Thesis

An Immunohistochemical Study of Syringeal Muscles in Songbirds

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY LINSEY CHRISTENSEN ENDTITLED "AN IMMUNOHISTOCHEMICAL STUDY OF SYRINGEAL MUSCLES IN SONGBIRDS" BE ACCEPTED AS FULFILLING PARTIAL REQUIREMENTS FOR THE THESIS IN ZOOLOGY.

Thesis Committee

Advisor

Department Chair

ABSTRACT

The avian syrinx is the vocal organ located at the base of the trachea and is comprised of four intrinsic pairs of muscles: ventral and dorsal tracheobronchialis and ventral and dorsal syringealis. These muscles work to regulate the sound that is produced by modulating airflow through the trachea. Syringeal muscles were previously found to possess fast and superfast muscle fibers. Superfast fibers are unique fibers that are found mostly in sound-producing muscles and have been shown to contract at 250 Hz in songbirds. Previous work in our lab revealed that in two sexually dimorphic species, Zebra Finches and Bengalese Finches, males possessed significantly more superfast fibers than in the non-singing females. In European Starlings, a non-sexually dimorphic species, males and females had comparable superfast fiber percentages. These results suggest that superfast fibers are correlated with singing behavior. However, in Brown-headed Cowbirds, a sexually dimorphic species, males and females had similar superfast fiber percentages. It was unclear why these non-singing females possessed comparable fiber percentages to those of males. I was interested to see if singing behavior was correlated with the number of superfast fibers present within the syringeal muscles. Using immunohistochemistry, I investigated the syringeal muscles from a variety of songbird species. All non-sexually dimorphic species had comparable superfast fibers percentages. In another sexually dimorphic species, African Silverbills, males and females possessed comparable superfast fibers. This suggests that singing behavior alone does not drive sexual dimorphism in syringeal muscles. Recent studies have proposed that female song is ancestral; this indicates that the loss of female song is more recently evolved and would explain the presence of male-like superfast fiber percentages in females.

INTRODUCTION

Songbirds produce their vocalizations through use of a complex phonatory organ, the syrinx. Located at the base of the trachea, the syrinx is responsible for regulating acoustic parameters and altering airflow through the trachea. Similar to the mammalian larynx, the syrinx consists of cartilaginous tissue, membranes and muscles. The shape and structure of the syrinx varies among bird groups, leading to variation in vocal control. Bird vocal behavior is among the most complex of communication signals, even beyond the vocal capacity of many mammals (Marler & Slabbekoorn, 2004) and frequently includes extended segments of rapidly altered sounds (Greenewalt, 1968). Song is used in female choice for mate selection and in male-male competition for mate attraction, territory defense and resource protection. Given these important roles of song, it is likely that vocal quality is under strong selective pressures (Marler & Slabbekoorn, 2004). Production of this song is the result of a combination of passive interactions from two independent sound generators in the syrinx (Nowicki & Caprianica, 1986) and direct muscular control from intrinsic sound generating structures (Fee et al., 1998). The syrinx is a bipartite structure, consisting of two distinct sound generators, one located in each bronchus. In most investigated species, each sound source is independently controlled, with the left side producing lower frequencies than the right. This independent control allows for complex overlap of simultaneous, but distinct sounds (Prince et al., 2011). The syrinx is composed of four intrinsic pairs of muscles: ventral and dorsal tracheobronchialis and ventral and dorsal syringealis (fig. 1). These muscles differentially contribute to regulate the sound that is produced by altering airflow through the trachea by opening and closing the airway. Electromyographic (EMG) recordings of syringeal muscles in Brown Thrashers (*Toxostoma rufum*) revealed that the tracheobronchialis dorsalis adducts and the tracheobronchialis ventralis abducts the airway

to regulate air movement (Goller & Suthers, 1996). The syringeal muscles are influential in vocalization by positioning the cartilages and membranes into the airway and modulating the tension as needed (Larsen & Goller, 2002).

Sexual dimorphism in singing behavior is accompanied by alterations in both the central nervous system (Cooke et al., 1998) and in the syrinx (Riede & Goller, 2010). Three areas of the brain are found to be larger in males than in females in Canaries (*Serinus* canaries) and Zebra Finches (*Taeniopygia guttata*) (Nottebohm & Arnold, 1976). Previous work investigating the syrinx morphology in Zebra Finches, a sexually dimorphic species, revealed that the syrinx of males weighed approximately twice as much as that of females (Wade & Buhlman, 2000). Similar results were found in European Starlings (*Sturnus vulgaris*). Male starlings had a larger syrinx attributable to a greater muscle mass for the ability to produce more complex song. Female starlings demonstrate less vocal range and sing at slower rates than males (Prince et al., 2011).

Prior work in our lab has established the existence of two distinct fiber types in the syrinx, fast and superfast (Meyers et al., 2005; Uchida et al., 2009). Superfast fibers are rare fibers found in sound-producing animals. These fibers are specialized for producing rapid contractions and are well characterized in the rattlesnake tail shaker muscles and the swim bladder of some fishes (Rome et al., 1996; Rome et al., 1999). In songbirds, superfast fibers have been shown to contract up to 250 Hz (Elemans et al., 2008). This performance of the syringeal muscles ranks them among the fastest recorded tetrapod muscles. The exceptionally quick contraction rate of the syringeal muscles allows for the rapid modulation of airflow and complex acoustic production in many songbird species (Elemans et al., 2008).

Previous work on syrinx fiber types using European Starlings, revealed both sexes possessed similar superfast fiber percentages (~70%) (Uchida et al., 2009). Members of the

Estrildidae family, Zebra Finches and Bengalese Finches (*Lonchura striata domestica*), were investigated next and revealed that the singing sex, males, had significantly more superfast fibers than the non-singing sex, females (70% vs. 30% and 85% vs. 25%, respectively) (Uchida et al., 2009). These results follow a pattern that allows superfast fibers to be attributed to singing abilities. However, in Brown-headed Cowbirds (*Molothrus ater*) and Brewer's Sparrows (*Spizella brewer*), where males sing and females do not sing, both sexes of both species displayed similar superfast fiber percentages (~67% and ~70%, respectively) (Uchida et al., 2009).

Based on the results from European Starlings, Zebra Finches and Bengalese Finches, superfast fibers were thought to play an important role in singing behavior (Christensen et al., 2014). However, findings from the Brown-headed Cowbirds and Brewer's Sparrows did not fit this original hypothesis. The goal of this study was to quantify the presence of superfast fibers in a broader range of songbird species representing multiple families to determine if there was a correlation with singing behavior and the presence or absence of superfast fibers.

