

Myosin heavy-chain isoforms in the flight and leg muscles of hummingbirds and zebra finches

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Velten BP, Welch KC, Jr. Myosin heavy-chain isoforms in the flight and leg muscles of hummingbirds and zebra finches. *Am J Physiol Regul Integr Comp Physiol* 306: R845–R851, 2014. First published March 26, 2014; doi:10.1152/ajpregu.00041.2014.—Myosin heavy chain (MHC) isoform complement is intimately related to a muscle's contractile properties, yet relatively little is known about avian MHC isoforms or how they may vary with fiber type and/or the contractile properties of a muscle. The rapid shortening of muscles necessary to power flight at the high wingbeat frequencies of ruby-throated hummingbirds and zebra finches (25–60 Hz), along with the varied morphology and use of the hummingbird hindlimb, provides a unique opportunity to understand how contractile and morphological properties of avian muscle may be reflected in MHC expression. Isoforms of the hummingbird and zebra finch flight and hindlimb muscles were electrophoretically separated and compared with those of other avian species representing different contractile properties and fiber types. The flight muscles of the study species operate at drastically different contraction rates and are composed of different histochemically defined fiber types, yet each exhibited the same, single MHC isoform corresponding to the chicken adult fast isoform. Thus, despite quantitative differences in the contractile demands of flight muscles across species, this isoform appears necessary for meeting the performance demands of avian powered flight. Variation in flight muscle contractile performance across species may be due to differences in the structural composition of this conserved isoform and/or variation within other mechanically linked proteins. The leg muscles were more varied in their MHC isoform composition across both muscles and species. The disparity in hindlimb MHC expression between hummingbirds and the other species highlights previously observed differences in fiber type composition and thrust production during take-off.

myosin; muscle; isoforms; hummingbird; zebra finch

IN VERTEBRATES, SKELETAL MUSCLE performs a diversity of actions, functioning at varying shortening speeds and contraction rates to maximize muscle power output and efficiency (20). Muscles are capable of performing a variety of movements due, in part, to the presence of different types of fibers with specific contractile and metabolic properties (20, 24). Accordingly, a muscle's given fiber composition is closely tuned to its contractile requirements (20, 24). For example, muscles that perform rapid, precise, short-term movements are typically composed of fast-twitch muscle fibers, while those utilized during long-term, slow, or postural actions tend to predominantly consist of slow-twitch or slow-tonic muscle fibers (23, 24).

The contractile properties of a muscle fiber, such as contraction rate and shortening velocity, are largely influenced by

differences in the subunit composition of the contractile protein myosin (18, 19, 24). Variation in the expression of different myosin heavy chain (MHC) isoforms has been well documented in numerous mammalian muscles and species, with each mammalian isoform generally corresponding to a specific fiber type with unique contractile properties (24). This relationship between a fiber's contractile characteristics and its MHC isoform expression permits mammalian muscle fibers to be classified by their predominant MHC isoform, such that slow-twitch fibers generally express MHC I, and fast-twitch fibers express any of the various MHC II isoforms (IIa, IIb, IIc/x) (24).

Comparatively, much less is known about the various MHC isoforms present in avian skeletal muscles, with our current understanding largely restricted to various muscles of the domestic chicken (*Gallus gallus*) (2, 5). Limited research suggests that, as in mammals, avian MHC isoforms differ between slow- and fast-twitch fibers (18), as well as across the various fast-twitch fiber types (22). Yet, this aspect of avian muscle morphology remains relatively poorly understood, especially with regard to how specific avian MHC isoforms may relate to a muscle fiber's contractile requirements. Thus, this study sought to comparatively examine the MHC isoform expression of avian locomotor muscles across species that perform under varied contractile conditions. A specific focus was placed on the flight and limb muscles of ruby-throated hummingbirds (*Archilochus colubris*) and zebra finches (*Taeniopygia guttata*) due to their unique contractile properties.

Zebra finches and ruby-throated hummingbirds are capable of achieving some of the highest known vertebrate wingbeat frequencies (25–60 Hz) (12). The muscles primarily responsible for powering flight at these high frequencies, the pectoralis and supracoracoideus, are composed exclusively of a single fast-twitch, highly oxidative fiber type (21, 31). This fiber type is histologically distinct from the other fast-twitch fiber types that may also be found in avian flight muscle (fast-twitch highly glycolytic and fast-twitch intermediate fibers) (21). While the predominant expression of highly oxidative, fast-twitch fibers is common in the flight muscle of several avian species (21, 31), wingbeat frequency varies greatly in this group of animals, even among species with flight muscles composed of this same histologically defined fiber type.

The variation in wingbeat frequencies observed across species requires avian flight muscle to operate over a wide range of contraction rates and shortening velocities (10, 29). Thus, larger birds with lower wingbeat frequencies have slower shortening velocities than small birds, such as hummingbirds and zebra finches, with more rapid wingbeat frequencies (10, 29). This trend is supported by the observed negative scaling of pectoralis maximum shortening velocity (V_{\max}) with body

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mass (9, 15). In some cases, differences in the fiber type of the flight muscle, with theoretically different MHC isoforms, may account for such variation in muscle shortening velocity. Yet for many species that have similar muscle fiber composition but vastly different wingbeat frequencies, histologically defined fiber type may not fully account for observed differences in muscle contractile performance.

