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RESEARCH ARTICLE

Is sexual dimorphism in singing behavior related to syringeal muscle composition?

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ABSTRACT

The avian vocal organ, the syrinx, is located at the base of the trachea and is controlled by 4 intrinsic pairs of muscles, ventral and dorsal tracheobronchial and ventral and dorsal syringeal muscles. These muscles facilitate active regulation of airflow and sound features, and show exceptionally fast contraction kinetics, stemming from superfast and fast oxidative fiber type composition. Here we investigated to what degree fiber type composition varies across songbird species and whether or not singing ability is related to fiber type composition. In all 10 species studied, syringeal muscles are composed of fast and superfast muscle fibers in males and females. Syrinx size and muscle fiber diameter show some dependence on body size. Content of superfast fibers in the syrinx does not appear to be correlated with singing ability, because non-singing females do not consistently show lower superfast fiber content. Instead, the percentage of superfast fibers in syringeal muscles may be connected to the involvement of neuromuscular control in the generation of the acoustic structure and the entire vocal repertoire of a species.

Keywords: syrinx, sexual dimorphism, superfast muscle fibers, vocalization, singing behavior

¿Está relacionado el dimorfismo sexual en el canto con la composición muscular de la siringe?

RESUMEN

El órgano vocal de las aves, la siringe, se localiza en la base de la tráquea y es controlado por cuatro pares de músculos intrínsecos, los traqueobronquiales ventral y dorsal y los músculos siríngeos dorsal y ventral. Estos músculos facilitan la regulación activa del flujo del aire y de las características del sonido, y presentan una cinética de contracción excepcionalmente rápida que se deriva de la composición de fibras musculares oxidativas de tipo súper rápido y rápido. En este estudio investigamos el grado de variación en la composición de tipos de fibras musculares entre varias especies de aves canoras y si la habilidad para el canto se relaciona con la composición de tipos de fibras musculares. Los músculos de la siringe de las diez especies se componen de fibras musculares rápidas y súper rápidas en machos y hembras. El tamaño de la siringe y el diámetro de las fibras musculares muestran cierta dependencia del tamaño corporal. El contenido de fibras súper rápidas en la siringe no parece estar correlacionado con la habilidad para el canto debido a que las hembras que no cantan no muestran consistentemente un contenido menor de fibras súper rápidas. Por el contrario, el porcentaje de fibras súper rápidas en los músculos de la siringe podría estar conectado con el control neuromuscular de la generación de la estructura acústica y del repertorio vocal completo de una especie.

Palabras clave: comportamiento del canto, dimorfismo sexual, fibras musculares súper rápidas, siringe, vocalizaciones

INTRODUCTION

Bird vocal behavior gives rise to a highly effective acoustic communication system (Marler and Slabbekoorn 2004). Song, the most complex acoustic signal, is used for mate attraction and selection, and in male–male competition, including territory defense (e.g., Searcy and Andersson 1986, Nowicki and Searcy 2004). Given these important roles of song, it is likely that vocal quality is subject to strong selective pressures (e.g., Marler and Slabbekoorn 2004). Singing birds may express quality by a variety of acoustic features of the vocal repertoire, among which are rapid temporal aspects such as trill and modulation rates (e.g., Podos 1997, Draganoiu et al. 2002, Schmidt et al. 2008, Suthers et al. 2012). Production of high trill rates encompasses rapid switching between sound production and silent periods, and often includes rapid frequency modulation rates (e.g., Suthers and Goller 1997, Suthers and Zollinger 2008, Riede and Goller 2010, Elemans 2014). If these features are linked to neuromuscular control, the muscle kinetics of the involved motor systems must be sufficiently fast.

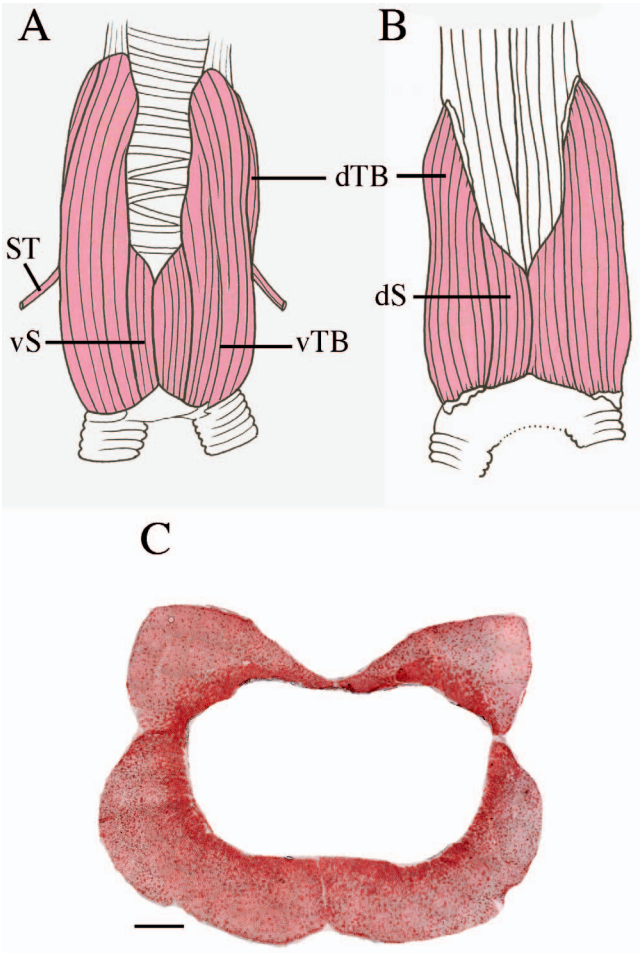

FIGURE 1. Schematic view of European Starling syrinx in ventral view (**A**) and dorsal view (**B**). Abbreviations: dS = dorsal M. syringealis; dTB = dorsal M. tracheobronchialis; dS = dorsal M. syringealis; ST = M. sternotrachealis; vS = ventral M. syringealis. M. sternotrachealis not illustrated on the dorsal view (modified from Uchida et al. 2010). In (**C**) a cross-section through the syrinx of a European Starling shows the immunohistochemical reaction using anti-fast antibody MY32. Red fibers indicate a positive reaction for fast fibers. White (unreacted) fibers are presumptive superfast fibers. Ventral is to the bottom of the page. Scale bar = 500 μm

The oscine syrinx is a bipartite structure, consisting of 2 distinct sound generators, one located in each bronchus. Each sound source is independently controlled by at least 4 pairs of intrinsic muscles, ventral and dorsal tracheobronchial and ventral and dorsal syringeal muscles (Figure 1; e.g., Larsen and Goller 2002, Düring et al. 2013). Song features arise as the result of the interaction between passive dynamic properties of the sound generating labia with physical parameters (airflow and driving pressure) as well as direct muscular control (e.g., Suthers and Zollinger 2008, Riede and Goller 2010, Goller and Riede 2013, Elemans 2014). Activity of the syringeal muscles permits direct control of airflow and labial tension and therefore affects acoustic parameters such as frequency and amplitude and facilitates rapid modulation of both (e.g., Vicario 1991; Goller and Suthers 1995, 1996; Larsen and Goller 2002; Riede and Goller 2010; Goller and Riede 2013).