MATERIALS AND METHODS

Tissue Collection

Fresh syringeal tissue was collected from three males and three females of each of the following species: European Starlings, Zebra Finches, Bengalese Finches, Red-winged Blackbirds (*Agelaius phoeniceus*), Brown-headed Cowbirds, Yellow-headed Blackbirds (*X. xanthocephalus*), White-crowned Sparrows (*Zonotrichia leucophrys*), House Sparrows (*Passer domesticus*), and Brewer's Sparrows. Due to limited availability, only one male and one female

were used for African Silverbills (*Lonchura cantans*). Species were chosen based on local availability and to represent a diversity of families. All songbird species were wild caught by Dr. Franz Goller of the University of Utah or raised at the University of Utah's aviary prior to tissue removal. In all species, all four pairs of intrinsic muscles (left and right vTB and dTB, left and right vS and dS) were collected.

Tissue Preparation and Histology

Immediately following an overdose with isoflurane, syringeal tissue was obtained by removing the portion of the trachea with the syringeal muscles intact and mounted on a 2x2 cm piece of cork using gum tragacanth (5%). Blocks were frozen in isopentane, cooled by liquid nitrogen to approximately -150° C and subsequently stored in an ultracold freezer at approximately -80° C. Serial cross sections were obtained by taking 10-12 μm sections using a cryostat (Tissue-Tek II, Microtome/Cryostat, models 4551 and 4553, respectively) set at -20° C and mounted onto microscope slides. Sections were collected along the entire length of the syrinx to ensure that the largest cross section could be identified.

Muscle fiber types were identified using a series of histochemical techniques described in McFarland and Meyers (2008) and Uchida et al. (2010). Serial sections were reacted for myosin ATPase using acidic (pH 4.2-4.3) preincubations. In addition, muscle sections were reacted with anti-slow antibody ALD58 (University of Iowa, Hybridoma Bank) and anti-fast antibody MY32 (Sigma Chemical Company, St. Louis, MO) as described in Meyers and Stakebake (2005). Antibody slides were incubated in a humid chamber for 2 hours at 25°C, rinsed with phosphate buffered solution, then incubated with anti-mouse antibody, and stained with peroxidase substrate system (IMMpact AEC Red kit, Vector Labs, Burlingame, CA). Acidic myosin ATPase and anti-slow antibody ALD58 give positive

reactions with slow muscle fibers. Alkaline myosin ATPase and anti-fast antibody MY32 give positive reactions with fast muscle fibers. Superfast fibers were identified by a negative reaction with both ALD58/acidic ATPase and MY32/alkaline ATPase.

Reacted whole syrinx cross-sections and appropriate scalebars were photographed using a Canon Rebel T1i EOS digital camera mounted on a Zeiss Axioskop 40/40 FL microscope at 10 or 20x. Adobe Photoshop CS2 was used to merge overlapping photographs into a complete photomontage of the entire muscle. The photomontage was printed and taped together to allow for analysis and quantification. Each identifiable muscle fiber of both fiber types was counted and the total percentage of fiber types (fast vs. superfast) as well as the standard deviation was calculated for each sex of each species.

Sryingeal Muscle Analyses

Average fiber diameters were also calculated by randomly selecting 50 fibers of each type from each of the four quadrants of the syrinx. Fiber diameters were calculated by averaging the widest and narrowest points of individual fibers using Mitutoyo digital calipers. Independent sample t-statistic analysis was conducted to determine if there was a significant difference in the percentages of superfast fibers using arcsine transformation values. T-statistics were also used to determine if there was a significant difference in fiber diameters between males and females and fiber types within a species.

RESULTS

The results given are for all songbird species, unless otherwise stated. Figure 2 shows representative cross sectional area images of male and female syringes from all ten studied species.

Fiber Composition

All syringeal muscles are composed of two distinct fiber types, fast and superfast (fig. 3). Fast fibers reacted like type IIA muscle fibers, with positive reactions to anti-fast antibody MY32 and negative reactions with anti-slow antibody ALD58 and acidic pH preincubations. The superfast fibers did not react like any typical type I, IIA or IIB or tonic muscle fibers. These fibers had a negative reaction with both MY32 and ALD58, and an intermediate reaction with acidic pH preincubations (fig. 4). These reactions were previously described by Uchida et al. (2010).

In two species that are sexually dimorphic in singing behavior (Zebra Finch and Bengalese Finch), comparisons of superfast fiber percentages between males and females showed that the singing sex, males, possessed significantly greater percentages of superfast fibers an the non-singing sex, females. However, in three sexually dimorphic species (Brown-headed Cowbird, Brewer's Sparrow and African Silverbill), both sexes possessed comparable superfast fiber percentages (fig. 5)(Table 1).

In all species in which males sing and females sing at least occasionally (European Starlings, House Sparrows, Red-winged Blackbirds, White-crowned Sparrows, and Yellow-headed Blackbirds), there was no significant difference in the percentage of superfast fibers between the sexes of each species (fig. 5)(Table 1).

Muscle Fiber Diameters

Fifty fibers of each fiber type were measured from each of the four muscles of the syrinx (vTB & dTB, vS & dS). Comparisons of fast and slow fibers revealed that in almost all species both males and females had superfast fibers that were significantly larger in diameter than the fast fibers. Several species showed that males had significantly larger fast or superfast fibers than females (Table 2).

DISCUSSION

This study investigates the muscle fiber identities of the syringeal muscles of males and females in ten songbird species. The results of this study show that the muscles of the syrinx are heterogeneously composed of superfast and fast muscle fibers as previously described in Uchida et al. (2010). This study also shows that the lack of song in female songbirds does not drive sexual dimorphism in the superfast fiber composition of the syringeal muscles. Superfast fibers were found to be larger than fast fibers in almost all cases.

Fiber Diameter

Traditionally, fast glycolytic fibers are considered to be the largest in diameter. McFarland and Meyers (2008) reported fast fiber diameters between 38-40 μm in the American Avocet (*Recurvitostra americana*) leg muscles. The pectoralis muscles of Rock Pigeons (*Columba livia*) were found to contain fast oxidative-glycolytic fibers with a diameter of 33 μm and fast glycolytic fibers with a diameter of 78 μm (Kaplan & Goslow, 1989). In contrast, slow oxidative fibers are considered to be the smallest in diameter. This smaller diameter contributes to an increase in overall surface area to allow for greater oxygen diffusion. Kovacs and Meyers (2000) found that in three flight muscles of the Atlantic Puffin (*Fratercula arctica*) the slow fibers were significantly smaller than the other fast fibers.

In almost all cases, superfast fibers were significantly larger in diameter when compared to fast fibers (except female Zebra Finches and Bengalese Finches; male Starlings had larger superfast fibers but not statistically significant). Measurements of fiber diameters revealed that fast fibers had a maximum diameter of 21 μm and superfast fibers had a maximum diameter of 36 μm. This shows that superfast fibers are in the range of typical fast fibers and that the fast fibers are smaller than in other muscles. Fibers are equipped with distinct physiological properties that allow muscles to be optimized for performing one

specific task at a time. If additional demands are placed on the muscle, there is a necessity to trade-off, or compensate, in physiological properties; this trade-off is generally between contraction force and velocity (Rome and Lindstedt, 1998). The force that a muscle fiber can produce is directly related to its cross sectional area, with larger cross sectional area allowing greater force capabilities. Interestingly, the superfast fibers, which contract at rates reaching 250 Hz, are, in some cases, double the size of fast fibers. This seems puzzling given the trade-off in contraction velocity and force; these fibers are contracting at rapid rates, which would not allow them to produce much force, so why do they possess the larger diameter? Perhaps the larger superfast fiber diameter is an attempt to increase force capabilities, while allowing for rapid contraction capabilities. Muscle size is limited by constraints from overall body size; the fast fibers may have reduced in size in order to adapt to the limitations of the syringeal muscle size.

