiation or overdose of ketamine/xylazine as part of other studies in our laboratory, and were immediately placed in sealed plastic bags and stored at -20°C . In cases where birds had received intramuscular injections of ketamine/xylazine, the injected muscles were excluded from use and the contralateral muscles were utilized instead. All birds were of apparent excellent health prior to sacrifice and were fully capable of sustained flight. All vertebrate animal procedures performed were approved by the Institutional Animal Care and Use Committee at the University of California Riverside.

2.2. Hummingbird flight muscle anatomy

Dissections were performed under a Stemi 2000-C stereomicroscope (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA) at magnifications ranging from $\times 2.5$ – 25 . Two hummingbirds were thawed overnight (at 4°C) and all the bones of the thoracic girdle

and forelimb, as well as flight muscles of interest, were then isolated and identified. Work by Zusi and Bentz (1984) describing the myology of the Purple-throated Carib hummingbird was used as a guide for muscle identification in the Anna's hummingbird. Muscle terminology follows that in the "Nomina Anatomica Avium" (Baumel et al., 1979). Skeletal and muscle dimensions were quantified relative to viewing angle under the dissection scope. The position of muscle origin and insertion were noted, and the thoracic skeleton and selected flight musculature were hand-drawn for diagrammatic purposes (Fig. 1).

2.3. Tissue preparation

Whole animal specimens were removed from the freezer and thawed overnight at 4°C . Whole muscles were then extracted, coated in Tissue-Tek O.C.T. compound (Sakura Finetek USA, Inc., Torrance, CA, USA), and frozen in 2-methylbutane cooled to -160°C by liquid nitrogen. The following muscles were dissected from each bird: M. pectoralis major (P), M. supracoracoideus (SC), M. biceps brachii (BB), M. tensor propatagialis pars brevis (TPB), lateral section of the M. gastrocnemius (G), and M. triceps brachii scapulotriceps (TBS) and M. triceps brachii humerotriceps (TBH). Both triceps muscles were distinguished prior to removal, and removed, frozen, and analyzed separately. The locations of the wing muscles are shown in Fig. 1. In the case of the M. pectoralis and M. supracoracoideus, the most distal and most proximal several millimeters of muscle were removed (cut perpendicular to the long axis of the muscle) prior to freezing. Similarly, the most distal several millimeters of the M. gastrocnemius (including most of the long tendon) were removed prior to freezing. In the Anna's hummingbird, only two replicates of the M. supracoracoideus, M. biceps brachii, M. triceps brachii (both scapulotriceps and humerotriceps), and M. gastrocnemius muscles were analyzed as there was tissue damage accrued during dissection or during freezing (cracking through the body of the muscle) in the third replicate.

From each available tissue block, transverse sections of 8–12 μm in thickness were cut in a cryostat maintained at -24 to -20°C . Three to six sections were picked up on each microscope slide (Superfrost® Plus, Fisher Scientific, Pittsburgh, PA, USA), with 18–54 serial sections obtained from each muscle. Slides were either stored in desiccating container at -20°C for up to 24 h before staining or were stained 2–5 h later. Just prior to staining, all slides were air-dried at room temperature for 1–2 h.

2.4. Fast/slow MHC labeling

Avian skeletal muscle fibers may be distinguished as either slow or fast-twitch by reacting muscle sections with antibodies specific for either slow or fast myosin heavy chain (MHC) isoforms (Rosser et al., 1996). The NA8 monoclonal antibody (Bourke et al., 1995; Cerny and Bandman, 1987) and F30 monoclonal antibody (Crow and Stockdale, 1986; Miller et al., 1985, 1989), each obtained from the Developmental Studies Hybridoma Bank (The University of Iowa, Department of Biological Sciences, Iowa City, IA, USA) were chosen for use in this study as each demonstrates general reactivity with avian slow and fast myosin heavy chain isoforms, respectively.

Immunocytochemical techniques modified slightly from those described by Rosser et al. (1996) and Shear et al. (1988), were used to identify fibers as either fast or slow-twitch. Briefly, sections from each muscle studied were first blocked for 30 min in a solution comprised of 5% goat serum, 1% bovine serum albumin and 5 mM EDTA in PBS (0.02 M sodium phosphate buffer, 0.15 M NaCl, pH 7.2). Sections were subsequently incubated overnight at 4°C with one of the two primary antibodies diluted in the blocking solution. The NA8 antibody was used at a dilution of 1:50. The F30 antibody was used at a dilution of 1:2. The next day, slides were rinsed with PBS three times for 5 min at a time. Presence of the primary antibody was then visualized by incubating sections for 30 min in the dark at room temperature with a