On the basis of these known differences in flight muscle contraction rate and fiber type, we hypothesized that flight muscle MHC isoform expression would differ across species to meet the varied contractile requirements of flight. Because of the uniform fiber type of the hummingbird and zebra finch flight muscles, both species were expected to express only one MHC isoform, yet this isoform would differ from that present in other histologically defined avian fast-twitch fiber types. Also, as the hummingbird and zebra finch flight muscles contract at such rapid rates to power flight at high wingbeat frequencies, the MHC isoform expressed by the hummingbird and zebra finch flight muscles was predicted to differ from that present in other avian species with lower wingbeat frequencies, but similar fiber type.

Unlike the flight muscles, which are predominantly responsible for performing the singular task of powering flight, the muscles of the avian leg are often more varied in the actions they perform, powering terrestrial, arboreal, and/or aquatic locomotion, generating thrust during takeoff, absorbing force during landing, and providing postural support (1, 6, 8, 11, 28). The diversity of movement these muscles are capable of performing is reflected in their mixed fiber composition, with single muscles often containing multiple fast-twitch fiber types, as well as slow-twitch fibers (13, 14, 26, 31). Hummingbirds, however, exhibit a marked difference from other avian species regarding the use of their hindlimbs. Belonging to the order Apodiformes, hummingbirds have relatively reduced hindlimbs and associated musculature. This uncommon morphology is reflected in the reduced reliance on hindlimbs for locomotion (28) and a difference in hindlimb muscle fiber composition compared with other avian taxa, including the zebra finch (13, 14, 31). As, the leg muscles of both hummingbirds and zebra finches contain multiple types of fibers, we hypothesized that these muscles would also express multiple MHC isoforms. However, on the basis of observed differences in hindlimb muscle fiber composition and use between the hummingbird and the zebra finch, the isoforms of the hummingbird leg muscles were expected to differ in type, as well as relative proportion of each isoform.

To test the hypotheses of this study, the MHC isoforms of the primary flight muscles, along with several superficial leg muscles, the flexor perforans et perforatus digiti II/III (flexor perforans), medial gastrocnemius, and lateral gastrocnemius, of the ruby-throated hummingbird and zebra finch were electrophoretically isolated. Domestic chicken was used as an identification guide (5), as well as a comparison, as chickens exhibit a much lower wingbeat frequency (~12.5 Hz) and a histologically distinct pectoral fiber type (fast-twitch, highly glycolytic) compared with that of hummingbird and zebra finch (21). Muscle samples from a juvenile yellow-bellied sapsucker (*Sphyrapicus varius*) and an adult house sparrow (*Passer domesticus*) were also opportunistically included in this study. These arboreal species have flight muscles similar in composition to those of the hummingbird and zebra finch (21, 30), but

with a lower range of wingbeat frequencies (10.5–22 Hz), providing further opportunity to examine how the MHC isoform may differ between muscles with varying contractile requirements.

MATERIALS AND METHODS

Specimens. All specimens utilized were adults, unless otherwise noted. Prior to use in this study, ruby-throated hummingbirds and zebra finch were cared for and killed following protocols approved by the University of Toronto Animal Care Committee. Hummingbirds were wild-caught under a permit issued by the Canadian Wildlife Service and zebra finches were purchased from a local supplier. A juvenile yellow-bellied sapsucker and adult house sparrow were opportunistically included in this study as they were discovered in locations where collectors had been less than an hour earlier. These locations were not heavily trafficked and no observable signs of tissue damage or rigor were observed at the time of their collection, providing further evidence that carcasses were discovered within 1 h after death and tissue was of high enough integrity for inclusion in this study. Care was taken to store and sample these specimens in the same fashion as those that expired in a laboratory setting, as outlined below. Use of such samples produced bands of similar quality to those of the hummingbirds and zebra finches. Diaphragm samples from a mouse (stored at -20°C) were run on each gel as a control to ensure proper sample preparation and band separation.

Sample preparation. Specimens were stored at -80°C as soon as possible following collection or death. Specimens were kept frozen until tissues were extracted. The muscles used and sample size for each are listed in Table 1 (4, 32). When possible, samples were taken from a superficial medial portion of the muscle, except for the leg muscles of the hummingbird, which, because of their small sizes, were used in their entirety. While the flexor perforans et perforatus digiti II was sampled in both the house sparrow and yellow-bellied sapsucker, the small size of this muscle in the hummingbird and zebra finch made it difficult to separate from the flexor perforans et perforatus digiti III. Thus, in these two species, it is likely that these flexor muscles were sampled together. Because of sampling of the flight muscle for

Table 1. *Species and muscles, along with sample size and sex ratio of each, included in this study*