In a few instances, there was a significant difference between the male and female superfast fibers and the male and female fast fibers (Zebra Finches, Bengalese Finches, Brown-headed Cowbirds, and Yellow-headed Blackbirds). It is unclear if these differences in fiber size between the sexes really exists or is a result of differences in body and thus, syrinx, size. Additional information, such as individual body size, will need to be collected before any valuable assessment can be made.

Fiber Composition

Muscle fiber performance is based on several structural factors including the volume of myofibrils, the volume of sarcoplasmic reticulum, and the volume of mitochondria present within each individual muscle cell. These factors determine the force of contraction, the contraction frequency, and the level of sustained performance of muscle fibers, respectively (Lindstedt et al., 1998). Superfast muscle fibers were found to contract at a

frequency of 250 Hz in European starlings (Elemans et al., 2008). This rate would require highly specialized muscle fibers to be able to compensate for this rapid activation and relaxation of the syringeal muscles.

In a previous study investigating syringeal muscle fiber cross-sectional area in Zebra Finches, no distinction was made between different populations of fibers (Wade & Buhlman, 2000). The current study found the presence of two distinct fiber types, fast and superfast, in all ten-songbird species. No slow fibers were found in any of the studied species. Superfast fibers are rare fibers that have infrequently evolved and are found in sound-producing muscles. These fibers are best described in the swimbladder muscles of some fish species and in the rattlesnake tailshaker muscles (Rome et al., 1996 and Rome et al., 1999). Superfast fibers have been described in the mammalian larynx, which functions analogously to the avian syrinx. The larynx is made of five intrinsic muscles that function to regulate sound frequency, as well as in quiet respiration and in producing vocalizations (Shiotani et al., 1999). However, these superfast fibers do not have the contractile capabilities of the superfast fibers (Hoh, 2005). Although superfast fibers are found in a variety sound-producing muscles, the avian and mammalian vocal organs differ from those of the swimbladder and tailshaker muscles. The muscles of the larynx and syrinx modify the sound as air passes through the trachea, causing the vocal organ to vibrate. In contrast, the muscles of the swimbladder and tailshaker muscles produce (not modulate) sound with each muscular contraction (Fine et al., 2001).

In species where males sing and females sing at least occasionally (European Starlings, Yellow-headed Blackbirds, Red-winged Blackbirds, White-crowned Sparrows, Brewer's Sparrows, and House Sparrows) superfast fiber percentages were comparable between males and females. The findings of male and female songbirds possessing similar

superfast fibers percentages suggests that superfast fibers play a role in singing behavior and aid in the rapid modulation of sound frequencies. Uchida et al. (2010) found similar superfast fiber percentages in male European Starlings. Here the authors suggest that fast (type IIA) fibers could be activated during quiet respiration in contrast to superfast fibers that would be activated during modulation or gating of vocal behavior.

In two sexually dimorphic species, Zebra Finches and Bengalese Finches, males had significantly more superfast fibers than the non-singing females. If superfast fibers are directly correlated with singing behavior, then this reduction, but not loss, of superfast fibers in females might suggest a non-vocalization function of the superfast fibers. Supplemental data was collected from Western Kingbirds (*Tyrannus verticalis*), which are suboscines. Histochemical reactions revealed that superfast fibers are present in the syringeal muscles of both males and females of this non-songbird, supporting the idea that these fibers might serve in other possible functions.

In two other sexually dimorphic species, Brown-headed Cowbirds and African Silverbills, males and females had comparable superfast fiber percentages despite females not singing. These perplexing findings suggest that superfast fibers are not directly related to singing behavior. It was unclear why non-singing females would retain superfast fiber percentages that are comparable to singing males.

Evolution of Female Song

It has long been accepted that male song is used to attract mates and compete for territory and resources. Female song has often been thought to be rare and atypical, with sexual selection driving the pronounced occurrence of only male song in many species (Price et al., 2009). However, recent studies have challenged this view and suggest that female song was more widespread in evolutionary history than previously thought. Several studies have

proposed that there is a geographical bias towards studying songbird species from temperate North America and Europe, where there are disproportionately more non-singing females than in other areas throughout the world (Price et al., 2009). Odom et al. (2014) suggested that female song is widespread and ancestral based on the observations that most of the songbird diversity exists in tropical regions of the world (Fjeldsa, 2013), where it is quite common to have songbird species in which male and females sing and in the area where songbirds are thought to have originated, Australasia, female song is widespread (Robinson, 1949). The authors of this study investigated a large diversity of songbird species from various geographic regions and found that in 71% of the 32 investigated families female song was present. This finding along with standard state reconstruction, allowed the authors to conclude that female song is ancestral.

Because song was originally present in both male and female songbirds, the presence of superfast fibers in non-singing females no longer remains a mystery. At one point in time, females used superfast fibers to produce their song. The results from Odom et al. (2014) suggest that losing female song occurred more recently in evolutionary history and that the current sexual dimorphism between many songbird species is a result of selective pressures against female song rather than sexual selection for male song. In the four sexually dimorphic species (Zebra Finches, Bengalese Finches, Brown-headed Cowbirds, and African Silverbills), the two finch species also showed sexual dimorphism in superfast fiber composition. In contrast, the Brown-headed Cowbird and African Silverbill did not show dimorphism in superfast fiber composition despite females not singing. This difference in fiber distributions could be explained if female Zebra Finches and Bengalese Finches lost their song earlier in evolutionary history allowing fibers ratios to be reduced as well. If female Brown-headed Cowbirds and African Silverbills lost their song more recently,then

superfast fibers have would not have had time to adjust for this recent loss. This prediction could be evaluated using detailed evolutionary timelines of these species.

CONCLUSIONS

Syringeal muscles are heterogeneously composed of fast and superfast muscles fibers. Superfast fibers are active during modulation of sound production, while fast fibers are likely active during quiet respiration. Superfast fibers are larger in diameter than the fast fibers within the syringeal muscles. This larger superfast fiber diameter might be in attempt to increase force capabilities, while allowing for rapid contraction rates. Sexual dimorphism in singing behavior is not the only predictor of superfast fiber compositions in the syringeal muscles. Two sexually dimorphic species were found to have comparable fiber percentages between the sexes. This puzzling result could be explained by female song being the ancestral state.

Investigating members of the shrike family, a primitive North American family with sexual dimorphism in singing behavior (Odom et al., 2014), will help to determine if superfast fibers are necessary for some other non-vocalization function given that the non-singing females likely lost their song earlier in evolutionary history.