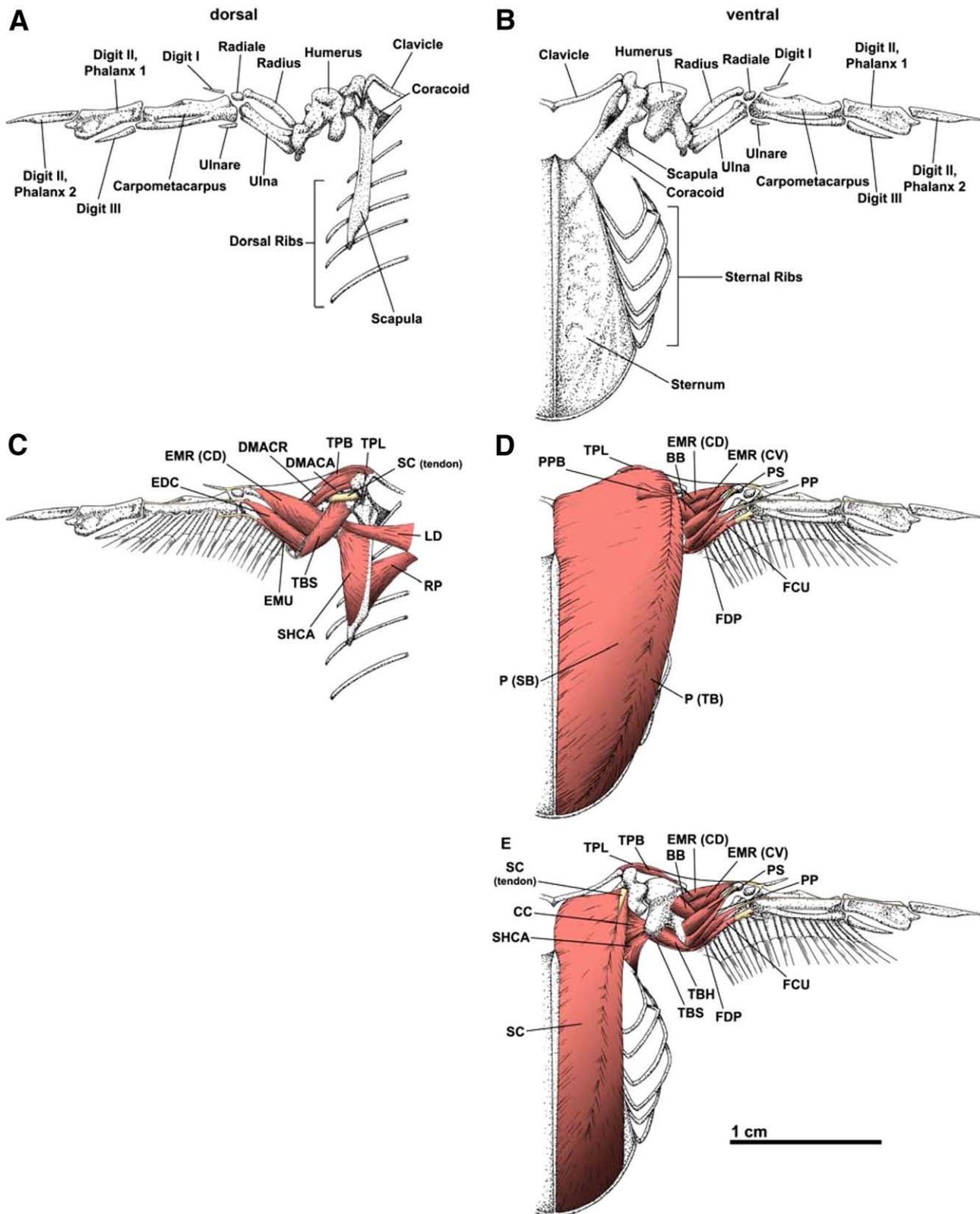


Fig. 1. Skeletal and superficial muscular (selected muscles only) anatomy of the wing and pectoral girdle of the Anna's hummingbird (*Calypte anna*). Skeleton only: dorsal view (A), and ventral view (B). Superficial muscles: dorsal view (C), ventral view including *M. pectoralis major* removed (D), and ventral view with *M. pectoralis major* removed to provide unobstructed view of deeper muscles (E). Abbreviations: BB, *M. biceps brachii*; CC, *M. coracobrachialis caudalis*; DMACA, *M. deltoideus major caudalis*; DMACR, *M. deltoideus major cranialis*; EDC, *M. extensor digitorum communis*; EMR (CD), *M. extensor metacarpi radialis (caput dorsale)*; EMR (CV), *M. extensor metacarpi radialis (caput ventrale)*; EMU, *M. extensor metacarpi ulnaris*; FCU, *M. flexor carpi ulnaris*; FDP, *M. flexor digitorum profundus*; LD, *M. latissimus dorsi (pars caudalis)*; P (SB), *M. pectoralis major (sternobrachialis)*; P (TB), *M. pectoralis major (thoracobrachialis)*; PP, *M. pronator profundus*; PPB, *M. pectoralis pars proapatagialis brevis*; PS, *M. pronator superficialis*; RP, *M. rhomboideus profundus*; SC, *M. supracoracoideus*; SHCA, *M. scapulohumeralis caudalis*; TBH, *M. triceps brachii humerotriceps*; TBS, *M. triceps brachii scapulohumeralis*; TPB, *M. tensor proapatagialis pars brevis*; TPL, *M. tensor proapatagialis pars longa*.

Table 1

Staining/labeling properties of each of the three, primary recognized avian fiber types

	SO	FOG	FG
NADH-TR stain	Dark	Dark	Light
Anti-slow MHC label	+	–	–
Anti-fast MHC label	–	+	+

Note. Each fiber type can be positively distinguished by combined application of these three stains/labels.

goat anti-mouse IgG secondary antibody conjugated to a compound that fluoresced at a peak excitation wavelength of 493 nm (Goat Anti-Mouse IgG, DyLight™ 488, Pierce Biotechnology, Rockford, IL, USA) diluted 1:200 in PBS. Sections were next rinsed in PBS three times for 5 min at a time and then fixed for 3 min in 4% buffered formalin. Sections were again rinsed in PBS three times for 5 min at a time and finally mounted in Fluoromount-G (Southern Biotech, Birmingham, AL, USA).

2.5. NADH-tetrazolium reductase stain