Sexual dimorphism in singing behavior is accompanied by differences in both the central nervous system (e.g., Nottebohm and Arnold 1976, Cooke et al. 1998, Wade and Arnold 2004) and in the syrinx (e.g., Wade and Buhlmann 2000, Wade et al. 2002, Riede and Goller 2010). Several areas of the central song control circuitry are larger in males than in females in the Island Canary (*Serinus canaria*) and the Zebra Finch (*Taeniopygia guttata*) (e.g., Nottebohm and Arnold 1976). In the Zebra Finch, in which only males sing, the syrinx of males weighs approximately twice as much as that of females (despite minimal body size dimorphism); this difference is attributed in large part to the increased muscle mass in males (Wade and Buhlman 2000). Similarly, the syrinx of male European Starlings (*Sturnus vulgaris*) is larger than that of females, and the greater muscle mass is thought to be correlated with the ability to produce more complex song. Female European Starlings demonstrate smaller vocal repertoires and sing at slower rates than males (Prince et al. 2011).

Superfast muscle fibers are known from sound-producing organs in species as diverse as fishes (e.g., Kéver et al. 2014), snakes (e.g., Rome et al. 1996), birds (Elemans et al. 2008, Fuxjager et al. 2016), and mammals (e.g., Elemans et al. 2011). These fibers contract faster than typical vertebrate "fast" muscle and display a trade-off between force generation and speed (Rome 2006). In songbirds, superfast fibers have been shown to produce positive work up to 250 Hz in European Starlings and up to 200 Hz in male Zebra Finches (Elemans et al. 2008), placing them among the fastest vertebrate skeletal muscles (e.g., Rome et al. 1996, Rome et al. 1999). Uchida et al. (2010) described both superfast and fast fibers in the European Starling syrinx. Interestingly, whereas there was no sexual dimorphism in fiber type composition (Uchida et al. 2010) and in contraction rate in the European Starling, the muscles of female Zebra Finches were significantly slower than those of males (Elemans et al. 2008). While female European Starlings sing occasionally, female Zebra Finches do not. This raises the question to what degree fiber type composition and resulting contraction speed are related to singing ability and activity in different oscine species. We address this question here by sampling fiber type composition of syringeal muscles across several oscine families. We find that sexual dimorphism is uncommon and explore to what degree body size relates to muscle fiber morphology. Some of these results have been

previously presented in abstracts (Uchida et al. 2009, Christensen et al. 2014).

METHODS

Tissue Collection

Fresh syringeal tissue was collected from males and females of each of the following species from a diversity of families: European Starling (Sturnidae); Zebra Finch, Bengalese Munia (caged-bird variety known as Bengalese Finch; *Lonchura striata domestica*), and African Silverbill (*Euodice cantans*) (Estrildidae); Red-winged Blackbird (*Agelaius phoeniceus*), Brown-headed Cowbird (*Molothrus ater*), and Yellow-headed Blackbird (*Xanthocephalus xanthocephalus*) (Icteridae); White-crowned Sparrow (*Zonotrichia leucophrys*) and Brewer's Sparrow (*Spizella breweri*) (Emberizidae); and House Sparrow (*Passer domesticus*) (Passeridae). All songbird species were wild caught or taken from the aviary at the University of Utah prior to tissue removal. In all species, the entire syrinx from 1–3 individuals was collected in spring or early summer.

Tissue Preparation and Histology

Immediately following an overdose with isoflurane, syringeal tissue was obtained by removing the portion of the trachea and bronchi with the syringeal muscles intact. The syrinx was then mounted with the cranial aspect facing up on a 2 × 2 cm piece of cork using 5% gum tragacanth. Blocks were flash-frozen in isopentane, cooled in liquid nitrogen to approximately −150°C and subsequently stored in an ultracold freezer at −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; Ames Division, Miles Laboratories, Elkhart, Indiana, USA) 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-sectional area 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) pre-incubations. In addition, muscle sections were reacted with anti-slow antibody ALD58 (Hybridoma Bank, University of Iowa) and anti-fast antibodies MY32 (Sigma Chemical Company, St. Louis, Missouri, USA) and EB165 (Hybridoma Bank, University of Iowa) as described in Meyers and Stakebake (2005). Primary antibody slides were incubated in a humid chamber for 2 hr at 25°C, rinsed with phosphate buffered saline, incubated with secondary antibody, and stained with a peroxidase substrate system (IMMpact AEC Red kit, Vector Labs, Burlingame, California, USA). Acidic myosin ATPase and anti-slow antibody ALD58 react positively for slow muscle fibers. Alkaline myosin ATPase and anti-fast antibodies MY32 and EB165 give positive reactions for fast muscle fibers. Superfast fibers were identified by negative reactions with both ALD58/MY32/EB165. Acidic and alkaline ATPase often showed an intermediate or moderate reaction staining (Figure 2).

Reacted whole syrinx cross-sections and appropriate scale bars were photographed using a digital camera (Rebel T1i EOS; Canon, Tokyo, Japan) mounted on a microscope (Axioskop 40/40 FL; Carl Zeiss, Oberkochen, Germany) at 10 or 20×. Graphics-editing software (Photoshop CS2; Adobe Systems, San Jose, California, USA) 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.

Syringeal Muscle Analyses

Cross-sectional areas from the largest serial section of each muscle were determined using CanvasX (ACD Systems, Miami, Florida, USA). Avian body masses were obtained from Dunning (2007).

Because the boundaries between tracheobronchealis and syringealis muscles are not distinctly demarcated in all species, average muscle fiber diameters were calculated by arbitrarily selecting 50 fibers of each type from each of the 4 quadrants of the syrinx. Regions representing tracheobronchealis and syringealis muscles were included. Fiber diameters were calculated by averaging the widest and narrowest points of individual fibers using digital calipers (Mitutoyo, Kanagawa, Japan).

Percentages of both fiber types were calculated by using the largest serial section and counting all of the muscle fibers present in the image; then the total percentage of fiber types (fast vs. superfast) as well as the standard deviation was calculated for both sexes of all species. A value of "diameter-corrected" superfast fiber percentages was also calculated to create a new percentage of total cross-sectional area (CSA) that takes into account the larger size of the superfast fibers, and represents a larger functional CSA than a simple percentage. It was calculated by taking the ratio of fast to superfast fiber diameters for a given species and multiplying that value by the percentage of fast fibers. That number was then subtracted from 100 to get the "diameter-corrected" superfast fiber percentage.

Two- and three-way ANOVAs were used to analyze the data, followed by LSD post-hoc tests. For the cross-sectional area and fiber percentage data, sex and species were used as factors. For the analysis of fast and superfast fiber diameters, fiber type, sex, and species were used as factors.