Species	Muscles Sampled	Sample Size (n)
<i>Archilochus colubris</i>	Pectoralis	n = 9 (7 M; 2 F)
	Supracoracoideus	n = 10 (8 M; 2 F)
	Lateral gastrocnemius	n = 4 (3 M; 1 F)
	Medial gastrocnemius	n = 4 (3 M; 1 F)
	Flexor perforans et perforatus digiti II/III	n = 4 (3 M; 1 F)
<i>Taeniopygia guttata</i>	Pectoralis	n = 6 (3 M; 3 F)
	Supracoracoideus	n = 6 (3 M; 3 F)
	Lateral gastrocnemius	n = 3 (1 M; 2 F)
	Medial gastrocnemius	n = 2 (2 F)
	Flexor perforans et perforatus digiti II/III	n = 3 (1 M; 2 F)
<i>Sphyrapicus varius</i> (juvenile)	Pectoralis	n = 1 (Unknown)
	Supracoracoideus	n = 1 (Unknown)
	Lateral gastrocnemius	n = 1 (Unknown)
	Medial gastrocnemius	n = 1 (Unknown)
	Flexor perforans et perforatus digiti II	n = 1 (Unknown)
<i>Passer domesticus</i>	Pectoralis	n = 1 (M)
	Supracoracoideus	n = 1 (M)
	Lateral gastrocnemius	n = 1 (M)
	Medial gastrocnemius	n = 1 (M)
	Flexor perforans et perforatus digiti II	n = 1 (M)

M, male; F, female.

another study, tissue collection at the middle of the muscle was not always possible. To control for the variation in sampling position and to ensure that the MHC isoform observed was illustrative of the muscle as a whole rather than the specific sample area, several spatially distinct positions of the two flight muscles were sampled for one hummingbird and one zebra finch. For the pectoralis, these samples included a cranial portion of the muscle near the muscle's insertion on the humerus, the middle of the muscle closest to attachment on the sternum, the middle of the muscle closest to its lateral edge, and the most posterior tip of the muscle. For the supracoracoideus, the spatially distinct samples included the most cranial portion of muscle near the tendon of insertion onto the humerus and the most posterior tip of the muscle, in addition to the superficial middle sample.

All muscle samples were prepared following the protocol of Blough et al. (5). Briefly, specimens were thawed on ice, and small (<5.0 mg) portions were excised from the muscle, cleaned of connective tissue, weighed, and placed in a 1.5-ml microcentrifuge tube. For every milligram of tissue, 30 μ l of sample buffer was added. Sample buffer was composed of 8 M urea, 2 M thiourea, 0.05 M Tris base, 0.075 M dithiothreitol, 3% (wt/vol) SDS, and 0.004% (wt/vol) bromophenol blue at pH = 6.80 [see Ref. 5 for sample buffer preparation]. Once in the sample buffer, the sample was sonicated on ice (Sonic Rupter 250, Omni International, Kennesaw, GA) 3–5 times, each for ~10 s, and then centrifuged (Beckman Coulter Microfuge 22R Centrifuge, Brea, CA) for 5 min at 14,000 *g*. The supernatant was transferred to a new microcentrifuge tube, heated in a water bath (VWR, Mississauga, ON, Canada) to at least 80°C for 2 min, and then immediately placed on ice for at least 5 min. Finally, samples were diluted to 1:10 or 1:100 with sample buffer and stored at –80°C. Most isoforms were successfully isolated using less than 10 μ l of the 1:100 sample dilution.

Protein electrophoresis. MHC isoforms were separated using SDS-PAGE electrophoresis, following a protocol modified from Blough et al. (5). All gels were run on a Hoefer SE600 gel apparatus with a Hoefer PS2A200 power supply (Hoefer, Holliston, MA). Gels measured 14 \times 16 cm and were 0.75 mm thick. The separating gel consisted of 9% acrylamide (wt/vol) [acrylamide:N,N'-methylenebis(acrylamide) (bis) ratio of 200:1], 12% (wt/vol) glycerol, 0.75 M Tris, pH=8.8, and 0.1% (wt/vol) SDS. Approximately 30 ml of separating gel solution was degassed for at least 30 min before 240 μ l of 10% (wt/vol) ammonium persulfate (APS) and 24 μ l of TEMED were added to induce polymerization. The separating gel was allowed to polymerize for at least an hour before pouring the stacking gel. The stacking gel consisted of 4% acrylamide (wt/vol) (acrylamide:bis ratio of 50:1), 0.125 M Tris, pH = 6.8 and 0.01% (wt/vol) SDS. Approximately 20 ml of stacking gel solution was degassed for at least 30 min before 300 μ l of 10% APS and 30 μ l of TEMED were added for polymerization. The stacking gel was allowed to polymerize for at least 30 min before samples were loaded.