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LITERATURE CITED

Cooke, B., C.D. Hegstrom, L.S. Villeneuve, and S.M. Breedlove. 1998. Sexual differentiation of the vertebrate brain: Principles and mechanisms. *Front. Neuroendocrin.* 19:323-362.

Christensen, L.A., R.A. Meyers, and F. Goller. 2014. Lack of song in females does not drive sexual dimorphism in syringeal muscle composition. *Integrative and Comparative Biology* 54(1): e253.

Elemans, C. P. H., A. F. Mead, L. C. Rome and F. Goller. 2008. Superfast vocal muscles control song production in songbirds. *Plos One* 3(7): e2581.

Fee, M. S., B. Shraiman, B. Pesaranm and P. P. Mitra. 1998. The role of nonlinear dynamics of the syrinx in the vocalizations of a songbird. *Nature* 395: 67-71.

Fine, M.L., K.L. Malloy, C.B. King, S.L. Mitchell, T.M. Cameron. 2001. Movement and sound generation of the toadfish swimbladder. *J. Comp. Physiol* 187: 371-379.

Fjeldsa, J. 2013. The global diversification of songbirds (Oscines) and the build-up of the Sino-Himalayan diversity hotspot. *Chinese Birds* 4:132-143.

Goller, F. and R. A. Suthers. 1995. Implications for lateralization of bird song from unilateral gating of bilateral motor patterns. *Nature* 373: 63-66.

Goller, F. and R. A. Suthers. 1996. The role of syringeal muscles in gating airflow and sound productions in singing brown thrashers. *J. Neurophysiol.* 75: 867-876.

Greenewalt, C.H., 1968. Bird Song: Acoustics and Physiology. Washington DC: Smithsonian Institution Press.

Hoh, J.F. 2005. Laryngeal muscle fiber types. *Acta Physiol. Scand.* 183: 133-149.

Kaplan, S.R., and G.E. Goslow Jr. 1989. Neuromuscular organization of the pectoralis (pars thoracicus) of the pigeon (*Columbia livia*): Implications for motor control. *Anat. Rec.* 224: 426-430.

Kovacs, C.E., and R.A. Meyers. 2000. Anatomy and histochemistry of flight muscles in a wing-propelled diving bird, the Atlantic Puffin, *Fratercula arctica. J. Morphology* 224: 109-125.

Larsen, O. N., and F. Goller. 2002. Direct observation of syringeal muscle function in songbirds and a parrot. *J. Experimental Biology* 205: 25-35.

Lindstedt, S.L., T. McGlothlin, E. Percy, and J. Pifer. 1997. Task-specific design of skeletal muscle: balancing muscle structural composition. *Comp. Biochem. Physiol.* 120: 35-40.

Marler P. and H. Slabbekoorn. 2004. Nature's Music: The Science of Birdsong. New York: Academic Press.

McFarland, J. C. and R. A. Meyers. 2008. Anatomy and histochemistry of hindlimb flight posture in birds. 1. The extended hindlimb posture of shorebirds. *J. Morphology* 269: 967-979.

Meyers, R.A., D. Schmutz and F. Goller. 2005. Histochemical analysis of songbird syringeal muscles. *Integrative and Comparative Biology* 45(6): 1167.

Meyers, R.A., and E.F. Stakebake. 2005. Anatomy and histochemistry of spreadwing posture in birds. 3. Immunohistochemistry of flight muscles and the "shoulder lock" in albatrosses. *J. Morphology* 263:12-29.

Nottebohm, F., and A. P. Arnold. 1976. Sexual dimorphism in vocal control areas of songbird brain. *Science* 194: 211-213.

Nowicki, S. and R.R. Capranica. 1986. Bilateral syringeal interaction in vocal production of an oscine bird sound. *Science* 231: 1297-1299.

Odom, K.J., M.L. Hall, K. Riebel, K.E. Omland, and N.E. Langmore. 2014. Female song is widespread and ancestral in songbirds. *Nature Communications* 5: 3379.

Price, J.J., S.M., Lanyon, and K.E. Omland. 2009. Losses of female song with changes from tropical to temperate breeding in the New World blackbirds. *Proc. Biol. Sci.* 276: 1971-1980.

Prince, B., T. Riede and F. Goller. 2011. Sexual dimorphism and bilateral asymmetry of syrinx and vocal tract in the european starling (*Sturnus vulgaris*). *J. Morphology* 272: 1527-1536.

Riede, T., and F. Goller. 2010. Functional morphology of the sound-generating labia in the syrinx of two songbird species. *J. Anatomy* 216: 23-36.

Robinson, A. 1949. The biological significance of bird song in Australia. *Emu* 48: 291-315.

Rome, L.C. 2006. Design and function of superfast muscles: new insights into the physiology of skeletal muscle. *Ann. Rev. Physiology* 68: 193-221.

Rome, L.C., Cook, C. Cook, D.A. Syme, M.A. Connaughton, M. Ashley-Ross, A. Klimov, and Y.E. Goldman. 1999. Trading speed for force: Why superfast crossbridge kinetics leads to superlow forces. *Proc. Natl. Acad. Sci.* 96: 5826-5831.

Rome, L.C., and S.L. Lindstedt. 1998. The quest for speed: muscles built for high-frequency contractions. *News Physiol. Sci.* 13: 261-268.

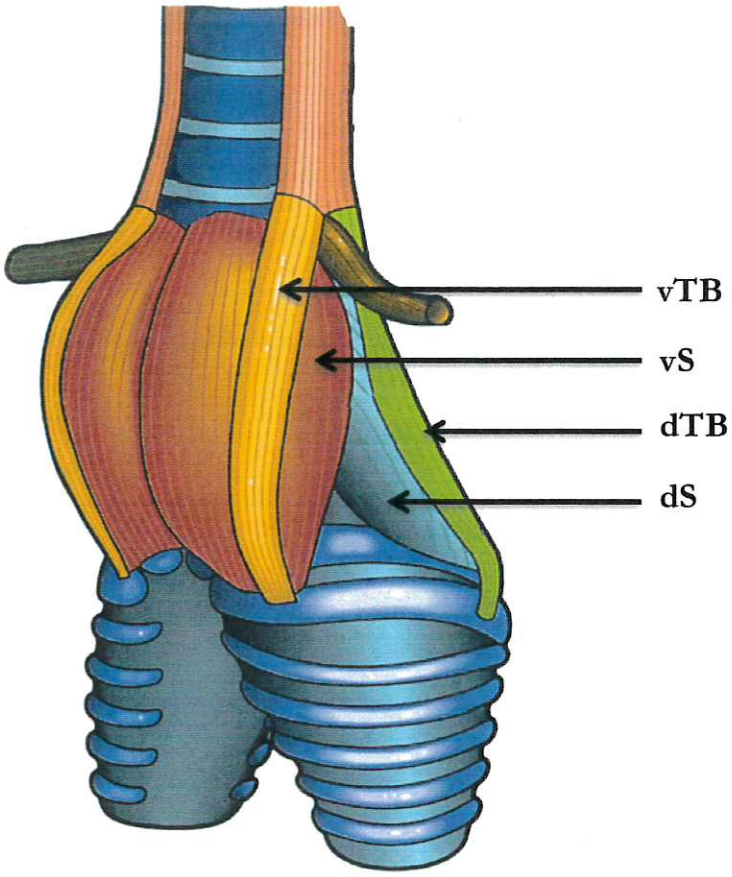
Rome, L.C., D.A. Syme, S. Hollingworth, S.L. Lindstedt, and S.M. Baylor. 1996. The whistle and the rattle: The design of sound producing muscles. *Proc. Natl. Acad. Sci.* 93: 8095-8100.