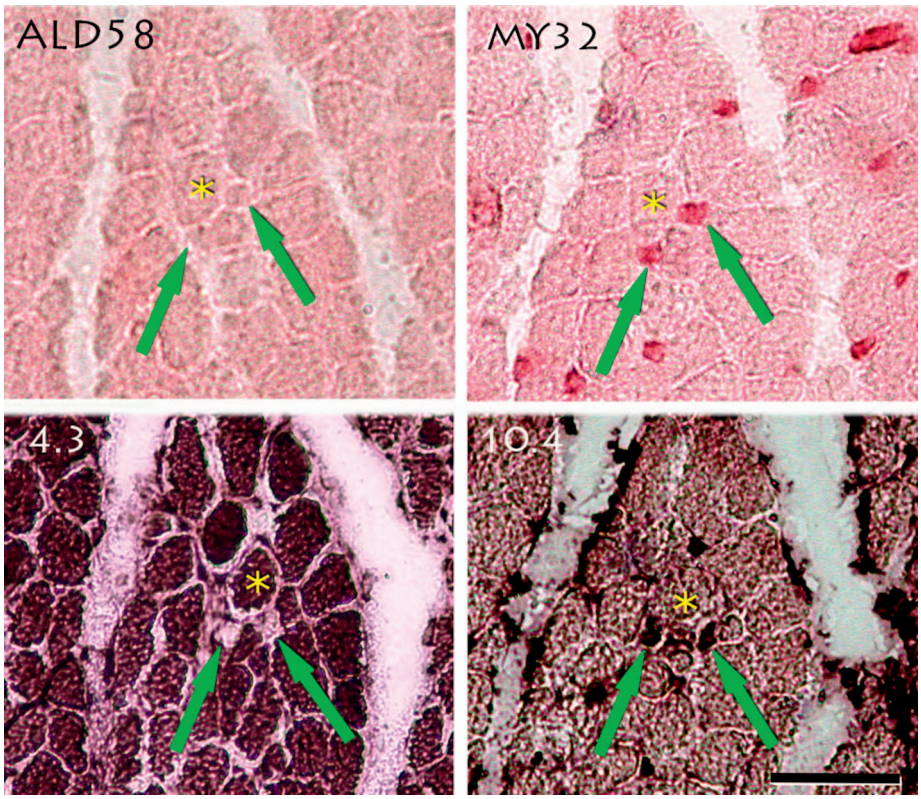

FIGURE 2. Serial sections showing muscle fiber staining profiles from a ventral tracheobronchealis (vTB) of a male European Starling. Fibers denoted by arrows are smaller, fast fibers whereas the fiber indicated by an asterisk represents the larger, superfast muscle fibers. Acid preincubation (pH 4.3) reacts darkly for slow-twitch fibers; no positive reaction was found in any syringeal muscle. Alkaline (pH 10.4) preincubation reacts positively for fast-twitch muscle fibers as shown by the arrowed fibers. The antibodies ALD58 and MY32 react positively with slow and fast muscle fibers, respectively. Note the uniformly negative ALD58 reaction indicating no slow fibers, and the positive reaction with the smaller fibers in MY32. Scale bar = 100 μm.

RESULTS

The syringeal muscles of all species were composed of 2 distinct fiber types, fast and superfast (Figures 1, 2, and 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 pre-incubations. The superfast fibers did not react like any typical type I, IIA, IIB, or tonic muscle fibers. These fibers had a negative reaction with both MY32 and ALD58, and a non-positive "intermediate" or "gray" reaction with acidic pH pre-incubations (Figure 2). These atypical reactions were previously described by Uchida et al. (2010) for the avian syrinx and DelGaudio et al. (1995) for the mammalian larynx.

Syrinx Cross-Sectional Areas

Cross-sectional area measurements revealed that in all species, males had a larger syrinx than females (Figure 3). A 2-way ANOVA with sex and species as factors revealed highly significant effects of species and sex and no significant interaction (species: $F = 16.008$, $p < 0.001$; sex: $F = 27.318$, $p < 0.001$; species × sex: $F = 1.48$, ns; Table 1). Cross-sectional area increased with increasing body size. Body mass differences explain 91% and 88% of the observed variation in area in males and females, respectively (Figure 4).

The number of syringeal muscle fibers scaled positively with body size in males (superfast: $F = 26.7$, $p = 0.0009$, $r^2 = 0.77$; fast: $F = 17.3$, $p = 0.003$, $r^2 = 0.68$) and females (superfast: $F = 40.6$, $p = 0.0002$, $r^2 = 0.83$; fast: $F = 7.98$, $p = 0.022$, $r^2 = 0.5$). Interestingly, the superfast fiber counts appear to be more tightly linked to body mass than fast fiber counts. This is particularly pronounced in females.

Syrinx Muscle Fiber Diameters

In all species, both males and females had superfast fibers that were larger in diameter than the fast fibers. A 3-way ANOVA with sex, species, and fiber type as factors indicated significant effects of all factors (species: $F = 6.075$, $p < 0.001$; sex: $F = 12.102$, $p < 0.001$; fiber type: $F = 154.6$, $p < 0.001$; all interactions are not significant). Differences between fiber types are fairly pronounced in all species (Table 2). For example, the mean diameter of

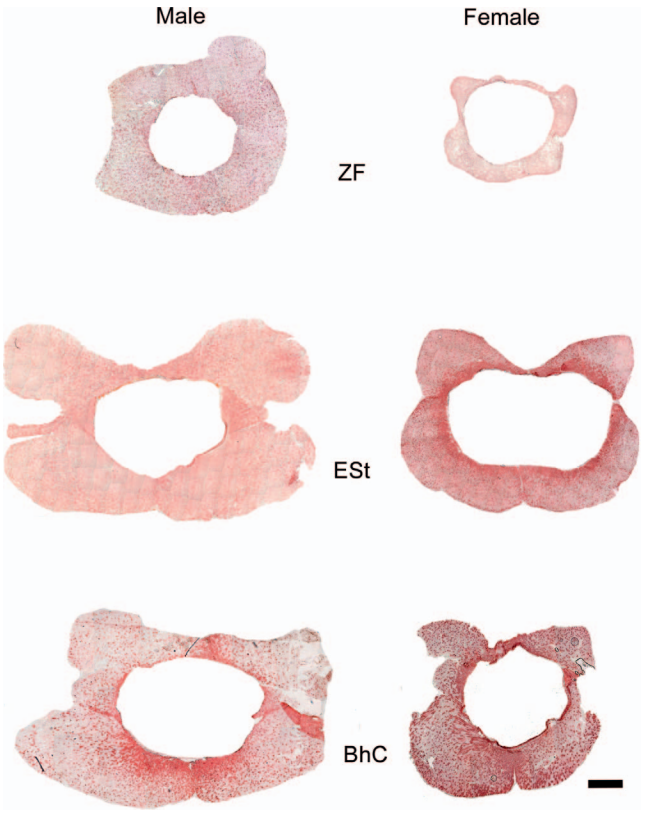

FIGURE 3. Representative syrinx cross-sections from males and females of 3 of the species examined in this study. Sections are reacted with MY32, which reacts the fast fibers red, and leaves superfast fibers unreacted. ZF = Zebra Finch, ESt = European Starling, BhC = Brown-headed Cowbird. All images are shown to scale. Scale bar = 500μm.

superfast fibers in male and female Brown-headed Cowbirds was about 32 μm and 23 μm, respectively, and mean diameter of fast fibers was 17 μm and 16 μm in males and females. In most species, post-hoc LSD tests revealed that superfast and fast fibers were not significantly different between males and females (Table 2). The most notable exceptions were found in Zebra Finches and Bengalese Finches, where mean diameter of superfast fibers was significantly larger in males (Zebra Finch: $p < 0.001$; Bengalese Finch: $p < 0.05$). In addition, mean diameter of fast fibers was larger in male Bengalese Finch ($p < 0.05$) and Yellow-headed Blackbird ($p < 0.01$).