The lower electrode running buffer consisted of 25 mM Tris, 192 mM glycine, and 0.1% SDS. The upper electrode running buffer was identical except for the addition of 800 μ l of 2-mercaptoethanol per liter. Gels were run for 44–45 h at 7–10°C. While migrating through the stacking gel, voltage was kept constant at 70 V. When the dye front crossed to the separating gel, voltage was increased to 200 V and held constant through the end of the run.

Identification of avian MHC isoforms. For reference and identification of MHC isoforms in study species, the superficial pectoralis, anterior latissimus dorsi, and lateral gastrocnemius muscles of the domestic chicken were used. These muscles express six different avian MHC isoforms with well-established gel migration patterns (5). Samples were collected from a fresh, never-frozen, chicken that was obtained from a local grocer and sampled on ice immediately following purchase. Care was taken to sample tissue from subsurface portions of the muscles to avoid any potential contamination on the muscle surface. Chicken MHC isoform migration patterns obtained in

this study were similar to those obtained previously (5), with the following exception: the superficial pectoralis of the chicken has previously been shown to contain only a single MHC isoform, labeled as the adult fast isoform (5, 18). In this study, two bands were observed in the superficial pectoralis. The prevalent band was assumed to be the adult fast MHC isoform, while the second, less prominent band migrated with the embryonic/neonatal isoforms present in the chicken lateral gastrocnemius. This second band may be a protein breakdown product due to the use of a grocer-supplied chicken (P. J. Reiser, personal communication). However, it may correspond to a neonatal isoform depending on the age of chicken when it was harvested, as transcripts of neonatal MHC are present in chicken pectoralis up to 35 days post hatch (27).

In avian muscle, many of the embryonic and neonatal isoforms have not been fully correlated to a specific band, and, thus, are often classified as unidentified embryonic/neonatal isoforms (5). Thus, in this study, these isoforms were simply classified as embryonic/neonatal (Emb/Neo) fast isoforms and identified by their migration speed through the gel, from slowest (Emb/Neo 1) to fastest (Emb/Neo 3).

Gel fixation and staining. Gels were fixed and silver-stained following Blough et al. (5) with minor modifications. Gels were rocked gently (ThermoScientific, MaxQ2000, Waltham, MA) during the entire fixation and staining process. Following electrophoresis, gels were immediately transferred to a solution of 50% methanol, 10% acetic acid, and bathed for 60 min. Gels were then transferred to a 5% glutaraldehyde solution and fixed for at least 60 min. Following fixation, the gels were rinsed thoroughly with distilled water using a double wash method (5) for a total of six double washes. Gels were placed in a staining solution (46 mM silver nitrate, 207 mM ammonium hydroxide, and 18.9 mM NaOH) for 10 min and then rinsed with distilled water 5 times, each for ~30 s. Once rinsed, gels were immediately placed in a developing solution of 0.24 mM citric acid and 0.125% (wt/vol) formaldehyde. Once bands began to develop, the original developing solution was discarded, and the gel was placed in fresh developing solution until bands were fully developed. Development was inactivated using 3.33% acetic acid.

Imaging, data analysis, and statistics. Gels were imaged immediately after staining using a Bio-Rad ChemiDoc XRS+ (Bio-Rad Laboratories, Hercules, CA) and the accompanying Image Capture software (version 3.0). For muscle samples that contained more than one MHC isoform, the intensity of each band was analyzed using the band percent tool of the Image Capture software to determine the relative percentage of each isoform present within each muscle. As staining sensitivity may vary across gels, as well as across specimens, relative isoform percentage was determined from at least three different gels and only gels with prominent bands and little background staining were used for analysis.

A Fisher exact test was used to determine whether the relative MHC isoform proportion of the hummingbird hindlimb muscles differed significantly ($P < 0.05$) from that of the zebra finch. Statistical analysis was not performed on data obtained from the sapsucker and house sparrow as the sample size for each was only one. Data are presented as mean \pm SD.

RESULTS

Hummingbird. The pectoralis and supracoracoideus of the hummingbird both displayed only a single MHC isoform that migrated with the predominant adult fast isoform of the chicken superficial pectoralis (Fig. 1A, Table 2). There was no difference in MHC isoform across muscle sites sampled for either of these flight muscles (data not shown).

The three superficial leg muscles of the hummingbird all contained the same three MHC isoforms, slow myosin (SM) 2, adult fast, and Emb/Neo 3, with the embryonic/neonatal fast