Shiotani, A., W.H. Westra, and P.W. Flint. 1999. Myosin heavy chain composition in human laryngeal muscles. *Laryngoscope* 109: 1521-1524.

Uchida, A. M., J. Green, S. Ahmad, F. Goller, and R. A. Meyers. 2009. Sexual Dimorphism of Syringeal Muscles in Songbirds. *Integrative and Comparative Biology* 49(1): e318.

Uchida, A. M., R. A. Meyers, B. G. Cooper, and F. Goller. 2010. Fibre architecture and song activation rates of syringeal muscles are not lateralized in the European starling. *J. Experimental Biology* 213: 1069-1078.

Wade, J. and L. Buhlman. 2000. Lateralization and effects of adult androgen in a sexually dimorphic neuromuscular system controlling song in zebra finches. *J. Comp. Physiol.* 426: 154-164.



Adapted from Larsen and Goller, 2002

Fig. 1. Ventrolateral view of a songbird syrinx illustrating the syringeal muscles (vTB & dTB: ventral and dorsal Treacheobronchialis; vS & dS: ventral and dorsal Syringealis)

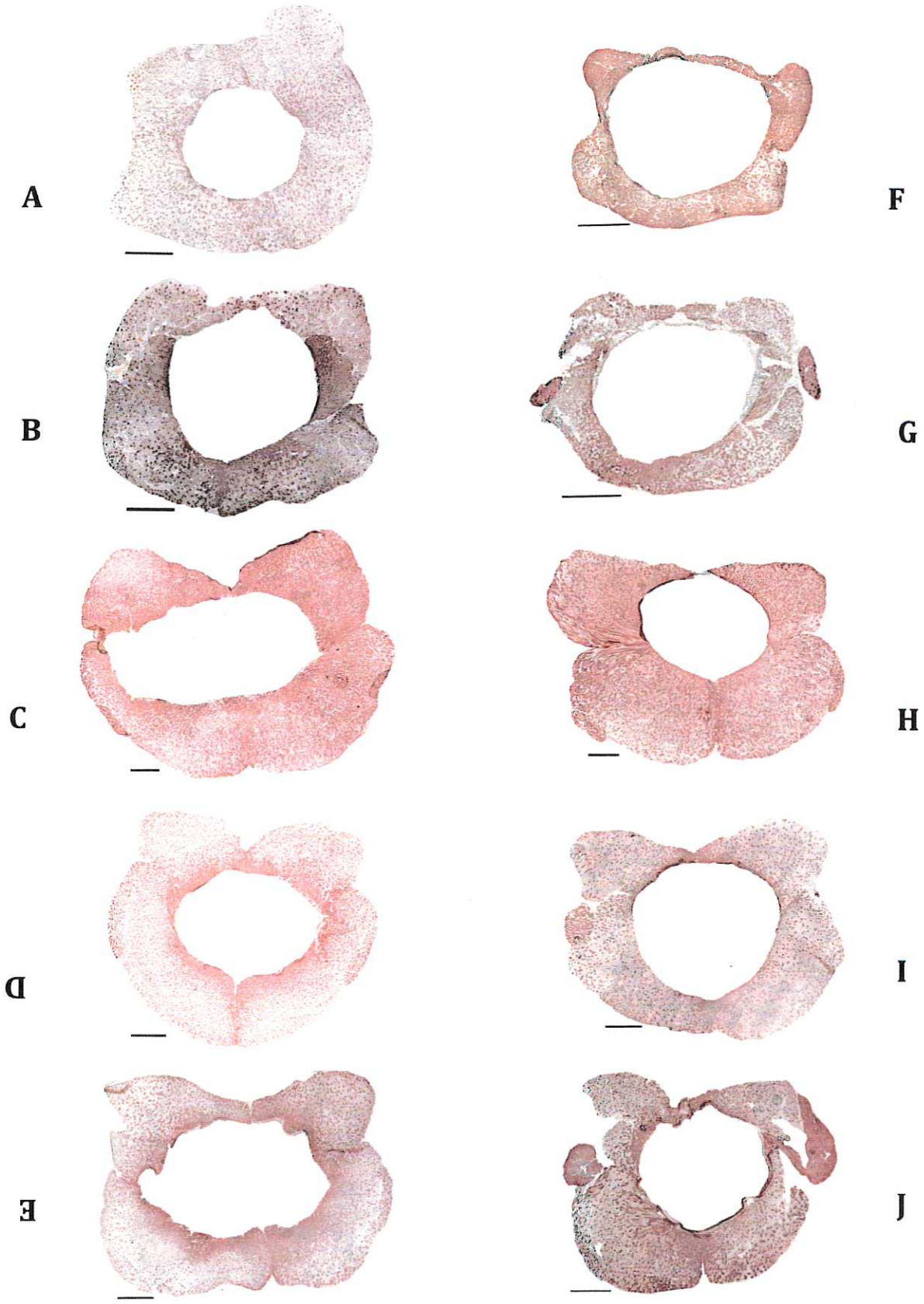


Fig. 2a Syrinx cross-sections of males and females from five songbird species (A-E) are males of each species and (F-J) are females of each species. A,F: Zebra Finch; B,G: Bengalese Finch; C,H: Yellow-headed Blackbird: D,I: Red-winged Blackbird; E,J: Brown-headed Cowbird. Images represent the thickest section of syringeal muscle. Ventral is towards bottom of the page. Scale bars, 0.5 mm.