The diameters of the 2 fiber types showed a different relationship with body size. No statistically significant relationship was found in fast fibers (Figure 5A; $r^2 = 0.19$, $p = 0.16$ for males and $r^2 = 0.36$, $p = 0.064$ for females), whereas the superfast fiber diameter in males and females increased significantly with body mass (Figure 5B; $r^2 = 0.47$, $p = 0.028$ for males, $r^2 = 0.45$, $p = 0.033$ for females). However, there is substantial variation in fiber diameters beyond the relationship with body mass. For example,

TABLE 1. Cross-sectional area (CSA) (mm^2) for each species.

Species	CSA	CSA (mean ± SD)
Brewer's Sparrow		
Male	2.63	
Male	4.19	3.64 ± 0.873
Male	4.09	
Female	1.55	1.55
White-crowned Sparrow		
Male	3.32	
Male	5.27	3.89 ± 1.198
Male	3.09	
Female	2.89	
Female	2.25	2.57 ± 0.453
African Silverbill		
Male	2.13	
Male	3.06	2.56 ± 0.56
Female	2.98	2.98
Bengalese Finch		
Male	2.92	
Male	2.48	3.01 ± 0.580
Male	3.63	
Female	1.97	
Female	1.18	1.51 ± 0.414
Female	1.36	
Zebra Finch		
Male	4.99	
Male	4.83	5.42 ± 0.887
Male	6.44	
Female	1.97	
Female	1.96	1.87 ± 0.164
Female	1.68	
Brown-headed Cowbird		
Male	10.75	
Male	11.01	10.51 ± 0.648
Male	9.78	
Female	4.01	
Female	5.07	4.48 ± 0.539
Female	4.37	
Red-winged Blackbird		
Male	12.63	
Male	9.44	10.19 ± 2.165
Male	8.50	
Female	5.67	
Female	4.34	5.68 ± 1.345
Female	7.03	
Yellow-headed Blackbird		
Male	21.53	
Male	11.42	14.76 ± 5.86
Male	11.33	
Female	6.64	
Female	10.01	8.83 ± 1.898
Female	9.84	
House Sparrow		
Male	3.72	
Male	3.57	3.65 ± 0.106
Female	2.33	2.33
European Starling		
Male	5.61	
Male	10.72	13.17 ± 3.458
Female	5.22	
Female	9.09	7.39 ± 1.976
Female	7.85	

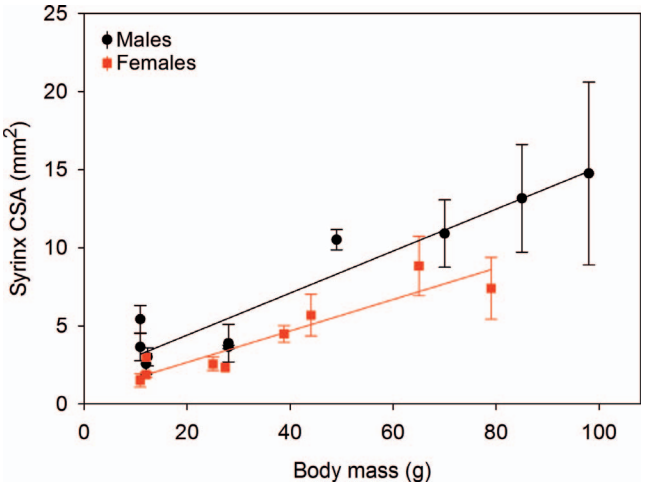

FIGURE 4. Plot of the relationship between the average syrinx CSA (in mm^2) vs. body mass for the males and females in this study. Muscle CSA increased with increasing body size, with males typically larger in both measurements. Linear regression: Males: diameter = 1.71 + 0.134 × mass, $F = 85.6$, $p < 0.0001$, $r^2 = 0.91$. Females: diameter = 0.651 + 0.1 × mass, $F = 57.4$, $p < 0.0001$, $r^2 = 0.88$.

TABLE 2. Diameter (μm) of fast and superfast fibers for each species.

		Diameter (μm) (mean ± SD)	
Species	*n*	Fast	Superfast
Brewer's Sparrow			
Male	3	16.66 ± 0.16	28.80 ± 5.09
Female	1	13.81	28.36
White-crowned Sparrow			
Male	3	16.32 ± 1.86	25.99 ± 5.36
Female	2	14.80 ± 1.25	26.50 ± 3.15
African Silverbill			
Male	2	12.24 ± 0.77	22.99 ± 0.76
Female	1	13.09	20.76
Bengalese Finch			
Male	3	18.74 ± 1.38	28.56 ± 1.51
Female	3	13.70 ± 1.43	17.84 ± 3.28
Zebra Finch			
Male	3	14.82 ± 1.31	26.51 ± 0.60
Female	3	13.96 ± 1.42	17.43 ± 3.14
Brown-headed Cowbird			
Male	3	16.91 ± 3.12	31.94 ± 3.01
Female	3	15.69 ± 1.60	23.38 ± 1.68
Red-winged Blackbird			
Male	4	15.58 ± 2.08	27.01 ± 1.17
Female	4	14.42 ± 1.02	24.60 ± 3.24
Yellow-headed Blackbird			
Male	3	22.64 ± 2.16	36.34 ± 9.02
Female	3	17.24 ± 0.77	30.18 ± 4.50
House Sparrow			
Male	2	20.98 ± 1.47	31.07 ± 0.71
Female	1	19.54	25.37
European Starling			
Male	2	16.73 ± 2.74	31.5 ± 5.61
Female	3	17.60 ± 3.8	30.00 ± 2.23