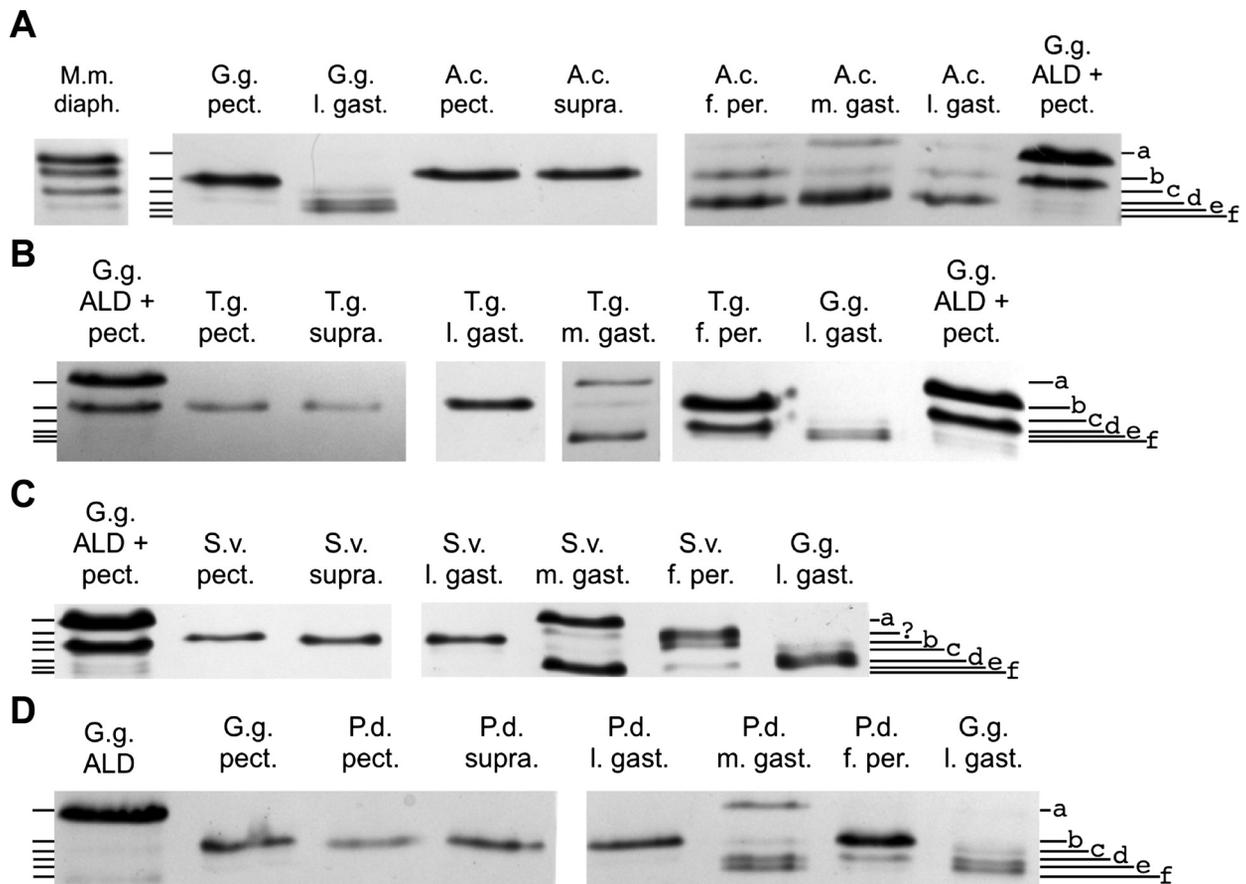


Fig. 1. Myosin heavy chain (MHC) isoforms present in the pectoralis (pect.), supracoracoideus (supra.), flexor perforans et perforatus digiti II/III (f. per.), medial gastrocnemius (m. gast.), and lateral gastrocnemius (l. gast.) of several avian species were identified by their migration pattern relative to known MHC isoforms present in chicken (G.g.) skeletal muscles, including the anterior latissimus dorsi (ALD). In order of migration speed, from slowest to fastest (from top to bottom), the MHC isoforms identified are slow myosin (SM) 2 (A), a previously unidentified myosin (?), adult fast myosin (B), embryonic/neonatal fast myosin (Emb/Neo) 1 (C), Emb/Neo 2 (D), Emb/Neo 3 (E), and SM1 (F). Mouse diaphragm (M.m. diaph.) served as a control to ensure proper band separation. Bands of the mouse diaphragm were identified on the basis of migration distance from top to bottom as follows: IID, IIA, IIB, and I. Although only pictured in panel A of the figure, this control was run on every gel. A: MHC isoforms present in hummingbird (A.c.) locomotor muscles. B: MHC isoforms present in zebra finch (T.g.) locomotor muscles. C: MHC isoforms present in yellow-bellied sapsucker (S.v.) locomotor muscle. D: MHC isoforms present in the house sparrow (P.d.) locomotor muscle. Sample lanes separated by a noncontiguous space were taken from either a separate gel or a separate portion of the gel.

isoform being the most prevalent in all three muscles (Fig. 1A, Table 2). The proportion of the SM2 and adult fast isoforms varied slightly between the muscles (Table 2).

For all occasions in which similar MHC isoforms were present in a hindlimb muscle in both zebra finches and hummingbirds, the relative proportion of the isoform in the hummingbird differed significantly from that of the same muscle in the zebra finch ($P < 0.05$) (Table 2).

Zebra finch. Both zebra finch flight muscles displayed a single MHC isoform that corresponded to the adult fast isoform of the chicken pectoralis (Fig. 1B, Table 2). As with the hummingbird flight muscle, no spatial variance in MHC isoform was observed in the zebra finch pectoralis or supracoracoideus (data not shown).