Fast-twitch muscle fibers may be further categorized based on relative oxidative capacity as either FOG or FG (Rosser and George, 1986a). The relative oxidative capacity of muscle fibers was evaluated by staining for nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) activity as in Rosser et al. (1996), following Dubowitz (1985). Fibers with high oxidative capacities contain high concentrations of NADH-TR whereas fibers with low oxidative capacities (glycolytic fibers) contain low concentrations of this enzyme (Dubowitz, 1985). Some previous studies have described fibers that stain with intermediate intensity for NADH-TR activity (e.g. George and Berger,

1966; Kiessling, 1977; Kovacs and Meyers, 2000; Rosser and George, 1986a). However, other authors have not positively identified 'intermediate' fibers in examined muscles, or possibly lumped such fibers with FOG fibers generally (e.g. Knust et al., 2000; Rosser et al., 1996; Torrella et al., 1999). Specimens included in the current study were stored in the freezer for 1–14 days. It has been suggested that variation in the duration of storage time could potentially affect quantitative, but not qualitative, analysis of oxidative enzyme activity. Thus, we chose to classify fibers as simply possessing either high (red) or low (white) oxidative capacity. Following air-drying, sections were incubated for 25 min at 39 °C in approximately 20 mL of a 0.05 M Tris buffer (0.686 g Trizma HCl, 0.2 g Trizma base in 120 mL H₂O at pH 7.4), 2.5 mM Nitro BT, and 2.35 mM NADH. Sections were then rinsed three times with dH₂O, and dipped for 10–15 s at a time in a series of beakers containing 30%, 60%, 90%, 60%, and 30% acetone. Slides were again rinsed three times with dH₂O and kept in dH₂O until mounted with Fluoromount-G.

2.6. Data collection

Muscle sections were examined at 20× or 40× magnification using a Zeiss Axiophot microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA). Photographs were taken of one to several areas of multiple sections of each muscle studied using a Nikon Coolpix E995 digital camera and MDC-A Relay Lens (Nikon USA, Melville, NY, USA) at maximum resolution. Section images were later examined on a computer screen so that individual fibers could be counted. Only qualitative observations were recorded for muscles presenting homogenous fiber staining/labeling. For sections in which staining/labeling was heterogenous, counted fibers were temporarily digitally