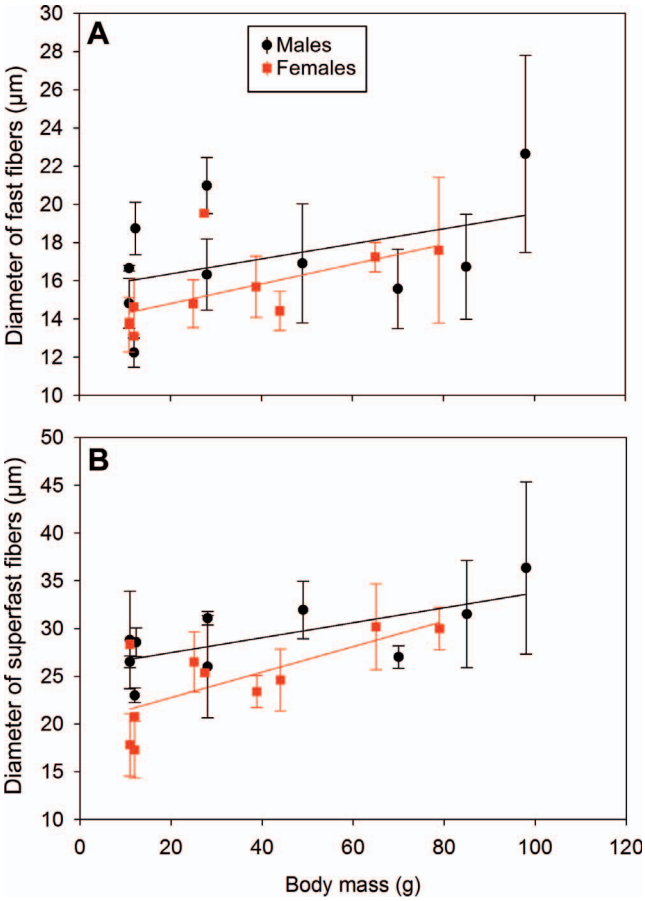

FIGURE 5. Plot of the relationship between the mean diameters of (**A**) fast fibers vs. body mass, and (**B**) superfast fibers vs. body mass. Males typically showed larger diameter fibers than females.

mean superfast fiber diameter was 27 μm in male Red-winged Blackbird, whereas that from male Zebra Finch was 26 μm.

Fiber Type Composition of Syringeal Muscles

Our initial 2-way ANOVA using data from all species with species and sex as factors yielded a significant effect of both on superfast fiber percentage and a significant interaction (species: $F = 9.595$, $p < 0.001$; sex: $F = 34.616$, $p < 0.001$). We further explored whether the 2 species with distinct sex differences in superfast fiber type percentage (Bengalese Finch and Zebra Finch) were driving the significant effect of sex as a factor. For the data set of 8 species (Bengalese Finch and Zebra Finch removed), sex no longer is a significant factor (sex: $F = 0.724$, n.s.), indicating that a clear sex difference in fiber type composition only occurred in Bengalese Finch and Zebra Finch (Figure 3, Table 3). Post-hoc LSD tests showed male superfast percentages significantly greater only in Bengalese Finch ($p < 0.001$) and Zebra Finch ($p < 0.01$).

TABLE 3. Total fiber numbers, superfast fiber percentages, and mean superfast fiber percentages for each species.

Species	Number of fibers		% superfast fibers	Mean % superfast fibers (mean ± SD)
	Fast	Superfast		
Brewer's Sparrow				
Male	1999	4660	69.98	
Male	1890	5308	73.74	70.74 ± 2.71
Male	1894	4116	68.49	
Female	1153	2867	71.32	71.32
White-crowned Sparrow				
Male	2443	3102	55.94	
Male	2372	3074	56.44	56.19 ± 0.35
Female	2007	2641	56.82	
Female	1512	3317	68.69	62.76 ± 8.39
African Silverbill				
Male	1750	3364	52.02	52.02
Female	1620	1798	52.76	52.76
Bengalese Finch				
Male	2104	3093	59.51	
Male	934	3244	77.64	69.76 ± 9.29
Male	1352	3500	72.14	
Female	2404	828	25.62	
Female	3494	1133	32.42	28.66 ± 3.46
Female	3917	1095	27.95	
Zebra Finch				
Male	1053	7219	85.52	
Male	901	5263	85.38	86.70 ± 2.17
Male	622	5145	89.21	
Female	4825	2067	29.99	
Female	3553	890	20.03	21.79 ± 7.47
Female	4922	893	15.36	
Brown-headed Cowbird				
Male	6427	7642	54.31	
Male	7905	7952	50.15	53.30 ± 4.45
Female	3865	4663	54.68	
Female	4762	5349	52.93	53.81 ± 1.24
Red-winged Blackbird				
Male	5192	11331	68.58	
Male	5335	15565	74.47	70.18 ± 3.76
Male	4035	8378	67.49	
Female	3347	6568	66.24	
Female	5223	9547	64.64	65.33 ± 0.82
Female	4607	8603	65.12	
Yellow-headed Blackbird				
Male	5308	9238	63.51	
Male	6530	10312	61.23	62.37 ± 1.61
Female	4901	6700	57.75	
Female	5607	7034	55.64	51.74 ± 8.65
Female	7264	5221	41.82	
House Sparrow				
Male	1523	3217	68.87	
Male	2165	4470	67.37	68.12 ± 1.06
Female	1455	3722	71.89	71.89
European Starling				
Male	11548	12576	51.91	
Male	10790	11094	50.69	51.30 ± 0.86
Female	5210	9970	65.68	
Female	1902	4056	68.08	65.52 ± 2.65
Female	7981	13469	62.79	

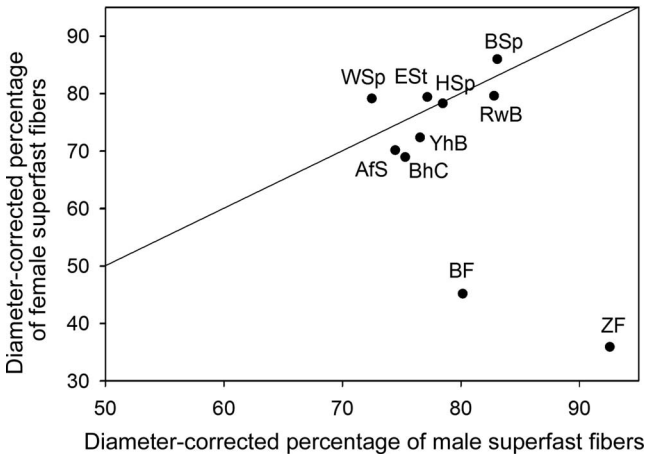

FIGURE 6. Plot of the relationship between the diameter-corrected male superfast fiber percentage vs. female superfast fiber percentage. Most species are clustered near the line of equal superfast contribution in both sexes, with the exception of Zebra Finches and Bengalese Finches displaying a markedly lower percentage in females compared to that in males. Zebra Finch males also have the highest percentage of all species, whereas male Bengalese Finches fall within the distribution of males in the other species. AfS = African Silverbill, BF = Bengalese Finch, BhC = Brown-headed Cowbird, BSp = Brewer's Sparrow, ESt = European Starling, HSp = House Sparrow, RwB = Red-winged Blackbird, WSp = White-crowned Sparrow, YhB = Yellow-headed Blackbird, ZF = Zebra Finch.