The three superficial leg muscles of the zebra finch each had a distinct MHC isoform composition, consistently exhibiting 1–2 dominant ($\geq 95\%$) isoforms that varied between the muscles (Fig. 1B, Table 2). Very faint bands corresponding to other MHC isoforms were observed for each of the zebra finch leg muscles, but the presence of these bands varied both across and within specimens and accounted for less than $\sim 5\%$ of total MHC composition (Table 2).

Yellow-bellied sapsucker. The flight muscles of the juvenile yellow-bellied sapsucker both displayed a single MHC isoform corresponding to the adult fast isoform of the chicken pectoralis. As in the zebra finch, the superficial leg muscles of the sapsucker each displayed a distinct MHC isoform pattern (Fig. 1C), exhibiting many of the same isoforms that were found in the zebra finch leg muscles (Table 2). The medial gastrocnemius and flexor perforans, however, displayed a band that did not appear to correspond to any isoform previously described in the chicken or any of the other study specimen. This isoform migrated between the SM2 adult fast isoforms (Fig. 1C) and was the predominant isoform present in the flexor perforans (Table 2). As it is not known whether this band corresponds to a slow or fast isoform at this time, it has been labeled as an unknown MHC isoform (Table 2).

House sparrow. As with the other species studied, the samples from both the flight muscles of the house sparrow exhibited only the adult fast MHC isoform (Fig. 1D, Table 2). The MHC isoforms of the sparrow's superficial leg muscles also exhibited a pattern very similar to those of the zebra finch (Fig. 1D, Table 2).

Table 2. Percent composition of myosin heavy chain (MHC) isoforms identified in the flight and superficial leg muscles of three avian species

Muscle	MHC Isoform	Percent Composition (mean \pm SD) Species			
		<i>Archilochus colubris</i>	<i>Taeniopygia guttata</i>	<i>Sphyrapicus varius</i>	<i>Passer domesticus</i>
Pectoralis	Adult fast	100 \pm 0	100 \pm 0	100	100
Supracoracoideus	Adult fast	100 \pm 0	100 \pm 0	100	100
Lateral gastrocnemius ^a	SM 2	2.3 \pm 2.6			
	Adult fast	19.2 \pm 25.3	99.7 \pm 0.8	100 \pm 0	100 \pm 0
	Emb/Neo 2	78.4 \pm 24.6	0.3 \pm 0.8		
Medial gastrocnemius ^a	SM 2	19.6 \pm 8.1	34.7 \pm 7.9	52.3 \pm 3.4	29.6 \pm 3.1
	Unknown adult			4.5 \pm 1.7	
	Adult fast	14.9 \pm 13.5	1.8 \pm 2.4		9.1 \pm 0.8
	Emb/Neo 1			0.7 \pm 0.7	
	Emb/Neo 2	65.2 \pm 11.8	2.7 \pm 4.4		36.1 \pm 4.4
	Emb/Neo 3		61.2 \pm 9.3	42.5 \pm 3.3	25.2 \pm 7.2
Flexor perforans et perforatus digiti II/III ^a	SM 2	3.8 \pm 1.6			
	Unknown adult			65.4 \pm 3.5	
	Adult fast	30.4 \pm 7.7	72.1 \pm 24.6	19.6 \pm 13.7	83.7 \pm 0.0
	Emb/Neo 2	65.9 \pm 19.8	26.3 \pm 8.9 ^a	15.0 \pm 11.1	16.3 \pm 0.0
	Emb/Neo 3		1.4 \pm 1.9		

Percent composition is expressed as mean \pm SD. MHC classification is based on muscles of the adult chicken (*Gallus gallus*), which is known to contain two slow myosin (SM), one adult fast, and several unidentified embryonic/neonatal (Emb/Neo) fast isoforms. In this study, embryonic/neonatal fast isoforms are identified by their migration distance from slowest (1) to fastest (3). ^aRelative proportion of isoforms within these muscles differed significantly ($P < 0.05$) between the hummingbird (*Archilochus colubris*) and the zebra finch (*Taeniopygia guttata*). Statistical analysis did not include the sapsucker (*Sphyrapicus varius*) or house sparrow (*Passer domesticus*) due to their sample size ($n = 1$).

DISCUSSION

MHC isoforms of the flight muscles. As we hypothesized, the pectoralis and supracoracoideus of both hummingbirds and zebra finches contained only a single MHC isoform. However, this isoform did not differ from that present in the superficial pectoralis of the chicken (Fig. 1), a muscle that differs from the zebra finch and hummingbird flight muscles in both contractile requirements and fiber type. The flight muscle of the sapsucker and house sparrow also exhibited the same single MHC isoform corresponding to the adult fast isoform of the chicken pectoralis. These results are similar to those of Dalla Libera and Carpena (7), who also predominantly found the adult fast MHC isoform in the pectoralis of several other avian species.