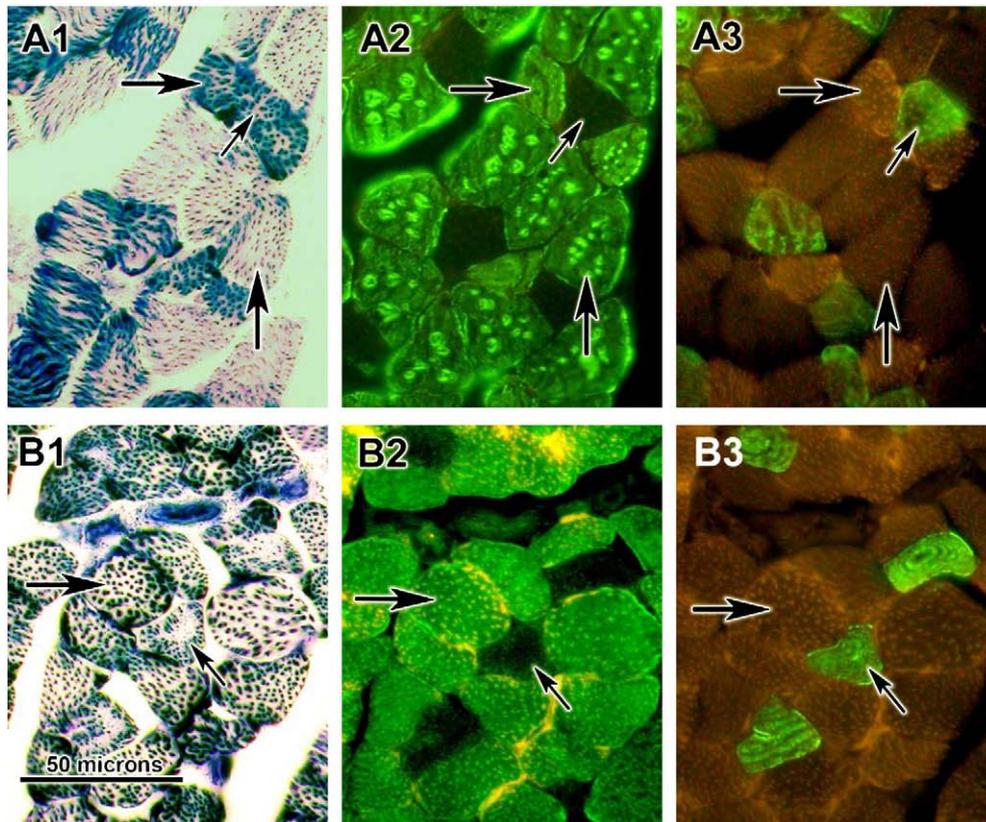


Fig. 2. Representative transverse serial sections of (A) zebra finch (*T. guttata*) and (B) Anna's hummingbird (*C. anna*) gastrocnemius muscle. A1. NADH tetrazolium reductase stain demonstrating heterogenous staining for oxidative and non-oxidative fibers. A2. Anti-fast myosin heavy chain antibody incubation demonstrating labeling of exclusive subset of fibers as fast. A3. Anti-slow myosin heavy chain incubation demonstrating labeling of exclusive subset of fibers as slow. Horizontally, diagonally, and vertically-oriented arrows indicate the same FOG, SO, and FG fiber in each section, respectively. B1. NADH tetrazolium reductase stain demonstrating relatively heterogenous staining for oxidative fibers. B2. Anti-fast myosin heavy chain antibody incubation demonstrating labeling of exclusive subset of fibers as fast. B3. Anti-slow myosin heavy chain incubation demonstrating labeling of exclusive subset of fibers as slow. Horizontally and diagonally-oriented arrows indicate the same FOG and SO fiber in each section, respectively.

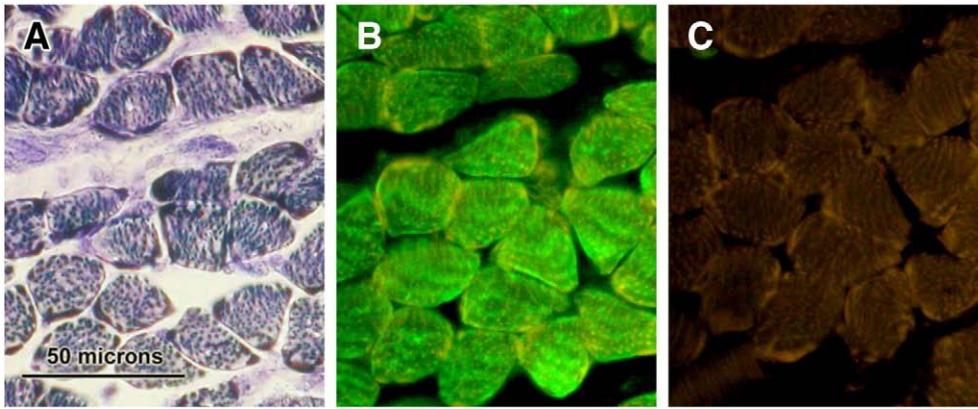


Fig. 3. Representative transverse serial sections of the biceps brachii muscle in the Anna's hummingbird (*C. anna*). A. NADH tetrazolium reductase stain demonstrating homogenous staining for oxidative fibers. B. Anti-fast myosin heavy chain antibody incubation demonstrating homogenous labeling of fast fibers. C. Anti-slow myosin heavy chain incubation demonstrating homogenous lack of labeling of slow fibers.