When corrected to reflect the larger size of the superfast fibers, a comparison of male vs. female superfast percentage was again most apparently different for Bengalese Finch and Zebra Finch (Figure 6). In these 2 species, superfast content was distinctly lower in females than in all the other species. Additionally, Zebra Finch male superfast content was the highest of all species.

In order to investigate whether superfast fiber content drives overall cross-sectional area, we regressed the residuals of the relationship between body mass and cross-sectional area (from Figure 4) on the diameter-corrected superfast percentage values (Figure 7). The weak positive relationship in males is not statistically significant ($F = 3.4$, $p = 0.1$, $r^2 = 0.30$), no relationship was found in females ($F = 0.08$, $p = 0.78$, $r^2 = 0.01$), and excluding Zebra Finch and Bengalese Finch also did not result in a significant linear relationship ($F = 1.94$, $p = 0.21$, $r^2 = 0.24$). The residual values for Brown-headed Cowbird and Zebra Finch are notably high. Unlike in the case of Zebra Finch, this large size cannot be attributed to very high superfast fiber content in Brown-headed Cowbird and must therefore reflect a greater contribution by fast oxidative fibers. The value for male House Sparrow is the lowest residual despite a medium superfast percentage, indicating a smaller contribution by fast oxidative fibers than in the other species.

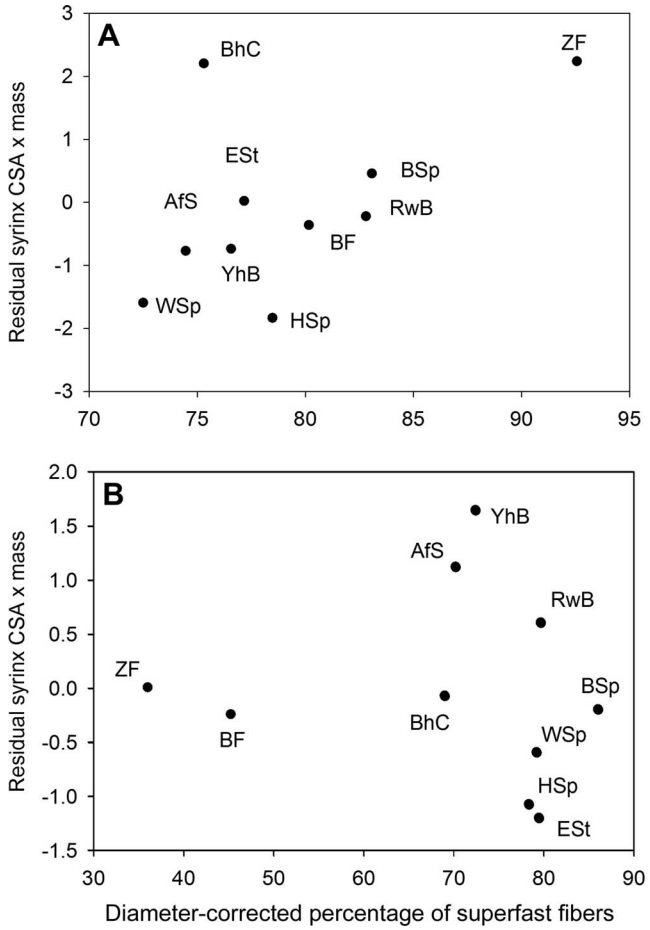

FIGURE 7. Residual syrinx CSA × mass (from Figure 4) vs. the diameter-corrected superfast fiber percentages shows a positive relationship in males (**A**) but not in females (**B**). Species abbreviations as in Figure 6.

DISCUSSION

In this study we investigate the composition of syringeal musculature in males and females of 10 songbird species. These species differ greatly in the extent of their sexual dimorphism in singing behavior, ranging from highly dimorphic singers in which males sing and females do not (e.g., Zebra Finch, Bengalese Finch, Brown-headed Cowbird, Brewer's Sparrow, African Silverbill) to those species where females at least occasionally sing (e.g., White-crowned Sparrow, European Starling, House Sparrow) or have different song-like vocalizations (e.g., Yellow-headed Blackbird, Red-winged Blackbird). The results show that the syringeal muscles of both sexes are heterogeneously composed of superfast and fast muscle fibers in all species examined, as previously described for European Starling (Uchida et al. 2010). Although there is variation between species in detailed muscle composition, the most striking result is low superfast fiber content in Zebra Finch and Bengalese Finch females.

Differences in cross-sectional area of the syrinx between species were largely explained by body mass. A clear sex difference independent of mass differences is indicated by the 3-fold higher intercept of the linear regression in males. A larger muscle mass in males has been measured consistently, and is at least in part regulated by androgens (e.g., Häcker 1900, Luine et al. 1980, Springer and Wade 1997, Wade et al. 1999). Variable levels of testosterone at the time of measurement may also explain some of the substantial inter-individual variation. The larger muscle mass in males may also reflect increased use of the syringeal muscles for vocal behavior. In many species in which females also sing regularly, males still sing much more frequently and loudly. The specific mechanisms by which higher muscle mass supports high vocal output, while often retaining high stereotypy of song, are not known.

We document substantial variation in fiber diameters of both fiber types between the different species and large variation within some species. In the syrinx, superfast fibers within a sex were found to be larger in diameter (max. diameter = 36 μm) when compared to fast fibers (max. diameter = 21 μm). Thus, superfast fibers seem to be within the normal range of avian fast fibers (see Kaplan and Goslow 1989), whereas the fast fibers of syringeal muscles are smaller than those found in avian leg muscles (see Suzuki and Tamate 1979, McFarland and Meyers 2008), though comparative data are minimal. Interestingly, the superfast fibers, which contract at rates reaching 250 Hz, are, in some cases, double the size of fast fibers. Perhaps this increased diameter compensates for otherwise low force production, which is likely to result from the trade-off between force and speed (Rome et al. 1996, Rome and Linstedt 1998, Rome et al. 1999, Rome 2006). Individual muscle size is limited by constraints of overall body size, but it is unclear whether the size of individual structures, such as the syrinx, also influences the maximal diameter of muscle fibers. The syrinx is situated in the interclavicular air sac and needs to move freely without contacting the sternum ventrally. Such restrictions may have exerted selective pressure for fairly small fiber diameters, while maintaining fine control through sufficient numbers of fibers and thus motor units. Although syringeal muscle fiber diameters do scale positively with body mass (Figure 5), there is little comparative data to suggest that this is a trend across muscles in a variety of species. Furthermore, it is well known that testosterone can increase muscle fiber sizes up to 50% (e.g., Volek et al. 1999, Sinha-Hikim et al. 2002) as can resistance training with protein supplementation (Andersen et al. 2005). Although male superfast fibers tended to be larger than those of females in all cases, this difference was only significant in Zebra Finch and Bengalese Finch. Similarly, fast fiber diameters were only significantly different in

Bengalese Finch and Yellow-headed Blackbird, but the trend was found in all species. Greater sample sizes would likely establish a distinct sex difference. How the differences in fiber size affect vocal behavior is unclear, because the biomechanics of vocal control are insufficiently understood (e.g., Goller 2016).