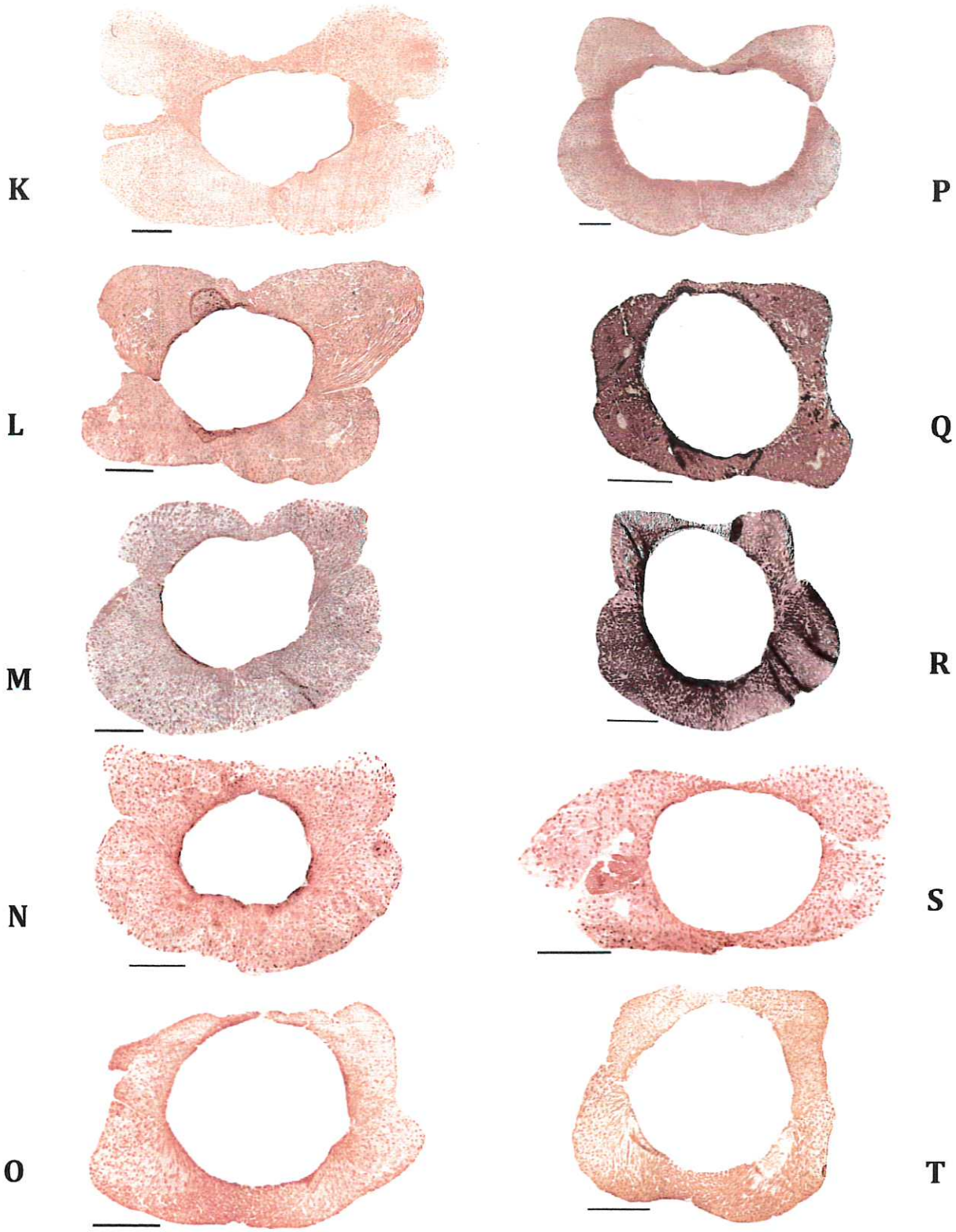


Fig. 2b Syrinx cross-sections of males and females from five songbird species (K-O) are males of each species and (P-T) are females of each species. K,P: European Starling; L,Q: White-crowned Sparrow; M,R: House Sparrow; N,S: Brewer's Sparrow; O,T: African Silverbill. Images represent the thickest section of syringeal muscle. Ventral is towards bottom of the page. Scale bars, 0.5 mm.

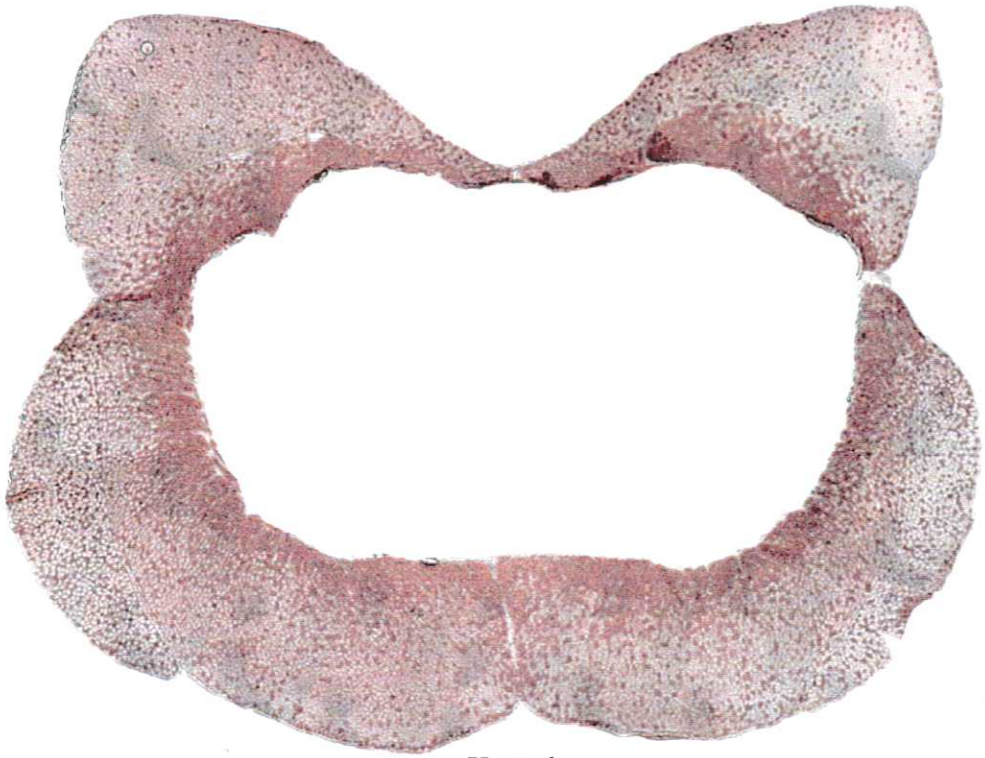
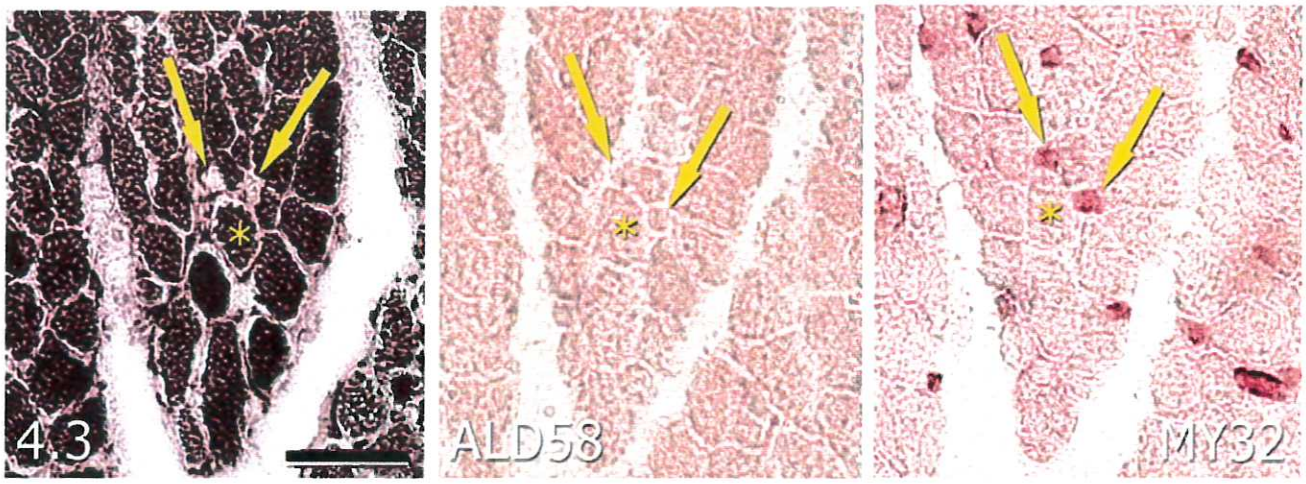
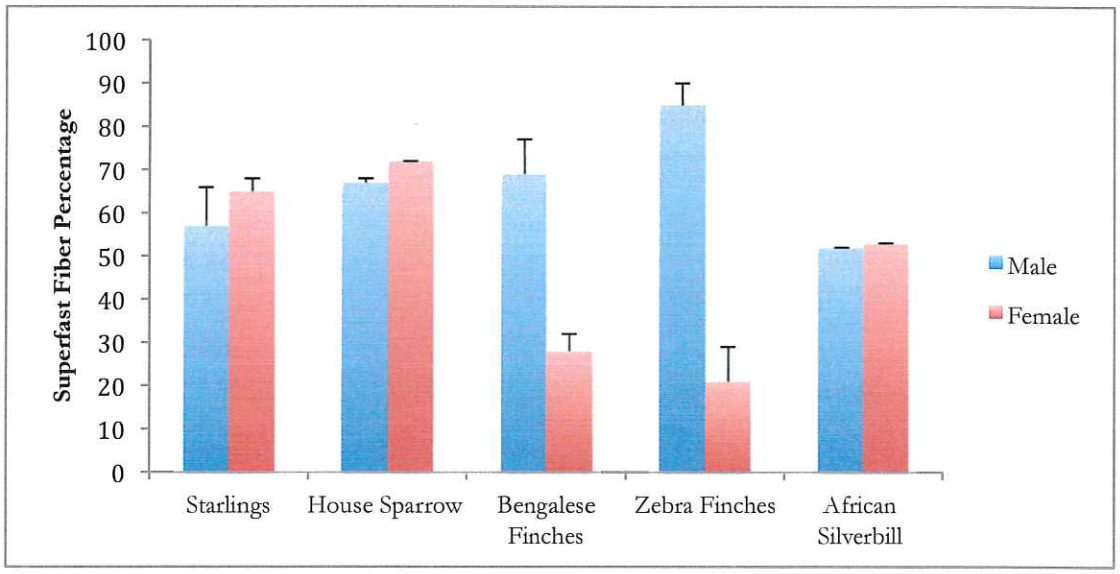