marked within an image viewing program so that they would not be counted twice. On average, 250 fibers were counted per section. In the case of smaller muscles, the entire cross-section of a given muscle was examined. For larger muscles, the same approximate area of each serial section was examined for each muscle from each individual. In the case of the gastrocnemius, the lateral one third of this muscle was targeted for examination. For the large bipennate muscles (pectoralis and supracoracoideus), representative sections of each division were examined. Careful examination of a subset of serial sections confirmed that all fibers could be positively classified as either SO, FG, or FOG using the methodology indicated in Table 1. Data are presented as mean (\pm SD) percent of fibers for a given muscle examined classified as either FOG, SO, or FG averaged across all specimens from which sections were successfully obtained.

3. Results

3.1. *M. gastrocnemius*

In the case of the zebra finch gastrocnemius, the NADH-TR stain yielded a combination of light and dark staining fibers, indicating the presence of both oxidative and non-oxidative (glycolytic) fibers (Fig. 2 image A1). Similarly, both the anti-fast MHC and anti-slow MHC antibodies reacted with mutually exclusive groups of fibers in the zebra finch gastrocnemius, indicating the presence of both fast and slow twitch fibers in this muscle (Fig. 2 images A2, A3, respectively).

Examination of a subset of serial gastrocnemius sections confirmed that all fibers that reacted with the anti-slow MHC antibody also darkly stained when subjected to the NADH-TR staining protocol. The fiber type composition of the lateral portion of the zebra finch gastrocnemius was $27.1 \pm 2.8\%$ fast oxidative glycolytic (FOG), $12.7 \pm 1.2\%$ slow oxidative (SO), and $60.2 \pm 1.6\%$ fast glycolytic (FG).

In the case of the hummingbird gastrocnemius, the NADH-TR stain yielded qualitatively homogenous dark staining of all fibers, indicating all fibers were of relatively high oxidative capacity (Fig. 2 image B1). As in the zebra finch gastrocnemius, exclusive sets of fibers reacted with either anti-fast MHC or anti-slow MHC antibodies, indicating the presence of both slow and fast twitch fibers in this muscle (Fig. 2 images B2, B3, respectively). The fiber composition of the lateral portion of the hummingbird gastrocnemius was $85.5 \pm 1.4\%$ FOG and $14.5 \pm 1.4\%$ SO.

3.2. Muscles of the wing and pectoral girdle

For all muscles of the wing and pectoral girdle examined in both the zebra finch and hummingbird, the NADH-TR stain resulted in homogenous dark blue staining of all fibers, indicating the fibers were exclusively oxidative (Figs. 3A and 4A). All fibers in each section from each muscle reacted strongly with the anti-fast MHC antibody (Figs. 3B and 4B) whereas none reacted with the anti-slow MHC antibody (Figs. 3C and 4C), indicating all fibers in these muscles were fast twitch. Collectively, these results indicate that all fibers in the flight muscles examined in both species were FOG.