The fiber type composition of syringeal muscles is marked by a high percentage (>50%) of superfast fibers. Whereas this high proportion of superfast fibers is found in males and females of most species, in Zebra Finch and Bengalese Finch the composition is sexually dimorphic. Although it is tempting to attribute this to the lack of song in females of these species, this behavioral difference cannot explain the absence of strong sexual dimorphism in other species in which females do not sing. In several other species of the set investigated here, females are not known to sing, yet their fiber type composition is not strongly dimorphic. So, why do we find such a distinct sexual dimorphism in fiber type composition only in these 2 species?

Superfast muscle content may be important for controlling the syringeal valves during nonvocal behaviors. In this case the low percentage of superfast fibers in female Zebra Finch and Bengalese Finch is rather puzzling and an explanation for why there should be less need for rapid respiratory control in the females of these 2 species than in all the other species is not obvious. Both Zebra Finch and Bengalese Finch are domesticated strains, and a possible relaxation of selective pressures on rapid valve control might have resulted from the domestication process. Investigations of the syringeal muscles of wild Zebra Finches and the White-backed Munia (*Lonchura striata*), the presumed parent species of the Bengalese Finch, would provide clarification of this possibility.

Alternatively and more likely, the sexual dimorphism may be related to the entire vocal repertoire of each sex, rather than just singing behavior or the lack thereof. If a relaxation in selective pressures to achieve high contractile speed of the syringeal muscles occurred, then we can expect that rapid control by syringeal muscles is not required to produce the call repertoire. Consistent with this interpretation is the fact that in female Zebra Finch syringeal muscles are not very active during distance call and soft call production (Goller, personal observation) and that these vocalizations do not contain acoustic features, such as fast frequency and amplitude modulation, whose production would require rapid muscle control. However, the situation is less clear in female Bengalese Finch, which generate trilled calls that may require muscular control. Detailed investigation of the role of syringeal muscles in the production of the vocal repertoire of Bengalese Finch females could clarify this issue.

The fact that there is no marked sexual dimorphism in fiber type composition in the other species most likely indicates that vocal repertoires are composed of calls and song that require contributions of rapid muscle contractions. Song production by both sexes is thought to be ancestral (Odom et al. 2014), and female song is more common in tropical species (Slater and Mann 2004). Most females possess complex call repertoires; the female Streak-backed Oriole (*Icterus pustulatus*) sings more than the male (Price et al. 2008). In addition, female traits (e.g., singing) can evolve rapidly and independently (Webb et al. 2016) and be under weakened selective pressure. One idea may be that weak selection may cause more variable content of superfast fibers in females across different species. However, if the development and maintenance of superfast fibers in muscles incurs a greater cost, their presence in females may also be under some positive selective pressure. Evidence for a potentially substantial cost of maintaining syringeal muscles may be seen in the fact that overall syrinx weight is increased by circulating testosterone (Luine et al. 1980), syringeal fiber size is increased by estrogen-blockers (Martin and Veney 2008), and superfast fiber percentages are increased by testosterone (Allred et al. 2011).

It is therefore likely that vocal behavior of males and females requires rapid muscle contractions and thus explains the absence of strong sexually dimorphic fiber type composition. Alternatively, selection for a phenotypic characteristic in one sex may also lead to expression of this feature in both sexes (Lande 1980).

The large inter-individual and interspecific variation in fiber type composition makes it difficult to interpret some of the more subtle differences between species. For example in Brown-headed Cowbird fast fibers appear to account for the larger than expected syringeal cross-sectional area, whereas in male Zebra Finch the high superfast fiber content does not lead to an exceptionally large cross-sectional area (Figure 7). Furthermore, there is no simple relationship between vocal repertoire size and fiber type composition. One could speculate that a large song repertoire containing rapid frequency and amplitude modulation, such as in the European Starling, would not only require very large syringeal muscles but also muscles that are most adapted for speed. However, this is not the case. While the syrinx musculature in European Starling is within the general pattern expected from the body size relationships, it falls distinctly off these relationships in other species. These trends indicate that more detailed studies of the relationship between acoustic behavior and syringeal muscle phenotype are likely to reveal how fiber type composition is finely tuned to the specific needs dictated by the unique acoustic features found in each vocal repertoire.

In conclusion, singing behavior or lack thereof in some female songbirds likely does not drive the strong sexual dimorphism in the superfast fiber composition of the

syringeal muscles in these 2 species, because fiber type composition is similar in other species in which females are not known to sing. The differences in muscle fiber type composition between species and sexes may instead reflect muscle use for generation of the entire vocal repertoire, including song and calls.

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Author Contributions: FG and RAM conceived the idea, design, and experiment; LC, LA, and RAM performed the experiments; FG, RAM, and LC wrote the paper; FG and RAM developed methods; all authors analyzed the data.

LITERATURE CITED

Allred, L. M., L. A. Christensen, R. A. Meyers and F. Goller (2011). Denervation and testosterone changes muscle fiber types in the Zebra Finch syrinx. Integrative and Comparative Biology 51(Supplement):e159.

Andersen, L. L., G. Tufekovic, M. K. Zebis, R. M. Crameri, G. Verlaan, M. Kjær, C. Suetta, P. Magnusson, and P. Aagaard (2005). The effect of resistance training combined with timed ingestion of protein on muscle fiber size and muscle strength. Metabolism 54:151–156.

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(Supplement):e253.

Cooke, B., C. D. Hegstrom, L. S. Villeneuve, and S. M. Breedlove (1998). Sexual differentiation of the vertebrate brain: Principles and mechanisms. Frontiers in Neuroendocrinology 19:323–362.

DelGaudio, J. M., W. R. Carroll, J. J. Sciote, and R. M. Esclamado (1995). Atypical myosin heavy chain in rat laryngeal muscle. Annals of Otolaryngology, Rhinology and Laryngology 104: 237–245.

Draganoiu, T. I., L. Nagle, and M. Kreutzer (2002). Directional female preference for an exaggerated male trait in Canary (*Serinus canaria*) song. Proceedings of the Royal Society of London B 269:2525–2531.

Dunning, J. B., Jr. (2007). CRC Handbook of Avian Body Masses, 2nd edition. CRC Press, Boca Raton, FL, USA.

Düring, D. N., A. Ziegler, C. K. Thompson, A. Ziegler, C. Faber, J. Müller, C. Scharff, and C. P. H. Elemans (2013). The songbird syrinx morphome: A three-dimensional, high-resolution, interactive morphological map of the Zebra Finch vocal organ. BMC Biology 2013 11:1.

Elemans, C. P. H. (2014). The singer and the song: The neuromechanics of avian sound production. Current Opinion in Neurobiology 28:172–178.

Elemans, C. P., A. F. Mead, L. Jakobsen, and J. M. Ratcliffe (2011). Superfast muscles set maximum call rate in echolocating bats. Science 333(6051):1885–1888.

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. doi:10.1371/journal.pone.0002581

Fuxjager, M. J., F. Goller, A. Dirkse, G. D. Sanin, and S. Garcia (2016). Select forelimb muscles have evolved superfast contractile speed to support acrobatic social displays. eLife 5:e13544.