The consistency of the MHC isoform in the flight muscles across study species was contrary to our hypothesis. Unlike the species included in the study by Dalla Libera and Carpena (7), which represented a relatively narrow range of wingbeat frequencies based on their similar body sizes, the birds included in this study had a range of body sizes and wingbeat frequencies (10.5–60 Hz). As both in vivo and maximum shortening velocity of the pectoralis scale negatively with body mass (9, 10, 15, 29), this variation of wingbeat frequency is likely achieved by the flight muscles of the study species operating at different shortening velocities during flight. Because maximum shortening velocity of a muscle fiber is known to correlate with its predominant MHC isoform in both mammals and birds (17, 18, 24), it was surprising that the MHC isoform of the hummingbird and/or zebra finch flight muscles did not differ from that present in the flight muscles of species with up to 5.2 times lower wingbeat frequencies and an estimated 1.7 times lower muscle strain rate (29).

The results of this study also demonstrated that the MHC isoform expressed by the fast-twitch, highly oxidative fibers of the hummingbird, zebra finch, house sparrow, and sapsucker flight muscles did not differ from that present in the fast-twitch,

highly glycolytic fibers of the chicken pectoralis. This finding is contrary to those of previous studies. Using similar methodology, Reiser et al. (18) found that the relatively more oxidative fast-twitch fibers (likely intermediate or highly oxidative fibers) of the deep portion of the chicken pectoralis contained an embryonic fast isoform rather than the adult fast isoform of the fast-twitch, highly glycolytic fibers in the superficial pectoralis. Rosser et al. (22) used differential antibody binding to also demonstrate variation of pectoral fast-twitch fiber MHC isoforms both in and across several avian species, suggesting differential isoform expression across species and/or fibers. As differences in antibody binding were only observed in a fraction of the species included in that study, it may be that our study species and/or muscle sampling location were not adequate to detect such differences. It may also be that our methodology was not sensitive enough to observe differences between the pectoral isoforms of these species. Within chickens, genes corresponding to the fast MHC isoforms are highly homologous (3), and some bands, such as those corresponding to the embryonic/neonatal fast isoforms, migrate very near to each other (Fig. 1), making them difficult to separate using gel electrophoresis. If high levels of MHC homology also exist between species, different pectoral isoforms may also exhibit relatively similar gel migration patterns, and, thus, be hard to distinguish from one another.

However, the technique used by Rosser et al. (22) does leave uncertainties as to whether variation in MHC composition is indicative of distinct isoform identities or simply differences in the amino acid sequence of a given homologous isoform. As each of the avian MHC isoforms identified to date is encoded by a separate gene (3), continued research into the avian MHC gene family will help resolve this matter. Without further evidence to suggest otherwise, the results of this study, along with those of Dalla Libera and Carpena (7), indicate that avian flight muscles predominantly contain the same adult fast MHC

isoform, regardless of differences in muscle contractile properties and fiber type. Such results suggest that the kinetic properties of this isoform are those necessary to achieve the basic contractile requirements of avian powered flight.

The diversity of contractile properties in avian flight muscles across species may, instead, be due to variations in the structural composition of this singular MHC isoform, as suggested by differential antibody binding (22). When examined at the level of the protein structure, the adult fast isoform of the chicken shares 93% similarity to that predicted for the same zebra finch isoform. Assuming the adult fast MHC isoform gene has been correctly identified in the zebra finch genome and encodes the MHC present in the flight muscle, this degree of protein similarity between these two species is similar to that found between Type IIX and IIB isoforms of mouse skeletal muscle (92%) and between embryonic and adult fast isoforms of the chicken (95%). Thus, potential interspecific sequence dissimilarity of the avian adult fast MHC isoform may be great enough to account for functional variation in contractile performance. Differences in the contractile performance of avian flight muscle may also be due to variation in other contractile proteins, such as the myosin light chain, which is known to differ between fiber types in both mammals and birds (7, 18, 23).

MHC isoforms of the superficial leg muscles. Given the mixed fiber-type composition of avian hindlimb muscles, including slow, as well as multiple types of fast-twitch fiber (13, 14, 31), the expression of several MHC isoforms in the hindlimb muscles was expected and was similar to what is observed in a mammalian skeletal muscle of mixed fiber type (23, 24). These results contrasted with those for the flight muscles, as MHC isoforms present in each of the three superficial leg muscles varied among both species and muscles.

Among species, the MHC isoform expression in the hummingbird leg muscles was the most distinct, differing significantly from that of the zebra finch, as hypothesized. The muscle with the most obvious disparity was the lateral head of the gastrocnemius, reflecting the known difference in fiber-type composition of the gastrocnemius (including both lateral and medial heads) between hummingbirds and other avian species (13, 14, 31). The hummingbird gastrocnemius is composed of mostly fast-twitch, highly oxidative fibers (31), while that of the zebra finch and house sparrow predominantly contains fast-twitch, highly glycolytic fibers (14, 31). Predominant expression of an embryonic fast myosin in the lateral gastrocnemius of the hummingbird, compared with the adult fast isoform found almost exclusively in the lateral gastrocnemius of zebra finches and the house sparrow suggests that, unlike the flight muscle, different fast-twitch fiber types of the hindlimb muscles may display specific, unique MHC isoforms. Unfortunately, the sampling technique used in this study does not permit us to associate a specific MHC isoform with a given fiber type at this time.