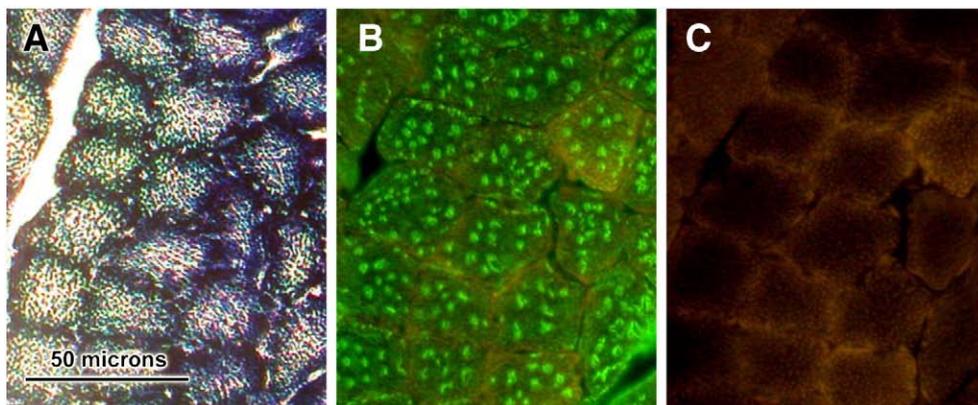


Fig. 4. Representative transverse serial sections of the pars tensor propretagialis pars brevis muscle in the zebra finch (*T. guttata*). A. NADH tetrazolium reductase stain demonstrating homogenous staining for oxidative fibers. B. Anti-fast myosin heavy chain antibody incubation demonstrating homogenous labeling of fast fibers. C. Anti-slow myosin heavy chain stain demonstrating homogenous lack of labeling of slow fibers.

4. Discussion

4.1. Pectoral muscles

This is the first study employing both histochemical and immunohistochemical techniques to identify fiber types in the locomotory muscles of hummingbirds, and is the first to apply such techniques to the smaller muscles of the zebra finch wing. In agreement with previously published studies employing alternative methodologies for the classification of fiber types (Grinyer and George, 1969; Lasiewski et al., 1965; Mathieu-Costello et al., 1992; Rosser and George, 1986a) we find that both the pectoralis and supracoracoideus of the hummingbird are composed exclusively of fast oxidative-glycolytic (FOG) fibers. Nearly all small-bodied passerines thus far examined have pectoralis and supracoracoideus muscles exclusively containing fast twitch fibers with moderate to high levels of staining for enzymes indicative of oxidative capacity (Lundgren and Kiessling, 1988; Rosser and George, 1986a; Swain, 1992). Given the general conclusion that FOG fibers are best suited for powering and sustaining the relatively high contraction frequencies associated with flapping flight in smaller birds (Goldspink, 1980, 1981), it is unsurprising that we find the zebra finch pectoralis and supracoracoideus muscles are composed entirely of FOG fibers.

4.2. Wing muscles

Fiber type compositions of other muscles of the avian wing are less well known. The homogenous fiber composition of all wing muscles in the hummingbird and zebra finch examined in this study stands in contrast to all other published studies. Specifically, studies by other groups have demonstrated that the triceps brachii of the English sparrow (triceps head not specified) and Atlantic puffin (scapulotriceps head) contains both fast and slow-twitch oxidative fibers (Geyikoglu and Ozkaral, 2000; Kovacs and Meyers, 2000). Torrella et al. (1998, 1999) report that the triceps brachii of the common coot, yellow-legged gull, and mallard duck are composed of both fast oxidative and fast non-oxidative (glycolytic) fibers.

Differences in flight muscle fiber type composition across species have been explored in relation to associated variation in flight behavior. In species noted for adopting a spread-wing posture and the extensive use of gliding flight, researchers have linked the relative abundance of slow twitch or tonic fibers in muscles responsible for holding wings outstretched (Meyers, 1997; Meyers and Stakebake, 2005). Gliding flight involves sustained, isometric contractions, and slow muscle fibers are believed to be better suited for this function due to greater efficiency during such operation compared to fast-twitch fibers (Goldspink, 1980, 1981). The California and yellow-legged gull, the double-crested cormorant, the black-footed and Laysan albatrosses, the White pelican, the Turkey Vulture, and the Red-tailed hawk all possess flight muscles that contain slow fibers (Meyers, 1997; Meyers and Mathias, 1997; Meyers and Stakebake, 2005; Rosser and George, 1986a,b; Rosser et al., 1994; Torrella et al., 1998). It is important to note, however, that relative proportions of slow fibers in any given flight muscle differ greatly among these species, thus confirming that the relationships between fiber composition and muscle use patterns are not so simply defined. For example, whereas the pectoralis muscles of the double-crested cormorant, albatrosses, White pelican, Turkey Vulture and Red-tailed hawk possess slow fibers (Meyers and Stakebake, 2005; Rosser and George, 1986a,b; Rosser et al., 1994), the pectoralis muscles of those Ring-billed, Herring, and Yellow-legged gulls possess only FOG fibers (Rosser and George, 1986a; Torrella et al., 1999). In the case of the gulls, it is clear that FOG fibers are recruited during gliding flight and are sufficient for this work (Meyers and Mathias, 1997). The absence of slow fibers in the pectoralis of the gull may reflect a lack of selection for, or selection against, the presence of these fibers stemming from the diverse demands for power production and contraction velocity or frequency (Meyers and Mathias, 1997).