Goller, F. (2016). Sound production and modification in birds – Mechanisms, methodology and open questions. In: Comparative Bioacoustics: An Overview (C. Brown and T. Riede, Editors). Bentham Science Publishers, Sharjah, United Arab Emirates. pp. 165–230.

Goller, F., and T. Riede (2013). Integrative physiology of fundamental frequency control in birds. Journal of Physiology-Paris 107:230–242.

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. Journal of Neurophysiology 75:867–876.

Häcker, V. (1900). Der Gesang der Vögel, seine anatomischen and biologischen Grundlagen. Gustav Fischer, Jena.

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. Anatomical Record 224:426–430.

Kéver, L., K. S. Boyle, B. Dragičević, J. Dulčić, and E. Parmentier (2014). A superfast muscle in the complex sonic apparatus of *Ophidion rochei* (Ophidiiformes): Histological and physiological approaches. Journal of Experimental Biology 217:3432–3440.

Lande, R. (1980). Sexual dimorphism, sexual selection, and adaptation in polygenic characters. Evolution 34:292–305.

Larsen, O. N., and F. Goller (2002). Direct observation of syringeal muscle function in songbirds and a parrot. Journal of Experimental Biology 205:25–35.

Luine, V., F. Nottebohm, C. Harding and B. S. McEwen (1980). Androgen affects cholinergic enzymes in syringeal motor neurons and muscle. Brain Research 192:89–107.

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

Martin, L. C., and S. L. Veney (2008). The specific estrogen receptor antagonist ICI 182,780 masculinizes development of the Zebra Finch syrinx. General and Comparative Endocrinology 156:434–439.

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

Meyers, R. A., and E. F. Stakebake (2005). Anatomy and histochemistry of spreadwing posture in birds. 3. Immuno-

histochemistry of flight muscles and the "shoulder lock" in albatrosses. Journal of 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 W. A. Searcy (2004). Song function and the evolution of female preferences: Why birds sing, why brains matter. Annals of the New York Academy of Sciences 1016: 704–723.
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.
Podos, J. (1997). A performance constraint on the evolution of trilled vocalizations in a songbird family (Passeriformes: Emberizidae). Evolution: 51:537–551.
Price, J. J., L. Yunes-Jiménez, M. Osorio-Beristain, K. E. Omland, and T. G. Murphy (2008). Sex-role reversal in song? Females sing more frequently than males in the Streak-backed Oriole. The Condor 110:387–392.
Prince, B., T. Riede, and F. Goller (2011). Sexual dimorphism and bilateral asymmetry of syrinx and vocal tract in the European Starling (*Sturnus vulgaris*). Journal of Morphology 272:1527–1536.
Riede, T., and F. Goller (2010). Functional morphology of the sound-generating labia in the syrinx of two songbird species. Journal of Anatomy 216:23–36.
Rome, L. C. (2006). Design and function of superfast muscles: New insights into the physiology of skeletal muscle. Annual Reviews in Physiology 68:193–221.
Rome, L. C., 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. Proceedings of the National Academy of Sciences USA 96:5826–5831.
Rome, L. C., and S. L. Lindstedt (1998). The quest for speed: Muscles built for high-frequency contractions. News in Physiological Science 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. Proceedings of the National Academy of Sciences USA 93:8095–8100.
Schmidt, R., H. P. Kunc, V. Amrhein, and M. Naguib (2008). Aggressive responses to broadband trills are related to subsequent pairing success in nightingales. Behavioral Ecology 19:635–641.
Searcy, W. A., and M. Andersson (1986). Sexual selection and the evolution of song. Annual Review of Ecology and Systematics 17:507–533.
Sinha-Hakim, I., J. Artaza, L. Woodhouse, N. Gonzalez-Cadavid, A. B. Singh, M. I. Lee, T. W. Storer, R. Casburi, R. Shen, and S. Bhasin (2002). Testosterone-induced increase in muscle size in healthy young men is associated with muscle fiber hypertrophy. American Journal of Physiology – Endocrinology and Metabolism 283:E154–E164.
Slater, P. J., and N. I. Mann (2004). Why do the females of many bird species sing in the tropics? Journal of Avian Biology 35: 289–294.
Springer, M. L., and J. Wade (1997). The effects of testicular tissue and prehatching inhibition of estrogen synthesis on the development of courtship and copulatory behavior in Zebra Finches. Hormones and Behavior 32: 46–59.
Suthers, R. A., and F. Goller (1997). Motor correlates of vocal diversity in songbirds. In Current Ornithology 14 (V. Nolan, E. D. Ketterson, and C. F. Thompson, Editors). Plenum Press, New York, NY, USA. pp. 235–288.
Suthers, R. A., E. Vallet, and M. Kreutzer (2012). Bilateral coordination and the motor basis of female preference for sexual signals in canary song. Journal of Experimental Biology 215:2950–2959.
Suthers, R. A., and S. A. Zollinger (2008). From brain to song: The vocal organ and vocal tract. In Neuroscience of Birdsong (H. P. Ziegler and P. Marler, Editors). Cambridge University Press, Cambridge, UK.
Suzuki, A., and H. Tamate (1979). Histochemical properties and fiber type composition of the pectoral and thigh muscles of the Japanese Quail. Acta Histochemica et Cytochemica 12(1): 69–74.
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. Journal of Experimental Biology 213:1069–1078.
Vicario, D. (1991). Contributions of syringeal muscles to respiration and vocalization in the Zebra Finch. Journal of Neurobiology 2:63–73.
Volek, J. S., N. D. Duncan, S. A. Mazzetti, R. S. Staron, M. Putukian, A. L. Gómez, D. R. Pearson, W. J. Fink, and W. J. Kraemer (1999). Performance and muscle fiber adaptations to creatine supplementation and heavy resistance training. Medicine & Science in Sports & Exercise 31:1147–1156.
Wade, J., and A. P. Arnold (2004). Sexual differentiation of the Zebra Finch song system. Annals of the New York Academy of Sciences 1016:540–559.
Wade, J., and L. Buhlman (2000). Lateralization and effects of adult androgen in a sexually dimorphic neuromuscular system controlling song in Zebra Finches. Journal of Comparative Neurology 426:154–164.
Wade, J., L. Buhlman, and D. Swender (2002). Post-hatching hormonal modulation of a sexually dimorphic neuromuscular system controlling song in Zebra Finches. Brain Research 929: 191–201.
Wade, J., D. A. Swender, and T. L. McElhinny (1999). Sexual differentiation of the Zebra Finch song system parallels genetic, not gonadal, sex. Hormones and Behavior 36:141–152.
Webb, W. H., D. H. Brunton, J. D. Aguirre, D. B. Thomas, M. Valcu, and J. Dale (2016). Female song occurs in songbirds with more elaborate female coloration and reduced sexual dichromatism. Frontiers in Ecology and Evolution 4:22. https://doi.org/10.3389/fevo.2016.00022