Fig. 3. Cross-section of the syrinx of a European Starling, showing an immunohistochemical reaction using anti-fast antibody MY32. Red fibers indicate a positive reaction for fast fibers. White (unreacted) fibers are superfast fibers.



Adapted from Uchida et al., 2010

Fig. 4. Serial sections of syringeal muscle from a male European Starling. Arrows indicate smaller fast muscle fibers. The asterisk indicates the larger superfast fibers. Acidic preincubation (pH 4.3) and anti-slow antibody (ALD58) react positively with slow fibers; no positive reaction was found in any syringeal muscles. The anti-fast antibody (MY32) reacts positively with fast fibers. Note the positive reaction with the smaller fast fibers. Scale bar, 100 μm.



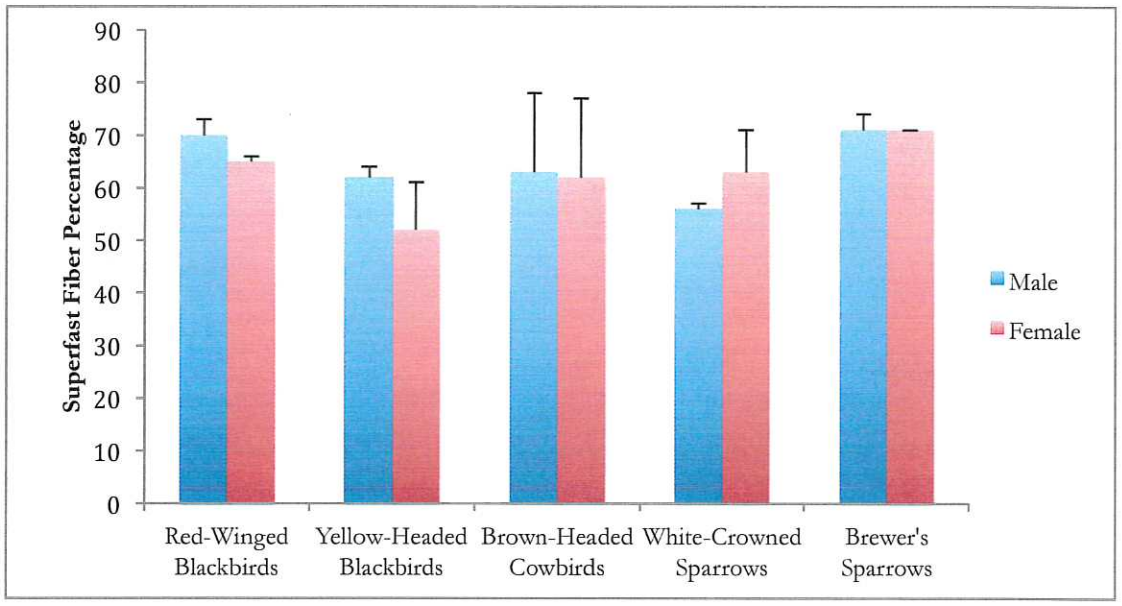


Fig. 5. The graphs show the percentage of superfast muscle fibers of the syringes of both sexes in ten songbird species. Asterisks indicate species that showed males with significantly greater superfast fibers percentages.