This differential isoform expression of the hummingbird hindlimb muscles may relate to the species' reduced hindlimb musculature and thrust production during takeoff. In many birds, including the zebra finch, the muscles of the leg are used to produce most of the thrust required for takeoff, contributing 80–90% to total takeoff velocity (8, 28). However, the legs of hummingbirds appear to generate much lower thrust forces during takeoff, contributing only 46–63% to the animal's total

takeoff velocity (28). The MHC isoform expression of the hummingbird hindlimb muscles, in particular, that of the lateral gastrocnemius, along with the reduced hindlimb muscle mass and the absence of forceful fast-twitch, highly glycolytic fibers in the gastrocnemius (28, 31), may contribute to this observed difference in hindlimb functional capacity in this group of birds. Yet, at this time, no conclusive connections between fiber type or MHC isoform and the mechanical properties of leg fibers have been made, requiring further research into these properties in avian limb muscle fibers, expressing specific MHC isoforms.

In the other study species, MHC isoforms varied across each of the three superficial leg muscles. However, one similarity in the medial gastrocnemius did emerge across the four species. Compared with the other leg muscles examined, this muscle contained the highest percentage of the SM2 isoform in each species examined. This isoform is associated with avian slow tonic muscles that perform a postural function (such as the chicken anterior latissimus dorsi) (5, 17). The presence and relative percentage of this isoform in the medial gastrocnemius support previous findings that this muscle is composed of more slow fibers and generally exhibits mechanical properties that are slower, more fatigue-resistant, compared with the lateral gastrocnemius (13).

In the yellow-bellied sapsucker, zebra finch, and house sparrow, the differential MHC isoform expression pattern of the three leg muscles, specifically the variation between the medial and lateral heads of the gastrocnemius, highlights the functional differences that exist among the muscles of the avian hindlimb. Both the medial and lateral heads of the gastrocnemius act to extend the ankle, an action that, along with playing a role in generating takeoff thrust, also functions to maintain posture during perching (16). Although both heads of the gastrocnemius can perform similar mechanical actions (25, 31), the distinct MHC isoforms expressed by these two muscles, along with previously reported differences in fiber composition and mechanical properties, supports the notion that these muscles perform relatively contrasting functions, with the medial head specialized to serve a more postural role (25).

Finally, the juvenile yellow-bellied sapsucker expressed a novel, unidentified MHC isoform in both its medial gastrocnemius and flexor perforans. In the flexor perforans, this unidentified isoform accounted for the greatest proportion of total MHC isoform expression. No avian MHC isoform has been previously described migrating between the SM2 and adult fast isoforms; thus, we conclude from the presence of a band at this position that there exists a new avian MHC isoform that has not been previously observed in other avian species examined to date. However, as we were not able to identify this MHC isoform, even its basic properties (e.g., fast or slow myosin) are currently unknown, and further research is required to identify the type and functional properties of this new isoform.

Perspectives and Significance

The flight muscles of the hummingbird, zebra finch, house sparrow, and yellow-bellied sapsucker operate over a range of drastically different contraction rates and are composed of a different histochemically defined fast-twitch fiber type than the chicken pectoralis, yet each exhibited only a single MHC isoform corresponding to the adult fast isoform present in the

chicken superficial pectoralis. These results suggest that despite quantitative differences in the contractile demands of flight muscles across avian taxa, this single MHC isoform appears necessary to meet the contractile requirements of flight. However, interspecific variation in the amino acid sequence of the pectoral MHC isoform, not resolvable using the methodology of this study, may exist across species. Such variation could account for the differences in contractile performance and function of flight muscles among birds.

Unlike the flight muscles, the superficial leg muscles of these species were more varied in their MHC isoform composition. The marked difference in hindlimb MHC isoform expression between hummingbirds and the other study species highlights previously observed differences in fiber type composition and leg thrust production during takeoff. Also, drastically different MHC isoform expression between the medial and lateral heads of the gastrocnemius in the zebra finch, yellow-bellied sapsucker, and house sparrow suggest that these two muscles perform different functions, with the medial gastrocnemius playing a more postural role. Finally, a new, previously undescribed MHC isoform was observed in the medial gastrocnemius and flexor perforans of the yellow-bellied sapsucker. Properties of this isoform remain unknown, but it provides evidence that, across avian species, new MHC isoforms await formal identification and description.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: B.P.V. and K.C.W.J. conception and design of research; B.P.V. performed experiments; B.P.V. analyzed data; B.P.V. and K.C.W.J. interpreted results of experiments; B.P.V. prepared figures; B.P.V. drafted manuscript; B.P.V. and K.C.W.J. edited and revised manuscript; B.P.V. and K.C.W.J. approved final version of manuscript.

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