Other wing muscles, such as the extensor metacarpi radialis, include multiple fiber types in many of the species previously examined. The California and yellow-legged gull, the common coot, the mallard duck, the double-crested cormorant, and the black-footed and Laysan albatrosses all have extensor metacarpi radialis muscles containing slow twitch or slow tonic fibers (Meyers, 1997; Meyers and Mathias, 1997; Meyers and Stakebake, 2005; Torrella et al., 1998, 1999). These birds are noted for employing gliding flight, or adopting a spread-wing posture during other behaviors such as the drying postures adopted by cormorants. The authors conclude that these more 'efficient' slow fibers are likely recruited specifically for these activities.

Dial et al. (1987) and Sokoloff et al. (1998) propose that FG fibers in the pigeon pectoralis may be recruited primarily during take-off, landing, and other transient behaviors that require high mechanical power output. This idea is consistent with the functional properties of FG fibers but explicit demonstration of fiber recruitment specificity is logistically challenging. For example, the estimated power output requirements for take-off in blue-breasted quail and other members of the Phasianidae family are relatively high compared to the requirements for steady-state flight in other birds (Askew and Marsh, 2001; Askew et al., 2001). The specific roles of the smaller wing muscles for controlling wing kinematics are not well understood (but see Raikow, 1985), but certain modes of flight could require wing motor elements that can transiently produce very high power.

The lack of both SO or FG fibers in the wing muscles of zebra finches and hummingbirds suggests either that 1) these birds rarely perform behaviors that require transient, high mechanical power or 2) FOG fibers are fully sufficient for powering such behaviors in these birds.

4.3. Leg muscles

The presence of the FOG, SO, and FG fibers in the zebra finch gastrocnemius is consistent with findings in both the sparrow and pigeon gastrocnemius (Marquez et al., 2006; Wada et al., 1999). In contrast, the apparent lack of non-oxidative (glycolytic) fibers in the hummingbird gastrocnemius is unexpected. This muscle was specifically chosen for examination to serve as an internal positive control (to demonstrate our ability to find all three fiber types) in both the zebra finch and hummingbird.

We have not exhaustively surveyed all skeletal muscles in the Anna's hummingbird and future studies may find glycolytic fibers in other skeletal muscles. Nonetheless, the results presented here suggest that the hummingbird might lack glycolytic skeletal muscle fibers entirely. This pattern is in contrast to most vertebrate endotherms, which possess FG fibers in at least some locomotor muscles. However, there are a few vertebrate endotherm species, including multiple species of Carnivora (Amann et al., 1993; Snow et al., 1982; Toniolo et al., 2007), opossums (Peters et al., 1984; Van De Graaff et al., 1977), and Etruscan shrews (Jurgens, 2002), which appear to lack FG fibers in any locomotor muscles. The possible lack of FG fibers in the locomotor muscles of both Etruscan shrews and Anna's hummingbirds is particularly striking in light of the fact that even slightly larger, and closely related species, such as the common shrew, English sparrow, and zebra finch possess such fibers in their locomotor muscles (Marquez et al., 2006; Savolainen and Vornanen, 1995); this study). Like hummingbirds, Etruscan shrews (mean body mass = 2.2 g; Jurgens, 2002) are among the very smallest vertebrate endotherms. Relative rates of heat loss are highest among these small-bodied animals because of their extremely high surface area to volume ratios. As a consequence, relative heat production rates, which are reflected in high resting metabolic rate and large increases in metabolic rate with cold exposure, are highest among these same species. Jurgens (2002) suggests that the relatively high ATP turnover rates and associated rates of heat production in oxidative muscle fibers underlie their important role as heat generating elements in these animals. Oxidative fibers are better suited than non-oxidative fibers for thermogenesis

due to the former's greater fatigue resistance and higher mitochondrial density (Block, 1994). Both Etruscan shrews and hummingbirds employ torpor and rely, at least in part, on shivering thermogenesis as a means of rewarming following torpor (Block, 1994; Dawson, 1975; Fons et al., 1997; West, 1965). Evidence from multiple sources now indicates that birds employ non-shivering thermogenesis, like mammals, during exposure to low ambient temperatures and during periods of re-warming (reviewed in Bicudo et al., 2001, 2002). However, birds, unlike mammals, do not possess brown adipose tissue. The recent characterization of an uncoupling protein homolog in the pectoralis of the swallow-tailed hummingbird and its purported role in non-shivering thermogenesis (Vianna et al., 2001), is further evidence that hummingbird skeletal muscles may serve to do more than simply power locomotion. The presence of uncoupling proteins in other skeletal muscles such as the gastrocnemius has not been explicitly demonstrated. Given the relatively high thermogenic requirements of small endotherms, and their employment of torpor and subsequent need for re-warming, it is conceivable that most or all hummingbird skeletal muscles are adapted not just to meet locomotory requirements, but also for high rates of thermogenesis.