Table 1. Total fiber numbers, superfast fiber percentages, and mean superfast fiber percentages for each species

Species	No. of Fibers		% Superfast Fibers	Mean % Superfast Fibers (±s.d.)	t-value
	Fast	Superfast			
European Starling					
Male (2)	2697	2773	50.69	57.20±9.20	a=0.203
	1791	3144	63.71		
Female (3)	1302	2492	65.68	65.52±2.65	
	1995	3367	62.79		
	475	1014	68.10		
Zebra Finch					
Male (3)	263	1554	85.53	85.86±3.17 [a]	a=0.00128
	272	1315	82.86		
	156	1286	89.18		
Female (3)	1206	517	30.01	21.37±8.06	
	888	223	20.07		
	1476	241	14.04		
Bengalese Finch					
Male (3)	255	811	76.08	69.24±8.66 [a]	a=0.00677
	338	875	72.14		
	526	773	59.51		
Female (3)	601	207	25.62	28.66±3.45	
	590	283	32.42		
	706	274	27.96		
Brown-headed Cowbird					
Male (3)	486	1927	79.86	61.66±15.94	a=0.485
	1976	1988	50.15		
	1565	1911	54.98		
Female (3)	1191	1339	52.92	62.33±14.79	
	966	1166	54.69		
	392	1509	79.38		
Brewer's Sparrow					
Male (3)	1999	4660	69.98	70.74±2.71	*
	1890	5308	73.74		
	1894	4116	68.49		
Female (1)	1153	2867	71.32	71.32	

Statistics performed using arsine-transformed values, data presented as original values
a- Indicates statistically significant result of comparison of % superfast fibers between males and females
*- Indicates no statistics, sample size too small
All t-values evaluated at $p>0.05$

Table 1 (cont). Total fiber numbers, superfast fiber percentages, and mean superfast fiber percentages for each species

Species	No. of Fibers		% Superfast Fibers	Mean % Superfast Fibers (±s.d.)	t-value
	Fast	Superfast			
House Sparrow					
Male (2)	541	1118	67.39	67.62±0.32	*
	381	804	67.85		
Female (1)	364	931	71.89	71.89	
Red-winged Blackbird					
Male (3)	1334	3891	74.47	70.18±3.75	a=0.0792
	1009	2095	67.49		
	1298	2833	68.58		
Female (3)	837	1642	66.24	65.33±0.82	
	1306	2386	64.63		
	1151	2150	65.13		
White-crowned Sparrow					
Male (2)	611	776	55.95	56.20±0.36	a=0.236
	593	769	56.46		
Female (2)	502	660	56.80	62.71±8.36	
	379	829	68.63		
Yellow-headed Blackbird					
Male (2)	1633	2578	61.22	62.37±1.62	a=0.0755
	1327	2310	63.51		
Female (3)	1401	1759	55.66	51.75±8.66	
	1816	1305	41.81		
	1225	1675	57.76		
African Silverbill					
Male (2)	404	438	52.02	51.77±0.36	*
	416	442	51.52		
Female (1)	403	450	52.75	52.75	

Statistics performed using arsine-transformed values, data presented as original value
a- Indicates statistically significant result of comparison of % superfast fibers between males and females
*- Indicates no statistics, sample size too small
All t-values evaluated at $p>0.05$

Table 2. Diameter and average diameter of fast and superfast fibers (microns ± s.d.) for each species

Species	Fiber Diameter (μm)		Mean Diameter (± s.d.) (μm)		t-value
	Fast	Superfast	Fast	Superfast	
European Starling					a=0.0602
	18.67	35.43			b=0.388
Male (2)	14.79	27.49	16.73±2.74	31.5±5.61	c=0.00692
	21.92	30.76			
Female (3)	14.79	27.49	17.60±3.80	30.00±2.23 [a]	
	16.09	31.74			a=0.00692
Zebra Finch					
	14.38	26.53			a=0.000557
Male (3)	13.79	25.9	11.12±1.31	26.51±0.60 [a b]	b=0.00310
	16.3	27.1			c=0.455
	14.43	15.33			
Female (3)	12.36	15.92	14.63±1.46	14.44±0.42	
	17.1	12.07			a=0.461
Bengalese Finch					
	17.16	27.11			a=0.000592
Male (3)	19.75	28.45	18.74±1.38	28.56±1.51 [a b c]	b=0.00832
	19.31	30.12			c=0.00597
	15.34	21.55			
Female (3)	13.08	15.35	13.7±1.43	17.84±3.28	
	12.68	16.61			a=0.0738
Brown-headed Cowbird					
	20.38	34.46			a=0.00194
Male (3)	14.12	28.61	16.91+3.12	31.94+3.01 [a b]	b=0.0106
	16.35	32.76			c=0.293
	14.16	21.45			
Female (3)	15.55	24.24	15.69±1.60	23.38±1.68 [a]	
	17.35	24.45			a=0.00228
Brewer's Sparrow					
	16.59	23.33			
Male (3)	16.84	33.39	16.66±0.16	28.80±5.09 [a]	
	16.55	29.7			a=0.0269
Female (1)	13.81	28.36	13.81	28.36	*

a- Indicates statistically significant result in comparison of fast and superfast fiber diameters
b- Indicates statistically significant result in comparison of male to female superfast fibers
c- Indicates statistically significant result in comparison of male to female fast fibers
*- Indicates no statistics, sample size too small
All t-values evaluated at $p>0.05$

Table 2 (cont.). Diameter and average diameter of fast and superfast fibers (microns ± s.d.) for each species

Species	Fiber Diameter (μm)		Mean Diameter (± s.d.) (μm)		t-value
	Fast	Superfast	Fast	Superfast	
House Sparrow					
Male (2)	19.94	30.57	20.98±1.47	31.07±0.71 [a]	
	22.03	31.58			a=0.0161
Female (1)	19.54	25.37	19.54	25.37	*
Red-winged Blackbird					
Male (4)	18.57	28.52	15.58±2.08	27.01±1.17 [a]	a=0.000143
	13.92	26.02			b=0.119
	14.46	27.34			c=0.165
	15.4	26.16			
Male (4)	14.46	25.47	14.32±1.02	24.60±3.24 [a]	
	12.99	26.52			
	14.35	19.81			
	15.47	26.61			a=0.00264
White-crowned Sparrow					
Male (3)	16.62	22.69	16.32±1.86	25.99±5.36 [a]	a=0.0379
	18.01	32.18			b=0.452
	14.32	23.12			c=0.178
Female (2)	15.68	28.72	14.80±1.25	26.50±3.15 [a]	
	13.92	24.27			a=0.0427
Yellow-headed Blackbird					
Male (3)	24.87	46.37	22.64±2.16	36.34±9.02 [a c]	a=0.0560
	20.55	33.77			b=0.185
	22.49	28.89			c=0.0186
Female (3)	16.36	26.41	17.24±0.77	30.18±4.50 [a]	
	17.7	28.99			
	17.67	35.16			a=0.0174
African Silverbill					
Male (2)	12.78	22.45	12.24±0.77	22.99±0.76 [a]	
	11.69	23.52			a=0.00251
Female (1)	13.09	20.76	13.09	20.76	*

a- Indicates statistically significant result in comparison of fast and superfast fiber diameters
b- Indicates statistically significant result in comparison of male to female superfast fibers
c- Indicates statistically significant result in comparison of male to female fast fibers
*- Indicates no statistics, sample size too small
All t-values evaluated at $p>0.05$