In birds, the gastrocnemius muscle acts to extend the ankle (Raikow, 1985). Thus, it potentially plays a postural role during perching, as well as a power-producing role during takeoff. Anna's hummingbirds may spend 80% of their time perching during active daylight hours (Stiles, 1971). In addition, both hummingbirds and zebra finches spend quiescent nighttime hours perched. Perching for extended periods may involve isometric contraction of the gastrocnemius resulting in extension and stabilization of the tarsometatarsus. Slow-twitch or tonic muscle fibers are most efficient at powering such isometric contractions and it is not surprising that SO fibers may be found in the gastrocnemius of both the hummingbird and the zebra finch.

Bursts of forceful isotonic contraction resulting in extension of the tarsometatarsus would be expected during takeoff. For example, both European starlings and quail produce 80–90% of initial takeoff forces using leg muscles (Earls, 2000), with the remaining lift produced by the wings. Initial work suggests that zebra finches also use their legs to generate a high proportion of lift during takeoff (Tobalske et al., 2004). Fast glycolytic fibers are well suited to providing much of this burst power and it is not surprising that such fibers comprise more than half of the fibers found in the zebra finch gastrocnemius. Hummingbirds, however, rely upon their legs to produce much less of the whole body force generated during takeoff (Tobalske et al., 2004). The relatively smaller leg muscle mass of hummingbirds, reflected in their placement in the order Apodiformes ('footless form'), at least partly accounts for the lower levels of force generation (Tobalske et al., 2004). Fast glycolytic fibers are capable of greater maximal force production per unit fiber cross-sectional area compared to oxidative fibers. Muscles lacking glycolytic fibers would be predicted to be capable of lower maximal force production compared to geometrically similar muscles containing these fiber types. Thus, lack of fast glycolytic fibers in the gastrocnemius of the hummingbird may further limit the maximal force production by their leg muscles.

The high mass-specific power output requirements of hovering flight place a great importance on the economy of muscle morphology, particularly at the lower limits of body size. There may be a greater premium placed on the minimization of muscle mass in hummingbirds compared to larger species. The lack of glycolytic fibers, even in a leg muscle, in the hummingbird suggests that the inclusion of FG fibers in their skeletal muscles may be unwarranted. For example, these birds may not engage frequently enough in behaviors that would be best powered by glycolytic fibers, as opposed to oxidative fibers, for the inclusion of such fibers to be advantageous. Alternatively, competing concerns, such as the need for the muscles to serve as high output thermogenic organs, in part drive hummingbird muscle fiber composition. In the absence of a clear advantage to the inclusion of FG fibers in hummingbird muscles, evolution may have favored their absence in response to thermogenic and mass concerns.

Additional studies are required to establish if hummingbirds lack FG fibers entirely, and to understand the adaptive value of a potential absence of these fibers. Specifically, investigations with the larger hummingbirds such as *Patagona gigas* (mean mass=22g; Altshuler et al., 2004) should be particularly informative because this bird's thermogenic requirements are likely to be lower, especially at the warmer temperatures experienced at lower elevations. Additionally, muscle composition data from the closely related swifts would greatly add to our understanding of the importance of leg muscles in generating takeoff forces and of muscles as thermogenic organs. Such comparisons should also help to reveal the relative unimportance of FG fibers as high-power producing elements in leg muscles in these groups.

4.4. Summary

In summary, and in agreement with previous work, we find that the fiber type composition of the wing musculature of small birds reflects the requirements for high frequency operation and resistance to fatigue associated with hovering and fast flapping flight. The species examined here have the lowest body mass and display higher wingbeat frequencies during flight than any other birds for which fiber composition of shoulder and wing muscles has been reported, such as the English sparrow (Greenewalt, 1962). We suggest that FOG fibers are necessary for the sustained, very high frequency operation (wingbeat frequency > 25 Hz) characteristic of flight in the zebra finch and hummingbird. Moreover, fast oxidative fibers are both necessary and sufficient for the full range of flight behaviors employed by these small (<15 g) birds. Hummingbirds in particular have the highest surface area to volume ratios of any birds and therefore must maximize the thermogenic capabilities of the limited available body mass. These high thermoregulatory requirements, coupled with reduced requirements for burst power production in leg muscles, may constrain fiber diversity in hummingbird skeletal muscles to a greater extent than in larger or non-hovering vertebrates.

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